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Ontogeny of sexual development in the roach (*Rutilus rutilus*) and its interrelationships with growth and age

Science report SC030299/SR1

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Steve Killeen

Head of Science

Executive summary

The roach (*Rutilus rutilus*) has become a sentinel species for the study of sexual disruption in wild fish populations as a consequence of exposure to endocrine disrupting chemicals (EDCs). However, little is known about the normal ontogeny of sexual development in this species and this is a major shortfall. Here, we analysed the ontogeny of sexual development and assessed how growth rate and fish size impacted on the timing of both sexual differentiation and sexual development in the roach.

Ovarian differentiation in roach was first recorded at 68 days post fertilisation (dpf) and this preceded testicular differentiation (first recorded at 98 dpf). In contrast, sexual maturation occurred at an earlier age in males (300 dpf) compared with females (728 dpf). No differences in body size (length or weight) were recorded between male and female roach until the fish were between 415 and 728 dpf. Studies on three populations of roach which grew at different rates showed that the timing of sexual differentiation was highly variable and more related to fish size than to fish age.

Time to sexual maturation was also variable between populations but, subsequent to their first year of life, gonadal status was less well associated with fish size. Interestingly, fish growth rate during early life appeared to affect the subsequent gender balance of the population, biased to females in more rapidly growing fish. The fact that captive populations of roach are able to complete sexual differentiation within 112 dpf, and sexual maturation within 728 dpf, makes the species practicable for studies in the laboratory assessing effects of EDCs on sexual differentiation and development, as well as for studies in the field on wild populations.

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1 Introduction

Endocrine disrupting chemicals (EDCs) in the aquatic environment have been associated with deleterious impacts on development, reproduction, and on wider aspects of health in a wide range of wildlife species (reviewed in Tyler et al. 1998, 2002) and, possibly, humans too (Sharpe and Skakkeback 1993, Colborn et al. 1996). In the UK, much of the evidence for endocrine disruption in wildlife has come from studies on wild populations of roach (*Rutilus rutilus*) exposed to EDCs present in effluent discharges from sewage treatment works (STWs).

Endocrine disruption in roach was discovered when Thames Water Authority, acting as a result of casual observations by anglers, found a 5% incidence of intersex (the simultaneous presence of both male and female germ cells) in wild populations living just downstream of a STW effluent discharge in the River Lea catchment, South East England (Sweeting 1981). This finding was deemed unusual because roach are a gonochoristic species (they have either a distinct testis or ovary and the sexes are stable) and this raised the possibility that compounds in STW effluents might be causing disruptions in sexual development. More recent studies have confirmed sexual disruption is widespread in UK roach populations living in STW effluent-contaminated waters (e.g. Jobling et al. 1998, 2002a, 2006). Furthermore, the intersex condition most likely results from the feminisation of male roach due to oestrogenic substances present in STW effluents, notably steroidal oestrogens and alkylphenols (Desbrow et al. 1998, Harries et al. 1999). Importantly, intersex roach have been found to have reduced fertility (Jobling et al. 2002b), which may in turn have population-level consequences.

Mesocosm studies exposing roach to treated STW effluents have confirmed that feminisation of the reproductive duct in male fish (formation of a female-like ovarian cavity in the testis) occurs as a result of exposure to STW effluent during early life (Rodgers-Gray et al. 2001, Liney et al. 2005). True intersex (germ cell disruption) in roach, however, has yet to be induced from controlled exposures to treated sewage effluent. Nevertheless, laboratory exposures of fish to the oestrogenic components present in STW effluents have been shown to cause intersex (e.g. Brion et al. 2004, reviewed in Mills and Chichester 2005), albeit these effects generally occur at concentrations higher than those found in the riverine environment.

Thus, the impacts of STW effluents on sexual development in roach are not fully understood and comprehensive assessments of the effects of STW effluents on sexual development can only be done effectively against a background of detailed information on the normal pattern and timing of gonad differentiation and development in this species. Detailed histological descriptions of normal gonad differentiation and development in fish used in studies on ecotoxicology have been conducted for relatively few species and for the most part have predominantly focused on so-called model species (e.g. medaka (*Oryzias latipes*, Satoh 1974, Hamaguchi 1992), fathead minnow (*Pimephales promelas*, van Aerle et al. 2004) and zebrafish (*Danio rerio*, Takahashi 1977, Maack and Segner 2003)). Information on the progression of sexual differentiation and development for roach and other fish species native to the UK waters is limited.

The roach is a particularly good representative species for studies on the effects of EDCs in fish. This is because it is widespread throughout the UK and found in a range of habitats (even including brackish waters), it is tolerant of poor water quality (and is, therefore, amenable for effluent exposures in which oxygen levels are typically low and sediment loads high), it is a cyprinid fish (one of the largest families of freshwater fish) and, like most other cyprinids, it is a group spawner, and, as such, the principles derived from work on roach are likely to be widely applicable to other group-spawning

species. Moreover, the roach can be easily cultured in the laboratory and as a native coldwater species there is no requirement to heat the tank water (as for the more commonly used fish species for studies on EDCs in the laboratory, including the medaka, fathead minnow and zebrafish), which may affect the stability/nature of the EDCs under study.

The principal aim of this study was to develop an atlas documenting the ontogeny of sexual differentiation and development in the roach. In addition, through the use of different populations of roach reared under varying conditions, the interrelationships between growth and age and the timing of sexual development were established. These studies provide the basic reference information against which the effects of EDCs can now be examined more effectively. The data gathered also allow for comparisons of sexual development (and effects of EDCs) between a wild indigenous UK fish species and non-native fish species, including the zebrafish, fathead minnow and medaka, that are used most extensively for assessing the impacts of EDCs under laboratory conditions and for the development of OECD test guidelines.

2 Methods

2.1 Fish source, culture and husbandry

For this study, fish were sampled from three independently maintained populations of roach. To produce these populations, pre-spawning, sexually mature male and female roach were obtained from the Environment Agency's National Coarse Fish Farm (Calverton, Nottinghamshire, UK) and were artificially induced to spawn using established procedures with carp pituitary extract (see Jobling et al. 2002b). The resulting embryos were deployed into the culture systems described below. Embryos hatched 7–10 days post fertilisation (dpf) in all three populations and the roach fry were fed with Cyprico Crumble EX (Coppens International bv, Helmond, The Netherlands) dry food (0.01–0.2 mm) and freshly hatched *Artemia* sp. nauplii until satiation. As the fish grew they were fed progressively larger grades of the Cyprico Crumble EX dry food fed, increasing from 0.01–0.2 mm to 0.2–0.3 mm to 0.5–0.8 mm and finally up to 1 mm Cyprico Start Premium EX. Any variation in feeding between the populations is highlighted below.

Population 1 was reared at the Environment Agency's National Coarse Fish Farm (Calverton, Nottinghamshire, UK), where the fish were maintained in the hatchery from May until November and then transferred into large (2 m x 2 m x 0.7 m) tanks on a recirculation system and held at 20–22 °C with a fixed photoperiod regime of 20 h:4 h light:dark for the remainder of the study.

Population 2 was reared in the fish culture unit at the University of Exeter in glass aquaria under flow-through conditions (exchanging one tank volume of dechlorinated tap water, filtered to 5 µm, per day). Embryos were hatched and maintained in 20 L tanks until approximately 140 dpf at which point approximately 150 fish were transferred into each of 2 x 45 L tanks. Fish were sampled periodically from these tanks until 300 dpf, when 60 fish were remaining in each tank. These fish were then transferred into 2 x 70 L tanks for the remainder of the study. All tanks were maintained at 18 ± 2 °C with a fixed photoperiod regime of 16 h:8 h light:dark. From December to February of the second year the temperature was gradually reduced down to 12 ± 2 °C before being increased again (in a stepwise manner) back up to 18 ± 2 °C by April. The photoperiod regime was also reduced during this same time period to 12 h:12 h light:dark. From 250 dpf onwards, Cyprico Start Premium EX (1 mm) was supplemented with frozen gamma-irradiated brine shrimp (*Artemia* sp.) and bloodworm (*Chironomus* sp.).

Population 3 was reared in the fish culture unit at the University of Exeter in glass aquaria under flow-through conditions (exchanging at least one tank volume of dechlorinated tap water, filtered to 5 µm, per day). Approximately 1000 embryos were hatched and maintained in 5 x 20 L tanks until 35 dpf at which point 250 fish were transferred into 10 x 45 L tanks (25 fish per tank). Fish were sampled at regular time intervals (keeping the density of fish approximately the same in each tank) until 154 dpf when the remaining 170 fish were transferred into 8 x 70 L tanks (approximately 20 fish per tank). These fish were again sampled at regular time intervals (keeping the densities approximately the same in each tank). All tanks were maintained at the ambient (incoming) water temperature (20 ± 2 °C May to October, 13 ± 2 °C November to December, 8 ± 2 °C January to March, 13 ± 2 °C in April and then gradually increasing to 20 ± 2 °C by May). The photoperiod regime was also adjusted from 16 h:8 h light:dark between May and September in a stepwise manner to 12 h:12 h light:dark between October and March, and then in a stepwise manner back to 16 h:8 h

light:dark by May, to simulate ambient seasonal changes. From 35 dpf onwards, Cyprico Crumble EX dry food was supplemented with frozen gamma-irradiated brine shrimp (*Artemia* sp.) and bloodworm (*Chironomus* sp.) and TetraMin® dry coldwater flake food (TetraMin, Tetra Werke, Melle, Germany).

2.2 Biological sampling, growth analyses and histological analyses

Fish were sampled randomly from each of the three populations at various time points. The age of fish and numbers of fish sampled are shown in Table 2.1.

Population 1		**Population 2**		**Population 3**	
Age (dpf)	Number of fish analysed	Age (dpf)	Number of fish analysed	Age (dpf)	Number of fish analysed
68	20	64	10	28	4
102	20	92	33	56	10
137	20	120	26	70	10
173	20	260	53	84	10
207	10	526	35	98	10
271	20	728	26	112	10
313	20			126	10
368	20			140	10
415	20			154	10
473	20			182	10
				210	10
				300	18
				400	30

Table 2.1 Age and number of fish sampled from each population.

Fish were sacrificed by terminal anaesthesia with benzocaine (ethyl-p-aminobenzoate; Sigma, Poole, UK), as approved by the UK Home Office (Animals (Scientific Procedures) Act 1986. Fork length (mm) and wet weight (g) were recorded for each fish sampled. Either fish as whole bodies or dissected gonads were fixed in Bouin's fixative (Raymond A. Lamb Ltd, Eastbourne, UK) for 4 h (dissected) or 24 h (whole bodies) and then transferred into 70% industrial methylated spirits (IMS; Fisher Scientific, Loughborough, UK) for processing. For the fish fixed as whole bodies, transverse sections were taken from the mid-body region between the base of the pectoral fin and the urogenital papilla, thus including the gonad. Body sections or dissected gonads were subsequently dehydrated and embedded in paraffin wax (Sigma-Aldrich, Gillingham, Dorset, UK) using a Shandon tissue processor (Thermo Electron Corporation, Runcorn, UK). Serial sections (5 µm thick) were cut throughout the gonad for early-life stage fish and at every 100 µm throughout the gonad of fish with well-developed testis or ovaries (the timing of which varied between each population), collected onto glass slides, stained with Shandon haematoxylin and eosin (Thermo Electron Corporation) and then treated with Histomount (National Diagnostics, Hessle, Hull, UK). Histological sections were then analysed by light microscopy (Zeiss

Ltd, Oberkochen, Germany) and digital images were obtained using an Olympus DP70 CCD camera (Olympus Optical, UK) coupled to analySIS 3.2 software (Soft Imaging System GmbH, Munster, Germany).

In general, the status of gonadal development in the fish was determined by the presence of primordial germ cells (PGCs), gonadal duct formation and, in later life, by staging of the germ cells (e.g. according to Nolan et al. 2001, van Aerle et al. 2004). However, in order to distinguish males from females in early life (prior to the presence of male/female-specific germ cells) the shape of the gonad (club-shaped for males; elongated for females), the number of points of attachment of the gonad to the body wall (one for males, two for females) and the distribution of somatic cells relative to primordial germ cells (interspersed throughout the developing gonad for males, localised around the periphery of the developing gonad for females) were all used as diagnostic features for assigning sex. At this early life stage where there was no distinction between the germ cells for gender these fish were classified as 'presumptive males' and 'presumptive females'. Fish that were described as 'undifferentiated' referred to those individuals whose sex could not be classified as male or female or as presumptive male or female using the above criteria, using light microscopy. Sexual maturity was defined as the presence of spermatozoa within the testis lumen of males and mature (diameter *c*. 1 mm) vitellogenic stage oocytes within the ovary of females.

The condition factor (K) was calculated for each fish as:

$K = \text{Weight (g)}/\text{Length (cm)}^3 \times 100$

Specific growth rates (SGR) based on weight (SGR_W; % body weight per day) and length (SGR_L; % body length per day) were calculated for each population (as described in Saillant et al. 2001) encompassing the period at which all fish had completed sexual differentiation irrespective of time (population 1, 68–271 dpf; population 2, 64–260 dpf; population 3, 28–112 dpf) and during sexual maturation (i.e. during the remainder if the study; population 1, 271–473 dpf; population 2, 260–728 dpf; population 3, 112–400 dpf) according to the following formulae:

$$SGR_W = 100[\ln(W_f) - \ln(W_i)]/(t_f - t_i),$$

$$SGR_l = 100[\ln(L_f) - \ln(L_i)]/(t_f - t_i)$$

W_f = final weight, W_i = initial weight, L_f = final length, L_i = initial length, t_f = final time (dpf), t_i = initial time (dpf).

2.3 Data analysis

Analysis of the data is divided into two main sections. In the first section a description of the ontogeny and timing of gonadal sexual development is presented and is based on the most advanced fish at the time of sampling for each of the three populations studied. In the second section an analysis of the interrelationship between growth and timing of sexual development was undertaken, based on the growth parameters (length, weight and condition) and associated germ cell development for every fish sampled in each of the three populations.

2.4 Statistics

Unless stated otherwise, all data are presented as means ± SEM. All statistical analyses were performed using Sigma Stat version 2.0 (Jandel Scientific Software,

USA). Differences between experimental groups were analysed by Student's t-test or One-Way Analysis of Variance (ANOVA), followed by Tukey's multiple comparison post-hoc test, or a non-parametric alternative (Kruskal-Wallis One-Way ANOVA on ranks) where data were not normally distributed. All data were considered statistically significant at $P < 0.05$.

3 Results

3.1 Section 1: Ontogeny and timing (age: days post fertilisation) of gonadal sexual development

3.1.1 Location of the gonad in the body cavity

The gonads of male (testis) and female (ovaries) roach were comprised of paired structures located in the dorsal region of the visceral cavity (either side of the swim bladder; Figure 3.1), extending approximately from the origin of the pectoral fin along the length of the body to the urogenital papilla. In males each testis was attached to the peritoneal wall by a single duct (sperm duct) and in contrast in females each ovary was attached to the peritoneal wall by two ducts (oviducts), one at each end of the ovary and forming an ovarian cavity.

3.1.2 Locating the gonad during early life stages

In order to identify the developmental stage of the gonad during early life, when the gonad was typically very small, serial whole body sections (5 µm thick) were cut, starting from the base of the pectoral fin and moving in a posterior direction along the length of the body. The gonad typically started to appear shortly after the gut had become divided into three parts (Figure 3.1). This provided us with an additional reference point from which to locate the gonad at the anterior end. Capturing sections across the entire length of the gonad during early life was important for staging the gonad; only very few cells occur in the gonad at this stage and these vary along its length. At the anterior end somatic cells only occur and at the periphery of the gonad, germ cells occur in the central region of the gonad, and then somatic cells only again at the posterior end. In addition, the placement of the gonad in relation to the peritoneal wall differed along its length and thus the further need to section the whole gonad to enable accurate reporting of duct formation.

3.1.3 Gonad development from early life to sexual maturity

3.1.3.1 Undifferentiated gonad: At 56 dpf and onwards, gonads were identifiable in all fish studied. However, it was not possible to determine the sex of these fish based on the histological appearance of the gonad. The gonads were very small (*c*. 40 µm in diameter), typically ovoid and consisted either of just a small cluster of somatic cells or of a few primordial germ cells [PGCs; identified by their large round to oval nucleus (with a diameter of *c*. 8 µm) with a granular cytoplasm] surrounded by somatic cells. The gonad at this stage was always in close proximity with both the liver (often nestled within it) and the peritoneal wall. There were no obvious duct formations between the gonad and the peritoneal wall at this life stage (Figures 3.2a–d).

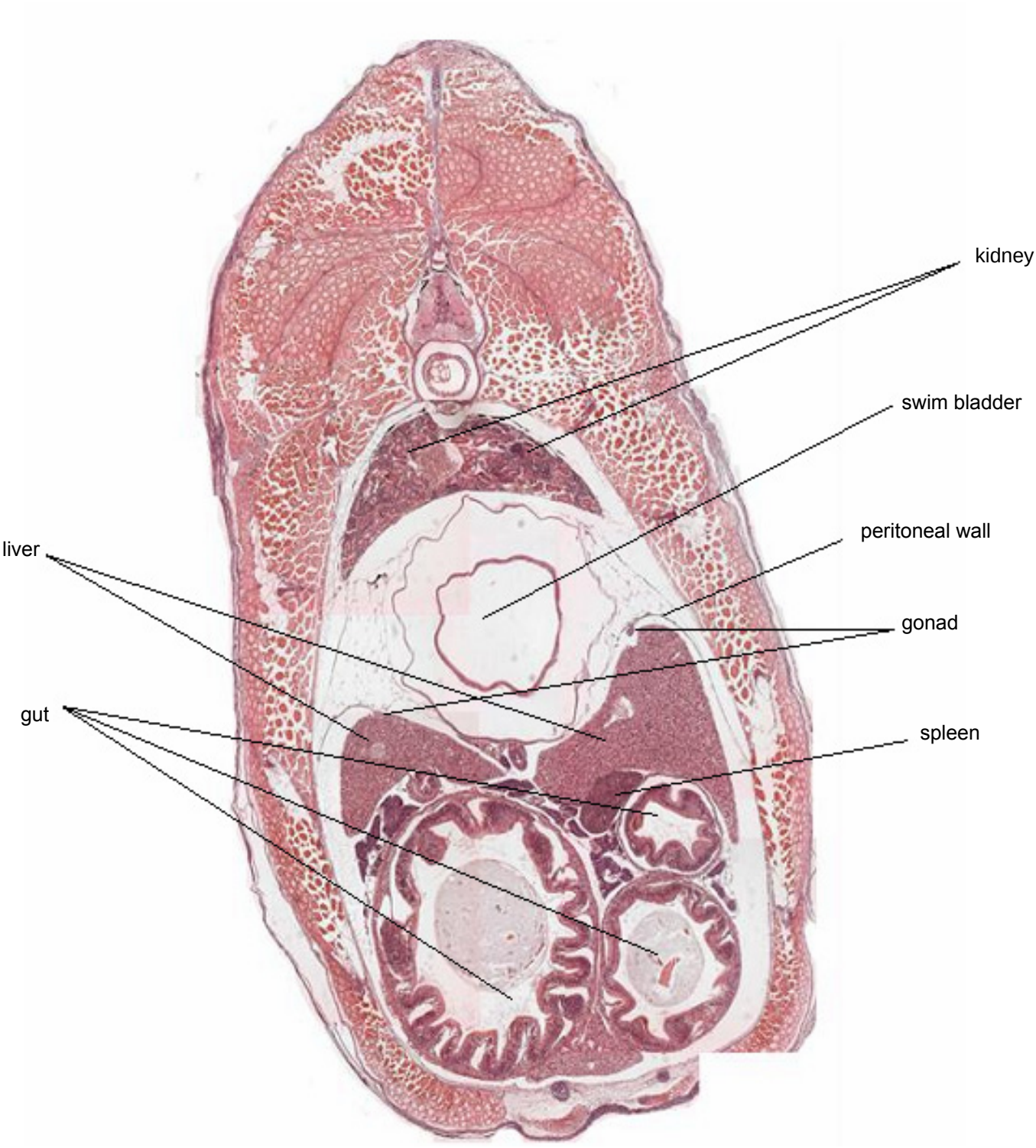
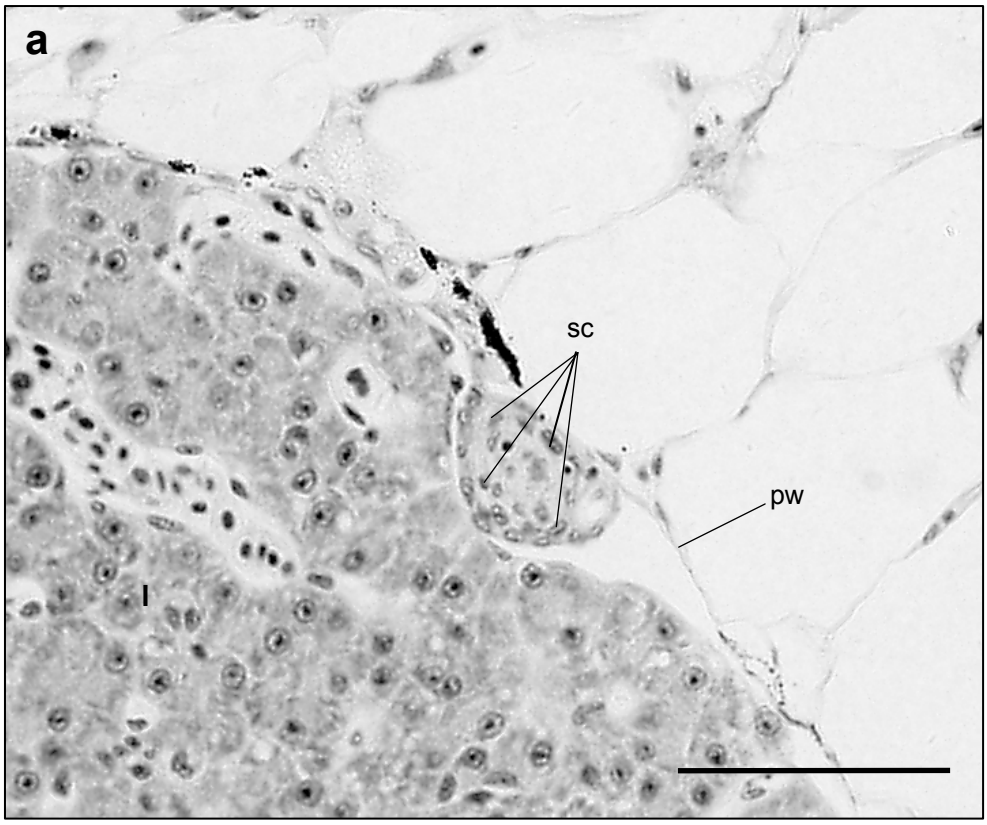
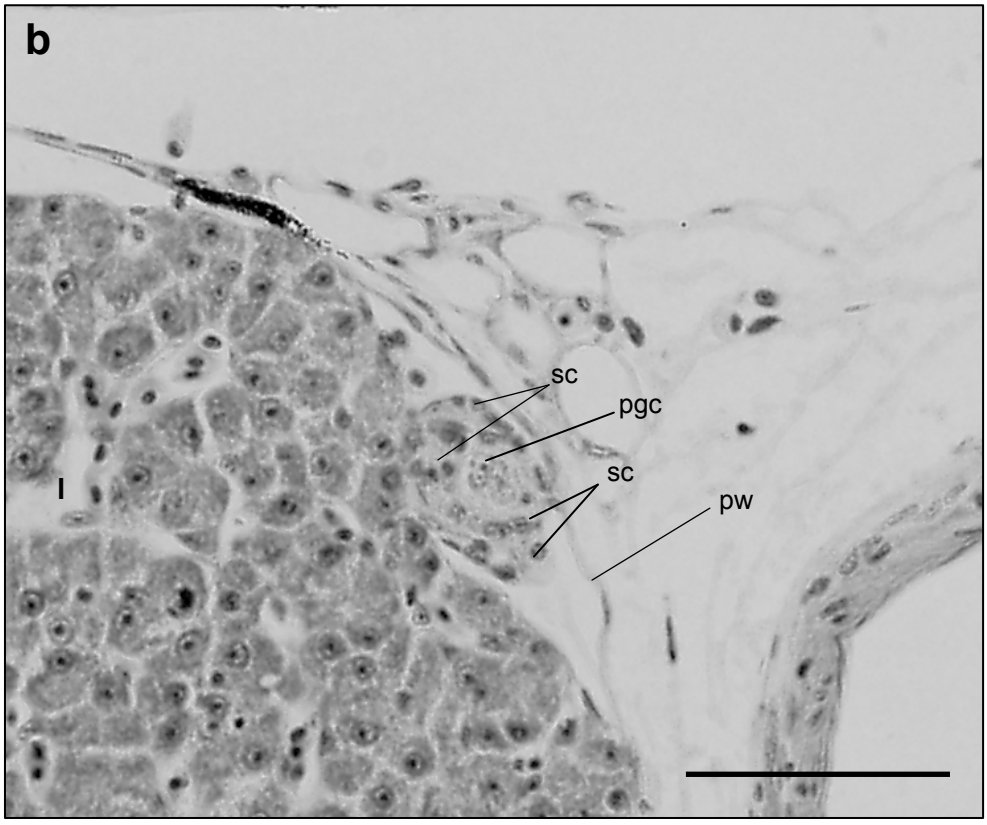
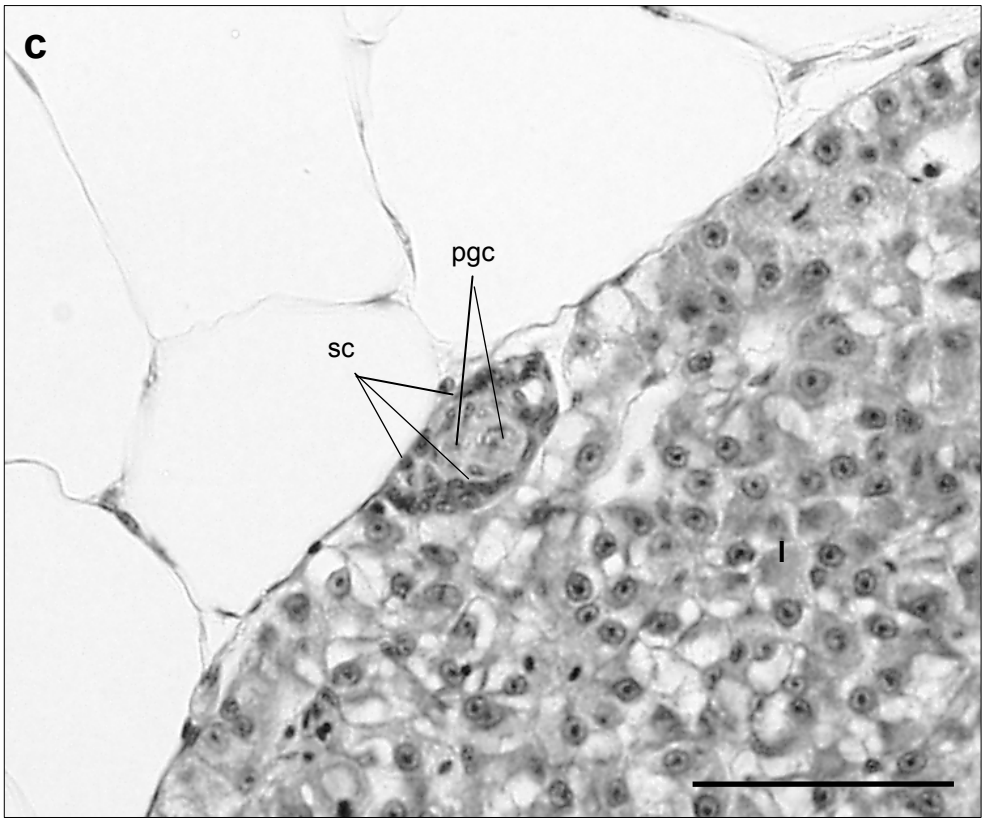


Figure 3.1 Whole body transverse section (5 µm thick) of a roach showing the position of the gonads within the body cavity.

Figures 3.2a–d Transverse sections (5 µm thick) of gonads (in fish from 56 dpf) classified as undifferentiated. l, liver; mgc, migrating germ cell; pgc, primordial germ cell; pw, peritoneal wall; sc, somatic cell. Bar 50 µm.



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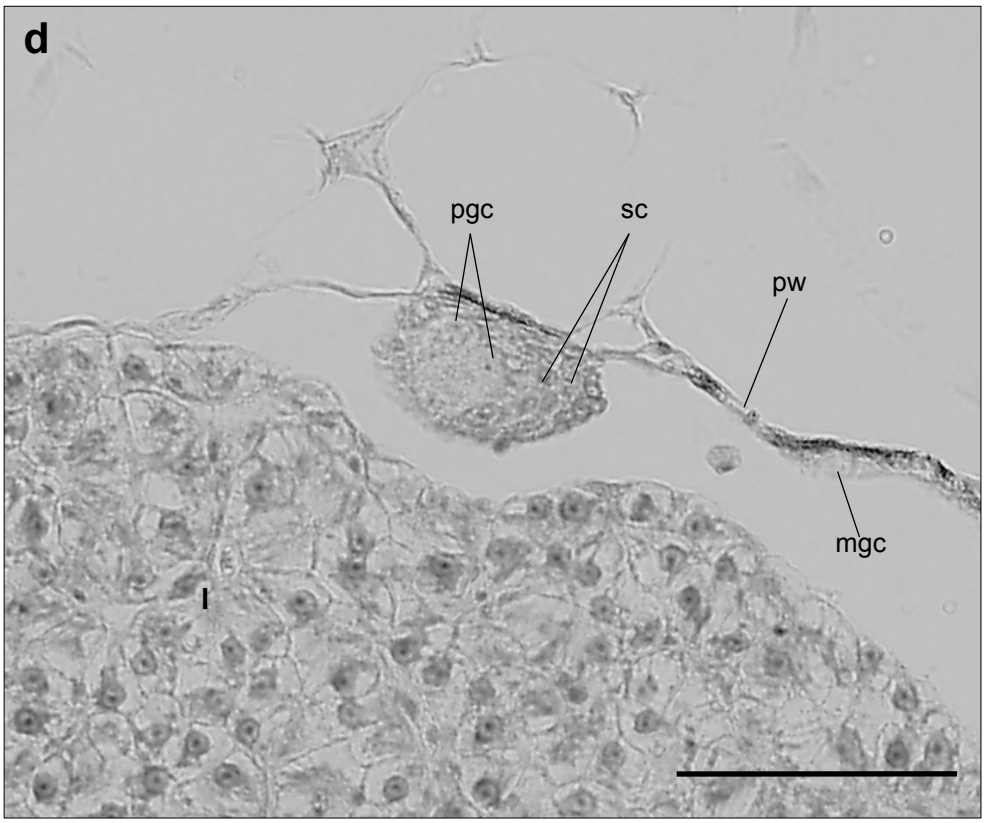
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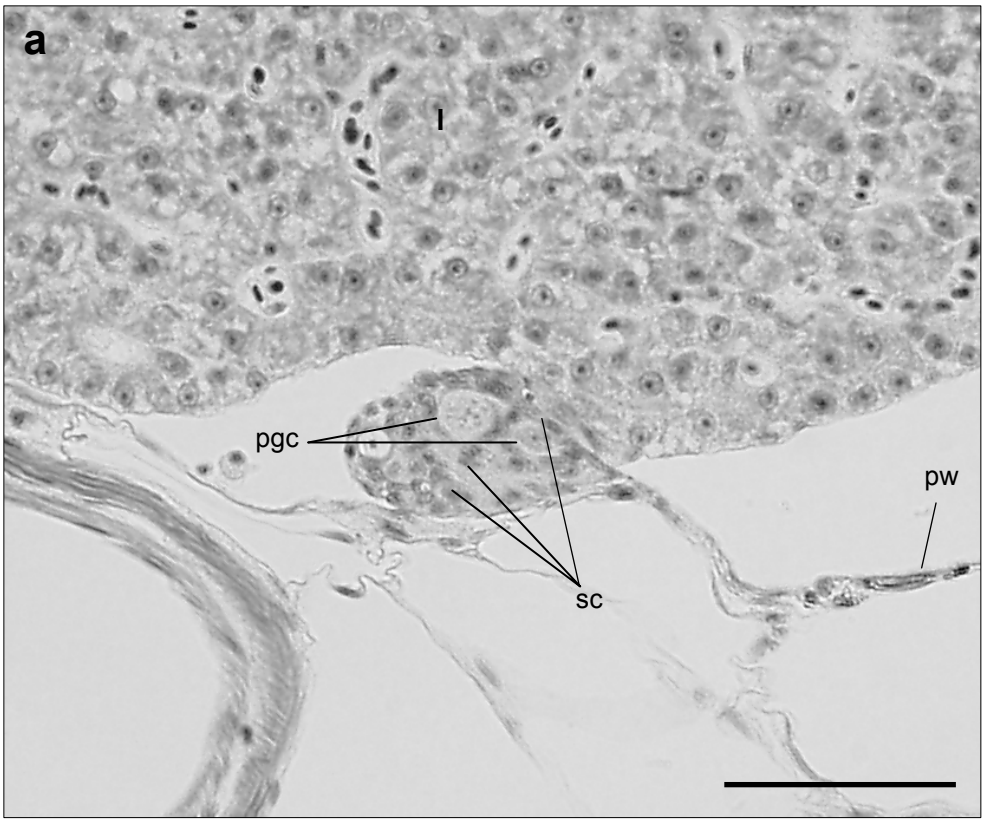
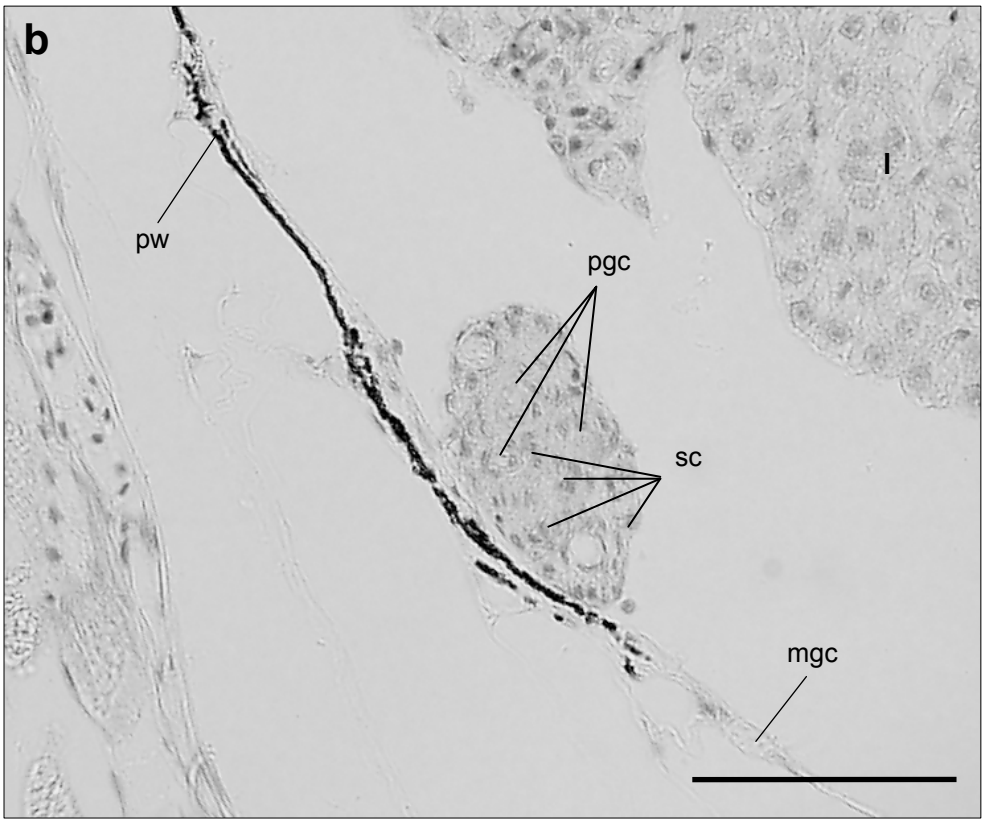
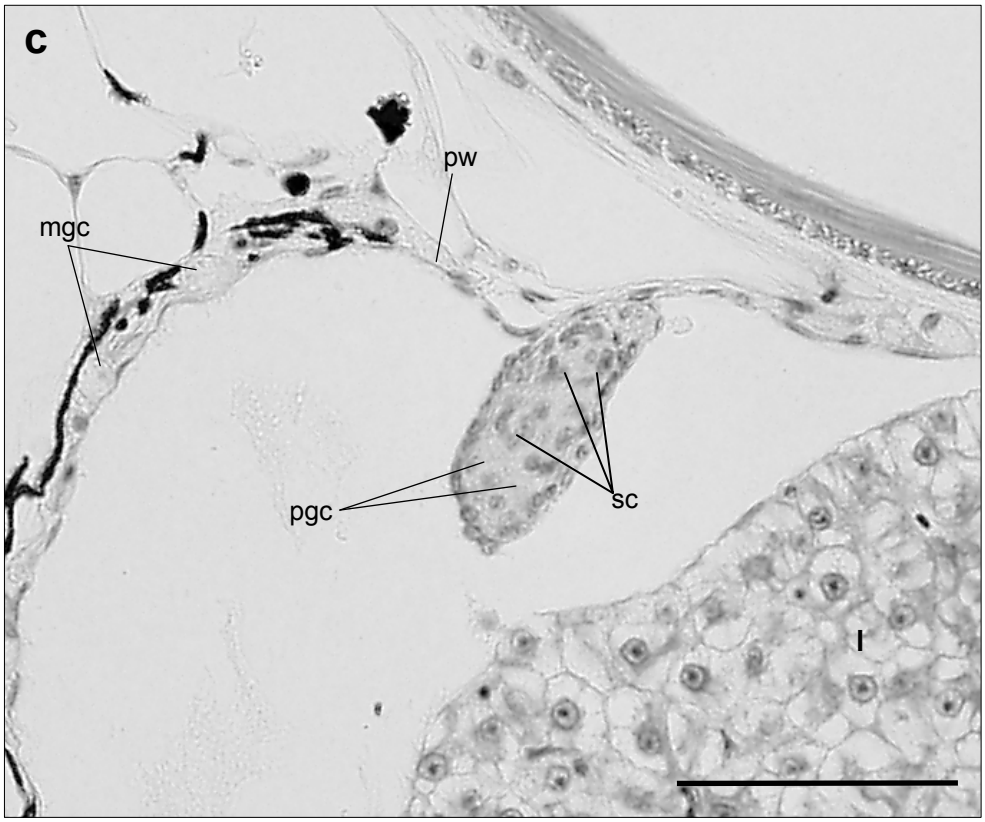
3.1.3.2 Development of a male gonadal phenotype: However, for other fish from 56 dpf, the gonads were more advanced and possessed certain morphological features that were characteristically associated with the male gonad. They included: (a) the positional location of the somatic cells relative to germ cells; somatic cells were observed scattered throughout the gonad and interspersed with PGCs (Figures 3.3a–b) which contrasts that in the gonad of developing females (see later) and (b) gonad types with a range of shapes and forms that included gonads that had partially peeled away from the peritoneal wall at one end but without any obvious sign of duct formation/attachment at the opposite end (Figures 3.3c–d); through to gonads that were completely detached from the peritoneal wall except for a very short presumptive sperm duct forming a single point of attachment (Figures 3.3e–f). The arrangement of the somatic cells relative to germ cells and the attachment of the gonad by a single point to the peritoneal wall were key features used to distinguish males at this early age. At this life stage, none of the gonad types had a combination of both of these morphological characteristics to categorise them as presumptive males (at the resolution of light microscopy). These fish were assigned as undifferentiated, rather than as presumptive males.

3.1.3.3 Presumptive males: From 70 dpf, there was both an increase in the number of fish with gonads containing PGCs and an increase in the total number of PGCs present within an individual gonad. The somatic cells were scattered throughout the gonad and there was now a clear single point of attachment between the gonad and the peritoneal wall formed by the sperm duct. The gonads had increased slightly in size (*c*. 50 µm in diameter), were no longer nestled within the liver, and typically had a club-shaped appearance. There was a clear distinction at this life stage between a male and female gonad structure for almost all fish; however, these fish did not possess male-specific germ cells (e.g. spermatogonia), and therefore they could only be classified as presumptive, rather than as definitive, males (Figures 3.3g–j).

Figures 3.3a–j Transverse sections (5 µm thick) of undifferentiated gonads showing the development of a male gonadal phenotype (a–f; in fish from 56 dpf) and of 'presumptive' testes (g–j; in fish from 70 dpf). l, liver; mgc, migrating germ cell; pgc, primordial germ cell; pw, peritoneal wall; sc, somatic cell; ⟶, points of attachment. Bar 50 µm.



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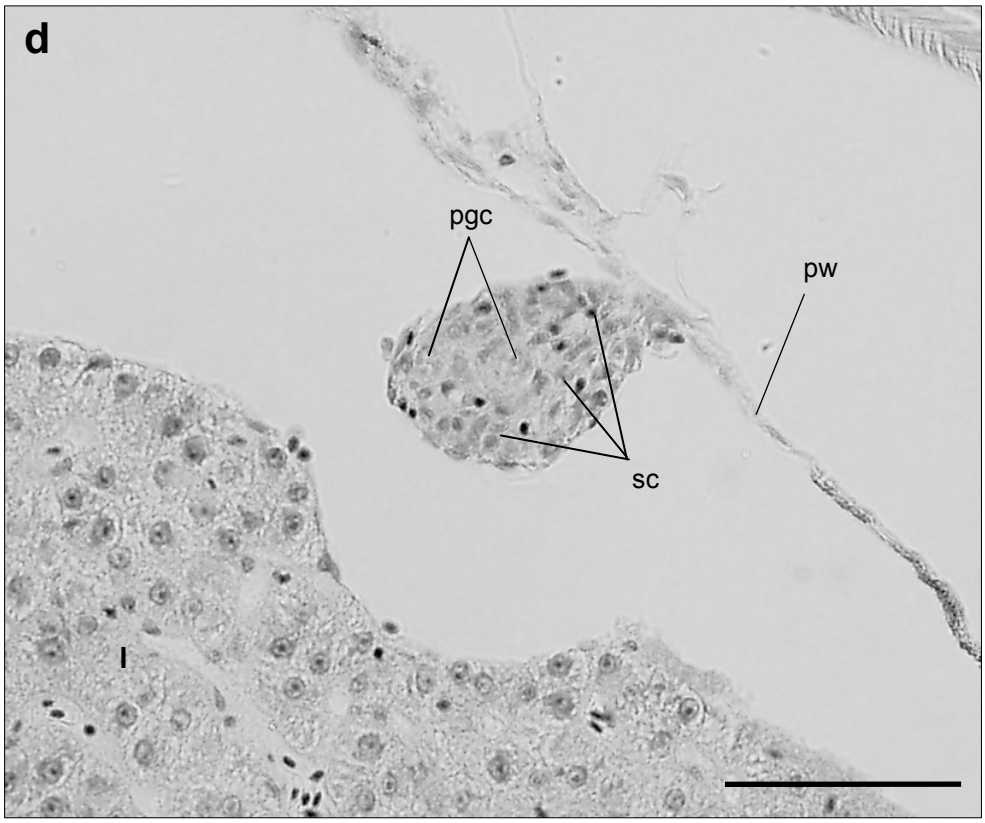
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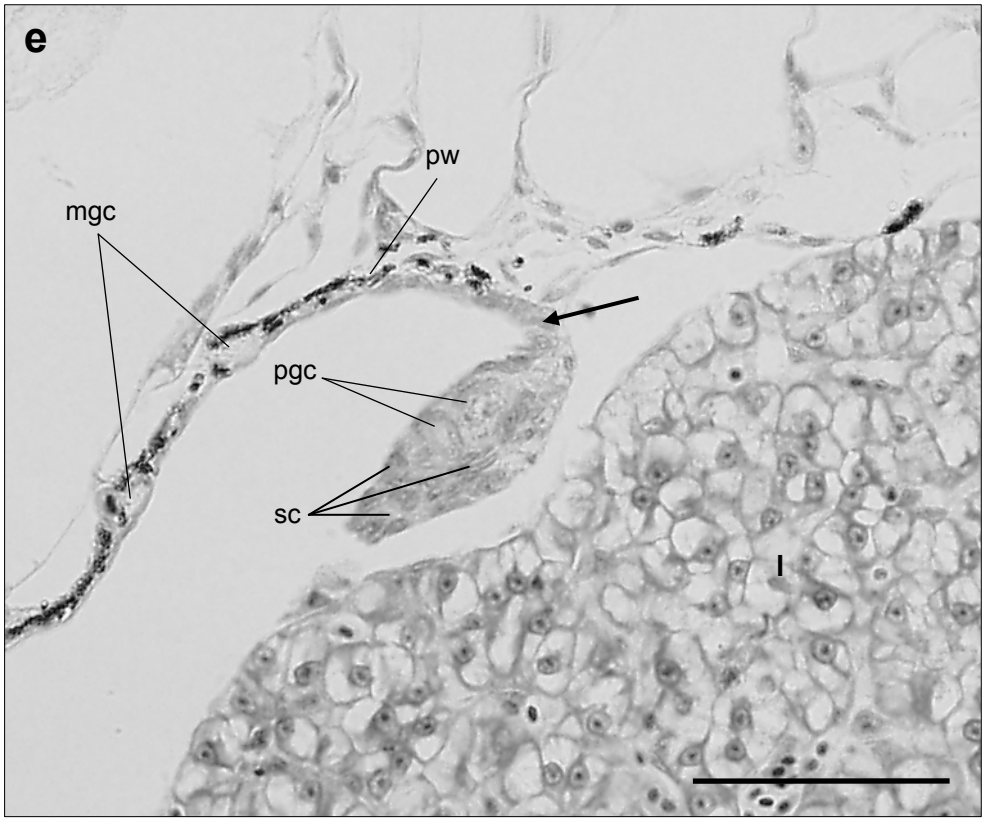
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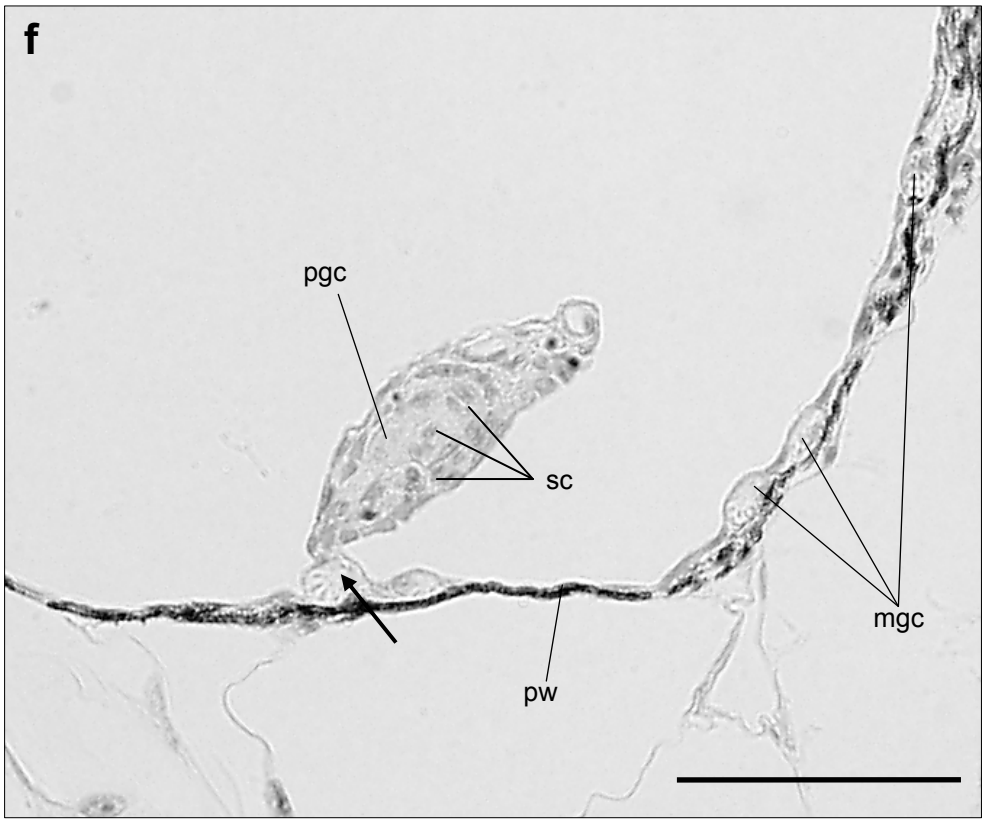
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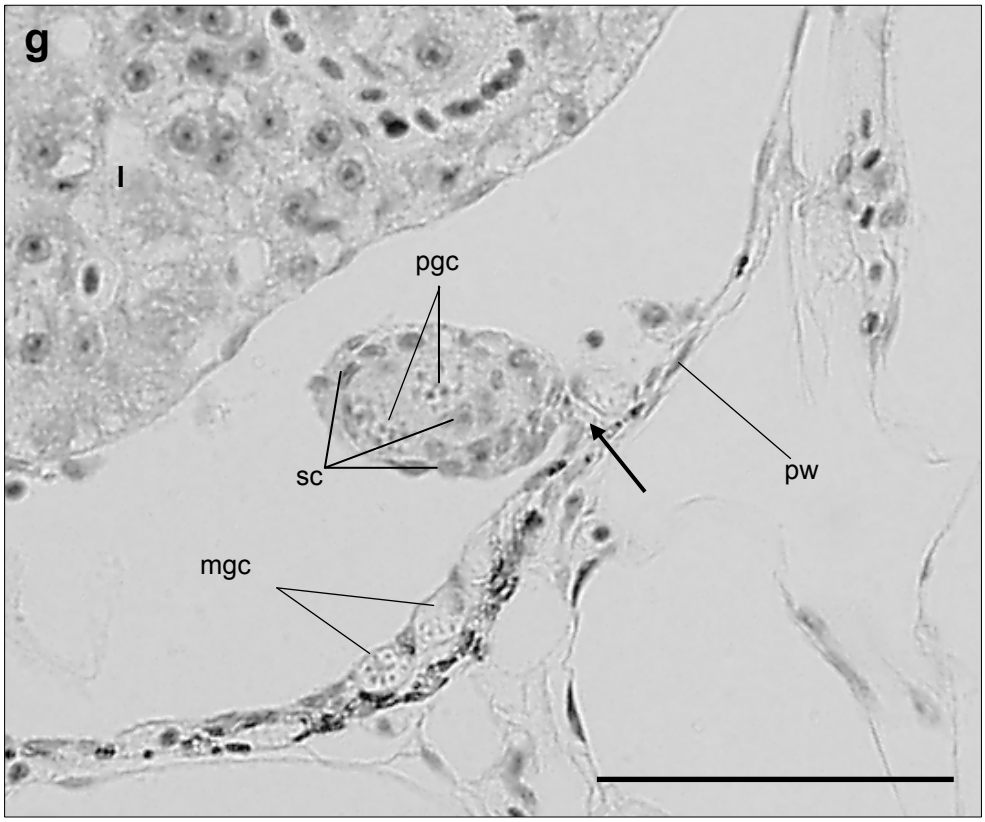
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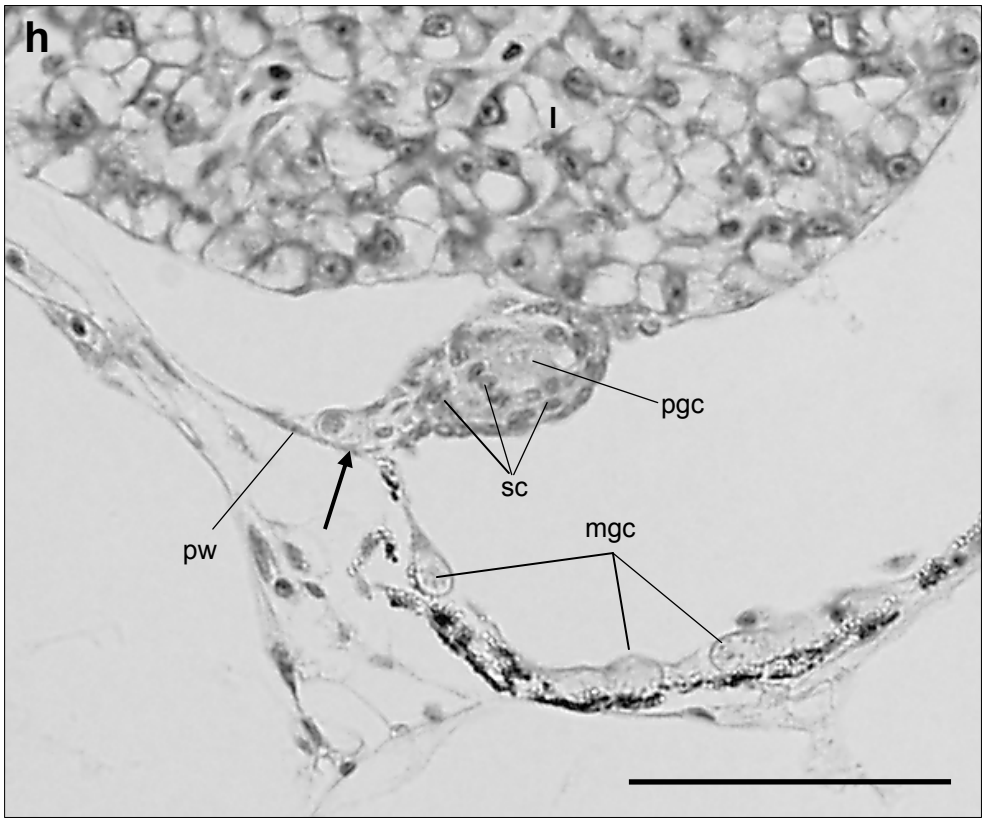
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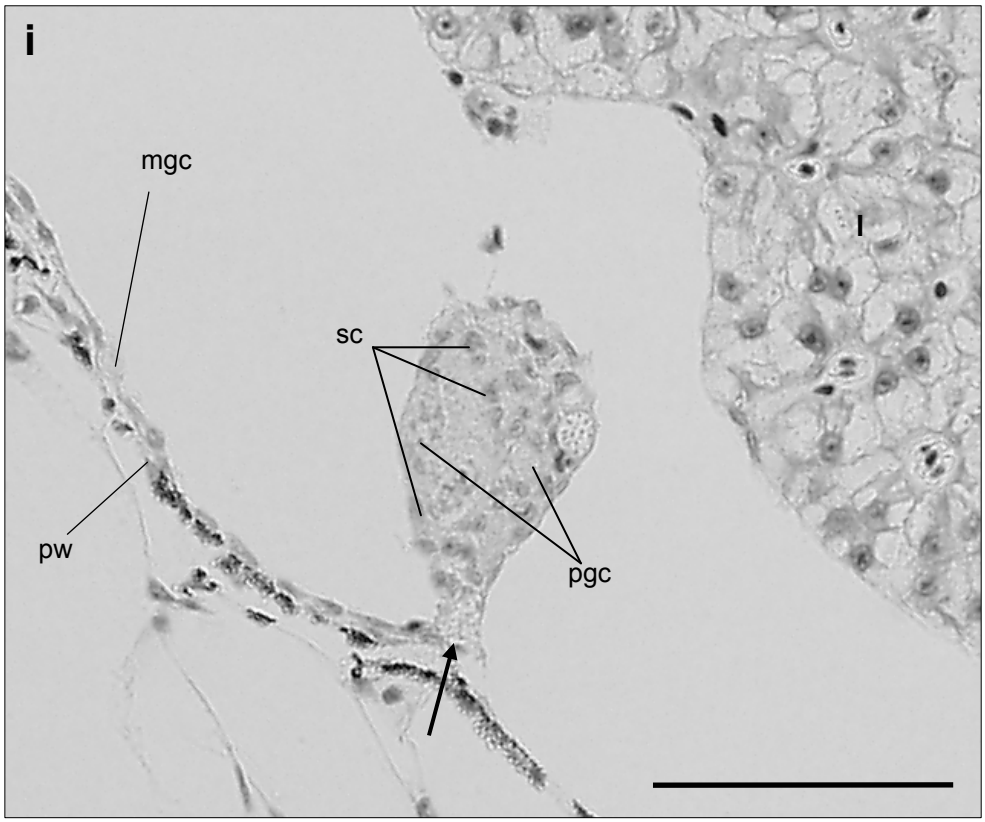
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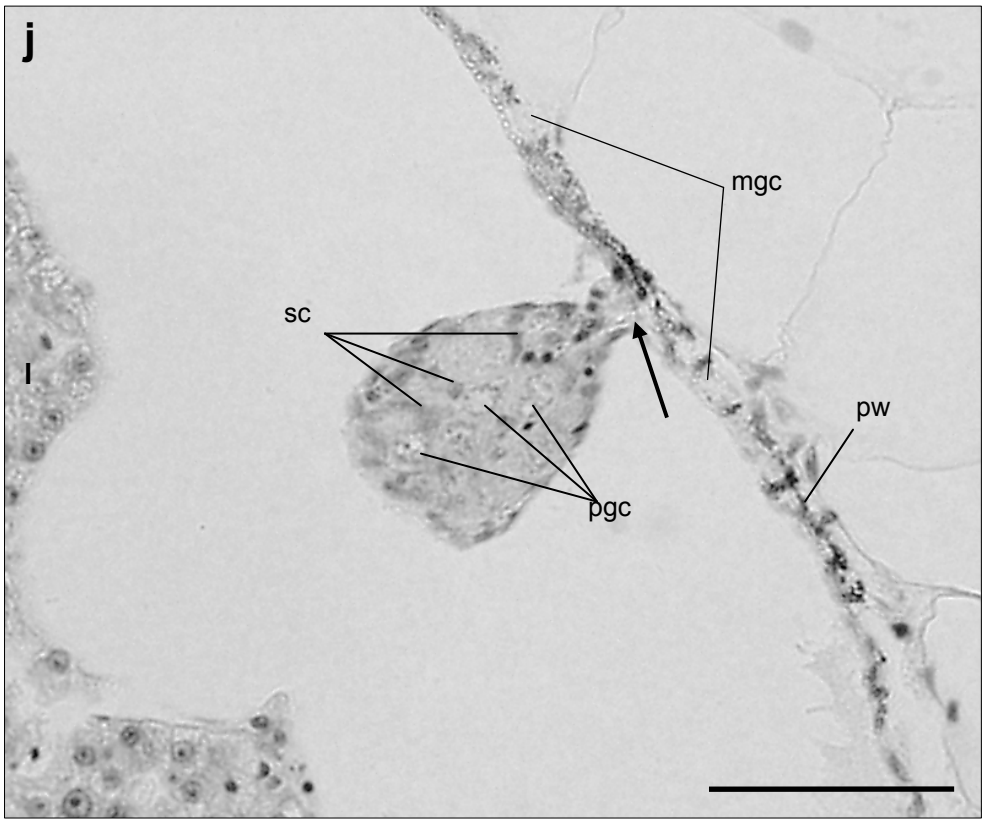
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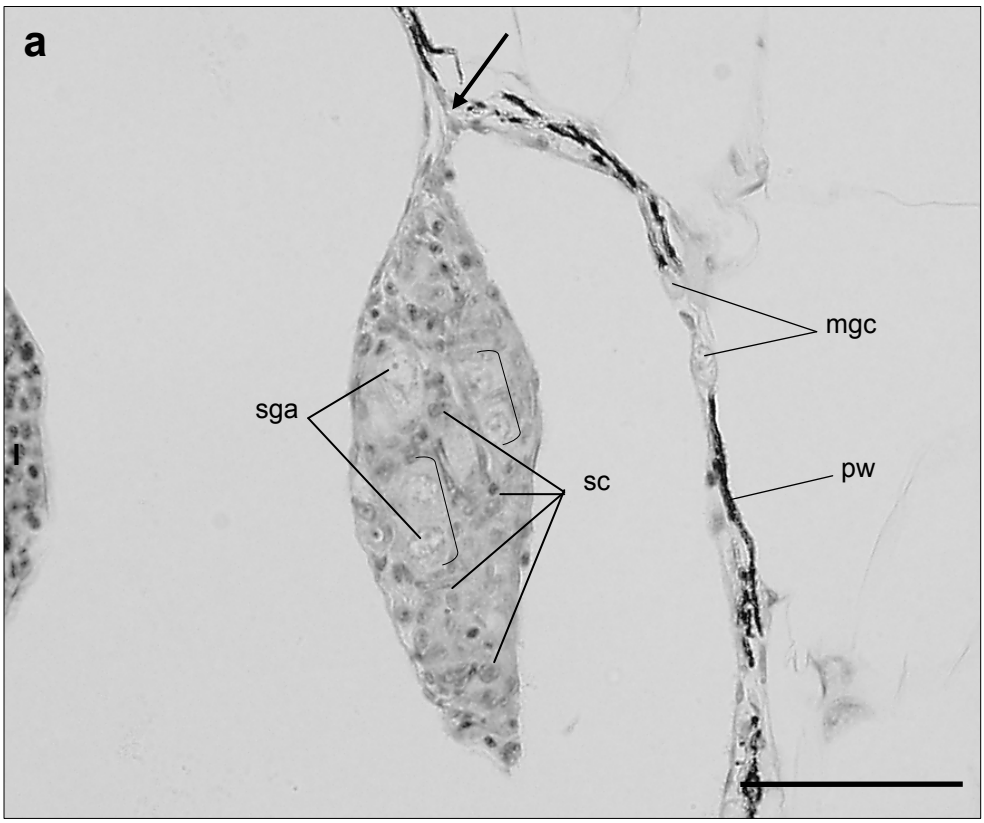
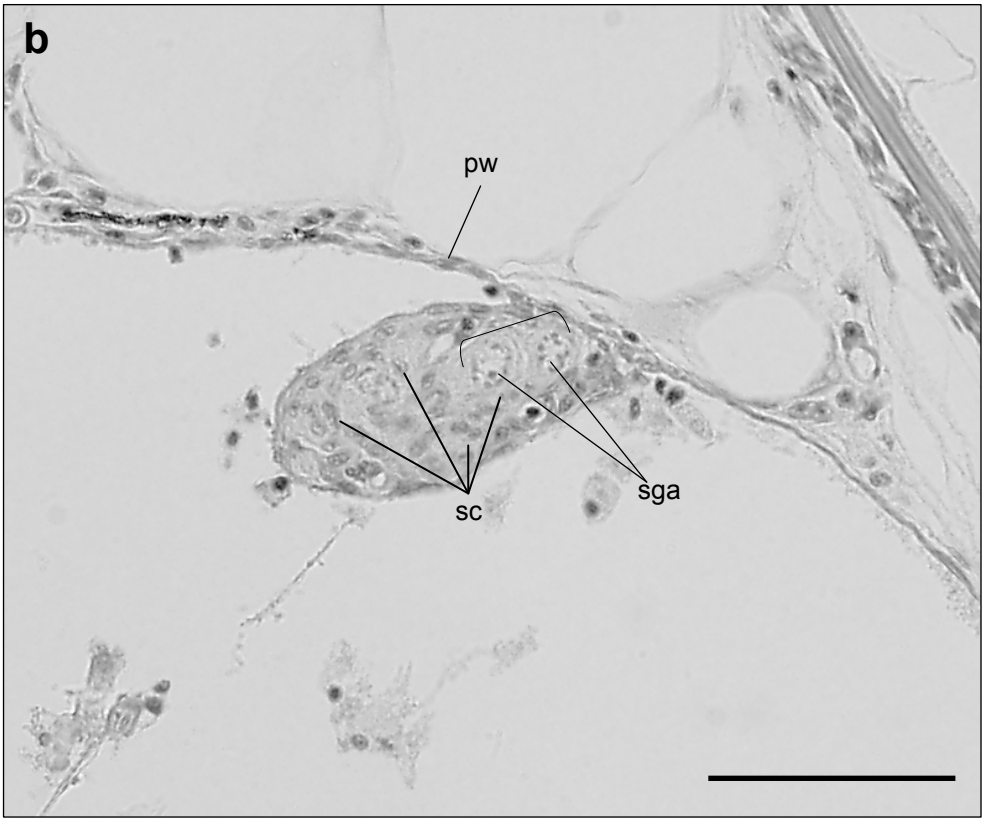
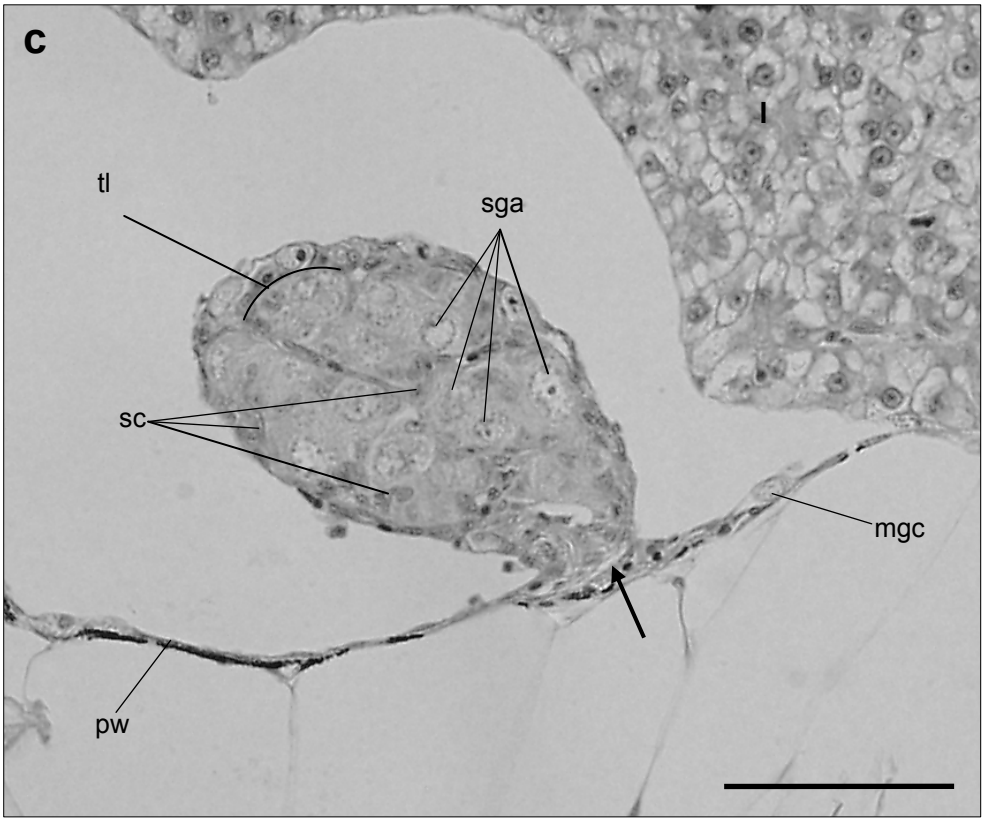
3.1.3.4 Definitive males: From 98 dpf, the gonads of some of the fish contained small numbers of spermatogonia A which, although sometimes difficult to distinguish from PGCs (at the resolution of light microscopy), were generally larger in diameter (nucleus diameter of *c*. 6–13 µm), had a clear cytoplasm, prominent circular nucleolus and developed in small groups in cyst-like structures (Figures 3.4a–c). The gonads continued to increase in size (*c*. 70–160µm in diameter) and lobule-like structures developed (presumptive testis lobules), interspersed by thin layers of somatic cells; these first became obvious from 126 dpf (Figure 3.4d). In conjunction with this increase in gonad size, there was a concurrent increase in the number of lobules within the testis and a corresponding increase in the number of spermatogonia A contained within each lobule. From 140 dpf, spermatogonia A were the dominant cell type throughout the gonads of some fish (Figures 3.4d–e). During this growth period, the sperm duct linking the gonad to the peritoneal wall increased in size from approximately 5–25 µm in diameter between 98 and 140 dpf. From 173 dpf, gonads of some males contained clusters of spermatogonia B (Figure 3.4f), which were easily identified from spermatogonia A by their smaller size (nucleus diameter of *c*. 3–6 µm in diameter), their higher nucleo-cytoplasmic ratio and thus their darker staining. Spermatogonia B increased in number and became the dominant cell type within the testis lobules, although spermatogonia A persisted in all the successive stages of spermatogenesis (Figure 3.4g). From 260 dpf, gonads of some fish contained spermatocyte As, which were irregular in shape, densely stained, and had a nucleus diameter ranging approximately between 3 and 5 µm. In contrast to spermatogonia A, and spermatogonia B, spermatocyte A were never found to be the dominant cell type, but rather they were found in small clusters within some testis lobules. At this stage the testis also contained spermatogonia A and B (Figure 3.4h). From 300 dpf the gonads of some fish contained all other stages of spermatogenesis: spermatocyte Bs, spermatids and spermatozoa. Categorisation of some of these later meiotic stages was difficult to discern with light microscopy. Spermatocyte Bs were smaller than spermatocyte As (nucleus diameter of *c*. 2–3 µm compared to *c*. 3–5 µm in diameter, respectively), while spermatids (identified as spherical cells with a darkly stained horseshoe-shaped nucleus) and spermatozoa (identified by the darkly stained spherical heads – condensed nuclear material) were even smaller (*c*. 2 µm). From 300 dpf spermatozoa had formed large swirls in the lumen between the testis lobules, while all other cell types of spermatogenesis were contained within the lobules (Figures 3.4i–j).

Figures 3.4a–j Transverse sections (5 µm thick) of testes (in fish from 98 to 300 dpf) showing the progression of testicular differentiation. l, liver; lm, lumen; mgc, migrating germ cell; pw, peritoneal wall; sc, somatic cell; scya, spermatocyte a; scyb, spermatocyte b; sga, spermatogonia a; sgb, spermatogonia b; spd, spermatids; sz, spermatozoa; tl, testis lobules. →, points of attachment; ⌜⌝, cyst structures. Bar 50 µm.



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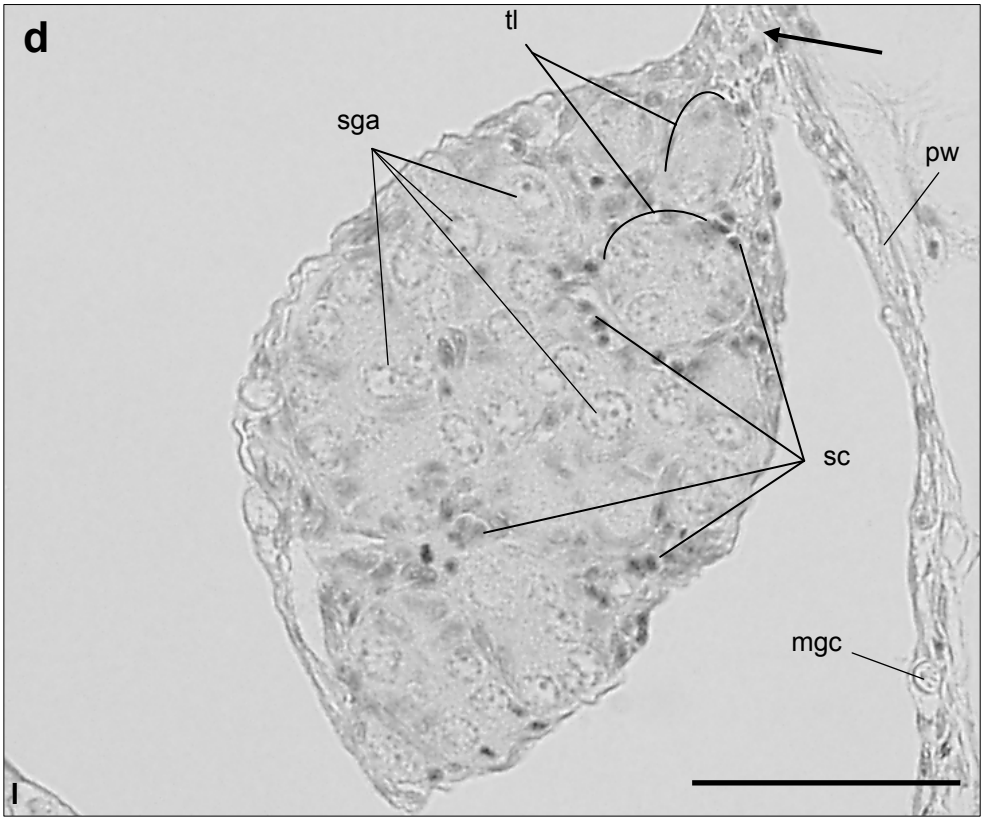
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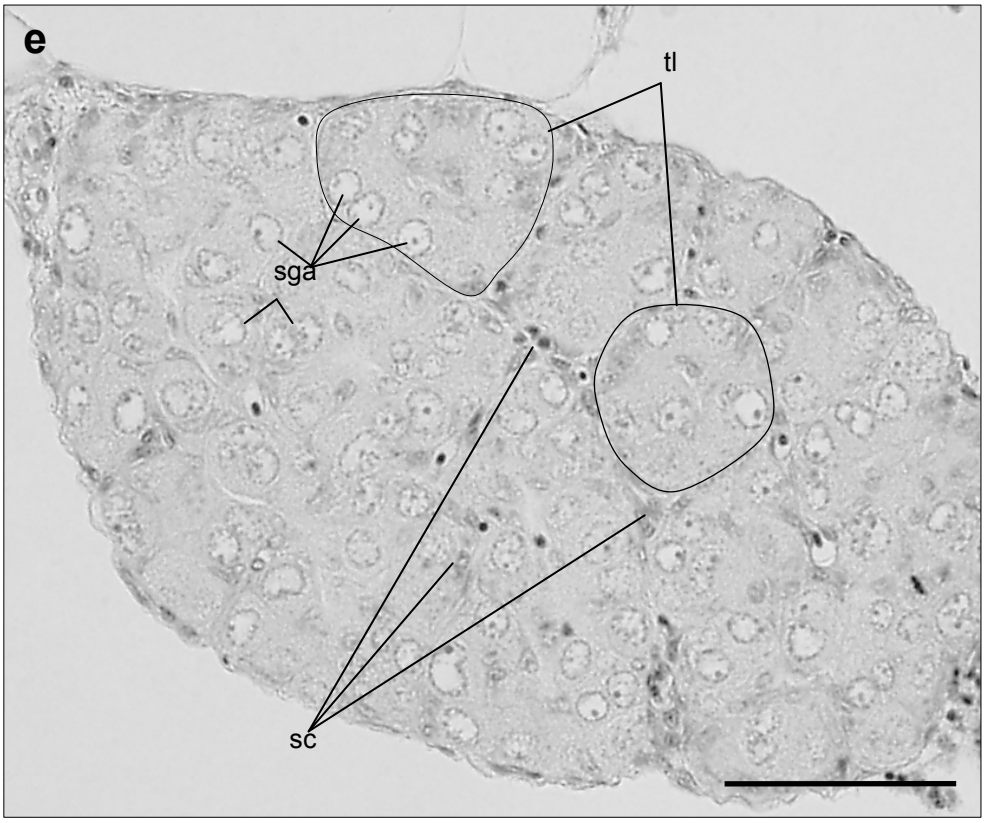
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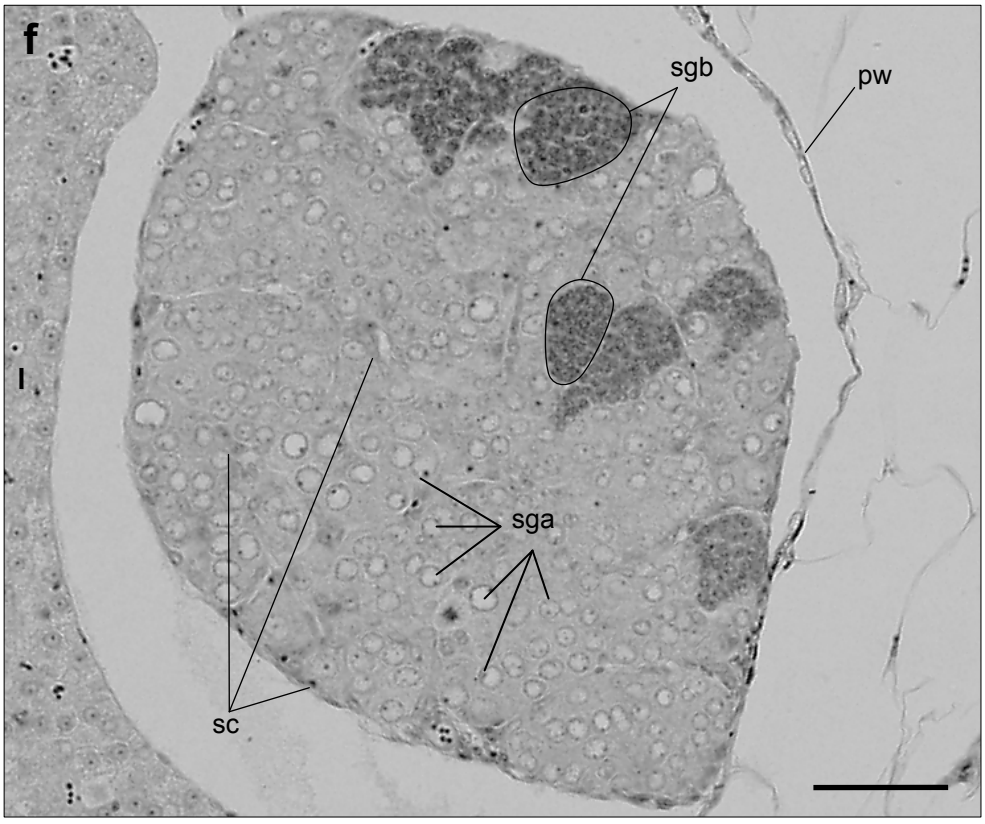
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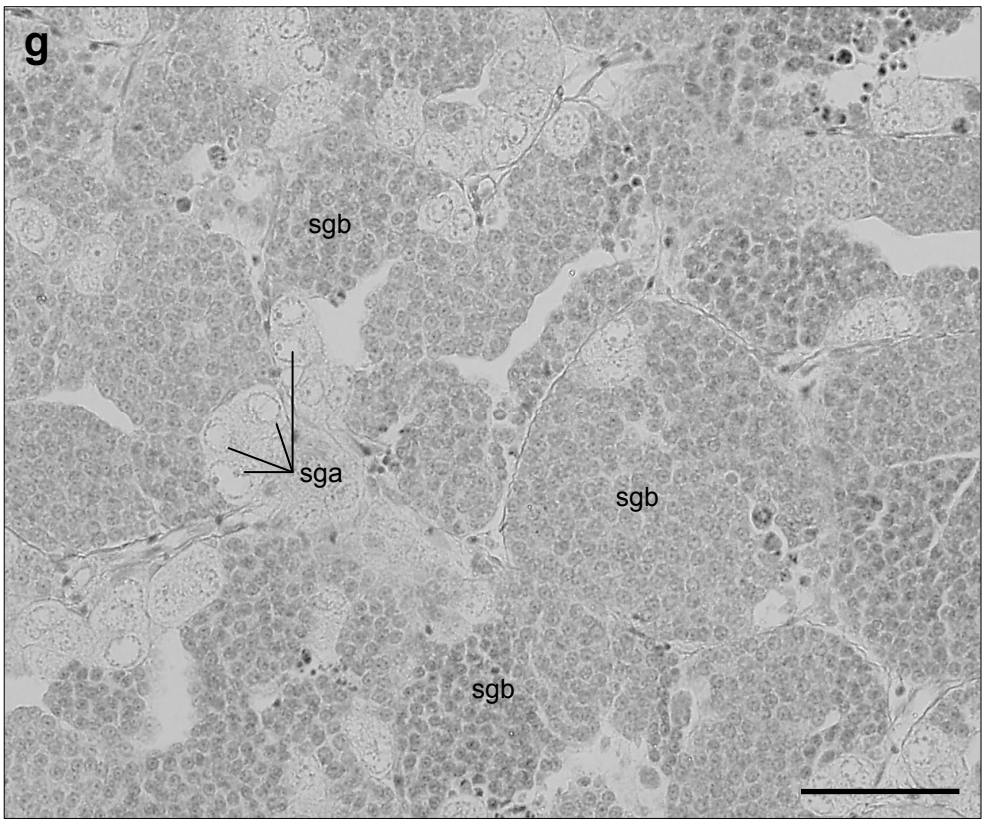
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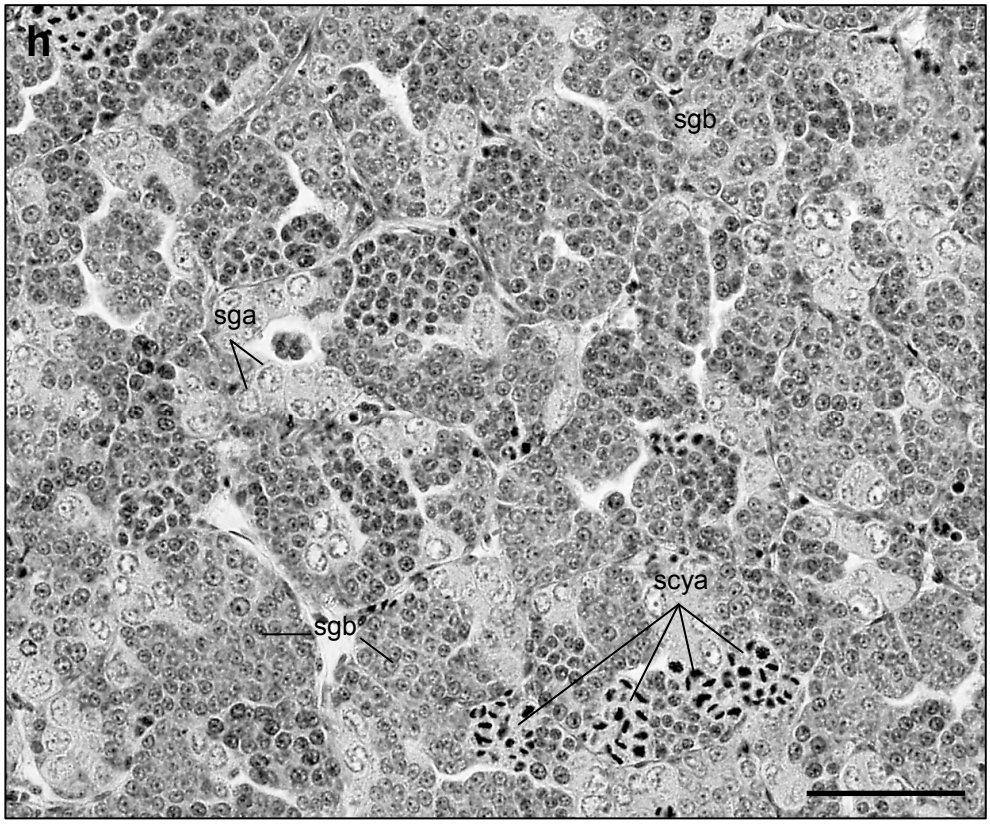
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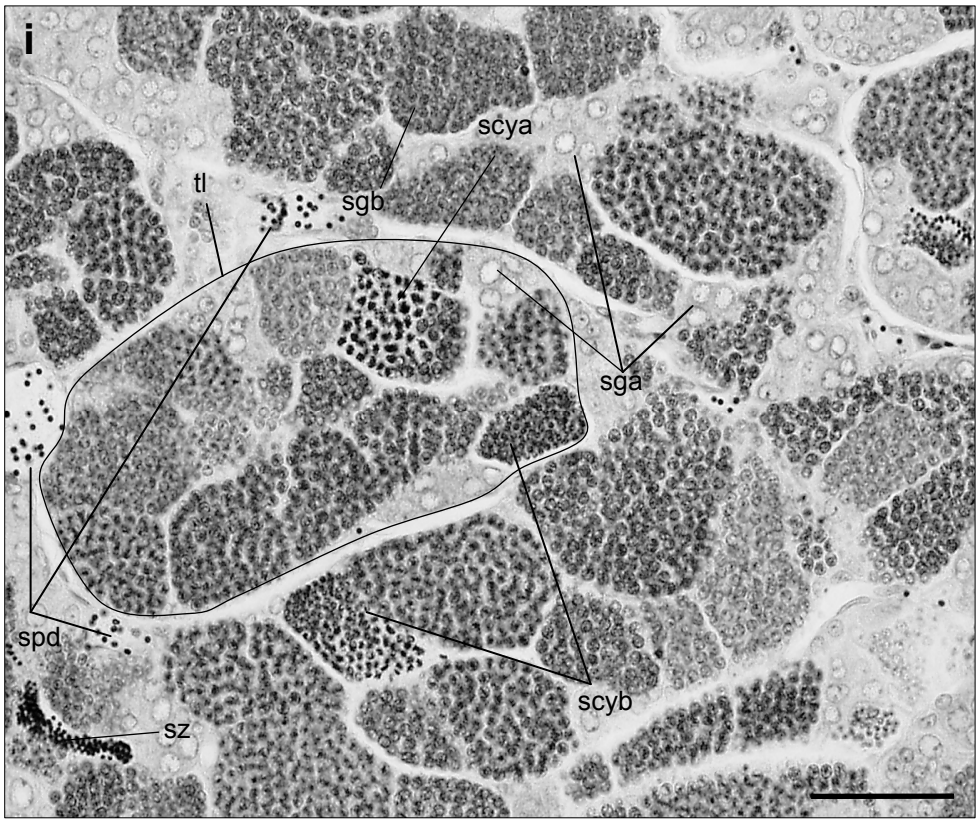
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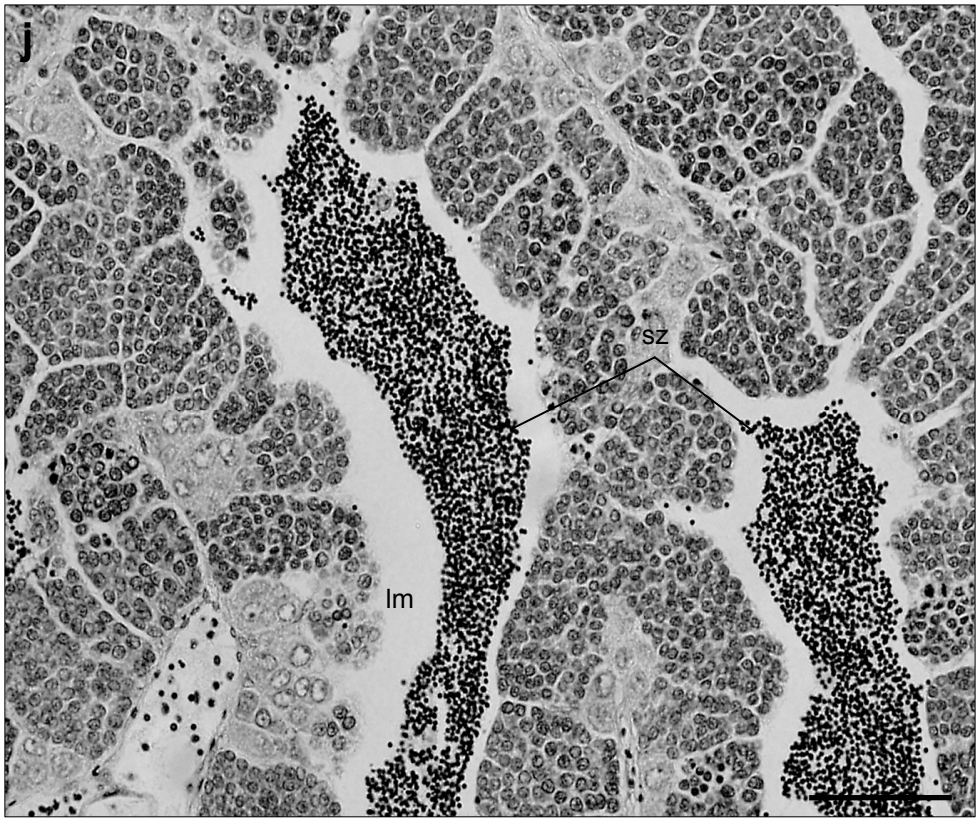
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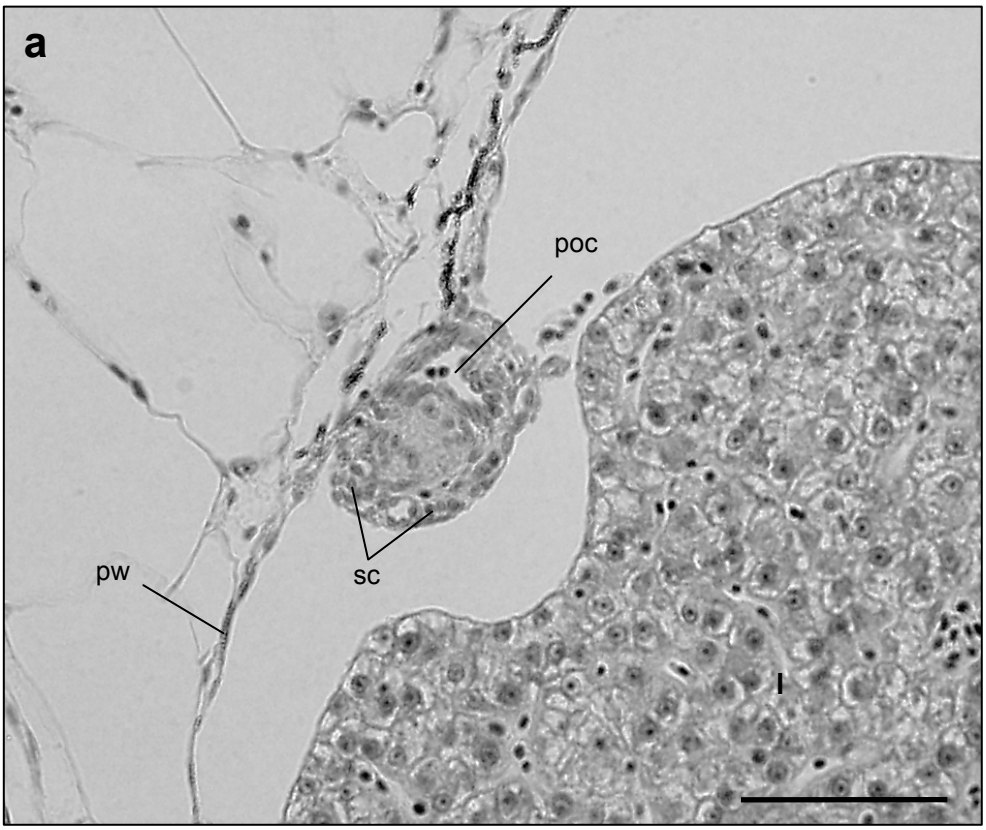
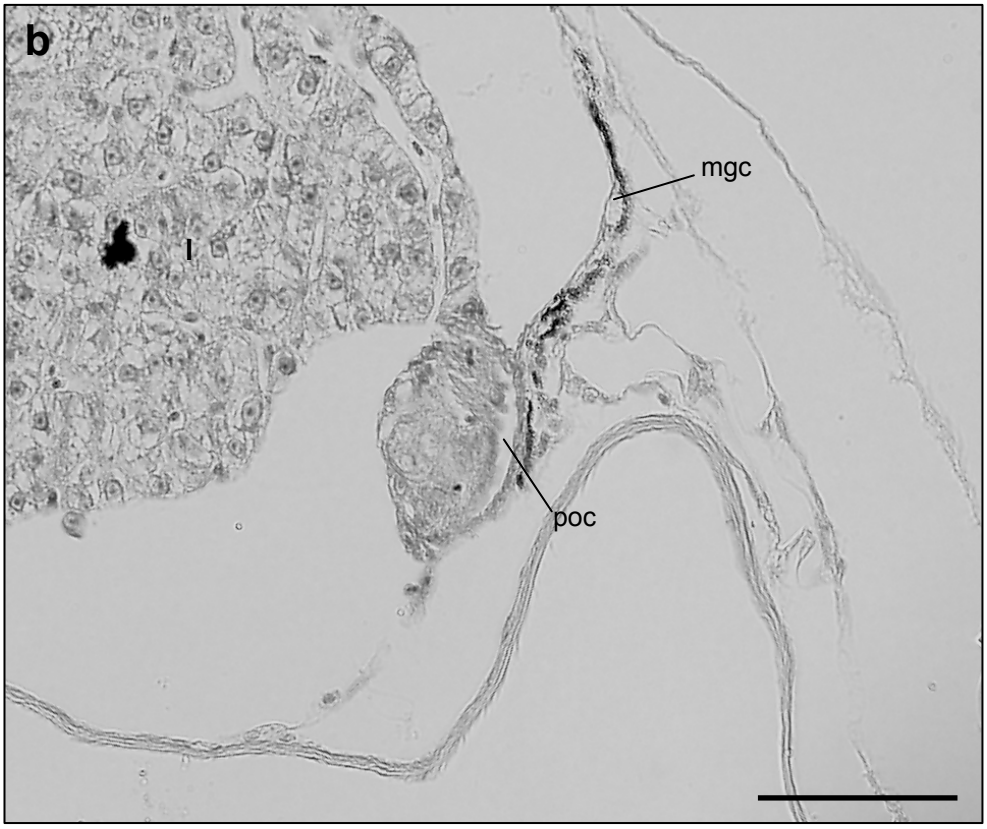
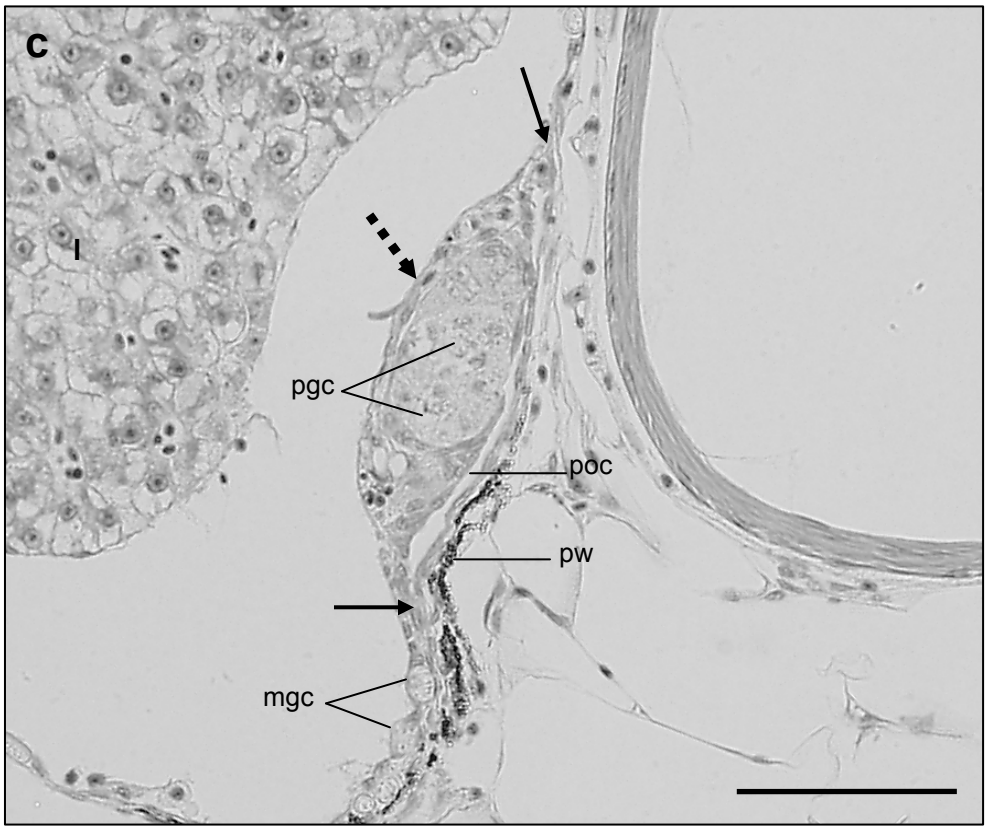
3.1.3.5 Development of a female gonadal phenotype: From 56 dpf onward, the gonads of some of the fish showed certain morphological characteristics associated with a female gonad (ovary). The somatic cells were, in contrast to those in the developing testis, localised more around the periphery of the gonad and, in some fish, a cavity appeared between the peritoneal wall and the gonad itself forming a presumptive ovarian cavity, albeit often very small. In these gonads, however, it was difficult to identify the presence of PGCs and the gonads themselves were very similar (in terms of their size, shape and lack of any obvious duct formations) to those gonads categorised as undifferentiated. It was concluded, therefore, that there was insufficient evidence to assign these fish as females and that they should also be classified as undifferentiated until such time that more definitive markers for gender assignment are developed (Figures 3.5a–b).

3.1.3.6 Presumptive females: From 70 dpf, the gonads of some of the fish now possessed a more obvious ovarian cavity formed by two clear points of attachment (developing oviducts) between the gonad and the peritoneal wall. The somatic cells were localised predominantly around the periphery of the gonad and surrounding the PGCs in the central region. The gonads had increased in size from approximately 50–60 µm at 56 dpf to approximately 70–90 µm at 70 dpf, were no longer nestled within the liver, and although they typically remained ovoid in shape they were becoming more elongate in shape. At this life stage in some fish there was a clear distinction in the gonad between females and males (although a few undifferentiated fish still remained) but, because these fish did not possess female-specific germ cells (e.g. oogonia), they could only be classified as presumptive, rather than as definitive, females (Figures 3.5c–d).

Figures 3.5a–d Transverse sections (5 µm thick) of ovaries showing the development of a female gonadal phenotype (a–b; in fish from 56 dpf) and of 'presumptive ovaries' (c–d; in fish from 70 dpf). l, liver; mgc, migrating germ cell; pgc, primordial germ cell; poc, presumptive ovarian cavity; pw; peritoneal wall; sc, somatic cell; ⟶, points of attachment; ⇢, somatic cells located at the periphery of the gonad. Bar 50µm.



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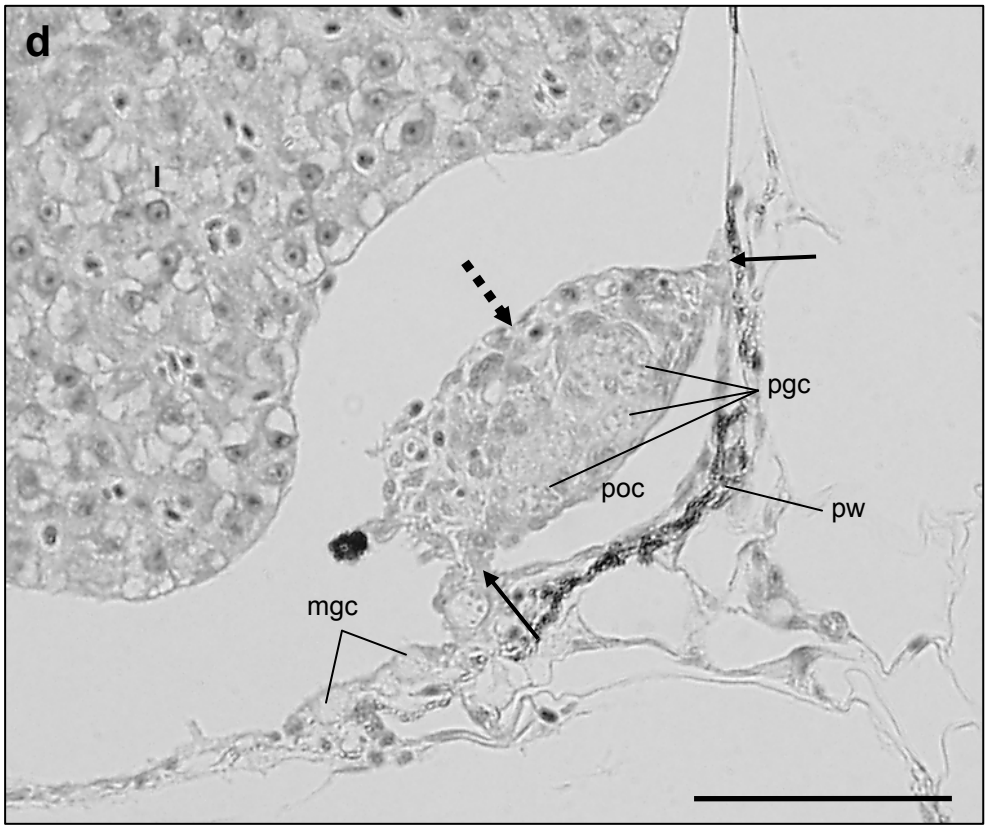
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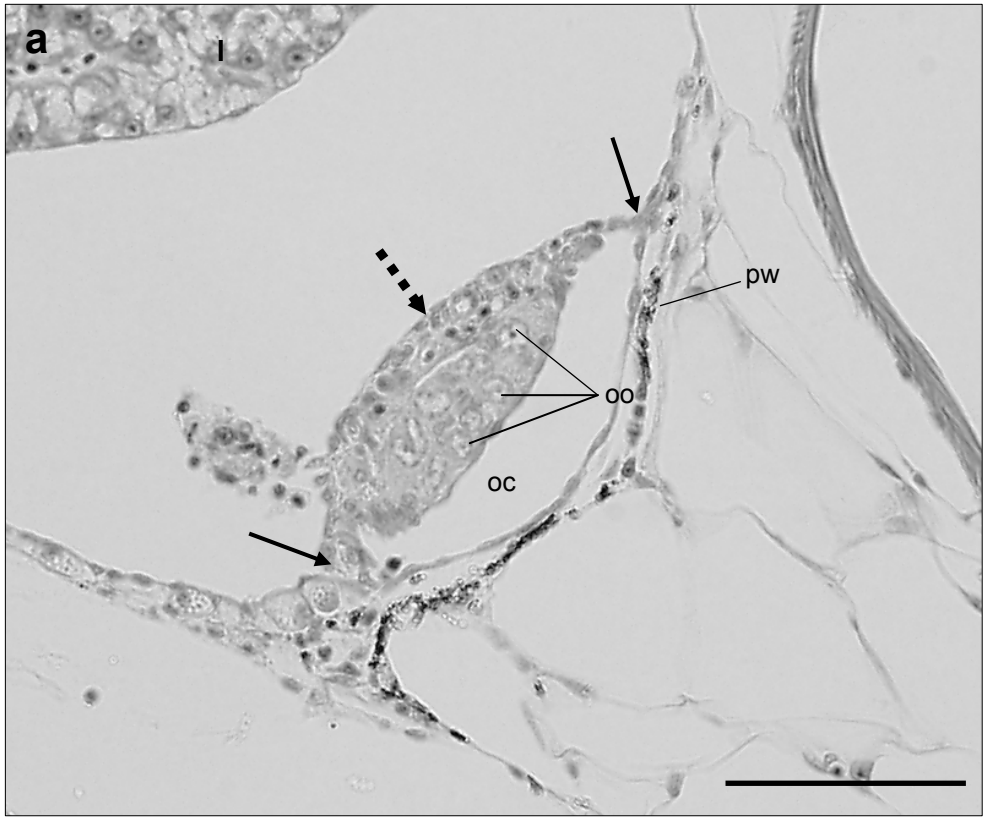
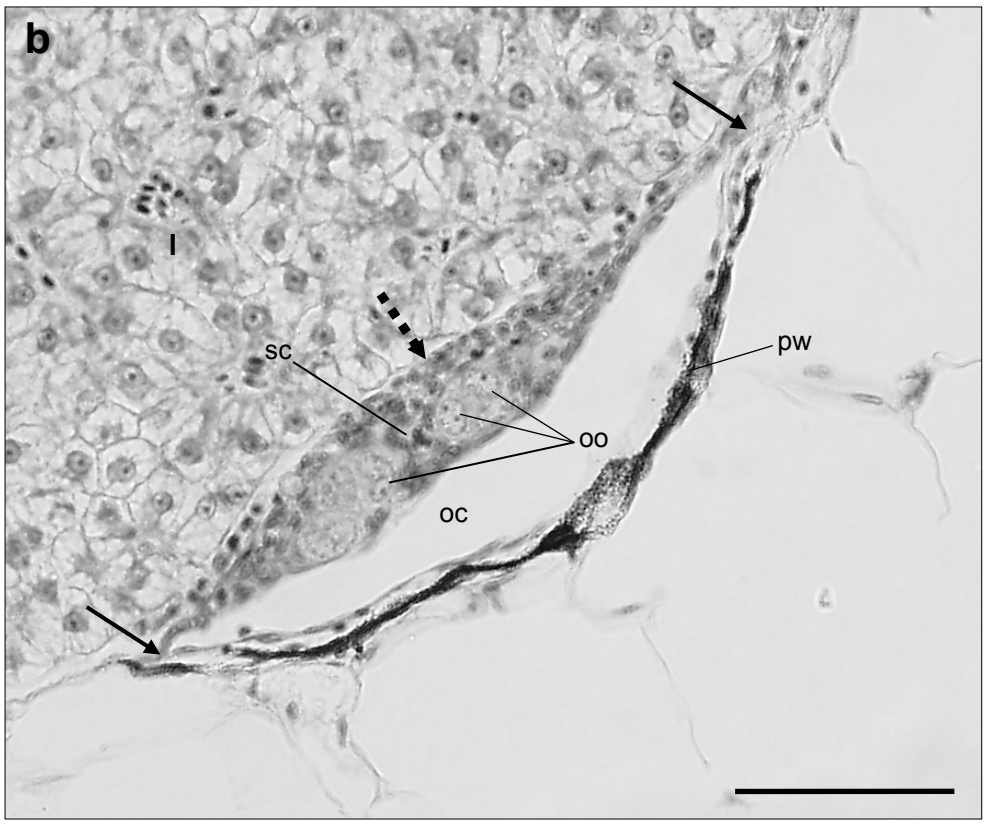
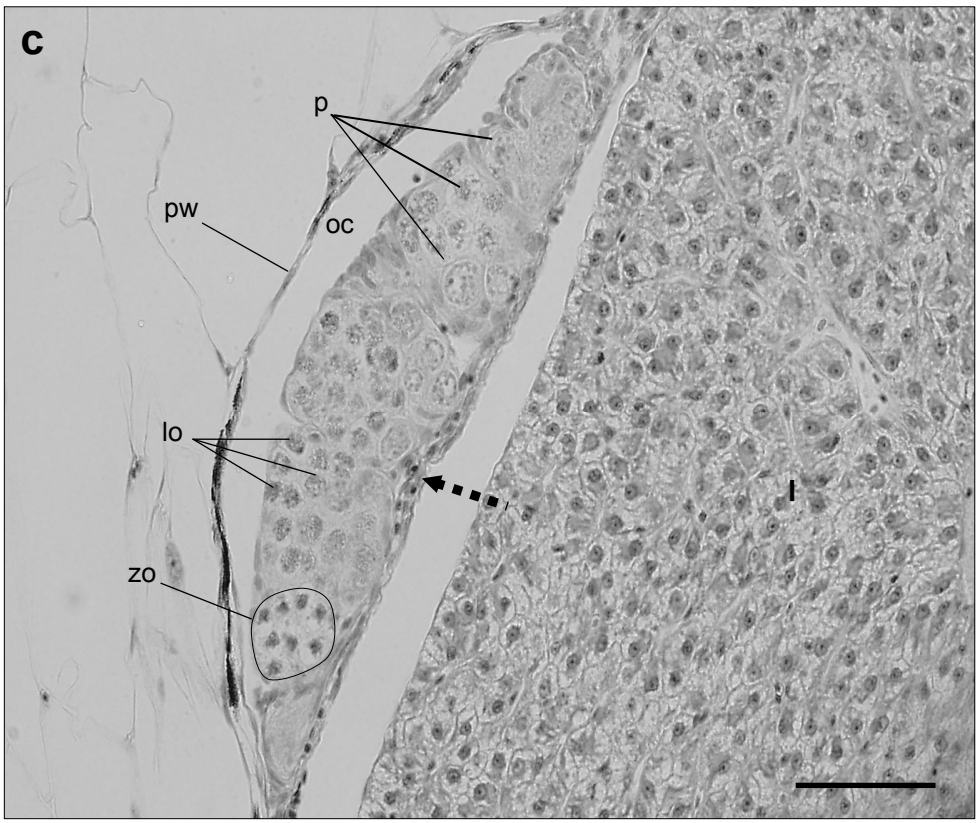
3.1.3.7 Definitive females: From 68 dpf (in one fish) but more commonly from 84 dpf, the gonads of some of the fish contained small numbers of oogonia (the first stage defining oogenesis), with a circular nucleus measuring approximately 6–10 µm, a prominent nucleolus and a clearly defined, darkly stained nuclear membrane. Oogonia developed in small cyst or nest-like structures and were very similar in appearance at this stage to developing spermatogonia A in males. The somatic cells were again localised more in the periphery of the gonad, notably along the edge of the gonad nearest the liver. Although, occasionally, some somatic cells were observed between nests of developing oogonia, in no instance were they scattered throughout the gonad, as observed in the developing testis (Figures 3.6a–b). At 98 dpf, the first early meiotic stage oocytes appeared: leptotene stage oocytes (identified by thread-like chromosome structures distributed throughout the nucleus and with a nucleus diameter of *c*. 6–8 µm), zygotene stage oocytes (identified by more clearly defined and localised chromosomes at one edge of the nucleus referred to as the bouquet stage and with a nucleus diameter of *c*. 7–11 µm) and pachytene stage oocytes (identified by the appearance of small chromatin clumps or nucleoli and the loss of the bouquet distribution of chromosomes and with a nucleus diameter of *c*. 9–14 µm) (Figures 3.6c–d). During this period the ovary continued to increase in size (*c*. 150–350 µm in diameter) typically becoming more elongate in cross section with a corresponding increase in the size of the ovarian cavity formed by the developing oviducts. The ovary continued to develop rapidly and by 102 dpf the ovary in some fish contained small numbers of primary oocytes at the early diplotene (peri-nucleolar) stage (identified by the presence of small nucleoli at the periphery of the nucleoplasm and with a nucleus diameter of *c*. 20–50 µm) (Figures 3.6e–f). There was a noticeable increase in gonad mass at this stage changing from an elongate structure to a much fuller almost semi-circular shape occupying more of the ovarian cavity. From 137 dpf, peri-nucleolar stage oocytes were the dominant cell type throughout the ovary of some females with the largest oocytes now measuring approximately 150 µm in diameter (Figure 3.6g). Primary oocytes at the next transitional stage, the Balbiani body stage (identified by the presence of Balbiani vitelline bodies in the cytoplasm and with a diameter of up to *c*. 200 µm) were first found from 154 dpf (Figures 3.6h–i). From 368 dpf the first cortical aveolus stage oocytes (identified by the presence of clear vacuoles, known as cortical alveoli, in the cytoplasm of the oocyte) occurred and have a diameter of *c*. 200–300 µm) appeared in some fish (Figure 3.6j). At 368 dpf the gonads of some fish also contained early vitellogenic stage oocytes (with a diameter from *c*. 300 µm), which were identified by a thickening of the zona radiata and the presence of yolk in the cytoplasm. All other stages of oogenesis from peri-nucleolar stage oocytes were also present in these fish (Figure 3.6k). Vitellogenic stage oocytes measured up to approximately 1000 µm in diameter in fish at 728 dpf (Figure 3.6l).

Figures 3.6a–l Transverse sections (5 µm thick) of ovaries showing the progression of ovarian differentiation (in fish from 68 to 728 dpf). bb, Balbiani bodies; bbo, Balbiani body stage primary oocyte; ca, cortical alveolus stage oocyte; evo, early vitellogenic stage oocyte; l, liver; lo, leptotene stage oocyte; oc, ovarian cavity; oo, oogonia; p, pachytene stage oocyte; pn, peri-nucleolar stage primary oocyte; pw; peritoneal wall; sc, somatic cell; so, secondary stage oocyte; vo, vitellogenic stage oocyte; zo, zygotene stage oocyte →, points of attachment; ⇢, somatic cells located at the periphery of the gonad. Bar 50 µm.



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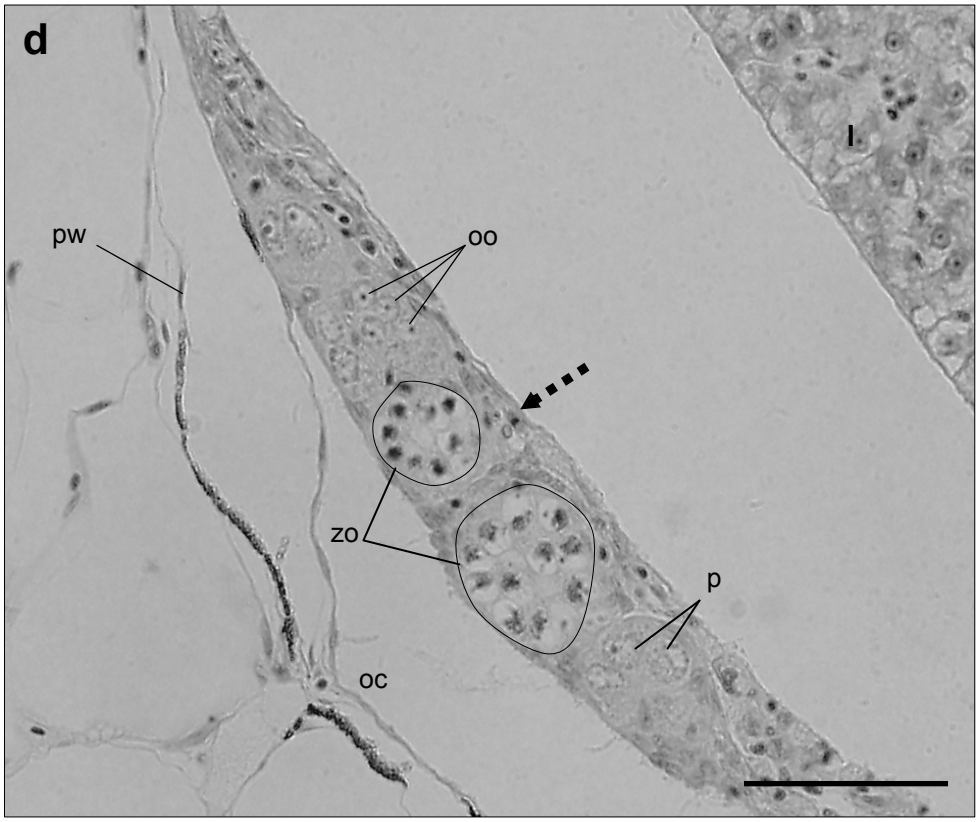
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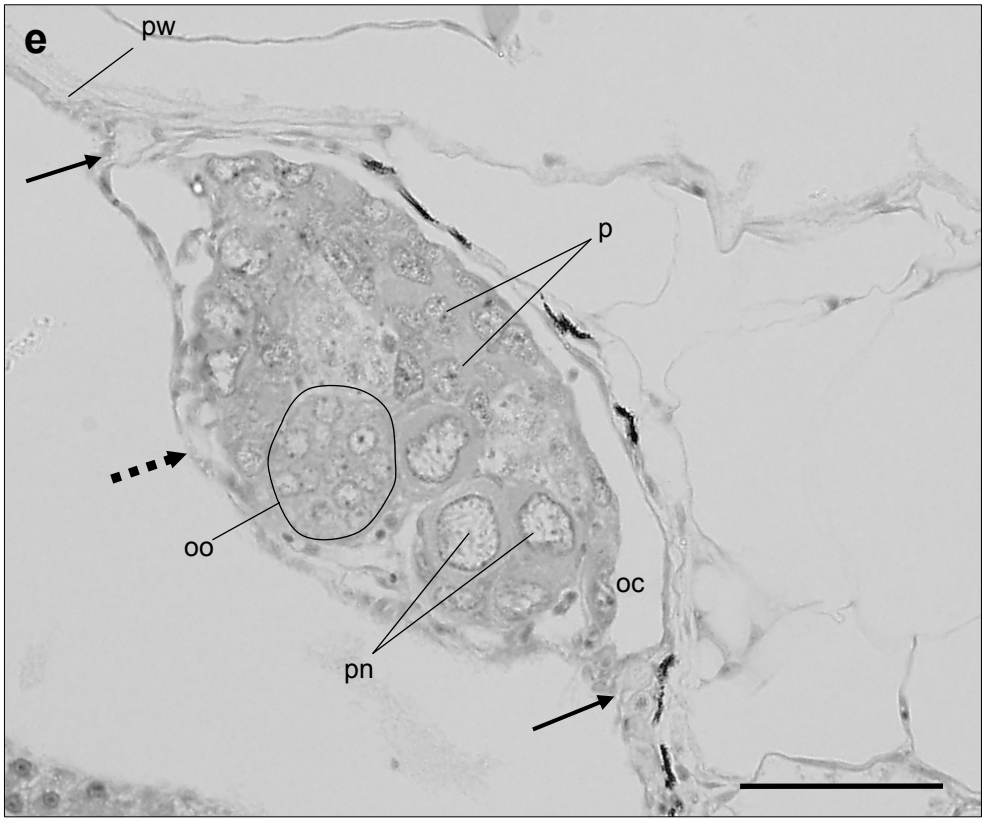
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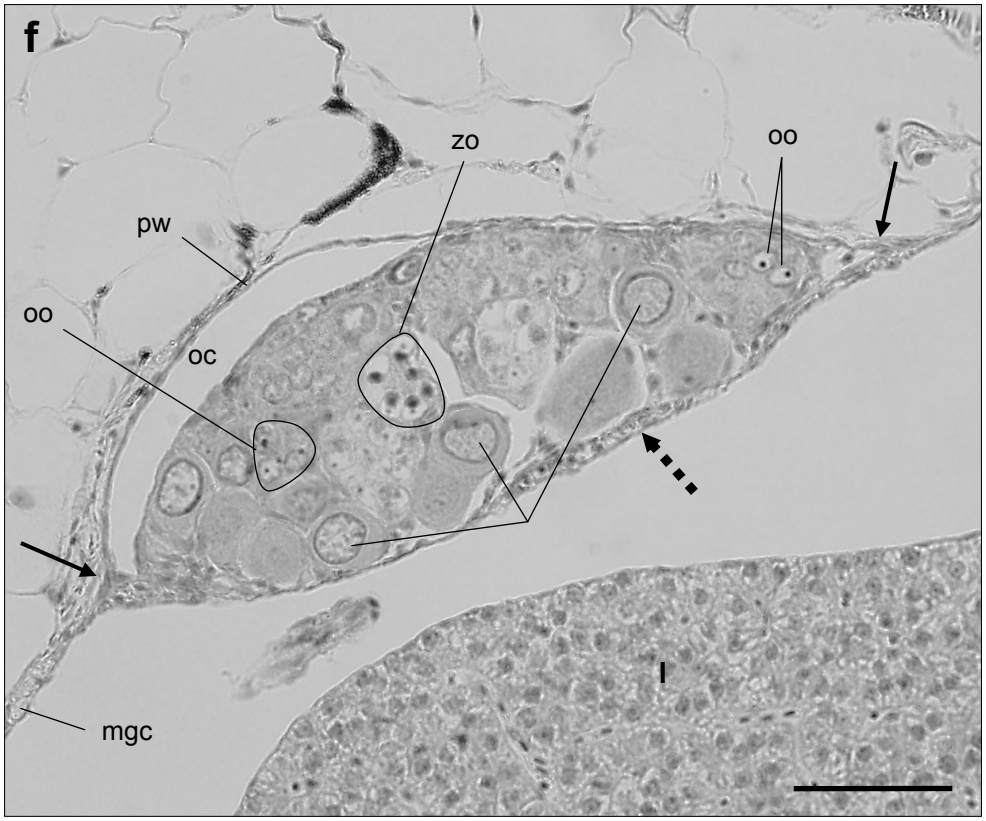
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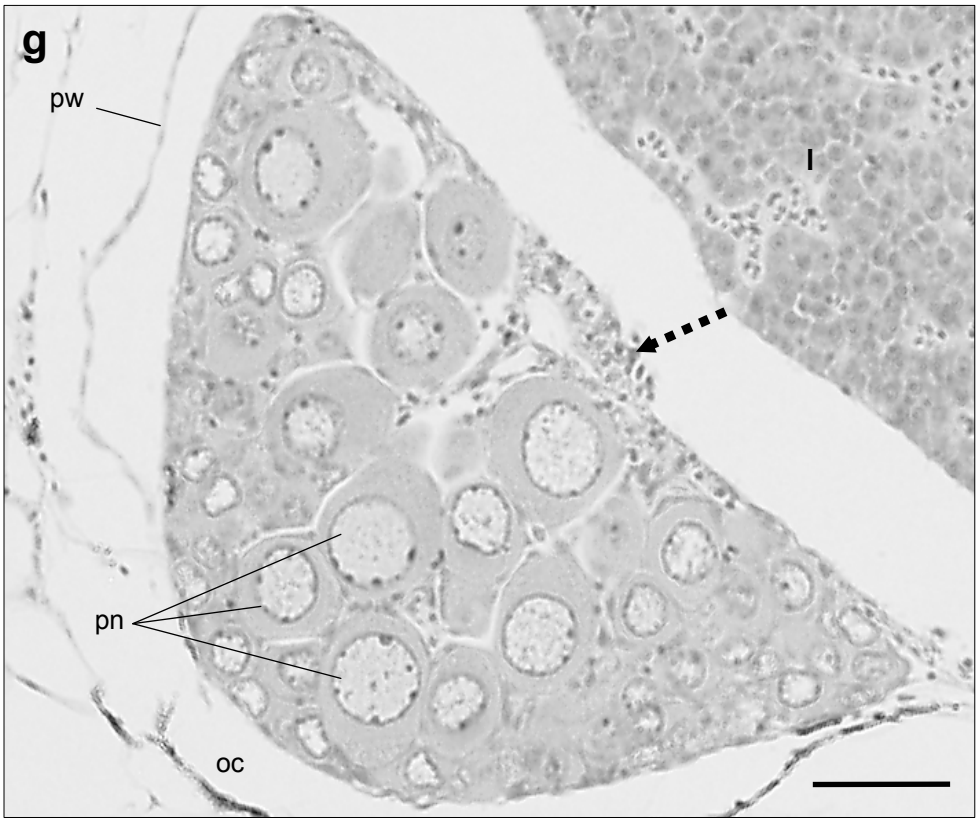
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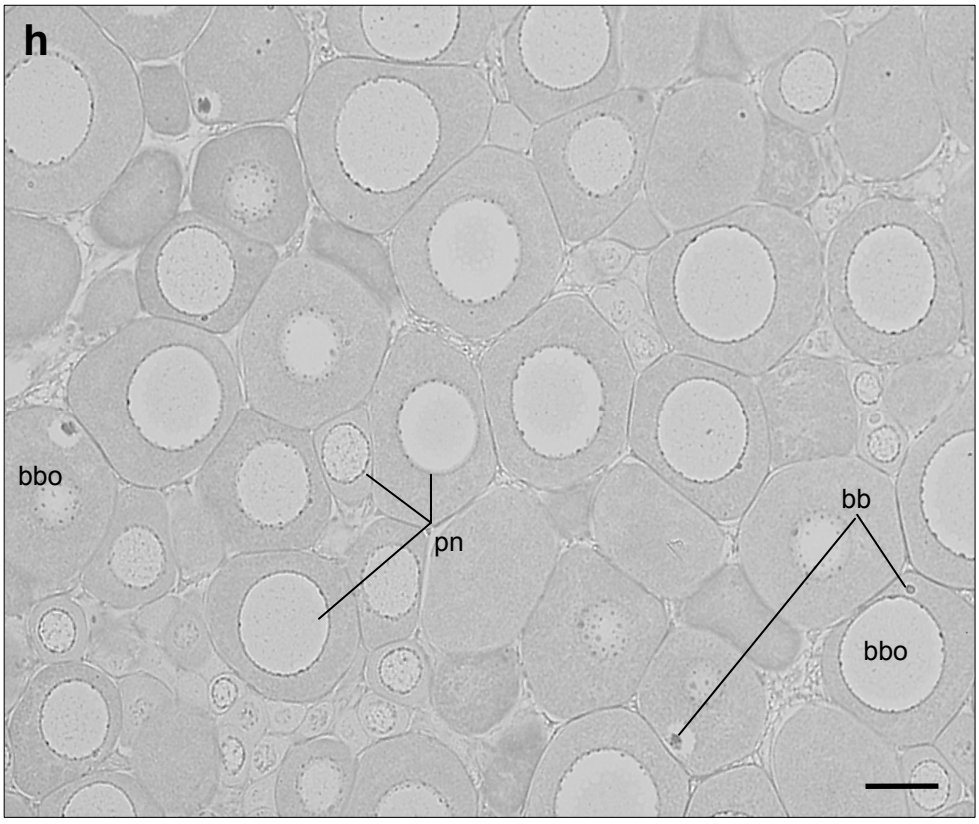
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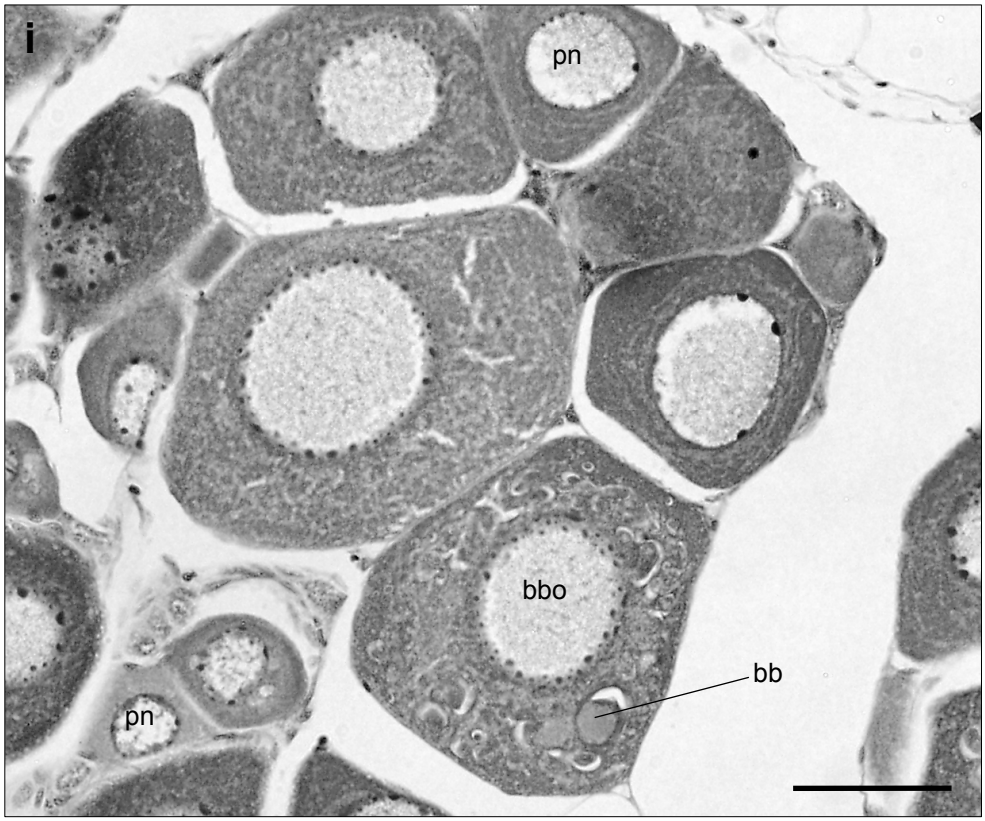
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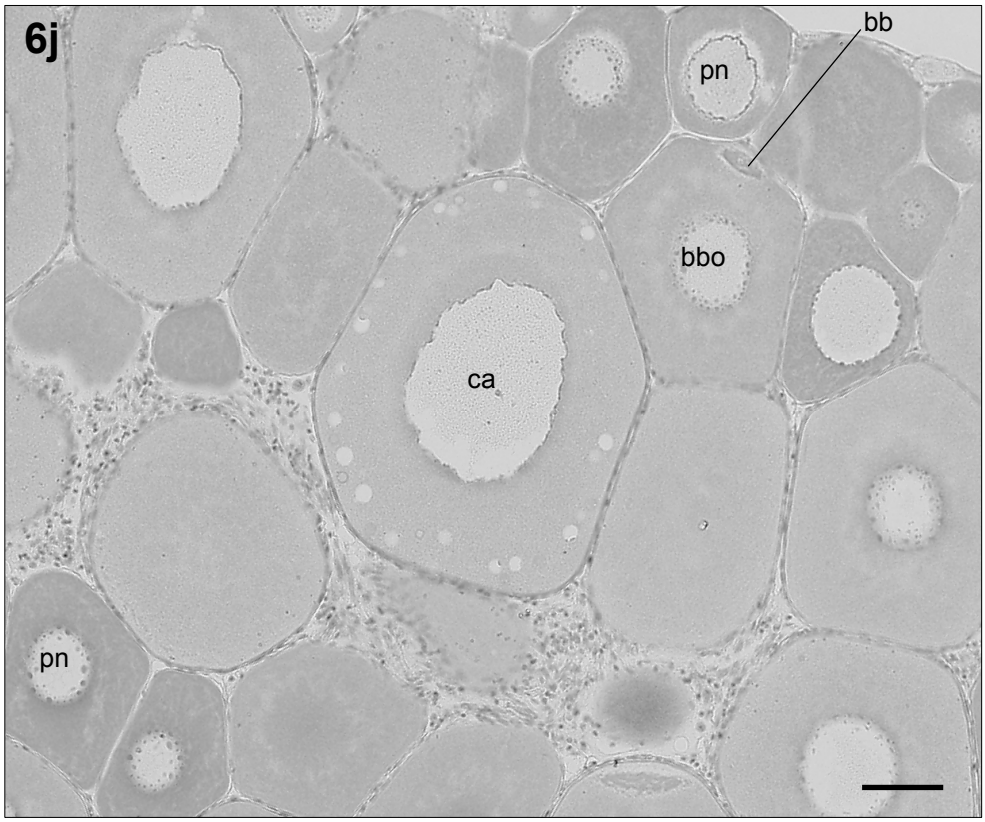
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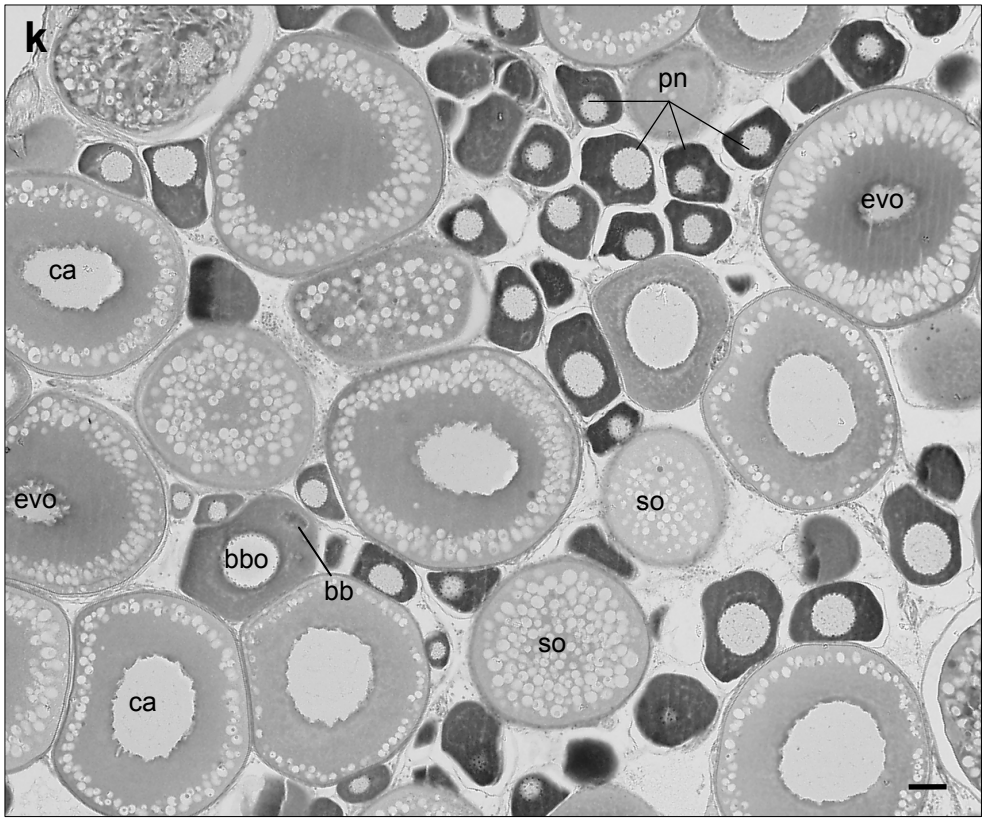
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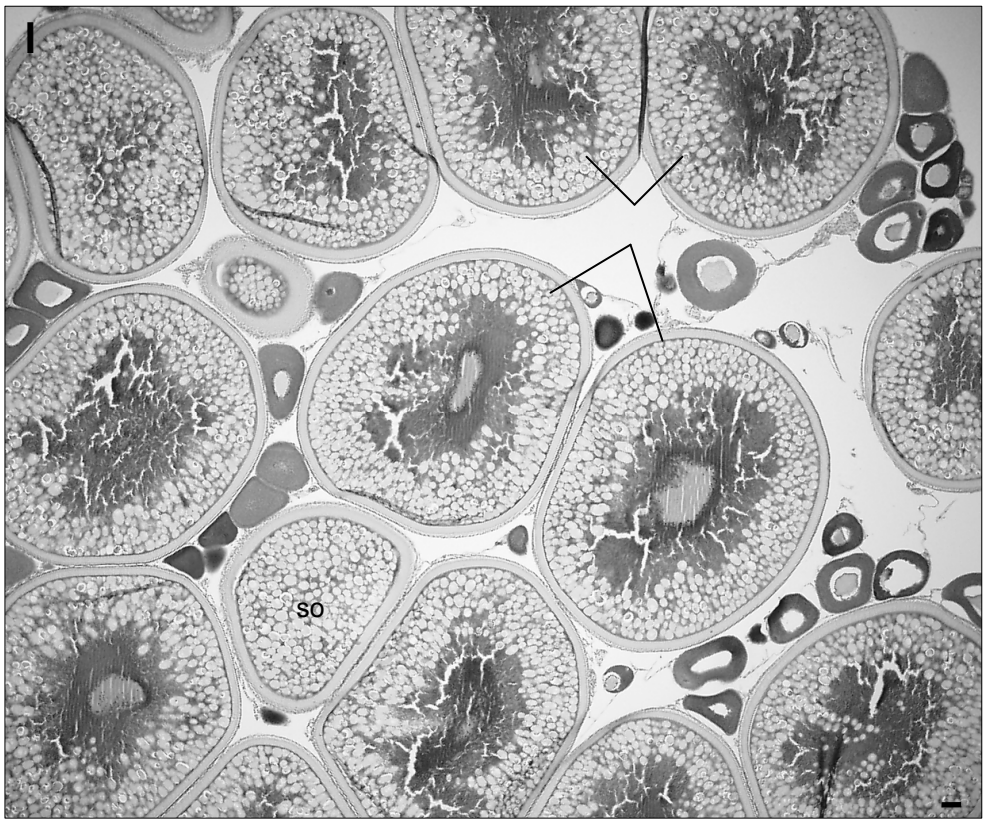
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3.2 Section 2: Interrelationships between growth and timing of sexual development

3.2.1 Growth

There was considerable variation in growth (length, weight and condition factor) between the three populations of fish (Figure 3.7). Overall length, weight and condition factor were consistently greatest in fish from population 3 until approximately 300 dpf when they became greatest in population 1, while the lowest lengths, weights and condition factors were generally observed in fish from population 2, especially after 260 dpf (Figure 3.7). During the period from the first sampling until all fish had completed sexual differentiation (population 1: 68–271 dpf; population 2: 64–260 dpf; population 3: 28–112 dpf), the SGR of fish was greatest in population 3, both in terms of length and weight (SGR_W 4.18% and SGR_L 1.65% for population 3 compared to SGR_W 1.59% and SGR_L 0.54% for population 2 and SGR_W 1.63% and SGR_L 0.49% for population 1). However, post sexual differentiation (i.e. during the remainder of the study), SGR was highest in population 1, both in terms of length and weight (SGR_W 0.95% and SGR_L 0.30%) compared to SGR_W 0.45% and SGR_L 0.13% for population 2 and SGR_W 0.43% and SGR_L 0.13% for population 3). In addition, fish in populations one and two overall showed a much greater variation in size (length and weight) and condition at each sampling point compared with fish in population 3 (Figure 3.7). This degree of variation was reflected in the co-efficient of variance (CV) statistic (CV = (standard deviation/mean)*100), which for length ranged between 7.71 and 26.46% for population 1, between 7.12 and 20.34% for population 2 and between 3.42 and 8.35% for population 3. For weight, the CV ranged between 23.99 and 101.30% for population 1, between 24.05 and 64.79% for population 2 and between 10.02 and 30.58% for population 3. For K, the CV ranged between 6.92 and 27.18% for population 1, between 6.53 and 23.53% for population 2 and between 3.33 and 10.75% for population 3. Across all three populations, weight was the most variable growth parameter while length was the most consistent.

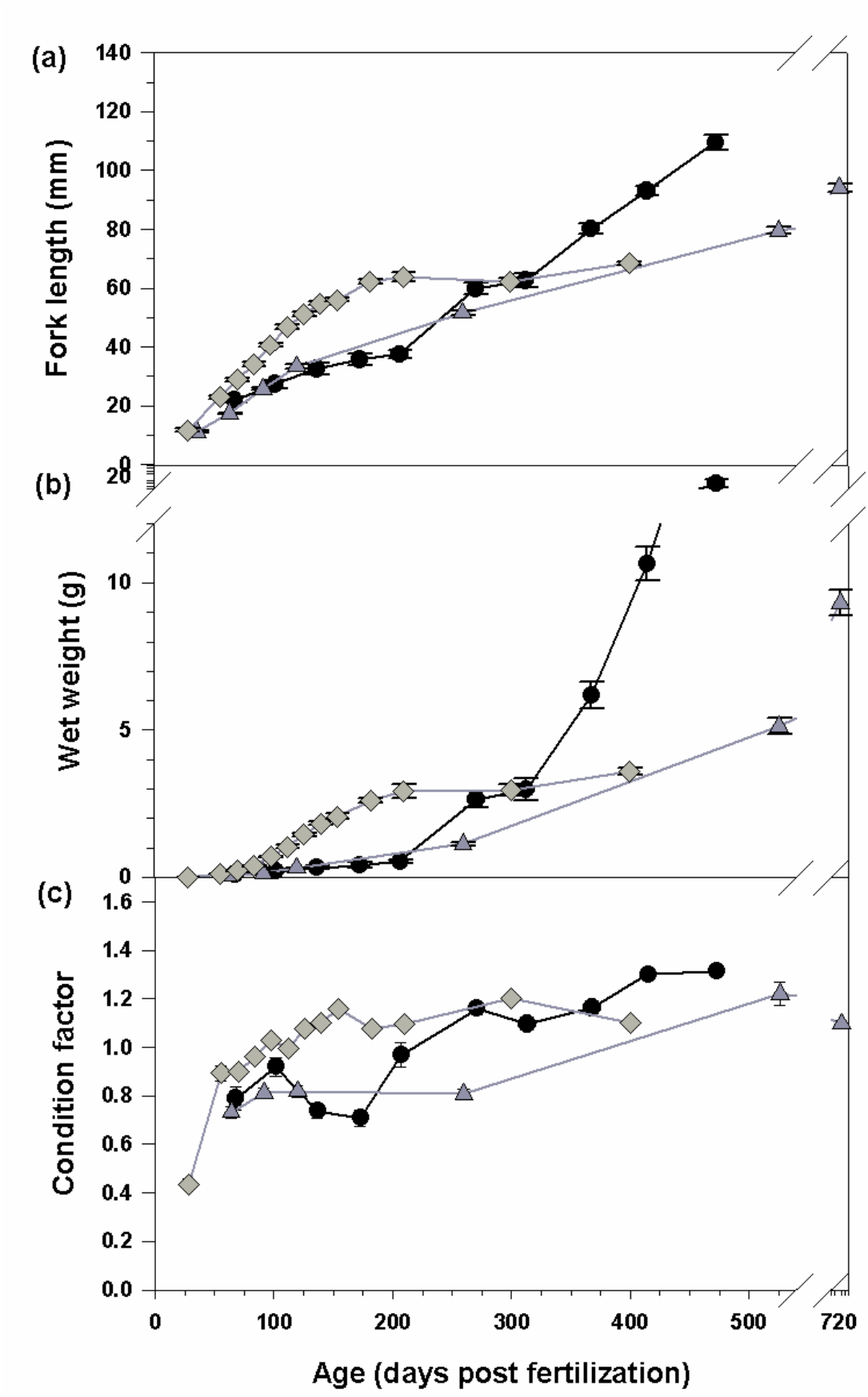

Figure 3.7 Measurements of growth of roach in the three populations studied (population 1, black line with circular data points; population 2, grey line with triangular

data points; population 3, grey line with diamond-shaped data points). (a) Fork length (mm), (b) wet weight (g), (c) condition factor (K). Data are shown as means ± SEM.

3.2.2 Sexual development

All three populations of fish showed 'normal' gonad development both in terms of gonadogenesis (the formation of ovaries or testis and associated gonoducts (ovarian cavity or sperm duct)) and gametogenesis (sex cell progression from germ cells to gametes); however, considerable variation was found in the timing of gonadal development between the three populations. Gonadogenesis (synthesis of a definable gonad) had already occurred by the first sampling points in population 1 (68 dpf) and population 2 (64 dpf) and by the second sampling point in population 3 (56 dpf). Female germ cells (primary oocytes) appeared from 68 dpf in population 1, from 84 dpf in population 3 and from 92 dpf in population 2. Male germ cells (spermatogonia) appeared later than female germ cells, from 98 dpf in population 3, from 102 dpf in population 1 and from 120 dpf in population 2. The sex of the roach could be distinguished in all fish by 84 dpf (population 3), 120 dpf (population 2) and 207 dpf (population 1), based on the presence of 'presumptive' as well as 'definitive' male and female fish (see section 1 for definitions) and by 112 dpf (population 3), 260 dpf (population 2) and 271 dpf (population 1) based solely on the presence of 'definitive' male and 'definitive' female fish. Sexual maturation first occurred in male fish (presence of spermatozoa) at 300 dpf in population 3, at 728 dpf in population 2, but did not occur in males in population 1 (terminated at 473 dpf). In female fish, early vitellogenic stage oocytes were present from 368 dpf in population 1 and from 526 dpf in population 2, but were still not present at 400 dpf (the last sampling point) in population 3. Mature vitellogenic stage oocytes were only present in fish in population 2 at 728 dpf.

Variation in the timing of gonadal development was also found between individual fish of the same age within a population. For example, in population 1 at 173 dpf the stage of gonadal development in fish ranged from undifferentiated gonads to males containing spermatogonia B and female gonads containing many peri-nucleolar stage oocytes. This variation in the stage of sexual development between individuals was greater for population 1 than for populations 2 and 3. For example, in population 3 the greatest variation between individuals of the same age was seen at 300 dpf when the sexual status of male fish ranged from gonads containing early clusters of spermatogonia B through to the gonads containing all stages of spermatogenesis including spermatozoa. At all other ages in this population very little variation was seen in either males or females.

Once all the fish within a population had undergone sexual differentiation, the sex ratios in the three study populations were as follows: Population 1, 76% males and 24% females (determined from 271 dpf onwards); population 2, 59% males and 41% females (from 260 dpf onwards), and population 3, 43% males and 57% females (from 112 dpf onwards).

3.2.3 Linking somatic growth with the timing of sexual development

For all three roach populations the gonad developed progressively with age and size of the fish (length and weight; Table 3.1 and Figure 3.8). Despite the large variation found in the timing of gonadal development both between and within the three populations studied, much less variation was evident when fish of a similar size (particularly length), irrespective of their age, were compared.

3.2.3.1 Males: In presumptive males no significant difference was found in the length (33.1 ± 1.8, 32.9 ± 1.7, and 36.3 ± 1.3 mm) of fish between the three populations or in the weight of fish between population 1 and 2 (0.310 ± 0.056 and 0.267 ± 0.035 g). The mean weight of fish in population 3 was, however, significantly higher (0.497 ± 0.058 g, $P < 0.05$) than both population 1 and 2 at this life stage. There was no significant difference in the size (length, 35.9 ± 1.8 and 53.5 ± 1.5 mm; weight, 0.390 ± 0.055 and 1.715 ± 0.150 g) of fish that contained spermatogonia A as the most advanced germ cell stage between populations 2 and 3. However, the fish in population 1 were significantly larger in both length (78.2 ± 6.4, $P < 0.001$) and weight (8.171 ± 1.892 g, $P < 0.05$) than fish in population 2 and 3 at this life stage. No significant difference was found in the length (73.7 ± 4.8, 61.6 ± 3.7 and 61.9 ± 2.4 mm) of fish between the three populations or weight of fish between populations 2 and 3 (2.488 ± 0.548 and 2.515 ± 0.315 g) in fish containing germs cells up to spermatogonia B. The mean weight of fish in population 1 only was significantly larger (9.474 ± 1.285 g, $P < 0.05$) than for both populations 2 and 3 in fish containing spermatogonia B. There was no significant difference in the size (length, 74.3 ± 2.0, 76.5 ± 2.6 and 66.4 ± 1.1 mm; weight 5.226 ± 0.450, 5.259 ± 0.552 and 3.268 ± 0.169 g) of fish containing germ cells up to spermatocytes between populations 1 and 2 and 1 and 3, respectively. However, the fish in population 2 were significantly larger both in length ($P = 0.024$) and weight ($P = 0.032$) than in population 3. Not enough fish from each population contained spermatozoa to compare their respective sizes.

For presumptive males in all three populations there was a significant increase in the length (25.4 ± 0.6 up to 34 ± 0.9 mm, $P = 0.019$) but not weight (0.148 ± 0.010 up to 0.367 ± 0.344 g) compared with undifferentiated fish. Male fish with spermatogonia A were significantly larger in size (length, 60.0 ± 3.7 mm; weight 4.15 ± 0.946 g, $P < 0.001$) compared with presumptive males. Fish containing spermatogonia B were not significantly bigger (length 67.0 ± 2.8 mm; weight 6.4 ± 0.906 g) than fish containing spermatogonia A. Fish containing spermatocytes, as the most advanced germ cell stage, were not significantly bigger (length 74 ± 1.5 mm, weight 4.8 ± 0.305 g) than fish containing spermatogonia B. Similarly, fish containing spermatozoa were not significantly bigger (length 71 ± 2.9 mm, weight 4.2 ± 0.496 g) than fish containing spermatocytes.

3.2.3.2 Females: In presumptive females no significant difference was found in the size of fish between populations 2 and 3 (length, 31.4 ± 2.6 and 28.5 ± 0.5 mm; weight, 0.273 ± 0.065 and 0.210 ± 0.002 g, respectively); no fish in population 1 were classified at this stage. For females containing pre-meiotic stage oocytes there was no significant difference in the length or weight of females between populations 1 (26.6 ± 2.3 mm; 0.214 ± 0.052 g) and 2 (33.0 ± 3.1 mm; 0.323 ± 0.057 g). In contrast, the females in population 3 were significantly larger in length than the fish in population 1 (40.4 ± 1.7 mm; $P < 0.001$) and significantly larger in weight (0.709 ± 0.090 g) than the fish in both populations 1 ($P = 0.002$) and 2 ($P = 0.022$) at this life stage. In fish containing primary stage oocytes, there were no significant differences in the size of females between populations 2 (length, 53.0 ± 2.2 mm; weight, 1.366 ± 0.212 g) and 3 (length, 60.3 ± 0.9

mm; weight, 2.579 ± 0.122 g). However, the females in population 1 were significantly larger in both length (71.7 ± 6.5 mm) and weight (7.117 ± 1.750 g) compared with population 2 ($P < 0.001$) and in both length ($P = 0.014$) and weight ($P < 0.001$) compared with population 3. For fish containing cortical alveolus stage oocytes there was a significant difference in the size of females between populations 1 (length, 108.7 ± 1.5 mm; weight, 18.437 ± 1.612 g) and population 2 (length, 88.0 ± 2.0 mm, weight, 7.255 ± 0.395 g, $P = 0.003$, $P = 0.013$, respectively, no female in population 3 had reached this developmental stage). For females containing vitellogenic oocytes there was no significant difference in the size of females between population 1 (length, 95.0 ± 14.0 mm, weight, 11.240 ± 4.53 g) and population 2 (length, 88.4 ± 2.8 mm; weight, 7.924 ± 3.865 g). No females in population 3 had reached this developmental stage. Comparing fish across all three populations there were no significant differences between the lengths or weights of undifferentiated fish and presumptive female fish (length 31.4 ± 1.7 mm, weight 0.255 ± 0.047 g), or between the lengths and weights of presumptive female fish and pre-meiotic stage females (length 35.3 ± 1.7 mm, weight 0.502 ± 0.066 g). Females containing primary oocytes were significantly bigger in length ($P < 0.001$) but not in weight compared with females with pre-meiotic stage oocytes (length 60.5 ± 1.6 mm, weight 3.1 ± 0.404 g), and significantly smaller in both length and weight ($P < 0.001$) compared with female fish containing oocytes at the cortical alveolus stage (length 100.4 ± 5.2 mm, weight 14.0 ± 2.9 g). There was also a significant difference in the size of females (smaller; see discussion) (in weight only, $P = 0.002$) containing vitellogenic oocytes compared with females with cortical alveolus stage oocytes as the most advanced germ cell stage (length 89.2 ± 2.8 mm, weight 8.3 ± 0.886 g).

There were no differences in the size of males compared with females until the sexes were well developed. In population 1, female fish were significantly larger in both length (10.0 ± 0.12 mm versus 8.99 ± 0.17 mm; $P = 0.002$) and weight (13.4 ± 0.66 g versus 9.45 ± 0.50 g; $P < 0.001$) but not condition factor compared with males at 415 dpf. In population 2, this dichotomy in size was not apparent until 728 dpf (length: females versus males; 9.8 ± 0.14 mm versus 9.23 ± 0.16 mm, respectively; $P = 0.042$, weight: females versus males; 10.74 ± 0.64 g versus 8.68 ± 0.51 g, respectively; $P = 0.027$). There was no difference between the sexes in condition factor at this time. No differences in size were seen between the females and males at any sampling point in population 3 (at 400 dpf; females: 6.87 ± 0.11 mm, 3.62 ± 0.19 g and K = 1.11 ± 0.01; males: 6.84 ± 0.89 mm, 3.56 ± 0.15 g and K = 1.11 ± 0.01).

		UD	Males					Females				
			PGC	**SA**	**SB**	**SCY**	**SPD/SZ**	**PGC**	**PM**	**PO**	**CA**	**VT**
Age (dpf)	**Population 1**	68–207	102–207	102–473	173–473	271–415	--------	--------	68–102	102–473	368–473	368–473
	Population 2	64–92	92–120	120–728	260–526	260–728	728	92–120	92–120	120–526	526	526–728
	Population 3	56–70	70–98	98–210	182–400	300–400	300–400	70	84–126	112–400	--------	--------
Length (mm)	**Population 1**	16–44	25–45	36–126	42–130	55–102	--------	--------	19–38	31–116	106–111	81–109
	Population 2	14–37	26–42	25–46	46–86	46–105	88.4	25–40	21–40	26–83	86–90	69–105
	Population 3	20–31	30–43	43–66	56–67	55–73	62–72	28–29	31–53	46–73	--------	--------
	Mean±SE	*25.4±0.6*	*34±0.9*	*60±3.7*	*67±2.8*	*74±1.5*	*71±2.9*	*31.4±1.7*	*35.3±1.7*	*60.5±1.6*	*100.4±5.2*	*89.2±2.8*
Weight (g)	**Population 1**	0.027–0.588	0.111–0.694	0.510–26.8	0.508–28.1	1.8–12.25	--------	--------	0.065–0.485	0.250–30.27	15.22–19.86	6.70–15.77
	Population 2	0.018–0.376	0.135–0.524	0.142–0.773	0.660–7.18	0.690–12.55	7.2	0.179–0.527	0.109–0.472	0.190–5.89	6.9–7.7	2.6–13.5
	Population 3	0.073–0.286	0.220–0.800	0.800–3.1	1.8–3.3	2.3–4.5	3.0–4.9	0.210	0.300–1.5	0.900–4.4	--------	--------
	Mean±SE	*0.148±0.105*	*0.367±34.4*	*4.1±0.947*	*6.4±0.906*	*4.8±0.305*	*4.2±0.496*	*0.255±0.047*	*0.502±0.066*	*3.1±0.404*	*14.0±2.9*	*8.3±0.886*

Table 3.1 Range in fish age and fish size (length and weight) recorded for each population at each progressive stage of gametogenesis. UD, undifferentiated gonad; PGC, primordial germ cell; SA, spermatogonia A; SB, spermatogonia B; SCY, spermatocyte; SPD/SZ, spermatid/spermatozoa; PM, pre-meiotic stage oocyte; PO, primary stage oocyte; CA, cortical alveolus stage oocyte; VT, vitellogenic stage oocyte.

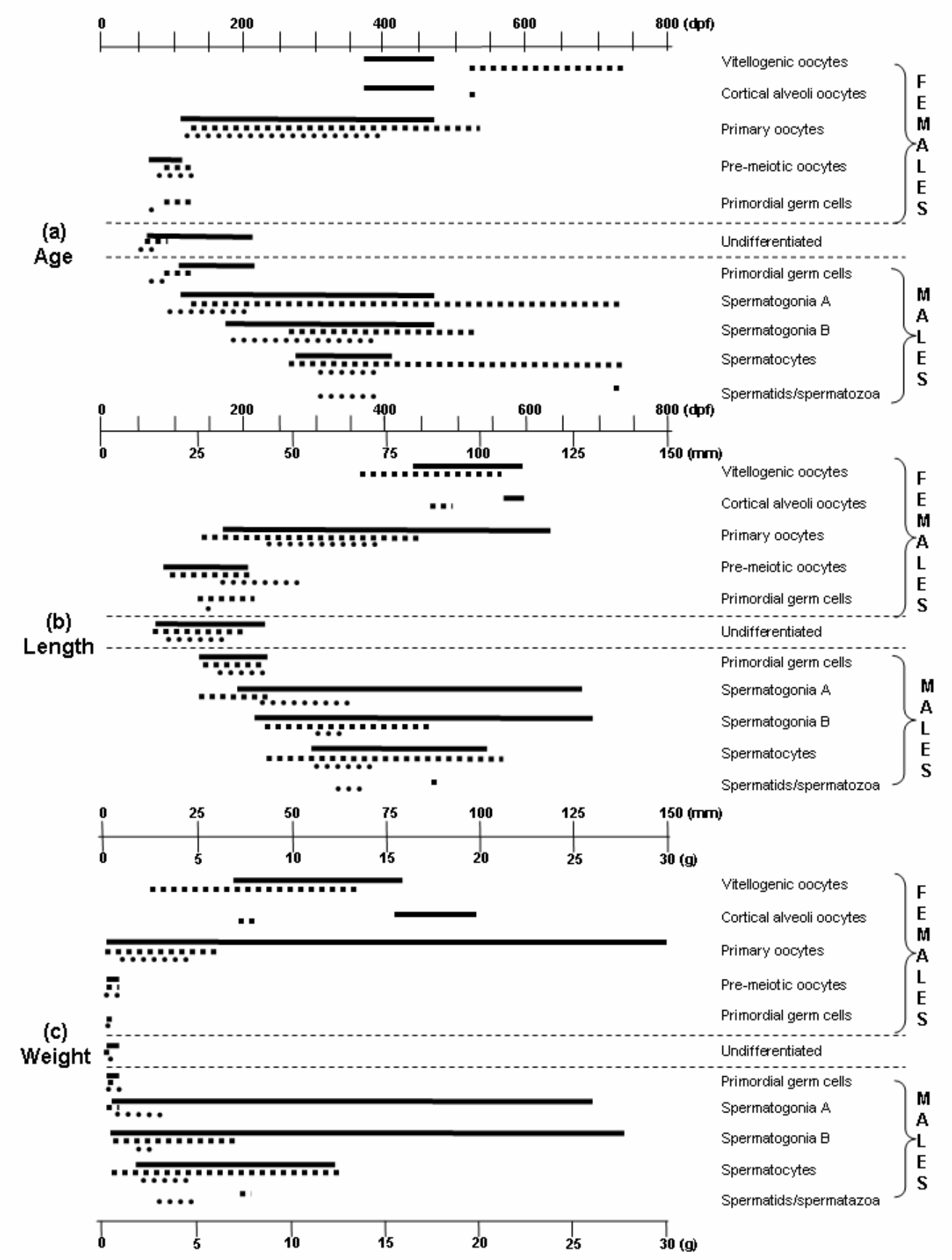

Figure 3.8 Schematic representation showing the range in (a) age (days post fertilisation; dpf), (b) fork length (mm), and (c) wet weight (g) of roach at which different stages of sex cell development were present in the gonads of males and females

comparing each of the three populations. Solid line, population 1; dashed line, population 2; dotted line, population 3.

4 Discussion

Histological analysis of gonadal development in fish has played a key role in establishing the fact that effluent discharges from sewage treatment works (STWs) induce sexual disruption. Histology of the gonad has also been used to determine the capacity of individual environmental oestrogens to induce sexual disruption in fish in the laboratory. The most comprehensive studies on sexual disruption in wild fish have come from studies on the roach in UK rivers. Nolan et al. (2001) described the intersex condition in roach, but relatively little has been documented on sexual development in this species. This study provides a detailed histological description of the ontogeny of sexual development in the roach and provides a comprehensive examination of how age, growth rate and size affected the rate of sexual development in this species.

The precise timing of the onset of gonadogenesis in the roach was not determined in this study because the fish in populations 1 and 2 had definable gonads at the first sampling point (64 and 68 dpf, respectively) and in population 3, although there was no definable gonad in fish at 28 dpf, at 56 dpf (the next sampling point) all the fish had a definable gonad. More appropriate to our investigation, however, was establishing the timing of germ cell differentiation. Germ cell differentiation in female roach was first recorded at 68 dpf and generally preceded germ cell differentiation in male roach (first recorded at 98 dpf). Definitive females, therefore, were distinguishable at an earlier age than definitive males for all three populations. This difference in the timing of sexual differentiation, where the ovary in the female develops in advance of the testis in males, is common in most fish species that are gonochorists (Nakamura 1978, Nakamura et al. 1998). The onset of ovarian and testicular differentiation in the roach is later than for many other fish species that are commonly used for studies into the effects of EDCs in the laboratory (medaka, 5–10 days post hatch [dph] [females], 25–45 dph [males]; Satoh 1974, Hamaguchi 1992; fathead minnow, 10–25 dph [females], 40 dph [males], van Aerle et al. 2004; zebrafish, 10–28 dph [females], 23–49 dph [males], Takahashi 1977, Maack and Segner 2003). This is not surprising, however, given that the roach is a coldwater species with a relatively long time to sexual maturity (2–4 years), whereas the medaka, fathead minnow and zebrafish are all small temperate/tropical species with short times to sexual maturity (2–3 months). Interestingly, although female roach consistently underwent sexual differentiation at an earlier age than males, sexual maturation in males (300 dpf, population 3) preceded that for sexual maturation in females (first recorded at 728 dpf, population 1). This is comparable with that known to occur in wild roach populations, where males are reported to mature after 2–3 years, compared with 3–4 years for females (Davies et al. 2004).

In this study, there were differences in the timing of sexual differentiation and sexual maturation between the three roach populations. Germ cell differentiation was complete in all fish at a much earlier age in population 3 compared with the fish in populations 1 or 2. Sexual maturation in male roach also occurred at a much earlier age in population 3 compared with populations 1 and 2. These findings may be as a result of the significantly faster growth rate of the fish in population 3 during the first phase of development, encompassing the period during sexual differentiation, compared with fish in populations 1 and 2. Sexual maturation in female roach occurred only in population 2 (which was the only population maintained for two full years). However, the gonads of female fish from population 1 first contained early vitellogenic stage oocytes at a much earlier age (368 dpf) compared with females in populations 2 and 3. The growth rate of this population was faster post sexual differentiation than for populations 2 and 3. Differences in growth rates between individuals within a population are common in both captive reared and wild populations and are typically

attributed to a combination of natural (genetic) variation as well as variation in fish density, feeding regime and water temperature. Some of the differences in growth between the study populations were probably due to differences in fish density and differences in the food provisioned (populations 1 and 2 were kept at a higher density during early life than population 3 and were not provided with such a varied diet from an early age).

There were differences in the sex ratios in the three study populations. Populations 1 (76% males and 24% females) and 2 (59% males and 41% females) had a higher proportion of males, while population 3 had a bias towards females (43% males and 57% females). Interestingly, there was a progressively higher proportion of females with an increased SGR during the first part of life (encompassing the period of sexual differentiation). In various fish species temperature, pH, density and social interactions can affect the subsequent sex ratio in fish populations (Baroiller and Cotta 2001), but nothing could be found in the literature for effects of growth rate on gender assignment in fish.

In previous studies on the roach that have investigated the effects of STW effluents on sexual differentiation and development, some fish were found to be undifferentiated even at 200–300 dph (*c*. 210–310 dpf; Rodgers-Gray et al. 2001, Liney et al. 2005). This finding might lead one to conclude that the roach is not a suitable species for studies investigating chemical effects on sexual differentiation because of the long exposure periods required and thus high associated costs of experimentation compared with the relatively short times to sexual differentiation in 'model' fish species. However, contrasting this, our findings have shown that captive populations of roach can complete sexual differentiation from as early as 84–112 dpf (*c*. 74–102 dph) and that male and female roach can reach sexual maturity from as early as 300 dpf (*c*. 290 dph) and 728 dpf (*c*. 718 dph), respectively. Our results demonstrate that it is practical to use roach in the laboratory for assessing the effects of EDCs on sexual differentiation, and feasibly also for studies on the effects of EDC exposure on subsequent gamete production/quality. It is of interest to note that the size of the fish in the fastest growing population (population 3) was comparable with roach of a similar age captured from several populations of wild fish in UK rivers (40–50 mm at *c*. 130 dph, Beresford et al. 2004; compared to 46–57 mm at 126–140 dpf from population 3). In addition, under favourable conditions, roach have been reported to reach 60–90 mm (Maitland and Campbell 1992) during their first year of life, which is comparable (in fact, slightly larger) than the fish for all three study populations. Importantly, in our study, male fish of this size reached sexual maturity during their first year (males, 62–72 mm), which would also suggest that some males in the wild, growing under favourable conditions, could reach sexual maturity during their first year.

In addition to fish size, our results suggest that seasonality played a role in defining the sexual status of the roach. This is not surprising considering most fish species exhibit an annual rhythm of reproduction related to environmental cues and events (Le Gac and Loir 1999). In most cyprinids the predominant environmental cues that stimulate reproduction are a combination of temperature and photoperiod (Hontela and Stacey 1990). Roach typically spawn between April and June, stimulated by an increase in day length and an increase in water temperature above 12 °C (Maitland and Campbell 1992, Davies et al. 2004). The three populations of roach used in this study were subjected to different environmental conditions and this may have had an influence on the timing of sexual development. Throughout, roach in population 3 were subjected to a water temperature and photoperiodic regime that simulated ambient seasonal changes and this may have induced the advancement of sexual maturation in males (which underwent sexual maturation after their first simulated winter period). The reason why females within this population did not undergo sexual maturation as one-year-old fish, unlike the males, may have been because they had not reached a critical

size in order to do so. Females in population 2 were maintained in a constant environment throughout their first year and showed no sign of undergoing sexual maturation as one-year-olds. This may be because of the failure to reach the critical size needed to do so. Following the simulation of a winter period (reduced water temperature and reduced photoperiod) in their second year, however, sexual maturation was stimulated in both males and females in population 2 despite their slow SGR. Fish from population 1 were kept under a constant temperature and photoperiod regime throughout their life until approximately 210 dpf when they were transferred to tanks held at elevated temperatures and with extended daylight hours. In population 1, despite a rapid increase in growth immediately after tank transfer, no females or males reached sexual maturity. In fact, after 368 dpf there was a noticeable regression in the sexual status of the fish in this population, particularly in the males (that had developed as far as the spermatocyte stage), but also in some females (degenerative oocytes occurred in two fish). It is possible that the elevated temperature (6–10 °C above the temperature at which roach normally spawn) and the extended daylight hours promoted growth only and not sexual maturation in these fish. These fish were larger compared with the fish in both populations 2 or 3 that had already undergone sexual maturation. The regression in their stage of sexual development in conjunction with their continued rapid growth also explains why, when all populations were pooled for an analysis of fish size versus status of sexual development, the mean size of mature male roach (containing spermatozoa) was smaller than the mean size of male roach containing spermatocytes, and why the mean size of mature female roach (containing mature vitellogenic stage oocytes) was significantly smaller than the mean size of female roach containing cortical alveolus stage oocytes.

5 Conclusions

In summary, this report provides a detailed histological description of the ontogeny of sexual development in the roach. Accordingly, this work will enable the design of more effective experiments to investigate for the effects of EDCs on gonadal sex differentiation and development in the roach. The data presented also provide a firm basis on which to analyse for disruption in the morphology and timing of gonadal sexual development in wild roach. The work presented has shown that sexual status is more related to fish size than to fish age. Clearly, there is likely to be a minimum age at which fish can advance through each stage of gonadal development. However, this minimum age is undoubtedly linked to the maximum SGR possible for the species, which, in turn, determines the rate (time) at which the critical size is reached to allow for progression to the next sexual stage. In this work, we found that fish length provided the best somatic growth measurement for predicting the likely status of sexual development in roach during their first year of life. Subsequent to this, seasonality is likely to play a more dominant role in defining germ cell development that, in turn, provisions gametes for each successive breeding season and size (length) of the fish becomes less instructive for informing on gonadal status. The variation in the growth and timing of sexual development between the three roach populations studied suggests that fish husbandry practices in the laboratory (including the simulation of environmental conditions such as seasonality) are important in determining not just size/growth rate of roach but also rates of sexual development. This is highlighted particularly well in the fish in population 3 (study still ongoing) that had all completed sexual differentiation within 112 dpf (*c*. 102 dph). Importantly, this work has shown that roach, in addition to their utility for studying effects of EDCs on wild fish populations, are practicable for studies on the effects of EDCs on sexual development/function in the laboratory. With the rich data sets available on sexual disruption in wild populations of roach, the ability to study roach populations in the wild, and apply them in both *in situ* studies and laboratory-based studies, the roach is arguably the most suitable fish species for integrative studies to define the impacts of EDCs on fish populations in our freshwater ecosystems.

References

Baroiller, J. F. and Cotta, H. D. (2001). Environment and sex determination in farmed fish. *Comparative Biochemistry and Physiology Part C* 130, 399–409.

Beresford, N., Jobling, S., Williams, R. and Sumpter, J. P. (2004). Endocrine disruption in juvenile roach from English rivers: a preliminary study. *Journal of Fish Biology* 64, 580–586.

Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J. and Flammarion, P. (2004). Impacts of 17 beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquatic Toxicology* 68, 193–217.

Colborn, T., Dumanoski, D. and Peterson Myers, J. (1996). *Our Stolen Future*. 306pp. UK: Little, Brown and Co.

Davies, C. E., Shelley, J., Harding, P. T., McLean, I. F. G., Gardiner, R. and Peirson, G. (2004). *Freshwater Fishes in Britain: The Species and Their Distribution*. pp. 86–88. UK: Harley Books.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P. and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent. I. Chemical fractionation and in vitro biological screening. *Environmental Science and Technology* 32, 1549–1558.

Hamaguchi, S. (1992). Sex differentiation of germ cells and their supporting cells in *Oryzias latipes*. *The Fish Biology Journal Medaka* 4, 11–18.

Harries, J. E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. P. and Tyler, C. R. (1999). Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environmental Toxicology and Chemistry* 18, 932–937.

Hontela, A. and Stacey N. E. (1990) Cyprinidae. In: Munro, A. D., Scott, A. P. and Lam, T. J., eds. *Reproductive Seasonality in Teleosts: Environmental Influences*. Boca Raton, FL: CRC Press, pp. 53–57.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G. and Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environmental Science and Technology* 32, 2498–2506.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G. C., Sumpter, J. P. and Tyler, C. R. (2002a). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biology of Reproduction* 66, 272–281.

Jobling, S., Coey, S., Whitmore, J., Kime, D. E., van Look, K. J. W., McAllister, B. G., Beresford, N., Henshaw, A. C., Brighty, G., Tyler, C. R. and Sumpter, J. P. (2002b). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction* 67, 515–524.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Tyler, C. R., van Aerle, R., Santos, E. and Brighty, G. (2006). Predicted exposure to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives* (Suppl. 1), 32–39.

Le Gac, F. and Loir, M. (1999) Male reproductive system, fish. *Encyclopedia of Reproduction 3*, pp. 20–30. Academic Press.

Liney, K. E., Jobling, S., Shears, J. A., Simpson, P. and Tyler, T. R. (2005). Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environmental Health Perspectives* 113, 1299–1307.

Maack, G. and Segner, H. (2003). Morphological development of the gonads in zebrafish. *Journal of Fish Biology* 62, 895–906.

Maitland, P. S. and Campbell, R. N. (1992). *Freshwater Fishes*. 368pp. UK: HarperCollins.

Mills, L. J. and Chichester, C. (2005). Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *The Science of the Total Environment* 343, 1–34.

Nakamura, M. (1978). *Morphological and Experimental Studies on Sex Differentiation of the Gonad in Several Teleost Fish*. PhD thesis, Hokkaido University, Japan.

Nakamura, M., Kobayashi, T., Chang, X. T. and Nagahama, Y. (1998). Gonadal sex differentiation in teleost fish. *Journal of Experimental Zoology* 281, 362–372.

Nolan, M., Jobling, S., Brighty, G., Sumpter, J.P. and Tyler, C.R. (2001). A histological description of intersexuality in the roach. *Journal of Fish Biology* 58, 160–176.

Rodgers-Gray, T. P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M. J., Sumpter, J. P. and Tyler, C. R. (2001). Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental Science and Technology* 35, 462–470.

Saillant, E., Fostier, A., Menu, B., Haffray, P. and Chatain, B. (2001). Sexual growth dimorphism in sea bass *Dicentrarchus labrax*. *Aquaculture* 202, 371–387.

Satoh, N. (1974). An ultrastructural study of sex differentiation in the teleost *Oryzias latipes*. *Journal of Embryology and Experimental Morphology* 32, 195–214.

Sharpe, R. M. and Skakkeback, N. E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 341, 1392–1395.

Sweeting, R. A. (1981). *Hermaphrodite Roach in the River Lee*. Internal report. Thames Water, Lea Division, Herts, UK.

Takahashi, H. (1977). Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bulletin of the Faculty of Fisheries Hokkaido University* 28, 57–65.

Tyler C. R., Jobling, S. and Sumpter J. P. (1998). Endocrine disruption in wildlife: a critical review of the evidence. *Critical Reviews in Toxicology* 28, 319–361.

Tyler, C. R., Ball, J., Maack, G., Paull, G. and Santos, E. M. (2002). Endocrine disruption in vertebrate wildlife – today's story. *Proceedings of the 21st Conference of European Comparative Endocrinologists*. Bonn, Germany, 26–30 August.

van Aerle, R., Runnalls, T. J. and Tyler, C. R. (2004). Ontogeny of gonadal sex development relative to growth in fathead minnow. *Journal of Fish Biology* 64, 355–369.

List of abbreviations

ANOVA	Analysis of variance
CV	Co-efficient of variance
dpf	Days post fertilisation
dph	Days post hatch
EDC	Endocrine disrupting chemical
K	Condition factor
PGC	Primordial germ cell
SGR	Specific growth rate
SGR_L	Specific growth rate based on fork length
SGR_W	Specific growth rate based on wet weight
STW	Sewage treatment works

Glossary

Endocrine disrupting chemical – An exogenous substance that interferes with the endocrine system.

Gametogenesis – Sex cell progression from germ cells to gametes.

Germ cell – A reproductive cell.

Gonadogenesis – The formation of ovaries or testis and associated gonoducts.

Gonochorist – A single sexed species.

Intersex – Condition where both male and female sex cells/structures occur simultaneously in the same gonad.

Ontogeny – The process of development of a tissue/organism/function.

Primordial germ cell – Germ cell precursor.

Reproductive duct – Ovarian cavity/oviduct – female reproductive duct carries eggs from the ovary to the exterior. Sperm duct – male reproductive duct, carries sperm from the testis to the exterior.

Somatic cell – Any cell forming the body of an organism that is not a germ cell.

Undifferentiated – A fish that has not undergone the process of translation of genetic sex into phenotypic sex.

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