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Gonad histology and vitellogenin concentrations in brown trout (*Salmo trutta*) from Danish streams impacted by sewage effluent

Lisette B. Bjerregaard · Allan H. Madsen · Bodil Korsgaard · Poul Bjerregaard

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Abstract Brown trout (*Salmo trutta*) collected from a number of Danish streams impacted by sewage effluent were examined for alterations to gonadal development and induction of vitellogenin synthesis. Among fish collected in June/July 2000/2001 and November 2002 higher levels of plasma vitellogenin were found in males from six streams impacted by sewage effluent compared to males from their respective reference sites. A direct non-competitive ELISA was developed for brown trout vitellogenin in order to perform the vitellogenin measurements. Intersex in females with no apparent relation to sewage effluent exposure was observed at all sites. In one stream, male brown trout with a very high level of vitellogenin were concomitantly found to have a high degree of vacuolation of the testes and a presence of only the early spermatogenic stage, spermatogonia. The cause of these alterations to the testis structure is unclear. However, as a high level of plasma vitellogenin in these males indicated estrogenic exposure, the vacuolation might also be a result of endocrine disruption causing delayed or disrupted spermatogenesis.

Key words Brown trout · ELISA · Vitellogenin · Testes · Intersex · Gonad histology · Sewage effluent · Estrogens · Vacuolation

L. B. Bjerregaard · A. H. Madsen · B. Korsgaard · P. Bjerregaard
Institute of Biology, University of Southern Denmark, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

L. B. Bjerregaard (✉)
Department of Environment, Aarhus County, Lyseng Allé 1, DK-8270 Højbjerg, Denmark
e-mail: lbc@biology.sdu.dk
Tel.: +45-6550-2770
Fax: +45-6593-0457

Introduction

Endocrine disruption in wild populations of fish has become an issue of growing concern concurrently with an increasing number of observations of reproductive abnormalities of fish in a number of countries across Europe (Jobling et al. 1998; Flammarion et al. 2000; van Aerle et al. 2001; Gercken and Sordyl 2002; Hecker et al. 2002; Solé et al. 2002a, b; Vethaak et al. 2002) , in USA (Folmar et al. 1996, 2001; Harshbarger et al. 2000), Canada (Aravindakshan et al. 2004) and Japan (Hashimoto et al. 2000; Ohkubo et al. 2003) during the past years. The reproductive abnormalities which primarily have been of feminising nature are suspected to be a result of endocrine disruption due to natural and synthetic estrogens and/or estrogen mimicking chemicals which reach the aquatic system via outlets of domestic and industrial sewage effluent. Ethinylestradiol, 17β-estradiol and estrone are mostly believed to be responsible for the observed feminisations and have been detected in a number of sewage effluents (Larsson et al. 1999; Hemming et al. 2001; Sheahan et al. 2002). In single cases alkylphenols compounds have also been suggested as possible causative agents (Sheahan et al. 2002). Induction of vitellogenin (vtg), a biomarker of estrogenic exposure, has been detected in various species of male fish collected downstream of sewage treatment works (STWs) (Jobling et al. 1998; Flammarion et al. 2000; van Aerle et al. 2001; Gercken and Sordyl 2002; Hecker et al. 2002; Folmar et al. 2001; Harshbarger et al. 2000; Minier et al. 2000) and in caged fish placed near STW outlets (Harries et al. 1997, 2000; Larsson et al. 1999). After high prevalences of intersex were first observed in roach (*Rutilus rutilus*) from UK rivers (Jobling et al. 1998), other populations of fish with intersex males have been observed downstream of STWs (van Aerle et al. 2001; Aravindakshan

et al. 2004). Intersex males have oocytes in the testes, and in male roach the ovarian cavity has also been seen. Reproductive disorders among wild populations of fish also include abnormal hormone levels (Folmar et al. 1996, 2002; Jobling et al. 2002a; Lavado et al. 2004), asynchrony in the development of germ cells in males and females as well as reduced spawning and fertilising capacity of male fish (Aravindakshan et al. 2004; Jobling et al. 2002a, b). Observed feminisation in wild populations of fish does now include the freshwater species roach (Jobling et al. 1998: Minier et al. 2000), gudgeon (*Gobio gobio*) (van Aerle et al. 2001), carp (*Cyprinus carpio*) (Folmar 1996; Solé et al. 2002a, b), bream (*Abramis brama* L.) (Hecker et al. 2002; Vethaak et al. 2002), chub (*Leucicus cephalus*) (Flammarion et al. 2000), shovelnose sturgeon (*Scaphirhychus platyorynchus*) (Harshbarger et al. 2000), walleye (*Stizostedion vitreum*) (Folmar et al. 2001), three spined stickleback (*Gasterosteus aculeatus*) and perch (*Perca fluviatilis*) (Gercken and Sordyl 2002) plus the marine species flounder (*Platichthys flesus*) (Lye et al. 1997, 1998; Allen et al. 1999; Stentiford et al. 2003), eelpout (*Zoarces viviparus*) (Mathiessen et al. 2002; Stentiford et al. 2003) and two species of sand gobies (*Pomatoschistus minutes* and *P. lozanoi*) (Mathiessen et al. 2002). Many of the freshwater species which have been examined for signs of feminisation belong to the carp family. The objective of the present study was to examine brown trout (*Salmo trutta*) from various Danish streams impacted by sewage effluent for reproductive abnormalities to see if endocrine disruption also exists among wild populations of a salmonid species. Brown trout is a territorial fish and therefore relatively stationary. Further, brown trout live in streams with higher water flow and less pollution compared to typical habitats for roach and many other cyprinid fish, and therefore a study of a salmonid species provides the opportunity to study a different habitat. A direct, non-competitive sandwich Enzyme Linked Immunosorbent Assay (ELISA) was developed in order to measure vtg in male brown trout plasma.

Materials and methods

Fish sampling

Brown trout (*Salmo trutta*) (n=683) were collected from eight Danish streams in June/July 2000 (plus a single collection in August) by electro fishing. Three streams receiving no or only small amounts of sewage effluent from scattered houses were chosen as reference sites. The remaining five streams received varying degrees of sewage effluent from STWs. From one of the streams impacted by sewage effluent (Voel Brook), fish collection was repeated in July 2001 (n=68). A second round of fishing was performed in November 2002 in which brown trout were collected from five new sites including one reference stream and four streams impacted by sewage effluent (n=287). Characteristics of the streams from which brown trout were collected are shown in Table 1. It should be noticed that water flow and therefore the load of sewage effluent changes thoughout the year and that a more massive pollution of streams exists in periods with low water flow. The fish were transported alive in an aerated tank to the University of Southern Denmark, Odense where sampling took place. At sampling the fish were stunned by a blow to the head. Body weight and total length were determined and a blood sample was taken from the caudal blood vessel. The blood was transferred to eppendorf tubes containing heparin and centrifuged for 4 min at 17,000×g and 4°C. Plasma was stored at −80°C until measurements of vtg were performed by ELISA. The fish were killed by decapitation and the gonads were dissected out, weighed and fixed in Bouin's fixative for approximately 24 h. The gonads were then transferred to 70% ethanol until preparation of histological sections.

Gonad histology

The gonads were dehydrated through series of graded ethanol (50–99.9%) and xylene, and embedded in paraffin. Three pieces of approximately 5 mm were embedded from each testis—one from the anterior, middle and posterior part—while the ovaries were cut in two pieces due to their relatively shorter length. Transverse sections of 5 μm were subsequently cut on a microtome and stained with hematoxylin and eosin (H&E). In total six sections of the testes or four sections of the ovaries were evaluated from each fish. The sections were examined for the presence of primary oocytes and ovarian cavity in the testes and other abnormalities by light microscopy.

ELISA

The ELISA for brown trout vtg was developed as a direct sandwich ELISA in accordance with an ELISA for rainbow trout vtg and zebrafish vitellin described in Christiansen et al. (2000) and Holbech et al. (2001). Only few modifications were made, primarily in the preparation of vtg and in the ELISA validation.

Preparation of vitellogenin

Brown trout (*Salmo trutta*) collected by electro fishing were injected with 5 mg/kg 17-β estradiol dissolved in

Table 1 Type of sewage treatment and degree of sewage load at the sites from which brown trout (*Salmo trutta*) were collected in June/July 2000/July 2001 and November 2002

Sampling time and site	Type of sewage treatment	PE	Percentage of sewage (%)		Adjusted PE		Waterflow (l/s)	
			Median minimum	Average	Median minimum	Average	Median minimum	Average
June–August 2000/2001								
Reference sites								
Granslev Brook	–	–	–	–	–	–	250	405
Assendrup/Sander Brook	–	–	–	–	–	–	12/6	60/50
Maren Mølle Brook	–	–	–	–	–	–	42	55
Streams impacted by sewage effluent								
Giber Brook	MBNDCF	5264	62/35*	11/2.4	3254/1845	592/128	30/10	652/128
Knubbro Brook	MBNDC	3345	40	16	1334	537	22	76
Voel Brook	MBNC	1040	8	5	81	47	35	62
Hoed Brook	MBN	636	8	2	54	15	35	135
Ørum Brook	MBN	1011	2	1	19	11	172	307
November 2002								
Reference site								
Bjergskov Brook	–	–	–	–	–	–	–	–
Streams impacted by sewage effluent								
Usserød Brook	MBNDCF	25,000–30,000	85	16	21,216–25,460	4106–4928	150	775
Babrekær Ditch	MBNC	831	52	5	435	45	11	102
Højen Brook	MBNC	500	19	1	93	7	12	167
Haldrup Mølle Brook	MBNC	176	9	1	16	2	14	125

Sewage treatment steps: M: mechanical; B: biological; N: nitrification; D: denitrification; C: chemical; F: filtration. The percentage of sewage effluent and the population equivalents (PE) in the respective streams are given at both the median minimum and the average water flow. Median minimum and mean water flows are based on observations of annual water flows (calculated from data in Århus Amt 1998; Århus Amt 2000). (1 PE is the amount of organic biodegradable load which has a biochemical oxygen demand (BOD_5) of 60 g per day). Adjusted PE values are the actual values in the respective streams after adjustment by the dilution of the effluent in the streams. *In Giber Brook fish were collected at two different stretches

peanut oil twice a week for 2 weeks. They were kept in 500-l tanks supplied with aerated freshwater (11–14 °C) at a 12 h:12 h light/dark period. The fish were anaesthetised in 0.02% phenoxyethanol prior to injections and blood sampling. After 2 weeks blood was collected from the caudal blood vessel and transferred to heparinised eppendorf tubes. Phenylmethylsulfonyl fluoride (PMSF) and aprotinin were added to a final concentration of 1 mM and 0.02%, respectively. Plasma was obtained by centrifugation at 2500×*g* for 15 min and the same amounts of PMSF and aprotinin as above were added again. Vitellogenin was then purified from the fresh plasma by gel filtration and ion exchange chromatography as described in Christiansen et al. (2000) with slight modifications in the ion exchange chromatography of the gel filtrate. The gel filtrate was diluted 1:4 in 50 mM Tris–HCl, pH 8.0 before application to the ion exchange column, and the diluted gel filtrate was loaded onto a 5 ml HiTrap Q (Amersham Pharmacia) ion exchange column. The sample was eluted with 50 mM Tris–HCl at a flow rate of 2 ml/min. The rest of the purification procedure followed the protocol of rainbow trout vtg (Christiansen et al. 2000). The purified brown trout vtg was then used for antibody production and vtg standards for the ELISA.

Antibody production and purification, native PAGE, SDS-PAGE and Western blotting

The antibodies were raised in rabbits at the Animal Unit of Odense University Hospital, Denmark. Purification of the antibodies was performed by affinity chromatography. Identification of vtg and verification of antibody specificity were made by native PAGE plus SDS-PAGE and Western blotting, respectively. Native PAGE, Western blotting as well as the immunisation scheme did not differ from those described in Christiansen et al. (2000). SDS PAGE was run in accordance with Holbech et al. (2001).

Preparation of dextran-horse radish peroxidase (Dex-HRP) conjugated antibodies and the ELISA procedure

The enzyme conjugated antibodies used as the second layer of the sandwich ELISA and the ELISA itself was performed in accordance with the methods described by

Holbech et al. (2001) with exception of a higher dilution of the conjugated antibodies. The optimal dilution of the conjugated antibody was determined with each new batch of antibodies.

ELISA validation

The lowest possible dilution of the plasma which could be used when measuring the samples and still avoiding matrix effects was determined by examining the recovery percentage of the ELISA. A standard curve of 0.2–10 ng/ml was added to a serially diluted plasma sample initially containing 768 ng/ml vtg. The plasma sample was diluted 20, 50, 100, 200 and 400 times. The recovery was calculated as the percentage of measured vtg relative to the expected value. The intraassay coefficient was determined by addition of 11 times the same plasma sample each in triple application to one plate. The interassay coefficient was calculated from 66 different measurements of an internal standard which was added to each plate diluted one million times. The internal standard was a blood sample from a sexually mature female brown trout. The plates were run on different days.

Statistics

Differences in plasma vtg concentrations among males from the various streams were tested by one-way ANOVA followed by Bonferroni adjusted Fishers Least Significance test where normality of data and homogenous variances could be obtained by log-transformation. Otherwise data were tested non-parametrically by Kruskall–Wallis followed by Dunn's test for multiple comparisons. Differences in frequency of gonadal effects in brown trout were tested by Pearsons chi-square. $P < 0.05$ was used as significance level. Statistical analyses were carried out in SYSTAT 7.0 or SigmaStat (SPSS Inc., Chicago, IL, USA).

Results

Purification of vitellogenin

Vitellogenin was purified by gel filtration and ion exchange chromatography of plasma from 17β-estradiol treated brown trout. At the gel filtration procedure, a major high molecular weight protein fraction was eluted at an elution volume of 113–122 ml. Further purification by ion exchange chromatography yielded a main component eluted at a chloride concentration of 0.37 M. Native PAGE demonstrated that this fraction contained a major single band of a molecular weight of approximately 540 kDa (Fig. 1A). SDS-PAGE of the same fraction produced four major bands with an estimated molecular weight of 171, 139, 104 and 98 kDa (Fig. 1B).

Specificity of antibodies

Western blotting demonstrated the specificity of the purified antibodies (Fig. 1C). All bands produced after SDS PAGE from the purified vtg (Lane 2,3) as well as a single band in the 17β-estradiol treated fish corresponding to the

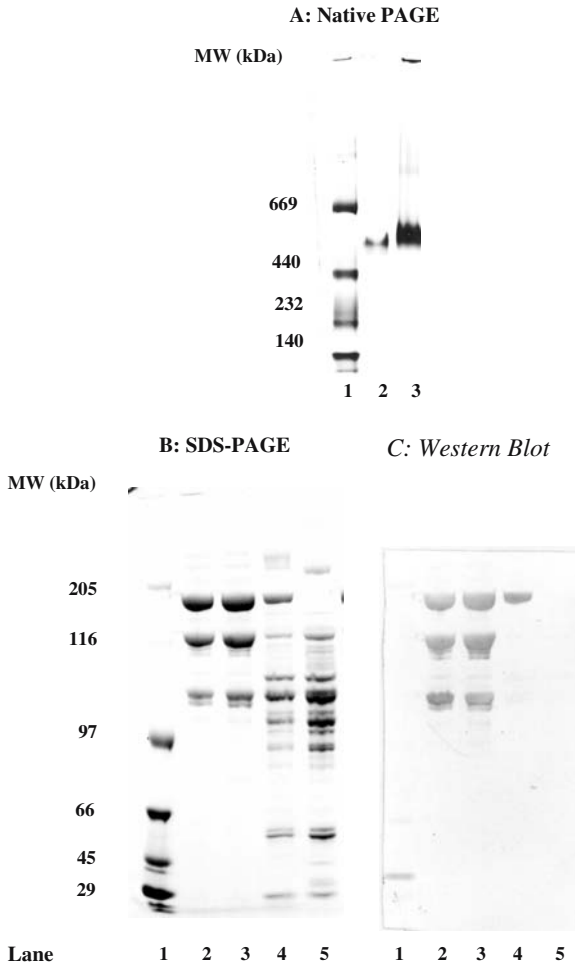

Fig. 1 Identification of vitellogenin (vtg) and verification of antibody specificity by native and SDS PAGE and Western blotting, respectively. **A** Native PAGE of gel filtration and ion exchange chromatography fractions of plasma from E_2 treated brown trout (*Salmo trutta*). Lane 1: Marker (High Molecular Weight Marker, Pharmacia LKB), Lane 2: Vtg containing fraction after ion exchange chromatography and lane 3 after gel filtration. **B** SDS PAGE and (C) Western Blotting. Lane 1: Marker (Sigma SDS-6H), Lane 2: Vtg containing ion exchange chromatography fraction, Lane 3: Lyophilised purified vtg, Lane 4: Plasma from 17β-estradiol treated brown trout (1:50). Lane 5: plasma from control male (1:20)

highest molecular weight protein fraction of the purified vtg (Lane 4) were recognised by the antibodies. The antibodies did not recognise any bands in plasma from a control male (Lane 5).

ELISA

The ELISA was constructed as a direct sandwich ELISA and had a detection limit of 0.2 ng/ml plasma. This low detection limit was obtained by the use of antibodies conjugated with a dextran chain containing several molecules of horse radish peroxidase (HRP). Standard addition at different dilutions of plasma initially containing 768 ng/ml vtg showed that a minimum plasma dilution of 200 times was needed to avoid matrix effect. Table 2 shows the recovery percentages of the standard vtg when added to diluted plasma. The quantification limit for the assay was therefore 40 ng/ml. During the second sampling of fish in 2002 the quantification limit was raised to 100 ng/ml since the lowest standards were no longer three times higher than the standand deviation of blanks. This was probably due to new batches of standards and antibodies. The ELISA had an intraassay coefficient of 8.1% (n=11) and an interassay coefficient of 16.7% (n=66).

Vitellogenin in male brown trout

Vitellogenin levels in male brown trout plasma are shown in Fig. 2. Among fish collected in 2000 (Fig. 2A) males from Voel Brook had a significantly higher concentration of vtg in plasma ($P < 0.001$) compared to males from the three reference sites. When sampling was repeated from Voel Brook at the same time the following year, plasma vtg was again higher in males inhabiting this stream compared to males from reference streams. However, the average concentration in 2001 was a factor of 10 lower than seen among the fish in 2000. A large proportion of males from Voel Brook (especially in 2000) had vtg levels which were typical of the female brown trout (results not shown), and individual concentrations up to 9 mg/ml were measured in males. The box plot illustrates that in some streams, a few individuals had an unusually high plasma vtg level relative to the rest of the males from the respective site. This gave an average vtg concentration much higher than the vtg concentration found in the major part of the male fish from these sites. This was especially the case among males from the three reference streams as well as males from the two streams, Hoed and Ørum Brook, impacted by sewage effluent. The typical vtg levels among these males were 40–1000 ng/ml.

Among the fish collected in 2002 males from Babrekær, Højen and especially Usserød Brook all had significantly higher vtg levels compared to males from the chosen reference brook, Bjergskov Brook. Male brown trout from Bjergskov Brook had the lowest blood plasma concentration of vtg of all reference groups in the present study. Bjergskov Brook is a stream running solely through a forest area and without any input of sewage effluent and influence from domestic animals. When compared to males from the reference sites of the sampling in 2000, only males from Usserød seemed to have higher plasma vtg. It must be noticed, however, that the fish from the two respective sub-studies were not collected at the same time of the year making the comparison between them difficult, as the seasonal variation of vtg levels in male brown trout is not known.

Table 2 Recovery (%) of vitellogenin in brown trout (*Salmo trutta*) ELISA at different degrees of plasma dilutions

Standard curve (ng/ml)	0.2	0.5	1	2	5	10
Dilution						
20×	17	18	18	15	20	25
50×	57	66	53	49	54	61
100×	64	71	64	73	98	88
200×	122	91	95	80	89	95
400×	119	98	91	88	100	101

The undiluted control plasma had an initial vitellogenin concentration of 768 ng/ml

Gonad histology

In the examined brown trout, some females were found to have small areas of spermatocytes, spermatids or spermatozoa in the ovaries (Fig. 3C–F). The areas of male germ cells were apparently delimited by Sertoli cells (Fig. 3E, F) and in some cases, what seemed to be an entire seminiferous lobule existed between the oocytes. These male germ cells were observed in 0–9% of the females from the various brooks including females from both reference streams and streams impacted by sewage effluent, and in females sampled in both June/July and November. In these individuals, the male tissue constituted a small area of the otherwise apparently normal ovarian tissue.

Male brown trout collected in June/July 2000 had—in the sexually mature fish—testes containing all spermatogenetic cell stages—spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 4A, B). A number of fish were, however, found to have a severe degree of vacuolation in all or part of the testes (Fig. 4C, D). The vacuolation often appeared together with a thickening of the testis wall (Fig. 4D). Where the vacuolation was very profound, spermatogonia were the only germ cell stage present in the gonad, and there was no or only a rare occurrence of the later germ cell stages, spermatocytes,

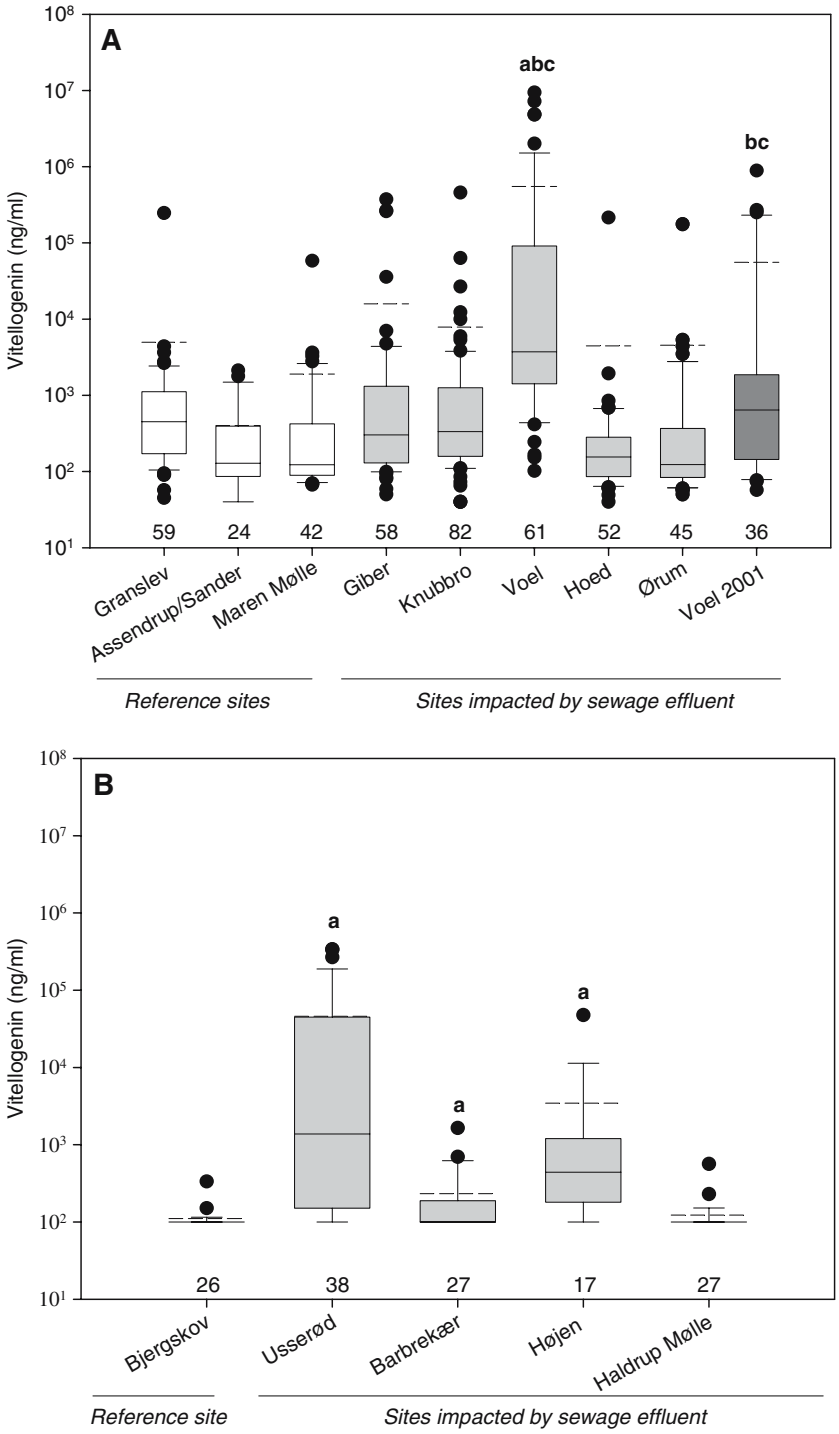

Fig. 2 Vitellogenin in plasma of male brown trout from reference and sewage effluent receiving Danish brooks shown in box plots. **A** Males collected in June/July 2000 and July 2001. a,b,c denotes significant difference from the reference sites Granslev Brook, Assendrup/Sander Brook and Maren Mølle Brook, respectively, $P < 0.001$. **B** Males collected in November 2002. a denotes significant difference from the reference site, $P < 0.001$. The concentration is shown as mean±SEM and depicted on a logarithmic scale. (The quantification limit for the ELISA was 40 ng/ml in study **A** and 100 ng/ml in study **B**). The upper and lower lines in the box show the 75th and 25th percentile, respectively, while the line within the box shows the median. The upper and lower whiskers show the 90th and 10th percentile, respectively, and the dotted line shows the average. The dots represent outliers. The number of fish in each group is shown under the boxes

spermatids and spermatozoa. The vacuoli seemed to appear in Sertoli cells and the testicular changes were apparently due to phagocytosis and resorbant activity by the Sertoli cells. In the lumen of several seminiferous lobules, debris could be seen (Fig. 4E). In some individuals there was also an increased amount of interstitial tissue in the testis (Fig. 4D). The degree of vacuolation varied between individuals. In most cases the vacuoli were distributed all over the testis as illustrated in Fig. 4C. In other individuals only half of the testis was affected (Fig. 5A, B) or the vacuolation was mild meaning that vacuoli were sparse and there was no visible effect on the appearance and occurrence of the later germ cell stages (Fig. 5C, D).

The presence of vacuoli in the testes was found in male brown trout from June/July 2000 in four streams, of which three received sewage effluent. Especially males from Voel

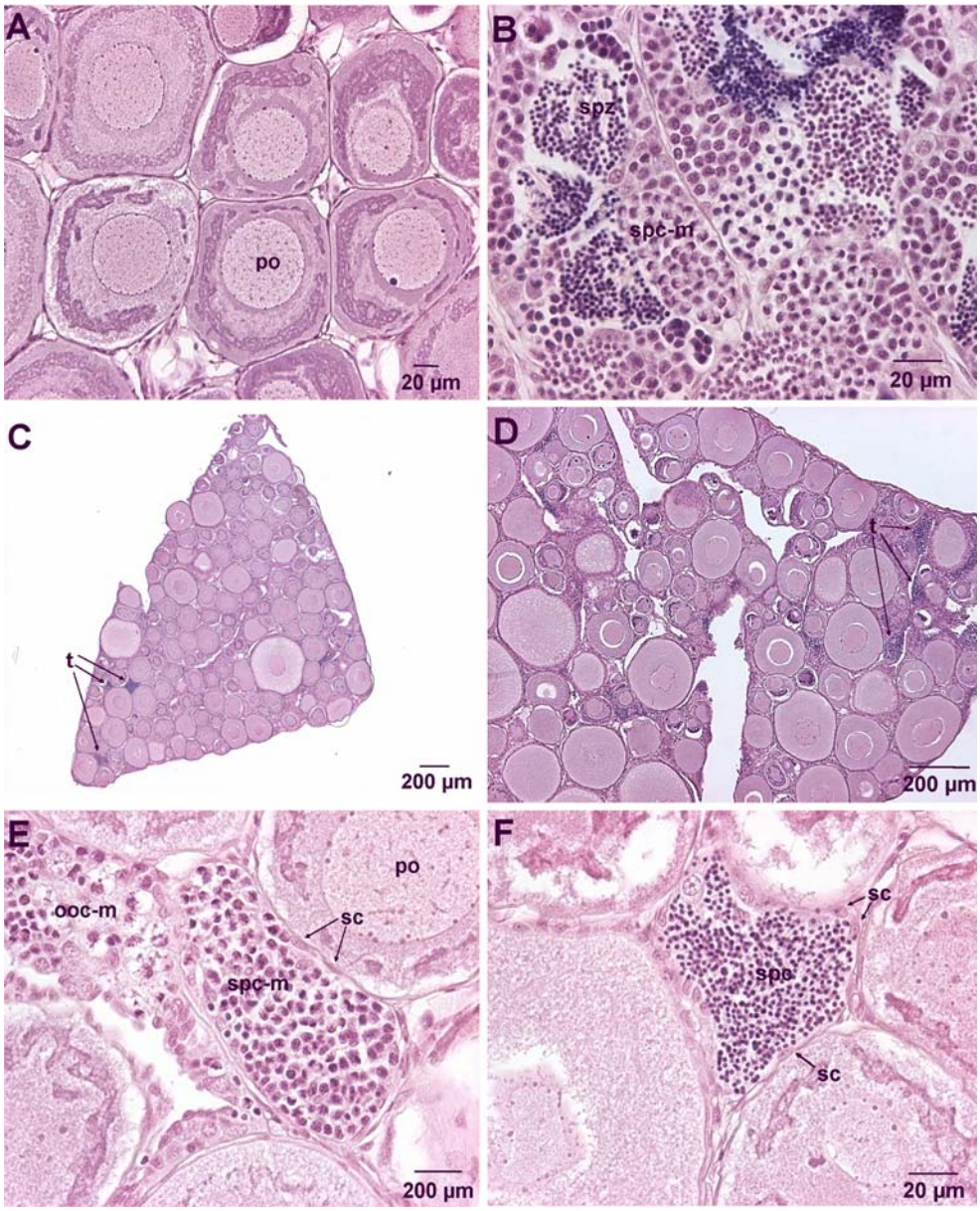

Fig. 3 Intersex in brown trout (*Salmo trutta*) females (cross sections (5 µm), H and E stained) June/July 2000/2001. **A**, **B** Normal female and male, respectively. **C**, **D** Ovaries showing patches of male germ cells. **E**, **F** Large magnification of ovaries with areas of meiotic spermatocytes and spermatozoa, respectively. Po: primary oocyte, o-m: oogonium undergoing meiotic division to primary oocytes, sc: Sertoli cell, spc-m: spermatocytes undergoing meiotic division, spz: spermatozoa

Brook had a high prevalence of the phenomenon which was observed in 44.3% of the males from this stream (Table 3) while the prevalence of the phenomenon was low (below 3%) in the other two streams impacted by sewage effluent. Vacuolation was also observed in approximately 5% of the males from one reference station, Granslev Brook, but not in males from the other reference streams. When sampling was repeated from Voel Brook at the same time the following year, vacuolation was again found in 41.7% of the male brown trout.

Among the sexually mature male brown trout caught later in the reproductive cycle in November 2002, spermiation, and in some individuals spawning, were initiated or completed. Spermiating males had fully mature testes in which most spermatozoa had been liberated from the cysts to the lumen of the seminiferous lobules (Fig. 6A) and the sperm duct. In these testes, small vacuoles were often observed in the interstitial tissue (Fig. 6A, B). Spawning males had some emptied or partly emptied seminiferous lobules which had some similarities in appearance with the vacuolated testes observed among some males from Voel Brook. However, they did not contain distinct vacuoles and were assumed to be presumptive spent testes in which spermatozoa had been fully released. This was supported by the observation that no germ cells besides occasional spermatogonia were present in the testis (Fig. 6C, D). From Usserød Brook 15.8% of the males had this testis structure and was presumptive post spawning.

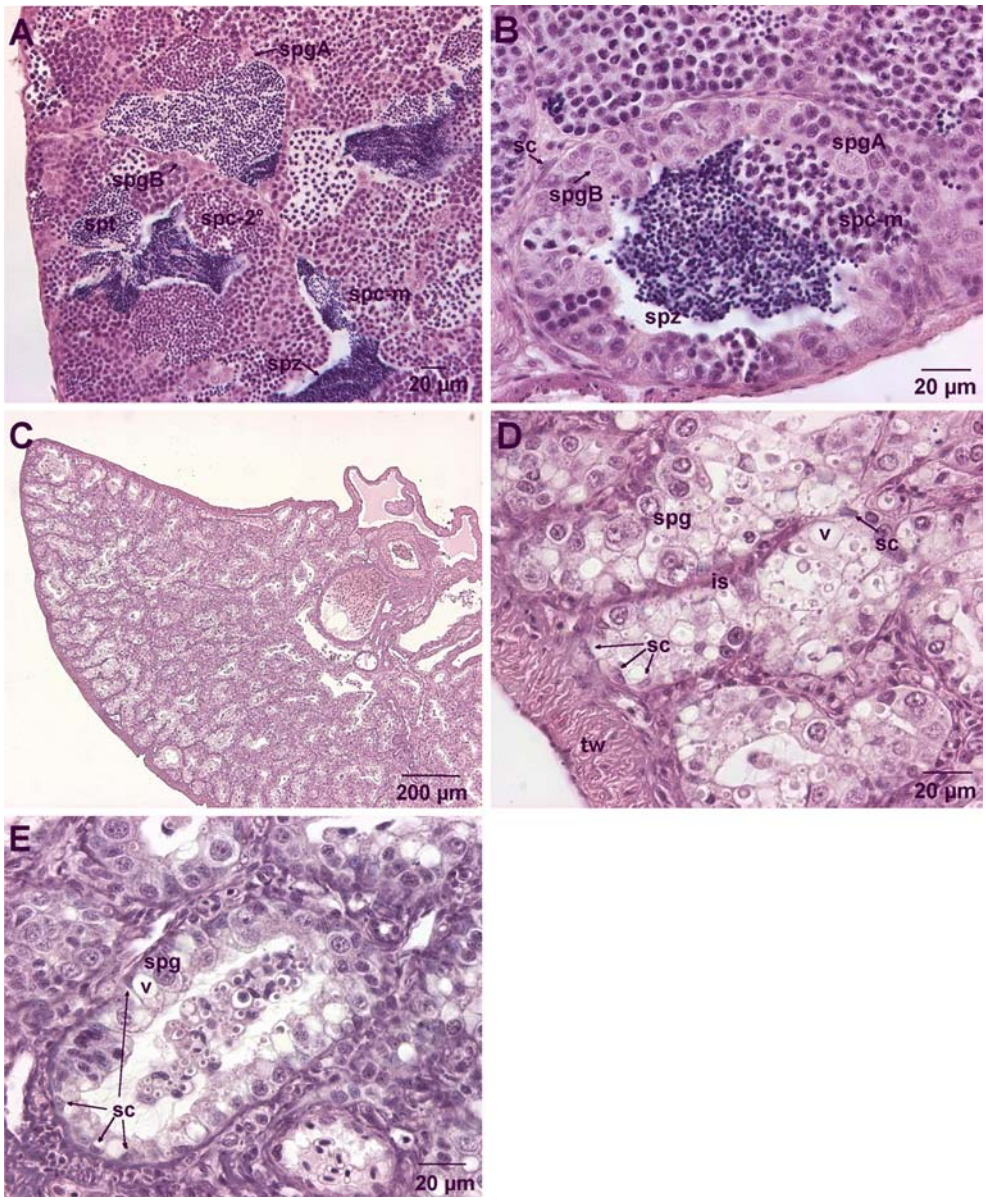

Fig. 4 Vacuolations in brown trout (*Salmo trutta*) testes (cross sections (5 µm), H and E stained) June/July 2000/2001. **A**, **B** Normal testis with presence of all stages of sperm cells in cyst structures. **C**, **D** Severe degree of testicular vacuolation and thickened testis wall. Note also increased amounts of interstitial tissue in D. The vacuoli seem to appear within the cytoplasmic space of the Sertoli cells. **E**. Cross section of a seminiferous lobule showing vacuolated Sertoli cells with intact nuclei and cytoplasmatic extensions but an empty space where germ cells normally reside. Debris is seen in the seminiferous lumen. V: vacuoli, Sc: Sertoli cell, tw: testes wall, spgA: spermatogonium A, spgB: spermatogonium B, spc-1°: primary spermatocytes, spc-2°: secondary spermatocytes, spc-m: spermatocytes undergoing meiotic division, spt: spermatids, spz: spermatozoa, is: interstitial tissue

Discussion

Development of ELISA

The elution profiles obtained by gel filtration and ion chromatography of plasma from E_2-exposed brown trout were homologous to the profiles previously seen at purification of vtg from rainbow trout (Christiansen et al. 2000). The identification of the purified protein as vtg was based on its inducibility by E_2, its absence in plasma of male fish and the high molecular weight of the protein. The molecular weight of 540 kDa corresponds well with the molecular weight of 535 kDa determined for rainbow trout by Maitre et al. (1985). The fragmentation obtained when running the protein under reducing conditions in SDS-PAGE is probably due to a high susceptibility of the protein to proteolysis which has often been seen with vtg from other species (Silversand et al. 1993; Christiansen et al. 2000; Holbech 2001). In the present study, the ability of plasma vtg from an E_2 treated fish to avoid fragmentation, when run under the reducing conditions, could be due to the higher stability of the non-handled protein compared to the double chromatographed purified protein. This, however, does not influence the measurements of vtg by the ELISA, since native PAGE demonstrated that the purified protein, when run under normal non-reducing conditions, was a single intact protein. The raised and purified antibodies were specific towards vtg as no protein bands from male plasma were recognised by Western blotting. The quantification limit of the direct sandwich ELISA was

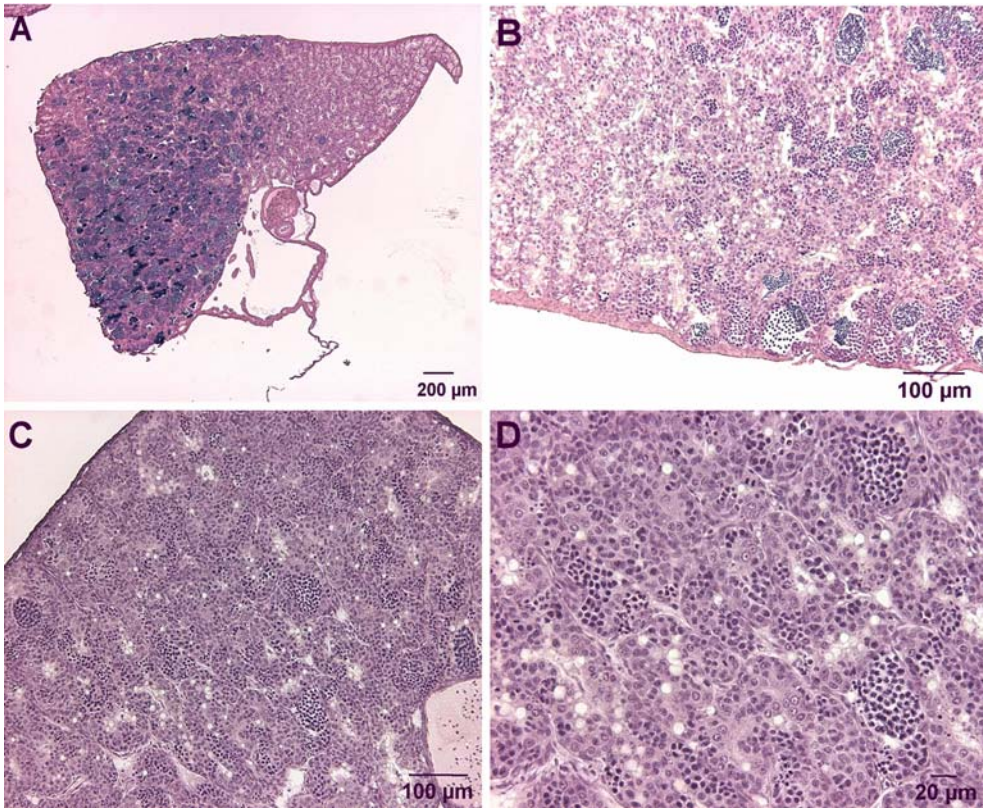

Fig. 5 Mild vacuolation in brown trout (*Salmo trutta*) testes (cross sections (5 µm), H and E stained) June/July 2000/2001. **A**, **B** Mild degree of testicular vacuolation. **C**, **D** Testis with normal structure and all sperm cell stages in part of the gonad while the other part is severely vacuolated and only containing spermatogonia

below the quantification limits which have been reported so far from two other ELISAs for brown trout vtg (Maisse et al. 1991; Sherry et al. 1999).

Vitellogenin production and gonad histology in brown trout from streams impacted by sewage effluent

The higher plasma vitellogenin levels found in males from Voel Brook in June 2000 and July 2001, and in Usserød, Højen and Barbrekær Brook in November 2002 compared to the respective reference sites, indicate that fish from especially Voel and Usserød Brook were exposed to estrogenic compounds in the water. The level of vtg in males from these sites corresponded to the average level observed among male and intersex roach from rivers in UK (Jobling et al. 1998). Vtg has earlier been detected in two brown trout males downstream a sewage treatment work in a Swiss river in a study involving relatively few fish and using immunohistochemical detection of vtg (Wahli et al. 1998).

Table 3 Frequency of male brown trout (*Salmo trutta*) collected in 2000 with vacuolated testes

	Site	Frequency of testicular vacuolation (%)
Reference streams	Granslev Brook	5.1
	Assedrup Brook/ Sander Brook	0
	Maren Mølle Brook	0
Streams impacted by sewage effluent	Giber Brook	0
	Knubbro Brook	2.4
	Voel Brook	44.3^{***} $^{\#}(41.7)^{***}$
	Hoed Brook	1.9
	Ørum Brook	0

$^{***}P < 0.001$. The frequency of testicular vacuolation in males from streams impacted by sewage effluent is tested against the frequency in males from the reference site Granslev Brook. $^{\#}$: Vacuolation of testes from brown trout collected in 2001

Among the examined trout in this study, some individuals had gonads which appeared as normal ovaries but which had sparse areas of germ cells reminiscent of spermatocytes, spermatids and spermatozoa in cyst like structures. This type of intersex among females is formerly described among non-exposed brown trout (Ashby 1965). The same type of intersex has also been seen among 2% of the female poeciliid *Heterandria formosa* in which spermatozoa and Sertoli cells were present in ovarian tissue (Riehl 1980). Macroscopically, the intersex gonads in this species of poeciliid fish had a predominant appearance of normal ovaries, and the female tissue dominated with approximately 100 times more female than male tissue. The same predominance of female tissue was seen in the apparently intersexed individuals among the brown trout in this study. It is, however, uncertain whether these individuals might be weakly masculinised females or severely

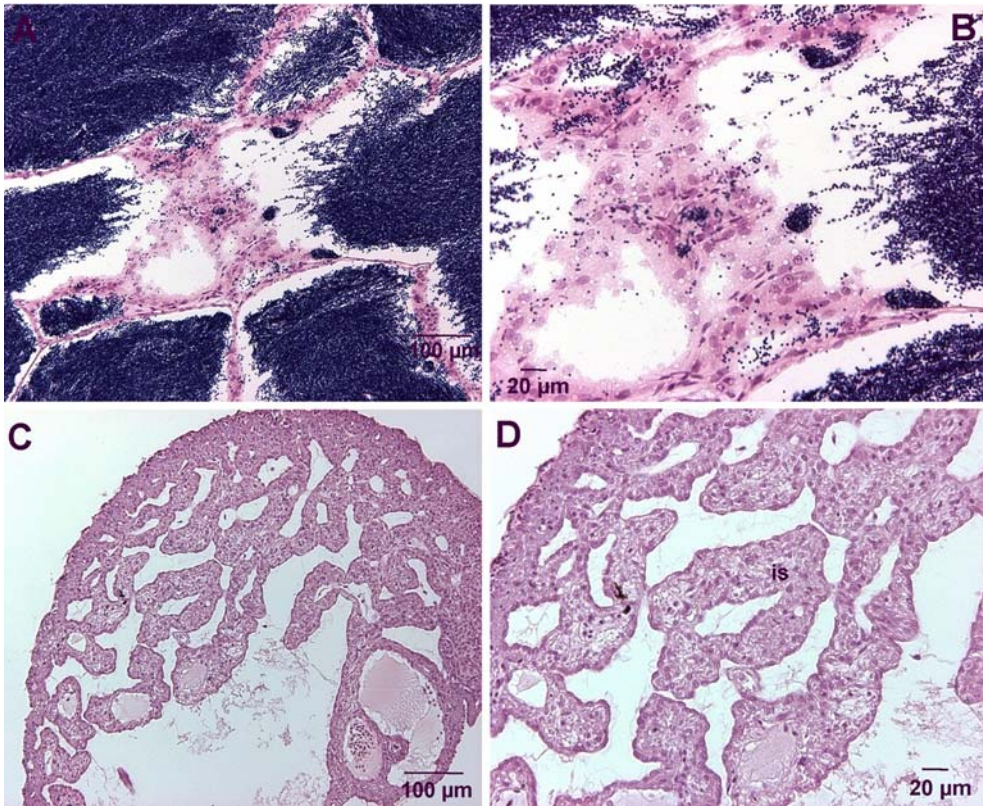

Fig. 6 Brown trout testes from fish collected in November 2002 (cross sections (5 µm), H and E stained). **A**, **B** spermiating males with small vacuoles in the interstitial tissue. **C**, **D** Presumptive postspawning males in which the testes primarily consist of interstitial tissue. is: interstitial tissue, v: vacuoles, spz: spermatozoa

feminised males, or if it is a normal condition found in a proportion of female brown trout.

An observation of vacuolated Sertoli cells and in some cases an inhibition (or delay) of spermatogenesis, manifested by the presence of spermatogonia only, was made in the testes of a number of male brown trout collected in June/July 2000/2001. The swollen Sertoli cells and the presence of debris in the seminiferous lobules indicated that a degeneration/necrosis and a subsequent phagocytosis of the later stages of sperm cells had taken place. Phagocytosis of degenerated sperm cells and spermatozoa, which have not been spawned, is a well-known function of Sertoli cells (Russel et al. 1990) and have been seen in a number of fish species (Henderson 1962; Billard and Takashima 1983). The observed thickening of the testis wall might be a result of contraction, since a large percentage of the germ cells in the cysts had disappeared.

The cause of the vacuolation which was observed in males from both reference streams and streams impacted by sewage effluent, and especially among males from one particular stream, Voel Brook, is not known. The presence of vacuoli in brown trout testes is a naturally occurring phenomenon during certain periods of the reproductive cycle (Billard 1983; Dziewulska and Domagala 2003). Dziewulska and Domagala (2003) have described the production of small vacuoli by the Sertoli cells at the end of spermatogenesis. This was also observed in the present study among males, collected in November, which had initiated spermiation. Further, larger vacuoli appear as a result of phagocytosis by the Sertoli cells of spermatozoa, which have remained in the testes after completion of the spawning season (Billard 1983; Dziewulska and Domagala 2003). In brown trout, vacuoles have been reported to remain in the Sertoli cells after the spermatogenetic cycle has been completed and even up to a couple of months afterwards. Spawning in brown trout lasts from November to January, and phagocytic activity of Sertoli cells has been reported to appear in December to February (Billard 1987). Minor phagocytic activity has also been seen in the periods February to August and October to December. In the present study, the widespread vacuolation observed among male fish from Voel Brook may be ascribed to population differences in the extent and timing of spawning between streams. Males from Voel Brook might have been delayed in their reproductive cycle compared to male fish from the other sampling sites. A delay in the reproductive cycle might, however, also be a result of endocrine disruption. Jobling et al. (2002a) reported delayed spermatogenesis resulting in asynchronous development of male and female germ cells in roach from sewage effluent receiving UK Rivers. The fact that males from Voel Brook in the present study had a high level of plasma vtg indicates that the fish

must have been exposed to estrogens and/or estrogenic chemicals, and vacuolated testes may also be a result of endocrine disruption by these compounds.

Vacuolation of brown trout testes can, however, also be a result of disrupted spermatogenesis caused by other xenobiotic exposures. This may be a direct hormonal effect, but a disturbance of the germ cell development such as necrosis of germ cells and other disturbances of normal germ cell development may also be an indirect toxic, non-hormonal effect on the endocrine system or a direct toxic effect on the germ cells themselves (Kime 1999). Further, it is difficult to assess whether the vacuolation in the Sertoli cells may be due to a damage/disturbance of the germ cell which thereafter are phagocytised, or if the damages may have been directly on the Sertoli cells. The germ cells may hence be degenerated due to lacking support from the Sertoli cells. It is, however, described that a number of xenobiotics can cause cytoplasmatic vacuolation in Sertoli cells and it is believed that it mostly is associated with a direct damage of the Sertoli cells (Russel et al. 1990; Russel and Griswold 1993). Vacuolation is a result of swelling and coalescence of intracytoplasmatic membrane-bound organelles, such as endoplasmatic reticulum and vesicles (Russel and Griswold 1993).

Thus, vacuolation is not a specific response which can be ascribed solely to exposure to estrogen active compounds but has been induced by a variety of xenobiotics. Vacuolation, thickening of the testis wall and loss of the late sperm cell stages have been seen in male cod (*Gadus morhus*) treated with PCB (Sangalang et al. 1981). Histopathological changes such as degeneration and necrosis of spermatozoa, hypertrophy of Sertoli cells, inflammation of interstitial tissue and lack of mature spermatozoa have also been found in the guppy (*Poecilia reticulata*) exposed to methyl mercury chloride (Wester and Canton 1992). Also copper sulphate and cadmium chloride have caused inhibition of spermatogenesis and hypertrofied Sertoli cells (Sengal et al. 1984). Degeneration, proliferation of Sertoli cells and presence of phagolysosomes in Sertoli cells containing degenerated spermatozoa have, however, also been found after treatment of fathead minnow (*Pimephales promelas*) with E_2 (Miles-Richardson et al. 1999a), and similar effects have also been seen after treatment with 4-NP (Miles-Richarson et al. 1999b). Phthalates are also known in general to exert reproductive toxicity (Sharpe et al. 1995; Wine et al. 1997; Gray et al. 2000). Presence of vacuolated cytoplasma of Sertoli cells and early degenerative changes of spermatocytes and spermatids, which resulted in loss of germ cells except spermatogonia, have thus been observed in testes of di-*n*-pentylphthalate exposed young rats (Creasy et al. 1983). The same effects have also been seen after 2,5-hexanedione exposure of rats (Boekelheide 1988; Boekelheide and Hall 1991). Due to the fact that vacuolation of Sertoli cells is a non-specific response to multiple xenobiotics and also a naturally occurring phenomenon at certain periods of the reproductive cycle, it is difficult to establish if estrogenic compounds might have caused the very high occurrence of vacuolated testes among males from Voel Brook. The measurement of quite high concentration of the estrogenic biomarker, vtg, in the blood of the male brown trout from this stream indicates, however, that the disturbance in the testis development might be caused by estrogens and/or estrogenic chemicals.

In conclusion, the observation of high vtg levels in male brown trout from some Danish streams impacted by sewage effluent indicates that the fish have been exposed to estrogen active compounds in the water. This paper, therefore, presents preliminary evidence of endocrine disruption in a salmonid species in the field. Whether endocrine disruption by the same compounds might be responsible for the observed testes structure found in males from one stream, Voel Brook, including widespread vacuolation and lack of more mature germ cell stages beyond spermatogonia is unclear. A better understanding of the seasonal and population variations in the reproductive cycle of wild populations of brown trout as well as of the underlying mechanisms of vacuolation in the fish testes is needed.

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