Parasitology Research Monographs 2

Heinz Mehlhorn *Editor*

Progress in Parasitology

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Preface or Why Parasitology Research

Parasitology is an **interdisciplinary science** combining the activities of biologists, human physicians, pharmacists and veterinarians in the fight against parasites introducing diseases in humans and animals. Since its early beginnings in the middle of the eighteenth century parasitologists have acted together in international campaigns, although nationalists in many countries wanted "*to cook their own soup*". These common efforts were based on the convincing insights that agents of diseases will not stop at the border of a country as was shown by the pandemics that "*knocked at the European doors*" at regular intervals killing millions of human beings and/or their live stock animals. Thus, bad experiences with flea-transmitted plaque disease, mosquito-transmitted malaria, or with faecally transmitted cholera forced the international scientific community into close cooperations in spite of the intensions of many local politicians. Through these efforts research over the last 150 years has revealed knowledge on the life cycles of the most important parasites, the discovery of their pathogenicity and physiology as well as deep insights into the pathways of transmission. On the basis of this knowledge and the development of very skilful methods of investigation the industry was able to produce potent products that allowed a quick and efficient diagnosis as well as the initiation of a powerful control. Without these success stories it would not have been possible to keep (rather) healthy and to nourish so many of the seven billion human inhabitants on earth. However, drug resistance, bad environmental conditions, wars and over-crowded towns always give parasites a new chance. Thus, the struggle for life affects both parasites and hosts – up to now there has been no winner!

Therefore it is required that parasites stay within the **focus of research**, since globalization and its effects – even those of low grade –and global warming may bring new dangers for the world community of humans. This makes it necessary that all disciplines of biological, veterinarian and human medical parasitology remain strong. The pandemics of several emerging diseases recently have proven that we "*all live on very thin ice*" or "*on an already rumbling volcano*".

This book, published soon after the occasion of the 50th anniversary of the German Society of Parasitology presents factual accounts of important parasites and reviews recent knowledge and needs. Especially in medicinal entomology and acarology many gaps have to be filled in order to withstand parasites, which have been successful for millions of years while the "newcomer" *Homo sapiens* has been around for less than 200,000 years.

Düsseldorf Heinz Mehlhorn
June 2011

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Heinz Mehlhorn (editor)
Düsseldorf, April 2011

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Chapter 1
50 Years German Society of Parasitology (DGP) (1961–2010)

Johannes Eckert

Abstract. The German Society of Parasitology (Deutsche Gesellschaft für Parasitologie; DGP) was founded in 1960 following an initiative of Professor Dr. Karl Enigk (Hannover, Germany) by a group of leading parasitologists. During the 50 years of its existence the number of DGP-members has increased from originally 12 to approximately 500 by the end of 2010. This society has regularly held biannual meetings and numerous symposia, in 1974 it organized the 3rd International Conference of Parasitology in Munich, and it has initiated several multicentric research programs supported by the German Research Foundation (DFG) or other funding organizations. These programs were of great significance for promoting basic parasitological research, training of young researchers and establishing some new research groups. Generally, the performance of the DGP is positive but modern problem-oriented research requires better promotion as indicated by the lack of specialists in certain fields, such as arachno-entomology, ecology, epidemiology and helminthology. In addition to DGP other societies are dealing with parasites, too. Therefore, an analysis of the current situation of the DGP with evaluation of their aims and options for cooperation with other societies is recommended. In this retrospective article a few proposals are presented which could be helpful for the further development of DGP.

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1.1 Introduction

The 50th anniversary of the foundation of the German Society of Parasitology (Deutsche Gesellschaft für Parasitologie, DGP), which was celebrated in 2010, was a happy event on which I congratulated the society and its members expressing my best wishes and thanks to everyone who helped to realize the collective aims during many years. The anniversary furthermore offerd a good opportunity to take a brief look at the society's development and to try to derive suggestions for the present from an evaluation of the past. Political development as well as the development of the parasitological societies in West- and East Germany temporarily took place on separate paths which luckily fused to a common one in 1990. First, there will be a report on the development of the DGP in the West, then Prof. Hiepe (Berlin) will outline the development of the parasitological society in the East until the merging of both societies in 1990.

I appreciate the honour of being asked to be a speaker on this day of celebration of the 50th anniversary of the DGP and I'd like to thank for the invitation. First I have to point out that even though I was witness to the foundation of the DGP and although I was a Committee Member of the Steering Board for 6 years in the period between 1970 and 1978, I do not regard myself as an intimate expert of the societies' history. I can therefore be rather categorized as an external observer, who has tried to freshen up some memories by looking at the records of the DGP with the friendly assistance of Prof. Dr. Brigitte Frank (Stuttgart).

The first 15 years after the end of the Second World War were darkened on one side by the consequences of the war and characterized on the other side by the development of Germany into a new democratic society with faith and hope for a better future. In this situation academics in both parts of Germany tried to provide research with new impulses and to become included again into the international academic society. This was also the case in the field of parasitology.

Back then, parasitology in Germany and in other European countries had to face big problems because parasitic diseases played a major role both for humans and animals, since for example endo- and ectoparasites in animal stocks caused massive economic losses. Effective and well-tolerated antiparasitic agents for the control of these parasitoses were lacking. In the years around 1961 a new era of broad-spectrum anthelmintics began with the launch of thiabendazole, followed by very successful industry research activities resulting in the development of highly effective antiparasitics that are available today (Campbell and Rew 1986). Although the existing parasitological research institutes took up their activities right after the end of the war, many of these institutions did not have sufficient staff and were only sparsely equipped with instruments and materials. Many of the research methods and accessories that are available today like computers or the internet weren't available at these times (for further information see Enigk 1986).

In view of the major and visible importance of parasitic diseases of animals and humans there was a great motivation for promoting parasitological research at this time. This motivation also found its expression in the foundation of the DGP.

1.2 Foundation of the DGP

On September 26, 1960, leading parasitologists held a memorable meeting in the Institute of Parasitology at the University of Veterinary Medicine Hannover (TiHo) ("Westfalenhof", Bünteweg 17) on the initiative of Prof. Dr. Karl Enigk, director of this institute. These parasitologists were Josef Boch (Berlin), Albert Erhardt (Brackwede), Georg Lämmler (Frankfurt-Hoechst); Rudolf Lehmensick (Bonn), Hans Liebmann (Munich), Otto Mattes (Marburg), Gerhard Piekarski (Bonn), Werner Reichmuth (Berlin), Curt E.W. Sprehn (Celle), Fritz Steiniger (Hannover) and Albert Westphal (Hamburg). The young assistant Dr. Dieter Düwel acted as a secretary at this meeting. Later – from 1967 to 1990 – he was DGP's secretary and had a major influence on the development of the society, (Fig. 1.1).

At this meeting, a proposal for the foundation of a "Society of Parasitology" was unanimously accepted (DGP 1960). Karl Enigk commented this step as follows: "The increasing relevance of parasitology requires an organization so that this area gains attention at official and private authorities" (DGP 1960). The foundation of a parasitological society was supported by other prominent parasitologists who couldn't take part in the meeting, such as Alfred Borchert (Berlin), Fritz Peuss (Berlin), Hans-Jürgen Stammer (Erlangen), Hans Werner (Berlin) and Rudolf Wetzel (Giessen) (DGP 1960).

During this meeting Albert Westphal of the Institute of Tropical Medicine in Hamburg suggested the foundation of a "Society for Parasitology and Tropical Medicine". This suggestion was declined for different reasons, some of which seem strange from a present-day perspective. According to the record one reason was the opinion that the term "Tropical Medicine" has to be considered as a remnant from colonial times and that the field of parasitology is wide stretching "from the tropics to the arctic". At this time it was hardly foreseeable that Tropical Medicine was about to blossom soon reaching enormous importance in our times of globalization. It is striking that the German Society for Tropical Medicine, which had existed since 1907, was not mentioned in the record.

At that time the creation of an own publication organ for the new society was not considered necessary. The "Journal of Tropical Medicine and Parasitology" had already existed since 1949/1950 and the "Zeitschrift für Parasitenkunde", which was later renamed "Parasitology Research" and which was the organ of the DGP from 1962 to 1986, existed since 1928 (being today one of the oldest parasitological journals in the world). Since 1981 this journal appears under Prof. Heinz Mehlhorn's (Düsseldorf) editorial care and is distributed as print and online versions worldwide by Springer Publishers (Heidelberg, Berlin, New York).

At the meeting on September 26, 1960, the participants agreed that the word "German" should not be added to the society's naming "because of potential economic consequences" and "political concerns", although it was secured in the record that the distinction should be expressed so comprehensively "that all German-speaking parasitologists feel addressed" (DGP 1960). Here one should remember that although Germany had been divided into two States – the Federal

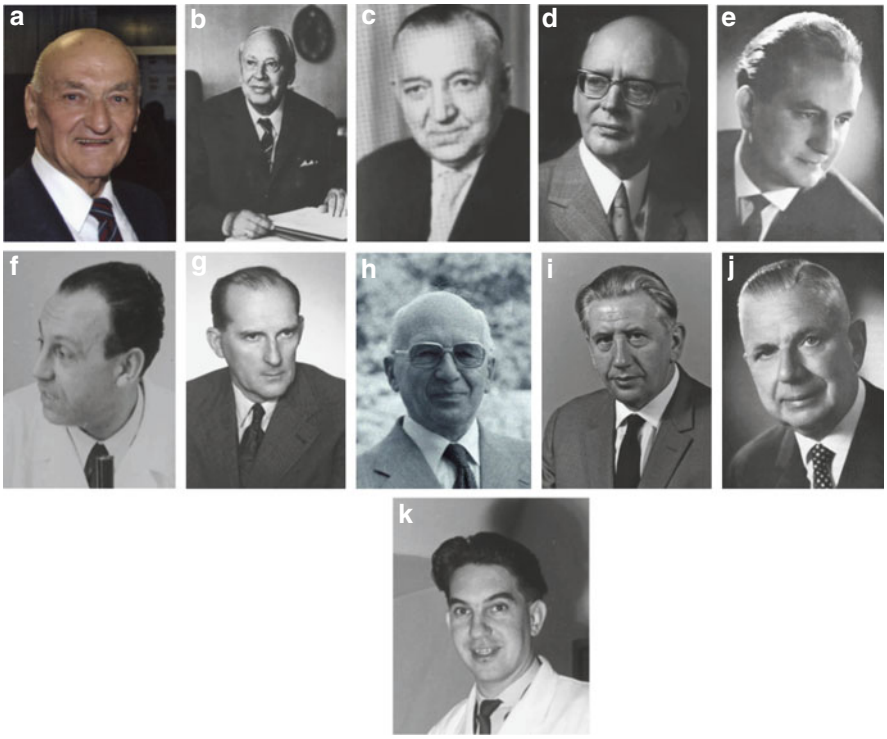

Fig. 1.1 Some of the DGP founders. (**a**) Josef Boch (*1916, †2007) (Origin: K. Pfister, Inst. Vergl. Tropenmed. u. Parasitol, Munich). (**b**) Alfred Borchert (*1886, †1976) (Origin: Th. Hiepe, Berlin). (**c**) Curt E.W. Sprehn (*1892, †1976) (Origin: Enigk, Hannover, 1986). (**d**) Karl Enigk (*1906, †1997) (Origin: J. Eckert. Zürich). (**e**) Georg Lämmler (*1925, †1981) (Origin: Enigk 1986). (**f**) Hans Liebmann (*1910, †1971) (Origin: K. Pfister, Inst. Vergl. Tropenmed. u. Parasitol, Munich). (**g**) Otto Mattes (*1897, †1975) (Origin: Archiv der Phillips-Universität Marburg, Hessisches Staatsarchiv Marburg). (**h**) Gerhard Piekarski (*1910, †1992) (Origin: H. Seitz, Inst. Med. Parasitology, Bonn). (**i**) Albert Westphal (*1909, †1987) (Origin: Bernhardt-Nocht-Institut für Tropenmedizin, Hamburg). (**j**) Rudolf Wetzel (*1895, †1983) (Origin: Enigk 1986). (**k**) Dieter Düwel (*1928) (Origin: J. Eckert, Zürich)

Republic (Bundesrepublik) and the GDR (German Democratic Republic) - since 1949, in 1960 it was not yet separated by the "Wall", which was built on the 13th August 1961 (and luckily fell in 1989).

As early as on January 9, 1961, another meeting was held in the Institute of Parasitology at the University for Veterinary Medicine Hannover (TiHo) where a draft constitution was discussed (DGP 1961a). At this meeting there was a change of opinion and nine participants of the meeting voted for the term "German Society of Parasitology". They decided upon a constitution and elected the first Steering Board which consisted of the following persons: Prof. Dr. K. Enigk, Hannover (1. chairman), Prof. Dr. Dr. R. Lehmensick, Bonn (vice-chairman), Dr. G. Lämmler, Frankfurt (Main) (secretary and treasurer), Prof. Dr. G. Piekarski, Bonn (vice secretary),

Prof. Dr. F. Weyer, Hamburg (1. committee member), Prof. Dr. O. Mattes, Marburg (2. committee member) and Dr. H. Werner, Berlin (3. committee member).

On April 25, 1961 the "German Society of Parasitology" with its place of residence in Frankfurt (Main) was enrolled into the register of German Associations and Societies and therefore was administratively established.

1.3 Objectives of the DGP

The intentions and tasks of the DGP are described in § 2 of the constitution from January 9, 1961 as follows: "The society aims at joining together of all scientifically interested parasitologists with the objective to promote progress in all areas of parasitology through professional cooperation, exchange of experience between home and abroad and promotion of junior scientists. To accomplish these tasks the society will hold scientific conferences and symposia. Their ambitions solely serve non-profit purposes" (DGP 1961b). As appears on the DGP's homepage, the society also conducts public relations to bring the importance of parasitoses and the work of parasitologists to light in the media and to the notice of research promoters. The society supports initiatives of their members which aim at better framework conditions for the scientific and practical work of parasitologists (DGP 2006a).

Special emphasis has to be placed on the fact that 50 years ago the DGP was founded as an interdisciplinary society, where researchers from the fields of biology, human medicine, veterinary medicine and other areas and subdisciplines work together. The DGP has therefore given itself a structure which also matches the contemporary requirements of interdisciplinary, national and international cooperations and which is essential for parasitology with its diverse research topics.

1.4 Development and Activities of the DGP

Looking at the development of the DGP retrospectively, the question arises if and how the objectives that were formulated in the constitution could be achieved. A number of selected indicators provide information in this regard.

1.4.1 Membership Figures

The fact of the 50 years' existence of the DGP can alone be regarded as an indication for DGP's successful development. Another evidence is provided by the membership figures, which developed from only 12 members on September 26, 1960 to about 500 at the end of 2010. According to the updates of February 2010, the members of the DGP consisted of the following groups: 63% biologists, 24%

veterinarians, 10% physicians and 3% scientists with other occupations; the percentage of women came to 34% (DGP 2010).

Scientists honored by the DGP by awarding Honorary Membership or the Leuckart Medal are listed in the Annex (Tables A1 and A2). Further information can be found in Mehlhorn et al. (2010).

1.4.2 Scientific Conferences

Further indicators of the DGP's activities are the conferences (held in 2-year-turns) (Table 1.1) and numerous symposia. A special event was the organization of the 3rd International Conference of Parasitology) ICOPA III in Munich 1974) (Table 1.1, Figs. 1.2 and 1.3).

Since many years, the basic structures and themes of scientific conferences have been discussed in the DGP. This has to do with the fact that in the course of years the research areas had been subject to change and that the society provides a roof for different subdisciplines of research, namely parasitology of medical and of biological disciplines (phytoparasitology is not considered here).

Table 1.1 Meetings of the DGP 1962–2010 (held in 2-year-turns)

Year	No.	Date	Town
1962	01	29.-31.03.	Hamburg
1964	02	18.-20.03.	Munich
1966	03	18.-30.04.	Berlin
1968	03	04.-06.04.	Bonn
1970	05	09.-11.04.	Tübingen
1972	06	10.-12.04.	Hannover
1974		25.-31.08.	ICOPA III Munich
1976	07	31.03.-01.04.	Berchtesgaden
1978	08	15.-18.03.	Freiburg/Brsg.
1980	09	26.-29.03.	Giessen
1982	10	30.03.-02.04.	Stuttgart-Hohenheim
1984	11	10.-13.04.	Bad Harzburg
1986	12	23.-25.04.	Vienna/Austria
1988	13	23.-25.03.	Neuchâtel/Switzerland
1990	14	03.-06.04.	Marburg
1992	15	30.03.-03.04.	Berlin
1994	16	21.-25.03.	Bochum
1996	17	17.-29.03.	Munich
1998	18	24.-28.03.	Dresden
2000	19	28.03.-01.04.	Stuttgart-Hohenheim
2002	20	20.-23.03.	Travemünde
2004	21	17.-20.03.	Würzburg
2006	22	22.-25.02.	Vienna/Austria
2008	23	05.-07.03.	Hamburg
2010	24	16.-20.03.	Düsseldorf

Fig. 1.2 ICOPA III in Munich, Germany, 25.-31.08.1974. (**a**) President of Congress Prof. Dr. J.-G. Baer (Switzerland). (**b**) Prof. Dr. G. Piekarski (Germany) delivering a welcome address. (**c**) Participants at the opening ceremony in historical surroundings (Origin: DGP and J. Eckert, Zürich)

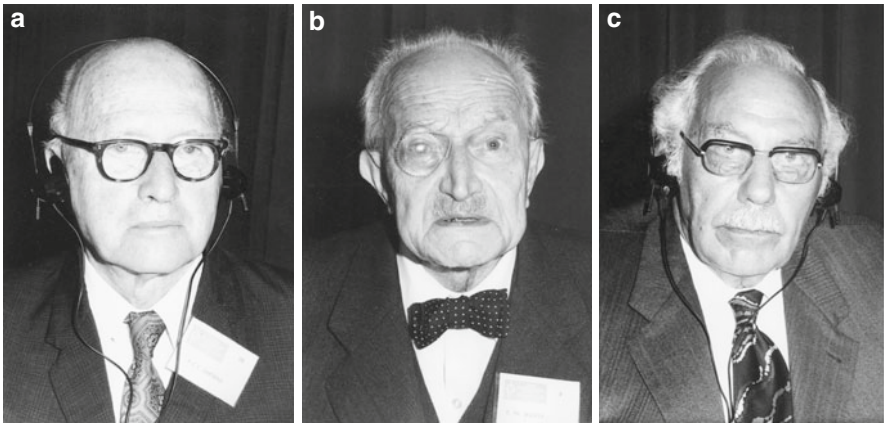

Fig. 1.3 Prominent scientists having attended at ICOPA III in Munich, 1974 (Origin DGP): (**a**) P.C.C. Garnham (UK) (*1901, †1994) (Leuckart-medal 1974). (**b**) R.Ph. Dollfus (F) (†1976) (Leuckart-medal 1974). (**c**) J.-G. Baer (CH), (*1902, †1975) (Congress President)

Medical and veterinary parasitology are concerned with parasites causing diseases (parasitoses) and their function as vectors of agents of diseases. The scientific objectives of these subdisciplines are inevitably problem-oriented and among

other aspects deal with pathogenesis, diagnosis, treatment, control and prevention of parasitoses of humans and animals.

In veterinary faculties, parasitology is represented by independent institutes. In the curriculum of veterinary medicine parasitology has its fixed place and is an examination subject for all students. In the winter semester 2008/2009 8,021 students were registered at the veterinary faculties in Germany (StB 2009). A significant percentage of the 34,000 (2006: 34,259) veterinarians registered in Germany is confronted with parasitological problems.

According to the subject catalogue of 2009 (IMPP 2009) parasitological questions concerning different disease patterns were taken into account in the second part of the final examination of **medical students** in approximately 30 German faculties for human medicine. This subject catalogue concerns about 79,000 students of human medicine (StB 2009). Regrettably, independent institutes of medical parasitology and/or tropical medicine with adequate research facilities exist only at a few faculties, for example in Hamburg, Berlin, Tübingen, Munich and Bonn.

With respect to **parasitology of biological orientation** there exists another situation. This area is represented by relatively few research institutes and departments in the area of biology. There are only a few and mostly small research groups which are in general integrated in the institutes of different subject areas. They are often very specialized and concerned with a broad range of basic parasitological questions, including aspects of genetics, biochemistry, immunology, molecular biology, physiology etc. Since it has been detected that parasites are excellent models for the exploration of basic principles of eukaryotic biology, some groups were able to line up in this field of research and to achieve international reputation. The teaching subjects in biological parasitology are not as distinct as in veterinary or medical parasitology.

It is evident that the main objectives of the mentioned subdisciplines of parasitology differ but it is wrong to draw a strict separation line between basic and problem-oriented research like some research promoters do. The development of the last years has clearly shown how closely intertwined both areas are and how important the mutual exchange of ideas and knowledge is.

Already Rudolf Leuckart[1] (1822–1898) whose head-relief decorates the Leuckart-medal (see Annex Table A1) that is granted by the DGP, wrote in the introduction to his famous book "The Parasites of Man and the Diseases Caused by Them" the following: "While wording the present work I had in mind the interests

[1]Karl-Georg Friedrich LEUCKART was Professor for Zoology in Giessen (Germany) and later Professor for Zoology and Compartive Anatomy in Leipzig. He was scientifically mainly a helminthologist opening deep insights in his field. His "cassical" book was written for "Naturforscher und Aerzte" (natural scientists and physicians) and was the starting point of parasitology as a separate, but interdisciplinary field of fundamental and applied research. Thus, he was chosen as name giver of the Leuckart medal of the DGP honoring outstanding parasitolgists (see Annex, Table A1).

of the medical practitioner as well as the ones of the zoologist. Both are not as far apart as might seem at first glance" (Leuckart 1863). He then carries on: "Without a complete knowledge of the parasites' structure and life it is almost impossible to recognize the nature and the range of diseases they cause and to find the medicine that protects us against the attacks of the evil guests".

From my point of view, the fascination of parasitology lies in the fact that it is a border area between biology and medicine. It is open for researchers from different disciplines and provides a wide field of research opportunities, from basic research to problem solving. With this in mind, the conferences of DGP ought to aim for a good balance between the mentioned functional areas and the different methodical orientations.

As an example of a successful DGP conference the 1998 meeting in Dresden is mentioned here (Table 1.1) (DGP 1998). The program (desigend by Prof. Dr. Rolf Entzeroth, Dr. Frank R. Mattig and Andreas Freud) was included in a clear and well-structured time frame, which in my opinion represents a good pattern for further conferences. At this conference 138 lectures (in plenary or parallel sessions) and 150 posters were presented. Furthermore, presentations in the fields of so-called "classical"[2] and "modern"[3] parasitology were well balanced. Some other conferences, for example those held in Stuttgart-Hohenheim and Vienna (Table 1.1) has a similar structure and quality as the meeting in Dresden (DGP 2000, 2006b).

In my opinion the conferences of the DGP should continue to serve as a forum for the interdisciplinary exchange of information within the wide field of parasitology and thereby including all subdisciplines. The value of such conferences increases if all participants try to speak a language that is also intelligible for nonspecialists, use as few abbreviations as possible, relinquish the extensive description of methodical details and concentrate on the presentation and discussion of results.

1.4.3 Promotion of Parasitology at Universities

Shortly after the foundation of the DGP, approximately between May and July 1962, the first chairman of the DGP, Prof. Karl Enigk, sent letters on behalf of the society to the directors of many medical and zoological university institutes as well as to some museums (Berlin, Hamburg, Kiel, Münster, Düsseldorf, Giessen, Marburg, Mainz, Frankfurt, Saarbrücken, Heidelberg, Freiburg, Stuttgart-Hohenheim, Tübingen, Würzburg, Munich) (DGP 1962a, b). In these letters he referred to the deficient representation of medical and zoological parasitology and emphatically called for the promotion these disciplines at West German universities. The basic tenor in the written replies was predominantly affirmation

[2]"Classical" parasitology: morphology, biology, ecology, epidemiology, diagnostics, therapy, control etc.

[3]"Modern" parasitology: biochemistry, molecular biology, genetics, cell biology, immunology etc.

for the situation analysis and the claims but contingent on indications of lacking resources. In the report of the board of the DGP from October 25, 1962 is a note to this: “The responses show that over and above the institutions which already exist in the Federal Republic there is large interest at some universities in the establishment of parasitology divisions or institutes. But since the establishment of the above-mentioned divsions or institutes lies within the competence area of the tenured professors and is basically dependent on their ambition and endeavours, there are no further steps planned on the part of the DGP” (DGP 1962c). With the persistence characteristic to him, Enigk referred repeatedly to the situation of parasitology at other occasions and thereby considerably contributed to a general sensitization for this topic.

1.4.4 Research Programs

Of particular importance for the development of parasitology in Germany were and still are the endeavours of the DGP to obtain research funds from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) and other institutions.

1.4.5 Priority Program “Physiological Parasitology”

In December 1961, K. Enigk mentioned in a letter to H.-J. Stammer, who at this time was the director of the Zoological Institute of the University of Erlangen, that he had opened negotiations with the DFG about a Parasitology Priority Program (DGP 1961c). After a resolution in favour of a priority program had been launched on March 30, 1962 at a general meeting of the DGP in Munich, Enigk made an application concerning this matter to the DFG on April 11. The application succeeded in 1964 and was entitled “Physiological Parasitology”. According to the DFG “research projects in which analytical investigations on the physiological interrelations between parasites and their animal hosts have priority should be supported. Research on life cycles and ultrastructure should only serve as a precondition for the work and should not be the main topic of the project”. The DGP assumed that the DFG would support 20–25 projects for 3 years each with 25,000–65,000 DM (about € 12,500–32,500) per year. No reliable information on the dimensions of the actual sponsorship for parasitology by the DFG could be gained upon a recent request (DFG 2009). One can deduce from a number of letters (documents of the DGP) that the sponsorship from September 1, 1964 was granted until 1970. The available funds back then may seem decent from today’s standards, but they constituted a valuable start up for some working groups. The author of this report was also a profiteer of this program being supported for investigations on physiology and in vitro cultivation of trichostrongylid larvae (Eckert 1967).

Fig. 1.4 Theodor von Brand (*1899, †1978) (Origin: J. Eckert, Zurich)

The results of this priority program were presented during a symposium, which took place from October 15 to 17, 1970 at the Bayer Convention Centre in Grosse Ledder close to Wermelskirchen/Germany. The realization of this priority program is also owed to the advice by Prof. Dr. Theodor von Brand from the National Institutes of Health in Bethesda/USA, who had to leave Germany during the Nazi period but still did not give up his affinity to German research and his home country (Fig. 1.4). He took part in the symposium in Grosse Ledder and observed the development of the priority program with great interest.

1.4.6 Symposium "Immune Reactions to Parasites"

Another step forward in research promotion was connected with the organization of an International Symposium on "Immune Reactions to Parasites". This symposium took place from October 6 to 9, 1981 in Mainz on the initiative of Prof. Dr. M. Lindauer, Würzburg. It was a combined meeting of the "Academy of Science and Literature Mainz" and the DGP (DGP 1981). According to Prof. Dr. M. Rommel "movement entered the scene" after this symposium (Rommel 2002). On the initiative of the DGP the BMFT (Bundesministerium für Forschung und Technologie; Federal Ministry of Research and Technology) granted 12 scholarships for training of young scientists abroad. A couple of the BMFT scholars were later able to establish parasitology working groups in Max Planck Institutes or in Federal Research Centres (Rommel 2002).

1.4.7 *Priority Program "Molecular and Immunological Mechanisms of Host-Parasite Interactions"*

Shortly after that, Prof. Dr. Werner Frank (Stuttgart) (Fig. 1.5), who then was the first chairman of the DGP, proposed a new priority program with the title "Molecular and immunological mechanisms of host-parasite interactions" to the DFG, which started on July 1, 1988 (DFG 1989). This program included 35 subprojects with the total sum of 5. 73 million Deutsche Mark (≈ € 2.86 million) (DFG 2009). The results of this program are published in scientific journals and a summary is documented by 20 authors in the book "Immunological and Molecular Parasitology", edited by Röllinghoff and Rommel (1994).

1.4.8 *Recent DFG – Programs and Other Possibilities for Research – and Promotion of Junior Scientists*

Also today the DGP is putting effort into achieving a new priority program that should be financed by the DFG. Apart from that, the DFG opened up new possibilities for parasitology, for example programs for supporting junior scientists collaborative research groups and graduate schools (DFG 2008). Support measurements were also offered by other institutions (e.g. Federal Ministry for Education and Research, foundations, industry).

Fig. 1.5 Werner Frank (*1926, †1991) (Origin: Verh. Dtsch. Zool. Ges. 84, 529, 1991)

It should be mentioned that the annual report of the DFG of 2008 listed 238 projects of individual and junior promotion in the area of "Medical microbiology, parasitology, mycology, hygiene and molecular infectiology" (area 204–03). Fifty-one (21.4%) of them belonged to parasitology. Eighty-four percent of the latter were concerned with protozoa, 14% with helminths and 2% with general parasitology, while arachno-entomological projects were missing (DFG 2008). The majority of the studied parasites were important for tropical countries, European parasites were represented by only 18%. Today, there are many possibilities for promoting junior scientists, but their employment for longer terms is difficult because of the lack of adequate positions.

1.4.9 "Memorandum Parasitology"

The "Memorandum Parasitology" which was published by the DFG and prepared by 15 experts from parasitology, microbiology and immunology under the aegis of Prof. Dr. P. Klein (Mainz) contributed to the approval of the priority program "Molecular and immunological mechanisms of host–parasite interactions" (DFG 1989).

In 1988 this Memorandum has listed in the Federal Republic 19 sites with 44 units (working groups, divisions, institutes), which investigated parasitological questions. The laboratories of industry and the German Armed Forces (Bundeswehr) were not taken into account during this examination (DFG 1989). Concerning parasitology in the biological departments it is noted that there were many "without institutional continuity perspectives". This meant a serious demotivation for young scientists (DFG 1989). Moreover, it was recorded that "classical parasitology"[4] had a satisfying performance level but still only a few areas were supported by the DFG. It was pointed out that this research is for the most part related to practice and was "directly financed by industry" (DFG 1989). To this day, the situation concerning support by the DFG does not seem to have changed much, which is probably one of the reasons why well-provided working groups and junior scientists are lacking in some areas of problem-oriented parasitology. Concerning "modern parasitology",[5] the Memorandum stated an unsatisfactory level of performance, which fortunately – also through the support of the DFG – improved impressively in the subsequent decades.

[4]"Classical" parasitology: morphology, biology, ecology, epidemiology, diagnostics, therapy, combat etc.

[5]"Modern" parasitology: biochemistry, molecular biology, genetics, cell biology, immunology etc.

The Memorandum contained the following recommendations concerning the further development of parasitology (shortened representation):

- Creation of interfaculty college centres by centralization and expansion of existing units.
- Establishment of research groups at universities.
- Expansion or establishment of several supraregional main institutes, each with medical, veterinary medical and/or biological orientation.
- Establishment of programs for promotion of projects and of junior scientists.

These recommendations had a particularly positive effect on the projects and on promotion of junior scientists. In the sense of the Memorandum and because of a relevant situation analysis it would in my opinion be important for the DGP to develop new activities and to support specifically developments which already started.

Such activities should include the creation of research networks (Dupouy-Camet et al. 2009). As a recent example I would like to mention the "MALSIG Consortium", an international association founded in 2009, which is dedicated to exploring the signal mechanism in the life cycle of malaria parasites (Doerig et al. 2009). In this context, one should mention as well: various COST[6]-actions at European level, to which members of the DGP made significant contributions (COST 89 1994; COST 820 1998; COST 857; Dupouy-Camet et al. 2009) as well as the working groups that have been established in the DGP (Ichthyoparasitology, Ecology, Drug design and development etc.) and the working group "Medical arachno-entomology", a collaborative project of the DGP and the DgaaE[7] (Fig. 1.6).

1.5 Other Activities of the DGP

1.5.1 Parasitological Expert (Fachparasitologe)

In a board meeting on April 3, 1968, Dr. Dieter Düwel suggested the awarding of a title "Fachparasitologe" (Expert Parasitologist) by the DGP because such a title might be helpful for younger colleagues when they apply for certain positions, also for positions abroad (DGP 1968). After the members had been informed of the conditions for the acquisition of the title in a newsletter in 1970, the title was introduced (DGP 1970); in 1971, the first applications concerning this matter were submitted to the DGP (DGP 1971).

[6]COST: European Cooperation in the field of Scientific and Technical Research.

[7]DgaaE: Deutsche Gesellschaft für Allgemeine und Angewandte Entomologie (German Society for General and Applied Entomology).

Vorsitz: Prof. Dr. P. Klein (Mainz)

15 Experten der Parasitologie, Immunologie, Mikrobiologie und Molekularbiologie

Parasitologie:
- **W. Frank (Stuttgart)**
- **J. Eckert (Zürich)**
- **H. Mehlhorn (Bochum)**
- **M. Rommel (Hannover)**
- **H.M. Seitz (Bonn)**

Andere Gebiete
- **H.D. Klenk (Marburg)**
- **P. Overath (Tübingen)**
- **M. Röllinghoff (Erlangen)**
- **R. Thomssen (Göttingen) u.a.**

Fig. 1.6 Memorandum on the future of parasitology

1.5.2 PID

From October 1963 to December 1989 the DGP published a "Parasitological Information Service" (PID; Parasitologischer Informationsdienst) in 25 volumes and 433 issues. Its aim was to inform experts and the public about important parasitological research results and problems. The messages in the PID attracted remarkable interest (DGP 1973). As editor of the PID served Prof. K. Janitschke, Berlin. He was awarded with Honorary Membership by the DGP in recognition of his achievements. One part of the aims of the PID was transferred to "DGP up to date" (www.dgparasitologie.de), but an expansion of public relations should be taken into consideration.

1.6 The DGP and Other Related Societies

With regard to the future orientation and development of the DGP, its standing in relation to other related societies, which are mainly or partially concerned with parasitological questions, is of considerable interest. Two of them were founded as late as 2004 and 2009, respectively. An increasing diversification of the spectrum

Table 1.2 Some German Societies mainly or partially concerned with parasitological issues (and the year of their foundation)

□ Deutsche Zoologische Gesellschaft (DZG), 1890
□ Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM), 1906
□ Deutsche Tropenmedizinische Gesellschaft (DTG), 1907
□ Deutsche Veterinärmedizinische Gesellschaft (DVG), 1951
□ Group "Parasitology and parasitic diseases"
□ Group "Tropical veterinary medicine"
□ Deutsche Gesellschaft für Allgemeine und Angewandte Entomologie (DgaaE), 1976[a]
□ Deutsche Gesellschaft für Protozoologie (DGP), 1981
□ Deutsche Gesellschaft für Medizinische Entomologie und Acarologie (DGME), 2004
□ Deutsche Gesellschaft für angewandte Humanparasitologie (DGAH), 2009

[a]Fusion of the societies: "Deutsche Entomologische Gesellschaft" (German Entomological Society) and "Deutsche Gesellschaft für angewandte Entomologie" (German Society for Applied Entomology)

of parasitologically oriented societies is on one hand a signal for an advancing specialization, on the other hand it can be recognized as a symptom for the fact that the DGP was not able to convey a "feeling of wellness" to all parasitological subdisciplines and to keep them under one roof. Thus, since several years, veterinary parasitologists have found an information platform in the section "Parasitology and Parasitic Diseases" of the German Veterinary Medical Society (DVG, Deutsche Veterinärmedizinische Gesellschaft) which obviously matches their demands better than the DGP-platform, as the very well-frequented annual conferences show. In my opinion this "separation" should be a reason to reconsider the tasks and objectives of the DGP and its relations to other societies. Furthermore, it should be considered if certain measures of coordination and task sharing at national and European levels would be useful. Another reason for reconsideration should be the fact that in 2009 a "German Society for Applied Human Parasitology" ("Deutsche Gesellschaft für angewandte Humanparasitologie") was founded (Table 1.2).

1.7 Problems and Challenges

Today, it can be stated that many parasitological groups in Germany have achieved a high standard – not least because of the application of modern research methods – and successfully perform in the international network of parasitological research. But it is also conspicuous that in the last decades several areas of parasitology have been neglected and desperately require promotion. Thus in 2007 Prof. Dr. Norbert Mencke pointed towards the precarious situation of arachno-entomology (Mencke 2007). The unexpected appearance (2006) of Blue tongue disease in Central Europe showed for example that the current knowledge on arthropod vectors is in general insufficient. In the same year, Prof. Dr. Brigitte Frank

complained about the decreasing interest in taxonomical questions and pointed out the negative consequences (Frank 2007). Among other areas that are in need of support are (inter alia) helminthology, ecology and epidemiology (the list is not complete!), which today apply new research methods and therefore could become more effective and attractive.

As we all know, there are on the one hand many fundamental questions about "parasitism", a common form of life in nature, which are still unanswered. On the other hand we notice that quite a number of the long-known parasitoses of animals and humans still cannot be sufficiently controlled and at the same time new problems appear. That is why even today, parasitology faces significant research tasks which have been explicitly discussed with regard to the 21st century in the literature (Thompson 1999; Coles 2001; Geary and Thompson 2003; Vickerman 2009).

Here are just some of the topics listed:

- Migration, organ tropism and survival of parasites in their hosts.
- Genetic principles of host resistance against parasites.
- Development of vaccines, antiparasitics and biological control measures.
- Identification and control of drug resistance of parasites.
- Improved surveillance of parasitic infections, especially of zoonoses and "emerging diseases".
- Parasites of wild animals and in food chains.
- Impact of climate change and globalization on parasitoses.
- Parasitic infections in the tropics.

As the famous Scottish parasitologist Keith Vickerman mentioned, parasitologists have often been confronted with the accusation that they failed to formulate "questions of bigger importance for biology" in their works (Vickerman 2009). In defence of this one can state that parasitological basic research has made important contributions which exceeded its own subject area. Here, one merely has to remember the beginnings of antiprotozoal chemotherapy by the German Nobel Prize winner Paul Ehrlich, the discovery of antigen variation in trypanosomes and the role of helminths as models for comparative metabolism studies. Other examples are recorded in a review by Peter Köhler, Zürich (Köhler 2001). Furthermore, the transfer of knowledge which constantly results from basic research, is fundamental for problem-oriented parasitological research.

At an increasing rate, not only basic research but also solution approaches for existing problems are demanded from science. The former Chancellor of Germany, Helmut Schmidt, once spoke of the "academics' obligation to provide information and solutions for the public and politics" (quoting G. Mack 2001). Parasitology cannot flinch from this demand. Therefore, parasitology should aim at an adequate promotion of both fundamental and problem-oriented research as well as at an interconnection of both fields of research.

1.8 Final Remarks

After 50 years of existence the DGP can present a remarkably positive balance of acitvities. The prospective tasks of parasitology and the general development of sciences should still be a motivation for the DGP to conduct a brain storming from time to time, to reveal existing weak points and to outline ways for improvements.

On the occasion of the 50th anniversary of the DGP a few suggestions were presented by J. Eckert and Th. Hiepe:

- Evaluation of the situation of parasitology in Germany (see also "Memorandum Parasitology" from 1989 and Memorandum on the situation of Tropical Medicine in Germany, 1995) (DTG 1995).
- Development of new initiatives for the preservation of existing institutions and for the foundation and support of new parasitology institutions.
- Actions of the DGP for a balanced promotion of basic and problem-oriented research.
- Establishment of networks of working groups for the processing of bigger, collective research projects.
- Promotion of junior scientists especially in areas that have been neglected so far.
- Improvement of public relations (examples: publication of statements on parasitological problems of public interest like the invasion of vectors, or on drug resistance of arthropods and helminths)
- Elaboration of guidelines for the organization of the biennial conferences. A Program Committee should arrange a program in which all subdisciplines are appropriately represented (presentation of reviews and original data, workshops). An Organizing Committee should plan congresses and cooperate closely with the Program Committee.
- Establishment of networks with other societies that are interested in parasitology and mutual reconciliation of activities.

Concluding I would like to wish the DGP continuing prosperity in an academic mind, which aims at the enrichment of knowledge as well as at the solution of problems for the benefit of man, animals and the environment.

Annex

Table A1 The German Society of Parasitology has honoured the following scientists by awarding the Leuckart Medal (Source: Mehlhorn et al. 2010)

Year	Name	Country	Town
1974	R. Ph. Dolfus, Prof. Dr. †	France	Paris
1974	P.C.C. Garnham, Prof. Dr. †	England	Ascot
1974	R. Geigy, Prof. Dr. †	Switzerland	Basel
1974	G. Poljanski, Prof. Dr. †	Russia	St. Petersburg
1974	H.W. Stunkard	USA	New York
1974	P.H. van Thiel	The Netherlands	Bilthoven
1980	W. Peters, Prof. Dr. †	England	London
1982	R.M. Cable, Prof. Dr †.	USA	Indiana
1982	W. Trager, Prof. Dr. †	USA	New York
1982	J. Weiser, Prof. Dr. †	Czech Republic	Prague
1984	Sheila M. Willmott, Dr.	England	St. Albans
1986	K. Enigk, Prof. Dr. Dr. †	Germany	Hannover
1986	R. Supperer, Prof. Dr. Dr.	Austria	Vienna
1987	L.J. Bruce-Chwatt, Prof. Dr.	England	London
1992	G. Piekarski, Prof. Dr. †	Germany	Bonn
1996	J. Eckert, Prof. Dr. h. c.	Switzerland	Zürich
2000	T. Hiepe, Prof. Dr. Dr. h. c.	Germany	Berlin
2002	M. Rommel/M. Röllinghoff, Profs. Drs.	Germany	Hannover, Erlangen
2004	H. Mehlhorn, Prof. Dr.	Germany	Bochum, Düsseldorf
2008	J. Boothroyd, Prof. Dr.	USA	Stanford
2010	Katja Becker, Prof. Dr. med.	Germany	Giessen

Table A2 The Honorary members of the German Society of Parasitology (in alphabetical order and indicating the year of the award) (Source: Mehlhorn et al. 2010)

Prof. Dr. med. vet. Dr. h. c. Josef Boch, Scheidegg † (1986)
Prof. Dr. phil., Dr. med. Theodor von Brand, Bethesda, USA † (1968)
Dr. Jean-Francois Dubremetz, Lille, France (2000)
Dr. med. vet. Dieter Düwel, Dänischenhagen (1988)
Prof. Dr. med. vet. et med. vet. h. c. Karl Enigk, Hannover † (1976)
Prof. Dr. rer. nat. A. Hase, Berlin † (1962)
Prof. Dr. med. vet. Klaus Janitschke, Berlin (2006)
Prof. Dr. Alan Johnson, Sydney, Australia (2000)
Prof. Dr. Tibor Kobulej, Budapest, Hungary † (1979)
Prof. Dr. med. H. E. Krampitz, Munich † (1988)
Prof. Dr. phil., Dr. med. Rudolf Lehmensieck, Bonn † (1974)
Prof. Dr. rer. nat. Otto Pflugfelder, Stuttgart † (1974)
Prof. Dr. phil. Gerhard Piekarski, Bonn † (1978)
Dr. med. vet. Hans Rüffer, Bonn † (1978)
Prof. Dr. M. D. Sonin, Moscow, Russia † (1989)
Prof. Dr. med. vet. Curt E. W. Sprehn, Celle † (1962)
Prof. Dr. phil, Dr. med., Dr. h.c. Hans Vogel, Hamburg † (1970)
Prof. Dr. rer. nat. Hans Werner, Berlin † (1988)
Prof. Dr. rer. nat.Albert Westphal, Ahrensburg † (1989)
Prof. Dr. med. vet. Rudolf Wetzel, Giessen † (1965)
Prof. Dr. rer. nat. Fritz Weyer, Hamburg † (1974)

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DGP (1962c) Vorstandsprotokoll vom 25.10.1962.
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Chapter 2
Parasitology of the German Democratic Republic (1961–1990): Memories of a Time Witness

Theodor Hiepe

Who does not accept his past,
Does not deserve the future
Wilhelm von Humboldt (1767–1833)

2.1 Introduction

The deliberate search for the unexplored is at an increasing rate based on communication between kindred spirits. Academic societies unquestionably offer a forum for that. They provide the possibility for exchange of ideas about new knowledge. In 1960 – i.e. 15 years after the end of the horrible Second World War – bright minds in parasitology thought that it was the right time to unite German-speaking parasitologists in a common Society of Parasitology.

A short time after the foundation of the "German Democratic Republic" (GDR) a strict political separation between East and West Germany occurred, which became even worse after the building of the "Wall" starting on the 13th of August 1961 (see Chapter 1). Until then several East German colleagues (including me) had already been members of the DGP (see Chapter 1). I had become the 43rd

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Fig. 2.1 First President of the Society of Parasitology of the GDR Prof. Dr. A. Borchert

member of the DGP just after was appointed to the Chair of Parasitology and Veterinary Zoology at the Humboldt University in Berlin.

On the initiative of Dr. Engelbrecht and Dr. Odening (Berlin) a group of East German parasitologists met in the year 1960 in East Berlin to consider the foundation of a parasitological society in the GDR. As a result of this meeting the "Working Group Parasitology" was established inside the already existing "Society for Biology of the GDR." However, it soon turned out that this position was not advantageous and that progress in parasitology could better be achieved by an independent "Society for Parasitology." The latter was finally founded on the 10th of October 1961 in Berlin and the first secretary became Dr. Odening (Berlin).

Already in the year 1960 Prof. Dr. WD. Eichler started together with colleagues (e.g. Profs. Jirovec, Prague; Z. Kozar, Wroclaw, Breslau and H. Peters, Heidelberg) a scientific journal at the Fischer Publishing House (Jena) called "Angewandte Parasitologie" (= Applied Parasitology, AP).

As soon as the Parasitological Society was founded, its steering board decided that the AP journal should become the official publishing organ of the new Society (Table 2.1 and Table 2.2).

The journal "Angewandte Parasitologie" (= Applied Parasitology) closed a gap in the international parasitological literature. As an official publication organ it accompanied the Society for Parasitology from day one with four volumes/year, with memorandums on important problems with parasites and parasitoses as well as including an intensive informational part. Every member of the Society for Parasitology was a free subscriber of the AP [6].

From a historical point of view, the documented foreword deserves closer attention, which reads as follows

> After the end of the Second World War, parasitology in Germany fell considerably behind the development of other countries. This becomes especially obvious if one thinks on the enormous boom of primarily the hygienic branches of parasitology during the last 10 years

Table 2.1 Foundation document of the Parasitological Society of the GDR

Parasitologische Gesellschaft der DDR

Mit dem ersten Heft des 3. Jahrganges beginnend wird die „Angewandte Parasitologie" von der Parasitologischen Gesellschaft der DDR als ihr offizielles Publikationsorgan herausgegeben. Damit konnte schon in den wenigen Monaten des Bestehens der Parasitologischen Gesellschaft eine ihrer wesentlichsten Aufgaben, die Förderung parasitologischer Veröffentlichungen, in die Tat umgesetzt werden.

Wie sehr die Gründung der Parasitologischen Gesellschaft der DDR einem dringenden Bedürfnis nach Zusammenschluß der auf den verschiedenen Gebieten der Parasitologie in der DDR arbeitenden Persönlichkeiten und Institutionen entspricht, geht schon allein daraus hervor, daß sich an der Gründungsversammlung im Oktober 1961 zahlreiche Parasitologen aus Wissenschaft und Praxis beteiligt haben. Deshalb verfolgt die Gesellschaft vor allem das Ziel, ihren Mitgliedern durch regelmäßige wissenschaftliche Veranstaltungen, Bildung von Sektionen und Arbeitskreisen, Erweiterung der Publikationsmöglichkeiten usw. einen regen Erfahrungs- und Meinungsaustausch auf breiter Basis zu ermöglichen. In diesem Sinne wird die Gesellschaft auch bestrebt sein, auf nationaler und internationaler Grundlage mit den Fachkollegen anderer Länder, sowohl mit Einzelpersonen als auch mit anderen parasitologischen Gesellschaften, durch gegenseitigen Besuch von Kongressen und Symposien, durch Austauschbesuche usw. zusammenzuarbeiten.

Ferner betrachtet es die Gesellschaft als eine ihrer dringendsten Aufgaben, die staatlichen Institutionen für die Planung und Durchführung groß angelegter parasitologischer Forschungsaufgaben zu gewinnen, insbesonderer solcher, die unter Zusammenfassung der besten Arbeitsmöglichkeiten und -kräfte nur kollektiv gelöst werden können.

Ein weiteres wesentliches Ziel sieht die Gesellschaft darin, sich zur Schaffung eines wissenschaftlichen Nachwuchses auf parasitologischem Gebiet für die Unterweisung von Studenten, Diplomanden und anderen Kräften in gleicher Weise einzusetzen wie für die Weiterbildung bereits auf Spezialgebieten tätiger Parasitologen.

Zur Erfüllung ihrer Aufgaben bedarf die Gesellschaft der Mitarbeit aller Persönlichkeiten, die wissenschaftlich oder praktisch parasitologisch tätig und gewillt sind, die Entwicklung der Parasitologie in ihren verschiedenen Fachrichtungen zu fördern. In diesem Sinne wendet sich die Gesellschaft an die Biologen, Human- und Veterinärmediziner ebenso wie an die Angehörigen der Land-, Forst-, Fisch- und Wasserwirtschaft, der Phytopathologie, Pharmazie und Schädlingsbekämpfung und nicht zuletzt an die Bearbeiter von Spezial- und Grenzgebieten der Parasitologie mit dem Aufruf zur tätigen Mitarbeit für das Wohl unseres Volkes.

Der Vorstand

Prof. em. Dr. Dr. h. c. A. Borchert

Dr. L. Britz	Dr. H. Engelbrecht	Dr. G. Hartwich
Prof. Dr. Th. Hiepe	Prof. Dr. Dr. K.-D. Rudat	Prof. Dr. G. Wildführ

Anschrift der Gesellschaft: Berlin-Friedrichsfelde, Am Tierpark 41. Fernruf: 55 00 12. Sekretär: Dr. K. Odening.

1 Angew. Parasitol. 3. Jg. 1

in those countries that were hit hard by the war like Poland, Czechoslovakia and Hungary. In consideration of the big tasks that we were set on one hand by the control of parasitic diseases of our domestic animals and on the other by the preventive human health protection, it proved necessary to strengthen parasitological research in every possible way. This shall also be the task of the present new journal.

Table 2.2 Document of the aims and targets of the Parasitological Society of the GDR

Parasitologische Gesellschaft der DDR

STATUT

§ 2

Aufgaben und Ziele

Die Gesellschaft hat zum Ziel, die Aufgaben der Parasitologie in Forschung, Lehre und Praxis zu unterstützen durch:

2.1. Förderung der allseitigen Entwicklung der Parasitologie in der DDR,
2.2. Organisierung von wissenschaftlichen Tagungen, Seminaren, Kolloquien, Arbeitssitzungen und Fortbildungsveranstaltungen,
2.3. Zusammenarbeit im nationalen und internationalen Rahmen insbesondere mit anderen wissenschaftlichen Gesellschaften der DDR, der Sowjetunion und der anderen sozialistischen Staaten sowie mit internationalen Organisationen, in denen die Gesellschaft Mitglied ist,
2.4. Förderung einer Zusammenarbeit der parasitologisch orientierten Biologen, Humanmediziner und Veterinärmediziner der DDR und Naturwissenschaftler anderer Disziplinen,
2.5. Orientierung der Mitglieder auf die prognostischen Schwerpunkte der Forschung und die strukturbestimmenden Hauptaufgaben unserer Volkswirtschaft,
2.6. Förderung der Veröffentlichung von wissenschaftlichen Publikationen, der Öffentlichkeitsarbeit und eines Literaturinformations- und Dokumentationssystems,
2.7. Einflussnahme auf die Anwendung parasitologischer Forschungsergebnisse im Interesse der Volkswirtschaft und Volksgesundheit,
2.8. Beratung bei der Berufslenkung von Parasitologen, Einflußahme auf den fachgerechten Einsatz,
2.9. Einflussnahme auf die Aus- und Weiterbildung der wissenschaftlichen Kader und der technischen Mitarbeiter auf dem Gebiet der Parasitologie,
2.10. Befürwortung von Studienreisen und Besuchen von Kongressen und Tagungen im In- und Ausland,
2.11. Unterstützung staatlicher Organe und Einrichtungen, wirtschaftsleitender Organe, von VE-Kombinaten und-Betrieben sowie von wissenschaftlichen Institutionen. Förderung von Vereinbarungen, Verträgen und Forschsaufträgen zwischen den genannten Institutionen und Mitgliedern.

Due to the choice of the title "Applied Parasitology" it is already expressed that our new journal should first and foremost deal with the practical issues of the parasitological sciences. Here, we mainly think on the problems of the medical and veterinary medical parasitology – including its border areas like fish parasites and pest control in the hygiene sector. The practical needs of these economically so significant fields of work are to be accounted for by the fact that apart from original works we also plan to include collective reports, reviews of literature and classification tables.

With this, we also wish to help our junior parasitologists, for whom the to some extent extremely scattered literature is often difficult to access. This means at the same time that it was aimed to take over some of the objectives of the scientific documentation into the area of responsibility of our journal.

May our new journal therefore offer in this way a positive contribution to the growth and prosperity of the parasitological science.

(April 1960, editor and publisher)

2.1.1 *Further Remarks*

From the beginning our main concern was to give a home to parasitologists, who were active as single researchers or worked in small groups mainly in institutes where medical or veterinary medical investigations were carried out or who worked at universities, regardless of whether they were biologists, physicians or veterinarians.

It should be mentioned that in the GDR only two professorships for parasitology existed at veterinary medical educational institutions [University of Leipzig – Prof. WD. Eichler, 1949–1955; Prof. E.A. Nickel, 1956–1985; followed by Prof. Regine Ribbeck (from 1986) and at Humboldt University/Berlin, Institute for Veterinary Medical Zoology and Parasitology from 1948 to 1960 Prof. A. Borchert; Prof. Th. Hiepe (since 1961)]. Furthermore, there was a personal chair for parasitology (without license), first in Kleinmachnow, from 1968 onwards at the Museum of Natural History Berlin: WD. Eichler [2, 3].

As early as 1926 parasitology had been an obligatory subject at the veterinary medical educational institution in Berlin [1], where also Karl Enigk, later Hannover, was active as an assistant and as a lecturer as well. By the way this was the first chair for parasitology in the German-speaking countries (W. Nöller, 1890–1964) (Fig. 2.2). From a historical point of view I would also like to remark the following: Already **Robert Koch** called for a chair for parasitology shortly before the turn of the nineteenth or twentieth century, after his second Africa expedition ". . .because there is something – the parasitic worms and the disease-causing protozoa – which go beyond the horizon of the bacteriologist." Not until a quarter-century later, this was realized. And when this had become reality it was W. Nöller and K.I. Skrjabin who postulated in Berlin in 1930: "parasitologists of all countries, unite (Fig. 2.3)." This was in those days (late phase of the Weimar Republic) a dangerous sentence with negative consequences: W. Nöller had to leave the Veterinary University in

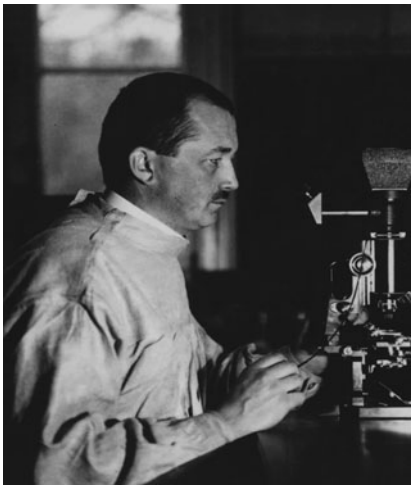

Fig. 2.2 Prof. Wilhelm Nöller. Founder of the Institute for Parasitology in Berlin

Fig. 2.3 K.I. Skrjabin (*left*) and W. Nöller (1930 in Berlin)

1930. Not until 34 years later, in 1964, the first World Congress for Parasitology took place in Rome – an important step beyond the national parasitological societies. On the former territory of the GDR were and still are no separate parasitological chairs in university biology and medicine. Thus, the parasitological educational level of the members of the society differed greatly.

In the field of human medicine the institutes for microbiology and hygiene of the Medical Faculty of Rostock [director at that time: Prof. K.-D. Rudat who was experienced in the field of protozoology but who soon left the GDR (in 1962)] and Leipzig (director: Prof. G. Wildführ) allowed parasitology a place as a subject in the frame of microbiological lectures and tutorials. A replacement chair was not available for this. The teaching of parasitology for physicians was usually "supplied" by the Biological Faculties, for example Prof. Freye/ Halle(Saale), Prof. Kämpfe/ Greifswald. Parasitology, either as an obligatory or as a facultative subject was represented at almost all biological faculties respectively universities of the GDR. Under the care of Prof. Reimer, applied parasitology could present remarkable achievments, especially in the area of parasites and parasitoses of fish, was developed at the pedagogical university Güstrow in the course of the years. At the **Academy of Sciences** (at today's **Leibniz Institute for research on zoo and wild animals**), a superb parasitological working group, which did special research on trematodes and protozoans, developed under the care of Prof. Odening [4, 5].

2.2 Structure of the Society

The newly founded Parasitological Society (PG, Parasitologische Gesellschaft) was at the beginning subdivided into three sections; the heads of the sections were at the same time associated presidium members. This basic structure was extended with a 4th section "general parasitology," starting in 1969. From 1989 the section Arachno-Entomology was complemented with the term study of vectors and an independent Committee Ichthyoparasitology was formed. On the whole, 14

Table 2.3 General meetings of the Parasitological Society of the GDR

	Gründungsveranstaltung	23.-24.11.1962	Berlin
1.	Haupttagung „Bedeutung der Biologie und Ükologie für die Bekämpfung wichtiger Parasiten“	02.-04.10.1963	Dresden
2.	Haupttagung „Parasit und Umwelt“	18.-20.11.1965	Berlin
3.	Haupttagung, ohne Generalthema	15.-17.11.1967	Leipzig
4.	Haupttagung, ohne Generalthema	29.10-01.11.1969	Rostock
5.	Haupttagung „Bekämpfung von Parasiten und Parasitosen“	11.-13.04.1972	Berlin
6.	Haupttagung „Zur Epidemiologie von Parasitosen“	24.-25.04.1974	Schwerin
7.	Haupttagung „Parasit und Umwelt“	23.-25.09.1976	Karl-Marx-Stadt
8.	Haupttagung „Parasit im Ökosystem“	19.-22.09.1979	Cottbus
9.	Haupttagung „Immunologie und Populationsbiologie in der Parasitologie“	11.-14.11.1981	Greifswald
10.	Haupttagung „Strategie der Bekämpfung von Parasiten und Parasitosen und ihre Grundlagen“	28.02-03.04.1984	Reinhardtsbrunn
11.	Haupttagung „Probleme der Diagnostik von Parasiten und Parasitosen“	21.-24.05.1986	Magdeburg
12.	Haupttagung „Parassiten – Pathogenität – Pathogenitätsmechanismen“	15.-18.03.1988	Frankfurt/Oder

Table 2.4 Sections of the Parasitological Society and their chairmen

Protozoology

Chairmen

Eble, Halle (1962–1965)
G. Wildführ, Leipzig (1965–1974)
G. Gräfner, Schwerin (1974–1986)
B.U. Knaus, Cottbus (1986–1990)

Helminthology

Chairmen

S. Nickel, Berlin (1962–1976)
H. Engelbrecht, Kleinmachnow (1976–1981)
B.U. Knaus, Cottbus (1981–1986)
R. Schuster, Berlin (1986–1990)

Arachno-Entomology, from 1989 AE + study of vectors

Chairmen

L. Britz, Leipzig (1962–1965)
H. Schumann, Berlin (1965–1979)
P. Müller, Kleinmachnow (1979–1990)

General Parasitology (constitution 1969)

Chairmen

L. Reimer, Güstrow (1969–1974)
K. Odening (1975–1990)

Ichthyoparasitology (foundation 1978)

Chairman

L. Reimer, Güstrow (1978–1990)

Table 2.5 Workgroups of the Parasitological Society of the DDR and their chairmen

Protozoology
Coccidiosis and nosematosis (G. Gräfer, Schwerin)
Blood Protozoan infections (1967–1974; R. Jungmann, Berlin)
Trichomoniasis and intestinal protozoa (1965–1978; Dr. W.A. Müller, Magdeburg)
Toxoplasmosis (1965–1974; G. Wildführ, Leipzig)
Immunoparasitology (1986–1990; W.A. Müller, Magdeburg)
Progress in parasitology
Helminthology
Fasciolosis (1962–1975; S. Nickel, Berlin, G.W. Demski, Potsdam, Frick, Potsdam)
Cysticercosis-taeniasis (1963–1982; H. Engelbrecht, Kleinmachnow, K.H. Müller, Karl-Marx-Stadt = Chemnitz)
Helminthic systematics, biology (1963–1969; G. Hartwich, Berlin)
Helminthic immunoparasitology (1983–1986; W.A. Müller, Magdeburg)
Arachno-entomology
Diptera (1963–1990; H. Schumann, Berlin)
Pesticides and repellents (1963–1974); P. Müller, Kleinmachnow)
Ectoparasitica (1974–1990; P. Müller, Kleinmachnow)
Acarina (1968–1979; R. Ribbeck, Berlin)
Ectoparasites (excl. Diptera) (1979–1990; R. Ribbeck, Berlin, Leipzig)
General parasitology
Key technologies (High standard techniques) in parasitology (1987–1990; H.F. Matthes, Berlin)

working groups were gradually (mostly on the occasion of recent events) formed in the departments.

At the foundation meeting of the Parasitological Society of the GDR, which took place on November 23 and 24, 1962 in Berlin and where lectures on all subdisciplines of parasitology were given, it was recommended to conduct main conferences at intervals of 2 or 3 years, planned by the board and the directors of the sections protozoology, helminthology and arachno-entomology (Table 2.3) [11, 12].

In total, 12 main conferences took place between 1963 and 1988 at 11 different locations. The general topics ranged from "parasite and environment" via "epidemiology of parasites," "pathogenicity mechanisms," "population biology," "immunology" through to "diagnostics" and "control"; twice without general topics. The lectures were without exception appealing and followed by lively discussions, the atmosphere was increasingly confident – in the course of the years a solidary group emerged (Tables 2.4 and 2.5).

2.3 Classification of the Parasitological Society in the Scientific Scene of GDR

It used to be custom in the GDR to assign academic societies to a central administrative or social institution. Originally, three possibilities were taken into consideration for parasitology: the Ministry for Healthcare, the Ministry for Agriculture and Forestry or the Ministry for University Education and Technical colleges. The

Table 2.6 Scientific Societies in the Academy of Sciences

- Astronautische Gesellschaft der DDR
- Biologische Gesellschaft der DDR
- Gesellschaft für physikalische u. mathematische Biologie der DDR
- Chemische Gesellschaft der DDR
- Geographische Gesellschaft der DDR
- Gesellschaft für Geologische Wissenschaft der DDR
- Historiker-Gesellschaft der DDR
- Mathematische Gesellschaft der DDR
- Meteorologische Gesellschaft der DDR
- Parasitologische Gesellschaft der DDR
- Physikalische Gesellschaft der DDR
- Gesellschaft für Psychologie der DDR

decision was made for the Ministry which was the most unfavorable for us, MHF (Ministerium für Fach-und Hochschulwesen; Ministry for University Education and technical colleges), a ministry which tended towards strict controls, that is to regulate or, to use stronger language, to patronize, and we weren't suited to that. In short: We consulted the internationally renowned parasitologists K.I. Skrjabin, Moscow, A. Kotlan/Budapest, J.Hovorka/Kosice and came to the conclusion that it would be purposive to put the Parasitological Society under the patronage of a well-known society. This worked out after tough negotiations. Starting from July 1, 1969, the Parasitological Society was under the care of the German Academy of Sciences (including the Physical Society, the Chemical Society, the Biological Society and others) (Table 2.6) [9, 10].

The Vice-president for plenum and classes was responsible for the scientific societies. It was the internationally accepted social scientist and humanities scholar, Prof. Heinrich Scheel, who soon took pleasure in parasitology.

Allow me to draw your attention to the divergent constitutions of scientific societies and cultural societies. The trait of the diversity consists of disciplinarity and transdisciplinarity. In the transdisciplinarily structured Academy the leading representatives are united whereas the scientific societies preferably include all scientists working in the same discipline.

Parasitology was allowed to enter into the ensemble of these scientific societies and thus was allowed to claim a seat in the plenum and in a class, first as a permanent guest with reporting duty, then personalized as a corresponding and finally as a proper member of the Academy of Sciences. Internationally, the Parasitological Society was integrated into the **World Federation of Parasitologists (WFP)** and was one of the seven foundation members of the EFP (European Federation of Parasitology). The foundation act took place in Poland at Jablonna palace near Warsaw on November 19, 1966. (The subscribers were Prof. Stefanski and Prof. Kozar/Poland, Prof. Garnham/GB, Prof. Fain/Belgium, Prof. Hovorka/CSSR, Prof. Pavlov/Bulgaria, Prof. Jansen/the Netherlands and Prof. Hiepe/GDR). At the end, a membership with a close link to the WAAVP (president at that time was J. Eckert/Zurich) was formed; as from 1969, a parasitologist from the GDR (Th. Hiepe/Berlin) was on the

board for many years (WAAVP Executive Committee) and then was elecetd as 1. Vice president. Therefore, the 13th WAAVP conference with more than 500 participants from 43 countries could take place in East Berlin in August 1989. For the first time it was possible to welcome a remarkable number of participants from the RGW-countries (= Eastern bloc countries); and thus the term **World Congress** was justified [8].

2.4 Postgraduate Teaching Process – Experts in Parasitology

The Parasitological Society undertook special efforts in the postgraduate teaching process of their members. Apart from the main conferences, the symposia at autochthonous and international levels as well as the activities of working groups for the purpose of finding solutions to "burning" parasitological problems, a process was sought to enable members to graduate as an expert in parasitology via postgraduate studies. This was in particular considered necessary for parasitologists who came from biology because it had been possible to acquire the rank "medical specialist" respectively "veterinary medical specialist" (for example for laboratory diagnostics, microbiology and others) in medicine as well as in veterinary medicine since the 1960s. To solve this problem, a special path was pursued: namely to acquire the term "Fachparasitologe/Expert in parasitology" after biennial postgraduate studies [7].

The teaching comprised the following topics:

- General Parasitology
- Protozoology (and related diseases)
- Helminthology (and related diseases)
- Arachno-Entomology (and related diseases)
- Zoonosis
- Diagnostic methods
- Chemotherapy and other control methods of parasitosis
- Basic parasitological methods

2.5 Honors

2.5.1 Honorary Members

Initially, the honors awarded by the Parasitological Society were restricted to the election of Honorary Members. According to § 3 of the Statutes honorary membership could be awarded to persons who rendered oustanding services to parasitology and to the fulfilment of the aims of the society.

During the 28 yeras of its existence the Parasitological Society awarded honorary membership - based on strict selection creteria- to 9 persons (Table 2.7)

Fig. 2.4 Eichler (*left*) congratulates A. Nestler as a new Honorary member of the society

Fig. 2.5 H. Splisteser (*left*) and Th. Hiepe (*middle*) honored K. Skrjabin (*right back*) on the occasion of his 90th anniversary. *Front*: Prof. Keldych, President of the Russian Academy

Table 2.7 List of honorary members of the society

Prof. Dr. Dr. h.c. A. Borchert/Berlin
Dr. habil. H. Engelbrecht/Kleinmachnow
Prof. Dr. sc. J. Hovorka/Kosice
Prof. Dr. Dr. h.c. O. Jirovec/Prague
Technical preparateur A. Nestler/Berlin
Prof. Dr. T. Kobulej/Budapest
Prof. Dr. Rosicki/Prague
Prof. Dr. Dr. h.c.mult. K.I. Skrjabin/Moscow
Prof. Dr. Dr. h.c. W. Stefanski/Warsaw

Fig. 2.6 Both sides of the K.A. Rudolphi Medal

2.5.2 Karl-Asmund Rudolphi Medal

Starting on the occasion of its 25 years of existence, the Parasitological Society of the GDR donated the Karl-Asmund Rudolphi Medal for outstanding scientific achievements in the field of parasitology. The Rudolphi Medal could be bestowed to parasitologists from the GDR as well as to those from other countries (Fig. 2.6).

2.5.2.1 Why the Karl-Asmund Rudolphi Medal?

K.-A. Rudolphi (1771–1832) was one of the most outstanding researchers and scientists of his time. Born in Stockholm (Sweden) and growing up in Stralsund (Germany), he first studied natural sciences with the major subject botany as well as human medicine in Greifswald and then in Jena. He returned to Greifswald, acquired doctoral degrees in philosophy and medicine and continued his studies in the areas of medicine and veterinary science in Berlin. From 1804 to 1808 he was Professor and head of the Veterinary Institute in Greifswald and afterwards Professor at the Medical Faculty of the University of Greifswald.

On the occasion of the foundation of the University of Berlin (1810) he was appointed to the Chair of Anatomy (today's discipline physiology was also included therein at that time) on the personal recommendation of Wilhelm von Humboldt. From a present-day perspective Rudolphi was a biologist, a physician and a veterinarian at the same time. He ranks among the pioneers of the bioscience parasitology, namely helminthology (a group of nematodes is named after him). K.-A. Rudolphi was undoubtedly one of the masterminds of sciences [Applied Parasitology 29: 105–106 (1988)].

The K.-A. Rudolphi Medal, created by us and designed by the renowned visual artist König/Suhl (the upper front shows a portrait of Rudolphi, the back the logo of the society) is coined in bronze and has a philatelic dearness; it has been bestowed seven times during GDR times to (Table 2.8):

The Rudolphi Medal was then taken over by the 1990 reunited German Society of Parasitology as an honor for outstanding achievements of young parasitologists. Instead of bronze it's now made of pure silver. The name of the excellent researcher is engraved on the rim.

Table 2.8 Persons honored by the Rudolphi Medal

R. Supperer/Vienna, 1986
U. Dashnjam/Ulan Bator, 1986
T. Kassai/Budapest, 1987
P. Müller/Kleinmachnow, 1987
Th. Hiepe/Berlin, 1989
G. Piekarski/Bonn, 1990
K. Enigk/Hanover, 1990

2.6 Evaluation of Achievements – Final Reflection

Contemplated in retrospect – this I allow myself to claim since I was repeatedly elected chairman of the Parasitological Society of the GDR from 1965 to 1990 – it was a fruitful society, which went its own way under complicated political circumstances.

The collectivization of agriculture, combined with extraordinary highly concentrated livestock populations (e.g. more than one million *Gallus domesticus* chickens, 200,000 pigs or 10,000 dairy cows, 30,000 young cows or 25,000 fattening lambs), each one of it on one farm with a relatively uniform genetic constellation of the species listed, caused highly explosive problems that were caused by parasites and were considered to be a direct threat to human health (parasitic zoonoses!).

Always when parasitological problems came up, they could be solved due to the highly developed will for communication of our members in the Parasitological Society. Especially the action of the working groups proved to be very functional. On behalf of the PG, a parasitological information service was established in the Institute for Parasitology at Humboldt University Berlin. One should also mention the short term eradication of hypodermosis in the area of the GDR (the damage reached the total of 100 million German Mark) on the basis of a mathematical model, the development of a biological control method against the fly plague in pig fattening stock (due to insecticide resistance!) by the use of *Ophyra aenescens* against *Musca domestica*, the *Sarcocystis* and *Toxoplasma* problems in the food chains or the *Enterobius* respectively lice problems in many kindergartens.

Finally, it should be mentioned that the integration of these parasitologists into the group of scientific societies under the patronage of the Academy of Science was very functional. The legacy of the PG is documented twice: in the **Academy archive** (of today's Academy of Science in Berlin Brandenburg) and in the journal **Applied Parasitology** (Angewandte Parasitologie).

2.7 Merging of the Parasitological Societies West – East

And in the fall of 1989: "With the end, the turnaround came" (the deliberate turnaround!), the wall partitioning Berlin was removed through a peaceful revolution and – despite the abundance of everyday problems – both chairmen of the Parasitological Societies of East and West Germany (Prof. Hiepe, Prof. Mehlhorn)

met together in Berlin on September 21, 1990 yet before the reunification of our home country, and adopted the resolution that only one German Society for Parasitology should exist thereafter, which on record looked like this (see Table 2.9):

Table 2.9 Document of the reunion of both German Societies of Parasitology on the 29 September 1990 in Berlin

PARASITOLOGISCHE GESELLSCHAFT DER DDR

DEUTSCHE GESELLSCHAFT FÜR PARASITOLOGIE e. V.

Berlin, den 29.09.1990

Sehr geehrte Kolleginnen!
Sehr geehrte Kollegen!

Am 21. September 1990 fand eine Sitzung der Vorstände der Parasitologischen Gesellschaft der DDR e.V. und der Deutschen Gesellschaft für Parasitologie e.V. in Berlin statt.

Auf Grund der Vereinigung Deutschlands am 03. Oktober 1990 ist von beiden Vorständen folgende Übereinkunft getroffen worden:

- den Mitgliedern der Parasitologischen Gesellschaft der DDR wird der Beitritt in die Deutsche Gesellschaft für Parasitologie empfohlen; auch Nichtakademikern und anderen Berufsgruppen, die in enger Beziehung zur Parasitologie stehen, wird ein Beitritt ermöglicht
- 2 Mitglieder des bisherigen Präsidiums der Parasitologischen Gesellschaft werden in den Vorstand der Deutschen Gesellschaft für Parasitologie bis zu den Neuwahlen 1992 kooptiert
- den Traditionen der Parasitologischen Gesellschaft folgend, werden von der Deutschen Gesellschaft für Parasitologie sowohl die

  Ehrenmitgliedschaften und die
  Rudolphi - Medaille

  übernommen und letztere weitergeführt
- das Postgradualstudium Parasitologie wird als Fachspezifische Weiterbildung anerkannt
- die Arbeiten der bisherigen Arbeitskreise können weitergeführt werden.

Prof. Dr. Dr. h. c. Th. Hiepe
Präsident der Parasitologischen Gesellschaft der DDR

Prof. Dr. H. Mehlhorn
Vorsitzender der Deutschen Gesellschaft für Parasitologie

(continued)

Table 2.9 (continued)

ANGEWANDTE PARASITOLOGIE

Herausgegeben von der Parasitologischen Gesellschaft der DDR

Schriftleitung:
A. Borchert · Wd. Eichler · G. Hartwich

Wissenschaftlicher Beirat:
L. Britz, Leipzig · V. Dyk, Brno · I. G. Galuzo, Alma-Ata · Th. Hiepe, Berlin · J. Hovorka, Košice · L. Hussel, Leipzig · O. Jírovec, Prag · Z. Kozar, Wrocław · G. Makara, Budapest · A. P. Markevič, Kiew · K. Matoff (Matov), Sofia · W. Michajłow, Warschau · L. Nemeséri, Budapest · K. Odening, Berlin · L. Pellérdy, Budapest G. Poljanskij, Leningrad · D. Popovic, Belgrad · R. S. Schulz (Šul'c), Alma-Ata H. Schumann, Berlin · E. M. Ungureanu, Iași · V. I. Vaškov, Moskau · G. Wildführ, Leipzig · E. Żarnowski, Lublin

3. JAHRGANG

MIT 21 ABBILDUNGEN IM TEXT

VEB GUSTAV FISCHER VERLAG JENA
1962

Hear a lot,
select the good things and follow them,
See a lot,
keep them in mind,
so you'll, sure, gain a great amount
of knowledge!

Confucius (551-479 before Chr.)

Acknowledgement The following secretaries of the Society should be honored for their important contributions:
K. Odening (1962–1965)
Renate Buchwalder (1965–1976)
S. Spiess – Akademie-Büro (1976–1981)
Bringfriede Flentje (+ Krüger, Akademie-Büro) (1981–1990)
Furthermore, the following colleagues contributed much to the daily work of the Society: Dr. B. Betke/Cottbus, Dr. Haupt/Leipzig, Prof. R. Ippen/Berlin, Heide Irmer/Berlin, Dr. Jutta Meichsner/Dresden, Dr.habil. Rehbein/Leipzig, Dr. Anngret Semlow/Rostock, Dr.sc. H. Splisteser/Potsdam, M. Buske und F. Coch/Kleinmachnow.

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Part I
Protozoa

Chapter 3
Parasites and Their World Records in Their Fight for Survival

Heinz Mehlhorn

Abstract Parasites are animals that enter plants, animals and humans – but are not welcome. Therefore they had to develop strategies for invasion and survival – outside and inside of hosts. For this purpose they developed amazing, often unbelievable skills during evolution. Thirteen of them are presented in this chapter showing that they are fit for the future thus threatening mankind, if there are no precautions in the future.

3.1 Introduction

In their fight for survival parasites depend absolutely on their ability to adapt themselves to the life cycle, behaviour, food and physiology of peculiar hosts and on their capacity to create sophisticated ways of host finding. Since all these tasks needed huge numbers of trials, evolution supported only the "winners" and suppressed as "losers" all less well-adapted specimens. Therefore it is not astonishing that these winners developed admirable skills that are listed as top records in the scorebook of nature being often unbelievable when compared to the range of human skills. However, the fight for survival is never finally decided, especially not for those parasites that may harm the health of humans and animals, since human science declared war on these "beasts" that may endanger human survival on earth. We are now in round 11 out of 12 in the fight for survival – nothing has been decided, since vector-transmitted viruses lurk everywhere in a globalized world. The sudden outbreak of the West Nile virosis in the USA (transmitted by mosquitoes), the unexpected outbreak of Blue Tongue virosis in 2006 in Central Europe (transmitted by Ceratopogonid midges), or the pandemics of the virus-based disease SARS (severe acute respiratory syndrome), "bird flu" and "pig flu" prove that we live on very thin ice or on a still silent volcano.

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H. Mehlhorn (ed.), *Progress in Parasitology*, Parasitology Research Monographs 2,
DOI 10.1007/978-3-642-21396-0_3,

3.2 *Giardia lamblia*: Twins in One Body with a Giant Holdfast System

Twofold keeps better
(Tailor's wisdom)

Greek mythology reports that at the beginning of social life on earth ancestors of humans lived as happy hermaphrodites uniting both sexes in one body. After human disobedience against divine rules this totipotent organism was divided by judgement of the gods into male and female organisms punished with the endless "pain" of reciprocal attraction. *Giardia* – named after the famous Italian scientist Alfredo Giardi (1846–1908) – is such an original organism in eternal inner peace.

Giardia species, races or strains – or whatever definitions will be used in future – are worldwide able to infect many hosts and act as **opportunistic agents of diseases** introducing severe symptoms of diarrhoea in immunosuppressed hosts, while none or low-graded ones occur in immunocompetent hosts. *Giardia* specimens are flagellated protozoans, which in evolution apparently once had missed a cell division, so that they have lived since then as a double organism with a longitudinal symmetry: both the left and right side of the drop-shaped cell each contain one genetically identical nucleus and four flagella arranged in an identical pattern (Fig. 3.1a–c). Their survival strategy follows two pathways: (1) The 10–20-µm long trophozoite is strongly attached with the help of its giant ventral holdfast system (disc) at the intestinal cells of the hosts (Fig. 3.1b) and feeds via food vacuoles at its dorsal surface (Fig. 3.1c). (2) The 15–20-µm sized, ovoid-shaped cyst is able to survive outside of the host due to its strong wall. The cell organelles of the cyst are doubled, since already four nuclei are present, so that immediately after the cyst has reached the intestinal lumen of a new host, two new trophozoites may emerge from each cyst. These trophozoites become attached by means of their ventral sucker so tightly at the surface of the intestinal cells, that the permanent intestinal compressions during food transportation do not detach them. As soon as they have left their attachment point, deep depressions become visible at the surface of the host cells (Fig. 3.1b).

Records of *Giardia*

1. ***Living sites***: inside and outside intestines
2. ***Stages***: Flagellated trophozoites, cysts
3. ***Size***: 15–20 µm
4. ***Characteristics***: all organelles are present at least in double feature, eight flagella, ventral disc
5. ***Reproduction***: Longitudinal fission
6. ***Hosts***: Worldwide occurrence in hundreds of millions of animals and humans
7. ***Transmission***: Orally by uptake of cysts from faeces of hosts, zoonotic activity
8. ***Prophylaxis***: Avoid human and animal faeces
9. ***Therapy***: Metronidazole

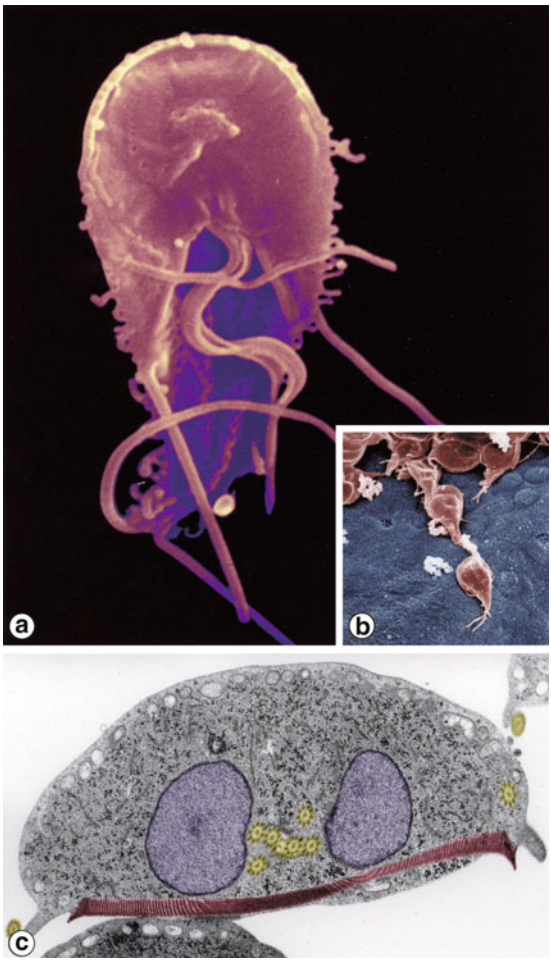

Fig. 3.1 *Giardia lamblia* (stages from humans and animals) – Transmission (TEM) and scanning (SEM) electron micrographs. (**a**) Ventral side (SEM) (**b**) Dorsal side of attached stages. Note depressions at formerly parasitized host cells. (**c**) TEM of a cross section showing the two identical nuclei, the stiff proteins of the ventral sucker and the dorsal food vacuoles

3.3 *Trypanosoma* and *Leishmania* Stages: The Inventors of the Cloak of Invisibility

A life undercover avoids detection
(Spy's wisdom)

In the German world of sagas a blond, blue-eyed hero named Siegfried snatched a cloak of invisibility from the potent dwarf Alberich who watched over an enormous treasure. This cloak of invisibility enabled Siegfried to perform further acts of heroism.

A similar system of invisibility was developed by single-flagellated protozoans of the genera *Trypanosoma* and *Leishmania*, which live as parasites in the blood and/or inside tissues of man and animals and thus are constantly attacked by the famous and mighty immune system of the hosts, if they are recognized. In order to block such attacks these parasites have developed a protective shield on their

cell membrane – a **surface coat** – which makes them invisible to the deadly arrows (= antibodies) of the immune system. However, since the host's defence is able to learn and thus produces "sharper arrows" after contact with the invaders, the latter change their shield during each division, when another of nearly 1,000 genes becomes active and produces another variation of the surface coat (layer of mucopolysaccharides) in order to hide perfectly the presence of the aggressors. Therefore these parasites are the "winners" in the struggle for life and pose severe problems to the relatively young species *Homo sapiens*, while African cattle and other ruminants have made their "peace" with the invaders, which live there at reduced reproductive rates in a surviving and rather untouched host.

Trypanosomes have developed a peculiar flagellum, which is attached to the surface of the cell (Fig. 3.2a) thus enabling the parasites to swim in the rather viscous blood. The *Leishmania* species have reduced the flagellum of intracellular stages to a stump, which does not overtop the surface while being anchored in a depression. Only the so-called promastigote stages in vectors have retained their free projecting flagellum (Fig. 3.2b). With respect to their non-intestinal parasitism in vertebrate hosts *Trypanosoma* and *Leishmania* stages had been obliged to change their invasion strategy and thus use blood-sucking insects as vectors to become

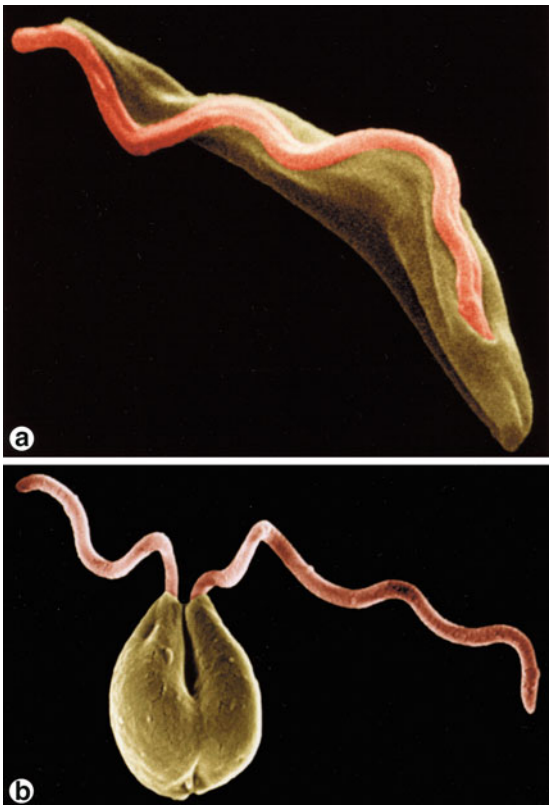

Fig. 3.2 (**a**, **b**) SEM micrographs of blood stage of *Trypanosoma brucei gambiense* (**a**) and a dividing stage of *Leishmania tropica* from the foregut of a sand fly

transmitted from one vertebrate host to another. This makes it necessary to again live in disguise in order not to become attacked by the insect's "defence system". Thus, they have to change their surface coat again, when they arrive in the insect's intestine. In addition they must adapt their physiology inside the vector. This is seen also in the shape of the cell, since *Trypanosoma* occurs as epimastigote stages in the intestine of the insect, but *Leishmania* as promastigotes (Fig. 3.2b).

Records of *Trypanosoma* and *Leishmania*

1. ***Living sites***: Inside blood and tissues of vertebrates, inside the intestine and salivary glands of insects
2. ***Stages***: Flagellated trophozoites, no cysts
3. ***Size***: *Trypanosoma* (8–40 μm), *Leishmania* (2–3 μm)
4. ***Characteristics***: One single flagellum, existence of a kinetoplast (= DNA-containing portion of the mitochondrion) at the basal body of the flagellum
5. ***Reproduction***: Permanent longitudinal fission
6. ***Hosts***: Vertebrates: humans, animals; insects: African trypanosomes: Tse-tse fly = *Glossina*, *Leishmania*: sand flies *Phlebotomus*
7. ***Transmission***: Transmission by blood-sucking vectors
8. ***Prophylaxis***: Use of repellents
9. ***Therapy***: *Sleeping sickness*: Suramin; *Leishmaniasis*: Diamidines

3.4 Cysts of Protozoans: An Invincible Castle

My home is my castle
(British feeling)

Protozoans that invade cells, tissues or organs are attacked by strong and numerous systems of host defence. In order to survive such constantly increasing attacks the parasites have to seek shelter in places, where they cannot be detected, or they have to produce walls, behind which they are protected from any attack. Thus, several protozoans developed successfully the following systems:

3.4.1 Parasitophorous Vacuole

During invasion of host cells coccidians (e.g. malaria parasites, *Toxoplasma*) push the cell membrane of the host cell forward thus forming a (finally closed) inner vacuole, within which their feeding and propagation is protected from recognition by the immune system via two membranes and by the cytoplasm of the host cell (Fig. 3.3a, b). This type of cell invasion needs a very skilful system of functional organelles, which produce substances that allow penetration and feeding (e.g. conoid, rhoptries, micronemes, dense bodies, apicoplast, micropores etc.).

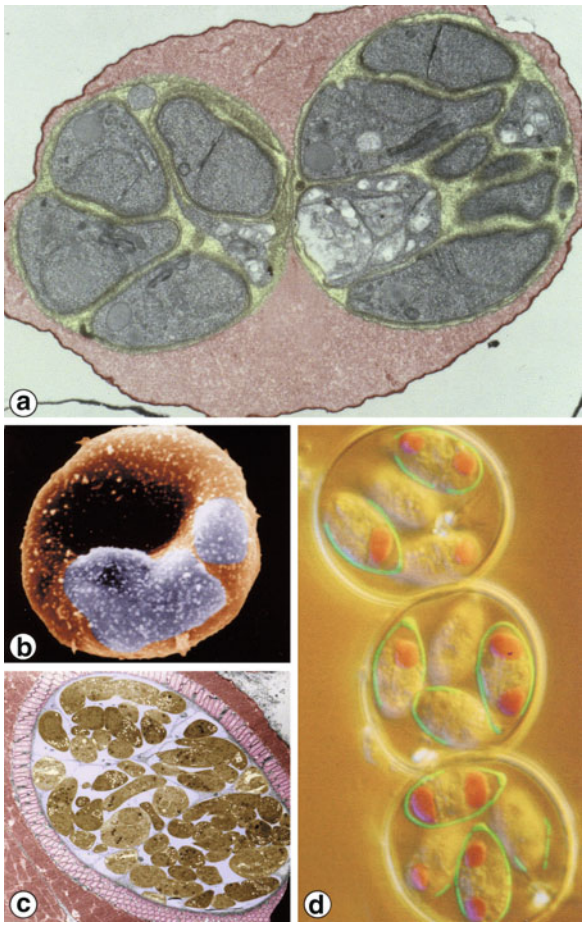

Fig. 3.3 (**a**–**d**) Micrographs of coccidians. (**a**, **c**) Transmission electron micrographs; (**b**) scanning electron micrograph; (**d**) light micrograph). (**a**) Two schizonts of *Plasmodium falciparum* (agent of malaria tropica) lying inside their parasitophorous vacuoles are included within a red blood cell. (**b**) Two schizonts of *P. falciparum* become visible through the surface of an infected red blood cell showing the typical knobs (= *white dots*). (**c**) Section through a *Sarcocystis* tissue cyst inside a muscle fibre showing the palisade-like protrusions of the primary cyst wall and containing numerous infectious cyst merozoites. (**d**) Three oocysts of *Eimeria tenella* (agent of the diarrhoeic disease = coccidiosis of chicken). They contain four sporocysts each with two sporozoites

3.4.2 Intracellular Cyst (= Tissue Cyst)

Increased protection is reached, when so-called tissue cysts are formed inside host cells (e.g. in muscles, brain cells) by some coccidians (e.g. genera *Toxoplasma*, *Sarcocystis*, *Besnoitia*). In these cases the membrane of an original parasitophorous vacuole becomes underlaid by dense material thus forming the so-called primary cyst wall (PCW), in addition this PCW may form a wall of upright protrusions, which block attacks from the outside, but also enable an increased uptake of food from the cytoplasm of the host cell (Fig. 3.3c).

3.4.3 Oocyst and Sporocyst Walls

The pinnacle of protection, however, is achieved by those coccidians (e.g. genera *Eimeria*, *Isospora*, *Toxoplasma*, *Sarcocystis*) that form besides intracellular stages

also extracellular ones with strong walls. The oocysts contain (like a Russian doll) other fortified systems such as wall-surrounded sporocysts, which offer excellent protection outside of a host and thus guarantee survival even during heat, dryness or coldness. Thus, these stages may survive for a long time outside of a host's body. While bacteria might harm the walls, these oocysts and sporocysts (Fig. 3.3d) are stored in laboratories inside fluids containing potassium bichromate or 1–4% of different acids, which would considerably harm human skin in case of contact.

Thus, these parasites have developed strong "castles" inside and outside of their hosts and thus remain protected in their "*home sweet home*".

Records of Self-protectors

1. ***Living sites***: In cells or inside cyst walls
2. ***Stages***: Merozoites, brady-, tachyzoites, sporozoites
3. ***Size***: 6–16 μm, depending on the species
4. ***Characteristics***: Penetration organelles, wall-forming bodies
5. ***Reproduction***: Endodyogeny, schizo-, sporogony, gamogony
6. ***Hosts***: All living species including humans
7. ***Transmission***: Oral uptake of cysts inside faeces or meat; other species use a vector-based transmission during blood sucking
8. ***Prophylaxis***: Avoid contact with faeces
9. ***Treatment***: Species-specific application of sulfonamides

3.5 Couples: Lifelong Undivorced

True love keeps forever
(Hope)

Trematodes of the genus *Schistosoma* (*Greek*: divided body) belong to the most common parasites in the subtropics and tropics attacking humans and many animals. About 500 million humans are infected and many of them are in danger of death from liver destruction and cancer, if they are not treated at an early stage of the infection. These 1–2. 5-cm long worms, which live in the blood vessels of the liver, intestine or bladder, were originally detected by a jobless German physician (Theodor Bilharz; 1825–1862), who left Germany in the 1850s and worked in Cairo, Egypt. He described in letters to his teacher von Siebold that he had found pairs of worms in livers of dead cancer patients. At first these worms were named *Distoma* referring to the two holdfast systems – a mouth sucker and a ventral sucker, which are situated close together at the anterior ends of both the female and male worm. Later the genus was named *Bilharzia*, honouring the discoverer, who is buried in Cairo. Although this genus name is the correct one with respect to the rules of Zoological nomenclature, the English literature changed the genus name to *Schistosoma*, so that today this name is retained. Humans are most commonly infected by the species *S. mansoni*, *S. intercalatum* and *S. japonicum*, which set their eggs free via human faeces, while the eggs of *S. haematobium* are excreted with the urine.

To survive inside their hosts these worms had to overcome many severe obstacles. First of all they must enter the skin of a host. This problem was solved by the development of very powerful proteolytic enzymes inside glands of the actively swimming and skin-penetrating larval stage – the bifurcated cercariae. Having successfully entered, the now tailless "schistosomulum" is immediately attacked by the host's immune system. To block these attacks this larva develops at high speed a surface coat. However, only the quickest larvae succeed and survive, while about 50% of the slower invaders are killed by the immune system leading to "not very nice reactions" in the skin of the hosts (e.g. strong inflammation and nasty itching). Then the tiny survivors (males or females) have to find a partner inside the giant tube system of the host's blood vessels. This is solved, when young worms enter the blood vessels and travel via lung and heart to the "Vena hepatica" that collects all blood arriving from the blood vessels of the intestine. The 1–2-mm-sized males and females "marry" – that is the leaf-like shaped male forms a groove, into which the young female enters and remains not only until maturity but for its whole life, which may last for 25 years or even more (Fig. 3.4a). In order to withstand the constant flow and pressure of the blood, the males of some species develop spines along their surface (Fig. 3.4b, c), which allow them to holdfast on the wall of the blood vessels, and they build up an enormous system of circular and longitudinal muscles below the syncytial tegument (neodermis), which covers as a single layer the whole body. Finally these "loving couples" have to protect themselves from the immune system of the host. This is done by wearing "foreign clothes", where male and female worms incorporate elements of the host serum into their shield. Their surface coat is demonstrated in Fig. 3.4c. Its composition is changed constantly in reply to the attacks of the rising immune system. Thus, this couple is perfectly equipped for its fight for survival having developed admirable capacities of penetration, shelter and reproduction that allow it to survive inside a hostile environment.

Records of Schistosomes

1. ***Living sites***: Adult worms: blood vessels
2. ***Stages***: Male and female adult worms
3. ***Size***: 1–2.5 cm
4. ***Characteristics***: Mouth and ventral sucker, non-cellular tegument, skin penetrating larvae (cercariae)
5. ***Reproduction***: *Adults*: egg-laying after fertilization, *Larvae*: division inside sporocysts in snails (intermediate hosts)
6. ***Hosts***: *Adults*: warm-blooded animals, *Larvae*: snails in water
7. ***Transmission***: Cercariae enter swimming mammals by skin penetration
8. ***Prophylaxis***: Avoid contact with water (lakes, river) in the tropics or subtropics
9. ***Treatment***: Praziquantel

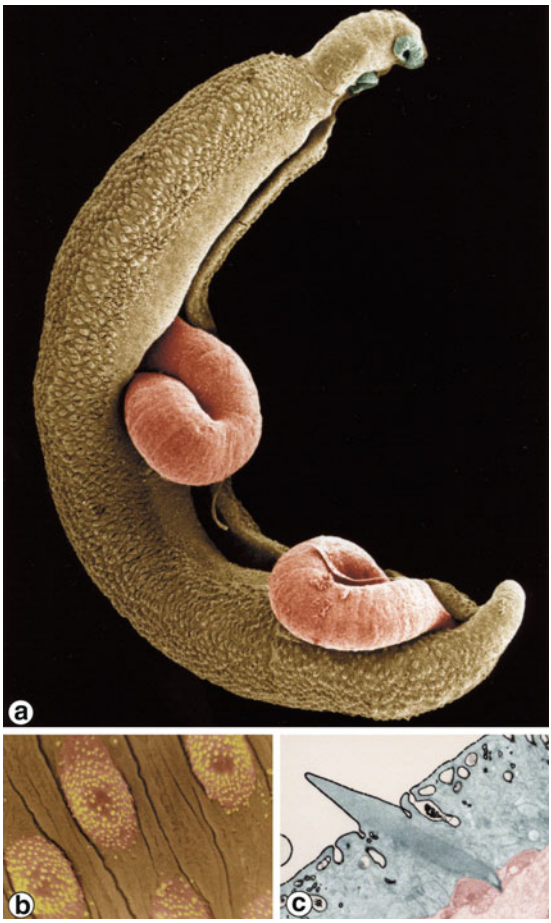

Fig. 3.4 Scanning (**a**, **b**) and transmission electron micrographs (**c**) of *Schistosoma mansoni*. (**a**) Couple; (**b**) Bosses at the surface of the male worm containing numerous hooks. (**c**) Section through the tegument of a male with spines along the surface, which is provided with deep invaginations (for additional uptake of nutrients) and the electron dense surface coat

3.6 Flat but Giant or Tiny: Tapeworms – The Intestine Inside an Intestine

Appétit vient en mangeant
The appetite comes while eating
(Rabelais)

Cestodes (*Greek*: cestos = belt, band) may reach up to 30 m in length (i.e. *Diphyllobothrium latum*, fish tapeworm of humans) and thus belong to the largest animals on earth. On the other hand the group of tapeworms also includes tiny, but very dangerous individuals of only 1–3 mm in length (i.e. *Echinococcus multilocularis* – the small tapeworm living in the intestines of foxes and dogs, which may produce eventually deadly cysts of more than 20 cm in diameter in the liver of humans). Thus, the size of tapeworms does not correspond to their pathogenous effects. Although some other human tapeworms also reach a giant size (*Taenia solium*:

4–6 m; *T. saginata*: 6–8 m), only a few people have seen them in full length. They live as adults in the darkness of their host's intestine, folded-back on themselves several times, so that even posterior portions of the worms come into contact with those at anterior regions. This is important, since the anterior proglottids contain the active testes of the hermaphroditic (dioecius) worms and their sperm are able to fertilize the eggs, which are produced in the female systems of the proglottids at the posterior end of the worms. The most terminal proglottids are finally pinched off from the worm's body (strobila) thus setting free the eggs, which contain the infectious oncosphaera larva. In order not to become expelled together with the host's faeces due to the constant contractions of the intestine, these worms have developed powerful holdfast systems at their head = scolex such as suckers and (often in addition) crowns of stable hooks, which guarantee a strong attachment at the intestinal wall of their hosts (Fig. 3.5a). Their giant size, however, is reached by members of the genus *Taenia* only by performing a "fratricide", which was

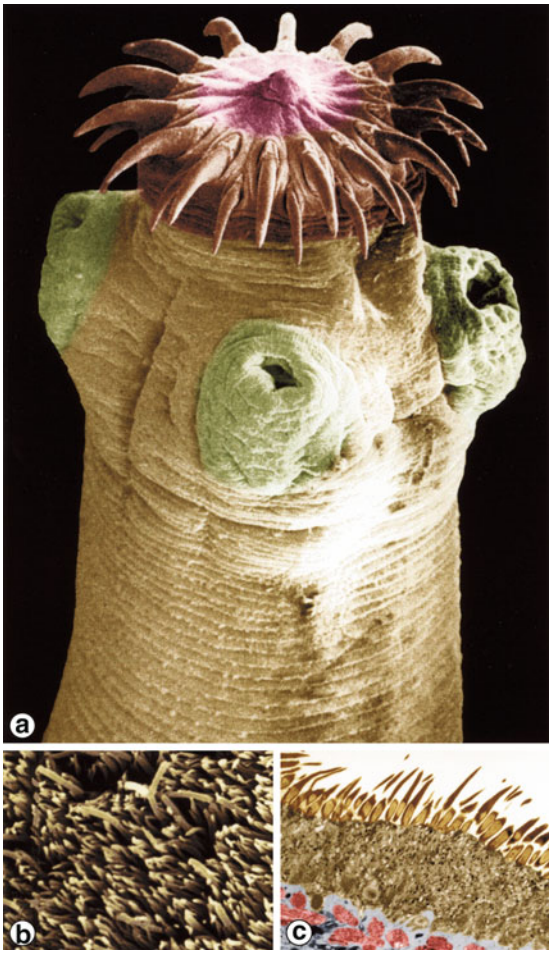

Fig. 3.5 (**a**–**c**): (**a**, **b**) Scanning electron micrographs of the scolex (**a**) with four suckers and a crown of hooks of a *Taenia* worm and of the outer surface (**b**) with the closely arranged microtriches. (**c**) Transmission electron micrograph of longitudinal sections through the microtriches protruding from the non-cellular tegument (neodermis)

developed during evolution apparently without remaining genetic damage. This "dark side" of the life of tapeworms was discovered in the eighteenth century by the parasitologist Gottlob (= Praise God) Heinrich (Henry) Küchenmeister (1821–1890) (master of the kitchen). This human physician asked people that had been condemned to death to allow their infection by several cysticerci (= infectious stages of tapeworms) in order to check later during autopsy, how many of these larvae had grown up to an adult and healthy worm. In nearly all cases only one adult tapeworm was found in the intestine of the hosts, who had received almost luxury food during the last weeks of their life as recognition of their participation in this painless "human experiment". But even today it is not clear, how these large tapeworms manage to live alone in the host's intestine, while from some other genera several up to many cestodes may have their seat there.

Since tapeworms already live inside an intestine with enormous digestive functions, they reduced their own inner intestine, but evolved a morphologically nearly identical system along their outer surface. The non-cellular tegument (= neodermis) developed protrusions (microtriches), which look very similar to the tiny, long microvilli of the human or animal intestine and which increase enormously the surface for resorption of the host's intestinal fluid components (Fig. 3.5b, c). However, both the microvilli and the microtriches are endangered by digestion or destruction due to the activity of the intestinal enzymes. Thus, both – worm and host – have developed practically the same system of protection: they created a system of mucopolysaccharides (a so-called surface coat), which protects their surface from destruction. If these surface coats on the host cells and on the worms are not permanently present (e.g. due to intestinal diseases of the hosts or due to drug-derived death of the worms), significant damage occurs along the surface of both. Thus, a dead *Taenia solium* would be quickly digested setting free the infectious oncosphaera larvae, which may induce life-threatening brain cysticercosis (neurocysticercosis) in humans.

Since tapeworms never know, whether their faecally excreted eggs will come into contact with an intermediate host (e.g. depending on the worm species: cattle, pig) they "decided" during evolution to establish a mass production of infectious eggs. Therefore, each of the faecally excreted proglottids (= terminal portion) contains several thousand eggs, which are in addition protected by a very thick wall, which allows survival even at very low temperatures (−40 to −50°C) or even against considerable heat. While *Taenia* species have survived due to this mass production of eggs – one egg will hopefully reach its goal –, the tiny *Echinococcus* worms developed another mass strategy. In these cases thousands of the tiny worms live simultaneously in the intestine of foxes or dogs (without harming them) producing only a few hundred eggs per proglottid. However, since hundreds of worms may be present within one intestine, again hundreds of thousands of eggs are produced in order to find an intermediate host. Thus, a persisting survival of these tapeworms until eternity could be possible, were it not for the "skilful dwarfs" of the species *Homo sapiens*, who at first used plant extracts as anthelmintics (such as extracts of seeds or squeezed worm ferns) and now have developed chemical compounds such as praziquantel, which paralyze the worms and thus prevent them from attaching any longer to the intestinal wall. Thus, worm survival is not easy in our days.

Records of Giant Tapeworms

1. *Living sites*: *Adults*: intestine of man and animals, *larvae*: muscles of animals
2. *Stages*: *Adults* and *larvae*
3. *Size*: Giants: 1–30 m, depending on the species, dwarfs: 1–3 mm
4. *Characteristics*: Hermaphrodites, intestineless, strong holdfast systems, protective surface coat
5. *Reproduction*: Mass egg production in female system
6. *Hosts*: Giant *adults* in humans, *larvae* in pigs, cattle or fish depending on the species
7. *Transmission*: Oral uptake of cysticerci in meat of cattle/pigs or plerocercoids in fish
8. *Prophylaxis*: Avoid eating raw meat/fish
9. *Therapy of hosts*: Praziquantel

3.7 Tooth by Tooth – Hookworm: The Intestinal Dracula

In sucum et sanguineum
(Cicero)

Although the hookworms of the genera *Ancylostoma* (*Greek:* bended mouth) or *Necator* (*Latin*: killer) reach only a length of around 1 cm, their "blood thirst" is enormous. With the help of cutting teeth (*Ancylostoma*, Fig. 3.6) or cutting plates (*Necator*) they attach themselves to the intestinal wall of their hosts, destroy blood vessels and engorge the flowing blood with the help of their muscular oesophagus. They waste blood and just let it run through their intestine (apparently they use the included oxygen). Thus, about 100 worms deprive the host of more than 5 mL blood per day. Since often loads of more than 500 worms occur inside the 1.2 billion human hosts, anaemia and/or death (especially in children) can occur, if treatment is not carried out consistently.

Female hookworms may live for 1–2 years and thus produce millions of eggs hoping that one of the developing larvae will find an appropriate host. Again the tactics of mass production are successful in order to overcome the hostile surroundings. The females produce up to 30,000 eggs per day. Larva 1 hatches from the eggs, lives on the soil, develops into Larva 2, which after another moulting becomes the sheathed Larva 3. This infectious stage creeps together with others at the tips of blades of grass there forming a "waving tree of larvae". This peculiar behaviour guarantees that in case of contact with a host that many larvae may enter the skin at the same time. This increases the chance that female and male larvae enter the host and may become couples in the intestine. The intestine, however, is only reached after a dangerous journey via blood vessels to the heart–lung–trachea and after being engorged into the throat to reach finally the intestine, where the stages become

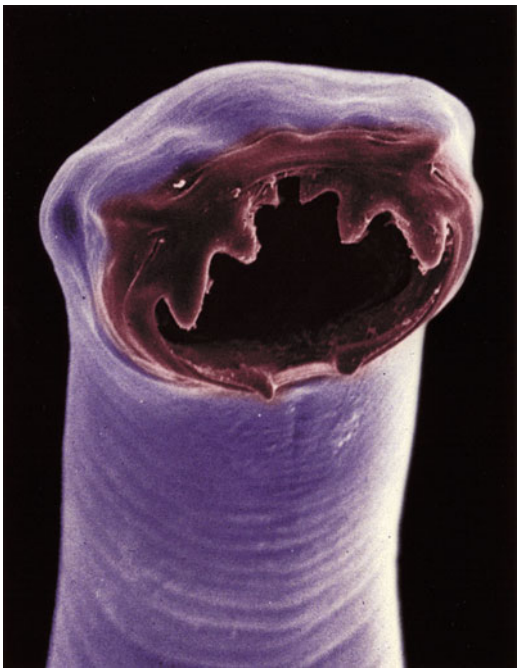

Fig. 3.6 Scanning electron micrograph showing the mouth of an adult dog hookworm (*Ancylostoma caninum*). If the larva enters human skin it does not succeed in reaching the intestine, but it wanders as a "*creeping eruption*" inside the skin

mature. The males firmly clutch with their terminal "bursa copulatrix" the laterally situated sexual opening of the females. While in permanent copulation both males and females constantly suck blood and produce sperms and/or eggs. The appearance of the mouth of hookworms and their blood sucking activity reminds one of the "romantic Count Dracula", who by means of his bite offered unlimited life to his female victims (of course without permanent copulation).

Records of Hookworms

1. ***Living sites***: *Adults*: inside the intestine of man and animals; *larvae*: on soil
2. ***Stages***: Three free-living larvae, wandering larvae 3 and 4, adults (♀, ♂)
3. ***Size***: *Adults*: 1 cm; *larvae 3*: ~1 mm
4. ***Characteristics***: Larvae 3 penetrate into the skin and wander via blood vessels, heart, lung, trachea and finally via the oesophagus into the intestine; adults have cutting teeth or plates in their mouth, suck blood. Males hold females tightly by a posterior grip system = bursa copulatrix
5. ***Reproduction***: Sexually, females produce 10,000–30,000 eggs per day
6. ***Hosts***: Humans, animals
7. ***Transmission***: Larvae 3 penetrate into the skin, eggs are excreted via host faeces

(continued)

8. ***Prophylaxis***: Avoid contact to faeces-contaminated soil or grass, use shoes
9. ***Therapy of hosts***: Bendazoles, avermectines

3.8 Ixodid Ticks: World Record Holders in Starvation and Blood Engorging

Feed as much as you can, you never know,
when you can do it again!
(unknown mother tick)

With respect to ticks there are a lot of nice but wrong fairy tales that are often repeated by different media and thus are held as true by the public. It is told that ticks drop from trees and that hats offer good protection. However, it is a fact that ticks mostly lurk for hosts (Fig. 3.7a) while sitting at the tips of blades of grass or on low plants. Furthermore, it is often told that protection can be given by making sure that the trouser leg opening is closed by a rubber band. However, it is a fact that unengorged ticks are so flat, that it is nearly impossible to squeeze them. Therefore, they easily wander below these bands onto the naked skin of the legs and seek a nice place for a blood meal. Because of this misinformation other true top records of the ticks often remain neglected, although only these skills made it possible for these rather slow animals, which are unable to fly and thus unable to leave unpleasant = hostless biotopes, to successfully survive for the last 300 million years.

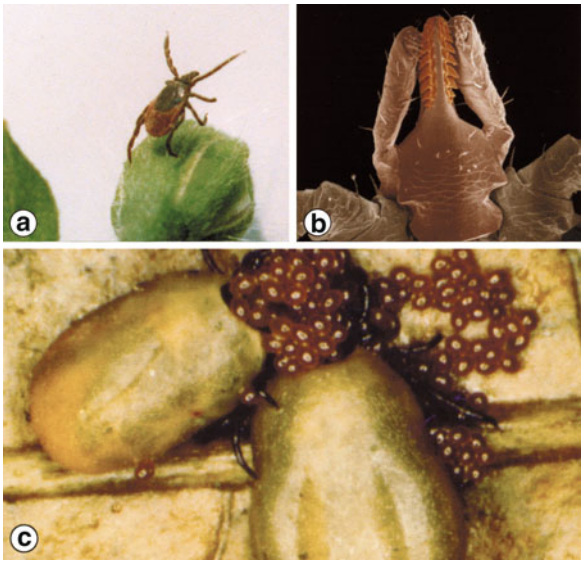

Fig. 3.7 (**a**–**c**) Light (**a**, **c**) and SEM (**b**) micrographs of stages of *Ixodes ricinus*. (**a**) Lurking female on a plant, (**b**) anterior of the tick showing the sucking channel with hooks, which keep the tick fixed inside the skin. (**c**) Two females laying eggs on leafs

Furthermore, their ability to starve and on the other hand the amounts of blood that they can take up within 10 days are unbroken records in the whole animal kingdom. Notably the ticks of the common genus *Ixodes* (*Greek*: ixodes = glueing) are able to suck blood from huge numbers of hosts (at least more than 250 species are attacked belonging to the groups of reptiles, birds and mammals). Thus, they may use practically any type of blood as a source of their food. Bloodsucking is done by any stage of the tick's life cycle, which comprises one six-legged larva, one eight-legged nymph and both of the adults. All of these three stages suck only once in their life. This is done by injecting the anterior sucking tube (with the hooked hypostome) into the hosts' skin (Fig. 3.7b). This is followed by the activity of the knife-like cheliceres, which produce a little "blood lake" inside the skin. This "pool of blood" is kept fluid due to the anticoagulant components of the saliva, which are constantly pumped for 5–10 days into this pool. Female ticks are able to feed up to 250-times more than their body weight. This would mean that a man would have to take up at least one ton of food within 10 days. The body of the female swells from 3 to 4 mm in length reaching finally 12–15 mm. Man would have to grow to a height of 5 m. Having taken up this giant amount of blood, fertilized females are able to produce up to 4,000 eggs of a considerable size within a very short period of about 4 weeks (Fig. 3.7c).

On the other hand these ticks are also masters of starvation. Larvae, nymphs and unfed males may starve for many months (record holders survived for 10 years in the laboratory without food). Especially nymphs or females may live extremely long with the blood that they had taken up before moulting (e.g. as larvae or as nymphs). This ability was developed during evolution, since these eyeless ticks have to wait until a new host passes by close enough so that they may attach to it with the help of the tiny claws on their feet. Other ticks with eyes, however, are able to walk into the direction of potential hosts. Although their eyes are not of high quality, they can recognize the movements of possible hosts and thus run in this direction. This for example is done by the members of the genera *Dermacentor*,

Records of Ticks

1. ***Living sites***: Sitting on hosts, moulting on soil
2. ***Stages***: Larva, nymph, ♀, ♂ adults
3. ***Size***: Unengorged: 3–5 mm, engorged: up to 1.5 cm
4. ***Characteristics***: Blood suckers with six legs as larva, eight legs as nymph and adults; mouthparts: cheliceres, pedipalps
5. ***Reproduction***: Sexually, ♀ lay fertilized eggs
6. ***Hosts***: Many species of reptiles, birds, mammals
7. ***Transmission***: All stages lurk on plants for new hosts
8. ***Prophylaxis***: Use of repellents
9. ***Therapy/treatment***: Acarizides are placed onto the hair of animals; pinching off ticks from skin with pincers.

Hyalomma or *Ambylomma*. Therefore, these ticks do not need as long starvation periods as those of the genus *Ixodes*, but in any case many months may be necessary to find a new host after moulting from one stage into another. In summary the survival of ticks on earth is based on their ability to starve for long periods, to feed on giant masses of blood in short periods and to produce giant amounts of eggs in the hope that some of them may become mature ticks and may reproduce successfully.

3.9 Fleas: The Record Jumpers

Jump as far as you can
(Unknown flea father)

Always when highly trained human athletes jump over 2.3 m or reach even 6 m with the help of a pole, the whole stadium shouts in pleasure. However, this is nothing compared to the performance of the tiny, only 2–3-mm long adult fleas, which are able to jump from 10–25 cm in height or length – a record flea jumper was told to have reached 60 cm (perhaps supported by a gust of wind). In order to attain a comparable record a man must jump 200 m in length and at least 60 m in height. Fleas developed this ability during evolution in order to find hosts like mice, birds etc., which of course are highly motile and change their place of feeding so often that "slowly walking fleas" would have only poor chances to attack them for a nice drink of blood. Therefore, fleas have developed very long and strong hind legs (Fig. 3.8a–c), which enable them to jump over considerable distances. Having found a suitable host – fleas are not very picky – male and female fleas suck blood at least once per day for (if undisturbed) 20–150 min. However, they are easily disturbed. Then meals take only minutes. During one meal they take up 0.5 mg blood, which corresponds to a multiple of their body weight. However, this huge amount is not used for the adults alone, but serves also for the larvae (Fig. 3.8d), since portions of the blood are released via the anus (about 20–40 times per hour). Thus adults "drink for their kids = larvae", which live on the soil, in human beds, in the nests of birds or on the sleeping place of pets. This type of blood meal, which benefits the survival of the progeny, may be repeated several times per day. On the other hand fleas are also masters in the discipline of "starvation", since adult fleas can wait inside the pupal cocoon for months (e.g. in bird nests) until a trembling announces the arrival of new hosts. Also unfertilized females may starve for long periods and – if getting blood – they may live for up to 5 years. Fertilized females, however, become exhausted after 3 months due to their production of about 450 of the 0.5-mm long, white, ovoid eggs.

Of course flea bites (that are often found in rows) are not nice due to their itching effects. However, fleas are really dangerous due to a particular non-polite behaviour during the blood-sucking act. If fleas are disturbed during blood sucking, they may change host and start another blood meal on the new host. However, since they suck hastily, their foregut becomes overfilled, and some portions of the blood masses

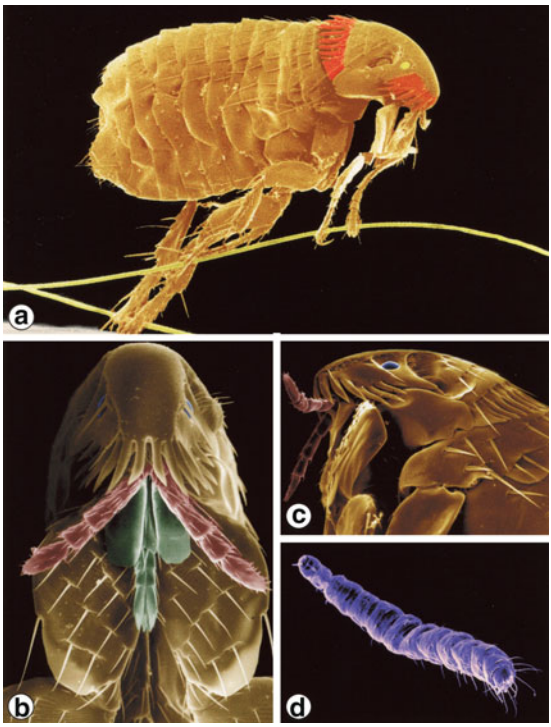

Fig. 3.8 (**a–d**) Scanning electron micrographs of the developing stages of the cat flea *Ctenocephalides felis*, which represents about 80% of the flea population on human cats and dogs

may become regurgitated into the wound. This blood may contain portions of the blood of the preceding host. In case this host contained agents of diseases such as plague bacilli or several viruses (as was shown 2007–2009 by the groups of Mencke and Mehlhorn) the second host may become infected, too. As a consequence fleas are serious vectors and represent a big epidemiological problem, especially in locations crowded by man. Therefore, treatment of animals with insecticides and flea eradication in hospitals, camps or in human family dwellings is very important.

Records of Fleas

1. ***Living site***: Adult and larvae: bed of man, rest places and nests of animals
2. ***Stages***: Larvae (three stages), pupa, ♂, ♀
3. ***Size***: *Adults*: 1–6 mm (depending on the species)
4. ***Characteristics***: Adults: wingless, six legs, last pair of legs are long and strong, blood suckers; *larvae*: without legs, many surface bristles
5. ***Reproduction***: Sexual, holometabolic life cycle including a pupa
6. ***Hosts***: Mainly birds and mammals
7. ***Transmission***: Jumping activity of ♂, ♀
8. ***Prophylaxis***: Repellents
9. ***Therapy***: Insecticides against *adults*, inhibitors of moult against *larvae*

3.10 Bed Bugs: The Stinking High-Speed Runners in the Dark

Running until blood comes
(Wisdom of recruits)

Bed bugs – about 6–8 mm in length (*Cimex lectularius*: *Latin*: lectulus = little bed) have been companions of humans since the days humans ceased their nomadic life and became sessile in common places and built homes for shelter. Apparently birds and rodents imported the blood suckers from their nests into human dwellings, where they became active during the night. Then this armada of heavy blood suckers (larvae and adults) leaves their protected places in beds, behind desks or wall coverings and runs towards their sleeping hosts. During one sucking act that takes about 15 min, each bed bug may take up about 7 mg of blood until its body is completely stretched. Unfed bugs even attack their "brothers" and suck blood from their abdominal bodies. The blood loss from hosts due to the attacks of numerous bed bugs may be considerable. For example, about 180 adult bed bugs may kill a mouse due to the enormous loss of blood. Initially the bed bug's bites are not painful and hosts do not notice them. However, after some hours itching at the biting site draws the attention of the host to the previous sucking act. When attacking hosts, bed bugs may run very quickly covering 1.25 m within 1 min. This distance represents the 200-fold of their length. Thus, a man of 1.8 m in length must run at least 360 m within 1 min in order to show the same performance. This can be done by highly trained male humans, who afterwards are completely exhausted, while bed bugs run further distances whilst maintaining the same speed. Bed bugs are very social animals. Their abdominal glands excrete an odour, which stinks to humans, but which holds the society of bed bugs together in a room. This makes it easy for bed bugs to find a sexual partner, but humans suffer twice: from the follow up of the wounds and from the stink in their rooms. The blood is imperative for the reproduction of bed bugs. Fed females are very busy and lay up to 12 eggs per day (in total about 500 during their life span). Bed bugs reach within 22 days via five larval stages (without pupa) maturity and the reproduction process starts again. Therefore humans, who have bed bugs in their rooms, will never remain alone (Fig. 3.9).

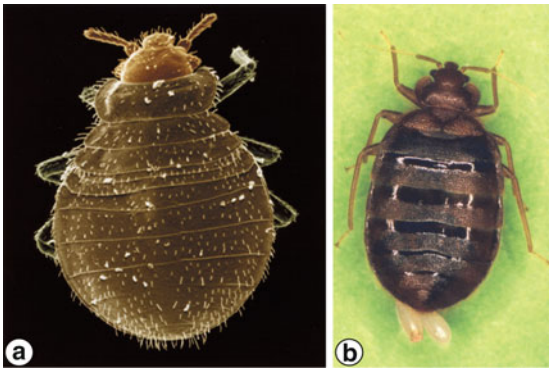

Fig. 3.9 (**a**, **b**) Scanning (**a**) and light micrographs (**b**) of bed bugs from their dorsal side. In (**b**) the female is about to lay eggs

Records of Bed Bugs

1. ***Living site***: Hidden in human dwellings, beds
2. ***Stages***: Five larval (nymphal) stages and adults (♂, ♀)
3. ***Size***: Up to 8 mm
4. ***Characteristics***: Brown, wingless insects with sucking mouthparts
5. ***Reproduction***: Hemimetabolic life cycle (development without pupa)
6. ***Hosts***: Humans, birds, mice
7. ***Transmission***: Bed bugs are imported into homes with luggage or furniture
8. ***Prophylaxis***: Check luggage and furniture before transportation into a home
9. ***Therapy***: Insecticides which have to be used at least twice

3.11 Head, Pubic and Body Lice: The Heavy Clingers

Hairless heads are too slippery for lice (Fact)

The blood-sucking lice of humans (Fig. 3.10a–d):

- *Pediculus humanus capitis* (head louse: *Latin:* pediculus: little foot)
- *Pediculus humanus corporis* (body louse)
- *Phthirus pubis* (pubic louse, *Greek*: phtheir: louse)

are excellent clingers. These wingless insects have developed a giant claw at each of their six legs, so that any of these claws is able to clutch a separate hair. This means that it is difficult to separate lice from their host. This is very important because these three lice species have lived for about 15 million years exclusively on the body of humans and their precursors. The first human lice apparently lived exclusively inside the hair, while later – when humans created clothes for cold periods – the body lice separated from the main development. Body lice often glue their eggs onto the inside of human clothes, while head lice exclusively attach the eggs to the hair (Fig. 3.10d), as is done by the pig species *Haematopinus suis* (Fig. 3.10c). Head lice only detach willingly from their hosts in case of two events:

1. The host dies and its surface temperature decreases. The same often happens during surgery, when the skin temperature decreases considerably. This is noted by the lice and viewed as a stimulus to look for another host.
2. Fertilized females of head lice run just after their blood meal towards the end of hair and lurk there for another host. If there is a hair-to-hair contact, the females change host and become the grand-grandmothers of a new population. If there is no hair-to-hair contact within the hour following sucking, they return to the head's surface, suck again and glue some eggs at the base of the hair of their present host.

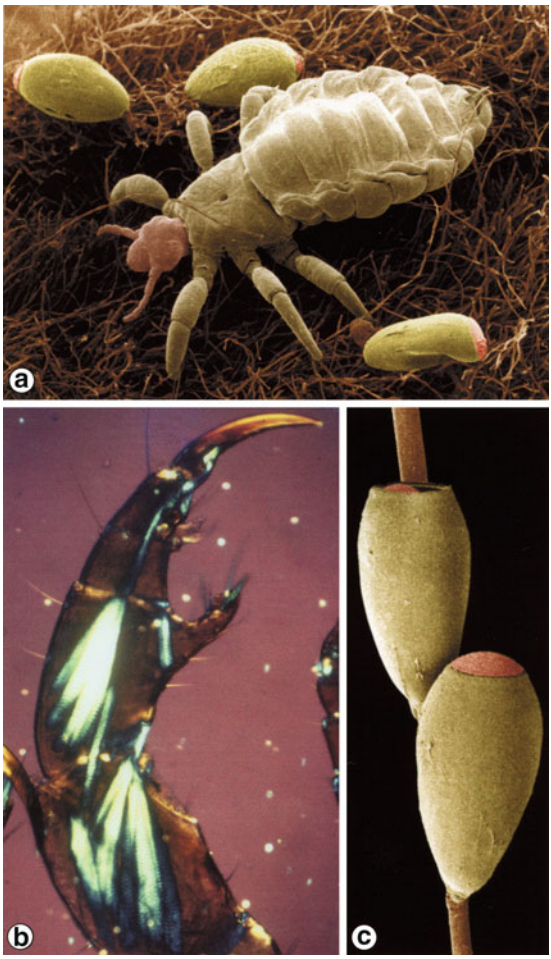

Fig. 3.10 (**a–e**) Scanning (**a**, **c**, **d**, **e**) and light micrographs (**b**) of lice. (**a**) Body louse with eggs glued to human clothes. (**b**) Nomarski-aspect of the strong muscles inside the legs of lice enabling them as clingers in the "hair forest". (**c**) Pig louse: *Haematopinus suis*: one egg with operculum, the second without – both being glued on a hair. (**d**) Egg of a human head louse showing the typical operculum with aeropyls. (**e**) Crab or pubic louse

Thus, head and pubic lice only change their hosts during body contacts. Therefore, the French named the pubic louse also "papillon of love" = butterfly of love. Richness in lice was not always considered a sign of bad hygiene, as in the sixteenth to eighteenth century it was believed that possession of many lice was an indication of high sexual potency in males, since it was believed that lice suck away "bad body fluids", while the "good" ones remain. All stages of the life cycle of lice suck blood: all three larval stages and both females and males. Females suck up to 1.1 mg per meal, which is ingested every 2–3 h. Males need less blood, since they do not lay 5–10 eggs per day as is done by the females. Males suck about 0.3 mg blood per meal. Starvation is bad for head lice. At 37°C they survive only for 24 h at the maximum without blood, while body lice may survive for up to 10 days – especially if temperatures are low (12–20°C). Thus, in the case of head lice there is no need to clean the bed or clothes of lice which occasionally drop down from a crowded head.

In some records more than 20,000 head lice had been counted on a single person – a real festival. The eggs – covered by an operculum (Fig. 3.10d) with aeropyles (for the entry of oxygen) – are miracles of stability. They become glued at the base of hair with the help of a water-insoluble substance and are protected by a chitinous shell (nit). Thus, even combs with very closely standing teeth are not useful to detach most of the eggs reaching a length of 0.8 mm and diameters of 0.2 mm. While head and pubic lice are surely nasty visitors of humans, body lice may become dangerous, since they may transmit agents of diseases such as *Rickettsia prowazekii*, which may introduce outbreaks of a disease of the spotted fever group (a possibly deadly disease in untreated cases).

Thus, the development of the strong claws has enabled the three human lice species to live truly for some millions of years with humans and their precursors.

Records of Lice

1. ***Living site***: Human body (exclusively)
2. ***Stages***: Three larvae, ♂, ♀
3. ***Size***: *Head louse*: 3 mm, *body louse*: 4 mm, *pubic louse:* 1.5 mm
4. ***Characteristics***: Strong claws, eggs become glued to hair or clothes
5. ***Reproduction***: Sexual intercourse
6. ***Hosts***: Exclusively humans
7. ***Transmission***: Hair-to-hair contact (*head louse*), contacts of clothes and/or bodies (*body* and *pubic lice*)
8. ***Prophylaxis***: Avoid hair-to-hair contact respectively body contacts with unknown people
9. ***Therapy***: Use of good and efficacious hair and body shampoo (such as Wash Away Louse or Lincin)

3.12 Antarctic Lice: The Inventors of the Diving Suit

Oh, how cold is your little hand
(La Boheme)

All those that believe lice like it warm will be sceptical on talk of the lice of seals belonging for example to the genus *Antarctophilus* (Fig. 3.11a–d). The Weddell seals and related species live and hunt in the very cold (−1.7°C) waters of Antarctica, so that their fur should be icy from the skin until its very end. However, lice of different blood-sucking species are often found closely attached to the skin while sucking blood. Considering that the fur of the seals is often exposed to temperatures as low as –40°C, after they have left the water and are exposed to the winds, it is nearly unbelievable that these lice survive in such an environment. This is, however, possible, since these lice have developed a multi-layered cuticular surface consisting of overlapping scales. In between these scales layers of the fat

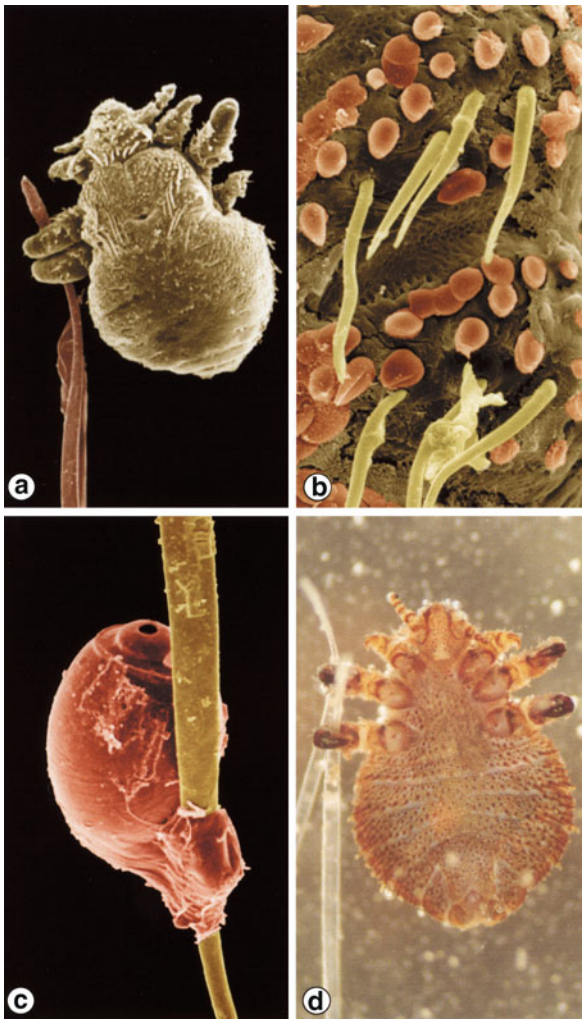

Fig. 3.11 (**a–d**) Scanning (**a–c**) and light (**d**) micrographs. (**a**, **d**) Adult lice clinging to a seal's hair. (**b**) Knobs and hair at the dorsal surface fixing the cover made by the seal's surface fat. (**c**) Egg being glued with water-insoluble material on a hair of the seal. Note the single opening (aeropyl) of the cover (operculum)

excreted by the body surface of the seal become mingled. In addition at several places air spaces persist thus producing a multi-layered cover, which surely protects against freezing. Furthermore the whole surface of the lice is topped by another thick layer of the seal's surface fat being fixed by short, knob-like protrusions and longer hair (Fig. 3.11b). The inner body of these lice is heated by the permanent uptake of the warm blood of the seal. In addition this multi-layered surface of the lice is very helpful, when the seals begin diving (up to 600 m deep). Then this layer becomes smoothly depressed and the surface fat is squeezed out from the space between the cuticular scales, as the seal dives down, and is refilled again, when the seal again reaches the surface of the water. Besides this perfect protection of the Antarctic lice another miracle remains, however, unsolved: from where does the

heat come from that starts and keeps running the "motor" of embryo development inside the nits, which are glued (as in the case of other lice) by *Antarctophilus* females onto the hair of the seals (Fig. 3.11c)?

Records of Antarctic Lice

1. ***Living site***: Inside the fur of diving seals
2. ***Stages***: Three larvae, ♀, ♂
3. ***Size***: Adults: 2 mm in length
4. ***Characteristics***: Surface scales creating a diver's suit; highly compressible; claws at their feet
5. ***Reproduction***: Sexual intercourse, nits are glued to the hair of the host
6. ***Hosts***: Antarctic and arctic seals
7. ***Transmission***: During body contacts of the hosts
8. ***Prophylaxis***: None in the wilderness
9. ***Therapy***: None in the wilderness, bath treatments with anti-lice shampoos in the zoo.

3.13 The Super Moms: Tse-tse Flies

Oh babe, I keep you in your dreams
(Folk song)

Tse-tse flies – the African relatives of our European biting flies (e.g. *Stomoxys calcitrans*: *Greek*: with pointed mouth) – are also called tongue flies (genus *Glossina* = *Latin* = tongue), since they protrude their mouthparts like a tongue and since their folded wings look like a tongue when seen from above, as the fly is at rest (Fig. 3.12a, b). Both sexes suck blood from motile hosts, being attracted by movement. The amount of blood taken up during a single blood meal varies depending on the species. For example *Glossina palpalis* sucks 30 mg of blood at short intervals. *Glossina morsitans* (*Latin*: the biting one) takes up 90 mg per meal and *G. brevipalpis* may ingest even up to 260 mg thus feeding 3–4-fold their own body weight. If man would do the same, he would have to eat up to 150 kg in one meal. Such blood meals are repeated by the *Glossina* individual at intervals of 3–6 days. During, before or after feeding they may meet their sexual partners on the host, while during the rest of the time they live as "lonely riders" far away from hosts. This behaviour leads to the fact that hosts are not really disturbed by *Glossina* specimens and their limited blood meals. Furthermore, this behaviour makes it difficult to kill them through application of insecticides, which must be sprayed at high dosages in order to reach a long-lasting protection. Another behavioural peculiarity brought an exceptional advantage in the fight for survival. While non-biting, normal flies rely on mass production of eggs – hoping that a few of them reach the adult stage – the glossines have developed a unique way of caring for their

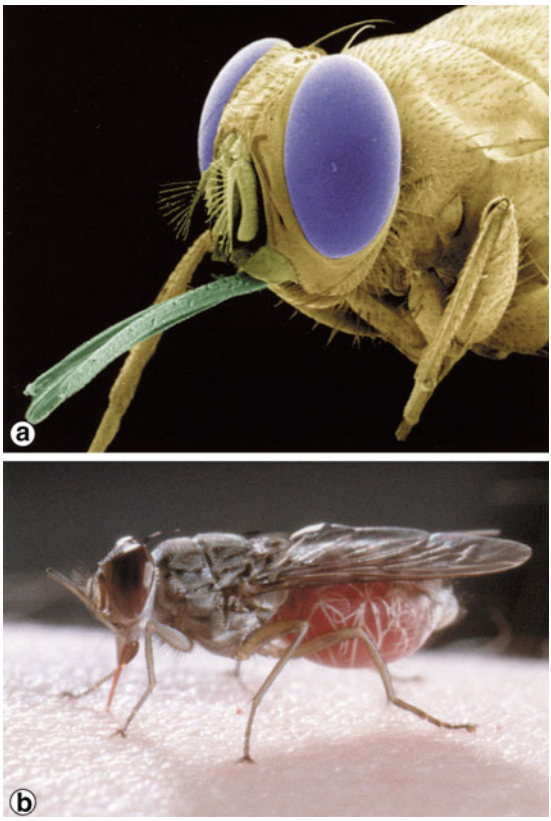

Fig. 3.12 (**a**, **b**) (**a**) Scanning electron micrograph of the anterior end of a *Glossina* specimen. (**b**) Light micrograph of a *Glossina* female during bloodsucking on human skin

progeny. The females do not deposit eggs somewhere on the soil, on faeces or on dead bodies, but keep them inside their body within a peculiar "brood chamber". The first hatched larva suppresses the development of the others, uses their resources and in addition is fed by excretions of the wall of the brood chamber. These glands are described as "milk glands". After a developmental period of 8–14 days the larva, which is ready to pupate, is finally deponed into sand. The pupa finally gives rise to the "ready-to-bite" adult female or male. Because of this excellent "brood-care-system" the progeny of the glossines has the "best cards" in the fight for survival. Therefore, it is sufficient that each female produces only 10–20 "kids" during its 3–7 month-long life span in order to maintain a reasonable population in a given biotope. These "super moms" even contribute to the expansion of the population, since they import – like aircraft carriers – their progeny into new biotopes. This peculiar behaviour endangers humans and their stock animals considerably, since glossines are the vectors of the agents of the deadly trypanosomiasis, which even today is not treatable with a reasonable survival rate.

Records of the *Glossina* Species

1. ***Living site***: Free in the savannahs of Africa, preferring dark hiding places
2. ***Stages***: Larval development inside the mother; free pupae and adults living for 3–7 months
3. ***Size***: Adults: ca. 10 mm
4. ***Characteristics***: Blood-sucking mouthparts directed straight forward
5. ***Reproduction***: Sexual intercourse
6. ***Hosts***: Cattle, antelopes, humans, rodents, crocodiles, other wildlife etc.
7. ***Transmission***: Adults search their hosts
8. ***Prophylaxis***: None in the wilderness
9. ***Therapy***: None in the wilderness, insecticides in farms

3.14 Having Beautiful Eyes: But Not Needing Them – Mosquitoes During Attack

Look into my eyes, babe
(Humphrey Bogart – in Casablanca)

Who does like the buzzing sound of attacking mosquitoes (from *Portuguese: mosquitos* = biting insect), while waiting for the benefits of the arriving god of sleep in a quiet bedroom? Surely nobody. Of course – the bite itself does not initiate pain, since the female mosquito injects its saliva, which contains besides anticoagulants and vessel-enlarging components also anaesthetics that at first make the bite unnoticeable. However, all these elements may induce intense immune system reactions, so that – depending on the host – quick or slow local symptoms occur such as intensive itching, formation of skin papulae, local inflammations etc. Only female mosquitoes suck blood via their two-channel-mouthparts directly from blood vessels. Thus, they are named "vessel-feeders", while other biting blood suckers such as ticks, simuliids or tabanids cut little "lakes" within the skin and thus are named "pool-feeders". The nasty "attacking music" of female mosquitoes is produced by the beating of their two large forewings and their two tiny back wings (halteres) at a magnitude of around 350–450 Hz (depending on the species). This frequency is even topped by the males, where in some species even 1,000 Hz may be reached. In many species males, which feed – if at all – on plant juices, form large swarms of "dancing" individuals. These swarms appear as clouds of dark smoke close to bushes along little creeks, lakes etc. The females fly into these clouds and become fertilized even during flight. After that they try to get a blood meal from a host and start about three days later with the deposition of 50–250 eggs mostly onto the surface of water. This procedure is repeated several times (depending on the temperature, availability of hosts and species peculiarities): for example the European fever mosquito *Anopheles maculipennis* produces about 2,500 eggs which are deposed during ten different acts. Mosquitoes may occur as part of a population of millions of individuals. These enormous reproduction rates, which prohibit human

settling in many regions of the earth, are only possible, since the mosquitoes have developed through the course of evolution many admirable world records in the field of physiological abilities. For example, adult mosquitoes may hibernate in temperate climates and even at very low temperatures for many months. This does not stop their fecundity in the next spring. Females of *Culex pipiens* (the piping mosquito inside houses) and those of the fever mosquito *Anopheles maculipennis* become fertilized for example in November, starve until March, then suck blood and deposit their eggs onto the surface of water. Other species hibernate as eggs (*Aedes cantans* = the singing one) or larvae (*Mansonia richardii, Aedes nemorosis*) in the mud of frozen ponds etc. The larvae of some species (e.g. *Aedes mariae*, several *Culicoides* species) have learnt to survive even inside salt water. All this needs a high level uptake of food in order to produce the needed energy: larvae filtrate large amounts of proteins from their biotope and female mosquitoes are able to suck within a few seconds up to the fivefold of their body weight (e.g. *Anopheles gambiae*: 2–5 mg; *Aedes aegypti*: 4 mg; *Culex quinquefasciatus*: 6–10 mg, *Psophora ciliata*: 25 mg), while pool-feeding midges only suck 0.001 mg. Sucking of mosquitoes is done through use of the pressure inside the blood vessels, into which they inject their two-channel-system mouthparts (one channel is used to introduce the saliva, the other to engorge the blood meal). These meals are repeated every three days enabling the females to lay eggs. However, one meal may also be sufficient to survive for a whole winter in a quiet, non-freezing location somewhere in a house or within a protected shelter in nature. Of course, many of these individuals will die – but the mass production of the progeny guarantees survival of sufficient specimens to generate the following generations of blood suckers. Mosquitoes possess large compound eyes with numerous ommatidia of the "apposition" type (i.e. being clearly separated from each other; Fig. 3.13a–d). They are of high importance for finding hosts in the case of daytime active mosquitoes such as many *Aedes* species = forest and bush mosquitoes. However, many species are active at night or during dawn and dusk and thus these species rely on further abilities. They therefore developed highly sensitive receptors for different odours, which enable them in the dark to find hosts over distances of up to 20 m. More than 40 different components have been determined to be attractive to mosquitoes, if they are mixed within the "skin perfume" of humans. This explains why some persons are more attractive than others possessing a varying coat along their skin. CO_2 and body warmth, however, are very important components that stimulate female mosquitoes to become nasty aggressors against peacefully sleeping persons. Since mosquitoes inject saliva into the blood vessels of their hosts in order to keep the blood meal fluid, this saliva may be contaminated with agents of diseases such as malaria parasites or many viruses that may introduce life-threatening diseases such as Yellow Fever, Dengue Fever, Rift Valley Fever etc. Happily this does not occur in temperate zones of the earth, but is not excluded in further times of intense globalization and global warming. Other blood suckers like midges (genus *Culicoides*) or black flies (genus *Simulium*) are already known as vectors of diseases in Europe. *Culicoides* species led to an outbreak of Bluetongue disease in ruminants in Europe during the years 2006–2009 and *Simulium* species (the vectors of onchocerciasis = river blindness in Africa) may transmit skin filariae or introduce painful

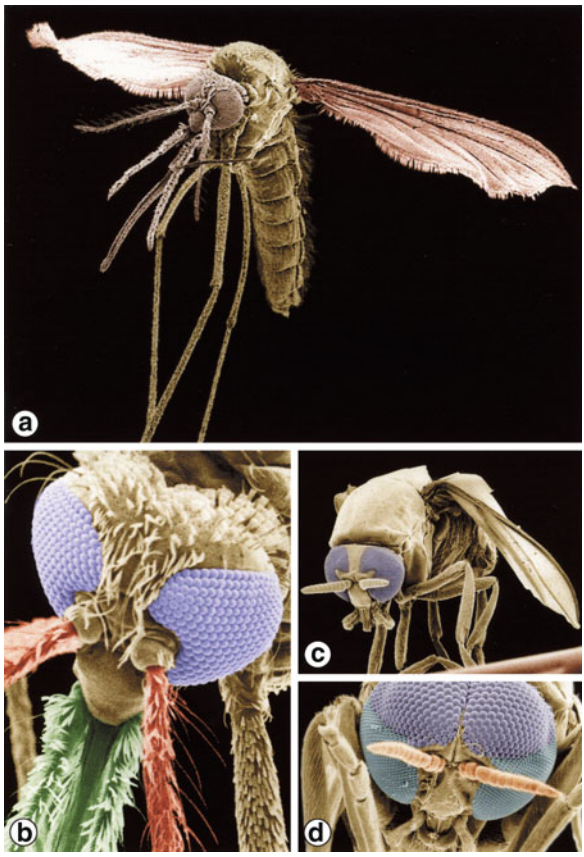

Fig. 3.13 (**a**–**d**) Scanning electron micrographs of mosquitoes. (**a**, **b**) *Anopheles stephensi* (8 mm), (**c**) *Simulium morsitans*, female (3 mm). (**d**) Head of a male showing smaller lower ommatidia

wounds at their biting sites. Both *Culicoides* species and simuliids are "pool-feeders" cutting tiny blood lakes into the skin, which often become additionally superinfected by bacteria.

The males of the genus *Simulium* developed two types of ommatidia inside their compound eyes (Fig. 3.13d). There are smaller ommatidia at the lower side of the eye and larger ones at its upper rim. Thus, these species apparently have developed a type of "glasses for reading", that is recognition of details at narrow distances, since these smaller ommatidia should produce sharper pictures in their tiny "brains".

Records in the World of Mosquitoes

1. ***Living site***: Larvae, pupae: water; adults: air, blood-sucking females, worldwide
2. ***Stages***: Larvae (4–5), pupa (1), ♀, ♂
3. ***Size***: *Adults: Aedes:* (2–4 mm), *Anopheles* (5–8 mm), *Culex* (4–6 mm)

(continued)

4. ***Characteristics***: Females suck blood (up to 5-fold of their body weight), large eyes, vessel feeder, attracted by body odours
5. ***Reproduction***: Sexual intercourse
6. ***Hosts***: Warm-blooded hosts, reptiles, amphibia
7. ***Transmission***: Females are attracted by body odours
8. ***Prophylaxis***: Humans may become protected by nets and repellents (such as Viticks Cool®, Autan®)
9. ***Therapy***: After bites: cooling of wounds, use of antihistaminica, medicaments against transmitted agents of diseases

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Chapter 4
Cryptosporidium parvum: The Veterinary Perspective

Arwid Daugschies

Abstract Apicomplexan parasites of the genus Cryptosporidium are increasingly considered as pathogens of zoonotic and animal health impact. However, many questions related to important aspects such as epidemiology, pathogenesis, immunology and efficient control measures remain to be answered. Advanced and conventional tools are available to study these parasites in depth which opens the door to multidisciplinary approaches combining expertise from different scientific disciplines. This short review highlights several aspects where particularly veterinary parasitologists are committed to provide relevant contributions to cryptosporidiosis research.

Cryptosporidium parvum is a **zoonotic protozoan parasite** (Table 4.1) that is increasingly regarded as an important intestinal pathogen in animals, particularly young ruminants. It is well documented that, in contrast to most other monoxenous apicomplexa, *C. parvum* is not restricted to a single host but may dwell in the intestines of a large number of vertebrate species. In spite of many attempts to develop pharmaceuticals or vaccines to allow control of cryptosporidiosis in animals and man the respective options are very limited. Thus, it will remain a challenge for the future to evolve efficient control options. Suitable experimental models are inevitable for this means. Considering these aspects *C. parvum* may serve as an excellent example to comment on the general tasks of veterinary medicine in current research on parasites which are:

1. To reduce the risk of zoonotic transmission of parasitic pathogens
2. To prevent and/or cure parasitic disease in animals

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Table 4.1 Case numbers for notifiable parasitic zoonotic infections in Germany

Year	2001	2002	2003	2004	2005	2006	2007
Giardiosis	3,894	3,097	3,216	4,621	4,519	3,661	3,654
Cryptosporidiosis	1,481	816	885	935	1,309	1,204	1,459
Toxoplasmosis (prenatal)	38	18	19	16	18	10	20
Echinococcosis	46	41	85	97	125	124	93
Trichinellosis	5	10	3	5	0	22	10

Source of data: Yearly report of Robert Koch Institute (http://www.rki.de/cln_160/nn_196658/DE/Content/Infekt/Jahrbuch/jahrbuch__node.html?__nnn=true)

3. To maintain productivity of livestock farming
4. To develop and provide models suitable to evaluate for example host–parasite interaction or parasite control options

This short review highlights these topics with particular emphasis on the author's personal experience in the area of *C. parvum* research and does not claim to comprehensively reflect the current state of knowledge.

In short, the **monoxenous life cycle** of *C. parvum* is relatively simple. After oral ingestion of sporulated oocysts from the environment each oocyst releases four sporozoites that invade the brush border of the intestinal lining and subsequently transform to trophozoites. Following asexual multiplication (merogony) the parasite undergoes sexual differentiation (gamogony). After fertilization the zygote develops into the oocyst stage. It is typical for cryptosporidia that oocysts are sporulated already before they are passed with the faeces into the environment, and thus they are fully infective upon excretion. The life cycle may be completed within as few as 3–7 days and infected animals may shed tremendous amounts of oocysts within a short time. Oocysts may survive, depending on the environmental conditions, for several weeks or even months, thus imposing a continuing infection risk to susceptible hosts. Surface water is believed to be an efficient carrier for oocysts and epidemic outbreaks of human cryptosporidiosis (the most cited reported from Milwaukee by MacKenzie et al. 1995) are explained by ingestion of contaminated water. In fact, several studies from different continents documenting oocysts in surface water have shown that zoonotic genotypes were present in the respective samples (e.g. Ono et al. 2001; Ward et al. 2002; Xiao et al. 2001).

Originally only two *Cryptosporidium* spp. were believed to represent the respective genus, namely *C. muris* (Tyzzer 1907) and *C. parvum* (Tyzzer 1912). Further on, intestinal infections with cryptosporidia were described for many animal species and were exclusively attributed to *C. parvum* which thus appeared to lack host specificity. Later it became evident, mainly based on oocyst morphology but also on host and/or site preference, that the genus is more divergent than previously thought.

For instance, other host-specific species of *Cryptosporidium* (e.g. *C. felis* in cats, *C. meleagridis* in poultry) may be zoonotic under certain circumstances (Morgan et al. 2000). Two *C. parvum* types, one being zoonotic and mainly found in animals and the other obviously adapted to humans, were described (Awad-El-Kariem et al. 1998) and the latter is meanwhile regarded as a different species, named *C. hominis*. Many other *C. parvum* genotypes have been defined and may or may not reach species status in the future. The various *C. parvum* genotypes are more or less adapted to certain host species, some representing zoonotic genotypes while others

appear to be of less or no zoonotic relevance. Immunocompromized persons, for example those infected with HIV, are particularly susceptible to cryptosporidia infection and may suffer from severe, potentially life-threatening disease. This may be less dramatic in industrialized countries due to the high level of medical care, for example improved treatment options for AIDS, however, in less developed areas and under conditions of poor hygiene cryptosporidiosis will remain a severe threat to exposed persons, particularly those belonging to a risk group. Unfortunately, our knowledge on the epidemiology and relevance of cryptosporidiosis in developing countries is very limited (Rahman et al. 1985; Jex and Gasser 2010).

Other cryptosporidia species being less important as zoonotic pathogens may occasionally infect humans. For instance, *C. felis* and *C. meleagridis* were found in AIDS patients (Morgan et al. 2000). Close contact to pet animals increases the risk of zoonotic transmission of *Cryptosporidium* spp. (Joachim and Daugschies 2004) such as *C. felis* or *C. canis*. The molecular taxonomy of the genus *Cryptosporidium* and particularly of *C. parvum* is still in progress (Jex and Gasser 2010) and new respective insights will have profound consequences for the elucidation of epidemiological questions, for example regarding the source of human infection and transmission routes.

In Germany **human cryptosporidiosis** belongs to the group of **notifiable infectious diseases** and appears to be more prevalent than other parasitic and potentially zoonotic infections in humans (Table 4.1). Since the clinical course is not very specific and disease is typically transient in immune-competent persons the prevalence estimated on the basis of notified cases (less than 1,500 per year in Germany) is without doubt extremely underestimated. The infectious dose for humans is low (30 oocysts) whereas one infected calf may shed as much as 10^{11} oocysts during patency (Joachim and Daugschies 2004) and thus it appears very likely, considering that *C. parvum* is a ubiquitous pathogen in ruminant herds, that livestock contribute to the contamination of surface water (Fayer 2004; Karanis et al. 2006) and that every person in the vicinity of young ruminants (e.g. animal keepers, veterinarians) will attract *C. parvum* infection sooner or later. However, data are fragmentary and mainly reflect the situation in developed countries whereas very little is known on the zoonotic transmission in economically less developed countries where the problems are supposed to be much more dramatic (Jex and Gasser 2010).

Wildlife may also serve as hosts for cryptosporidia including *C. parvum* as the host range of the latter is extremely wide (Fayer 2004; Jex and Gasser 2010). European hedgehogs are often kept during the winter season under human care if they are found to be in a condition (e.g. young or diseased hedgehog) that is related to a risk of nonsurvival. Around 20–40% of these animals excrete oocysts of different genotypes of *C. parvum*. Further genotyping of GP60, HSP70 and actin revealed three subtypes, namely IIa (bovine), IIc (human) and a proposed new genotype VIIa (Dyachenko et al. 2010). The former two indicate the possibility of zoonotic transmission by hedgehogs whereas the latter one remains to be studied further.

Besides its role as an agent of **zoonotic disease** *C. parvum* in particular attracts considerable attention as a cause of **enteritis** in animals. Mainly young animals such as suckling calves or lambs are susceptible to cryptosporidiosis which displays as transient watery diarrhoea, sometimes resulting in rapid dehydration and death. However, most affected animals survive and build up protective immunity but may show sustained reduction of productivity, although this aspect is not well documented.

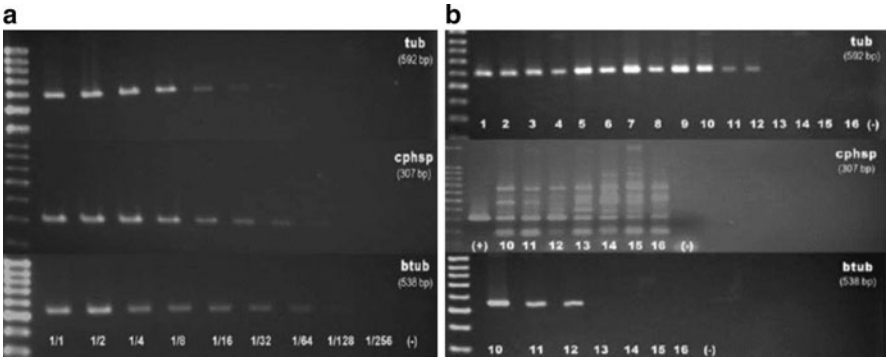

Fig. 4.1 Banding pattern of amplicons after PCR with various primer pairs (tub: Caccio et al. 1999; cphsp: Rochelle et al. 1997; btub: Widmer et al. 1998). (**a**) Pure oocyst suspension; (**b**) amplification from faecal samples

Diagnosis of cryptosporidiosis in animals is classically done by microscopic evaluation of oocysts. Because diseased animals tend to shed tremendous amounts of oocysts, stained (e.g. carbol fuchsine, Ziehl Neelsen stain) or unstained faecal smears are generally suitably sensitive for diagnostic purposes. Alternatively, immunological test kits are commercially available. PCR is very sensitive, however, not all primer combinations published are suited for direct detection of parasite DNA in faecal samples. In our experience, primers targeted against the β-tubulin gene are specific in faecal specimen whereas the heat shock protein gene locus (HSP70) resulted in many unspecific bands (Fig. 4.1). Calves experimentally infected with *C. parvum* were positive by conventional microscopy from 4 days postinfection (dpi) until 12 dpi whereas PCR delivered respective signals from 4 dpi until 14 dpi (Kar et al. 2010). Thus, molecular methods are without doubt powerful and useful tools in epidemiological research on cryptosporidiosis but it appears that the additional technological effort and costs related to PCR are not justified for routine diagnostic purposes in veterinary medicine.

It has long been questioned whether cryptosporidia are primary pathogens or whether they exert relevant insult only under certain conditions (e.g. altered immunity or in combination with other pathogens). Meanwhile it is clear that infections with only *C. parvum* induce diarrhoea in otherwise healthy young calves or lambs under experimental conditions as well as in livestock herds. Of course, the severity and course of disease depend on other factors in field situations, for example sufficient access to colostrum or exposure to other germs. For instance, lambs shed more pathogenic *E. coli* if raised colostrum-deprived and simultaneously infected with *C. parvum* (La Ragione et al. 2006). It has been estimated that the prevalence of *C. parvum* in calves ranges between 20% and 40% in Europe, which in the light of own experience appears to be a rather conservative approximation. Farmers (and veterinarians) tend to accept diarrhoea in young calves as long as this is not related to considerable mortality, or the self-limiting disease is attributed to other causes (e.g. bacterial infection, feeding). Consequently, specific

diagnosis is often neglected and awareness is low. Therefore, more epidemiological data on *C. parvum* in ruminant livestock herds are urgently needed.

Attempts to protect young ruminants by vaccination have been published. Because clinical cryptosporidiosis may already develop in the first week after birth application of a *C. parvum* vaccine to exposed new-born animals appears not to be a promising option. Goat lambs were reported to be well protected against *C. parvum* challenge when their mothers were treated with a plasmid vaccine in the 3rd/4th month of gestation (Sagodira et al. 1999). However, commercial vaccines are still not available.

Cleaning and disinfection of areas where susceptible (young) animals are kept are obviously necessary measures to keep the infection pressure at an acceptable level. Chemical disinfectants that are able to inactivate oocysts are available on the market and represent cresol-based products in most cases (Shahiduzzaman et al. 2010). However, in a conventional cow herd chemical disinfection with a cresol alone did not efficiently control cryptosporidiosis in calves. In this herd prevalence was 100% and thus the level of contamination with oocysts was probably very high. Considering the low oocyst dose necessary to induce infection and short prepatent period of *C. parvum* it appears that reduction of environmental contamination by increased hygiene alone to a level preventing cryptosporidiosis may be an exhausting challenge in farms with high initial prevalence (Keidel 2004).

Only **few drugs** have been registered for control of cryptosporidiosis in animals worldwide. Halofuginone is on the market in several European countries (Halocur^{R}, Intervet). Calves need to be treated with this drug on 7 consecutive days starting early (within 24 h) after birth to achieve metaphylactic control. Even under these laborious conditions oocyst excretion is not completely suppressed within groups of naturally exposed calves (Fig. 4.2) and some may still present diarrhoea (Keidel 2004). Interestingly, the combination of cresol disinfection and halofuginone

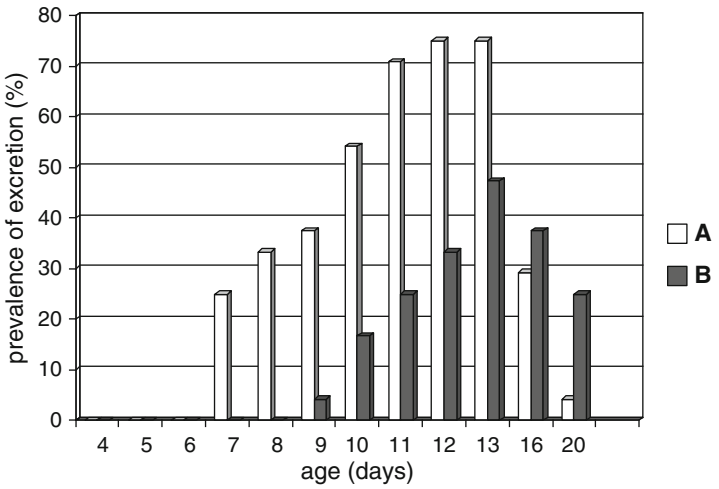

Fig. 4.2 Oocyst excretion by calves ($n = 24$) treated with placebo (**a**) or halofuginone (**b**) 100 μg/kg body weight daily over 7 days starting within 24 h after birth)

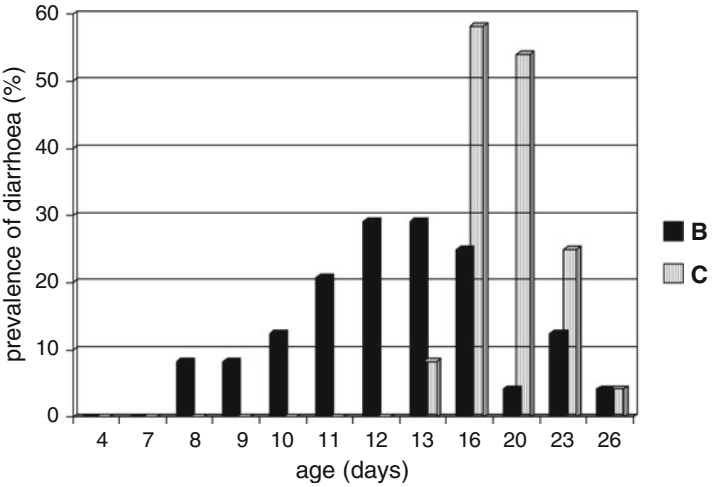

Fig. 4.3 Diarrhoea prevalence in calves ($n = 24$) treated with halofuginone (**b**, **c**) 100 µg/kg body weight daily over 7 days starting within 24 h after birth) and kept in crates either disinfected (**c**) with a cresol-based product or not chemically disinfected (**b**)

treatment almost completely controlled cryptosporidiosis during the period of particular risk in conventional calves, i.e. the first and second week after birth. The protected calves were more susceptible thereafter than calves that experienced cryptosporidiosis before, indicating insufficient immune stimulation in the former group (Fig. 4.3). Although older animals suffering from cryptosporidiosis appear to be less prone to develop a severe disease than younger ones it remains to be evaluated whether optimal protection of young animals is a worthwhile strategy in terms of economic benefit and reduced oocyst excretion.

Despite significant steps forward major gaps in knowledge on *Cryptosporidium* still exist. This includes taxonomy and epidemiology, risk analysis, host–parasite interaction and immunology and, particularly important from a veterinary point of view, control options. To assess most of these aspects advanced molecular tools are now available and the genome of *C. parvum* is completely sequenced (Abrahamsen et al. 2004).

To evaluate new control options animal infection models, particularly in mice, are commonly used in laboratories all over the world whereas studies in experimentally infected natural hosts such as calves are subject to practical constraints. *Cryptosporidium parvum* can be cultured in vitro in permanent cell lines and HCT-8 cells appear to be particularly suitable. They are easily grown to confluence in a simple medium (RPMI-1640 supplemented with fetal calf serum, L-glutamate, sodium pyruvate and antibiotics) at 37°C and 5% CO_2. Recently a combination of *C. parvum* in vitro culture and quantitative PCR ("cc-qPCR") to screen drugs and inactivation measures (e.g. chemical disinfection) at a laboratory scale has been published. The system is well standardized and results are reproducible thus allowing quantification of reproduction (viability, infectivity) of the parasite under strictly defined conditions (Fig. 4.4, Shahiduzzaman et al. 2009a, b, 2010).

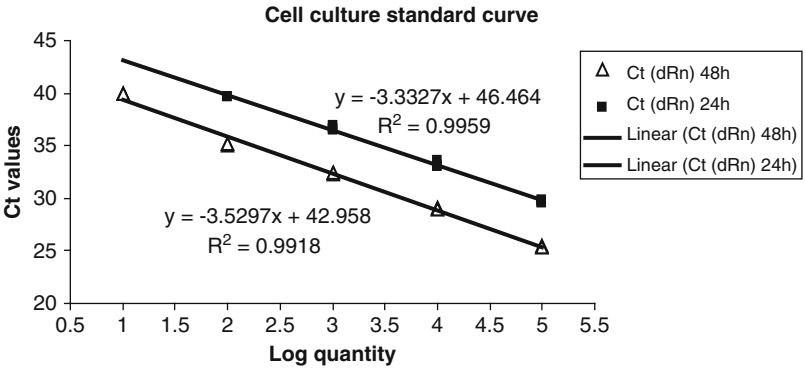

Fig. 4.4 Linear association of infection dose of *C. parvum* oocysts and Ct values in cc-qPCR

Table 4.2 Comparison of conventional assessment of oocyst inactivation by commercial chemical disinfectants (incubation period: 2 h) according to DVG-guideline (in vivo, *E. tenella*) and cc-qPCR

		Inactivation (%)	
	Product concentration (%)	cc-qPCR (*C. parvum*)	In vivo (*E. tenella*)
	3	99.40	92.9
	4	99.93	96.8
	3	99.11	90.6
Neopredisan® 135-1	4	99.91	95.2
Aldecoc® TGE	4	99.91	95.5
	3	99.86	95.5
KokziDes®	4	99.87	99.93
Threshold value		≥99.5%	≥95%

In Germany anti-parasitic chemical disinfectants have to be tested according to guidelines published by the German Veterinary Association (DVG) to obtain respective certification (see www.dvg.net). The ability of chemical disinfectants to inactivate coccidia oocysts is currently being evaluated with *Eimeria tenella* oocysts in a chicken infection model (Daugschies et al. 2002). Excretion of oocysts by experimentally infected birds is assessed and results for birds infected with treated/untreated oocysts are compared. Inactivation is considered sufficient if oocyst excretion in birds infected with disinfected oocysts is reduced by at least 95%. When *C. parvum* oocysts were treated with the same disinfectants in an identical manner (time, concentration) a 99.5% reduction of viability was always recorded in cc-qPCR. On the other hand, insufficient inactivation in the chicken model (<95%) was paralleled by <99.5% inactivation in cc-qPCR (Table 4.2, Shahiduzzaman et al. 2010). Therefore, it is proposed to replace the standard animal infection model by cc-qPCR and respective further testing is currently being performed.

A similar in vitro model has been applied to test curcumin (herbal extract isolated from *Curcuma longa*) for its potential as an anti-cryptosporidial drug. It was clearly

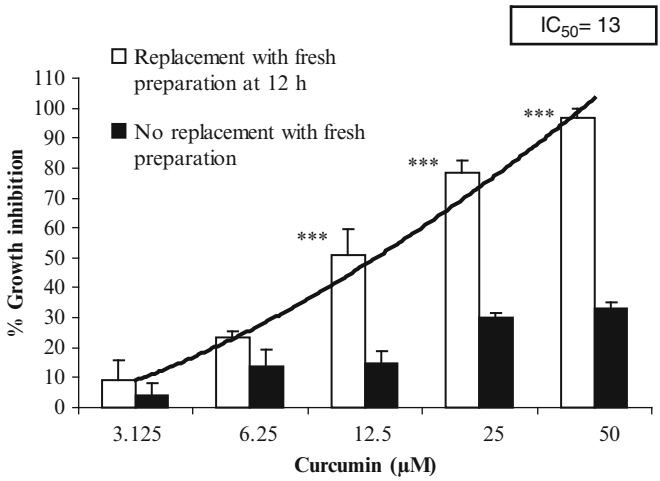

Fig. 4.5 Suppression of multiplication of *C. parvum* in HCT-8 cells by 24 h incubation with curcumin

seen, that curcumin inhibits *C. parvum* multiplication in a dose-dependent way with an IC_{50} of 13 µM provided that the drug-containing medium was replaced every 12 h (Fig. 4.5). This indicates that the herbal extract is not very stable under the given conditions, however, further exploration of curcumin may be rewarding (Shahiduzzaman et al. 2009b).

The demand for new drugs that may hopefully allow better control of *C. parvum* infections in animals and man has stimulated the search for respective targets. For instance, calcium-dependant protein kinases (CDPK) are not expressed in mammalian cells but have been demonstrated in *C. parvum*. They appear to be constitutive in the parasite and treatment of *C. parvum* cultures with specific antiserum inhibits parasite replication dose dependently. Although the functions of CDPK remain to be characterized in *C. parvum* it appears that this may be a promising drug target (Etzold et al. 2009).

Altogether, cryptosporidia and particularly *C. parvum* are a rewarding area for research in many respects. Veterinary parasitologists have the duty to improve animal health and livestock productivity, to protect man from zoonotic transmission and to make use of any modern and traditional tools to achieve these ambitious goals. The multiple aspects to be considered demand inter-disciplinary networks based on mutual respect and understanding thus strengthening collaboration between researchers from the fields of veterinary, medical, biological and other sciences.

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Chapter 5
Neonatal Porcine Coccidiosis

Hanna Lucia Worliczek and Anja Joachim

Abstract ***Isospora suis***, the causative agent of **porcine neonatal coccidiosis** (isosporosis), was identified as an important pathogen of pigs only in the 1970s with the intensification of pig production in industrialised countries. The parasite is diagnosed with high prevalences in neonatal piglets and is associated with considerable economic problems due to diarrhoea and subsequent weight loss. While details of the life cycle and the more general features of the parasite have been described in detail, little is known about the host–parasite interplay which determines clinical and pathological outcome as well as the development of immunity. The dynamics of transmission and the pathogenesis of the disease are poorly understood, as are phenomena such as age resistance to the disease in piglets older than 3 weeks. In recent studies infection models mimicking the natural situation have been established for research on the host–parasite interactions and population dynamics of *I. suis*. The results indicate complex actions of the developing immune system in porcine neonates involving both innate and acquired immunity in the control of the parasite. Interactions of the parasite with the gut flora of the fast-developing neonatal pig are still not resolved, although an increase of clostridiosis is implied in outbreaks of isosporosis with increased mortality. Treatment with coccidiocides considerably improves piglet health; however, sustainable control regimes are still to be developed. New technologies like custom-made species-specific commercial immunological assays, in vitro propagation of *I. suis* in suitable host cells, genotyping of both host and parasite and the use of germ-free piglets for defined infection models will throw light on the unresolved questions surrounding *I. suis* and porcine coccidiosis.

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5.1 Introduction

Swine can be infected by several species of intestinal coccidians of the genera ***Eimeria*** and ***Isospora***. *Eimeria* spp. are usually found in weaned and adult pigs and are considered to be of minor importance as pathogens (e.g. Damriyasa and Bauer 2006; Daugschies et al. 2004). *Isospora suis*, on the other hand, is the causative agent of neonatal porcine coccidiosis, a disease affecting piglets up to the age of 3 weeks. It is spread worldwide and affects pig breeding units independently of the farm management system (Lindsay et al. 1992; Meyer et al. 1999). *I. suis* was first described in 1934 (Biester and Murray 1934) and beginning with the 1970s it was recognised as one of the most important causes of diarrhoea of suckling piglets, maybe due to an increasing importance of pig breeding since that time (Harleman 1977; Martineau and del Castillo 2000; Stuart et al. 1979; Stuart et al. 1980). While the infection is rarely fatal it can severely affect weight gain with all its economic consequences, including considerable treatment costs on farms with persevering problems.

In the field of comparative research on intestinal protozoa *I. suis* may serve as a model for mammalian coccidiosis, including human infections with *Isospora belli*, the causative agent of human isosporosis which is considered an emerging disease (Curry and Smith 1998). *Isospora suis* is also a model pathogen for diarrhoeal diseases in newborns which frequently affect neonates including piglets and are characterised by a complex interaction of primary pathogens, the maturing immune system, maternal and nutritional influences and the developing intestinal flora (Bailey et al. 2001, 2005; Grierson et al. 2007; Solano-Aguilar et al. 2001; Stokes et al. 2004). All these considerations led to an increased interest in piglet coccidiosis in clinical and basic research.

5.2 *Isospora suis*: A Coccidian Parasite Like Any Other?

Like other intestinal coccidia *I. suis* sporozoites excyst after ingestion and invade their host cells, in this case the epithelial lining of the small intestines, primarily the jejunum, to multiply asexually. In contrast to members of the genus *Eimeria*, however, the merozoites of *I. suis* cannot be characterised by subsequent generations but merely by types (Lindsay et al. 1980; Matuschka and Heydorn 1980). The asexual stages multiply by endodyogeny and the occurrence of schizogony is discussed (Harleman and Meyer 1984; Lindsay et al. 1980; Matuschka and Heydorn 1980). The intestinal development ends with gamogony and the production of a zygote which is excreted as an unsporulated oocyst (Fig. 5.1). Merogony and gamogony can frequently be found in parallel (Matuschka and Heydorn 1980), and both meronts (Niestrath et al. 2002) and gamonts (Vítovec and Koudela 1987) are described as the pathogenic stages. The development of *I. suis* is extremely rapid; oocysts are excreted as early as 4–5 days after infection (Lindsay et al. 1980;

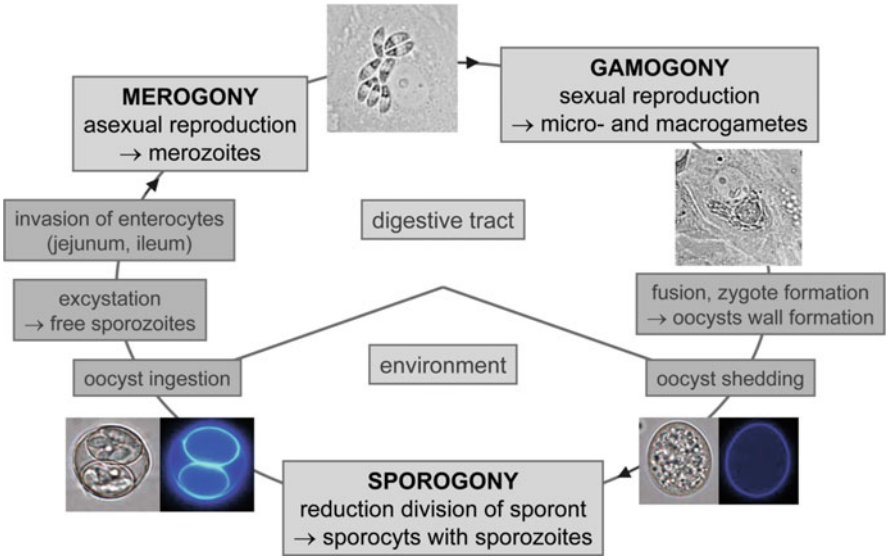

Fig. 5.1 Life cycle of *I. suis*. Oocysts are shown in bright-field and epifluorescent microscopy. Merozoites and microgametes with residual body are shown in phase-contrast microscopy of *I. suis* infected IPEC-J2

Mundt et al. 2006; Vítovec and Koudela 1990) and require only between 1 and 2 days for sporulation in the warm and humid environment of a farrowing unit (Lindsay et al. 1982). Consequently, the life cycle is completed within 5–6 days postinfection (p.i.). Survival of sporulated oocysts is supported by high humidity (75–100%) (Langkjaer and Roepstorff 2008). Characteristically the excretion of oocysts takes place in waves with several (two, sometimes more) peaks at day 5–9 and 11–14 p.i. (Harleman and Meyer 1984; Matuschka and Heydorn 1980; Vítovec and Koudela 1990; Worliczek et al. 2010a). The cause for this undulating excretion pattern is not known; the interruption of excretion can even lead to the absence of parasite stages in the faeces (Mundt et al. 2006; Worliczek et al. 2009b). It was postulated that the intestinal development is interrupted and re-initiated leading to this multiphasic development (Christensen and Henriksen 1994; Matuschka and Heydorn 1980), other authors claim that the return of extra-intestinal stages has this effect (Harleman and Meyer 1984). The latter statement is based on the finding that other *Isospora* species can be found in extra-intestinal tissues (Lindsay et al. 1997); however, unequivocal proof of this hypothesis is still lacking for *I. suis*.

Unlike with other mammalian coccidians infections with *I .suis* on the first day of life lead to severe clinical outcomes with decreased severity as the animals grow older (Koudela and Kučerová 1999; Matuschka and Heydorn 1980; Stuart et al. 1982a, b; Worliczek et al. 2009b). In contrast, infections with *Eimeria* spp. in the first days of life are sometimes less prolific compared to animals at the age of a few weeks as shown in experimental infections of rabbits and cattle (Coudert et al. 1995; Daugschies and Najdrowski 2005; Pakandl et al. 2008a). The reasons for this

are not known; turnover rates of the epithelial lining or the presence of certain gut bacteria may be crucial for optimised development of the parasites in the epithelium.

5.3 Veterinary Aspects of Piglet Coccidiosis

While pigs of all age groups can excrete oocysts only suckling piglets develop the typical signs of disease, i.e. transient pasty or watery diarrhoea which is characterised by a sudden onset with high morbidity and low mortality within a litter. For reasons still unknown, clinical outcome and oocyst shedding vary greatly between individual piglets (Martineau and del Castillo 2000; Mundt et al. 2006). Clinical observations with increased mortality may be related to cases of clostridiosis (Mundt et al. 2008) or other co-infections (Vítovec et al. 1991) which could be favoured by the destruction of the intestinal lining. However, studies in gnotobiotic piglets have shown that *I. suis* is a primary pathogen that can cause excessive damage in the absence of synergistic pathogens (Harleman and Meyer 1985).

By the time the life cycle of *I. suis* is terminated – usually within 10–14 days p.i. – the pathological changes, characterised predominantly by villous atrophy, are still noticeable at that time (Mundt et al. 2006). Interestingly, the occurrence of pathological alterations is not always associated with the presence of parasites in the investigated section of the small intestine (Harleman and Meyer 1985). Additionally, the amount of endogenous stages in the epithelial cells seems to be relatively low in relation to observed pathological effects (Lindsay et al. 1980). This might explain why the weight losses can be severe in affected piglets while the number of excreted parasites is low (Mundt et al. 2006). Whether immunological influences (promoting inflammation and further destruction of intestinal tissue) or disturbances in the gut flora play a supportive role in this remains to be determined.

While surveys targeting suckling piglets constantly reveal the high **prevalence** of *I. suis* in this age group worldwide (Lindsay et al. 1992; Meyer et al. 1999; Mundt et al. 2005; Torres 2004), the diagnosis of the parasite in practice still remains a challenge, partly because individual excretion is sometimes restricted to a very short time period of only a few days (Mundt et al. 2006). Autofluorescence microscopy is more sensitive than McMaster counting for detection of oocysts in faeces and can be performed on small amounts of samples (Daugschies et al. 2001). Therefore, this method is preferable in laboratories equipped with a fluorescence microscope. PCR is the most sensitive method (Joachim et al. 2004) but generally considered too expensive for routine application.

Coccidiosis in livestock is of great economic importance and causes significant losses (Figs. 5.2 and 5.3), especially with eimeriosis in poultry and cattle as well as isosporosis in pigs. *Isospora suis* infections are common, with prevalences of more than 70% on the farm level in Germany, Austria and Switzerland (Mundt et al. 2005). Good hygiene can significantly reduce the infection pressure but does not

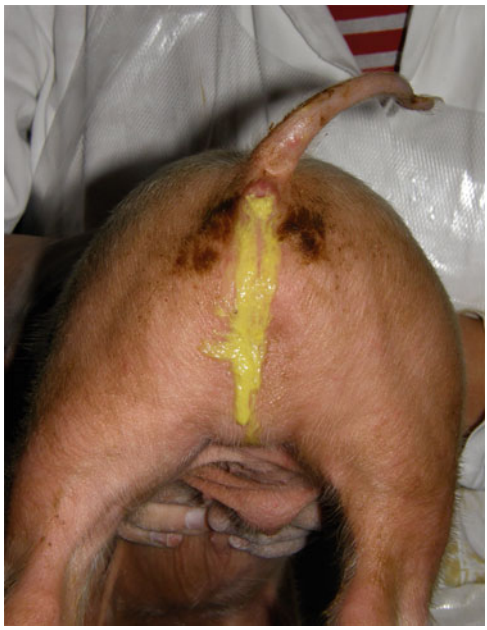

Fig. 5.2 Non-haemorrhagic, creamy to watery diarrhoea during isosporosis

Fig. 5.3 Uneven weaning weights in litter mates (3 weeks of age)

prevent infection (Sotiraki et al. 2008). Kresol-based disinfectants inactivate oocysts of *I. suis* and can therefore contribute to further reduction of infectious stages in the pens (Straberg and Daugschies 2007). Effective chemotherapy is achieved with toltrazuril (Baycox® 5%; Toltranil®). This agent prohibits intracellular development of coccidia including *I. suis* (Haberkorn and Mundt 1988; Mundt et al. 2007) and consequently abrogates oocyst excretion and effects on animal health such as diarrhoea, enteritis and weight loss (Bach et al. 2003; Mundt et al. 2007). Currently, it is the only coccidiocide registered for piglets in the EU for the metaphylactic control of the disease. Treatment of experimentally infected piglets during the prepatent period reduces diarrhoea caused by *I. suis* and thus

increases the weight gain significantly (Mundt et al. 2006) which results in an increase in profit per animal (Scala et al. 2009).

However, recently concerns have arisen regarding the **loss of efficacy** of treatment and the subsequent lack of control of piglet isosporosis in veterinary practice. Little is known about the fundamentals of pathogenesis and immunology, slowing progress for the development of sustainable control concepts. Further research is required to be able to tackle problems with the obscure transmission patterns of porcine coccidia and to exert sufficient control of the parasite. This requirement can be extrapolated to basic research on host–parasite interaction and the development of the immune response.

5.4 Immunology of Porcine Isosporosis

The mechanisms of **host–parasite interactions** in *I. suis* infections are still poorly understood. Earlier studies led to the belief that the parasite induces immunity (Stuart et al. 1982b), and it is assumed that this immunity is based on cellular components, since supernatants from lymphocytes sensitised with *I. suis* antigen induce chemotaxis in leukocytes. Antibodies on the other hand do not seem to be protective (Schlepers et al. 2009; Taylor 1984). It has been speculated that the ability of piglets to mount an IgA antibody response is poorly developed in the first weeks of life (as reviewed by Baker et al. 1994), but this remains to be investigated further. The enlargement of the mesenteric lymph nodes during the infection is an indication for a parasite-specific immune response (Harleman and Meyer 1985; Vítovec and Koudela 1990). Reinfection of previously infected piglets results in reduced oocyst excretion; however, an age-related resistance to isosporosis in piglets has also been described where animals at 3 weeks of age excreted fewer oocysts after primo infection compared to piglets in the first week of life while there was no significant difference in oocyst excretion between a primo or a reinfection in 3-week-old animals (Koudela and Kučerová 1999; Stuart et al. 1982a). Age resistance could be related to the development of innate as well as antigen-specific immune responses and/or with accelerated cell turnover in the gut mucosa of older piglets. The effect of parasite-specific immunity versus age-related resistance on parasite shedding and clinical outcome in challenge infections cannot easily be dissected due to this phenomenon.

Our understanding of the immune responses and the mechanisms of **immunopathogenesis** in porcine infectious diseases has greatly improved with the characterisation of surface proteins characteristic for distinct leukocyte populations (termed clusters of differentiation, CD, or swine workshop cluster, SWC) and consequently a growing immunological toolkit for pigs is available (see Lunney 1993; Saalmüller 1996; Saalmüller et al. 1998). Certain T-cell subpopulations are defined by their expression of CD4 and CD8. In swine the functions of these subpopulations are similar to those of other species but there are some peculiarities. In the majority of immunologically investigated species

CD8 molecules consist of an α and a β chain and are expressed as heterodimers. In swine a substantial proportion of cells expressing CD8α monomers and also homodimers exist. In contrast to other mammals, pigs show a high percentage of extrathymic $CD4^+CD8\alpha^+$ T lymphocytes and also $CD4^-CD8\alpha^-$ lymphocytes – mostly with a co-expression of T-cell receptor(TcR)-γδ – in addition to $CD4^+$ $CD8\alpha^-$ and $CD4^-$ $CD8\alpha^+$ lymphocytes (Saalmüller et al. 1987, 1989, 2002; Summerfield et al. 1996). Classical MHC-I restricted cytotoxic T-lymphocytes (CTL) express high proportions of CD8α and also CD8β but no CD4 (reviewed in Gerner et al. 2009). T-helper (TH) cells in pigs are defined as $CD3^+CD4^+$ cells with or without the co-expression of CD8α. The $CD8\alpha^-$ population seem to comprise resting TH cells which acquire CD8α and upregulate MHC-II upon activation. The $CD4^+CD8\alpha^+$ population consists of activated TH cells with the expression of CD8α and $CD25^+$ but also of memory-TH cells without CD25 (Gerner et al. 2009;0 Saalmüller et al. 2002).

T cells expressing a TcR with a γ and a δ chain (TcR-$\gamma\delta^+$ T cells) show in some cases a cytolytic activity independent of MHC-presentation and are able to recognise unprocessed non-peptide antigen, as is found in parasites, fungi and plant extracts (Kamath et al. 2003; Saalmüller et al. 1994, 1999; Tanaka et al. 1994). TcR-$\gamma\delta^+$ cells are involved in epithelial wound repair, the induction of tolerance, and they are able to produce pro- and anti-inflammatory cytokines and may be able to present antigen by MHC-II. The production of IFN-γ by TcR-γδ T cells in the gut mucosa attracts macrophages and induces the production of nitric oxides by enterocytes as a defence mechanism against pathogens. Moreover, TcR-$\gamma\delta^+$ cells are able to express a variety of pattern-recognition receptors associated with the adaptive as well as the innate immune system. Porcine TcR-γδ T cells express cytokines and chemokines similar to that of humans and mouse and might therefore have the same functional properties (Takamatsu et al. 2006). In pigs, TcR-γδ T cells are able to proliferate in the intestines and recirculate to peripheral blood; therefore they seem to be an important pool of circulating effector cells (Thielke et al. 2003). The proportion of porcine TcR-γδ T cells (15–30%) among circulating lymphocytes of adult swine is considerably higher than in mice and men and is even higher in piglets (Takamatsu et al. 2006).

Regarding **neonatal porcine coccidiosis**, TcR-$\gamma\delta^+$ cells were identified as the most promising target for further research of the local immune response in the gut. A strong increase of this cell population is observed in the jejunal lamina epithelialis (from 0.5 to 15% area fraction of lymphocyte profiles per mucosa profile) and the lamina propria (from 5 to 19%) of infected piglets (age 7–16 days). On the other hand, TcR-$\gamma\delta^+$ cells have a severely reduced frequency in the peripheral blood and the MLN of these animals. This leads to the hypothesis of a migration of lymphocytes from blood and secondary lymphoid organs to the site of infection, and probably also specific local proliferation. Especially in the lamina propria other T cells have to be involved since the increase in T cells in this compartment is not only caused by TcR-$\gamma\delta^+$ cells. These T cells might have been drained from blood, spleen or the MLN where $CD3^+$ cells are reduced in infected piglets (Worliczek et al. 2010a). TcR-γδ T cells are seen as a linkage between

adaptive and innate immune response and an important line of defence in young animals. The ability of lymphocytes to respond to mitogens is limited in piglets in the investigated age group (Becker and Misfeldt 1993; Schwager and Schulze 1997), reflecting the immature status of the neonatal porcine immune system. Therefore, a defence strategy which is at least partly independent of MHC-restricted antigen presentation seems likely.

NK cells ($CD3^{-}CD8\alpha^{high}CD4^{-}$) were shown to have a higher frequency in the spleens of *I. suis* infected piglets (Worliczek et al. 2010a). This might lead to the assumption that they are involved in the early immune response to *I. suis*, as shown for other infections with coccidia such as *Toxoplasma* or *E. papillata* (Korbel et al. 2004; Schito and Barta 1997). Also T-helper cells (including resting TH and activated and memory TH) might be involved in the primary immune response to *I. suis* as it was already shown for *Eimeria* infections in other hosts (reviewed in Worliczek et al. 2007). They are significantly reduced in infected animals and might migrate to the gut mucosa during the infection (Worliczek et al. 2010a).

The involvement of CTL in the immune response to coccidiosis and especially their requirement for the development of a protective immunity is reported for infections with *E. tenella* and *E. acervulina* in chicken (Lillehoj and Trout 1996; Swinkels et al. 2006), but also for infections with *E. intestinalis* in rabbits (Pakandl et al. 2008b) and for *E. bovis* in calves (Hermosilla et al. 1999). For *I. suis* a significant age influence on the distribution of this cell population but not of the infection itself can be detected in the spleen (Worliczek et al. 2010a). This indicates that the distribution of CTL in this organ depends on the age of the piglets and they are probably not involved in the primary immune response to *I. suis*.

Since *I. suis* cannot be propagated in large numbers in vitro, antigen material processed for functional studies on the cell populations in question is currently produced from oocysts excreted with the faeces. These must be purified to a high degree to avoid effects of bacterial contaminants on assay outcome (Worliczek et al. 2010b). Pure material and corresponding mock controls can be produced by flotation and coupled FACS sorting of oocysts. Preliminary investigations have shown that T cells of infected animals produce IFN-γ, a key effector molecule in cellular immune response to intra-cellular pathogens (Taylor et al. 2007), upon stimulation with such purified antigen material (Worliczek et al. 2010b). The application of this technique in, for example an ELISPOT assay combined with magnetic cell sorting provides an additional tool for functional analyses of cell populations which might be involved in the immune response to *I. suis* (Worliczek et al. 2009a).

5.5 Unresolved Questions

Although our knowledge on *I. suis* infections in piglets has increased considerably in recent years, a number of questions still need to be addressed. These include **interactions of the parasite with the host cell**; this would be feasible by in vitro

cultivation of the parasite. Propagation of *I. suis* has been achieved previously in various cell lines (Fayer et al. 1984; Lindsay and Blagburn 1987; Lindsay et al. 1991, 1998) and in chicken embryos (Lindsay and Current 1984). Recently, complete development was demonstrated in porcine intestinal epithelial cells (IPEC-J2) from the jejunum of a neonatal pig, which closely mimics the in vivo situation (Ruttkowski et al. 2009). Moreover, the (functional) characterisation of the immune response needs to be elucidated further. This includes interaction with cells of the innate and humoral immune systems. This research will be the basis for the development of non-chemical intervention strategies. Sustainable control also includes the prevention of the development of drug resistance (the mechanisms of which are yet unknown), the development of new drug classes and the reduction of drug use based on detailed knowledge on transmission patterns and pathogenesis. The latter includes research on the relationship between the host, the parasite and the gut flora. More refined animal models such as germ-free piglets infected with sterile *I. suis* oocysts can be utilised for such studies as demonstrated previously (Harleman and Meyer 1985; Vítovec and Koudela 1990; Vítovec et al. 1991). Finally, strains of *I. suis* with defined biological properties (e.g. prepatent periods, clinical and pathological outcome or reproductive capacity or susceptibility to anticoccidial compounds) should be compared on the genetic level to unravel the molecular mechanisms of development, pathogenesis and drug resistance. Finally, when the host–parasite relationship is approached from the porcine side, the genetic basis of clinical variation in disease outcome can be investigated by trait mapping of pigs of different breeds with varying susceptibility to the parasite as shown for other coccidia (Reiner et al. 2002, 2007).

As research on porcine isosporosis progresses further questions will most likely arise on this complex and important animal disease, which could be tackled with the help of biotechnological tools as demonstrated in recent years.

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Chapter 6
Post-translational Modifications in Apicomplexan Parasites

Esther Jortzik, Sebastian Kehr, and Katja Becker

Abstract Post-translational protein modifications are covalent modifications of amino acid side chains that give rise to a high number of proteins deriving from a relatively small number of genes. The biological functions of post-translational modifications in cells are as diverse as their nature and play a significant and widespread role in the regulation of parasitic proteins. Parasites belonging to the phylum *Apicomplexa* are the causative agents of fatal diseases including malaria, toxoplasmosis, and cryptosporidiosis. In apicomplexan parasites, post-translational modifications are crucial for various processes, such as invasion into and egress from host cells (phosphorylation, prenylation, and palmitoylation), intracellular signaling (*S*-nitrosylation), trafficking (ubiquitination, prenylation, palmitoylation), hemoglobin degradation (proteolytic processing), regulation of gene expression (methylation and acetylation), cell cycle regulation (ubiquitination), and many more. Due to their essential functions, several post-translational modifications attract considerable attention with regard to antiparasitic drug development. A range of enzymes that mediate certain post-translational modifications are studied as highly interesting antiparasitic drug targets, including prenyltransferases, proteases, methyltransferases, histone acetytransferases and histone deacetylases, and deubiquitinating enzymes. In this article, we aim to summarize the current knowledge on post-translational modifications, their regulation, and their functions in apicomplexan parasites.

6.1 Introduction

Apicomplexan parasites cause severe diseases worldwide, with *Plasmodium falciparum* and *Toxoplasma gondii* being the most prominent representatives. Malaria accounts for 225 million clinical cases with nearly 1 million deaths per

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year mainly in young children. The malaria parasite *Plasmodium falciparum* undergoes a complex life cycle both in the *Anopheles* mosquito vector and the human host with major metabolic and morphological changes (for a review on the biology of *Plasmodium* please see (Sherman 2005)). The protozoan parasite *Toxoplasma gondii* infects approximately one third of the human population worldwide. Although most *Toxoplasma* infections are benign, severe opportunistic diseases affect immunodeficient or immunosuppressed individuals or children infected *in utero*. *T. gondii* is an obligate intracellular organism that uses an actin-myosin-based motility system to actively invade nucleated host cells (Keeley & Soldati 2004). Like *T. gondii*, the apicomplexan parasite *Cryptosporidium* spp. cause infections that are especially harmful for immunocompromised individuals.

Posttranslational modifications were shown to play a versatile role in the regulation of parasitic proteins (Le Roch et al. 2004; Llinas et al. 2006; Foth et al. 2008). Post-translational protein modifications comprise a diverse spectrum and can be found on 15 out of the 21 proteinogenic amino acids (Uy & Wold 1977). The enzymatic modifications of proteins provide powerful mechanisms to regulate protein function both rapidly and reversibly, and are required for immediate adaptation to varying conditions in the cell. In parasites, post-translational modifications have been implicated in various processes such as gliding motility and invasion into host cells, growth and cell cycle regulation, and gene expression.

6.2 Phosphorylation and Dephosphorylation

The **reversible phosphorylation** of proteins represents a ubiquitous post-translational modification balanced by the antagonistic activities of protein kinases and protein phosphatases. Protein phosphorylation is the major way to regulate protein activity and a crucial part of cellular signaling cascades. Phosphorylation has been intensely studied, including phosphorylation events in several apicomplexan parasites. Both protein kinases and phosphatases have been in the focus of drug discovery in recent years and inhibitors of phosphorylation events have been demonstrated to exhibit antiparasitic activity (Cohen 2002; Easty et al. 2006; Tautz et al. 2006; Doerig and Meijer 2007).

Protein kinases (PK) catalyze the transfer of a phosphoryl group from ATP to a hydroxyamino acid of the residues serine, threonine, and tyrosine (Hanks 2003). The size of the protein kinase family, the so-called kinome, differs between organisms with approximately 500 members in human and 99 members in *Plasmodium* (Manning et al. 2002; Anamika et al. 2005). According to phylogenetic trees, protein kinases cluster into seven major groups: the tyrosine kinases (TK), the tyrosine kinase-like group (TKL), cyclic nucleotide- and calcium-phospholipid-dependent kinases (AGC, including the PKA, PKG and PKC families), calmodulin-dependent kinases (CAMK), the casein kinase 1 (CK1), STE (PKs functioning in the MAPK kinase cascades), and the CMGC group (including cyclin-dependent kinases, mitogen-activated protein kinases) (Doerig et al. 2008). Protein kinases

Table 6.1 Summary of post-translational modifications in *Apicomplexa*

Amino acid residue	Modification	References	Functions
	Methylation	Miao et al. (2006)	Transcriptional regulation, RNA metabolism
Arginine	Citrullination	nd	
	Glycosylation	Mendonca-Previato et al. (2005)	Anchoring proteins to lipid bilayers
Asparagine	Deamidation	nd	
Aspartate	Succinimide formation	nd	
	Nitrosylation	Venturini et al. (2000a, b); Colasanti et al. (2001)	Signaling
	Glutathionylation	nd	
	Palmitoylation	Rees-Channer et al. (2006); Russo et al. (2009)	Gliding motility, cell cycle processing
	Prenylation	Chakrabarti et al. (1998); Wiesner et al. (2004)	Cell cycle transition
Cysteine	Dolichylation	D'Alexandri et al. (2006)	Intracellular localization?
	Polyglutamylation	Fennell et al. (2008)	Merozoite biogenesis
	Methylation	nd	
	Carboxylation	nd	
Glutamate	Polyglycation	nd	
Glycine	Myristoylation	Stafford et al. (1996); Rahlfs et al. (2009); Russo et al. (2009)	Trafficking, energy metabolism
Glutamine	Transglutamination	nd	
	Acetylation	Fan et al. (2004), (2009); Trelle et al. (2009)	Regulation of gene expression through histone modification, actin modification
Lysine	Methylation	Xiao et al. (2010); Chookajorn et al. (2007); Lopez-Rubio et al. (2007)	Gene silencing, tubulin modification (new)
	Ubiquitination	Hunt et al. (2007); Philip and Haystead (2007); Ponts et al. (2008)	Protein degradation, drug resistance, chromatin remodeling, transcription, vesicular trafficking
	Sumoylation	Issar et al. (2008)	Invasion, immune escape, gene regulation
	ADP-ribosylation	French et al. (2008)	
Proline	Hydroxylation	nd	
	Glycosylation	Khan et al. (1997); Stwora-Wojczyk et al. (2004)	
Serine/threonine	Phosphorylation	Yokoyama et al. (1998); Anamika et al. (2005)	Growth regulation, cell cycle progression
	Sulfation	Choe et al. (2005)	Erythrocyte invasion
Tyrosine	Phosphorylation	Shiu and Li (2004); Andreeva and Kutuzov (2008)	No tyrosine phosphorylation in *Apicomplexa*

regulate nearly all activities of eukaryotic cells, including proliferation, gene expression, metabolism, membrane transport, and apoptosis.

Protein kinases have crucial roles in all stages of the life cycle of malaria parasites: CDPK6 is involved in sporozoite infectivity (Coppi et al. 2007). Erythrocytic schizogony depends on PK7 (Dorin-Semblat et al. 2008), CDPK1 (Kato et al. 2008), and the mitogen-activated PK Map-2 (Dorin-Semblat et al. 2007). The initiation of male gametogenesis of malaria parasites is controlled by the cGMP-dependent PK PKG (McRobert et al. 2008), the calcium-dependent PK CDPK4 (Billker et al. 2004), and the mitogen-activated PK Map-2 (Rangarajan et al. 2005). The NIMA-related kinases Nek4 and CDPK3 are involved in ookinete maturation and motility (Reininger et al. 2005; Siden-Kiamos et al. 2006), while PK7 is important for the development of oocysts (Dorin-Semblat et al. 2008). Experiments with protein kinase inhibitors show that protein kinases are essentially involved in the erythrocyte invasion process of malaria parasites by phosphorylation of the glideosome complex (Ward et al. 1994; Jones et al. 2009). There is increasing evidence that protein kinases are involved in host–parasite interactions: PKA influences the permeability of the erythrocyte plasma membrane and affects the activity of an erythrocyte anion channel (Dorin-Semblat et al. 2008; Merckx et al. 2009). In *T. gondii*, a rhoptry protein with kinase activity is secreted into the host cell cytoplasm and is responsible for the parasites' virulence (Taylor et al. 2006). Although an increasing number of protein kinases are identified in apicomplexan parasites, the knowledge about their cellular targets is limited.

Protein phosphatases (PP) catalyze the hydrolyzation of monophosphate esters from phosphorylated proteins, thereby restoring the hydroxyamino acid to its unphosphorylated state. Phosphatases are divided according to different classifications based on substrate type (proteinaceous or nonproteinaceous), pH optimum (alkaline versus acid phosphatases), size (high versus low molecular weight phosphatases), on the identity of the phosphorylated amino acid (serine, histidine, or cysteine phosphatases), or on signature motifs and their substrate specificity. Since this article focuses on post-translational modifications, we concentrate on protein phosphatases. Compared to the number of kinases, the number of protein phosphatases – the phosphatome – is relatively small. In *Plasmodium*, 27 protein phosphatases are complemented by seven phosphatases that dephosphorylate nonprotein substrates (Wilkes and Doerig 2008). This is in part compensated by their low substrate specificity and a high catalytic activity. Additionally, a large repertory of accessory proteins act as regulatory subunits of the limited number of phosphatase catalytic subunits, and influence the specificity and activity of the enzymes (Cohen 1997; Gallego and Virshup 2005). *Apicomplexa* have homologues of most human protein phosphatase subfamilies, mainly as single isoforms. In contrast to that, not all apicomplexan protein phosphatases have homologs in their vertebrate host: *Apicomplexa* have two bacterial-like phosphatase families, the kelch repeat domain-containing phosphatases (PKLL) and Ca^{2+}-binding phosphatases with EF-hand domains (EFPP) (Kutuzov and Andreeva 2008). In general, four large families of protein phosphatases can be identified: the phosphoprotein phosphatases (metallophosphatases, PPP), protein tyrosine phosphatases

(PTP), serine/threonine phosphatases (PPM), and NLI interacting factor-like phosphatases (NIF) (Wilkes and Doerig 2008).

Members of the phospho-protein phosphatase (PPP) family are involved in various processes in *Apicomplexa*: Protein phosphatase 2C of *Plasmodium* dephosphorylates the translation-elongation factor 1-β and thereby inhibits its nucleotide exchange activity (Mamoun and Goldberg 2001). YHV1 phosphatase of malaria parasites, a dual-specificity phosphatase with a Zn finger motif, is involved in nuclear protein activity (Kumar et al. 2004). Both *Plasmodium* and *Toxoplasma* employ PPP for invasion: *Plasmodium* PP1 interferes with the release of merozoites by dephosphorylating skeleton-binding protein 1, which influences the erythrocyte membrane stability (Blisnick et al. 2006), while a type one serine/threonine phosphatase of *Toxoplasma* invasive tachyzoites is involved in host cell invasion (Delorme et al. 2002).

Serine/threonine phosphatases are classified either in the PPP superfamily (comprising PP1, PP2A, PP2B, and P5 families) or the PPM superfamily (including Mg^{2+}- and Mn^{2+}-dependent phosphatases) and have crucial functions in apicomplexan parasites: PPM are essential for the survival of *Plasmodium* and regulate growth during the intraerythrocytic stage (Yokoyama et al. 1998). A type 2C PPM of *T. gondii* influences cell cycle progression and plays a key role in controlling actin dynamics (Delorme et al. 2003; Jan et al. 2009).

Protein tyrosine phosphatases (PTP) are of great importance for cellular signaling, cell cycle control and differentiation in eukaryotes. Interestingly, *Apicomplexa* do not have PTP homologs (Andreeva and Kutuzov 2008), which is consistent with the absence of tyrosine kinases (Shiu and Li 2004).

In addition to the catalytic subunits of protein phosphatases, the analysis of regulatory subunits of apicomplexan protein phosphatases needs further research; nevertheless, phosphatases of apicomplexan parasites are of comparable interest for the development of antiparasitic drugs as protein kinases.

6.3 Lipidation

The post-translational lipid modifications of proteins comprise *S*-acylation in the form of *N*-myristoylation and palmitoylation, as well as isoprenylation. Lipid modifications facilitate the membrane attachment of soluble proteins and subcellular targeting, stabilize protein–protein interactions, and can act as reversible switches to modulate signaling processes. Furthermore, proteins are partitioned into specific membrane domains by lipid modifications, allowing them to activate signaling cascades at specific subcellular domains. Additionally, combinations of diverse lipid modifications enable homologous proteins to be differentially targeted to different membrane compartments.

***N*-myristoylation** is a co-translational modification of N-terminal glycine residues. The $C_{14:0}$ fatty acid myristate is linked via an amide bond to the N-terminal glycine residue which is often part of a MGCCC(S/T) consensus sequence (Maurer-Stroh et al. 2002). *N*-myristoylation increases the hydrophobicity of polypeptides thereby enhancing the association with membranes and other proteins. *N*-myristoylation is required but insufficient for anchoring proteins in membranes and often occurs in combination with the palmitoylation of proximal cysteine residues (Martinez et al. 2008). Myristoylation can also support reversible membrane binding via conformational changes with so-called myristoyl switch mechanisms (McLaughlin and Aderem 1995). *N*-myristoylation has been shown to be essential for the survival of yeast and fungi (Duronio et al. 1989; Weinberg et al. 1995). Several proteins of the malaria parasite *Plasmodium* are known to be myristoylated such as adenylate kinase 2, calpain, and ADP-ribosylation factor 1 (Stafford et al. 1996; Rahlfs et al. 2009; Russo et al. 2009), and a functional myristoyl-CoA:protein *N*-myristoyltransferase of the parasite has been characterized (Gunaratne et al. 2000). Although some proteins are known to be myristoylated, the function of *N*-myristoylation in apicomplexan parasites remains to be studied in detail.

Palmitoylation is the post-translational addition of the $C_{16:0}$ fatty acid palmitate to the side chain of cysteine through a reversible thioester linkage. Palmitoylation has no single sequence requirement besides the presence of a cysteine residue and is often associated with either prenylation or *N*-myristoylation. The reversibility of palmitoylation regulates the stability of protein interactions with membranes, partitions proteins into distinct membrane domains and modulates protein structure (Linder and Deschenes 2007). Palmitoylation is known to occur on a wide variety of proteins, including peripherally associated and integral membrane proteins. Furthermore, palmitoylation is involved in protein trafficking and signaling, organelle inheritance, and vesicle fusion (Smotrys and Linder 2004). Palmitoylation events occur in apicomplexan parasites, but are rarely studied. Palmitoylation appears to be involved in the gliding motility of *Toxoplasma* and *Plasmodium*, since the gliding associated protein GAP45, a component of the glideosome complex involved in binding the actin–myosin motor complex to the membrane, is both myristoylated and palmitoylated (Rees-Channer et al. 2006). Furthermore, myristoylation-dependent palmitoylation is known to control the localization of the cysteine protease calpain from *Plasmodium*, which is critically involved in cell cycle progression during trophozoite development (Russo et al. 2009).

Protein prenylation includes the formation of covalent thioester bonds between farnesyl or geranylgeranyl groups and cysteine residues at the carboxy-terminal end of proteins via the activity of protein farnesyl- or geranylgeranyl-transferase. Protein prenylation generally promotes membrane association and protein–protein interactions by creating a hydrophobic tail. Following prenylation, proteins undergo two additional post-translational modifications, which are termed CaaX processing (Young et al. 2000). Protein prenylation has

been shown to be critical for various cellular activities, including proliferation and apoptosis (Sebti 2003). Although the knowledge about the targets of protein prenylation is limited, the post-translational modification has been observed in several parasites, including *T. gondii* and *P. falciparum* (Ibrahim et al. 2001; Chakrabarti et al. 2002). While animals, fungi, and archaebacteria employ the classical mevalonate pathway for isoprenoid synthesis, *Apicomplexa* synthesize the isoprenoid precursor via the 1-deoxy-xylulose 5-phosphate pathway in the apicoplast, a plastid-like organelle (Rohrich et al. 2005). Both farnesyl- and geranyltransferase are in the focus of drug development for cancer therapy (Sebti and Hamilton 2000), while inhibitors of farnesyltransferase are studied intensely for the treatment of parasitic diseases. Protein farnesyltransferase inhibitors affect the transition of malaria parasites from ring stage to trophozoite stage, demonstrating that protein prenylation is crucial for the growth of protozoan parasites (Chakrabarti et al. 1998; Wiesner et al. 2004).

6.4 *S*-Nitrosylation

Several oxidative reactions on cysteine residues can influence a variety of protein functions. The redox state of cysteine residues represents a dynamic and tightly regulated balance that crucially influences the activity of a protein as well as its subcellular distribution and its interaction with other proteins. Among the different oxidative protein modifications, *S*-nitrosylation is a post-translational modification with emerging influence on cellular functions and signaling events. *S*-nitrosylation is one of the major nitric oxide (NO) signaling pathways. NO acts as an intracellular messenger, hormone, and neurotransmitter that regulates cellular processes such as smooth muscle relaxation, neurotransmission, platelet inhibition, and immune regulation (Lowenstein and Snyder 1992; De Caterina et al. 1995; Shin et al. 1996). Therefore, *S*-nitrosylation represents the prototype of redox-based thiol-dependent cellular signaling mechanisms. The analysis of *S*-nitrosothiols *in vivo* is difficult due to the low stability and low concentration of this modification. *S*-nitrosothiols undergo photolytic degradation or are reduced by reducing agents such as glutathione (Singh et al. 1996).

NO-donors interrupt the life cycle of several parasites, including *Plasmodium* (Clark and Rockett 1996). It has been shown that the cysteine proteases falcipain and cruzipain are inactivated by *S*-nitrosylation of cysteine residues, which results in the death of the parasite (Venturini et al. 2000a, b; Colasanti et al. 2001). Additionally, *Plasmodium* plasmepsin, a pepsin-like aspartic protease involved in hemoglobin degradation, is inhibited by NO donors, which is most likely due to *S*-nitrosylation of a cysteine residue in the catalytic site (Sharma et al. 2004). *S*-nitrosylation is a young and mostly unexplored research field. NO production and *S*-nitrosylation as fundamental mechanisms for inter- and intracellular signaling might be of great importance when studying the biology and pathophysiological mechanisms of apicomplexan parasites. Strategies for upregulating

NO-mediated *S*-nitrosylation of proteases in order to block hemoglobin degradation should be considered for antiparasitic chemotherapeutic approaches.

6.5 Protein Processing

All pathogens of the phylum *Apicomplexa* spend a part of their life inside a host cell or cyst. Apicomplexan parasites produce a large number of proteases, which are essential for the parasite's survival since they have crucial function in host cell invasion and egress, nutrition, and processing of precursor proteins. Due to the fact that *Apicomplexa* are obligate intracellular parasites, their survival depends on their ability to invade cells and propagate intracellularly, which in turn critically depends on secreted proteases that create a hospitable environment for the parasites.

6.5.1 Host Cell Invasion and Egress

Entry into (invasion) and exit from (egress) host cells require a broad range of specialized parasite molecules. Invasion of malaria parasites into host cells comprises contact, interaction, and junction between the erythrocyte surface and the merozoite leading to active entry of the parasite into the host cell. This is mediated by adhesins and several GPI-anchored proteins (PfAMA-1 and the merozoite surface proteins MSP) (reviewed in Cowman and Crabb 2006). For invasion of apicomplexan parasites into the respective host cells, several serine proteases of the subtilisin and rhomboid families play the most important roles. The subtilisin-like protease PfSUB-1 from *P. falciparum* possesses a dual role in both erythrocyte invasion and egress. PfSUB-1 is responsible for proteolytic maturation of three merozoite surface proteins (MSP1, 6, and 7), which form the major protein complex on the merozoite surface and are essential for a successful invasion process (Koussis et al. 2009). The cysteine protease falcipain-1 is supposed to be specifically involved in host cell invasion (Greenbaum et al. 2002), while another study suggests that falcipain-1 is not essential for the intraerythrocytic stage, but for oocyst formation in the mosquito vector (Eksi et al. 2004). Several proteases are involved in host cell invasion by *T. gondii*: Serine protease inhibitors prevent *T. gondii* tachyzoites from penetrating host cells (Conseil et al. 1999). The cysteine protease cathepsin L, one of five cathepsins in *T. gondii*, mediates invasion by preventing the release of invasion proteins from microneme secretory organelles. Cathepsin B is essential for the processing of rhoptry proteins and cellular invasion by tachyzoites (Que et al. 2002), while the three cathepsin Cs are critical for *T. gondii* growth and differentiation (Que et al. 2007).

Rupture of the host red blood cells allows the release of *Plasmodium* merozoites into the blood stream (egress), where they invade fresh erythrocytes. Egress is a rapid and therefore highly regulated process, which is currently explained by

different models and not yet characterized in detail (Glushakova et al. 2005). The egress of *P. falciparum* blood-stage and liver-stage merozoites as well as mosquito midgut sporozoites critically depends upon the activity of a set of proteases. At least three proteases have been implicated in the egress of malaria parasites from their host cells via the degradation of host cell structures. The aspartatic protease plasmepsin II and the cysteine protease falcipain-2 are involved in hemoglobin degradation, but they also degrade host erythrocyte cytoskeleton proteins and are expressed in mature schizonts (Le Bonniec et al. 1999; Dua et al. 2001). This is supported by the finding that inhibition of falcipain-2 blocks parasite maturation and egress (Dhawan et al. 2003), while knockout studies show that both plasmepsin II and falcipain-2 are not essential for egress indicating redundancy in protease function (Omara-Opyene et al. 2004; Sijwali and Rosenthal 2004). Another family contributing to merozoite egress are the papain-like cysteine proteases serine-repeat antigens (SERA), which are highly expressed in the schizont stage (Miller et al. 2002). Proteolytic egress requires a cascade of proteolytic processing. The cathepsin-C like cysteine protease DPAP3 is required for the maturation of a subtilisin-like protease called PfSUB1 (Arastu-Kapur et al. 2008), which in turn processes the precursor of SERA5 into its active form (Yeoh et al. 2007). Calpain-1, a host calcium-dependent protease, is also required for both *Plasmodium* and *Toxoplasma* to egress from human host cells (Chandramohanadas et al. 2009). Compared to *P. falciparum*, little is known about the egress process of other *Apicomplexa*, including *T. gondii*.

6.5.2 Hemoglobin Degradation

Proteases targeted to the *Plasmodium* food vacuole, a unique organelle required for hemoglobin degradation, are critical for the parasites' survival. During the erythrocytic life cycle stages, malaria parasites degrade hemoglobin in an acidic food vacuole as a major source of amino acids required for protein synthesis. This process requires hemoglobin transport from the erythrocyte cytoplasm to the parasitic food vacuole, the precipitation of heme, and the hydrolysis of hemoglobin into small peptides, which is catalyzed by proteases (Francis et al. 1997). The acidic food vacuole contains four plasmepsins (aspartic proteases) (Banerjee et al. 2002), four falcipains (cysteine proteases) (Salas et al. 1995), falcilysin (metallo protease) (Eggleson et al. 1999), and a dipeptidyl aminopeptidase (DPAP1) (Klemba et al. 2004) that systematically degrade hemoglobin in an ordered pathway (as outlined in Fig. 6.1) (Gluzman et al. 1994). Initial cleavage in the hinge region of the domain responsible for holding the hemoglobin tetramer together exposes the protein to subsequent degradation into smaller peptides catalyzed by both plasmepsins and falcipains (Francis et al. 1997). Falcilysin cleaves small polypeptides of up to 20 amino acids, producing short oligopeptides (Eggleson et al. 1999), while DPAP1 cleaves off dipeptides from hemoglobin-derived oligopeptides in the food vacuole

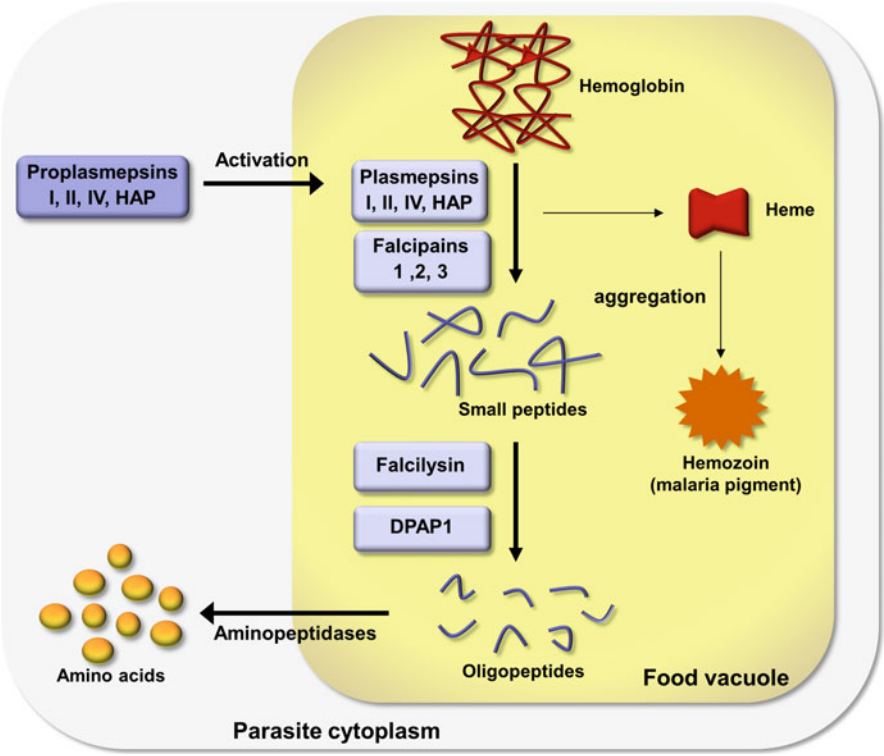

Fig. 6.1 Proteases of hemoglobin metabolism in *Plasmodium falciparum*

(Klemba et al. 2004). The hydrolysis to free amino acids is supposed to occur in the parasite cytoplasm by aminopeptidases (Gavigan et al. 2001).

Malaria parasites possess four hemoglobin-degrading plasmepsins (plasmepsin I, II, IV, and a histo-aspartic protease HAP), which are active in the food vacuole (Banerjee et al. 2002). Plasmepsins are synthesized as integral membrane proenzymes and are activated and released from the membrane by proteolytic cleavage catalyzed by falcipains or alternatively through autoprocessing (Drew et al. 2008). Cysteine protease inhibitors block hemoglobin hydrolysis, indicating that cysteine proteases play a key role in this process (Gamboa de Dominguez and Rosenthal 1996). The cysteine proteases falcipain-2 and falcipain-3 are currently a focus of drug development due to their essential role in hemoglobin hydrolysis (Rosenthal 2004). Falcipain-2 is critical for hemoglobin hydrolysis in malaria parasites since a disruption of the falcipain-2 gene leads to accumulation of undegraded hemoglobin in trophozoites (Sijwali and Rosenthal 2004). Disruption of the falcipain-3 gene could not be achieved, but the gene was readily replaced with a tagged functional copy, strongly indicating that falcipain-3 is essential for the survival of intraerythrocytic parasites (Sijwali et al. 2006).

6.6 Glycosylation

Glycosylation is the enzymatic process that produces glycosidic linkages of saccharides to other saccharides, proteins, and lipids (reviewed in Varki and Lowe 2009). Glycans belong to the four fundamental macromolecular structures of the cell together with nucleic acids, lipids, and proteins, and are a common feature in all domains of life. The diverse and abundant repertoire of glycans, the glycome, has important biological functions in protein maturation and turnover, cell adhesion and trafficking, and receptor binding and activation (reviewed in Varki and Lowe 2009). In recent years, glycan moieties attached to proteins and lipids on the surface of pathogens have received considerable attention, because they play important roles in the biology of host–pathogen interactions. Here, we will focus on the glycosylation of proteins from apicomplexan parasites.

Surprisingly, the extent of **protein glycosylation** in the asexual stage of *P. falciparum* is controversially discussed in the literature (Kimura et al. 1996; Gowda et al. 1997; Berhe et al. 2000). The glycosylphosphatidylinositol (GPI) anchor is the major type of glycosylation found in the parasite (Gowda et al. 1997). Whether or not proteins are glycosylated at other positions has only been ambigously solved to date. While some investigators identified a 14-sugar *Plasmodium N*-glycan resembling that of the human host, others identified no *N*-glycans (Kimura et al. 1996; Gowda et al. 1997; Berhe et al. 2000). Considering that in *Plasmodium* there is an extensive secondary loss of glycosyltransferases involved in precursor formation for *N*-glycosylation and that *Plasmodium* is missing proteins involved in *N*-glycan-dependent quality control of protein folding, it seems that *N*-glycosylation is unlikely to occur in the parasite (Samuelson et al. 2005; Banerjee et al. 2007). A recent paper starts to shed light on these uncertainties. It provides suggestive evidence for a Darwinian selection against *N*-glycans in protists with apicoplasts, which are likely to interfere with the import of apicoplast-targeted proteins into the organelle (Bushkin et al. 2010). Their assumption is supported by the fact that in *Plasmodium* and *Toxoplasma* the existing *N*-glycosylation sites in apicoplast-targeted proteins show a significantly lower occupation compared to other secretory proteins (Bushkin et al. 2010). Furthermore, *Plasmodium* synthesizes a severely truncated *N*-glycan precursor composed of one or two *N*-acetylglucosamine(s) (GlcNAc), and *Toxoplasma* has reduced numbers of glycosylation sites (Bushkin et al. 2010). This hypothesis also makes sense in view of the disagreement between former glycosylation studies and marks a new starting point in the analysis of protein targeting and glycosylation in the apicomplexan parasites.

However, the major glycolytic protein modification is via the glycosylphosphatidylinositol (GPI) anchor that is attached to the C-terminus of proteins in order to tack them to lipid bilayers (Gowda et al. 1997; Mendonca-Previato et al. 2005; von Itzstein et al. 2008). Analysis of the biosynthesis of plasmodial GPIs led to the discovery of unique structural features, for example the inositol 2-O-myristoylation (the inositol ring of a GPI is modified by the acyl group myristoyl) (Gerold et al. 1994, 1999; Lu et al. 2004). The characteristics of GPI biosynthesis are currently

being explored for the development of parasite-specific inhibitors (Azzouz et al. 2008). Further research mainly focuses on the clinically relevant blood stage of the life cycle, with most attention being paid to merozoite surface proteins, because of their role in causing pathology (Li et al. 2008; von Itzstein et al. 2008; Hinds et al. 2009). Analysis of the stoichiometry of GPI-anchored membrane proteins in *P. falciparum* showed that merozoite surface protein 1 (MSP-1) and MSP-2 make up approximately two-thirds of the total membrane-associated surface coat in merozoites, rendering them most important in generating pathology during asexual *Plasmodium* infection (Gilson et al. 2006). The parasite GPIs are an important endotoxin in malaria disease with several studies showing that the GPI anchor from *Plasmodium* induces the host immune response, leading to the production of proinflammatory cytokines and nitric oxide (Krishnegowda et al. 2005; Zhu et al. 2005). Furthermore, people living in malaria-endemic regions often have high levels of *anti*-GPI antibodies (Naik et al. 2000). Synthetic GPI was shown to work as a carbohydrate antitoxic vaccine against malaria in mice and the *anti*-GPI antibody response is subject of further investigation to aid the design of an efficient carbohydrate-based antitoxin vaccine (Schofield et al. 2002; Kamena et al. 2008).

6.7 Methylation

Methylation is a form of **alkylation** where a methyl group (one carbon) is enzymatically transferred to a substrate via methyltransferases. Such a post-translational modification increases both the hydrophobicity and steric bulk of proteins and can affect protein–protein interaction, protein function, and gene expression. The best known substrates of methyltransferases in *Plasmodium* are histones. Especially gene families that encode hypervariable surface antigens, such as *var*, are strictly controlled by histone methylation (Chookajorn et al. 2007; Lopez-Rubio et al. 2007; Deitsch 2009). It has been shown that histone H3 lysine 4 (H3K4) di- and trimethylation marks for *var* gene activation (Lopez-Rubio et al. 2007). In contrast, histone H3K9 trimethylation was linked to gene silencing of clonally variant gene families (Chookajorn et al. 2007; Lopez-Rubio et al. 2007, 2009). Interestingly, in *P. falciparum* this mark seems to be devoted almost solely to the silencing of variant antigen encoded genes, whereas in other organisms, including plants and yeast, H3K9 trimethylation is a more general epigenetic mark (Grewal and Moazed 2003; Deitsch 2009; Lopez-Rubio et al. 2009). Only recently could it be shown that H3K9 trimethylation might also be involved in the silencing of genes involved in erythrocyte invasion (Jiang et al. 2010). Further findings suggest that H3K4 trimethylation is cycle-regulated (Salcedo-Amaya et al. 2009). This brings into question the opinion that this mark is solely reflective of the transcriptional status of the parasite.

Histone lysine methylation is regulated by the opposing actions of histone lysine methyltransferases (HKMTs) and histone lysine demethylases (HDMs). In a

bioinformatic study at least nine putative SET-domain-containing *Plasmodium* HKMTs were identified and separated into five subfamilies with different putative substrate specificities (Cui et al. 2008a). For two of the nine putative SET-domain-containing HKMTs activity could be shown *in vitro* (Cui et al. 2008a). Also two genes coding for putative Jumonji C-domain-containing HDMs (JHDMs) have been identified (Cui et al. 2008a). Because HKMTs and JHDMs generally have narrow substrate specificities and also differ in their preferences for different methylation states (mono-, di-, trimethylation), it is not clear how the two putative JHDMs are involved in controlling the dynamics of histone methylation in the parasite (Cui et al. 2008a). One possibility is that the plasmodial JHDMs could have broader substrate specificity. Another possibility is that there might be other yet unidentified plasmodial HDMs. Therefore, it will now be interesting to experimentally verify the potential set of HKMTs and HDMs in the malaria parasite and to investigate how these proteins are involved in the modulation of the plasmodial epigenome.

Besides lysine residues, arginine residues can be methylated. *Plasmodium falciparum* encodes three conserved protein arginine *N*-methyltransferases (PRMTs) (Fan et al. 2009). Experiments have shown that recombinant PfPRMT1 can methylate histones H4 and H2A and several conserved substrates in the RNA metabolism (Fan et al. 2009). There is evidence that histone-arginine methylation may play a role in chromatin-mediated gene regulation in *P. falciparum* (Fan et al. 2009). Further investigation is necessary to clearly elucidate the role of arginine methylation in the malaria parasite.

Tubulin methylation has been recently detected for *T. gondii* (Xiao et al. 2010). This PTM was not previously described for tubulin in any other organism. If further investigation were to show that this modification is specific for *Apicomplexa*, the methyl transferase involved would represent a potential new therapeutic drug target (Xiao et al. 2010).

6.8 Acetylation

Lysine acetylation, or the transfer of an acetyl group from acetyl coenzyme A to the ε-amino group of a lysine residue, is a reversible post-translational modification, that neutralizes the positive charge of this amino acid, changing protein function in diverse ways (reviewed in Kouzarides 2000). Lysine acetylation regulates many diverse functions, including DNA recognition, protein–protein interaction, and protein stability, and exerts its effects through "**loss-of-function**" and "**gain-of-function**" mechanisms (Kouzarides 2000; Yang 2004). It has been known for a long time that lysine acetylation has a key role in the regulation of gene expression through the modification of core histone tails by histone acetyltransferases (HAT) or histone deacetylases (HDAC) (Lee and Workman 2007). Although predicted for a long time it was only recently that striking evidence could be provided that lysine acetylation provides a major regulatory switch in cells, equally important to

phosphorylation (Norvell and McMahon 2010; Wang et al. 2010; Zhao et al. 2010; Kouzarides 2000; Choudhary et al. 2009). For example, apart from the regulation of nuclear proteins, lysine acetylation seems to be an evolutionarily conserved mechanism involved in the regulation of metabolism in response to nutrient availability and cellular metabolic status (Norvell and McMahon 2010; Wang et al. 2010; Zhao et al. 2010).

Characterization of *P. falciparum* histone PTMs revealed a high content of acetylated lysine residues at their N-terminus (Miao et al. 2006; Trelle et al. 2009). Because of its vital role in the modification of chromatin structure and the binding of transcriptional regulators to the DNA, histone lysine (de-)acetylation was intensely studied as a potential drug target. GCN5 was identified as the first part of a large multimeric histone acetyltransferase complex in *Plasmodium* (Fan et al. 2004b). Recombinant PfGCN5 has been analyzed to preferentially acetylate histone H3 at K9 and K14 (Fan et al. 2004b). Furthermore, recent data suggest that PfGCN5 forms a complex with PfADA2, a yeast transcriptional coactivator homolog, and that this complex may have conserved functions in chromatin remodeling and gene regulation (Fan et al. 2004a, b). This hypothesis is supported by findings that acetylation of histone H3 at lysine residue 9 (H3K9) is enriched in active stage-specific genes in the parasite, including active *var* genes (see also Methylation) (Cui et al. 2007; Lopez-Rubio et al. 2007). Natural components for inhibiting histone acetylase activity are under investigation as potential antimalarials (Cui et al. 2008b).

The genome sequence of *P. falciparum* has revealed five HDAC homologs (Gardner et al. 2002). Two HDACs, PfHDAC1 and PfSir2, have been partially characterized (Joshi et al. 1999; Freitas-Junior et al. 2005). The presence of PfHDAC1 transcripts in both asexual and sexual stages of malaria parasites suggests that PfHDAC1 is functionally important for the parasite in both mosquito vector and human host (Joshi et al. 1999). PfSir2 co-localizes with subtelomeric clusters at the chromosome ends (Freitas-Junior et al. 2005). Its binding to the promoter region was found to be associated with histone deacetylation and silencing of the *var* gene family (Freitas-Junior et al. 2005). Studies dealing with the effectiveness of HDAC inhibitors in malaria chemotherapy have been carried out emphasizing the potential of HDACs as drug targets for malaria chemotherapy (Chaal et al. 2010; Andrews et al. 2008; Dow et al. 2008). A very recent study showing growth inhibition of *Plasmodium* cells after treatment with HDAC inhibitors becomes especially interesting in the light of two other very recent publications, providing strong evidence for a physiological role of dynamic acetylation in metabolic regulation (Chaal et al. 2010; Wang et al. 2010; Zhao et al. 2010). Therefore, the observed growth inhibition of *Plasmodium* after treatment with HDAC inhibitors might not be just caused through a general de-regulation of the global transcriptional regulation, but also through the disturbance of the general metabolism in the parasite. However, apart from the acetylation of actin at its N-terminus and the discussed histone acetylation, this PTM has not been investigated in *Plasmodium* (Schmitz et al. 2005). The global and important role of lysine acetylation in metabolic regulation renders the elucidation of the

"**acetylome**" of *Plasmodium* exceptionally interesting and would enormously contribute to the essential understanding of the functions and interactions of plasmodial PTMs, a major target for chemotherapeutic interventions.

6.9 Ubiquitination

Eukaryotic proteins are subject to a huge variety of PTMs, extending the functional variety and dynamics of the proteome. The first such protein-based modification described was ubiquitin (Ub) in the 1970s by Avram **Hershko**, Aaron **Ciechanover** and Irwin **Rose**, who received the **Nobel Prize** in Chemistry for their discovery in 2004 (Giles 2004). Ubiquitin is a small (76-residue) ubiquitous, eukaryotic cellular protein (reviewed in Hershko and Ciechanover 1998; Kerscher et al. 2006). In eukaryotes, the ubiquitin–proteasome system accounts for the bulk of cellular protein degradation, including short-lived, regulatory, and misfolded/denatured proteins (Hershko and Ciechanover 1998; Kerscher et al. 2006). But beyond being a mere signal for protein-waste degradation, ubiquitin attachment plays a crucial part in a variety of essential cellular processes, for example cell cycle regulation, DNA repair, cell growth, immune function, and vesicular trafficking (Hershko and Ciechanover 1998; Kerscher et al. 2006; Mukhopadhyay and Riezman 2007). The reversible ATP-dependent conjugation of ubiquitin to the ε-amino group of a protein substrate lysine is catalyzed via a three-step mechanism and involves activating enzymes (E1), conjugating enzymes (E2), and ligases (E3) (reviewed in Kerscher et al. 2006). E1 activates ubiquitin and transfers it to E2, which either transfers ubiquitin directly to the substrate by associating with E3, or E3 becomes ubiquitinated and is the one to transfer ubiquitin to the substrate. Deubiquitinating enzymes (DUBs) hydrolyze the isopeptide bond between Ub molecules and free Ub-conjugated proteins by removing Ubs from their lysine residues (Reyes-Turcu et al. 2009).

As mentioned above, the variety of processes regulated by ubiquitination is enormous (Hershko and Ciechanover 1998; Kerscher et al. 2006; Mukhopadhyay and Riezman 2007). This is achieved through multiple Ub and Ub-like proteins and their ability to form diverse protein modifications, each having different consequences for the Ub-modified substrate (Kerscher et al. 2006; Mukhopadhyay and Riezman 2007). Monoubiquitination, the attachment of a single Ub molecule to a protein, and multiubiquitination, the addition of multiple Ub molecules, can have nonproteolytic functions such as in endocytosis and DNA repair (Hicke 2001; Bergink and Jentsch 2009). The linkage between multiple Ub molecules can also be via different lysine residues. Whereas the linkage of Ub molecules through lysine 48 is mainly used for targeting to the proteasome, lysine 63 linked Ub molecules seem to have important roles in DNA damage control, inflammatory response, the endocytic pathway, and ribosomal protein synthesis (Mukhopadhyay and Riezman 2007).

In vitro and *in vivo* proteasome inhibitor studies have substantiated the essentiality of the ubiquitin–proteasome system in *Plasmodia* (Gantt et al. 1998; Kreidenweiss et al. 2008; Prudhomme et al. 2008; Czesny et al. 2009). Furthermore, *in silico* studies on the ubiquitin-mediated pathways in apicomplexan parasites, including the genus *Plasmodium*, have provided evidence that there are a number of proteins involved in the ubiquitination reaction that may serve as parasite-specific drug targets due to their diversity and divergence from the eukaryotic system (Ponder and Bogyo 2007; Ponts et al. 2008). In the following paragraphs, we highlight major findings about the plasmodial ubiquitin system.

Ubiquitin is encoded by a multigene family with three primary members, giving rise to the precursor protein polyubiquitin and two ubiquitin moieties, Ub_{L40} and Ub_{S27a} (Catic and Ploegh 2005). The polyubiquitin gene of *P. falciparum* (PfpUB) consists of five tandem repeats of the ubiquitin coding sequence (Horrocks and Newbold 2000). PfpUB is present as a single copy on chromosome 12 with only one intron at the 5′ end interrupting the gene sequence (Horrocks and Newbold 2000). The ubiquitin monomer of *P. falciparum* has a 98.6% sequence identity to bovine ubiquitin (Horrocks and Newbold 2000). The two remaining ubiquitin-fusion genes, Ub_{L40} and Ub_{S27a}, encoding N-terminal ubiquitin fused to one of the two ribosomal proteins (L40 and S27a), have also been identified in the genome of *P. falciparum* (Catic and Ploegh 2005; Ponts et al. 2008). Expression data available for *P. falciparum* indicate that all three ubiquitin genes are expressed throughout the parasite's life cycle (Bozdech et al. 2003; Le Roch et al. 2003). Additionally, polyubiquitin translation also appears to be induced on response to heat shock in late blood-stage parasites (trophozoites and schizonts) (Horrocks and Newbold 2000).

Apart from Ub, the common ubiquitin-like modifiers SUMO, NEDD8, HUB1, URM1, and ATG8 have been identified in *Apicomplexa*, and gene expression data suggest that these Ub-like modifiers are expressed at all life stages investigated (Ponder and Bogyo 2007; Ponts et al. 2008). But a number of Ub-like proteins typical for higher eukaryotes (ISG15, FAT110, UFM1, FUB1) could not be detected (Ponder and Bogyo 2007; Ponts et al. 2008).

The Ub-like modifier SUMO has recently been characterized in *P. falciparum* by the group of Artur Scherf (Issar et al. 2008). By using a mass spectrometry approach they identified 23 putative targets for sumoylation. The putative SUMO-substrates are involved in a variety of essential cellular functions, for example chromatin remodeling, transcription, and vesicular trafficking, suggesting a wide regulatory role for SUMO (Issar et al. 2008). Especially intriguing is that sumoylation of PfSir2, a telomere-associated nuclear protein involved in *var* gene silencing, could be detected (see also Acetylation) (Freitas-Junior et al. 2005; Issar et al. 2008). Moreover, through immunofluorescence microscopy, SUMO could be localized to the nucleus, the parasite cytosol and to Maurer's clefts, parasite-derived structures in the host cell cytoplasm (Issar et al. 2008).

The first step in the activation and conjugation cascade of Ub and Ub-like proteins is mediated via **E1 (Ub-activating)** enzymes. In the latest computational genome analysis of the *P. falciparum* Ub-system, the following eight putative E1s

have been identified: one paralog for UBA1, two paralogs for UBA1-like proteins, and additional paralogs for UBA2-4 and ATG7 proteins, which activate the Ub-like proteins SUMO, NEDD8, URM1, and ATG8, respectively (Ponts et al. 2008). Furthermore, a high level of primary sequence identity in the core domain of the E1 enzymes was identified (Ponts et al. 2008). However, outside this core domain, sequences diverged rapidly (Ponts et al. 2008). This is a consequence of the functional requirement for each E1 enzyme to react with its specific subset of E2 conjugation enzymes (Ponts et al. 2008). The available proteomic and transcriptomic data for *P. falciparum* provides strong evidence for the expression of E1 enzymes throughout the parasite's life cycle (Bozdech et al. 2003; Le Roch et al. 2003; Ponts et al. 2008).

E2s (Ub-conjugating enzymes) accept the activated Ub or Ub-like protein from E1 via a transesterification reaction. Each E2 isoform can interact with a distinguished set of E3s; therefore E2s have a distinct role in regulating downstream function (Pickart 2001). In *P. falciparum* 14 putative E2 paralogs have been identified (Ponts et al. 2008). Interestingly, in this analysis two atypical E2s with a long N-terminal extension have only been found in *Plasmodium* and *Toxoplasma* (Ponts et al. 2008). This extension might play a role in the recognition and association with specific E3s and succeeding downstream protein targets and therefore may be a specific adaption of *Plasmodium* and *Toxoplasma*, which should be validated as a potential drug target (Ponts et al. 2008). Furthermore, analysis of gene expression data for 9 of the 14 predicted *P. falciparum* E2s showed a changing pattern of steady-state mRNA levels at different intraerythrocytic stages of the parasite, suggesting a potential temporal control level for the E2s of the parasite (Ponts et al. 2008). Recently, one of the 14 predicted Ub-conjugating enzymes of *P. falciparum*, PfUBC13, was identified as an endogenous substrate for *P. falciparum* protein kinase 9 (PfPK9) (Philip and Haystead 2007). Strong evidence was provided that PfPK9 phosphorylates PfUBC13 at serine 106, leading to the suppression of its ubiquitin conjugating activity (Philip and Haystead 2007). Further *in vitro* and *in vivo* analysis of phosphorylation as a general regulatory mechanism of *P. falciparum* E2 enzymes is necessary.

E3 (Ub-ligases) are involved in the specific transfer of Ub and Ub-like proteins to a given substrate. In *P. falciparum* 54 putative E3 enzymes have been predicted; therefore the group of Ub-ligases exceeds greatly the E1 and E2 enzymes, reflecting the high specificity required for substrate recognition (Ponts et al. 2008). The superfamily of *P. falciparum* E3s is very diverse, and for some of the predicted E3s no homologs in other eukaryotes could be detected (Ponts et al. 2008). Such proteins could have parasite-specific physiological functions essential for parasite survival, for example invasion of hepatocytes or erythrocytes and evasion of the host immune system. Therefore, detailed functional analysis of the parasite-specific E3s would enormously contribute to the understanding of the Ub-mediated pathways in *Plasmodium* species.

Deubiquitinating enzymes (DUBs) are proteases that process ubiquitin or ubiquitin-like gene products, reverse the modification of proteins by a single Ub or Ub-like protein, and remove or remodel poly-ubiquitin or poly-ubiquitin-like

chains on target proteins. Computational predictions range between 18 and 29 putative DUBs in *P. falciparum,* depending on the prediction method used (BLASTP vs. HMM search) (Ponder and Bogyo 2007; Ponts et al. 2008). Like ubiquitination, deubiquitination is a highly regulated process that has been implicated in numerous cellular functions, including cell cycle regulation (Song and Rape 2008), gene expression (Daniel and Grant 2007), DNA repair (Kennedy and D'Andrea 2005), and kinase activation (Adhikari et al. 2007; Komada 2008). Recent analysis of antimalarial drug resistance in the rodent malaria parasite *P. chabaudi* identified mutations in a DUB enzyme with strong genetic linkage to drug resistance (Hunt et al. 2007). Subsequent analysis of drug resistance in *P. falciparum* did not identify mutations in a similar *P. falciparum* DUB enzyme (Hunt et al. 2007). Further work is necessary to determine and validate the potential role of this and other DUBs in the development of parasite drug resistance. The first two proteolytically active DUBs identified in *P. falciparum*, PfUCH54 and PfUCHL3, were found to react both with ubiquitin and NEDD8 (Artavanis-Tsakonas et al. 2006; Frickel et al. 2007). Though the exact function of PfUCHL3 is not known, a recent study provides strong evidence that distinct differences in the Ub binding site between PfUCHL3 and its human counterpart exist and that PfUCHL3 is essential for parasite survival, rendering it a promising drug target (Artavanis-Tsakonas et al. 2010).

In closing, detailed functional elucidation of Ub-mediated pathways in *Plasmodium* both *in vitro* and *in vivo* promises to lead to the discovery and validation of new urgently needed drug targets. Especially intriguing for the development of new drugs are the E3s and DUBs due to their diversity and potential involvement in parasite-specific pathways.

6.10 Conclusions

Besides the post-translational modifications presented here, several other modifications have been demonstrated in apicomplexan parasites. These include protein dolichylation (D'Alexandri et al. 2006), *S*-sulfonation (Medzihradszky et al. 2004), protein formylation (Bracchi-Ricard et al. 2001), and polyglutamylation (Fennell et al. 2008). In addition, malaria parasites possess several chaperone proteins that assist in correct protein folding and mediate malaria pathogenesis (for a review please see Shonhai 2009).

Interestingly, *Plasmodium* shows only minor transcriptional changes in response to external stimuli, suggesting that it may have a rigid transcription machinery and proteins are not mainly regulated on the transcriptional level (Ganesan et al. 2008). During the intraerythrocytic life cycle of *Plasmodium*, the malaria parasite compensates a less flexible control of gene transcription by versatile post-transcriptional and post-translational modifications. This implies that life cycle regulation largely depends on post-translational modifications that allow rapid morphological and metabolic changes and adapt the parasite to changing environmental conditions. Therefore,

post-translational modifications play a significant and widespread role in the regulation of parasitic proteins. What we currently know about the functions of post-translational modifications in apicomplexan parasites is limited since most studies on life cycle regulation are focused on the transcriptional level. Because of the variety of post-translational modifications, the possibilities of post-translational protein regulation are huge and provide excellent starting points for antiparasitic drug discovery.

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Chapter 7
Development of the RTS,S/AS Vaccine Candidate from Concept to Phase III

Joe Cohen, Sarah Benns, Johan Vekemans, Amanda Leach, and Lode Schuerman

Abstract This review describes the developmental history of the RTS,S/AS vaccine. Selection of the circumsporozoite protein (CSP) as the target antigen was key to the successful development of the vaccine so far, from concept to the initiation of Phase III testing. CSP, a pre-erythrocytic protective antigen against *Plasmodium falciparum*, has been demonstrated to be immunodominant and protective in pre-clinical studies both in animals and humans. The vaccine antigen was designated "RTS,S"; RTS being a hybrid polypeptide consisting of a portion of the CSP antigen and S the surface antigen of Hepatitis B virus (HBsAg). The RTS,S/AS candidate vaccine has been evaluated in multiple Phase I/II studies and shown to have a favourable safety profile and to be well tolerated in both adults and children. Consistent and significant efficacy against *Plasmodium falciparum* infection and disease was observed in the target population of infants and children in a range of age groups and in different malaria transmission settings. The RTS,S/AS01$_E$ malaria vaccine candidate has recently entered Phase III testing. Reaching this important milestone is the culmination of more than 20 years of research and development by GlaxoSmithKline, their partners and collaborators. If the Phase III results confirm the observations made during Phase II testing, the RTS,S/AS01$_E$ vaccine, when broadly implemented and judiciously integrated with other malaria-prevention measures, could have a major public-health impact in sub-Saharan Africa.

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7.1 Introduction

The RTS,S/AS candidate vaccine is currently the most advanced anti-malarial vaccine in clinical development. Research into the RTS,S/AS vaccine was initiated in 1987 at GlaxoSmithKline (GSK), as part of an ongoing collaboration with the Walter Reed Army Institute of Research (USA). The RTS,S antigen is based on a large segment of the *Plasmodium falciparum* circumsporozoite protein (CSP; Amino Acids 207–395 of the CSP from the NF54 strain of *P. falciparum*) fused to the hepatitis B virus surface protein (S) produced in genetically engineered *Sacharomyces cerevisiae* yeast cells. Immune responses to the RTS,S antigen were optimised by the development of new innovative Adjuvant Systems (AS). This review article provides an overview of the major milestones that the pre-erythrocytic RTS,S/AS vaccine candidate has successfully achieved from conception to ongoing Phase III clinical assessment.

7.1.1 Circumsporozoite Protein: A Valuable Vaccine Candidate Antigen

The critical role for **CSP** as a pre-erythrocytic protective antigen against *P. falciparum* has been demonstrated in pre-clinical studies in animals and humans using sporozoites whose life cycle is stopped at the liver stage (Nussenzweig and Nussenzweig 1989; Doolan and Marinez-Alier 2006; Mueller et al. 2005). In humans, bites from mosquitoes infected with radiation-attenuated *P. falciparum* protected against a subsequent infection with non-irradiated sporozoites (sporozoite challenge model). Protection was specific for *falciparum* species, required repeated immunisation sessions and, in a few volunteers, was shown to last up to 42 weeks (Hoffman et al. 2002). In mice, sterile immunity has also been demonstrated by injection of irradiated or genetically attenuated sporozoites (Nussenzweig and Nussenzweig 1989; Mueller et al. 2005). In these different models, CSP was shown to be immunodominant and protective (Kumar et al. 2006, 2009).

The important role of **antibodies** in protection was first shown in rodent models (Potocnjak et al. 1980). The repeat domain of *P. falciparum* CSP contains a B-cell epitope composed of a four amino acids motif, $(NANP)_n$. Sera from individuals "vaccinated" with radiation-attenuated parasites contain antibodies that bind to the sporozoite surface, and neutralise its infectivity (Sinnis and Nussenzweig 1996). Sera from individuals living in endemic areas of Africa also contain low titres of antibodies to the surface of *P. falciparum* sporozoites as detected by immunofluorescence assays. In general, there is an excellent correlation between the titres measured using immunofluorescence assays and the titres of antibodies to the (NANP)n motif of CSP (Zavala et al. 1985). To date, the only antibodies that consistently neutralise sporozoite infectivity of rodent or human sporozoites are directed to the CSP repeats (Sinnis and Nussenzweig 1996). The NANP repeats were therefore logical targets for the development of *P. falciparum* vaccines.

As the sporozoites injected by mosquitoes travel rapidly to the liver to infect the hepatocytes, a short time is left for the induction of a humoral response and the time that antibodies have to neutralise sporozoites before they enter hepatocytes is limited. Protective mechanisms other than antibody responses to *P. falciparum* sporozoites were thus explored for developing pre-erythrocytic vaccines. There is evidence for a protective role of interferon-γ secreting $CD4^+$ T lymphocytes, and the major epitope that is recognised is the "promiscuous" conserved T-cell epitope at the C-terminus of CSP (Ferreira et al. 1986; Reece et al. 2004).

In order to induce **T-cell recognition**, liver stage antigens need to be processed into short peptides and presented by antigen-presenting cells. Several liver cell types have such an antigen presentation capacity: Kuppfer cells, endothelial cells, dendritic cells, and hepatocytes themselves (Frevert and Nardin 2008). The continuous shedding of CSP off the sporozoite surface upon migration through the liver has been described (Singh et al. 2007). CSP is thus found in the cytoplasm of infected hepatocytes, where it inhibits cellular metabolic pathways, is processed and antigenic peptides derived from the CSP antigen are presented on the cell surface (Singh et al. 2007). Both $CD4^+$ and $CD8^+$ T cells may then recognise these antigenic peptides, which may lead to the release of cytokines such as interferon-γ or expression of other effector functions in the proximity of the infected hepatocytes, resulting in the inhibition of the parasite liver stage development (Sinnis and Nussenzweig 1996).

7.1.2 From the Laboratory to the Clinic Setting

Although several plasmodial antigens were investigated, the major focus was the CSP of *P. falciparum*. Over a decade of research spanning from the mid-1980s to the mid-1990s, nearly a dozen vaccine formulations were tested preclinically and six candidate vaccines were tested in Phase I/II clinical trials with little success (Ballou et al. 1985, 1987; Young et al. 1985, 1987; Hockmeyer et al. 1986; Wirtz et al. 1987; Hollingdale et al. 1987; Fries et al. 1992; Brown et al. 1994; Sherwood et al. 1996; Ballou and Cahill 2007; Vekemans and Ballou 2008).

In the late 1970s and early 1980s, the recombinant hepatitis B vaccine (*Engerix-B*™[1]) was in development. This genetically engineered vaccine is based on the gene encoding the hepatitis B virus surface protein, HBsAg or S. Crucially, the S proteins produced in genetically modified *Saccharomyces cerevisiae* yeast cells spontaneously assemble into virus-like particles (Rutgers et al. 1987). This led to the use of HBsAg as a carrier matrix for *P. falciparum* CSP by fusing the part of the CSP genetic sequence that encodes the B and T-cell epitopes to the sequence of HBsAg and expressing the chimeric gene in *S. cerevisae* (Gordon et al. 1995).

[1]Engerix-B is a trademark of the GlaxoSmithKline group of companies.

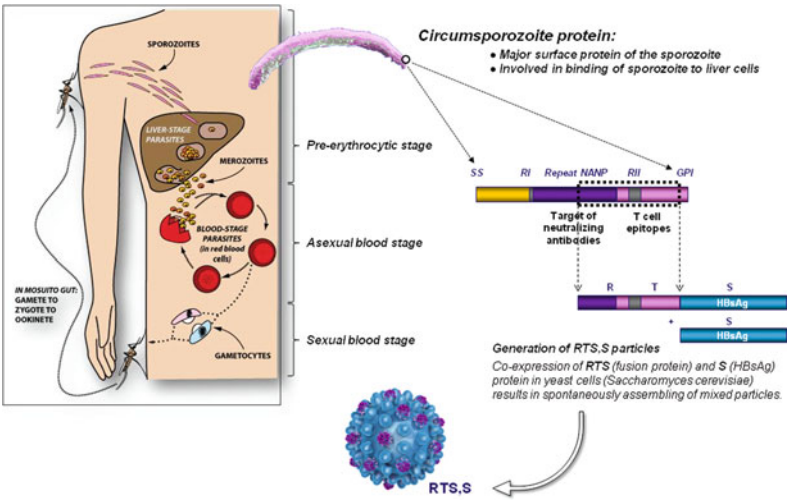

Fig. 7.1 The RTS,S vaccine antigen

The resulting fusion protein assembled into virus-like particles similar to those formed by the unfused viral HBsAg surface protein. The vaccine construct was designed to induce antibody responses against the dominant $(NANP)_n$ B-cell epitope as well as cellular immune responses against several T-cell epitopes identified in the C-terminal non-repetitive region of CSP (Gordon et al. 1995; Good et al. 1988). The vaccine antigen was designated RTS,S to indicate the presence of the CSP repeat region (R), T-cell epitopes (T) fused to the hepatitis B virus surface antigen (S), and assembled into virus-like particles with unfused copies of S antigen (Fig. 7.1).

A number of innovative **Adjuvant Systems** were assessed in extensive pre-clinical studies, in rodents and primates. This work led to the identification of a promising vaccine candidate combining the RTS,S antigen with the adjuvant system $AS02_A$ (proprietary oil-in-water emulsion with MPL® and QS21 immunostimulants) (Garçon et al. 2003). In several clinical trials involving healthy subjects experimentally challenged by the bites of *P. falciparum*-infected mosquitoes, RTS,S/$AS02_A$ consistently conferred full protection against infection in about 40% of the volunteers (Stoute et al. 1997; Kester et al. 2001, 2007, 2008). Table 7.1 provides a summary of the RTS,S/$AS02_A$ vaccine components and subsequent formulations.

7.1.3 Proof of Concept from Adult Clinical Trials in Malaria Endemic Countries

Field evaluation followed the promising results achieved in the **laboratory-based challenge trials**. Safety, immunogenicity and efficacy of RTS,S/$AS02_A$ was

Table 7.1 Formulations of RTS,S/AS

Formulation	Freeze-dried fraction	Liquid fraction				
	RTS,S (μg)		MPL (μg)	QS21 (μg)	Dose volume (mL)	Key milestones
RTS,S/AS02$_A$ (0.5 mL dose)	50	Oil-in-water emulsion	50	50	0.5	Efficacy demonstrated in adults in challenge model (Stoute et al. 1997) and in endemic countries (Bojang et al. 2001)
RTS,S/AS02$_A$ (0.25 mL dose)	25	Oil-in-water emulsion	25	25	0.25	Efficacy against clinical disease in children in Mozambique (Alonso et al. 2004, 2005; Sacarlal et al. 2009)
RTS,S/AS02$_D$	25	Oil-in-water emulsion	25	25	0.5	Efficacy in infants. Paediatric formulation (Macete et al. 2007b; Aponte et al. 2007; Abdulla et al. 2008)
RTS,S/AS01$_B$	50	Liposomes	50	50	0.5	Efficacy in adults in challenge model (Kester et al. 2009) and in endemic countries (Polhemus et al. 2009)
RTS,S/AS01$_E$[a]	25	Liposomes	25	25	0.5	Paediatric formulation. Efficacy against clinical disease in children in Kenya and Tanzania (Bejon et al. 2008)

[a]Formulation selected for Phase III clinical development

confirmed in adult men from The Gambia (Doherty et al. 1999; Bojang et al. 2001), where three doses of RTS,S/AS02$_A$ (administered according to a 0, 1, 5-month schedule) conferred significant protection against infection over a 15-week period (34%; 95% CI: 8–53; $p = 0.014$). Although efficacy appeared to wane during the surveillance period, a booster dose at 19 months demonstrated 47% (95% CI: 4–71; $p = 0.037$) efficacy over a 9-week period, which corresponds to the malaria transmission season in this region. Furthermore, the efficacy conferred by RTS,S/AS02$_A$

did not appear to be specific for the genotype of the parasite strain used to generate the vaccine construct (Allouèche et al. 2003). In this study, long-term safety and persistence of anti-CSP and anti-HBsAg antibodies induced by the RTS,S/AS02$_A$ vaccine candidate were subsequently documented over a 5-year period (Bojang et al. 2009).

As the work with the RTS,S/AS02 vaccine was progressing, additional pre-clinical studies in mice and primates identified a new Adjuvant System, AS01 (liposome suspension, MPL® and QS21), which improved humoral responses to both CSP and HBsAg antigen components of the vaccine candidate, and improved Th1-type cell-mediated immune responses against CSP (Stewart et al. 2006a, b; Mettens et al. 2008). These pre-clinical results were subsequently confirmed in malaria-naïve adults from the USA in whom anti-CSP antibody responses and multifunctional CD4$^+$ T-cell immune responses were found to be superior with RTS,S/AS01$_B$ compared to RTS,S/AS02$_A$. There was also a trend towards greater protection against infection with RTS,S/AS01$_B$ than with RTS,S/AS02$_A$ (50% vs. 32%; $p = 0.11$) following experimental sporozoite challenge (Kester et al. 2009). In a field trial conducted in adults in Kenya, RTS,S/AS01$_B$ induced greater anti-CSP humoral responses and similar efficacy compared to RTS,S/AS02$_A$ (Polhemus et al. 2009).

7.1.4 Assessment of the Vaccine in the Paediatric Population

The encouraging results obtained in studies in adults justified progression to development of the RTS,S/AS candidate vaccine in the paediatric population. This development was undertaken through a **Private/Public partnership** between GSK and the Malaria Vaccine Initiative (MVI) from the Program for Appropriate Technology in Health (PATH), funded by the Bill and Melinda Gates Foundation. Initial Phase I/II trials focussed on age de-escalation in the paediatric populations, dose optimisation, and evaluation of vaccine immunogenicity and safety profiles. Half the adult dose of RTS,S/AS02$_A$ (i.e. 0.25 mL) was shown to be highly immunogenic for both the CSP and S antigens and to have an encouraging safety profile in children aged 1–11 years from The Gambia and Mozambique (Bojang et al. 2005; Macete et al. 2007a).

Proof-of-concept of efficacy in the paediatric population was demonstrated in children aged 1–4 years from Mozambique (Table 7.2). Three doses of RTS, S/AS02$_A$ administered at monthly intervals showed that vaccine efficacy was 30% (95% CI: 11–45; $p = 0.004$) against the first clinical episode and 58% (95% CI: 16–81; $p = 0.019$) against severe malaria over a 6-month surveillance period. Vaccine efficacy was 35% (95% CI: 22–47; $p < 0.001$) and 49% (95% CI: 12–71; $p = 0.02$) over an 18-month surveillance period, against clinical episodes and severe malaria, respectively respectively (Alonso et al. 2004, 2005). Crucially, when considering the total follow-up period of 42 months, the vaccine conferred a protection of 26% against all clinical episodes of

Table 7.2 RTS,S/AS Phase II efficacy results in the paediatric population

Country/ reference	Vaccine formulation	Age subjects	Endpoints	Duration of follow-up post vaccination	Vaccine efficacy (%)	95% CI	*p*-value
Mozambique (Alonso et al. 2004, 2005)	RTS,S/AS02	1–4 years	Clinical disease	6 months	29.9	11.0–44.8	0.004
				18 months	35.3	21.6–46.6	<0.001
			All episodes	6 months	27.4	6.2–43.8	0.014
				18 months	29.8	13.8–42.8	0.001
			Severe disease	6 months	57.7	16.2–80.6	0.019
				18 months	48.6	12.3–71.0	0.020
			Hospitalised malaria	6 months	32.3	1.3–53.9	0.053
				18 months	30.5	4.1–49.9	0.032
			Infection	6 months	45.0	31.4–55.9	<0.001
Tanzania/ Kenya (Bejon et al. 2008)	RTS,S/AS01	5–17 months	Clinical disease	8 months (mean value)	52.9	28.1–69.1	<0.001
Mozambique (Aponte et al. 2007)	RTS,S/AS02	10–18 weeks	Infection	3 months	65.9	42.6–79.8	<0.001
Tanzania (Abdulla et al. 2008)	RTS,S/AS02	6–10 weeks	Infection	6 months	65.2	20.7–84.7	0.012

malaria (95% CI: 12–37; $p \leq 0.001$) and of 38% against severe malaria (95% CI: 3–61; $p = 0.045$) (Sacarlal et al. 2009). Furthermore, at the end of the follow-up period, the prevalence of *P. falciparum* parasites was 34% lower in RTS,S/AS02$_A$ recipients (12% compared to 19% in children in the control group; $p = 0.004$). Over the 42-month follow-up period, RTS,S/AS02$_A$ had an acceptable safety profile, with significantly fewer serious adverse events and a trend towards reduced all-cause mortality compared to recipients of control vaccines. As in the adult trial conducted in The Gambia, the protection conferred by RTS,S/AS02$_A$ was not strain-specific and reduced the genotypic multiplicity of infections by *P. falciparum* (Enosse et al. 2006).

A paediatric version of the RTS,S/AS02$_A$ vaccine candidate, RTS,S/AS02$_D$ (which contains half the RTS,S/AS02$_A$ dose in a volume of 0.5 mL, Table 7.1), was then developed and shown to be equivalent to RTS,S/AS02$_A$ (half dose) in terms of safety and immunogenicity (Macete et al. 2007b). The promising results obtained in field studies of RTS,S/AS02$_A$ in children led to the assessment of the RTS,S/AS02$_D$ vaccine candidate in infants within the Expanded Program for Immunisation (EPI) age range. In infants from Mozambique, staggered administration of RTS,S/AS02$_D$ at 8, 12, and 16 weeks of age and EPI vaccines diphtheria, tetanus, pertussis, and *Haemophilus influenzae* type b vaccine (DTPw/Hib) and oral polio vaccine (OPV) at 10, 14, and 18 weeks of age showed that the safety and tolerability profile of RTS,S/AS02$_D$ was similar to that of hepatitis B control vaccine (Aponte et al. 2007). In this study, vaccine efficacy against *P. falciparum* infection was demonstrated over 3-month's follow up (66%; 95% CI: 43–80; $p < 0.001$).

When co-administered with EPI vaccines (DTPw/Hib and OPV) in infants from Tanzania, low-grade fever and rash were reported more frequently in infants receiving the RTS,S/AS02$_D$ vaccine than in the control group receiving the hepatitis B vaccine, also co-administered with EPI vaccines (Abdulla et al. 2008). However, there were no cases of high-grade fever or rash in recipients of RTS, S/AS02$_D$ and there was no clinically significant difference in the incidence of any other solicited adverse events between the two groups. Remarkably, there was a trend towards fewer all-cause hospitalisations and hospitalisations due to pneumonia in the RTS,S/AS02$_D$ group than in the control group. In this study, vaccine efficacy against *P. falciparum* infection was 65% (95% CI: 21–85; $p = 0.012$) over a 6-month follow-up period. The study also demonstrated the noninferiority of responses against the EPI antigens upon RTS,S/AS02$_D$ vaccine co-administration.

Across all studies conducted in children and infants from malaria-endemic countries, the RTS,S antigen formulated with AS02 was highly immunogenic for anti-CSP and anti-HBsAg antibodies, irrespective of the age of the children or the region where the studies were conducted. Geometric mean titres for anti-CSP antibodies were generally higher in paediatric populations than in adult subjects from endemic regions of Africa.

Following the promising results with the AS01 adjuvant system in adults (see 7.1.3), comparative studies of RTS,S/AS02$_D$ and RTS,S/AS01$_E$ (the paediatric version of RTS,S/AS01$_B$; Table 7.1) were initiated. In children aged 18 months to 4 years in Gabon and in children aged 5–17 months in Ghana, vaccination with RTS,S/AS01$_E$ demonstrated better anti-CSP humoral responses than vaccination with RTS,S/AS02$_D$ (Lell et al. 2009; Owusu-Agyei et al. 2009). Both RTS,S/AS02$_D$ and RTS,S/AS01$_E$ vaccine candidates were well tolerated and had a favourable safety profile. Vaccine efficacy of RTS,S/AS01$_E$ was then assessed in children aged 5–17 months in Tanzania and Kenya over an average 8-month follow-up period and was shown to be 53% (95% CI: 28, 69; $p < 0.001$) against malaria disease (Bejon et al. 2008). Furthermore, there was a trend towards fewer serious adverse events leading to hospitalisation and fewer non-malaria morbidity among subjects in the RTS,S/AS01$_E$ group than in the control group.

7.1.5 Onwards to Phase III Testing

The encouraging Phase II efficacy, immunogenicity, and safety evaluation of RTS, S/AS01$_E$ in the paediatric population supported progression of the candidate vaccine to Phase III development.

The pivotal Phase III study of RTS,S/AS01$_E$ was initiated in May 2009 (ClinicalTrials.gov). This is a multicentre, double-blind, randomised controlled trial conducted in 11 centres in seven African countries, thereby representing diverse malaria transmission settings (Fig. 7.2). Children in two age categories, 6–12 weeks and 5–17 months at first vaccination, will participate in the study and will be followed for a total of 32 months. In February 2011, enrolment was

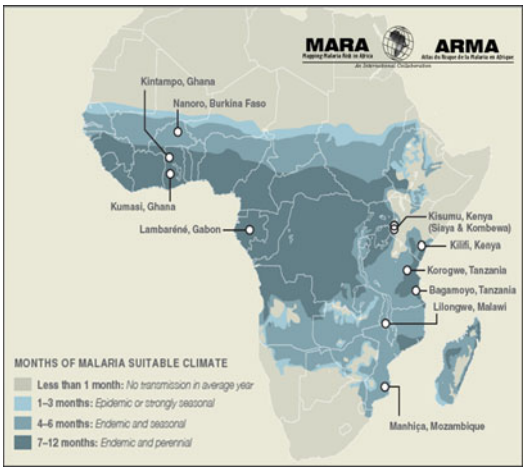

Fig. 7.2 Pivotal Phase III trial of RTS,S/AS01$_E$. The map used in this figure was adapted from the "Duration of Malaria Transmission Season" map (2001) published by MARA (Mapping Malaria Risk in Africa; http://www.mara.org.za/)

completed with 15460 children included in the study. The primary objective of the study is the evaluation of vaccine safety and its efficacy against clinical malaria disease. Secondary objectives will assess, among other endpoints of public health relevance, efficacy against severe malaria disease, severe anaemia, malaria hospitalisations, fatal malaria and all-cause mortality. Analysis by site will evaluate efficacy under different conditions of malaria transmission. Evolution of vaccine efficacy will be assessed during a 32-month follow-up period, and the potential benefit of a booster dose will be evaluated in a subgroup of subjects. Finally, the impact of RTS,S/AS01$_E$ co-administration with the current EPI vaccines and the vaccines that should be included in EPI programmes shortly, such as the rotavirus vaccine and the pneumococcal conjugate vaccine, will additionally be evaluated in Phase III clinical trials.

7.2 Conclusions

A collective effort by the malaria vaccine scientific community, coordinated by the World Health Organisation (WHO) Institute for Vaccine Research (IVR), led to the publication by WHO of a "Malaria Vaccine Technology Roadmap" in 2006 (Malaria Vaccine Technology Roadmap 2006). The document states that the communities first landmark goal is to: "by 2015, develop and license a first-generation malaria vaccine that has a protective efficacy of more than 50% against severe malaria and death and lasts longer than 1 year".

In Phase II evaluation of the RTS,S/AS vaccine, consistent and significant efficacy has been observed against *P. falciparum* infection, clinical episodes of malaria and severe malaria in different transmission settings and in the different age groups evaluated. In one study, the clinical benefit conferred by the vaccine has been demonstrated to extend up to 42 months following vaccination. Finally, it was

shown that the RTS,S/AS vaccine candidate can be co-administered with existing EPI vaccines.

In conclusion, major milestones have been achieved in the development of the RTS,S/AS vaccine over the last two decades. Provided that the results of the RTS,S/AS01$_E$ Phase III trial confirm the promising Phase II data, and that the challenges in vaccine registration, procurement and implementation are met, the ambitious goal set by the WHO in 2006 seems therefore achievable.

Acknowledgments The results reported in this review are the fruit of many years of very hard work by literally hundreds of highly dedicated individuals in research institutions and in organisations involved in the development of the RTS,S/AS malaria vaccine, in Europe, the USA, and Africa.

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Conflicts of Interest: J. Cohen, J. Vekemans, A. Leach and L. Schuerman are employees of GlaxoSmithKline Biologicals. A. Leach, J. Vekemans, J. Cohen, L. Schuerman and S. Benns hold shares in GlaxoSmithKline. J. Cohen is listed as an inventor of patented malaria vaccines, including RTS,S.

Comment of editor: In the mean time the clinical trial was successfully finished!

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Chapter 8
Small Ruminant Theileriosis

Jabbar Ahmed, Hong Yin, Mohammed Bakheit, Zhijie Liu, Heinz Mehlhorn, and Ulrike Seitzer

Abstract Theileria parasites, the causative agents of theileriosis, infect a vast number of wild and domestic animals and are transmitted transstadially by various members of tick vectors of the family Ixodidae. Three Theileria species infecting small ruminants have been shown to be responsible for losses in small ruminant production. Two of them, *T. uilenbergi* and *T. luwenshuni* have been described to occur in China, whereas *T. lestoquardi* causes malignant theileriosis in a number of countries like the Sudan, Iran and Iraq. Three other small ruminant *Theileria* species, *T. ovis*, *T. separata* and *T. recondita* are considered as non-pathogenic.

8.1 Introduction

Ticks and tick-borne diseases (TTBDs), including theileriosis, constitute a major constraint of livestock production. *Theileria* parasites, the causative agents of theileriosis, infect a vast number of wild and domestic animals and are transmitted transstadially by various members of tick vectors of the family Ixodidae. Generally, the life cycle of *Theileria* involves both the transmitting invertebrate tick vector, in which sexual reproduction and sporogony takes place, and the vertebrate host, in

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which asexual reproduction by schizogony and merogony occurs (Barnett 1968; Mehlhorn and Schein 1984, 1993).

A number of excellent reviews on the life cycle of *Theileria* have been given by Barnett (1968), Mehlhorn and Schein (1984, 1993) and Mehlhorn et al. (1994), which can be consulted for more detail on this subject. Briefly, after being inoculated by the tick, the sporozoites of these parasites invade host mononuclear cells (Fawcett et al. 1982; Jura et al. 1983) where they subsequently differentiate to schizonts. The infected cells undergo clonal expansion, a process referred to as "*Theileria*-induced reversible transformation". In this case, the host cell division is accompanied by simultaneous division of the schizonts, which usually results in the infection of daughter cells. Later, the schizonts differentiate to microschizonts, which are released after the rupture of the host leukocytes. Merozoites are capable of infecting erythrocytes and develop to piroplasms, which are then taken up by and are infective for the vector tick (Mehlhorn and Schein 1984; Fig. 8.1a, b).

The pathogenesis of theileriosis is dependant on the stage of the parasite and type of cells it infects. For the leukoproliferative Theileria species, *T. parva*, *T. annulata* and *T. lestoquardi*, the macroschizont infecting and proliferating in the host's leukocytes is considered to be the most pathogenic parasitic stage. In *Theileria* species which are characterized by a short schizogony period, for example *T. mutans*, the pathogenesis is related to the tissue injuries and anaemia caused by the merozoites infecting erythrocytes.

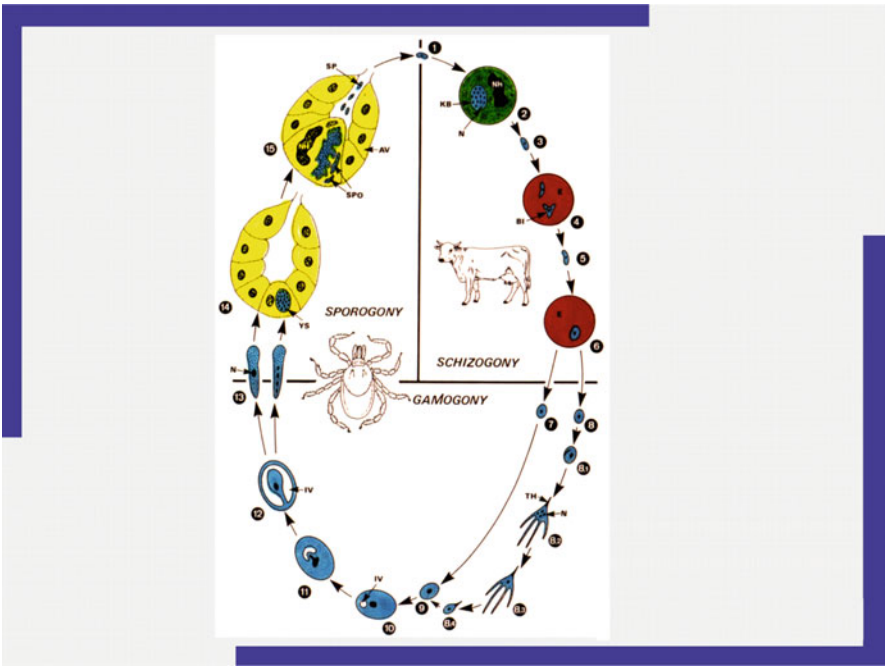

Fig. 8.1 (continued)

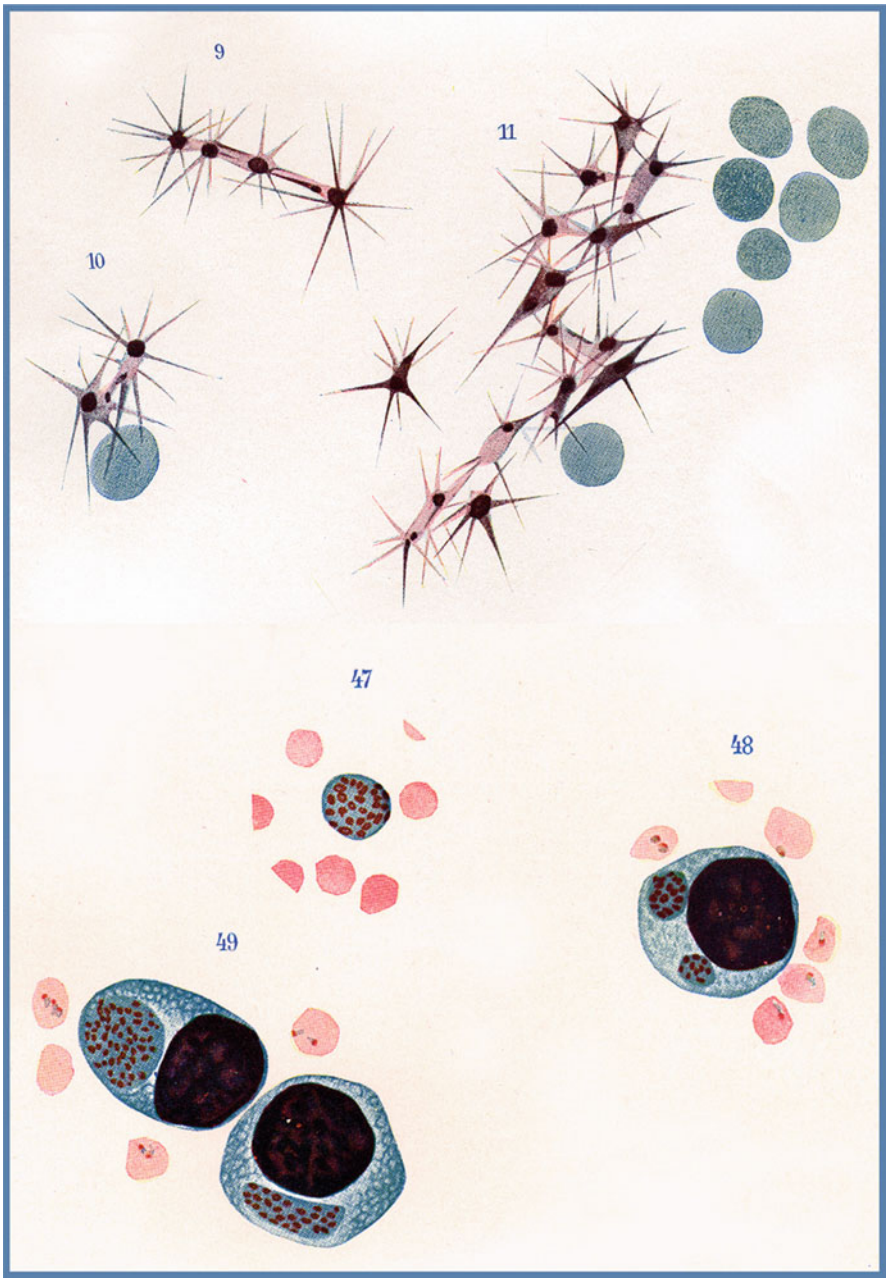

Fig. 8.1 (**a**) Life cycle of *Theileria parva*. At first lymphocytes are infected, later erythrocytes. In the tick's intestine gamogony occurs and in the salivary glands the formation of sporozoites. (**b**) Original drawings of Koch and Kleine showing *Theileria*-stages in lymphocytes and the ray-stages = gamonts

8.2 Leucoproliferative Bovine Theileriosis

The leucoproliferative *Theileria* (*T. annulata, T. parva*) cause diseases known as *Tropical Theileriosis* and *East Coast fever* (ECF), respectively.

8.2.1 Tropical Theileriosis

Tropical theileriosis is caused by *T. annulata* and is associated with *Hyalomma* vector ticks. It is known to occur in Southern Portugal, Spain, Italy, Bulgaria, Greece, Turkey, Southern Russia, Central Asia, the Near and Middle East, Pakistan, India and North-East China. In Africa, the distribution is limited to the Northern littoral, Morocco, Algeria, Tunisia and Libya, except in North-eastern Africa where it reaches down through Egypt to at least as far South as the 13th parallel in the Sudan and is also reported in Eritrea and Mauritania (Uilenberg 1981; Jacquiet et al. 1990, 1994). Susceptible cattle kept in areas where the tick vectors occur are in principle at risk of contracting theileriosis. Serological surveys carried out in the early 1980s showed that the prevalence of *T. annulata*-specific antibodies decreased from 90% in Khartoum State to less than 13% in the Southern parts of the Blue Nile (Um Benien). This was related to the distribution and abundance of *Hyalomma a. anatolicum* in these areas (FAO 1983). In Southern Sudan, antibodies to *T. annulata* were detected (FAO 1983), but as the parasite vectors were not reported to occur there, it was suggested that these antibodies, shown by the Indirect Fluorescent Antibody Test (IFAT) (Burridge et al. 1974), might have been due to cross-reactivity with *T. parva*. More recently, molecular evidence based on the sequence of the polymorphic region of the *T. annulata* TaSP gene has shown the presence of this parasite in southern Sudan (Salih et al. 2007a). This is an interesting finding which depicts southern Sudan as the only region in the world where *T. annulata* overlaps with *T. parva*. Nevertheless, more detailed studies would be required to establish the field vector of this parasite in Southern Sudan.

More recently, RLB (Radioligand Binding) was used for the first time to investigate the prevalence of tick-borne pathogens in Khartoum State, Sudan, showing a prevalence of *T. annulata* DNA of 65% (Ali et al. 2006). A second study using RLB was carried out in Southern Sudan detecting the presence of eight different piroplasms, in which *T. parva* and *T. annulata* were found with a prevalence of 71.2% and 0.2%, respectively (Salih et al. 2007a).

In Tunisia as well as in the rest of North Africa, *T. annulata* is mainly transmitted by the barn tick *Hyalomma detritum*. However, the situation of tropical theileriosis in Egypt is less documented. According to Osman and Al-Gaabary (2007), the disease is considered as one of the most important obstacles to livestock production. Clinical cases were described in Holstein cattle and buffalo. Investigations on *T. annulata* and its transmission revealed that cattle harbouring piroplasms could

reach 8% of the investigated population (Mazyad and Khalaf 2002). Several tick species were recorded on cattle, mainly *Hyalomma* ticks such as *H. dromedarii*, *H. impeltatum* and *H. excavatum* (El Kammah et al. 2001). Among these species, *H. dromedarii* could represent a potential vector as already found in Mauritania (Jacquiet et al. 1990). The limited knowledge on tropical theileriosis in Egypt emphasizes the need for more comprehensive epidemiological investigations to assess the requirements for specific control strategies in different production systems (buffalo and dairy exotic cattle).

8.2.2 East Coast Fever

Similar to *T. annulata*, the distribution of *T. parva* infection is directly linked to the distribution of its vectors (*Rhipicephalus appendiculatus*, *R. zambeziense* and, possibly *R. duttoni*). These species of ticks are present almost exclusively in the eastern and central African countries including Kenya, Tanzania and Uganda and extending down to the eastern part of Kwazulu Natal in South Africa (corridor disease of buffalo). ECF was first reported in Southern Sudan in 1950 (Hoogstraal 1956), then serological evidence by IFAT was provided (Morzaria et al. 1981; Julla 1994). A first study provided molecular evidence for the presence of TBDs in cattle in Southern Sudan, namely *T. parva*, *T. mutans*, *T. annulata*, *Babesia bigemina*, *B. bovis*, *T. velifera*, *T. taurotragi* and *T. buffeli*, the last three of which were reported to be present in the region for the first time (Salih et al. 2007a). High infection of *T. parva* was reported among young calves compared to older cattle, a finding that could be attributed to the acquired immunity.

Initial transmission dynamic modelling of *T. parva* infections focused on an epidemiological state described by the term "endemic stability" (Norval et al. 1992), in which African Zebu (*Bos indicus*) cattle previously exposed to *T. parva* over several generations, and subjected to little or no tick control, are continuously challenged by infected ticks, and either become immune or die by day 150 of exposure. In endemically stable situations, the majority of ticks are believed to exhibit relatively low levels of infection in terms of both prevalence (% of ticks infected) and abundance (the mean number of infected acini/tick), both of which are frequently low in these epidemiological circumstances. A contrasting situation is endemic instability, in which the percentage of infected cattle and ticks is lower, either because of tick control measures or because conditions are ecologically marginal for the vector (Young et al. 1986; Young et al. 1996). This results in periodic epidemic disease outbreaks. Endemic stability is thought to be the prevalent situation in western and central Uganda. Whereas the epidemiological situation in Southern Sudan has not yet been fully investigated, the *T. parva* prevalence in zebu cattle in a recent longitudinal study in Southern Sudan was as high as 70% (Salih et al. 2007b).

8.3 Theileriosis of Small Ruminants

Small ruminants have a great relevance for the economy of a significant proportion of the world. Two billion of altogether 3 billion ruminants are sheep and goats. A report by FAO Expert Consultants (1997) strengthened "the global significance of the small ruminant livestock industry from the viewpoint of both national economists and individual resource-poor farmers in semi-arid zones". It seems that diseases of small ruminants are among the least recognized problems in veterinary science; this is true especially for ticks and tick-borne diseases. Despite recent progress, the identity of some *Babesia*, *Theileria* or *Anaplasma* species or their vectors is often uncertain. The economic impact of TTBDs is still unclear and as mentioned in the report by the FAO Expert Consultants "even where it seems that the significance for indigenous breeds in enzootic areas is slight, there may be a great loss of potential production because of the difficulties in promoting up-grading schemes using more productive but susceptible exotic breeds".

For a long time it was assumed that there are four *Theileria* species infecting sheep and goats and that only one of them, namely *Theileria lestoquardi*, is highly pathogenic to sheep and in some cases to goats, causing a disease called malignant theileriosis (Uilenberg 1981; Friedhoff 1997). However in the last years, two newly identified parasites have been shown to be responsible for theileriosis in small ruminants in China. These have been designated *Theileria luwenshuni* (previously referred to as *Theileria* sp China 1) and *T. uilenbergi* (previously referred to as *Theileria* sp. China 2) (Ahmed et al. 2006; Yin et al. 2007). Thus, at least six *Theileria* species infect small ruminants, three of which are non-pathogenic: *T. ovis*, *T. separata* and *T. recondita* (Table 8.1) (reviewed in Bishop et al 2009).

8.3.1 Non-pathogenic Theileria *Species of Sheep and Goats*

8.3.1.1 *Theileria separata*

Uilenberg 1977 (syn., *Haematoxenus separatus*) was described as a non-pathogenic parasite of sheep in eastern and southern Africa transmitted transstadially by *Rhipicephalus evertsi* (Uilenberg and Andreasen 1974). Erythrocytes infected by

Table 8.1 *Theileria* species infecting small ruminants

Species	Vector tick	Pathogenicity	Cultivable schizonts
T. lestoquardi	*Hyalomma*	Yes	Yes
T. luwenshuni	*Haemaphysalis qinghaiensis*	Yes	No
T. uilenbergi	*Haemaphysalis qinghaiensis*	Yes	No
T. ovis	*Hyalomma species*	No	No
T. recondita	*Haemaphysalis punctata*	No	No
T. separata	*Hyalomma species*	No	No

piroplasms of this species usually contain a veil as is the case with *T. velifera* of cattle and wild ungulates in several African countries (Young and Mchinja 1977).

8.3.1.2 *Theileria ovis*

Rodhain, 1926 has been described to infect sheep and goats from Africa, Europe and Asia (Neitz 1957; Uilenberg 1981). According to Uilenberg (1981), the identity of the parasite, and thus the validity of the name *T. ovis*, is not clear, as most of the reports assign this name to non-pathogenic small domestic ruminant theileriosis, which could mean that this species may constitute a complex of different species. In contrast, Schnittger et al. (2003) stated that *T. ovis* isolates originating from three entirely different geographic regions, namely Tanzania, Sudan and Turkey could cluster together in a phylogenetic tree based on the sequences of the 18S rRNA gene, suggesting that *T. ovis* does represent a single species.

8.3.1.3 *Theileria recondita*

Lestoquard 1929: The identity and validity of this parasite is less clear. In some literature the name *T. recondita* was used synonymously with *T. ovis*. Thus, it is an invalid name and as such cannot be used again for a theilerial parasite (Uilenberg 1981). The species discovered in Germany and the UK, and which was called *T. recondita* by Enigk (1953) in Germany and by Alani and Herbert (1988) in Wales could well be an unnamed species. It has been shown that this parasite is transmitted by the adults of *Haemaphysalis punctata* (Enigk 1953). However, field-collected nymphal stages of this tick failed to transmit the parasite (Alani and Herbert 1988).

The aim of this article is to review the progress achieved regarding small ruminant theileriosis with a special emphasis on *T. lestoquardi*, *T. luwenshuni* and *T. uilenbergi*.

8.3.2 Pathogenic Theileria *Species of Sheep and Goat*

8.3.2.1 *Theileria lestoquardi*

Malignant theileriosis is an emerging constraint to the small ruminant industry especially for sheep. The disease is caused by *T. lestoquardi* (*Theileria hirci*) which is highly pathogenic for sheep and to a lesser degree for goats (Fig. 8.2). *Theileria lestoquardi* has been shown to be transmitted by *H. a. anatolicum* (Hooshmand-Rad and Hawa 1973b). Accordingly it is expected to occur in areas where this tick species is found, mainly in Northern Africa, Southern Europe and the Middle East (Uilenberg 1981). However, other tick species were also suspected to be vectors

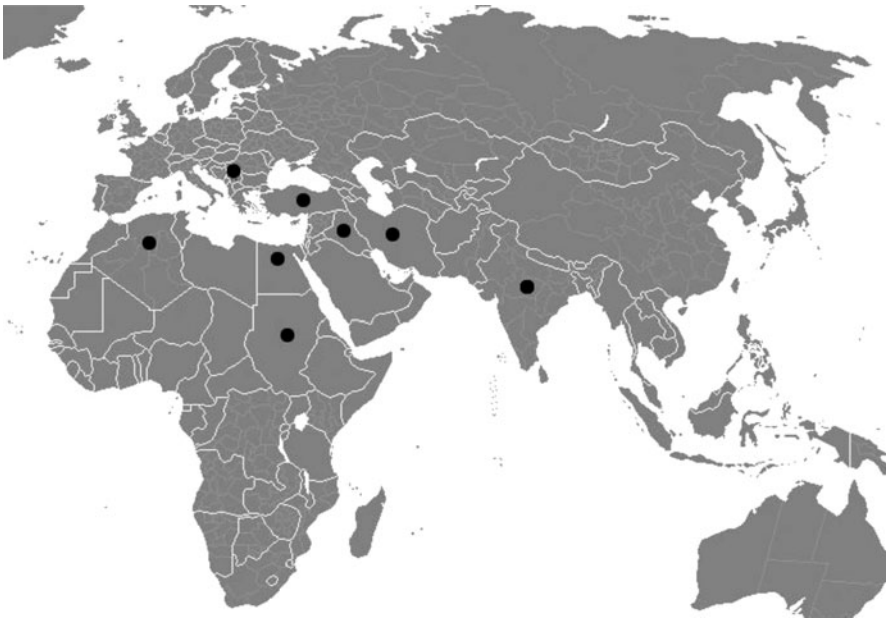

Fig. 8.2 Distribution map of *Theileria lestoquardi* (*T. hirci*)

and the parasite has also been reported to occur outside the *H. a. anatolicum* zone (Friedhoff 1997).

Historically, the first report on *T. lestoquardi* infection was in Egypt in 1914 involving two Sudanese sheep with the description of small, pleomorphic intra-erythrocytic piroplasms and extra-erythrocytic bodies found in the spleen, supra-scapular and other lymph nodes (Littlewood 1915). One year later, the disease was reported to be common also in Egyptian sheep (Littlewood 1916). Thereafter, *T. lestoquardi* and malignant theileriosis of sheep and goats were diagnosed and identified in many other countries, for example, Serbia (Dschunkowsky and Urodschevich 1924), Algeria (Lestoquard 1927), Turkey (Baumann 1939), Iraq (Khayyat and Gilder 1947) and Iran (Hooshmand-Rad and Hawa 1973a). Interestingly, it has never been reported from Israel, despite the presence of the tick vector (Pipano 1991).

Whether all these disease records can be attributed to the same pathogen is not clear (Brown et al. 1998) as the diagnosis, in most cases, was merely on the basis of the combination of severe disease with the presence of piroplasms and sometimes schizonts in blood and lymph node biopsy smears. In China, for example, a pathogenic *Theileria* species of sheep was first thought to be *T. lestoquardi* (Luo and Yin 1997) but lately it was shown to be a distinct entity (Schnittger et al. 2000). In fact, since the earlier work reviewed by Neitz (1957), *T. lestoquardi* has generally been inadequately studied despite its pathogenic nature.

The parasite is phylogenetically most closely related to *T. annulata* (Katzer et al. 1998; Schnittger et al. 2003) and is transmitted by the same vector *H. a. anatolicum* (Hooshmand-Rad and Hawa 1973a). This close relationship between *T. lestoquardi* and *T. annulata* is also reflected by the biological characteristics of these two *Theileria* species, for example:

1. Both parasites are transmitted by one known Hooshmand-Rad and Hawa (1973b)
2. Both *T. annulata* and *T. lestoquardi* parasitize the similar cell phenotypes of their respective hosts (Leemans et al. 2001)
3. *T. annulata* and *T. lestoquardi* are capable of infecting and transforming sheep and goat PBMCs (peripheral blood mononuclear cells) in vitro (Steuber et al. 1986; Leemans et al. 1999a) and in vivo (Brown et al. 1998; Leemans et al. 1999b)
4. The vast cross-reactivity in IFAT indicates the presence of common antigens between the two parasites (Leemans et al. 1997).

On the other hand, fundamental differences were reported to occur between these two parasites, for example:

1. *T. lestoquardi* infection of sheep can give rise to severe disease, whereas the course of *T. annulata* infection in these animals is subclinical and piroplasms are not detectable (Leemans et al. 1999b).
2. Cattle inoculated with a stabilate of *T. annulata* sporozoite suffer from severe or lethal disease, whereas calves injected with the same dose of *T. lestoquardi* sporozoites do not develop disease (Leemans et al. 1999a, b).
3. *T. annulata* can infect and transform sheep PBMC in vitro, whereas *T. lestoquardi* has not been observed to do likewise in bovine PBMC (Steuber et al. 1986; Leemans et al. 1999b).

Diagnosis of *T. lestoquardi* Infection

Like with other leukoproliferative *Theileria* species, the diagnosis of clinical infection by *T. lestoquardi* in small ruminants is usually based on clinical signs, knowledge of disease and vector distribution, and identification of the piroplasm and schizont stages of the parasite in Giemsa-stained blood and lymph node smears (Hooshmand-Rad and Hawa 1973a). However, these methods are reliable for the detection of acute cases but have limited value for chronic and long-lasting carrier cases, where only low numbers of *Theileria* piroplasms exist (Neitz 1957) and it needs great expertise to differentiate microscopically between pathogenic and non-pathogenic *Theileria* species that may simultaneously infect the same host (d'Oliveira et al. 1995; García-Sanmartín et al. 2006).

To overcome this problem, a number of serological tests such as IFAT have been developed for the diagnosis of different *Theileria* species including *T. lestoquardi* (Hawa et al. 1981; Leemans et al. 1997; Salih et al. 2003; Taha et al. 2003). Although, this test has been reported to be more sensitive than microscopic examination of blood smears, it is, however, not always sufficiently sensitive and in

addition, it shows cross-reactivity with antibodies directed against other species of *Theileria*. These observations limit the specificity of IFAT (Burridge et al. 1974; Kiltz et al. 1986; Darghouth et al. 1996; Papadopoulos et al. 1996).

Recently, major progress was achieved by using a recombinant parasite protein for the development of an enzyme-linked immunosorbent assay (ELISA) (Bakheit et al. 2006b). Bakheit et al. (2006a) succeeded to identify, clone, characterize and recombinantly express a gene (clone 5) which encodes for an immunodominant *T. lestoquardi* protein and compared to IFAT, ELISA has the following advantages:

1. The use of recombinant proteins would minimize the chance for cross-reactivity
2. A large number of animals can be tested in a quite short time and thus it is very useful for the conduction of epidemiological studies
3. The test is easy to perform, economic and amenable to standardization.

The huge progress in molecular biology has allowed the development of sensitive PCR-based diagnostic assays for the detection of several pathogens including *Theileria*, *Anaplasma* and *Babesia* (Bishop et al. 1992; Kawazu et al. 1992). This technique is based on an in vitro primer-directed enzymatic amplification of DNA by a thermostable DNA polymerase. Using parasite-specific primers, PCR was also established for the detection of small ruminant infecting *Theileria* species. For *T. lestoquardi* primers were derived from the 30-kDa merozoite surface gene of *T. lestoquardi*, the sequence of which was analyzed alongside the corresponding genes of several *T. annulata* isolates. The important aspect of this test is its ability to differentiate between *T. lestoquardi* and *T. annulata* in the transmitting Hyalomma vector and in sheep and goats (Leemans et al. 1999a).

The PCR assay was further developed not only for detection but also for differentiation between pathogens present in a given sample. Using the characteristic sequence signatures within the hypervariable region 4 (V4 region) of the 18 small ribosomal RNA gene of ovine/caprine piroplasm species and based on the ascertained gene variations, Schnittger et al. (2004) developed a reverse line blotting (RLB) assay. All probes bound with high specificity to their respective target sequence, therefore, no cross-reaction was observed resulting in clear recognition of either individual strains, species or groups. No signal was observed when ovine and caprine genomic DNA was used as the control, demonstrating that the signals are due to the presence of parasite DNA in investigated samples.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid detection method which allows a target DNA to be efficiently amplified with high specificity and sensitivity under isothermal conditions. This method can amplify a few copies of DNA to 10^9 copies in less than 1 hour. Moreover, LAMP can be applied using a non-denatured template, and DNA extraction may also be neglected, since a drop of blood spotted on filter paper meets the requirements to initiate the reaction (Nagamine Mori et al. 2001). The LAMP technique allows visual detection of amplified products through the addition of fluorescent dyes such as SYBR Green and measurement of turbidity. These characteristics make this method potentially useful to be employed as a rapid test for use in the field. Recently, a LAMP protocol was established for detection of *T. lestoquardi* based on

the clone-5 DNA sequence, which remains to be validated (Ali et al. abstracts TTP6; STVM09).

Immunity to *T. lestoquardi*

T. lestoquardi is known to be highly pathogenic to sheep and causes high morbidity and mortality rates, even among indigenous sheep populations (Hooshmand-Rad and Hawa 1973a; Tageldin et al. 1992). So far, little is known about the mechanisms involved in the pathogenesis of the disease and the immune response to *T. lestoquardi* has not been adequately studied. Nevertheless, it is known that animals that survive infection are resistant to challenge (Hooshmand-Rad 1985). On the basis of the similarities between *T. annulata* and *T. lestoquardi*, particularly regarding their host target cells, it is reasonable to speculate that similar immune mechanisms as observed for *T. annulata* are also operating in *T. lestoquardi* infection. These are MHC class I and II specific cytotoxic and helper T-cells, respectively. Moreover, it was shown that *T. lestoquardi* is, like *T. annulata*, able to infect monocytes/macrophages of its host sheep (Leemans et al. 2001), which also express mRNA for TNF-alpha (Ali et al. 2008).

It is also widely accepted that goats show significant resistance to the disease compared to sheep (Hooshmand-Rad and Hawa 1973a; Sisodia 1981; Brown et al. 1998). However, the mechanisms underlying this phenomenon are not known.

Like in other *Theileria* infections, animals recovering from *T. lestoquardi* infections produce antibodies that are detectable by serological means such as IFAT and ELISA. In an experimental infection of sheep with *T. lestoquardi*, Leemans et al. (1997) could demonstrate that the pattern of antibody production and titre durations in *T. lestoquardi* infection resemble those described previously for *T. annulata*.

Regarding immunoprophylaxis of malignant theileriosis, immunization of sheep with attenuated *T. lestoquardi* schizont-infected ovine cells has been carried out successfully in Iraq and Iran (Hawa et al. 1981; Hooshmand-Rad 1985; Hashemi-Fesharki 1997). Although the tissue culture vaccine might be the best method to control malignant theileriosis of sheep and goats, the testing, the long culture periods and the cold chain needed to produce the vaccine make the production expensive. The culture itself might be a risk of introducing other infections. Another limiting factor is that protected animals become carriers and a source of infection.

Identification and Characterization of Potential Immunologically Relevant Proteins

Little is known about the mechanisms of the immune response to *T. lestoquardi* infection. This is also due to the lack of knowledge regarding parasite proteins involved in the induction of the immune response of the host. In this respect,

attempts have been made in the last years to identify such parasite antigens using different approaches, such as immunoscreening of cDNA libraries with serum from infected animals, random screening and bioinformatic analysis of genes from cDNA libraries, and PCR strategies used to search for homologues of proteins described for *T. annulata* or *T. parva*.

With respect to *T. lestoquardi*, screening of a schizont cDNA library resulted in the identification of clone-5 protein (Bakheit et al. 2006b). PCR experiments and sequencing demonstrated the presence of two transcript forms of the gene, resulting from splicing variation at an intron in the gene. Both gene products, clone-5 long and clone-5 short variants with calculated molecular weights of 99.9 and 72.7 kDa, respectively, were expressed in a *T. lestoquardi* infected cell line. BLAST searches suggested the presence of homologues of the gene in both the *T. parva* and *T. annulata* genomes, with identities of 53% and 62% on the DNA level, respectively. The intron was preserved in size, sequence and location within the gene in these parasites. Analysis of the subcellular localization of the clone-5 proteins showed a predominant parasite membrane association in *T. lestoquardi* infected cells. Both recombinantly produced forms were found to be reactive with sera from infected animals. Bioinformatic analyses were employed to address the possible function of the gene products in the biology of *T. lestoquardi*. A partially expressed polypeptide of clone-5 was successfully used to establish an indirect ELISA for the detection of *T. lestoquardi* infection (Bakheit et al. 2006).

A PCR to search for a homologue to the TaSP protein previously identified in *T. annulata* led to the identification of the *T. lestoquardi* surface protein TlSP (Schnittger et al. 2002). The cDNA of this homologue was cloned and recombinantly expressed and found to be reactive with sera from *T. lestoquardi*-infected sheep, making this protein also a suitable candidate for ELISA development. Furthermore, the *T. lestoquardi* homologue of the *T. parva* P32 gene could be partially cloned and sequenced. The antigenicity of this protein is under evaluation.

8.3.2.2 Small Ruminant Theileriosis in China

Ovine theileriosis has been described to occur in the Gannan Tibet region of Gansu Province in China for more than 100 years but was however first documented in 1958 (Yang et al. 1958). Small ruminant theileriosis in China is caused by two *Theileria* species, which are morphologically and biologically very similar. They can only be differentiated by molecular biological means which clearly affiliate both parasites in distinct phylogenetic clusters (Yin et al. 2004; Ahmed et al. 2006). According to phylogenetic analysis, they appear to be most closely related to the *T. sergenti/T. buffeli* group (see below).

Investigations using microscopic examination of blood smears showed that the infection is widely distributed in northwest China including Gansu (Qiu and Yuan 1982; Lu et al. 1994), Ningxia (Li et al. 1985), Inner Mongolia (Yu et al. 1982), Qinghai in 1963 (Wang et al. 1980), Sichuan (Yang et al. 1958) and Shanxi

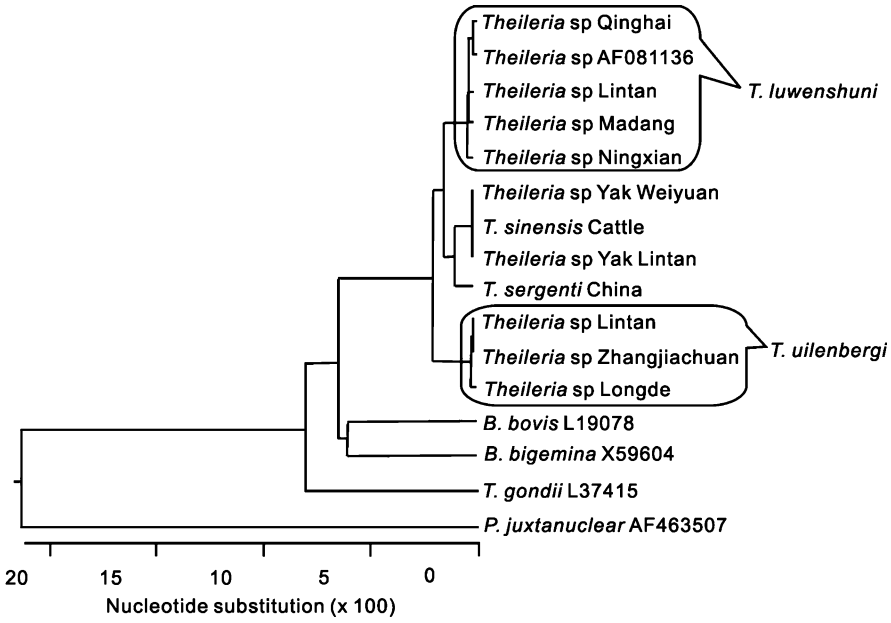

Fig. 8.3 Distribution of *Theileria* species in China according to molecular determination of probes

(Li et al. 1985) Provinces as shown in Fig. 8.3. These areas are considered to be the major ovine theileriosis endemic regions in China.

The disease occurrence is closely related to the distribution of the transmitting vectors. Among the ticks found in the endemic regions, *Haemaphysalis qinghaiensis* and *H. longicornis* were tested experimentally and proved to be able to transmit the disease, whereas *Dermacentor silvarum* and *D. abaensis* did not (Li et al. 1986, 2007; Guo et al. 2002). Whether or not other ticks existing in the area can transmit the disease is unknown to date. Apart from China, the occurrence and distribution of this disease in other parts of the world is unclear, although recently a parasite showing close identity on the 18S RNA gene level was described in Spain and was described not to be pathogenic (Nagore et al. 2004).

Sickness and death rates vary considerably with age and breed of animals. Studies on 1,144 sheep and goats in the mountain districts in the South of Ningxia Province showed that the incidence and lethality were 28.3% and 75.3%, respectively. Most cases were lambs and animals aged 1–2 years (Li et al. 1985), whereby exotic animals are more susceptible to this infection (Luo and Yin 1997). The incidence rate in cross-bred animals was almost the same compared to that of local herds (Guo et al. 2002). The incidence and death rate in young animals was higher than in adults. Unlike the case with *T. lestoquardi*, where goats are known to be more resistant than sheep (Hooshmand-Rad and Hawa 1973a), there was no evidence that sheep and goats differ in their susceptibility to the infection (Yin et al. 2002).

Diagnosis

The conventional diagnostic methods for ovine theileriosis in China depend on clinical signs, microscopic examination of blood smears and biopsy smears, and epidemiological history. The schizonts were distributed throughout the lymphocytes and were not surrounded by cytoplasm. During the later period of the disease, many schizonts were found outside the cells (Guo et al. 2002; Luo and Yin 1997). In recent years a number of more sensitive and specific assays have been developed, including molecular-based techniques such as reverse line blot (Schnittger et al. 2004), LAMP (Liu et al. 2010) and recombinant protein-based ELISA (Miranda et al. 2006; Liu et al. 2010; Abdo et al. 2010).

Pathology

The examination of smears prepared from liver, spleen, lung, kidney, lymph nodes and peripheral blood showed that macroschizonts and microschizonts could be found in all these samples (Yin et al. 2003). An interesting observation was that the schizonts were rarely seen in the host cell cytoplasm and that most of them were located outside the cells. To date, there is no explanation for this observation, however it may be hypothesized that an apoptosis-related mechanism leading to the release of the parasite from the host cell, as has been described for the malaria parasite (Sturm et al. 2006), may lead to the extracellular localization of the *Theileria* schizont. Three days after the appearance of the schizonts in the lymph nodes, the most important stage seems to be the piroplasm stage, which is detectable in erythrocytes.

Identification and Characterization of Potential Immunologically Relevant Proteins

As mentioned above, different strategies were also used to identify antigens of *T. luwenshuni* and *T. uilenbergi*. Using immunoscreening of a *T. uilenbergi* merozoite cDNA library led to the identification of the *T. uilenbergi* immunodominant protein (TuIP), which was successfully used to establish a diagnostic ELISA (Liu et al. 2010). Homology searches of the TuIP sequence showed the highest identity to a hypothetical protein of *T. parva* (Gene ID: 3503410 TP01_0987) and its *T. annulata* orthologue (Gene ID: 3864034 TA16685). Using a prediction server for antigenicity the average antigenic propensity for this protein was given as 1.0253, consisting of 27 antigenic determinants. Moreover, analysis of MHC-I binding epitopes based on bovine class I A20 specificity predicted four nonamer peptides with a disassociation half time of 1,000 min (Parker et al. 1994).

Random sequencing and bioinformatic analyses of clones from the *T. uilenbergi* merozoite library also led to the identification of a potentially antigenic clone-2 gene

family (Liu et al. 2008) and a member of this family, clone-9, was successfully implemented in an indirect ELISA (Abdo et al. 2010).

To investigate the immune response to infection with *T. uilenbergi*, parameters of cellular and humoral immunity of experimentally infected sheep against two recombinantly expressed *Theileria* proteins were examined. The in vitro proliferative response of blood mononuclear cells to rTaD and rTcSP, both putative membrane proteins, was significantly elevated and significant amounts of specific immunoglobulins were produced against both (Seitzer et al. 2008).

Phylogenic Position of *Theileria* Infecting Small Ruminants in China

The analysis of the 18S rRNA gene sequence and of a number of ovine and bovine *Theileria* species revealed that *T. lestoquardi*, *T. uilenbergi* and *T. luwenshuni* are affiliated to the genus *Theileria*. The established phylogenetic tree of *Theileria* splits into two monophyletic branches: one comprising *T. annulata*, *T. parva*, *T. taurotragi* and *T. lestoquardi,* the latter being closely related to *T. annulata,* while the other branch includes *T. sergenti*, *T. buffeli* and the Chinese isolates of *T. luwenshuni* and *T. uilenbergi* (Fig. 8.3). The group of *T. annulata, T. lestoquardi, T. parva, T. taurotragi* represent *Theileria* parasites with a marked intra-leukocytic phase in contrast to the group formed by *T. sergenti*, *T. buffeli*, *T. luwenshuni* and *T. uilenbergi*, indicating that the parasites of the latter group are not able to transform their host cells.

Three further *Theileria* genotypes have been identified, sharing 96.7–97.0% similarity between their 18S rRNA gene sequences: *Theileria ovis*, *Theileria* sp. OT1 (99.6% similarity with the recently described pathogenic piroplasm *Theileria luwenshuni*) and *Theileria* sp. OT3.

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Chapter 9
Interactions of *Trypanosoma cruzi* and Triatomines

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Abstract Triatomine bugs are vectors of *Trypanosoma cruzi*, the etiologic agent of Chagas disease in Latin America. The flagellate colonizes the intestinal tract of the insect, especially the rectum. *T. cruzi* changes the composition of amino acids and proteins/peptides in the rectum and affects the intestinal innate immune homeostasis. Since it induces only adverse effects on larval developmental times and mortality rates if starvation as a second stressor is present, the flagellate is classified as "subpathogenic" for the vector. Effects of the vector on the flagellate are obvious in the differing competence for different strains of *T. cruzi*. In addition, the development of the flagellate is affected by different nutritional stages of the vector, i.e. starvation and feeding induce changes in the population density and the percentages of the different developmental stages, especially of spheromastigotes and giant cells which usually occur rarely. Compounds in the urine which is secreted rapidly after feeding induce the development of metacyclic trypomastigotes, the human-infectious stage.

9.1 Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease, one of the "Big six" of tropical diseases (Schaub and Wülker 1984). It is endemic in Latin America and mainly transmitted by triatomines (Coura 2007). These insects are night-active, and especially poor living conditions, for example houses in rural areas made of adobe bricks or wooden frames covered with mud, offer hiding places during the daytime (Schaub 2009). Of the 140 species of triatomines, only some are strongly adapted to houses, especially *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongylus megistus* and *T. dimidiata* (Fig. 9.1). These species are in the focus of control campaigns,

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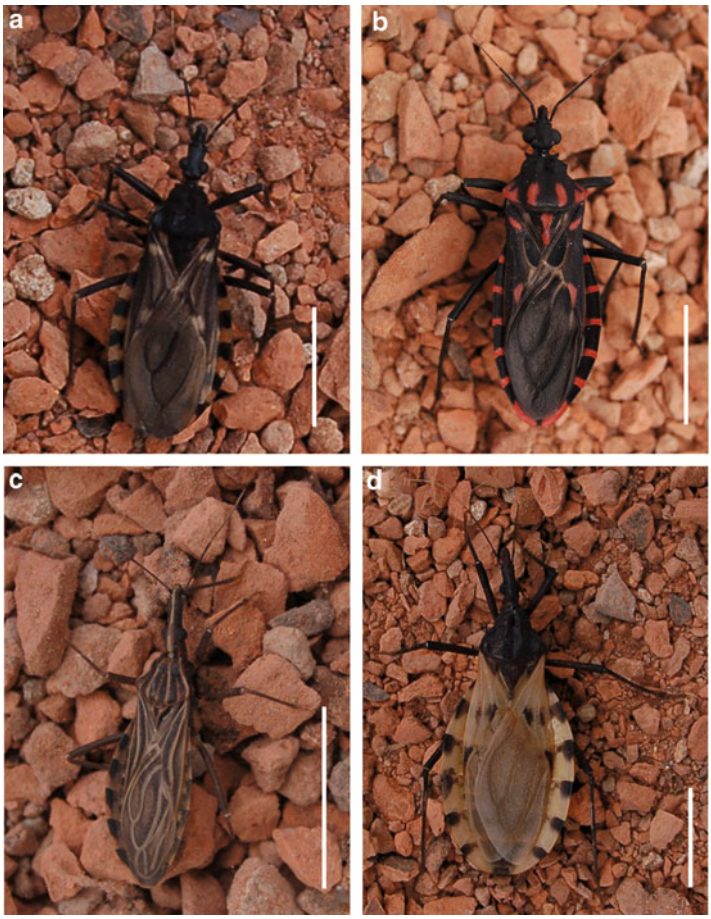

Fig. 9.1 Adults of *Triatoma infestans* (**a**), *Panstrongylus megistus* (**b**), *Rhodnius prolixus* (**c**), and *Triatoma dimidiata* (**d**), (Scale bar: 1 cm)

since only two compounds for chemotherapy which were developed about 1970 are still available, often inducing severe side effects in the patients. Beside house improvements and education, mainly intense insecticide campaigns against the domestic populations of the most important vector *T. infestans* are performed. This has strongly reduced the prevalence from about 20 million chronically infected people in 1982 to about 8 or 12 million in 2007, the latter estimations differing according to the source (WHO 1982, 2007; Dias 2007). Eradication is impossible since Chagas disease is an anthropozoonosis circulating also in many wild mammals as reservoir hosts. In addition, after eradication of domestic *T. infestans* sylvatic populations or other species of triatomines invade the houses (Abad-Franch and Monteiro 2005). Therefore, vector surveillance is of high priority. A suitable and promising immunologically based monitoring technique was recently developed to screen the sera of peridomestic and domestic animals for antibodies against saliva of the vectors (Schwarz et al. 2009, 2010).

9.2 Triatomines

Triatomines are the biggest blood-sucking insects (Schaub 2008), ingesting about 6–12-times their own body weight. Thereafter, some larval instars can starve for up to about 1 year. One full engorgement or several smaller volumes of blood are necessary for the development to the next larval instar or adult stage. The blood is stored in the distensible stomach, concentrated by withdrawal of ions and water and only processed by a lysis of erythrocytes and resorption of sugars (Schaub 2008) (Fig. 9.2). Small portions of blood are digested in the following small intestine where perimicrovillar membranes develop to thick staples after feeding (Billingsley and Downe 1986). Digestion of haemoglobin starts immediately, as indicated by the colour change from red to brown.

The digestion of triatomines is different from that of other blood-sucking insects, for example mosquitoes and lice. Whereas the latter have an alkaline intestinal pH and digest the blood mainly by the serine proteases trypsin and chymotrypsin, Hemiptera and therefore triatomines have an acidic pH in the lumen of the gut and digest mainly by at least three different cathepsins (Kollien et al. 2004a, b). Originally, the activity of cysteine proteases was attributed to cathepsin B (summarized by Terra 1990), but according to molecular biological identifications, also cathepsin L is synthesized in the gut (Lopez-Ordoñez et al. 2001; Kollien et al.

Region	Function	Digestive Enzymes[a]
1 Foregut **2 Cardia**	protection of symbionts	1 salivary enzymes 2 lysozymes[b]
3 Stomach (anterior midgut)	storage and concentration of the bloodmeal lysis of erythrocytes	3 glycosidases lysozymes alkaline and acidic phosphatases aminopeptidases sialidases[c] lipases[d] amylases (derived from symbionts)
4 Small intestine (posterior midgut)	protein digestion nutrient absorption	4 glycosidases lysozymes alkaline and acidic phosphatases cathepsin B cathepsin L cathepsin D aminopeptidases carboxypeptidases lipases[d] amylases (derived from symbionts)
5 Rectum (hindgut)	absorption	

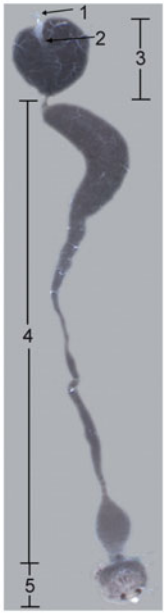

Fig. 9.2 Digestive tract of triatomines: regions, functions and digestive enzymes. [a]Modified from Kollien and Schaub (2000); [b]Araújo et al. (2006); [c]Amino et al. (1995); [d]Canavoso et al. (2004); Grillo et al. (2007)

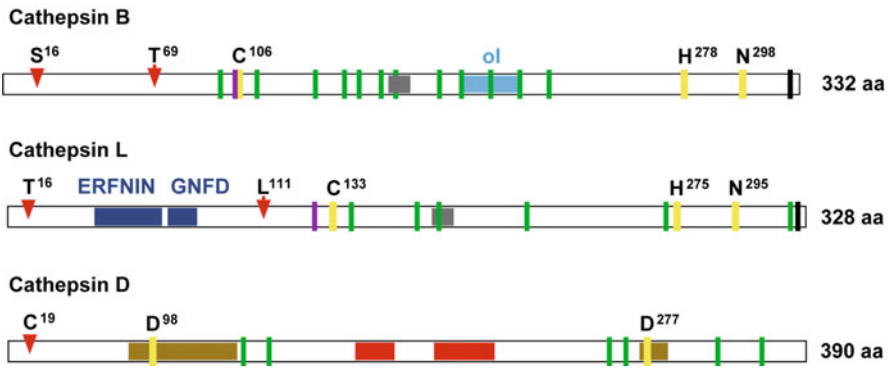

Fig. 9.3 Scheme of primary structures of cathepsin B, L and D from *Triatoma infestans*. Putative cleavage sites of signal peptide and activation peptide are indicated by *red arrow-heads* and *arrows*, respectively. Active site residues and cysteine residues forming disulphide bonds are marked in *yellow* and *green*, respectively. *Black bars* represent substrate specificity determining amino acid residues in cathepsin B and L. The "oxyanion hole" in cathepsin B and L is built by a glutamine residue (*purple*) and specific conserved regions classifying the different cathepsins are highlighted in *light blue* (occluding loop in cathepsin B, ol) and *dark blue* (ERFNIN and GNFD motif in cathepsin L). The common GCDGG motif of cysteine proteases cathepsin B and L is indicated in *grey*. Conserved regions of cathepsin D proteases are indicated by *red* and *brown boxes*, the latter containing the catalytic aspartate residues

2004b). This cathepsin shares many conserved regions in the deduced amino acid sequence with cathepsin B, but can be identified by the ERFNIN and GNFD motifs (Rawlings and Barrett 1994; Sajid and McKerrow 2002) (Fig. 9.3). Whereas both cysteine proteases possess an activity optimum at about pH 5, the aspartic protease cathepsin D has its major activity at about pH 3 (summarized by Garcia et al. 2010). Carboxypeptidases and aminopeptidases continue the digestion of the blood proteins (summarized by Garcia 1987).

After absorption of the nutrients in the small intestine, the remains of the blood are stored in the rectal sac before being defaecated (Terra 1990). Blood ingestion induces a rapid 1000-fold increase of the diuresis rate by the Malpighian tubules (Maddrell 1991). The urine sweeps off the remains of digestion, changing the conditions in the rectum rapidly (Kollien et al. 2001). Between 1 and 10 days after feeding, yellow-white urate crystals from the Malpighian tubules predominate in the rectum, followed by dark-brown remains of digestion.

9.3 *Trypanosoma cruzi*

9.3.1 Strain Peculiarities

Investigations of isoenzymes of strains of *T. cruzi* indicate a predominantly clonal genetic structure of the populations and only restricted genetic recombinations

(Tibayrenc et al. 1986). Until last year, strains of *T. cruzi* were classified into 2–3 groups (Anonymous 1999). Strains of the two major groups differ strongly in terms of biological characteristics, for example virulence and pathogenicity for mice, multiplication rate in in vitro cultures or metacyclogenesis rate in the vector (summarized by Schaub 2009). In some regions, lineages of *T. cruzi* are associated with infections in domestic vectors and humans, sylvatic vectors and humans, and sylvatic vectors and wild mammals (summarized by Vallejo et al. 2009; Schaub 2009). In vectors and mammalian hosts, often mixed infections occur. After experimental infections of mice and triatomines with strains belonging to *T. cruzi* I and *T. cruzi* II, a host-dependent selection towards one of the groups seems to occur (summarized by Schaub 2009). Recently, six genetic subgroups were classified based on molecular markers, named TcI–VI (Zingales et al. 2009). The characteristics of strains of these subgroups have to be determined.

9.3.2 *Developmental Cycle of* T. cruzi

Infectious stages in the faeces of the vector initiate the infection of the mammalian host. These metacyclic trypomastigotes are phagocytized, transform to amastigotes and multiply intracellularly in all cells of the mammalian host, except erythrocytes, because the parasites require purines from the host cell. If the host cell is exhausted, the amastigotes transform to non-dividing blood trypomastigotes. After rupturing the host cell they infect new cells or circulate in the blood (Schaub and Wunderlich 1985).

If a bug ingests blood containing blood trypomastigotes, these are aggregated, and some seem to fuse, enabling a genetic exchange (Brener 1972). They transform in the stomach to a-, sphero- and epimastigotes that multiply and colonize the whole intestinal tract and the Malpighian tubules (Fig. 9.4). The development of non-dividing metacyclic trypomastigotes seems to be restricted to the rectum. In the vector, *T. cruzi* reaches high population densities: 3 months after infection of second instars of *T. infestans*, the small intestine of fifth instar larvae contains about 500,000 parasites and the rectum about 1,500,000 (Schaub 1989a).

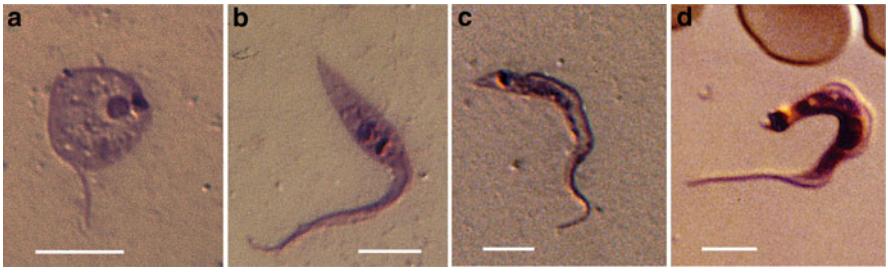

Fig. 9.4 Different developmental stages of *Trypanosoma cruzi*: spheromastigote (**a**), epimastigote (**b**), metacyclic trypomastigote (**c**), blood trypomastigote (**d**); Giemsa stained. (Scale bar: 5 μm)

9.4 Effects of *T. cruzi* on Triatomines

9.4.1 Effects on the Development of Larvae and Starvation Capacity

Effects of *T. cruzi* on the developmental rates of larvae were found rarely (summarized by Schaub 1992). Using a system in which vector and the *T. cruzi* strain originate from the same village, no retardation of development of regularly fed larvae occurs. Also the mortality rates are not affected by *T. cruzi* if the groups are maintained under optimal conditions (summarized by Schaub 2009). Under such conditions, the volume of ingested blood seems to be sufficient to compensate the metabolite losses of triatomines to the parasite.

This compensation theory is supported by investigations of the starvation resistance. After an infection in the first instar and the last feed in the second, third and fourth instar, the resulting third, fourth and fifth instar larvae survive about 5, 11 and 9 months, respectively, and the mean starvation resistance is reduced respectively by 3%, 14% and 32% relative to uninfected bugs (Schaub and Lösch 1989). Since more food remains are present in the intestine of infected than in uninfected bugs not the availability of proteins from the blood determines the starvation capacity, but the concentration of essential metabolites for which *T. cruzi* and vector compete. An accumulation of toxic products by *T. cruzi* seems to be unlikely since many flagellates die in starved bugs (Schaub and Böker 1986; Kollien and Schaub 1998a). Since no effects are obvious under optimal conditions and infections induce only adverse effects if starvation as a second, synergistic stressor is present, *T. cruzi* is classified as "subpathogenic" (Schaub 1989b, 1992).

9.4.2 Effects on Behaviour

Disturbances of probing in infected bugs have been found several times (summarized in Schaub 1992, 2009). However, also long-term starved bugs probe more often before engorging and ingest lesser amounts of blood than short-term starved bugs (Schaub, unpublished). If infected and uninfected bugs are compared at the same time after the last feeding, the infected bugs are in a more progressed state of starvation and thus the effects attributed to *T. cruzi* might have been effects of starvation and of minor relevance under natural conditions.

9.4.3 Effects on the Intestinal Border Face, Digestion and the Composition of the Intestinal Contents

In the small intestine, no effects on the perimicrovillar membranes or the cells of the intestinal wall are evident (Kollien et al. 1998). In the rectum, the flagellates attach

to the rectal cuticle and insert the flagellum between the layers of wax, also at the rectal pads, which are suggested to absorb metabolites from the remains of blood digestion (Schmidt et al. 1998). The disturbance of the wax layer might affect absorption processes, but more important effects should result from the colonization density, a carpet of three or four layers on the rectal pads (summarized by Kollien et al. 1998).

The composition of the contents of stomach, small intestine and rectum of uninfected and infected bugs has not been compared in detail, for example by two-dimensional electrophoresis, but the haemolysis of erythrocytes in the stomach and the protein digestion by cathepsin B in the small intestine are not affected by the flagellate (summarized by Schaub 2009). However, more detailed investigations are necessary, since molecular biological assays also identified another cysteine protease, cathepsin L (Lopez-Ordoñez et al. 2001; Kollien et al. 2004b). Such investigations should also consider that the flagellate possesses cysteine proteases, cruzipains, not only intracellularly but also as membrane-bound proteases and also the respective inhibitors (summarized by Schaub 2009). The activity of the third protease, cathepsin D, is increased at 1 and 3 days after an experimental infection with epimastigotes (Borges et al. 2006).

In the rectum, the strong colonization should induce effects. Therefore, it is not surprising that in infected bugs the composition of the rectal contents is changed: the concentrations of free amino acids are reduced and the composition of protein/peptide bound amino acids is changed, indicating that proteins or peptides from the rectal contents are hydrolyzed in the rectum also by surface proteases of *T. cruzi* or intracellularly in the flagellate (Kollien and Schaub, unpublished).

9.4.4 Effects on the Immune System and Intestinal Microorganisms

Ingestion of trypomastigotes stimulates immune reactions in the intestine of the triatomine *R. prolixus*. The expression of the gene of the more intestinally active lysozyme RpLys-A is increased after an infection and not that of RpLys-B, the gene of which is primarily expressed in the fat body (Ursic-Bedoya et al. 2008). The production of the only known antimicrobial peptide of triatomines, defensin, has not been investigated in infected triatomines, but another factor, nitric oxide, is also synthesized in the intestine after an infection. This has been shown by the upregulated expression of the gene of the nitric oxide synthase in the stomach after an infection with blood trypomastigotes, but also by the increased concentrations of a metabolite of nitric oxide, nitrite, in the small intestine (Whitten et al. 2007). In addition to lysozymes, defensins and nitric oxide, several unidentified bacteriolytic compounds are present, which are visible in zymograms using *Micrococcus luteus* (syn *M. lysodeikticus*) (Waniek et al. 2009). Since the expression rates of the genes of lysozymes, defensins and nitric oxide synthase are also increased after blood ingestion (Araújo et al. 2006; Whitten et al. 2007), these short-term reactions to

T. cruzi can partly be a general defence reaction against microorganisms and the increase of cathepsin D activity (see 4.3) could also be a part of this concerted action.

Effects of *T. cruzi* are also evident in long-term infected *T. infestans*. After an experimental infection via a mixture of blood and different microorganisms, high numbers of fungi and bacteria develop only in infected bugs not in uninfected controls, indicating an immune suppression in the intestine (Eichler 1998). The number of symbionts is not affected in these long-term infected larvae.

9.5 Effects of the Triatomine on *T. cruzi*

9.5.1 Susceptibility and Refractoriness

So far, all species of triatomines which were maintained in the laboratory could be experimentally infected with *T. cruzi*. Therefore, probably all species are potential vectors (Schofield 1994). However, susceptibility varies depending on various factors, and not all infections remain established (Garcia and Azambuja 1991). Usually natural infections of triatomines are not lost, indicating a strong co-evolution of *T. cruzi* and the respective species/strain of the insect host (summarized by Schaub and Lösch 1989). In endemic regions, the infection rates of triatomines vary strongly, for example between 79% in a local survey in Bolivia and 5% in a general survey in Brazil (Medrano-Mercado et al. 2008; Dias 2002). The latter value is not only due to a limited access of triatomines to an infected host, but to refractoriness mechanisms in the gut of the vector (summarized by Garcia et al. 2010). This is especially important in xenodiagnosis: Blood of patients suspected to be infected with *T. cruzi* in the chronic phase of the disease, in which the parasite cannot be found in microscopical blood examinations, are fed to laboratory-bred uninfected triatomines. Low numbers of parasites in the blood multiply in the triatomine and can more easily be found after some weeks (Dias 1940). In such diagnoses, sometimes parasites do not multiply. Therefore, the use of local vectors is suggested (summarized by Meiser and Schaub 2011).

9.5.2 Effects of Factors in the Stomach – Components of the Saliva of Triatomines and Host-Derived Factors

The saliva of triatomines contains many different pharmacologically active compounds that inhibit blood coagulation and enable rapid blood ingestion. For the majority of these compounds the function is unknown. In some of them the activity is controlled by the cleavage of the activation peptide via serine proteases in the saliva (Amino et al. 2001; Assumpção et al. 2008; Meiser et al. 2010b).

Usually such serine proteases act very specifically. Their activity in the stomach should be limited by Kazal-type inhibitors of serine proteases, which usually inhibit the activation of thrombin and thus blood coagulation (summarized by Meiser et al. 2010a). Possible interactions of these proteases and inhibitors with the surface proteins of *T. cruzi* have not been investigated.

One of these proteolytically activatable proteins, the alkaline, lysine-rich protein trialysin of *T. infestans*, lyses bacteria and mammalian cells, but also the cell culture-derived trypomastigotes and epimastigotes of *T. cruzi* (Amino et al. 2002, Martins et al. 2008). In addition to other factors, it seems to impair the transmission of *Trypanosoma rangeli* by *T. infestans*, since the main vector, *R. prolixus,* does not possess this lytic activity in the salivary glands (Gregório and Ratcliffe 1991).

Another protein of the saliva of *T. infestans*, a sialidase, targets sialic acids that are involved in blood coagulation as well as in inflammatory processes (Amino et al. 1998). A sialidase activity is not only found in the saliva, but also in the stomach contents, the enzyme rapidly desialylating the ingested blood cells and also the epimastigotes in the midgut (summarized by Amino et al. 1995). Therefore, sialic acid on the surface is not required for the development of epimastigotes in the intestine. Since stationary phase epimastigotes and metacyclic trypomastigotes possess high levels of a unique transsialidase to transfer sialic acid to their surface, sialic acids appear to be important for the survival of trypomastigotes in the mammalian host (Amino et al. 1995).

Another component of the saliva, an inhibitor of the complement activation, is suggested to be necessary for the protection of the intestinal cells of the vector against this host-derived system (Cavalcante et al. 2003; Barros et al. 2009). However, the activity of the complement in the stomach is not totally, but only strongly reduced within 2 h after blood ingestion and remains slightly active up to 1 day later (Garcia, unpublished). Within this period of time ingested blood trypomastigotes are agglutinated and transform to epimastigotes. This stage of *T. cruzi* is lysed by the complement. The sensitivity of epimastigotes is evident in the initial phase of an infection of humans: If faeces of the vector with the different stages of development of *T. cruzi* gets access to the mammalian host, all stages except metacyclic trypomastigotes are killed by the complement system. The sensitivity is also evident in established infections in which the stomach has been re-colonized from the population in the small intestine. After the larval moults, nearly all blood passes to the small intestine, and the red colour of the remaining blood in the stomach changes to brown, indicating a re-flux of contents with digestive enzymes from the small intestine into the stomach. Via this re-flux, also *T. cruzi* re-establishes in the stomach. Then the ingestion of blood from chickens and rats but not from mice (which possess a weak complement system) results in a lysis of epimastigotes (Schaub 1988; Schaub, unpublished). After such a feeding on mice, another factor from mammalian blood, plasminogen, binds to the epimastigotes (Rojas et al. 2008). The advantage or disadvantage of the presence of this inactive, usually fibrinolytic serine protease remains to be investigated.

A third host-derived factor, antibodies against *T. cruzi*, is also ingested together with the blood of the host. They can be a reason for the failure of the development of *T. cruzi* in xenodiagnosis triatomines (see Sect. 9.5.1), but an experimental proof is required. An indication is offered by in vitro assays in which, after an immunization of mice or rabbits with homogenates of epimastigotes, the decomplemented sera agglutinate the flagellates and induce ultrastructural damage (Fernández-Presas et al. 2001).

9.5.3 *Effects of Factors in the Stomach – Agglutinins and Hemolysins*

Whereas the general tasks of agglutinins/lectins in the stomach are unknown, hemolysins have to lyse the erythrocytes in the stomach. Lectins were suggested to be involved in the development of the different stages of *T. cruzi* in the vector because they differed between the stomach and the small intestine of *R. prolixus* and the respective receptors were present in epimastigotes but not in blood trypomastigotes (Pereira et al. 1981). The respective lectins in the small intestine were not verified, but the levels of lectins seem to be affected by the blood source (Ratcliffe et al. 1996). Whereas the effects of agglutinins on the long-term development of the different stages require new investigations, the initial establishment of *T. cruzi* in the vector is determined by agglutinins and hemolysins. After ingestion of *T. cruzi* they determine the susceptibility or refractoriness of the respective species or population of triatomines but also for a specific strain of *T. cruzi*. In the initial transformation of *T. cruzi* the blood trypomastigotes are often agglutinated, but not in all strains of *T. cruzi*. In incubations in stomach extracts of *R. prolixus*, epimastigotes of the strain Cl and the clone Dm28c are agglutinated but not lysed and develop in the bug. However, epimastigotes of strain Y are not agglutinated but lysed and thereby are unable to develop in *R. prolixus* (Mello et al. 1996). The agglutination is not induced by a reaction of a peanut-like lectin with the disaccharide D-Gal-β(1 → 3)D-GalNAc, since not only epimastigotes of strain Y but also of strain Cl do not react with this lectin (Schottelius 1982). The purified hemolytic factor of *R. prolixus* lyses the majority of epimastigotes of strain Y, but much less of the clone Dm28c (Azambuja et al. 1989).

9.5.4 *Effects of Factors from Small Intestine and Rectum – The Border Face*

Although the digestive small intestine contains higher concentrations of nutrients than the rectum, in regularly fed fifth instars of *T. infestans* only about 0.5 million flagellates/bug develop there, about one third of the population in the rectum (Schaub

and Lösch 1988; Schaub 1989a; Kollien and Schaub 1998a, b). After infecting fourth instars of *T. brasiliensis* and feeding the fifth instar, the small intestine even contains only one tenth of the rectal population (Araújo et al. 2008). Using two other strains, only one strain mainly colonized the small intestine of *T. brasiliensis* (Araújo et al. 2007). Except in the last investigation, the concentration of nutrients seems not to be relevant for these differences in the colonization. Since small intestine and rectum are of different ontogenetical origin – entodermal or ectodermal – the border face is different, i.e. the cells of the midgut possess apical microvilli that are covered by perimicrovillar membranes, whereas the rectal cells are covered by a cuticle.

In transmission electron microscopy of infected small intestines, all epimastigotes are in intimate contact with the perimicrovillar membranes (Kollien et al. 1998; Gonzalez et al. 1999). No ultrastructural modifications of the flagellum of trypanosomatids are evident, and the epimastigotes attach via glycoinositol phospholipids at their surface (Alves et al. 2007; Nogueira et al. 2007). Since the development of perimicrovillar membranes is hormonally regulated, 10 days after decapitation of *R. prolixus*, *T. cruzi* is present only occasionally in the gut; the same effect occurs after the feeding of blood containing antiserum against the membranes and midgut tissue (Alves et al. 2007; Gonzalez et al. 2006).

In the rectum, *T. cruzi* prefers to attach to the cuticle and initially colonizes the four rectal pads (summarized by Schaub and Böker 1986). In established infections, about one third of the rectal population is attached to this region, which covers – roughly estimated – only about 20% of the rectal surface (Schaub and Lösch 1988; Kollien and Schaub 1997, 1998b). Another third is attached to the remaining rectal wall and the final third colonizes, unattached, the rectal lumen. The preference for the rectal pads might have a physiological or mechanical basis (summarized by Schaub 2009). As a mechanism in the attachment by the flagellum originally lectins at the surface of the flagellum and chitin residues were supposed to be involved (summarized by Schmidt et al. 1998). Especially, the use of wheat-germ lectin-gold conjugates for the detection of chitin in transmission electron microscopy verifies that chitin is not accessible for *T. cruzi* and that the superficial layer at the luminal surface of the rectum is covered by a wax layer (Schmidt et al. 1998). The attachment of hexadecane droplets to a small region near the tip of the flagellum of epimastigotes identifies a hydrophobic interaction as attachment mechanism (Kleffmann et al. 1998). After the initial attachment to the rectal wall, the flagellum is modified. At the attachment site to the rectal cuticle, epimastigotes develop enlargements of the flagellum (summarized by Kollien et al. 1998).

A species-specific composition of the rectal border face is indicated by different attachment rates of epimastigotes of *T. cruzi* strain Y and strain Berenice: Epimastigotes of the latter strain adhere better to recta from *Rhodnius neglectus* than to recta from *Triatoma pseudomaculata* (Carvalho-Moreira et al. 2003). This corresponds with a higher metacyclogenesis rate in vivo. The epimastigotes of the *T. cruzi* strain Y show no differences in the attachment rate to the rectum of the two triatomines in vitro, but also develop more metacyclic trypomastigotes in *R. neglectus*. In general, attachment strongly enhances the transformation of epimastigotes to trypomastigotes in vitro (summarized by Kleffmann et al. 1998).

9.5.5 *Effects of Factors from Small Intestine and Rectum – Effects of Proteases*

All parasites developing in digestive regions of the intestine must possess a refractory surface and/or a rapid shedding or inactivation of attached proteases and/or inhibitors of digestive enzymes. The surface coat of trypanosomatids is refractory against many adverse compounds, and specific mucins are suggested to protect the epimastigotes against proteases (Acosta-Serrano et al. 2001). In addition, *T. cruzi* possesses a cysteine protease inhibitor, chagasin, at the surface (Monteiro et al. 2001, 2008; Ljunggren et al. 2007). The target of this inhibitor can be cathepsins in the direct neighbourhood of the flagellate and/or in the lumen of the gut (see Sect. 9.2). However, tissue culture-derived trypomastigotes possess higher levels of this inhibitor than epimastigotes, the latter producing inversely correlated more papain-like cysteine proteases, cruzipains. Therefore, in epimastigotes chagasin might mainly regulate the endogenous and not the surface bound cruzipain (Monteiro et al. 2001).

Direct or indirect effects of proteases have only been considered once. After feeding blood supplemented with the cathepsin D inhibitor pepstatin, the number and metacyclogenesis rate of *T. cruzi* in the gut of *R. prolixus* is not affected compared to bugs fed solely on blood (Garcia and Gilliam 1980).

9.5.6 *Effects of Factors from Small Intestine and Rectum – Influence of Starvation*

An effect of starvation on *T. cruzi* in a field population of triatomines was first detected in *Triatoma dimidiata*, of which more starved than regularly fed larvae lost the infection (Vargas and Zeledón 1985). In experimental infections and a scanning electron microscopical follow-up of the colonization density on the rectal wall (Schaub and Böker 1986), throughout the first 16 weeks after feeding of fifth instar larvae, no changes in the colonization pattern were evident: minimal colonizations around the entrance into the rectum, highest on the rectal pads and at a similar level in the other three regions. At 20 weeks after feeding, many regions were free of flagellates, but a residual population always remained attached to the rectal pads.

According to quantifications of the population density in small intestine and rectum, a starvation period of 3 or 4 weeks kills many flagellates in the small intestine of fifth instar larvae of *T. infestans* and reduces the population density (Schaub 1989a; Schaub and Lösch 1989). However, if infected triatomines die of starvation, some flagellates are still alive (Schaub and Lösch 1989). Longer starvation periods of 2 months, eliminate the population in the small intestine, but not in the rectum, which still contains about one third of the population of short-term

starved bugs (Kollien and Schaub 1998a, b). Four months after the last feeding, only 1% of the initial population are present, but in all bugs some parasites are alive.

Not only the number of flagellates is affected, also the composition of the population changes (Kollien and Schaub 1998a). The percentages of the respective intermediate forms and of spheromastigotes which only are present up to 2% of the total population in well-fed bugs increase during starvation to about 20% at 2 and 3 months after the last feeding (Kollien and Schaub 1998a).

9.5.7 Effects of Factors from Small Intestine and Rectum – Influence of Blood Ingestion and Excretion

Blood ingestion also affects *T. cruzi*, not only under certain circumstances in the stomach (see Sect. 9.5.2). In the small intestine, the population density increases after feeding (Schaub 1989a). Considering the whole intestine, this increase in the number of epimastigotes is correlated to the volume of blood ingested by the triatomine (Asin and Catalá 1995).

Initially opposite effects of blood ingestion are evident in the rectum. There, a low percentage of the attached population, but nearly all of the population in the lumen – about one third of the total population – is washed out by urine (summarized by Kollien and Schaub 1997). Identifying the different stages in the deposited drops of faeces and urine (see Sect. 9.2), the percentage of metacyclic trypomastigotes is low in the first drop of faeces, and the urine often contains pure populations of metacyclics (summarized by Schaub and Lösch 1988; Zeledón 1997). This is presumably based on the inability of trypomastigotes to attach, and especially metacyclic trypomastigotes of *T. cruzi*, lying on the carpet or in the upper layers of the carpet, are washed out. In addition, metacyclogenesis is induced (see Sect. 9.5.8). Thereby, in the remaining population the percentages of epi- and trypomastigotes are changed. The percentages of spheromastigotes and their intermediate forms are reduced from about 20% – the starvation effect – to about 2–3%, the level of regularly fed bugs (Schaub and Lösch 1988; Kollien and Schaub 1997).

After feeding of long-term starved bugs the population in the rectal lumen is also washed out, but an interesting phenomenon is evident in the composition of the population (Kollien and Schaub 1998b). In fifth instars which have starved for 60 days, the rectum contains about 20–30% spheromastigotes and the respective intermediate stages, about 20% epimastigotes and 40–50% trypomastigotes. One day after feeding, these forms represent 2%, 70% and 10%, respectively. However, about 10% are the so far only occasionally seen giant cells, containing many nuclei, kinetoplasts and flagella. In the following 2 days, the percentages of this form increase to 30–50% of the total population, and then decrease to 0%. These giant cells in long-term starved bugs originate from epimastigotes, those in the initial development after the infection from blood trypomastigotes (summarized by Schaub 2009).

9.5.8 *Effects of Factors from Gut and Malpighian Tubules – Induction of Metacyclogenesis*

Of all stages of *T. cruzi* deposited in the faeces/urine, only metacyclic trypomastigotes can survive after invasion through mucous membranes or skin lesions and initiate an infection in the mammalian host (Schuster and Schaub 2000). Therefore, elucidation of the mechanisms inducing the development of this stage has been the topic of many investigations. However, the investigations mainly focus on in vitro assays, simplified by the easy cultivation of *T. cruzi* in different media and cell cultures. In axenic in vitro cultivations first metacyclics appear after about 5 days (Chiari and Camargo 1984).

Using extracts of the small intestine or stomach of adult *T. infestans*, dissected 24–48 h after feeding, as a supplement to Grace medium, metacyclogenesis is induced after 4–7 days (de Isola et al. 1981). Extracts of adults fed 3 weeks before the dissections are inactive. Extracts of the rectum induce metacyclogenesis already within 15 min after incubation, and an additional supplementation with inhibitors of the ADP-ribosyltransferases inhibits this induction (de Isola et al. 1986, 1987). The inductory factor, which is present in the rectum of fifth instars and adults 2 days after feeding on chicken, increases the activity of the adenylate cyclase of *T. cruzi* and thereby induces metacyclogenesis. This factor is a 10-kDa peptide fragment from the amino terminus of chicken α^{D}-globin (Fraidenraich et al. 1993); pure chicken haemoglobin is inactive. A decrease of the concentration of this peptide in the rectum during the subsequent days after feeding explains the failure of induction using extracts of adults 3 weeks after feeding (see above). The reaction is not restricted to chicken α^{D}-globin since after feeding on mice, the rectum of *T. infestans* contains a similar active compound. Since 1–2 days after feeding, only urine or uric acid granules are present in the rectum, but no dark remains of digestion of haemoglobin (Schaub 2009), these data indicate a rapid passage of minor amounts of small molecules along the whole small intestine or an excretion of such compounds via the Malpighian tubules. However, the α^{D}-globin should also be present in the small intestine in which haemoglobin is digested, but extracts of this region induce metacyclogenesis much more slowly than extracts of the rectum (see above). In addition, metacyclic trypomastigotes rarely develop in the small intestine (Schaub 1989a).

According to in vitro incubations with synthetic peptides, which cover different parts of the respective α^{D}-globin fragment, the peptide corresponding to residues 1–40 at the amino terminus possesses the highest activity at concentrations higher than 10^{-10} M (Fraidenraich et al. 1993). In vivo, i.e. after feeding blood or plasma with different concentrations of haemoglobin and the synthetic peptides to *T. cruzi*-infected *R. prolixus,* higher concentrations of haemoglobin but not pure blood increase metacyclogenesis (Garcia et al. 1995). Although not all peptides show identical individual or synergistic effects in vitro and in vivo, the α^{D}-globin fragment seems to be a biologically relevant factor.

However, not only the usual adenylate cyclase pathway is activated during metacyclogenesis. In an induction via free fatty acids, especially oleic acid, and at concentrations similar to those found in the intestinal tract, protein kinase C isoenzymes are translocated to the membrane of culture-derived epimastigotes (Parsons and Ruben 2000; Wainszelbaum et al. 2003; Belaunzarán et al. 2009). It remains to be investigated whether or not one of these metacyclogenesis-inducing factors interacts with GP72, a major surface glycoprotein of *T. cruzi*; monoclonal antibodies against this glycoprotein strongly inhibit metacyclogenesis of epimastigotes (Snary 1985).

Whereas these assays identified specific factors, another approach uses nutritional stress. Epimastigotes in the late exponential growth phase of axenic in vitro cultures, shortly before an increase in the number of metacyclics, are incubated for 2 h in saline, named "artificial urine", and then in this saline supplemented with glutamate, aspartate, proline and glucose (Contreras et al. 1985a, b). This procedure strongly increases metacyclogenesis rates (Contreras et al. 1988), but most intensively only in one specific clone of *T. cruzi* (Dm28c) (Schaub, unpublished). Compared to the urine excreted by *T. infestans*, the "artificial urine" has other ionic strengths, another pH and contains neither amino acids nor peptides (Kollien et al. 2001). In addition, in the supplemented saline glucose is necessary for the transformation (Tyler and Engman 2001), but the presence of glucose in the rectum of a triatomine is doubtful. However, the respective clone and the specific conditions enable well reproducible studies, and the good timing of events enables the identification of steps in this cAMP-mediated process and of genes specifically expressed at each step of metacyclogenesis (e.g. Krieger and Goldenberg 1998; Gonzales-Perdomo et al. 1988; Ávila et al. 2003). The changes in the concentrations of the mRNA of the respective genes can also be found in *T. cruzi* populations in the vector (Cordero et al. 2008).

Can these in vitro assays be correlated to the metacyclogenesis in the vector? In the triatomines, metacyclogenesis seems to be restricted to the rectum. Although intermediate stages sometimes develop in the small intestine, only in the rectum do concentrations of metacyclic trypomastigotes increase up to 50% of that population, in part being enhanced by the attachment (summarized by Schaub 2009) (see Sect. 9.5.4). Metacyclogenesis already starts about 1–2 weeks after infection, some days after colonization of the rectum. After infection of second instar larvae and regular feeding, the percentages of trypomastigotes remain at this level for about 8 weeks (e.g. Schaub 1989a). Then in fifth instar larvae, the percentages increase to a higher level. However, an identification of factors acting 8–9 weeks after infection is difficult.

A better chance is offered by the induction of metacyclogenesis after blood ingestion (Schaub and Lösch 1988; Kollien and Schaub 1997) since it occurs in a short period of time, about 20 min after blood ingestion. Such a rapid induction is important for the population of *T. cruzi*. Urine washes out the majority of the population in the lumen. However, only metacyclic trypomastigotes or intermediate stages which possess the surface of the metacyclic trypomastigote can survive and develop in the mammalian host. Therefore, a rapid induction of metacyclogenesis increases the chance for survival. Since the surface coat does not change rapidly in

total (Schaub et al. 1989), a quantification of stages via these changes in a cell sorter is difficult. Easier, but more time-consuming is a morphological quantification in stained smears, also enabling a quantification of the four different ways of metacyclogenesis. Metacyclic trypomastigotes develop from spheromastigotes via drop-like or rarely ring forms and from epimastigotes as slender forms via a translocation of the kinetoplast to the posterior end or after unequal divisions resulting in an epi- and a trypomastigote daughter cell (Brener and Alvarenga 1976; Schaub 1989a). Before blood ingestion of larvae starved for 6 weeks, unequal divisions and ring forms are rarely found in the rectal lumen, drop-like and slender forms each make up about 50% of the intermediate forms. Within the first four drops of faeces/urine, only the percentages of slender intermediate forms increase, afterwards remaining at 100% (Schaub and Lösch 1988). Since this also occurs in vitro in incubations of the isolated complex of the rectum and the four Malpighian tubules after induction of diuresis by the artificial diuretic hormone 5-hydroxytryptamine (Kollien and Schaub 1997), the inducing factors seem to originate from the urine. This hypothesis is supported by incubations of pieces of recta with attached *T. cruzi* either in saline with different pH, or in faeces or urine of triatomines. Using different salines, the strong changes of pH, osmolality and ionic composition in the rectum (Kollien et al. 2001) do not induce metacyclogenesis. Also a mixture of remains of digestion and urine deposited in the first drop after feeding does not induce metacyclogenesis. Metacyclogenesis is increased only in incubations with urine (Kleffmann 1999).

Summarizing these investigations, they indicate the presence of different factors that induce metacyclogenesis. A factor in the urine enhances rapid metacyclogenesis in those epimastigotes which have already started to transform. About 1 or 2 days after blood ingestion, the $\propto^{D}$-globin fragment and oleic acid induce metacyclogenesis in the population remaining in the rectum after blood ingestion. This population might be sensitive to the enriched "artificial urine" which requires a previous nutritional stress that also occurs in the vector. It should be emphasized that all these factors are relevant for the metacyclogenesis of epimastigotes via a translocation of the kinetoplast. So far there is no indication which factors might induce metacyclogenesis of epimastigotes via unequal divisions and of spheromastigotes via drop and ring-like forms.

9.5.9 *Effects of Microorganisms and Antimicrobial Compounds*

The intestinal tract of triatomines is colonized by many different fungi and bacteria, the latter containing non-symbiotic and symbiotic species (summarized by Vallejo et al. 2009). Effects of non-symbiotic bacteria on *T. cruzi* have only been investigated for *Serratia marcescens,* which produces a red pigment, prodigiosin, and has been found in wild populations of *R. prolixus*. It is apathogenic for the triatomine (Azambuja et al. 2004). In *S. marcescens*-infected larvae, a subsequent infection with epimastigotes of *T. cruzi* strain Y and clone Dm28c results in strain/

clone-specific differences: the population density of strain Y decreases, whereas the density of clone Dm28c in the stomach remains unchanged (Azambuja et al. 2004, 2005). In incubations of the bacterium with epimastigotes of the strain/clone in vitro, the same differences arise. The bacteria develop long filamentous structures that connect the bacteria with the epimastigotes and kill them (Castro et al. 2007a, b). Using different strains of *S. marcescens*, only the strain which produces the red pigment kills *T. cruzi* strain Y. However, recent data exclude a direct effect of the pigment, since promastigotes of *Leishmania* are also lysed, if the production of prodigiosin is inhibited by changes in the growth conditions (Moraes et al. 2009). For lysis, attachment of the bacterium alone is sufficient. Lectin interactions seem to be involved since the effect is excluded if the epimastigotes are incubated in D-mannose (Castro et al. 2007a, b).

Effects of symbionts on the development of *T. cruzi* have only been considered in one investigation (Mühlpfordt 1959). The initial development of *T. cruzi* is stronger in bugs containing symbionts, but after a longer period is stronger in aposymbiotic bugs. However, the composition of the population, i.e. the percentages of the broad and slender epimastigotes, amastigotes and metacyclic trypomastigotes, remains unaffected. Since symbionts multiply in triatomines after blood ingestion and are suggested to produce vitamin B, the short-term results with *T. cruzi* correlate with results of another flagellate infecting triatomines, *Blastocrithidia triatomae*. A supplementation of blood with B-group vitamins (folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine) supports the initial development of *B. triatomae* in the small intestine of young larvae (Jensen and Schaub 1991).

Possible effects of antibacterial compounds of triatomines on *T. cruzi* have not been investigated. The RNAi technique for a knockdown of genes of antimicrobial factors and the incubation with heterologously synthesized antimicrobial compounds like lysozymes and defensins or enzymes of the immune system like the prophenoloxidase or nitric oxide synthase can elucidate possible effects on *T. cruzi*. Antibacterial compounds not belonging to the humoral response of triatomines affect the development of *T. cruzi* in vitro, for example mellitin, magainin, dermaseptin and tachyplesin (summarized by Löfgren et al. 2008). However, such in vitro assays usually use higher concentrations of peptides than those present in the respective insect. They might be usable in other approaches: The production of the lepidopteran cecropin by transformed symbionts kills all *T. cruzi* in the gut (Beard et al. 2002). Although *T. cruzi* induces the synthesis of several antibacterial factors, an effect on *T. cruzi* remains to be investigated.

9.6 Conclusions

Investigations of the interactions of *T. cruzi* and triatomines will be strongly supported by the genome project of *R. prolixus*. Although species of the tribe Rhodniini possess many differences in comparison to species of the other tribes (summarized by Schaub 2009), the increasing number of expressed sequence tags

and the RNAi technique offer many possibilities. Investigations of the effects of *T. cruzi* on triatomines mainly focus on the intestinal innate immune reactions, representing a second model system beside the Diptera. Two important open questions remain to be clarified on the effects of triatomines on *T. cruzi*: reactions inducing the initial killing of some strains of *T. cruzi* in the stomach and the molecular basis of metacyclogenesis in the rectum of the vector.

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Part II
Helminths

Chapter 10
Sex in Schistosomes – Signaling Mechanisms in the Female Gonads

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Abstract Besides their great medical importance as causative agents of schistosomiasis, an infectious disease affecting humans and animals in tropical and subtropical regions worldwide, schistosomes exhibit distinctive biological features. Living in the blood vessels of infected hosts, these blood flukes survive permanent attacks of the immune system over many years. Furthermore, schistosomes represent the only genus of the class trematoda which live doeciously. Their most remarkable attribute, however, is the continuous pairing-contact which is both obligatory for the development of the female reproductive organs and prerequisite for egg production

Over decades great efforts have been undertaken to develop an effective vaccine against schistosomes, but without fundamental success. In addition, due to the upcoming fear for resistance against the commonly used drug praziquantel, there is a pressing need to find new drugs to fight this parasite. In this respect, the understanding of basic processes of schistosome biology, especially its reproductive development, is fundamental. Since egg production is closely associated with the clinical progression of schistosomiasis, elucidating the molecular principles of the male-induced sexual maturation of the female may lead to new strategies intervening in these processes to control parasite spread on the one hand and to limit egg-induced pathology on the other.

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10.1 Schistosomiasis (Bilharzia) and Its Causative Agent

Second only to malaria, schistosomiasis represents an infectious disease of high global health priority for humans, but also for animals, and is caused by trematode parasites of the genus *Schistosoma* (Chitsulo et al. 2004; Engels et al. 2002; Quack et al. 2006; Ross et al. 2002; Steinmann et al. 2006). In honor of the German physician Theodor Bilharz, who first reported on this disease in 1851, it was initially named Bilharzia. This debilitating disease occurs in 77 countries worldwide affecting a population of about 600 million people. More than 200 million people are infected, 10% of which suffer from severe illness (Chitsulo et al. 2004; Engels et al. 2002; Mayer and Fried 2002; Ross et al. 2002; Xiaonong et al. 2002). About 85% of infected people live in sub-Saharan Africa, where the number of deaths was estimated at 200,000 per year (Engels et al. 2002). In the light of accumulating evidence from experimental and field studies for resistance against the commonly used drug Praziquantel (PZQ) (Botros et al. 2005; Doenhoff et al. 2008; Melman et al. 2009; Messerli et al. 2009; Pica-Mattoccia et al. 2009), and since no vaccine is available yet (McManus and Loukas 2008), great international research efforts have been initiated by governmental and educational institutions, industry, and WHO programs. Their aims are to create innovative ideas with the potential to be translated into safe, effective, affordable and widely utilized interventions to protect against this parasite. Among these efforts are comprehensive approaches to obtaining information on the genome, transcriptome, proteome, and the glycome of schistosomes with the aim of identifying new candidates for vaccine or drug development (Berriman et al. 2009; El-Sayed et al. 2004; Haas et al. 2007; Hokke et al. 2007a, b; Hu et al. 2004; Oliveira 2007; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium 2009; van Hellemond et al. 2007; Wilson et al. 2007).

10.2 Biology of Male–Female Interaction

Apart from their medical importance, schistosomes exhibit fascinating biological features. Living in the circulation of their hosts, these blood flukes face and withstand immune attacks over years and even decades. Furthermore, as the only family within the trematodes, schistosomes have evolved separate sexes. A nearly unique feature, however, is the sexual maturation of the female, which requires a close and constant pairing contact with the male (Grevelding 2004; Kunz 2001; Popiel and Basch 1984). Pairing induces mitoses and differentiation processes in the female leading to the full development of the ovary and the vitellarium, which is accompanied by a remarkable increase in the size of the females (Den Hollander and Erasmus 1985; Erasmus 1973; Knobloch et al. 2002a; Kunz 2001; Popiel 1986). The development of the female gonads is a prerequisite for egg production, which finally causes the pathological consequences of the disease. About 50–70% of the eggs penetrate the walls of the veins of the intestine or the urinary bladder leaving the body for maintenance of the

life cycle. However, about 30–50% of the eggs remain inside the body of a final host being trapped mainly in the spleen and liver (Moore and Sandground 1956). This provokes hyperimmune reactions resulting in inflammatory processes and chronic progression of the disease that can be lethal (Ross et al. 2002).

As typical for trematodes, schistosome eggs consist of one oocyte produced within the ovary and 30–40 vitelline (nurse) cells synthesized within the vitellarium. Both cell types are combined within the ootype to form eggs. Viable eggs are exclusively produced by paired, mature females, but not by virgin-like, immature ones (Shaw 1987). Mitoses and differentiation processes of stem cell-like precursor cells are induced in the ovary and vitellarium of the female upon pairing (reviewed in Kunz 2001). Furthermore, the stimulus of the male is not only necessary for the initiation of the reproductive development of the female, but also for the maintenance of her mature state (Clough 1981). After separation from its partner, the female regresses to an immature state. Mitotic activity and differentiation decline, and egg production stops (Popiel et al. 1984). However, upon re-pairing with the male the female resumes normal reproductive activity. Although many experimental approaches have been performed to identify the male stimulus in the past, its nature still remains unknown. Classical studies had demonstrated that female growth and development are independent of sperm development, sperm transfer or fertilization (Armstrong 1965; Popiel et al. 1984). Tactile stimulation or chemical communications, which is the favored hypothesis, are still discussed today (Kunz 2001; LoVerde 2002). Hormones, growth factors, or lipids were also proposed as being secreted from the male to induce the initial events in female maturation (Kunz 2001; Popiel 1986; Shaw et al. 1977; Silveira et al. 1986). The gynecophoral canal protein (GCP), an 80 kDa cell surface glycoprotein which had been located in the gynecophoral canal of male schistosomes, was discussed to play a key role in the male–female interaction. In unpaired males, GCP was only found in low amounts, which significantly increased following pairing (Bostic and Strand 1996; Jin et al. 2004). In paired females, GCP was found on the entire surface, whereas GCP expression was not observed in immature females. Since the maturation of vitellarium and ovary is limited to the regions of the female lying within the gynecophoral canal of the adult male (Popiel and Basch 1984), it was speculated that this surface protein may be associated with the male-induced maturation of the female during pairing (Cheng et al. 2009). RNAi approaches knocking down GCP in adult *Schistosoma japonicum* led in vitro to an abrogation of worm pairing and in vivo, using infected mice, to an inhibition of early parasite pairing, indicating that GCP might play an important role in pairing and subsequent development (Cheng et al. 2009). Furthermore, it was speculated that the female transmits a signal to the male during pairing which is presumably mediated by a TGFβ pathway within the male resulting in the expression and the release of GCP into the gynecophoric canal of male schistosomes (LoVerde et al. 2009). However, no conclusive evidence for the male signaling responsible for the induction of the female's maturation has been found yet.

Early studies on the regulation of female-specifically expressed genes had demonstrated that the male even exerts its influence at the transcriptional level of

the female. For genes expressed in the reproductive organs of paired females such as the eggshell precursor protein genes p14 (Koster et al. 1988) and p19 (Michel et al. 2003), the iron storage protein gene ferritin I (Schussler et al. 1995), and a mucin-like protein gene (Menrath et al. 1995) an influence of the male on transcription was documented. After separation from the male partner, the transcriptional activity of these genes declined, but was restored after re-pairing (Chen et al. 1992; Grevelding et al. 1997; Michel et al. 2003). Recently, male–female interaction was also studied using a microarray approach for global transcriptional analyses (Fitzpatrick et al. 2004; Fitzpatrick et al. 2005). Comparison of the transcriptomes of females at different stages of maturation showed that immature females transcribe a broader spectrum of genes than mature females. In immature females about 180 genes were found to be expressed more abundantly than in mature females. In these females about three times less genes were up-regulated after pairings, which included genes involved in egg formation or red blood cell consumption (Fitzpatrick and Hoffmann 2006). From these results it was speculated that the contact to the male streamlines the gene transcription profile of females on reproductive activities such as egg production.

10.3 Morphological Differences Between Mature and Immature *S. mansoni*

According to the physiological differences, former and recent studies have revealed significant morphological differences in the ovary and vitellarium between mature (paired) and immature (virgin-like) schistosome females (Erasmus 1973; Kunz 2001; Neves et al. 2005; Popiel et al. 1984; Popiel and Basch 1984; Shaw 1987).

In a mature schistosome female the ovary consists of oocytes at different stages of maturation – small, immature oogonial cells in the anterior part and bigger germ cells in the posterior part (Fig. 10.1a, b). The latter represent primary oocytes. They originate from mitoses of oogonial cells, accumulate cytoplasm and grow filling the center and the posterior portion of the ovary (Gresson 1964; Nollen 1997). In some trematode species, meiosis is still ongoing when the oocytes move into the oviduct. Fertilization finally induces meiotic progression. In other species meiosis is not initiated until fertilization has happened (Nollen 1997). Primary oocytes of schistosomes are hexagonal containing large amounts of cytoplasm. They are the biggest cells in the ovary but can also be found within the oviduct where fertilization takes place. Following carmine red staining and confocal laser scanning microscopy (CLSM), the big central nucleus appears dark and the nucleolus bright (Fig. 10.1a–c). Primary oocytes leave the ovary and enter into the oviduct, which forms close to the ovary the *receptaculum seminis* by local wall expansion. Here, mature sperms are stored (Fig. 10.1a) ready to fertilize primary oocytes. The oviduct finally leads to the ootype (Fig. 10.1c) which is lined in its periphery with elongated cells representing the Mehlis' glands (Neves et al. 2005). The ootype is the egg-forming organ. To make one composite egg, one fertilized oocyte and

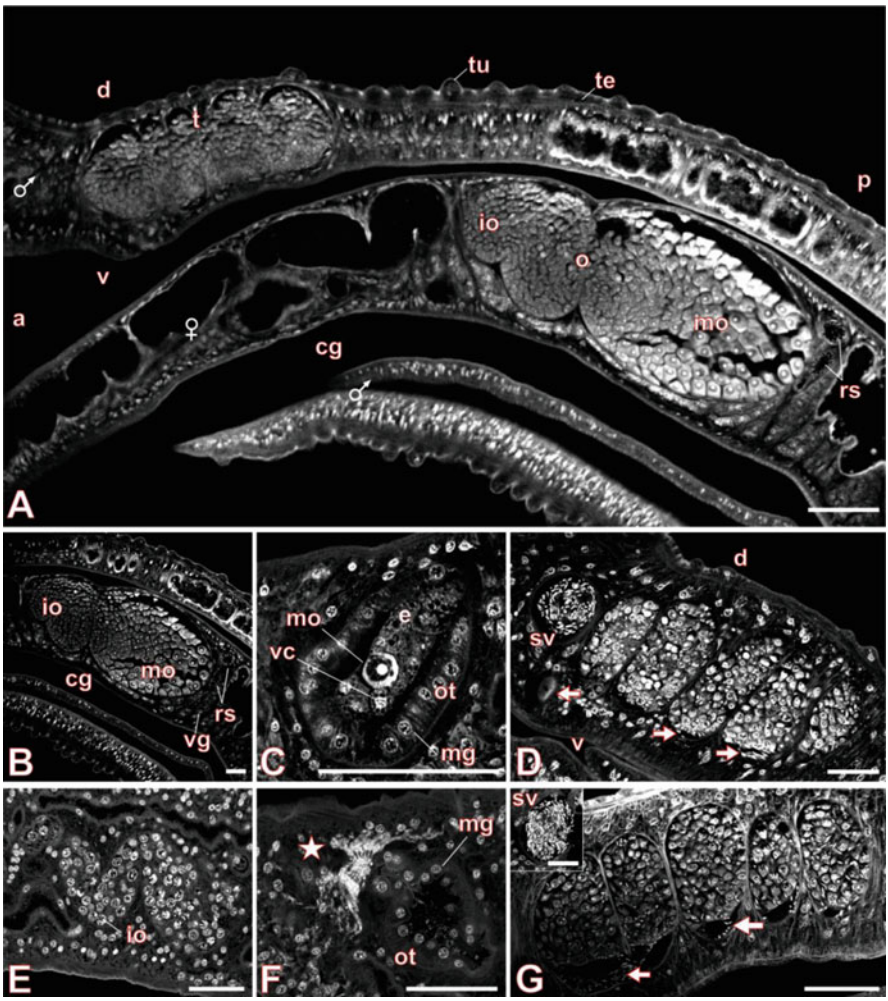

Fig. 10.1 Confocal laser scanning microscope (Leica TSC SP2) images of *S. mansoni* couples from a mixed infection (**a**–**d**), of immature *S. mansoni* females (**e**, **f**), and pairing-inexperienced puerile males (**g**) from unisexual infections stained with carmine red and analyzed by reflection mode. (**a**): Overview, (**b**): ovary with immature and mature oocytes, (**c**): ootype with egg and Mehlis' glands, (**d**): testicular lobes and sperm vesicle, (**e**): ovary with immature oocytes, (**f**): ootype with Mehlis' glands (*asterisk*: "hymen"), (**g**): testicular lobes with spermatocytes (*arrows*: mature sperms), sperm vesicle filled with mature sperms (Abbreviations: *a* anterior, *c* cirrus, *cg canalis gynaecophorus*, *d* dorsal, *e* egg, *io* immature oocyte, *mo* mature oocyte, *mg* Mehlis' glands, *o* ovary, *od* oviduct, *ot* ootype, *p* posterior, *rs receptaculum seminis*, *sv* sperm vesicle, *t* testes, *te* tegument, *tu* tubercle, *v* ventral, *vc* vitelline cell, *vg* vitelline gland; scale bar (**a**–**d**): 60 μm, scale bar (**e**–**g**): 30 μm) (Figure modified from Beckmann et al. (2010b), with permission from Cambridge University Press)

30–40 vitellocytes are needed. These cells provide egg-shell precursor proteins for egg-shell synthesis and energy resources for the developing miracidium. Vitellocytes are delivered from the vitellarium (Fig. 10.1c) and transported to the ootype via the vitelloduct. After formation, the eggs are transported via the uterus to the genital pore below the ventral sucker to be discharged.

Morphology and content of the ovary of an immature female are fundamentally different from that of a mature female. In its immature state, the ovary is small containing only a low number of oocytes, which probably represent stem cell-like oogonia in an undifferentiated stage of development (Fig. 10.1e) (Erasmus 1973; Neves et al. 2005; Popiel et al. 1984). The ootype is also smaller compared to that of mature females and contains no eggs (Fig. 10.1f). During our studies we have discovered a new structure located at the posterior end of the ootype, where oviduct and vitelloduct end (Fig. 10.1f). To our knowledge, this filamentous network-like assembly has not been described before. According to its location this structure seems to seal the entrance into the ootype of immature females preventing undifferentiated vitellocytes or oocytes from entering. In the biological sense this filamentous structure may fulfill the role of a hymen (Beckmann et al. 2010b), which corresponds to the observation that there is no egg production in immature females. Since we have never seen this structure in mature females, even if they were separated for 7 days from males, it is tempting to speculate that the hymen disintegrates as a direct consequence of pairing. It may be alternatively possible that the removal of this structure occurs indirectly by contact with mature vitellocytes and/or oocytes. In mature but separated females, undifferentiated vitellocytes and oocytes are able to enter the ootype since the hymen was removed during a former pairing contact. This leads to the production of abnormal eggs, as observed in earlier studies (Neves et al. 2005; Shaw and Erasmus 1981).

In contrast to females there are no obvious morphological differences between paired and puerile males, which have never been in contact with a female before. Testes of schistosome males consist of several testicular lobes enclosed by a thin albuginea-like capsule, containing germ cells at different stages of maturation. Spermatogenesis occurs from the dorsal to the ventral part in each lobe. In the dorsal part of the lobes, big, round spermatogonial cells are located (Fig. 10.1a, d). They are mitotically active and become primary spermatocytes, which enter meiosis differentiating via primary and secondary spermatocytes (meioses I) to spermatids (meioses II). These round cells elongate to become fully differentiated spermatozoa, which are visible in the ventral part of the testicular lobes (Fig. 10.1d). Via the *vas deferens* they move to the seminal vesicle (Machado-Silva et al. 1998), which is anterior of the testicular lobes (Fig. 10.1d). This vesicle is connected to the ejaculatory duct ending in a cirrus posterior of the ventral sucker. From here spermatozoa are continuously released into the gynecophoric canal during pairing (Kitajima et al. 1976).

Compared to pairing-experienced males, puerile males have the same number of testicular lobes containing spermatocytes in different stages of maturation and

a filled sperm vesicle (Fig. 10.1g). Furthermore, the production of sperm seems to occur at a similar level, although Neves et al. (2005) observed a slight size reduction of the testicular lobes of puerile males compared to their experienced counterparts.

10.4 Molecular Aspects of Male–Female Interaction

What we know today with respect to the male–female interaction of schistosomes is:

1. As confirmed by physiological, biochemical and molecular studies, the male influences the sexual development of the schistosome female, probably via transcriptional regulation.
2. Following pairing, mitoses and differentiation processes are initiated in the female.
3. Conserved molecules with functions during signal transduction processes have been identified and characterized in *S. mansoni*. Since homologues of these molecules are known to control mitoses and differentiation processes in other organisms, and since the expression of most of the signaling molecules of *S. mansoni* has been documented in the female reproductive organs, they may contribute to processes involved in male-induced differentiation processes in the female.

If the male factor is indeed of chemical nature, one possibility for its action could be that it diffuses or is actively transported as a ligand across the female tegument and through the parenchyma to find its way to target tissues. Alternatively, the male factor could bind to a surface receptor of the female inducing subsequent signaling events by second messengers (Kunz 2001).

Using molecular methods, various signal transduction molecules have been identified by homology-based cloning strategies or by *in silico* identification. Among those are kinases localized in reproductive organs of the female for example serine-threonine kinases of the transforming growth factor β receptor (TGFβ) family (Davies et al. 1998; Forrester et al. 2004), tyrosine kinases of the epidermal growth factor receptor (EGFR) family (Ramachandran et al. 1996), further receptor tyrosine kinases (RTKs) (Vicogne et al. 2003), and cytoplasmatic tyrosine kinases (CTKs) of the Src or Syk families (Beckmann et al. 2010a; Kapp et al. 2001, 2004; Knobloch et al. 2002b). To characterize these molecules in more detail a combination of novel techniques was applied. These included the identification of interaction partners by yeast two/three-hybrid (Y2/3H) analyses, the in vitro culture of adult schistosomes and their treatment with chemical inhibitors to block specific enzyme activity, the use of RNAi to silence gene function post-transcriptionally, and CLSM to examine the morphology of adult worms after inhibitor or RNAi treatment. Results of these approaches led us to propose first models of protein networks active in the reproductive organs of the female regulating mitoses and

differentiation processes. Some of these networks are also active in the testes of the male, where they probably exert similar roles as in the female gonads.

10.5 Signaling Cascades in the Vitellarium

The TGFβ-signaling cascade is known to play a key role in cell growth and proliferation (Krauss 2008; Massague 1998) and is initiated by transmembrane serine/threonine kinase receptors from type I (TβRI) and II (TβRII). TGFβ-ligand binding leads to the formation of a heterodimer composed of a type I and II receptor. This is a prerequisite for further signaling events, which include the activation of Smad proteins. These cytoplasmatic proteins enter the nucleus to regulate the transcription of specific genes in response to the ligand (Derynck et al. 1998). All pivotal TGFβ-pathway members like TβRI, TβRII, R-Smad, Co-Smad as well as the TβRI-regulating protein FKBP12 have been identified in schistosomes (Beall et al. 2000; Davies et al. 1998; Knobloch et al. 2004, 2007; Osman et al. 2001, 2004, 2006) so that the TGFβ/Smad pathway is one of the best characterized signaling pathways in schistosomes. The expression of the TGFβ-pathway members has been detected by in situ-hybridization and/or immunolocalization among other tissues in the vitellarium of *S. mansoni* females suggesting a role in vitellarium development (Fig. 10.2).

To unravel this postulated role of the TGFβ pathway, in vitro-cultured schistosome couples were treated with the synthetic TGFβRI kinase inhibitor (TRIKI; Sawyer et al. 2003). TRIKI led to a significant reduction of mitogenic activity in females (over 46%), and in correspondence with its antiproliferative activity also to the decrease of egg production (over 28%) (Knobloch et al. 2007).

A similar approach with the Src-kinase-specific inhibitor Herbimycin A also led to a significant reduction of mitogenic activity (down to 27%) as well as egg production (down to 14%) in female schistosomes, indicating that a TGFβ pathway as well as a Src-kinase pathway are presumably cooperatively involved in the regulation of cell proliferation in the vitellarium. In correspondence to this hypothesis, the combination of both inhibitors resulted in a higher reduction of mitogenic activity and egg production as with one of the inhibitors alone (Knobloch et al. 2007). At the morphological level, Herbimycin A treatment of adult schistosomes led to a porous structure of the vitellarium of the female, presumably caused by a reduced number of vitelline cells. Additionally, in the ovary the number of big mature oocytes was increased and in the testicular lobes of the male, the number of mature sperms was reduced (Beckmann et al. 2010b). From these results it was concluded that Herbimycin A influences mitogenic activity of cells in the gonads of both sexes.

Besides receptor tyrosine kinases (RTKs), also cytoplasmic tyrosine kinases (CTKs) belong to the superfamily of protein tyrosine kinases. They are activated by transmembrane receptors and transduce signals from the cell surface to a number of different downstream acting molecules, thereby regulating cytoskeleton

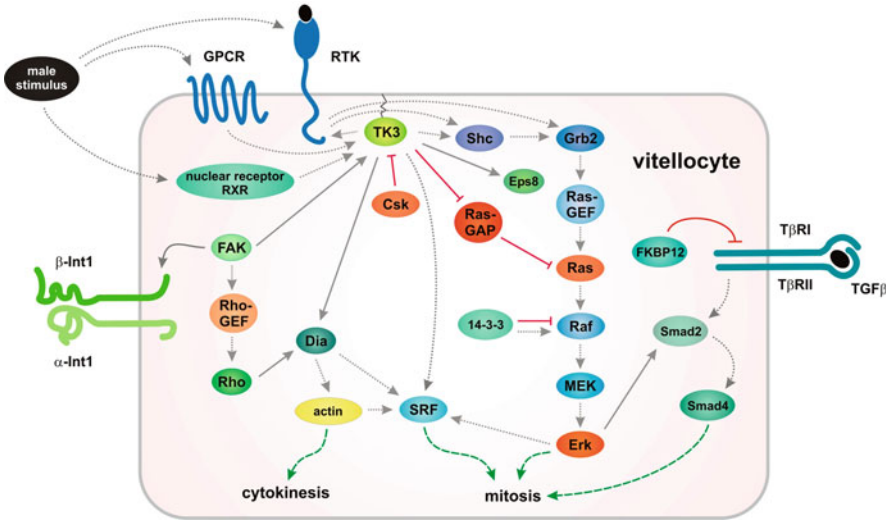

Fig. 10.2 Schematic model of signaling cascades regulating cytoskeletal rearrangements and mitogenic activity in vitellocytes. [*Green*: cloned molecules localized to vitellocytes; *red*: cloned molecules more abundantly expressed in mature than in immature females; *yellow*: cloned molecules not yet localized in vitellocytes; *blue*: not completely cloned molecules with homologues in the schistosome genome databases; *black*: molecules not cloned and not identified *in silico* yet; *gray continuous lines*: direct interactions confirmed by yeast two-hybrid analyses; *gray dotted lines*: direct interactions known from other organisms, not proven for schistosomes yet; *green lines*: activating function; *red lines*: inhibitory function]

rearrangement and cell proliferation (Hubbard and Till 2000; Tatosyan and Mizenina 2000). In schistosomes, several CTKs have been identified and characterized. Among these are a Fes-like kinase (SmFes; Bahia et al. 2007), the Src kinases SmTK3 (Kapp et al. 2004) and SmTK6 (Beckmann et al. 2010a), the Src/Fyn-type kinase SmTK5 (Kapp et al. 2001; Knobloch et al. 2002b), and the Syk kinase SmTK4 (Beckmann et al. 2010a; Knobloch et al. 2002b). With exception of SmFes, transcripts of all these CTKs were localized by in situ-hybridizations in the gonads of both sexes including the vitellarium. Only SmTK4 was detected to be expressed exclusively in ovary and testes, but not in the vitellarium (Knobloch et al. 2002b).

Since Src kinases are known to be involved in signaling pathways regulating cell proliferation and differentiation by controlling cell-cycle progression, transcriptional activity of mitogenic genes, and cytoskeleton rearrangements (Barone and Courtneidge 1995; Frame 2002; Ishizawar and Parsons 2004; Tatosyan and Mizenina 2000; Thomas and Brugge 1997), we postulated for Src kinases like SmTK3 a key role in cellular processes during the development of the vitellarium.

To analyze such a potential function, one possibility is the elucidation of the signaling pathway(s) in which these molecules are involved. To this end, yeast two-hybrid (YTH) screenings of a *S. mansoni* adult-stage YTH-library were performed with the aim to identify signaling molecules acting up- or downstream of the Src kinase SmTK3 in a signal transduction cascade. As a potential upstream interaction

partner we identified a schistosome homologue of the epidermal growth factor receptor pathway substrate 8 (Eps8) (Beckmann et al. 2010b; Burmeister et al., unpublished). The expression of Eps8 was localized to the vitellarium, the ovary and the testes of adult schistosomes corresponding to the expression of SmTK3. Eps proteins fulfill diverse functions in signaling pathways leading to cytoskeleton remodeling as well as the proliferation and differentiation of cells. They interact with EGFRs, but also with other transmembrane receptors like integrin receptors (Calderwood et al. 2003; Fazioli et al. 1993). The interaction of Eps8 with schistosome receptors has not been analyzed yet, but it can be speculated that Eps8 may link transmembrane receptors and Src kinases (SmTK3) in signaling pathways controlling mitogenic activity and differentiation of gonadal cells of schistosomes. As a strong downstream interaction partner of SmTK3, we recently identified a schistosome homologue of diaphanous (SmDia) (Quack et al. 2009). Diaphanous proteins often interact with Rho GTPases in signaling pathways regulating cell-cycle progression and cell proliferation. We performed binding experiments of SmDia with the known schistosome Rho-GTPase homologue SmRho1 and showed that SmRho1 can bind GTP-dependently to the Rho-binding domain of SmDia (Quack et al. 2009). The expression of SmRho1 and SmDia1 was localized to the gonads of both sexes, which perfectly corresponds with the expression pattern of SmTK3. Therefore, we postulate a functional trimeric complex of SmRho1, SmTK3 and SmDia, regulating cytoskeleton rearrangement and so differentiation processes in the reproductive organs of schistosomes (Quack et al. 2009).

10.6 Signaling Cascades in the Ovary

Ontogenetically, vitelline cells and oocytes arise from common mother cells (Kunz 2001). Since they are closely related it seems feasible that similar signaling pathways regulate their proliferation and differentiation. Accordingly, most of the signaling molecules expressed in vitelline cells of the female, supposed to be involved in their proliferation and differentiation, were also found to be expressed in oocytes. Among these molecules are SmTK3, SmDia, SmRho1, SmEps8, and TGFβ-pathway members. With regard to the distinct function of cells generated in vitellarium and ovary, also differences have to exist. In this respect it was not surprising to find molecules exclusively expressed in one of both organs, like the Syk kinase SmTK4 or the receptor tyrosine kinase SmVKR, which were only expressed in oocytes but not in vitellocytes (Fig. 10.3) (Knobloch et al. 2002b; Vicogne et al. 2003).

Because of the specialized function of Syk kinases in cells of the hematopoietic system of mammals, the presence of the Syk kinase SmTK4 in schistosomes, which is expressed in ovary and testes, was very surprising. To elucidate the function of SmTK4, in vitro-cultured worm pairs were treated with the Syk-kinase-specific inhibitor Piceatannol. This inhibitor caused significant physiological and morphological changes. First, Piceatannol reduced the egg production (down to 51%). Second, Piceatannol led to an increased number of big mature oocytes in the ovary

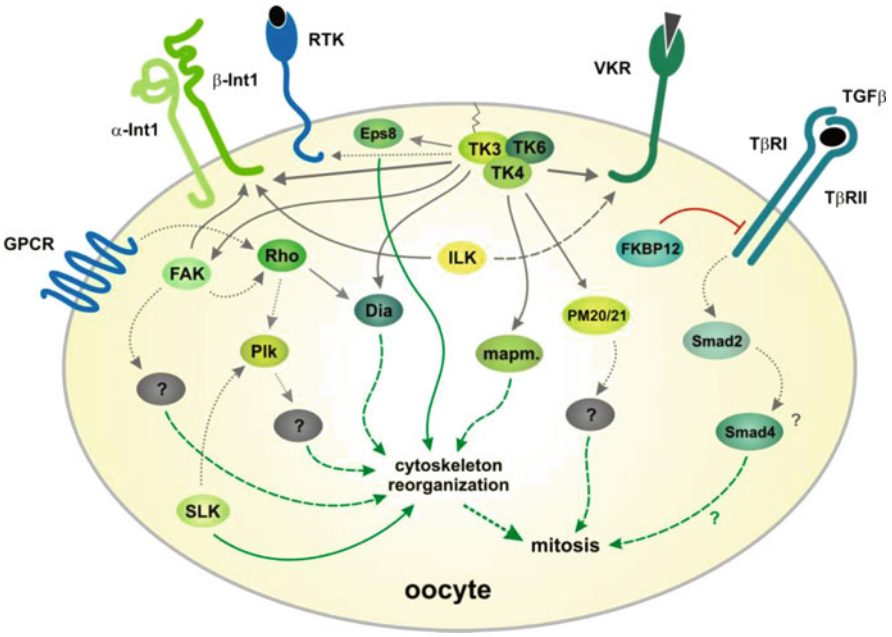

Fig. 10.3 Schematic model of signaling cascades regulating cytoskeletal rearrangements and mitogenic activity in oocytes. [*Green*: cloned molecules localized to oocytes; *yellow*: cloned molecules not yet localized in oocytes; *blue*: not completely cloned molecules with homologues in the schistosome genome databases; *black*: molecules not cloned and not identified *in silico* yet; *gray continuous lines*: direct interactions confirmed by yeast two-hybrid analyses; *gray dotted lines*: direct interactions known from other organisms, not proven for schistosomes yet; *gray dashed lines*: indirect interactions via adapter molecules known from other organisms; *green lines*: activating function; *red lines*: inhibitory function] (Figure modified from Beckmann et al. (2010b), with permission from Cambridge University Press)

of the female and to a lack of mature sperms in the testes of the male. These results were confirmed by SmTK4-specific RNAi approaches, which resulted in similar phenotypes (Beckmann et al. 2010a).

To further elucidate the signaling pathway in which SmTK4 is involved, we performed yeast two-hybrid screenings of a *S. mansoni* adult-stage YTH-library with the aim to identify up- or downstream interacting molecules. As strongest upstream interaction partners of SmTK4, the Src kinases SmTK6 and SmTK3 were identified. The expression of both Src kinases co-localized with the expression of SmTK4 in ovary and testes. Also for SmTK6 we showed by yeast two-hybrid analysis an interaction with SmTK3 leading to the postulation of a multi-kinase complex in cells of ovary and testes (Beckmann et al. 2010b; Beckmann et al. in preparation). From mammals it is known that transmembrane receptor-activated Src kinases can phosphorylate downstream-acting Syk kinases leading to the subsequent stimulation of downstream molecules (Geahlen 2007; Kurosaki et al. 1994). As potential downstream effectors of SmTK4 we identified a schistosome

homologue of a MAP kinase-activating protein of the PM20/21 type and a mapmodulin homologue, whose transcripts were localized in ovary and testes (Beckmann et al. 2010a). The MAPK-activating protein is supposed to activate a MAPK (mitogens-activated protein kinase) cascade regulating the proliferation and differentiation of cells in the ovary and testes, whereas the mapmodulin homologue may influence the reorganization of the microtubule-based cytoskeleton of the oocytes and spermatocytes. Taken together, the results of the inhibitor/RNAi studies and the yeast two-hybrid experiments led to the postulation of a key role for SmTK4 in oogenesis and spermatogenesis of schistosomes (Beckmann et al. 2010a).

Potential transmembrane receptors, which are able to activate the postulated kinase complex consisting of SmTK4, SmTK6 and SmTK3, are integrin receptors and/or receptor tyrosine kinases. Integrins are heterodimers composed of one α- and one β-receptor. They bind components of the extracellular matrix (ECM) or cell surface molecules to mediate cell attachment. Integrin receptors also play a role in cell signaling transmitting signals from the ECM to the cell thereby regulating cellular processes such as proliferation, differentiation, migration, cytoskeletal organization, or apoptosis (Giancotti and Ruoslahti 1999; Hynes 2002; van der Flier and Sonnenberg 2001). The intracellular domains of the integrin receptors lack catalytic activity but they can interact with a variety of intracellular signaling molecules like for example Syk and Src kinases (Arias-Salgado et al. 2003; Harrison 2003; Schlaepfer and Hunter 1998; van der Flier and Sonnenberg 2001; Woodside et al. 2002; Zaidel-Bar et al. 2007). Thus, interactions between the schistosome Syk kinase SmTK4 and the Src kinases SmTK3 and SmTK6 with integrin receptor homologues were speculated. In schistosomes, we identified and cloned one α- and one β-integrin receptor (Smα-Int1, Smβ-Int1), whose transcripts were detected in ovary, ootype, vitellarium, and testes of adults (Beckmann et al. 2010b; Beckmann et al. unpublished). Accordingly, the expression of the integrins co-localizes with the expression of the CTKs, among other tissues, in the ovary of the female. By direct interaction studies in the yeast two-hybrid system we finally showed binding of all three kinases to the intracellular part of the β-integrin receptor. This led to the postulation of the following model. Upon ligand binding, clustering of the integrin receptors Smα-Int1 and Smβ-Int1 and conformational changes in the intracellular domains occur, which is intracellularly accompanied by an increase in the local Src concentration. Binding to the β-integrin receptor and phosphorylations *in trans* then result in a full activation of the Src kinases SmTK3 and/or SmTK6, a prerequisite for subsequent Syk (SmTK4) activation (Beckmann et al. 2010b).

In signaling cascades downstream of integrin receptors, also focal adhesion kinases (FAKs) can play a role (Mitra and Schlaepfer 2006; Schlaepfer and Hunter 1998). The activation of FAK is facilitated by the β-integrin receptor, leading to the binding of a Src kinase to FAK and finally to Src activation. This is an alternative way of Src activation downstream of integrin receptors. A FAK homolog of schistosomes (SmFAK) was identified in our laboratory, cloned, and transcripts were localized in the ovary, ootype and vitellarium of the female (Beckmann et al. 2010b, Beckmann

et al., unpublished). Finally, we showed binding of the Src kinase SmTK3 to SmFAK as well as binding of SmFAK to the intracellular domain of Smβ-Int1 using direct interaction studies in the yeast two-hybrid system (Beckmann et al. unpublished).

It is well known that integrin receptor-induced signaling cascades "cross talk" with RTK-pathways involving Syk and Src kinases. Furthermore, it has been discussed that this co-operation potentiates downstream signaling (Bromann et al. 2004; Geahlen 2007; Giancotti and Ruoslahti 1999; Jakus et al. 2007; Wu et al. 2008). In *S. mansoni* four RTKs have been characterized (Dissous et al. 2006, 2007), among these is SmVKR1 (SmRTK1), whose expression products were detected in mature oocytes of female schistosomes (Vicogne et al. 2003). Here SmVKR1 co-localizes with the CTKs SmTK4, SmTK6, SmTK3, and the integrin receptors Smα-Int1 and Smβ-Int1. Yeast two-hybrid analysis performed in our laboratory confirmed binding of SmTK4, SmTK6 and SmTK3 to the intracellular part of SmVKR1 (Beckmann et al. unpublished). Since the three CTKs were also able to interact with Smβ-Int1, a co-operation of the Smβ-Int1 and SmVKR1 pathway via CTKs (SmTK3, SmTK6, SmTK4) and/or SmFAK has been suggested (Beckmann et al. 2010b). Interactions between integrin receptors and RTKs may also be associated with an integrin-linked kinase (ILK; Li et al. 1999). A schistosome homologue, SmILK2, was identified and cloned in our laboratory (Beckmann et al. unpublished). Interaction studies in the yeast two-hybrid system showed binding of SmILK2 to the intracellular part of Smβ-Int1, thus also an involvement of SmILK2 in linking the integrin receptor and RTK pathways may be possible.

Beside tyrosine kinases further kinases, like Polo-like kinases (Plks) and Ste20-like kinases (SLKs) have been shown to be expressed in reproductive organs of schistosomes and were supposed to play a role in development. Plks regulate the cell-cycle and thus are essential for progression through mitosis. The schistosome homologue of Plk1, SmPlk1, was shown to be expressed in the vitellarium and the ovary of the female (Long et al. 2010). To elucidate its function, schistosome pairs were incubated with the Plk1-specific inhibitor BI 2536 (Steegmaier et al. 2007). This resulted in an increased number of big mature oocytes in the ovary of the female. Additionally, the morphology of the small immature oocytes was significantly altered exhibiting a more elongated form with a loose arrangement (Beckmann et al. 2010b; Long et al. unpublished). The inhibitor presumably led to a mitotic arrest in the oocytes and the induction of apoptosis, an effect which was described before for this inhibitor (Steegmaier et al. 2007). A similar effect of BI 2536 was observed in the testes of the schistosome male, which showed a decreased size of the testicular lobes and a reduced number of spermatocytes and mature sperms after treatment, indicating that SmPlk1 might also be involved in the mitosis of spermatogonial cells (Beckmann et al. 2010b; Long et al. unpublished).

Plks are activated by phosphorylation. A potential activating kinase is xPlkk1 (polo-like kinase), a member of the Ste20-like kinase (SLK) family (Qian et al. 1998). In schistosomes, such a kinase has been indentified and characterized (Yan et al. 2007), which in correspondence with SmPlk1 is also transcribed in the ovary of the female. It could be shown that SmSLK can phosphorylate SmPlk1 and thus is supposed to be involved in the regulation of cell proliferation in schistosomes, too

(Dissous and Long unpublished). Besides this function, an involvement of SmSLK in cytoskeletal rearrangement associated with reproductive organ development and function has been hypothesized (Beckmann et al. 2010b; Dissous and Long unpublished).

10.7 Signaling Cascades in the Testes

Pairing of schistosomes seems not to influence maturation processes in the male. Thus, proliferation and differentiation of spermatocytes and thus the continuous production of mature sperms seem to be independent from the contact with a female (Armstrong 1965; Basch and Basch 1984). Accordingly, the testes of unpaired males are already fully developed, and they produce mature elongated sperms (Fig. 10.1g).

With respect to the evolutionary ancestry from hermaphroditic blood flukes, it seems conceivable that both genders still use similar signaling pathways to regulate cellular processes in the gonads. This is supported by the fact that nearly all molecules expressed in the ovary of the female were also expressed in the testes of the male, indicating a function in both genders and both gonadal cell types. Therefore, we hypothesized that signaling pathways involving the Syk kinase SmTK4 and upstream receptors like integrins and RTKs as well as further signaling molecules such as the Src kinases SmTK3 and SmTK6, are not only involved in the proliferation and differentiation of oogonial cells and oocytes in the female, but also in the proliferation of spermatogonial cells in the males (Beckmann et al. 2010b). Accordingly, the inhibitor treatment of adult schistosomes pairs with Herbimycin A, Piceatannol, and BI 2536 and thus the inhibition of Src, Syk, and Plk negatively influenced not only the differentiation of cells in the ovary, but also the proliferation of spermatogonial cells in the male, leading to a dysfunction in the production of mature sperms (Beckmann et al. 2010b).

10.8 Conclusions

With respect to the worldwide importance of schistosomes as infectious agents and the need to find new ways to fight these parasites, their unusual reproduction biology is one focus of research activities. During the last years comprehensive genome information has been generated for schistosomes (Berriman et al. 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium 2009) and research on this parasite is now at the beginning of the postgenomic era. A combinatory application of new techniques like yeast two-hybrid analysis (Beckmann et al. 2010a; Quack et al. 2009), transient transfection or RNAi (Beckmann et al. 2007; Correnti and Pearce 2004; Kines et al. 2006; Ndegwa et al. 2007; Wippersteg et al. 2002), inhibitor treatment and morphological analysis using confocal microscopy (Beckmann et al. 2010b; Beckmann et al. 2010a; Long

et al. 2010), as well as laser microdissection for tissue-specific transcriptome analyses (Gobert et al. 2009; Jones et al. 2007) now allows functional analyses of signaling molecules involved in reproductive biology of this parasite, and the elucidation of signal transduction pathways these molecules are involved in. The understanding of the cellular processes during gonad differentiation and the identification of molecules controlling these processes contribute to our knowledge about the unusual biology of this parasite, thus providing a promising starting point for the development of new strategies to fight schistosomes. In the light of first studies describing the emergence of partial resistance to Praziquantel (Botros et al. 2005; Cioli and Pica-Mattoccia 2003; Doenhoff et al. 2008; Kusel and Hagan 1999; Melman et al. 2009; Messerli et al. 2009; Pica-Mattoccia et al. 2009), and due to the fact that there is still no vaccine available ready to use, there is an urgent need for the development of alternative medication.

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Chapter 11
Anisakid Nematode (Ascaridoidea) Life Cycles and Distribution: Increasing Zoonotic Potential in the Time of Climate Change?

Sven Klimpel and Harry W. Palm

Abstract Parasitic nematodes are known as important pathogens that cause problems for human and animal health. Some of them naturally inhabit the marine environment, where they are widespread and can be found in a variety of different hosts. Food-borne zoonoses via aquatic animals are most often linked to anisakid nematodes of the genera *Anisakis* Dujardin, 1845, *Contracaecum* Railliet and Henry, 1912, and *Pseudoterranova* Mozgovoi, 1951. These are commonly found in the digestive tract of marine mammals, and infect aquatic invertebrates and vertebrates as intermediate hosts. The most widely distributed whale worms *Anisakis* spp. involve cetaceans as final and planktonic copepods, euphausiids, squids and teleosts as intermediate or paratenic hosts. Painful infections of the digestive tract in humans originate through consumption of raw or semi-raw fisheries products, for example fish and squid. Recent molecular studies revealed the existence of morphologically similar but genetically different cryptic species ('sibling species') within the anisakids. Among these, *A. simplex* (s.s.) is responsible for the highest number of recorded human infections. Molecular studies of *Anisakis* larvae from various parts of the world Oceans demonstrate an uneven species distribution, with *A. simplex* (s.s.) being limited to the northern hemisphere. Another species, *A. typica*, has not yet been connected to this disease, and seems to be restricted to the tropical regions. This chapter presents the present state of knowledge about this widespread group of fish parasites, including the importance as human pathogens, their life cycle biology, biogeography and phylogeny. The distribution of the currently recognized *Anisakis*

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species is summarized and combined with the number of known cases of human anisakiasis. We suggest that pathogenicity for humans is different among the *Anisakis* siblings, providing a possible explanation for uneven disease records worldwide. The possibility of a changing risk of anisakidosis in the time of climate change is discussed.

11.1 Introduction

Parasitism, a form of symbiosis, is one of the most successful modes of life (Palm and Klimpel 2007). More than half of all plant and animal species on earth are parasites, and probably no organism avoids parasitic infection during its lifetime (Palm and Klimpel 2007). Including approximately 256 families and more than 40,000 known species, the phylum Nematoda is one of the most species rich and abundant invertebrate Taxon (Anderson 2000; McClelland 2005). Beside free-living nematodes in freshwater, marine and terrestrial habitats (McClelland 2005), their parasitic forms use plants, animals and humans as host organisms at a global scale (e.g. Blaxter et al. 1998).

Gastrointestinal parasitic nematodes are known to cause a wide range of diseases and have consequences for human and animal health. They impose a significant economic burden as parasites of domestic animals, reduce productivity, and require elaborate and expensive control methods (e.g. Parkinson et al. 2004; Audicana and Kennedey 2008). Infections of humans cause substantial mortality and morbidity, resulting in about 2.9 billion infected people worldwide (Parkinson et al. 2004). Most important are the hookworms (e.g. *Ancylostoma* spp.), ascarids (*Ascaris* spp.), whipworms (e.g. *Trichuris trichiura*) and filarial nematodes that cause lymphatic filariasis (e.g. *Brugia malayi*) or elephantiasis (*Wuchereria bancrofti*) and African river blindness (e.g. *Onchocerca volvulus*) (e.g. Parkinson et al. 2004). Humans also become accidental hosts for nematodes that cannot complete their life cycles inside them, but can cause disease problems or initiate immune hypersensitivity states or allergies. The consumption of raw or undercooked fish regularly leads to food-borne zoonoses, most commonly caused by larvae of the anisakid nematode genera *Anisakis*, *Contracaecum* and *Pseudoterranova* (Sakanari and McKerrow 1989; Kaneko 1991; Audicana et al. 2002; Palm 2004).

Since the 1960s, the term anisakiasis had been used for a human disease caused by the third-stage larvae (L3) of members of the family Anisakidae. In 1988, a standardized nomenclature recommended three different terms: (1) anisakidosis caused by any members of the family Anisakidae, (2) anisakiasis caused by members of the genus *Anisakis*, and (3) pseudoterranovosis caused by members of the genus *Pseudoterranova* (e.g. Audicana et al. 2003; Audicana and Kennedey 2008). The first case of a human infection with *Anisakis* sp. was reported for the Netherlands (Van Thiel 1962) from an eosinophilic intestinal lesion in a patient. Ishikura and Kikuchi (1990) recorded 12,586 cases of anisakiasis between 1968 and 1989 in Japan. The number of cases is increasing worldwide, with ~50 cases annually in the USA and

~500 cases in Europe, over 95% of them from The Netherlands, Germany, France and Spain (e.g. Plath et al. 2001; Audicana et al. 2002; Fuentes et al. 2002). To date, over 14,000 anisakiasis cases have been reported, approximately 95% from Japan (Audicana et al. 2002). The parasite transmission is clearly related to the consumption of raw or semi-cooked fish. Especially Japanese sushi and sashimi, Dutch salted or smoked herring, Nordic gravlax (dry, cured salmon), Hawaiian lomi-lomi (raw salmon), German rollmop (rolled fillet of marinated/pickled herring), South American cebiche and Spanish boquerones en vinagre (pickled anchovies) are regular pathways of infection (e.g. Petersen et al. 1993; Audicana et al. 2002; Palm 2004).

The present communication summarizes the current state of knowledge on zoonotic anisakid nematodes, their pathogenicity, life cycle biology, biogeography and phylogeny. We suggest that in addition to different special dishes and food preferences, a characteristic distribution pattern of the currently recognized *Anisakis* species is responsible for an unequal regional distribution of known cases of anisakiasis so far. We assess the risk that might result from potentially changing infection levels in the time of increasing anthropogenic influence and climate change. This is especially important for an increasing number of people that use marine food products for their daily needs.

11.2 Genetic Identification

An accurate identification of nematodes at any particular life cycle stage is essential for the diagnosis of nematode infections, and consequently an important part of disease surveillance and control. Identification of nematodes from marine vertebrates has been based on morphological characters, such as the size and shape of the spiculae (sexual organs) in adult males, and head structures and papillae that regularly occur on the body surface. Larval identification used the orientation of the excretory pores, the arrangement and separation of the digestive tract into oesophagus, ventricle and attaching structures such as caeca and appendices, and the shape of the tail (Fig. 11.1). Even accompanied with morphometric information, generic and especially species identification has been difficult, leading to a high number of erroneous identifications. This promoted molecular methods for a better and more reliable species diagnosis.

Molecular techniques have the advantage that they allow analyses of the parasite DNA, securing species identification and providing data for phylogenetics. Genomic DNA sequences evolve at different rates, with non-coding, non-transcribed sequences of ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) evolving faster than those that encode essential proteins or nuclear DNA (nDNA), respectively. Molecular anisakid nematode identification started with allozyme analyses including restriction fragment length polymorphism techniques (PCR-RFLPs of ITS-DNA, e.g. D'Amelio et al. 2000; Kijewska et al. 2002; Pontes et al. 2005). The next approach was direct sequencing of rDNA, including the highly variable

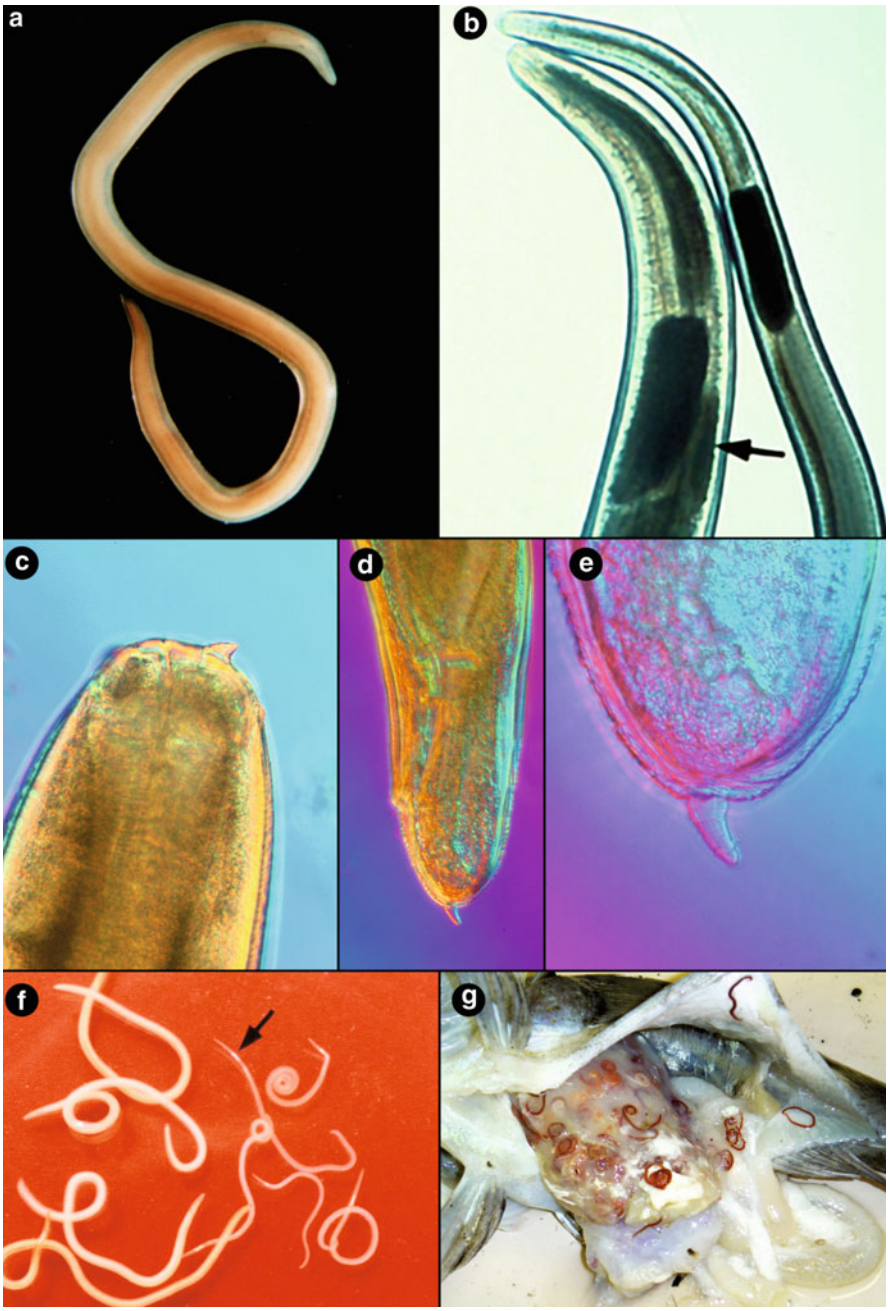

Fig. 11.1 (**a**) Habitus of the anisakid nematode *Pseudoterranova decipiens* E isolated from the liver of the icefish *Chaenocephalus aceratus*. (**b**) Anterior end of the third-stage larvae (L3) of *P. decipiens* s.l. (*left*) from smelt (*Osmerus eperlanus*) and *Anisakis simplex* s.l. from herring

internal transcribed spacers ITS1-2 and the conserved 5.8S rDNA region; direct sequencing of the 28S (LSU rDNA) and complete internal transcribed spacer (ITS-1, 5.8S, ITS-2) rDNA (e.g. Li et al. 2005; Nadler et al. 2005; Zhu et al. 2000a, b, 2001, 2002) and mitochondrial cytochromoxidase 1 and 2 (mtDNA *cox1*, *cox2*) sequence analyses (e.g. Valentini et al. 2006; Cross et al. 2007; Mattiucci and Nascetti 2008; Mattiucci et al. 2008a, b) followed. Also micro-satellites can be used to distinguish the species among populations. These studies identified the existence of "sibling species" within the ascaridoids, being morphologically very similar but genetically different, having distinct host preferences, life cycles and geographical distribution (e.g. Mattiucci et al. 1997, 2005; Zhu et al. 2002; Nadler et al. 2005; Marques et al. 2006; D'Amelio et al. 2007; Klimpel et al. 2007, 2008, 2010; Mattiucci and Nascetti 2008).

Within the family Anisakidae the genus *Contracaecum* includes two sibling species complexes, the (1) *C. osculatum* complex with the five species *C. osculatum* A, B, C (*C. osculatum* s.s.), D, E, and the (2) *C. ogmorhini* complex with the two species *C. ogmorhini* (s.s.) and *C. margolisi*, and additionally *C. radiatum* (e.g. Mattiucci and Nascetti 2008; Shamshi et al. 2009a, b). The *Pseudoterranova decipiens* complex consists of six different species (*P. decipiens* s.s. = *P. decipiens* B, *P. krabbei*, *P. bulbosa*, *P. azarasi*, *P. decipiens* E, *P. cattani*) (Mattiucci et al. 2007; Mattiucci and Nascetti 2008). Most recently Klimpel et al. (2007, 2008, 2010) and Palm et al. (2008) studied the species identity within the genus *Anisakis* by using the following protocol.

Genomic DNA isolation and purification followed amplification of the rDNA region (ITS-1, 5.8S, ITS-2), and flanking sequences (=ITS+), using the primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al. 2000a). The PCR reaction (50 µL) includes 25 µL of Master-Mix (Peqlab Biotechnology GmbH, Erlangen, Germany) containing dNTPs, $MgCl_2$, buffer, and Taq-Polymerase, 3 µL of each primer, 14 µL aqua dest. and 5 µL genomic DNA. Each PCR reaction is performed in a thermocycler (Biometra or Peqlab, Germany) under the following conditions: after initial denaturation at 91°C for 1 min, 40 cycles of 94°C for 45 s (denaturation), 55°C for 45 s (annealing), and 72°C for 45 s (extension), followed by a final extension at 72°C for 10°min. PCR products were examined on 1% agarose gels. A 100-bp ladder marker (peqGOLD, Erlangen, Germany) is used to estimate the size of the PCR products. For anisakid nematode identification, the PCR products must be purified with an E.Z.N.A. Cycle-Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany), followed by sequencing of a total volume of 7 µL, including 2 µL of primer (individually) and 5 µL of the PCR product (250 ng/µL). Both spacers and the 5.8S gene from each PCR product are sequenced in both directions,

Fig. 11.1 (continued) (*Clupea harengus*). (**c**) Anterior end of the third-stage larva of *A. typica* with the boring tooth. (**d**, **e**) Posterior end of *A. typica* with the mucron. (**f**) Nematode larvae (L3) isolated from Wadden Sea fish; *left* – *P. decipiens* s.l., *right* – *A. simplex* s.l. (**g**) Numerous anisakid nematode larvae in the viscera of the icefish *C. aceratus*

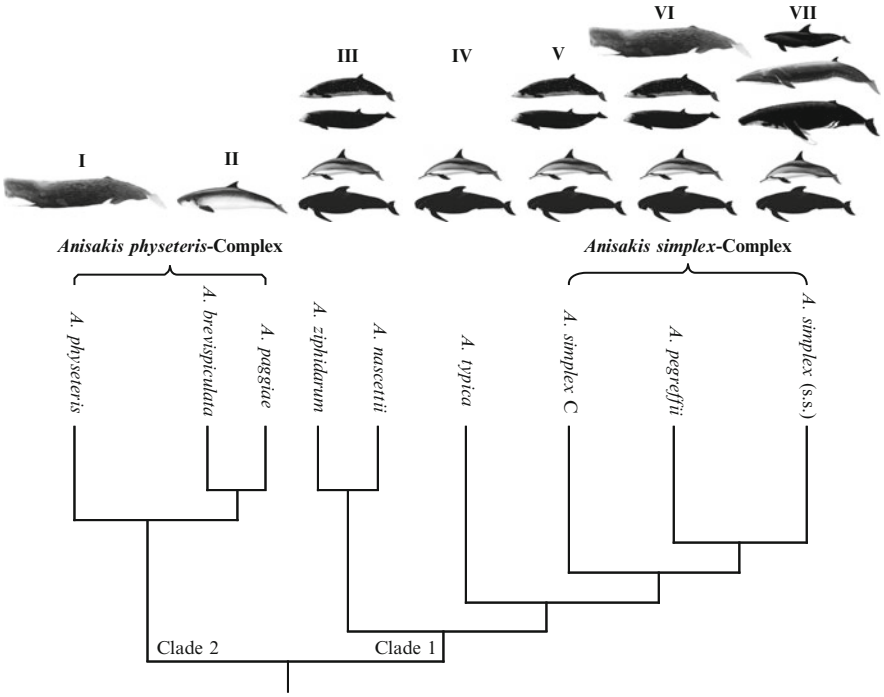

Fig. 11.2 *Anisakis* spp. final cetacean host distribution in the *A. physeteris* and *A. simplex* complexes (Cetacea-families; I Physeteridae; II Kogiidae; III Ziphiidae, Delphinidae; IV Delphinidae; V Ziphiidae, Delphinidae; VI Physeteridae, Ziphiidae, Neobalaenidae, Delphinidae; VII Phocoenidae, Balaenopteridae, Monodontidae, Delphinidae)

using the primers NC5, NC13 (forward; 5′-ATC GAT GAA GAA CGC AGC-3′), NC13R (reverse; 5′-GCT GCG TTC TTC ATC GAT-3′), XZ1R (reverse; 5′-GGA ATG AAC CCG ATG GCG CAA T-3′), and NC2.

These studies revealed the existence of nine species, six belonging to two sibling species complexes, the (1) *A. simplex* complex with *A. simplex* (s.s.), *A. pegreffii*, and *A. simplex* C, the (2) *A. physeteris* complex with *A. physeteris*, *A. brevispiculata* and *A. paggiae*, and the three species *A. typica*, *A. ziphidarum* und *A. nascettii* (e.g. Klimpel et al. 2008, 2010; Mattiucci and Nascetti 2008; Fig. 11.2).

11.3 Life Cycle Ecology of Anisakid Nematodes

According to Anderson (1984, 1996), parasitic nematodes first evolved in terrestrial hosts and were only able to invade aquatic environments after the development of heteroxeny (the use of intermediate hosts) and paratenesis (the use of transport hosts). Intermediate hosts support larval growth and development to a stage where

the nematode is capable of infecting its definitive host. Both intermediate and paratenic hosts participate in the temporal and spatial dispersal of the parasite, thereby increasing the likelihood of transmission into the final host (e.g. McClelland 2005). Heteroxeny is the common life cycle pattern of marine ascaridoid nematodes such as *Anisakis*, *Contracaecum*, and *Pseudoterranova*. Transmission pathways are habitat-dependent and usually involve a broad spectrum of invertebrates and intermediate or paratenic fish hosts (McClelland 2005; Klimpel and Rückert 2005; Palm and Klimpel 2007).

The life cycle of anisakid nematodes follows the general nematode life cycle pattern, including four larval stages (L1–L4) and the adults in the final host. The heteroxenous life cycle involves a variety of hosts that are transferred through the marine food chain. Most important are the three genera *Anisakis* (whales), *Contracaecum* (bird, seals), and *Pseudoterranova* (seals), that can be distinguished morphologically and according to their final host spectrum (e.g. Klimpel et al. 2008, 2010; Mattiucci and Nascetti 2008). Life cycle studies of these nematodes have been limited by difficulties in maintaining them alive in the laboratory, culturing sufficient numbers of parasite-free experimental hosts, and creating effective exposure (e.g. Køie and Fagerholm 1995; Køie et al. 1995; Køie 2001; Klimpel et al. 2004, 2008, 2010; Mattiucci and Nascetti 2008). However, empirical studies on the distribution and abundance of anisakid larvae in the intermediate and final hosts have revealed important insights into the life cycle biology of these parasites. Being responsible for anisakiasis and of high importance for human health, the whaleworms *Anisakis* spp. mainly infect toothed whales and a range of pelagic schooling fish worldwide. Thus, the life cycle can be considered to take place in the pelagic environment, with some seals and baleen whales getting accidentally infected (e.g., Køie et al. 1995; Hays et al. 1998a,b; Køie 2001; Klimpel et al. 2004, 2010). Life cycle stages include four larval stages (L1–L4), within the eggs (L1–L3) and subsequently in the intermediate or paratenic hosts (L3), and as preadults (L4) and adults in the cetacean final hosts (Fig. 11.3). The nematode eggs are excreted with the faeces and embryonate in seawater (Køie 2001). Køie et al. (1995) found larvae surrounded by two cuticles prior to hatching. They were surrounded by sheaths with lateral extensions and were able to float, enabling them to use mainly pelagic crustaceans as intermediate hosts (Køie et al. 1995). During ingestion by the crustacean first intermediate host, the larvae are most probably released from the second stage cuticle by the action of the mouthparts. This allows the third stage larvae to penetrate the gut prior to establishing themselves in the haemocoel (Køie et al. 1995). Larger invertebrates (mainly copepods, euphausiids) and smaller fish are thought to be important second intermediate hosts, and various predatory fish species and cephalopods serve as paratenic hosts. If small fishes are preyed upon by larger piscivorous fishes, the larvae are capable of re-infecting the latter without further moulting. Consequently, piscivorous hosts may accumulate enormous numbers of larvae (Lile 1998). Cetaceans acquire the nematodes by preying upon the intermediate hosts. To date, a total of 34 cetacean species have been found to harbour *Anisakis* spp. (e.g. Klimpel et al. 2008; Mattiucci and Nascetti 2008; Fig. 11.4).

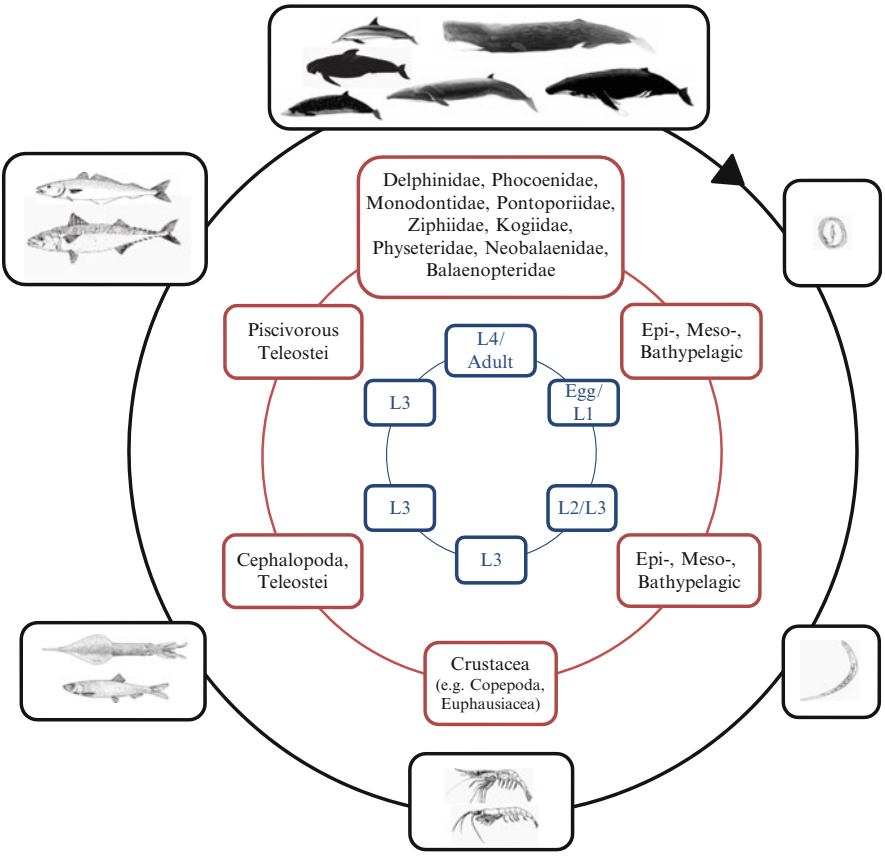

Fig. 11.3 Schematic life cycle of *Anisakis* species. The pelagic life cycle of *Anisakis* spp. follows the general nematode life cycle pattern, including four larval stages (L1–L4) and the adults in the cetacean final host. The heteroxenous life cycle involves a variety of invertebrate and vertebrate hosts that are transferred through the marine food chain (Kuhn 2010)

In contrast to the whaleworms, the sealworms of the genus *Pseudoterranova* seem to be restricted to a benthic life cycle (e.g. Palm et al. 1994; Køie et al. 1995; Palm 1999; McClelland 2002). Partially embryonated eggs, passed in the faeces of a seal, settle on the sea bed where they complete development to the third stage larvae (L3) and hatch. Newly hatched larvae are still ensheathed in the cuticle of the previous second larval stage (L2) and attach to the substrate caudally (e.g. Køie et al. 1995; Anderson 2000; McClelland 2002, 2005). When ingested by benthic crustaceans (e.g. amphipods, gammarids, isopods, harpacticoid copepods), they exsheath inside the first intermediate host, penetrate into the haemocoel and begin to grow. These hosts serve to enhance transmission to a larger array of benthic macro-invertebrates as second intermediate hosts, where the larval sealworms grow in length (e.g. Anderson 2000, McClelland 2002, 2005). At this point they become infective to fish and also to seals. The invertebrate hosts are usually ingested by

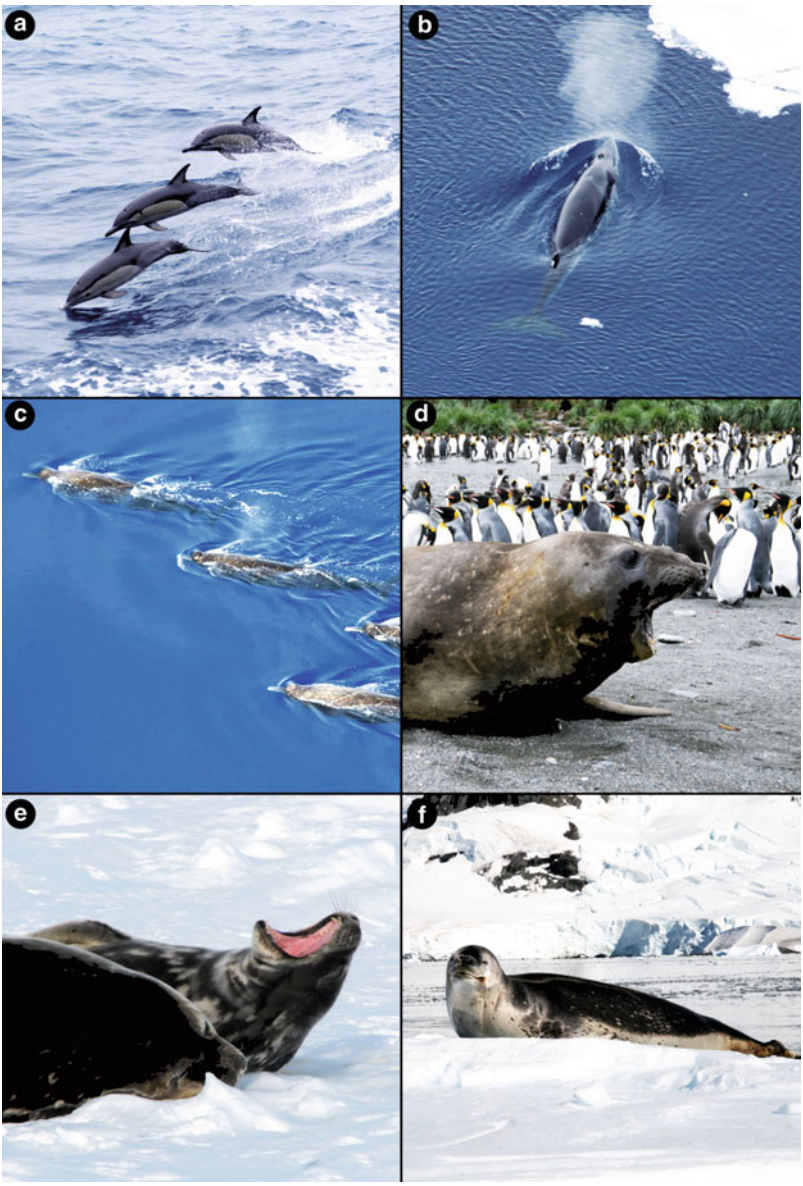

Fig. 11.4 Cetacean and pinniped final hosts of anisakid nematode species. (**a**) Dolphins (Fam. Delphinidae) are the most common final hosts of *Anisakis typica* from subtropical and tropical marine waters. (**b**) Minke whale (*Balaenoptera bonaerensis*, Fam. Balaenopteridae) as potential final hosts for *Anisakis* spp. in the Southern Ocean (Antarctica). (**c**) Beaked whales (Fam. Ziphiidae) in the Southern Ocean (Antarctica) are final hosts of *Aniskais ziphidarum*. (**d**) Southern elephant seal (*Mirounga leonina*), (**e**) Weddell seal (*Leptonychotes weddellii*) and (**f**) Leopard seal (*Hydrurga leptonyx*) are final hosts of the nematode genera *Contracaecum* and *Pseudoterranova* in the Southern Ocean (Antarctica)

primary benthic teleosts, including juveniles of larger demersal fish species. The larvae penetrate the gut wall of the fish and establish themselves in the internal organs or the musculature, where they continue to grow in length. Large, piscivorous fish may serve as second/third fish or paratenic hosts that accumulate the larval worms (Palm 1999; Anderson 2000; McClelland 2002, 2005). Following ingestion by the seal definitive host, infective third stage larvae (L3) escape from the bodies of the fish or invertebrate, embed their anterior part into the gastric mucosa, mature and reproduce. Ten marine mammal species belonging to the Otariidae and Phocidae have been recorded as final hosts (e.g. Mattiucci and Nascetti 2008; Fig. 11.4).

Nematodes of the genus *Contracaecum* seem to have equally complex life cycles involving benthic and pelagic invertebrates (e.g. crustaceans, squid) and fish (e.g. Klöser et al. 1992; Køie and Fagerholm 1995; Køie et al. 1995). They are also transmitted to pelagic and demersal fish by for example euphausiids, shrimps and small fish that are found near bottom in daytime but feed pelagically at night. In total 12 marine mammal species including specimens from the families Ottariidae and Phocidae and different fish-eating sea birds (e.g. of the genera *Larus*, *Pelecanus*, *Phalacrocorax*) have been identified as final hosts in the *Contracaecum* life cycle (e.g. Torres et al. 1983; Farjallah et al. 2008; Mattiucci and Nascetti 2008; Shamshi et al. 2009a; Fig. 11.4).

11.4 Phylogeny and Host Range of Anisakid Nematodes (*Anisakis* spp.)

Traditional nematode taxonomy was based on a limited number of criteria, such as the shape of the oesophagus, male and female reproductive organs and life cycle patterns (e.g. Wijova et al. 2006). Recent morphology-based classifications split the nematodes into the classes Secernentea (= Phasmidea, = Rhabditea) and Adenophorea (= Aphasmidea, = Enoplea), including most terrestrial and parasitic species and most marine species, respectively (Dorris et al. 1999). There were considerable obstacles for an accurate identification especially of the larval forms. For this reason, earlier classification systems were not compatible with each other, and there was no universally accepted nematode phylogeny during the last century (e.g. Blaxter et al. 2000; De Ley and Blaxter 2002; Meldal et al. 2007). A study of the small subunit (SSU) or 18S rRNA for a wide range of major nematode taxa concluded convergent evolution in many lineages, requiring revision of the morphology-based higher-level classification (Blaxter et al. 1989; Dorris et al. 1999). Although most recent datasets of the SSU rRNA genes comprise more than 300 taxa (Blaxter 2003), sampling remains strongly biased towards some groups and poor for other important taxa.

The nematodes divide into the three major clades Dorylaimia (clade I, free-living, invertebrate, vertebrate and plant parasites from the marine and terrestrial environment), Enoplia (clade II, marine and plant parasite species) and Chromadoria, which include the Rhabditida (marine free-living species, few terrestrial/freshwater representatives). The Rhabditida separate into the Spirurina (clade III, only animal parasites), Tylenchina (clade IV, animal-, plant- and fungus-parasitic and free-living groups) and Rhabditina (clade V, free-living and parasitic species) (e.g. De Ley and Blaxter 2002; Parkinson et al. 2004; De Ley 2006). Parasitism of both plants and animals seems to have arisen multiple times within nematode evolution, and all major clades include parasites (Blaxter et al. 1989; De Ley and Blaxter 2002; Parkinson et al. 2004). The Spirurina include five infraorders (Ascaridomorpha, Spiruromorpha, Rhigonematomorpha, Oxyuridomorpha, Gnathostomatomorpha) and one additional superfamily, the Dracunculoidea. With exception of the invertebrate parasites of the Rhigonematomorpha, all others include both invertebrate and vertebrate parasites (De Ley 2006; Wijova et al. 2006). The Ascaridomorpha are most closely related to the Spiruromorpha and Rhigonematomorpha, a clade that also comprises terrestrial and marine parasites (De Ley and Blaxter 2002; De Ley 2006), among these the family Anisakidae with *Anisakis, Contracaecum* and *Pseudoterranova*. While the former infects whales as final hosts, *Pseudoterranova* occurs in seals and *Contracaecum* in a range of aquatic hosts, including seals and birds.

Most *Anisakis* siblings have been identified from toothed whales, especially from the Delphinidae and Ziphiidae. The phylogenetic relationships within *Anisakis* together with their most common final hosts are illustrated in Fig. 11.2. The nine known species divide according to their host range into two major clades, the *A. physeteris* sibling species complex and the other six species. Three of them form the clade of the *A. simplex* sibling species complex. They are sister to the other three species, *A. typica*, *A. ziphidarum* and *A. nascettii*, the latter two combined on a single clade. *Anisakis typica* is restricted to dolphins (Delphinidae) from subtropical and tropical waters and to a single species of the family Pontoporidae (e.g. Mattiucci et al. 2002, 2005; Klimpel et al. 2008; Palm et al. 2008). *Anisakis ziphidarum* and *A. nascettii* have been reported so far only from the Ziphiidae. The *A. simplex* sibling species complex typically infects toothed but also baleen whales. *Anisakis simplex* (s.s.) parasitizes oceanic cetaceans of the families Delphinidae, Monodontidae, Phocoenidae, and Balaenopteridae mainly in the North Atlantic and Pacific Oceans. *Anisakis pegreffii* also utilizes the family Delphinidae as final hosts, however, additionally infecting the Ziphiidae, Physeteridae, and Neobalaenidae (Mattiucci et al. 1997) mainly in the entire Atlantic and Mediterranean but also in Australia. *Anisakis simplex* C infects toothed whales of the families Delphinidae and Ziphiidae in the southern hemisphere, extending its range of distribution into the North Pacific. Species within the *A. physeteris* sibling species complex are host specific for the Kogiidae and Physeteridae. *Anisakis brevispiculata* and *A. paggiae* have been recorded from kogiids mainly in the Mid- and Southern Atlantic Ocean (Mattiucci and Nascetti 2006; Valentini et al. 2006), and the cosmopolitan *A. physeteris* is known from physeterids (Fig. 11.5).

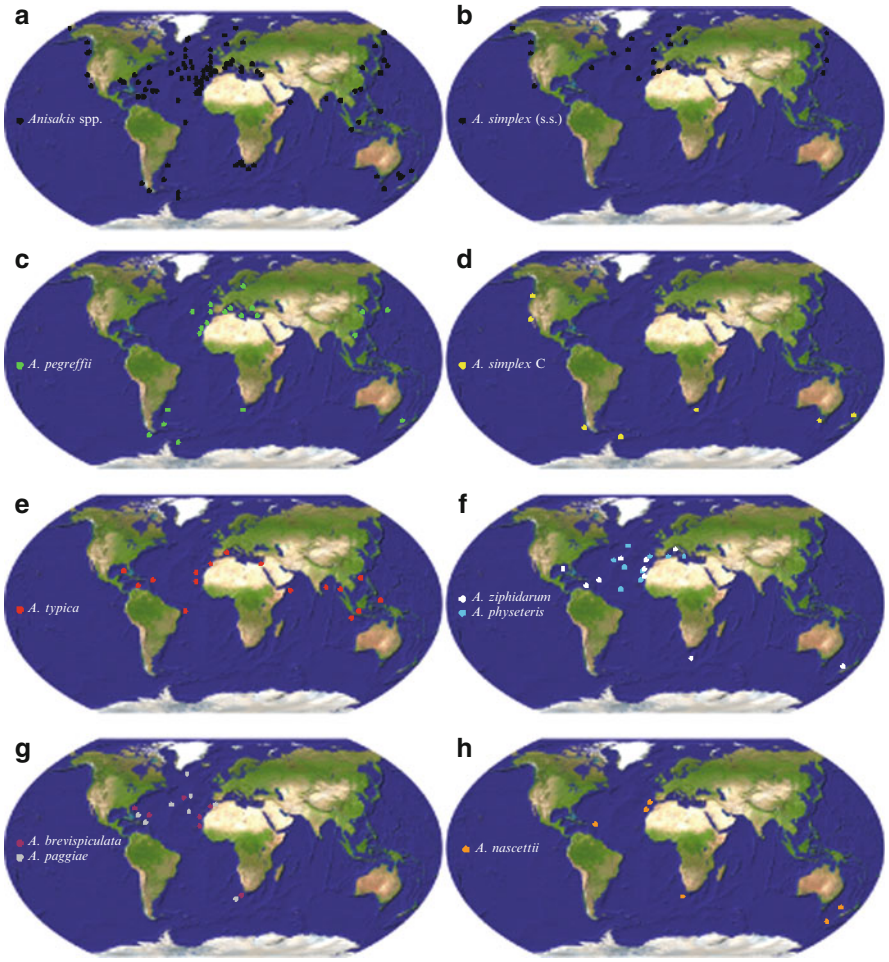

Fig. 11.5 Geographical distribution of *Anisakis* species. (**a**) *Anisakis* spp.; (**b**) *A. simplex* (s.s.); (**c**) *A. pegreffii*; (**d**) *A. simplex* C; (**e**) *A. typica*; (**f**) *A. ziphidarum*, *A. physeteris*; (**g**) *A. brevispiculata*, *A. paggiae*; (**h**) *A. nascettii*

11.5 Biography of *Anisakis* spp.

Species of the genus *Anisakis* are distributed worldwide. The biogeography of *Anisakis* spp. follows a variety of factors that, in combination, lead to zoogeographical distribution patterns: (1) the final host distribution, (2) the host specificity in the final and intermediate hosts, (3) migration patterns of second and paratenic hosts, and (4) the characteristic life cycle (Kellermanns et al. 2007; Klimpel et al. 2008, 2010; Palm et al. 2008). These factors enable *Anisakis* siblings to explore all kinds of marine environments, from the shallow seas and open ocean into the

deep-sea (e.g. Palm and Klimpel 2008). Most *Anisakis* siblings have been reported from the temperate, subtropical, and tropical waters between the equator and 35° North and South, while some species seem to be most common in the boreal regions of the Atlantic and Pacific (Fig. 11.5). Within Antarctic waters (Southern Ocean), these nematodes are at the most southern range of their distribution and to our knowledge extremely scarce (Klimpel et al. 2010).

Most detailed records on the zoogeography have been reported for *A. simplex* (s.s.), a common and highly abundant nematode in the North Atlantic and Pacific Oceans. According to the genetic identification, this species is limited to the northern hemisphere. The most closely related species, *A. pegreffii*, is known from the central Atlantic and the Mediterranean Sea, with some records from the most southern tips of the South American, African and Australian continents. Klimpel et al. (2010) identified specimens from migrating myctophids from the Southern Ocean off the South Shetlands that were genetically identical to specimens from China. The third species within the *A. simplex* complex, *A. simplex* C, has been also recorded from South America, Africa and Australia and around the South Shetland Islands in the Antarctic Ocean. Klimpel et al. (2010) analyzed the ITS-1, 5.8S and ITS-2 rDNA regions of *A. simplex* C specimens from the Antarctic Ocean, finding them identical to specimens from Pacific Canada and California, confirming the extensive range of distribution for this species. Because the parasites were found only in migrating myctophids coupled with rare findings from other teleosts in the Antarctic Ocean (also *A. pegreffii*), the authors concluded that these specimens originated from outside the Antarctic. Consequently, they can be considered at the most southern range of distribution in the Southern Ocean, and an earlier molecular record of *A. simplex* C from the elephant seal *Mirounga leonina* was interpreted as an accidental case of infection.

The two most closely related species within the *A. physeteris* complex, *A. paggiae* and *A. brevispiculata*, have been recorded so far only from the Atlantic Ocean, with most records in the northern hemisphere and a single record from the South African coast. *Anisakis physeteris* has been recorded only from the central and north Atlantic and the Mediterranean. However, because this species typically infects sperm whales that are known for an extensive zoogeographical distribution, the parasite might follow the distribution pattern of their final hosts. According to Klimpel et al. (2008) and Mattiucci and Nascetti (2008), *A. ziphidarum* and *A. nascettii* are typical parasites of ziphiid whales. The former has been recorded from the same localities as *A. paggiae* and *A. brevispiculata*, only from the Atlantic Ocean, and only a few records have reported *A. nascettii* from the waters of the central Atlantic Ocean, South Africa (SE Atlantic Ocean) and New Zealand (SW Pacific Ocean) (Mattiucci and Nascetti 2008; Mattiucci et al. 2009). A unique distribution pattern is known so far for *A. typica*, a species that has been described as circumtropical. According to Palm et al. (2008), several genotypes exist for this anisakid, however, there is no information on morphological differences between them. It can be concluded that following the extensive range of distribution of their mammalian final hosts and the low host specificity in migrating intermediate and paratenic hosts, anisakid nematodes have extensive ranges of distribution.

This may explain why they are among the most common fish parasites recorded during common fish parasitological examinations.

11.6 Pathogenicity and Zoonotic Potential

The research interest in anisakid nematodes is based on the ability of the parasite larvae to survive in humans when ingested alive. Besides having zoonotic potential, anisakid larvae in the teleosts as well as the adults in marine mammals are several centimetres in length, and can cause pathological effects in their hosts. According to Dailey (2001), gastritis or ulcers have been often found in association with aggregations of L3, L4 and adult stages of anisakids (*Anisakis*, *Contracaecum*, *Pseudoterranova*) in the stomach and upper intestine of pinnipeds, cetaceans and sea otters. The symptoms of heavy infections include diarrhoea, dehydration and anaemia (e.g. McClelland 2005). Intestinal perforations leading to peritonitis and death have been attributed to *Contracaecum* and *Pseudoterranova* infections in sea lions and sea otters, respectively.

Nematode parasites of marine vertebrates may also be pathogenic to their intermediate hosts. Larval *Pseudoterranova* spp. caused erratic behaviour and death in experimentally infected marine crustaceans (McClelland 1990). Various larval anisakids (*Anisakis, Contracaecum, Pseudoterranova*) have been connected to mechanical compression or necrosis of the liver, lesions in the gut wall, viscera and musculature, depletion of lipids and mortality in heavily infected marine fish (Rohde 1984; Williams and Jones 1994; McClelland 2005). However, even a high intensity of infestation with *Pseudoterranova decipiens* and *Contracaecum* spp. in Antarctic fish (Klöser et al. 1992; Palm 1999; Palm et al. 1994, 1998, 2007) or the frequent infestation of the Atlantic and Baltic Sea herring (e.g. Szostakowska et al. 2002; Levsen and Lunestad 2010) with *A. simplex* (s.s.) has no visible effect on the host's fitness. Some fish populations are commonly associated with very high anisakid burden, closely related to the abundance of their final hosts in the area of investigation (e.g. Des Clers 1991; Des Clers and Andersen 1995; Lile 1998).

Most recent research activities relate to the zoonotic potential of anisakids to infest humans. Epidemiological studies in Japan have indicated that anisakiasis was most frequent in coastal human populations (Audicana et al. 2002). Most common transmission routes are raw, undercooked and lightly marinated seafood (see Petersen et al. 1993; Palm 2004), for example of the spotted chub mackerel (*Scomber japonicus*) and Japanese flying squid (*Todarodes pacificus*) in Japan (Audicana et al. 2002; Audicana and Kennedey 2008). In western Europe, herring (*Clupea harengus*) is the main species involved, and in Spain, most cases can be related to the consumption of pickled anchovies (*Engraulis encrasicolus*) and raw sardines (*Sardina pilchardus*) (Audicana et al. 2002; Audicana and Kennedey 2008). Archetypal cases of anisakiasis or anisakiosis (Couture et al. 2003), involving the penetration of the alimentary tract and associated organs and causing clinical symptoms (e.g. nausea, severe epigastric pain, vomiting, allergy, diarrhoea), have

been reported largely from Japan and other Asian countries (e.g. McClelland 2005). *Anisakis* larvae have been diagnosed as the disease-causing pathogen in most cases, the remainder being attributed to an infection with *Pseudoterranova* larvae (McClelland 2005). According to Smith (1999) and McClelland (2005) most cases of *Pseudoterranova* infection in Europe and the US have been largely asymptomatic, being diagnosed after the expulsion of the nematodes by coughing, vomiting or defaecation.

Helminth infections often induce chronic rather than acute disease, even in cases of very high levels of parasites. This results from parasite's adaptations to evade the host immune response to secure their own survival. Human anisakidosis is peculiar because these parasites are not adapted to humans, and more than 90% of cases are caused by a single larva (Kagei and Isogaki 1992; Daschner et al. 2000; Audicana and Kennedey 2008). Differences may therefore be expected between *A. simplex* pathogenesis and that caused by other helminths in humans. An example of this is filariasis, in which there is a high and persistent burden of parasites, possibly resulting from host–parasite coevolution in order to optimize their mutual survival (e.g. Mitchell 1991; Taylor et al. 2005; Audicana and Kennedey 2008). Overt hypersensitivity reactions are rare unless provoked by natural or drug-induced death of the parasites residing in tissues. This contrasts with *Anisakis* infections, where allergic reactions seem to be common in humans.

Over the last few years, studies have indicated that, as with other helminth infections, the pathological changes occurring within the gastrointestinal tract are the combined result of the direct action of the larva during tissue invasion and the complex interaction between the host immune system and the substances released by, or contained within, the parasite. Allergies caused by the anisakid larvae in fish consumers have been of major concern. In the reported allergic cases of people from northern Spain, cooked hake (*Merluccius merluccius*) closely followed anchovies as the main pathway of infection. In Germany especially rolled fillet of marinated herring (rollmop) and fried smelt (*Osmerus eperlanus*) are a common source of infection with allergic reactions to *A. simplex* (s.s) in the former and *P. decipiens* in the latter.

Human infection by anisakid nematodes, especially *Anisakis* species, induces stimulation of both T helper type 1 and 2 (Th1, Th2) responses, and provokes a strong specific immune response by antibody isotypes, the immunoglobulin (Ig) IgE, IgG, IgA and IgM (Kennedey 2000; Cho et al. 2005; Audicana and Kennedey 2008). According to Anadón et al. (2009) more than 10.0% of gastrointestinal anisakiasis may be accompanied by allergic symptoms. Some studies have detected the presence of anti-*Anisakis* IgE antibodies in more than 10.0% of healthy subjects, suggesting the existence of a high number of infected patients who do not develop clinical symptoms (Anadón et al. 2009). In contrast to marine mammals, *Anisakis* larvae do not usually reach the adult stage in humans and the larvae die over a specific period after infection. Therefore, it is likely that the immune response against *Anisakis* allergens from the third and/or fourth-stage larvae occurs in response to two consecutive antigenic stimuli, for example (1) the excretory/secretory (ES) and cuticle antigens while the larvae is alive and (2) the cuticle

and protease-resistant somatic and ES antigens, after the larvae die (Anadón et al. 2009). Previous studies have shown that the *Anisakis* ES allergens are the most clinically important, as they are targeted by most of the anti-*Anisakis* IgE antibodies induced during infections by this parasite (Anadón et al. 2009). To date several ES and somatic *Anisakis* allergens have been characterized and cloned, including Anis s 1 and Anis s 7 as probably the most important ES allergens, and have been reported in 85–100.0% of infected humans (Anadón et al. 2009). The Anis s 2 (paramyosin) and Ani s 3 (tropomyosin) are somatic *Anisakis* allergens that cross-react with other common allergens. Other allergens such as Ani s 4 (cystain), Ani s 6 (serine protease inhibitor), Ani s 5, Anis s 8 and Ani s 9 (the latter three among to the SXR/RAL-2 family proteins) are minor ES allergens which were reported from fewer than 50.0% of infected humans (Anadón et al. 2009).

11.7 Ascaridoid Nematodes and Climate Change

We are living in a period of climate change. Temperatures have increased by at least 0.33°C since 1990 (ocean and land combined), and ice fields on Greenland and parts of the Antarctic continent, for example the Larsen and Wilkins shelf ice, are melting at alarming rates (e.g. Rahmstorf et al. 2007). Though proceeding at a moderate pace in terms of human life span, climate change is transforming the world's oceans by increasing the temperature and acidity of seawater and altering atmospheric and oceanic circulation. This has consequences for species distribution and composition in the marine ecosystems, changing the biogeography and biodiversity in aquatic habitats.

The natural variability of abiotic factors such as water temperatures (resulting e.g. in frontal zones, e.g. Klimpel and Rückert 2005) and ocean circulations is relatively high, often following non-linear or cyclic patterns. Similarly, aquatic habitats suffer significant anthropogenic habitat change, mainly caused by overexploitation and unsustainable use. Because these fluctuations overlay the so far subtle effects of temperature changes that are caused by anthropogenic-induced global warming, direct studies of the future consequences for the major ecosystems are difficult. However, the study of the effects of natural climate variability on selected organisms and environments can provide valuable insights into the possible impact of global warming. Compared to terrestrial systems, marine ecosystems are expected to react more sensitively and quickly to changes in climatic conditions, with unpredictable consequences for the species composition, spatial population shifts, or a restructuring of the food webs involved (e.g. Steele 1998, 2004; Hsieh et al. 2005; Jiao 2009). For example, many Atlantic and Pacific fish stocks exhibit a close correlation with climate patterns over many decades (Klyashtorin 2001). Even small natural climatic changes can have significant effects on the marine ecosystems and their organisms.

Fish parasites can be used as biological indicators for environmental impact and change (Palm 2010). However, their potential to indicate global change

scenarios has not been tested. Palm (2010) suggested that oceanic and remote marine ecosystems such as the central Pacific, mid Atlantic, Antarctic or the Polar Sea are the best candidate localities to link an effect of a changing climate directly to aquatic parasite communities at a larger scale. These regions are less affected by factors such as anthropogenic species introduction, pollutants and for example seasonal migrations. Most informative are metazoan helminths as parasite bioindicators because they are embedded within the marine food web and live in oceanic and remote environments. Ascaridoid nematodes combined with larval cestodes and possibly acanthocephalans are useful as biological indicators for host abundance (Palm 2010). The anisakids especially, due to their omnipresence, wide distribution and dependence on the availability of the large predatory final hosts such as seals and whales, are potential candidates to indicate environmental change at a larger scale. According to Marcogliese (2008), warming of coastal waters will result in a higher number of pelagic fish species that follow warmer currents northwards, resulting in increasing *Anisakis* spp. infection of fish. Other effects are a general shift in host ranges and the introduction of pathogens into formerly uninfected regions.

Climate change might have a direct affect on the parasite species but also indirect effects through changes in the distribution and abundance of their intermediate and final hosts (Marcogliese 2008). Especially parasites with complex life cycles, or those in poikilothermic hosts, may be disproportionately affected by global warming (Marcogliese 2008). Induced ice melting in most northern and southern habitats must have consequences for the polar seal and whale populations, and also their parasites. Under global change scenarios, increased sea surface temperature in the northern Arctic might shrink seal populations, reducing the number of final hosts and consequently the number of worms in the fish. On the other hand, more ice-free waters allow the large migrating whale populations to extend their range of distribution into more northern and southern habitats, into formerly ice-covered regions. It has been demonstrated that *A. simplex* C and *A. pegreffii* are at the most southern range of distribution in the Southern Ocean (Klimpel et al. 2010). Higher water temperatures in the high Antarctic might lead to a higher abundance of the whaleworm *Anisakis* in formerly unrepresented regions. Consequently, while the numbers of the sealworms *Pseudoterranova* and possibly *Contracaecum* decrease under shrinking and changing final host populations, other anisakids (*Anisakis*) might be able to extend their numbers. This would also have consequences for the zoonotic potential of these worms. A higher worm abundance of *A. simplex* (s.s.) in fish of the North Atlantic and Pacific waters will result in higher transmission rates to humans, and probably cause an increasing conflict potential through the consumption of marine fish.

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Chapter 12
Fish Parasites as Biological Indicators in a Changing World: Can We Monitor Environmental Impact and Climate Change?

Harry W. Palm

Abstract Global warming scenarios combined with political and public awareness have led to increasing funding and research efforts on the measurement and prediction of effects of a changing world on the ecosystems. Fish parasites represent a major part of aquatic biodiversity, and consequently become affected either directly through the environment or indirectly through their respective hosts. On the basis of a conservative estimate of an average of 3–4 fish parasites in each existing fish species alone and a current number of 31,400 described fish species, we can estimate the existence of up to 120,000 fish parasite species, including both protozoans and metazoans. Combined with a number of life cycle stages that may infect all aquatic hosts and organs, this vast biodiversity represents a widely neglected tool for a variety of ecology-based applications. Studies have demonstrated that fish parasites can serve as biological indicator organisms to illustrate the ecology of their infected hosts, including feeding, migration and population structure. Parasite metrics have been connected to specific environmental conditions, and they can indicate different pollutants such as heavy metal concentrations, industrial and sewage pollution, and also eutrophication. Most recently, parasite infections have been connected to anthropogenic impact and environmental change also in marine habitats. This book chapter summarizes the use of fish parasites as biological indicators, and discusses their potential and the requirements in the utilization of fish parasites as biological indicators of climate change.

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12.1 Introduction

Aquatic ecosystems along the costal zones belong to the most vulnerable systems on earth and face increasing anthropogenic stress in terms of pollution and environmental degradation. About 2.75 bn people are expected to live within 60 mi of the coastline in 2025, living from or indirectly using the coastal environments. This, however, is the region that harbours the highest aquatic biodiversity, especially in tropical coastal waters. It is obvious that extensive anthropogenic activities directly affect the species composition and diversity of the aquatic biota, possibly negatively influencing the long-term perspectives and sustainability of these ecosystems.

Fish parasite biodiversity and species composition in the aquatic realm depends on species richness of the final hosts and their ecosystem. The global fish fauna comprises more than 31,400 species (Froese and Pauly 2010), about half of them (14,970 species) live in marine waters. Because of the long-term stability of marine ecosystems, fish parasite diversity per host is higher than in freshwater. Rohde (2002) estimated 100,000 fish parasites in about 30,000 known fish species, resulting in an average of 3.3 parasite species in each fish studied. Margolis and Arthur (1979) and McDonald and Margolis (1995) recorded 925 different fish parasites on 292 marine and freshwater fish species from Canadian waters, including protozoans and metazoans (3.2 parasite species/fish species). Palm et al. (1999) reported 191 different metazoan parasite species from another northern habitat, the coastal waters of Germany. A total of 62 wild fish species from the North and Baltic Sea coast harboured an average of 3.1 metazoan parasite species per fish species. This contrasts the deep-sea, where the average number of parasites per fish species is 1.5, a value that did not increase in the last 8 years (Klimpel et al. 2001, 2009). On the basis of the existence of more than three metazoan fish parasites in each existing fish species alone, we can estimate the existence of up to 120,000 fish parasite species, including both protozoans and metazoans.

The aquatic environment can be studied either directly by a regular monitoring of water quality parameters or indirectly by using bioindicators (also see Palm and Rückert 2009), such as fish parasites (Galli et al. 2001). These organisms react on specific environmental conditions or change, leading to a wide range of applications (bioindication for water quality, MacKenzie et al. 1995; environmental stress, Landsberg et al. 1998; pollution, Khan and Thulin 1991; Yeomans et al. 1997). Vidal-Martínez et al. (2010) generally distinguished between accumulation or effect bioindicators, where organisms efficiently take up substances in the former or are used to detect environmental impact in the latter. This is done while recording a definite change in their physiology, chemical composition, behaviour, or number. Also other parasite metrics such as diversity indices or species richness can be a source of information (e.g. Rückert et al. 2009a), demonstrating a possible effect of specific environmental conditions on the fish parasite community. Among others, Lafferty (1997), Marcogliese and Cone (1997), Overstreet (1997), Williams and MacKenzie (2003), Marcogliese (2005) and most recently Vidal-Martínez et al. (2010) summarized the published literature on how to use fish parasites as bioindicators in the aquatic environment.

In the context of long-term and climate change scenarios, rising sea-level and water temperatures may have direct effects on the fish parasite composition within a respective habitat. Only very few freshwater habitats, however, are under pristine conditions, and anthropogenic species introduction connected to fisheries combined with regular migration events of neozoons alter the regular fish and parasite fauna. Most marine environments have suffered heavy fishing pressure over the last century. Anthropogenic changes have greatly altered the fish species composition, especially of large predators at high trophic levels (Hutchings and Baum 2005; Baum and Worm 2009). This has measurable effects even on life history traits, substantially changing age and size at maturation (Sharpe and Hendry 2009). Consequently, fish parasite numbers that are related to their changing host numbers may also change with a shift in environmental conditions. A conclusive description of the circumstances under which parasites can be used as indicators of environmental impact, however, still remains difficult (Vidal-Martínez et al. 2010).

The present chapter describes a simplified method of routine fish parasitological investigations, presents an overview on the available literature, and provides different examples on how to use the resulting fish parasite metrics as biological, accumulation and effect indicators in different aquatic environments. Differences of the freshwater and sea water ecosystems are discussed. A perspective on how to use natural fish parasite populations as biological indicators for climate change is presented, focusing on different problems involved in the freshwater and marine environments.

12.2 Applied Methodology

The application of fish parasites as bioindicators requires the routine study of fish for the presence of a variety of fish parasites. The parasitological samplings must follow standard protocols. To use fish parasites as effect indicators for environmental change, the study of both protozoans and metazoan parasites is suggested. Consequently, the external examination should be done directly after catch from the living fish when the protozoan parasites are still alive on the body surface. They disappear soon after the death of the fish. Smears for the detection of ectoparasitic trichodinid ciliates must be taken directly from the gills, surface and the inner opercula of the living fish, followed by the study of the skin, fins, eyes, gills, mouth- and gill cavity for other metazoan ectoparasites, such as monogeneans and crustaceans. The inner organs, digestive tract, liver, gall bladder, spleen, kidneys, gonads, heart and swim bladder should be subsequently separated and transferred into saline. While a stereomicroscope must be used to study the internal organs, a phase-contrast microscope or better with Nomarski (DIC = differential interference contrast) enables recognition of parasitic life cycle stages in the gall bladder and urogenital system. Belly flaps and musculature must be examined on a candling table with a light source from beneath.

Most useful fixatives are 4% formalin and 70% ethanol, or absolute ethanol for molecular analyses (see Klimpel and Palm 2011). The smears from the gills, surface and opercula must be stained by using a silver nitrate impregnation after Klein (1926, 1958), rinsed and covered with 5% silver nitrate solution and impregnated for 30 min in the dark. The $AgNO_3$ has to be removed and the slides must be covered with distilled water, exposed to ultraviolet light for 40–50 min, and dried after exposure. Acanthocephala should be transferred to freshwater until the proboscis everts prior to fixation. For identification purposes, Nematoda and Acanthocephala should be dehydrated in a gradated ethanol series and transferred to 100% glycerine (Riemann 1988). Digenea, Monogenea and Cestoda are stained with acetic carmine, dehydrated, cleared with a clearing agent such as eugenol and mounted in Canada balsam (Palm 2000, 2004). Directly fixed nematodes in 100% ethanol can be identified by using molecular tools, such as described for the ascaridoid genus *Anisakis* (see Palm et al. 2008; Klimpel and Palm 2011).

The parasitological terms (prevalence, intensity and mean intensity) are standardized, and should follow Bush et al. (1997), where the prevalence (P) is the number of fish with one or more individuals of a particular parasite species (or taxonomic group) divided by the number of hosts examined (expressed as a percentage) [Prevalence (P) = No. of hosts infested / No. of hosts examined * 100]. Intensity (of infection, I) is the number of individuals of a particular parasite species in a single host (expressed as a numerical range); and mean intensity (of infection, I_m) is the total number of parasites of a particular species found in a sample divided by the number of infected hosts [Mean Intensity (I_m) = Total no. of a particular parasite / No. of infected hosts]. The diversity of the parasite fauna (endoparasite fauna according to Rückert et al. 2009a) should be determined by using the Shannon–Wiener diversity index (H') and the evenness index (E) of Pielou (Magurran 1988) [Shannon-Wiener Index (H') = $\sum P_i \ln P_i$, Evenness (E) = $H'/\ln S$, with H' being the diversity index, P_i the proportion of the individual (ith) species to the total and S is the total number of species in the community (species richness)]. The ecto- versus endoparasite ratio (E/E) has been introduced by Palm and Rückert (2009). Any other ecological measures or parameters might also be applied to compare the different sampling sites.

12.3 Fish Parasites as Biological Indicators

One of the first applications to utilize fish parasites as biological indicators using standard parasitological methodologies (see above) was related to fisheries, including the separation of fish stocks (e.g. Lester 1990; Khan and Tuck 1995; MacKenzie 1990, 2002; Moser 1991; Moser and Hsieh 1992; Williams et al. 1992; Pascual and Hochberg 1996). Herrington et al. (1939) was the first to use the crustacean parasite of a marine fish in the North Atlantic as a biological tag in a population study. The abundance and occurrence of the parasites directly relates to the distribution, migration patterns and population biology of their hosts

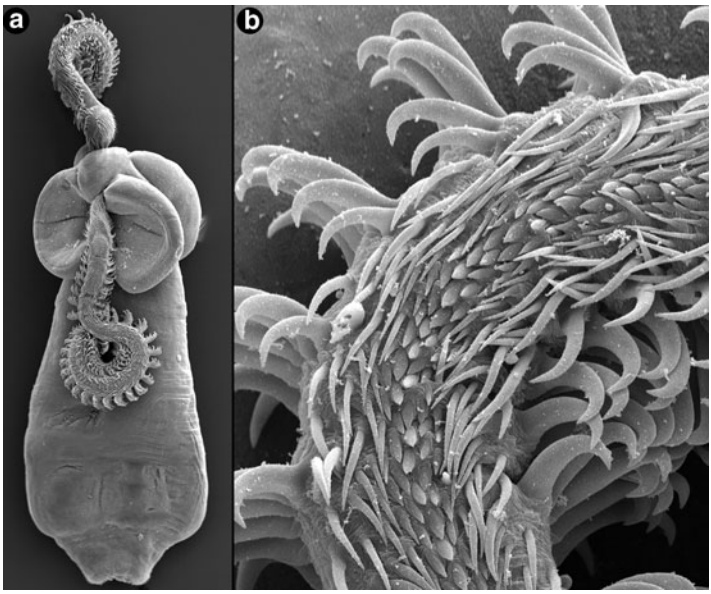

Fig. 12.1 (**a**) Scolex of the trypanorhynch cestode *Grillotiella exile* from Indonesia, with two bothria and armed tentacles (worm length = 0.8 mm). This species has been recorded from the tiger shark (*Galeocerdo cuvier*), infests the gills of the narrow-barred Spanish mackerel (*Scomberomorus commerson*) and has been used as a biological tag for stock analyses. (**b**) Tentacular armature of *G. exile* from Indonesia. The arrangement of the tentacular hooks is species specific and enables identification (Palm and Klimpel 2007; Palm 2008)

(Kabata et al. 1987; MacKenzie 1985; Boje et al. 1997; Klimpel et al. 2010), and can be used for stock identification (Fig. 12.1a-b) and even risk assessment of fish food-borne zoonoses. MacKenzie (1983) listed different criteria to decide on the usefulness of parasites as biological indicators that he applied for the Atlantic herring *Clupea harengus* and the mackerel *Scomber scombrus* in the North Sea and Northeast Atlantic (MacKenzie 1985, 1987, 1990). Larval trypanorhynch cestodes (Fig. 12.2b) and anisakid nematodes were most useful for stock recognition and separation. It is important that the parasites are easy to collect and can be easily recognized, excluding systematic errors based on erroneous species identification (Table 12.1). Suitable parasites should also infect their hosts at a particular age or in a definite region and are differently abundant in different regions, allowing the original locality of infection to be traced. *Anisakis simplex* infection of herrings in the Baltic Sea off the coast of Poland has identified the western spring herring that migrates from the North Sea, the origin of the infection, into the Baltic Sea (Grabda 1974). Klimpel et al. (2010) found that migrating myctophids around the South Shetland Islands, Antarctica, were infected with *Anisakis simplex* C and *A. pegreffii*, having identical ITS-1, 5.8 S and ITS-2 sequences to specimens from Pacific Canada or California (*A. simplex* C) and the Mediterranean Sea (*A. pegreffii*). The authors suggested that the nematodes originated from more

Table 12.1 Important factors for the selection of fish parasites as biological indicators

Parasite characteristics	Biological indicator	Accumulation indicator	Impact indicator
1. Non-pathogenic in free-living host populations	√	√	√
2. Attached to the host that it is unlikely to be removed within the short period until examination	√	√	√
3. Easy to collect (e.g. large body size, availability, time to dissect)	√	√	√
4. Easy to find (to obtain quantitative data, e.g. abundance and prevalence)	√	√	√
5. Can be identified to species level (e.g. for host identification)	√	√	–
6. Easy to identify at least to the genus level (as a single definite species)	(√)	–	√
7. Infection data can be analyzed acc. to prevalence, intensity and abundance of infection, as defined by Bush et al. (1997)	√	–	√
8. Long life-span (longevity)	√	√	–
9. Differently abundant in various localities	√	–	√
10. The parasite infects a host at a particular age/region (e.g. young fish on the nursery ground)	√	(√)	–
11. Widespread (to facilitate comparison among areas)	–	√	(√)
12. Accumulation potential and resilience to high levels of pollutants	–	√	–
13. Knowledge on the physiology and life cycle ecology (e.g. intermediate/paratenic host involvement)	–	√	√
14. Ability to react under changing environmental conditions	–	–	√
15. Detailed knowledge on the study area	–	–	√
16. Conjunction with other information and methods for biological effect monitoring, incl. the possibility for experimental studies	–	–	√

northern waters, and became introduced to the Southern Ocean by migrating myctophids and whales. Marine fish parasite larvae have been recorded in freshwater up to 3,000 km away from the coast (Lühe 1910; Shulman 1957). Anadromous fish species such as salmonids transport these parasites upstream, explaining the presence of trypanorhynch cestodes within the German rivers Rhine, Elbe and Weser as well as within a lake in Switzerland (Lühe 1910; Palm 2004). These records provide evidence for the extensive fish migrations from the Ocean also upstream into the freshwater environments.

Gibson (1972) separated flounders (*Platichtys flesus*) from two estuaries and the sea within a 40 mi range. The fish had dissimilar parasite-faunas, and individual fish from each locality could be recognized by its parasites, especially by the level of certain indicator species. Similarly, "foreign" flounders moving into a flounder population could be picked out by the markedly different composition of their parasite-fauna. Using two species of myxozoans, *Myxobolus arcticus* in the brain and *Henneguya salmonicola* in the musculature, Margolis (1982) was able to

distinguish three different spawning stocks of sockeye salmon, *Oncorhynchus nerka*, off Vancouver Island, Canada. These stocks came from three different lakes, where the parasites infect the juveniles in freshwater. Juvenile fish from the Great Central Lake were virtually free of these parasites, those from Sproat Lake had only *M. arcticus* and sockeye from Henderson Lake were infected with both species. These results permitted for the first time in-season management of a fishery (Moser 1991). MacKenzie and Abaunza (1998), MacKenzie (2002) and Lester and MacKenzie (2009) later summarised and discussed the procedures and methods for the stock discrimination of marine fish, and Latama (2006), Lester et al. (2001) and Charters et al. (2010) recently applied these methods for Spanish and grey mackerel (both genus *Scombermorus*) populations even in the widely distributed stocks off the northern Australian coast and in tropical Indonesia (Fig. 12.1). It was demonstrated that Spanish mackerel from Australia and Indonesia clearly separate, and harbour different metazoan parasites. Four different clusters of Spanish mackerels were found in Sulawesi waters alone, four stocks or populations of grey mackerels around the northern and eastern coastline of Australia, indicating the presence of different local stocks in the areas that do not entirely mix. Such results are of importance for the fishing industry in the attempt to better utilize and sustainably use these commercially important fish stocks.

Relevant information also results from the knowledge of parasites that might be transferred into humans via the musculature of fishes from our markets (e.g. Palm and Overstreet 2000). The prevalence and abundance of zoonotic parasites is different among most fish stocks and geographical regions, leading to a different risk resulting from fish food that is meant for human consumption. Petersen et al. (1993) indicated that based on a parasitological survey the risk of acquiring parasitoses from eating raw fish from central Philippine waters is low. Consequently, the abundance of fish parasites can be used as an indicator for the risk of fish food-borne zoonoses. This human health aspect has implications not only for the fishing but also for the tourism industry within a region (e.g. *Anisakis* spp., see Palm et al. 2008; Klimpel and Palm 2011).

Another interesting use of fish parasites as bioindicators concerns their potential to elucidate aspects of the biology of the host organism (Caira 1990), for example feeding ecology and behaviour. Many metazoan fish parasites are transmitted through the food chain. Because many life cycle stages are long-lived and can maintain themselves over a longer time inside the host, a parasite record integrates over time and has significant advantages to time-consuming stomach content analyses. Parasitological studies of metazoan parasites with a long life span (Table 12.1) combined with an analysis of the stomach contents are useful for ecological studies of fish that are difficult to study in vivo, because they live in inaccessible and extreme environments (Fig. 12.2c, f). For example, Klimpel et al. (2003, 2006a, b) and Palm and Klimpel (2008) analyzed the parasite fauna and feeding ecology of deep-sea fish around the Great Meteor sea mount and the Mid-Atlantic Ridge, and Palm (1999) and Palm et al. (1998, 2007) analyzed the food web in the Southern Ocean. On the other hand, the occurrence of fish parasites associated with specific prey organisms in the host can help to identify the life cycles of the parasite

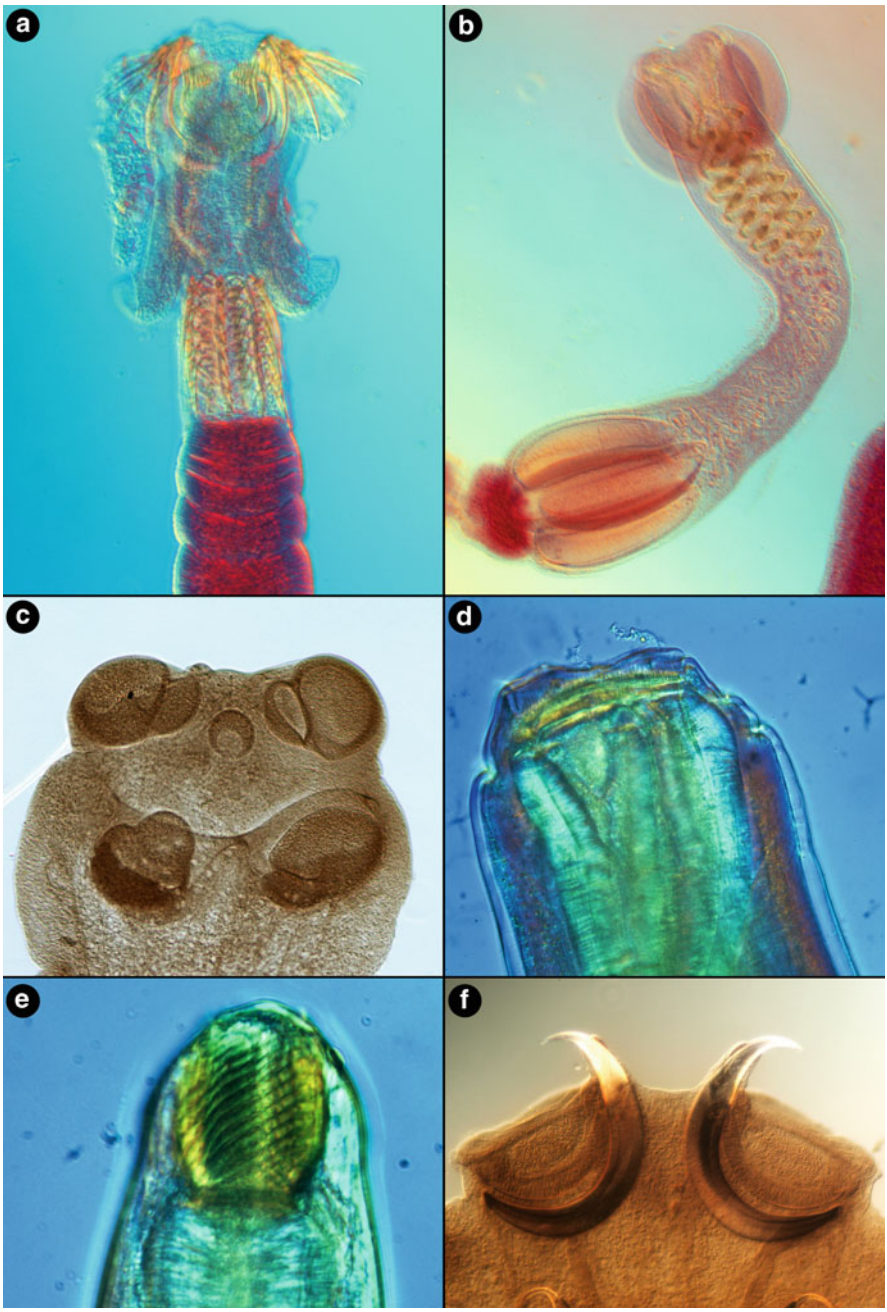

Fig. 12.2 (**a**) Diphyllidean cestode from *Raja* sp., Pelabuhan Ratu, Indonesia. These parasites are highly host specific and can be used for host species identification and possibly phylogeny. (**b**) Trypanorhynch cestode *Lacistorhynchus* sp. from *Hemipristis elongata*, Pelabuhan Ratu, Indonesia. These parasites can be used for stock identification, host abundance, migration,

species involved. An analysis of the parasites and the stomach contents of the pearlside *Maurolicus muelleri* from the Norwegian Deep revealed the typical life cycle of the anisakid nematode *Anisakis simplex* (Klimpel et al. 2004), and Klöser et al. (1992) and Palm (1999) studied the life cycles of the anisakids *Contracaecum* spp. and *Pseudoterranova decipiens* in the Southern Ocean. Palm and Schröder (2001) postulated, from a study of cestode parasites, that deep-water elasmobranchs of the genus *Deania* can serve as food fish for larger oceanic sharks around the Great Meteor Seamount in the central North Atlantic Ocean.

Finally, fish parasites can be used as biological indicators for host identification and phylogeny (Fig. 12.2a), and its systematic position (Rokicki 1983). Palm (2007), Palm and Klimpel (2007) and Palm et al. (2009) used trypanorhynch cestodes as a model system to better understand the ecology and co-evolutionary history (cumulative evolution) of parasitic life cycles in the ocean. Olson et al. (2010) estimated the original hosts of two primary parasite lineages in the cestode order Trypanorhyncha to be alternatively rajiform batoids and carcharhiniform sharks. This fundamental split provided independent support for rejecting the notion that rays are derived sharks, and supported the most recent molecular phylogenies of the Neoselachii. Beyond the basal split between shark- and ray-inhabiting lineages, no pattern was found to suggest that the trypanorhynchs have closely tracked the evolutionary histories of these host lineages, but instead, it appears that host-switching has been common (Palm et al. 2009) and that the subsequent evolution of the parasites has been ecologically driven primarily through overlap in the niches of their shark and ray hosts. This was possible by relaxed host specificity within the group (Palm and Caira 2008), making these parasites of interest in the analysis of the feeding ecology of their hosts. A number of other fish parasites in the marine ecosystem have high host specificity, living only on one or a few closely related fish species. They have thus developed in close host–parasite co-evolution, and illustrate the evolutionary history and phylogeny of their hosts, as exemplified for anisakid nematodes of the *Anisakis simplex* species complexes and their whale hosts (Klimpel et al. 2011) and the phylogeny of deep-sea trematodes in fish (Bray et al. 1999).

12.4 Environmental Indicators

The presence of parasites within the environment often becomes evident after a massive infestation causing clinical signs or leading to mortality of the infected hosts. Such a situation can be combined with biotic or abiotic changes in the

Fig. 12.2 (continued) elasmobranch ecology and phylogeny. (**c**) Tetraphyllidean cestode *Scolex pleuronectis* from Hawaiian waters. Biological indicators for assessing spatial variation in shark distribution. (**d**, **e**) Fish parasitic *Cucullanus* sp. from *Balistapus undulatus* and *Pro(spiro) camallanus* sp. from *Gomphosus varius*, French Polynesia. These parasites might be useful as effect indicators for environmental change. (**f**) Opisthaptor of the monogenean *Dasyonchocotyle dasyatis* from *Dasyatis lata*, Hawaii. Potential bioindicator for the host distribution

environment (Möller 1987), in the application of fish parasites as environmental indicators. Knowledge of the biology of the parasite and its host(s), the host–parasite relationship and the environment can help to detect environmental change (Table 12.1). Particularly long-living species (some digenean trematode, cestode, nematode life cycle stages) provide information on the seasonal migration of their hosts and migration habits of different age groups (feeding area/spawning area, see above, Table 12.2). They, however, also change their abundance in the host if some of their life cycle stages become affected through the disappearance of their intermediate hosts, provoking disappearance of some parasite species under polluted conditions (Fig. 12.2d–e). Consequently, the occurrence of heteroxenous (multi host life cycle) parasites in an area affected by pollution can be related to the number of intermediate hosts at the studied sites (Xinghua 1987; Overstreet et al. 1996). The intermediate hosts may be more sensitive to environmental changes than the parasite, which in the case of

Table 12.2 Tools for the utilization of fish parasites as biological indicators, distinguishing biological, accumulation and effect or impact indicators

Parasite taxa:	Ciliophora/ Myxosporidea	Monogenea	Digenea	Cestoda	Nematoda	Acanthocephala	Crustacea
Biological indicator							
Population biology/ migration	+	+	+	+	+	+	+
Host abundance/ density	+	+	+	+	+	+	n
Feeding ecology	=	=	=	+	+	+	=
Host identification/ phylogeny	n	+/−	+/−	−/+	−/+	−/+	+/−
Accumulation indicator							
Bioaccumulation (heavy metal)	n	n	=/+	+	=/+	+	n
Effect and ecosystem indicators							
Eutrophication/ bacterial biomass	+	=	+	+	+	+	−
Industrial waste	+	+/−	−/+	−/+	−/+	=/−	−
Thermal pollution	+	+/−	−	=	+	+/−	n
Paper/pulp mill effluent	+	+/=	−/+	=	−	+	−
Sludge	n	=	−/+	+	=	−	n
PCB/pesticides	+	=	=/−	−	−	=/−	−
Heavy metals (field study)	+/−	+/−	−	−	=	+	−
Crude oil/oil spill	+/=	−	−	=	−	=	=
Acidic rain	n	−	−	−	n	+	n
Anthropogenic influence	n	−/=	−	−	+/−	n	−/+

Altered after Lafferty (1997), Sures (2004) and Vidal-Martínez et al. (2010). Positive abundance or effect term (+), negative (−), positive trend (+/−), negative trend (−/+), without effect (=), not enough data (n), from laboratory and field experiments.

endoparasites is buffered from the environment by the host physiology (Paperna and Overstreet 1981). On the other hand, Jeney et al. (2002) related an increasing experimental infection rate with the digenean *Rhipidocotyle fennica* cercariae in roach from a eutrophic lake contaminated with bleached paper mill effluent and an oligotrophic lake to a decreased resistance of the fish to the parasite. Short-living species combined with a direct life cycle and high reproduction rates (protozoan ectoparasites, monogenean trematodes) may react to the environmental conditions of the host (Lester 1990). Pettersen et al. (2006) demonstrated that aqueous aluminium of 200–260 µg Al/l at pH 5.8 had a negative effect on ectoparasites (elimination of gyrodactylids, decreased abundance of duck mussel glochidia, increased mortality rate in the fish louse), without any apparent negative effect on the fish hosts. Consequently, a proper choice of the parasite or the parasite metrics to be used is the prerequisite for the application of fish parasites as bioindicators for environmental change (Table 12.2).

12.4.1 Accumulation Bioindicators

Living inside the fish and using the host as food resource, fish parasites closely interact with the metabolisms of the host. Thus, parasite infrapopulations can be affected by changes of the host physiology and substances accumulated with the host´s food. Early studies of Read (1951) and Roberts (1961) with the adult rat tapeworm *Hymenolepis diminuta* demonstrated the decrease of weight, total tissue carbohydrate and reduced numbers of mature and gravid proglottids after the hosts were exposed to a low carbohydrate diet (cited in Esch et al. 1975). If the host is living within a polluted environment where some pollutants also enter the fish, the concentration of these substances in the immediate surroundings of the parasite can also increase. In such cases, some fish parasites can accumulate pollutants in a much higher concentration as their host organisms, and serve as accumulation indicators (Table 12.2). For example, some acanthocephalans specifically accumulate certain heavy metals in greater amounts than their host, and can be used as accumulation indicators of heavy metal pollution (Sures and Taraschewski 1995; Sures et al. 1994; Sures 2003; Sures and Siddall 2003). Adults of the acanthocephalans *Pomphorhynchus laevis* and *Paratenuisentis ambiguus* accumulate lead and cadmium in a greater amount than their hosts (*Anguilla anguilla*, *Leuciscus cephalus*, *Perca fluviatilis*). However, there is some dependence on the parasitic life cycle stage. According to Sures et al. (1994), adults of the acanthocephalan *Acanthocephalus lucii* accumulated Cd in higher amounts than the larvae. Sures and Reimann (2003) compared the heavy metal concentration of the acanthocephalan *Aspersentis megarhynchus* with the muscle of the Antarctic rock cod *Notothenia coriiceps*. Most of the elements were found in significantly higher concentrations in the acanthocephalan than in the muscle of its host. Levels of Ag, Co and Ni in the muscle of *N. coriiceps* were even below the detection limit, and were only found in the worm. Other metals commonly associated with human activities (e.g. Pb, Cd, Cu) were accumulated to a high degree in the parasite,

demonstrating that pollutants of anthropogenic origin are dispersed within this remote, fairly unpolluted environment. Cestodes can also be used as accumulation indicators. For example, lead and cadmium is found in significantly higher concentrations in the tissues of the cestode *Monobothrium wageneri* than in their fish host tissues (*Tinca tinca* from Ruhr River). The marine cestode *Bothriocephalus scorpii* (Cestoda) from *Scophthalmus maximus* (Gdansk Bay) was found to accumulate these heavy metals especially in the posterior part of the proglottids, while the anterior part revealed the same amounts of the heavy metals as the fish host tissues (Sures et al. 1997). Jirsa et al. (2008) detected a higher amount of Cd, Pb and Zn in *Caryophyllaeus laticeps* compared with the cyprinid host *Chondrostoma nasus* in river sites in Austria, and according to Tekin-Özan and Kir (2005), the plerocercoids of *Ligula intestinalis* provided reliable information on the amount of heavy metals in a freshwater reservoir in Turkey. According to Sures (2004) and Barus et al. (2007), some fish parasitic nematodes such as *Anguillicola crassus* from eel and *Philometra ovata* from bream can also have an increased level of heavy metal concentration compared to their host organisms, and Ruus et al. (2001) recorded accumulation of another environmental contaminant, lindane, in metacercariae of the trematode *Bucephaloides gracilescens* in the central nervous system of *Myoxocephalus scorpius*.

12.4.2 Effect Bioindicators

One of the broadest applications of fish parasites is their use as effect bioindicators. A summary of effects combined with different environmental factors is given in Table 12.2, and was reviewed by Williams and MacKenzie (2003). Protozoan mobiline peritrichous ciliates (genus *Trichodina*, phylum Ciliophora) have been experimentally examined for their function as biological indicators of water quality (Voigt 1993). They were also reported as indicators for petroleum hydrocarbons (Khan and Thulin 1991). *Trichodina cottidarum* and *T. saintjohnsi* infected *Myoxocephalus octodecemspinosus* that had been exposed to crude oil in laboratory experiments, as well as at an oil-receiving terminal site, in a significantly higher intensity than in uncontaminated control experiments/sites. Pulp and paper mill effluents showed similar effects on these trichodinid ciliates (Khan 1990; Khan et al. 1993). Also oil-contaminated sediments caused increasing infestation with *Trichodina* sp. in *M. octodecemspinosus* after exposure over a time of 3 and 6 months. The prevalence of *Trichodina* spp. gill infection increased significantly on fish from inside the heavily polluted local fishing port Pelabuhan Ratu on the southern Java coast, Indonesia, compared to specimens that were caught outside the harbour (*Epinephelus* sp., 45.5%, n = 11 vs. 0%, n = 23). Palm and Dobberstein (1999) suggested the use of prevalence and density data of *Trichodina* spp. as a biological indicator for the bacterial biomass in the environment, because peritrichous ciliates are primarily filter feeders on small algae and bacteria. The first record of *Trichodina rectuncinata* from the gills of *Melichthys vidua* in Moorea,

French Polynesia, might be connected to the degraded reef habitat where the fish was caught (Fig. 12.3). Ogut and Palm (2005) demonstrated the relationship between *Trichodina* spp. infection on *Merlangius merlangus* and organic pollution, measured as levels of nitrite, nitrate and phosphate in the surrounding environment. These relationships suggest the usefulness of fish parasites as biological indicators for ecosystem change (see below; Palm and Rückert 2009; Rückert et al. 2009a).

Also other pollutants can have effects on the parasites within or attached to the host. For example exposure to petroleum aromatic hydrocarbons (PAH) caused increasing prevalence of *Ceratomyxa acadiensis* (Myxosporida) in the gall bladder of the winter flounder *Pseudopleuronectes americanus* (Khan 1986, cited in Khan and Thulin 1991). PAHs are known to cause lesions and hyperplaisia of the secondary gill lamellae in fish. Long-time exposure of cod to aromatic hydrocarbons (max. 30 weeks) caused increasing infestation with monogenetic flatworms (prevalence as well as intensity), which was attributed to the already damaged gill tissue, and therefore to the better living conditions for monogenetic trematodes (Khan and Kiceniuk 1988). Bayoumy et al. (2008) showed conflicting evidence for the reaction of monogeneans to heavy metal concentrations in the Red Sea. While the lead concentration correlated negatively with the prevalence of five different monogenean species, nickel and also temperature were positively correlated. Gheorghiu et al. (2006) reported improved parasite *Gyrodactylus turnbulli* (Monogenea) population growth under water-borne zinc concentration up to 120 lg/L, while Gheorghiu et al. (2007) indicated reduced parasite survival and reduced reproduction over this range of Zn concentrations, rejecting the suggestion that water-borne Zn improves parasite growth and reproduction. These examples demonstrate the difficulties involved in finding the best host–parasite systems as effect bioindicators for pollutants in aquatic environments.

Parasitic endohelminths with a heteroxenous life cycle can be more tolerant to an increased level of water pollutants (see accumulation indicators above). However, an effect on the intermediate hosts prevents parasite transmission and reduces the observed prevalence and intensity of infection in the definite host. For example, the gastrointestinal parasites of *Pseudopleuronectes americanus* (*Steringophorus furciger*; Trematoda) and *Gadus morhua* (*Echinorhynchus gadi*; Acanthocephala) exposed to oil had a lower infection compared to the control groups. It was thought that water-soluble fractions of the crude oil were especially involved in causing this effect (Khan and Kiceniuk 1983; Khan 1987).

Another interesting biological-effect monitoring study was carried out in the context of eutrophication, in both freshwater and marine habitats. Hartmann and Nümann (1977) reported an increasing level of infection with parasites of various species, especially *Diplostomum* sp., with progressive eutrophication in Lake Constance. According to Lafferty (2008), parasites that increase under eutrophic conditions tend to be host generalists with local recruitment. Athanassopoulou and Ragias (1998) studied the parasites and diseases of wild fish populations from a lake in Greece over two long periods (1984–1990 and 1994–1997). The study concluded that the pollution in the lake increased together with the prevalence, intensity and pathology of most parasites. Tumours and bacterial infections were directly

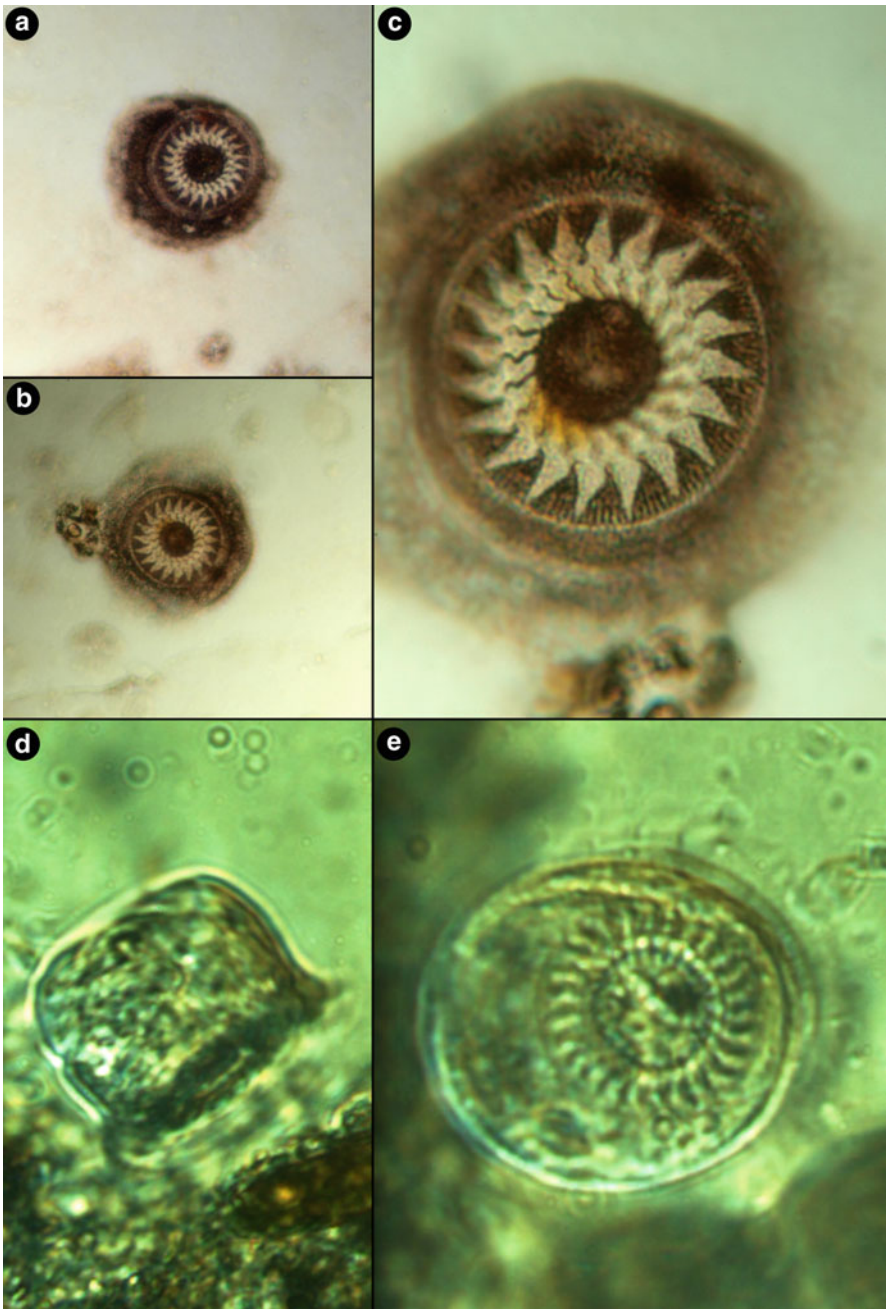

Fig. 12.3 *Trichodina rectuncinata* from the gills of *Melichthys vidua* from Moorea, French Polynesia. (**a-c**) Silver nitrate impregnation after Klein. (**d**, **e**) Living specimens on the gills. The prevalence of infestation with trichodinid ciliates is related to the bacterial biomass in the water body, and can be used as an eutrophication effect indicator. The above specimens were found on a single fish from a degraded reef in Oponohu Bay

associated with increased pollution, especially industrial and sewage waste values. In the Baltic Sea, Reimer (1995) used parasites of piscean and other hosts as eutrophication indicators. The different infestation of gobiid fish with helminth parasites from different sampling sites in the western Baltic Sea was associated with the eutrophication level, influencing the abundance of herbivorous intermediate hosts (Zander and Kesting 1996, 1998). Kesting and Zander (2000) related the impoverishment of the metazoan parasite fauna in the Schlei Fjord over an 18-year period to increasing eutrophication. In the marine environment Moser and Cowen (1991) studied the nototheniid *Trematomus bernacchi* from the eutrophic east and oligotrophic west side of McMurdo Sound in Antarctica. The fish from eutrophic localities revealed high prevalences of the acanthocephalan *Echinorhynchus* sp., the nematode *Ascarophis nototheniae*, the trematode *Dinosoma* sp. and the cestode *Phyllobothrium* sp. The investigation of the abundance of arthropods at these localities demonstrated that these are more abundant at the eutrophic east side of McMurdo Sound. The higher number of available intermediate hosts of these parasites in the region triggers their prevalence in the fish as the definitive host (Moser and Cowen 1991).

Environmental monitoring programs using fish parasites in open water have not been applied, though preliminary data provide promising results. Recently, Lafferty et al. (2008) proposed that the sampling of larval cestodes in small teleosts (Fig. 12.2c) is a convenient method for assessing spatial variation in shark distribution, and that the lower parasitism at Kiritimati compared to Palmyra in the Central Pacific Ocean resulted from a simplified food web due to overfishing. Low biodiversity could impair parasite transmission by reducing the availability of hosts required by parasites with complex life cycles. This would imply that these fish parasites can be used to monitor food web composition and elasmobranch abundance in oceanic habitats. The latter is probably influenced by shark fisheries, significantly reducing the number of available oceanic top predators over time. This result supports the notion by Marcogliese (2005) that perturbations in ecosystem structure and function that affect food web topology also impacts upon parasite transmission, thus affecting parasite species abundance and composition. As such, parasite populations and communities are useful indicators of environmental stress, food web structure and biodiversity.

12.4.3 Ecosystem Bioindicators

In marine coastal areas, human activities directly influence living communities, which may result in a heavier parasite infestation compared to less polluted sites (Skinner 1982). On the other hand, some parasite species can disappear, and thus reduce parasite diversity within the system (see above). While it remains difficult to find the best host–parasite system that can indicate a specific parameter change, MacKenzie (1999) proposed the use of fish parasites as an early warning system for pollution and environmental change. With the identification of particular parasite

species whose ecology is so delicately balanced, that environmental changes may lead to transmission failure, regular studies can monitor and indicate environmental change much earlier than using other methods. He suggested that infections with endoparasitic helminths tend to increase while those of ectoparasitic heminths tend to decrease with increasing levels of pollution. This would allow the use of entire parasite communities and their changing infection levels as environmental bioindicators (also see Marcogliese 2005).

To date, ecological aspects of infestations by parasites and their relationship to anthropogenic factors such as urban pollution, overfishing and fish-farming (mariculture) are only just beginning to be understood. Recent studies demonstrate, however, the relatedness of parasite abundance and the environmental condition. Sasal et al. (2007) utilized the entire parasite community (as larval Cestoda, adult Crustacea, larval and adult Digenea, larval Nematoda) of apogonid fish to detect anthropogenic influences (urban and industrial pollution) on two coral reef lagoons in New Caledonia. Pech et al. (2009) used fish physiological biomarkers and the parasite infracommunity characteristics to evaluate the effects of chemical pollutants on the fish host and in the environment. Rückert et al. (2009a) applied three different parasite parameters to describe the environmental situation in Segara Anakan lagoon, an extensive mangrove ecosystem in Indonesia, and Rückert et al. (2009b, 2010) compared free living *Epinephelus coioides* and *E. fuscoguttatus* with specimens from mariculture farms. The most striking result was reduced parasite diversity within the lagoon ecosystem compared with outside the lagoon, probably linked to the changing hydrographic conditions and not eutrophication. This would follow Hudson et al. (2006) who considered a healthy system to be one that is rich in parasite species. According to Hechinger et al. (2007) and Lafferty (2008), trematode diversity and prevalence in snails correlates with macro-invertebrate diversity and abundance. This may result from birds being attracted to areas with diverse invertebrate prey communities (particularly if the invertebrates present also serve as intermediate hosts for trematodes). By the same reasoning, trematodes in snails may indicate aspects of the fish community. Lafferty (1997) and Marcogliese (2005) argued that helminth infracommunity patterns are useful tools to assess the impact of anthropogenic pollution on ecosystems. Among others, Oros and Hanzelová (2009) demonstrated the re-establishment of the fish parasite fauna in a Slovakian river system after a catastrophic pollution event. Beside total abundance and prevalence of specific parasite species or their life cycle stages, parasite metrics (Diamant et al. 1999) such as the Shannon-Wiener diversity index as calculated for the endoparasites and the ratio of ecto- vs. endoparasites are suitable measures to compare the parasite community in different aquatic systems (Rückert et al. 2009a).

Palm and Rückert (2009) developed a new method to visualize environmental differences between habitats, among them a grouper mariculture facility in the Thousand Islands, Indonesia. A star graph was used to visualize the parasite composition for the different fishes, using (1) the prevalence of trichodinid ciliates, (2) the ecto-/endoparasite ratio and (3) the endoparasite diversity as bioindicators. This was found a suitable method to visualize and monitor environmental health under high parasite biodiversity conditions in tropical ecosystems, and to better

communicate the scientific results to involved stake holders and decision makers. Palm et al. (2011) studied fish from floating net cages of a commercially run mariculture facility after its opening in 2001 for six consecutive years. Tetraphyllidean larvae *Scolex pleuronectis* (Fig. 12.2c) and the nematodes *Terranova* sp. and *Raphidascaris* sp. 1 were highly abundant in 2003/04–2005/06 (max. prevalence *S. pleuronectis* 40%, *Terranova* sp. 57%, *Raphidascaris* sp. 1 100%, Fig. 12.4a), and drastically reduced subsequently (up until 2008/09) (Fig. 12.4b). These parasites, especially the nematodes (e.g. Fig. 12.2d, e), together with the above parasite metrics illustrate a significant change in holding conditions over the years. This can be either referred to a definite change in management methods (such as feed use and fish treatment) or more likely to a transition of a relatively undisturbed marine environment into a more effected habitat caused by the anthropogenic activities.

12.5 Fish Parasites as Bioindicators for Climate Change

Global climate change with rising sea-levels and water temperatures that may result in changes in (e.g.) ocean circulation and a decrease in salinity may also cause measurable effects on fish parasite composition and biogeography. According to Harvell et al. (2002), climate warming can affect host–pathogen interactions by (i) increasing pathogen development rates, transmission and number of generation times per year (ii) raising the overwinter survival rate of the pathogen and (iii) increasing the host susceptibility to thermal stressors. More importantly, increasing temperatures alter the seasonality and biogeographical range of many species, including the hosts and the parasites. Consequently, with the potential to indicate environmental change and host migration or pollution, fish parasites as an important component of the aquatic biodiversity may well be useful as bioindicators of climate change. This application must utilize fish parasites that are widely distributed, easily monitored (Table 12.1), and can indicate long-term changes within the aquatic environment, being related either to natural or anthropogenic influenced environmental conditions.

MacKenzie (1987) recorded changes in the prevalence of metazoan parasites, the trypanorhynch cestodes *Grillotia angeli* Dollfus, 1969 in mackerel (*Scomber scombrus*) and *Lacistorhynchus* sp. in herring (*Clupea harengus*) over the periods of 8 years (1978–1985) and 11 years (1974–1984), respectively (e.g. Fig. 12.1b). Both data sets showed sharp decreases in parasite prevalence from periods at relatively high levels to others at much lower levels. The changes in prevalence occurred at the same time in both host–parasite systems and coincided with the end of a hydrographic phenomenon known as the mid-70s salinity anomaly, with a salinity reduction in the upper 1,000 m of water over most parts of the eastern Atlantic. The author explained these results by invoking changes in the abundance of first intermediate and definitive hosts, host diet and variations in year class strength, and changes in hydrographic conditions. Rising sea-water temperatures and a possible change in currents and resulting hydrography must have consequences

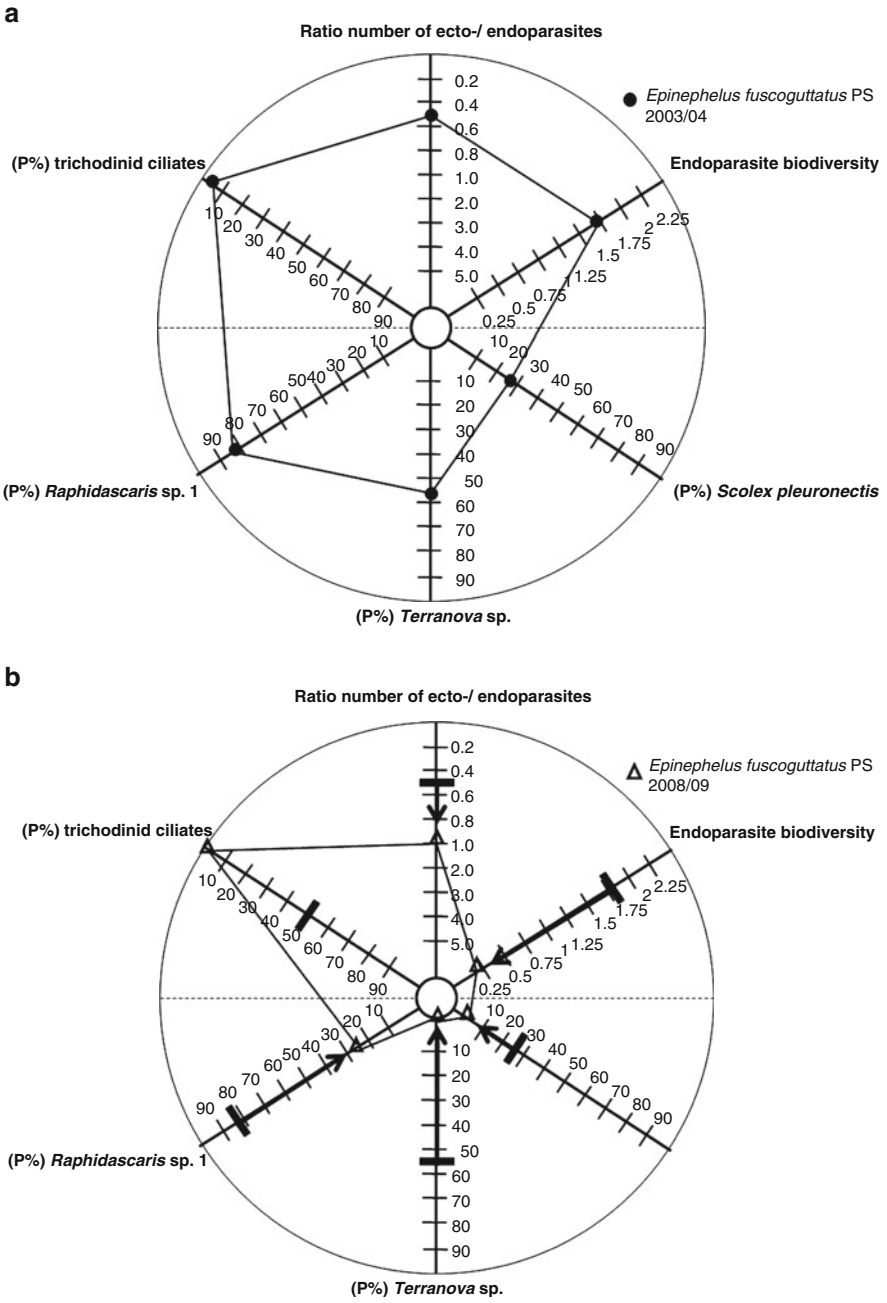

Fig. 12.4 (**a-b**) Stargraph with parasitological metrics to monitor cultured groupers (*Epinephelus fuscoguttatus*) from a mariculture farm. The parasite infection of the sampled groupers from the same net cages in the rainy seasons 2003/4 and 2008/9 was significantly different (note the change with *black arrows*), indicating different holding conditions, feed use or environmental change (see Palm et al. 2011)

for parasite composition and abundance. Poulin (2007) emphasized the importance of latitude as a convenient surrogate measure for temperature. As a rule, fish parasite diversity increases from higher latitudes towards the tropics. Among marine fish, the mean species richness of ectoparasites per fish population sampled increases with increasing water temperature towards lower latitudes, a pattern that is not detectable for endoparasitic helminths. Among freshwater fish species, species richness of parasites seems to peak at temperate latitudes, and not in the tropics. However, as stated by Vidal-Martínez et al. (2010), the exact circumstances under which parasites can be used as indicators of environmental impact or change remain difficult, especially at the large scale of consideration. In coastal regions with a variety of different underlying factors such as river-based pollution, salinity change, industrial impact, sewage and other anthropogenic waste, or in intensive fisheries, climate change-related abnormalities in the fish parasite fauna will be difficult to assess. Consequently, such studies should be carried out in remote environments such as the Southern or Arctic Oceans, the open oceanic waters or the deep-sea, where recorded effects can be better linked to a change of the environment at a larger scale and not to local variation.

There is no doubt that climate is decisive for a variety of ecological processes, concerning the individual, populations and species biogeography and composition (e.g. Mouritsen and Poulin 2002; Mouritsen et al. 2005). Cattadori et al. (2005) concluded that specific climatic events may lead to outbreaks of infectious diseases or pests that may cause dramatic, synchronized changes in the abundance of their hosts. The size of red grouse populations in northern England either increased or decreased in synchrony to correlated climatic conditions during May and July, influencing the density-dependent transmission of the gastrointestinal nematode *Trichostrongylus tenuis*, a parasite that reduces grouse fecundity. This in turn forced both, the grouse and the parasite populations into synchrony. Temperature is a key factor that regulates such processes, determining species distribution and occurrence also in aquatic habitats. Because freshwater and marine environments are generally different in terms of their size, stability and physical conditions, these systems must be treated differently. While the observation of climate change-related scenarios in the large marine ecosystems may be possible (see above) and should be dealt with in future studies, it has to be questioned how small-scale parasitological data from freshwater environments can be unequivocally related to climate change by temperature increase. For example, Esch et al. (1976) related epizootic outbreaks of the facultative episymbiont *Epistylis* sp. and *Aeromonas hydrophila* in centrarchid fish in some freshwater reservoirs to a combination of eutrophication and rise of temperature. However, Kennedy et al. (2001) demonstrated that the larval cestode *Ligula intestinalis* (L., 1758) together with the roach *Rutilus rutilus* regulated the size of the other fish populations inhabiting that lake. Thus, neither temperature nor other environmental factors but this parasite–host system consisting of two generalistic, widely distributed species was the driving factor for the fish and parasite population structure within the entire ecosystem. It must be kept in mind that according to Bagge et al. (2004), the total fish population size of the crucian carp *Carassius carassius* alone directly influenced the mean number of parasite (monogenean) species per fish and the mean total abundance.

Other factors such as the fish population density, mean fish length per pond, number of fish examined per pond, distance to the nearest lake, and several water quality measures did not influence the monogenean infection within that system.

12.6 Conclusions

Fish parasites can elucidate problems in a variety of applications. They can be used as biological indicators for their hosts, accumulation indicators to detect pollutants, impact indicators to describe the relationship between for example pollution or eutrophication and the parasite, and finally as systemic indicators that provide information on the health status of the environment. A proper selection of the parasites utilized and their specific life cycle stages is a prerequisite for all these applications, and requires careful planning and some background information on the parasite diversity within each habitat. Sixteen different criteria have been formulated to support selection of the best parasite indicator species for any particular purpose (Table 12.1).

The published literature demonstrates that parasites can be successfully used as biological indicators in both freshwater and marine environments. Protozoan and metazoan parasites likewise are useful, though they react differently according to their needs and characteristic life cycle biology. Trichodinid ciliates react positively to environmental pollutants while most metazoan parasites with their indirect life cycles, requiring the availability of invertebrate intermediate hosts, are negatively affected (Table 12.2). The widest range of applications has been demonstrated for the heteroxenous digeneans, cestodes, nematodes and acanthocephalans that can serve as biological, accumulation and impact indicators. Though several open questions still remain, fish parasites are ready to be used as indicator organisms for environmental change, and should be included in regular monitoring programs that will provide long-term data sets. In combination with other indicator measures, fish parasites will enable a better estimate of the causes and consequences that underlie environmental change in aquatic habitats.

While there can be no doubt that particular fish parasite species and metrics describe the environmental conditions in the aquatic realm (impact/ecosystem bioindicators), the use of fish parasites as biological indicators of climate change is more unclear. Distinction must be made between freshwater, coastal and oceanic environments, with systemic differences in biodiversity, stability and already existing anthropogenic influences. There is practically no freshwater habitat under pristine conditions (e.g. most of anthropogenic origin in the state North Rhine-Westfalia), and anthropogenic species introduction, migrations of neozoons connected to fisheries and habitat change combined with natural migration events already altered the regular fish and parasite fauna. Many freshwater parasite species are opportunistic generalists that can infect a variety of different fish hosts, use reservoir habitats, and underlie small-scale variability according to the ecological conditions in the freshwater systems. This can explain the conflicting evidence

provided by Kennedy (1997) who considered eel parasites in a freshwater environment to be useful to explain observed environmental change. However, he stated that in his case the helminth communities provide no clear indication of the nature of these changes. This contrasted with his results in an earlier study (Kennedy et al. 1994). A change in species diversity and composition at a sampling site does not necessarily result from slight temperature increase over a long time period, but merely from a shift in intermediate and final host abundances at a small scale. The monogenean species richness and abundance in a freshwater lake system was directly dependent on the total fish population size (Bagge et al. 2004). Consequently it will be difficult to directly relate the observed parasite infection levels in freshwater ecosystems to changing parameters at a large scale such as under climate change, and not to local factors or naturally/anthropogenic caused species invasion events.

With high population density along the coasts and significant anthropogenic stress in terms of pollution and environmental degradation, fish parasites can describe the environmental conditions of their habitat, such as a tropical lagoon ecosystem (e.g. Rückert et al. 2009a). However, like in freshwater habitats, it will be difficult to relate parasite abundances with effects that are due to climate change and not to local factors. Marcogliese (2008) stated that though much evidence suggests that parasite and disease transmission, and possibly virulence, will increase with global warming, these effects will be superimposed on a multitude of other anthropogenic environmental changes. On the other hand, Poulin and Mouritsen (2006) developed a model to predict that rising sea surface temperatures in the Wadden Sea will lead to regular local extinction events in amphipods, affecting the broader intertidal system. Lafferty and Kuris (2005) argued that aquatic helminths vary in their optimal temperature, making it impossible to make a general prediction about the effect of warming. Single parasite species models as predictors of possible multidimensional environmental change scenarios, considering entire communities are still to be tested within the aquatic habitat.

Finally, oceanic and remote marine ecosystems such as the central Pacific, mid Atlantic, Antarctic or the Polar Sea are the best candidate localities to link an effect of a changing climate directly to aquatic parasite communities at a larger scale. These regions are less affected by factors such as anthropogenic-influenced species introduction, pollutants and for example seasonal migrations. Such studies are entirely missing, and long-term parasitological monitoring programs in these regions have not been done. Melting sea ice and increasing ice-free areas in the Southern and Arctic Oceans open up new habitats that can be newly explored for existing parasite populations. Poulin (2007) concluded that more emphasis should be placed on the geographical scale in studies of parasite communities of fish, and a little less on local scales, to assess spatial variation on a level relevant for the sort of changes ahead. It is suggested that such data sets should be collected in future studies, in order to assess the use of fish parasites as bioindicators for long-term or global climate change.

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Part III
Vector Transmitted Diseases

Chapter 13
Infectious Diseases Under the Influence of Changing Environmental Factors

Franz J. Conraths and Thomas C. Mettenleiter

Abstract Changing environmental conditions are not only the result of the direct impact of climate change, but represent also the consequences of migration, urbanisation and the globalisation of trade and human mobility. In consequence, changes in the occurrence of infectious diseases in humans and animals ensue. Several infectious diseases which were hitherto considered "exotic" in Europe have largely lost this feature and can now occur nearly everywhere. These include a number of arthropod-borne diseases such as Bluetongue, which mainly affects ruminants, West Nile Fever in humans, horses and birds, as well as Chikungunya Fever in humans. There is a trend towards a global spread of Dengue Fever and Japanese Encephalitis, associated with extension of the habitats of the respective arthropod vectors. In addition, transportation of animals and products of animal origin has caused the spread of animal diseases, notably of Rift Valley Fever from Africa to the Arabic peninsula and of African Swine Fever from East Africa into the Caucasus region where it shows a clear tendency of spreading in a northerly and westerly direction. Therefore, we propose to stop using the term "exotic" for these diseases, because infections that are today considered "exotic", may tomorrow be part of our daily life.

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13.1 Introduction

Humans and animals have always been exposed to infectious diseases. Environmental factors and anthropogenic activities have influenced their distribution in the past and continue to do so. Areas in southern Europe, for example, were endemic for **malaria** and **yellow fever** until the early twentieth century. Animal diseases, which are today considered "exotic", such as **Rinderpest**, now close to global extinction, led two centuries ago to the introduction of veterinary measures which are still valid today, like veterinary certificates and movement restrictions. Because of the implementation of these regulations and further measures including herd hygiene and immune prophylaxis, control of infectious animal diseases improved. However, the debate over future **influenza pandemics**, irrespective of their cause (H5N1, "bird flu" or H1N1, "swine flu" or any other influenza virus), clearly illustrates the threat which infectious diseases still pose today. Two-thirds of the infectious diseases that emerged in the past 20 years had their origin in animals, i.e. represent **zoonotic infections** (Jones et al. 2008). Obviously, also the "new" pandemic influenza virus A/H1N1 originated in an animal host reservoir. However, the role of wildlife as a reservoir for particular pathogens is often largely unknown and the risk of transmission of the infections into the human population difficult to assess.

Increasing urbanisation in the absence of appropriate infrastructure, inadequate medical care and veterinary surveillance in particular in crisis regions, flight, migration and the progressive destruction of habitats lead to alterations in the exposure of humans and animals to several pathogens (Woodhouse 2008). Globalisation, including world-wide trade and travel, facilitates the introduction of pathogens from remote countries over large distances within short periods of time (Mettenleiter and Böhle 2008).

Changing environmental factors also modulate the exposure to pathogens. The increasing number of cases of tick-born encephalitis in Germany until 2007 can be attributed equally to the expansion of tick vector habitats, to an increase in tick activity due to climate change and to the altered leisure behaviour of humans, in particular outdoor activities. The outbreaks of infections with highly pathogenic avian influenza virus of the subtype H5N1, which was first described in Hong Kong in 1997, in Europe in spring 2006, showed how fast "exotic" infections can spread and establish themselves under favourable conditions. This also applies to **Bluetongue disease**, which was originally endemic in Africa, but occurred in central Europe for the first time in history in August 2006, and was rapidly transmitted by indigenous *Culicoides* spp. (Mehlhorn et al. 2007; Hoffmann et al. 2009). In the following, several examples of the recent past are presented.

13.2 Bluetongue Disease

Bluetongue is mainly a disease of ruminants, but affects also camelids. It is caused by an **orbivirus** (bluetongue virus [BTV], family Reoviridae), which is transmitted by biting midges of the genus *Culicoides*. At least 24 serotypes of BTV have been

described. Bluetongue has spread along with its main "African" vector *Culicoides imicola* into the European part of the Mediterranean basin since the mid 1990s, possibly due to the expansion of the habitat range of *C. imicola* caused by long-term climatic changes (Purse et al. 2005). Nevertheless, the occurrence of BTV in Belgium, Germany and the Netherlands in August 2006 was unexpected. Not only had Bluetongue never before occurred in central Europe, but the serotype 8, which had caused the infections, had never been detected in Europe at all. *C. imicola,* the prime vector of Bluetongue in Africa and the Mediterranean, was not found in Central Europe despite intensive monitoring. This suggested that indigenous biting midges were capable of transmitting BTV-8 effectively. Comprehensive entomological studies showed that biting midges with a Palaearctic distribution, in particular members of the *C. obsoletus* complex, represent competent vectors for BTV-8 (Hoffmann et al. 2009). It remains unclear, however, whether the extreme weather conditions observed in summer 2007, when temperatures exceeded the long-term averages considerably, had an essential effect on establishment and spread of the disease in a region of temperate climate. The available data suggest, however, that increased temperatures may have triggered the Bluetongue epidemic after initial introduction of BTV, and led to a lasting, perhaps irreversible, establishment of the disease in central Europe (Conraths et al. 2010).

The fact, that vaccines against BTV-8 became available just before or at the beginning of the Bluetongue transmission season in 2008, reduced the incidence and the economic losses caused by the disease considerably (Conraths et al. 2008). However, a few infections with BTV serotypes 6 and 11 were also detected, possibly due to illegal use of life vaccines. By contrast, the northward spread of BTV-1 from southern France to regions bordering Germany raised concern that this serotype might also establish in Germany. In conclusion, it has become obvious that a disease which had never occurred in central Europe until 2006 seemed suddenly to be in the process of establishing itself permanently in this region.

13.3 African Horse Sickness

African Horse Sickness (AHS) is transmitted in Africa by the same vectors as Bluetongue. AHS virus, of which nine different serotypes are known, is a close relative of BTV. Zebras represent the prime reservoir for AHS. Normally, infections in zebras remain asymptomatic or subclinical, or lead to only mild symptoms. By contrast, horses develop a severe, acute or peracute disease associated with high mortality. Historically, AHS has been endemic mainly in central Africa from where it spread to northern Africa and Europe. At least some introductions of AHS were due to the import of infected zebras into European zoos. Fortunately, the virus has not established itself in Europe so far. It cannot be excluded, however, that biting midges transmitting BT in central Europe can also spread AHS, which could lead to an epidemic of AHS similar to the one that occurred with BTV-8. Consequently, the European Commission has established a vaccine bank for AHS for rapid use to

contain any primary AHS outbreak. It remains to be seen if this strategy is successful in the case of an emergency.

13.4 Chikungunya Fever

Chikungunya infections are caused by an alphavirus from the family **Togaviridae**. The virus was first described in East Africa in 1952 and remained confined to the African continent for a long time. Since 2005 it spread eastwards into countries around the Indian Ocean and to Indonesia. In the meantime, almost the entire territory of Southeast Asia is endemically affected (Enserink 2008). The virus is transmitted by mosquitoes of the genus *Aedes*. It is important to note that the Asian tiger mosquito or forest day mosquito, *Aedes albopictus*, has been spreading massively in recent years. It has reached Africa, America and southern Europe (Enserink 2008). Consequently, the first Chikungunya epidemic in Europe occurred in Italy in the region of Rimini-Ravenna during summer 2007. The virus had been introduced by an infected tourist from India and was spread by *A. albopictus*, which had already established itself in the region. Although there were no further cases in 2008, it cannot be excluded that the infection may re-occur in this region as the virus is transmitted transovarially. It is also possible, however, that vector control programmes, which were started immediately, could have diminished the vector population under the critical limit.

13.5 African Swine Fever

African Swine Fever (ASF) is caused by a large double-stranded DNA virus of the family **Asfarviridae**. It occurs primarily in sub-Saharan Africa, where it has its reservoir in warthogs and bush pigs. For these animals, the ASF virus is harmless, while domestic pigs are highly susceptible to ASF. Leather (soft) ticks of the genus *Ornithodoros* can transmit ASF, but the disease is also spread by direct contact. *Ornithodoros moubata*, the prime vector in south-eastern Africa (Madagascar, Mozambique, Zambia), usually lives in burrows made by the warthog, where it relies on this animal for its blood meals and can transmit ASF virus.

Until recently, the only existing focus in Europe was on the Italian Island of Sardinia. In 2007, the disease was first detected in domestic pigs in Georgia, spread rapidly in the Caucasus region and into southern Russia, where wild boar contracted the infection with the result of the potential formation of a reservoir in this wildlife species.

The virus was probably introduced via the harbour of Poti on the Black Sea by ship from East Africa, since the virus found in the Caucasus region shows the highest degree of homology with isolates from East Africa. Transmission to domestic pigs may have occurred through food waste of porcine origin deposited on

dumps outside Poti, where the contaminated material was apparently accessible to free-ranging pigs. In the Caucasus, ASF seems to be primarily transmitted by direct contact. It appears that ticks do not play a role in the transmission of the disease in this region.

Although domestic pigs and wild boar are usually highly susceptible to ASF and die with haemorrhagic symptoms, the persistence of ASF virus in the swine population cannot be excluded, as the disease situation on the island of Sardinia has shown. In the meantime, the infection has spread westwards towards the Crimean peninsula and northwards towards Siberia. In October 2009, ASF was detected close to St. Petersburg in northern Russia, approximately 2,000 km away from the region where the disease had occurred before in the Russian Federation.

Rapid eradication of the disease in these regions is unlikely, since there is no vaccine against ASF, and only the systematic culling of potentially infected animals remains to control the disease. However, due to the rural farming conditions in the Caucasus, a systematic culling strategy is difficult to implement, with the consequence that a new endemic focus of ASF may have been established in this region.

13.6 West Nile Fever

The family **Flaviviridae** contains a number of viruses causing important diseases in humans or animals, such as **Yellow Fever** and **Dengue** as human infections, and zoonotic viruses, causing for example **Japanese Encephalitis**, **Tick-borne encephalitis** and **West Nile Fever** (WNF). West Nile Fever virus (WNV) was first detected in the West Nile district of Uganda, Africa, in 1937. WNV is an **arbovirus** ('**ar**thropod-**bo**rne') as it is transmitted by blood-feeding arthropods. It has long been present in large areas of Asia, Eastern Europe, Africa and Australia, but gained centre stage in 1999, when it suddenly occurred in New York, USA, causing widespread deaths among corvids, in particular American crows (*Corvus brachyrhynchos*), and birds of prey as well as infections in humans and horses, some of which were lethal. In the meantime, the virus has spread over the entire North-American continent and is continuing to expand into South America. In Europe, cases of WNF were recorded in birds, horses and humans in France, Romania, Hungary and Italy in the past decade. Infections detected in wild birds (mainly raptors) in Austria in 2008 may suggest that the virus is spreading northwards.

Wild birds represent the natural reservoir of the virus which is mainly transmitted by mosquitoes of the genus *Culex*. Several *Culex* spp. occurring in central Europe, such as *C. pipiens*, may be capable of transmitting **WNV**. Infections in wild birds normally remain asymptomatic, although some bird species, for instance crows and some birds of prey, can develop severe disease and die in large numbers upon infection with **WNV**. Horses and humans represent dead-end hosts. They become infected by mosquitoes which blood-feed on birds and mammals. The spread of WNF via Hungary to Western Austria suggests that WNV may also invade Germany. While a vaccine for horses has been registered in Europe, vaccines for

humans are not available yet. Thus, WNF may have become established as another arbovirus infection in Europe. However, our knowledge on the factors that determine the spread of WNF in Europe is still sparse.

13.7 Conclusions

Alterations in environmental conditions such as climate change, globalisation in trade and human mobility, urbanisation on the one hand, and increasing contact with wildlife on the other hand have a major impact on the spread of infectious diseases. The term "exotic disease" has therefore become inappropriate as virtually every disease can be rapidly introduced from anywhere on the globe into our region. To improve risk assessments, a better knowledge of the impact of the different parameters mentioned above on the spatial distribution of infectious agents and their vectors, where applicable, is required. Our understanding of the reservoir function of wildlife for various pathogens needs to be improved with research focussing on the biology and epidemiology of infectious agents and their vectors, including the causative agents of those diseases which have so far been regarded as "exotic". It is necessary to establish science-based prevention and control strategies, preferably prior to the arrival of the respective pathogens and their vectors in our region.

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Chapter 14
Arthropod Vectors and Their Growing Importance in Europe

Helge Kampen and Doreen Werner

Abstract After the eradication of malaria in the middle of the past century, Europe felt relatively safe from vector-borne diseases due to the availability of effective insecticides and progress in medical diagnostics and treatment. In fact, except for Lyme disease, no vector-borne disease of cross-national distribution and far-reaching epidemiological relevance has been registered in Europe for decades. Nevertheless, not only have microorganisms with pathogenic potential increasingly been found to circulate among haematophagous arthropod populations but indigenous arthropods have also been demonstrated to possess vector competence for one pathogen or the other. Also, single cases and even localized outbreaks of mosquito-borne and further tick-borne diseases have occurred. But only in the last 10–20 years or so, probably as a consequence of ecological and climatic changes as well as of continuing globalization, some alarming vector- and vector-borne disease-related developments have taken place in Europe: the establishment and spread of invasive arthropod-vectors of disease, the importation of vector-associated pathogens, and outbreaks of emerging vector-borne diseases. The most striking examples of these incidents are the bluetongue disease epidemic in central Europe which started in 2006, and the chikungunya fever outbreak in Italy in 2007. In the first case, indigenous biting midges that had previously not been known to be vector-competent served as bluetongue virus vectors, while in the second case a human traveller carrying chikungunya virus infected vector-competent *Aedes albopictus* mosquitoes that had established in Italy some years before and had been spreading since then. In this contribution, major trends of the recent past concerning arthropod-vectors and the disease agents they transmit in Europe are

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reviewed. Because of their growing scientific importance and increasing public attention, the focus will be on mosquito, tick, sandfly and biting midge vectors.

14.1 Introduction

In the last half century or so, emerging infectious diseases have been on the rise globally, driven by socio-economic, environmental, ecological and behavioural factors. Zoonoses contribute significantly to them, and among these, vector-borne diseases play an important role (Jones et al. 2008). This is not only true for tropical and subtropical regions of the world but also for temperate and colder zones including the majority of the European territory. So, when discussing the growing importance of vector arthropods in Europe, it is necessary to mention changes in climate, in environment, and in human behaviour. Socio-economic factors may cause problems regionally, but are not relevant on a general European scale.

According to a report from the European Environmental Agency, European mean temperature has increased by about 1.2°C over the past 100 years. Global mean temperatures are projected to increase by 1.4–5.8°C until 2100, with larger increases in eastern and southern Europe (EAA 2003). Climate warming will have many effects on most arthropod vectors, such as expansion of distribution areas, acceleration of life cycles and reproduction rates, increase in biting frequencies and population sizes, and extension of seasonal activities. Several of these factors contribute to the vectorial capacities of potential vectors so that not only the nuisance caused by haematophagous arthropods but also the risk of acquiring a vector-borne disease after an arthropod's bloodmeal will be increased under a rising temperature scenario. Changes in environment may be climate-driven or man-made and include deforestation, construction of irrigation schemes, land fragmentation, loss of biodiversity and introduction of new species, just to mention some important elements. Environmental changes produce new habitats and improved living conditions for vector arthropods in many cases, and may facilitate the spread of present vectors or the establishment of new ones. Renaturation of river systems or rice cultivation, for example, are usually beneficial to arthropods with aquatic developmental stages such as mosquitoes, as new breeding sites are usually generated (e.g. Ponçon et al. 2007). The probability of contact with blood-feeding arthropods is, at least in part, dependent on human behaviour. An obvious modern change in human behaviour is that people increasingly spend their leisure time in natural environments where they are much more exposed to blood-feeding arthropods, such as ticks and mosquitoes. Enforcing this trend, urban sprawl is taking place with more and more people settling in those natural habitats (Bonnefoy et al. 2008). Most crucial for the introduction of new vector species and new vector-borne pathogens, however, is probably globalization. Technical progress in transportation and increasing global travel and trade in the past three decades have paved the way for outbreaks of vector-borne diseases in non-endemic regions of the world. During the last 50 years, air travel passenger numbers have grown by nearly 9%

annually, and shipping traffic has increased by some 27% since 1993 (Tatem et al. 2006a). Along with the passengers, animals and goods come the vectors and pathogens.

14.2 Mosquitoes (Diptera, Culicidae)

There are roughly 100 mosquito species in Europe including former malaria vectors, efficient virus vectors and vectors of filarial worms. While malaria is unlikely to become a health problem in Europe again in the foreseeable future, viral and filarial vectors seem to be spreading, followed by the pathogens.

14.2.1 Malaria

Endemic malaria was completely eradicated from Europe in the last century due to the comprehensive control programmes that had been implemented after the development of new and efficient insecticides and drugs. According to the WHO, the last autochthonous case of malaria was reported from Greek Macedonia in the early 1970s (Bruce-Chwatt and de Zulueta 1980). Mosquito populations have recovered since then, and several former malaria vectors, such as *Anopheles sacharovi, An. atroparvus* and *An. labranchiae*, are nowadays still widely distributed in Europe. Locally acquired cases of malaria therefore have continued to occur from time to time in various European countries (Sartori et al. 1989; Nikolaeva 1996; Baldari et al. 1998; Krüger et al. 2001; Cuadros et al. 2002; Kampen et al. 2002; Zoller et al. 2009). In view of the increasing travel activities to and from tropical regions where malaria is endemic and a growing number of *Plasmodium*-infected people entering Europe who may serve as infection sources for indigenous *Anopheles* mosquitoes, the number and frequency of autochthonous cases of malaria must be expected to rise in the future (Kampen and Maier 2008). A recurrence of endemic malaria, however, is rather unlikely, given the high standard of medical care in Europe.

Increasing air traffic might also lead to a rise in the frequency of inadvertent transport and importation of alien mosquitoes including *Plasmodium*-positive *Anopheles* (Karch et al. 2001; Tatem et al. 2006b). Being released at the place of destination, they may bite humans and cause cases of "airport malaria" or "baggage malaria" (Isaäcson 1989; Castelli et al. 1993; Thang et al. 2002).

14.2.2 Mosquito-Borne Viral Infections

Ten mosquito-borne viruses have been found circulating in Europe during recent decades. At least three of them are endemic and have pathogenic potential: West Nile virus, Sindbis virus and Ťahyňa virus (Hubálek 2008).

West Nile virus (WNV), a virus of the family Flaviridae and a member of the Japanese encephalitis virus complex, is generally considered an emerging pathogen although it was first observed in Europe in the early 1960s in the French Camargue (Panthier 1968). From then on, epidemics were noticed every now and then in the Mediterranean area, but intervals were long and disease symptoms generally mild. It was only in the 1990s that West Nile fever (WNF) became more and more associated with severe and neurological fatal disease, probably due to new and more aggressive virus strains entering Europe (Gubler 2007). Migratory birds are reservoir hosts to the virus, and these are responsible for spreading it over long distances and even transcontinentally. The virus is vectored by numerous mosquito species of several genera, most importantly *Culex pipiens*, that are indiscriminant between feeding on birds and on mammals (Reiter 2010). Humans and horses are especially susceptible to infections, but high numbers of dead crows may be a first indication of local virus transmission. Major WNF outbreaks in the Mediterranean basin during the past 15 years occurred in Algeria in 1994 (Le Guenno et al. 1996), in Romania in 1996 (Tsai et al. 1998), Tunisia in 1997 (Feki et al. 2005), Russia in 1999 (Platonov et al. 2001), Israel in 2000 (Green et al. 2005), France in 2000 (Murgue et al. 2001), and Italy in 2002 (Autorino et al. 2002). More recent European episodes including fatal human cases occurred in Italy in 2008 and 2009 (Macini et al. 2008; Barzon et al. 2009; Rizzo et al. 2009; Angelini et al. 2010).

Although in more northern European countries no cases of WNF have been diagnosed so far, there is serological evidence of viral infection in resident birds, for example in Great Britain and Germany (Buckley et al. 2006; Linke et al. 2007). It has been suggested that indigenous birds may be natural moderators of viral circulation as their immune system is able to cope with the infection (Pauli 2004; Reiter 2010). In North America, however, where a relatively virulent WNV strain encountered a naïve bird population in 1999, the virus was able to spread over the whole continent within a few years, with tremendous morbidities and mortalities among birds, horses and humans (Kramer et al. 2008).

Sindbis virus (SINV), a member of the western equine encephalomyelitis virus complex belonging to the family Togaviridae, is another mosquito-borne virus that is reservoired by birds. Among bird populations, it is transmitted by ornithiphilic *Culex* and *Culiseta* species whereas the bridge vector *Aedes cinereus* is mainly responsible for transmission to humans (Lundström 1999). The disease SINV causes comprises headache, myalgia, arthralgia, malaise, conjunctivitis, pharyngitis and rash. It has been named "Ockelbo disease" in Sweden, "Pogosto disease" in Finland and "Karelian fever" in north-western Russia. In Scandinavia (Sweden and Finland), thousands of human clinical cases were reported during the past decades but the virus has also been found to occur in eastern and southern Europe (Lundström 1999; Brummer-Korvenkontio et al. 2002). In Great Britain, seroconversion against SINV was demonstrated in sentinel chickens (Buckley et al. 2006) and, most recently, the virus was isolated from mosquitoes in Germany (Jöst et al. 2010).

Ťahyňa virus (TAHV) belongs to the family Bunyaviridae and is a member of the California encephalitis virus complex. It has been reported from many parts of Europe (Lundström 1999; Hubálek 2008) where it is transmitted by numerous

mosquito species, predominantly *Ae. vexans*. Principal vertebrate hosts are lagomorphs, rodents and insectivores (Medlock et al. 2007, Hubálek 2008). Infections usually cause febrile illnesses in humans, sometimes accompanied by CNS involvement and bronchopneumonia.

In 2001, **Usutu virus** (USUV; Flaviviridae, Japanese encephalitis group) was detected for the first time in Europe when examining uncommonly high numbers of dead blackbirds in Austria (Weissenböck et al. 2002). Later, the virus was also found in Hungary (Bakonyi et al. 2007) and Spain (Busquets et al. 2008) while antibodies could be demonstrated in birds from Great Britain, Switzerland and Italy (Buckley et al. 2006; Weissenböck et al. 2007). USUV originates from Africa where it is transmitted mainly by *Culex* spec. among birds. Despite two human cases of neuroinvasive infection in immunocompromized patients in Italy (Cavrini et al. 2009; Pecorari et al. 2009), there is still some debate on its human pathogenic potential.

Although **Rift Valley fever virus** (RVFV; Bunyaviridae) has not yet appeared in Europe, the increasingly frequent occurrence of infections in endemic areas and its geographic expansion are being carefully observed by European epidemiologists. Originally, Rift Valley fever (RVF) was restricted to eastern and southern Africa but, since several outbreaks were reported from Egypt and the Arabian peninsula (Ahmad 2000; Gerdes 2004), a possible further spread and a leap into southern Europe is a matter for concern (Martin et al. 2008; Chevalier et al. 2010).

RVF is a serious disease of domestic animals and humans. Epidemics are often characterized by sudden abortion storms and close to 100% mortality in newborns. In older humans infection causes an influenza-like illness (Abdel-Wahab et al. 1978). A large range of mosquito species is capable of transmitting the virus, and historically outbreaks have often been associated with heavy rainfall, floods and the construction of dams. Some of the African vector species (Turell et al. 2008) also occur in Europe, where several of them are thought to be potential vectors or have been demonstrated to be susceptible to RVFV (Moutailler et al. 2008; Chevalier et al. 2010). A peculiarity with RVFV is that certain *Aedes* vector species in some African regions are at the same time the viral reservoirs, as the virus is transmitted vertically from one mosquito generation via the eggs to the larvae of the next generation (Gerdes 2004). Thus, vertebrate reservoir hosts are not necessary for the maintenance of the natural cycle, and the virus can remain endemic for a while without appearing in vertebrates (Linthicum et al. 1985).

14.2.3 Dirofilaria spec.

In the Mediterranean, various mosquito species of the genera *Culex*, *Aedes* and *Anopheles* (Pampiglione et al. 1995; Cancrini et al. 2003) also transmit the filarial worms *Dirofilaria immitis*, the etiological agent of heartworm disease, and *D. repens*, which parasitizes the subcutaneous tissue. Their natural hosts are canines and sometimes felines, but human infections with *D. repens* have lately been on the

rise in France, Greece, Spain and Italy (Pampiglione et al. 1995; Muro et al. 1999; Pampiglione and Rivasi 2000; Simón et al. 2005). Also, the first cases of autochthonous infections in dogs have been reported from The Netherlands and Germany (Hermosilla et al. 2006; Overgaauw and Van Dijk 2009). In humans, an infection with *D. repens* may lead to subcutaneous and pulmonary nodules or parenchymal lesions. Climatic change, the spread of possible vectors, and increased movement of cats and dogs across Europe are supposed to be responsible for a continuing expansion of the geographic range of *Dirofilaria* parasites (Genchi et al. 2005, 2009).

14.2.4 Invasive Mosquitoes

Although there are numerous indigenous mosquito species in Europe that may become vectors of endemic or imported pathogens under certain circumstances, there are some mosquito species of tropical or subtropical origin that have been shown to be much more efficient in transmitting. Unfortunately, several of those have recently invaded Europe and some of them have even succeeded in establishing and spreading. The most important one undoubtedly is the Asian tiger mosquito ***Aedes albopictus***, which was introduced into southern Europe at least twice in the late 1970s and in the late 1980s through the used tyre trade (Sabatini et al. 1990; Adhami and Reiter 1998). During the second introduction, a mosquito strain was imported from the USA that was adapted to moderate climates and, in contrast to Asian *Ae. albopictus* strains, is now able to hibernate in southern Europe by egg diapausing. It successfully established populations in Italy and has started spreading northwards (Knudsen et al. 1996; Romi and Majori 2008). *Aedes albopictus* was also reported from France and Belgium in 2000 (Schaffner and Karch 2000; Schaffner et al. 2004), Serbia/Montenegro in 2001 (Petrić et al. 2001), Switzerland in 2003 (Flacio et al. 2004), Greece in 2003 (Samanidou-Voyadjoglou et al. 2005), Croatia in 2004 (Klobučar et al. 2006), Spain in 2004 (Aranda et al. 2006), The Netherlands in 2005 (Scholte et al. 2007), Germany in 2007 (Pluskota et al. 2008) and Malta in 2009 (Gatt et al. 2009). It had been imported into most places via the tyre trade (cf. Reiter 1998), but introductions via the lucky bamboo trade to greenhouses and via truck traffic also play a role (Flacio et al. 2004; Scholte et al. 2008). Fortunately, established outdoor populations of *Ae. albopictus* do not appear to exist at present in those more northern latitudes, but a further northward spread combined with an ecological adaptation cannot be excluded. *Aedes albopictus* is an efficient vector of at least 22 arboviruses including Dengue and yellow fever viruses (Mitchell 1995; Gratz 2004). In 2007, it was responsible for a chikungunya fever outbreak in northern Italy with more than 200 human clinical cases (Angelini et al. 2008). It was found later that a visitor from India who was unknowingly carrying chikungunya virus must have been the infection source to the local *Ae. albopictus* mosquitoes (Rezza et al. 2007).

In addition to *Ae. albopictus*, there have been further invasions of exotic mosquito species into Europe. The Asian rock pool mosquito ***Aedes japonicus japonicus***,

an efficient vector of WNV and Japanese encephalitis virus, was found in France in 2000 (Schaffner et al. 2003), in Belgium in 2002 (Versteirt et al. 2009) and in northern Switzerland/southern Germany in 2009 (Schaffner et al. 2009).

Aedes atropalpus, a North American vector of WNV, was demonstrated in Italy in 1996 (Romi et al. 1997), in France in 2003 and 2005 (Adege-EID Méditerranée 2003, 2006), and in The Netherlands in 2009 (Scholte et al. 2009). It could, however, be completely eliminated by the control measures directed against *Ae. albopictus*.

Another mosquito species that is vector-competent to several arboviruses is the yellow fever mosquito, ***Aedes aegypti***, which was once widely distributed in subtropical southern Europe. It caused yellow fever outbreaks in the 18th and 19th centuries (Eritja et al. 2005), and a major dengue fever outbreak in the 1920s in Greece (Cardamatis 1929; Rosen 1986). It is not clear why it disappeared from Europe, but as it reappeared on the island of Madeira in 2004 (Almeida et al. 2007) and, although not European, on the Georgian Black Sea coast (Iunicheva et al. 2008), it is not unlikely that it will also reconquer the southern European continental areas, supported by climate warming.

14.3 Ticks (Acari, Ixodidae)

Next to mosquitoes, ticks are the most important group of arthropods that are agents of pathogen transmission. According to Süss and Schrader (2004) and Süss et al. (2004), there are 31 tick species in Europe from which viruses, bacteria and parasites have been isolated. Most of the pathogens are not very common but some are widely distributed, may be serious threats to health, or are just emerging, such as the *Borrelia burgdorferi* sensu lato complex, tick-borne encephalitis virus (TBEV), or *Babesia* spec., *Anaplasma phagocytophilum* and *Rickettsia* spec.

14.3.1 Spread of Ticks

Not only do several tick-borne pathogens seem to be emerging and spreading, but their vector ticks are spreading too (e.g. Hartelt et al. 2008; and chap. 16). The most common tick species in Europe is the hard tick ***Ixodes ricinus***, the main vector of *B. burgdorferi* sensu lato and TBEV. With few exceptions (e.g. regions of Scandinavia), this tick species is distributed over much of Europe and beyond (Gern 2005). In areas of central and northern Sweden, increasing population densities and a northwards distribution shift were observed between the 1980s and the 1990s (Tälleklint and Jaenson 1998) which has been attributed to climate change (Lindgren et al. 2000). In addition, an altitude shift from about 700–800 m above sea level to 1100 m and more has been registered for *I. ricinus* in the Czech Republic and Austria within the recent past, which has also been linked to a rise in temperature (Danielová et al. 2008; Materna et al. 2008; Holzmann et al. 2009).

There is also evidence for the spread of other tick species. For example, the taiga tick ***Ixodes persulcatus***, an efficient vector of TBEV, seems to be expanding its Asian distribution area westwards. It has recently been found in Finland, several hundreds of kilometres further to the northwest than previously recorded (Jääskeläinen et al. 2006). Another hard tick species, ***Dermacentor reticulatus***, the major vector of *Babesia canis* in Europe, is nowadays found at considerably more sites in Germany than only a few years ago (Heile et al. 2006; Dautel et al. 2006). In The Netherlands, questing specimens of this tick species were found in the field for the first time ever in 2006 (Nijhof et al. 2007). Environmental and climatic changes as well as an increase in blood host populations are among the causes being discussed. The former absence of *D. reticulatus* from Germany had in part been assigned to a lack of adequate humid biotopes such as swampy lowlands and floodplain forests (Enigk 1958).

Much more alarming would be a geographic expansion of ***Hyalomma marginatum***, as this tick species is the principal vector of Crimean-Congo haemorrhagic fever virus (CCHFV). It is a two-host tick that remains on the same host for up to 26 days, from the unfed larval stage through to the fed nymphal stage. Pre-mature stages of the subspecies *H. m. marginatum* are frequently imported by migratory birds from the tick's distribution areas in the southern European/North African Mediterranean and the southwestern Palaearctic regions into central and northern Europe (Hoogstraal et al. 1961; Papadopoulos et al. 2002). Laboratory studies on temperature requirements (Emelyanova 2005), however, suggest that they cannot survive and do not reach the adult stage under present central European conditions. Notwithstanding, a questing adult female of *Hyalomma m. marginatum* was found in southern Germany in 2006 (Kampen et al. 2007).

A further factor that needs to be considered in the context of pathogen transmission by ticks under changing environmental conditions is the extension of seasonal tick activity periods. Such an extension has in fact been observed in Sweden as a consequence of mild winters (Tälleklint and Jaenson 1998). In a German study, *I. ricinus* could even be collected throughout the winter (Dautel et al. 2008).

As with other ectoparasites, ticks may also take advantage of globalization and increased travel activities in terms of expanding their distribution area. The brown dog tick ***Rhipicephalus sanguineus***, for example, a thermophilic hard tick species and the major vector of *Rickettsia conorii* in Europe, is often imported while attached to, and feeding on, dogs. In recent decades, *Rh. sanguineus* has successfully established indoor populations in more northern European countries, such as Germany, the UK and Belgium (Gothe 1968; Fox and Sykes 1985; Sibomana et al. 1986).

14.3.2 Lyme Disease

The most significant tick-associated disease in Europe and worldwide is Lyme borreliosis (LB). At least four of the seven genospecies of the *B. burgdorferi*

complex occurring in Europe are pathogenic to humans and are causative agents of LB: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* and *B. spielmanii* (Baranton and De Martino 2009). In contrast to North America, it cannot really be said that LB or the spirochetes are spreading geographically in Europe (except for northern Scandinavia where its vector, *I. ricinus*, is spreading). *Borrelia burgdorferi* s.l. probably occurs everywhere where *I. ricinus* occurs, which by preference is in mixed and deciduous woods (Gern 2005), where borrelial reservoir hosts are available and where contact between ticks and humans is possible. Infection prevalences vary regionally as does the composition of the different genospecies (Hubálek and Halouzka 1997, 1998). There is evidence, however, that infection prevalences have been increasing within populations, possibly depending on tick and blood host densities (Kampen et al. 2004). In many countries, LB is not a notifiable disease and so disease incidences are hard to obtain. The symptoms are manifold, ranging from slightly feverish to skin lesions and the involvement of heart, joints and the nervous system (Strle and Stanek 2009).

14.3.3 Tick-Borne Encephalitis

Less frequent and regionally more focussed, but at the same time more critical with regard to human health and geographic spread, is tick-borne encephalitis (TBE). TBEV (family Flaviviridae) is endemic in central and eastern Europe, Russia and the Far East. It occurs in three subtypes, the far eastern (formerly RSSE = Russian spring-summer encephalitis), the Siberian, and the central European (formerly CEE = Central European encephalitis) (Gritsun et al. 2003). The far eastern and the Siberian subtypes are transmitted predominantly by *I. persulcatus*, the central European subtype by *I. ricinus*. An infection with the central European type often has a biphasic course (Haglund and Günther 2003). The first acute phase usually presents with fever, headache, joint and back pain, nausea and vomiting, whereas in the second chronic phase, following a short symptom-free interval, neurological signs occur and 20–30% of the patients suffer from meningoencephalitis. The fatality rate is ca. 1%. Infections with the far eastern subtype are regarded as much more severe than those with the other types, and case fatality rates of 20–60% have been described (Pogodina et al. 1986). *Ixodes persulcatus* often also reaches much higher virus infection prevalences than *I. ricinus*. While *I. ricinus* infection prevalences are mostly quoted as being about 0.5–5% (e.g. Dumpis et al. 1999; Randolph 2001; Süss et al. 2006), locally 30–40% of *I. persulcatus* may be found infected with TBEV (Smorodintsev 1958; Korenberg et al. 1999).

In Europe, TBE is endemic in many western and central countries and Scandinavia, particularly Austria, Croatia, Czech Republic, Estonia, Finland, Germany, Hungary, Latvia, Lithuania, Poland, Slovakia, Slovenia, Sweden and Switzerland. Although incidences vary and reporting is not standardized internationally, an overall increase in case incidences during the past 30 years can clearly

be seen (Süss 2005, 2007). This trend is generally thought to be related to global warming, as warmer weather leads to larger rodent populations and more active ticks. However, sophisticated models and analyses show that climate alone cannot explain the spatio-temporal heterogeneities in TBE epidemiology (Sumilo et al. 2007; Randolph 2008).

Incidences of TBE have not only risen but TBEV-positive ticks and human infections after tick bites have recently been recorded in areas and altitudes previously free of TBEV (e.g. Jääskeläinen et al. 2006; Skarpaas et al. 2004; Brinkley et al. 2008; Stefanoff et al. 2008; Fomsgaard et al. 2009; Holzmann et al. 2009).

14.3.4 Babesia spec., *Anaplasma phagocytophilum,* Rickettsiae

Tick-borne pathogens in Europe which, from a medical point of view, have been the subject of enhanced awareness in recent years and may become even more relevant in the future are *Babesia* spec., *Anaplasma phagocytophilum* and *Rickettsia* spec.

In Europe, the genus *Babesia* contains several species with zoonotic potential including *B. divergens*, *B. bovis, B. venatorum*, *B. microti* and *B. canis*. They are transmitted by various species of ticks, primarily *I. ricinus*, which may regionally reach infection prevalences of up to 20% (Halos et al. 2005). *Babesia divergens* and *B. ovis* have a reservoir in cattle, *B. microti* in mice, *B. canis* in dogs and *B. venatorum* in deer (Hunfeld et al. 2008). The majority of the human cases detected so far in Europe are traced back to *B. divergens* (Gorenflot et al. 1998), but cases of disease following infections with the other species have been described. *Babesia microti*, for example, formerly thought to occur only in North America (Homer et al. 2000), was recently diagnosed in humans in Switzerland and Germany (Meer-Scherrer et al. 2004; Hildebrandt et al. 2007). Additionally, a newly emergent species, *Babesia venatorum* (formerly *Babesia* EU1), was isolated from patients in Austria, Italy and Germany (Herwaldt et al. 2003; Häselbarth et al. 2007). Most patients infected with *Babesia* parasites were asplenic and immunocompromized. Symptoms were generally life-threatening and mortality rates high (Hunfeld et al. 2008).

Canine babesiosis is another growing problem in Europe, with an increasing number of cases in recent years (e.g. Bourdoiseau 2006; Matjila et al. 2005; Welc-Faleciak et al. 2009). The principal causative agent, *Babesia canis*, is transmitted mainly by *D. reticulatus* ticks, and the spread of *B. canis* is probably directly associated with the spread of its vector. In contrast to *B. canis*, *B. gibsoni* is supposed to be transmitted mainly by *Rh. sanguineus*. Recently, several cases of canine babesiosis caused by *B. gibsoni* (Asian genotype) have been found in Italy, Spain and Germany (Casapulla et al. 1998; Suarez et al. 2001; Hartelt et al. 2007).

Anaplasma phagoytophilum is the etiological pathogen of another emerging *I. ricinus*-borne disease in Europe, human granulocytic anaplasmosis (HGA;

Strle 2004). The first case of HGA was described in 1997 in Slovenia (Petrovec et al. 1997), but up to early 2003, 65 more patients were diagnosed with the disease, mostly in Slovenia and Sweden (Strle 2004). Isolated cases, however, occurred in other European countries. Clinical features are usually mild to moderately severe, but may be severe, particularly in the elderly, when there is a concomitant chronic illness (Bakken and Dumler 2000; Olano and Walker 2002). Small mammals have been identified as reservoir hosts for *A. phagocytophilum* (Liz 2002). Infection prevalences in ticks may be as high as 30% (Christova et al. 2001).

Mediterranean spotted fever caused by *R. conorii* was for a long time thought to be the only tick-borne rickettsiosis prevalent in Europe. During the last decade, however, several other rickettsial species transmitted by ticks, such as *R. aeschlimannii*, *R. slovaca*, *R. sibirica* subsp. *mongolitimonae, Rickettsia massiliae* and *R. helvetica*, have emerged (Brouqui et al. 2007; Dobler and Wölfel 2009). They are associated with different tick species and pathogenicities, but there are as yet no detailed European studies on their distribution and prevalences.

14.3.5 Crimean-Congo Haemorrhagic Fever

Together with the spread of *Hyalomma* ticks, the spread of Crimean-Congo haemorrhagic fever (CCHF) virus (family Bunyaviridae) is anticipated. CCHFV is enzootic in Africa, the Middle East, central and southwestern Asia (Hoogstraal 1978), and has also been shown to circulate in parts of southern and southeastern Europe, such as Bulgaria, Albania, Kosovo and Turkey (Ergönül 2006; Vourou 2009). Human infections may be coupled with severe haemorrhagic manifestations and case fatality rates of more than 30% (Charrel et al. 2004). CCHF has become a most severe public health problem in Turkey since 2002, with more than 3,000 fatal cases in Anatolia since 2007 alone (Yilmaz et al. 2009; Maltezou et al. 2010). The reasons for these dramatic developments are unknown, but it has been suggested that changes in land use have ameliorated the living conditions of both small and large mammals as potential tick blood hosts and, consequently, of the ticks themselves (Randolph and Ergönül 2008).

14.4 Sandflies (Diptera, Psychodidae)

14.4.1 Leishmaniasis

Sandflies are vectors of *Leishmania* parasites and several arboviruses. In Europe, they transmit *Leishmania infantum*, *L. tropica*, Toscana virus, sandfly fever Naples

virus and sandfly fever Sicilian virus. Numerous species of sandflies occur in the Mediterranean, with some of them, such as *Phlebotomus perfiliewi* and *P. perniciosus*, spreading northwards (Kuhn 1999). *Phlebotomus perniciosus* and *P. mascittii* have sporadically been reported from northern France, the British island of Jersey, Belgium and Germany (Callot 1950; Naucke and Pesson 2000; Naucke and Schmitt 2004; Depaquit et al. 2005). The northernmost finding of a phlebotomine sandfly in Europe was that of *P. mascittii* in Rhineland-Palatinate, central-western Germany (Naucke et al. 2008).

Leishmania infantum zoonotic cutaneous and visceral leishmaniases are endemic in almost the whole Mediterranean, with major foci in Italy and Albania (Gramiccia and Gradoni 2005). *Phlebotomus perfiliewi* and *P. perniciosus* are reportedly able to transmit *Leishmania infantum* (Maroli and Khoury 1998), while *P. mascittii* has not definitively been proven to be a vector (Depaquit et al. 2005). A few years ago, two cases of *L. infantum* leishmaniasis were diagnosed in Germany, in a 15-month-old baby and in a horse that had never been outside of Germany (Bogdan et al. 2001, Koehler et al. 2002). Naucke et al. (2008) even reported 11 autochthonous *Leishmania* infections in Germany since 1991, mainly in dogs.

Anthroponotic cutaneous leishmaniasis caused by *L. tropica* sporadically occurs in Greece and probably in neighbouring countries where *P. sergenti* is likely to be the main vector. Because of migration and travelling, *L. tropica* has a high potential to be introduced into the rest of the EU (Ready 2010).

A particular problem with leishmaniasis and its potential spread in Europe is the significant increase in the movement of dogs together with their owners to endemic southern countries, and the exploding and often uncontrolled importation of dogs from those areas (Shaw et al. 2008; Menn et al. 2010). In some Mediterranean regions more than 50% of dogs are infected with *Leishmania* parasites (e.g. Brandonisio et al. 1992; Ciaramella et al. 1997), and imported and returning dogs are not necessarily medically examined. Since dogs are important reservoir hosts for the parasite, they may become infection sources to vector-competent sandflies occurring in non-endemic areas (Zahner and Bauer 2004; Mettler et al. 2005).

14.4.2 Sandfly Virus Fever

Toscana virus (TOSV; Bunyaviridae) is transmitted by sandflies in the European Mediterranean whereas sandfly fever Naples virus and sandfly fever Sicilian virus (both family Bunyaviridae), also referred to as “papatacci fever” viruses, have been reported from more eastern parts of the Mediterranean and some north African areas (Depaquit et al. 2010). Infections often present with influenza-like symptoms but, in the case of TOSV, can also lead to acute meningitis and meningoencephalitis. Important vectors are *P. perniciosus* and *P. ariasi* but potential vectors appear to be more widely dispersed than the viruses. At present, no reasons are known why the viruses should not extend over the entire distribution ranges of their vectors.

Little is known, however, about the vertebrate reservoirs and the impact of climatic factors on the viral replication in their vectors.

14.5 Biting Midges (Diptera, Ceratopogonidae)

14.5.1 Bluetongue Disease

Bluetongue disease (BTD), a viral infection of ruminants, in particular of sheep, cattle and goats, became endemic in southern Europe in the late 1990s (Purse et al. 2005). Prior to that, its main vector in its African and Asian distribution areas, *Culicoides imicola*, had been introduced and established in the Iberian peninsula and had spread to other European Mediterranean countries (Mellor et al. 1983; Purse et al. 2005). This ceratopogonid species has mainly been responsible for the transmission of five serotypes of the bluetongue virus (BTV; family Reoviridae) that have been circulating and causing BTD outbreaks in southern Europe. In August 2006, however, BTD unexpectedly broke out in central Europe (Belgium, The Netherlands, Germany, France, Luxembourg) from where it spread to numerous other countries of the continent during the following three years, thereby affecting tens of thousands of animal holdings and causing enormous damage to animal health and economic losses (Kampen and Werner 2010). Strangely, the newly emerging virus strain was serotype 8 (BTV-8) and different from the serotypes circulating in southern Europe. It later transpired that it was most closely related to a strain isolated in Nigeria in 1982 (Enserink 2006; Maan et al. 2008), but the way in which it entered Europe has remained obscure (Mintiens et al. 2008). Also, it has been demonstrated that the culicoid species responsible for BTV transmission in Europe so far, *C. imicola*, did not occur in the northern outbreak areas although a spread to the north had been observed (Purse et al. 2005). Instead, evidence has accumulated that indigenous *Culicoides* species, notably those of the Obsoletus complex, are the vectors of BTV in Europe north of the distribution area of *C. imicola* (Meiswinkel et al. 2007; Carpenter et al. 2008; Dijkstra et al. 2008; Hoffmann et al. 2009).

In addition to BTV-8, short episodes of BTV-6 and BTV-11 transmission occurred in late 2008 in a Dutch/German border region and in Belgium, respectively, and these are equally unsolved as regards their origin and background (De Clercq et al. 2009; Eschbaumer et al. 2009; Kampen and Werner 2010).

Last but not least, BTV-1 is spreading in Europe (Kampen and Werner 2010). This serotype jumped to the Iberian peninsula from northern Africa in 2007, and since then has caused disease outbreaks in Spain and Portugal, and, subsequently, France. During 2008, close to 5,000 animal holdings were affected in France, and despite the national vaccination programme several new cases occurred north of the restriction zone in 2009 (EU-BTNET 2009, 2010).

14.5.2 African Horse Sickness

BTD is only one of two dangerous viral animal diseases transmitted by *Culicoides* biting midges. African horse sickness (AHS) is even more devastating to equids than BTD is to ruminants. In AHS, mortality rates may exceed 90% in susceptible equids. The disease also originates from sub-Saharan Africa where Zebra are the reservoir hosts and where the same *Culicoides* species as in BTD, basically *C. imicola*, are the viral vectors (Mellor and Hamblin 2004). From 1959 to 1961, a massive AHS outbreak swept over the Arabian peninsula and the Middle East as far as India and Pakistan, with hundreds of thousands of horses dying (Anwar and Qureshi 1972). Another major epidemic beyond the sub-Saharan endemic zone was registered in North Africa and Spain in 1966 (Diaz Montilla and Panos Marti 1968). The Iberian peninsula was once again struck by an outbreak in 1987 after the introduction of subclinically infected Zebra to a zoological garden close to Madrid (Lubroth 1988). Because of the mild climate in southern Spain and Portugal and the vector activity throughout the year, the virus was able to overwinter and produce further incidents in 1988, 1989 and 1990 (Mellor and Hamblin 2004).

14.6 Conclusions

The BTV-8 epidemic is a remarkable, unprecedented and unique example of an emerging vector-borne disease and of the importance of public health preparedness in Europe. Prior to the BTD outbreak in 2006, entomologists had slowly disappeared from the European university scene as vector research was no longer thought to be sufficiently competitive and innovative (cf. Cuisance and Rioux 2004). At the time of the outbreak, hardly any of the few remaining entomologists dealt with ceratopogonids and so, when the disease broke out, specialists were not available and knowledge on the occurrence, distribution, biology, ecology and vector competence of biting midges, if present at all, was outdated. On the governmental side, a delusory feeling of safety, paired with ignorance concerning vector-borne diseases, had become commonplace. Interestingly, only shortly before the outbreak, a few reports and publications had explicitly pointed out the risk of BTD outbreaks in central and northern Europe (Wittman and Baylis 2000; Maier et al. 2003; Koslowsky et al. 2004).

The outbreak, disastrous as it was, resulted in some positive developments. Medical entomology has become attractive again and some research positions have even been established in Europe. As the epidemic was border-crossing, a fruitful international research network has been initiated and supported by the EU under the umbrella of the MedReoNet programme (Cêtre-Sossah 2010).

Since 2006, we have learnt much about European biting midges, but there are still tremendous gaps in our knowledge of breeding habitats, vector competences and overwintering, for example. Indigenous ceratopogonids cannot even be reared

in the laboratory in order to conduct controlled experimental studies. Thanks to LB and TBE, there is a considerable body of knowledge on *I. ricinus*, but other tick species, mosquitoes and further groups of haematophagous arthropods have been recklessly neglected for a long time. Furthermore, we must recognize that we know a great deal more about alien and invasive potential arthropod vectors and vector-transmitted pathogens than about the indigenous ones. Both groups are important of course, and, depending on the ecological conditions, any combination between endemic or imported/invasive vector, and endemic or imported/invasive pathogen may be most efficient in causing vector-borne disease. A research gap that has accumulated over decades has to be filled, and this will only be possible with adequate personnel and research funds.

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Chapter 15
A Look at the World of Ticks

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Ticks are one of the best known groups of parasites. They have accompanied humans and their domestic animals throughout recorded history (Hoogstraal 1970 and subsequent volumes) and have become a major focus of medical and veterinary research, not only because of their direct pathogenic influence on hosts, such as blood loss and tick-induced paralysis (Gothe 1999; Pfäffle et al. 2009), but more importantly because of their role as vectors of a very wide range of viral, bacterial and protozoan diseases (Nicholson et al. 2009). Indeed, ticks are of considerable economic importance as a constraint to animal production in most of the countries

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where they occur (Jongejan and Uilenberg 2004). Despite this sinister background, ticks are a fascinating, highly successful group, manifesting a wide variety of adaptations to their hosts and the environments in which they live.

In this contribution we will present the world of ticks both from the perspective of a biologically highly successful and interesting group and from their dark side as parasites and carriers of diseases.

15.1 Taxonomy and Systematics

Ticks are an ancient lineage. Specimens from Burma have been found entombed in amber from the Cretaceous Period, about 100 million years ago, indicating that the two most important tick families extant today, the Argasidae or soft ticks and the Ixodidae or hard ticks, had differentiated by this time. Indeed, two extinct genera of the Ixodidae, *Compluriscutula* and *Cornupalpatum*, are known only from Burmese amber (Grimaldi et al. 2002; Poinar and Brown 2003; Poinar and Buckley 2008), while the earliest argasid, from 90 to 94 mya, was found in New Jersey amber (Klompen and Grimaldi 2001).

Ticks are large, obligately hematophagous mites and, like all mites, are members of the arthropod class Arachnida, subclass Acari. This is a remarkably large and diverse group, with estimates of nearly 50,000 described species, probably only about 10% of those that actually exist (Halliday et al. 2000). Ticks make up a very small proportion of the suborder Ixodida but have a significance out of proportion to their numbers. To date, a total of 893 tick species have been described, divided among the Ixodidae (701 spp., 14 genera), Argasidae (191 spp., the number of genera is controversial and currently under discussion), and Nuttalliellidae (one species) (Guglielmone et al. 2010).

All ticks have two body segments, the capitulum comprising the mouthparts and sensory palps, and the idiosoma or body containing most of the organs as well as the anus and (in adults) genital aperture. The first active life history stage of all ticks, the larva, has six legs, while nymphs and adults have eight legs, all originating from the idiosoma (Figs. 15.1 and 15.2). The first pair of legs possesses Haller's organ, which contains a variety of sensory structures largely associated with finding an appropriate host (Oliver 1989).

The Ixodidae are characterized by a hard sclerotized shield covering the anterior part of the idiosomal dorsum or, in males, the entire dorsal surface. Additionally, ixodid mouthparts project forward and are visible from above during all life history stages (Fig. 15.1). In the Argasidae there is no dorsal shield, and the integument is leathery, wrinkled, granulated, mammillate, or tuberculate. Argasid mouthparts are on the ventral surface of the body and, except during the larval stage, are not visible from above (Fig. 15.2). About 10% of the species in these two families are of medical or veterinary importance (Oliver 1989; Jongejan and Uilenberg 2004).

The third family, the Nuttalliellidae, is the most unusual of the three, containing only the single species *Nuttalliella namaqua*. In many ways this tick is intermediate between the Ixodidae and the Argasidae, with the mouthparts extending anterior to the body and a dorsal shield present but with a body structure suggestive of the

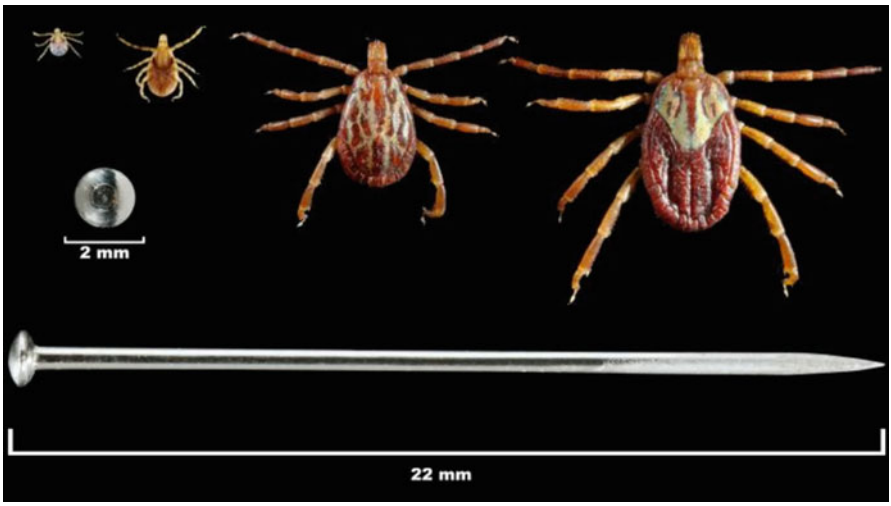

Fig. 15.1 Dorsal view of the different life history stages of *Ambylomma maculatum*, belonging to the family Ixodidae. From *left to right*, this group of four comprises a larval tick, a nymph, an adult male, and at *far right*, an adult female (courtesy of the CDC)

Fig. 15.2 Dorsal view of an *Ornithodoros kelleyi*, belonging to the family Argasidae (courtesy of the CDC)

Argasidae. *Nuttalliella namaqua* will not be dealt with below because it remains almost unknown today, nearly 80 years after its discovery. Less than 30 female and nymphal specimens have been collected, all from southern or eastern Africa (Keirans 2009).

15.2 Ecology

Argasid and ixodid ticks are found throughout the world, thriving in tropical rain forests, in barren, windswept deserts, and within both sub-Arctic and Antarctic latitudes, including isolated islands such as Tristan da Cunha and Kerguelen

(Dumbleton 1961). Even penguins are known to be infected by tick-borne pathogens, for example *Babesia piercei* (Jones and Shellam 1999).

Some species, such as the European sheep tick, *Ixodes ricinus*, occur on hundreds of different hosts, ranging from snakes and lizards through birds to mammals, while others are so specific that they are usually only found on a single species of host (Hoogstraal and Aeschlimann 1982). *Ixodes lividus*, for example, is found almost exclusively on the sand martin *Riparia riparia* (Guglielmone et al. 1963). Although amphibians are rarely infested, some tick species, such as *Amblyomma rotundatum* from the New World tropics, are regularly found on this group (Guglielmone et al. 2003). Those species that only occur on endangered hosts, such as *Amblyomma crenatum* from Asian rhinoceros, are threatened with coextinction should their hosts die out (Durden and Keirans 1996).

In addition to their morphological differences, there are considerable ecological differences between argasid and ixodid ticks. There are four stages in the life history of ixodid ticks: eggs, larvae, nymphs and adults (Fig. 15.3). Once the eggs have hatched, all subsequent stages require a blood meal before they can continue

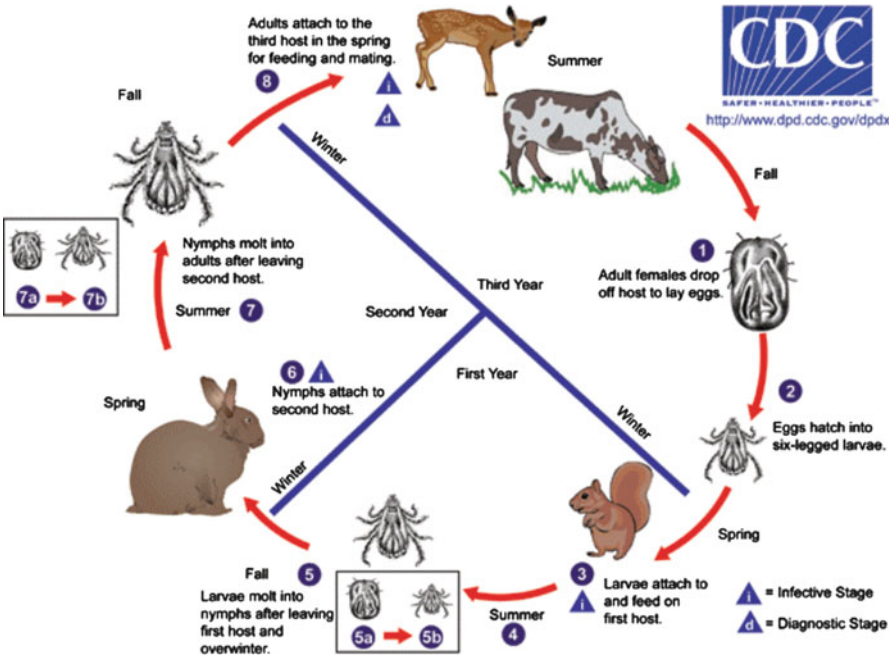

Fig. 15.3 The life cycle of a three-host ixodid tick. Adult females drop off the third host to lay eggs after feeding ①, usually in autumn. Eggs hatch into six-legged larvae ②. These seek out and attach to the first host, such as a rodent ③. Once fully engorged, the larvae detach from the first host ④ and moult to nymphs ⑤a–⑤b. The nymphs then seek out and attach to the second host ⑥. The nymphs feed on the second host ⑦, detach and moult into adults ⑦a–⑦b, which then seek out and attach to a third host, usually a large herbivore ⑧. Mated, fully engorged females drop off the host and lay their eggs on the ground, under litter or in nests to continue the cycle (courtesy of the CDC)

their development. In most species, the fully engorged tick will detach from its host and fall to the ground before moulting, or, in the case of females, laying eggs before dying. In fact, most of the tick's life is not spent attached to the host but free-living. Each feeding session takes a few days to a week. Usually the tick detaches after each engorgement, requiring three hosts to complete its life cycle; however, about 12 species moult from larva to nymph on the same host before detaching and moulting to adults on the ground (two-host ticks), and an equal number moult from larvae to nymphs to adults with only the fully engorged females detaching to lay eggs on the ground (one-host ticks). *Hyalomma scupense* can even switch between a one- and two-host life cycle (Oliver 1989). Most one- and two-host tick species are found in arid areas, where the off-host environment is particularly harsh and survival is difficult (Oliver 1989).

The argasid cycle (Fig. 15.4) differs in certain details from that of ixodids. Once the larval stage is over, 2–8 nymphal stages occur, with each stage detaching before feeding again on a new host. The number of nymphal stages varies both within and between species (Oliver 1989). In some species the larvae do not feed, nor do the first nymphal instars in subgenus *Alectorobius* of *Ornithodoros*. In addition, adults of the genus *Otobius* do not feed. The duration of feeding in nymphal and adult argasids is much shorter than in ixodid ticks, usually taking from minutes to a few hours. Adults can feed more than once, and a number of egg batches may be laid. Although they are usually relatively short lived, some argasid species have been recorded to survive for up to 18 years (Hoogstraal 1985).

The ecology of ticks can be considered from the standpoint of environmental conditions, onto which are superimposed the interactions between ticks and their hosts. Tick survival and rate of development are dependent on temperature, humidity and, for many species, photoperiod (Norval 1977; Belozerov and Naumov 2002; Randolph 2004). Low and high temperatures and high saturation deficits preclude successful hatching. For example, the rate of development increases for *I. ricinus* with increasing temperature up to 30°C, after which it decreases, while the pre-oviposition period and the time for eggs to develop to larvae decrease with temperature up to this limit (Randolph et al. 2002).

At the local level, the distribution of ticks (and their hosts) can be influenced by a variety of factors. Microclimate, particularly temperature and humidity as indicated above, is of particular significance. Microclimate is dependent on a variety of biotic and abiotic factors, such as the amount of vegetation present (shade and leaf litter provide lower temperatures but higher humidities) and the type of soil (water retention and potential interstitial refuges) (Merler et al. 1996; Schwarz et al. 2009). These factors are components of the habitat occupied by ticks. Hosts, on which the ticks are dependent, also have specific habitat preferences. Thus, each habitat must be considered separately in studies involving tick population and community dynamics.

In one of the most comprehensive studies to date, Estrada-Peña (2001) compared the abundance of *I. ricinus* in 18 different habitats, defined by the vegetation present, over 3 years in Spain. He found that this species was absent from open, grassy habitats and hillsides as well as young pine forest monocultures and

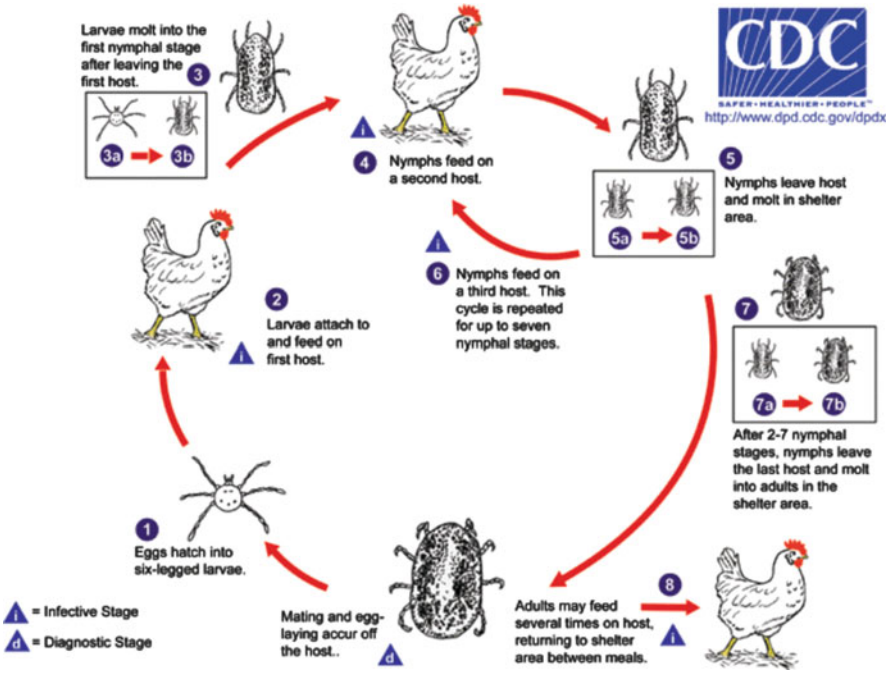

Fig. 15.4 The life cycle of an argasid tick. Mating usually occurs, and egg-laying always occurs, off the host in a sheltered area, such as an animal nest. Eggs hatch into six-legged larvae ① in the parents' sheltered area. They quest for a host in the vicinity of the sheltered area. Once a suitable host is found, they feed for anywhere from 1 h to several days, depending on the species ②. After feeding, the larvae leave the host and moult into first nymphal instars in the sheltered area ③a–③b. The nymphs quest for, and feed on, the second host ④ rapidly (usually about an hour). The second host is usually the same species, and often the same individual, as the first host. The first nymphal instar leaves the host and moults into the next nymphal instar in the sheltered area ⑤a–⑤b. This cycle can continue to accommodate up to eight nymphal instars ⑥, depending on the species. After the last nymphal instar has fed, it leaves the host and moults into an adult ⑦a–⑦b in the sheltered area. Adults may continue to feed on the host ⑧, feeding rapidly and detaching after each blood meal. Females of some species lay egg batches after each meal. Humans are usually only incidental hosts for argasid ticks and may be fed upon by any of the stages (courtesy of the CDC)

preferred sites with substantial secondary plant growth, in particular forests with oak species and fragmented forests with many ecotones. Estrada-Peña was able to show that 50% of the variation in tick abundance could be accounted for by temperature and vegetation characteristics of the habitat.

In the same area, a long-term study over 9 years considered the dynamics of *I. ricinus* populations (Estrada-Peña 2005). Collections were made by flagging over a 30 min period. Larvae usually showed only a single sharp peak around July/August; nymphs consistently showed two peaks in spring and autumn, of which the spring peak was usually higher. Adults also showed bimodality but, unlike Central Europe, the autumn peak was usually the highest. Of considerable interest was the

consistent increase in population size for all life history stages throughout the study period, indicating that the population studied was in a process of dynamic, directed change. This change could not, however, be related to a regular pattern of climate change, although certain periods or components of the system correlated with climate. Thus, the mild winters between 1999 and 2002 were correlated with nymphal abundance in the following years. Estrada-Peña et al. (2004) suggest that a combination of factors involving climate and host population densities is responsible for the variation found. In general, in areas where hosts are rare, tick populations are also low, independent of whether the environment is suitable (Estrada-Peña 2003).

15.3 Medical, Veterinary and Economic Significance

In Europe and North America, ticks are among the most important vectors of human and animal diseases. They are responsible for transmitting a wide variety of viral, bacterial and protozoan pathogens causing substantial morbidity and occasional mortality in at-risk populations (Goodman et al. 2005). In Germany, an estimated 60,000–100,000 people suffer from borreliosis every year (Fischer and Siegmund 2007) with an unknown but potentially large number experiencing chronic illness. Data from several German states indicate that the number of cases is increasing steadily (Talaska 2002). The costs of *Borrelia* infections are variable. Rapid diagnosis usually involves visits to the local physician, blood tests and antibiotics (Talaska 2002). For disseminated borreliosis cases, treatment was estimated to cost 10,000€ (>US $12,000)/case in 2002 (Talaska 2002). A study conducted in the United States estimated that the total 5-year expenditure for Lyme disease in the human population in the late 1990s was $2.5 billion (Maes et al. 1998). To this must be added the economic and social costs of ticks and tick-borne diseases to the livestock industry, particularly in tropical and subtropical developing countries where they are a major constraint to animal production (Jongejan and Uilenberg 2004). In Africa, for example, many communities lack adequate dietary protein (protein-energy malnutrition, Müller and Krawinkel 2005), but livestock improvement, particularly by import of rapidly growing, high milk producing European cattle (*Bos taurus*), is hampered or blocked by the presence of such tick-borne diseases as heartwater (*Ehrlichia ruminantium*), East Coast fever (*Theileria parva*) and redwater (*Babesia bigemina* and *B. bovis*) (Jongejan and Uilenberg 2004).

15.4 Human Involvement

Human contact with ticks is directly related to human activity in habitats occupied by ticks and their hosts. Most tick species have only limited mobility, either horizontally or vertically (Petney et al. 1983; Carroll and Schmidtmann 1996).

Thus, moulting and egg laying usually take place near the place where ticks detach from their hosts. We have also seen that different habitats are likely to be inhabited by different tick species or to have different densities of ticks present, and that this density is dependent on season; i.e., the chances of being bitten by a particular tick species are dependent on where you are and when you are there.

15.5 Pathogenicity for Humans and Animals

Because humans usually find and remove ticks soon after they attach, the ticks alone are of relatively limited medical importance except when they cause paralysis or induce an allergic response. *Ixodes holocyclus* from eastern Australia, for example, injects a potent neurotoxin into the site of its bite. Children between the ages of one and five are most commonly affected. The symptoms develop slowly as a flaccid ascending paralysis, which can result in death due to respiratory failure if treatment is not obtained (Grattan-Smith et al. 1997). The case of *I. holocyclus* is unique in that, unlike North American paralysis ticks, removal does not lead to rapid recovery of the patient (Felz et al. 2000). Only with the development of an antitoxin in the mid 1930s was an effective cure for severe paralysis realized (Stone et al. 1989).

Allergies to tick bite are more common than tick-paralysis, and potentially as harmful (Stone et al. 1989). In Europe, many cities harbour large populations of domestic pigeons, *Columba livia*, which may be infested with the pigeon tick *Argas reflexus* (Dautel et al. 2009). When control programs aimed at reducing the number of pigeons are carried out, the natural host of the tick is removed and a new host is required (Dautel et al. 2009). This frequently leads to *A. reflexus* biting humans and causing serious allergic responses, including anaphylactic shock (Quercia et al. 2005; Spiewak et al. 2006; Dautel et al. 2009).

Domestic and wild animals are also subject to paralysis and allergic responses to ticks, depending on the species present (Gregson 1973). However, they are also more likely to be subject to intensive infestations with large numbers of ticks. These can cause a variety of problems, such as anaemia due to blood loss, loss of condition, reduced growth and reproductive rate, and lowered milk production, while the lesions at the site of the tick bite can provide an entry point for secondary infections.

15.6 Ticks as Vectors

Ticks are extremely important vectors of diseases to humans as well as to stock and companion animals worldwide (Parola and Raoult 2001; Piesman and Eisen 2008). In recent years our knowledge of the number of pathogens transmitted by ticks has increased remarkably; in Germany alone at least eight new human pathogens

or potential pathogens have been reported over the last 15 years, including several species of *Borrelia*, *Rickettsia* and *Anaplasma* (Süss and Schrader 2004; Süss et al. 2004). Some recently discovered, common species, such as *Rickettsia kotlanii* (Sreter-Lancz et al. 2006; previously *Rickettsia* RpA4) from *Dermacentor reticulatus* (Dautel et al. 2006), remain ecologically and epidemiologically virtually unknown. There is also considerable evidence to show that the distribution and density of tick populations is increasing and that tick-borne diseases are spreading for a number of medically and economically important species such as *I. ricinus* (Lindgren et al. 2000). The reasons for these changes remain controversial and include global climate change as well as changing land-use and human behaviour patterns.

15.7 Epidemiology

Ticks, their hosts and the pathogens that they transmit cannot be considered as single entities that are independent of one another. Rather, they represent a complex system of interactions and are intimately connected (Ostfeld et al. 1996). In order to unravel this epidemiological complexity, it is necessary to know how common the individual host species are, what the infestation rates are, the success of tick feeding on each host species, and their rate of infection and susceptibility to the individual pathogen. The population density of non-susceptible hosts and their use by ticks as hosts is particularly important because such hosts cannot pass the infection further and potentially act to dilute the overall degree of successful transmission (Begon 2008; Keesing et al. 2009).

Although it is well known that perturbations in host-vector-pathogen systems can produce substantial changes at the population level, the long-term effects of such changes on wildlife, livestock and disease management within ecosystems remain unknown (Anderson and May 1991; Chemini and Rizzoli 2003). There is substantial evidence that interactions across the pathogen-vector-host system control tick-borne disease epidemiology. In European alpine areas, the decline of agriculture has led to increases in the area occupied by forests and shrubby ecotones (Chemini and Rizzoli 2003). This in turn has increased the habitat available for tick hosts (e.g., rodents, other small mammals and deer) and is correlated with an increase in the number of human cases of TBE and borreliosis in these areas (Chemini and Rizzoli 2003).

15.8 Control

Tick-borne diseases are notoriously difficult to control (Piesman and Eisen 2008). Many economically important species are very widely distributed and are capable of infesting a variety of host species, making long-term control, even in limited

areas, almost impossible. *Ixodes ricinus*, for example, is common in many European habitats, such as forests, forest edges, and areas with shrubs and low vegetation, i.e., areas where hosts are available, including rural, peri-urban and urban environments. Any large-scale control measure would invariably have to cover vast areas, potentially influencing other species present, and even if control were obtained, it would only be short-term because the hosts would still be present or could migrate into the area again, and ticks would accompany these hosts. Even long-term, local eradication would be associated with continual costs for the control agent, its application and determination of its effectiveness. In addition to being unsuccessful, the bulk application of acaricides over large areas could have a devastating effect on the local ecology due to their lack of specificity (Graf et al. 2004; Ashley et al. 2006). Local application in gardens can, however, be successful (Piesman and Eisen 2008). Vegetation management, such as the removal of shrubs and undergrowth that provide suitable habitat for rodent hosts can be successful on a small scale but is costly and time-consuming over larger areas and destroys the habitat of a wide variety of other species, including those beneficial to humans.

Biological control by fungi (Hartelt et al. 2008), parasitic nematodes (Hartelt et al. 2008) and parasitoids (Mwangi and Kaaya 1997) has been successful in laboratory experiments, and estimates exist of substantial natural reductions in tick populations caused by certain of these organisms (Samish and Rehacek 1999). However, their use as biocontrol agents seems less promising. The addition of non-host specific organisms to the environment automatically causes changes in the dynamic relationships within local food webs. These webs are of such complexity that changes cannot be predicted; thus, a natural stability or dynamic may be modified with unknown consequences. Such modifications can have severe negative impacts on the natural ecology of an area. In addition, the introduction of tick pathogens or parasites is fraught with complexities. It is known, for example, that the parasitoid *Ixodiphagus hookeri* is capable of reducing populations of *Ixodes scapularis* in North America, but that a minimum host deer density (and therefore tick density) is required before *I. hookeri* can attain viable populations (Stafford et al. 2003).

15.9 Personal Protection

The most effective and obvious means of preventing exposure to ticks is to avoid their known habitats. Some professions, however, such as forestry workers, require contact with tick habitat. If this is the case, appropriate clothing which reduces the amount of exposure as well as the use of repellent chemicals is recommended (Estrada-Pena and Jongejan 1999; Faulde and Hoffmann 2001). In the case of *Ixodes ricinus*, the colour of clothing may be important. Although ticks are easier to see on light-coloured cloth (Kahl 1996), evidence from Sweden suggests that nymphal *I. ricinus* prefer people wearing such cloth to those wearing dark fabric. After individuals alternately wearing light and dark clothes walked through a

tick-infested area, 62% of the ticks were found on the light cloth and only 38% on the dark cloth (Stjernberg and Berglund 2005).

If ticks are encountered, they can be removed from clothing by hand (Armed Forces Pest Management Board 2009). However, crushing them with your fingernails should be avoided because their body fluids may contain pathogens and therefore be infective (Armed Forces Pest Management Board 2009). Removal of ticks as soon as possible is recommended to reduce the likelihood of disease transmission (Faulde and Hoffmann 2001). After removal, disposal may pose a problem. If returned to the immediate area, ticks may reattach to the clothing or attack another individual. They can be destroyed by placing them in alcohol or by securing them within a piece of folded tape (Armed Forces Pest Management Board 2009). It is important to control for ticks which have already attached. Once clothing is removed, it is important to carefully check all areas of the body for ticks. Re-examine the clothing, inside and out, and remove and dispose of all ticks. Fine, pointed forceps are the best means of removing attached ticks (Armed Forces Pest Management Board 2009).

A wide variety of effective repellents have been tested, including permethrin (Faulde et al. 2008), DEET (N,N-diethyl-m-toluamide) alone and in combination with other compounds (Staub et al. 2002; Faulde and Hoffmann 2001) and dodecanoic acid (Schwantes et al. 2008).

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Chapter 16
What Else Besides TBE and Borreliosis? Tick-Transmitted Pathogens in Germany and Beyond

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Abstract **Tick-borne diseases** are an important problem in European countries. In this chapter, we review the biology and distribution of five tick-transmitted pathogens in Germany and neighbouring countries Austria and Switzerland. We focus on the bacterial pathogens *Rickettsia* spp., *Anaplasma phagocytophilum* and *Francisella tularensis*, the protozoan parasite *Babesia* spp. as well as on Eyach virus. The diagnosis of these pathogens is difficult, because the majority of the infections result in mild or self-limited diseases. However, all these pathogens may induce severe clinical symptoms; therefore, their importance is not to be underestimated in the diagnosis of tick-borne diseases.

16.1 Introduction

Tick-borne encephalitis (TBE) and **Lyme Borreliosis** are probably the most common and best-known tick-borne diseases in Germany, Austria and Switzerland. Other pathogens have however gained more and more attention in recent years, because their potential to cause diseases in humans or their endemic occurrence in natural foci has been clearly demonstrated. Many tick species may act as vectors of bacterial, viral and protozoan pathogens. However, four species are of particular

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Fig. 16.1 Unfed female of *Ixodes ricinus* (From Mehlhorn and Mehlhorn 2009)

Fig. 16.2 Unfed female of *Dermacentor reticulatus* (From Mehlhorn and Mehlhorn 2009)

medical importance in central Europe: *Ixodes ricinus*, *Dermacentor marginatus*, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* (Figs. 16.1–16.3).

The most prevalent tick-species in Germany, Austria and Switzerland is ***I. ricinus***. Typically, it inhabits mixed forests with herbaceous vegetation (Kimmig et al. 2000). Because of the wide distribution and the broad host range as well as its high affinity for humans, *I. ricinus* is the main vector of tick-transmitted pathogens in Europe.

Ticks of the genus *Dermacentor* (i.e. *D. marginatus* and *D. reticulatus*) are not as abundant as *I. ricinus* and prefer different habitats. *D. marginatus* needs warm and dry conditions; it mainly occurs on pastures and grasslands in river valleys. In Germany, its distribution is currently limited to the Rhine valley and parts of the

Fig. 16.3 Brown dog tick *Rhipicephalus sanguineus* (From Mehlhorn and Mehlhorn 2009)

Main valley (Liebisch and Rahmann 1976). *D. reticulatus* is adapted to colder and more humid areas and, therefore, shows a wider distribution. Both species are also distributed in Austria and Switzerland, but data about their precise distribution in these countries are not available.

Rhipicephalus sanguineus is a thermophilic tick, which is widely distributed in the Mediterranean area, but it has also spread to many other parts of the world. The occurrence of *R. sanguineus* in Austria is uncertain, but natural foci were detected in the canton Ticino in Switzerland (Bernasconi et al. 2002). The colder climate in central and northern Europe prevents this tick from breeding in the environment, but it can survive and breed inside houses and may become a pest problem. Up to now, the occurrence of *R. sanguineus* in Germany is mainly related to animal transports from Mediterranean countries. However, *R. sanguineus* reveals a different vector competence compared to *I. ricinus* and *Dermacentor* spp., and this has to be taken into account in the diagnosis of tick-borne diseases.

In order to study the distribution of tick-borne pathogens, ticks and potential mammalian hosts are examined. The results of such studies indicate (a) if the pathogen is established in natural foci and (b) if these pathogens may pose a risk for humans if these natural foci exist. To estimate if humans are actually exposed to these pathogens, seroepidemiological studies are conducted. These studies can be carried out with members of the general population or can be focussed on "high risk groups". The latter includes people that are constantly exposed to ticks and, therefore, have an increased risk to acquire tick-borne diseases. Hunters and forestry workers are members of these high risk groups and participate in such studies.

In this chapter, we review some tick-transmitted pathogens that are (or might be in the near future) of medical importance in Germany as well as in Austria and Switzerland. We focus on the biology and distribution of the bacterial pathogens *Rickettsia* spp., *Anaplasma phagocytophilum* and *Francisella tularensis*, the protozoan parasite *Babesia* spp. and the Eyach virus. To our knowledge, these data summarize the current status of distribution of these pathogens. The "status quo" is important because it provides the basis for further studies in order to elucidate and

to evaluate any changes in the distribution of these pathogens, which may occur in the future.

16.2 *Rickettsia* spp.

Rickettsia spp. are bacteria with worldwide distribution that are transmitted by various arthropod species. These pathogens have been known as causative agents of human diseases for several hundred years (Raoult and Roux 1997).

16.2.1 Taxonomy

Bacteria of the genus *Rickettsia* belong to the **α-Proteobacteria**, order *Rickettsiales*. Traditionally, the genus *Rickettsia* was divided into two subgroups (Raoult and Roux 1997). The **typhus group** includes only two species: *Rickettsia prowazekii*, the causative agent of epidemic typhus, and *R. typhi*, the causative agent of murine typhus. These species are transmitted by lice and fleas, respectively. The **spotted fever group** comprises more than 20 species that are transmitted by hard ticks (*Ixodidae*). For a long time, *R. akari* and *R. felis* were also included in the spotted fever group, although they are transmitted by mites and fleas, respectively. Recently, phylogenetic analyses revealed new insights into the taxonomic relationships in the genus *Rickettsia* and resulted in the reorganisation of subgroups. Gillespie et al. (2007) showed that *R. akari* and *R. felis* represent a cluster of its own called the transitional group. Additionally, non-virulent species such as *R. bellii* and *R. canadensis* are grouped in the ancestral group (Gillespie et al. 2007; Dobler and Wölfel 2009). Other studies propose even more subgroups, as they include also *Rickettsia* species that are symbionts of non-bloodfeeding arthropods (mainly beetles) and other animals such as amoeba and leeches (Weinert et al. 2009; Perlman et al. 2006). In summary, the classification of the numerous *Rickettsia* species is still a matter of controversy.

16.2.2 Biology

Rickettsiae are gram-negative, short rod-shaped or coccoid bacteria that are about 0.8–2 μm in length and about 0.3–0.5 μm in diameter (La Scola and Raoult 1997). The bacteria are obligate intracellular organisms and infect endothelial cells of small blood vessels as their main host cells (Parola et al. 2005).

Rickettsial species can be pathogenic or non-pathogenic for humans, and many species of (yet) unknown pathogenicity have been described (Raoult and Roux 1997). In recent years, several species that were thought to be non-pathogenic were

demonstrated to be associated with human diseases, for example *R. raoultii* (Parola et al. 2009) and *R. helvetica* (Nilsson et al. 1999). It is assumed that all rickettsiae may be pathogenic to humans, as long as their arthropod hosts are capable of feeding on humans (La Scola and Raoult 1997).

Spotted fever group rickettsiae can be transmitted both transovarially and transstadially in their arthropod hosts (Azad and Beard 1998); therefore, all developmental stages of ticks can serve as vectors.

16.2.3 Disease

After inoculation of the rickettsiae into the host, the bacteria proliferate in endothelial cells close to the inoculation site. This leads to dermal and epidermal necrosis, resulting in skin reactions that are called **eschar** or "**tache noire**". Further proliferations cause vasculitis and a typical rash that is characteristic for most rickettsioses (Martinez and Cossart 2004). Other common symptoms include fever, headache, muscle pain and local lymphadenopathy. The bacteria may be disseminated via lymph and blood to various organs including lungs, liver, spleen, kidney and heart (Martinez and Cossart 2004). The clinical symptoms and the severity of human diseases are different for the various rickettsial species. Rickettsioses may induce only mild symptoms if the infection remains localised, but they can also become life threatening or even lethal if infestations of inner organs result in internal bleeding (Parola et al. 2005). **Antibiotic therapy** is used to treat rickettsial infections, and **doxycycline** is the treatment of choice for all tick-transmitted rickettsioses (Parola et al. 2005).

16.2.4 Vertebrate Hosts

It is assumed that a broad range of mammals, including rodents, dogs, rabbits and deer, are susceptible hosts for rickettsiae (Azad and Beard 1998; Raoult and Roux 1997). But studies about the actual prevalence of *Rickettsia* spp. in wild animals are scarce and exist only for few species.

Dogs are known to be important hosts for *R. conorii*, the causative agent of **Mediterranean spotted fever** (Rovery et al. 2008). Recently, Menn et al. (2010) detected antibodies against *R. conorii* in 68.2% of dogs in an endemic area in Portugal. Wild boars seem to be important hosts for *R. slovaca*. Ortuno et al. (2007) showed that 52.2% of wild boars in northeastern Spain developed antibodies against *R. slovaca*. In 1992, Rehacek et al. identified different rodent species (including *Microtus arvalis* and *Apodemus flavicollis*), which are susceptible to infections with rickettsiae. Recently, spotted fever group rickettsiae were identified in 9.1% of rodents in China. Zhan et al. (2009) demonstrated by means of PCR of spleen samples that both *Arvicolidae* and *Muridae* were infected with rickettsiae.

Rodents from Bavaria and Baden-Wuerttemberg were studied for antibodies against *Rickettsia* spp. by serological methods (Schex and Essbauer, personal communication; Pluta, unpublished data). The preliminary results of these still ongoing projects indicate that rodents serve as hosts for *Rickettsia* spp. in Germany. Comparable data about the importance of certain vertebrates as hosts for rickettsiae in Austria or Switzerland are not available.

16.2.5 Distribution in Germany

16.2.5.1 Rickettsiae in Ticks

The first isolation of ***R. slovaca*** was published in Slovakia in 1969 and it was characterised as a new species in 1978 (Sekeyova et al. 1998). Since then, it has been found in ticks in several European countries (Parola et al. 2005). Rehacek et al. (1977) identified *R. slovaca* in 8 out of 51 *D. marginatus* ticks from southern Baden-Wuerttemberg in 1977.

From 2006 to 2009, we collected about 1,100 questing *D. reticulatus* and *D. marginatus* ticks in southern Germany. By PCR methods and DNA sequencing, we were able to identify *R. slovaca* in 16% of *D. marginatus* ticks from Aschaffenburg (Bavaria) and in 0.9% of *D. marginatus* ticks from Karlsruhe (Baden-Wuerttemberg), respectively (Pluta et al. 2009; Pluta, unpublished data). For a long time, *R. slovaca* was considered to be non-pathogenic for humans. However, Raoult et al. (1997) identified *R. slovaca* in a patient with febrile illness in France by PCR and serological methods. Because of the main symptom, enlarged and painful lymph nodes close to the tick bite, this rickettsiosis was subsequently named tick-borne lymphadenopathy (TIBOLA) (Lakos 1997). Other common symptoms include an eschar and/or erythema at the site of the tick bite, fever and local alopecia, whereas a rash is present only in few cases. Besides our detection of *R. slovaca* in *D. marginatus* ticks, we could recently identify this pathogen in a tick removed from a patient in Rhineland-Palatinate (Pluta et al. 2009). The patient was bitten by a *D. marginatus* tick and subsequently fell ill with fever, lymphadenopathy of submandibular lymph nodes and an exanthema at the site of the tick bite. IgG and IgM antibodies against rickettsiae of the spotted fever group were detected by an immunofluorescence assay (IFA), whereas an IFA detecting antibodies against typhus group rickettsiae remained negative. *R. slovaca* was identified in the tick removed from the patient by means of PCR and DNA sequencing. Therefore, we were able to confirm the first autochthonous case of tick-borne lymphadenopathy in Germany.

In 2008, ***R. raoultii*** was characterised as a new species (Mediannikov et al. 2008).

31.6% of *Dermacentor* ticks collected in the Rhine valley near Lörrach harboured *R. raoulti* (Pluta et al. 2010). This was confirmed by PCR methods followed by DNA sequencing. Additionally, 38.5, 39 and 3.2% of *Dermacentor*

ticks collected in the districts Karlsruhe (Baden-Wuerttemberg), Freiburg (Baden-Wuerttemberg) and Aschaffenburg (Bavaria), respectively, and five out of six ticks in the district Esslingen (Baden-Wuerttemberg) were infected with this pathogen (Pluta, unpublished data). In 2006, Dautel et al. studied *D. reticulatus* ticks that were removed from deer in southern and eastern Germany. They detected *R. raoultii* in 23% of ticks collected in the federal states of Bavaria, Brandenburg, Saxony and Saxony-Anhalt.

On the basis of these data, it can be assumed that *R. raoultii* is widely distributed in *Dermacentor* tick populations in Germany. *Rickettsia raoultii* has been regarded as non-pathogenic. However, Parola et al. (2009) studied samples of 86 patients with tick-borne lymphadenopathy in France. *Rickettsia raoultii* was the causative agent of the disease in 8% of the cases, whereas the other infections were due to *R. slovaca* infections. On the basis of these results, Parola et al. (2009) believe that *R. raoultii* can cause tick-borne lymphadenopathy but seems to be less pathogenic than *R. slovaca*, as it was shown to be prevalent in fewer patients. Although the prevalence of *R. raoultii* in *Dermacentor* ticks in Germany is very high, no cases of *R. raoultii* infection in humans have been reported until now.

Rickettsia helvetica was isolated for the first time from *I. ricinus* ticks in Switzerland (Burgdorfer et al. 1979). These bacteria have been frequently found in *I. ricinus* ticks in many areas of Germany with infection rates up to 13%. Hartelt et al. (2004) studied about 1,100 *I. ricinus* from various districts in southern Baden-Wuerttemberg. In these areas, the *R. helvetica* infection rate was 8.9% on average, ranging from 5.6 to 13.3%. In Bavaria, a prevalence of 12% was detected in 2006 (Wölfel et al. 2006), whereas Silaghi et al. (2008b) revealed a lower prevalence of 4.8%. In a forest near Berlin, 14.2% of *I. ricinus* nymphs were infected with *R. helvetica* (Pichon et al. 2006).

Hildebrandt et al. (2010) detected *Rickettsia* spp. in 14.7% of *I. ricinus* ticks in Thuringia and identified *R. helvetica* by DNA sequencing in some of the samples. In Germany, *R. helvetica* has been detected in *I. ricinus* ticks only, but *Dermacentor* ticks are also possible vectors of this pathogen. Dobec et al. (2009) detected *R. helvetica* in 10% of *D. reticulatus* ticks in Croatia. *Rickettsia helvetica* was considered a non-pathogenic species for a long time (Raoult and Roux 1997). However, it was associated with two cases of perimyocarditis and sudden cardiac death in Sweden in 1999 (Nilsson et al. 1999). It has also been detected in a patient with meningitis in Sweden (Nilsson et al. 2010) and in patients with febrile illnesses in France and Italy (Fournier et al. 2000, 2004). Up to now, no cases of *R. helvetica* infection have been reported in Germany.

Rickettsia monacensis was isolated for the first time in 2002 from an *I. ricinus* tick collected in the English Garden in Munich, and was subsequently characterised as a new species (Simser et al. 2002). Since then, this pathogen has been found now and then in *I. ricinus* ticks in different parts of Germany. Silaghi et al. (2008b) detected *R. monacensis* in 0.5% of ticks from southern Bavaria, and Hildebrandt et al. (2010) demonstrated this pathogen in ticks in Thuringia. This indicates that *R. monacensis* is distributed in ticks throughout Germany, but apparently with a low prevalence. In Spain, Jado et al. (2007) reported infections of two patients with

R. monacensis. Both patients had a rash and unspecific flu-like symptoms after tick bites. The authors isolated the pathogen and characterised it by DNA sequencing as *R. monacensis*, showing that it is able to cause disease in humans. Up to now, there are no reports about *R. monacensis* infections in humans in Germany.

Antibody Prevalences in Humans

Until now, there is only one report of an autochthonous rickettsiosis caused by *R. slovaca* in Germany (Pluta et al. 2009). In contrast, no cases of infection with *R. raoultii*, *R. helvetica* or *R. monacensis* have been found, although they were frequently detected in ticks in Germany. However, in 2008, Jansen et al. conducted a seroepidemiological study with 286 hunters from all over Germany. They detected antibodies against *Rickettsia* spp. in 9.1% and antibodies specific for *R. helvetica* in two of the participating hunters. Nearly all participants noted at least one tick bite within the year before the study, and some of them remembered having symptoms that are compatible with rickettsiosis, but none of them was diagnosed with an acute rickettsial infection.

16.2.5.2 Distribution in Austria

In Austria, only very few data about the occurrence of *Rickettsia* spp. in ticks are available.

Until now, only *R. helvetica* was detected in *I. ricinus* ticks. Dobler et al. (2008) identified *R. helvetica* in 5.6% of *I. ricinus* ticks from the federal state of Burgenland in the eastern part of Austria. Blaschitz et al. (2008b) collected ticks in different areas of Austria and showed that 35.6% of the ticks harboured *Rickettsia* spp. Further studies revealed that the ticks were infected with *R. helvetica* only, although not all of the samples were sequenced. To our knowledge, the antibody prevalence in humans is not known and there are no reports about rickettsial infections.

16.2.5.3 Distribution in Switzerland

Rickettsiae in Ticks

Three different rickettsial species have been identified in *I. ricinus* and *Rhipicephalus* spp. ticks. In 1979, Burgdorfer et al. detected a new *Rickettsia* species in 8.4% of *I. ricinus* ticks, which was subsequently characterised as ***R. helvetica***. Boretti et al. (2009) identified this species in 12% of unfed and 36% of engorged *I. ricinus* from the canton of Zurich. Additionally, ***R. monacensis*** was found, but only in one tick. Furthermore, Bernasconi et al. (2002) identified ***R. massiliae*** in three *R. sanguineus* ticks from southern Switzerland (canton Ticino). This species has recently been linked to human disease (Parola et al. 2005).

Human Infections

In 2003, Baumann et al. examined patients with febrile illnesses following tick bites for various tick-borne pathogens. In eight patients, they were able to show antibodies against spotted fever group rickettsiae, and assumed that these antibodies were directed against *R. helvetica*. This study indicates that infections with tick-borne rickettsiae occur in Switzerland.

16.2.6 Conclusions

The numerous detections of various pathogenic species of rickettsiae in ticks in Germany, Austria and Switzerland revealed that there are considerable risks for humans to acquire rickettsioses. This is supported by the detection of autochthonous cases of rickettsioses in Germany and Switzerland as well as by seroepidemiological studies of hunters in Germany. The results of these studies emphasize that tick-borne rickettsioses have to be included in the differential diagnosis of tick-borne diseases in these countries.

16.3 *Anaplasma phagocytophilum*

The bacterial pathogen *A. phagocytophilum* is distributed in the northern hemisphere, and ixodid ticks of the genera *Ixodes* and *Dermacentor* act as vectors. The bacterium is well known as an agent of veterinary importance, but in the last years, it has received more and more attention as a pathogen causing disease in humans.

16.3.1 Taxonomy

Anaplasma phagocytophilum is classified as **α-Proteobacterium** and belongs to the order *Rickettsiales*, family *Anaplasmatacae*. Recent studies (Dumler et al. 2001; Taillardat-Bisch et al. 2003) based on the analysis of the 16S rDNA gene, the heat shock protein (GroEL) and the β-subunit of the RNA-polymerase, initiated a reorganisation within the genera *Ehrlichia, Anaplasma* and *Neorickettsia*. The former genus *Ehrlichia* was divided into three groups based on the nucleotide sequence of the 16S rDNA gene: *Ehrlichia canis* genogroup, *Ehrlichia phagocytophila* genogroup and *Ehrlichia sennetsu* genogroup. All members of the *E. phagocytophila* genogroup, which included *E. phagocytophila, Ehrlichia equi* and the agent of human granulocytic anaplasmosis (HGA) (formerly known as **human granulocytic ehrlichiosis**), are now described as *A. phagocytophilum*.

16.3.2 Biology

Anaplasma phagocytophilum is a small (0.4–1.9 μm), coccoid, **gram-negative bacterium**. It infects granulocytes and multiplies in intracytoplasmatic vacuoles, so-called morulae. According to its target cells, the disease is known as human granulocytic anaplasmosis (Maurin et al. 2003).

16.3.3 Disease

Infections with *A. phagocytophilum* remain asymptomatic in 60% of cases. Symptomatic infections are usually mild and self-limited with unspecific symptoms. After an incubation period of 2–7 days, the infection is characterised by **flu-like symptoms** with fever, arthralgia and malaise; thrombocytopenia and elevation of liver enzymes are typically observed. An exanthema can be present in some cases, and severe disease courses can lead to neurological disorders such as meningitis or meningoencephalitis. *Anaplasma phagocytophilum* may suppress the immune system; this is often followed by secondary infections such as candidiasis and pneumonia. The severity of disease depends on the age of the patients, the capability of their immune system and the timely administration of **antimicrobial therapy** that consists of **tetracyclines, fluoroquinolones or rifampicin** (Maurin et al. 2003). Severe and fatal infections mainly occur in patients with a deficient immune system. According to criteria of the WHO, human granulocytic anaplasmosis is confirmed if (a) an at least fourfold increase of IgG antibodies is observed, (b) *A. phagocytophilum* is detected by PCR, or (c) *A. phagocytophilum* can successfully be grown in cell culture (Bakken and Dumler 2000).

16.3.4 Vertebrate Hosts

It is supposed that *A. phagocytophilum* may infect a broad range of mammals, including wild and domestic animals as well as several rodent species (Strle 2004).

Anaplasma phagocytophilum infections were detected by PCR in 5.3% of rodents from southern Germany and in 7% of rodents from western Switzerland (Hartelt et al. 2008; Liz et al. 2000). In both studies, infection rates of *Myodes glareolus* were considerably higher than those of *Apodemus* spp. or *Microtus* spp. This indicates that *M. glareolus* is a particularly suitable host for *A. phagocytophilum.* Recently, Skuballa et al. (2010) studied different organs of 31 hedgehogs (*Erinaceus europaeus*) from various regions in Germany by means of PCR and sequencing. They detected *A. phagocytophilum* in eight of these hedgehogs (25.8%) and discussed the importance of hedgehogs as hosts for this pathogen. *Anaplasma phagocytophilum* was also detected in cervids in Switzerland and Austria with prevalences ranging from 29 to 60% (Liz et al. 2002; Petrovec et al. 2003; Polin

et al. 2004). However, sequencing revealed differences in the amplified genes of the pathogens isolated from cervids and humans. Therefore, the question whether *A. phagocytophilum* of cervids are responsible for human infections remains open.

16.3.5 Distribution in Germany

16.3.5.1 Ticks

In different studies carried out in Baden-Wuerttemberg, *A. phagocytophilum* was detected in 1.2, 2.8 and 1% of *I. ricinus*, respectively (Baumgarten et al. 1999; Oehme et al. 2002; Hartelt et al. 2004). The pathogen was also identified in *I. ricinus* in different parts of Bavaria. Baumgarten et al. (1999) described an infection rate of 2.6% in ticks from Frankonia in the northern part of Bavaria. Silaghi et al. (2008a) collected about 3,000 ticks in different areas in the south of Bavaria and observed a similar prevalence of 2.9%. Several reports confirmed that *A. phagocytophilum* is also widespread in eastern parts of Germany (Hildebrandt et al. 2002, 2010; Pichon et al. 2006). The infection rates vary between 2.3 and 5.4%. In 2003, von Loewenich et al. collected *I. ricinus* ticks in different parts of Germany and described a prevalence of 4.1%.

16.3.5.2 Antibody Prevalences in Humans

Hunfeld and Brade (1999) studied serum samples of 270 patients from the Rhine-Main area with history of a tick bite or confirmed Lyme Borreliosis and detected antibodies against *A. phagocytophilum* in 5.5% of all samples. In 2002, Oehme et al. screened sera from 4,368 forestry workers from Baden-Wuerttemberg for antibodies against *A. phagocytophilum*. The prevalences ranged from 5 to 16% in different areas with a mean prevalence of 10.7%. In a similar study, Bätzing-Feigenbaum et al. (2002) detected antibodies against *A. phagocytophilum* in 3.1 and 3.9% of forestry workers from Hesse and Berlin, respectively. In Berlin and Brandenburg, Kowalski et al. (2006) demonstrated *A. phagocytophilum* antibodies in 4.5% of patients with confirmed Lyme Borreliosis.

16.3.6 Distribution in Austria

16.3.6.1 Ticks

Anaplasma phagocytophilum was detected in 5.1 and 8.7% of questing *I. ricinus* ticks from various areas in Austria (Sixl et al. 2003; Polin et al. 2004). Additionally, Sixl et al. (2003) collected 178 *D. reticulatus* ticks, but none of them revealed an

infection with *A. phagocytophilum*, indicating that *D. reticulatus* is not a suitable vector for this pathogen in Austria.

16.3.6.2 Antibody Prevalences in Humans

Walder et al. (2003b) detected antibodies against *A. phagocytophilum* in 9% of sera from healthy blood donors from western Austria. Deutz et al. (2003) studied blood samples of 149 hunters from southern Austria. Interestingly, the prevalence of IgG antibodies against *A. phagocytophilum* was 15% and, therefore, remarkably higher than in people less exposed to ticks.

Human infections: Several cases of HGA were reported in Austria. Walder et al. (2003a) detected very high levels of both IgG and IgM antibodies in a patient with fever, flu-like symptoms and thrombocytopenia after a tick bite in Tyrol (southern Austria). The IgG antibody titre increased further in the following 6 weeks, whereas the IgM titre decreased. Therefore, Walder et al. confirmed the first autochthonous case of human granulocytic anaplasmosis in Austria. Further cases of HGA were identified by Walder et al. (2006) in five patients with febrile illness after tick bites and by Vogl et al. (2010) in a patient from eastern Austria.

16.3.7 Distribution in Switzerland

16.3.8 Ticks

In the cantons Zürich and Schaffhausen, *A. phagocytophilum* was detected in 1.3 and 2% of questing *I. ricinus* ticks, respectively (Pusterla et al. 1999; Liz et al. 2000). Additionally, the pathogen was identified in 1.4% of ticks collected in western Switzerland (cantons Bern and Neuchatel) (Liz et al. 2000). Wicki et al. (2000) screened 6,071 *I. ricinus* from various regions of Switzerland. They demonstrated *A. phagocytophilum* infections in 2.95% of adult ticks and in 0.94% of nymphs, respectively.

16.3.8.1 Human Infections

In 2000, Weber et al. detected antibodies against *A. phagocytophilum* in 8 out of 80 patients with febrile illness following tick bites and in 12 out of 48 patients who were seropositive for TBE or Lyme Borreliosis. Additionally, Baumann et al. (2003) revealed antibodies in seven patients with fever after tick bites.

Neither Weber et al. (2000) nor Baumann et al. (2003) were able to detect the pathogen by microscopic or molecular biological methods, and no fourfold increase in IgG antibody titres was observed. All of the described cases were probably due to

A. phagocytophilum infections, but it has to be pointed out that these infections did not match the WHO case definitions completely.

16.3.9 Conclusions

Anaplasma phagocytophilum was frequently detected in ticks and mammals in Germany, Austria and Switzerland. Moreover, high seroprevalences especially in persons that are highly exposed to tick bites were reported. Surprisingly, only few cases of HGA occurred in Austria and Switzerland, and no case has been reported from Germany so far. Because of the often unspecific flu-like symptoms, it might be possible that human infections are overlooked or misdiagnosed in some cases. However, *A. phagocytophilum* infections should be considered in cases of febrile illness after tick bites in these countries.

16.4 *Babesia* spp.

Babesiosis caused by the protozoan parasite *Babesia* spp. is a well-known disease of veterinary importance that attracts increasing attention as an emerging tick-borne disease in humans (Homer et al. 2000; Kjemtrup and Conrad 2000). More than 100 *Babesia* species are known, but only a few were identified as pathogens for humans.

16.4.1 Taxonomy

Parasites of the genus *Babesia* belong to the phylum Apicomplexa, order *Piroplasmida*.

Traditionally, the genus *Babesia* is divided into two groups based on their morphology and their size: the large *Babesia* species, including *B. bigemina* and *B. canis*, and the small *Babesia* species, for example *B. microti* and *B. gibsoni* (Homer et al. 2000). Phylogenetic analyses confirmed the separation of large *Babesia* species (also referred to as *Babesia* sensu stricto) and small *Babesia* species (or *B. microti*-like *Babesia*) (Gray et al. 2010).

16.4.2 Biology

Babesia spp. are obligate intracellular, pleomorph organisms that infest erythrocytes. Large *Babesia* species are about 2.5–5.0 μm in diameter, whereas small *Babesia* measure 1.0–2.5 μm in diameter (Hunfeld et al. 2008).

The life cycle of *Babesia* spp. includes an obligate host switch with ticks as definitive hosts and vertebrates as intermediate hosts. The asexual reproduction (merogony) takes place in the erythrocytes of the vertebrate, whereas sexual reproduction (gamogony) and further asexual proliferation (sporogony) occur in the tick. In contrast to small *Babesia* species, large *Babesia* species are transmitted transovarially in their tick vectors (Gray et al. 2010). All European *Babesia* species use *I. ricinus* as definitive host, but their intermediate hosts differ. *Babesia divergens* mainly infects cattle, whereas *B. microti* is found in rodents. The main host for *B. venatorum* seems to be the deer (Gray et al. 2010).

16.4.3 Disease

Babesiosis in Europe is mainly caused by *B. divergens*. Most patients are splenectomised, and severe, often lethal cases are observed frequently. *Babesia microti*, which causes many infections in North America, is rarely diagnosed in Europe. However, infections with this species also affect immunocompetent persons, and the course of disease is usually mild (Granström 1997; Kjemtrup and Conrad 2000). *Babesia venatorum* (previously known as strain *Babesia* sp. EU1) is also linked to human babesiosis in Europe. This species belongs to the large *Babesia* species and is closely related to *B. divergens* (Hunfeld et al. 2008). Most infections with *Babesia* spp. remain subclinical. Clinical manifestations have been observed mainly in immunocompromised patients and are often found in splenectomised patients. However, the number of infections in otherwise healthy persons has increased in the last years (Granström 1997; Kjemtrup and Conrad 2000).

After an incubation period of 1–3 weeks, a flu-like illness with fever, arthralgia and weakness emerges. Haemolysis leads to the main symptoms of acute infection, which are haemoglobinuria, haemolytic anaemia, hepatosplenomegaly and jaundice. In mild cases, the disease is self-limited and patients recover without any further problems. In immunocompromised or splenectomised and elderly patients, complications including acute pulmonary failure, congestive heart failure and renal failure may occur and may be lethal (Homer et al. 2000; Wang et al. 2000; Hunfeld et al. 2002a). Patients with severe babesiosis need **rapid treatment** with a combination of either **clindamycin** and **quinine or atovaquone** and **azithromycin** (Krause 2003). Additionally, a blood exchange transfusion may be necessary to reduce the number of parasites in the blood and to prevent further haemolysis. *Babesia* spp. infections are mainly due to tick bites, but transmission via blood transfusion may also occur (Leiby 2006).

16.4.4 Distribution in Germany

The information about the distribution of *Babesia* species in ticks as well as mammals is often restricted to certain areas in Germany. Hartelt et al. (2004)

screened more than 3,000 *I. ricinus* ticks in Baden-Wuerttemberg and identified *B. divergens* and *B. microti* in 0.9 and 0.1% of ticks, respectively. Additionally, they trapped 508 rodents in the same areas (Hartelt et al. 2008). 1.6% of *Arvicolidae* (*M. glareolus* and *M. arvalis*) were infected, but none of the *Muridae*. In 2002, Hunfeld et al. showed that exposure to *Babesia* spp. must frequently occur in Germany. They screened serum samples of 467 persons from the Rhine-Main area for antibodies against *Babesia* spp. IgG antibodies against *B. microti* and *B. divergens* were detected in 5.4 and 3.6% of participants, respectively. Additionally, antibody prevalences were considerably higher in persons with previous tick bites (11.5%) than in persons without tick exposure (1.7%) (Hunfeld et al. 2002b). The first report about an autochthonous case of human babesiosis in Germany was published in 2005 (Häselbarth et al. 2007). The splenectomised patient living in the south of Baden-Wuerttemberg showed typical signs of babesiosis, including haemolytic anaemia and haemoglobinuria. *Babesia venatorum* was detected in the peripheral blood by microscopy and PCR. Hildebrandt et al. (2007) confirmed another case of human babesiosis due to *B. microti* in a patient with acute myeloid leukaemia in Thuringia by PCR and microscopy of peripheral blood smears. They assumed that the patient acquired the infection via blood transfusion. This was confirmed by the detection of *B. microti* antibodies in serum samples of the blood donor. Neither the patient nor the donor travelled recently to countries with high risk of *B. microti* infections (especially North America). The authors assumed that this is the first autochthonous case of a *B. microti* infection in Germany.

16.4.5 Distribution in Austria

Blaschitz et al. (2008a) studied 853 *I. ricinus* from different sampling sites in Austria. The results of their study revealed a surprisingly high infection rate with *Babesia* spp. of 51.7%. Infection rates ranged from 0 to 100% in the different sampling areas. All positive samples were sequenced and identified as *B. divergens* or *B. divergens*-like strains, respectively. Blaschitz et al. assumed that the striking differences in prevalences might be due to a very focal distribution of the pathogen, and that the high overall infection rate could be explained by high tick population densities. In 2003, Herwaldt et al. reported the first autochthonous case of human babesiosis in Austria. According to PCR and DNA sequencing, the disease of a splenectomised patient was due to an infection with *B. venatorum*.

16.4.6 Distribution in Switzerland

Several studies concerning *Babesia* spp. infections in ticks are available. Foppa et al. (2002) detected *B. microti* in 3.4% of *I. ricinus* nymphs from eastern Switzerland. Casati et al. (2006) examined *I. ricinus* ticks from various regions in Switzerland and identified *B. microti*, *B. divergens* and *B. venatorum* in 0.2, 0.2 and

0.4% of ticks, respectively. Hilpertshauser et al. (2006) studied ticks that were removed from wild and domestic ruminants (sheep, goat, cattle and deer) in southern Switzerland. They identified *B. divergens* and *B. venatorum* in 0.9 and 2% of ticks, respectively. Additionally, they found strains of *Babesia* spp. of yet unknown pathogenicity.

In 2002, Foppa et al. confirmed that the exposure of humans to *Babesia* spp. occurs in Switzerland more frequently than expected, because they detected IgG antibodies against *B. microti* in 1.5% of healthy blood donors from eastern Switzerland.

The first autochthonous case of **human babesiosis** in Switzerland was published in 2004 (Meer-Scherrer et al. 2004). *Babesia microti* was identified as the causative agent by PCR. Additionally, the patient suffered from concurrent *Borrelia burgdorferi* infection.

16.4.7 Conclusions

Pathogenic *Babesia* species are prevalent in ticks in all three countries and cases of human babesiosis were reported from Germany, Austria and Switzerland. The detection of antibodies against *Babesia* spp. indicates that people are frequently exposed to this pathogen without developing symptomatic infections. *Babesia divergens*, *B. microti* and *B. venatorum* were identified as causative agents not only in immunocompromised or splenectomised patients but also in healthy people.

16.5 *Francisella tularensis*

16.5.1 Biology

Francisella tularensis belongs to the **γ-proteobacteria**, family *Francisellaceae*. It is a facultative intracellular, small gram-negative bacterium. Because of its high infectivity and low infection dose, it was classified as a possible bioterrorist agent and, therefore, has gained increasing attention in recent years. Two subspecies are responsible for human infections. *Francisella tularensis* subspecies *tularensis* occurs only in North America, whereas *F. tularensis* subsp. *holarctica* is distributed in the northern hemisphere (Ellis et al. 2002).

16.5.2 Disease

Generally, *F. tularensis* subsp. *holarctica* induces mild infections, whereas severe forms of tularaemia (typhoid and pneumonic forms) are almost exclusively related to infections with *F. tularensis* subsp. *tularensis*.

The **symptoms of tularaemia** differ depending on the route of infection. Ulceroglandular tularaemia is the most common form and usually follows transmission by arthropod bites or inoculation of the bacteria through skin lesions. It is characterised by a sudden onset with flu-like symptoms such as fever, chills and headache, followed by the development of an ulcer at the site of infection that can persist for several months. Dissemination of the bacteria via lymph fluid leads to an enlargement of regional lymph nodes and lymphadenopathy. This form is usually mild and self-limited, but a spread of the pathogen to tissues of inner organs may lead to complications such as pneumonia. Ingestion of contaminated food or water leads to oropharyngeal tularaemia with a painful sore throat, enlargement of tonsils and swollen cervical lymph nodes or to gastrointestinal infection. Depending on the infection dose, a gastrointestinal infection can lead to mild and persistent diarrhoea or to severe intestinal ulceration that can be lethal. The most severe forms of tularaemia are the typhoid form without an identified route of infection, characterised by septicaemia and a mortality rate of 30–60%, and the pneumonic form that follows inhalation of *F. tularensis* and appears as pneumonia with very variable clinical signs (Ellis et al. 2002).

Tularaemia is usually treated with **streptomycin** or **gentamicin**. Because of the unspecific symptoms, the diagnosis of mild infections might often be missed.

16.5.3 Epidemiology

Francisella tularensis has been identified in a broad range of mammals, but only some species seem to be able to maintain the pathogen in enzootic cycles. *Francisella tularensis* subsp. *tularensis* mainly infects lagomorphs (rabbits and hares), while *F. tularensis* subsp. *holarctica* is detectable in lagomorphs as well as in rodent species. Various arthropods are able to transmit the pathogen to animals and humans. Tabanids and mosquitoes (*Aedes* spp., *Culex* spp., *Anopheles* spp.) are frequently involved in larger outbreaks of tularaemia. They often bite multiple hosts in short time periods, and, therefore, contribute to a rapid spread of the disease. It is assumed that both tabanids and mosquitoes are mechanical vectors and that the bacteria cannot survive in these arthropods for more than a few days. In hard ticks, however, the bacteria are present and multiply in the gut and in haemolymph, and they are transmitted transstadially. Therefore, ticks are capable of maintaining *F. tularensis* for a long time in nature. Transovarial transmission may occur exceptionally (Petersen et al. 2009).

In North America and Scandinavia, arthropod bites are the most important mode of transmission (Petersen et al. 2009). Only few studies were conducted in central Europe. Hubalek et al. (1998) detected *F. tularensis* in 2.8% of *D. reticulatus* in Austria, whereas Wicki et al. (2000) identified this pathogen in 0.12% of *I. ricinus* nymphs in Switzerland. In 2009, *F. tularensis* was found in 0.9% of *I. ricinus* ticks from Baden-Wuerttemberg (Oehme et al. unpublished data). Moreover, Hanke et al. (2009) reported a case of tularaemia in a 1-year-old

child living in the Southwest of Germany. They assumed that the toddler acquired the infection via a mosquito bite. These studies indicate that *F. tularensis* is distributed in arthropods in Germany, and that transmission of this pathogen via arthropod bites may occur.

However, in central Europe, hares and rodents seem to be the most important sources of human infections (Ellis et al. 2002). In Germany, the pathogen has been detected in 4.92% of rodents (*M. glareolus*, *Arvicola terrestris*, *Microtus* spp., *A. flavicollis*) trapped in areas with previous tularaemia outbreaks (Kaysser et al. 2008). Since 2005, the number of *F. tularensis* infections in hare increased in Germany. In summary, 25 infected hare were found between 2005 and 2009 in various federal states (P. Otto, personal communication). Additionally, specific antibodies were detected in 3.1% of wild boar (*Sus scrofa*) from Mecklenburg-Western Pomerania (Al Dahouk et al. 2005). It was also detected in 3 out of 167 hares in Switzerland (Friedl et al. 2005). Data about *F. tularensis* infections in mammals are not available for Austria so far. Besides sporadic cases of tularaemia, there are outbreaks with high numbers of patients from time to time. Such outbreaks often occur concurrently in humans and wild animals (Ellis et al. 2002). Infections are mainly acquired via skin lesions while handling infected animals, or by inhalation of aerosolised bacteria. In 2005, there was an outbreak of airborne tularaemia among participants of a hare hunt in Hesse (Hauri et al. 2010). It was subsequently shown that nine hunters got infected by inhalation of aerosols containing *F. tularensis* while they rinsed carcasses of infected hare.

Hunters have an increased risk of acquiring *F. tularensis* infection via contact to infected animals. Jenzora et al. (2008) detected antibody prevalence of 1.7% in hunters from western Germany, and Winter et al. (2010, unpublished data) showed a very high prevalence of 4.5% in hunters from Baden-Wuerttemberg. In Austria, 3.0% of hunters from the federal states of Styria and Burgenland revealed antibodies against *F. tularensis* (Deutz et al. 2003).

Similar seroprevalence studies in the general population indicate that infections with *F. tularensis* occur frequently, despite the low number of reported cases. In Germany, seroprevalences of 0.23, 3.0 and 2.32% in the general population were reported (Porsch-Özcürümez et al. 2004; Schmitt et al. 2005; Splettstoesser et al. 2008).

The seroprevalence data of both hunters and the general population contradict the very low number of reported cases. In Germany, an average number of three cases are reported per year, with most infections occurring in Baden-Wuerttemberg, Hesse and North Rhine-Westphalia (Grunow and Priebe 2007). However, the number of human cases in Germany has increased in the last years; in 2007, 21 cases were reported, which is the highest case number since 1958 (Splettstoesser 2008). The average number of infections in Austria is stated as 10–15 cases (Deutz et al. 2003). In Switzerland, only sporadic cases occur (Friedl et al. 2005).

16.5.4 Conclusions

The distribution of *F. tularensis* in arthropods in central Europe is still not known. Therefore, it is not certain if transmission by arthropod bites plays an important role in transmitting *F. tularensis* in these countries. More studies are necessary to clarify the epidemiology of this pathogen and its occurrence in natural foci.

16.6 Eyach Virus

In 1976, Rehse-Küpper et al. isolated a virus from *I. ricinus* in the Eyach valley in Baden-Wuerttemberg, which was subsequently named the Eyach virus. Sequence analyses revealed a close relationship with the Colorado tick bite fever virus (CTF virus) that was isolated in Colorado in the 1940s and identified as a disease agent causing a febrile illness after tick bites.

16.6.1 Biology and Disease

CTF and Eyach virus belong to the family **Reoviridae,** genus *Coltivirus*. They are non-enveloped, spherical, often icosahedral particles that measure about 75–80 nm in diameter. Target cells for the CTF virus are human haematopoietic precursor cells. The virus can persist in the erythrocytes and can be transported in other organs such as brain, spleen, bone marrow and myocardium. It is assumed that the Eyach virus shows the same characteristics (Charrel et al. 2004). To date, nothing is known about the disease course in human Eyach-virus infections, but it is supposed that it resembles that of CTF-virus infections. Colorado tick bite fever is usually a mild and self-limited disease with a characteristic biphasic fever that is present in half of the patients (Bowen 1988). After an incubation period of 3–6 days, a febrile illness with unspecific symptoms such as retroorbital headache, myalgia and arthalgia, pharyngitis and anorexia develops. Typically, fever lasts for 2–5 days, followed by an afebrile and asymptomatic interval of 2–7 days. Subsequently, a second febrile period with higher temperatures and involvement of inner organs may follow (Klasco 2002). Complications due to secondary infections, such as meningitis, encephalitis or myocarditis, are relevant in about 5% of patients and are mainly seen in children (Braun et al. 1999). No specific treatment against Colorado tick bite fever exists at the moment.

It is suspected that the Eyach virus causes similar symptoms in humans. This was supported by studies with experimentally infected new-borne mice that showed neurological symptoms, and most of them died due to encephalitis (Rehse-Küpper et al. 1976; Chastel et al. 1984).

16.6.2 Epidemiology

The Eyach virus was first isolated in Germany in 1976 (Rehse-Küpper et al. 1976). In 2004, Hartelt screened 3,260 ticks in Baden-Wuerttemberg and detected the virus in three of them, i.e. 0.1%. The pathogen was also identified in *I. ricinus* and *Ixodes ventalloi* from northwest France (Chastel et al. 1984). Up to now there have been no reports about this pathogen in other European countries. At the moment, nothing is known about mammalian hosts of the Eyach virus. Dobler et al. (2006) studied 166 sera of brown hare from northern Germany, but did not find any evidence for Eyach-infected animals. The pathogenicity of the Eyach virus for humans is unknown, for no human infections have been reported so far. But antibodies against this pathogen were detected in patients with meningoencephalitis in the Czech Republic (Malkova et al. 1980). This indicates that the Eyach virus is indeed pathogenic for humans. However, as no cases of Eyach-virus infection were reported in Europe and the infection rate of ticks in Germany seems to be rather low, it is very likely that an infection risk for humans does not exist at the moment. But the virus should be kept in mind for future studies.

16.7 General Conclusions of the Chapter

The data reported in this review summarise the current status of the distribution of some tick-transmitted pathogens in natural foci. However, these distribution patterns may change in the future, for example due to climate change and increasing average temperatures. It is generally assumed that changes in temperature have an important influence on ticks (Gray et al. 2009). If temperatures rise due to climate change, the distribution and abundance of some tick species (especially *Dermacentor* spp. and *R. sanguineus*) may be extended, and tick-borne pathogens might spread with them. Therefore, continuing studies are necessary to monitor any changes in the distribution and abundance of tick-transmitted pathogens. Future infection risks for humans can only be evaluated on the basis of these studies.

Ticks and pathogens that are not endemic in central Europe may establish in natural foci if the climate conditions become more suitable for them. For example, *R. sanguineus* could be imported and build up populations in Germany if the average temperatures rise in the future, and *Rickettsia conorii* may be introduced together with its main vector. Dogs are the main hosts for both *R. sanguineus* and *R. conorii*; therefore, dogs from endemic areas in the Mediterranean region can act as carriers for both the vector and the pathogen. Menn et al. (2010) detected antibodies against *R. conorii* in 68.2% of dogs from an endemic area in Portugal. This shows that the risk of introducing this specific pathogen with imported or travelling dogs is quite high.

All tick-borne diseases mentioned in this review can manifest as mild, self-limited illnesses with unspecific symptoms. Therefore, their diagnosis might often

be missed, and the current infection risk for humans may be underestimated. But infections with the above-mentioned pathogens can also lead to severe or even life-threatening disease courses. Hence, their medical importance should not be treated lightly, and they have to be kept in mind in the differential diagnosis of tick-borne diseases.

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Chapter 17
Tick-Borne Encephalitis: From Microfocus to Human Disease

G. Dobler, F. Hufert, M. Pfeffer, and S. Essbauer

Abstract Ticks transmit a number of pathogens to humans and animals. Among them, the most important arboviral human disease in Central Europe and Northern Asia is tick-borne encephalitis (TBE). The Western subtype of TBE virus (TBEV) in Central Europe is mainly transmitted by the tick *Ixodes ricinus*. The incidence and the numbers of human cases are thought to be correlated to tick activity. Two different, but closely located TBEV endemic foci in South Eastern Germany were studied. The results of our longitudinal studies in both foci showed that the areas, where positive ticks could be repeatedly detected, were relatively small in comparison to earlier descriptions. The data of two endemic foci of TBEV imply that the natural circulation of TBEV between ticks and rodents or other small mammals occurs in rather small areas, named microfoci. From these microfoci, TBEV-bearing ticks are dispersed eventually, probably by larger forest animals with a greater radius of activity than rodents. Human infection occurs if humans enter the microfocus area or if infected ticks are dispersed and occasionally come into contact with humans, for example in gardens near forests or on forest ways within the area of activity of the larger forest animals, named macrofocus or endemic area. Further studies are needed to show whether this concept of TBEV microfocus and TBEV macrofocus will also apply to other endemic areas such as for example in Southwestern Germany.

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17.1 Introduction

Tick-borne encephalitis (**TBE**) is caused by Tick-borne encephalitis virus (**TBEV**), a member of the tick-borne flavivirus subgroup of the family Flaviviridae. Three subtypes can be distinguished between the TBEV, a European subtype (TBEV-EU), a Siberian subtype (TBEV-SI), and a Far Eastern subtype (TBE-FE) (Ecker et al. 1999). All three subtypes of TBEV are circulating in nature in endemic cycles involving ticks as the natural vectors (*Ixodes ricinus* for TBEV-EU, *Ixodes persulcatus* for TBEV-SI and TBEV-FE) and rodents as the main natural hosts, mainly the yellow-necked mouse (*Apodemus flavicollis*) and the bank vole (*Myodes glarolus*) (Labuda et al. 1997). The geographical distribution of the virus subtypes is closely associated with the geographical range of the vector ticks. The role of other forest animals is not known exactly or is thought to be marginal for the transmission cycle.

During the last years many factors were determined to influence the occurrence of TBE endemic cycles (Dobler et al. 2005). Among these factors, climate changes and especially global warming were accused as the main causative factors for the fluctuating increase of TBE vector activities and of the increasing geographic distribution of the TBEV foci (Eisen 2008). While extensive models were developed for the understanding of the influence of climatic factors on TBEV activity, only limited information is available on the understanding of the activity of single natural foci. Studies on the characterization of natural TBE foci and the longitudinal studies on these cycles are still missing or date back to the 1960s (Loew et al. 1963, 1964; Radda et al. 1963; Pretzmann et al. 1963, 1964). Another classical work was published by Czech researchers describing the spatial distribution and stability of natural foci of TBEV in the former Czechoslovak Republic (Nosek et al. 1978). In these former studies the concept of the natural transmission of TBEV from rodents to ticks was based on the short-termed viremic phase of these rodents. Meanwhile, non-viremic transmission by co-feeding ticks is thought to play a major role in the transmission of TBEV between ticks and rodents (Labuda et al. 1993).

In a universal model of arbovirus transmission in nature, Aspöck showed the importance of the three major factors for the arbovirus transmission cycle: the virus, the arthropod (vector) and the vertebrate (host) (Aspöck 1970) (Fig. 17.1). In the calculations based on 2 years of observation of an Austrian TBEV focus it was estimated that a TBEV endemic focus needed a minimal size of more than 50,000 m^2 (Pretzmann et al. 1963). In this area the number of rodents high enough to sustain the TBEV transmission cycle was estimated to be about 700. The number of ticks living in this focus was estimated at 1.5–2.5 million ticks of all three developmental stages (Pretzmann et al. 1963). Already in those former studies a correlation between the occurrence of human clinical TBE and the highest number and activity of *Ixodes ricinus* was detected (Radda et al. 1963). A more recent comparison of human TBE cases and prevalence of TBEV in *Ixodes ricinus* in Southern Germany did not show a clear correlation, however, the absolute number

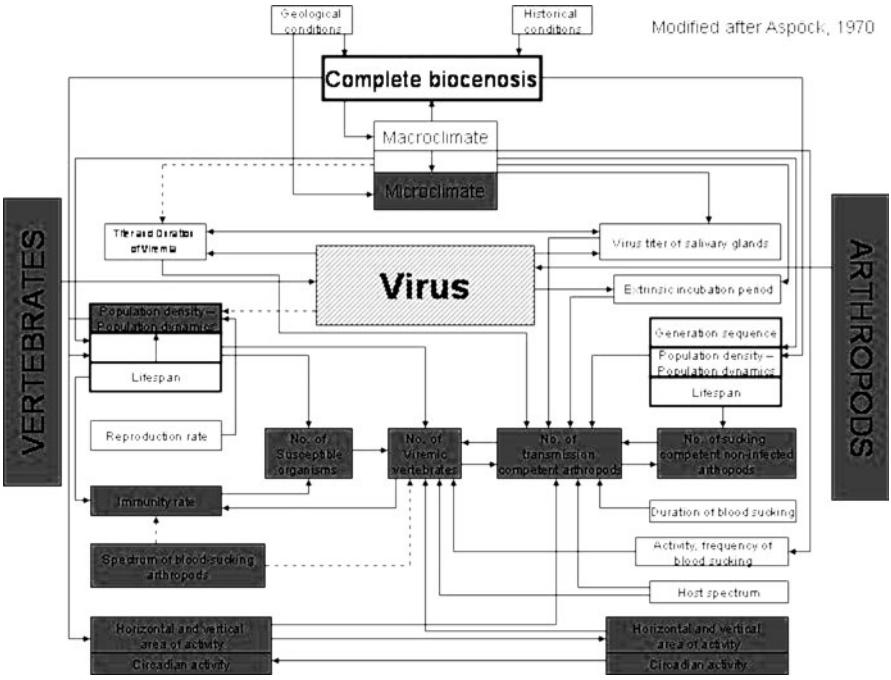

Fig. 17.1 The synecological relationship of factors of an arbovirus transmission cycle (Modified after Aspöck (1970))

of ticks was not reported in this study (Süss et al. 2004). Therefore, our knowledge on the activity and dynamics of individual TBE natural foci still relies mainly on data and models from the 1960s, except for the preliminary characterization of focus A presented here (Kupca et al. 2010).

17.2 Methods

We present first data on the longitudinal observation of **two TBE natural foci in Germany**. Both foci are located in the Administrative District of Amberg in the region of Upper Palatine in Eastern Bavaria, South Germany. In 2005, one of the two foci (focus A) was detected after the occurrence of an unusually high number of human TBE cases with unusual severity (Kupca et al. 2010) in a small village. A TBE virus strain was isolated from this focus showing a unique nucleotide and amino acid sequence of the E gene. Therefore, this strain could be easily identified and distinguished from any other known TBEV strain by sequencing of the E gene. A second focus (focus B) was detected in 2009 when two human cases occurred in autumn 2008 and spring 2009, respectively. Focus B is about 10 km south of focus

A. The identified virus does not have the unique nucleotide sequence of the E gene of the TBEV strain of focus A. We present the results of the longitudinal and spatial tick abundance and TBEV prevalence rates in the two foci. An experimental model is developed based on the data found and this model is discussed in respect of other current transmission models.

Ticks were collected by flagging the vegetation. In focus A ticks were collected once per year from 2005 to 2008. They were processed individually or in pools of five individuals. Focus B was sampled monthly from April to October 2009. In focus B ticks were sampled once per month. Sampling was conducted for 2 h at identical times (2 h before dawn) and places of collection. Therefore, the monthly sampling data of this focus are comparable regarding the sampling techniques. The location of tick sampling could be identified within the sampling area in areal grids. Ticks were pooled in groups of five (adult males, adult females) and ten animals (nymphs, larvae) and were processed for detection of TBEV by real time-Reverse Transcriptase-PCR (rt-RT-PCR) and for virus isolation as described previously (Kupca et al. 2010).

17.3 Results

In focus A from 2005 to 2008 a total of 2,150 ticks, mainly nymphs and adults were collected (Table 17.1). A total of five TBEV-positive ticks were detected. One tick in 2005 in sector N was found to be positive by rt-RT-PCR and a virus was isolated showing a unique nucleotide sequence of the E gene. In subsequent years no virus could be detected in section N in a total of 360 ticks collected (Table 17.1). However, repeatedly, TBEV RNA-positive ticks were detected by rt-RT-PCR in subsequent years in sector K. In these TBEV-positive ticks, also the unique E gene nucleotide sequence could be determined and therefore the ongoing circulation of this unique TBEV strain could be confirmed.

In focus B, in the six monthly sampling activities a total of 1,929 ticks were collected (Table 17.2). In focus B nine TBEV-positive ticks were detected by rt-RT-PCR during 2009. Seven of the nine positive ticks were found in sector II,

Table 17.1 Tick samples per sector and year in TBEV focus A. Rt-RT-PCR-positive ticks are in parentheses

Sector	2005	2006	2007	2008
F	39	40	n.d.	9
I	44	171	n.d.	n.d.
J	60	n.d.	n.d.	n.d.
K	n.d.	n.d	699 (2)	147 (2)
L	12	n.d.	n.d.	129
M	n.d.	n.d.	109	n.d.
N	48 (1)	52	182	78
O	22	n.d.	82	120
Total	225	263	1.072	483

Table 17.2 Tick stages sampled per sector and per month in 2009 in TBEV focus B. Rt-RT-PCR-positive ticks are in parentheses

Sector	Year	♂	♀	N	L
I	2008	33	31	144	40
II	2009	101(1)	219 (6)	726	92
III	2009	103 (1)	123	260 (1)	6
IV	2009	4	1	12	34
Total		241 (2)	374 (6)	1142 (1)	172

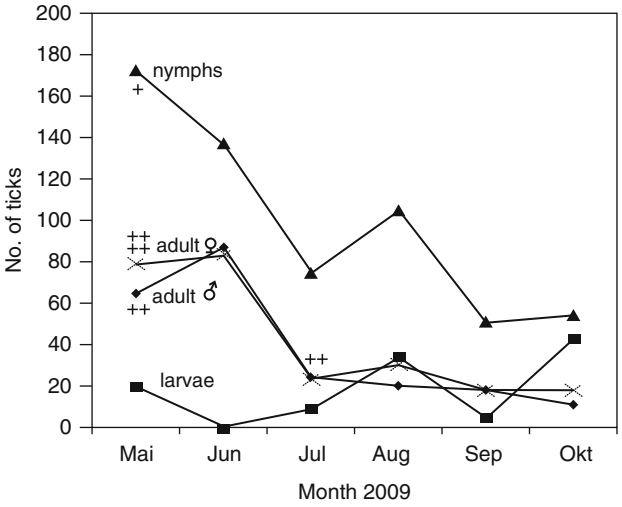

Fig. 17.2 Absolute number of sampled tick stages per month of focus B in 2009. ▲ nymphs; × adult females; ♦ adult males; ■ larvae; + TBEV-positive ticks. Total numbers of ticks are sampled per standardized sampling activity for 2 h before dawn

while the remaining two positive ticks were collected in section III which is adjacent to section II. It was surprising, that eight out of nine positive ticks were adults. Only one nymph was found to be positive, although twice as many nymphal stages than adult stages were found in total. No positive larvae were found. The ratio of the different tick stages during the year and the appearance of TBEV-positive ticks is shown in Fig. 17.2. The minimum infection rate for the sampled female adults was found to be 1.6% (6/374), followed by adult males with 0.8% (2/241), and 0.08% (1/1,142) for nymphs. Positive ticks appeared early in 2009, in the first sampling activity in May 2009 and again in July 2009. It was interesting that in June 2009, where similar amounts of adults were sampled as in May 2009, no rt-RT-PCR-positive ticks could be detected. After July no further positive ticks were sampled. However, the total number of ticks sampled in August , September, and October was lower than that of spring and early summer (May, June, July).

17.4 Discussion

The presented data for the first time show data on active TBE foci associated with human diseases. In focus A more than 2,000 ticks were collected within a time period of 4 years. In 2005 an isolate was generated from sector N. However, during the following years it has not been possible to re-detect TBEV in ticks in this sector. We therefore assume that grid N is not the location of the natural transmission cycle. We hypothesize that a wild animal dispersed the infected tick into this section and it was accidentally detected during our sampling. In addition, in two instances TBE patients remembered the location of their tick bites in their gardens. Intensive sampling only 2 weeks after the time of infection around the two gardens of these patients did not reveal any TBE-positive ticks. This also indicates that TBEV was not transmitted between hosts and vectors at the locations of these infections. In contrast, in focus A repeated detection of positive ticks was made in section K in 2007 and 2008. In the years 2005 and 2006, no sampling was performed in section K as it was several hundred metres away from the patients' homes and also from section N (location of virus isolation in 2005). Therefore, at the beginning of the sampling no TBEV was expected to be found in section K.

These results pose the following hypothesis for focus A: The detected focus with active circulation of TBEV is a relatively small area within a larger area where TBE-positive ticks can be detected. We name this small area the "microfocus". This microfocus is the area where continuous transmission of TBEV at least for a period of several years takes place. The ecological conditions that distinguish this area from adjacent areas with obviously similar vegetation and also ecological conditions is unclear so far and warrants further studies.

The concept of microfocus was already postulated by Czechoslovakian scientists (Nosek et al. 1978). There, they state that the transmission of TBEV within the tick and rodent populations is occuring in small areas. They showed that TBEV is transported outside of the microfocus by rodents, mainly male bank voles (*Myodes glareolus*) showing a home range of up to 200 m in diameter and the yellow-necked field mouse (*Apodemus flavicollis*) showing a home range of up to about 500 m in diameter (Niethammer and Kapf 1978). Either the natural hosts or other animals, for example the European mole (*Talpa europaeus*) or larger animals like foxes, roe deer or deer serve in the dispersion of infected ticks over the observed area of the macrofocus of about 1 km in diameter (Radda et al. 1968).

The second focus, named focus B, was also detected by occurrence of two human clinical cases of TBE (case 1 and case 2). Focus B was studied over a period of 6 months in 2009. The data of sampled ticks are comparable as the sampling methods for each sampling were identical: sampling was conducted for 2 h before dawn in exactly the same way. The ticks sampled during the first sampling activity of the year in May 2009 already yielded seven positive tick pools. However, among those rt-RT-PCR positive pools only one pool consisted of ten nymphs, while six pools consisted of adult ticks (four pools of five females each and two pools of five males each). This was unexpected as the number of

nymphal ticks was about twice that of the adult male and female ticks. All positive ticks were detected in sector I or the adjacent areas of sector II. As in July again two adult female ticks of the same sector I were TBEV positive, we assume that the area of sector I forms the hypothesized microfocus. For patient 1 of focus B the location of infection was identified. This place of tick acquirement is some 500 m away from the microfocus. This observation again supports the theory on TBEV microfoci and macrofoci. Also for this case we assume that either the natural hosts (*Apodemus flavicollis*) or, more probably, medium sized or large wild animals dispersed the infected tick from the microfocus to the location of tick acquisition. As the place of tick acquisition was directly in the village, animals entering villages like hedgehogs or foxes seem to be possible transporters of ticks. For patient 2 of focus B the location of tick acquisition could not be reported. The patient's home however is also several hundred metres away from the microfocus of focus B. Focus B has an estimated size of about 50 × 50 m. Therefore, with a total of about 2,500 m^2 it seems to be much smaller than the modelled size of 50,000 m^2 postulated by Pretzmann et al. (1963). However, in 1963 the only important mode of transmission was thought to be transmission by viremic rodents. Non-viremic transmission might intensify the transmission and therefore much smaller areas and numbers of ticks and rodents might be necessary for maintaining the transmission cycle of TBEV.

For focus B, for the first time the temporal composition of different tick stages during the year and the TBEV incidence in the tick population could be studied. It was surprising that in spring relatively high numbers of adult ticks were found in comparison to the absolute numbers of nymphs. In total, the absolute numbers of male and female ticks was comparable to the total number of nymphs. However, for the month of May the TBEV prevalence of ticks was found to be 5% (4/80) for adult female ticks and 3.3% (2/61) for adult males, while the prevalence of infected nymphs was five to eight times lower (0.68%). This contradicts the presumed predominant role of nymphs for the transmission of TBEV to humans (Pretzmann et al. 1963). Two TBEV rt-RT-PCR-positive female adults in July increased the TBEV minimum infection rate of adult females for the month July to 7.1% (2/28). Whether the unusual increase of the number of sampled larvae in October was unique for focus B in the year 2009 or is a general phenomenon, has to be confirmed by further studies. In earlier works an increase of larval activity was found in early autumn in a TBE focus in Austria (Pretzmann et al. 1963). Also, Randolph et al. summarized the synchronous increases and decreases of questing nymphs and larvae in various TBEV endemic and non-endemic areas (Randolph et al. 2000; Gray 2008). In their reviews they showed increased numbers of larvae mainly in August and September, while for the year 2009 in focus B, an increase was only detected in October. However, this delay of several weeks might have been dependent on the actual weather conditions of the year.

The extremely low number of infected nymphs also argues against the hypothesis of transmission via non-viremic co-feeding on rodents (Randolph et al. 1996). For focus B, an explanation for the high infection rates of adult female ticks and also of male ticks is that they may have resulted from a single transmission (viremic or non-viremic co-feeding) event from one single rodent to several blood-sucking

nymphs which then were detected in spring 2009 as infected adults in a small area (microfocus). At least one of the TBEV rt-RT-PCR-positive adult females in July did not belong to this potential transmission event as the TBEV of this female pool contained a TBEV with a nucleotide sequence in the E gene distinct from the other TBEV sequences (data not shown). Therefore, one of the July positives may also be still from the 2008 autumn infected nymphs. However, it is unusual, that no other infected adult ticks were detected in June. Another explanation may be that the TBEV-positive female adult in July with another E gene sequence resulted from an early moulting in spring 2009 appearing only in June.

17.5 Conclusions

In summary, the comparison of two TBE foci showed that the transmission between ticks and rodents may occur in a relatively small area, called the microfocus. Humans may be infected by acquiring TBEV-infected ticks by entering and/or passing through a microfocus, or more likely by acquiring TBEV-infected ticks over a larger area called a macrofocus with a diameter of up to 1 km around the microfocus. The dispersal of TBEV-positive ticks may proceed by transport by their natural hosts or by larger wild animals with a larger radius of activity. In this respect the increase of animal populations like foxes and their increasing adaptation to human dwellings may increase the risk of dispersal of infected ticks into villages and towns. Adult ticks were found to be of major importance as vectors. The concept of non-viremic co-feeding transmission on rodents for focus B has to be proven in further studies.

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