Research Article

Ontogeny and Dynamics of the Gonadal Development, Embryogenesis and Gestation in Xenotoca eiseni (Cyprinodontiformes, Goodeidae)

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Short Title: Ontogeny of gonadal development, embryogenesis and gestation in *X. eiseni*

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1. Abstract

We characterized the ontogeny and dynamics of gonadal development, embryogenesis and gestation in captive stocks of the viviparous red tailed splitfin, *Xenotoca eiseni*. Using histology, we show that gonads were fully differentiated at the time of birth with a male:female sex ratio of 1:1 in the captive stock. External secondary sex features included a modified tail fin and a distinctive orange tail coloration. These features first appeared at 4 weeks after birth and were discriminative for males thereafter. There was no sex related dichotomy in body size and *X. eiseni* reached sexual maturity at approximately 12 weeks in age. We found no evidence for sperm storage in females. Gestation normally took 6 weeks and there was a positive correlation between female body size and the number of offspring produced, with up to 27 offspring for a single pregnancy. Yolk is the main food source for developing embryos for the period up to 2 weeks and thereafter, trophotaeniae in embryos act as nutrient exchange surfaces in the ovarian lumen, which subsequently undergo complete regression within two weeks of birth. In our final analysis, we discuss the great potential of *X. eiseni* as a model for studying the effects of chemicals on sexual development.

2. Introduction

Redtail splitfin (*Xenotoca eiseni*) is a viviparous freshwater fish of the goodeid family, which originates and is restricted to the Central Mexican Plateau [Turner, 1937; Fitzsimons, 1972; Webb, 1998; Contreras-Balderas, 2005; Domínguez-Domínguez et al., 2005; Domínguez-Domínguez et al., 2016]. The center of abundance for the species lies in the Río Lerma basin [Miller and Fitzsimons, 1971; Nelson, 2006], but it is also known to inhabit the Santiago River basin, the endorheic Ameca-Magdalena basin and the Río Coahuyana basin [Domínguez-Domínguez et al., 2005; Domínguez-Domínguez et al., 2016]. It is a species considered to be endangered [Koeck, 2019; Lyons et al., 2019], but it can occupy a great variety of habitats ranging from warm to cold climates, (spanning approximately 15-33 °C; [Alderton, 2012]) clear to turbid waters of lakes, ponds, rivers, streams, canals and marshes [Miller and Fitzsimons, 1971; Fitzsimons, 1972; De la Vega-Salazar and Macías-García, 2005; Iida et al., 2015].

Considering its reproductive biology, the sexes of the red-tailed splitfin are dimorphic and the female chooses the mate based on visual traits, including body coloration and size [Macıas Garcia and Valero, 2010; Greven, 2013]. Furthermore, they have internal fertilization which demands a high degree of synchrony in the mating process [Macıas Garcia and Valero, 2010; Greven, 2013]. The reproductive system of the redtail splitfin is typical of the goodeids with females having a single hollow structured ovary divided by a median septum [Turner, 1937; Hubbs and Turner, 1939; Mendoza, 1965; Uribe et al., 2005] and males having paired testes. Male goodeids produce spermatozeugmata, un-encapsulated sperm bundles containing thousands of sperm cells [Meyer and Lydeard, 1993; Bisazza, 1997; Grier et al., 2005] that they transfer to the female for internal fertilization. Males lack a specialized copulatory organ (andropodium) that occurs in some other viviparous fish species, but they have a modified anal fin where the anterior six to seven fin rays are shortened and separated from the rest of the fin and is used to aid gamete transfer to the female [Parenti, 1981; Bisazza, 1997]. Little is known about fertilization in goodeids. Many assumptions on reproduction in goodeids were made in the first half of the previous century but most remain unverified due to a lack of studies on them [Greven, 2013]. Examples of uncertainty in their reproductive development include whether oocytes undergo fertilization within the follicle, how sperm penetrates the oocytes (as the sperm have no acrosome) or for how long spermatozeugmata remain viable within an ovary [Greven, 2013].

Species that retain fertilized eggs internally for a significant period of time during which embryos develop to an advanced stage are generally referred to as viviparous [Wourms, 1981; Wourms et al., 1988; Wourms and Lombardi, 1992; Blackburn, 2005]. Based on the trophic relationship between the mother fish and embryo, the viviparous mode of reproduction can be classified as either lecithotrophic, if nutrients come from yolk exclusively, or matrotrophic, if there is an alternative supply route of

maternal nutrients [Wourms, 1981; Knight et al., 1985; Blackburn, 2005]. Matrotrophy requires considerably modified maternal and embryonal structures [Wourms, 1981]. If embryos do not establish a direct connection with maternal body tissues for the passage of nutrients, they have to be otherwise supplied by means of dissolved nutrients [Wourms and Lombardi, 1992]. Embryonic uptake of maternally derived nutrients takes place via pinocytosis or phagocytosis [Schnorr and Kressin, 2011] across two major types of epithelial surfaces, either integument, such as general body surface, gills, fins and fin folds, yolk sac and pericardial sac, or via gut associated tissues, that can include gut, a branchial system and trophotaeniae [Wourms and Lombardi, 1992]. Among viviparous fish there are four different placenta types where gestation takes place and nutrients are exchanged, namely yolk sac placenta, follicular placenta, branchial placenta or trophotaenial placenta [Wourms and Lombardi, 1992]. Two viviparous teleost families exhibit complete intrafollicular gestation [Nelson, 2006] and six other families show intrafollicular retention before the embryos are then ovulated and gestation continues intraluminally [Hoar, 1969; Meisner and Burns, 1997; Nelson, 2006]. In the remaining five families, including Goodeidae, gestation takes place mainly within the ovarian lumen [Hoar, 1969; Meisner and Burns, 1997; Nelson, 2006], in which nutrients can be derived by yolk supply or by means of maternal provisioning [Chernyayev, 1974; Meisner and Burns, 1997; Love et al., 2002; Nelson, 2006]. Goodeid embryos take up nutrients from the ovarian fluid through trophotaeniae [De La Vega Salazar et al., 1997] which are external structures that extend from the perianal region of an embryo and are embryological and evolutionary derivatives of the hindgut [Wourms and Lombardi, 1979; Wourms, 1981; Wourms and Lombardi, 1992; Wourms, 2005].

In a trophotaenial placenta, the dynamic apposition of organs includes a modified ovarian lumen epithelium as the maternal component and trophotaeniae as an embryonal structure [Wourms and Lombardi, 1992; Uribe et al., 2005; Wourms, 2005; Schindler, 2015]. Nutrients are transported from the maternal vascular system to the ovarian fluid via the specialized ovarian epithelium [Wourms, 2005] and then taken up by the hatched embryos within the ovary [Schindler, 2015]. Trophotaenial placentas can be found in all three ophidiiform viviparous families [Turner, 1936; Mead et al., 1964] as well as in 33 of 34 goodeid species [Nelson, 2006]. The point in time at which trophotaeniae start to develop during embryogenesis is not known, and also the gestation period in viviparous Ophidiiformes has not been established. Gestation in Goodeinae is reported to take several weeks [Schindler and de Vries, 1987, 1988; Schindler, 2015] and for *X. eiseni* , eight weeks [Alderton, 2012]. Viviparous poeciliid fish, on the other hand, are pregnant for approximately one month [Nelson, 2006]. Some viviparous fish species in other orders are reported to have similar gestation periods, as for example in hemiramphid [Nelson, 2006] or sebastid species [Love et al., 2002], whereas other fish may be pregnant for a significantly longer period of time. Gestation in Comephoridae takes three to four months [Nelson, 2006], zoarcids are pregnant for four to five months [Kristoffersson et al., 1973;

Korsgaard and Petersen, 1979; Skov et al., 2010] and Embiotocidae have a gestation period of six to twelve months [Hoar, 1969].

In this study, we applied histology to investigate the ontogeny and dynamics of gonadal development, embryogenesis and gestation in subpopulations of a captive stock of redtail splitfin (*Xenotoca eiseni*). In this work, we sought to characterize the morphological features that distinguish the sexes, provide a detailed description of gametogenesis in this species and describe how the embryos are retained and develop within pregnant females. We discuss our findings both in the context of the evolution of placental systems in other fish species and the potential utility of *Xenotoca eiseni* as a model organism for studies in ecotoxicology.

3. Materials and Methods

3.1. Fish maintenance

Xenotoca eiseni (redtail splitfins) were supplied from stocks raised at the University of Exeter, UK and held in aerated glass aquaria (50 L) with biological under gravel filters and a flow-through regime of between 200 and 300 litres per day. The reconstituted water had a conductivity of 300 µS/cm, a pH value of 6.8 to 7.2 and the temperature was kept at 26 ± 1 °C. The photoperiod was 12h:12 h light:dark with artificial dawn and dusk transition of 30 minutes. We recognize that in natural populations temperature and light conditions will be more variable, and this will differ also across the species breeding range. Fish were fed daily to satiation with a combination of gamma-irradiated brine shrimp (*Artemia salina*, Tropical Marine Centre, Chorleywood, UK) and gamma-irradiated bloodworm (*Chironomidae*, Tropical Marine Centre) and TetraMin flake food (Tetra GmbH, Melle, Germany). Young fish were fed with freshly hatched *Artemia* (cysts from ZM Ltd., Hampshire, UK).

Adult fish were generally kept in tanks separated by sex and age. For breeding purposes, male and female fish of the same age (and approximately equal size) were placed together for three to ten days before being separated again. In order to prevent filial cannibalism, gestating females close to parturition were transferred into small groups of two to three individual females to give birth. After parturition, adult females were placed back into stock tanks and broods of the same age were held together until the expression of their secondary sexual characteristics allowed their separation by sex.

Ontogeny of gonad development was studied through sample collections from two separately maintained subpopulations of a captive stock (3.5 and 4.5 months old). In the first study group, 62 fish (31 males and 31 females) were housed in three glass aquaria (50 L) for breeding, and in the second group, 112 fish (56 males and 56 females) were housed in four glass aquaria (50 L). The offspring, born

43 to 49 days after the initial housing of males with females, was thereafter kept in glass aquaria (20 L) in groups of equal sizes and studied over a period of six months.

To study embryo development and gestation, two breeding trials were performed using two separate groups of adult fish of seven to nine months of age. Across both trials, a total of 93 male and 93 female fish were placed (1:1 ratio) in glass aquaria to initiate breeding before the pregnant females were studied subsequently over a period of six weeks. In the two breeding trials, groups of between 10 and 11 females were housed with the same number of males in tanks for a period of 3 days (trial 1) or 5 days (trial 2). Trial 1 had 40 females and trial 2 had 53 females.

3.2. Sampling procedures

Fish were checked daily for any visible signs of ill health and examined at incremental time periods during development and gestation. At each sampling point, wet weight and total body length were recorded for each fish.

To study the ontogeny of gonadal development, fish were sampled at the day of birth (day 1), at 1, 2, 4, 8 and 12 weeks, and then 4, 5 and 6 months after birth (Tab. S1). For the period up to and including week four, 19-20 randomly selected fish were sacrificed and prepared for gonadal histology. From week eight, sex could be determined by the presence of external secondary sexual characteristics and from this time point 16-20 fish were sampled to provide (approximately) a 50%:50% sex ratio for histological processing. Euthanization was carried out by terminal anaesthesia with benzocaine (50 mg/L, inhalative) followed by destruction of the brain. All animal work and protocols used in this research were approved by the University of Exeter's Animal Welfare and Ethical Review Body, and undertaken under project and personal licences granted by the UK Home Office under the United Kingdom Animals (Scientific Procedures) Act, and in accordance with the University of Exeter's ethical policies. Fish were fixed *in toto* in Bouin's solution for 1.5 to eight hours, depending on the size of the fish and a small abdominal incision was made in fish with a total body length of more than 3.0 cm to enable effective fixative penetration.

During gestation, between 22 and 33 fish were randomly selected and sampled at 2, 3 and 5 days, and between 21 and 22 fish were randomly selected and sampled at 1, 2, 4 and 6 weeks after fertilization (Tab. S1) and sacrificed as detailed above. Their ovaries were dissected out, weighed, measured and fixed in Bouin's solution for between one and two hours. At weeks 1, 2 and 4 after fertilization, an additional 63, 42 and 21 fish were measured for body length and weight.

3.3. Histology

After fixation, tissues were rinsed with and stored in 70% IMS until processing. Fixed tissues were dehydrated and embedded in paraffin wax using a Shandon Citadel 2000 tissue processor. Serial transverse or longitudinal sections at 3 to 5 µm were cut through the entire lengths of the bodies or gonads, respectively. The sections were collected onto glass slides and stained with haematoxylin and eosin (both from Shandon, Cheshire, UK) using a Shandon Varistain 24-4 slide stainer. The slides were covered with a cover slip using Histomount (National Diagnostics, Hessle Hull, UK). The sections were examined by light microscopy using an Axioskop 40 microscope (Zeiss, Oberkochen, Germany) coupled with an Olympus DP70 CCD camera (Olympus Optical, UK) and analysed using analySIS docu software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Whole body sections were examined for the presence of gonads to identify the sex and stage of gonadal development and to determine the position of the gonads within the body. Gonads of gestating females were analyzed to assess the development of structural features associated with the gravid ovary and the stage of embryonic development.

3.4. Statistics

Growth data are presented as mean ± SD with a sigmoidal fit curve. Non-linear regression analysis with a least squares method and a t-Test (unpaired, non-parametric) were applied to compare male and female growth data. Correlation between brood sizes and total body length of females was tested for significance (two-tailed). All statistical analyses were performed using GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).)

4. Results

4.1. Growth and Secondary Sexual Characteristics

At birth, *Xenotoca eiseni* embryos had a total body length of 13.5 ± 0.9 mm and a total body weight of 30.6 ± 7.1 mg. Their body length and weight increased in a non-linear manner over time (Fig. S1A). The sexes could not be distinguished based on growth or on external secondary sexual characteristics up to 4 weeks-old. Between four and eight weeks after birth, external secondary sexual characteristics developed that allowed for visual differentiation between males and females. The first external feature appearing that distinguished males from females was a notched anal fin in males (Fig. S1D) and this was proceeded (between a few days to a few weeks later) by an orange coloration in the tail of

males (Fig. S1E). A more subtle difference in the dorsal fin that distinguished males from females, included the darker color in males. From about two months in age, the body shape and coloration were also clearly different between males and females. Males developed a hump behind their head that became more distinctive in older fish, and female fish had a mark of dark skin pigmentation on their lower abdomen (Fig. S1E).

At six months in age, non-pregnant female fish appeared to be slightly larger in size: 43.0 ± 1.2 mm and 1,800 ± 340 mg in body length and weight, respectively versus 39.1 ± 1.7 mm and 1,280 ± 200 mg in males, but this apparent difference was not statistically significant ($p = 0.53$ for wet weight and $p = 0.62$ for total length).

4.2. Gonadal Development

Gonads of *X. eiseni* were located in the visceral cavity below the swim bladder, attached to the dorsal body wall by a gonadal mesentery. The ovary in adult females aligns with the start of the swim bladder, extends through the length of the abdomen, and ends at the genital pore (Fig. S2). In adult males, the testis extends from a position approximately halfway down the length of the swim bladder to the genital opening (not shown).

At birth, the gonads of *X. eiseni* were fully differentiated (Fig. S3), but always the structures of ovary and testis were similar in appearance until the onset of gametogenesis. The female ovary however was generally distinguishable appearing as a single lobed structure, separated into two compartments by a highly folded median septum. At two weeks, the structure of the gonads became more distinct (Fig. S4). Oogenesis was first observed at between two and four weeks after birth and females reached full sexual maturity at approximately twelve weeks in age (Fig. S4), when their total body length was at least 3 cm. Male fish had a two lobed testis, spermatogenesis started around four weeks after birth and sperm bundles were first seen at between four and eight weeks (Fig. S4).

4.2.1.Testis

The paired testis consisted of a right and a left lobe joined together at the anterior end. Testes of adult males were between 3.6 and 8.6 mm in length with a lobe diameter of between 0.5 and 2.8 mm at its widest section. Testicular tubes appeared shortly after birth and they ended blindly at the periphery of the gonad. At four weeks old, spermatogenesis was initiated. Spermatogenesis progressed through three main stages: an immature testis with spermatogonia only (stage 0), the onset of spermatogenesis (stage 1; Fig. 1A) and the presence of germ cells at all stages of maturation, including sperm packages or spermatozeugmata (stage 2, Fig. 1B). In a stage 2 testis, nests of spermatogonia occurred at the periphery of the gonad and they underwent synchronous development

with the germ cells migrated towards the centre of the gonad (Fig. 1C and Fig. S5). During this process, spermatocytes, spermatids and sperm were present as clusters of the same cell type (Fig. 1D). These clusters were encapsulated by Sertoli cells, forming spermatocysts (Fig. 1D). During the final stage of spermatogenesis, spermatozeugmata were formed within the cysts and these underwent release into the efferent duct system where they remained stored. The countless efferent ducts merged to form bilateral ducts (Fig. 1C) which eventually joined to form a single sperm duct leading to the urogenital pore just anterior to the anal fin. In some fish, the bilateral ducts were visible from birth.

4.2.2.Ovary

The single lobed ovary of a mature non-gravid female was between 7 and 16 mm in length with a diameter of between 0.5 and 2 mm, depending on the body size of the female. The lumen was divided into two more or less equal lateral halves by a highly folded longitudinal septum (Fig. 2A&C; for a schematic illustration see Fig. S6). Oocytes at different stages of maturation were present in the ovarian wall and the septum, located mainly in the anterior part of the ovary. The ovarian wall of the posterior part formed the gonoduct opening to the exterior at the genital pore (Fig. 2C)**.**

Oogenesis and oocyte maturation were continuous processes that can be divided into two growth stages: primary growth comprising stages from the onset of oogenesis to oocytes containing oil droplets, followed by secondary growth characterized by vitellogenesis and the generation (and thus inclusion) of yolk. Staging in females was characterized based on the presence of specific cellular and morphological characteristics as described previously [Uribe and Grier, 2011; Uribe et al., 2012]. We have only limited information on the size of oogonia which on average were approximately 5 µm in diameter (range between 3.27 and 5.95 µm, n=4). Chromatin nucleolus stage had an average diameter of 20 µm (range between 13.24 and 25.89 µm, n=68). Early primary growth oocytes (stage 1) had an average diameter of 35 ± 7 µm (range between 23 and 58 µm, n=175) and contained one or multiple nucleoli (Fig. 2D). Mid primary growth oocytes (stage 2) had an average diameter of 75 ± 16 µm (range between 45 and 116 µm, n=90) and were characterized by the presence of Balbiani bodies (Fig. 2E). Late primary growth oocytes (stage 3) were characterized by the occurrence of large oil droplets (Fig. 2F) and had an average diameter of 150 ± 22 µm (range between 96 and 189 µm, n=96). With the appearance of the first yolk globules, oocytes entered the early secondary growth stage (stage 4; Fig. 2G) and reached an average diameter of 200 ± 20 µm (range between165 and 232 µm, n=59). Late secondary growth oocytes (stage 5) had an average diameter of 260 ± 36 µm (range between 206 and 359 µm, n=32). During this late vitellogenic stage, yolk droplets fused together finally forming one large yolk globule that occupied most of the oocyte's volume. At that time, oil droplets were at the periphery of the ooplasm (Fig. 2H). Fully grown oocytes (stage 6; Fig. 2I) had an average diameter of 380 ± 86 µm, with a range between 234 and 506 µm (n=45). At this stage, female fish were ready to

mate. During secondary growth, the germinal vesicle migrated to the periphery of the oocyte and its surrounding somatic tissue including the follicle cells became clearly visible. When oocytes had not been fertilized, they were ovulated into the ovarian lumen where they underwent resorption.

In both, gravid and non-gravid ovaries, high numbers of oocytes in stages 1 and 2 were present with the former usually present in clusters. Exact numbers could not be determined, as the sectional spacing of 60 µm was larger than the sizes of the oocytes. However, taking the probability of capturing an oocyte in a section into account allowed for an estimation of the oocyte numbers. These calculations gave numbers between 176 and 342 with an average of 243 for stage 1 (early primary growth) oocytes and between 53 and 190 (average 87) for stage 2 (mid primary growth) oocytes. Late primary growth oocytes (stage 3) were also often present in numbers of between 30 and 140 (average 57) per ovary, whereas numbers for vitellogenic oocytes were much lower at between 5 and 30 in an ovary. In pregnant females, the most developed oocytes observed were usually at stage 3 with a few exceptions where early vitellogenic oocytes were also present.

4.3. Embryogenesis and Gestation

Two breeding trials were performed to study embryogenesis and gestation. In the first trial, with 40 females, 10 (25%) became pregnant and in the second trial, 28 of the 53 females used became pregnant (52.8%).

It was not possible to determine visually when an individual female was ready to mate and there was no external sign to indicate pregnancy. A gestating female could be recognized only three to four weeks after fertilization by a swelling of the abdomen. We found no evidence for sperm storage. Fertilized eggs were always seen free in the ovarian lumen and it could therefore not be determined whether the fertilization took place within the follicle or just after ovulation.

Embryos first developed within their egg envelope (Fig. S7) and were provisioned with nutrients via their yolk supply. In the first week of gestation, there were no obvious changes in yolk reserve but there was a subsequent rapid depletion after the second week. During week two, embryos developed pigmentation and some embryos began to hatch within the ovary. At this time, trophotaeniae had started to form (Fig 3), implying that the embryos had started to draw on maternal provisioning.

During the following two weeks, the yolk reserve was absorbed completely and trophotaeniae reached their maximum length. Embryos generally had four to six trophotaeniae, extending from the perianal region and consisting of a vascularized tissue core with surrounding epithelial cells. The trophotaeniae were either ramified or unbranched. During weeks two to four, embryos also grew substantially and this increase in brood volume resulted in the swelling of the abdomen of the gestating

female. Four weeks into gestation, embryos had a total body length of approximately 10 mm (Fig. 3) and were viable in water, if dissected out at this time point. Growth of the embryos caused the ovary to expand further and the ovarian wall became thin and transparent, but retained the septum folding into which some of the embryos were nestled (Fig. 4).

Gestation normally took six weeks. In the ovarian lumen, most embryos were aligned and orientated in the same direction as their mother. After six weeks, differentiated gonads were visible below the swim bladder of some embryos (Fig. S8), although it was not always possible to determine the sex of the fish. At that stage, the trophotaeniae had become partly reabsorbed and within two weeks after birth underwent complete regression.

The duration of the birth process (Fig. S9) was variable and in part depended on the brood size. In this study, brood size ranged between 1 and 27 (pregnant females in this study had a total body length of 32 - 49 mm). There was a significant positive correlation between size and the number of offspring produced ($r^2 = 0.4661$, n = 50, including pregnant fish and their offspring for the ontogeny study also; $P < 0.0001$; Fig. 5).

At birth, embryos were 13.5 ± 0.9 mm in length and weighed 30.6 ± 7.1 mg. Development was generally synchronous within broods and also across different broods at the same stage of gestation. All broods comprised of both male and female embryos and the overall sex ratio was approximately 50%:50%. During gestation, new oocytes continued to mature within the ovarian walls and septa and female fish were ready to mate again a few days after parturition.

5. Discussion

5.1. Ontogeny and gonadal development

The presence of fully differentiated gonads at birth developing as a spindle-shaped hollow single ovary in female and paired testes in male *Xenotoca eiseni* is typical to that reported for other goodeid species [Turner, 1933; Mendoza, 1965; Grier et al., 1978; Greven, 2013]. It was difficult, however, to distinguish the sexes at birth and prior to the onset of gametogenesis via histology, because in males the two testes merge together for about a quarter of their length from the anterior end, before they separate into two lobes. Therefore, they appeared similar across this fused area to that of the single lobed ovary in females.

Spermatogenesis started approximately four weeks after birth of the fish when sperm developed synchronously in cysts surrounded by single layers of Sertoli cells. This, as well as the appearance of the main duct of the testes with branched ramifications, are characteristic features for goodeid

teleosts [Grier et al., 1978; Nelson, 2006]. The latter is morphologically similar to poeciliid testes [Grier et al., 1978]. Whilst the formation of spermatozeugmata is similar between goodeids and poeciliids, the un-encapsulated sperm bundles differ in various ways including the manner in which sperm are orientated between these two families: in Goodeidae, as we show, flagella are located at the periphery of the sperm packages and irregularly organized [Grier et al., 1978], whereas in Poeciliidae they are directed towards the center of the sperm bundles [Nelson, 2006]. Spermatozeugmata also occur in the perciform family of Embiotocidae, whereas species of the two ophidiiform families of Aphyonidae and Bythitidae produce spermatophores (encapsulated sperm packages) [Nelson, 2006]. The notched anal fin that functions as an andropodium in *X. eiseni* is unique to Goodeidae [Fitzsimons, 1972; Parenti, 1981; Bisazza, 1997]. Species of the hemiramphid family also possess an andropodium, whereas other male viviparous teleosts generally have an intromittent organ for internal fertilization [Nelson, 2006].

Female *X. eiseni* had a hollow single median ovary which is common for Goodeidae [Turner, 1933; Wourms, 1981; Wourms and Lombardi, 1992; Greven, 2013], Poeciliidae, Embiotocidae and Zoarcidae [Nelson, 2006; Wootton and Smith 2014], whereas females of other viviparous teleost families normally have a paired gonad system [Nelson, 2006]. The average size of an ovary of a mature female *X. eiseni* in this study compares well with that reported previously [Mendoza, 1965]. We were not able to find evidence for sperm storage in mated females again supporting that seen for other goodeids and unlike that which occurs in poeciliid and embiotocid fish.

Oogenesis was initiated at between two and four weeks after the birth of the fish and females reached full sexual maturity at a total body length of at least 3 cm and an age of around twelve weeks. Gonads of mature females comprised of oocytes at different developmental stages with earlier stages normally occurring in higher numbers. Oocytes lost during their growth process through resorption – often referred to as atresia [Tyler and Sumpter, 1996; Uribe Aranzábal et al., 2006] - tended to occur at a very early stage and once an oocyte has passed through previtellogenesis (primary growth stage) most appeared to be retained until reaching full maturity. This is supported by observations on the brood sizes of *X. eiseni* (1-44, data from this and another unpublished study) and the corresponding number of vitellogenic oocytes estimated in the developing ovaries. However, upon reaching maturity, most of the fully-grown oocytes were atretic in the present study suggesting that, unless fertilized, mature oocytes were resorbed. Atretic follicles have been observed in both pregnant and non-pregnant goodeid females and all non-fertilized mature oocytes appear to undergo degradation [Uribe Aranzábal et al., 2006]. Other researchers have suggested that for Goodeidae roughly half of the original number of embryos area normally absorbed during gestation [Turner, 1933], but this may be as a consequence of the provision of insufficient food or undue stress imposed on those animals.

348 The average diameter of fully grown *X. eiseni* oocytes was 380 µm, which is similar to the diameter
349 of mature oocytes in the viviparous poeciliid, Least Killifish (*Heterandria formosa*) [Uribe and Grier,
350 2011]. There are, however, noticeable differences in the yolk content between these the two species.
351 *H. formosa* produces microlecithal oocytes which, at the final stage of secondary growth, are
352 characterized by a large oil globule occupying most of the cell and only very little yolk at the periphery
353 of the oocyte [Uribe and Grier, 2011], whereas mature oocytes of *X. eiseni* are almost completely filled
354 with yolk, a feature typical for egg-laying species, including the oviparous goodeids White River
355 springfish (*Crenichthys baileyi*) and Pahrump poolfish (*Empetrichthys latos*) [Uribe et al., 2012]. The
356 large yolk supply in *X. eiseni* compared with other viviparous species, suggests that embryos may not
357 depend on maternal provisioning during early gestation. This assumption is supported by the finding
358 that embryos of *X. eiseni* start to develop trophotaeniae only after two weeks of gestation.

359

360 *5.2. Embryogenesis and Gestation*

361 In cases of successful insemination, fertilized eggs were always observed in the ovarian lumen and
362 it was therefore not possible to determine whether or not fertilization took place within the follicle. If
363 eggs are fertilized in their follicles, it is likely that this more or less coincides with the time of ovulation,
364 as concluded previously for the goodeid, *Neotoca bilineata* [Mendoza, 1943]. Furthermore, since
365 spermatozeugmata were observed only in one female during the housing period and in this case,
366 sperm packages were undergoing disintegration, our study supports an absence of sperm storage in
367 goodeids [Mendoza, 1941; Greven, 2013]. The question of whether a female could possibly breed with
368 multiple males is an intriguing one and genetic analyses would need to be carried out to provide an
369 answer to this question.

370 Rates of embryonal development between the first and second week and also between the second
371 and fourth week of gestation were considerable, while changes occurring in the last third of the
372 gestation period were less obvious. After the first two weeks of gestation, the yolk supply of the
373 embryos had largely been absorbed. Together with the presence of budding trophotaeniae, this
374 implies that embryos then start to depend on a supply of maternal nutrients from histotroph. In
375 studies on other goodeid fish species, histotroph has been shown to be composed of a protein mixture
376 similar to that in the maternal blood serum [Schindler, 2015].

377 A trophotaenial placenta is common in Ophidiiformes and Cyprinodontiformes and occurs in
378 almost all viviparous goodeids. While this placenta type has been described in *X. eiseni* by various
379 authors [Mendoza, 1965, 1972; Schindler, 1990; Iida et al., 2015; Schindler, 2015] a branchial placenta
380 has not been mentioned before in association with *X. eiseni*. A few viviparous teleosts have a branchial
381 placenta and the histological structure of a branchial placenta was described for the first time in the

goodeid *Ilyodon whitei* [Uribe et al., 2014]. It is described as ovarian folds entering through the embryonic operculum into the branchial chamber, sometimes extending into the pharyngeal cavity. Here, for the first time, a branchial placenta was also identified in a number of *X. eiseni* embryos. If, or to what extent, the branchial placenta may complement the trophotaenial placenta remains to be determined. However, the main site of nutrient uptake in *X. eiseni* are the trophotaeniae. After their appearance in the first two weeks of gestation, they then grew to their maximum length over the next two weeks before starting to regress in the last phase of gestation. According to Iida and colleagues [2015] trophotaenial regression is caused by apoptotic cell death, but whether this is triggered maternally or is regulated embryonically is not known.

There was a positive correlation between female size (body weight) and the number of offspring produced in *X. eiseni*, with smaller (or younger) fish also tending to be less successful in breeding. Future studies measuring reproduction as an outcome in *X. eiseni* will need to take into account their size-related fecundity. The lack of external signs indicating whether a female *X. eiseni* has been impregnated and the fact that pregnancies only become visible after three weeks, make the prediction of fertilization outcome extremely difficult. In our studies we found that groups of at least ten females and ten males housed together for five days produced the highest breeding success rate (at over 50%). Housing smaller groups of adult fish (five females and five males) for a shorter period (e.g. three days) resulted in poor breeding success. We also found that fish maturing at a very young age were less likely to breed successfully. To maximize breeding in *X. eiseni* we therefore suggest housing males with females together in relatively large groups for periods of five days or more as a sensible approach. If breeding to produce fertilized females for experimental purposes, holding males and females together for more than five days will also result in a greater divergence in the embryonal stages between the different pregnant females, albeit also a higher number of successful matings.

Trophotaeniae take up many substances non-specifically from the ovarian fluid and therefore any substance passed from the maternal system to the histotroph may end up in the embryonal system. *X. eiseni* thus offers a novel model for investigating effects of maternal exposure to chemicals during critical developmental periods, and notably for effects on sex differentiation and development. It has been established that exposure to various so-called endocrine disrupting chemicals (EDCs), for which there is considerable environmental and human health concerns [Tyler et al., 1998; Korsgaard et al., 2002; Ankley and Johnson, 2004] can result in significant disruption to reproductive function for exposures during early life. In support of such ecotoxicology studies it would be useful to gain information on the development of the sexual organs during gestation within the pregnant female.

In summary, we show *X. eiseni* has a viviparous breeding system common to those of other goodeids and the female of the species has a relatively short gestation time of around six weeks.

Breeding is possible at any time of the year, with an interbreeding interval of less than two months and is most successful where fish are housed in large groups. The information presented on the basic biology of the reproductive system in *X. eiseni* provides an excellent platform for the potential development of this species as a model for studies into maternal transfer and biological effects of contaminants on fish embryos and early life stages.

6. Statements

6.1. Acknowledgements

We thank the Aquatic Resources Centre at the University of Exeter for their support in maintenance of the fish and Victoria Jennings, Eliane Bastos, Jan Shears and Steven Cooper at the University of Exeter for their technical support and help with fish sampling.

6.2. Statement of Ethics

All animal work and protocols used in this work were approved by the University of Exeter's Animal Welfare and Ethical Review Body, and undertaken under project and personal licenses granted by the UK Home Office under the United Kingdom Animals (Scientific Procedures) Act, and in accordance with the University of Exeter's ethical policies.

6.3. Disclosure Statement

The authors have no conflicts of interest to declare.

6.4. Funding Sources

This work was funded by the University of Exeter.

6.5. Author Contributions

ST and CRT conceived the project. ST conducted the practical work with support from AL for the histology. ST, CRT and AL wrote the manuscript.

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589

8. Figure Legends

Figure 1. Histological sections of *Xenotoca eiseni* testes showing the different stages of spermatogenesis. **A:** Stage 1 gonad containing spermatogonia and spermatocytes. **B:** Stage 2 gonad containing sperm packages. **C:** Longitudinal section of a fully mature testis. **D** Transverse section of a testis showing the process of spermatogenesis and all stages of germ cells. **Key**: aw: abdominal wall, eds: efferent duct system, se: Sertoli cells, sc: spermatocyte, sg: spermatogonium, sp: sperm package, st: spermatid.

Figure 2. Histological sections of *Xenotoca eiseni* ovaries and oocytes at different growth stages. **A:** Transverse section of the ovary of an adult female. **B:** Oocytes at primary and secondary growth stages. **C:** Lateral section of an adult female. **D:** Stage 1 – early primary growth oocyte with one or multiple nucleoli (average diameter: 35 µm). **E:** Stage 2 – mid primary growth oocyte with multiple nucleoli and Balbiani bodies (average diameter: 75 µm). **F:** Stage 3 – late primary growth oocyte containing oil droplets and cortical alveoli (average diameter: 150 µm). **G:** Stage 4 – early secondary growth oocyte with small yolk droplets (average diameter: 200 µm). **H:** Stage 5 – late secondary growth oocyte containing large yolk droplets (average diameter: 260 µm). **I:** Stage 6 – fully grown oocyte filled with yolk (average diameter: 380 µm). **Key:** aw: abdominal wall, bb: Balbiani body, cells, gv: germinal vesicle, od: oil droplet, os: ovarian septum, ow: ovarian wall, pge: early primary growth oocyte, pgl: late primary growth oocyte, pgm: mid primary growth oocyte, sge: early secondary growth oocyte, sgf: fully grown secondary oocyte, sgl: late secondary growth oocyte, y: yolk, yd: yolk droplet.

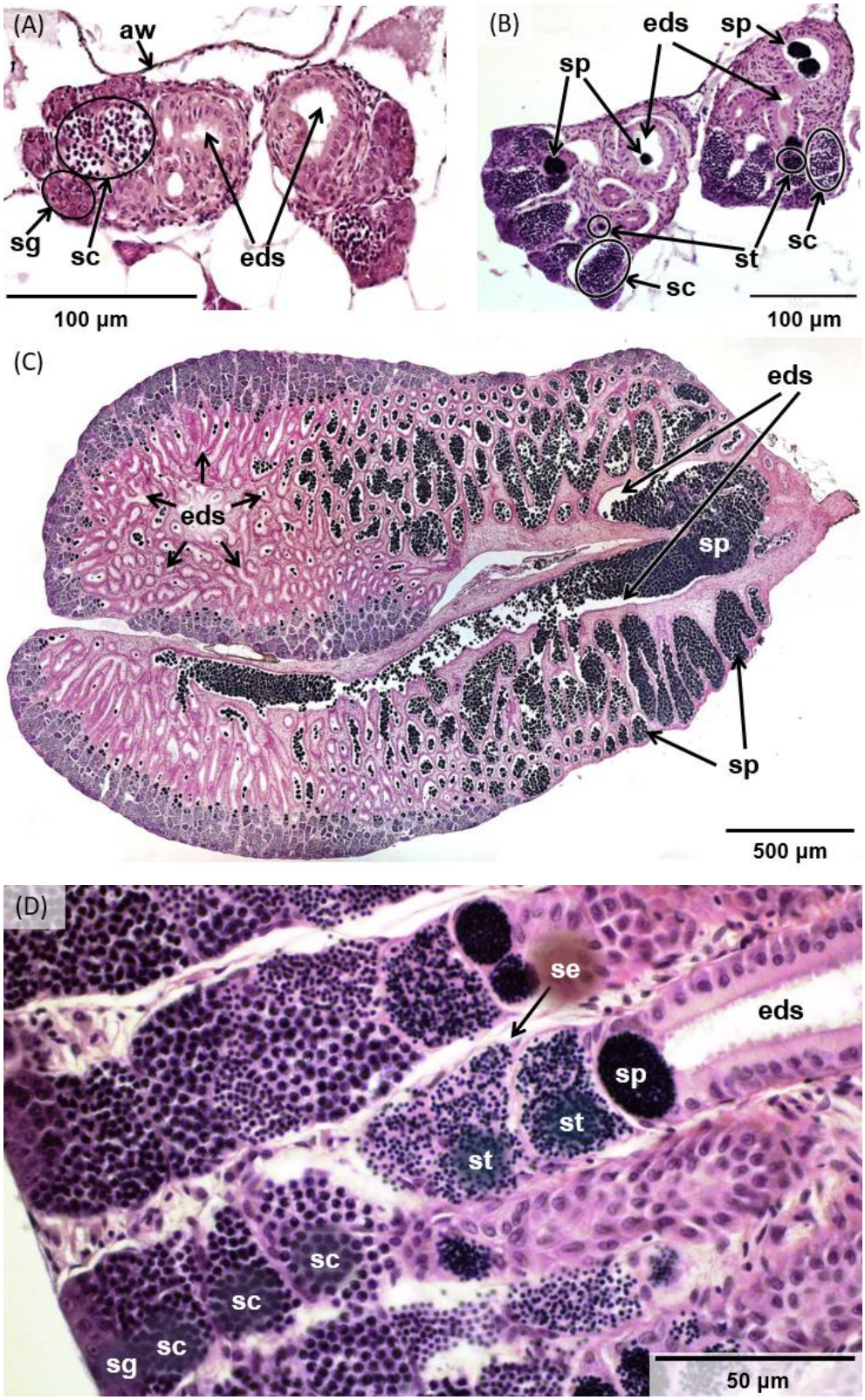
Figure 3. Ovaries of pregnant females and developing embryos during gestation. **A:** Egg with an embryo at one week. **B:** Lateral histological section of an ovary with embryos at one week (a magnified view of the embryo is shown in Figure S7, supplementary material). **C:** Embryo at two weeks, still in the egg envelope. **D:** Embryo at two weeks, partly hatched. **E:** Embryo at four weeks with fully-grown trophotaeniae. **F:** Embryo at six weeks with partly regressed trophotaeniae. **G:** Lateral histological section of an ovary with embryos at six weeks, separated by the ovarian septum (dotted line). **Key:** e: embryo, env: envelope, h: heart, od: oil droplets, os: ovarian septum, ow: ovarian wall, pg: primary growth oocyte, tr: trophotaeniae, y: yolk.

Figure 4. Histological sections of four weeks old embryos within an ovary. **A:** Lateral section showing two embryos separated by the ovarian septum (dotted line) and the connection between trophotaeniae and the embryonal gut (dashed line). **B**: Transverse section of an embryo within an ovary. The section was cut behind the embryo's eyes showing folds of the ovarian septum (dotted

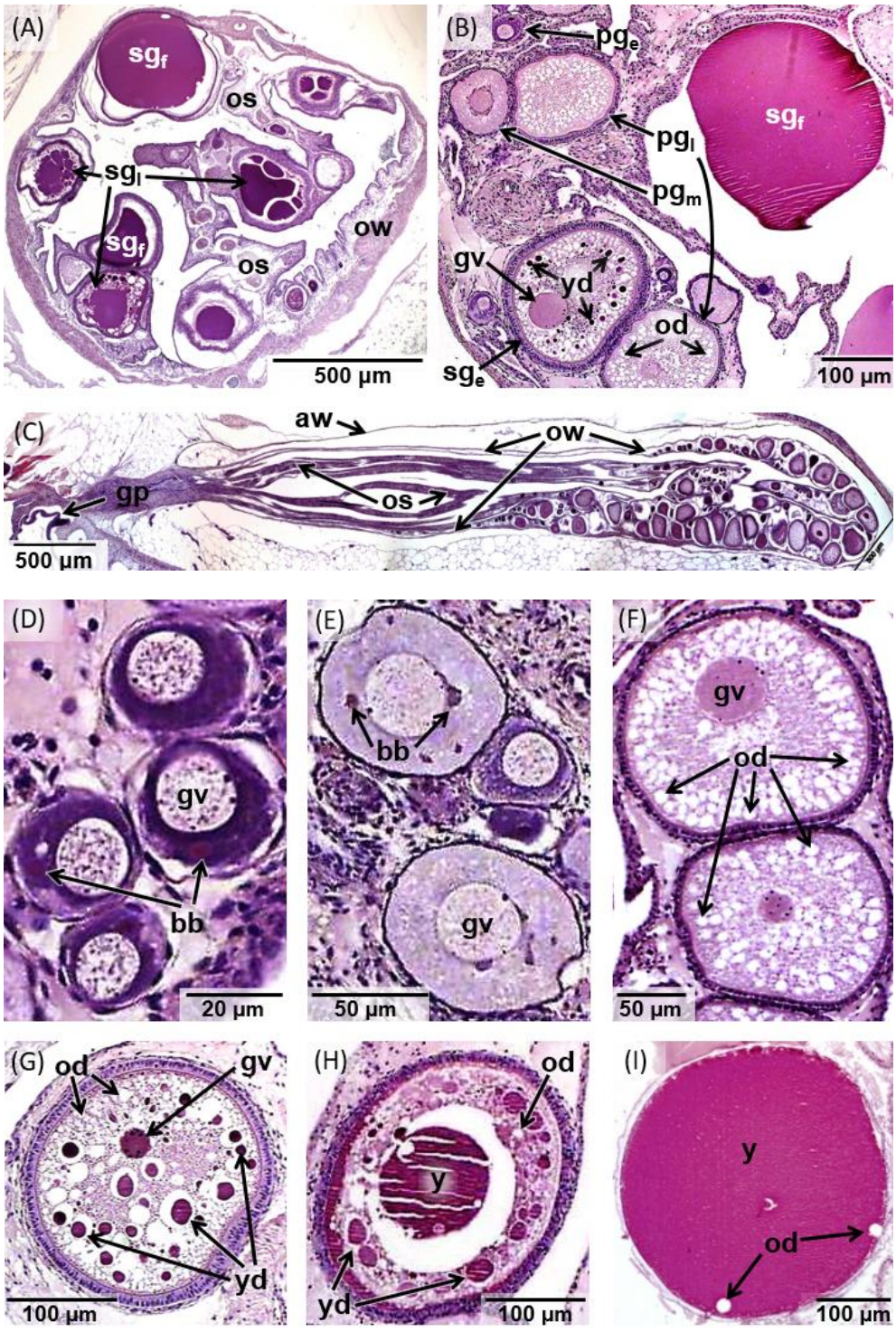
line) that migrated into the gill cavity of the embryo. **Key**: b: brain, g: gut, h: heart, k: kidney, li: liver, op: operculum, os: ovarian septum, ow: ovarian wall, sb: swim bladder, tr: trophotaeniae.

Figure 5. Correlation between brood size and total body length of the mother fish in *Xenotoca eiseni,* showing how body size relates to the number of offspring produced ($r^2 = 0.4661$, n = 50, $P < 0.0001$).

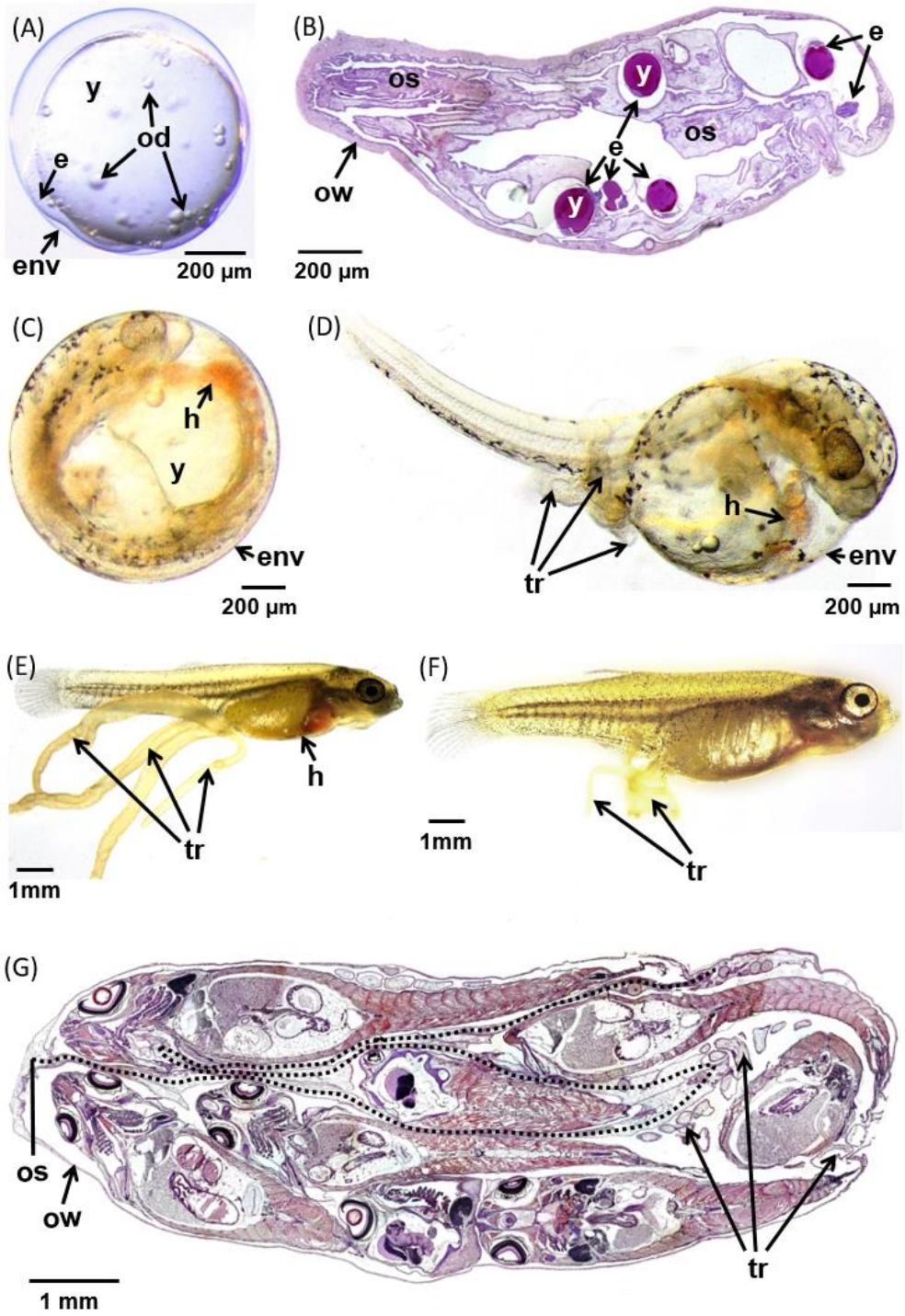
Tinguely et al_Figure 1



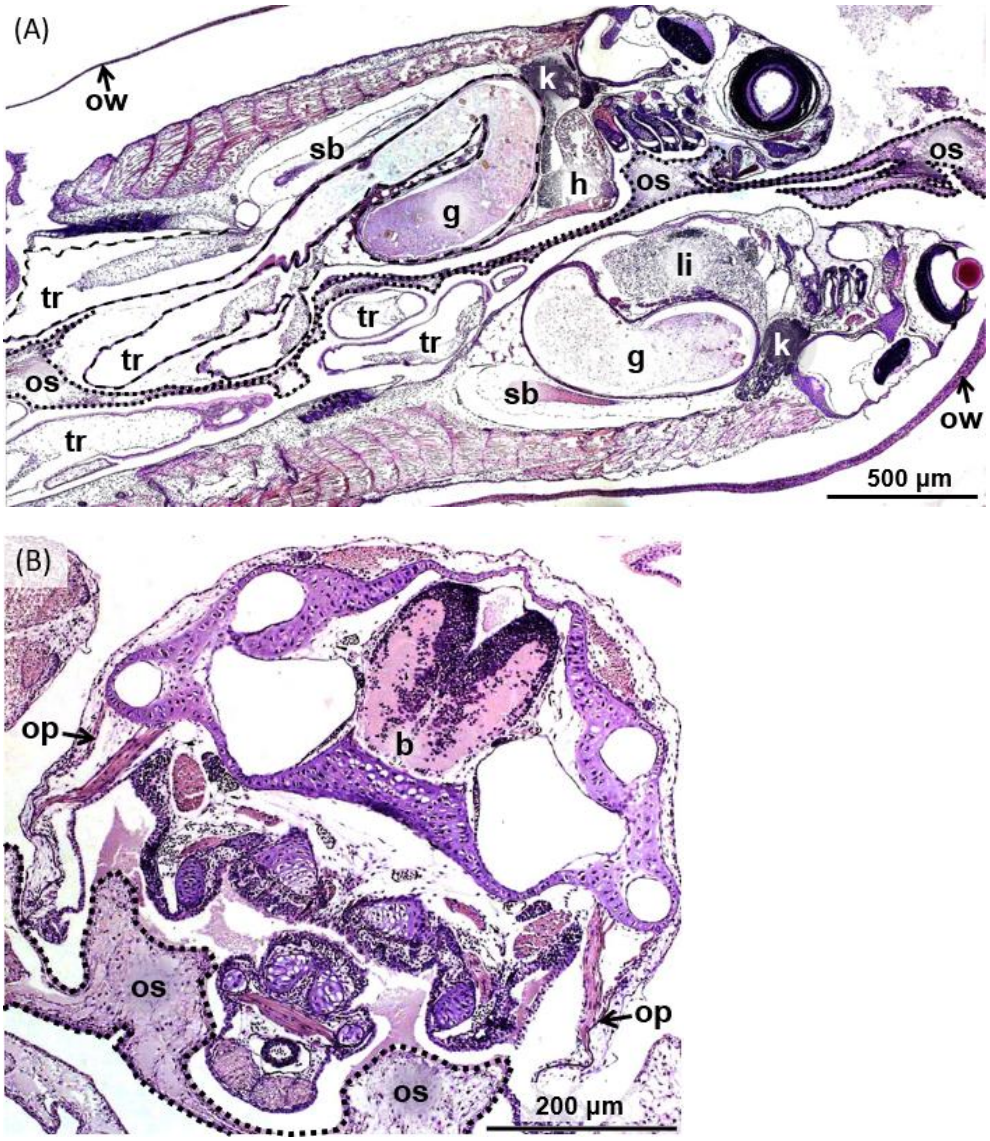
Tinguely et al_Figure 2



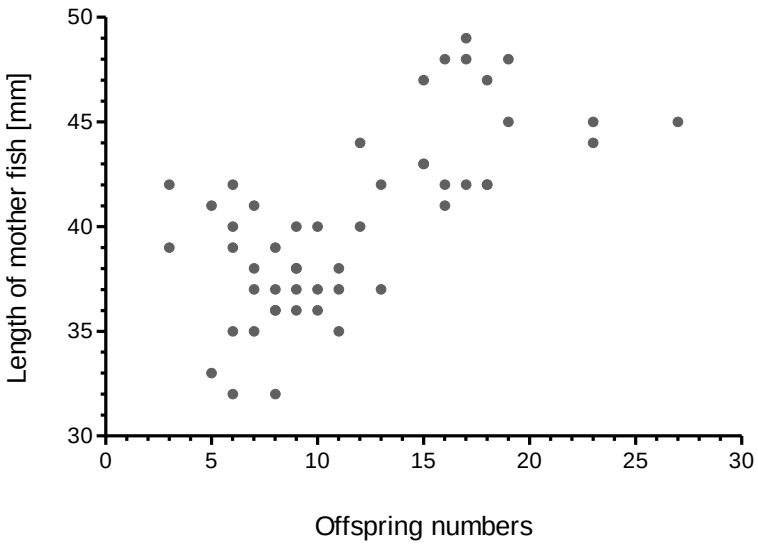
Tinguely et al_Figure 3



Tinguely et al_Figure 4



Tinguely et al_Figure 5



Supplementary Material

Ontogeny and Dynamics of the Gonadal Development, Embryogenesis and Gestation in Xenotoca eiseni (Cyprinodontiformes, Goodeidae)

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This supplementary material contains:

Supplementary Material

Table S1: Numbers of fish analyzed for the various endpoints on the specified sampling dates. Various numbers of randomly sampled fish were sacrificed at each sampling point for histological analysis of gonadal development and gestation. Body weight and total body length were measured for all fish remaining in the study at the respective sampling dates. A total of four fish died during the first experiment: two in the first week, one between week 2 and week 4 and one between week 4 and week 8. **Key**: f: female, m: male.

Study	Sampling Time	Weight and Length	Histology
Ontogeny of gonadal development	**Day 1**	172 fish	19 fish
	Week 1	151 fish	20 fish
	Week 2	131 fish	20 fish
	Week 4	110 fish	19 fish
	Week 8	90 fish (41 f + 49 m)	16 fish (8 f + 8 m)
	Week 12	74 fish (33 f + 41 m)	19 fish (9 f + 10 m)
	Month 4	55 fish (24 f + 31 m)	20 fish (9 f + 11 m)
	Month 5	35 fish (15 f + 20 m)	16 fish (8 f + 8 m)
	Month 6	19 fish (7 f + 12 m)	19 fish (7 f + 12 m)
Embrygenesis and gestation	**Day 2**	33 fish	33 fish
	Day 3	33 fish	33 fish
	Day 5	22 fish	22 fish
	Week 1	85 fish	22 fish
	Week 2	63 fish	21 fish
	Week 4	42 fish	21 fish
	Week 6	21 fish	21 fish

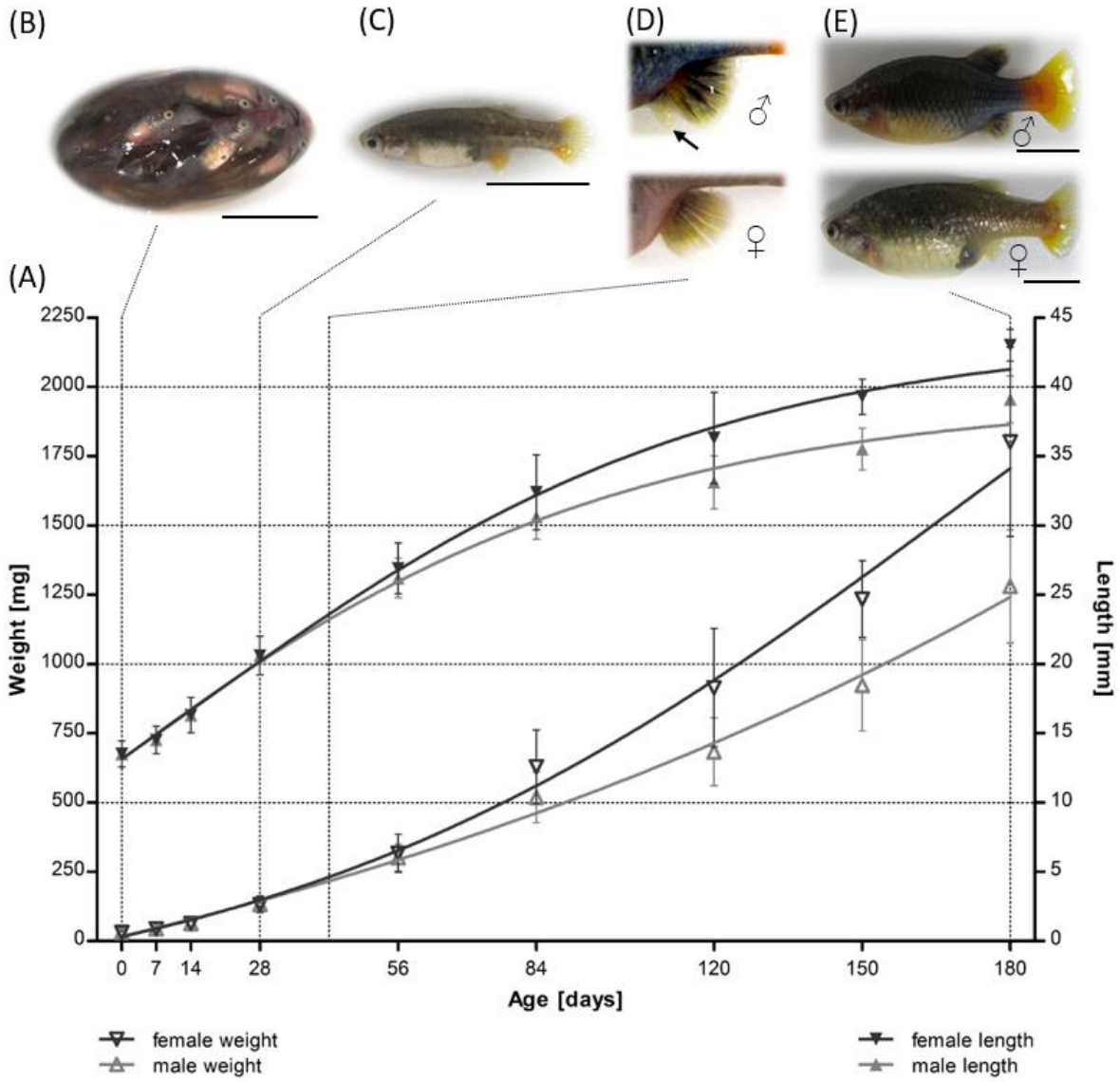


Figure S1: Growth and development of secondary sex characteristics of *Xenotoca eiseni* from birth until the age of six months. **A** Wet weight and total body length of male and female fish. Unpaired t-tests revealed no significant differences between male and female (non-pregnant fish only) growth. **B-E** Illustrations of *X. eiseni* at different ages (at birth (**B**), at four weeks (**C**) and six months of age (**E**)). **D** Anal fins at the age between four and eight weeks (period indicated by the vertical dotted lines) show the notch and the andropodium in the male indicated by the arrow. Bars = 1 cm.

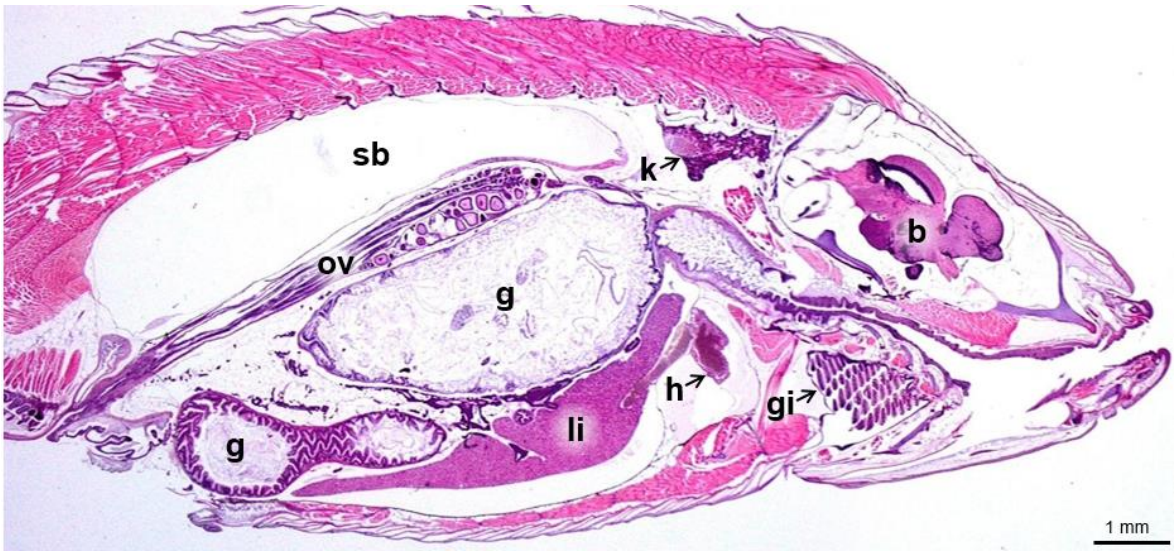


Figure S2: Lateral section of an adult (12 week old) female *Xenotoca eiseni* showing the position of the gonad. **Key**: b: brain, g: gut, gi: gills, h: heart, k: kidney, li: liver, ov: ovary (with oocytes at different developmental stages), sb swim bladder.

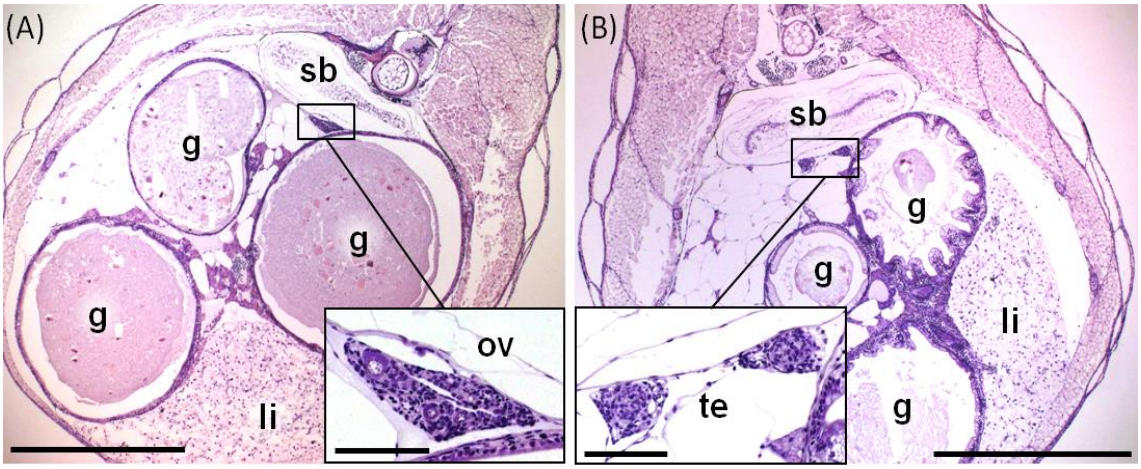
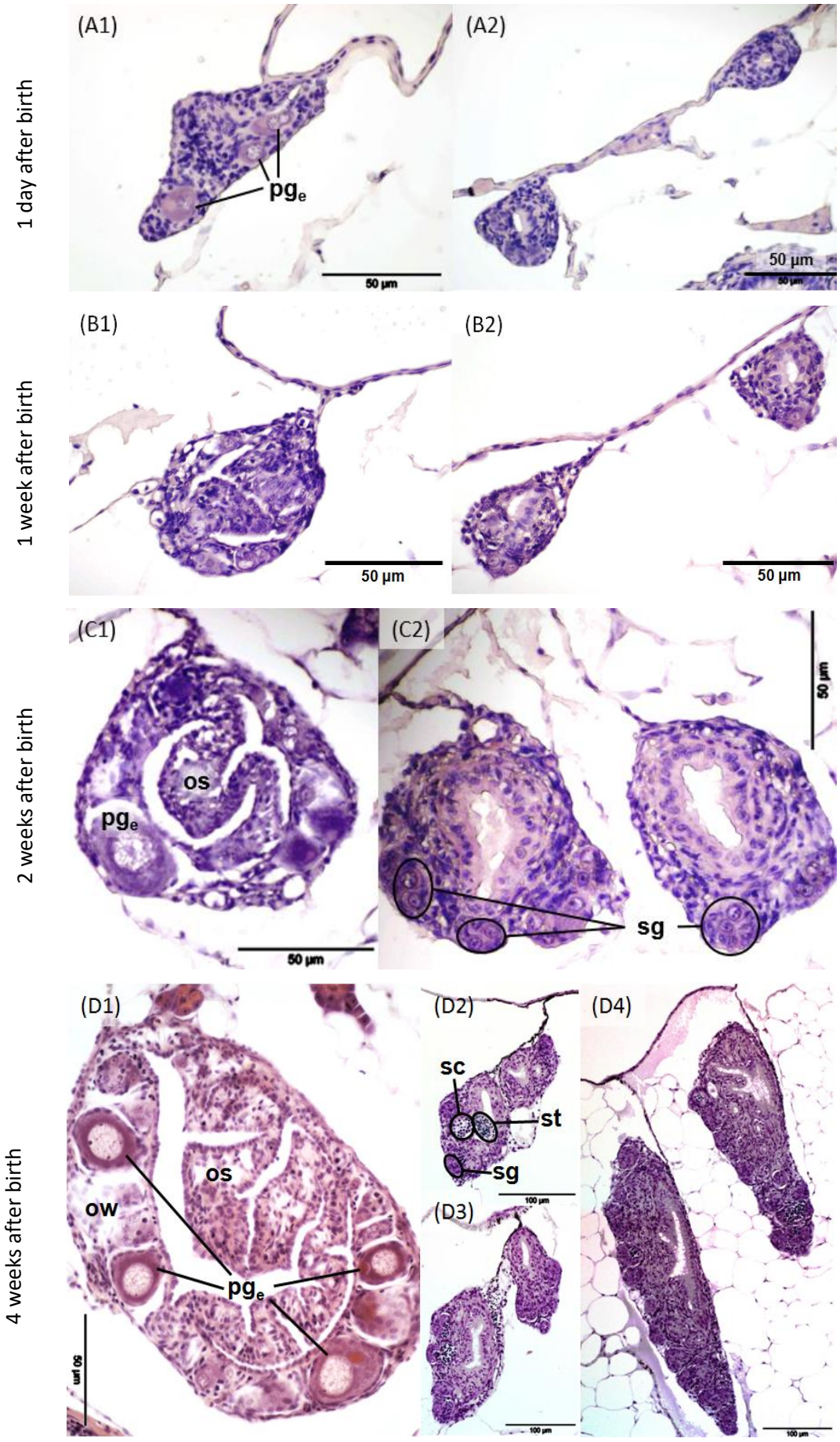


Figure S3: Transverse whole body sections of *Xenotoca eiseni* immediately after birth showing the position of the gonads and a more detailed view of these gonads (inset). **A** Section of a female fish. **B** Section of a male fish. **Key**: g: gut, li: liver, ov: ovary, sb swim bladder (not yet inflated), te: testis. Bars = 500 µm and 50 µm (insets).


1 day after birth
(A1)
(A2)
pg$_e$
50 µm
1 week after birth
(B1)
(B2)
50 µm
2 weeks after birth
(C1)
(C2)
os
pg$_e$
sg
50 µm
4 weeks after birth
(D1)
(D2)
(D3)
(D4)
sc
st
sg
os
ow
pg$_e$
100 µm

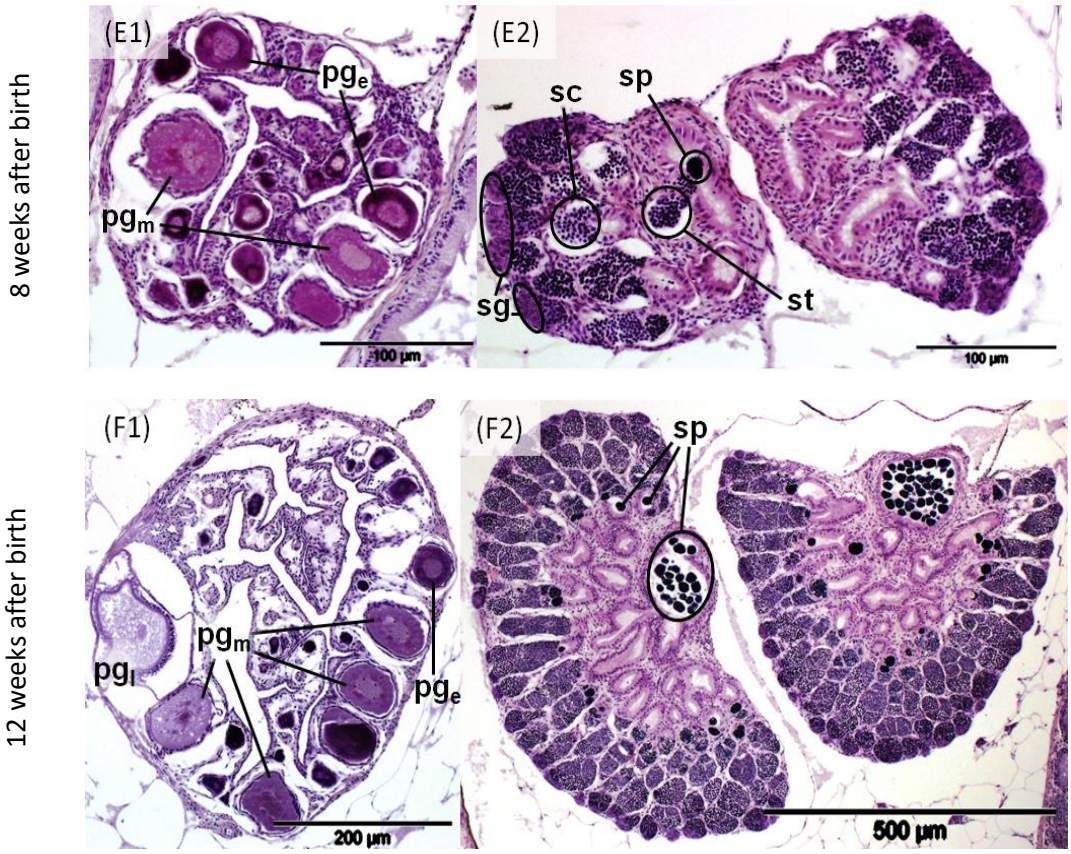


Figure S4: Sections of *Xenotoca eiseni* at different ages, focused on the gonads. **Female** fish: A1, B1, C1, D1, E1, F1. **Male** fish: A2, B2, C2, D2-4, E2, F2. **A1-2**: Transverse sections of gonads of one day-old fish. **B1-2**: Transverse sections of gonads of one week-old fish. **C1-2**: Transverse sections of gonads of two week-old fish. **D1-4**: Transverse sections of gonads of four week-old fish. **E1-2**: Transverse sections of gonads of eight week-old fish. **F1-2**: Transverse sections of gonads of twelve week-old fish. **Key**: os: ovarian septum, ow: ovarian wall, pge: early primary growth oocyte, pgl: late primary growth oocyte, pgm: mid primary growth oocyte, sc: spermatocyte, sg: spermatogonium, sp: sperm package, st: spermatid.

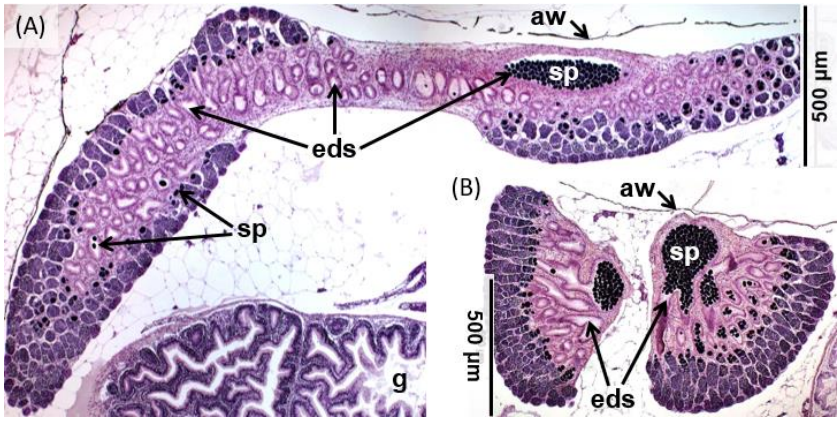


Figure S5: Histological sections of *Xenotoca eiseni* testes. **A** Lateral section of an adult male fish. **B** Transverse section of an adult male fish. **Key**: aw: abdominal wall, eds: efferent duct system, g: gut, sp: sperm package.

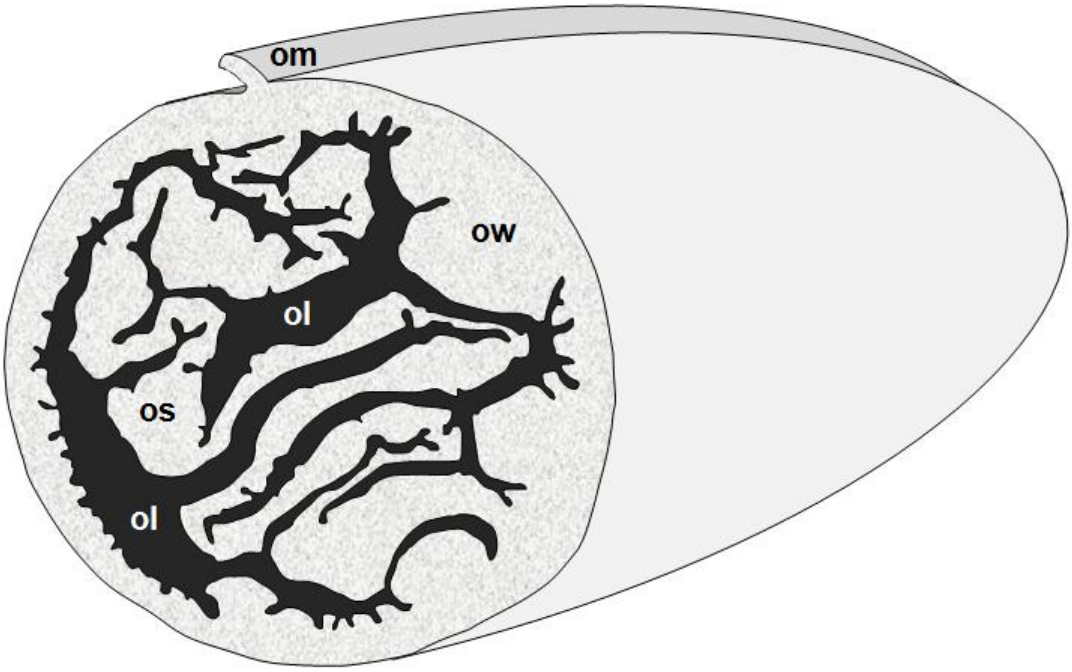


Figure S6: Scheme of a mature, non-gravid ovary; oocytes not shown. **Key**: ol: ovarian lumen, om: ovarian mesentery, os: ovarian septum, ow: ovarian wall.

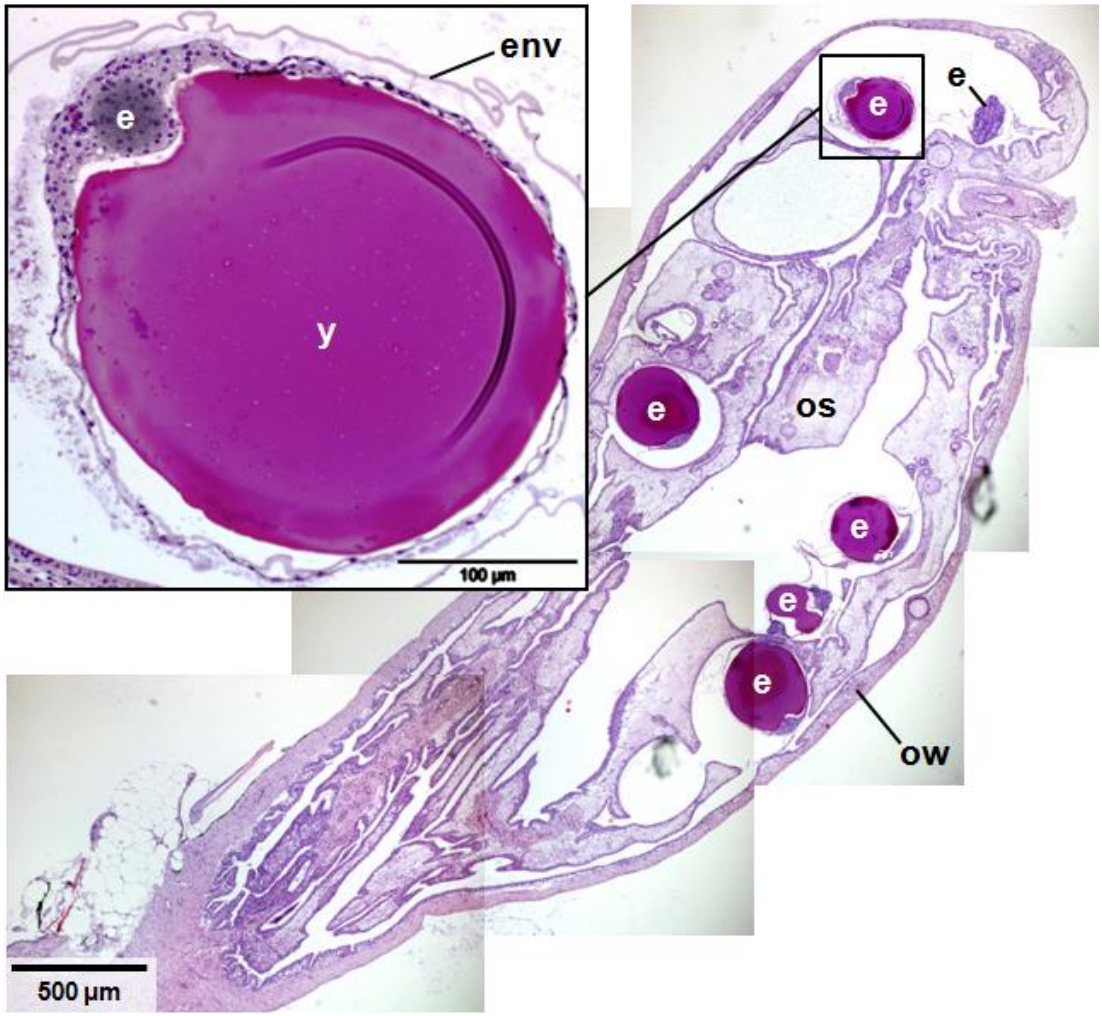


Figure S7: Lateral histological section of an ovary of *Xenotoca eiseni* one week after fertilization. Embryos develop freely in the ovarian lumen but are still within the egg envelope. **Key**: e: embryo, env: egg envelope, os: ovarian septum, ow: ovarian wall, y: yolk.

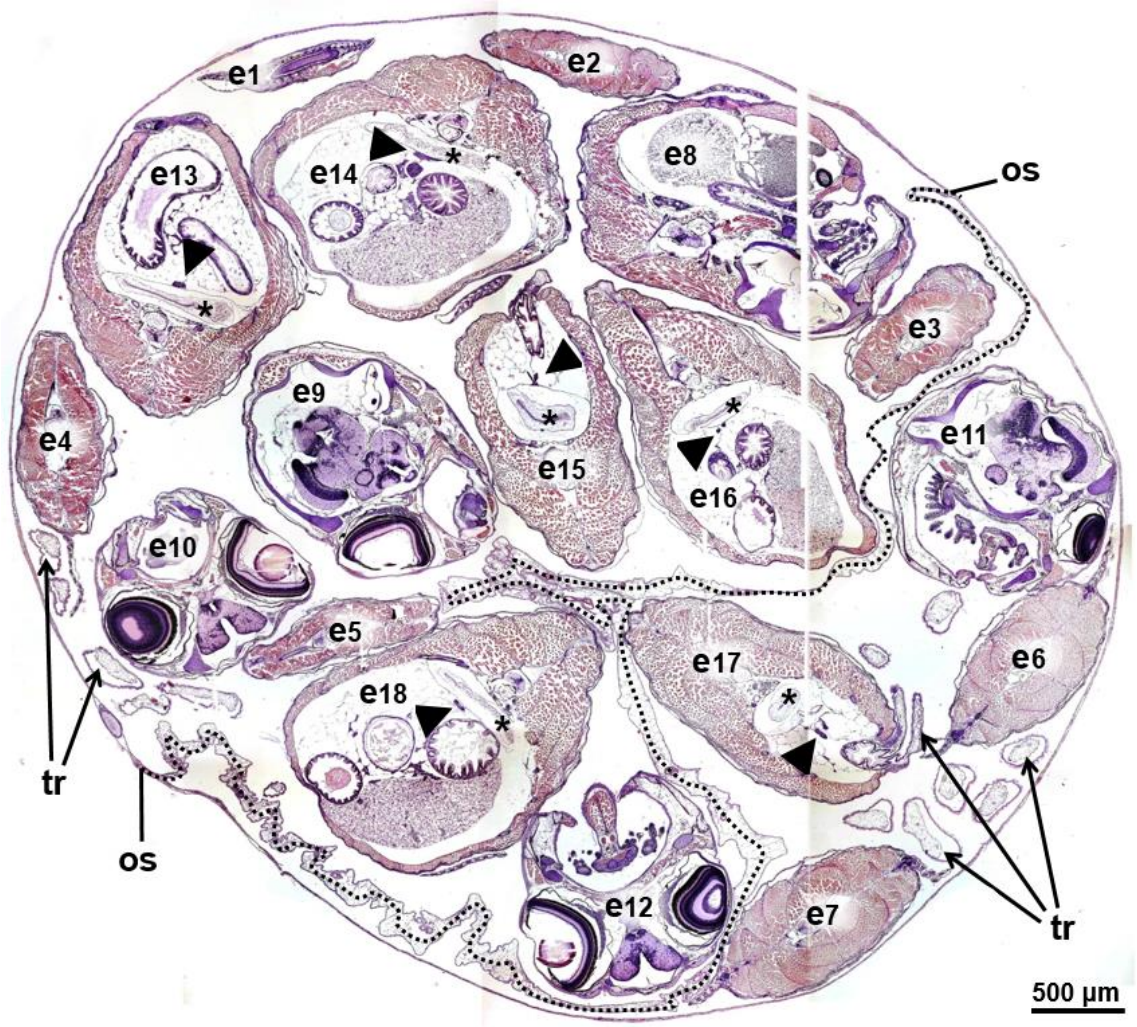


Figure S8: Transverse histological section of an ovary (six weeks into gestation) of *Xenotoca eiseni* with 18 embryos. The image shows seven embryos (e1-7) sectioned through the tail, five embryos (e8-12) sectioned through the head and six embryos (e13-18) sectioned through the abdomen. Of the last six embryos, two (e13-14) have presumptive female gonads, three (e15-17) have presumptive male gonads and one (e18) has a gonad that is not determinable. The dotted line highlights the folds of the ovarian septum. **Key**: e: embryo, os: ovarian septum, tr: trophotaeniae; arrows are pointing to the gonads, asterisks are marking the (not yet inflated) swim bladders.

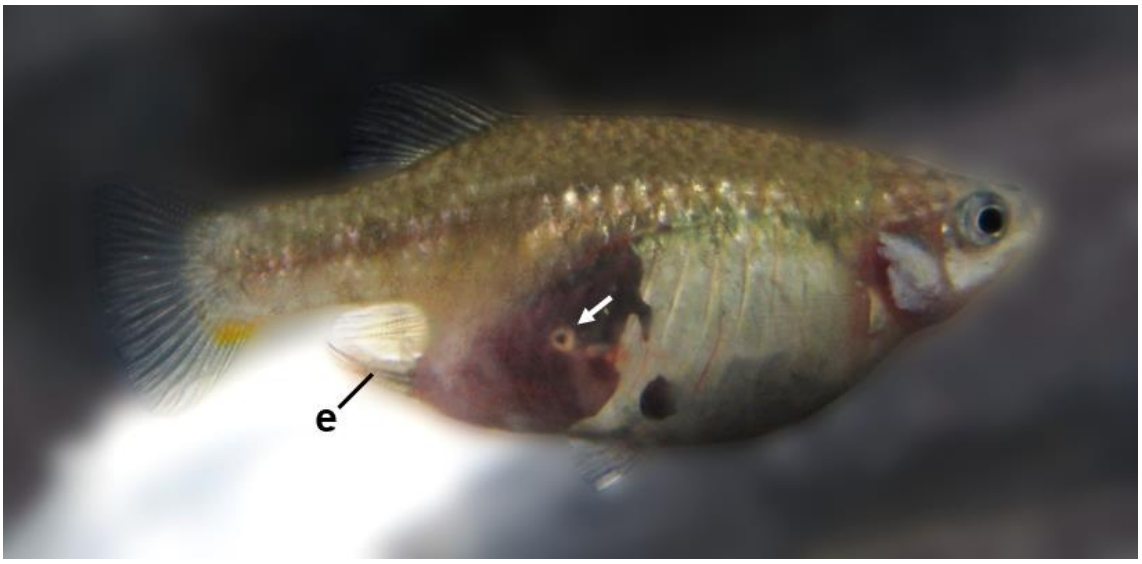


Figure S9: Female *Xenotoca eiseni* at parturition. One embryo (e) is being born while the eye of another one can be seen through the abdominal and the ovarian walls of the mother fish.