Regressive and Constructive Traits in *Astyanax* Surface and Cave Fish

6

Abstract

The surface and cave forms of the characid *Astyanax* have become a model system of outstanding importance in evolutionary research. It is extraordinary, because this diurnal day-active fish only exhibits minimal preadaptive traits allowing life in continuous darkness. In *Astyanax*, transition into the new environment took place abruptly and without a phase of gradual adaptation, as is usual when night-active, so-called troglophile surface forms start colonizing caves. Because of the specific characteristic of the surface fish, a large discrepancy and a large number of different traits have evolved in the cave form. The comparatively quick change into a drastically different environment is probably one of the reasons why surface and cave fish are still interfertile and can be genetically analyzed and phenotypically compared. Furthermore, cave colonization has been performed, at least in part, in parallel at different geographically separate locations and at different times. As a result, variously advanced stages of phenotypic evolution and their genetic bases can be studied.

The traits concerned can be categorized into different groups. Some, like eyes and dark pigmentation, regress because of the absence of light. Others, like the lateral line and taste sense, are submitted to directional selection to compensate for the loss of vision. Another group of traits, like altered sleep and activity phases, are ambiguous because they appear as loss and regressive in light but are constructive in permanent darkness. Entirely independent from the direct consequences of missing light, species living in temperate and subtropical caves must adapt to a continuously or temporarily low food supply.

Possibly because of regular bottlenecking events or even continuously low population density, *Astyanax* cave fish seem to tend to be K strategists with the egg yolk content enhanced in comparison with the surface fish. Because of such bottlenecking

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events, sex determination changed from polygenic in *Astyanax* surface fish to monogenic in the cave fish, based on one autosome exhibiting a gonosomal characteristic. By this, odd sex ratios or even the complete absence of one sex, which tends to occur in low population density under polygenic sex determination, are avoided.

Classical crossing analyses have revealed that regressive and constructive traits do not differ in their phenotypic manifestation. They are polygenic, and the phenotypic manifestation in the various crosses is identical. The gene effect underlying the phenotypic manifestation may exhibit an exponential increase at differing amounts in the various traits and crossings. An important role in constructive evolution is attributed to a threshold-like increase of gene effect, which is expressed after a minimum number of polygenes are recombined. By this, a disproportionate enhancement of number, size, or intensity of a specific trait is achieved, which surpasses the modifying and not genetically based environmental influence on trait expression, which hinders selection to act. The absence or presence of such genetic interaction helps determine whether the variability of regressive traits like eyes or pigmentation, exhibited by phylogenetically younger variably eye- and pigment-reduced *Astyanax* cave fish populations like Micos, is due to a more recent origin or to secondary hybridization with surface fish.

The eye is the trait best studied in *Astyanax* cave fish and provides a prime example of the genetic analysis of the evolution of complex constructive and regressive traits. Crossing experiments revealed that the eye is built by two developmental subunits, the dioptric lens subunit and the sensory retinal one. Both inherit independently from each other. It is proposed that the genes responsible for eye development divide into two groups: the first expresses around the sonic hedgehog (*shh*) genes during a first step of eye regression and determines the size of the primordial eye cup through regulation of *shh* expression. A second group of eye genes is suggested to regulate structural genes responsible for both the lens and the retina subunit. The identification of snake-specific sequence changes within the otherwise highly conserved long-range limb ZRS enhancer as being responsible of the progressive reduction of limbs from basal snake species provides a possible explanation for the complex pattern, how *shh* may be regulated, and how embryonic eye size is determined. It also provides a model of how all the other constructive and regressive traits evolve in *Astyanax* cave fish.

6.1 General Remarks

Caves are mostly colonized by so-called troglophiles. Such species are characteristically small-eyed and exhibit a nocturnal lifestyle. They are preadapted to life in complete darkness, and the number of traits and their degree of constructive improvement developed after the complete transition into the cave habitat is comparatively low. This was demonstrated for the cave catfish *Rhamdia zongolicensis* and *R. reddelli* in relation to their surface sister species *R. laticauda* (Hepapteridae) (Wilkens 2001) (Fig. 3.13), for the cave swamp eel *Ophisternon infernale* and its surface sister counterpart *O. enigmaticum* (Synbranchidae) (Fig. 3.19), and between the surface reef-dwelling *Ogilbia cayorum* and the closely related cave cusk eel *Typhliasina pearsei* (Bythitidae) (Fig. 3.16).

Among the cave-colonizing teleost species, surface *Astyanax* represents an exception because this large-eyed fish is diurnal and therefore, in contrast to nearly all ancestors of cave fish, cannot be characterized as a troglophile (see Sect. 6.14). This is unique among cave-colonizing species. Surface *Astyanax* did not actively invade caves, but during the rainy seasons was washed into the karstic underground with the floods of captured streams and creeks. The fact that *Astyanax* surface fish are not active cave invaders is nicely demonstrated by the distribution pattern of this species in Northern Yucatan, where surface rivers are absent and the troglophile hepapterid catfish *Rhamdia guatemalensis* has penetrated to every cenote via the vast underground system, whereas surface *Astyanax* is exclusively found in locations close to the coast, which it reached via coastal lagoons and marine transgressions (Fig. 4.5). During cave colonization in Northern Mexico, surface *Astyanax* was submitted to an abrupt change from light to continuous darkness. It was able to survive here because of a "coincidental" set of preadaptations such as non–visually released reproductive behaviour or a comparatively well-developed lateral line sense. Cave colonization was not the result of a "slow" transition, as is characteristic of troglophile species. This may explain why a higher number of quite different traits than those in troglophile species were subjected to evolutionary change.

The traits concerned can be categorized into different groups. Those directly depending on the information of light are no longer submitted to stabilizing selection and regress. The best known and most obvious ones are the eyes and dark melanin pigmentation, but behaviours such as schooling, dorsal light reaction, phototaxis, or optically released aggression are also affected (see Sects. 6.21, 6.20, 6.18, 6.13, 6.14, 6.12). Others are submitted to directional selection to compensate for the loss of vision. For example, orientation in space or food finding is changed by adaptive improvement of senses like lateral line, taste, and olfaction or behavioural traits like feeding posture (see Sects. 6.5–6.8). A third group exhibits an ambiguous character. For example, reduction of the amount of sleep may be characterized as regressive, but it becomes constructive under conditions of constant darkness (see Sect. 6.10). Entirely independent from the direct consequences of the absence of light, particularly cave species living in moderate and subtropical zones must adapt to a continuously or temporarily low food supply. In *Astyanax* cave fish, this adaptation is brought about by traits such as the enhanced ability to store fat and starvation resistance (see Sect. 6.9).

After the description of the first cavernicolous characid *Anoptichthys jordani*, the Chica cave fish, by Hubbs and Innes (1936) it was soon realized that this species was closely related to the widely spread Mexican *Astyanax* surface fish. Curt Kosswig (1902–1982), a German biologist who had started studying the genetic basis of complex traits like eyes and pigmentation by crossing surface and cave populations of isopodes from the Istrian and Slovenian karst (Kosswig and Kosswig 1940), also initiated crossing analyses of surface and cave *Astyanax* to gain insight into the genetics and evolution of complex traits in vertebrates. He could not immediately pursue his idea, though, because he had to emigrate from Germany to Turkey for political reasons in 1937 (Dzwillo 1982; Franck 2012). Nonetheless, about 20 years after the discovery of this cave fish, his Turkish student Perihan Şadoğlu performed for the first time crosses between the surface fish and fish from the Chica, Pachón, and Sabinos caves in the laboratory of Charles M. Breder Jr. at the New York Aquarium in the mid 1950s (Kosswig 1949, 1967; Sadoglu 1955, 1957) (Fig. 6.1).

Interfertility of surface fish and their cave derivatives is also found in other species. Examples are the Mexican live-bearing cave toothcarp *Poecilia mexicana* (Poeciliidae) and its surface sister species (Parzefall 1979), as well as surface and cave populations of the cyprinid *Garra barreimiae* from Oman (Wilkens, unpublished). Crossings were also performed between the surface troglophile catfish *R. laticauda* (Hepapteridae) and two of its cave sister derivatives, *R. zongolicensis* and *R. reddelli*, as well as between both cave species (Wilkens 2001). In *Astyanax*, all cave populations can be successfully crossed with surface fish and each other in the laboratory (Sadoglu 1957; Wilkens 1988; Wilkens and Strecker 2003) (Fig. 6.1) (see Sect. 5.5). Post-mating hybridization barriers as claimed by Borowsky and Cohen (2013) could not be confirmed in crossing experiments.

6.2 Reproductive Behaviour

In *Astyanax*, the sexes cannot be discriminated by sex-specific traits like body colour and, except during spawning, no specific and divergent sexual behaviour is exhibited. The adult males of all surface and cave populations can be discriminated from females, though, by their slightly more slender phenotype and above all by short serial hooklets they carry along the anal fin rays on both sides. These can be felt as a raw sensation when seizing the fin between two fingers (Wilkens 1972).

Caves can only be successfully colonized by species with reproductive behaviour that does not rely on visual cues. In darkness and in light, spawning in *Astyanax* begins when females ready to ovulate start slowly circling within a rather limited area around vertical structures like stones or plants. Male spawning behaviour is released after a male has by chance touched the female genital region with its snout. At that moment the male immediately starts quickly swimming around, trying to make contact with the ovulating female again. In the light, stimulated surface males are favoured by sight, because they can directly swim at conspecifics to try to find out whether they are ready to spawn. Male propagation behaviour is solely released by an as-yet unknown chemical cue secreted by spawning females, which is not perceived at distance. It is hypothesized that the chemical immediately decays in the water, but may persist for some time in the female body mucus. Possibly, as in goldfish (*Carassius auratus*), a postovulatory prostaglandin pheromone emitted through the female genital opening releases male propagation behaviour (Stacey 2003). By intersecting the olfactory nerves, it was revealed that this cue is perceived by olfaction and not by taste, because propagation behaviour was not performed by such males though they were kept together with synchronously spawning females and males (Wilkens 1972).

Surface and cave *Astyanax* have a promiscuous mating system (Wilkens 1972). The spawning process of a female may extend over several hours. Finally, all males are whirling around the ovulating female trying to come into lateral body contact with her. When this is achieved, the male and female lay their bodies side by side and turn up their ventral sides, simultaneously emitting sperm and a clutch of eggs using shivering body movements. Possibly the male anal fin hooklets, by hooking at the female body, help to concentrate the male sperm and the simultaneously yielded

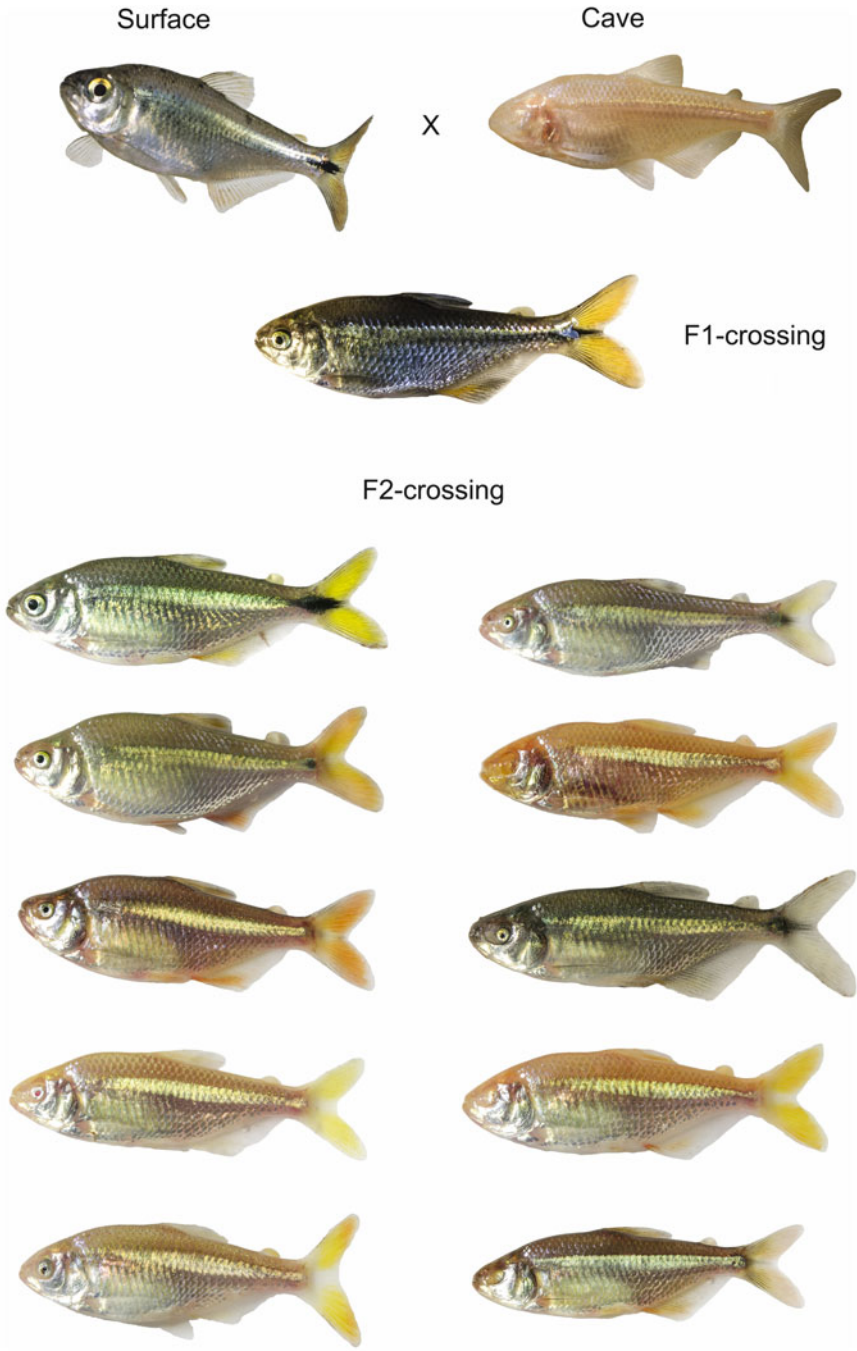

Fig. 6.1 *Astyanax* surface and Pachón cave fish, their F1- and F2-crossing hybrids

eggs in the region of the female genital opening where the external insemination takes place. *Astyanax* surface and cave fish do not exhibit parental care, and the fertilized eggs are whirled around and, because they become adhesive in the water, stick to plants or rocks, or slowly sink to the ground.

Thus, female surface and cave *Astyanax* only choose a mate during this actual spawning process, when the ovulating female selects a male to nestle with, and not by non-ovulating females, as incorrectly claimed by Plath et al. (2006).

6.3 Sex Determination

Sex determination is the mechanism by which sexual organisms direct gonad development towards distinct but reproductively compatible outcomes (Moore and Roberts 2013). The most widely known genetic sex determination is the monogenic male heterogametic system of most mammals, whereby one gene on the Y chromosome determines male sex. In contrast, in polygenic sex determination, multiple and independently segregating genes determine the sex. Polygenic sex determination was detected and primarily described by Kosswig (1935, 1964) for platyfish such as *Platypoecilus maculatus* and the swordtail *Xiphophorus helleri* (Poeciliidae). In both species, males with different numbers of sex genes exist. Dwarf males containing a higher number of sex genes show rapid sexual differentiation and produce more male progeny than other males. In contrast, so-called late males differentiate more slowly, become larger, and have fewer male offspring because they have fewer sex genes.

For *Astyanax*, laboratory breedings revealed a different proportion of sexes in surface and cave fish populations. In strongly eye- and pigment-reduced (SEP) (Pachón, Piedras, Yerbaniz, Curva) and the variably eye- and pigment-reduced (VEP) cave fish (Micos, Molino), male and female specimens exhibit one-to-one sex ratios (Table 6.1). In contrast, in surface fish significant deviation from the one-to-one sex ratio of male and female specimens was found in breedings, independent from their origin from Northern or Southern Mexico. Either the male or the female specimens may alternatively represent the larger group.

The unexpected odd and varying proportions of sexes found in laboratory breedings of *Astyanax* surface fish are probably the result of polygenic sex determination. It is proposed that the number of sex genes differs in the various specimens. In nature this is not disadvantageous because in the vast space of the rivers and other water bodies populated by the surface fish, the spawning females mate successively with large numbers of males. Through this participation of many specimens, the finally advantageous one-to-one ratio of sex is probably sustained, because the varying numbers of sex genes contained in different specimens are mixed at random. However, when breeding just a single pair of surface fish in the laboratory, as done for these studies, odd proportions will develop. For example, recombining one male and one female specimen, both containing few sex genes, will result in an overwhelming number of females, whereas breeding a pair with many sex genes provides the contrary.

Table 6.1 Sex ratio in different breedings of *Astyanax* surface and cave fish

Breedings	No. of females	No. of males	χ^2	Significance
Surface fish				
Rio Coy 1	40	81	13.8926	***
Rio Coy 2	177	108	16.7053	***
Rio Coy 3	11	75	47.6279	***
Rio Coy 4	26	70	20.1667	***
Rio Teapao	184	70	51.1654	***
Cave fish				
Pachón1	20	25	0.5556	ns
Pachón 2	17	13	0.5333	ns
Pachón 3	12	14	0.1538	ns
Pachón 4	20	23	0.2093	ns
Piedras 1	66	58	0.5161	ns
Piedras 2	34	33	0.0149	ns
Piedras 3	20	12	2.0000	ns
Curva 1	22	25	0.1915	ns
Curva 2	15	18	0.2727	ns
Yerbaniz	13	13	0.0000	ns
Molino 1	65	64	0.0078	ns
Molino 2	8	13	1.1905	ns
Micos 1	55	72	2.2756	ns
Micos 2	15	16	0.0323	ns
Micos 3	44	72	6.7586	**
Micos 4	17	15	0.1250	ns

Surface fish from Northern (Rio Coy) and Southern Mexico (Rio Teapao); cave fish populations from the Sierra de El Abra (Pachón, Piedras, Curva, Yerbaniz), Sierra de Guatemala (Molino), and Sierra de la Colmena (Micos)

Significance levels for sex ratio deviating from one to one: *ns* not significant, $^{**}p < 0.01$, $^{***}p < 0.001$

In contrast, the cave fish populations exhibit a one-to-one sex ratio that is suggested to rely on monofactorial heterozygote sex determination. It is proposed that this change results from and is an adaptation to the temporarily or continuously small population of the cave fish, as it is also exemplified by the diminished variability of molecular markers (Bradic et al. 2012; Strecker et al. 2003, 2012) (see Sect. 4.3.3). Temporary lower population density results from bottlenecks and at such occasions the polygenetic basis of sex determination is unfavourable. Odd sex ratios or, in the extreme, even the complete absence of one sex will occur. Thus, during their evolution *Astyanax* cave fish have changed from polygenic to monogenic sex determination to prevent odd sex combinations and provide constancy for the equal proportion of both sexes favourable in the cave environment.

Whereas for zebrafish and cichlids it is assumed that sex determination likely results from a combination of additive and epistatic effects at many loci (Beukeboom and Perrin 2014; Moore and Roberts 2013), in *Astyanax* cave fish

the monogenic sex determination system has probably emerged from a quantitative shift caused by a threshold-like epistatic gene effect of sex-determining genes, as it was also found to manifest in all constructive and regressive traits in the *Astyanax* cave fish (Wilkens 2016) (see Sect. 6.23). In this way, an autosomal chromosome became gonosomal.

6.4 Auditory Capacities

Fish, like all vertebrates, possess an inner ear, in which two saclike structures—the sacculus and the lagena—in the pars inferior together with the enclosed otoliths, primarily function in sound reception (Popper 1970). In ostariophysians like the surface and cave forms of the catfish genus *Rhamdia* or characids like *Astyanax*, to increase hearing sensitivity the inner ear is connected with the swim bladder by the Weberian apparatus. Both act as amplifiers of sound waves that would otherwise be only slightly perceivable by the inner ear alone. One could therefore expect that, at least in some cave-living fish species, constructive improvement of auditory sensitivity has taken place in order to detect the movement of small prey.

One example of this might be provided by the cave cusk eel *T. pearsei*, in which the sacculus and its otolith, the sagitta, are enormously enlarged in relation to the other parts of the inner ear. This indicates an improvement in hearing sensitivity (Schemmel 1977) (Fig. 3.16). In amblyopsid cave fish, not only the sacculus and its otolith but also the whole inner ear are enlarged in comparison with ancestral surface fish (Niemiller and Poulson 2010; Poulson 1963; Poulson and White 1969).

Further comparative studies between closely related surface and cave sister forms were performed in the live-bearing toothcarp *Poecilia mexicana,* in which alterations in hearing sensitivity were not revealed. Only otolith morphology was changed (Schulz-Mirbach et al. 2010). In the *Astyanax* surface fish and the Pachón and Sabinos cave fish it was shown that no morphological differences exist between the inner ears of these fish (Schemmel 1967). Studies revealed, however, that both surface *Astyanax* and specimens from the Chica cave are able to hear a wider range than other fish species, but there was no evidence that the auditory capacities of the cave fish are improved (Popper 1970).

6.5 Lateral Line System

The mechanosensory lateral line system of fish provides a highly sensitive hydrodynamic sense that is used in a wide range of behaviours. Hydrodynamic stimuli originate from both abiotic and biotic sources. They include water currents and water disturbances caused by own body movement, prey, predators, and conspecifics (Montgomery et al. 2001, 2009). The responsible sensory organs are neuromasts, each of which consists of a sensory bud composed of support cells and of nervous hair cells, the apical ends of which are enclosed by a gelatinous cupula produced by the support cells. Depending on their location on the body, two

types can be discerned: so-called superficial or epidermal neuromasts are distributed all over the body surface whereas canal neuromasts are located within subdermal canals developed within bones or bony tubes filled with gelatinous fluid. In principal, the canal system consists of a trunk lateral canal alongside each body side and several head canals. The canals are connected with the outside through pores, with one canal neuromast regularly situated between two pores.

Superficial and canal neuromasts exhibit a functional dichotomy which is not absolute, however (Montgomery, personal communication). Superficial neuromasts are low-pass filters encoding direct current (DC) and low-frequency oscillating flows, such as those generated by currents in the water surrounding the animal or movements of the animal itself, so-called noise. In comparison, canal neuromasts do not respond to DC flows, but respond to the higher frequency signals generated by other animals such as prey and mediate prey detection or orientation to a small vibrating source (Kanter and Coombs 2003; Patton et al. 2010). The functional difference between canal and superficial neuromasts is further supported by the behavioural findings that superficial neuromasts mediate rheotaxis, the orientation to water flow (Montgomery et al. 2009).

The mechanosensory lateral line system has been improved in cave fish to enable orientation in the dark and is one of the basic prerequisites of many of the fish species colonizing caves. Cave specimens may sense their surroundings using self-generated water movements, an ability termed hydrodynamic imaging (Bleckmann et al. 2004; Windsor et al. 2008). In a "head on" approach to a wall, the *Astyanax* cave fish reacted to avoid collision at a distance of about 4.0 mm. Furthermore, it was found that they possess and can develop and encode order in a spatial map (Burt de Perera 2004a, b; Sutherland et al. 2009).

The two components, lateral line canal neuromasts on the one hand and free neuromasts on the other, may evolve quite differently and independently in the dark cave environment. An example for a unique highly developed specialization of the head canal system in fish is developed in the cave cusk eel *Typhliasina pearsei* (Bythitidae) from Yucatán (Figs. 3.5, 3.16c, d) (Schemmel 1977; Wilkens 1982). It consists of a series of widened chambers separated from each other by small openings, the lumen of which is occupied by a neuromast cupula. Additionally, the canals are not enclosed in bones but the superficial parts of the chambers are transformed into thin membranes. In stagnant calm underground waters the functional combination of a velocity (cupula of canal neuromast) and a pressure receiver (membrane-covered chamber) mediates even the weakest damming phenomena for orientation in space and seems to signal approaching live prey. The exceptionally huge head of *T. pearsei* compared with the rest of the body correlates with the enlargement of the canal chambers. Optimal function of this sensory system is completed by the method of locomotion of this fish species, which by gliding almost motionlessly through the water driven by its gently undulating long dorsal and ventral fin fringes it is avoiding noise caused by its own locomotion. *T. pearsei* has also developed superficial neuromasts, which are concentrated on the head, with only a few being found on the body (Fig. 3.16a–d). These neuromasts are located on dermal protrusions and carry enlarged cupulas (Fig. 3.16c). In contrast to

the highly specialized lateral line, the sense organs of chemoreception are poorly developed in this species. No taste buds were found on the body or on the filamentous pelvic fins, and the olfactory epithelium is small in size (Schemmel 1977).

In contrast, the lateral line system of the cave catfish *Rhamdia reddelli* and *R. zongolicensis* (Hepapteridae), which are closely related to the troglophile surface sister species *R. laticauda*, was not subjected to any constructive adaptation process. This can be attributed to the fact that the troglophile ancestor of these cave fish was already adapted to life in darkness (Figs. 2.7 and 3.13c–e) (Weber 1995). In the North American family Amblyopsidae, the surface swampfish still possess head canals, which no longer exist in the closely related cave fish. Instead, an extensive system of free neuromasts arranged on elevated distinct ridges is developed (Niemiller and Poulson 2010; Poulson 1963; Soares and Niemiller 2013).

In *Astyanax*, the lateral line system also consists of enclosed canal and superficial free neuromasts. The surface *Astyanax* belongs to those fish species characterized by a high number of superficial neuromasts, with four to eight of them on every scale and more developed on both sides of the head. The course and position of the lateral line canals and the location of the canal neuromasts enclosed in them do not differ between surface and cave forms except for the infraorbital canal, which may be shortened in cave fish (Fig. 6.2). The lateral canal extending along the whole body trunk side between head and tail regularly penetrates the scales through a pore. Reaching the head it divides into the occipital canal, which branches off dorsally connecting with that of the other body side, and in the preopercular canal, which descends down within the preopercle bone ventrally entering into the maxillary canal. The latter continues to the tip of the lower jaw and is enclosed by the articular and dentary bones. After the occipital canal has branched off dorsally, it divides into the supra- and the infraorbital canals which run above and below the eye orbit. Within the frontal bone, the supraorbital canal extends as far as to the premaxilla in the upper jaw, while the infraorbital canal takes a course enclosed by the bony orbitalia into the prefrontal bone (Schemmel 1967). For the infraorbital canal, partial fragmentation was observed in some cave specimens (Schemmel 1967). It can be assumed, though, that these are artefacts resulting from injuries, as was also erroneously observed for the lateral line (Wilkens 1977).

Whereas the canal neuromasts of the cave fish do not differ from the surface fish, alterations were found concerning the free neuromasts. The sensitive areas of individual neuromasts are two times larger than in the surface form and the cupulas are much longer (300 μm vs 50 μm) (Teyke 1990). Their sensitivity is enhanced when compared with the surface fish (Yoshizawa et al. 2010). This divergent development can already be observed at early life stages between 1 and 4 months (Yoshizawa et al. 2010). The neuromasts located in the head region are the longest, whereas they become progressively shorter caudally (Montgomery et al. 2001).

In surface *Astyanax* the superficial neuromasts on the head are restricted to narrow areas, which in part follow the position of the head canals (Fig. 6.2). In the cave fish, the number of neuromasts developed on the head has increased by extending into areas devoid of them in the surface fish. They cover almost all the space between the infraorbital and preopercular/maxillary head canals. In this

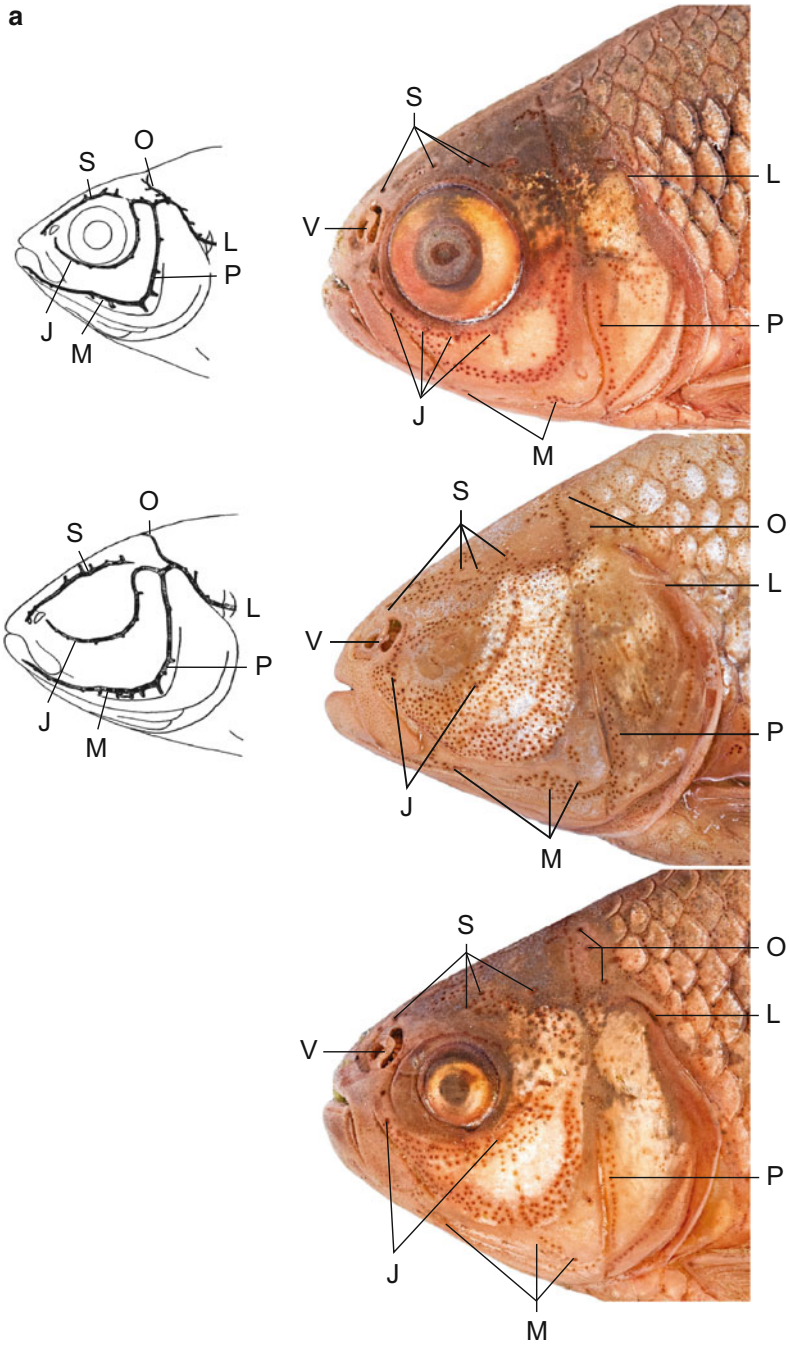

Fig. 6.2 (**a**) Distribution of the superficial free neuromasts (*large brown dots*) and course of the head lateral line canals in *Astyanax* surface (*above*), the SEP Pachón cave fish (*middle*), and an F1 hybrid between surface and SEP Piedras cave fish (*below*).

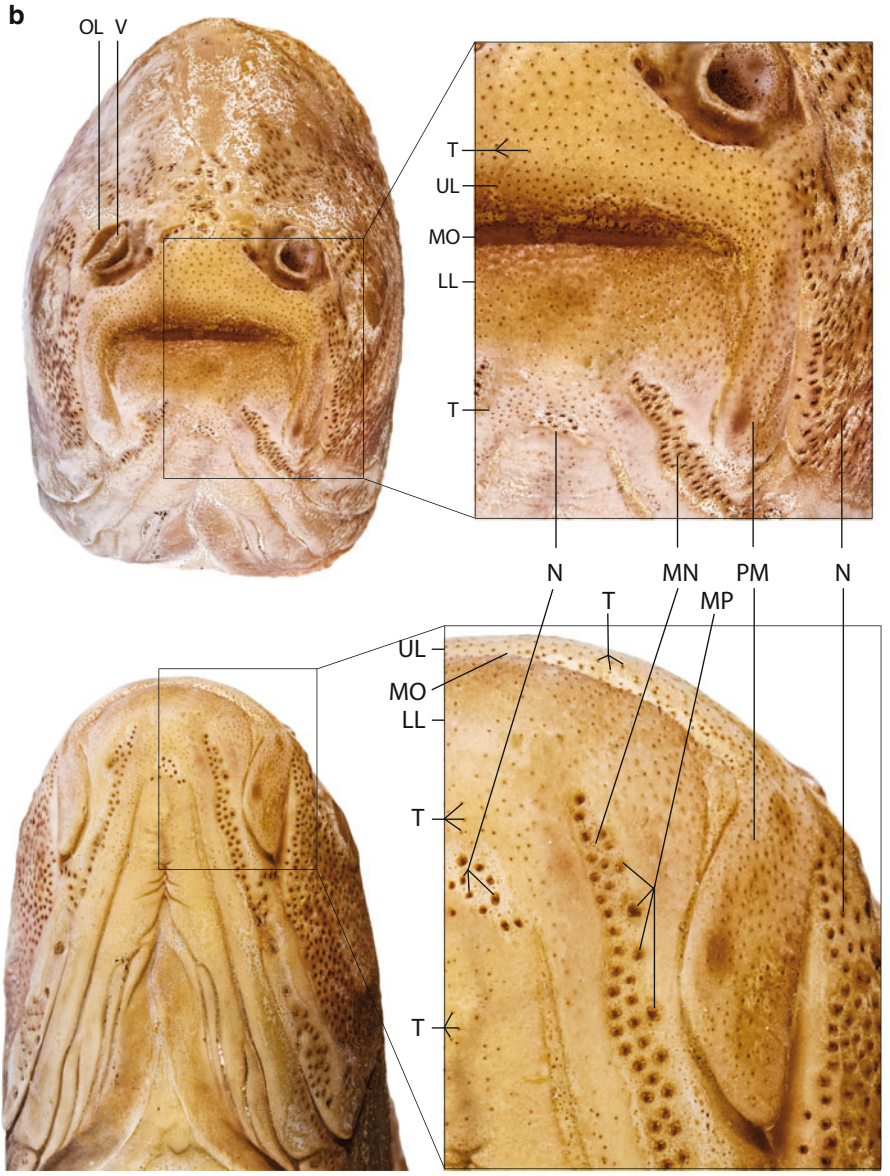

Fig. 6.2 (continued) (**b**) Distribution of the taste organs (*tiny brown dots*) in frontal view of the head (*above*) and underneath the jaw (*below*) in the SEP Pachón cave fish. Head canals are externally recognizable by their pores (some indicated by *lines*). Staining of free neuromast cupulae and taste organ receptor areas with silver nitrate. *J* infra-/suborbital canal, *L* trunk canal, *LL* lower lip, *Mo* mouth opening, *M* mandibular canal, *MN* course of mandibular canal indicated by cupulae of free neuromasts, *MP* canal pores, *N* cupulae of neuromasts, *O* occipital canal, *Ol* nose pit with velum (*V*) over nose pit, *P* preopercular canal, *PM* premaxilla, *S* supraorbital canal, *SEP* strongly eye- and pigment-reduced, *T* taste organ, *UL* upper lip

so-called cheek area, their number has nearly doubled from about 180 in the surface fish to 350 in adult Pachón cave fish (measured at 4.5 cm standard length). Most interestingly, differences between cave fish populations were found; for example, the increase is less in the Curva (190) and the Molino (240) cave fish. In close correlation to the number of superficial neuromasts developed on the head, that following the maxillary canal below the lower jaw is also enhanced (Fig. 6.2 continued) (Wilkens, unpublished).

Although prey detection (or orientation to a small vibrating source) was found to be mediated by the canal neuromasts (Coombs et al. 2001; Kanter and Coombs 2003; for review see Montgomery et al. 2009), experimental external vibration stimuli were observed to be perceived by the free neuromasts releasing vibration attraction behaviour (VAB) (Yoshizawa et al. 2010, 2012, 2015). In contrast to most surface fish, cave fish populations were found to be able to detect the experimental vibration stimuli within a low frequency range at about 35 Hz. VAB is assumed to represent a potential foraging behaviour by which the cave fish can better locate moving prey. However, this ability is variable within and among cave populations. Whereas the SEP Sabinos and Piedras show improvement, some of the Pachón specimens lack this behaviour and it was not at all observed in the SEP Tinaja and the VEP Molino cave fish (Yoshizawa et al. 2010, 2012, 2015). The kind of prey detected by VAB in the cave is as yet unknown. Planctonic crustaceans, which should emit such vibrations, do not occur here (Elliott 2015), because green algae do not exist.

The number of superficial neuromasts developed in the cheek area in the F2 and the backcross generations between surface and Pachón cave fish range between the respective parental forms (see Sect. 6.23, Fig. 6.55e). Furthermore, it was found that the F1 hybrids between surface and SEP cave fish also exhibit an intermediate number of free neuromasts as well as intermediate abilities for the detection of water disturbances (VAB) (Fig. 6.2) (Yoshizawa et al. 2010). These results indicate a multiple polygenic mode of inheritance for the number of superficial neuromasts and also for this specific behaviour. In contrast, however, the size of the superficial neuromasts of F1 hybrids was not intermediate but equivalent to that of the Pachón cave fish (Yoshizawa et al. 2010), which can be explained by genetic epistasis. This suggests that size and number of superficial neuromasts inherit separately.

6.6 Taste

In fish, taste is perceived by taste buds and solitary chemosensory cells (SCCs). Taste buds are compound sensory organs that respond to food-related and tactile stimuli, whereas SCCs are single cells reacting to taste only (Hara 1994; Kotrschal 2000; Ogawa et al. 1997). In contrast to taste buds, which can only be found in the head region, they are not restricted to certain areas, but may occur all over the body surface. Their density is higher at the forehead and along the dorsal trunk (Kotrschal 1992, 1996). Taste information functionally overlaps with olfaction and both converge in the telencephalic and diencephalic nuclei of the brain.

Usually the number of taste buds is enhanced in cave fish. In species like the swamp eel *Ophisternon infernale* (Synbranchidae) (Fig. 3.19) (Parzefall and Wilkens 1972), the live-bearing toothcarp *Poecilia mexicana* (Poeciliidae) (Parzefall 1970), or the Northern blind fish *Amblyopsis spelea* (Amblyopsidae) (Poulson 1963; Poulson and White 1969), the regions covered by them are extended in comparison to their surface sister forms. In cave catfish, more taste buds probably come about by the prolongation of the barbels (Fig. 3.13) (Wilkens 2001).

In *Astyanax*, the study of the number of taste buds is incomplete and needs further investigation, because Schemmel (Schemmel 1967, 1974a, b) did not discriminate between taste buds and SCCs (Yamamoto et al. 2009; own observation). All taste organs, including taste buds and SCCs, are developed in the internal mouth cavity as well as on the upper and lower jaws with the highest densities on the labial margin surrounding the mouth opening (Fig. 6.2). Compared with the surface fish, the areas covered by taste buds have been expanded and exhibit a higher density in the cave fish. Their number has particularly increased on the upper jaw maxillary and premaxillary bone areas as well as on the ventral side of the lower jaw, where their distribution is additionally extended further caudally (Fig. 6.2). According to Schemmel's study, the SEP Pachón contains more taste organs than the SEP Sabinos, whereas the VEP Micos cave fish exhibits an intermediate position (Fig. 6.55f) (Schemmel 1967, 1974a, b).

During ontogeny of *Astyanax* larvae the taste buds start developing at 5 days post-fertilization and surface and cave forms start off with similar numbers. However, differences become notable at an age of 12 days and are significant at 22 days. At this stage the increase was 3.3- and 2.0-fold in the upper and lower jaws, respectively, in the cave fish in comparison with the surface fish. This improvement of the gustatory system is largely the result of increased taste bud density (Varatharasan et al. 2009). It is not due to the enlargement of the upper and lower jaws of the cave fish, as suggested by Yamamoto et al. (2009).

The sensory receptors make up the bulk of the cells building the taste buds (Boudriot and Reutter 2001) (Fig. 6.3). The nerve fibre plexuses of part of the taste buds contain significantly more axon profiles than in the surface fish, which was interpreted as possibly being associated with the improvement of taste. The cave fish appear to have an accelerated rate of taste bud development as well as an increased number of receptor cells per taste bud (Varatharasan et al. 2009). Unfortunately, the existence of structural histological differences between cave and surface *Astyanax* has not been sufficiently studied.

SCCs have as yet not been histologically analyzed and identified in *Astyanax* cave fish. However, as mentioned before, they may have been erroneously counted by Schemmel (1967, 1974a, b) together with the taste buds, because the number of taste buds on the chin he revealed was much higher than that actually existing (Yamamoto et al. 2009; Wilkens, own observation). The SCCs are spread all over the body surface and function by perceiving odours. They may release the fright reaction (see Sect. 6.17) when the fish are zigzagging to sample the predator's odour plume with the SCCs as described for an epibenthic fish species (Kotrschal 1996).

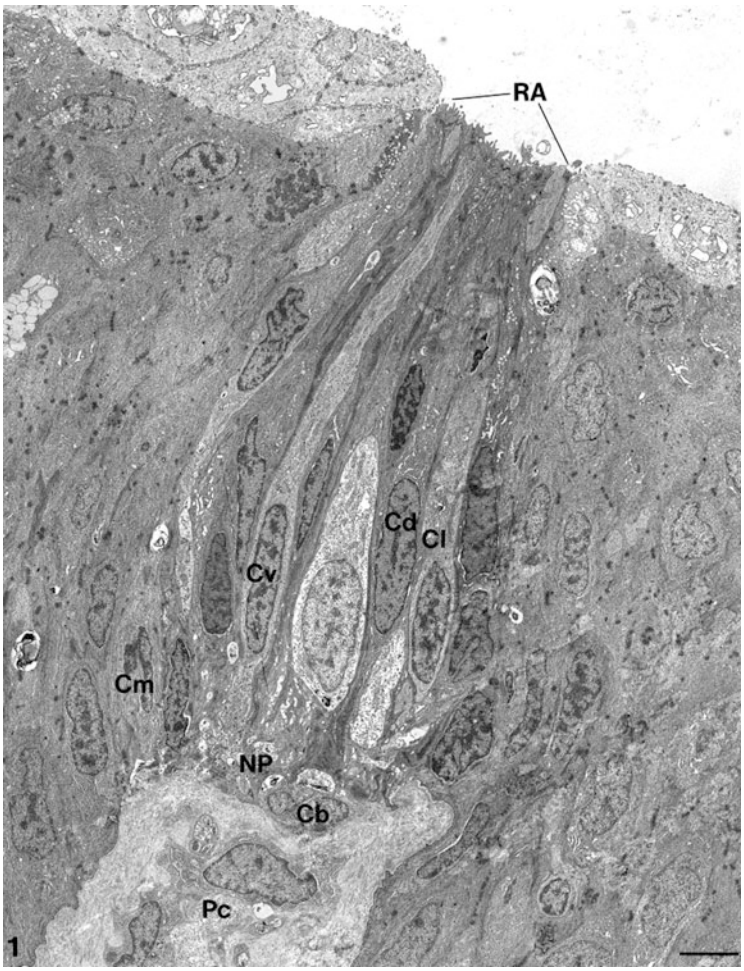

Fig. 6.3 Longitudinal section of a taste bud of an *Astyanax* cave fish. The taste bud is situated on top of a dermal papilla (*Pc*). The sensory epithelium of the taste bud consists of elongated (*Cl*), dark (*Cd*), and dense-cored-vesicles cells (*Cv*). Their apical endings build the taste bud receptor area (*RA*). At the base there are basal cells (*Cb*) and axons of the nerve fibre plexus (*NP*). Marginal cells lie between the sensory epithelium and the unspecialized epithelium (*Cm*) (Boudriot and Reutter 2001)

The differing numbers of taste organs (buds and SCCs) between surface and cave fish are based on a polygenic system (Schemmel 1974a, b). This was confirmed by the finding of at least three different quantitative trait loci (QTL) involved (Protas et al. 2007). Crossing experiments also revealed that the increase of the taste organ number in the *Astyanax* cave fish, like all constructive and regressive traits, exhibits epistatic gene effect (see Sect. 6.23, Fig. 6.55f).

It was suggested that eye reduction in *Astyanax* cave fish might pleiotropically be driven by the increase of taste bud numbers (Yamamoto et al. 2009). However,

comparison of eye size and number of taste organs in the F2 crosses did not reveal correlations between the manifestation of small eyes and large numbers of taste buds. In extreme combinations, large-eyed specimens with many taste buds as well as small-eyed ones with few of them were found. Therefore, it was concluded that the formation of eyes and taste buds is submitted to different independent genetic systems (Schemmel 1974a, b), which is also corroborated by QTL analysis (Protas et al. 2007) and lens ablation experiments (Dufton et al. 2012).

6.7 Olfaction

Olfaction plays an important role in fish. It may be decisive in food location, discrimination between individuals of the same or different species, defence against predators, parental care, orientation, or reproductive synchronization. Olfaction in fish is mediated by the olfactory epithelium, which is located on the lamellae. These are arranged in rosettes situated on the bottom of paired olfactory pits. Each nasal pit has an anterior inflow and a posterior outflow opening, which are partially divided by a median superficial velum directing the water flow through the anterior opening into the pit (Fig. 6.2). In synbranchid swamp eels like surface and cave *Ophisternon*, however, the nose pits are covered and closed by a dermal epithelium that separates the anterior and posterior openings (Fig. 3.19).

In *Astyanax*, the nasal pits are shallower in the cave (Pachón, Piedras, Sabinos, Micos) than in the surface fish. As a result, the olfactory epithelium might be more exposed (Fig. 6.2). In general, the nasal opening is significantly larger in the cave fish, but to varying degrees (Bibliowicz et al. 2013; Schemmel 1967). For example, in Pachón and Micos its length diameter is increased by 25%, in Curva by 17%, and in Molino by 21% compared with the surface fish (Wilkens, unpublished results). Study of 1-month-old juvenile surface and SEP Pachón cave fish using the amino acids alanine and serine resulted in a strong attractive response in both. Compared with the surface fish, the Pachón cave fish were able to perceive amino acid concentrations 105-fold lower. In contrast to the cave fish, the surface fish are only attracted for about 4 minutes. This can be explained by olfactory-driven behaviour being disrupted by vision: when they do not see any food, the surface fish will not continue searching for it (Hinaux et al. 2016).

Crossings revealed that the increase of the nasal pit size in the *Astyanax* cave fish is polygenic and, like all constructive and regressive traits, exhibits an epistatic gene effect (see Sect. 6.23).

6.8 Feeding Behaviour and Food Uptake

Usually cave fish derive from troglophile surface species, which are night active and therefore well adapted to life in light-poor environments. In contrast, the surface *Astyanax*, as a rare exception, is active during the day. It catches prey and food particles in the three-dimensional open water space by visual orientation

(Parzefall 1983). In caves, therefore, it has changed its behaviour and in darkness is restricted to a two-dimensional feeding approach, by solely being able to pick up food particles from the ground (Hüppop 1987). Under these conditions, the surface fish take a steep-angled feeding posture of approximately 74° (Kowalko et al. 2013a, b) to 82° (Schemmel 1980) between body axis and bottom (Fig. 6.4). This feeding posture is disadvantageous, because the swimming process is halted while food items are taken up.

The *Astyanax* cave fish mainly search for food on the ground but also feed at the water surface using an additional two-dimensional area (Hüppop 1987). In contrast to the surface form, their feeding posture on the ground is characterized by a lower angle. As a result, they are able to continuously swim, search, and swallow food particles, which in the caves are mostly found on the bottom (Fig. 6.4). Schemmel (1980) observed an average angle of about 55.7° between body axis and bottom when picking up food from the ground, which was the same in the SEP Pachón, Piedras, Yerbaniz, and Sabinos cave fish. In principle, this was confirmed by the studies of Kowalko et al. (2013a, b), although with slightly differing results, with angles of 38° in Pachón and 49° in Tinaja found. Most remarkably, the VEP Molino cave fish exhibits an angle of 66°, which is in between the surface and the SEP cave fish angles (Kowalko et al. 2013a, b) (Fig. 6.5).

In the laboratory, under darkness, the cave fish reacted much faster to food offered to them than the surface fish did. A comparable observation was made when just food odour was offered to Micos cave and surface fish caught in the Micos cave. In this study, only the cave fish started food searching behaviour and it was suggested that this was due to improved olfaction resulting from the larger naris size (Bibliowicz et al. 2013). Possibly, in the predominantly visually orientated surface fish, olfaction does not play that much of a role in food finding in its lit environments. This was confirmed by studying 1–month-old juveniles (5–6 cm body length) of the surface and the SEP Pachón cave fish, which revealed that the amino acids alanine and serine resulted in a strong attractive response in both

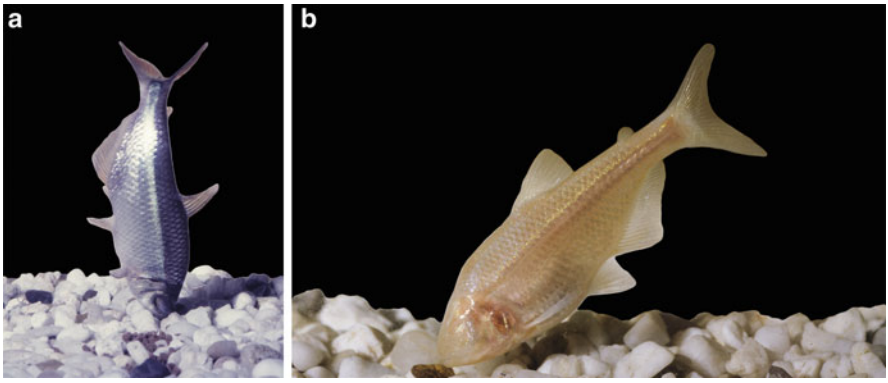

Fig. 6.4 *Astyanax* surface (**a**) and cave fish (Pachón) (**b**) exhibit different angles of feeding posture when picking up food from the ground

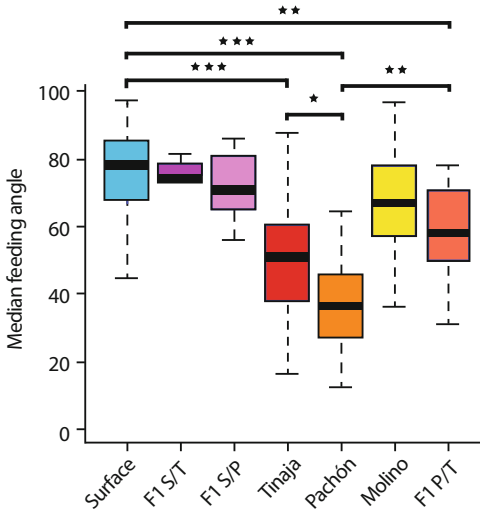

Fig. 6.5 Feeding angle in the *Astyanax* surface, the Pachón, Tinaja, and Molino cave fish, and their crossings. *F1 S/T* surface × Tinaja, *F1 S/P* surface × Pachón, *F1 P/T* Pachón × Tinaja, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (adapted from Kowalko et al. 2013a, b)

(Hinaux et al. 2016). However, in the light, in contrast to the cave, the surface fish are only attracted for about 4 minutes (see Sect. 6.7).

Furthermore, specific hectic food searching movements, performed by single cave fish specimens having detected food particles, immediately stimulate food searching behaviour of other cave fish nearby. In darkness, the surface fish start such behaviour much later (Hüppop 1987; Lüling 1954; Parzefall 1983).

When kept together in darkness in laboratory experiments, adult cave fish find 80% of food particles at the bottom whereas surface fish are only successful at finding 20% (Hüppop 1987). Field studies in the Pachón and the Micos cave revealed that, when small clay balls mixed with food of 5 mm in diameter are offered, the falling food balls released a higher swimming activity within a radius of 0.80 m. A rising number of cave fish from the wider periphery swimming by chance into this area began concentrating there. They started searching for food near the bottom or at the water surface. Little reaction was exhibited, however, when just clay balls of the same size were introduced, which indicates the importance of olfaction in food finding (Fig. 6.6) (Parzefall 1983). When the above-mentioned clay balls mixed with food were introduced into rivers and pools, all surface specimens (which detect the falling balls by vision) leave the school and concentrate around the food ball trying to catch particles. Thus, in surface fish active feeding behaviour is primarily visually released, but is only continued after the food particles have directly been checked by olfaction or taste. Like in the cave fish, feeding behaviour ceases when pure clay is offered.

Under the premise of food scarcity, it is often claimed that the *Astyanax* cave fish are food addicts and because of this they continuously swim in search of food

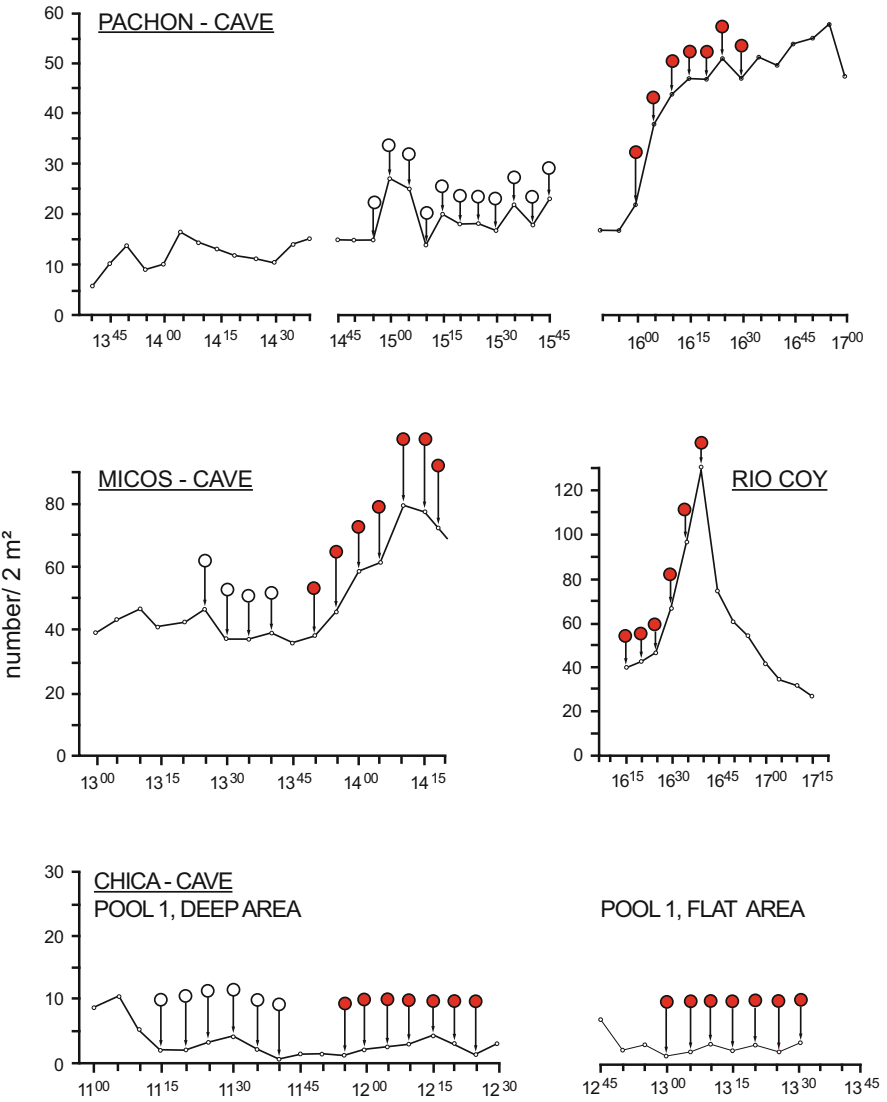

Fig. 6.6 Density of fish in the Pachón, Micos, and Chica cave fish as well as in a surface river fish (Río Coy) before, during, and after introduction of balls of clay mixed with food (*red*) or without food (*empty circles*) within a 2 m^2 area (adapted from Parzefall 1983)

(Elipot et al. 2013). However, comparing surface and cave fish shows that they both show two different behavioural phases. In the first phase, low activity is characterized by slowly swimming within schools or shoals in the surface fish or within a specific home range in the cave fish. This behaviour is exhibited while the surface fish (based on vision) and the cave fish (relying on chemical senses) are both

searching for food. In the second phase, high activity is released in both after a food source has been identified.

During the field experiments it was observed that neither the surface nor the Micos and the Pachón cave fish became satiated. Most cave fish even continued searching in the test area for hours. However, different observations were made in the Chica cave fish, which did not react to either pure or "mixed with food" clay balls (Parzefall 1983). This shows that the Chica fish are satiated due to the high food offer provided in the Chica cave by bat guano. This is not in accordance with the hypothetical assumptions that the cave fish were food addicts and would be insatiable (Aspiras et al. 2015; Elipot et al. 2013). Like every other species, the cave fish will probably stop feeding when saturated.

Among the above-mentioned feeding traits, only the change in the feeding posture has been genetically studied. A polygenic basis exhibiting epistatic gene effect was revealed (see Sect. 6.23, Fig. 6.55c) (Kowalko et al. 2013a, b; Schemmel 1980). Whether the genes regulating feeding posture are the same in the different cave populations is not finally resolved. The F1 cross between Sabinos and Pachón fish does deviate from the parental forms (Schemmel 1980). In contrast, the F1 cross between Pachón and Tinaja was intermediate and ranged from surface-like to cave-like feeding postures (Kowalko et al. 2013a, b). This F1 cross was significantly different from surface and Pachón fish, but not from Tinaja fish. According to these data, the genetic basis of feeding posture is suggested to have evolved independently in these two cave populations and it is suggested that several distinct QTL representing different loci are regulating feeding angles in the Pachón and Tinaja cave fish populations. Furthermore, no genetic correlations were detected between feeding posture on the one hand and morphological features like distribution and number of taste buds on the chin, altered craniofacial morphology, or eye loss on the other, either by classical crossing experiments or by QTL mapping (Kowalko et al. 2013a, b; Wilkens 1988, 2010).

6.9 Metabolic Adaptation to Permanent or Periodic Low Food Supply

The caves inhabited by the *Astyanax* cave fish are situated in the subtropical region, where almost all depend on periodic energy supply during the rainy season. At that time, large amounts of organic debris as well as aquatic surface species are washed into the underground. A well known exception among the *Astyanax* caves is the Chica cave, in which large masses of guano are introduced by thousands of roosting bats providing a high energy input all year long. Here, numerous specimens of the fully eyed cambarid surface crayfish *Procambarus acutus cuevachicae* even coexist with the cave fish (Mitchell et al. 1977; Wilkens and Burns 1972).

Because of the permanently or temporarily low food supply in many caves, diverse adaptations to these circumstances have been acquired by cave-dwelling animals (Hüppop 2000, 2012). The most prominent adaptations are improved starvation resistance, lowered metabolism, and enhanced fat storage. Improved

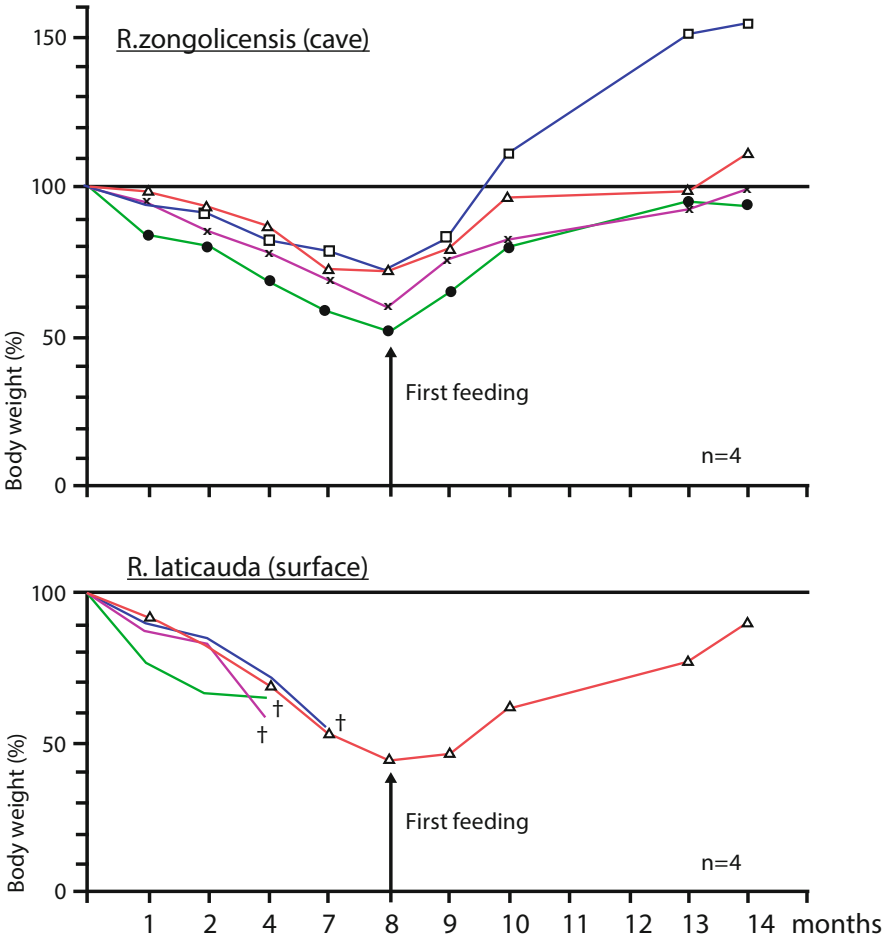

Fig. 6.7 Development of body weight in the cave catfish *Rhamdia zongolicensis* and its surface sister species *R. laticauda* during and after a starvation phase

starvation resistance occurs in the cave catfish *Rhamdia zongolicensis*, specimens of which, due to enhanced fat deposits, are able to survive during longer starvation periods than their closely related surface sister species *R. laticauda*. The cave fish also recover more quickly (Fig. 6.7) (Wilkens 2001). The study of starvation resistance in *Astyanax* revealed that the Pachón, Tinaja, and Molino cave populations lost only half as much weight as individuals from the surface population during a 2-month fast (Fig. 6.8a). This is concordant with the finding that only after a starvation period of almost half a year does the condition factor of individual Pachón cave fish fall below that of well-fed *Astyanax* surface fish (Hüppop 2000). The study of body fat content of the Micos, Pachón, and Chica cave fish revealed that it surpasses that of the surface fish (Hüppop 1986a, b, 1989). The highest mean values were found in Pachón cave fish with 34.9% fat in wet body mass compared with

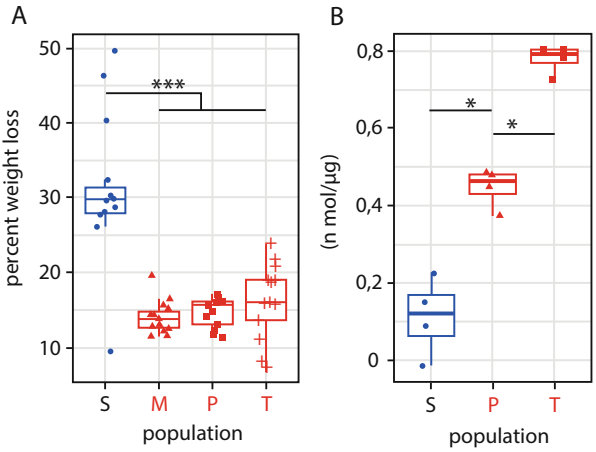

Fig. 6.8 Adaptation of *Astyanax* surface (*S*) and cave fish (*M* Molino, *P* Pachón, *T* Tinaja) to starvation. (**a**) Percentage of weight loss after 60 days of starvation (***$p < 0.0001$, Tukey honest significant difference [HSD] test). (**b**) Total triglyceride content/protein in adult fish (*$p < 0.05$, Tukey HSD test) (adapted from Aspiras et al. 2015)

8.9% in the surface form. Consistent with this observation, the triglyceride fat content was increased in regularly fed adult Pachón and was even higher in the Tinaja cave fish compared with the surface fish (Fig. 6.8b). The only exception was observed in the Tinaja cave fish, in which fat storage takes place in the greatly enlarged liver (Apiras et al. 2015).

Food consumption of individuals from the cave and surface populations was studied to reveal how cave fish acquire increased fat content. Differences in appetite were studied by measuring the amount of freshwater oligochaetes (*Lumbriculus variegatus*) consumed over a certain period in cave and surface fish (Aspiras et al. 2015). The results suggest that there are significant differences in appetite regulation between populations. For example, although Pachón cave fish reach higher fat levels than surface fish, a difference in their appetite from their surface counterparts was not detected (Fig. 6.9a–c). Furthermore, the appetite of well fed Tinaja cave fish was not significantly different from that which surface fish reached after 3 weeks of fasting (Fig. 6.9a). Neither Pachón nor Tinaja cave fish appetites were affected by a 3-week fast, whereas surface fish that have fasted display a significant elevation in appetite (Fig. 6.9a). However, the Tinaja cave fish exhibit the highest appetite among all populations (Fig. 6.9a–c). These data suggest the possibility that there are significant differences in appetite regulation between populations.

Study of F1 hybrids between Pachón and Tinaja cave fish as well as between surface and Tinaja cave fish revealed that both exhibit appetites similar to Pachón and surface fish during a fed state. This suggests the excessive Tinaja appetite to be a recessive trait (Fig. 6.9b–c). The improved ability of the Pachón cave fish to store fat, as measured by its mean routine oxygen consumption rate over 24 hours (Vo_2), is intermediate in the F1 crossing and shows a slightly bimodal or at least left-

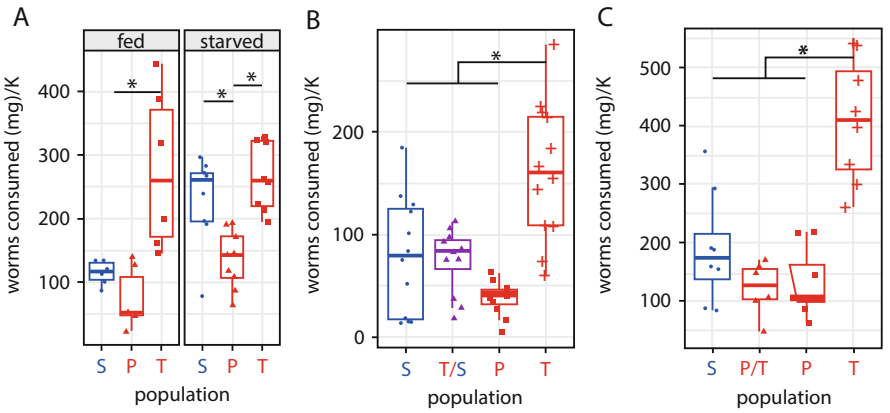

Fig. 6.9 Differential appetite regulation. Comparison among fed and starved *Astyanax* surface (*S*), Pachón (*P*), and Tinaja (*T*) cave fish over a 3-wk period (**a**), among fed surface (*S*), Tinaja/surface F1 hybrids (*T*/*S*), Pachón (*P*), and Tinaja (*T*) fish over a 36-h period (**b**), and among fed surface (*S*), Pachón/Tinaja F1 hybrids (*P*/*T*), Pachón (*P*), and Tinaja (*T*) cave fish over a 48-h period (**c**). *K* condition factor (adapted from Aspiras et al. 2015)

oblique distribution in the F2 crossing between the surface and the Pachón cave fish, insinuating epistatic gene action (see Sect. 6.23) (Hüppop 1989).

A candidate gene approach led to the identification of coding mutations in conserved residues of the melanocortin 4 receptor (*MC4R*) gene in the Pachón, Molino, Arroyo, Yerbaniz, Tinaja, Piedras, Micos, and Sabinos cave populations, which is assumed to contribute to the increase in appetite and starvation resistance (Apiras et al. 2015). Most notably, the mutated *MC4R* is heterozygous in the Pachón cave fish and also present at very low frequency in the surface fish. This corroborates the view that standing variation may have played an important role in the initial phase of cave colonization by surface *Astyanax*.

Besides fat storage, starvation resistance, and increased appetite, lowering of metabolism is another adaptation made by cave species to survive during starvation periods. Measurements of oxygen consumption in total darkness in an absolutely silent room (camera silens) over a 24-hour period after 2 days of acclimatization revealed no difference between *Astyanax* surface fish and the Pachón cave fish, when calculating fat-free body mass (Hüppop 1989). These conditions were necessary to allow comparisons because of a different maintenance metabolism in specific body tissues, which in fat tissue is much lower than in nerve tissue, in which it is the highest (Ball and Jungas 1965; Hüppop 1989; Moran et al. 2015).

In contrast to the observation over a 24-hour period, and not considering the before-mentioned caveat (Hüppop 1989), measurements of oxygen consumption in darkness over a longer period of 7 days showed high energy savings (Moran et al. 2014). In constant darkness, the surface fish exhibited a circadian rhythm in metabolism with an increase in oxygen demand during the subjective daytime, whereas cave fish did not. It was concluded that the lack of circadian rhythm in

metabolism leads to a 27% energy savings for Pachón cave fish compared with the surface fish in their natural photoperiods. Under constant darkness, the Pachón cave fish will consume 38% less energy than the surface fish. Moran et al. (2014) suggest that the lower energy demand can be explained by the fact that the metabolism in the surface fish is still subjected to circadian rhythmicity with an increase in oxygen consumption during the subjective daytime, whereas because of the lack of circadian activity in the cave fish, no increase occurred. Most notably, the metabolic cycle was uncoupled from locomotor activity.

As neural tissue exhibits the highest energetic costs of maintenance metabolism, it was often claimed that eye regression in cave fish would be driven by energy savings, particularly because the reduction of the visual system consisting of the eye and the tectum opticum are correlated. Based on experimental studies, the cost of vision was calculated to be 15% of resting metabolism for a 1 g larval fish, decreasing to 5% in an 8.5 g adult fish as relative eye and tectum size decline during growth. For the SEP Pachón and the VEP Micos cave fish, it was shown that the loss of the visual system substantially lowered the amount of energy expended on neural tissue, in particular for juvenile fish. The cost of neural tissue contained in eyes and the whole brain for a 1 g surface fish represented 23% of resting metabolism, whereas for Micos and Pachón cave fish the cost was 13 and 10%, respectively (Moran et al. 2015).

6.10 Overall Sleep and Activity Patterns

Circadian sleep and activity patterns were studied in juvenile fry of *Astyanax* surface fish, the SEP Pachón and Tinaja as well as the VEP Molino cave fish over a period of 48 hours (Duboué et al. 2011). Sleep profiles were based on 60 seconds of inactivity as the criterion for sleep. It was revealed that the surface fish are diurnal, sleeping at night and largely awake during the day, but with some sleep toward the middle of the day (Fig. 6.10a).

The three cave populations were found to have diverged from the surface sleep pattern and show a drastically reduced sleep phenotype both in the day and at night time (surface: 800 min vs cave: 110–250 min per 24 h) (Fig. 6.10b). The phenotype of reduced sleep has independently evolved in the cave fish as shown by the study of the SEP Pachón and Tinaja and the VEP Molino cave fish (Duboué et al. 2011). To differentiate between true sleep and reduced activity, quantification of activity per waking minute (velocity) was performed and revealed that the active waking velocities of Pachón and Tinaja but not Molino cave fish exceed those of the surface fish. However, Pachón and Tinaja are much more active both day and night compared with surface fish and Molino cave fish (Fig. 6.10c).

Sleep efficiency is generally characterized by the number and length of undisturbed bouts or periods. Whereas the number of nighttime bouts did not differ significantly between surface and cave fish, during the daytime the Pachón and Tinaja cave fish exhibited fewer bouts than the surface fish (Fig. 6.10d). Both

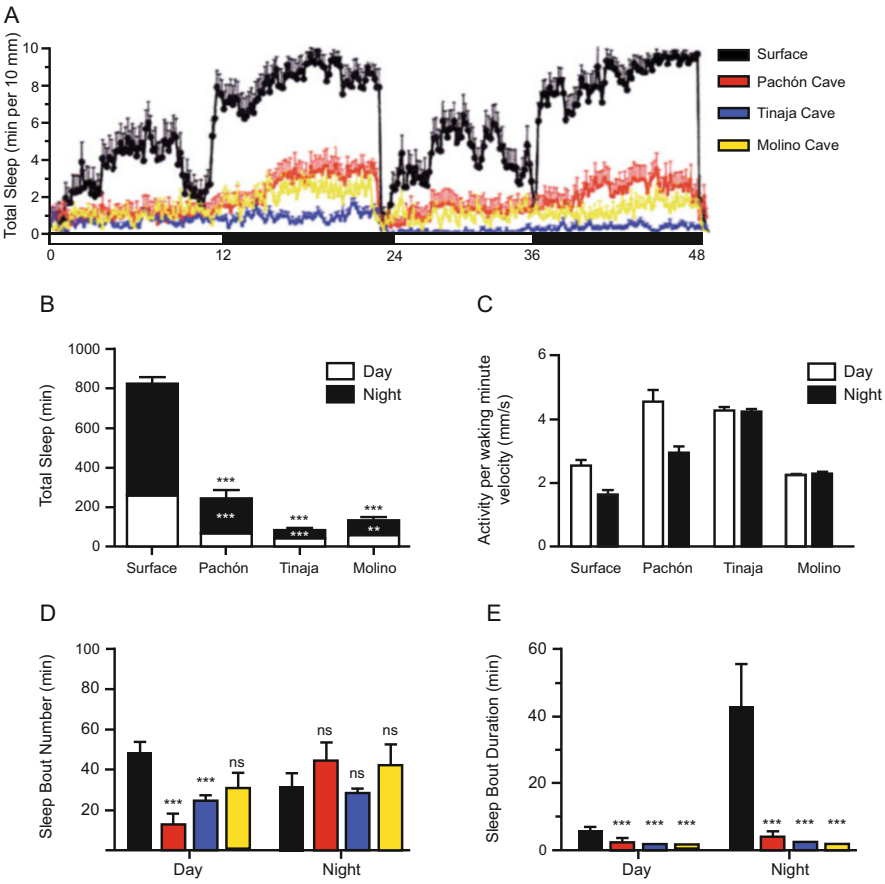

Fig. 6.10 Amount of sleep in 21- and 24-day-old larvae of the *Astyanax* surface fish and three independently evolved cave populations. (**a**) Sleep profiles graphed as number of minutes of sleep per 10-min period over 48 h, (**b**) amount of sleep per 24 h, (**c**) average waking activity, (**d**) average number of sleep bouts, (**e**) average bout duration, *asterisks* represent significance relative to surface fish (adapted from Duboué et al. 2011)

daytime and nighttime bout lengths were shorter in all cave populations studied compared with the surface fish (Fig. 6.10e).

The studies dealing with the resting and activity patterns performed with larval fry could be stated for adult cave fish too (Yoshizawa et al. 2015). Crossing analysis revealed that the difference in total sleep between surface and cave fish has a polygenic basis (see Sect. 6.23, Fig. 6.55d) and that the genes responsible for the cave fish sleep phenotype are inherited independently from those underlying eye and pigmentation reduction (Duboué et al. 2011). It is claimed that changes in adrenergic signalling may underlie the reduced amount of sleep in *Astyanax* cave fish (Duboué et al. 2012).

Sleep is a homeostatically regulated physiological state marked behaviourally by prolonged periods of quiescence and a reduced responsiveness to external stimuli (Campbell and Tobler 1984). In mammalian species, the amount of sleep was found to vary greatly and primarily reflects ecological constraints acting on total sleep time (Capellini et al. 2008). For example, in whales, pinnipedes, and sirenia, unihemispheric slow-wave sleep (USWS) is exhibited, in which only one hemisphere of the brain is sleeping. It is proposed that within the specific ecological framework of the *Astyanax* cave fish, reduced sleep is no loss but may instead be an adaptation to an environment, where the change between night and day has lost its biological function. It is as yet unknown whether the reduced sleep duration might be compensated for by the physiological intensity of sleep in the cave fish. Thus, sleep in *Astyanax* is an independent peripheral outcome of circadian rhythmicity, which changed constructively under altering environments in the cave fish.

Taken altogether, the reduced sleep pattern might be a constructive rather than a regressive trait. The cave fish have evolved a dramatically reduced sleep phenotype to increase the time available for foraging (Duboué et al. 2012), which would be a gain and no loss. Resting and activity patterns have been uncoupled from circadian rhythm by destructive mutations, because they have lost their biological function in constant darkness and are no longer submitted to selection.

6.11 Egg Yolk Content

Few cave species like the cusk eels of the genus *Typhliasina* and *Lucifuga* (Bythitidae) or the live-bearing toothcarp *Poecilia mexicana* (Poeciliidae) produce live offspring. They do so in rather low numbers of 2–15 juveniles. However, most cave fish lay large numbers of eggs (up to about 1000) in every spawning process. At the beginning, the fish embryos exclusively subsist on yolk. Comparing the amount of egg yolk content it was found that between the troglophile surface catfish *Rhamdia laticauda* and its cave sister species *R. zongolicensis* and *R. reddelli* no differences exist (Wilkens 2001). In contrast, *Astyanax* cave fish provide an example of the yolk content being significantly increased (by about 50%) in comparison to the surface sister form. This was shown by the study of five different cave populations (Micos, Chica, Pachón, Piedras, Yerbaniz) (Figs. 6.11 and 6.12). The chemical composition of yolk (e.g. fat, protein) is nearly identical between surface and cave forms.

Due to the higher quantity of yolk reserves in the egg, the *Astyanax* cave fish juveniles grow for a longer time and are larger than the surface fish (Hüppop and Wilkens 1991) when they start external feeding. For example, Pachón cave fish larvae do so at an age of 4 days after fertilization, compared with 3 days in the surface fish. Because of their size they are probably able to catch slightly larger prey (Fig. 6.12) (Hinaux et al. 2011; Hüppop 1987, 1989; Yamamoto et al. 2009).

Mean and distribution of yolk content in the F1 crossings show a more or less intermediate position between surface and phylogenetically old Pachon or Piedras

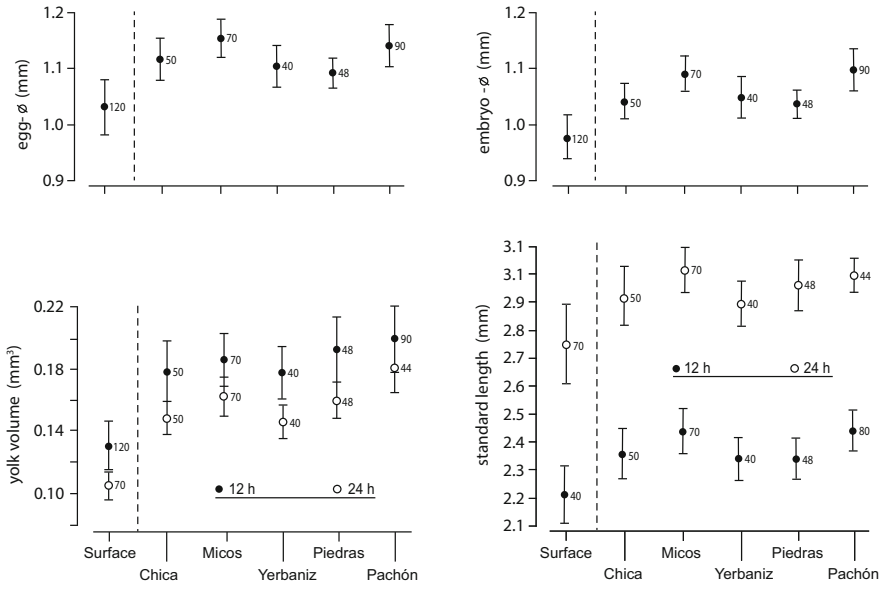

Fig. 6.11 Means and standard deviation of egg and embryo size of *Astyanax* surface fish and five cave fish populations at 12 (*closed circle*) and 24 h (*open circle*) after fertilization, numbers give sample size (adapted from Hüppop and Wilkens 1991)

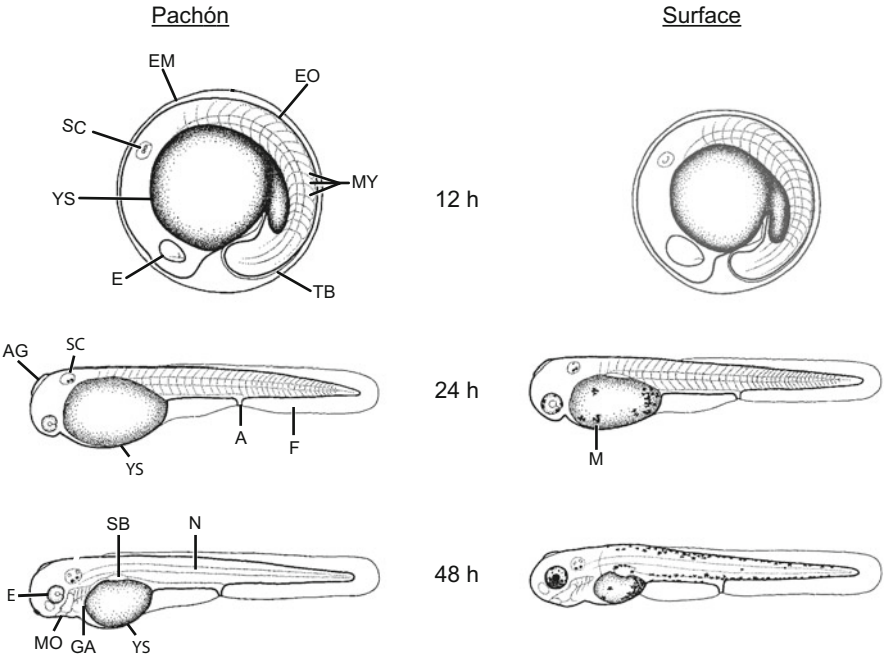

Fig. 6.12 Comparison of yolk content and growth in *Astyanax* surface and Pachón cave fish at 12, 24, and 48 h after fertilization. *A* anus, *AG* adhesive gland, *E* eye, *EM* egg membrane, *EO* embryo, *F* fin fold, *M* melanophore, *MY* myomeres, *N* notochord, *NP* olfactory pit, *SB* swim bladder, *SC* statocyste, *TB* tail bud, *YS* yolk sac (adapted from Hüppop and Wilkens 1991)

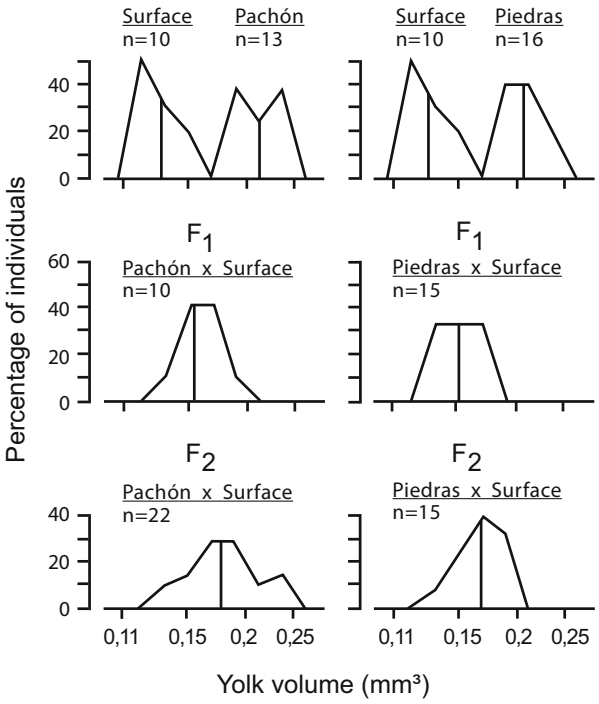

Fig. 6.13 Yolk content of *Astyanax* surface, the Pachón and Piedras cave fish as well as in their F1 and F2 crossings (adapted from Hüppop and Wilkens 1991)

cave fish. These findings insinuate that the increase of yolk content is polygenic, showing epistatic gene effect (see Sect. 6.23) (Fig. 6.13) (Hüppop and Wilkens 1991).

6.12 Aggressive Behavioural Patterns in *Astyanax*

Animals competing for food and mates display species-specific aggressive behaviours independently of whether they live in dark caves or in lit environments. Visual signals often play an important role in aggressive behaviour, but cannot be used in darkness (Parzefall 2000). Caves are mostly colonized by troglophile species such as catfish, which are already preadapted to darkness and do not alter their aggressive behaviour in the new environment. However, when such preadaptations are lacking, changes in aggressive behaviour occur, originally visually triggered traits get lost and new ones are developed (Parzefall and Trajano 2010).

Astyanax surface fish exhibit a visually released complex aggressive behaviour consisting of threatening postures and attacks, which may be followed by fights if the attacked individual does not take flight (Parzefall 1983; Parzefall and Trajano 2010). The threatening surface fish in general enlarges its body size by spreading its fins (aggressive fin spreading) and snake swimming (Fig. 6.14a, b). In escalated fights, tail beating (Fig. 6.14c), circling (Fig. 6.14d), ramming attempts, ramming,

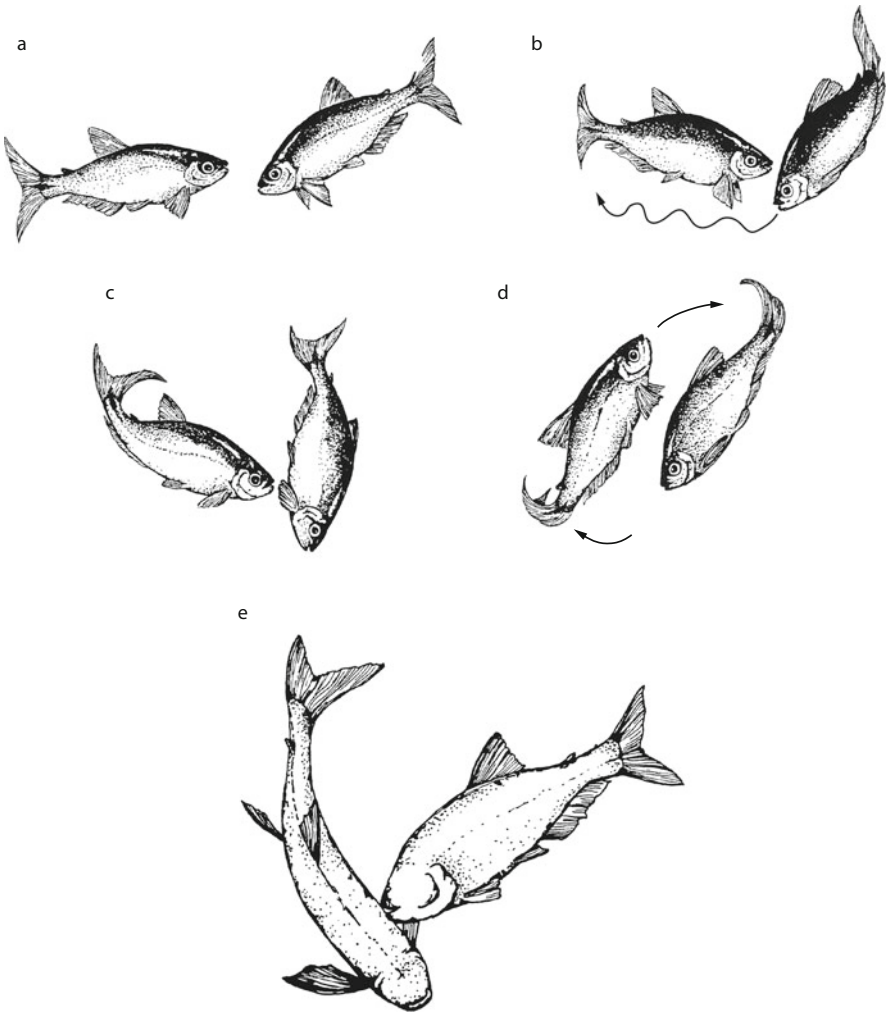

Fig. 6.14 Aggressive patterns in *Astyanax* surface fish. (**a**) Fin erection: the head-down position of the right fish expresses a higher aggressive motivation, (**b**) snake swimming of the right fish and aggressive fin erection by the left one, (**c**) ramming attack of the left fish, (**d**) circling and tail beating, (**e**) ramming and circling in the Pachón cave fish (adapted from Parzefall and Hausberg 2001)

and biting is performed by the attacking individual (Parzefall and Hausberg 2001). The intensities of the aggressive patterns of fin spreading, ramming attempts, ramming and tail beating are positively correlated (Fricke and Parzefall 1989).

In contrast to the surface fish, strongly eye-reduced cave populations like the SEP Pachón, Yerbaniz, and Piedras cave fish are no longer able to perceive and exhibit visually released aggressive behaviour like fin spreading and snake swimming. Instead, other elements of surface aggressive behaviour like biting are

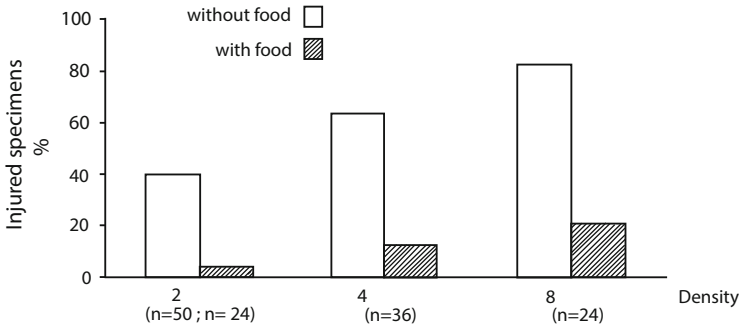

Fig. 6.15 Percentage of injured specimens of the Pachón cave fish in dependence of food supply and density. n = number of specimens studied (adapted from Hausberg 1995)

exhibited (Fig. 6.14e). As close proximity and direct body contact were observed to be the premise of such aggressive actions in the SEP cave fish, it is assumed that a different sense like the lateral line is involved in triggering it. As a consequence of these fights the cave fish also get hurt, shown by a loss of scales and parts of the fins, especially of the caudal one (Hausberg 1995). The amount of injuries depends on the population density (Fig. 6.15).

Aggressive behaviour in surface and cave *Astyanax* is a density controlling, sex-independent mechanism (Parzefall 1983). In the surface fish, escalated fighting usually does not take place as long as the fish have enough space while schooling in their natural habitats like rivers, cenotes, and lakes. The threatening behaviour of fin spreading and snake swimming will suffice to avoid injuring fights. However, during the dry season when food gets scarce and the surface fish get separated in shrinking pools, small feeding territories are defended (Parzefall and Trajano 2010). When there is no chance to escape or hide, fights occur leading to severe injuries, and the weaker opponent may even get killed. In the cave fish populations, territories are only built and defended under food deprivation (Hausberg 1995).

In absolute darkness, neither juvenile nor adult surface fish display aggressive behaviour or establish territories at all (Fig. 6.16). The finding that surface specimens do not show scale loss or fin injuries supports the theory that no fights take place under such conditions (Hausberg 1995; Parzefall 1983, 2000; Wilkens 1988). Therefore, it is proposed that the aggressive behaviour of the *Astyanax* surface fish is exclusively visually released.

The optical releasers for aggression were studied by observing the choice-preference behaviour of single specimens with respect to two simultaneously presented signals (Langecker et al. 1995). Three experimental tanks were arranged in a linear order. The experimental set-up ensured that only optical signals could be transmitted. The central tank was marked at its centre by a line dividing it into left and right compartments. Two alternative signals were presented at the left and the right side of the central tank. As signals, live surface and cave fish as well as surface dummies were tested. Among the observable agressive behavioural patterns, only

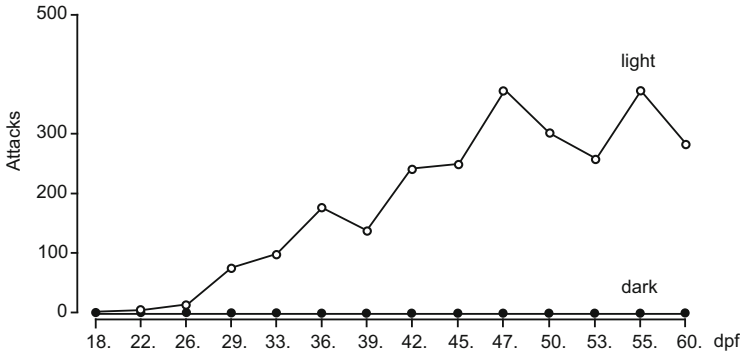

Fig. 6.16 Ontogeny of aggressive behaviour of *Astyanax* surface fish at light and in absolute darkness (number of attacks/12 min). *dpf* days post-fertilization (adapted from Hausberg 1995)

"ramming attempt" performed at the left or the right side of the central tank was counted as a general measure for aggression because of its high frequency and distinctness.

All of the 20 surface individuals tested attacked the moving, naturally coloured live surface signal fish rather than the alternatively presented live Pachón cave fish (Fig. 6.17a). A significantly lesser number of tested surface specimens (79%) preferred the moving, naturally coloured 3D surface dummy when presented alternatively with a stationary surface dummy (Fig. 6.17b). This shows that both "natural shape and colouration" as well as "locomotion" are effective releasers of aggression, but that the combination of both is important in the surface fish. Movement alone is less effective. These findings are also stated when testing the signal combination of a live moving Pachón cave fish presented as an alternative to a stationary surface dummy (Fig. 6.17c) as well as a moving 2D square plastic sheet presented as an alternative to a stationary dummy (Fig. 6.17d). In both experiments the moving object was preferred by a very low number of tested surface specimens, however.

Regression of the releaser "natural shape and colouration" has obviously occurred in the cave fish. It can be assumed that this ability of perceiving visual releasers got reduced because it is no longer controlled by stabilizing selection. Whereas all surface specimens tested attacked the surface signal fish when alternatively presented with the Pachon signal fish, a significantly lower number of the F1 hybrids (surface × SEP Pachón) (65%) (Fig. 6.17a) as well as of the VEP Micos fish (72%) (Fig. 6.17a) attacked the live surface signal fish. From the nearly intermediate number of attacks in the F1 it can be concluded that the releaser is completely reduced in the SEP Pachón cave fish and from the lower number found in the VEP Micos fish that it is on the way to reduction.

The choice experiments also revealed a change of the releaser "locomotion" in cave specimens. Whereas only some of the surface specimens (22%) attacked a moving object that had no natural shape but consisted just of a square piece of plastic (Fig. 6.17d), higher numbers of the tested F1 hybrids (65%) and of the Micos

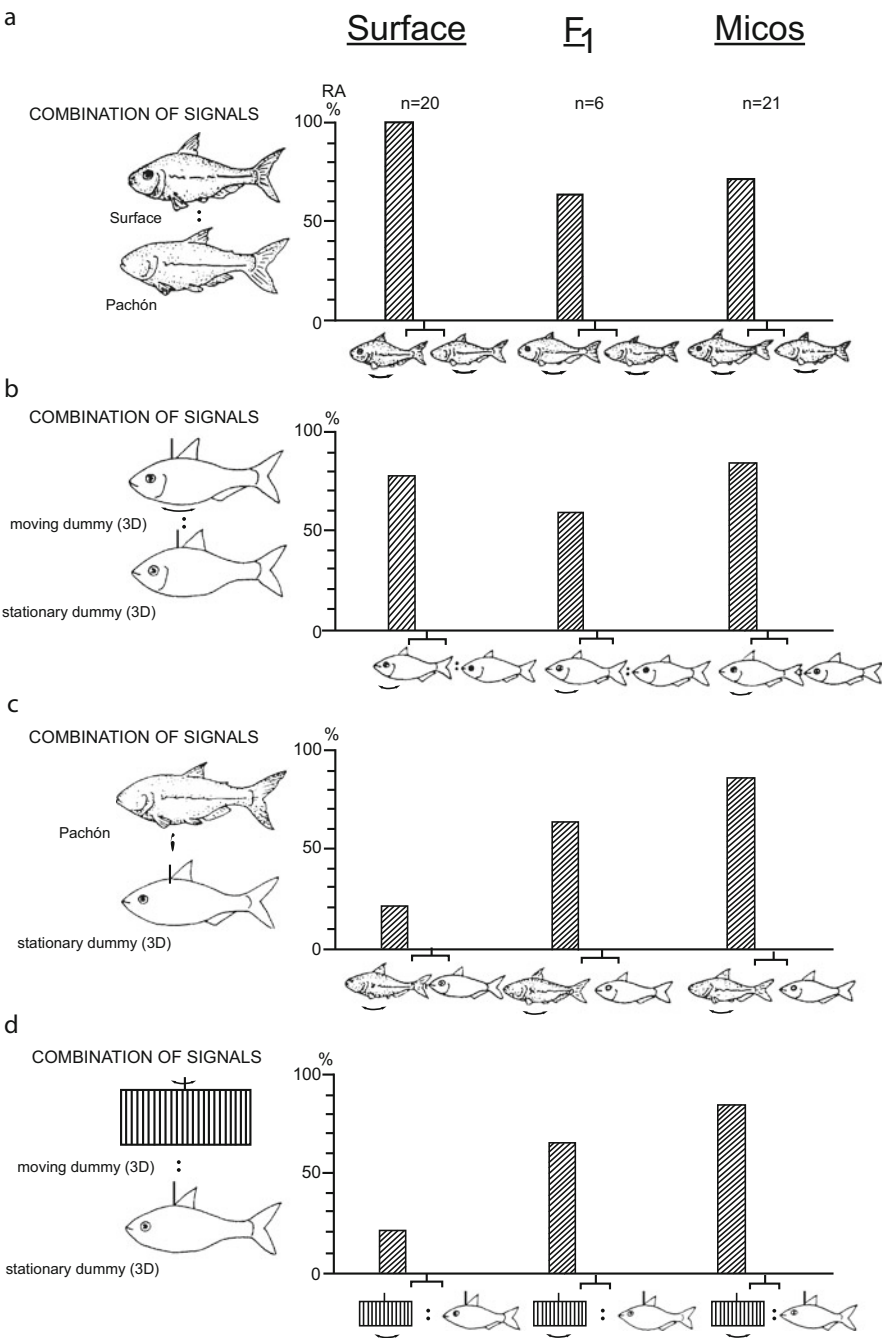

Fig. 6.17 Percentage of surface, F1 crossing (SEP Pachón cave × surface fish), and VEP Micos cave fish showing a significant preference for aggressive ramming attempts to distinct combinations of signals. *n* Total number of specimens tested, *RA* ramming attempts, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Langecker et al. 1995)

fishes (85%) preferred to attack all moving objects regardless of their shape (Fig. 6.17c, d). From this is concluded that the ability of perceiving the releaser "locomotion" has been improved in the cave fish.

Thus, in all cave fish populations the releaser has changed, because aggression is almost exclusively triggered by visual stimuli in the surface ancestor. The stimulus "locomotion" is probably perceived by the lateral line organ and has come to play a new role in releasing the aggressive behaviour of *Astyanax* cave fish. Whereas it was found that in the SEP Pachón and Piedras cave fish the visually released trigger of aggressive behaviour is reduced and has been completely replaced by the trigger "locomotion", the VEP Micos cave fish has an intermediate position between surface and SEP cave fish. Testing of well sighted Micos cave fish revealed differing degrees of intensity of the visually triggered aggressive attacks performed by them. In some specimens they are even lacking (Hoffman and Hausberg 1993). In the mean, however, the intensity of the visually triggered aggressive behaviour is lower than in the surface fish. Like in eyes and pigmentation, the visually triggered aggressive behaviour is lower in the VEP Micos cave fish and comprises an intermediate stage between the SEP and surface fish.

The number of aggressive reactions measured as ramming attacks in the F1 as well as the F2 crossings between the surface and the SEP cave fish indicates that the reduction of the visually triggered aggressive behavioural traits in the SEP cave fish relies on a genetic basis. The mean number of attacks is lower and they become completely manifested only at an ontogenetically later stage compared with the surface fish (Fig. 6.18) (Hausberg 1995; Langecker et al. 1995). As all Micos specimens tested had large eye size and good vision ability, but showed differing degrees of visually triggered aggressive behaviour, it was concluded that eye size and aggressive behaviour inherit independently (Hoffman and Hausberg 1993; Langecker et al. 1995; Parzefall and Hausberg 2001).

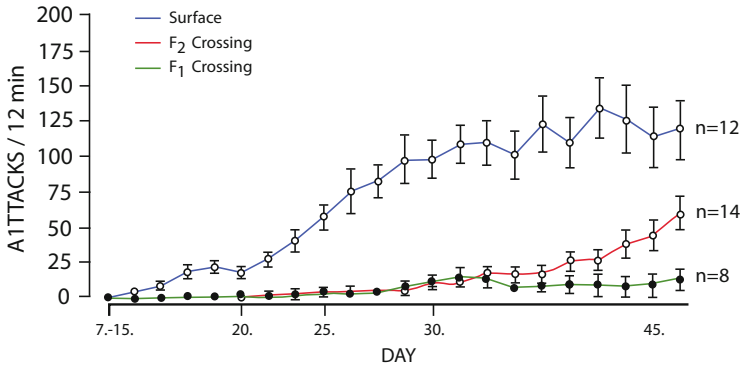

Fig. 6.18 Ontogeny of the attack rate of *Astyanax* surface fish and F1- and F2-crossing hybrids with the Pachón cave fish (mean and standard deviation, adapted from Parzefall and Hausberg 2001)

In several studies it was claimed that surface *Astyanax* would be aggressive in darkness. For example, Burchards et al. (1985) observed that surface fish show aggressive behaviour in darkness. It was later found that the experiments were not conducted in complete darkness and that the light from the control lamp for the heat rod and the infrared devices applied provided enough illumination for the surface fish to build and defend territories (Hausberg 1995; Burchards, personal communication). Aggressive behaviour was also observed in lens-extirpated surface fish, because it was assumed that they would mimic reduced cave fish eyes not able to see (Espinasa et al. 2005). However, it was not considered that these eye residuals still contain intact visual cells (see Fig. 1D in Espinasa et al. 2005) and operate like a pinhole camera. They may perceive moving objects at least as diffuse images, which in *Astyanax* surface fish suffices to release aggressive reactions. These findings rely on inappropriate experimental conditions.

It was furthermore claimed that the SEP cave fish had lost the aggressive behaviour and hypothesized that during their evolution in darkness they had made an "evolutionary shift from fighting to foraging" under the bias of the cave fish being "food addicts" (Elipot et al. 2013). These results may have come about by applying a too-short acclimatization time of only few hours, not considering that adult cave fish start performing aggressive behaviour only after 2 days (Hausberg 1995). Besides this, aggressive behaviour in the crossings between surface and SEP cave fish starts at later ontogenetic stages (Fig. 6.18) and it is as yet unknown whether it is the same in the cave fish. Therefore, it cannot be excluded that the alleged foraging behaviour observed in these experiments performed with juvenile cave fish are only random clashes caused by swimming activity performed by the cave fish to get spatial orientation after having been introduced into a new environment (see Sect. 6.5).

6.13 Dorsal Light Reaction

The dorsal light reaction enables fish to orientate the body in a vertical position according to two factors, namely the light input from above and earth gravity from below. This behaviour furthermore serves to camouflage the fish against aerial predators by adapting the dorsal melanophore and iridophore colouration to that of the albedo of the underground. Simultaneously, it provides camouflage against aquatic predators attacking from below, because the silvery shine caused by guanophore colour cells on the ventral side clear away the body contours of the fish at the water surface.

When kept in darkness, both surface and cave fish swim in an upright position, which shows that solely one of the two factors, namely earth gravity, is sufficient for an upright orientation. However, when a light beam is applied in the dark at a 90° angle from the side, the surface fish will incline its body to a 45° angle. Because of the missing eyes, blind SEP cave fish like Pachón or Piedras show no reaction (Fig. 6.19). The eyed F1-crossing hybrids between both forms manifest an intermediate tilt under these experimental conditions. In F2 hybrids equipped with good vision, the tilt angles range between nearly missing and the mean exhibited by the

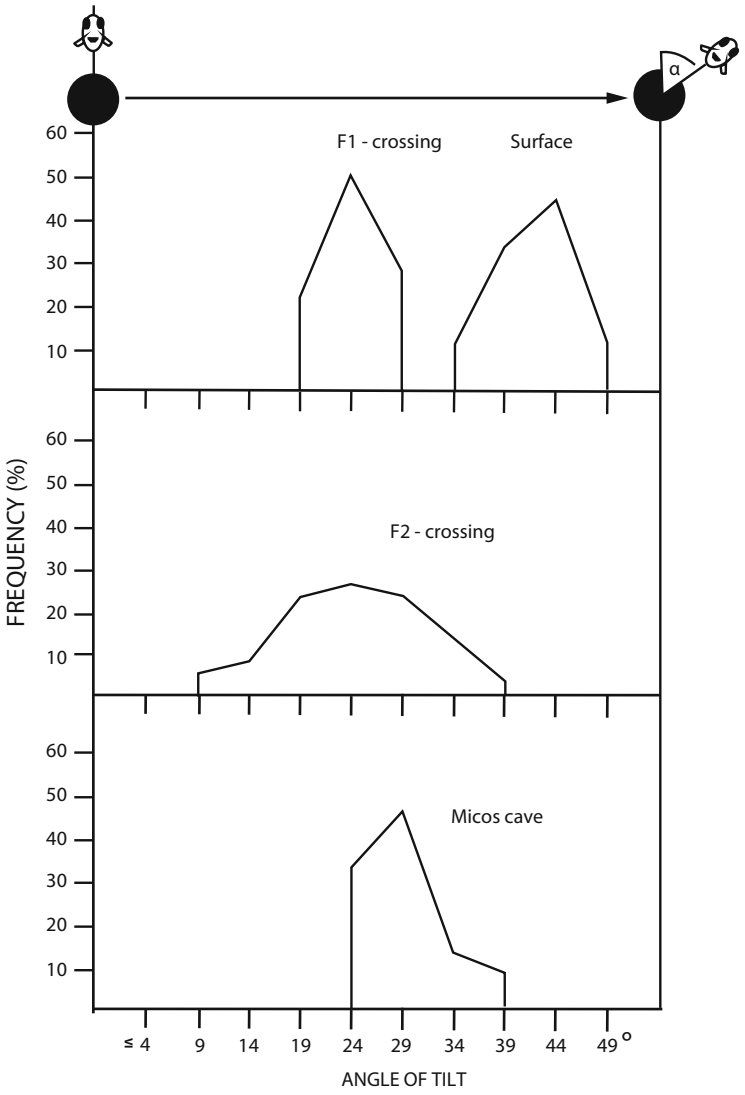

Fig. 6.19 Frequency distribution of the angle of tilt of *Astyanax* surface fish, the F1- and F2-crossing hybrids with the SEP Pachón, and the VEP Micos cave fish, when illuminated at an angle of 90° from the side. *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Langecker 2000)

surface form. These results suggest that part of the dorsal light reaction is strongly reduced in the SEP cave fish and only the earth gravity part is still functional. The VEP Micos cave fish lie between the mean angle of the F1 crossing between the surface and SEP cave fish, and the surface fish. The intermediate distribution of the angles in the F2 and in the F1 crossing between surface and SEP cave fish shows that the dorsal light reaction relies on a polygenic basis (Langecker 1993).

6.14 Phototactic Behaviour

The mostly troglophile surface ancestors of cave species derive from forms already preadapted to living in darkness. Avoiding light they show strong photonegative behaviour and mostly prefer a nocturnal way of life. For example, photonegativity was found in the Somalian cave fish *Phreatichthys andruzzi* (Cyprinidae) and *Uetgitglanis zammaroni* (Claridae) (Ercolini and Berti 1975). In *P. andruzzi* it was revealed that encephalic photoreception could explain the observed behavioural spectral sensitivity (Tartellin et al. 2012).

It is also described for the Mexican surface catfish *Rhamdia laticauda* (Hepapteridae) (Langecker 1992a; Wilkens 2001). Therefore, photonegative behaviour has long been looked upon as a prerequisite of surface species for cave colonization as well as an adaptation of cave species to prevent them from leaving the dark environment. However, the study of two cave sister species of surface *R. laticauda*, *R. zongolicensis* and *R. reddelli*, revealed that, in comparison with the surface species, photonegativity is less strong in them and shows high interindividual variability (Fig. 6.20), although photosensitivity is most probably still functional because of the pineal containing visual cells (Eilertsen et al. 2014; Langecker and Wilkens 1992). This could imply that the phototactic behaviour has lost its biological function in these cave species and is regressive, because in the vast

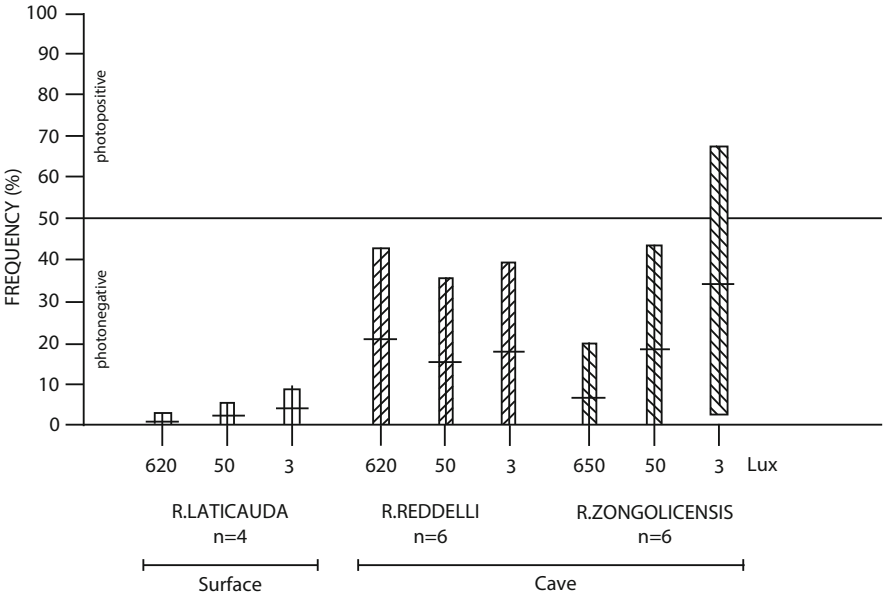

Fig. 6.20 Phototactic reactions of the surface catfish *Rhamdia laticauda* and its cave sister species *R. reddelli* and *R. zongolicensis* at different light intensities (650, 50, 3 lux). Shown are mean response, standard deviation (*vertical line within box*) and 95% confidence limits of the mean (*box*) (adapted from Langecker 1992a)

underground cave system the fish rarely, if at all, make contact with the influence of surface light.

In *Astyanax* surface fish it has been shown that phototactic behaviour changes during life history and therefore has to be evaluated in its ecological context (Langecker 2000). Whereas juveniles prefer lighter zones, even reacting photopositively, adult specimens are photonegative in bright light but indifferent in low light intensities. This differing behaviour may favour the utilization of different foraging areas by both groups as well as save the juveniles from being preyed on or attacked by the larger adult fish (Parzefall 1983), which prefer to hover in lesser illuminated places (Langecker 2000; Romero 1985) (Fig. 6.21).

For a comparison of phototactic behaviour between adult *Astyanax* surface and cave fish, the visual orientation was experimentally excluded by the complete enucleation of the eye ball in the surface fish. Under these conditions, the intensity of the photonegative reaction of the adult surface fish increases when compared with untreated surface fish and—except for low light intensity—is much stronger than in the cave fish. After additional ectomy of the pineal organ, the photonegative reaction in the surface fish is nearly neutral and the same as in normal, untreated cave fish (Fig. 6.22) (Langecker 1992a, b). The slightly negative phototactic behaviour observed in pineal-ectomized cave specimens possibly relies on the existence of melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) (Lucas et al. 2014).

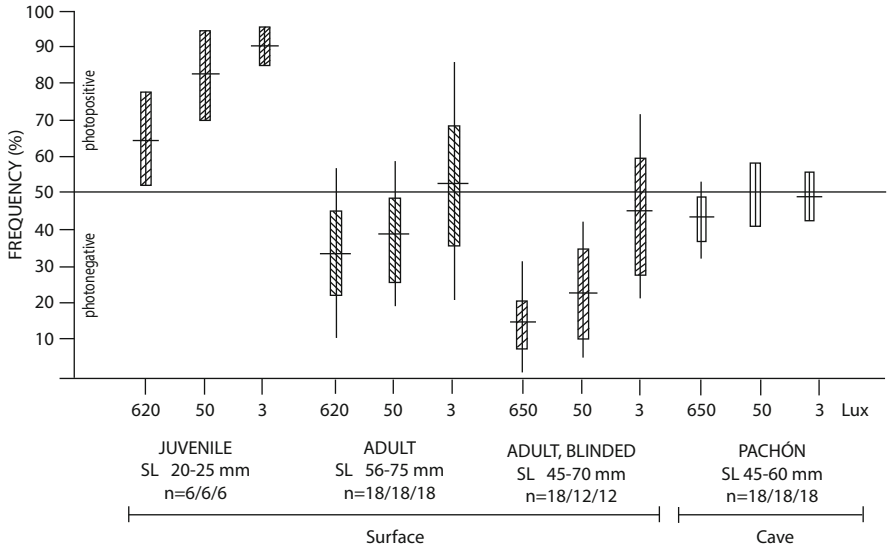

Fig. 6.21 Photonegative behaviour of juvenile eyed and blinded (eye enucleated) adult *Astyanax* surface fish and of adult Pachón cave fish. For explanation see Fig. 6.20 (adapted from Langecker 1992a, 2000)

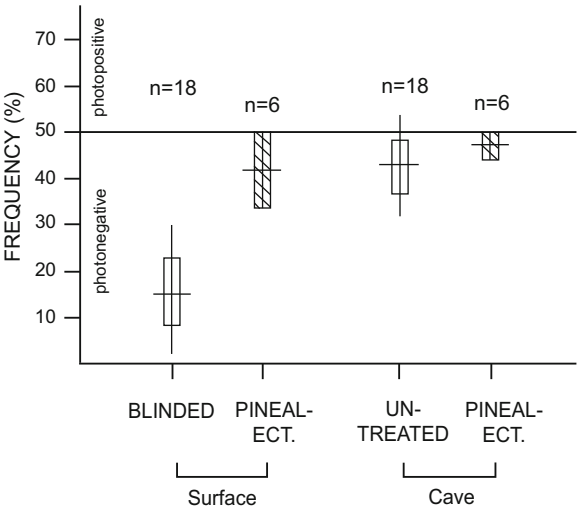

Fig. 6.22 Influence of the pineal organ on the phototactic behaviour in solely eye enucleated and eye enucleated as well as pineal ectomized *Astyanax* surface fish and in solely pineal ectomized Pachón cave fish. For explanation see Fig. 6.20 (adapted from Langecker 1992a)

It is suggested that the generally lower degree of photonegativity in the Pachón cave fish compared with the surface fish is not due to morphological degeneration of the light-perceiving structures, but is caused by the regression of the photonegative behaviour (Langecker 1992a).

This was also corroborated by studies of the phylogenetically old Tinaja cave fish, which showed no preference for the dark or the light (Kowalko et al. 2013b).

In crossings between surface and SEP cave fish, it was revealed that the F1 hybrids display strong negative phototactic behaviour comparable to the surface fish whereas the distribution curve in the F2 crossing is bimodal (Kowalko et al. 2013b). From this it can be interpreted that a polygenic system exhibiting epistatic gene effect is responsible for the phototactic behaviour (see Sect. 6.23).

6.15 Pineal Organ

The pineal organ is a small endocrine gland in the vertebrate brain that is photoreceptive because of the presence of exorhodopsin and melanopsin in photoreceptor cells structurally resembling the cones of the lateral eyes (Fig. 6.23) (Eilertsen et al. 2014). They regulate the melatonin synthesis important for photoentrainment of the circadian rhythm directly (Falcón et al. 2007, 2010). The nonvisual opsin exorhodopsin, which has been shown to be a pineal-specific opsin expressed from early in development of teleosts and has been indicated to have a role in the circadian rhythm, was evidenced in the *Astyanax* cave fish (Parry et al. 2003; Pierce et al. 2008).

Comparative histological studies of the pineal of adult *Astyanax* surface fish, the SEP Pachón and the VEP Chica cave fish revealed only minor differences between each other (Herwig 1976; Langecker 1992a). In the Chica and the Pachón cave

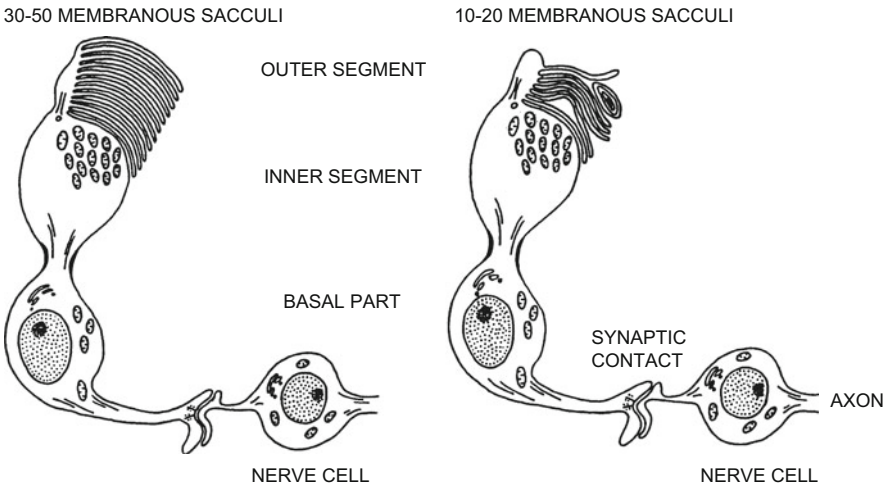

Fig. 6.23 Scheme of a single pineal photoreceptor cell of the *Astyanax* surface fish (*left*) and the Pachón cave fish (*right*) (adapted from Langecker 1992a, b)

populations, it was found that the size of the end vesicle was diminished when compared with the surface fish. Furthermore, the visual cells' outer segments have a reduced number of discs and are characterized by a large structural variability containing well differentiated as well as disorganized outer segments (Fig. 6.23) (Herwig 1976; Langecker 1992a). Also, in the blind cave salamander *Proteus anguinus* and the cave catfish *Rhamdia zongolicensis* such regressive processes restricted to the outer segments were observed whereas the surface sister species *R. laticauda* shows a well developed pineal organ (Langecker and Wilkens 1992; Kos and Bulog 2000). Study of the Chica and Pachón cave fish revealed that the outer segments of the pineal seem to be submitted to ontogenetic regression (Herwig 1976; Langecker 1992a). Thus, like the visual cells of the cave fish eye, those of the pineal organ seem to be submitted to a process of ontogenetic regression, but which does not end in total reduction. Compared with other light-dependent morphological structures, the degree of morphological reduction of the pineal organ as studied in the Pachón and the Chica cave fish is low, which can be attributed to the extant secretory activity and its function in regulating circadian rhythmicity (see Sect. 6.16).

Photosensitivity of the pineal was also confirmed by studies of the phototactic behaviour of adult cave and surface fish (see Sect. 6.14) (Langecker 1992a). Photoreception has already been detected in larval fish. *Astyanax* surface and cave fish larvae swim upward to the water surface when shaded from above, exhibiting the so-called shadow response. It is strongest at 1.5 days post-fertilization and has almost vanished at 6.5 days (Yoshizawa and Jeffery 2008).

6.16 Circadian Rhythm

The control of circadian rhythms and the coordination of different oscillators of fish are regulated by the oscillating melatonin production in the pineal organ. Melatonin as a common output signal of the vertebrate circadian clock is produced in the pineal photoreceptors and also in the retina. Different from retinal melatonin, pineal melatonin is released into the blood stream and the cerebrospinal fluid as soon as it is synthesized. However, the pineal organ is considered to serve as the central pacemaker in fish. Here the circadian clock drives rhythmic synthesis of the hormone melatonin. Studies in the pike (*Esox lucius*) have shown that each pineal photoreceptor cell contains a circadian mechanism, so that one pineal organ is made of multioscillatory cellular units (Bolliet et al. 1996). The rhythms of gene expression, enzyme activity, and melatonin release free run under constant conditions. Melatonin levels are high at night and low during the day. Photoreceptor cells, obtained from dissociated pineal organs and cultured either alone or together, maintain a rhythmic secretion of melatonin under alternating light and darkness (LD) and continual darkness (DD) for up to a week (Falcón 1999; Falcón et al. 2007, 2010; Foulkes et al. 2016; Idda et al. 2012).

In *Astyanax*, radioimmunassays of extirpated pineals in superfusion experiments were performed in the surface fish, the SEP Piedras, and the VEP Micos cave fish. These studies revealed a well detectable melatonin production. Rhythmic modulation can still be observed, indicating its role in endogenous circadian rhythmicity (Missal 1994; Wilkens et al. 1993) (Fig. 6.24). In surface and cave fish, the melatonin production was found to be high during the subjective night phase, lowered at the beginning of the subjective day phase, and rose again at the subjective night phase. After entrainment in LD 12:12 h the melanin production oscillates free running in DD for several days. The three *Astyanax* populations differ concerning the secreted amount of melatonin as well as concerning the secretional profile. The period length (τ) was 23.8 h in the surface fish, 26.4 h in the Piedras cave fish, and 24.2 h in the Micos cave fish. After light stimulation, melatonin production in all pineals is immediately lowered (Fig. 6.25). This can be attributed to regulated transcription and stability of serotonin-*N*-acetyl-tranferase (AANAT), a key enzyme in melatonin synthesis (Ziv et al. 2005), still functioning.

The melatonin levels in the pineals of the three *Astyanax* populations differed significantly (Wilkens et al. 1993). The pineal end vesicle of surface *Astyanax* contained on average 6.3 pg melatonin during the light and 119.5 pg during the dark period in February at LD 12:12 and did not significantly change at differing day length in June at LD 18:6 (Fig. 6.26). In contrast, the melatonin content of the SEP Piedras cave fish was high during daytime and not significantly different at night in February. In June, however, the daytime level was much lower, although still higher than in the surface fish. This could be caused by the light perception of the cave fish not being equally efficient as that of the surface fish, because of eye loss and the position of the light sensitive pineal in the interior of the head. Further studies should reveal whether, under permanent darkness, the SEP Piedras cave fish might exhibit more or less constant melatonin levels.

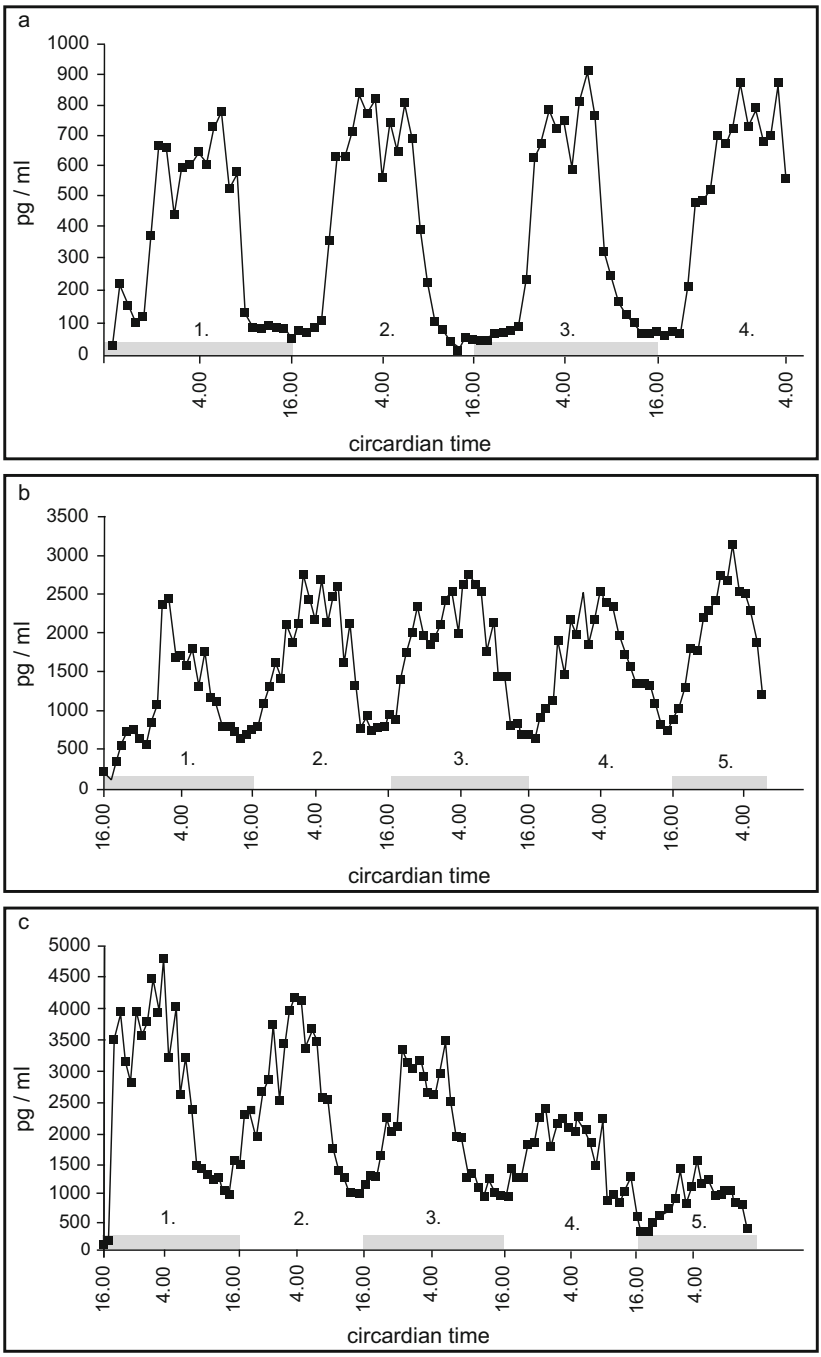

Fig. 6.24 Melatonin production of an isolated individual pineal organ of an *Astyanax* surface fish (**a**), the VEP Micos cave fish (**b**), and the SEP Piedras cave fish (**c**) all kept in DD after LD 12:12.

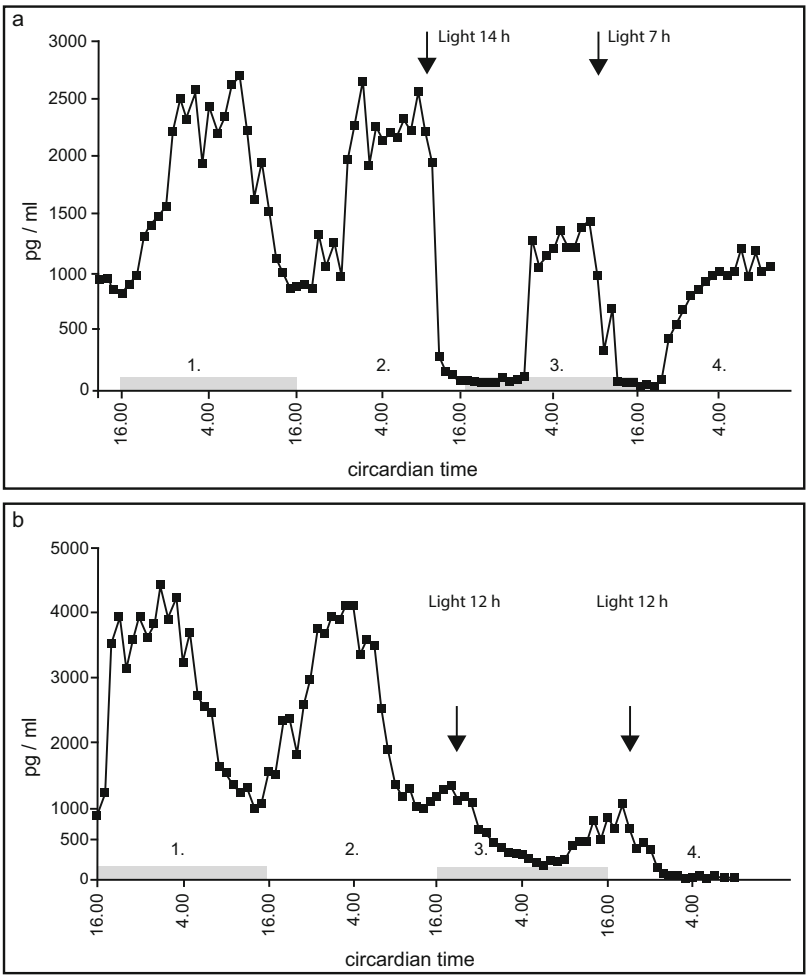

Fig. 6.25 Influence of light stimulation on melatonin production of an isolated individual pineal organ of the VEP Micos cave fish (**a**) and the SEP Piedras cave fish (**b**) kept in DD after LD 12:12. Light stimulation is procyclic in Micos for 14 and 7 h, respectively, and anticyclic in Piedras for 12 h. Days were measured in Micos from 1 pm to 1 am and in Piedras from 4 pm to 4 am. *DD* continual darkness, *LD* alternating periods of light and dark, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Missal 1994)

Fig. 6.24 (continued) Analysis was by superfusion and radioimmunoassay, probes were taken every hour for 4 days (surface fish) and 5 days (cave fish), days were measured from 4 pm to 4 am. *DD* continual darkness, *LD* alternating periods of light and dark, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Missal 1994)

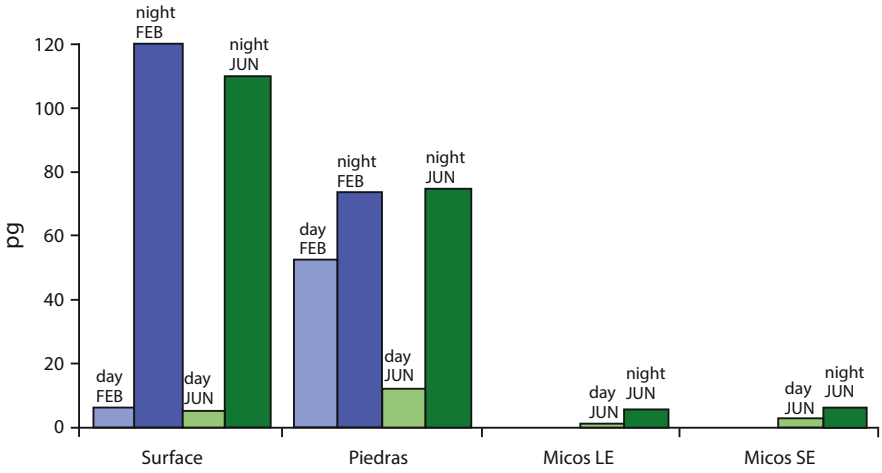

Fig. 6.26 Circadian changes of the mean melanin content in the pineal end vesicle of the *Astyanax* surface, the SEP Piedras, and the VEP Micos cave fish in February (LD 12:12) and June (LD 18:6). Micos LE and SE indicate large and small eyed specimens, respectively. *LD* alternating periods of light and dark, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Wilkens et al. 1993)

In these studies, the melatonin content in the pineal of the VEP Micos cave fish diverged even more from the surface fish than the SEP Piedras cave fish. Regardless of the variable eye size of the Micos specimens analyzed, the day-time value was minute in February and June (1.5 pg/3.1 pg) and the night-time value for the respective seasons (5.8 pg/6.6 pg) was within the range of the low day-time values of surface fish. These unexpected discrepancies of and between the SEP Piedras and the VEP Micos cave fish remain to be studied. However, altogether the results have shown that the cave fish melatonin level has changed in both cave populations.

Rhythmicity between day and night time was conserved in all experiments. The *Astyanax* surface and cave fish pineals were found to still be oscillating systems with endogenous circadian rhythmicity, which may be influenced by external factors like light stimuli. This extant functionality might explain why, in contrast to the cave fish retina, the pineal photoreceptors show almost no regression (see Sect. 6.15). As all fish from the SEP Piedras and the VEP Micos populations are able to exhibit circadian melatonin production, it is suggested that this feature is conserved in the cave fish. Further research will need to reveal, however, whether the nearly equal content of melatonin found in the SEP Piedras during the conditions of poor day light in February correlates with altered behavioural activities like loss of sleep as observed in the SEP Pachón cave fish (see Sect. 6.10) (Fig. 6.24). Possibly it is even a prerequisite for breeding success in the cave fish (see Sect. 6.2).

Molecular studies have revealed that circadian rhythms in the clock gene period 1 (*per1*), which represents a key element of the core clock mechanism and shows

high amplitude circadian oscillations in most teleosts, are retained in the Pachón and Chica cave fish but exhibit substantial alterations (Fig. 6.27) (Beale et al. 2013). In LD, *per1* levels oscillate in the two cave populations, confirming that the ability to detect light and generate molecular circadian oscillations still exists. However, there are differences between cave and surface fish. First, the cave fish *per1* rhythm is lower in amplitude and, second, the entrained phase is different with peak expression 6 hours later in both cave populations. These differences then persist in DD (Fig. 6.28). In the light-induced clock repressor *per2*, significantly raised levels compared with surface fish in DD are present in the cave fish. This might be responsible for the reduced amplitude and altered clock phase of molecular clock rhythms in the cave fish. It appears that there is a clear perturbation of the light input pathway in the *Astyanax* cave fish with the light-responsive *per2* gene constitutively expressed at high levels.

In the Chica cave fish, expression levels of *per1* under DD do not show rhythmicity and are continuously lower in the wild than in Chica fish kept in the laboratory as well as in surface fish, whereas *per2* is raised in the field comparable to the laboratory. Thus, no entrained molecular oscillation of *per1* in Chica exists in fish from the natural cave (Fig. 6.27). It can be concluded that the lack of rhythmicity is not due to the absence of sufficiently strong entraining signals within the cave, because they are provided by the circadian activity of an enormous number of bats roosting there. Rather, the core clock mechanism is hypothesized to be tonically repressed or damped as a consequence of overactivation of the light input pathway (Beale et al. 2013). Possibly the differing *per1* expression levels in the wild and under laboratory conditions are indicative of an existing variability resulting from the phylogenetically young age of the VEP Chica cave fish. Enhanced variability is a characteristic of regressive traits and also exhibited by other regressive features like the eye, body pigmentation, or fright reaction in the Chica fish (see Sects. 6.17, 6.21.3, 7.8.1).

In previous studies, behavioural activity of cave animals like amphipods and salamanders has been examined to analyze whether circadian rhythms are lost. It is therefore an interesting possibility that in cave fish the molecular circadian rhythm is somehow uncoupled from the control of locomotor activity (Fig. 6.28) (Beale et al. 2013). For example, as an output of the circadian clock, *Astyanax* surface fish show opposite circadian rhythms with respect to their activity in different water depths (Erckens and Martin 1982a, b). In nature, this behaviour is probably performed as a means to avoid predators, being different during the day and night. Activity close to the water surface peaks in the D-phase, whereas activity near the bottom is in the L-phase. Based on the study of this behaviour, two endogenous time-measuring mechanisms were found to be responsible in the surface fish (Erckens and Weber 1976; Erckens and Martin 1982a, b). Firstly, a foreign-sustained (passive) one can be induced by LD (1:0 lux) of any period length. It damps out in DD within one or a few cycles with decreasing amplitude, but unchanged frequency. Secondly, free-running circadian activity rhythms, which are obviously under the control of a self-sustained clock system, exhibit in DD, LL (constant light), and also in LDs that deviate from 24 h LDs by several hours. The

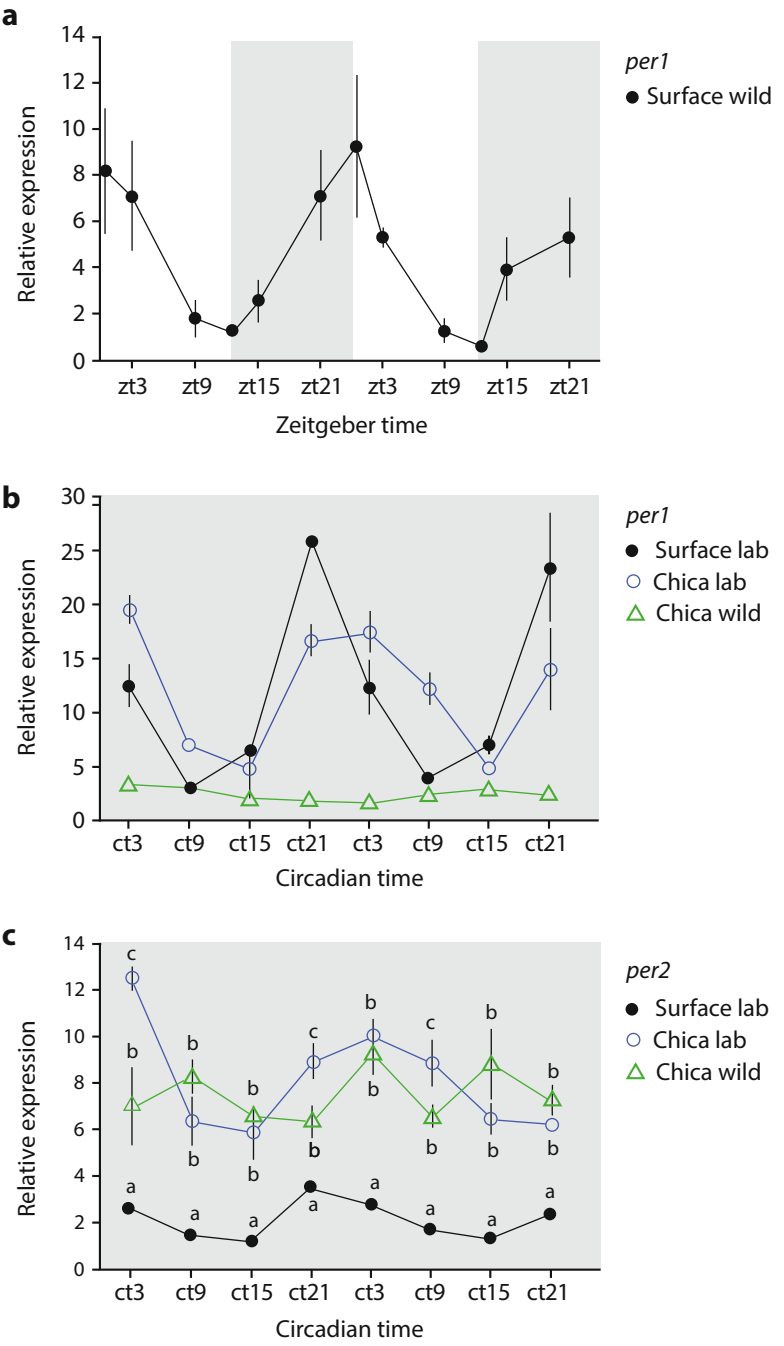

Fig. 6.27 Circadian clock gene (*period 1* and *2*) expression in fin samples from laboratory-kept and wild *Astyanax* surface fish and Chica cave fish. *White* and *grey* areas indicate light and dark periods. (**a**) Surface fish from the wild, (**b** and **c**) surface fish and Chica fish entrained in the lab and Chica fish from the wild (Different *lower case letters* in (**c**) indicate significant differences [$p < 0.05$] between comparisons) (adapted from Beale et al. 2013)

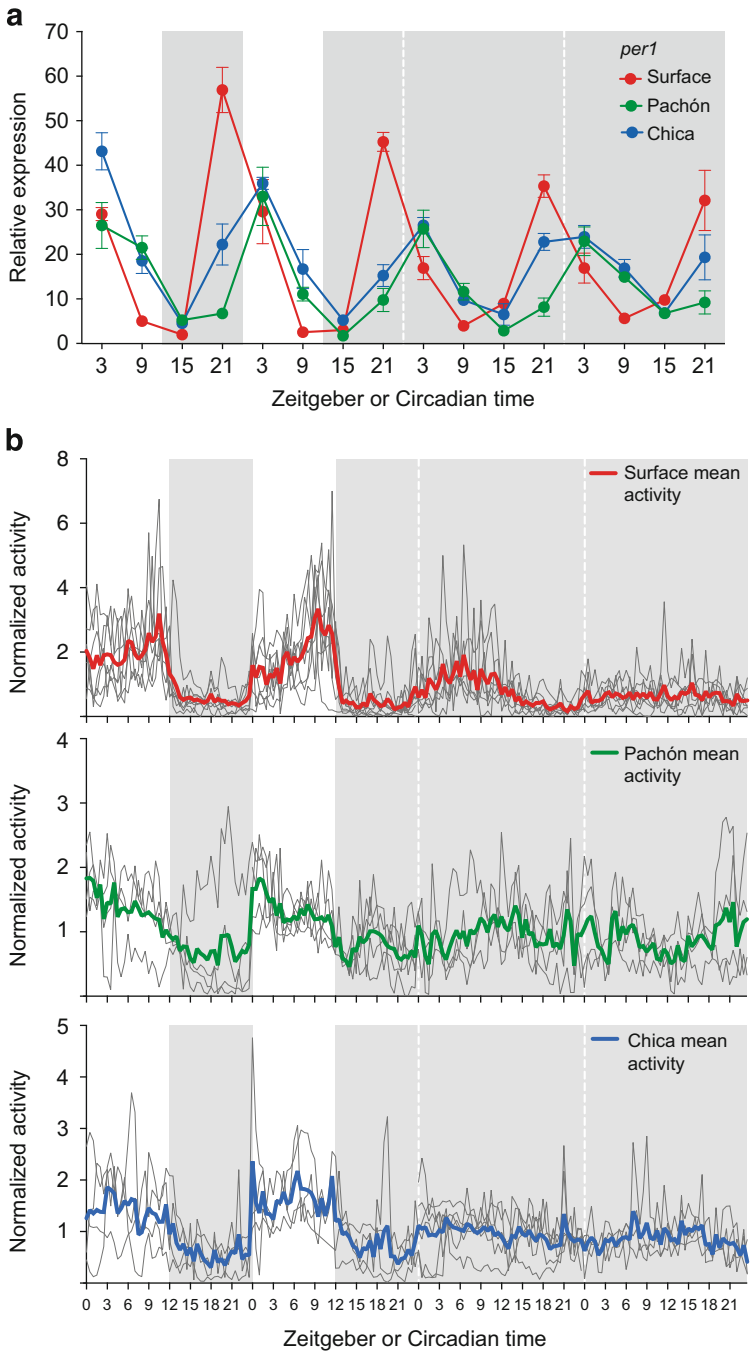

Fig. 6.28 Molecular and locomotor analyses of the *Astyanax* surface fish, the SEP Pachón and the VEP Chica cave fish in LD and DD. (**a**) Expression of clock gene *per 1*, (**b**) swimming activity of

free-running activity was mostly expressed only in the surface activity, while the bottom activity was arrhythmic. Compared with the surface fish, the Pachón cave fish has similar, but much less pronounced circadian rhythms in surface- and bottom-directed activities in LD. Its time-measuring ability was found to be regressive but the circadian clock system has not been reduced (Erckens and Martin 1982a, b; Erckens and Weber 1976).

The study of swimming behaviour corroborates these findings (Beale et al. 2013). Surface fish show a clear, diurnal circadian rhythm in LD (23.95 ± 1.01 h), which persists on the first day in DD (29.77 ± 2.49 h), but then becomes arrhythmic on the second day in DD (Fig. 6.28). Pachón and Chica cave fish also show significant circadian rhythms in LD (23.95 ± 0.82 h and 25.6 ± 1.30 h, respectively), but after entry into DD, both populations, like the surface fish, are behaviourally arrhythmic and show substantial variation between individuals. Cave fish activity remains at median-to-peak levels in DD, whereas surface fish activity drops closer to basal lower levels (Beale et al. 2013).

For the SEP Tinaja cave fish, it was shown that it retains its ability to show light entrainable circadian rhythms of locomotor activity (Caballero-Hernandez et al. 2015). It was found to persist during the 10 days before being exposed to light cycles and some of the specimens were able to exhibit phase control with the previous LD when released in DD, indicating a true entrainment. Like for sleep loss (Duboué et al. 2012) (see Sect. 6.10), Caballero-Hernandez et al. (2015) propose for the Tinaja cave fish an endogenous behavioural adaptation to an environment with extremely low food resources, in order to stay in a quasi-permanent state of alert at all times, but with potential induction of cycles when environmental conditions change. The entrainment responses vary between different Tinaja individuals indicating that the possible fading of the rhythmic circadian activity is variable and may occur differentially among specimens of the same population.

Sleep and activity periods also provide examples for the uncoupling from circadian rhythmicity (see Sect. 6.10) (Duboué et al. 2012). The same was reported to happen to the circadian rhythm of the aerobic metabolism, which is uncorrelated to locomotor activity in the Pachón cave fish (see Sect. 6.9) (Moran et al. 2014).

The conservation of circadian rhythmicity during regressive evolution was also found in the eye rudiments of embryonic *Astyanax* cave fish. Light-entrained shifting rhythms of pigment granules take place in the retinal pigment epithelium of the degenerating eyes of the SEP Pachón and the VEP Chica cave fish (Espinasa and Jeffery 2006). After LD, the granules continue shifting under DD for two cycles. Entrainment is probably by light, perceived by photosensitive ganglion cells of the inner nuclear layer, which, in contrast to the visual cells (including

Fig. 6.28 (continued) the surface fish, the Pachón cave, and the Chica cave fish. *White* and *grey* shaded areas indicate light and dark periods, *DD* continual darkness, *LD* alternating periods of light and dark, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Beale et al. 2013)

outer nuclear and outer plexifom layers), is retained in part of the eyes of the Pachón cave fish (see Sect. 6.21.1) (Peirson et al. 2009; Wilkens 1988, 2007).

Together, these results show that *Astyanax* cave fish possess a functional circadian clock, but one that is less robust than that of the surface fish. In contrast with the reduced eye, the circadian clock seems largely conserved in *Astyanax* cave fish. This is confirmed by transcriptome studies of surface and cave *Astyanax* (Gross and Wilkens 2013; Hinaux et al. 2013). In the Somalian cave fish *Phreatichthys andruzzi*, more severe regressive disturbances were revealed. Mutations in two circadian photoreceptors sequences, melanopsin and Teleost Multiple Tissue-opsin (TMT), have led to the loss of light entrainment of the clock ("blind clock", Cavallari et al. 2011). An examination of the TMT-opsin sequences between *Astyanax* surface and Pachón cave fish did not show such alterations (Beale et al. 2013). In *P. andruzzi* it was found, however, that feeding time has replaced light as a zeitgeber and a food-entrainable pathway exists. An infradian 47-hour free-running period is exhibited, by which it could be insinuated that the 24-hour period in principle still exists but in a doubled period to conserve homeostasis.

In contrast to *P. andruzzi*, visual light can still be used to entrain the circadian clock in a 24-hour rhythm via the pineal in *Astyanax* cave fish. As in most cave species, it can only be speculated about the nature of the zeitgeber by which the circadian clock is entrained in *Astyanax* cave fish in the natural continuously dark environment. It was proposed to be food-entrained, like in *P. andruzzi*, because bats roost in the caves (Mitchell et al. 1977; Wilkens and Burns 1972). However, although the Chica cave fish live in a cave that is settled by thousands of bats, no entrained molecular oscillation of *per1* was detected in the wild. This observation is explained by the hypothesis that the oscillator possibly does not run under cave conditions (Beale et al. 2013). It is suggested that in order to regulate homeostasis, the *Astyanax* cave fish cannot afford to lose the basic principles of circadian rhythmicity such as the rhythmic production of melatonin in the pineal, which consequently is submitted to stabilizing selection. In contrast, traits like surface water- and bottom-directed activity, which have lost the biological function, are no longer subjected to selection and therefore this behaviour as well as its rhythmicity may become reduced. Induced rhythms deviating from 24 hours were shown to disappear in DD immediately without any post-oscillations in the Pachón cave fish (Erckens and Martin 1982a, b). Furthermore, the circadian rhythmicity of aerobic metabolism found in surface *Astyanax* is no longer exhibited in the Pachón cave fish (Moran et al. 2014, 2015) and sleep rhythmicity has changed, too (Duboué et al. 2011; Erckens and Martin 1982a, b). These traits have become adaptive in *Astyanax* cave life, however.

Several light-induced genes, including *cry1a* (cryptochrome) and *per2*, are known to be critical for light resetting of the circadian pacemaker (Albrecht and Ripperger 2009). It was therefore proposed that the basally increased levels, particularly of *per2* in cave fish, are likely to lead to the extremely reduced *per1* expression detected under natural conditions and stop the clock (Beale et al. 2013). The authors furthermore argue that these molecular changes in the light input

pathway contribute to the phase differences they observed between surface and cave populations in the laboratory, and especially to the reduced amplitude of the core clock oscillation. They conclude that the light input pathway exists in a more activated state in cave populations, as if the fish were actually experiencing constant light rather than the perpetual darkness of the cave, and it is hypothesized that this might be responsible for a selective advantage. The data analyzed in wild Chica specimens show complete repression of clock function (Fig. 6.27), while in the Pachón and the Chica cave fish, two genes involved in DNA repair, *CPD phr* (cyclobutane pyrimidine dimer-photolyase) and *ddb2* (damaged DNA-binding), show raised basal levels of expression. In field samples of wild Chica cave fish, the level of *CPD phr* and *ddb2* expression is increased over both the surface fish and laboratory cave strains, although they were never exposed to light. It is speculated that CPD photolyase in wild cave populations might have a functional role in DNA repair within the complete darkness of the cave and that altered expression of light-inducible genes provides a selective advantage to cave fish. This speculation relies on the finding that CPD photolyase can repress the mammalian circadian oscillator (Chaves et al. 2011). The high levels of CPD photolyase in the cave fish are assumed to have the same effect and are claimed to be responsible for the highly repressed levels of clock function in the Chica cave fish. By tonically activating light-dependent signalling pathways and increasing DNA repair activity, individuals in the cave would reduce deleterious mutational events, whereas the circadian oscillator would be damped as a byproduct. However, this hypothesis is predicated on the questionable assumption that the mutation rate would be higher in cave environments and above all would provide some kind of indirect selection pressure being responsible for the regression of the circadian clock.

6.17 Fright Reaction

Von Frisch was the first to describe that the European minnow (*P. phoxinus*) when injured releases flight in conspecifics. This alarm or fright reaction is found in Ostariophysan fish like *Astyanax* and is caused by an alarm pheromone, the *Schreckstoff*, produced in the skin epithelial alarm substance club cells (von Frisch 1941; Pfeiffer 1960). These cells do not have pores and the alarm substance is therefore not released voluntarily but after the epidermis has been hurt by an aggressor or a predator (Chivers et al. 2007; Fricke 1988; Peters et al. 1990).

To analyze this behaviour the experimental fish were subjected to an acclimatization time of 2 weeks, during which once every day 20 ml of tap water was squeezed with a glass pipette below the water surface and dry food flakes were immediately offered on the water surface. During the subsequent experimental period, water containing alarm substance prepared from an *Astyanax* surface fish was introduced in the same manner (Fricke 1988).

After the introduction of the alarm substance, the surface fish immediately exhibited a fright reaction, which consists of swimming a zigzag path, rapid swimming, hiding, and avoidance of the area where the alarm substance

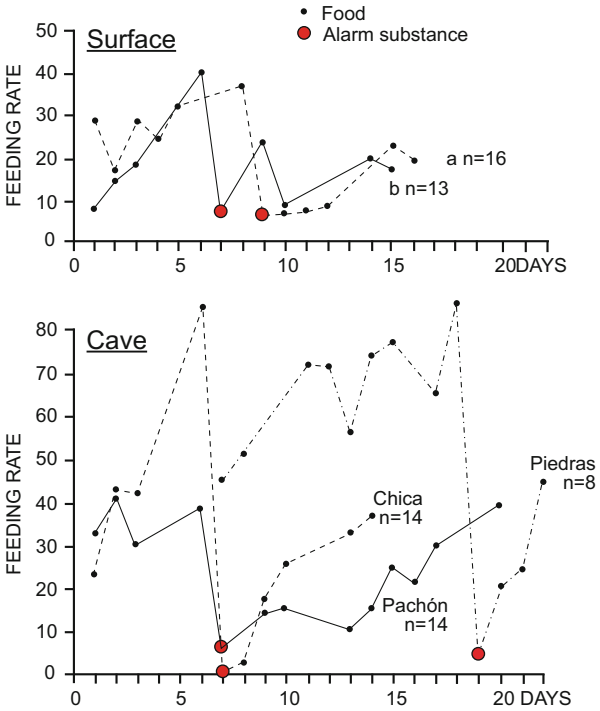

Fig. 6.29 Feeding rate in two *Astyanax* surface fish [(**a**) Cenote Dzibilchaltún (Yucatán) and (**b**) Rio Teapao (Tabasco)], the VEP Chica and the SEP Pachón and Piedras cave fish before and after introducing a conspecific alarm substance at the water surface (*black dot* introduction of food, *red dot* introduction of conspecific alarm substance, feeding rate = snaps at food per 5 min). *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Fricke 1988)

was set free. Furthermore, the feeding rate decreased significantly for several days (Fig. 6.29).

In the SEP Piedras and Pachón cave fish, only one of these reactions, namely avoidance, is exhibited whereas all others are lost. The specimens strictly do not swim into a layer of approximately 6 cm below the water surface, while they continue feeding on the ground. The VEP Chica cave fish shows the same behaviour but differs to a degree from the SEP Piedras and Pachón cave fish. A few of the Chica specimens still show reactions only exhibited by the surface fish. In all surface and cave populations, the fright reactions were found to persist over a period of nearly 6 days (Parzefall and Fricke 1991). As the alarm substance is not stable in water (Pfeiffer 1982) the persistence of fright reactions seems to be due to memory (Fricke 1988) (Fig. 6.29).

The alarm substance has not been altered in the cave fish and is perceived by olfaction (Chivers et al. 2007). However, in the surface fish, vision obviously plays an additional role, because specimens that have not noticed a danger by olfaction are alarmed by the behaviour of their conspecifics. The differences in behaviour between surface and cave fish can be explained by the absence of light in the caves. Whereas surface fish at daylight can be visually alarmed by conspecifics swimming rapidly or in a zigzag path, in cave fish visual alarm signals are useless and became completely reduced in the SEP cave fish. Only in the Chica cave fish a few of the

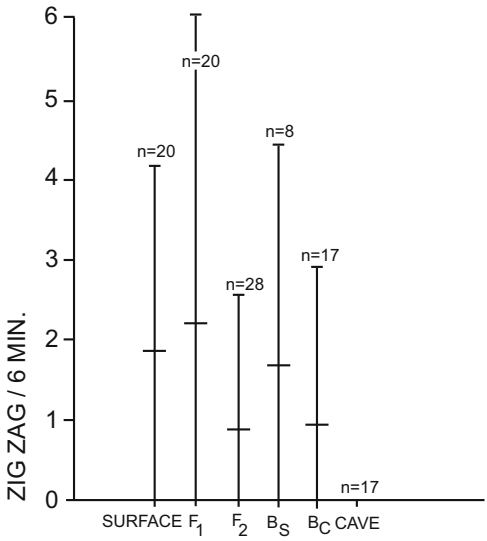

Fig. 6.30 Means and ranges of the fright reaction zigzag movement in the *Astyanax* surface fish, the SEP Piedras cave fish and their F1, F2, and backcrossings demonstrate additive polygenic inheritance. B_S backcrossing to surface, B_C backcrossing to cave fish, *SEP* strongly eye- and pigment-reduced (adapted from Parzefall and Fricke 1991)

visually effective elements still occur. This is concordant with the variability of other regressive traits like the eye exhibited in this population.

It is proposed that, in contrast to visual releasers, only avoidance still has a biological function in cave fish. By this behavioural trait, protection is provided against foraging conspecifics or terrestrial predators like bull dog bats (Noctilionidae), which catch fish at or in a narrow layer below the water surface. Crossings between the surface and the VEP Piedras cave fish have revealed that the zigzag swimming behavioural trait relies on polygenic inheritance, because the mean of the F2 crossing is intermediate and the distribution of the intensity of this trait ranges between surface and cave fish. The means of the F1 and the backcrossing to the surface fish equate to that of the surface fish, indicating that epistatic gene effect is involved (Fig. 6.30) (see Sect. 6.23).

6.18 Schooling and Shoaling Behaviour

By definition, groups of fish aggregating with other fish of the same species are termed shoals, and such groupings are called schools when they synchronize and polarize swimming activities (Pitcher 1983). Under daylight conditions, surface *Astyanax* fish may alternate between these two types of social grouping but also may become territorial under conditions of limited space (Parzefall 1979, 1983, 1993; Gregson and Burt de Perera 2007). Schooling tendency is measured by the time a fish is following a moving model school. Shoaling is measured by distance and time one or more specimens aggregate to each other (Kowalko et al. 2013b; Parzefall 1992).

In darkness, surface fish are unable to form shoals or schools (Fig. 6.31a, b) (Parzefall 1993). Similar to surface fish in darkness, the visionless Pachón, Tinaja,

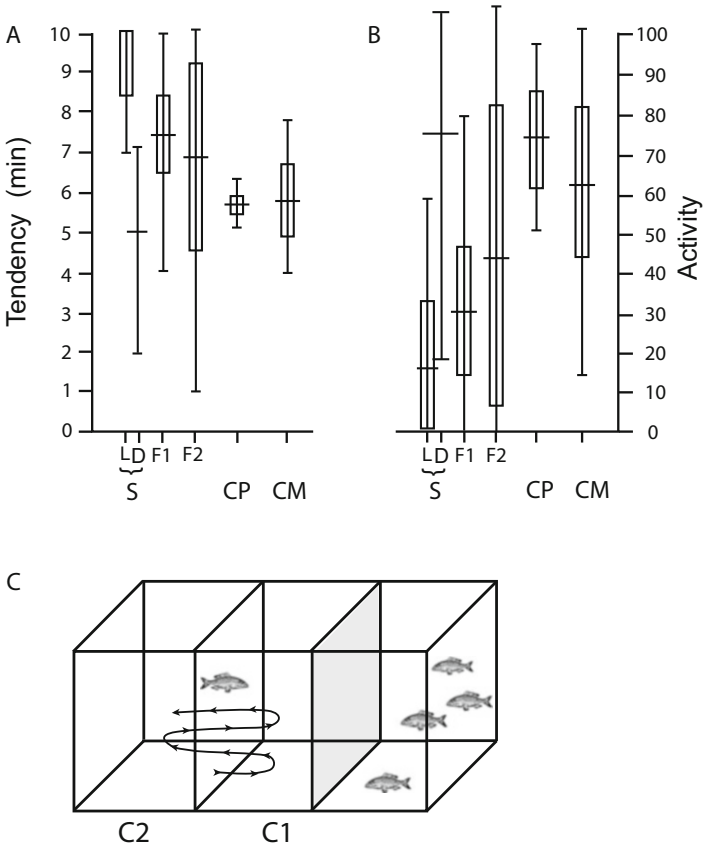

Fig. 6.31 Tendency to shoal (**a**) and swimming activity (**b**) of the *Astyanax* surface fish (*S*) and the SEP Piedras cave fish (*CP*), the F1 and F2 crossings between them, and of the VEP Micos cave fish (*CM*). F2 hybrids and Micos specimens are selected for a good optomotor response and functional eyes, mean of $n = 10$ specimens in each group, *box* standard deviation, *vertical line* range, *L* study at light, *D* study in darkness. Experimental set up (**c**): tendency to aggregate to a group of fish = time staying in C1 (measured in minutes/10 min); swimming activity = number of changes per 10 min between chambers C1 and C2; *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Parzefall 1993)

Piedras, and Molino cave fish do not aggregate to shoal or to school, but continuously swim without contact throughout the whole space available. This behavioural trait loss has evolved convergently in the different separate cave populations (Kowalko et al. 2013b; Parzefall 1993).

A study of specimens of the VEP Micos cave fish equipped with good vision revealed that the tendency to aggregate to a shoal is as low as that of the SEP cave fish (Fig. 6.31a, b). The variability of the tendency to shoal is about half that of the F2 crossing between surface and cave fish, but much higher than in the SEP Piedras cave fish (Parzefall 1993), which could be explained by the more recent origin of the VEP Micos compared with the SEP Piedras cave fish.

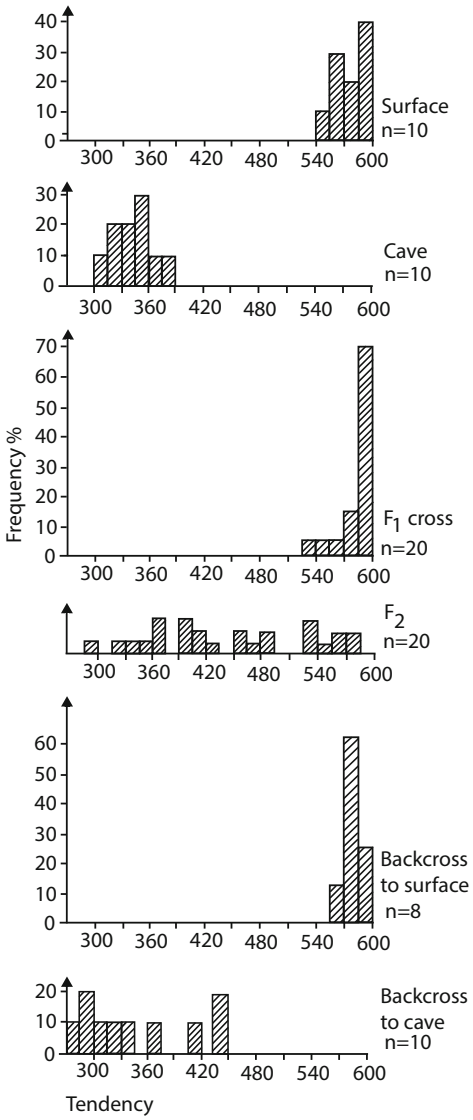

Fig. 6.32 The distribution of the tendency to shoal (measured in seconds) of the *Astyanax* surface fish, the SEP Piedras cave fish and their F1, F2, and backcrossings indicate polygenic inheritance and epistatic gene effect. *SEP* strongly eye- and pigment-reduced (adapted from Parzefall and Fricke 1991)

Loss of vision in the cave fish is not solely sufficient to explain the reduction of shoaling and schooling behaviour. Also, the genetic basis of this behaviour is deteriorated. This was revealed by the distribution of the tendencies to school or to shoal in the crossings between surface and cave fish selected for good vision (Fig. 6.32) (Kowalko et al. 2013b; Parzefall and Fricke 1991). The F2 hybrids distribute between both parental forms and that of the backcross to the cave fish between the cave fish and the mean of the F2 crossing. Some F2 hybrids with good vision do not even aggregate. This indicates that a polygenic mode of inheritance is responsible. In addition, however, the distribution curves of the F1 crossing and the

backcrossing to the surface fish equate to that of the surface fish due to epistatic gene effect (Fig. 6.32) (see Sect. 6.23) (Parzefall and Fricke 1991). QTL analysis has revealed that loss of shoaling behaviour in the *Astyanax* cave fish is based on at least two loci and that it has independently evolved from eye loss. Furthermore, neither the increased number nor size of sensory lateral-line free neuromasts are correlated with loss of shoaling behaviour (Kowalko et al. 2013b).

6.19 Scales

Fish scales provide protection against intra- and interspecific interaction and against parasites. Several cave fish species are characterized by their regression or even total loss (Banister 1984; Banister and Bunni 1980; Berti et al. 2001; Freyhof et al. 2016).

The body of *Astyanax* surface and cave fish is covered by the cycloid type of scales and their number along the lateral line is correlated to that of their vertebrae. In comparison with the surface fish, the scales of the cave fish show some characteristics of reduction. For example, in the Pachón cave fish they are shorter (Fig. 6.33) and the cranial scale part is not as deeply anchored in the scale pocket of the scale anterior to it, as is the case in the surface fish. Therefore, there is less overlap between the scales and the protective function is lowered. This effect is even intensified because the scales are less ossified and therefore are thinner. Furthermore, fixation of the scales of the cave fish is not as firm as in the surface fish, because they do not lodge as deeply in the scale pocket as they do in the surface form.

The partial reduction of the scales observed in *Astyanax* cave fish species is probably due to several facts. One reason may derive from the cave ecosystems being comparatively species-poor. For example, interspecific fights between different fish species competing for food do not occur because they mostly do not exist in the caves. Furthermore, the number of epidermal parasite species is lower, because

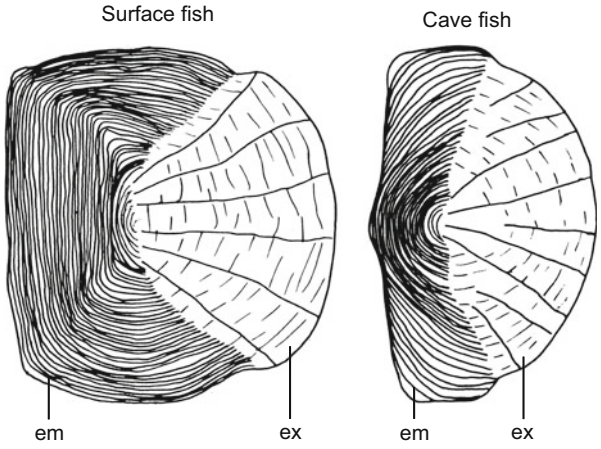

Fig. 6.33 Comparative morphology of the scales of *Astyanax* surface and cave fish. Cranial parts to the left, *em* embedded part, *ex* exposed part

they cannot rely on alternate hosts in their life cycle. Also, intraspecific aggressive fights may occur less often.

6.20 Pigmentation

6.20.1 Melanophores

In *Astyanax* surface fish, males and females look similar and do not show sexually different or nuptial colouration. Their colour pattern mainly serves as camouflaging. An important part of body colouration is provided by the melanophore colour cells, which enable the fish to adapt to dark or bright background albedos (Fig. 6.34). This process may rapidly proceed within a few minutes by concentrating or expanding the melanin pigment granules within the melanophore colour cells (physiological colour change) or may take a longer time (several days) by additionally enhancing or lowering the number of melanophores (morphological colour change) (von Frisch 1911). During long-term background adaptation, alterations in melanophore morphology and density follow physiological colour change with the same factors involved in both phenomena (Sugimoto 2002). The most effective pigment-cell dispersing factor, the peptide α-Melanocyte-stimulating hormone (α-MSH), is thought to be the driving force responsible for the increase in melanophore dendricity and density by up-regulating melanogenic gene expression. Melanophore density in adult fish skin is likely to be maintained by a balance between differentiation and apoptosis (Fujii 2000; Sugimoto 2002). In surface *Astyanax* specimens being kept for a long time over a white background, the number of melanophores is statistically significantly lower compared with that developed over a black underground (Wilkens 1970b) (Fig. 6.55b). In darkness, the number of melanophores of the surface fish was revealed to be the lowest (Wilkens 1970b).

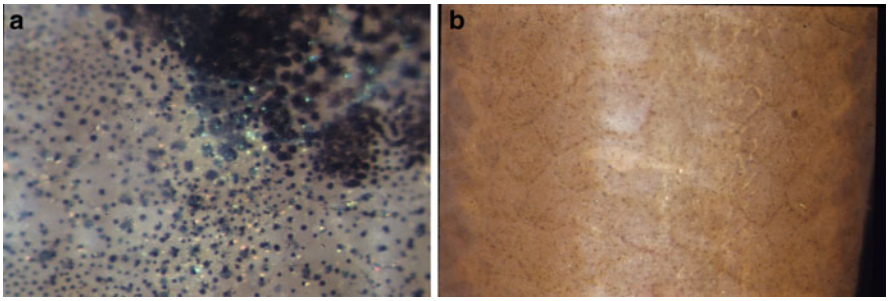

Fig. 6.34 Sections of the back of *Astyanax* surface fish (**a**) exhibiting melanophores with dispersed and contracted melanin granules due to physiological colour change, and of the SEP Sabinos cave fish (**b**), in which they are reduced in number and contain less melanin due to the brown *Mc1r* mutation. The shiny spots are iridophores. *SEP* strongly eye- and pigment-reduced

Several cave species enhance the number of melanophores and melanin content when kept in daylight. For example, the hepapterid cave fish species genus *Rhamdia* achieve a brownish pigmentation, and the amphibian cave urodele *Proteus anguinus* may even exhibit intensively black body pigmentation. However, the SEP *Astyanax* cave fish does not darken when kept at daylight and exhibits the same number of melanophores as in total darkness. Contrary to this, the VEP Micos cave fish becomes darker at light because of the enhancement of melanophore number and melanin content.

The pattern of melanophore distribution on the body surface, with the highest density on the back and their number slowly declining down the sides, has not changed in the cave fish populations. The colour cells are still capable of performing a physiological colour change, too (Fig. 6.34). In the SEP cave fish, the pale colouration by an important part is achieved by the reduction of the number of melanophores. Among the SEP cave fish populations, the degree of reduction of the number of melanophores ranges between 70% and 85% compared with the surface fish (Wilkens 1970b, 1988; Wilkens and Strecker 2003). In contrast to the SEP cave fish, the VEP Micos cave fish develops the same number of melanophores as the surface fish when kept in daylight (Fig. 6.35) (Wilkens 1976). To assess the number of melanophores in the albinotic VEP Molino cave fish, crossing experiments with non-albinotic SEP cave fish were performed. As the albino gene is recessive, melanin production is then enabled and the melanophores become visible. As a result, it was revealed that the number of melanophores is about 50% of that in the surface fish (Fig. 6.36) (Wilkens and Strecker 2003). Thus, the number is higher than in the SEP, but lower than in the VEP Micos cave fish. The population-specific number of melanophores is already manifested at early embryogenesis by the number of melanoblasts, the precursors of the melanophores, which are developed in the neural crest of the cave fish (McCauley et al. 2004).

Crossings of the SEP Sabinos and Pachón cave fish show that the differing numbers of melanophores developed in different cave populations is mirrored by the means in the respective F2 and backcrossings to the cave fish (Wilkens 1970b; Wilkens and Strecker 2003). The method of inheritance of the melanophore number can be explained by polygeny and is characterized by epistatic gene effect (see Sect. 6.23, Fig. 6.55b). It relies on at least eight QTL or two genes found by classical crossing experiments (Protas et al. 2007; Wilkens 1988).

6.20.2 Brown Gene (*Mc1r*)

Besides the reduction of the number of melanophores, the paleness of the cave fish is additionally caused by partial reduction of the melanin content in the body melanophores. This so-called "brown gene" is found in the SEP Pachón, Yerbaniz, Japonés, Sabinos, Tinaja, Piedras, and Curva as well as in the VEP Chica cave populations. It is also responsible for lower melanin content in the retina pigmentary epithelial cells, due to which the eyes of preserved specimens no longer appear black but brown (Fig. 6.34b, Sadoglu and McKee 1969) (Table 4.1). In contrast, the

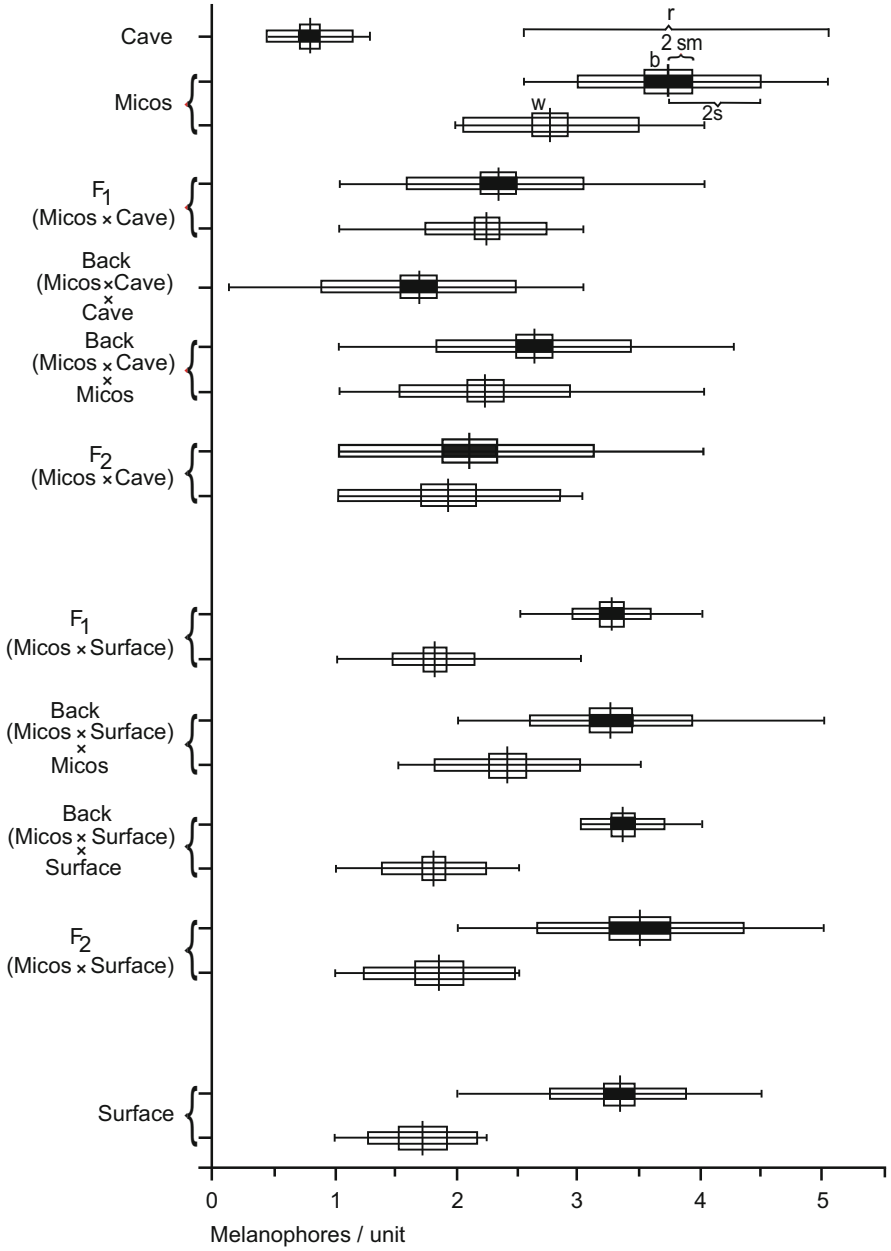

Fig. 6.35 Mean, standard error (*sm*), standard deviation (*s*), and range (*r*) of the melanophoral densities of the VEP Micos cave fish over a white (*w*, *open*) or a black (*b*, *solid*) background. *Cave* SEP Pachón cave fish, *back* backcrossing, *Micos* Micos cave fish, *SEP* strongly eye- and pigment-reduced, *surface* surface fish, *VEP* variably eye- and pigment-reduced (adapted from Wilkens 1976)

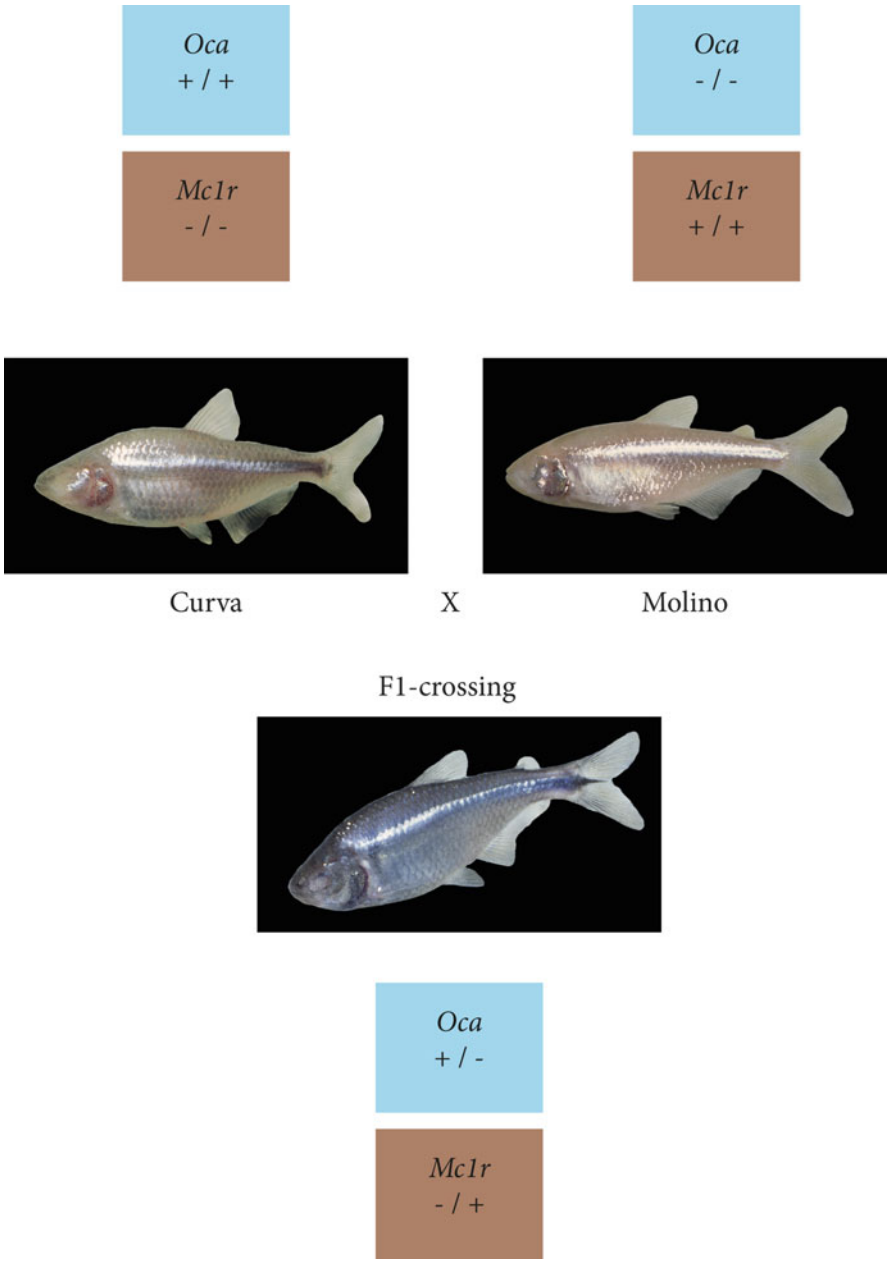

Fig. 6.36 The F1-crossing hybrids of strongly reduced SEP cave fish like Curva with the albinotic VEP Molino cave fish show dark colouration, because the Molino cave fish still carries the unmutated wild allele of the brown gene (*Mc1r* gene), which solely does not manifest in this population because the Molino cave fish is albinotic and the recessive albino gene (*Oca2* gene), when homozygous, blocks melanin synthesis, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced

VEP Molino cave fish does not possess the "brown gene", but exhibits pale body colour, nonetheless, because it is albinotic and melanin production is completely blocked (Wilkens and Strecker 2003) (Fig. 6.36).

The "brown" mutation relies on a single recessive gene, which is inherited independently from those responsible for the number of melanophores as well as that for albinism (Sadoglu and McKee 1969; Wilkens 1970b). Crossing experiments revealed that the brown phenotypes manifesting in different SEP cave populations are all caused by mutations at the same locus and do not show genetic complementation in the crossings.

Using a candidate gene approach, the *Mc1r* gene (melanocortin type 1 receptor) was characterized as the likely locus controlling this trait. Sequence analyses revealed a 2-base-pair deletion in Pachón cave fish, which is predicted to cause a frame-shift leading to the introduction of a premature stop codon and to destroy normal functioning of the receptor protein. A mutation discovered in the Yerbaniz and the geographically closely linked Japonés populations causes a cysteine substitution, by which diminished function of the Mc1r protein in these populations is likely to be caused, explaining the presence of the brown mutation in these fish (Gross et al. 2009). Crossings demonstrate non-complementation of the brown phenotype in hybrid individuals derived from these two independent groups (Pachón vs Yerbaniz/Japonés), once again indicating that the same locus is responsible for reduced pigmentation in these fish (Gross et al. 2009). In the other SEP Sabinos, Tinaja, Piedras, and Curva cave populations, no differences in *Mc1r* coding sequence relative to the surface (wild type) populations were found, suggesting that these populations likely exhibit regulatory mutations leading to a decrease or loss of *Mc1r* activity (Gross et al. 2009; Stahl and Gross 2015). Thus, among 12 cave populations studied, the brown phenotype has arisen independently, mediated through different mutations of the same gene in the SEP Pachón, the Yerbaniz/Japonés, and in all other SEP cave populations that do not harbour a sequence alteration to the open reading frame. In that case, the brown phenotype may have evolved through the accumulation of sequence mutations affecting the 5′ regulatory region. Thus, the brown phenotype in *Astyanax* cave fish may have evolved through a combination of both coding and cis-regulatory alterations (Stahl and Gross 2015).

6.20.3 Albino Gene (*Oca2*)

Whereas the "brown gene" only causes a reduction of melanin, in those specimens carrying the albino mutation, the melanin of the melanophores is completely missing. The albino gene is recessive (Sadoglu 1957; Wilkens 1970b). It seems to be responsible for the inability to convert L-tyrosinase to L-dopa and melanin (Bilandžija et al. 2013; McCauley et al. 2004). Among the 27 *Astyanax* cave fish populations, albinism is rare and has only been found in the SEP Pachón, Yerbaniz/Japonés, and the VEP Molino populations. It appeared homozygous in Yerbaniz and Molino and for short time in Pachón (see Sect. 5.2.1). Melanophores in the

albinotic populations are extant, but not visible due to the complete failure of melanin synthesis. However, they phenotypically manifest in the crossings with the surface fish (Sadoglu 1957; Wilkens 1970b; Wilkens and Strecker 2003). Albinism is linked to the *Oca2* gene (ocular and cutaneous albinism) and inherits independently from the genes responsible for the "brown" phenotype as well as from those for the number of melanophores (Wilkens 1988). On a molecular basis, in Pachón three differences from surface fish were found: two point mutations resulting in conserved amino acid substitutions each and a large deletion, which extends from within intron 23 through most of exon 24. Due to this deletion, the Pachón OCA2 protein would contain only part of intron 23 and lack most of exon 24. In Molino cave fish, a single change was found, namely a large deletion encompassing exon 21 that significantly shortens the OCA2 protein. Both differing coding sequence alterations affecting the Pachón and the Molino cave populations cause loss of function in the corresponding protein OCA2. From this can be concluded that the two cave populations evolved albinism independently through mutations at the same locus. It was also shown that the Yerbaniz/Japonés cave fish was deficient in *Oca2*. This seems, therefore, to be the third case of albinism detected, which separately arose, probably in a regulatory region (Protas et al. 2006; Protas and Patel 2008). A fourth case of albinism found in *Astyanax* cave fish appeared spontaneously in a captive stock of the VEP Micos cave population (Gross and Wilkens 2013), but has not, as yet, been detected in the Micos cave itself (Espinasa et al. 2014). Interestingly, it relies on the same loss of function alleles previously identified in the SEP Pachón cave fish (Gross and Wilkens 2013).

6.20.4 Guanine and Carotinoids

Besides the light-absorbing melanophores, light-reflecting, guanine-containing iridophores are developed as a second type of colour cell in the surface fish. They are developed in the lateral scales and protect the fish from being seen by predators attacking in the free water column from below. In addition, iridophores also exist in large numbers on the back, which helps camouflaging the surface fish over bright underground. This colour cell type is also submitted to regression in the cave fish. In the Molino cave fish, a slight reduction can additionally be found in the scales. However, the strongest reduction appeared in a captive stock of the Piedras cave fish, in which the guanine in the scales was totally lost. Because of this, the fish appear transparent although the scales are still present. This feature relies on a single recessive gene (Culver and Wilkens 2000) (Fig. 6.37a–c).

As a third type of colour cells, light-absorbing xanthophores are developed in the surface fish, which are particularly noticeable in the anal and caudal fins. The carotinoid pigments responsible for the colouration cannot be synthesized by the fish, but have to be ingested with food. A remarkable phenotype appeared in a captive stock of the SEP Piedras cave fish, the complete body of which was homogeneously coloured yellow, showing more intensive colouration in male than in female fish. It was suggested that this monogenic recessive mutant was

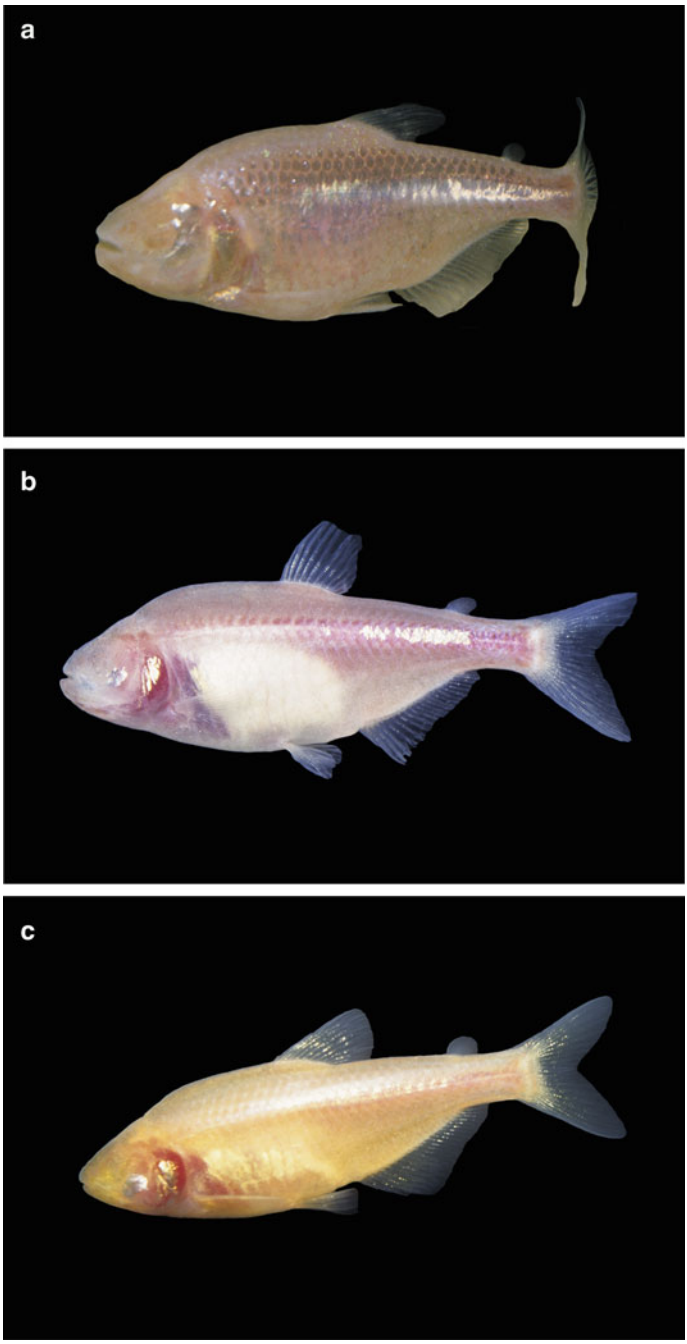

Fig. 6.37 Two recessive monogenic colour mutations as yet not found in nature appeared in the SEP Piedras laboratory strains, most probably due to standing variation: besides the normal phenotype (**a**), specimens exhibiting total loss of guanine in the scales (**b**) and a yellow mutant (**c**) developed. *SEP* strongly eye- and pigment-reduced

unable to break down the ingested pigments and that they were stored in the fatty body tissue (Culver and Wilkens 2000) (Fig. 6.37a–c).

6.21 Eye

6.21.1 Morphology and Histology of the Eyes of the Surface and the SEP *Astyanax* Cave Fish

Eye reduction is a key characteristic of cave animals and has always fascinated scientists. In different cave fish species, all stages of reduction from the slightest decrease in size not yet afflicted by any structural defects to a degenerate rudiment deeply sunken into the head may be found (Fig. 6.38). Only a few species like the Somalian cave fish *P. andruzzi* (Cyprinidae) and *Uegitglanis zammaroni* (Clariidae) are known to have completely lost even the tiniest eye rudiment in adult specimens (Berti et al. 2001).

Whereas the eyes of the mostly troglophile surface sister species of cave fish are relatively small and adapted to perceive light in twilight or nocturnal environments, those of the diurnal surface *Astyanax* are large and well differentiated (Peters and Peters 1966; Wilkens 1988). They are characterized by a wide pupil and a voluminous lens, which can be pro- and retracted for accommodation by the lens muscle inserting on the distal tip of the falciform process. The retina is well developed, containing rods as well as single and double cones, the outer segments of which deeply extend between the cubic pigmentary epithelium cells. The chorioid or chorioidal gland is large, forming a horseshoe-shaped body of capillaries between retina and sclera. Guanine-containing light-reflecting layers build the argentea, improving light perception in shaded environments (Peters and Peters 1966).

The eye rudiments of SEP cave fish are sunken into the orbit and covered by epidermal body tissue, to which they are connected by a thin duct of tissue. Their diameter is about 10–20% of that of the surface fish. As concerns the various SEP cave populations, the eye is smallest in the Piedras and largest in Pachón, with Yerbaniz and Curva cave fish lying somewhere in between (Wilkens and Strecker 2003). The VEP Micos cave fish exhibit variable eye sizes, ranging from being almost as small as that of the F1 crossing between the SEP and the surface fish and as large as that of the surface fish (Wilkens and Strecker 2003). In the cave fish, eye size and degree of rudimentary structural differentiation are correlated (Wilkens 1970c). In adult SEP cave specimens, the largest and best developed eyes still possess a tiny rudimentary retina, whereas the visual sensory cells including outer nuclear and outer plexiform layers are lost (Fig. 6.39a). In contrast, the clearly separated ganglionic, inner plexiform and inner nuclear layers are extant and still connected to the brain by a thin optic nerve. Horizontal and Muller glia cells are developed. The retina rudiment attaches to the pigmentary epithelium, the cells of which are flattened and do not exhibit the typical cubic form exhibited in the surface fish. The extant vitreous body is almost completely separated by the ventral falciform process into a larger and a smaller chamber and the lens is always completely

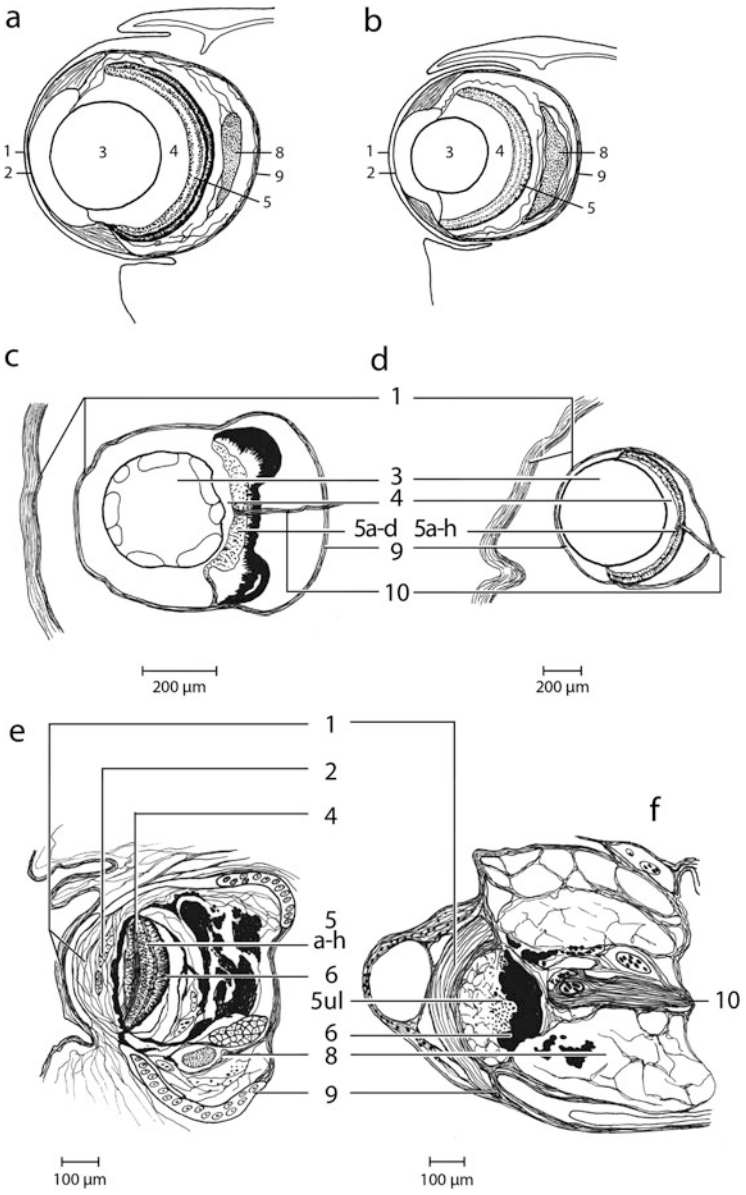

Fig. 6.38 At its beginning, cavefish eye reduction is just a diminution of overall eyeball size and that of all single structures (**a** *Poecilia mexicana*, surface; **b** *P. mexicana*, cave). Only at later stages certain structures may get lost completely and considerable variability develops. In the cave cusk eel *Lucifuga spelaeotes* from the Bahamas, lens and retina are only reduced in size in the one from Grand Bahama (**d**), whereas in specimens from Abaco both show structural reduction (**c**). In the Oman cave fish *Garra barreimiae* the lens may be reduced whereas the retina still contains all characteristic layers of a functional eye (**e**) or the retina is no longer layered at all (**f**). Epithelial and scleral part of cornea (1), anterior eye chamber/spongiosum (2), lens/lens capsule (3), vitreous

missing in adults. The chorioid is reduced in size, too, and consists of connective tissue, blood vessels, and fragmented pieces of the argentea. A large part of the space enclosed by the sclera contains fat tissue. The rudimentary anterior eye chamber is entirely filled with the so-called spongiosum, which is spongious tissue deriving from the ligamentum annulare of the surface fish eye, which is located in the outer circular margin of the anterior eye chamber.

In the smallest eye rudiments of the SEP cave fish, specific characteristics are no longer identifiable. Neither layered retina, vitreous body, falciform process nor flattened pigmentary epithelial layers exist. Only remnant vessels of the choroidal gland are left in the voluminous space enclosed by the sclera (Wilkens 1970c). As well as the relatively well and the poorly differentiated eye rudiments, intermediate degrees of differentiation are developed (Fig. 6.39b) (Wilkens 1970c). Whereas the sclera of the surface fish is reinforced with an additional ring of bone derived from scleral ossicles (Yamamoto et al. 2003), scleral ossification has regressed convergently at least three times in the SEP Pachón, Sabinos, and Curva as well as in the VEP Chica and Molino cave populations (O'Quin et al. 2015). The eye coats are cartilaginous or consist of connective tissue (Fig. 6.39c).

6.21.2 Visual Pigments

Visual pigments function in light perception by transforming light energy into electrical (nerve) potentials. The study of the visual pigments of *Astyanax* has shown that the surface fish exhibits tetrachromacy and, in addition to a rod pigment, possesses one ultraviolet and violet sensitive, at least two rhodopsin, one rhodopsin-like, one blue, and two green cone opsin genes. The surface fish varies widely with respect to the ratio of Rhodopsin (A_1): Porphyropsin (A_2) chromophores, which divide into two groups with either 70% A_1 or 20% A_1 retinal in their cones. The basic spectral distribution of the cone pigments in the surface fish, with $Longwave_{Green}$/$Longwave_{Red}$ double cones and short wave sensitive (SWS) and possibly violet sensitive (VS) single cones is typical of many shallow water teleosts. The complement of cone pigments supports tetrachromatic colour vision (Parry et al. 2003; Yokoyama et al. 1995).

Sequence analysis of one red (*r007*) and two green sensitive opsin (*g101-2* and *g103-1*) genes was performed in the surface fish, the VEP Micos, and the SEP Pachón cave fish (Yokoyama et al. 1995). The coding regions exhibited 15, 7, and 16 polymorphic nucleotide sites, respectively. Eight out of these 38 changes are non-synonymous. Sixteen sites contained a specific type of nucleotide residue found only in one cave population. Intrapopulational nucleotide differences are

Fig. 6.38 (continued) body (4), retina (5), retina rudiment (5a = inner limiting membrane, 5b = ganglionic layer, 5c = inner plexiform layer, 5d = inner nuclear layer, 5e = outer plexiform layer, 5f = outer nuclear layer, 5g = outer segments of visual cells, 5h = pigmentary epithelium, ul = unlayered), pigmentary epithelium (6), chorioid (8), sclera (9), optic nerve (10)

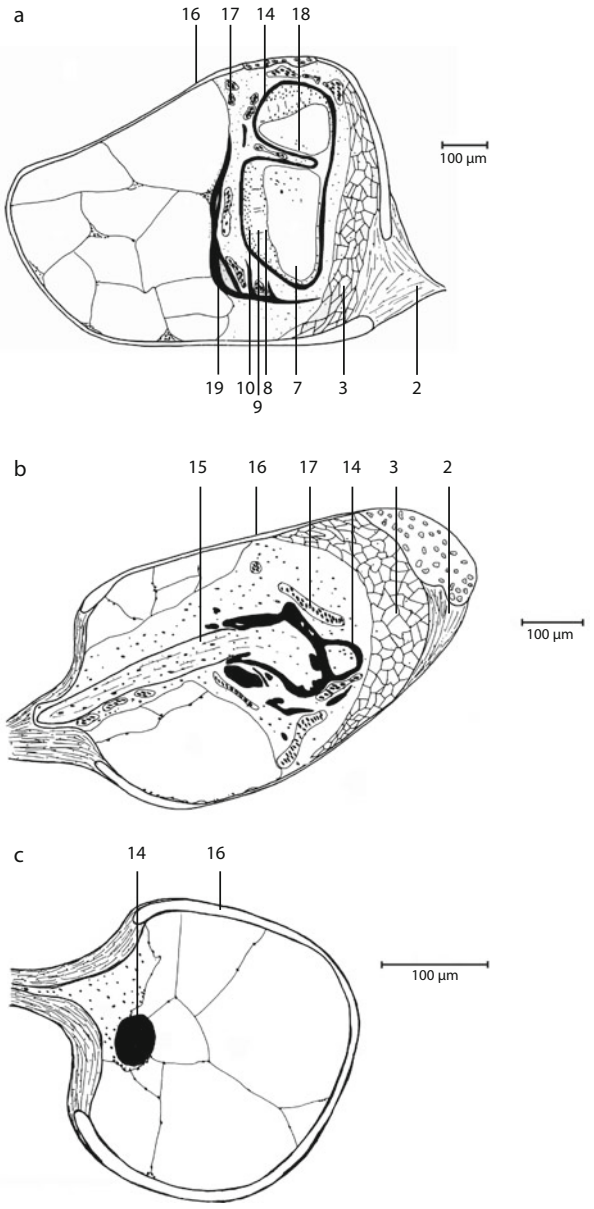

Fig. 6.39 Schematic cross sections showing the range of structural variability of eye rudiments in adult SEP cave fish like Pachón and Sabinos: developed best (**a**), intermediate (**b**) and strongly reduced (**c**). (1) cornea (epithelial), (2) cornea (scleral) and/or connective duct, (3/3a) anterior eye chamber in part filled with spongiosum, (4) iris, (5) lens capsule, (5a) lens muscle, (6) lens, (7) vitrous body, (8) ganglionic layer, (9) inner plexiform layer, (10) inner nuclear layer, (11) outer plexiform layer, (12) outer nuclear layer, (13) visual cell outer segments, (14) pigmentary epithelium, (15) optic nerve, (16) sclera, (17) chorioid, (18) falciform process, (19) argentea; *R* retina unlayered, *SEP* strongly eye- and pigment-reduced (adapted from Wilkens 1970c)

the highest in the surface fish, decrease in the Micos, and are lowest in the Pachón cave fish. The lower levels in the cave fish are consistent with lower variability found in other molecular markers (see Sect. 4.3.3). However, in comparison, nucleotide diversities are higher between the surface and the Micos cave fish than between the surface and the Pachón cave fish in all three opsin genes. Of the

14 directed mutations in the cave fish, 12 belong to the VEP Micos and only 2 to the SEP Pachón. Pachón and Micos cave fish exhibit a high frequency of cytosine to thymidine changes in *r007* and *g103*, of which most have occurred in the Micos cave fish. The unexpected higher nucleotide divergence as well as the higher rate of C to T changes in the VEP Micos compared with the SEP Pachón cave fish could be explained by a different mutation rate for *r007* and *g103* in the two cave populations. However, it could be due to their different surface origin. In any case, the high frequency of C to T changes resembles that of pseudogenes and their functional constraint is relaxed in the cave fish. In addition to the nucleotide differences, a deletion of 12 consecutive nucleotides is exhibited in *g101* of all Pachón cave fish. This deletion is expected to induce strong deleterious effects on the ability to perceive colour.

Microspectrophotometry of *Astyanax* surface fish and its F2 crossing with the VEP Molino cave fish revealed that over half of the F2 hybrids possessed almost pure A_1 chromophores in their cones. The opsin genes remain as intact open reading frames in this cave fish (Parry et al. 2003). Nonetheless, a high incidence of "anomalous" pigments was detected. Such "anomalous" cone pigments are spectrally intermediate to the normal red and green (L_R and L_G) pigments and are expressed by so-called hybrid genes. They are assumed to cause colour vision anomalies. The maintenance of the opsin genes found in the Molino cave fish might have different causes. It may be due to its phylogenetically young age with not enough time to accumulate deleterious mutations. However, as at least a red-like opsin was found to still briefly be transcribed in the embryonic retina of the SEP Piedras cave fish (Langecker et al. 1993), this may mean that opsin genes are under selection because they are required during ontogenetic retinal development and are responsible for the formation and folding of the outer segment disc membranes (Vinnikow 1982). Nonetheless, the majority of opsin genes are most probably on a course toward degeneration (Yokoyama et al. 1995).

6.21.3 Size and Histology of the Eye in the VEP Cave Fish and in the Crossings Between Surface and Cave Fish

6.21.3.1 Crossings of the SEP Cave Fish

In the crossings between the *Astyanax* surface and SEP cave fish, different eye sizes ranging from small rudiments to normal sized eyes found in the surface fish develop (see Sect. 6.23, Fig. 6.55a). The F1 crossings are about intermediate with the means shifted towards the surface fish. The single eye structures like the retina or lens are intact and about half the size of that developed in the surface fish. Eye size variability is the same as in the surface fish. In relation to body size, the eye ball grows isometrically. Whereas the correlation between lens and pupil growth is isometrical, the relation of the lens to the eye ball is positively allometric. Because of this, juvenile hybrids have relatively smaller lenses and pupils than adult ones (Wilkens 1970a).

In the F2 and the backcrossing hybrids with the surface fish the growth of the individual lens and pupillary opening is positively allometric (Wilkens 1970a). Similarly, when considering hybrids of the same body size, the closely correlated lens and pupil sizes are positively allometric in relation to eye ball size. This shows that allometric growth correlations within the eye are determined by relative eye size—independently from whether they rely on the ontogenetic growth stage or a different individual genetic basis. However, particularly in the backcross hybrids with the SEP cave fish, the growth process of lens and pupil is submitted to the process of ontogenetic eye reduction. Thus, the mean relative lens and pupil sizes are relatively larger in juvenile backcross hybrids than in adults. In very small-eyed hybrids, the lenses may even become completely reduced and the closely correlated pupils are lost.

6.21.3.2 VEP Cave Fish and Crossings

Whereas the SEP cave fish populations show strong reduction, the eyes of the VEP Micos, Chica, and Caballo Moro cave fish are less reduced, more variable in size, and, except for the smaller ones, externally visible. For example, eye size of the Micos cave fish approximately ranges between that of the F1 crossing of the surface and the SEP cave fish on the one hand and the surface fish on the other. When the distribution curve includes comparatively larger eyes, it exhibits bimodality (Fig. 6.55a). In the VEP Molino cave fish, the eye rudiment is sunken into the orbit and overgrown by tissue. Its size distribution partly overlaps with the larger ones of the SEP Pachón, but in contrast surpasses that of the SEP Piedras, Curva, and Yerbaniz cave fish (Wilkens and Strecker 2003).

The eye sizes developed in the crossings of the Micos cave fish with the SEP cave fish or with the surface fish depend on that of the respective parental Micos cave fish. The larger the eye, the higher the mean of the crossings. The variability of the lens size and the closely correlated pupil of the Micos cave fish itself are lower than in its F2 crossings with the surface fish or with the SEP cave fish. It is also lower than in the F2 crossings between the surface and the SEP cave fish (Fig. 6.40) (Wilkens 1976, 1988).

The mean eye size of the F1 crossing between the Molino and the surface fish converges with those of the F1 crossings between the surface and SEP cave fish. However, the variability is about three times higher, because eye balls smaller or larger than in the before-mentioned F1 crossings are developed. It can be concluded that this derives from comparatively high variability of eye genes.

6.21.3.3 Histology of Eye Structures in the Crossings Between SEP Cave and Surface Fish and in VEP Cave Fish

Histological studies of the eyes in the various crossings between the surface and the SEP cave fish as well as in the VEP Micos and the Chica cave fish have revealed that, like in the SEP cave fish, size and degree of differentiation are in principal correlated (Wilkens 1972, 1988; Fack and Wilkens 1989). For example, the largest eyes are the best developed ones. They contain well differentiated retinas and transparent crystalline lenses and are comparable to those of the surface fish. In general, it was found that, on the one hand, the anterior eye chamber and the

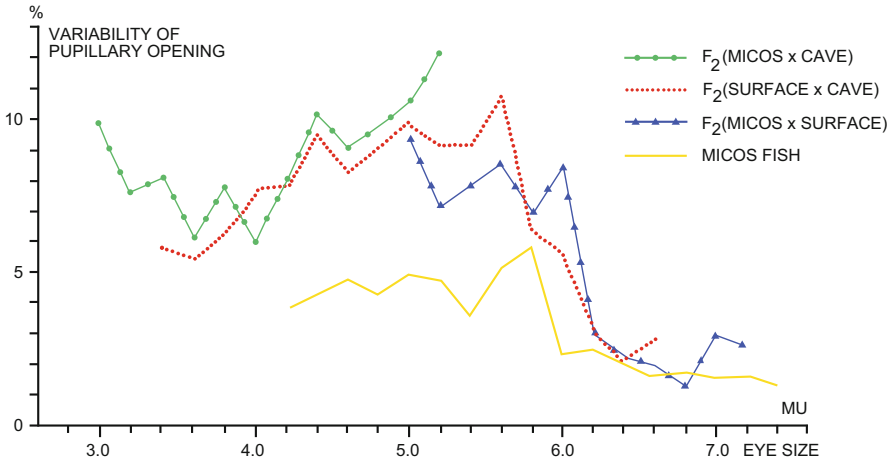

Fig. 6.40 Variability of the pupil diameter of eye balls of the same size in the *Astyanax* VEP Micos cave fish (*yellow line*) and its F2 crossings with the SEP cave fish (*green line*), the surface fish (*blue line*), and in the F2 crossing between surface and SEP cave fish (*red dotted line*) at 2.5 cm standard body length, *MU* units of measurement, *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Wilkens 1976)

ligamentum annulare, the thickness of the cornea, and the diameter of the pupil are correlated with the size of the lens. On the other hand, correlations exist between size and degree of differentiation of retina, pigmentary epithelium, lens muscle, and optic nerve. Both units seem to influence the volume of the vitreous body and the development of the sclera.

However, specific disturbances and developmental aberrations develop in the eyes of a lot of hybrids as well as in the VEP cave fish. In the extreme, eyes with large crystalline lenses may be combined with small retina rudiments (Fig. 6.41a) or well developed retinas may be found in eyes just containing rudimentary lens capsules (Fig. 6.41b). Furthermore, due to an oversized lens inducing the development of a vitreous body being too large for a retina being too small, part of the retina may be pressed as a funnel-shaped formation through the falciform process (Fig. 6.41c). Or, when the lens is too small to produce a sufficiently voluminous vitreous body adequate for a large retina, the retina may become undulated due to a lack of space (Fig. 6.41b). The same phenomenon was observed in chicken eyes, when the vitreous body was artificially prevented from accumulating (Coulombre and Coulombre 1963). Furthermore, in accordance with being part of the retina unit, a large lens muscle may exist while the functionally actually associated lens, which is the central part of the second subunit, only consists of a lens capsule (Fig. 6.41d). From this it can be concluded that two separate developmental sub-units exist within the eye, which show closer correlation within each and inherit independently.

Whereas the eyes of the hybrids between surface and SEP cave fish exhibit intermediate sizes between the parental forms, the crossings of the different SEP

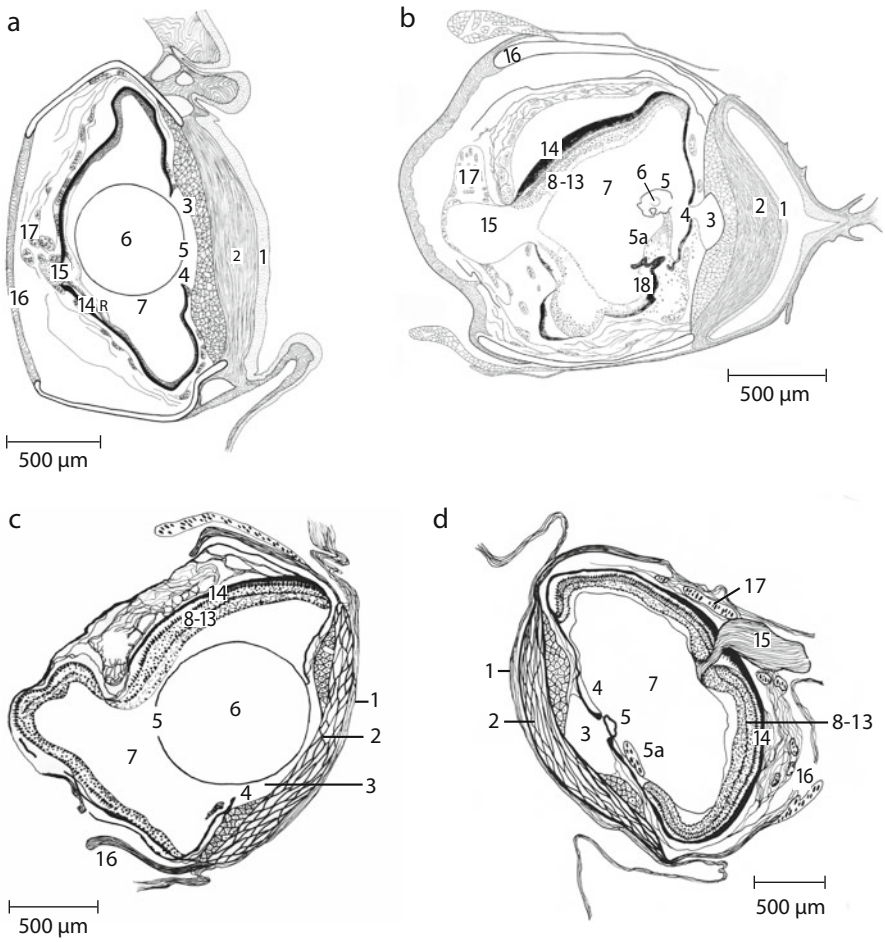

Fig. 6.41 Schematic transverse sections of the eyes of two specimens of the *Astyanax* VEP Chica cave fish (a = large lens combined with undifferentiated retina, b = undifferentiated lens combined with lens muscle and partly well developed or distorted retinal parts) and two F2-hybrids between surface and the SEP Pachón cave fish (c = large lens combined with retina protruded into the chorioid and d = rudimentary lens combined with large lens muscle and well developed retina). *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Wilkens 1972 and Fack and Wilkens 1989, for abbreviations see Fig. 6.39)

cave fish populations with each other as well as with the VEP Molino cave fish deviate from this. They may develop larger eyes than the parental cave fish. In the F1 crossings, specimens containing lens capsules may appear (Wilkens 1971, 1988, 2007; Wilkens and Strecker 2003). An exceptional situation is found in the F2 crossing between the Pachón and the Molino cave fish, in which "back to surface eyes" develop. Together with specimens exhibiting smaller eyes that are sunken into the orbital cavity, as in the parental generation, several of the adult F2

specimens develop externally visible eyes with large lenses and wide pupils (Fig. 6.42). Histological sections of the eyes from these latter specimens revealed the presence of large transparent crystalline lenses as well as retinas containing all optical layers with morphologically intact visual, horizontal, and Müller cells including pigment epithelium, optic nerve, and lens muscle. The number of visual cells is lower, though, and therefore the retina is not as thick as in the surface fish (Wilkens and Strecker 2003). This particular observation was only made in crossings with the SEP Pachón cave fish and can be explained by the Pachón cave fish exhibiting an eye genetic background less affected by regressive mutations than the other SEP cave fish (Wilkens and Strecker 2003). This could be caused by genetic introgression of an unknown VEP cave population, which is claimed to be responsible for the mitochondrial capture in the SEP Pachón cave fish (see Sects. 5.2.1, 5.4).

6.21.4 Ontogeny of Eye Development in the SEP Cave Fish

Cahn (1958) was the first to compare the ontogenetic eye development of two *Astyanax* cave forms, the Chica and the Sabinos cave fish, with the surface sister fish. These studies revealed that the larval cave fish eye and its single structures are not only smaller than in the surface fish, but already exhibit regressive development (Wilkens 2007; Wilkens and Meyer 1992). For example, neither of the lens cells will ever differentiate into transparent fibres nor will the visual cells develop outer segments. Thus, the SEP *Astyanax* cave fish do not start with a "complete eye", as is often inferred (Alunni et al. 2007; Jeffery 2001).

The ontogenetic growth of the eye ball of surface *Astyanax* starts positively allometrical in juveniles until about 4 cm body length and declines to negative allometry in adult stages after 6 cm body length is reached. In between, the eyes grow isometrically (Fig. 6.43) (Table 6.2). In the cave fish, four ontogenetic stages with specific histological and allometric growth characteristics can be differentiated (Figs. 6.43 and 6.44). At stage 1 (<4 mm body length), smaller primordia than in the surface fish are formed, but the relative growth correlations between eye and body size are similar (Table 6.2). In spite of its smaller size, the retina of the cave fish eye differentiates in the same manner as that of the surface fish. Usually the chorioid fissure closes and forms the falciform process. Lens fibre development, which in the surface fish begins at these early stages, fails in all strongly eye-reduced SEP and the VEP Molino cave fish, and the lens stays undifferentiated too (Wilkens 2007).

Occasionally the falciform process does not completely grow together in its ventral part. By this defect the vitreous body in cave fish is not able to properly accumulate, which is a prerequisite of eye growth (Coulombre and Coulombre 1963). Such cave fish eye rudiments cannot differentiate properly and remain smaller. In humans, this pathological defect is called coloboma iridis. In *Astyanax* cave fish it is an outlier (Figs. 6.12, 6.45, 6.46) (Wilkens 2007), but was falsely diagnosed as the ventral sector of the retina being reduced or missing and

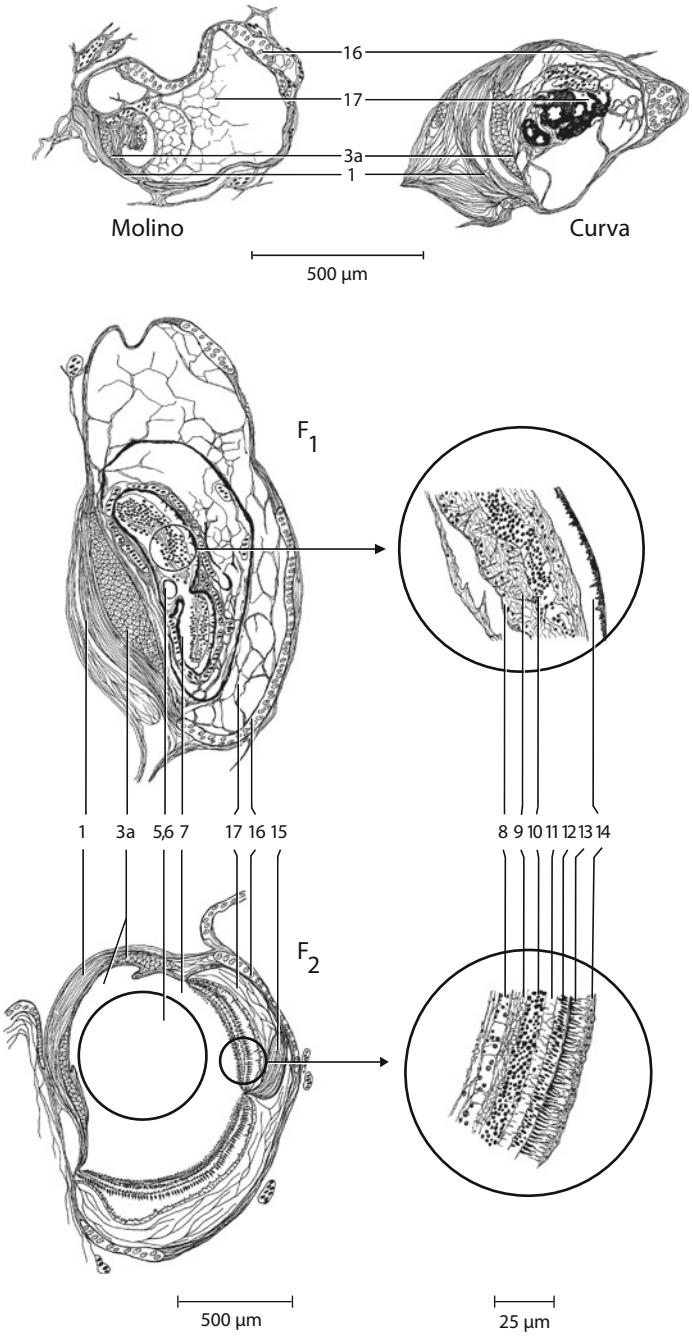

Fig. 6.42 Transverse sections of the eyes of the VEP Molino, the SEP Curva cave fish and of F1-and F2-crossing hybrids between VEP Molino and SEP Pachón cave fish (for abbreviations see Fig. 6.39, adapted from Wilkens and Strecker 2003)

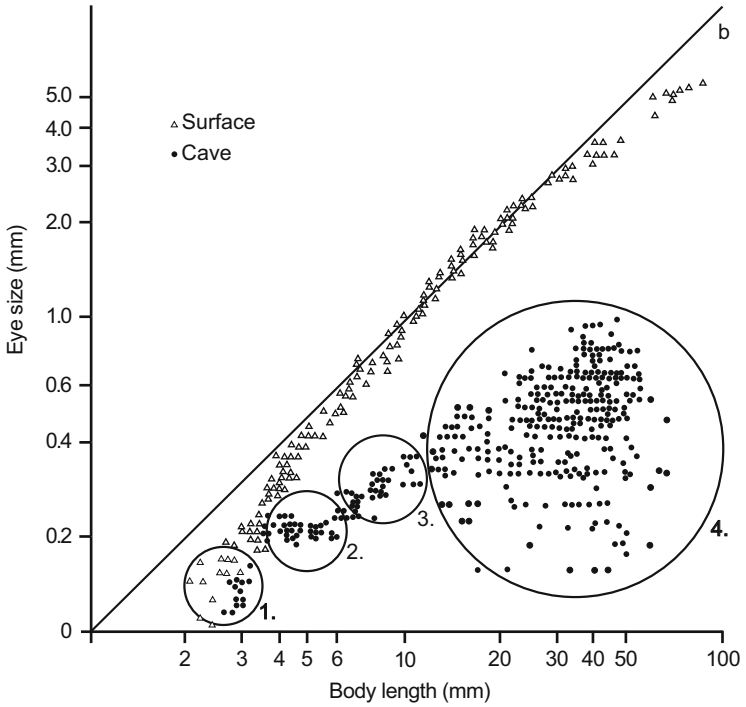

Fig. 6.43 Allometry of ontogenetic eyeball growth in correlation with body size (double logarithmic) in *Astyanax* surface (*triangles*) and the SEP Sabinos cave fish (*dots*). *Circles* 1–4 indicate different growth stages in cave fish, *SEP* strongly eye- and pigment-reduced. (For allometrical coefficients see Table 6.2, adapted from Wilkens 2007)

interpreted as a general characteristic of eye regression (Jeffery et al. 2003; Pottin et al. 2011).

At the beginning of stage 2 ($\geq$4 mm to <6 mm body length), the eyes of the SEP cave fish are smaller than the surface fish eye, which is maximally 0.4 mm in diameter. In the cave fish, average eye sizes are the largest in VEP Molino and the SEP Pachón cave fish (maximal sizes 0.32 and 0.28 mm, respectively), whereas the SEP Piedras, Sabinos, Curva, and Yerbaniz populations show smaller sizes (maximal size 0.25 mm). Most importantly, visual cell ellipsoids are rarely found in the retina and lamellar visual cell outer segments are always absent in the strongly eye-reduced SEP cave populations (Figs. 6.45a and 6.47a). As an exception, the VEP Molino cave fish, which possess relatively strongly reduced eyes compared with the VEP Micos and Caballo Moro cave fish, develop perfectly structured layers of visual cell ellipsoids containing rich accumulations of mitochondria in large numbers and even small primordia of lamellar outer segments (Fig. 6.45b).

A temporary stop or even a decline of eye growth and relative eye size can be observed during stage 2 in the cave fish (Figs. 6.43 and 6.44), whereas the surface fish eye continues growing. Such a temporary growth stop is also not found in the

Table 6.2 Allometrical coefficient (*b*), coefficient of correlation (r^2) and number of individuals (*N*) of *Astyanax* surface and cave populations as well as F1, F2 and backcrossings (*R*) with Pachón at the different ontogenetic stages (adapted from Wilkens 2007)

	Stage 1 (<4.0 mm)			Stage 2 (≥4.0 < 6.0 mm)			Stage 3 (≥6.0 < 15.0 mm)			Stage 4 (≥15.0 mm)		
	b	r^2	*N*	*b*	r^2	*N*	*b*	r^2	*N*	*b*	r^2	*N*
Surface	0.998	0.676	45	0.814	0.748	56	1.223	0.962	84	0.737	0.968	166
F_1	1.026	0.344	22	0.972	0.848	22	1.321	0.987	16	0.768	0.856	31
F_2				0.647	0.380	203	1.000	0.787	135	0.717	0.607	338
R				0.442	0.482	58	1.028	0.841	94	0.833	0.642	40
Piedras	0.923	0.703	30	−0.112	0.029	34	0.830	0.840	38	−0.480	0.302	34
Curva				0.250	0.035	40	0.621	0.846	86	0.597	0.263	40
Yerbaniz				−0.760	0.154	35	0.657	0.715	60	0.663	0.545	25
Pachón				−0.140	0.034	24	1.102	0.931	22	0.271	0.240	34
Molino				0.238	0.165	39	0.865	0.893	93	0.202	0.044	45

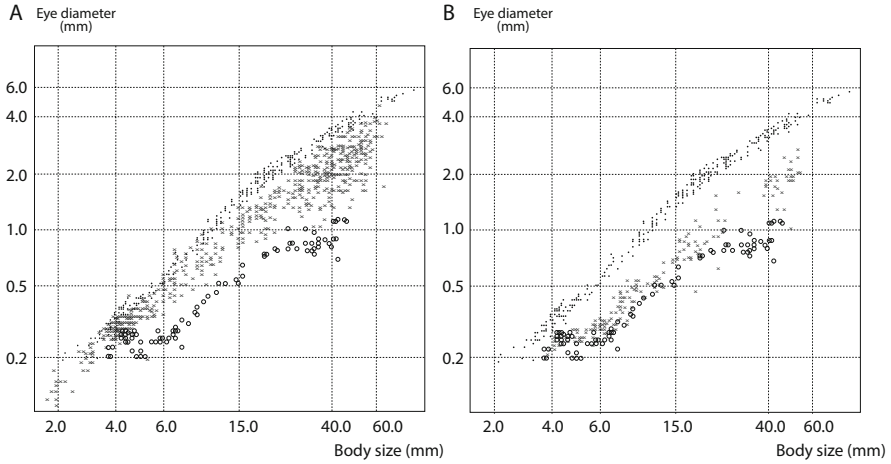

Fig. 6.44 Relative eye growth (double logarithmic) in the F2 and the backcrossing between the *Astyanax* surface and the Pachón cave fish. (**a**) Surface fish (*dots*), F2 crossing (*crosses*), and Pachón cave fish (*open circles*). (**b**) Surface fish (*dots*), backcrossing to cave fish (*crosses*), and Pachón cave fish (*circles*), for further data see Table 6.2 (adapted from Wilkens 2007)

F2 crossing between surface and SEP cave fish (Fig. 6.44). It is exhibited, though, in the backcrossing with the SEP cave fish. This stop is correlated with further differences. In all cave fish, holes of lytic zones, caused by apoptosis, appear in both retina and lens (Fig. 6.45a). Apoptotic processes are of general importance in cave fish eye regression and were also detected in the retina rudiment of the cave cyprinid *P. andruzzii* (Berti et al. 2001; Stemmer et al. 2015). However, whereas in this species these processes are correlated with the complete loss of the eye rudiment, in *Astyanax* cave fish, cell death processes cease during stage 3 ($\geq$6.0 mm to $<$15.0 mm bs) in the retina, which, along with the whole eye ball, increases in size through further growth (Figs. 6.43 and 6.44). Thus, eye growth does not arrest as recently claimed again by Krishnan and Rohner (2017).

Now, except for visual cells including outer nuclear and plexiform layers, all layers incorporating fine structures such as horizontal and Müller cells as well as the outer limiting membrane are developed in the SEP cave fish (Fig. 6.46a). Extremely rarely, single ellipsoids containing low numbers of mitochondria but no outer segments were found. The optic nerve, which is connected with the tectum opticum, as well as the falciform process are developed. The pigmentary epithelial cells have a cubic form in the central retina with their nuclei at the cell basis. The lens stagnates in its development and does not differentiate further. It consists of a capsule enclosing a noncrystalline nucleus of undifferentiated cells. In spite of only forming a rudimentary lens, the VEP Molino cave fish develops a large number of visual cells equipped with ellipsoids rich in mitochondria and outer segments at this stage (Fig. 6.46b). The lamellar arrangement of the discs of the outer segments of this population is completely regular and undisturbed (Fig. 6.47).

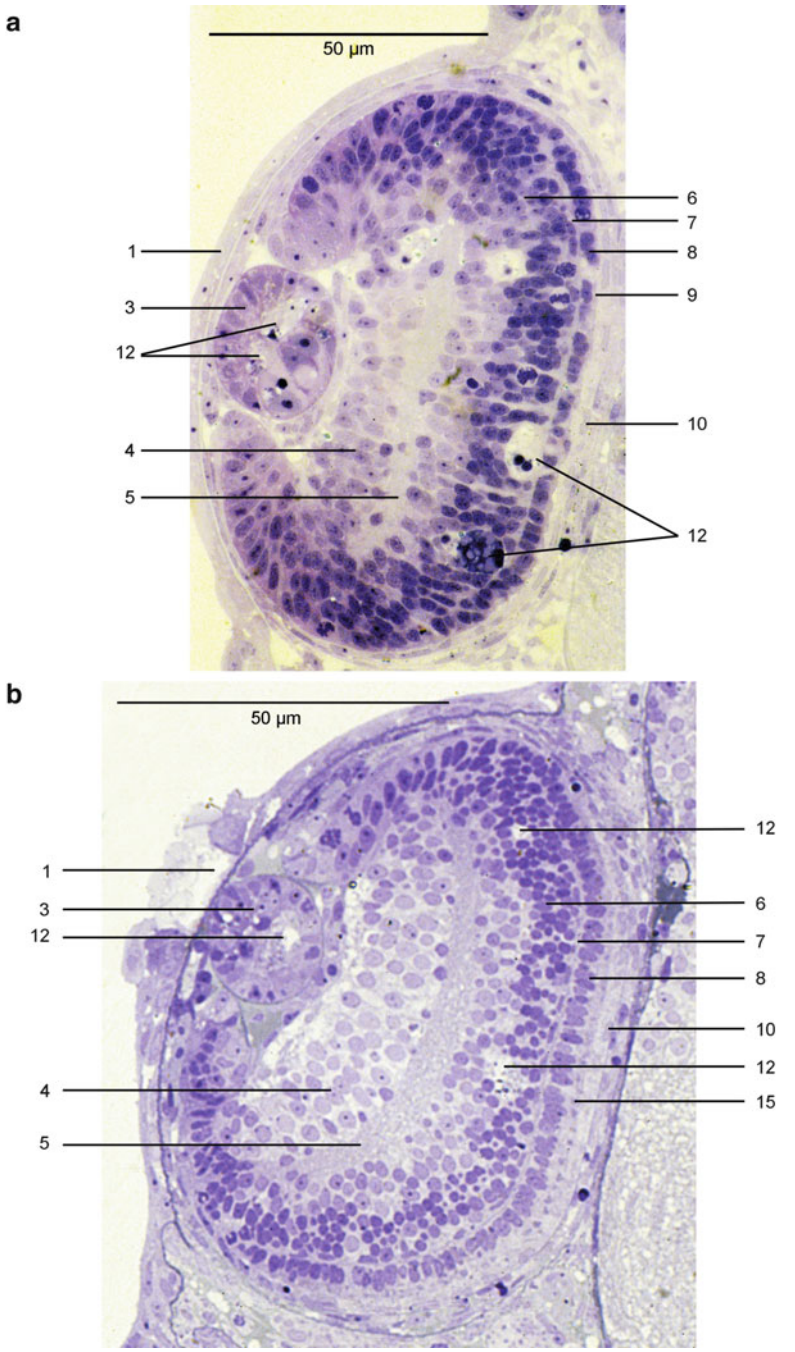

Fig. 6.45 Semi-thin sections of the larval eye at stage 2 (5 mm body length) of the SEP Curva (**a**) and the VEP Molino (**b**) cave fish. 1 = cornea; 2 = anterior eye chamber; 3 = lens; 4 = ganglionic layer; 5 = inner plexiform layer; 6 = inner nuclear layer; 7 = outer plexiform layer; 8 = outer nuclear layer; 9 = outer limiting membrane; 10 = pigmentary epithelium; 11 = optic nerve;

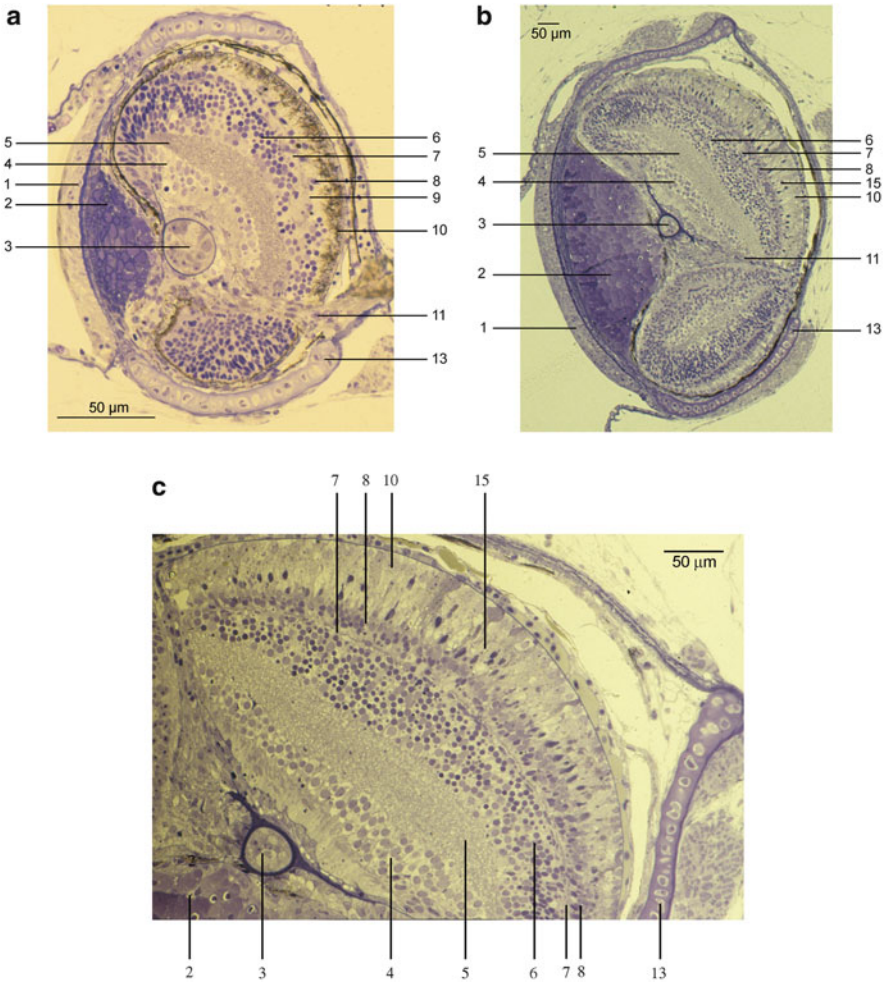

Fig. 6.46 Semi-thin sections of the larval eye at stage 3 (12 mm body length) of the SEP Curva (**a**) and the albinotic VEP Molino (**b**, **c**) cave fish. At this stage the melanin pigmentation in the pigmentary epithelium has developed (adapted from Wilkens 2007, for abbreviations see Fig. 6.45)

During stage 4 (>15 mm bl), the degree of histological differentiation characteristic of the eye of the adult phylogenetically old SEP cave fish develops and its final process of rudimentation is reached (Fig. 6.39, 6.43, 6.44) (Wilkens 1988, 2007). The relative growth of individual eyes decreases even more to different

Fig. 6.45 (continued) 12 = apoptotic centre; 13 = sclera, 15 = ellipsoids; *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Wilkens 2007)

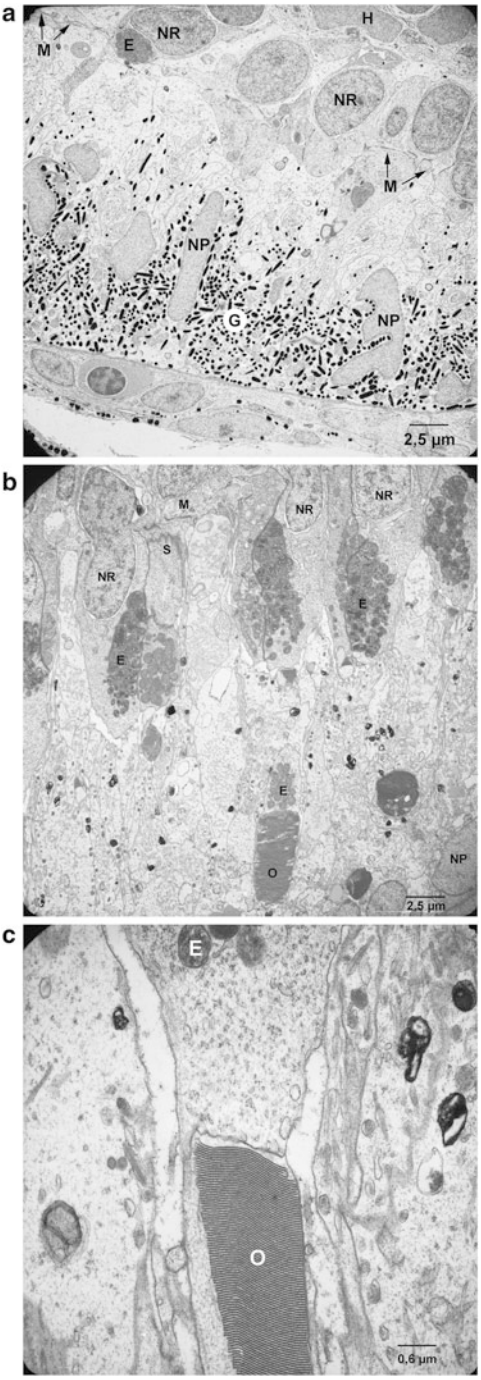

Fig. 6.47 Ultra-thin sections of outer nuclear and pigmentary epithelial layers at ontogenetic stage 3. (**a**) Piedras cave fish (7 mm body length), (**b**) Molino cave fish (12 mm body length), (**c**) ultra-thin section of outer segment of the Molino cave fish (12 mm body length). *E* ellipsoid, *G* melanin granules in pigmentary epithelium, *H* horizontal cell nucleus, *M* outer limiting membrane, *NP* nucleus pigment epithelial cell, *NR* nucleus photoreceptor cell, *O* outer segment, *S* synaptic body (adapted from Wilkens 2007)

degrees. Due to this, the characteristic high variability of the rudimentary eye of adult cave fish develops during stage 4, but it is low and does not surpass that of the surface fish eye during stages 1–3. In the VEP Molino cave fish, the visual cells, which in contrast with the SEP cave populations were developed during stage 3, become secondarily reduced (Wilkens and Strecker 2003).

In contrast to the retina, the lens in all SEP cave fish populations will not surpass the undifferentiated histological state acquired during stage 2 and completely vanish in the last ontogenetic phase of eye reduction (stage 4). However, the VEP Molino cave population showed that visual cells with lamellar outer segments may differentiate (Fig. 6.47c), although the lens remains in the same poorly developed state characteristic of all SEP cave populations. During their ontogenetic development, the fish of this population transitorily develop retinae, which are structurally able to perceive light.

Quite generally, there are two areas of cell proliferation in the growing teleost retina. (1) Stem cells proliferate in the ciliary marginal zone of the retina (CMZ) and their progeny differentiate into various neural and glial cells except rod photoreceptors. They also provide new cells to the pigmentary epithelium (Johns 1977; Negishi et al. 1990). (2) Cell proliferation also occurs in the inner nuclear layer of the retina, where stem cells are located that give rise to rod precursors, which intercalate into the outer nuclear layer and eventually become rod photoreceptors (Kwan et al. 1996; Otteson et al. 2001). Cell proliferation is not inhibited in the CMZ of the cave fish (Strickler et al. 2007). However, whereas during stage 2 eye growth stops because it is negated by apoptotic processes in the retina, the eyes of all cave fish specimens start growing again during stage 3, although to a lesser extent than in the surface fish, as exhibited by negatively allometric growth. During stage 4, in which the variability of eye size and eye histology starts to develop, growth is only exhibited by the larger eyes whereas the smaller ones keep the same size as observed during stage 3. In summary, it can be concluded that programmed cell death (PCD) and not inhibition of cell proliferation causes the decline of retina growth in cave fish (Strickler et al. 2007). These processes almost exclusively occur during stage 2, when apoptosis develops to a large extent and the cave fish eyes temporarily stop growing (Wilkens 2007, 2010).

Also, the growth of the eye of the cave-living hepapterid catfish *R. zongolicensis* and *R. reddelli* follows the principles described for *Astyanax* cave fish. Whereas in juveniles up to about 4 cm body length the variability of eye size does not surpass that of the closely related surface sister species *R. laticauda*, it drastically increases in adult specimens and often exhibits extensive left-right asymmetries (Fig. 6.48) (Wilkens 2001). Comparable observations were made in amblyopsid cave fish (Niemiller and Poulson 2010).

6.21.5 Genetic Basis of Eye Development

Crossing analyses between surface and the phylogenetically old SEP cave fish revealed that eye size relies on a polygenic basis and it was calculated that in

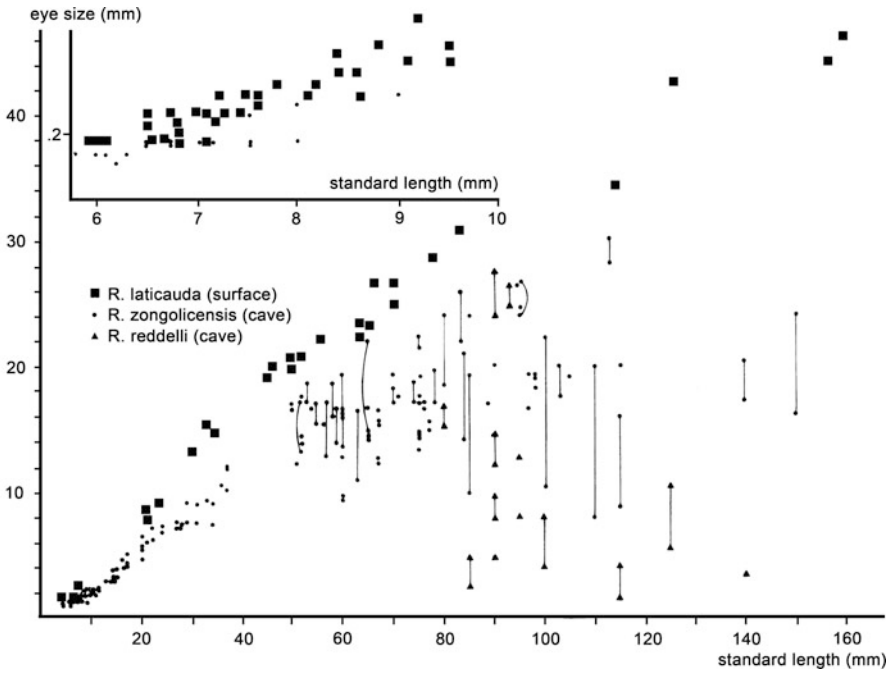

Fig. 6.48 Ontogenetic growth curves of the eyes of the surface catfish *Rhamdia laticauda* (Hepapteridae) and the cave sister species *R. zongolicensis* and *R. reddelli*. Each *symbol* indicates eye size (measured as eye diameter) and body length (measured as standard length) of one specimen. *Connecting lines* indicate left–right asymmetry of the eye rudiments of a specific single specimen exhibiting exceptionally high divergence. Insert showing eye growth up to 10 mm body length (adapted from Wilkens 2001)

total at least eight genetic factors, the so-called "eye genes", are responsible for this difference (Wilkens 1970a, 1988; Lande 1981). The polygenic basis of eye formation was confirmed by QTL analysis (see Sect. 6.23) (Protas et al. 2007).

On the molecular level, a large number of candidate genes involved in eye development and potentially in eye reduction have been identified (Fig. 6.49) (for review see Casane and Rétaux 2016). However, in developmental control genes responsible for eye formation like *pax6*, *shh*, or *sox*, no regressive mutations have been detected in *Astyanax* cave fish (Behrens et al. 1997, 1998; Ma et al. 2014; Stemmer et al. 2015; Strickler et al. 2001).

The *Pax6* gene, which encodes a paired-class homeodomain transcription factor, is assumed to be a prime candidate for mediating eye regression in cave fish (Behrens et al. 1998; Strickler et al. 2001). In surface fish embryos, two bilateral *pax6* expression domains are present in the anterior neural plate, which extends across the midline, and fuse to form the forebrain and optic primordia. In embryos deriving from different cave fish populations, these *Pax6* domains are diminished in size and remain separated, resulting in an anterior gap in *Pax6* expression and

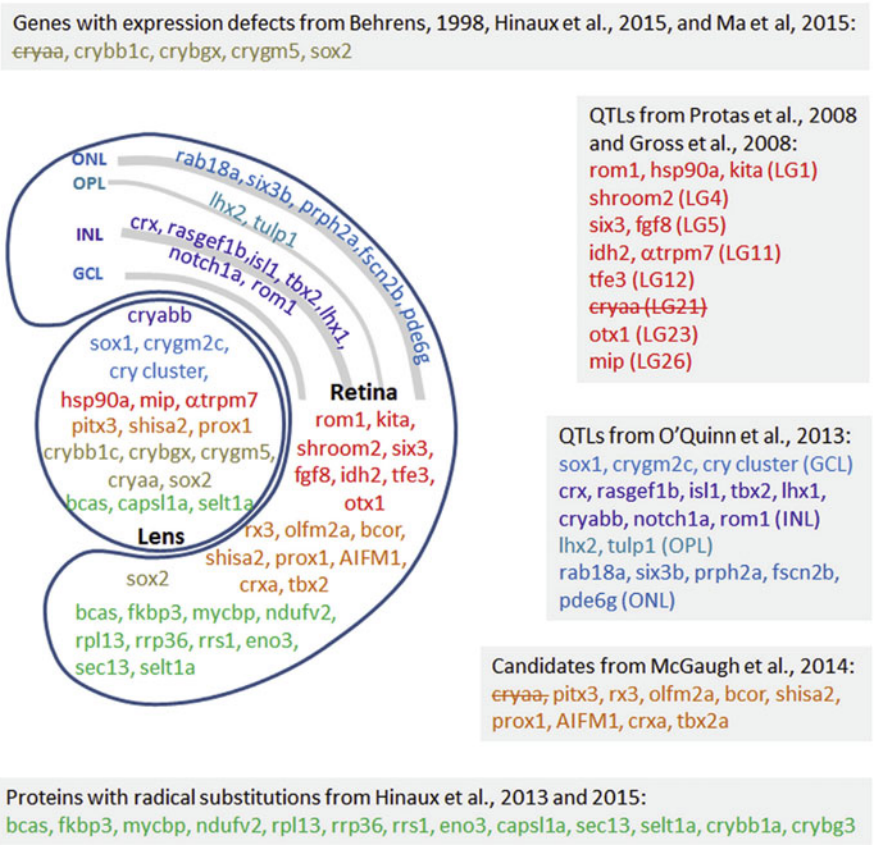

Fig. 6.49 Genes potentially involved in eye regression of *Astyanax* cave fish indicated according to their expression patterns in the retina, the lens, or both. As *alpha-A-crystallin* (*cryaa*) expression is missing in the cavefish lens, *cryaa* is crossed in all cases because there is evidence that it is not the causal gene in the identified quantitative trait loci (QTL) interval, although absence of *cryaa* expression in the cavefish lens is deleterious to the lens. *GCL* ganglionic layer, *INL* inner nuclear layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer (adapted from Casane and Rétaux 2016)

presumably the formation of smaller optic primordia (Strickler et al. 2001). *Hedgehog* proteins diffusing from the prechordal plate are known to regulate the size of the optic primordia by suppressing *Pax6* expression in the neural plate (Ekker et al. 1995; Varjosalo and Taipale 2008). Thus, the diminutive *Pax6* domains and optic primordia in cave fish embryos are suggested to be a consequence of a higher level of *hedgehog* signalling emanating from the prechordal plate. *Hedgehog* genes (*sonic hedgehog* [*shh*] and *Tiggy-winkle hedgehog* [*twhh*]) are thus obvious candidates for acting upstream of *Pax6*. As expected, the expression of these genes in several different cave fish populations was found to be expanded along the anterior embryonic midline (Yamamoto et al. 2004) and to arrest eye growth and development. These features can be phenocopied in surface fish by overexpression of *twhh*

and/or *shh*, supporting a role for *hh* signalling in cave fish eye regression (Yamamoto et al. 2004; Jeffery 2005). However, mapping of candidate genes *shh*, *twhh*, and *Pax6* revealed that no eye quantitative trait loci are located near them. This result makes it unlikely that mutations in any of these genes are directly responsible for eye regression (Protas et al. 2007).

The lens plays the central role for the development of one of the two subunits responsible for eye formation (see Sect. 6.21.6) (Wilkens 2010, 2016). It was found that *alpha-A-crystallin* (*cryaa*) expression is missing in the cave fish lens (Behrens et al. 1998; Hinaux et al. 2015; Ma et al. 2014; Strickler et al. 2007). As the *cryaa* gene is located in a QTL genomic region responsible for eye loss (Gross et al. 2008; McGaugh et al. 2014), it has been proposed as a candidate for regulating cave fish lens degeneration. To reveal the underlying reason for the absence of expression in cave fish, *Sox2* was studied, which is one of the transcription factors that regulate lens crystallin genes during eye development in other species. However, *Sox2* is not expressed in the cave fish lens and it is therefore assumed that downregulation of *cryaa* in cave fish is caused by an evolutionary change in an upstream gene in the lens differentiation pathway, either *sox2* itself or a gene regulating *sox2* (Casane and Rétaux 2016; Ma et al. 2014).

Visual pigment plays a central role in the second subunit within the eye, the retina, because the disc membranes of the outer segments consist of phospholipids in which *rhodopsin* is embedded. For example, the functionality of *rhodopsin* has repeatedly been lost in different lineages of Amblyopsid cave fish (Niemiller et al. 2012) and, dependent on time and phylogenetic age, the number of retinal pseudogenes increase in eye-reduced fossorial mammals (Emerling and Springer 2014). However, knowledge regarding *Astyanax* cave fish is poor as yet, possibly because of the generally accepted prejudice of the lens being causal for promoting the regression of the eye as a whole (Wilkens 2010, 2016) (see Sect. 6.21.6). Disturbances also exist in *Astyanax* cave fish, however. In the Micos cave fish, red and green opsin gene sequences showed a high frequency of C to T changes. In Pachón, a deletion of 12 consecutive nucleotides occurs, which would disturb colour vision (Yokoyama et al. 1995). Multiple photopigments were detected in a microspectrophotometric study of the Molino cave fish (Parry et al. 2003). However, a red-like opsin was found to be still expressed in the outer nuclear layer of the Piedras cave fish for a limited period of time during early ontogeny (Langecker et al. 1993).

Gene expression and sequencing data of cave fish imply that destructive loss-of-function mutations have only occurred to a limited extent in cave fish structural eye genes. This is supported by the observation that all the different-sized eyes developed in crossings between cave and surface fish as well as in phylogenetically young VEP cave fish in principle exhibit all the structures characteristic of an eye such as lens, lens muscle, pupil, and retinal layers (Peters et al. 1975; Wilkens 1988, 2007). In crossings between strongly eye-reduced cave fish like the SEP Pachón and the VEP Molino, eyes may develop that by far outclass those of the parental cave fish by size and degree of differentiation (Fig. 6.42) (Wilkens and Strecker 2003).

Histological analyses of crossing hybrids have shown that two developmental subunits build the eye, the lens dioptric and the retina sensorial apparatus, which were suggested to inherit independently from each other, although, as in all vertebrates, the primordial eye cup induces the formation of the lens placode and both are closely correlated in size (see Sects. 6.21.3.3, 6.21.6). This apparent discrepancy can be explained by different gene systems being active during eye ontogeny. One system, which acts at early ontogeny during step 1 of eye differentiation, is responsible for the formation of a smaller eye cup (Fig. 6.50, step 1). The genes included in this group would determine its size through regulation of *hh* expression and by induction, that of the lens placode. However, in the subsequent step 2 of eye differentiation (Fig. 6.50, step 2), which includes the stages two to four of ontogenetic eye regression (Fig. 6.43), two more gene systems, one responsible for the formation of the dioptric lens subunit and another for the sensory retinal apparatus subunit, act independently from each other (see Sect. 6.21.5, Fig. 6.50).

Since the discovery that neither the *pax6*, nor the *alpha a crystalline* (*cryaa*) (Behrens et al. 1997, 1998), nor the *hedgehog* genes (Yamamoto et al. 2004) were submitted to regressive mutations, no real progress in elucidating the genetic basis of eye regression in the *Astyanax* cave fish has been made. The question of which genes regulate *shh* expression remains unanswered. In this context, the genetic study of a comparable process in snakes might provide insight. For limb regression in this group, it was shown that the ZRS enhancer of *shh* underwent a rapid increase in substitution rate from basal snakes with vestigial limbs to advanced ones without any skeletal remnants (Kvon et al. 2016). It is suggested that eye loss relies on a similar genetic basis. In that case the appearance of "back to surface eyes" in the F2

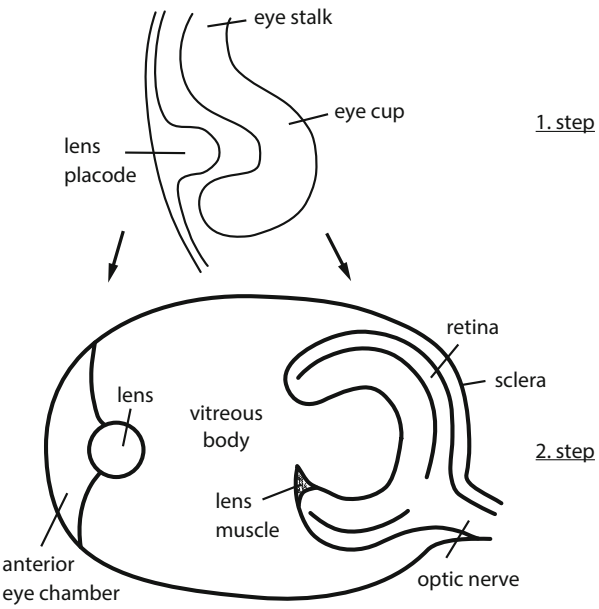

Fig. 6.50 Scheme of the ontogenetically early first and second step of eye development in *Astyanax* cave fish (1. step and 2. step correlate to *circles* 1 and 2–4, respectively, in Fig. 6.43)

crossing between Pachón and Molino cave fish could be explained by the complementary restitution of their function brought about by the recombination of such an enhancer gene having been submitted to different mutations in geographically separate cave fish populations (Fig. 6.42) (Wilkens 1971, 2007; Wilkens and Strecker 2003).

6.21.6 The Role of the Lens in Eye Development

During the phylogeny of the vertebrate eye, the neuronal retina and the dioptric lens subunits have subsequently evolved (Lamb et al. 2007), which may explain their relatively large developmental independence from each other after the lens placode has been induced. This is confirmed by developmental physiological studies in chicken eyes (Coulombre 1969) and by stem cell cultures showing that the formation of the optic cup in mice occurs in the absence of a lens depending on an intrinsic self-organizing programme (Eiraku et al. 2011; Nakano et al. 2012). Like for *Astyanax*, independent development of lens and retina has also been revealed in other cave fish species like *Rhamdia* catfish (Wilkens 2001), *Sinocyclocheilus specs*. (Meng et al. 2013), and *P. andruzzi* cyprinids (Stemmer et al. 2015).

Dissenting from histological and crossing analyses in *Astyanax* that revealed that there are two independent units within the eye, a central and exclusive role of the lens for eye development as a whole in *Astyanax* was claimed (Yamamoto and Jeffery 2000; Yamamoto et al. 2003). A large number of inconclusive genetic and developmental analyses have been performed with the objective of substantiating this hypothesis (for review see Ma et al. 2014). Meanwhile, it has become a virtual axiomatic uncritically accepted fact that "Cavefish eyes are lost through apoptosis of the lens, which in turn promotes the degeneration of other optic tissues" (Casane and Rétaux 2016; Hinaux et al. 2016; Krishnan and Rohner 2017).

Based on lens transformation experiments, it was claimed that the cave fish eye can be completely "rescued" by the transplantation of a surface lens (Yamamoto and Jeffery 2000). As a proof for the asserted "rescue" of the eye, just a small number of cells expressing *rhodopsin* in the cave fish retina rudiment are provided. This finding and the conclusion drawn from this are unsubstantiated, however, because transitional expression of an opsin gene was also detected in SEP cave fish like Piedras, which never during its ontogeny forms an intact retina with visual cells (Langecker et al. 1993). Furthermore, in the reciprocal experiment in which a cave fish lens was transplanted into the surface eye or the lens vesicle was deleted from the optic cup of a surface fish embryo, the retina will develop all its characteristic layers, nonetheless. Programmed cell death and apoptosis do not occur in the surface fish larval and adult retina under these experimental conditions either (Strickler et al. 2007). Similarly, the transplantation of a surface lens into a cave fish eye did not increase the volume of the tectum opticum, in which the optic nerve fibres originating in the rescued retina would be expected to grow into. Neither did the tectum in the surface fish become thinner in specimens after excision of the embryonic lens (Rodrigues 2013).

Cave fish with transplanted surface lenses are actually developing large crystalline lenses and wide pupils (Yamamoto and Jeffery 2000). The erroneous conclusion of the complete "rescue" has probably come about by this observation. However, this finding only proves the existence of the dioptrical lens apparatus as an independent unit, as also shown by crossing experiments, but does not support the central role of the lens for retina differentiation.

Lens removal in surface fish keeps the eye smaller by about 30% (Dufton et al. 2012). As the lens influences the volume of the vitreous body the latter cannot properly develop in lens-ectomized surface fish eyes. Due to this it remains smaller in size and the eye sinks into the orbit. As a result, a cave fish eye is only superficially phenocopied. The retina, which independently develops, is malformed because of its relative oversize compared with the vitreous body, but still contains all layers including visual cell outer segments. Such retinas are still functional and even suitable for vision. Moving objects, which are sufficient for the release of aggressive behaviour in surface fish, are still recognized. This was proven by the performance of aggressive behaviour in lens-enucleated surface fish (see Fig. 1D in Espinasa et al. 2005) (see Sect. 6.12).

Also, apoptosis does not occur in lens-ectomized surface retinas. In order to explain this, the so-called "dual signal model" for retinal growth and development was secondarily introduced (Strickler et al. 2007), suggesting that the lens acts in concert with another unknown component, possibly the pigmentary epithelium. As a result, the existence of two separate units within the eye is unintentionally corroborated (Wilkens 2010, 2016). Summarizing, "complete eyes", which should contain lenses as well as retinas with fully developed visual cell outer segments, are not restored in cave fish after transplantation of a surface fish lens as is untiringly cited (e.g. Casane and Rétaux 2016; Jeffery et al. 2003; Krishnan and Rohner 2017; Yamamoto and Jeffery 2000; Yamamoto et al. 2004).

6.21.7 Ontogenetic Eye Regression and Head Formation

Although never achieving their genuine function, many structures that have become biologically functionless develop more or less completely during early ontogeny, but become reduced or even finally lost entirely after ontogeny has finished. Such processes are characterized as ontogenetic regression. Examples are provided by structures like the reduced pelvic girdles and hind legs of whales and sirenia, which, as small and no longer externally visible remnants, are deeply sunken into the adult body after having been almost fully developed during early ontogeny (Deimer 1977). The eyes of cave-living fish and amphibians can be quoted among such rudimentary structures, too. This was shown in species like the Cuban and Bahamian cusk eels (Bythitidae) (Eigenmann 1909; Wilkens et al. 1989), in the Mexican heptapterid catfish genus *Rhamdia* (Wilkens 2001), in the Somalian cyprinid cave fish *P. andruzzi* (Berti et al. 2001), or cave-living newts like the European cave *Proteus anguinus* (Durand 1971).

The developing eye plays a non-visual role during development as an important "organizer" of craniofacial morphogenesis (Kish et al. 2011). It directs the proper migration of cranial neural crest cells (CNCs), a transient population of migratory stem cells (Kish et al. 2011; Langenberg et al. 2008). In the developing head, retinoic acid (RA), which is synthesized in the telencephalon, eye, and nasopharynx at different times, is required for patterning. In the eye, RA is produced in a spatiotemporally regulated fashion in the dorsal and ventral fields of the retina (Matt et al. 2008). However, RA is not required for early patterning of the dorsal-ventral retina, but from the developing retina it targets RA receptors in the neural crest-derived periocular mesenchyme (Matt et al. 2008). The effects of the paracrine RA signal gradients are known to regulate important gene expression and signalling pathways. For example, altering RA signalling during early ontogeny can change a lower jaw into an upper jaw. Since RA signalling is important in craniofacial development, and since a significant amount of RA is synthesized in the retina in a tightly regulated fashion, it follows that RA from the developing eye helps to establish the RA gradient that is necessary for optimal craniofacial morphogenesis (Kish et al. 2011).

Although never achieving their genuine function, early ontogenetic anlagen may be well developed because they are responsible for the appropriate formation of other adjacent ones during early ontogeny. Consequently, they are at least transitorily still submitted to stabilizing selection. In the SEP *Astyanax* cave fish this is proven by the low variability of eye size at early ontogeny (stages 1–3), which increases drastically in adult cave fish (stage 4) (Figs. 6.43 and 6.48). The embryonic eye is supposed to play an inductive role in head and/or brain formation. In contrast, in adult cave fish, stabilizing selection for eye development relaxes and the high variability of eye ball size arises at these later stages. It is suggested that the temporary cessation of eye growth exhibited by *Astyanax* cave fish at stage 2 probably comes about by the inductive function of the eye in head and/or brain formation sinking to a lower level, because this process has been completed (see Sect. 6.21.4).

In several cave catfish, cave amblyopsids, the cave cyprinids genus *Sinocyclocheilus* (Meng et al. 2013) or cave salamanders (Weber 2000), eye reduction causes the flattening of the anterior head skeleton as a by-product.

In contrast, the outer shape of the head in *Astyanax* cave fish has not been much altered in comparison with the surface fish. Studies in F2 crossings between *Astyanax* surface and the SEP Pachón cave fish revealed that in correlation with eye size there is only a small decrease in the vertical head height of the cave fish (Wilkens, unpublished). The neurocranium is unaltered (Dufton et al. 2012) and the wide space of the orbital cavities formerly occupied by the large eye balls in surface *Astyanax* is extant in the cave fish and instead completely filled with adipose tissue (Fig. 6.1). Only the jaws are slightly elongated (Atukorala et al. 2013; Yamamoto et al. 2009).

In surface *Astyanax*, the eye is surrounded by the supra- and a semicircle of six infra- or suborbital bones, which mechanically protect it (Schemmel 1967). Number, form, and position of the orbital bones differ slightly between *Astyanax* cave populations (Dufton et al. 2012; Gross et al. 2014; Yamamoto et al. 2003) and were

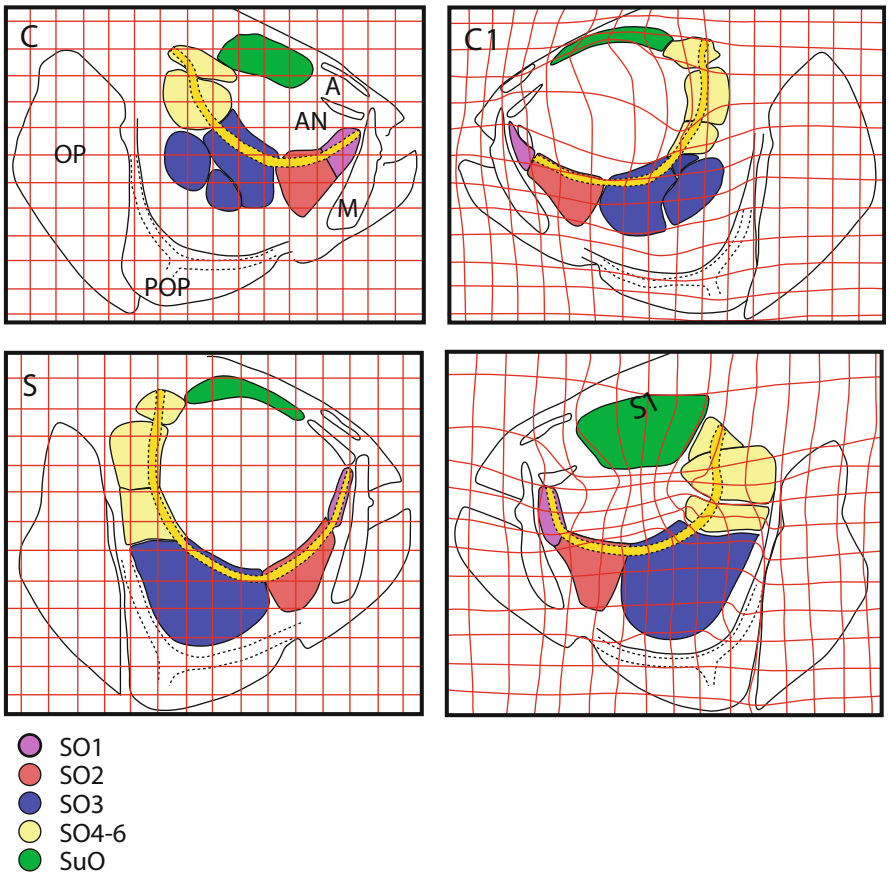

Fig. 6.51 Craniofacial skeletons and the effects of early lens transplantation (1 day post-fertilization): Pachón cave fish (*C*) and Pachón cave fish with transplanted surface lens (*C1*), surface fish (*S*) and surface fish with transplanted cave fish lens (*S1*), *SuO* supraorbital bone, *OP* opercular, *POP* preopercular, *M* maxillary bone, *A* nasal bone, *AN* antorbital bone, *SO1–SO6* sub- or infraorbital bones (adapted from Yamamoto et al. 2003)

used for taxonomic purpose (Fig. 6.51) (Alvarez 1946, 1947; Mitchell et al. 1977). These bones also enclose the sensorially important supra- and sub- or infraorbital lateral line canals. Lentectomy and grafting of the embryonic eye between surface and cave fish and vice versa revealed the extent to which craniofacial phenotypes were influenced by experimental removal of the eye or the lens. Certain traits were affected, including the distance between the nasal and antorbital bones, the inner sectors of the third suborbital bone (SO3), the supraorbital bone, and the position of SO3 relative to the orbit of the eye. However, other craniofacial traits were not affected by eye loss, such as number of suborbital bony elements, positioning of the suborbital bones SO4 to SO6 relative to the opercular bone, and opercular bone

shape (Yamamoto et al. 2003). Very often the infraorbital canal in the *Astyanax* cave fish is interrupted and shortened, because the infraorbital bones are fragmented and move in the direction of the orbit (Schemmel 1967). However, this fragmentation may be due to an artefact resulting from injuries, as was also erroneously observed for the lateral line (Wilkens 1977).

However, more severe defects in head formation like lack of the dorsal orbit and most of the ethmoid bone as observed in certain eyeless zebrafish mutants (Langenberg et al. 2008) were not developed in the experiments dealing with *Astyanax* cave fish. This may be due to the fact that an important player in craniofacial patterning was still functioning. The retina, which produces RA targeting RA receptors in the neural crest-derived periocular mesenchyme (Matt et al. 2008), is still present in the lens-ectomized surface specimens treated in these experiments and may prevent drastic cranial deformation (Wilkens 2010).

In nature, specimens exhibiting severe defects of the cranium, which, for example, might negatively impact feeding, have never been found in *Astyanax* cave fish because the specimens would have been inviable and would therefore have immediately been eliminated by selection. In contrast, minor defects observed in the *Astyanax* cave fish head skeleton, like fragmentation of the largest circumorbital bone (SO3) or partial fusion of the postorbital bones (SO4 to SO6), resulting from eye regression, do not disturb the biological function and would persist (Gross et al. 2014). Also, lateral asymmetries of the cave fish skull resulting from premature bony fusions are biologically irrelevant and therefore not eliminated.

6.21.8 Root Effect

The root effect is characteristic of ray-finned fishes. It is important for the supply of the retina with oxygen and for the inflation of gas into the swim bladder. Its main specificity relies on a pH-dependent reduction in hemoglobin-oxygen-carrying capacity. The underlying physiology of oxygen secretion involves an elaborate vascular arrangement, the rete mirabile, which builds the chorioidal gland in the eye. It is situated between the sclera and the retina and releases oxygen into the tissue. The choroid rete evolved only once, some 250 million years ago. In contrast, the rete mirabile of the swim bladder developed independently in different systematic groups and were secondarily lost again several times (Berenbrink et al. 2005; Rummer et al. 2013).

The chorioid in the eye is well developed in *Astyanax* surface fish but reduced in the cave fish (Figs. 6.39 and 6.41). The magnitude of the root effect has been shown to be lowered to the same degree in the VEP Micos and the SEP Pachón cave fish in comparison with the surface fish, although the size of the chorioid is variable in the Micos and much smaller in the Pachón cave fish. This indicates that the root effect magnitude inherits independently from chorioid size (Damsgaard 2016).

6.22 Brain

Studies of mammalian brains have revealed that the different regions develop independently from each other, which is in agreement with the "Mosaic Brain Evolution" hypothesis (Barton and Harvey 2000; Crish et al. 2006). Similar observations can be made when analyzing the specific brain parts of *Astyanax* cave fish, which also mirror sensory and behavioural adaptations to the environment differing from the surface fish. The study of 5-day-old larval and adult specimens revealed that all typical brain parts can be identified and that the cranial nerves are topologically conserved (Riedel 1997; Rodrigues 2013) (Fig. 6.52).

The most obvious finding is that the paired tecta optica along with the optic nerves are smaller in the VEP Micos and Chica as well as in the SEP Pachón cave fish in comparison with the surface fish (Figs. 6.53 and 6.54). Differences were also observed between surface and cave fish like the amblyopsids (Niemiller and Poulson 2010; Poulson 1963) or *Poecilia mexicana* (Eifert et al. 2014). In the SEP Pachón cave fish, the difference amounts were between about 50% and 65%, respectively, of the surface tectum opticum superficial area and volume (Peters

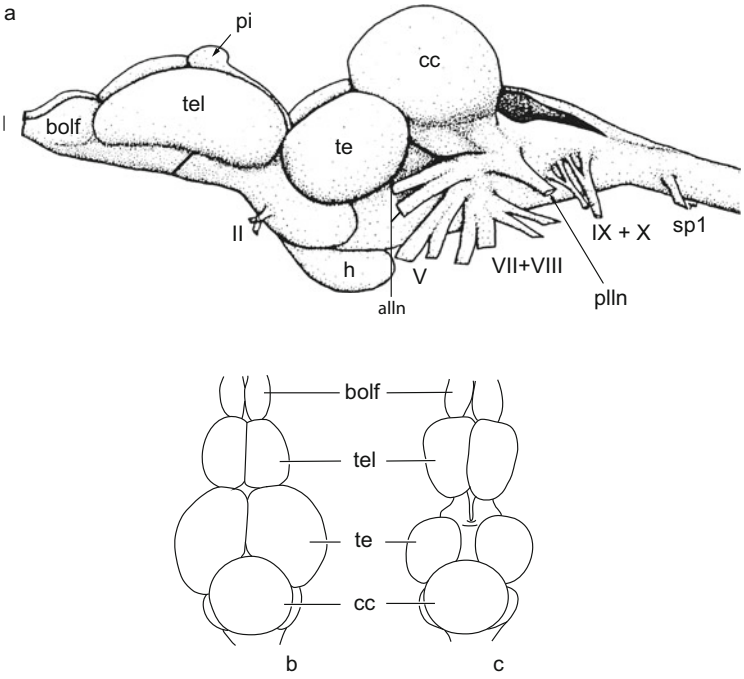

Fig. 6.52 Lateral and dorsal view of the brain of *Astyanax* cave (**a**, **c**) and surface fish (**b**). *alln* anterior lateral line nerve, *bolf* bulbus olfactorius, *cc* cerebellum, *h* hypothalamus, *pi* pineal organ, *sp1* first spinal nerve, *tel* telencephalon, *te* tectum opticum, *plln* posterior lateral line nerve; *I* Nervus olfactorius, *II* N. opticus, *V* N. trigeminus, *VII* N. facialis, *VIII* N. vestibocochlearis, *IX* N. glossopharyngeus, *X* N. vagus (adapted from Peters et al. 1993 and Riedel 1997)

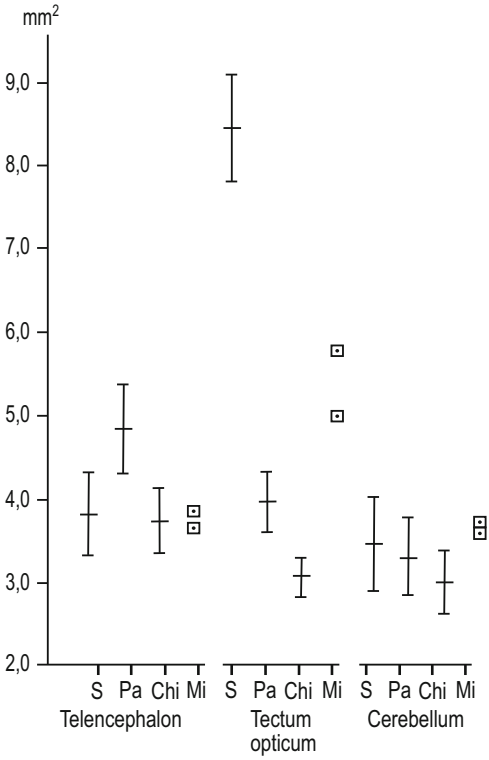

Fig. 6.53 Mean and range of dorsal area size of telencephalon, tectum opticum, and cerebellum in 15 specimens of each of the following: *Astyanax* surface (*S*), the SEP Pachón (*Pa*), as well as the VEP Chica (*Chi*) and two Micos cave fish (*Mi*). *SEP* strongly eye- and pigment-reduced, *VEP* variably eye- and pigment-reduced (adapted from Peters et al. 1993)

et al. 1993; Rodrigues 2013), whereas in correlation to the larger eye size in the VEP Micos and Chica cave fish, the reduction is less and depends on the eye size of the specific specimens studied (Fig. 6.53) (Peters et al. 1993; Moran et al. 2015). In the SEP Pachón cave fish, the lowered volume of the tectum is already evident as early as at 5 days post-fertilization (Rodrigues 2013). This can be explained by the fact that visual cells are never developed and therefore optic innervation of the tecta does not take place. Consequently, compared with the surface fish, the tecta optica of *Astyanax* cave fish are separated by broad gaps between each other as well as from the telencephalon from the beginning of ontogeny due to their smaller size.

The olfactory bulbs are involved in the reception of olfactory input, which is then relayed to the telencephalic lobes. No differences between cave and surface fish were found in the volume of the lobes or in the number of olfactory lamellae in the naris. Therefore, it is usually assumed that the olfactory sense is not particularly enhanced in the cave fish (Riedel 1997; Schemmel 1967). However, Rodrigues (2013) observed that in contrast to the decrease in volume of the whole brain, the olfactory bulbs remain unmodified in the Pachón cave compared with the surface fish. It was therefore suggested that the olfactory bulbs are more likely enhanced (Fig. 6.54). Also, in the amblyopsid cave fish (Niemiller and Poulson 2010) and in

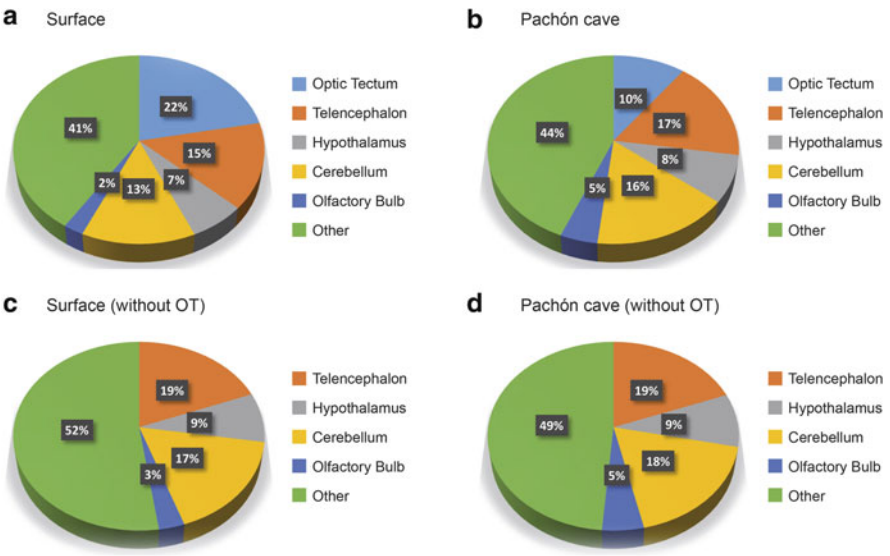

Fig. 6.54 Ratios of the volume of different brain parts in the *Astyanax* surface fish and the SEP Pachón cave fish including (**a** and **b**) and excluding (**c** and **d**) the optic tectum. No differences except for the olfactory bulbs exist. *SEP* strongly eye- and pigment-reduced (adapted from Rodrigues 2013)

cave populations of *Poecilia mexicana* (Eifert et al. 2014), an increase of the olfactory bulbs occurs.

However, in general when excluding the tectum from that of the whole brain in surface and Pachón cave fish by calculating the percentage volume size of the different brain regions, no remarkable differences were found (Fig. 6.54) (Rodrigues 2013). Nor did Rodrigues (2013) detect a proliferation of the hypothalamus as claimed by Rétaux et al. (2008).

Also, no increase in cerebellum size was found, although this could have been expected, given its role in spatial cognition and somatosensory input from the improved lateral line system in the cave fish (Fig. 6.52). Such increase occurs in the amblyopsid cave fish as well as in *Poecilia mexicana* cave forms (Eifert et al. 2014; Niemiller and Poulson 2010; Peters et al. 1993; Rodrigues 2013). However, the anterior lateral line nerves show an enhanced diameter in the Pachón cave fish, which correlates with the improvement of the lateral line sense by number and size of neuromasts in the head region (see Sect. 6.5). In contrast, no differences were found between the surface and Pachón cave fish posterior lateral line nerves, which is in accordance with the lateral line organ not being improved along the body.

When excluding the volume of the tectum opticum, larval as well as adult Pachón cave fish exhibit a brain volume that is approximately 20% smaller than in the surface fish. It was hypothesized that this may be related to energy consumption to save high metabolic costs caused by neural tissue and information (Rodrigues 2013). It was not considered, however, that this might result from

environmental modification. For example, for wild and common garden-reared surface and cave populations of *Poecilia mexicana*, a high amount of phenotypic plasticity of brain size was revealed (Eifert et al. 2014). This can not be excluded for *Astyanax* cave fish either.

6.23 Comparison of the Genetics of Complex Regressive and Constructive Traits

6.23.1 Phenotypic Manifestation and Gene Expression

The morphological and histological study of complex traits of surface and cave forms has revealed that the difference between them has quantitative character. This was shown for constructive traits like number of taste organs (Schemmel 1974a, b), feeding posture (Schemmel 1980; Kowalko et al. 2013b), and neuromasts (Wilkens, unpublished) as well as sleep duration (Duboué et al. 2011), amount of yolk content (Hüppop and Wilkens 1991), or improved ability to store fat (Hüppop 1989) and in regressive traits like eye size, melanophore number (Wilkens 1970a, b, 1988, 2010), degree of dark preference, schooling (Kowalko et al. 2013a), inclination of dorsal light reaction (Langecker 1993), sex determination, or scleral ossicles (O'Quin et al. 2015). In detail, in fish, eye regression starts with a slight decrease of total size including that of all the single structures like lens, vitreous body, or retina. Destructive histological processes can only be observed after the overall size has been considerably diminished. Loss of single structures like lens or visual outer segments only occurs in very small eyes at the end of the process of reduction (Figs. 3.17 and 6.38). A similar process was shown to take place in the decapod compound eye. At the beginning of reduction just the number of ommatidia is lower, although no structural defects are manifested. In close correlation to this, the optic ganglia or optic neuropiles (Elofsson and Dahl 1970) in the eye stalk become smaller, too. The end of this process is reached when only single or no ommatidia at all are left and also the optic ganglia are lost (Fig. 3.12).

All of these morphological, behavioural, or physiological traits differing between cave and surface fish studied in classical crossing experiments and by QTL analyses to date exhibit polygenic inheritance (Kowalko et al. 2013a, b; Protas et al. 2007; Wilkens 1988). In the following examples, the genetic basis of two regressive and three constructive traits are presented in detail.

6.23.1.1 Eye

It was found that the eye size in the F2 and backcross hybrids lies between those of the respective parental forms (Fig. 6.55a). The mean of the F1 progeny is about intermediate and only slightly shifted towards the surface fish. Its distribution exhibits a much lower range than the F2 and the backcrosses. Whereas all crossings exhibit Gaussian distribution, it is bimodal in the backcross to the surface parent (Wilkens 1970a, 1988). Based on classical crossing experiments, at least eight loci, so-called "eye genes", were calculated to be responsible for eye size (Lande 1981;

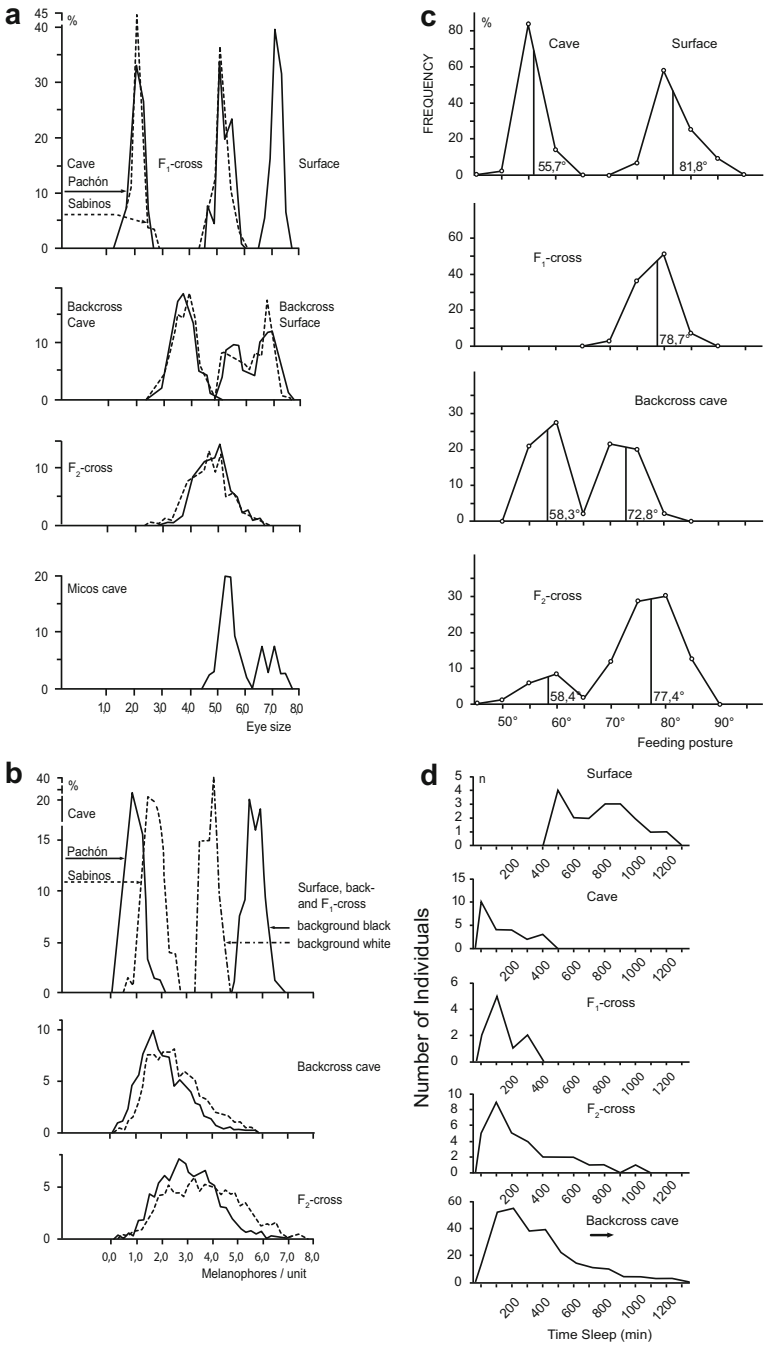

Fig. 6.55 Frequency distributions of regressive and constructive traits in the crossings between *Astyanax* surface and cave fish. (**a**) Eye size, (**b**) melanophore number (crossings of the Sabinos and the Pachón cave fish in *dotted and continuous lines*, respectively), (**c**) feeding posture, (**d**) sleep duration, (**e**) neuromast number, (**f**) taste organ number (adapted from Wilkens 1970a, b; Schemmel 1974a, b; Duboué et al. 2011)

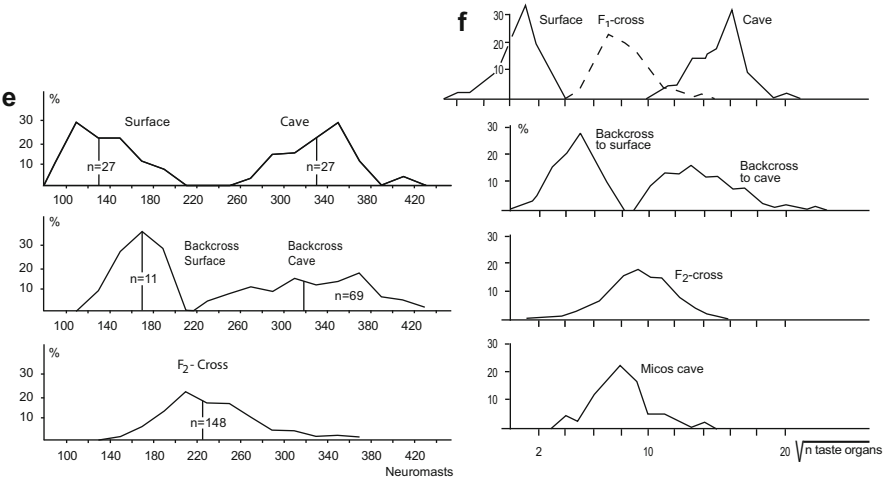

Fig. 6.55 (continued)

Wilkens 1970a). In QTL studies it was found that 14 in total were affecting eye and lens size (Protas et al. 2007).

6.23.1.2 Melanophore Number

Besides the amount of melanin within the melanophores ("brown gene", see Sect. 6.20.2), the colouration of *Astyanax* surface and cave fish is also influenced by melanophore number. Mean and range do not deviate from the surface fish in the F1 and the backcross to the surface fish (Fig. 6.55b). In contrast, however, the number of melanophores developed in the F2 progeny ranges between that in the cave fish and surface fish, which are kept under a black background. Whereas across F2 progeny it is normally distributed, the progeny of the backcross to the cave fish shows positive skewness with a tail extending as far as the surface fish (Wilkens 1970b). The difference in number of melanophores found between the parental SEP Sabinos and Pachón cave fish is manifested in the F2 and the backcross to the respective cave fish population, too (Wilkens 1970b). Because of their ability to perform the morphological colour change, the surface fish only develop half the number of melanophores under a white background than under a black background (see Sect. 6.20.1). This ability was found to manifest in the F1 progeny between surface and cave fish and in the backcross to surface fish as well. In contrast, the F2 and the backcross to the cave fish do not exhibit morphological colour change, but exhibit more or less the same distributions of melanophores over a dark background as they do over a white background (Wilkens 1970b, 1988). In classical crossing experiments, at least one or two loci ("melanophore genes") were calculated to be responsible for the difference between the number of melanophores in the SEP Sabinos and Pachón cave populations, respectively. QTL mapping revealed at least 18 affecting pigmentation (Protas et al. 2007).

6.23.1.3 Feeding Posture

The SEP cave fish have improved food search ability by modifying the feeding posture exhibited while taking up food from the ground (see Sect. 6.8). The classical crossing analysis revealed that the F1 progeny between surface and different phylogenetically old SEP cave fish populations exhibit similar postures to the surface fish phenotypes. The F2 progeny ranges between surface and cave fish, but the distribution is centred near the mean angle observed in the surface fish with the tails of the distribution overlapping the cave fish phenotype (Pachón and Tinaja, Kowalko et al. 2013b) or shows bimodality (Pachón and Sabinos, Schemmel 1980) with a second smaller peak weighted toward the cave fish (Fig. 6.55c). The progeny from the backcross to the cave fish exhibits bimodality with two peaks, one approximately corresponding with the cave fish and the other one partially overlapping the F1 cross. The backcross to the surface fish was not performed but is assumed to resemble the F1 cross.

Based on classical analysis, at least three loci were calculated as being involved in this difference ("feeding posture genes") (Schemmel 1980). QTL analysis confirms that the evolution of feeding posture is controlled by multiple genetic loci (Kowalko et al. 2013b). Some of them may not be shared between different cave fish populations as, for example, was revealed for the SEP Pachón and Tinaja cave fish. Consistent with these data, hybrid individuals in an F1 cross between Pachón and Tinaja cave fish have an intermediate phenotype, significantly different from the Pachón parental population. Thus, it appears that distinct QTL-representing loci are regulating feeding posture in the Pachón and Tinaja cave fish populations (Kowalko et al. 2013b). This difference was not found by classical crossing analysis between the Pachón and the Sabinos population cave fish (Schemmel 1980).

6.23.1.4 Number of Free Neuromasts

For orientation and improved food finding in darkness, the number of free neuromasts is enhanced in the cave fish (Teyke 1990; Yoshizawa et al. 2012; unpublished own results). The means of the F1 and the F2 cross between Pachón cave and the surface fish are intermediate. In the F2 the distribution ranges between the cave and the surface fish with a tail extending towards the cave fish (Fig. 6.55e) (Kowalko et al. 2013b; Wilkens 2016). The mean number of neuromasts in the backcross to the surface fish is only slightly lower than in the surface fish itself, because the distribution is weighted in those surface fish exhibiting a higher number of neuromasts. The neuromasts in the backcross to the cave fish exhibit negative skewness and show a distribution reaching toward the cave fish.

6.23.1.5 Number of Taste Organs

The number of taste sense organs developed below the ventral jaw is increased in the SEP cave fish compared with the surface fish (Fig. 6.55f) and was found to cover a larger area containing more organs in the Pachón than in the Sabinos cave fish. However, this difference between the two cave fish populations is not manifested in their crossings (Schemmel 1974a). In the SEP Sabinos cave fish the means of the

backcrossing to the cave fish and of the F2 crossing between surface and SEP cave fish are shifted towards the cave fish. The numbers of taste organs exhibit Gaussian distributions ranging between the means of the respective parental forms. However, the backcross to the cave fish shows a broader distribution overlapping almost entirely with the cave fish. Three loci were calculated by classical crossing analysis and by QTL studies to be responsible for the enhancement of the taste organs ("taste bud genes") (Protas et al. 2007). Their number in the phylogenetically young VEP Micos cave fish ranges between the means of the surface and the Sabinos cave fish with a tail extending to the latter (Schemmel 1974b).

6.23.1.6 Sleep Duration

Change in ecological conditions, from surface to cave, was found to be correlated with a change in sleep duration and activity, which was revealed by studies of three independently derived *Astyanax* cave populations, the SEP Tinaja, Pachón, and the VEP Molino cave fish (see Sect. 6.10, Duboué et al. 2011). Classical crossings were performed between surface and Pachón cave fish. It was found that the range of the F1 crossing is congruent to the cave fish. In the F2 and the backcrossing to the cave fish, a peak is weighted in the cave fish with a tail overlapping the surface fish (Fig. 6.55d). QTL analysis showed that multiple loci ("sleep genes") are involved in the difference of sleep duration.

6.23.2 Comparison of Phenotypic Manifestation

Classical crossing experiments revealed that the complex traits studied in *Astyanax* surface and cave populations rely on polygenic systems. This was supported by QTL analyses, in which the number of genes was usually higher (e.g. 8 vs 14 for eye plus lens size, 4 vs 18 affecting dark pigmentation) (Protas et al. 2007). This can probably be attributed to the fact that groups of genes involved may be located on one chromosome, which cannot be differentiated by classical crossings.

The classical crossings of *Astyanax* do not exhibit fundamental differences in the phenotypic manifestation between constructive and regressive traits, but are submitted to principally comparable patterns of distribution and phenotypic manifestation. They exhibit an exponentially rising epistatic gene effect, the amount of which differs in the various traits. The contribution of gene effect may continuously increase in an "asymmetrical" manner towards the specific larger parental trait of the surface (e.g. eye size or melanophore number) or the cave fish (e.g. taste bud or neuromast numbers). When just skewness of distribution curves is observed, epistatic gene effect size is only slightly enhanced, building a distributional tail (e.g. Fig. 6.55b, backcross to cave; Fig. 6.55d, F2 and backcrossing to cave). As a result, the range is considerably enhanced in these crossings. In contrast, bimodality is caused when, by an abrupt "threshold-like" increase of effect, a second additional distribution peak appears, which converges with the distribution of the larger traits (e.g. Fig. 6.55a, backcross to surface and Micos cave fish; Fig. 6.55c, backcross to cave and F2 crossing). Finally, complete "dominance" due to large epistatic gene

effect is observed. In this third case, the critical epistatic "genic threshold" of gene effect is so large that the intermediate traits do not build one of the two peaks found in the stage presented before (e.g. Fig. 6.55b, F1 and backcrossing to surface; Fig. 6.55c, F1 crossing; Fig. 6.55d, F1 crossing). By these epistatic gene effects the means of most traits are shifted towards the larger trait. The "threshold" might imply that a monogenic mode of inheritance would be responsible for this. This was rejected by crossings, however, as shown for feeding posture (Schemmel 1980).

However, in some traits the pattern of exponential asymmetrical increase of gene effect unexpectedly does not manifest in the F2 crosses between surface and cave fish. For example, trait sizes do not develop bimodality for eye size as expected with respect to the distribution curve in the backcross to the surface fish (Fig. 6.55a). Neither can this be observed in the F2 crossing for melanophores, although the F1 cross converges with that of the surface fish (Fig. 6.55b). For both traits, nearly Gaussian distribution is exhibited in these F2 crosses. The latter cannot be explained by the loss of gene balance as might be the expected result from the recombination of two genetically separated populations, because, for example, other traits like feeding posture exhibiting bimodality (Fig. 6.55c) or sleep durance skewness (Fig. 6.55d) develop enhanced gene effect in the respective F2 crosses. I suggest that the manifestation of epistatic gene effect in these F2 crosses is inhibited, because both eye size and melanophore number are based on two independent subunits. These are in the eye lens and retina subunits (see Sect. 6.21.3.1) and in pigmentation melanophore number and the ability of morphological colour change (Sect. 6.23.1.2). The alleles of the different genes responsible for the different subunits are recombined at random (Wilkens 2010). In addition to eye and melanophores, Gaussian distribution is also found for taste organs in the F2 crossing between SEP cave and surface fish. It is suggested that this might also be caused by the involvement of two independently inheriting traits, namely taste buds and solitary chemosensory cells (SCCs), which were incorrectly counted as the same organ by Schemmel (1967) (Fig. 6.55f).

In case of the eye, a series of findings have corroborated the existence of two developmental units. For example, histological analyses revealed that two units built by the dioptric lens apparatus and the sensory retina apparatus form the eye (see Sect. 6.21.3.3, Wilkens 2010). Further support is provided by the lens size of the F2 progeny still exhibiting bimodality, when measured separately, although the eye ball size shows Gaussian distribution (Wilkens 1970a). As concerns the retina, it is difficult to detect and quantify such deviating phenotypic manifestations. However, studies of the surface fish, the SEP Pachón, and the VEP Micos cave fish revealed that brain and eye mass are closely correlated in these forms (see Sect. 6.22) (Moran et al. 2015; Peters et al. 1993; Pfeiffer 1967). In contrast, the brain mass is largely invariant despite a ten-fold difference in eye mass in the F2 crossing between surface and Pachón cave fish, and no clear correlation of external eye size and brain tectum size as expected from the findings in pure cave fish was detected (Moran et al. 2015). As tectum opticum mass directly depends on the amount of retinal nerve cells, this result indicates that like the lens, the retina also shows a more independent manner of manifestation from that of the eye ball in the variable

F2 crossings. Furthermore, the VEP Molino cave fish, which never develops a differentiated lens, but nonetheless completely independently develops complete visual cells containing outer segments at early ontogeny, substantiates this (Wilkens 2007). The existence of two independent subunits within the eye is also supported by the so-called "dual signal model" (Strickler et al. 2007) for retinal growth and development, which suggests that the lens acts in concert with another unknown component and it is hypothesized that this would be the pigment epithelium, which is part of the second retinal, but not of the lens subunit.

Two separate units are also responsible for the melanophore system. In addition to the formation of melanophores, the number developed on the body surface is influenced by a second independent unit, the morphological colour change over different coloured backgrounds. As mentioned before, this ability to change colour is lost in the SEP cave fish (see Sect. 6.20.1). Both the formation of the melanophores and the morphological colour change traits are influenced by a large number of genes (Protas et al. 2007; Wilkens 1970b, 1988). Therefore, due to their independent recombination and assortment, the epistatic effects, which are responsible for the functioning of the morphological colour change, also do not manifest.

In addition, the Gaussian distribution of the number of taste organs found by Schemmel (1974a, b) in the F2 crossing between SEP cave and surface fish is probably caused by the disturbed manifestation of epistatic gene effect. This may well be explained by the involvement of two units. Schemmel (1974a) did not differentiate between real taste buds and solitary chemosensory cells (SCCs), the existence of which was unknown at that time (see Sect. 6.6) (Yamamoto et al. 2009, own observations). I argue therefore that the distribution curve originally found in the F2 crossings is based on counting both types of organs. The bimodal distributions assumed to exist for both units are supposed to be blurred because of the independent inheritance.

6.23.3 Genetics of Phylogenetically Young VEP Cave Fish

The most obvious feature of the VEP populations Micos, Chica, and Caballo Moro is that their externally visible eyes exhibit variable size and are less reduced than those of the SEP cave fish. As mentioned above, these cave fish populations have long been thought to have a more recent origin or are just hybrids between the surface and SEP cave fish (Bradic et al. 2012; Mitchell et al. 1977; Strecker et al. 2012; Wilkens 1988; Wilkens and Burns 1972).

Classical genetic analyses of the VEP Micos cave fish revealed that the inheritance of regressive and constructive traits in this fish itself, as well as in its crossings with the SEP cave fish and the surface fish, is in principle subjected to the same rules of manifestation, as they were analyzed by crossing SEP cave fish and the surface fish (Wilkens 1976).

The melanophore number of the Micos cave fish kept at daylight does not differ from that of the surface fish and the morphological colour change is also still functioning. Consequently, none of the crosses of the Micos cave fish with the

surface fish (Fig. 6.35, F1 (Micos × Surface), Back (Micos × Surface) × Micos, Back (Micos × Surface) × Surface) diverge from the parental surface fish and Micos cave populations. They can perform the morphological colour change and show equivalent melanophore densities. The same is valid for the F2 crossing between Micos and surface fish (Fig. 6.35, F2 Micos × Surface).

In contrast, however, to the F1 cross between the SEP cave fish (Sabinos and Pachón) and the surface fish (Fig. 6.55b), the F1 cross between the Sabinos and Pachón cave fish and the Micos cave fish does not exhibit the same number of melanophores as the surface fish, but only shows an intermediate number. Also, the melanophore number in the backcross of the F1 (Micos × Cave) to the Micos fish is more or less intermediate (Fig. 6.35, Back (Micos × Cave) × Micos). In contrast, the backcross of the F1 (Micos × Cave) to the SEP cave fish (Fig. 6.35, Back (Micos × Cave) × Cave) lies in between the F1 cross and the SEP cave fish. As can be derived from the slight difference of means, only a few of the F1 and backcross hybrids are able to perform the morphological colour change over white and black backgrounds. The melanophore number of the F2 crossing between Micos and SEP cave fish ranges between both (Fig. 6.35 = F2 Micos × Cave). The small difference of the means over black or white backgrounds in the before-mentioned crossings indicates that the morphological colour change only functions in a few of the hybrids. Although the Micos cave fish is still able to perform a morphological colour change, the previously described failures of its manifestations in the different crossings with SEP cave fish can be explained by the genetic basis of the morphological colour change in the Micos cave fish already carrying regressive mutations. Whereas the high exponential increase of gene effect necessary to develop the full number of melanophores is still manifested in the Micos cave fish, the number of non-mutated genes does not suffice to develop this epistatic increase to perform the morphological colour change in the above crossings with the SEP cave fish.

The eye size of the laboratory-bred Micos cave fish is distributed between the surface fish eye and half its size. However, in Micos cave fish "surface eyes" can only be achieved by selectively crossing specimens equipped with comparatively larger eyes (Wilkens 1976), which are rare under natural conditions (Fig. 5.7). Most remarkably, the distribution of eye size is bimodal (Figs. 5.5 and 6.55a). This indicates that in contrast to the F2 crossing between surface and SEP cave fish, enhanced epistatic gene effect can manifest in the Micos cave fish. Such enhanced epistatic gene effect is also exhibited in the distribution curve of the Micos cave fish taste organs, which exhibits skewness. Its peak correlates with that of the F1 cross between surface and SEP cave fish, but a skew distributional tail extends to the latter (Schemmel 1974b) (Fig. 6.55f). In addition, the variability of pupillary opening and lens size (which are correlated) of the Micos cave fish is lower than in its F2 crossings with the surface fish or with the SEP cave fish. It is also lower than in the F2 crossings between the surface and the SEP cave fish (Fig. 6.40).

The results of classical crossing experiments performed with the VEP Micos cave fish show that the basal principles of manifestation of regressive as well as constructive traits are the same as in the crossings between phylogenetically old

SEP cave and the surface fish. There are some differences, though, corroborating that this population is no hybrid but phylogenetically young. This is above all the undisturbed manifestation of the discontinuous increase of epistatic gene effect in eye size and melanophores as well as the low variability of the pupil size.

6.23.4 Significance of Unproportional Epistatic Gene Effect

The existence of VEP cave fish populations like Micos exhibiting intermediate stages of regressive traits (eyes, melanophores, dorsal light reaction, or aggressive behaviour) and constructive traits (taste organs, free neuromasts, feeding posture, egg yolk content, naris size, or fat storage ability) comparable to those of the SEP cave fish provides an exceptional tool to evaluate whether the genetic findings revealed by classical crossing experiments between surface and SEP cave fish can also be observed and above all are relevant during the natural evolutionary process (Aspiras et al. 2015; Bibliowicz et al. 2013; Hüppop and Wilkens 1991; Wilkens 1988, 2010). This could be confirmed in the Micos cave fish, in which classical genetic analyses showed that the inheritance of regressive and constructive traits is subjected to the same rules as were revealed by the crossing analyses between the surface and the phylogenetically old SEP cave fish (Wilkens 1976, 1988, 2010).

For example, the bimodal distribution of eye size observed in the VEP Micos cave fish (Fig. 6.55a) derives from a threshold-like increase of epistatic gene effect. The recombination of up to a certain number of eye genes all exhibit the same amount of small gene effect. However, after the contribution of additional eye genes, a second peak is formed because epistatic gene effect increases manifesting a threshold. This may be achieved by breeding large-eyed specimens in the laboratory. It may also happen in nature as long as there is a sufficient number of alleles present in the gene pool. The probability of such recombination depends on the number of unmutated genes left. If it is low, large eyes will only rarely appear.

In general, a quantitative trait locus may exhibit dominance, additive, non-additive, or recessive gene effect (Shao et al. 2008). The crossings between the *Astyanax* surface and cave populations have revealed that the genes responsible for the phenotypic manifestation of regressive and constructive traits in principle act in a quantitative manner. The phenotypic manifestation comes about by the additive effect of single genes. The extent of each gene effect is regulated by interaction of the whole genetic system, whereby one gene interferes with the phenotypic expression of one or more non-allelic ones. The amount of increase may be the same or be unproportional and in the extreme show discontinuous threshold-like increases. However, it is the phenotypically manifested trait that is submitted to selection and therefore the underlying epistatic effects of each single gene of the gene system as a whole are shaped by selection.

Therefore, the suggestion of a potential significance of the differing epistatic gene effects and in particular of the threshold-like one arises. In some traits, a biological function was detected. For example, the phenotypic manifestation of the number of melanophores is reversible after the total number of melanophore genes

is recombined. As a result, the adaptive morphological colour change can be performed. The threshold-like epistatic gene effect obviously also plays a role in monogenic sex determination, which is acquired by the cave fish. They have switched from the additive polygenic sex determination occurring in the surface ancestor to a quasi-monogenic one in the cave fish. In *Astyanax* cave fish, monogenic sex determination results from a threshold-like increase of epistatic gene action, by which an autosomal gonosome originates (see Sect. 6.3). However, for most other traits the threshold-like epistatic gene effect is suggested to play a role in constructive evolution. With the discontinuous quantitative increase of traits by number, size, or intensity in the case of behaviour, selection is enabled to promote a phenotype more rapidly the larger the constructive phenotypical step achieved is. This is advantageous because small quantitative changes of constructive traits, as provided by small gene effect, are overruled by modificatory influence. Thus, unproportional increase of epistatic gene effect may be an important means of constructive evolution.

This finding may furthermore explain why the evolution of regressive traits in *Astyanax* cave fish is often characterized as proceeding rapidly. For example, due to the "threshold-like" decrease of gene effect at the beginning of eye reduction, its size abruptly gets smaller by about 25% of the surface fish eye. In contrast, the subsequent phenotypic regressive process of eye reduction ending in complete regression proceeds much more slowly, because gene effects are smaller.

6.23.5 Nature of Genes Responsible for Complex Traits

The analyses of crossing experiments suggest that the genetic expression and phenotypic manifestation of regressive traits such as the reduced eye or melanophore numbers, and of constructive traits such as feeding posture or neuromast numbers, as well as all other complex traits analyzed, are polygenic and show similar principles of manifestation. Therefore, it is suggested that the above-described findings provide insight into the evolution of complex regressive and constructive traits in general.

Because of their quantitative nature of expression, the "eye genes" or "melanophore genes" found by classical crossing experiments in regressive traits as well as "neuromast genes" or "feeding posture genes" found in constructive ones are suggested to be regulatory genes. Apart and independently from the structural genes which are responsible for each specific trait, they strongly determine the final size of a trait already at early ontogeny (Wilkens 2010). This assumption gets support from findings in eye regression. They show that in the *Astyanax* cave fish eye regression is due to expanded *hedgehog* (*hh*) gene expression along the anterior midline (Yamamoto and Jeffery 2000; Yamamoto et al. 2004, 2009). It suggests that already the very first anlage of the eye is smaller. Mapping of candidate genes *shh*, *twhh*, and *Pax6* revealed that no eye QTLs are located near these loci. This result makes it unlikely that mutations in any of these genes are directly responsible for eye regression (Protas et al. 2007). However, the question of what causes

downregulation of s*hh* genes and consequently determines the initial eye size remains unsolved. It is suggested that as-yet unidentified genes are regulating *shh* expression (Wilkens 2010; Yamamoto et al. 2009), which would be equivalent to those genes called "eye genes" found by crossing experiments and which are hypothesized to be responsible for eye size (Wilkens 2010). These genes would also determine the variability of the embryonic eye sizes characteristically found in the diverse crossings already at early ontogeny, because they regulate *shh* expression to different extents.

In this context, the progressive reduction of limbs from basal snake species only carrying rudimentary ones to advanced snakes in which all skeletal structures have disappeared provides an example (Kvon et al. 2016). During limb development a ZRS enhancer (Zone of Polarizing Activity Regulatory Sequence) is active in the limb bud mesenchyme where it is critically required for normal limb development in vertebrates. The ZRS is a limb-specific enhancer of the *sonic hedgehog* (*shh*) gene that is located at the extreme distance of nearly one million base pairs from its target promoter. It is highly conserved in basal snakes, whereas it underwent a rapid increase in substitution rate in advanced ones. The identification of snake-specific sequence changes within the otherwise highly conserved long range limb enhancer of *sonic hedgehog* provides a possible explanation of the complex pattern of how *sonic hedgehog* may be regulated and embryonic eye size is determined. It may also exemplify how all the other constructive and regressive traits evolve in the *Astyanax* cave fish.

It was therefore proposed that the genes responsible for eye development are divided into two groups: the first group expresses around the s*hh* genes during the first step of eye regression and determine the size of the primordial eye cup through regulation of *shh* expression (Fig. 6.50) (Wilkens 2010). The second group of eye genes is suggested to regulate structural genes responsible for the lens and the retina subunits, respectively (see Sect. 6.21.4, 6.21.5).

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