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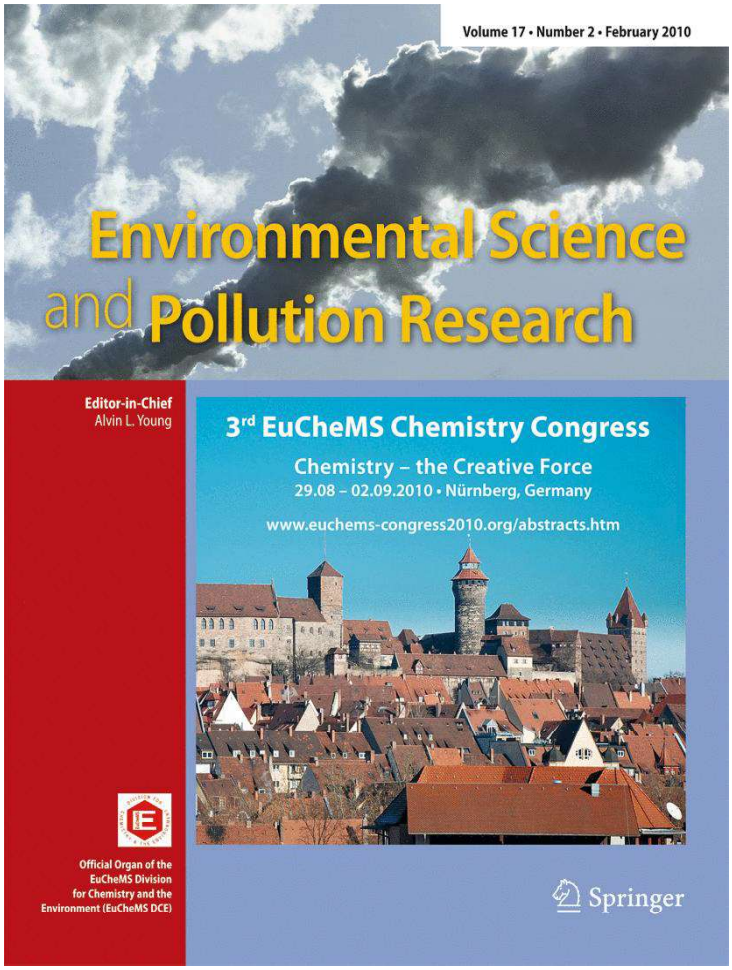

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AREA 7.2 • IMPACTS OF CHEMICALS / FATE IN BIOTA • RESEARCH ARTICLE

Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment

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Abstract
Background, aim, and scope Impacts on the reproductive health of wild fish are thought to be suitable early-warning tools indicating contamination of surface waters with endocrine-disrupting compounds. Ecotoxicological assessment of these field observations depends on the availability of reliable biomarkers to enable a discrimination of natural variations of reproductive functions from anthropogenic impacts.
Materials and methods Roach and perch were caught at eight sampling sites by electrofishing twice a year in summer (July–September) and late autumn/winter (November–December) over a 2-year period. The sites are characterized by different degrees of anthropogenic impact and are situated within the greater Upper Rhine catchment. Age growths, parasitization and gonadal histology of more than 3,000 fish were examined.
Results The two dominant fish species in German surface waters perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) differ considerably regarding their suitability for biomonitoring. Even in pristine habitats, perch show several variants of sex differentiation in terms of (1) the time of first sexual maturation, (2) the course of seasonal gonadal recrudescence, and (3) the occurrence of heterologous germ cells (testes ova). A statistically significant elevated proportion of males were observed in fish obtained from a TBT-contaminated marina and suppression of gonadal ripening was observed in females caught in a sewage-contaminated brook. Both effects appear to be due to chemical contamination. The only "natural" alteration of sex differentiation in roach was related to parasitization with *Ligula intestinalis* (Eucestoda, Pseudophyllidea). Other deviations from the normal pattern of sex differentiation were (1) suppression of ovarian ripening and (2) asynchronic seasonal gonadal recrudescence. These are strong indicators of an anthropogenically induced impact on reproductive health. Feminization phenomena were not observed at either the individual or the population level.
Discussion Interpretation of field monitoring results concerning reproductive health requires large numbers of samples and detailed knowledge of the natural plasticity of sex differentiation in the species under investigation. A better understanding of the mechanisms underlying the plasticity of sex differentiation in perch is indispensable to enable perch to be used as a bioindicator.
Conclusions Deviation from the strict and probably endogenous control of sex differentiation in roach is a strong and unequivocal warning signal.
Recommendations and perspectives The subject of fish monitoring should be addressed in the context of a broader spectrum of potential risks. Seasonal and ontogenetic integrity of gonadal development and recrudescence are potent biomarkers, provided the natural process is well documented for the species under investigation.

Keywords Endocrine disrupters · Feminization · Follicle · Intersexual gonads · Oestradiol syntheses · Reproductive health · Sex ratio · TBT · Tributyltin · Vitellogenin

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1 Background, aim, and scope

In the last decade, xenobiotics thought to act as endocrine disrupters have been at the center of ecotoxicological research. There was growing concern that industrial chemicals and drugs that interact with the vertebrate endocrine system were distributed through the aquatic environment. Specific endpoints/biomarkers for endocrine disruption were measured in aquatic vertebrates (Gross-Sorokin et al. 2006; Jobling et al. 1998, 2002; Liney et al. 2006). Induction of the estrogen-dependent hepatic yolk protein precursor vitellogenin (vg) in male fish was used as an endpoint to assess exposure to exogenous estrogens in vivo. The results of these studies showed that exposure to trace amounts (in ng/L range) of synthetic estrogens and industrial chemicals (in µg/L range) may elevate the vg-serum concentration in a number of laboratory test fish species (Allner et al. 1999; Liney et al. 2005; Nash et al. 2004). Estrogen treatments in the same concentration range affected the differentiation of the somatic gonadal component. Considerably higher concentrations of exogenous xeno-estrogens are required to affect the generative gonadal tissue (germ cells) (Gimeno et al. 1996; Zha et al. 2007). For most of the compounds tested, the concentrations leading to phenotypic sex reversal were found to be very close to or even above the LC 50 threshold (Hill and Janz 2003; Kashiwada et al. 2002). First of all, hormone treatment studies were conducted with a view to enhancing the effectiveness of fish aquaculture (Katz et al. 1976). In consequence, only a small spectrum of species was examined.

Intersexual gonads showing male and female germ cells (testes ova) within the same gonad were first observed in wild roach caught in UK rivers. The incidence of this phenomenon was found to be elevated downstream of sewage treatment plants. Gonadal intersexuality was accompanied by induction of estrogen-dependent yolk protein in male fish. It was concluded that the coincidence of these two female characteristics occurring in male fish indicated the onset of a feminization process. This effect was thought to be due to exposure to sewage plant effluents. Feminization of fish at the individual and the population level therefore seemed to be caused by sewage components with an affinity to the vertebrate oestradiol receptor (Jobling et al. 2002; Rodgers-Gray et al. 2001). The observation of a sex ratio of 7:3 (female/male) in wild-living zander *Stizostedion lucioperca* (L.) in German rivers confirmed this assumption (Hansen & Dizer, 1998).

No data on sex differentiation and sex ratio are available for the two dominant fish species in the river Rhine, i.e. perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) in the wild. To provide appropriate reference data, sex differentiation and gonadal recrudescence in roach and perch were studied in natural and in anthropogenically impacted habitats. Endocrine-disrupting compounds are neither defined by their chemical structure nor are they related to individual industrial sectors. Some compounds are effective in trace amounts known to be below the detection limits of standard chemical analysis. This means that it is not possible to forecast effects on the reproductive health of fish on the basis of environmental chemistry. We therefore defined "anthropogenic impact" as a high sewage burden (of industrial and municipal origin). In order to consider impacts with a potential to cause a shift in the sex ratio toward a surplus of males, we also included marinas known to be contaminated with tinorganyls in our study. These compounds cause masculinization of gastropods by inhibition of oestradiol biosynthesis and testosterone overproduction (Oehlmann et al. 1991). The conversion of testosterone to oestradiol plays an important role in feedback control of testicular functions in vertebrates (Allner et al 2000; Stahlschmidt-Allner et al. 1997).

Special emphasis was put on identifying influences on sex differentiation that are related to the taxonomic differences between the perciformes perch and the cyprinid roach. Feeding and habitat preferences, and, thus, different exposure to sediment or water-borne contaminants, were also considered.

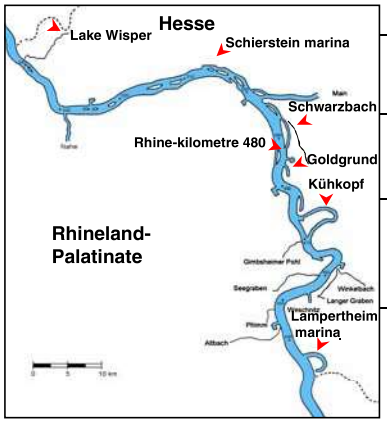
2 Materials and methods

Roach and perch were caught at eight sampling sites by electrofishing. The sites are characterized by different degrees of anthropogenic impact (Table 1) and are situated within the greater Upper Rhine catchment. Data on pesticide and typical background contamination (Schwarzbach and Rhine-kilometer 480) (Tables 4 and 5/electronic supplementary material (ESM)) show an elevated pollutant burden for Schwarzbach. Tinorganyl suspended matter burden for marinas and Schwarzbach are given in Table 6/ESM.

From 2000 to 2002, spot checks of randomly selected individuals per sampling site were carried out twice a year in summer (July–September) and late autumn/winter (November–December) to examine early and late stages of gonadal differentiation and gonadal recrudescence. Based on a confidence interval calculation, a spot check size of 40 individuals per sampling site and fishing campaign was considered conducive. Perch spawned 4–6 weeks earlier (in April) than roach (in May) so that their gonadal development was more advanced in the summer months when the monitoring was carried out. In order to get an impression of the early steps of gonadal differentiation in perch, small juveniles were caught solely at the Kühkopf sampling site, using nets, at the beginning of June in 2001 and 2002.

Preparation and ex-vivo sexing Fish were killed by cervical dislocation and examined for macroscopically visible skin

Table 1 Sampling sites and anthropogenic impact (map: Hessian river Rhine catchment sampling sites and Rhine-kilometer 480)



Sampling site	Position	Altitude	Anthropogenic impact
Lake Guckai Lake Wisper	50,48°N/ 9,92°E 50,15°N/ 7,96°E	691 m 360 m	natural reserves, artificial lake without agricultural or sewage impact
Taubergießen Kühkopf Goldgrund	48,29°N/ 7,69°E 49,82°N/ 8,47°E 49,88°N/ 8,34°E	163 m 86 m 86 m	natural reserves without sewage impact, river Rhine oxbow
Lampertheim marina Schierstein marina	49,61°N/ 8,43°E 50,04°N/ 8,20°E	88 m 84 m	boats, marina (TBT burden of sediments> 1100 µg Tributyltin / kg dry weight)
Schwarzbach	49,95°N/ 8,35°E	84 m	brook, high degree of treated sewage (up to 80% municipal and industrial sewage)

The classification is based on continuous water quality measurements of the Hessian Agency for Environment and Geology from 1998 to 2000 (Allner et al. 2000; Allner 2005; HLFU 1998a, b)

parasites and malformations. After weighing (body, gonads for gonado-somatic index (GSI) calculation) and measuring the length of the fish, the gonads of roach >4 cm were taken together with the lateral peritoneum, where the gonads are attached. Small pieces of the string-like gonadal tissue were examined histologically using 100–200-fold light microscope magnification. The criteria for female sexing were ovarian tissue consisting mainly of clearly visible oocytes and small cells arranged in groups and the absence of oocytes (males). In addition to gender, the state of gametogenesis was recorded from native tissue. The remaining gonadal tissue of each individual of both species was fixed in Bouin–Holland solution for later histological examination. Apart from microscopic examination of the native tissue, histological gonadal preparations (8–15 individuals/species/sampling sites/pot check) were examined. A careful histological examination of the gonads for testes ova was carried out with gonads of individuals (males or juveniles) showing paradoxical vitellogenesis (in an accompanying study) (Allner 2005).

The gender of perch is recognizable by gonadal anatomy (paired testes, unpaired ovary). As gonads of individuals from both species smaller than 4 cm are difficult to locate in the body cavity, these specimens were fixed in toto for histological sex determination. In toto, more than 600 fixed gonad and liver preparations were examined to record gonadogenesis in relation to the sampling site, the season, and the age of the individual. The average number of slides taken from each preparation was eight. The string-like gonads of juveniles and adults caught in summer become twisted during the preparation treatment. Cross-sections from anterior to posterior gonadal lobes were therefore considered. From ripening gonads, five aliquots were taken along the cranio-caudal axis.

The parasitological examination of fish <4 cm was restricted to a microscopic check of the gill epithelia. In fish >4 cm, the gills were examined microscopically for parasites, and the body cavity was examined macroscopically for encysted parasites, *Ligula intestinalis* (L.) (Eucestoda, Pseudophyllidea), and malformation.

Age was determined by analyzing 20–30 scales taken from the dorsal trunk region. To check the number of annual growth rings microscopically, cleaned scales were fixed between two microscope slides. Age-growth classes were determined by the Peterson method (Bagenal and Tesch 1978), based on the age determination of 35–40 individuals of each sampling site. Data on demography are given by Allner (2005).

Histological preparation Fixed torsos of small individuals and tissue were treated by standard histological techniques using paraffin (Histosec, Merck, Germany) as embedding medium and hematoxylin/eosin staining of 6-µm cross-sections (Romeis 1989).

Vg immunostaining was carried out using polyclonal anti carp vg antibody for roach tissues, anti-perch vg antibody for perch tissues (Hennies et al. 2003), peroxidase anti peroxidase technique, and diaminobencidine labeling.

Cell isolates Suspensions of isolated cells to visualize mobility were gained from two male and two female fish caught in June. Minced gonadal tissue was treated with 2 ml phosphate-buffered saline/ethylenediaminetetraacetic acid buffer containing 40 µL of 2.5% trypsin (Roth, Germany) for 10 min and washed with phosphorus buffer to remove the lytic enzyme. The cells were examined histologically after a 2-h recovery period in a Leibowitz cell culture medium covered slide.

Data analysis was carried out by using an Excel file containing the complete data set of individuals examined in this study. Histological results were transferred into nominal categories reflecting the stage of germ cell differentiation and peculiarities of gonadal anatomy, e.g., presence of gonadal secretion or mesenchymal cells. Parasitization was recorded on the parasites species level as far as the description was available. For "unknown" parasites, the taxonomic order was recorded. Detailed information on parasitization is given in Fig. 31/ESM. Pivot data analysis was carried out to correlate data on sampling sites, age, gender, GSI, and vg blood content growth. A deviation from the normal development was defined as difference of more than one differentiation step (nominal category) from the predominant category observed at reference sites.

Statistics In order to detect differences in the sex ratio of uncontaminated and contaminated sites for roach and perch populations, we carried out a three-step procedure: (1) For each individual spot check, we tested for deviations from an equal sex ratio and examined whether spot checks performed at the same site in different seasons showed homogeneous sex ratios; if so, we pooled the respective samples; otherwise, samples from uncontaminated sites were omitted from further analyses. (2) We tested whether the sex ratios of pooled uncontaminated sites comprised homogenous sex ratios. Sites that fulfilled these conditions were pooled to a reference population for uncontaminated sites. (3) We finally compared the sex ratio of this reference population to each of the contaminated sites to check whether sex ratios observed at contaminated areas differed from the ratio in the reference population. For deviations from an equal sex ratio, we applied the exact test, expecting equal proportions of sexes and assuming a binomial distribution (Sokal and Rohlf 2000). For homogeneity of data, we used the 2I-test (Sokal and Rohlf 2000). The three-step analysis was only applied to 0+individuals because low sample sizes of older individuals did not allow for an age-class-dependent analogous analysis, and the inclusion of older individuals in this analysis could cause a random bias in sex ratios of populations. To check for differences in gonadal differentiation of juvenile male perch, we performed pairwise H-Tests following a Kruskal–Wallis ANOVA (Sokal and Rohlf 2000). In these tests, we compared pooled data of uncontaminated sites and contaminated sites (Lampertheim, Schierstein, Schwarzbach) for each of the spot samples. For limiting the overall type 1 error rate, we applied the Bonferoni method (Sokal and Rohlf 2000).

Further, we applied the confidence interval model. The confidence interval indicates the range of possible values of sex ratio within the population in relation to the spot check at the 95% level. Intervals were calculated as $p \pm \frac{\sqrt{np(1-p)}}{n} * 1.96$, where n is the total number of individuals sampled at the site and p is the frequency of males or females, respectively, in the sample (Sachs 1992). A spot check size of 40 individuals represented a compromise, considering the estimated "fish population size" in the areas under investigation (Prof. Lellek, personal communication) and the desired statistical significance level. Since the relationship between confidence interval and spot check size is not linear, the examination of additional individuals per sampling did not contribute effectively to the significance of the entire study. The confidence interval model was applied to the 0+cohorts, as well as to the complete spot check comprising all age classes.

3 Results

Sex ratio In 0+perch, the sex ratio differed significantly from the expected equal ratio for five out of 28 samples: Goldgrund, Taubergießen, and Lake Wisper as uncontaminated sites in the winter of 2002, and at the contaminated Lampertheim marina, a surplus of males was observed in winter 2001 and winter 2002 (Fig. 1; Table 2/ESM). The 2I-test showed homogeneity of spot checks over the monitoring period for all sites except for Goldgrund, which is uncontaminated (Table 3/ESM). Therefore, the latter site was omitted from the further analyses and individual samples obtained over the monitoring period were pooled for all other sites. The sex ratios of the other uncontaminated sites (Lake Guckai, Kühkopf, Taubergießen, and Lake Wisper) were again statistically homogeneous (Table 3/ESM). We pooled these populations and used them as a reference population for uncontaminated sites. The reference population had an equal sex ratio of 196 males and 200 females (exact distribution test, N=396, p=0.44). The sex ratio found at the contaminated site Lampertheim marina differed significantly from the sex ratio of the reference population and had a surplus of males (Table 2/ESM). In contrast, the contaminated sites Schwarzbach and Schierstein marina were not statistically different from the reference population and showed an equal sex ratio (Table 2/ESM). The confidence class analyses led to the same results for the complete spot check, as well as for the 0+individuals. A significant shift towards a higher proportion of males at the Lampertheim marina site was also proven using this method (Fig. 1).

In 0+roach, the sex ratio of individual samples differed significantly from the expected 1:1 ratio in four out of 26 cases: Goldgrund in summer 2001 (male surplus), Kühkopf in summer 2001 (female surplus), Lampertheim marina in winter 2001 (female surplus), and Lake Guckai in winter 2002 (male surplus) (Table 2/ESM).

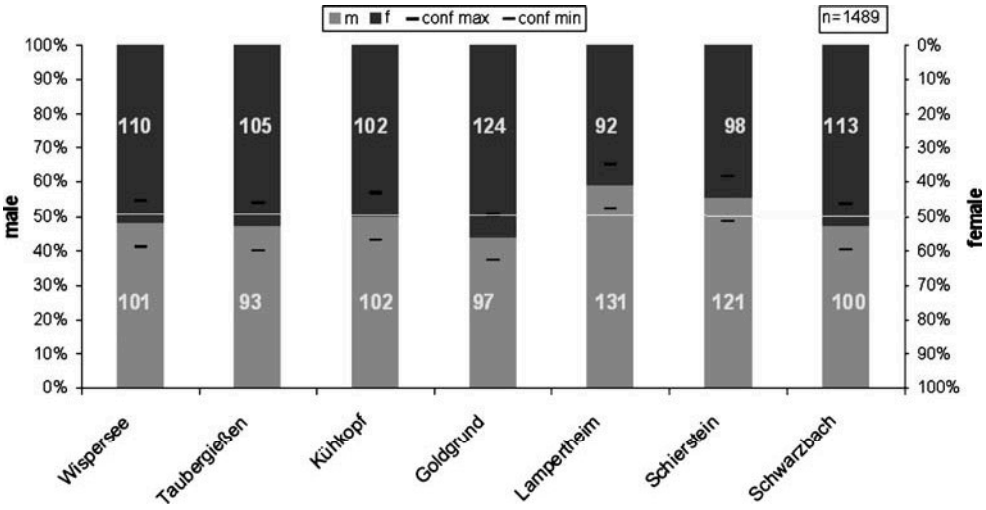


Fig. 1 Sex ratios of perch. Internal rectangles give 95%-confidence intervals of ratios

Samples of spot checks obtained over the monitoring period at the same site were homogeneous for all sites and were therefore pooled for further analysis (Table 3/ESM). Pooled samples of the sites Goldgrund, Kühkopf, Taubergießen, and Lake Wisper (belonging to the river Rhine catchment area) were homogeneous and were used as a reference population of uncontaminated populations (Table 3/ESM). Spot checks from Lake Guckai differed significantly from an equal sex ratio (male as well as female surplus, Table 2/ESM). Due to lower temperature in spring at 600 m above MSL spawning, the early steps of sex differentiation could thereby be retarded. The pooled, uncontaminated river Rhine catchment sampling sites showed a sex ratio with 185 males and 168 females, which did not differ significantly from the expected equal ratio (exact distribution test, $p=0.169$). For none of the contaminated sites (Lampertheim marina, Schierstein marina, Schwarzbach) did we find a significant deviation of the sex ratio from the 1:1 ratio of the reference population (Fig. 2; Table 2/ESM). This is underlined by the fact that the 1:1 ratio lies within all of the 95% confidence levels at all sites (Fig. 2).

Sex differentiation The basic pattern of sex differentiation and gonadal recrudescence in both species conforms to the known features of synchronous germ cell differentiation of seasonal spawning fish (Figs. 3 and 5).

Gonadal differentiation in perch In juvenile perch, gonads are located asymmetrically in the right body cavity. On the left side, a further organ is differentiated, which corresponds to a structure found in an ambisexual anemonefish, and is thought to represent the urinary bladder (Fig. 4a; Fig. 8/ESM) (Stahlschmidt-Allner and Reinboth 1991). This unpaired, duct-like structure had a prominent muscularis and a folded inner epithelium showing secretion activity. The duct opens in a postanal position. In juvenile perch caught in the beginning of June, the gonadal primordium was visible in the caudal body cavity (Fig. 4a).

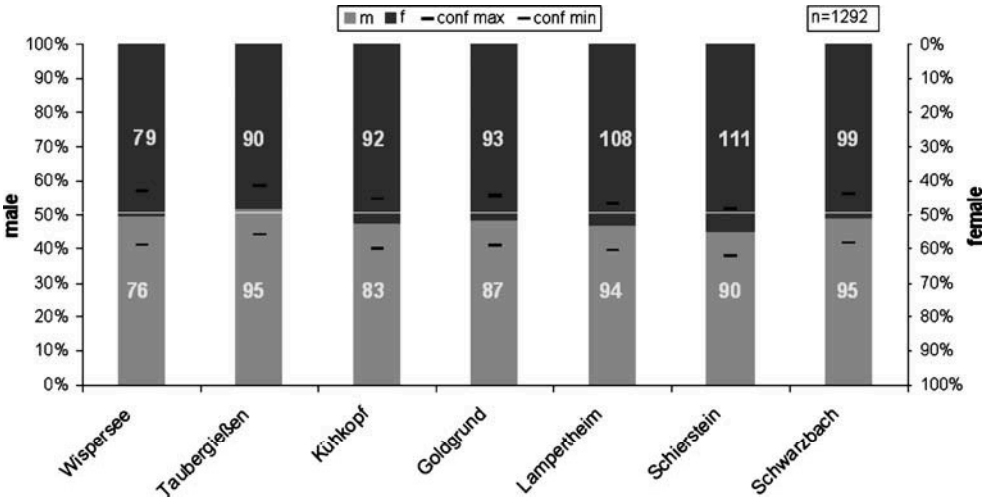


Fig. 2 Sex ratios of roach. Internal rectangles give 95%-confidence intervals of ratios

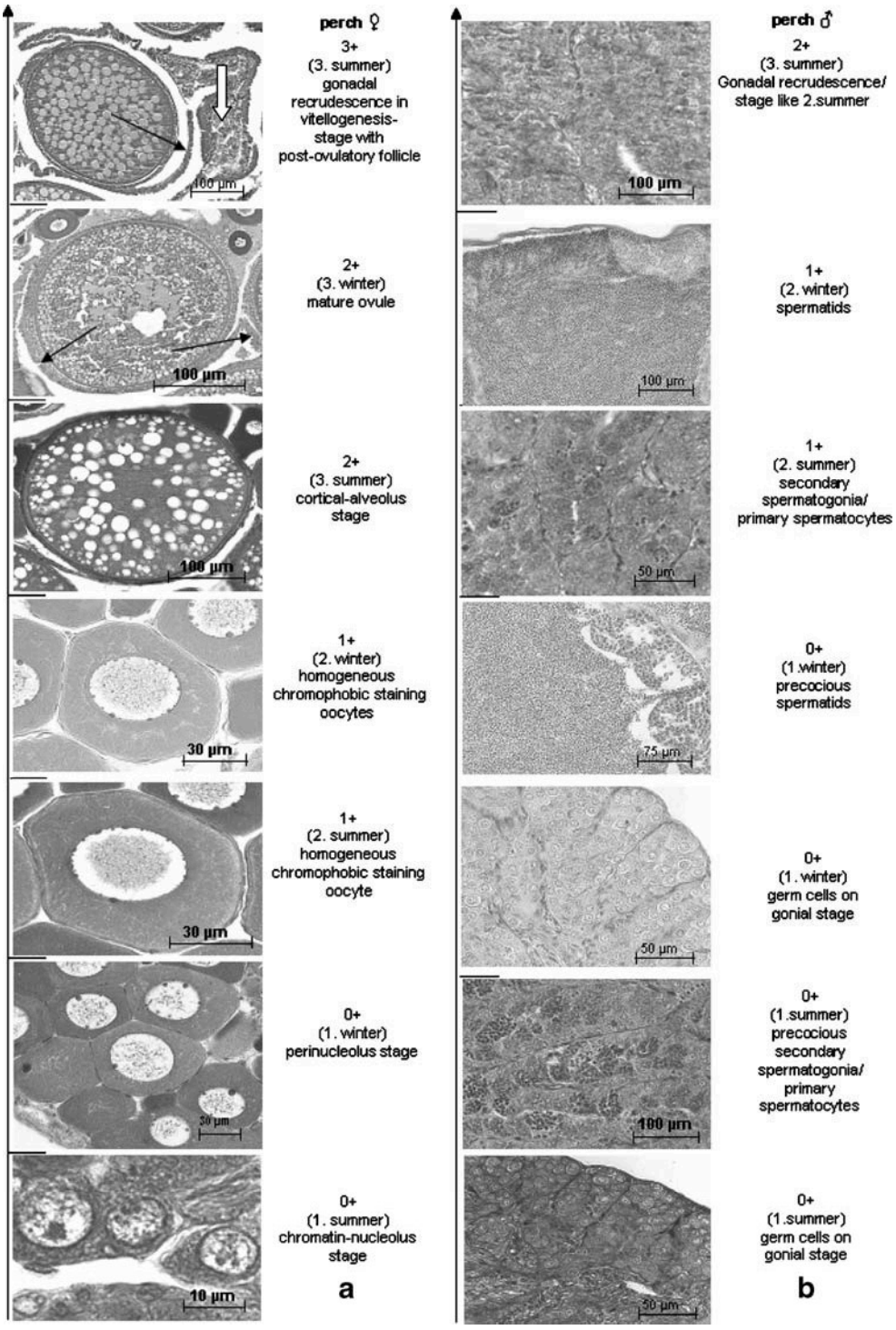


Fig. 3 Overviews on gonadogenesis/gametogenesis and gonadal recrudescence in perch; **a** ovary/oogenesis, *black arrows* point to perch specific ovarian epithelium, *white arrow* points to post-ovulatory follicle, 3+ winter showing ripening oocytes to be spawned next spring and auxocytes to be spawned the year after; **b** testis/spermatogenesis

The gonads were attached to the peritoneum by a peritoneal duplication (Fig. 4a–c). The prospective cavity of the unpaired ovary is lined by an epithelium, which undergoes considerable changes during the different ontogenetic and seasonal ovarian differentiation steps (Figs. 7a, 11, and 12/ESM). A prominent ovarian wall, consisting of connective tissue and muscular layer, is differentiated before the onset of the formation of the generative gonadal component (Fig. 4c). This structure persists and becomes strongly innervated and vascularized in maturing females (Fig. 9/ESM). Only a very few cells in the prospective gonads of both sexes showed the typical histological features of germ cells (oogonia, spermatogonia). It therefore seems likely that the status of the histological gonadogenesis in the beginning of June does not proceed beyond the stage of differentiation of a somatic component of the prospective

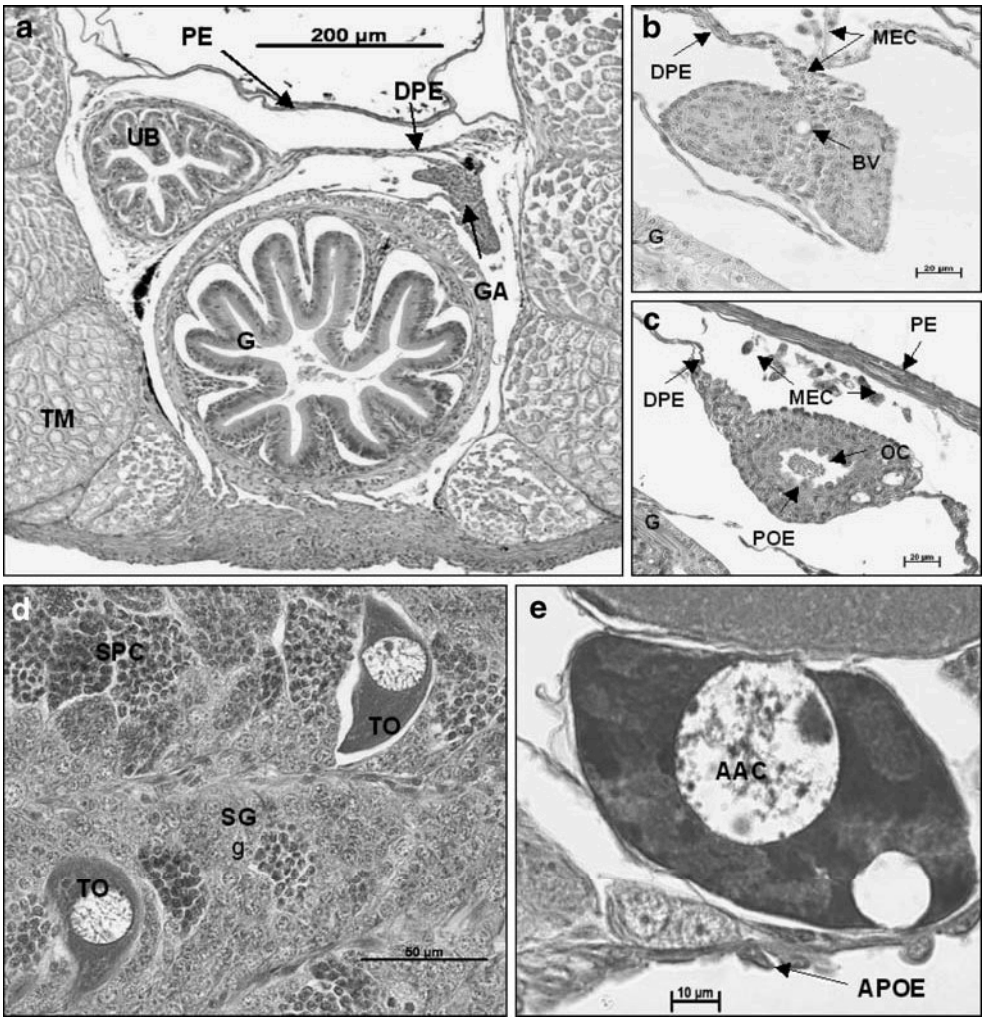


Fig. 4 **a** Cross section through the caudal body cavity of a juvenile perch caught in June. **b** Prospective paired testes in June. **c** Prospective unpaired ovary showing an ovarian cavity June 0+. **d**: Testis ova in early ripening male perch in summer 0+**. e** Histopathological alteration of auxocyte cytoplasm (*arrow*) of nonripening females from Schwarzbach. *AAC*: altered auxocyte, *APOE*: altered perch specific ovarian epithelium, *BV*: blood vessel, *DPE*: peritoneal duplication, *GA*: gonadal anlage, *G*: gut, *MEC*: mesenchymal cells, *OC*: ovarian cavity, *PE*: peritoneum, *POE*: perch specific ovarian epithelium, *SG*: spermatogonia, *SPC*: spermatocytes, *TM*: trunk muscles, *TO*: testis ovum, *UB*: urinary bladder

gonads. Male gonadal anlagen were built up by two blastemata representing the prospective paired gonadal lobes that were connected by mesenchymal tissue (Fig. 4b; Fig. 7b/ESM).

A survey on female sex differentiation is given in Fig. 3a. Oocyte differentiation began in late summer and resulted in an ovary containing many oocytes in the chromatinnucleolus stage. In winter, the perinucleolus stage was predominant (Fig. 10a, b/ESM). Growing oocytes with chromophobic staining properties were observed in the second summer. Thirty percent of female 1+3 perch (originating from uncontaminated sites) completed gonadal ripening during the second summer and autumn (early ripening females) (Fig. 11a/ESM). Ovaries of the other part of the cohorts showed a specific winter feature with characteristic inhomogeneous staining of the ooplasma (Fig. 11b/ESM). In late ripening females, a bilayered follicle epithelium appeared in the periphery of the oocytes during the second summer. Oocyte growth started with the differentiation of cortical alveoli at the oocyte periphery. Simultaneously with oocyte ripening, the inner ovary lining epithelium folded up between the growing oocytes. In the lumina, which are limited by this epithelium, an acellular secretion became visible (Fig. 12/ESM). No deviation from the well known final steps of oocyte ripening (germinal vesicle breakdown, formation of zona radiata) in teleost fish was observed. In a minor proportion of the females older than 2+ (23% at uncontaminated sampling sites), rebuilding of the gonads rested. The occurrence of postovulatory follicles in the specimens showed (in addition to scale age analyses) that these individuals were "adults" in terms of age and first sexual maturity, but the seasonal differentiation process (gonadal recrudescence) had not taken place (Fig. 13/ESM). Since these non-ripening gonads were observed at all sampling sites and no pathological features were recognizable, this phenomenon was assumed to be natural and not the consequence of chemical contamination. A second type of non-maturing females was observed at the highly contaminated Schwarzbach site. All ovaries from fish caught there that were older than 2+ (n=15) showed no features of previous ripening events, such as atretic or postovulatory follicles. Oocytes are not surrounded by

ovarian epithelium. Additional histopathological alterations of the oocyte plasma, like atypical lipid inclusions, which were removed during the fixation procedure, were observed (Fig. 4e; Fig. 14/ESM).

Male sex differentiation in the first year varied between the individuals of the same population (fish originating from the same sampling site). Depending on the sampling sites, 0–50% of the male population matured during the first year (Fig. 15d/ESM). The complete sequence of maturing, beginning with gonial proliferation and followed by spermatogenesis and the initial steps of spermiohistogenesis, was performed before winter (Fig. 3b). These early maturing individuals were ready to span the following spring. Gonadal differentiation of late maturing perch differed from precocious males in a strongly prolonged period of gonial proliferation, which lasted until the second summer of their life (Fig. 15/ESM).

The statistical analyses performed for early maturing males showed that the higher incidence of precocious males at the Lampertheim marina (50%) and the lack of early maturing individuals at the Schwarzbach site represent a significant deviation from the "normal" population structure, which is assumed to be established at sampling sites without a contamination impact. Testes ova (Fig. 4d) were found in 7% of the male individuals examined. The histological feature shown in Fig. 4d was only observed in early ripening males.

Male perch caught in late winter immediately prior to the spawning season showed a considerable alteration of the epithelium lining the urinary bladder. The epithelial cells exhibited secretory activity. The cell plasma contains strongly eosinophilic matter. Given the sex specificity of this very striking transformation of urinary bladder to a glandular organ, we suggest that it is based on a reproductive pheromone-secreting function (Fig. 16/ESM). Gonadal recrudescence in male perch takes place during the summer months. Mitotic division of cells showing gonial shape was observed very rarely during the period of spermatogonial proliferation. This very striking feature confirms the assumption that metamorphosis of cells with different morphological features is the underlying mechanism of gonadal growth during this period (Bruslé 1980, 1982). Mitosis seems to be related to the transition of primary to secondary spermatogonia, exclusively (Fig. 17/ESM).

Gonadal differentiation in roach In 0+ roach, no clear sex differentiation was recognizable during the summer months. In both genders, two clearly separated bilateral symmetric aggregations of somatic cells at the dorsolateral peritoneum represent the prospective gonads (Fig. 18/ESM). In female fish caught in July, morphological sex differentiation had taken place. In limited areas of serial cross-sections of the dorsolateral body cavity, the formation of an oviduct was recognizable (Fig. 6a–c; Fig. 19/ESM). The lateral parts of each gonadal primordium grew towards the dorsal peritoneal epithelium and built up the prospective oviduct. Concomitant with duct formation, the first histological features of oogenesis appeared. Cells with gonial shape and staining properties showed nucleoli attached to chromatin threads (Fig. 5a). Chromatin nucleoli represent the fixation equivalent for the amplification of ribosomal RNA genes. Multiplication of ribosomal genes is required to equip the future oocyte with ribosomes to sustain embryonic protein biosynthesis. The course of gonoduct and chromatin nucleolus formation is discontinuous along the dorsocranial axis (Fig. 6b). Serial cross-sections are required to avoid overlooking the very early steps of female sex differentiation. Correct early sexing is, therefore, a precondition for estimating the correct number of males. In late autumn of the first year (0+), the female gonad contained only a few oogonia (Fig. 5a). Most of the germ cells entered meiosis and transformed into auxocytes. This state of oogenesis was characterized by strong basophilic staining properties, reflecting the deposition of ribosomal RNA in the cytoplasm. The number of oocytes in female roach increased during the first autumn and winter. Oocyte development never proceeded beyond the auxocyte stage. The somatic gonadal component is built up by lymphatic and mesenchymal cells (Fig. 19/ESM). During the second year, oocytes grew and lost their basophilic staining properties, indicating a deposition of proteins in the cytoplasm. The hepatic yolk precursor vg was only detectable in the blood of a minority of the females examined (Allner 2005). This indicates that the alteration of staining properties is not due to incorporation of hepatic yolk proteins. A spatial, as well as temporal, compartmentalization of vitellogenesis (endogenous oocyte borne/exogenous hepatic) existed. The number of somatic cells between the basophilic oocytes increased considerably in late spring and summer, which was a striking ovarian feature of roach older than 1+. Two different cell types appeared: (1) mobile cells showing a typical granulation and an exocentric nucleus; in native preparations (cell isolates) of gonadal tissues, hyaloplasm-based movement of these cells could be observed by means of light microscopy; (2) mesenchymal cells with condensed nuclei and long cytoplasmatic protrusions (Fig. 20/ESM). During the second winter (1+winter), the basophilic staining properties of ooplasma disappeared completely. A single-layered follicle epithelium was established. In addition to growing oocytes, many cells appeared in the transition phase from oogonia to oocytes (initial step of meiosis). A resting oogonial population was not recognizable (Fig. 21/ESM). Oocyte ripening started in all females aged 2+. Many blood vessels became visible. The gonad was colonized by undifferentiated cells, which seem to contribute to the formation of a very tightly packed bilayered follicle

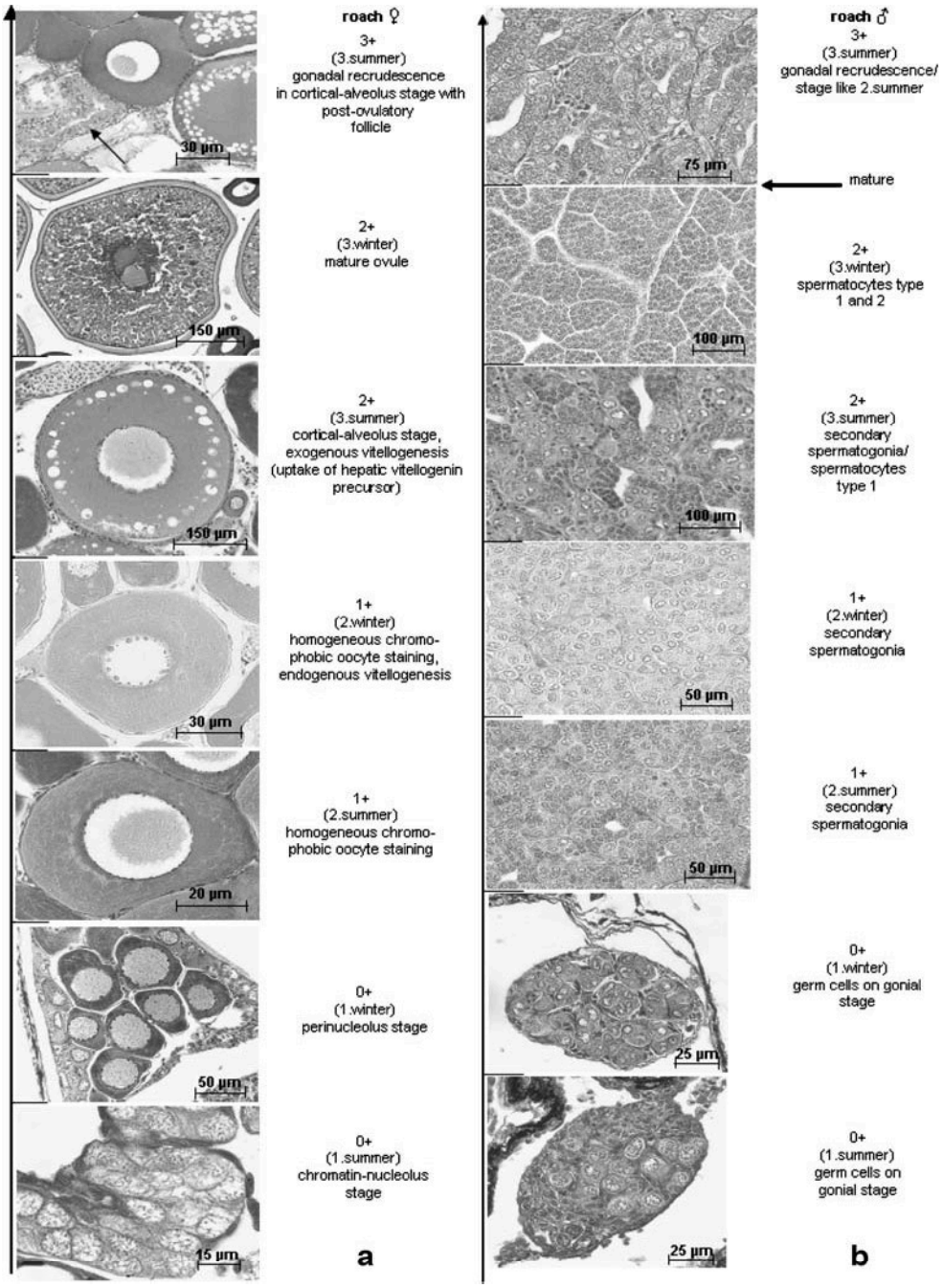


Fig. 5 Overviews on gonadogenesis/gametogenesis and gonadal recrudescence in roach. **a** Ovary/oogenesis, *black arrow* points to post-ovulatory follicle**,** 3+ winter showing ripening oocytes to be spawned next spring and auxocytes to be spawned the year after; **b** testis/spermatogenesis

epithelium surrounding the growing oocytes at the cortical alveoli stage (Fig. 22a–b/ESM). The accompanying study of vg induction showed that a rise of vg content in blood to values above 11 µg/ml was strongly related to this differentiation process (Allner 2005). Figure 22a/ESM shows two oocyte populations (auxocyte and cortical alveoli stage) in the maturing ovary in late summer. On the cellular level, there were no differences regarding the following steps of oocyte maturation (cortical alveoli, formation of zona radiata, germinal vesicle breakdown) between perch and roach. A folded secretory epithelium lining the ovarian cavity was not developed in roach. A resting oogonial population was not present. In the ventral region of the roach ovary, a ciliated seam appeared in late summer/autumn (Fig. 22c–d/ESM). Germinal vesicle breakdown and exogenous vitellogenesis took place in the third winter. GSI is rapidly increasing from values <1.5 in summer to >3 in autumn. Deviations from this pattern of sex differentiation were observed in fish caught in the highly sewage-contaminated brook Schwarzbach (Fig. 23/ESM). Histopathological alterations were observed in all gonadal preparations of 1+ females (n=11) and 2++ females (n=8). 1+ and 2++ females showed precocious maturation in summer in terms of the ontogenetic schedule,

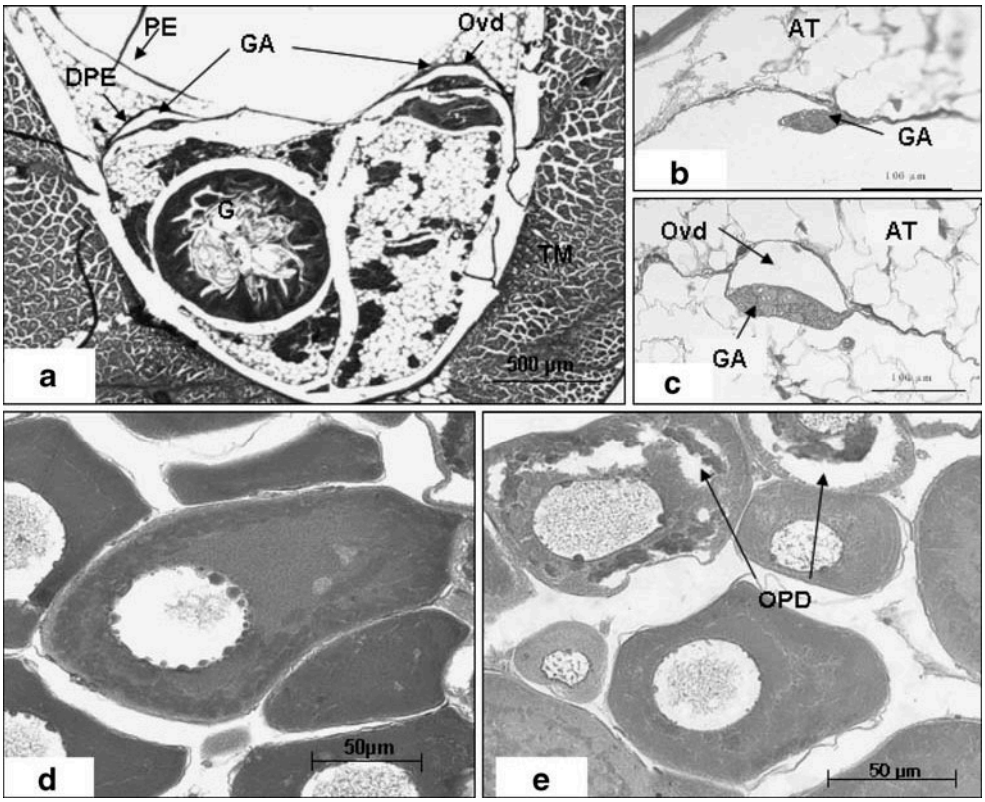


Fig. 6 **a** Cross-section through the caudal body cavity of a juvenile roach caught in August; **b**, **c** differences in gonoduct formation along the cranio-caudal axis, **b** caudal, **c** cranial; **d** *L. intestinalis*-related suppression of oogenesis in a 2+ summer female (missing cortical alveoli). **e**: Roach ovary (2+ winter): Disturbed oogenesis on Schwarzbach site, *AT*: adipose tissue, *DPE*: peritoneal duplication, *G*: gut, *GA*: gonadal anlage, *OPD*: oocyte plasma degeneration, *Ovd*: oviduct, *PE*: peritoneum, *TM*: trunk musculature

as well as on the seasonal level of periodical gamete ripening. This means, in contrast to the other sampling sites showing synchronous gamete ripening, that all developmental stages of oocytes were present in the ovary (Fig. 23i–j/ESM). This alteration is not consistently reflected by an elevated GSI, whereas, at uncontaminated sites, maturing oocytes were only observed in females with a GSI >3 in autumn and winter. GSI >1.8 during summer was observed for 35% of the 1+ females from Schwarzbach and for 4.8% of the 1+females from reference sites in summer. Gonadal blood vessels contained immature blood cells (erythroblasts), which are normally found in renal haematopoetic tissue (Fig. 23a–b/ESM). In terms of developmental biology, it is important to note tumourous malformation of follicle epithelium, as shown in Fig. 23k/ESM, and ontogenetic precocious differentiation of the gonoduct. In 1+ females, gonoduct epithelium is thickened and differentiates into cilia. Cilia are also differentiated from individual gonadal cells (Fig. 23d–e/ESM). Due to the highly variable expression of these malformations, a reliable assessment of incidences was not feasible. In 2++ individuals caught at this sampling site during winter, oogenesis (Fig. 23h/ESM) or oocyte ripening was suppressed and ooplasma showed histopathological alterations (Fig. 6c).

Suppression of oogenesis could also be observed in individuals infected by *L. intestinalis*. In contrast to females from the Schwarzbach site (where *L. intestinalis* was not found), no additional histopathological features were observed (Fig. 6d; Fig. 23g/ESM). Data on the prevalence of *Ligula* infections are given in Fig. 31/ESM.

In male roach, the dorsal peritoneal epithelium was not involved in gonoduct formation; a second attachment side was therefore not differentiated (see Fig. 5b). The absence of oocytes is the only clear male sex characteristic (Fig. 24/ESM). Gonia-like germ cells appeared in the juvenile male gonad in late summer (Fig. 25/ESM). The number of these cells increased during autumn and winter until the next spring. Histological features of mitotic cell division, which were clearly related to gonial proliferation, were not recognizable (Fig. 26/ESM). It therefore cannot be excluded that gonadal growth is caused by the metamorphosis of already available or immigrating cells, which differed in shape and size from gonial germ cells.

The arrangement of germ cells changed during the second summer. Gonia formed a spherical structure enclosing a small lumen. Each sphere represented a prospective spermatogenic cyst. During the second summer and winter, spermatogonia underwent mitotic cell division and proliferated secondary spermatogonia (Fig. 27/ESM). Transition from secondary spermatogonia to spermatocytes was completed during the second winter (Fig. 28/ESM). Spermatogenesis never proceeded beyond the spermatocyte stage in autumn and winter. The final steps of sperm development took place in spring immediately prior to spawning.

Gonadal recrudescence in male roach started from gonads mainly consisting of the collapsed connective tissue of the former emptied gonoduct. A considerable population of residual spermatogonia functioning as stem cells for a new generation of germ cells was not recognizable. The seasonal reconstruction of the gonads resembled in many ways the differentiation of the primary gonadal anlage. There were no differences in gonadal morphology and cytology between individuals undergoing first sexual maturation (1+) and gonadal recrudescence taking place immediately after spawning in late spring (Fig. 29/ESM). The typical features of mitotic cell division were mainly related to the transition of spermatogonia to spermatocytes (Fig. 30/ESM).

The description of the differentiation of a primary gonadal anlage was based on serial histological cross sections of gonads from 360 individuals and microscopic investigation of about 1,500 roach gonads. None of the juvenile fish showed features of intersexuality. Two attachment sites occurred exclusively in individuals showing a clear female pattern of meiosis and early stages of oogenesis.

4 Discussion

This study was motivated by growing concern about the presence of endocrine disrupting compounds in the environment that are thought to cause feminization of wild fish. The data presented in Table 2/ESM show clearly that the experimental design for sex ratio determination of wild fish populations calls for multiple sampling and cumulated spot checks. We assume that single spot checks deviating significantly from 1:1 are due to seasonal sex-specific schooling behavior. Considerable effort was devoted to checking the hypothesis of feminization of wild cyprinid fish, involving expert analysis of the histological preparations and adequate spot checks. The differentiation of a second attachment site represents an unmistakable feature of the natural and the hormone-induced feminization of the prospective gonads (Gimeno et al. 1996). In the present study, the differentiation of a second attachment site was, without exception, concomitant with female meioses and oogenesis. In particular, chromatin nucleoli reflecting the amplification of rRNA are a very reliable sex characteristic. In all the examined specimens, both features always occurred together. Post larval feminization of the somatic gonadal component, as shown in roach after exposure to sewage treatment plant effluents (Jobling et al. 2002; Rodgers-Gray et al. 2001), can be excluded for the areas where the sampling was carried out. Nor did gonadal feminization take place on the germ cell level, as chromatin nucleolus stages were not observed in prospective testes. Only one testis ovum was observed in roach, whereas, in the case of perch, single oocytes surrounded by functional testicular tissue seem to be a phenomenon occurring in 7% of the male individuals. A correlation between the incidence of testes ova and the chemical contamination of the sampling sites was not recognizable.

The complete lack of intersexual features in the roach examined in this study, the high doses of estrogen required to induce sex reversal on the germ cell level, the absence of induced testes ova after exposure to treated sewage, and the overall restriction of the phenomenon to UK surface waters point to a need for a critical evaluation of the biomarker "intersexual gonad" in wild fish. On the basis of our data, reasons other than endocrine-disrupting compounds could cause gonadal feminization. The findings of Wiklund et al. (1996) in particular, showing that infection by microsporidia may cause the differentiation of testes ova, provide a much more plausible explanation for intersexual gonads in roach observed in UK surface waters. The higher incidence of intersexual gonads downstream of UK sewage treatment plants could be due to reduced immune competence of the fish as a consequence of elevated contaminant burden (Hecker 2006).

The second biomarker thought to indicate endocrine disruption—the paradoxical induction of vg in male fish—also requires very careful interpretation. Significant vg production (>11 µg VG/ml serum) has been shown to depend on a bilayered oestradiol-producing follicle epithelium (Allner 2005; Bun and Idler 1983; Nakamura et al. 1993). To recognize exogenous vg induction caused by estrogenic compounds, the absence of function means that bilayered follicles surrounding testis ova have to be checked.

Gonads from males showing paradoxical vg syntheses in this study did not show features of gonadal feminization. Testis ova were not observed in these individuals. This result is in accordance with laboratory experiments showing that treated sewage caused hepatic yolk protein syntheses in male fish but did not induce testis ova (Rodgers-Gray et al. 2001). Therefore, the assumption of a causal connection between the phenomena of vg induction and the presence of testis ova seems to be questionable. The shifts in sex ratio towards a higher portion of females found elsewhere could be due to the sampling design. As shown in Table 2/ESM, studies based on a single sampling may feign a shift in sex ratio. We assume that sex-specific behavior (female schooling, sex-specific habitat preference) may cause uneven distribution of males and females in the area under investigation. Lower mortality of females due to sex-specific excretion of xenobiotics via eggs could potentially cause a real shift in sex ratio.

This study did not support the thesis that sewage-borne, endocrine-disrupting chemicals cause a purposeful directed

diversion of sex differentiation in roach, resulting in the phenotype contradicting the (genetically?) determined gender. However, we observed serious impairments of ovarian development/recrudescence in roach caught in sewage-contaminated Schwarzbach. Deviation from the seasonal and ontogenetic schedule of synchronous oogenesis concerning only a restricted number of oocytes clearly indicates a disturbance of the normal endogenous hormonal control of oogenesis. The observed gonadal malformation reflects an endocrine disruption resulting in female infertility of the population under investigation. Trace amounts of the xeno-estrogen ethinylestradiol induce differentiation of a ciliated gonoduct epithelium in zebra fish (*Danio rerio*) (Nash et al. 2004). The precocious differentiation of cilia observed in female roach in this study is the only deviation from normal development potentially reflecting a non-physiological estrogen exposure of the gonadal tissue. Therefore, we conclude that estrogen receptor activation is not the predominant mode of action causing infertility of roach from Schwarzbach. This observation is in accordance with data on riverine environments showing a decline of fish populations. Apart from estrogens, high burdens of geno-, embryo-, and cytotoxic and mutagenic compounds are suspected to contribute to impairments of fish reproductive health (Braunbeck et al. 2003; Keiter et al. 2006). Sex differentiation in both indicator species did not differ essentially regarding germ cell development. Female perch caught in Schwarzbach showed the same "endocrine disruption" as roach. More surprising results were the considerable differences regarding the ontogenetic schedule of sexual maturation, development of the gonad, and the resulting anatomy of the organ. A striking feature of the perch gonad is an asymmetric localization of gonadal primordium in the right body cavity and the urinary bladder on the left side. As far as we are aware, a corresponding organ is only described in ambisexual anemonefish *Amphiprion frenatus* (Stahlschmidt-Allner and Reinboth 1991). In this species the urinary bladder shows considerable variation regarding secretion activity of the lining epithelium and shape of the muscularis during sex inversion. The organ was suspected to play a role in the social control of sex differentiation via chemical (pheromonal) cues. The considerable seasonal changes of glandular activity of this organ in male perch led to the conclusion of a corresponding potentially pheromone-producing function in riverine perciformes. The innervated muscular gonadal wall and the seasonal sexual dimorphism of the urinary bladder led us to presume a pronounced control of reproductive functions via the central nervous system in perciformes. In the light of this working hypothesis, the high plasticity of perch sex differentiation (male and female pubertal praecox, facultative biennial spawning period) could be due to pheromonal communication (social control) between the members of a perch population. This implicates an additional exogenous access to sex differentiation via chemoperception. Since pheromones are known to be effective in the 10^{-14}-mol range in fish, chemical communication represents a potential target through which reproductive functions could be exogenously impacted in a way that is not properly understood (Sorensen et al. 1987; Cardwell and Liley 1991). The significant male surplus at Lampertheim marina may reflect an anthropogenic impact mediated via the same pathways enabling the high natural plasticity of sex differentiation in perciformes. Lampertheim marina is excessively polluted by organic tin compounds (Allner 2005; HLFU 1998a, b). Different types of contamination—organic tin compounds at the Lampertheim marina and sewage at the Schwarzbach site—were accompanied by different alterations of sex differentiation, namely, masculinization and inhibition of maturation. These findings may indicate that the sex differentiation of perch is a very sensitive and specific biomarker.

5 Conclusions

The data of the two dominant species of the riverine fish fauna, roach and perch, cannot be used alternatively in field monitoring studies. Deviation from the strict and probably endogenous control of sex differentiation in roach is a strong and unequivocal warning signal. A normal table approach regarding sex differentiation seems to be applicable in this species, whereas a better understanding of the mechanisms underlying the plasticity of sex differentiation in perch is indispensable to enable perch to be used as a bioindicator.

From the investigation of more than 1,500 individuals, we conclude that biomonitoring of the reproductive health of wild roach could be based on the histological examination of gonads. Depending on the season when the monitoring is carried out, the following endpoints were assessed as the main checkpoints in a broader normal table approach.

Natural (healthy) sex differentiation of roach is reflected by (1) strict linkage of formation of a second attachment site and female meioses during the first winter; (2) correct settlement of spermatogonia in the primary gonadal anlage and during male gonadal recrudescence; (3) appearance of prominent areas of mesenchymal cells between the residual auxocytes, resulting in a new population of oogonia and a functional bilayered oestradiol-producing follicle epithelium; (4) appearance of cortical alveoli (lipid droplets) in the growing oocyte taking place in late summer and autumn in females aged 2+; and (5), in male fish, the transition from secondary spermatogonia to spermatocytes (male meioses) represents an important step of male sex differentiation in late summer. (6) The differentiation of a ciliated epithelium

lining the ventral part of the caudal ovarian wall is a clear female sex characteristic occurring prior to spawning. This structure is known to be induced by trace amounts of ethinyloestradiol in male cyprinids (Nash et al. 2004).

6 Recommendations and perspectives

Our results showed that several reliable biomarkers can be derived from fish germ cell differentiation to identify impairments related to contamination impact on the aquatic environment. Since gametogenesis follows the same basic pattern in all vertebrates, alterations in this process can be used as an early warning tool for reproductive toxicant contamination. Focusing on paradoxical induction of vg and intersexual gonads entails a risk of misinterpretation and a reduced level of monitoring success using early warning tools based on contamination by estrogenic compounds. The present study did not provide any evidence that this type of compound caused the adverse effects observed. The subject of fish monitoring should be addressed in the context of a broader spectrum of potential risks. Seasonal and ontogenetic integrity of gonadal development and recrudescence are potent biomarkers, provided the natural process is well documented for the species under investigation.

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ESM-Electronic supplementary material / table 2-6, fig. 7-31

Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment

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table 2 Deviations from equal sex ratios in perch and roach. Sex ratios were tested with the exact test assuming equal sex ratios and a binomial distribution. Contaminated sites are marked with an asterisk.

species	site	season	sex ratio
perch	Lake Wisper	winter 2002	male surplus (N = 6, df = 1, p = 0.016)
	Taubergießen	winter 2002	female surplus (N = 40, df = 1, p < 0.001)
	Goldgrund	winter 2002	male surplus (N = 39, df = 1, p = 0.01)
	Lampertheim marina*	winter 2001	male surplus (N = 37, df = 1, p = 0.01)
	Lampertheim marina*	winter 2002	male surplus (N = 41, df = 1, p = 0.03)
	Lampertheim marina pooled*	summer 2001 through winter 2002	male surplus (N = 123, df = 1, p = 0.004)
roach	Kühkopf	summer 2002	male surplus (N = 7, df = 1, p = 0.008)
	Goldgrund	summer 2002	male surplus (N = 9, df = 1, p = 0.02)
	Lake Guckai	winter 2002	female surplus (N = 40, df = 1, p = 0.001)
	Lampertheim marina*	winter 2001	female surplus (N = 35, df = 1, p = 0.02)
	Lake Guckai pooled	winter 2001 and winter 2002	male surplus (N = 79, df = 1, p < 0.001)

table 3 Homogeneity analysis for sex ratios in perch and roach. Homogeneity of samples was tested with the 2I-test. Contaminated sites are marked with an asterisk. n.s. p > 0.05.

Species	Samples	Test result
perch	Lake Wisper summer 2001 through winter 2002	G_H = 5.425, df = 3, N = 65, n.s.[#]
	Taubergießen summer 2001 through winter 2002	G_H = 0.446, df = 3, N = 136, n.s.
	Kühkopf summer 2001 through winter 2002	G_H = 0.920, df = 3, N = 137, n.s.
	Goldgrund summer 2001 through winter 2002	G_H = 8.297, df = 3, N = 141, p < 0.05
	Lake Guckai winter 2001 and winter 2002	G_H = 1.665, df = 1, N = 58, n.s.
	Lampertheim marina * summer 2001 through winter 2002	G_H = 1.642, df = 3, N = 122, n.s.
	Schierstein marina * summer 2001 through winter 2002	G_H = 1.987, df = 3, N = 132, n.s.
	Schwarzbach* summer 2001 through winter 2002	G_H = 1.116, df = 3, N = 142, n.s.
	Pooled samples of Kühkopf, Lake Guckai, Taubergießen and Lake Wisper (reference population of uncontaminated sites)	G_H = 0.645, df = 3, N = 396, n.s.
	Reference population vs. Lampertheim marina *	G_H = 5.412, df = 1, N = 512, p < 0.05
	Reference population vs. Schierstein marina *	G_H = 0.063, df = 1, N = 528, n.s.
	Reference population vs. Schwarzbach*	G_H = 1.237, df = 1, N = 538, n.s.
roach	Lake Wisper summer 2002 and winter 2002	G_H = 1.908, df = 1, N = 36, n.s.
	Taubergießen summer 2001 through winter 2002	G_H = 1.136, df = 3, N = 122, n.s.
	Kühkopf summer 2001 through winter 2002	G_H = 1.592, df = 3, N = 92, n.s.
	Goldgrund summer 2001 through winter 2002	G_H = 4.806, df = 3, N = 103, n.s.
	Lake Guckai winter 2001 and winter 2002	G_H = 1.078, df = 1, N = 82, n.s.
	Lampertheim marina * summer 2001 through winter 2002	G_H = 6.529, df = 3, N = 92, n.s.
	Schierstein marina * summer 2001 through winter 2002	G_H = 0.841, df = 3, N = 124, n.s.
	Schwarzbach* winter 2001 and winter 2002	G_H = 0.631, df = 1, N = 72, n.s.
	Pooled samples of Goldgrund, Kühkopf, Taubergießen and Lake Wisper (reference population of uncontaminated sites)	G_H = 0.529, df = 3, N = 353, n.s.
	Reference population vs. Lampertheim marina *	G_H = 2.337, df = 1, N = 445, p < 0.05
	Reference population vs. Schierstein marina *	G_H = 2.389, df = 1, N = 477, n.s.
	Reference population vs. Schwarzbach*	G_H = 0.236, df = 1, N = 425, n.s.

[#] n.s. = not significant

table 4 pollutant burden Schwarzbach and river Rhine (water) (HLFU 1998).

	Schwarzbach	Rhine (km 480)
µg/L		
Chlorine pesticides:		
HCH (LQ:0,01)	0,03	<0,01
o,p´DDT (LQ: 0,01)	<0,01	<0,01
o,p´DDE (LQ: 0,01)	<0,01	<0,01
p,p´DDE (LQ: 0,01)	<0,01	<0,01
p,p´DDD (LQ: 0,01)	<0,01	<0,01
Aldrin (LQ: 0,01)	<0,01	<0,01
Dieldrin (LQ: 0,01)	<0,01	<0,01
Bromocyclen (LQ: 0,01)	<0,01	<0,01
Endosulfan (LQ: 0,01)	<0,01	<0,01
ng/L		
Alkyl phenol (LQ: 5):		
p-iso NP	30	240
iso OP	<5	8

(LQ: limit of quantitation)

table 5 suspended matter pollutant burden - means of 14 measurements (HLFU 1998). ,

	Schwarzbach	Rhine (km 480)
mg/kg dry weight		
AOX	130	133
Alkyl phenol (1996) (LQ: 0,08)	2,82	-
%		
TOC	14,7	4,9
µg/kg TS		
PCB (LQ: 1):		
6 DIN PCB	165	43
coplanare PCB 77	2	<1
coplanare PCB 105	2	<1
HCB (LQ: 1)	13	4
Chlorine phenol:		
PCP (1996) (LQ:7-14)	46	-
Chlorine pesticides:		
HCH (LQ: 1-7)	12	<3
o,p´DDT (LQ:1-7)	<3	<2
o,p´DDE(LQ: 1-2)	1	<2
p,p´DDE (LQ: 4-7)	22	7
p,p´DDD (LQ: 1-9)	11	<1
Aldrin (LQ:1-9)	<2	<1
Dieldrin (LQ:1-23)	<1	<3
Bromocyclen (LQ: 1)	7	<1
Endosulfan:		
(LQ: 1-48)	<4	<14
(LQ: 4-20)	<5	<7
Heptachlor (LQ: 1-3)	<1	<1
ng/kg dry weight		
Dioxine and Furane (1994):		
PCDD tetra-octa	2653	2478
PCDF tetra-octa	1140	600
µg/kg dry weight		
PAK:		
Fluranthen (LQ: 1-10)	1360	583
Benzo(a)pyren (LQ: 1-10)	887	307
Anthracen (LQ: 1-10)	119	60

Phenanthren (LQ: 1.10)	538	279
Pyren (LQ: 1-10)	941	431
Naphthalin (LQ: 1-10)	133	102
16 EPA PAKs	9248	3710
6 TVO PAKs	5470	2012
Musk compounds:		
M-Xylol (LQ: 1-8)	6	2
M-Keton (LQ: 1-8)	78	<2
HHCB (Galaxolid) (LQ: 10)	1915	45
AHTN (Tonadolid) (LQ: 10)	2062	53

(LQ: limit of quantitation)

table 6 Tinorganic compounds in suspended matter (µg/kg dry weight) (HLFU 1998). (LQ: limit of quantitation)

	Schwarzbach	Rhine (km 480)	Schierstein marina	Lampertheim marina
Monobutyltin (LQ: 1-5)	78	17	51	33
Dibutyltin (LQ: 1-5)	130	33	58	61
Tributyltin (LQ: 1-5)	26	8	143	206
Triphenyltin (LQ:1-5)	17	2	14	<1
Dioctyltin (LQ: 1-5)	17	2	7	136

(LQ: limit of quantitation)

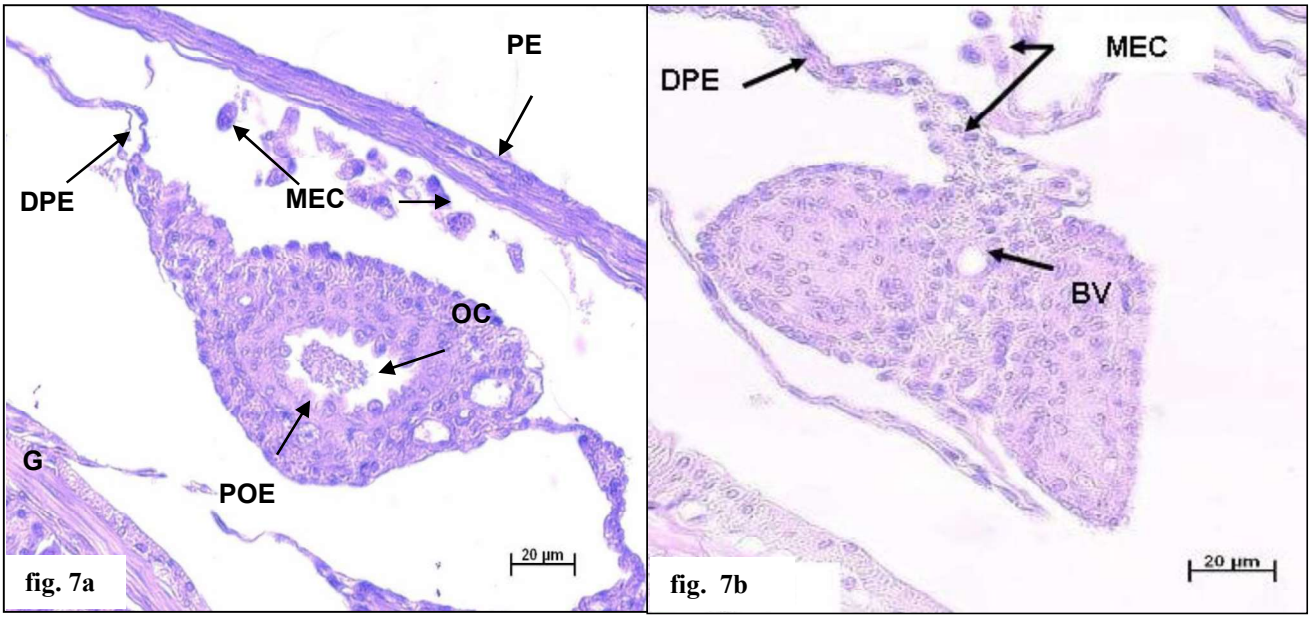


fig. 7 Gonadal anlage of juvenile perch in June. **fig. 7a** Overview: prospective unpaired ovary showing an ovarian cavity
fig. 7b prospective paired testes. BV: blood vessel, DPE: peritoneal duplication, G: gut, MEC: mesenchymal cells, OC: ovarian cavity, PE: peritoneum, POE: perch specific ovarian epithelium

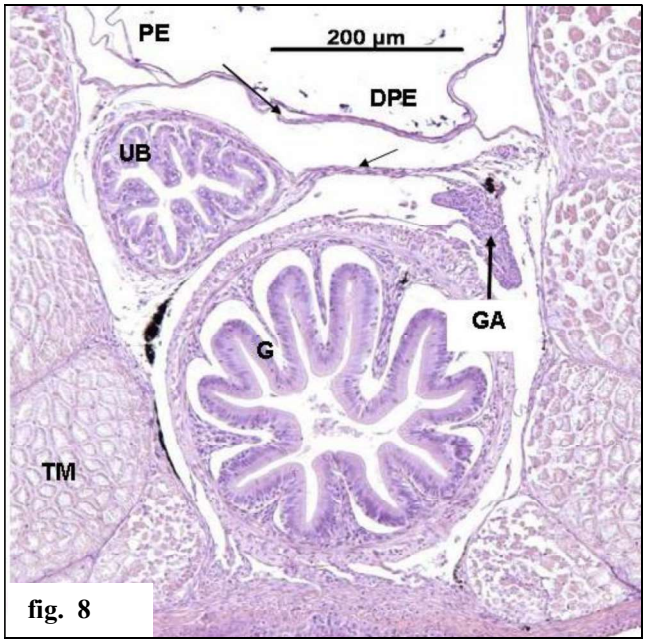


fig. 8 Cross-section through the caudal body cavity of a juvenile perch caught in June. DPE: peritoneal duplicature, GA: gonadal anlage, G: gut, PE: peritoneum, UB: urinary bladder, TM: trunk muscles

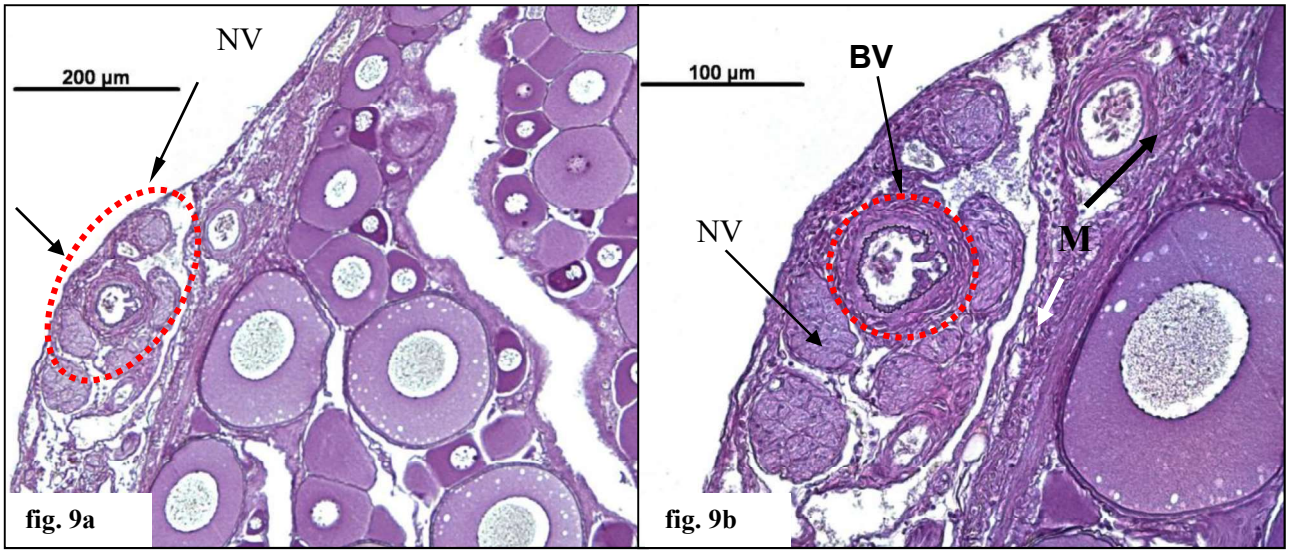


fig. 9 Cross-section of perch ovary. Arrows: cross-sections of nerve fibers that to innervate the muscularis of ovarian wall**.**
fig. 9a: higher magnification, different sections of nerve fibers**, Fig. 9b** BV: blood vessels, M: muscularis NV: nerve fibers

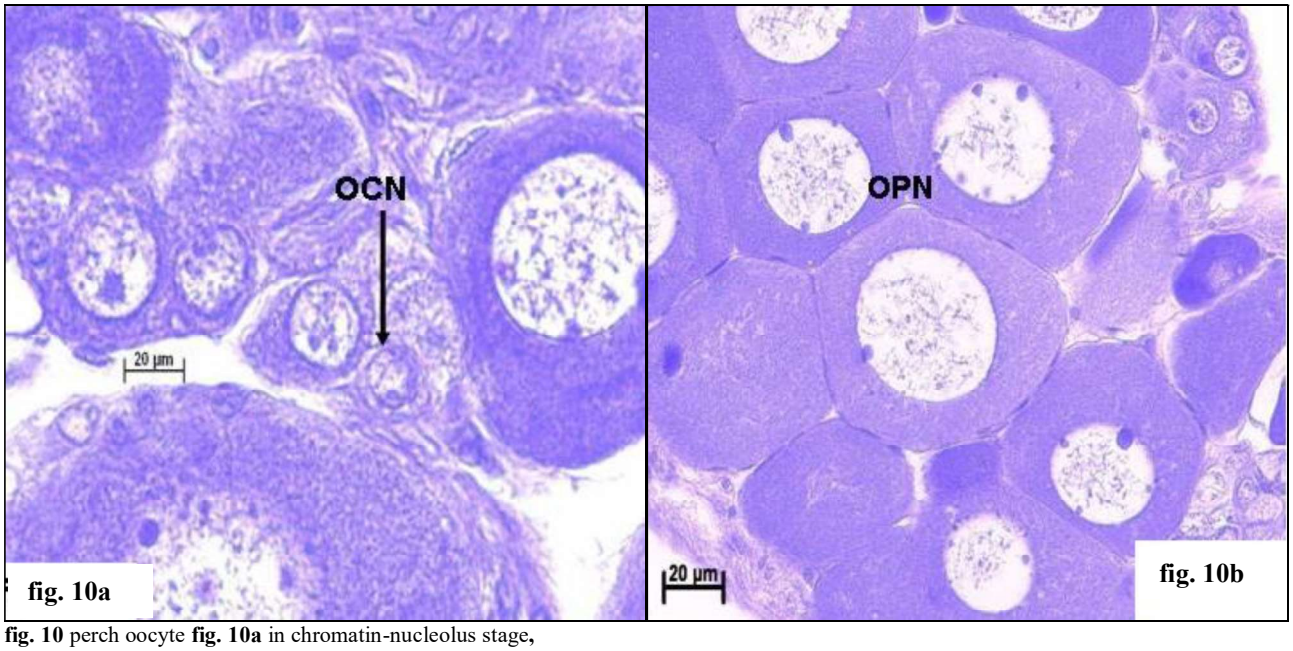


fig. 10 perch oocyte **fig. 10a** in chromatin-nucleolus stage**,**
fig. 10b in perinucleolus stage. OCN: oocyte in chromatin-nucleolus stage, OPN: oocyte in perinucleolus stage

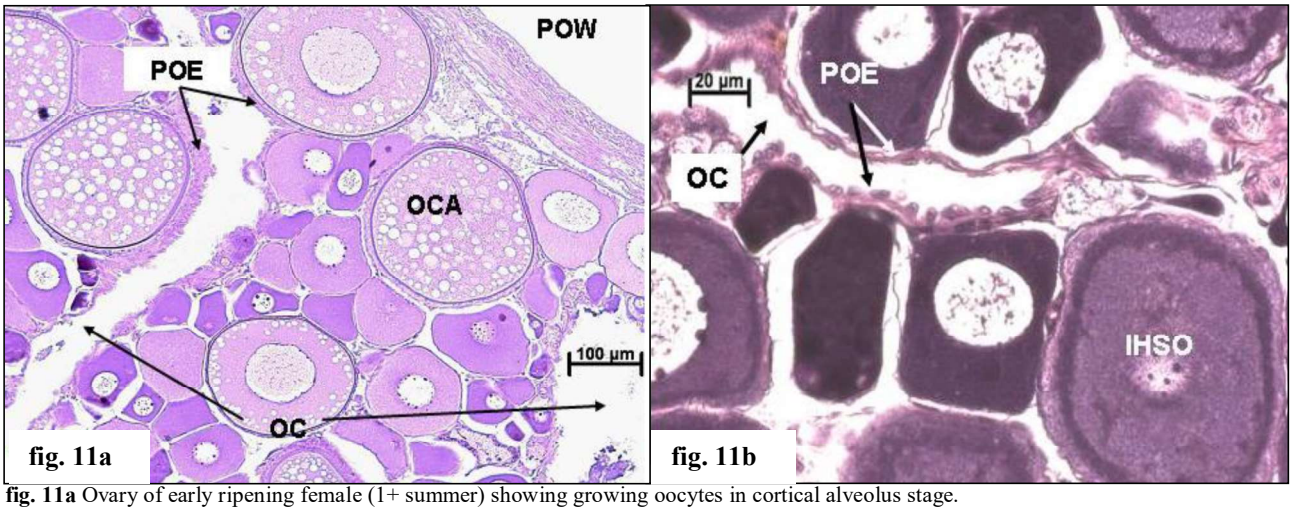


fig. 11a Ovary of early ripening female (1+ summer) showing growing oocytes in cortical alveolus stage.
fig. 11b Ovary of a late ripening male (1+ winter) showing oocytes with inhomogeneous ooplasma staining properties. IHSO: inhomogeneous staining ooplasma, OC: ovarian cavity, OCA: oocytes in cortical alveoli stage, POE: perch specific ovarian epithelium, POW: perch specific ovarian wall

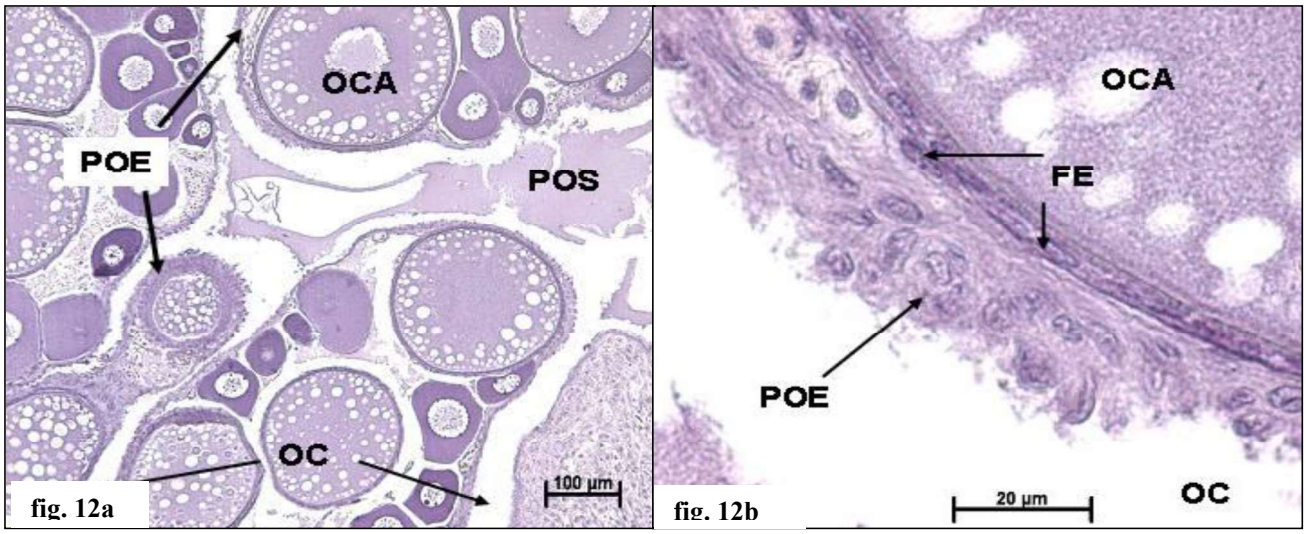


fig. 12a Ovary of late ripening female (2+ summer) showing oocytes in cortical alveoli stage, ovarian epithelium surrounding individual, growing oocytes. **fig. 12b** Higher magnification of oocyte periphery showing gonadal epithelium partly surrounding growing oocytes, peripheral ooplasma with lipid droplets, bilayered follicle cells and gonadal epithelium. FE: follicle epithelium OCA: oocyte in cortical alveoli stage, POS: perch specific ovarian secretion, OC: ovarian cavity, POE: perch specific ovarian epithelium,

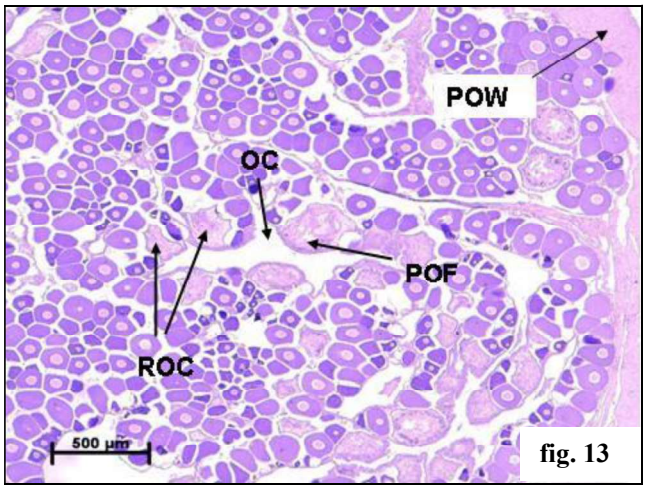


fig. 13 Resting ovary (summer) clearly showing histological features of previous spawning events (overview). OC: ovarian cavity, POF: postovulatory follicle, POW: perch specific ovarian wall, ROC: resorbtion of oocyte

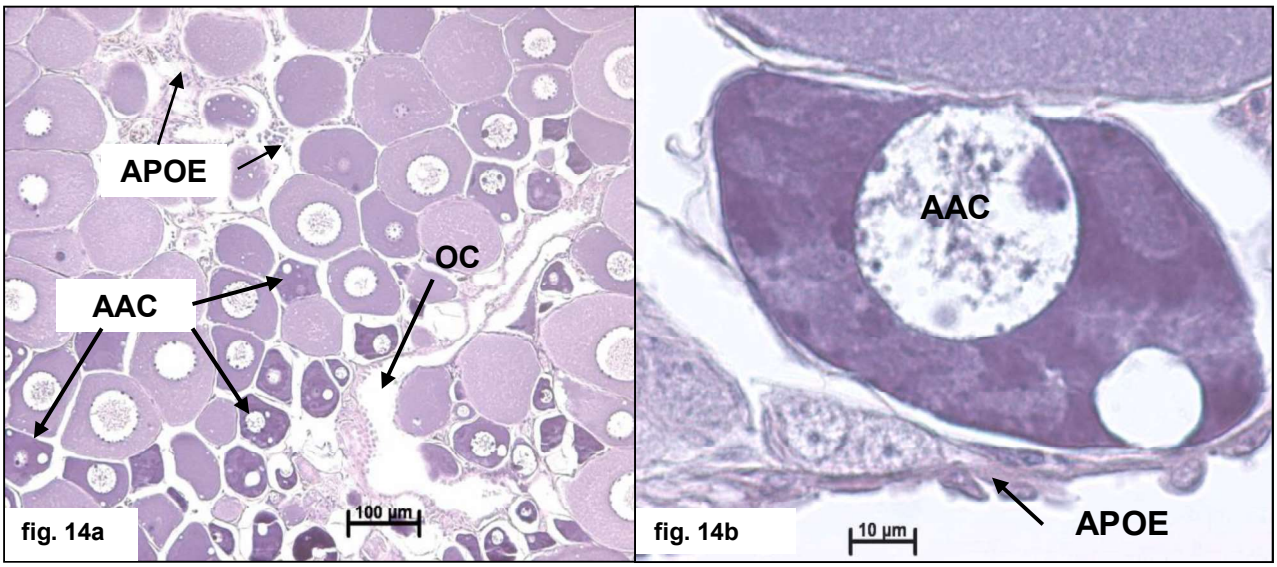


fig. 14 Ovary (Schwarzbach, 2+ winter) showing suppression of ovarian development on previtellogenic stage of oogenesis instead of mature oocytes. **fig. 14a** survey, **fig. 14b** Histopathological alteration of auxocyte cytoplasm.

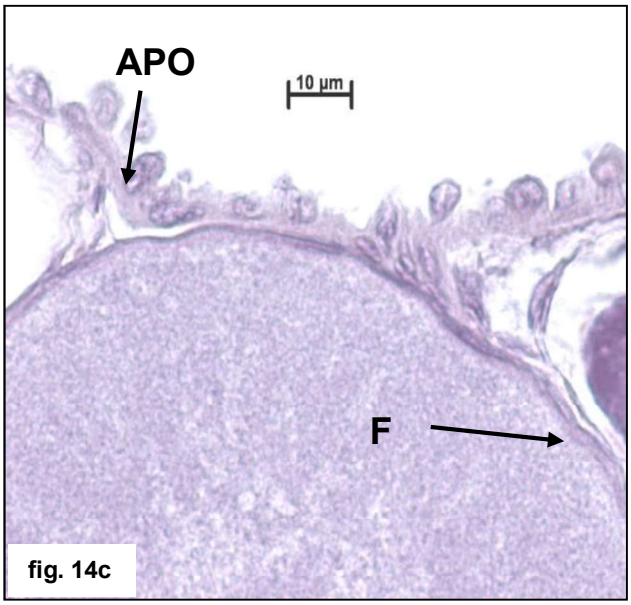


fig. 14c gonadal epithelium does not surround individual oocytes, follicle epithelium is single layered. AAC: altered auxocyte, APOE: altered perch specific ovarian epithelium, FE: follicle epithelium,

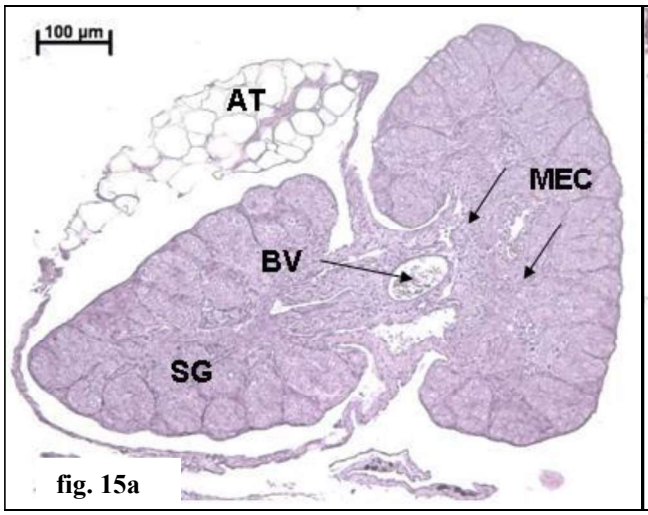


fig. 15a

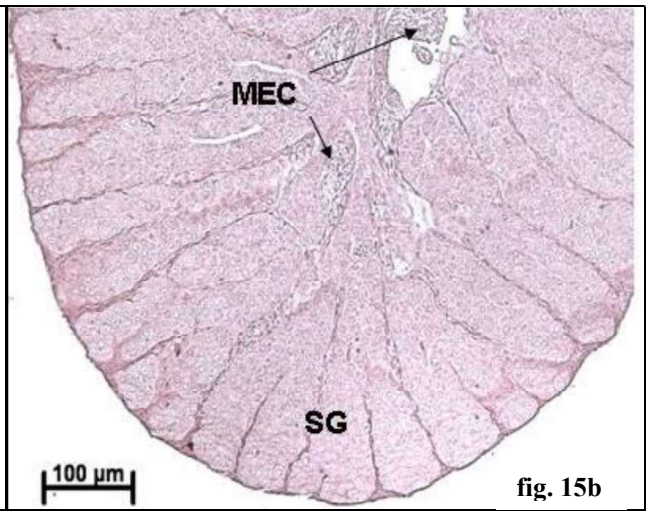


fig. 15b

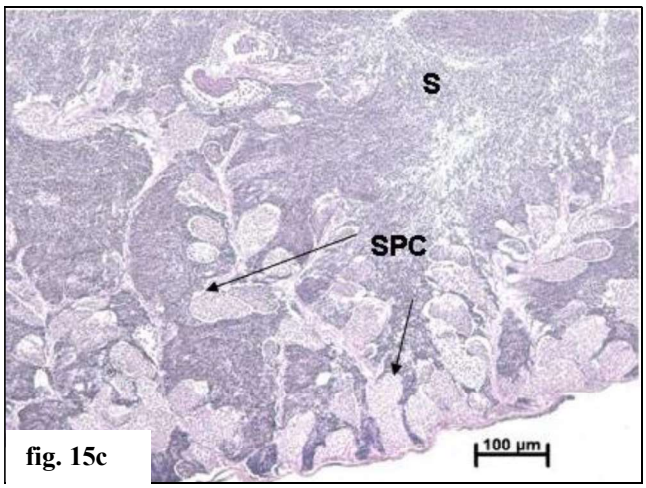


fig. 15c

fig. 15 Histological features of spermatogenesis. **fig. 15a** Caudal region of late maturing testes (0+ summer), juvenile testes. **fig. 15b** Caudal region of late maturing testes (1+summer), proliferation of secondary spermatogonia. **fig. 15c** Caudal region of late maturing testes (1+ winter) spermiohistogenesis.

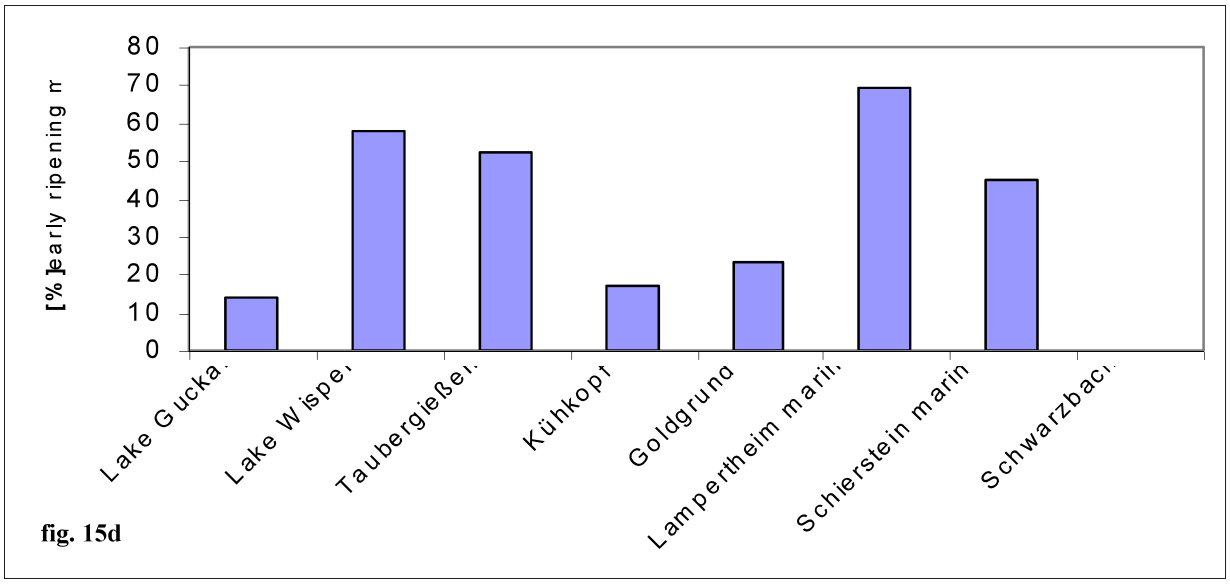
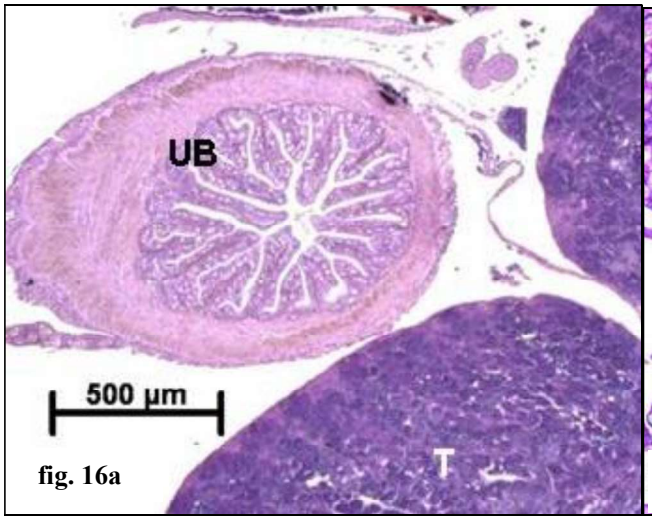


fig. 15d

fig. 15d Early ripening males in perch [%].AT: adipose tissue, BV: blood vessel, MEC: mesenchymal cells, SG: spermatogonia, SPC: spermatocytes, S: sperm.



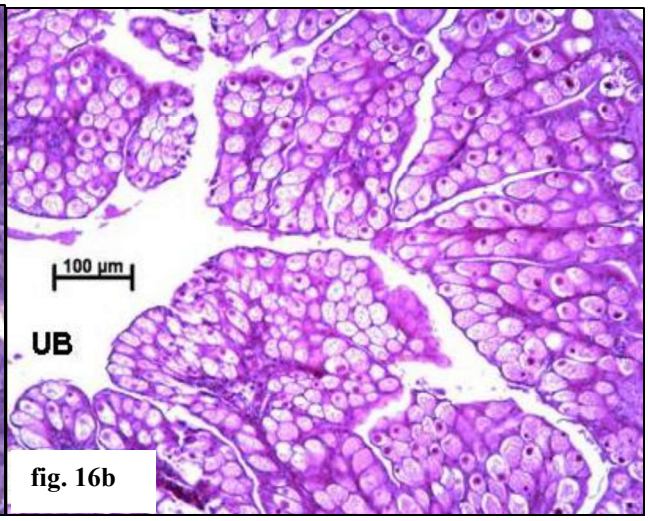


fig. 16a Urinary bladder of male perch in summer. **fig. 16b** Urinary bladder of male perch in march immediately prior to spawning.

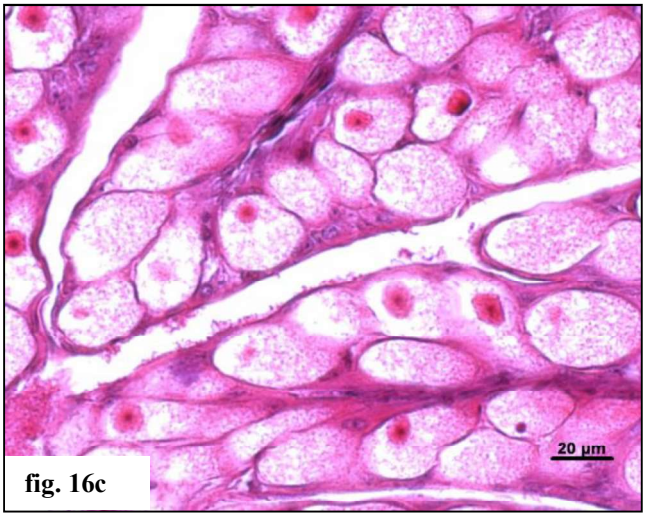
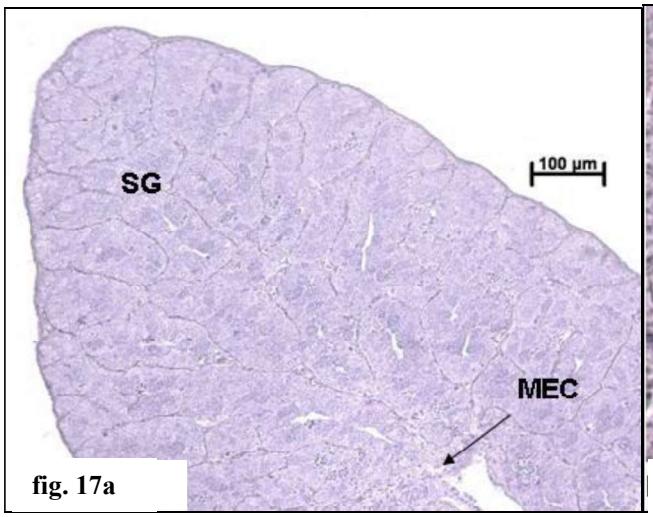


fig. 16c higher magnification. UB: urinary bladder.



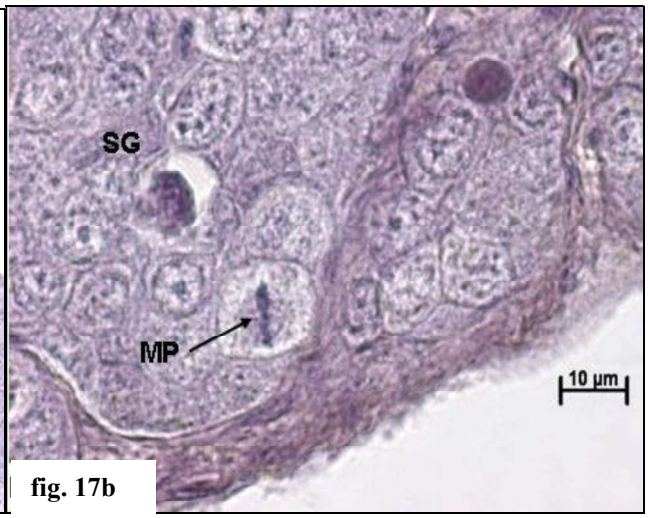


fig. 17 Gonadal recrudescence (2+ summer). **fig. 17a** Proliferation of secondary spermatogonia **fig. 17b** Higher magnification showing gonial mitosis primary- to secondary spermatogonium; MEC: mesenchymal cells, SG: spermatogonia, MP: metaphase plate

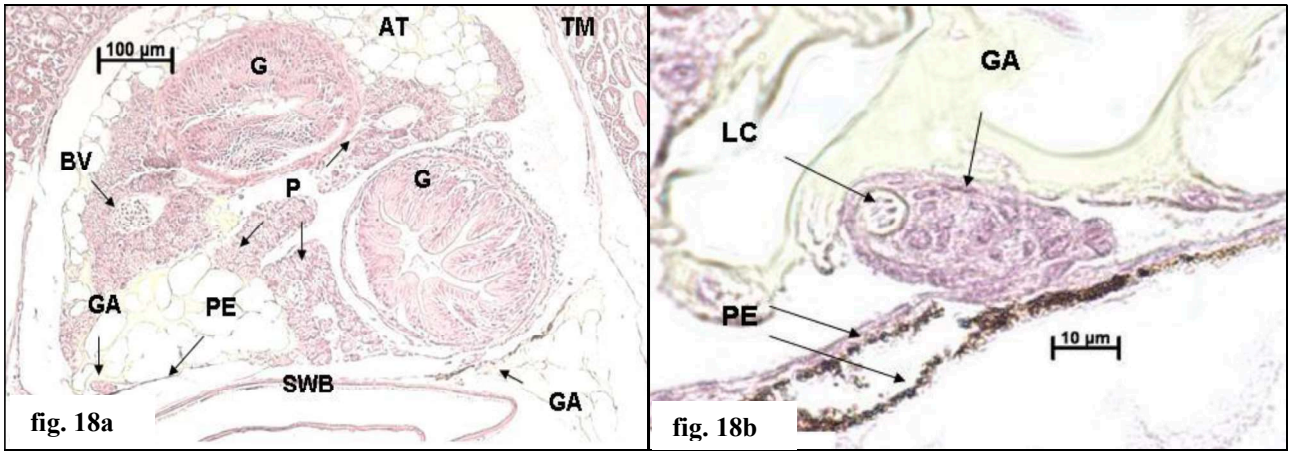


fig. 18a Cross-section through caudal body cavity of roach larvae (2 cm total length, 0+ summers). The gonadal anlage comprises only a few cells attached to the dorsal peritoneal epithelium.
fig. 18b Higher magnification of gonadal anlage. AT: adipose tissue, BV: blood vessel, GA: gonadal anlage, G: gut, LC: lymphatic cell, PE: peritoneum, P: pancreas, SWB: swim bladder, TM: trunk muscles

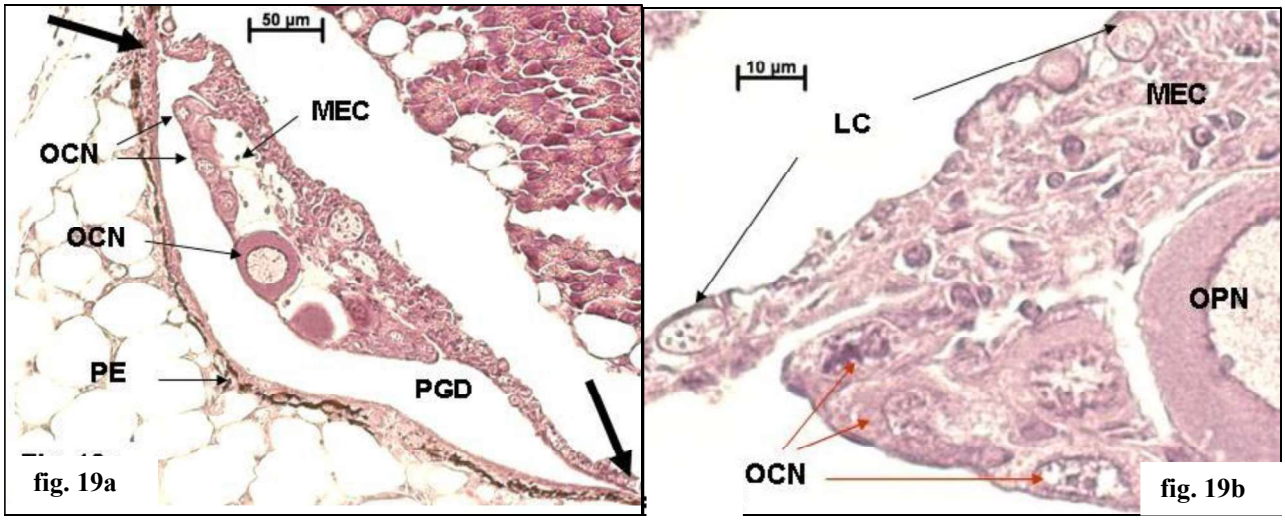
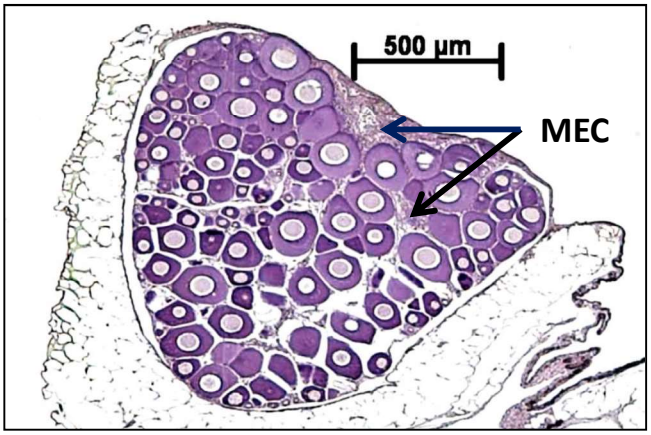


fig. 19 Roach fingerling (0+ summer). **fig. 19a** Ovary with two attachments to the peritoneal wall (arrows). Germ cells persisting on the oogonium stage are not visible indicating an immediate transformation to oocytes.
fig. 19b Higher magnification showing oocytes in chromatin- nucleolus stage; LC: lymphatic cell, MEC: mesenchymal cells, OCN: oocytes in chromatin-nucleolus stage, OPN: oocytes in perinucleolus stage, PE: peritoneum, PGD: prospective gonoduct

fig. 20a



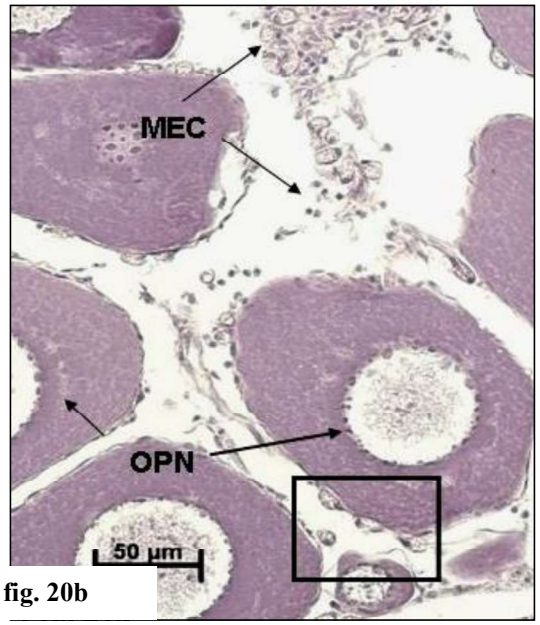


fig. 20b

fig. 20 Roach ovary in second summer (1+ summer), transition from the auxocyte to early vitellogenic step of endogenous oogenesis,
fig. 20a overview.
fig. 20b During the second summer a prominent mesenchymal tissue consisting of granulated lymphatic cells and undifferentiated motile cells appear in the gonadal interstitium.

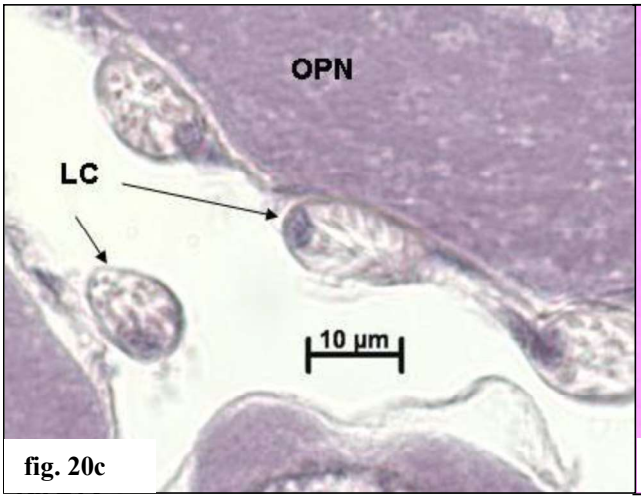


fig. 20c

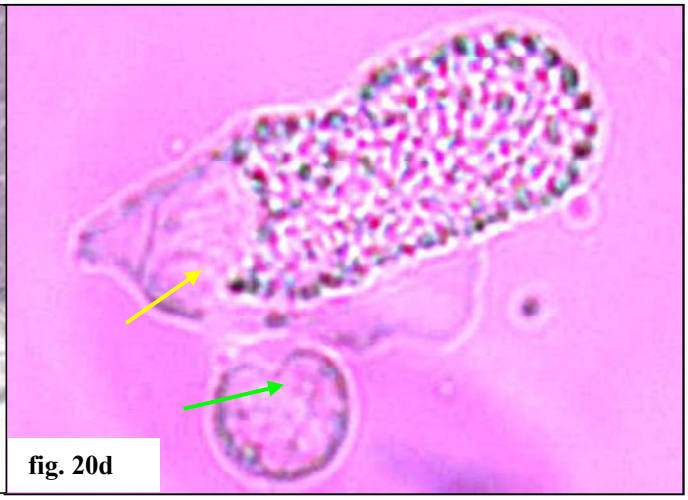
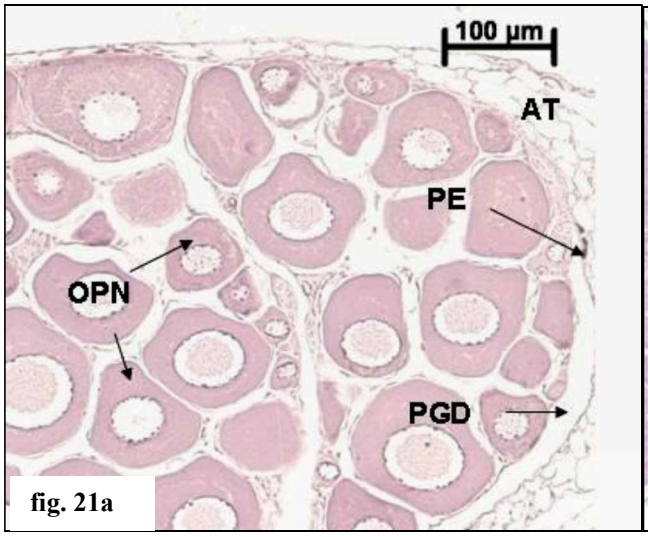


fig. 20d

fig. 20c Higher magnification (section of fig. 20b). AT: adipose tissue, LC: lymphatic cell, MEC: mesenchymal cells, OPN: oocytes in perinucleolus stage, PCD: prospective gonoduct,
fig. 20d Cells originating from *ex-vivo* preparations of (1+ summer) roach ovarian tissue. Green arrow: undifferentiated mesenchymal cell, yellow arrow: eccentric nucleus of granulated lymphatic cell, black arrow: hyaloplasm belonging to the granulated cells (enables motility of the cell type).



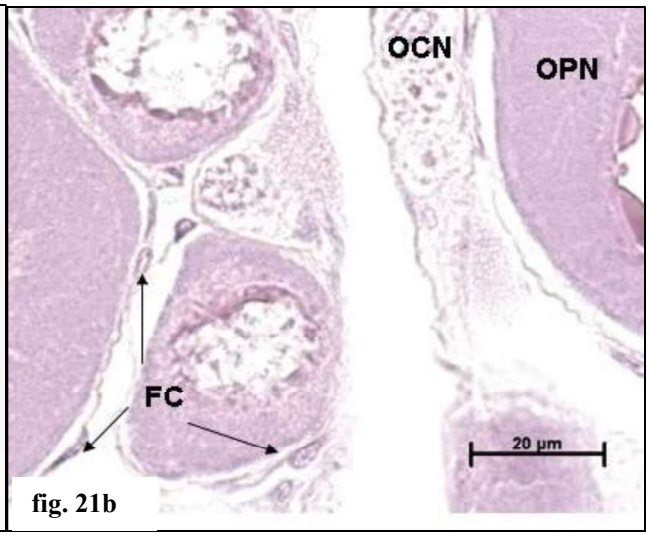
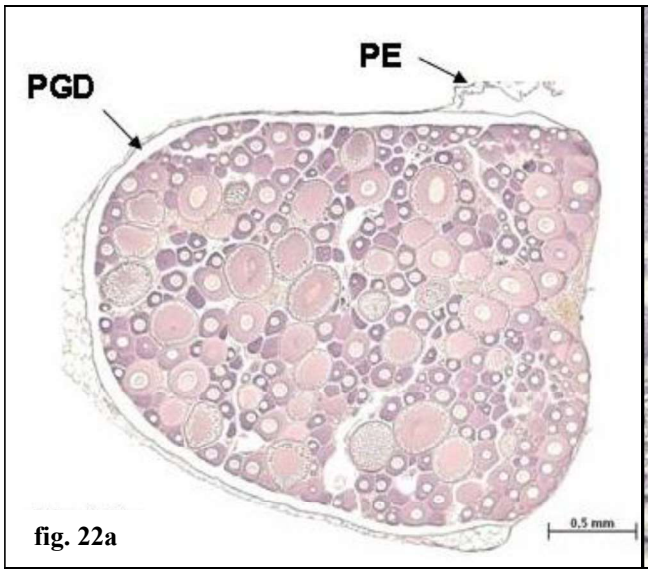


fig. 21a Roach ovarian tissue in second winter (1+winter), overview. During the second winter the oocyte cytoplasm becomes more eosinophilic.
fig. 21b Higher magnification, a single layered follicle epithelium becomes visible. AT: adipose tissue, FC: follicular cells, OCN: oocytes in chromatinnucleolus stage, OPN: oocytes in perinucleolus stage, PE: peritoneum, PGD: prospective gonoduct



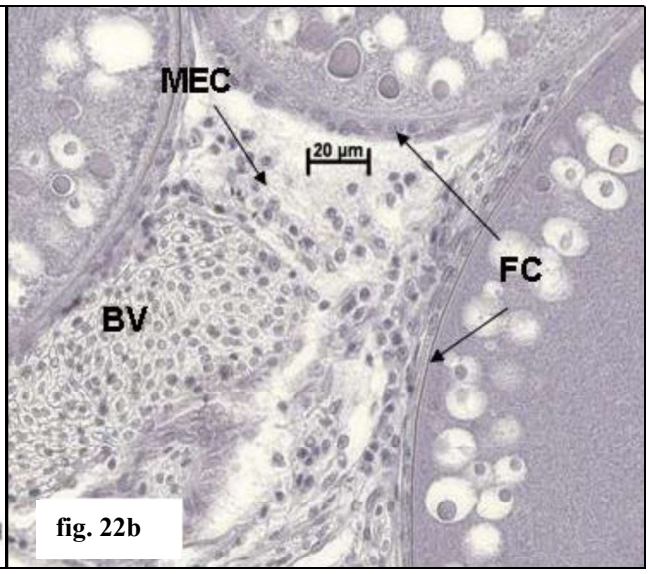
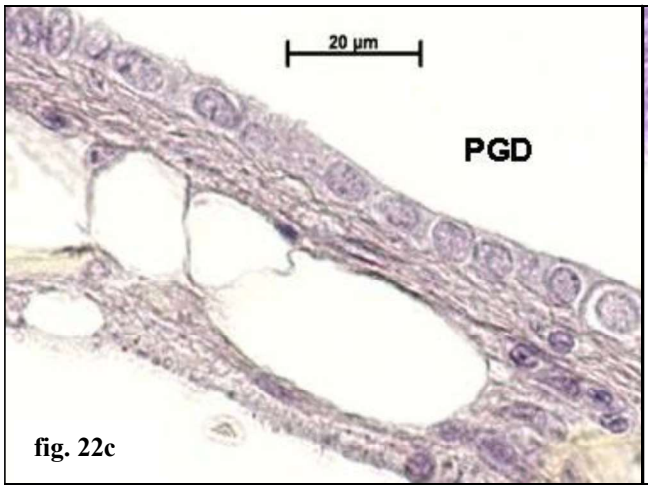


fig. 22 Ovary in the third summer (2+ summer), **fig. 22a** Ovary overview oocyte ripening starts in all females,
fig. 22b the gonad is colonized by undifferentiated cells contributing to the formation of a very tight packed bilayered follicle epithelium surrounding the growing oocyte in cortical alveoli stage, many blood vessels become visible



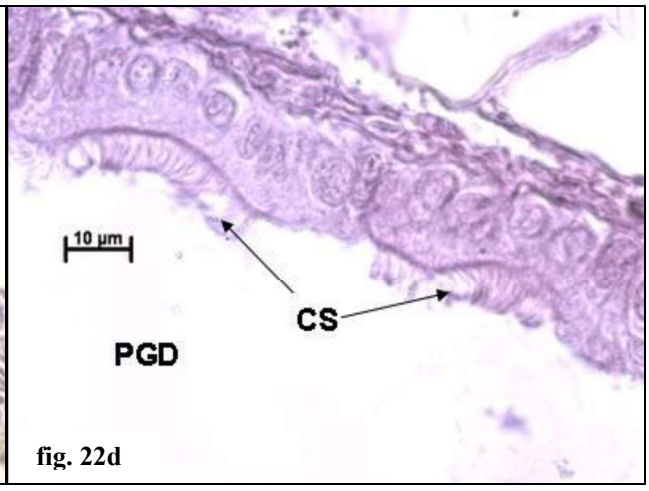


fig. 22c Oviduct epithelium. Oviduct epithelial cells become isoprismatic during summer
fig. 22d Temporary ciliated oviduct epithelium in late summer/autumn. BV: blood vessel, CS: cilia seam, FC: follicular cells, MEC: mesenchymal cells, PE: peritoneum, PGD: prospective gonoduct.

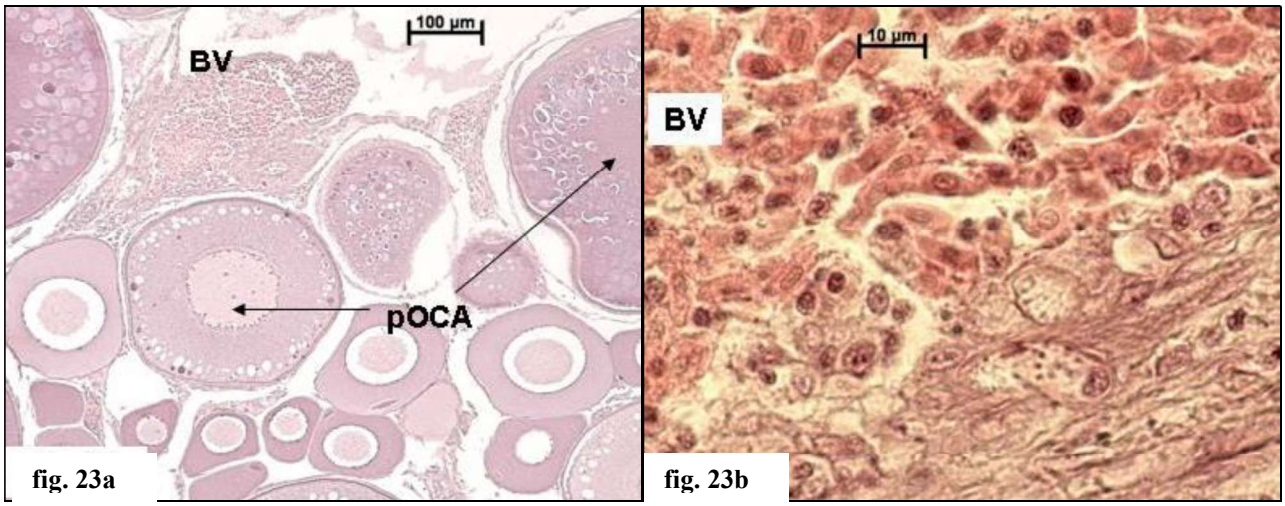


fig. 23 a-e Roach ovary (1+ summer) originating from a highly polluted creek
fig. 23a Seasonal and ontogenic precocious ripening of individual oocytes (cortical alveoli stage).
fig. 23b Immature blood cells.

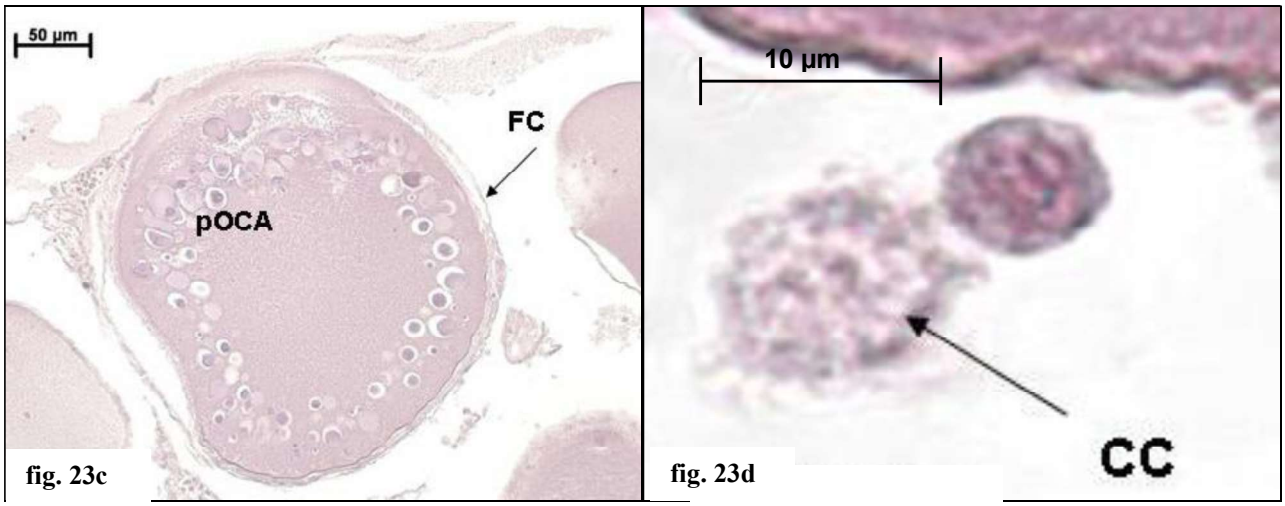


fig. 23c Deviations in forming of bilayered follicular epithelium.
fig. 23d Individual ciliated cells appear between the oocytes.

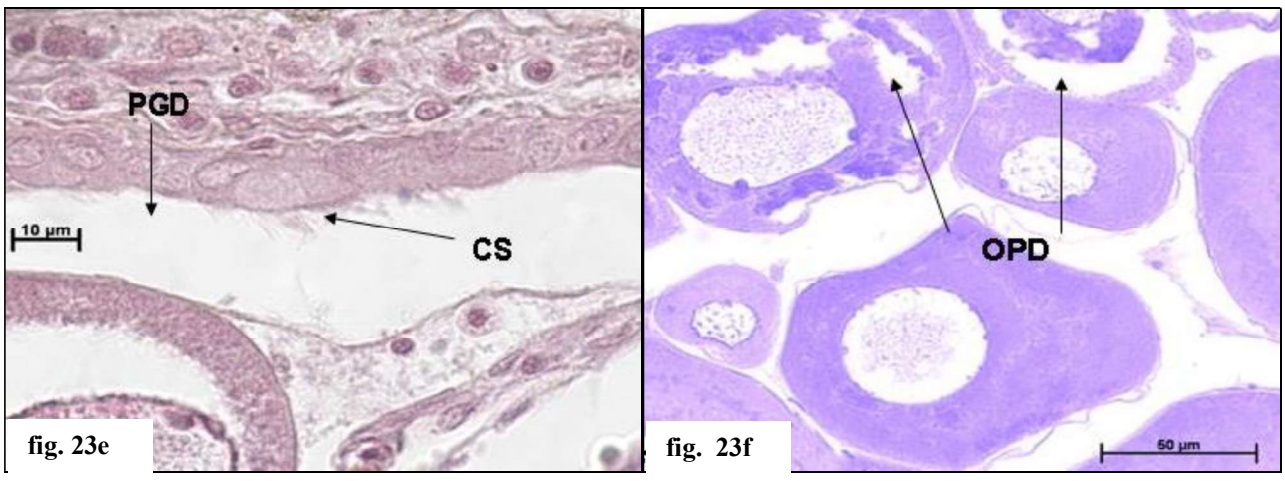
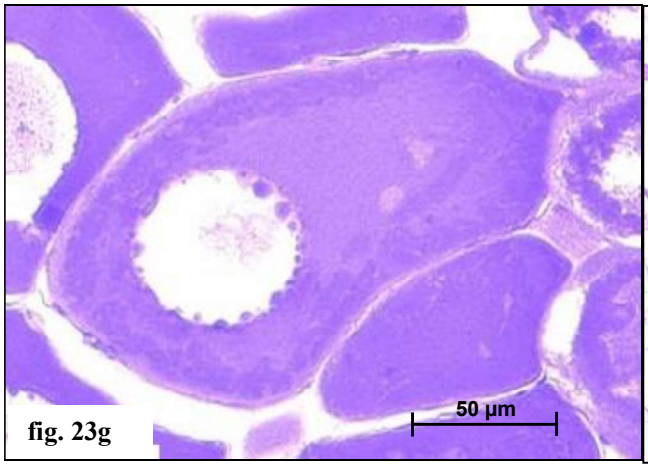


fig. 23e Gonoduct epithelium is thickened and shows cilia.
fig. 23f Roach ovary (2+ winter): Disturbed oogenesis on Schwarzbach site



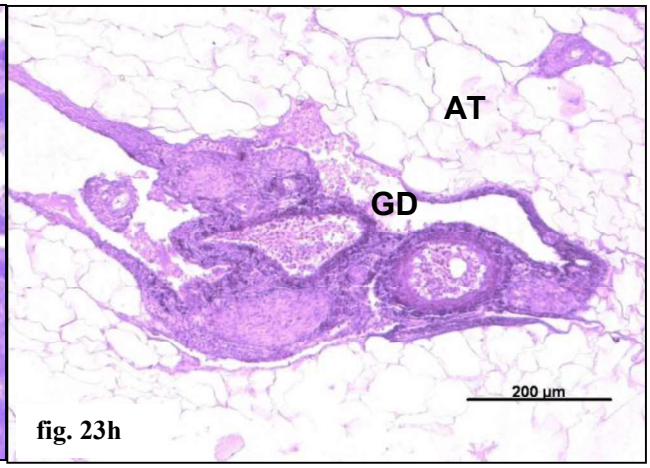
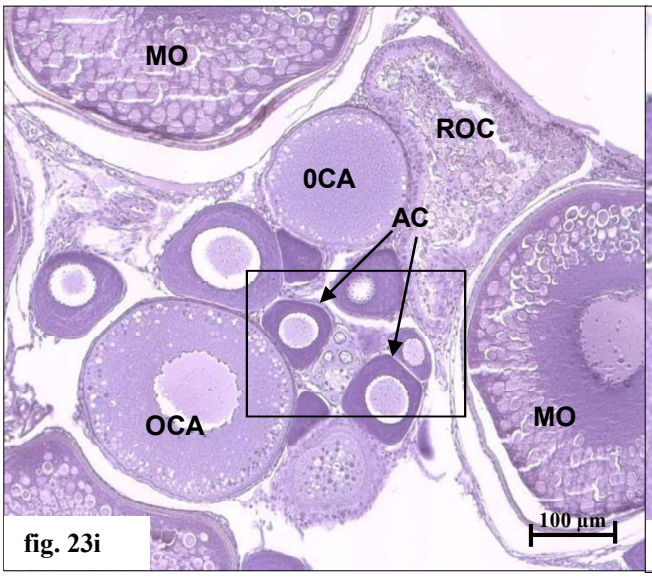


fig. 23g *Ligula intestinalis* related suppression of oogenesis.
fig. 23h Degenerated ovary of roach (3+ summer) from Schwarzbach-site.



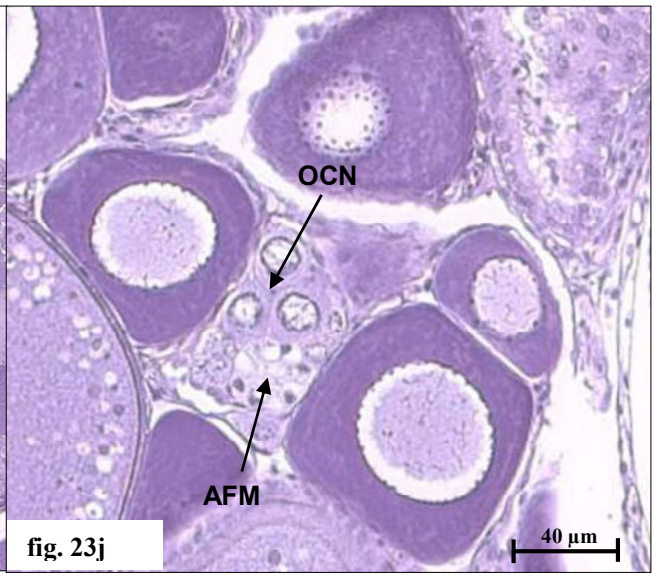
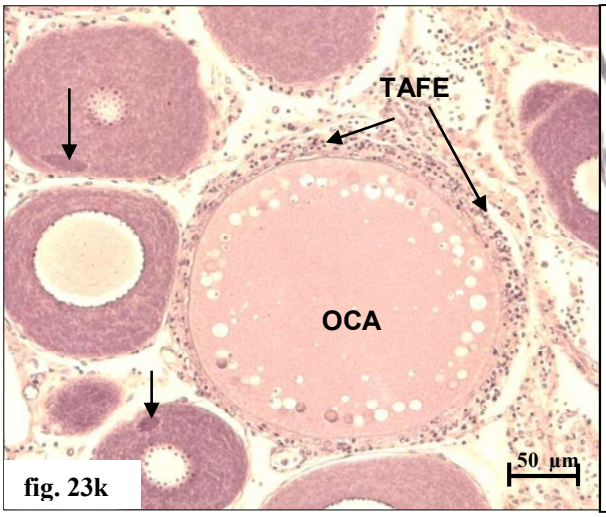


fig. 23i Roach 3+ summer caught in Schwarzbach asynchronous oogenesis comprising juvenile and adult winter and summer stages,
fig. 23j magnification of 22 e,



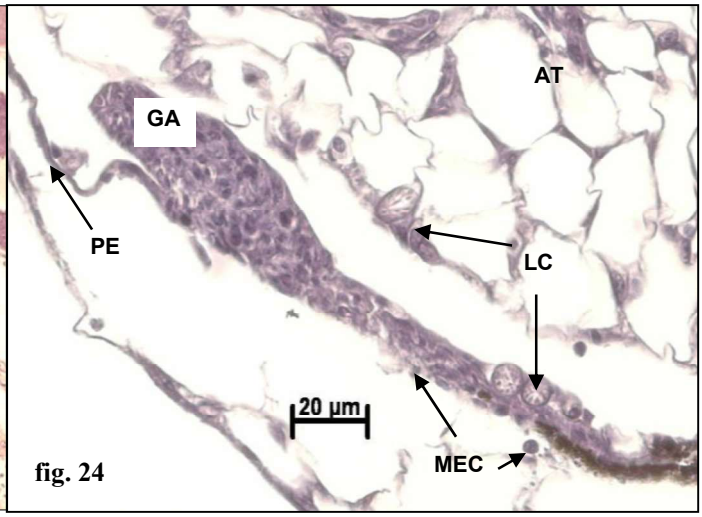


fig. 23k Roach 3+ summer caught in Schwarzbach single growing oocyte with tumourous alteration of follicle epithelium, arrows: atypical inhomogeneous cytoplasm staining in summer. AC: auxocyte, AFM: atypical female meioses, AT: adipose tissue, BV: blood vessel, CC: ciliated cells, CS: cilia seam, FC: follicle cells, GD: Gonoduct, MO: mature ovule, OCA: oocyte in cortical alveoli stage, OCN: oocyte in chromatin-nucleolus stage, OPD: oocyte plasma degeneration, PGD: prospective gonoduct, pOCA: precocious oocyte in cortical alveoli stage, ROC: resorbtion of oocyte, TAFE: tumourous altered follicle epithelium.

fig. 24 Testes of roach in late spring (0+ summer); male gonads are characterized by the absence of oocytes. Spermatogonia showing typical germ cell features are also missing. AT: adipose tissue, GA: gonadal anlage, LC: lymphatic cell, PE: peritoneum, MEC: mesenchymal cells

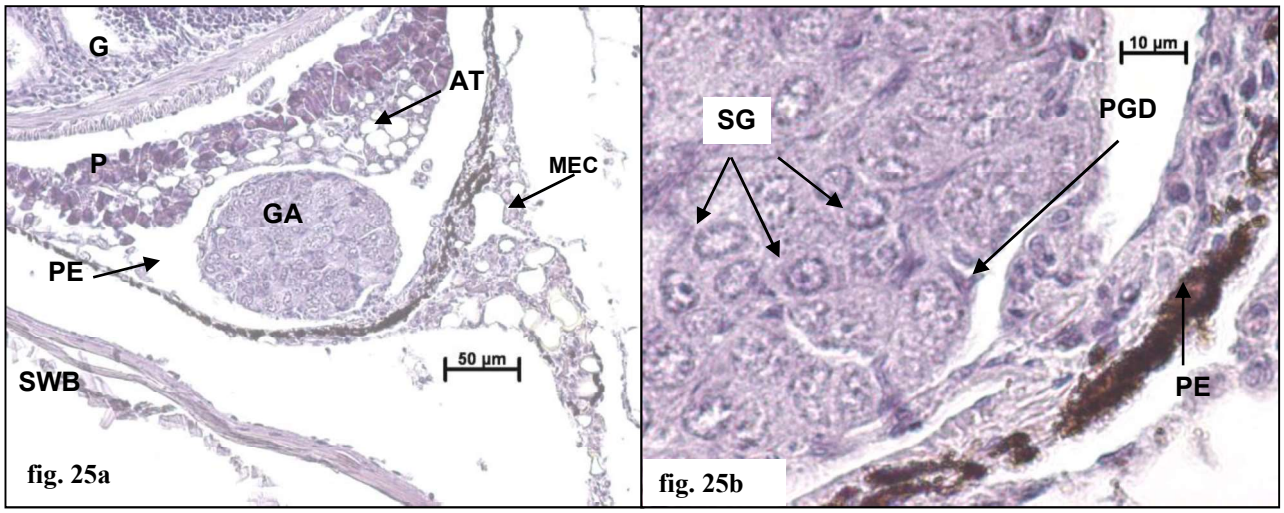


fig. 25 Testes of roach in late summer (0+ summer); Differentiation of spermatogonia, a typical gonadal hilum showing one attachment site to the peritoneum and a prospective sperm duct takes place until autumn of the first year, **fig. 25a** Dorsolateral overview of the body cavity. **fig. 25b** Higher magnification of the attachment site. AT: adipose tissue, GA: gonadal anlagef, G: gut, M: mesenchymal cells, P: pancreas, PE: peritoneum, PGD: prospective gonoduct, SG: spermatogonia, SWB: swim bladder

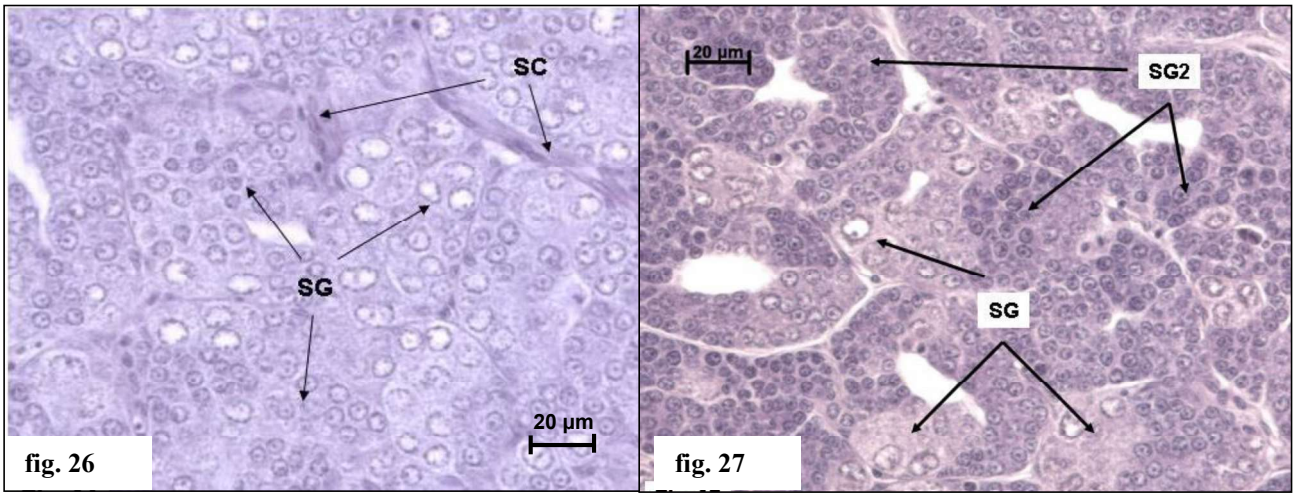


fig. 26 Testes of roach in second summer (1+ summer). The number of gonia increased during the second summer. Gonia were arranged in groups, pronounced mitotic activity is not recognizable. SG: spermatogonia, SC: somatic cells

fig. 27 Testes of roach in second summer (1+ summer). Transition of primary spermatogonia to secondary spermatogonia starts in late summer and results in sphere like gonial aggregations. SG: (primary) spermatogonia, SG2: secondary spermatogonia

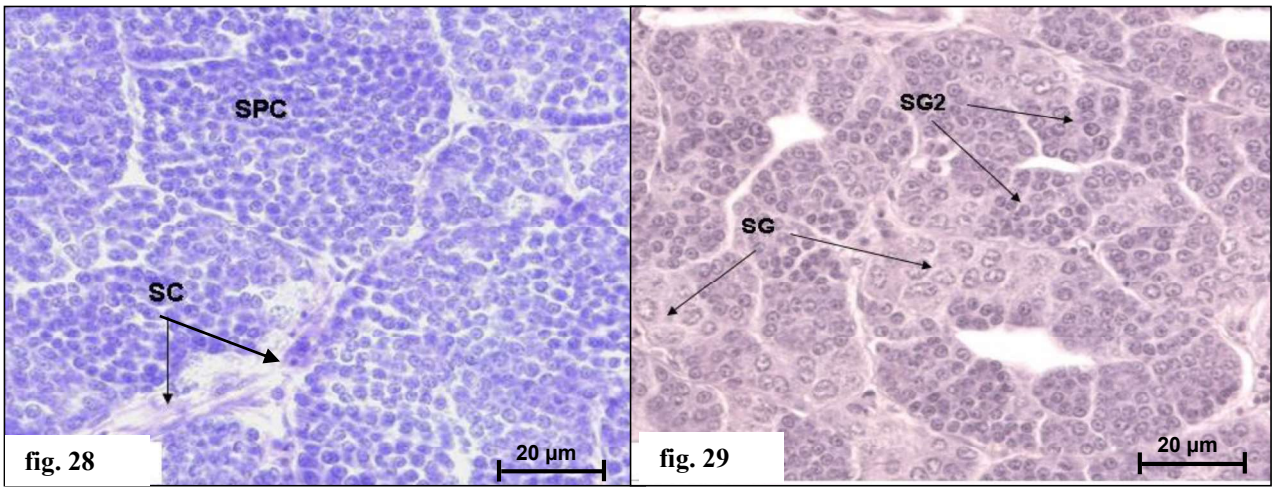


fig. 28 Testes of roach in second winter (1+ winter). Transition from secondary spermatogonia to spermatocytes is completed during second winter. SC: somatic cells, SPC: spermatocytes

fig. 29 (6+ autumn). Histological features of gonadal ripening are very similar in 1+ and elder fish during late summer and winter. SG: (primary) spermatogonia, SG2: secondary spermatogonia

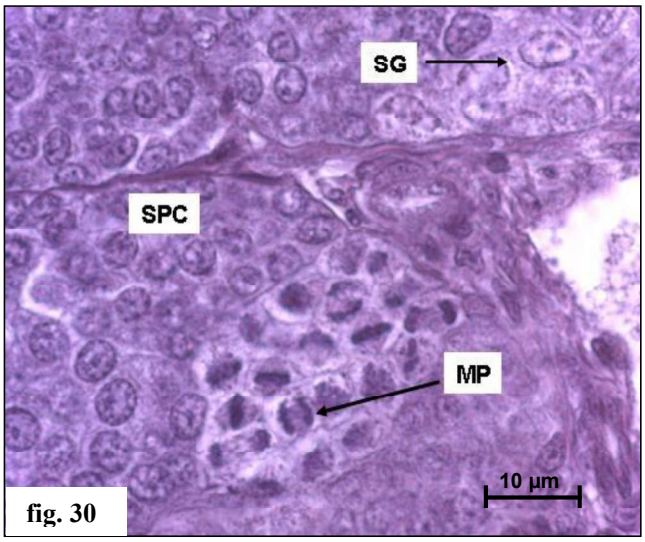


fig. 30 Testes of adult roach (4+ late summer). Pronounced mitotic activity was only related to transition of spermatogonia to spermatocytes (meiosis). MP: metaphase plates, SG: spermatogonia, SPC: spermatocytes

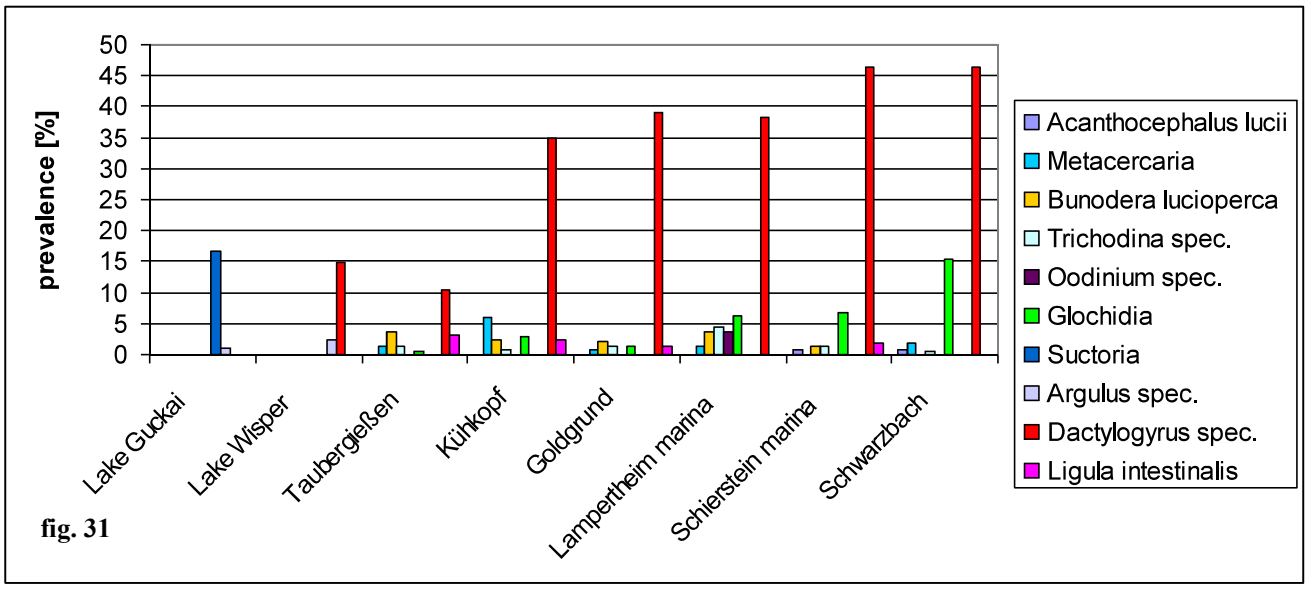


fig. 31 Prevalence of parasitical infects in roach.