Xenotoca eiseni (Cyprinodontiformes, Goodeidae) as a Potential New Model for Studies on Maternal Transfer of Environmental Contaminants

Submitted by Simone Monique Tinguely

to the University of Exeter
as a Thesis for the Degree of
Master of Philosophy in Biological Sciences

June 2015

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

This thesis studies aspects of the reproductive physiology and embryogenesis in the viviparous fish *Xenotoca eiseni* with a view to increasing the knowledge on its basic biology and development of the species as a model for studying maternal transfer and embryo susceptibility to toxicants.

The ontogeny of gonad development was studied via histology to identify sex, stage of gonadal development, structure of the gravid ovary and stage of embryonal development. In males, the testis was comprised of two lobes merged at the anterior end. Spermatogenesis started around four weeks after birth and spermatozeugmata were first seen at between four and eight weeks. In females, there was a single hollow ovary divided into two compartments by a highly folded septum. Oogenesis was first observed between two and four weeks after birth and females reached full sexual maturity at around twelve weeks, when their total body length was at least 3 cm.

Fertilisation and gestation took place in the ovary. Two weeks after fertilisation, embryos hatched within the ovarian lumen, at which time part of the yolk reserve had been depleted and trophotaeniae (hindgut extensions) had started to grow in the fish larvae. The trophotaenial placenta is typical for goodeids, but there was also evidence of a branchial placenta in a number of the embryos studied. Gestation normally took around six weeks and under the appropriate conditions breeding was possible at any time of year, with an interval of less than two months between pregnancies for females.

In a four-week exposure to 17α-ethinyloestradiol (EE2, nominal 1 ng/L and 5 ng/L) there were no discernible effects on morphological endpoints in the exposed female fish or on somatic growth, gonadal development or sex partitioning in their developing offspring. Exposures of the adult females to EE2 (at the highest exposure concentration) however, did induce a 10-fold up-regulation of hepatic vitellogenin (*vtg B)*.

The studies presented provide a foundation of data for the use of *X. eiseni* as a new model for studies on maternal transfer in ecotoxicology. However, further work is required to elucidate more about sensitivity and maternal transfer efficacy in this species.

List of Contents

List of Figures

List of Tables

Abbreviations

a	anus
aw	abdominal wall
bb	balbiani body
bv	blood vessel
ct	connective tissue
cy	cytotrophoblast
e	embryo
eb	embryonal blood
eds	efferent duct system
EE2	17α-ethinyloestradiol
env	egg envelope
ep	epidermis
F	fertilisation
fb	foetal blood
fc	follicle cell
g	gut
gv	germinal vesicle
H	hatching
h	heart
hc	heterologous (trinucleate) cell
hg	hindgut
ioe	inner ovarian epithelium
iue	inner uterine epithelium
k	kidney
li	liver
mb	maternal blood
O	ovulation
od	oil droplet
og	oogonium
ol	ovarian lumen
om	ovarian mesentery
os	ovarian septum
ov	ovary
ow	ovarian wall
P	parturition
pge	early primary growth oocyte
pgl	late primary growth oocyte
pgm	mid primary growth oocyte
sb	swim bladder
sc	spermatocyte
sd	sperm duct
sg	spermatogonium
sge	early secondary growth oocyte
sgf	fully grown oocyte
sgl	late secondary growth oocyte
sp	sperm package
st	spermatid
sy	syncytiotrophoblast
t	theca cell
TACs	trophotaenial absorptive cells
te	testes
tec	trophectoderm
tr	trophotaeniae
tre	trophotaenial epithelium
vtg	vitellogenin
y	yolk
yd	yolk droplet
zp	zona pellucida

1 General Introduction

This thesis is a composite of papers focused on research studying the basic reproductive biology of the viviparous fish *Xenotoca eiseni*. The thesis includes a literature review giving an up to date account of the research and understanding of viviparity in fish, including the study species *X. eiseni,* and subsequently a series of chapters detailing empirical studies on the ontogeny of reproductive development. In addition, the effects of the synthetic pharmaceutical oestrogen 17α-ethinyloestradiol (EE2) on reproductive outcome in *X. eiseni* were studied. This thesis introduction serves to set out the individual chapters, each of which is a self-contained research study.

1.1 Small Fish Models

Fish are commonly used for toxicological studies since they offer particular advantages including efficiency, cost and duration of tests. Small fish find frequent application in aquatic toxicity testing. Well-established laboratory model organisms in ecotoxicology include zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and three-spined stickleback (*Gasterosteus aculeatus*) (Ankley and Johnson, 2004; Helfman et al., 2009; Lange et al., 2012). Fish generally show conservancy across species in terms of mode of action, thus allowing effect extrapolation (Ankley and Johnson, 2004).

None of the more standard test fish species, however, gives birth to live young. Maternal transfer is potentially a major route for chemical uptake into developing organisms and this is a life stage when an animal is at its most vulnerable to effects (Wourms and Lombardi 1992; Hutchinson et al., 1998; Rasmussen et al., 2002; Brion et al., 2004; Hedman et al., 2011; Brande-Lavridsen et al., 2013; Cazan and Klerks 2014). Even though, viviparity in fish has generally received little attention in research.

1.2 Viviparous Fish Models

The live-bearing eelpout (*Zoarces viviparus*) is a teleost species which has been used for studies on maternal transfer of chemicals into developing offspring (Rasmussen et al., 2002; Hedman et al., 2011; Brande-Lavridsen et al., 2013). Thus far, reports on this species have focused on effects of endocrine disrupting chemicals and their effects on developing embryos within the exposed mother fish.

A very recent publication from Cazan and Klerks (2014) describes metal transfer in the viviparous poeciliid fish *Heterandria formosa* and *Gambusia affinis*. These authors showed both essential and non-essential metals were transferred from exposed gravid females to their developing young. It has also been shown that maternal exposure to contaminants can adversely affect the embryos' life history (Cazan and Klerks, 2015).

The eelpout and two poeciliid fish species all have different reproductive strategies, with some of them including sperm storage and superfetation.Yet, all of them have a relatively long gestation period of four to over six months (Rasmussen et al., 2002; Skov et al., 2010; Hedman et al., 2011; Brande-Lavridsen et al., 2013, Cazan and Klerks, 2014, 2015). For (eco-) toxicological studies on maternal transfer, a more favourable model organism would have a shorter gestation period. The goodeid fish *X. eiseni* has a gestation time of a few weeks only (Parenti, 2005; Skov et al., 2010) and may therefore be a more suitable species for testing adverse effects of maternally transferred toxicants.

1.3 Endocrine Disrupting Chemicals

Environmental pollutants have been shown to have negative impacts on the normal physiological functions of aquatic organisms, in some cases at concentrations found in the natural environment. Over 1,000 chemicals have now been reported to have endocrine modulating activity, 200 of which are active as environmental oestrogens. Over the past decades, natural and synthetic

oestrogens have been observed to be endocrine disruptive chemicals (EDCs), and have been extensively studied for their adverse effects on sexual development, reproduction and growth (Tyler et al., 1998; Korsgaard et al., 2002; Ankley and Johnson, 2004). It is well known that natural oestrogens are essential for oogenesis and the induction of vitellogenin synthesis (Sumpter and Jobling, 1995; Tyler et al., 1998; Denslow et al., 1999). Synthetic pharmaceutical oestrogens like 17α-ethinyloestradiol (EE2) are strong modulators of the vitellogenin gene expression (Tyler et al., 1998; Denslow et al., 1999), which is why vitellogenin has become one of the most widely used biomarkers for measuring oestrogen exposure in animals (Denslow et al., 1999; Lange et al., 2012).

1.4 Details on the Structure of the Thesis

The first part of this thesis provides an introduction to *Xenotoca eiseni* in form of a literature review. After addressing the evolutionary aspect of viviparity in bony fish, the review then centres on reproduction of *X. eiseni* in order to set the basis for the following experimental chapters.

The experimental part of the thesis is divided into four chapters. The first of these chapters introduces the general materials and methods used in all three experimental studies within this thesis. Chapters 4 and 5 focus on ontogeny and dynamics of the gonadal development and on embryonal development, including timing of sexual differentiation in *X. eiseni*. Chapter 4 details the ontogeny of the gonadal development using histology from birth up to full sexual maturity at six months of age. This chapter also investigates the relationship between somatic growth and sexual development. In chapter 5, embryogenesis is described by means of histological examinations that were carried out on gonads of pregnant fish throughout gestation to investigate the structure of the gravid ovary and the stage of the embryonic development.

These studies on reproductive processes in *X. eiseni* provided the groundwork for the studies undertaken in chapter 6, which concentrated on investigating

maternal transfer and biological effects in developing offspring resulting from exposure of pregnant females to the oestrogen EE2.

1.5 Research Goals

This thesis aims firstly to critically review the literature on viviparous reproduction in teleosts and provide a comprehensive understanding of the biology of the live-bearing goodeid species *X. eiseni*.

Secondly, it is intended to provide a detailed understanding of the ontogeny of sexual development and gestation in *X. eiseni* in order to establish the required knowledge to develop this species as an experimental model for studies in ecotoxicology. The work in chapters 4 and 5 are of a descriptive nature.

The third goal is to establish the effects of exposure of pregnant female *X. eiseni* to EE2. It is hypothesised that there would be significant maternal transfer of this pharmaceutical oestrogen, affecting the (sexual) development of offspring.

2 Literature Review on the Viviparous Fish *Xenotoca eiseni* (Cyprinodontiformes, Goodeidae)

2.1 Introduction

Viviparity is a form of parental care that evolved from oviparity in order to enhance offspring survival (Blackburn, 2005a). Live-bearing in teleost fish is rare, occurring in 2-3% of fish species only, but it includes a wide spectrum of complex adaptations with a broad range of specialisations. Although piscine viviparity has been the subject of previous reviews (Wourms, 1981), there are very few publications that detail basic reproductive systems in Goodeidae (Greven, 2013).

This literature review presents a compilation of information on viviparous teleost fish, addressing viviparous reproduction in general in the context of an evolutionary background, and later focuses specifically on *Xenotoca eiseni*. Reproductive modes have been classified in various ways and authors frequently use different definitions. As a consequence, distinctions between one reproductive mode and another can be blurred and various different subdivisions have been made. With regards to the evolution of Goodeidae, the literature is even more complex as various authors have given different viewpoints. In formulating the evolutionary tree for Goodeidae, some fish species have been synonymised, others have been relatively newly discovered, families have been added or removed from orders and new orders have been defined. In this review the system developed by Nelson (2006) was adopted as this arguably has been built on the most comprehensive available information.

The final two sections in this review are focused on the viviparous goodeid *Xenotoca eiseni* to provide a comprehensive understanding of the biology of this species to support the following experimental chapters.

2.2 Reproductive Systems in Fish

2.2.1 Multiplicity and Diversity of Reproduction

Reproduction in vertebrates is typically classified as either egg-laying or live-bearing, and the latter can be further characterised by the mode of maternal input (Wourms, 1981; Blackburn, 1992; Dulvy and Reynolds, 1997). Fish, like all the other vertebrates, reproduce sexually and they do so in numerous ways (Hoar, 1969). Chondrichthyan fish mostly reproduce via viviparity (especially sharks and rays), whereas live-bearing is much less common in osteichthyes (Wourms, 1981, 2005; Dulvy and Reynolds, 1997). Helfman and co-workers estimated in 2009 that more than 26,000 of the 28,000 known living fish species (Nelson, 2006) belong to the osteichthyans (bony fish). Approximately 20,000 bony fish are teleost species (Rosa-Molinar, 2005) whose sexual determination is controlled by genetic, environmental and/or social factors (Guerrero-Estévez and Moreno-Mendoza, 2012). While the vast majority of teleosts expel eggs and sperm into the surrounding water, where fertilisation occurs and is directly followed by the development of the offspring, the alternative extreme is internal fertilisation and subsequent embryonal development within the parent's body (Hoar, 1969). This mode of reproduction – viviparity – occurs in 2-3% of teleosts (Wourms, 1981, 2005; Wourms and Lombardi 1992; Meyer and Lydeard, 1993; Grier, 2005), which corresponds to just over 500 species (Guerrero-Estévez and Moreno-Mendoza, 2012; Iida et al., 2015).

Sexual reproduction in fish, can be characterised further according to the degree of protection given by adult fish during incubation. (Hoar, 1969). Fish have evolved various manners of parental care, including construction of nests, cleaning and protecting eggs, as well as provisioning nourishment for the young. According to Helfman et al. (2009), teleost fish can be divided into reproductive guilds. Non-guarding species include open substrate spawners and brood hiders, guarders encompass substrate choosers and nest spawners and bearers consist of different kind of brooders (e.g. mouth, gill chamber or pouch), as well as internal bearers.

Reproductive modes have often been subdivided into intermediate stages between oviparity and viviparity, one of them being ovo-viviparity. This form was

defined as “bearing of young developed from eggs which have been retained in the body of the mother but without the maternal organism providing additional nourishment for the embryo” (Wourms et al., 1988). The distinction between ovo-viviparity and viviparity is imprecise, as studies for very few species have established whether nourishment is provisioned by the mother in these different forms of viviparity (Wourms, 1981). As a consequence it has been suggested that only the terms “viviparity” and “oviparity” are used in order to reduce confusion about inconsistent definitions of subdivisions (Wourms et al., 1988).

Oviparity

Wourms and Lombardi (1992) defined oviparity as a reproductive mode in which eggs are laid and a young organism subsequently hatches from the egg. Oviparity can further be distinguished according to the time of fertilisation. An ovum that is released and subsequently fertilised is referred to as ovuliparity, whereas zygoparity is used to describe the release of a fertilised egg. The release of a well-developed embryo surrounded by an egg envelope is referred to as embryoparity. Since the time of hatching from the egg is variable, there is an area of overlap between embryoparity and viviparity (Wourms and Lombardi, 1992).

Viviparity

The term viviparity is generally now used for species that retain their fertilised eggs within the maternal system for a significant period of time during which embryos develop to an advanced stage, and birth of live young succeeds or coincides with hatching (Wourms, 1981; Wourms et al., 1988; Wourms and Lombardi, 1992; Blackburn, 2005a). Live-bearing is a highly successive mode of reproduction and occurs in all vertebrate classes except in birds. For this form of reproduction a series of adaptations was needed, including a reduction in the number of eggs produced, perfection of systems for internal fertilisation and internal care of developing embryos (Wourms, 1981).

In viviparous chondrichthyans, the female reproductive system consists of single or paired ovaries and one or two oviducts (Wourms, 1981). The posterior part of the oviduct is highly modified and has become to all intense and purposes a uterus. Ovaries in osteichthyans, however, differ from other vertebrates inasmuch

as they are the site of egg production and internal fertilisation as well as gestation (Wourms and Lombardi, 1992; Uribe et al., 2005). In teleost fish, ovaries begin to develop as paired structures and subsequently may remain as separate structures or they may fuse partially, or completely (Wourms, 1981, 1988). Oviducts or a uterus is absent in these fish (Wourms and Lombardi, 1992).

Depending on where gestation takes place – uterus, ovarian lumen or ovarian follicle – Wourms (1981) identified different developmental sequences. The sequence includes **O**vulation, defined as the event when a mature egg or embryo leaves the follicle, **F**ertilisation in which an egg and a sperm fuse and initiate development of a young, **H**atching, where the embryo leaves the egg envelope, and **P**arturition, where the embryo leaves the maternal body. The order of these events is part of a fixed sequence. Fertilisation always precedes hatching but can occur before ovulation, and ovulation always precedes parturition. Typical orders for these events that characterise the different modes of reproduction in fish are shown in Tab 2.1.

The most prevalent mode of gestation in teleosts is intraluminal gestation (Wourms and Lombardi, 1992). While it has been repeatedly emphasized that fertilisation takes place within the follicle and development proceeds to completion in the lumen (Wourms, 1981, 2005; Dulvy and Reynolds, 1997), fertilised oocytes have rarely been observed in species with intraluminal gestation (Greven, 2013). It is therefore assumed that fertilisation and ovulation must happen very close together.

Tab 2.1: Developmental sequences according to Wourms (1981). Events occur in fixed sequences which are distinct for different reproductive modes. **Key**: F: fertilisation, H: hatching, O: ovulation, P: parturition.

			Fertilisation Type	
			Internal	External
Reproductive Mode	Oviparous		**O-F-P-H**	**O-P-F-H**
	Viviparous	Uterine Gestation	**O-F-H-P**	
		Intraluminal Gestation	**F-O*-H-P**	
		Intrafollicular Gestation	**F-H-O-P**	

* In some species a temporal blurring of the first two events has been observed

Lecithotrophy, matrotrophy, different placental systems

Viviparous embryos are retained in the mother fish, although the degree of maternal dependence varies greatly (Wourms, 1981). Among live-bearing fish are species whose eggs have sufficient yolk for the nourishment of the developing embryos and the female body provides protection only. In other species, the yolk content of the eggs is greatly reduced and the embryos depend on maternal nourishment during development (Hoar, 1969; Wourms, 1981). Based on the trophic relationship between mother fish and embryo, the viviparous mode of reproduction can be classified as either lecithotrophic, if nutrients come from yolk exclusively, or matrotrophic[1], if there is an alternative continuous supply of maternal nutrients (Wourms, 1981; Knight et al., 1985; Blackburn, 2005a).

While lecithotrophy demands little or no alteration compared to oviparous functional relationships, matrotrophy requires considerably modified maternal and embryonal structures (Wourms, 1981). If embryos do not establish a direct connection with the maternal body for the passage of nutrients, they have to be otherwise nutritionally supplied by their mother by means of dissolved nutrients (Wourms and Lombardi, 1992). Such nutrients are generally referred to as embryotroph. Depending on the maternal source, the nutrients can be classified as haemotroph, if they are derived directly from maternal blood, or histotroph, if they are derived from maternal tissue. Histotroph is secreted from surface epithelia and may contain material broken down from mucosa or extravasated blood (Schnorr and Kressin, 2011). Moreover, nutrient transfer might also be accomplished by oophagy and adelphophagy (ingestion of eggs that are periodically ovulated during gestation and intra-uterine / intra-ovarian cannibalism) (Wourms, 1981). Embryonic uptake of maternally derived nutrients takes place via pinocytosis or phagocytosis (Schnorr and Kressin, 2011) across two major types of epithelial surfaces. This may be either integument, such as general body surface, gills, fins and fin folds, yolk sac and pericardial sac, or gut associated tissues like gut, branchial system and trophotaeniae (Wourms and Lombardi, 1992). All of these structural adaptations for maternal-embryonal nutrient transfer evolved first among fish and can be regarded as part of placental analogues (Wourms, 1981, 1988).

[1] In pregnant male seahorses and pipefish, the analogue mode of reproduction that is thought to occur would be called patrotrophic (Helfman et al., 2009).

A definition of a placenta was first proposed by Mossman (1937) as: “an intimate apposition or fusion of the fetal organs to the maternal (or paternal) tissues for physiological exchange.” Among viviparous fish there are four different placenta types: yolk sac placenta, follicular placenta, branchial placenta and trophotaenial placenta (Wourms and Lombardi, 1992). The most common systems in teleost fish are the follicular placenta, trophotaeniae and the absorption of histotroph (Wourms, 1981). The trophotaenial placenta comprises of the maternal ovarian luminal epithelium and the embryonal trophotaeniae (Wourms and Lombardi, 1992). This placenta type will be further discussed in chapter 2.4.3 (Trophotaenial Placenta).

As mentioned above, the female reproductive system in teleost fish consists of either a single ovary or a pair of ovaries. The ovarian wall is continuous with a single gonoduct that extends posteriorly and opens to the exterior at the genital pore (Wourms, 1981, 2005; Wourms and Lombardi, 1992). Most viviparous teleosts characteristically have a single median hollow ovary and the interior surface is lined by a germinal epithelium. Within this germinal epithelium, follicles are produced from oogonia (Uribe et al., 2005). Together with its associated connective tissue it undergoes complex folding to form ovigerous folds that project into the ovarian lumen into which eggs are released at ovulation (Wourms, 1988). In case of intraluminal gestation, the stroma beneath the germinal epithelium is highly vascularised and the germinal epithelium itself is involved in transport and secretory functions (Wourms and Lombardi, 1992; Uribe et al., 2005). In fish with intrafollicular gestation, the luminal epithelium assumes a less important role, whereas the morphology of the follicle must have been structurally modified (Wourms and Lombardi, 1992).

2.2.2 Internal Fertilisation

There are two major requirements for viviparity to be possible: firstly the retention of eggs in the female reproductive tract (Wourms, 2005) and secondly the features for internal fertilisation (Wourms, 1981; Wourms and Lombardi 1992; Meyer and Lydeard, 1993). Reproduction in teleosts is generally oviparous and fertilisation takes place externally, and only a few groups fertilise internally

(Wourms, 1981). Although internal fertilisation may be followed by laying of newly fertilised eggs (Hoar, 1969), the great majority of internally fertilising teleosts are viviparous. Structural modifications that make internal fertilisation possible in an aquatic environment are extremely complex and viviparity amongst teleosts is rare. For chondrichthyans, the transition from oviparity to viviparity poses less of a problem because all recent species, including oviparous fish, employ internal fertilisation (Wourms, 1981).

The internal environment of a live-bearing female must be physiologically adapted to internal fertilisation (Meyer and Lydeard, 1993). The female gonoduct is the region of the ovary involved in internal fertilisation (Wourms, 2005). Insemination is typically accomplished through an intromittent organ (Meyer and Lydeard, 1993). Male viviparous teleosts have developed a range of modifications that serve as intromittent organs, including an enlarged genital papilla, a specialised anal fin, or an internal muscular organ (Hoar, 1969; Dulvy and Reynolds, 1997; Contreras-Balderas, 2005; Meisner, 2005). These devices are referred to as gonopodium, if there is a direct involvement in sperm transfer, or andropodium, if it appears to have little to do directly with the transfer of sperm (Dulvy and Reynolds, 1997). Both structures, however, are associated with directing a stream of spermatozoa or spermatozeugmata towards the female's reproductive tract (Hoar, 1969; Meyer and Lydeard, 1993; Meisner, 2005). Spermatozeugmata are un-encapsulated sperm bundles that contain thousands of sperm cells, the heads of which are embedded in an outer gelatinous matrix and their tails extend into the lumen, or vice versa (Meyer and Lydeard, 1993). Such sperm bundles are a morphological adaptation linked with internal fertilisation, as it would not be efficient for males to release free sperm into the water (Grier et al., 2005).

Superfetation

Superfetation is a condition in which two or more broods of embryos of different ontogenic stages simultaneously develop within an ovary. It is an unusual feature in viviparous reproduction as it is dependent on the storage of sperm for successive fertilisations (Greven, 2013). In goodeids, remaining sperm are phagocytised soon after fertilisation and sperm storage does not occur (Uribe et al., 2005).

2.2.3 General Advantages and Disadvantages of Viviparity

Viviparity is the most advanced reproductive strategy in fish and has many advantages. Firstly, the female body carrying the developing offspring protects the developing offspring from predation, disease and other hazards (Wourms et al., 1988; Wourms and Lombardi, 1992; Meyer and Lydeard, 1993). Besides protection, the mother fish also serves as means of transportation. Parental care is not constrained to a specific place as broods can be moved to an environment with the most favourable conditions (Wourms et al., 1988; Meyer and Lydeard, 1993). Mobility also allows parents to escape competition (Wourms et al., 1988) which is especially beneficial in habitats with a high density of individuals (Wourms and Lombardi, 1992). Furthermore, viviparous species might be more resilient to extinction, as a single pregnant female is more able to colonise a new habitat (Wourms et al., 1988; Wourms and Lombardi, 1992; Meyer and Lydeard, 1993).

In the more advanced viviparous reproduction mode of matrotrophy, females provide nourishment for the embryonal development on demand. This not only frees the embryos from the constraints imposed by the fixed metabolic content of the egg (Wourms and Lombardi, 1992) but also leads to offspring weighing more at birth than the fertilised ova (Meyer and Lydeard, 1993). The larger size of born viviparous fish increases their survivorship (Meyer and Lydeard, 1993) as it reduces the risk form predation and increases their food resources due to their higher trophic level (Wourms and Lombardi, 1992). In addition to their larger size, neonates of live-bearing fish are also at a more advanced developmental state than newborns of oviparous fish (Meyer and Lydeard, 1993). This would imply an increased swimming speed and locomotor control as well as fully differentiated sensory organs, which again results in better fitness and therefore enhances the survival of offspring (Wourms and Lombardi, 1992). Some female viviparous species are able to store and possibly even nourish sperm, which ensures that sperm will be available when ova are released. At the same time, male viviparous fish have the advantage of a higher assurance of paternity (Meyer and Lydeard, 1993).

Monopolisation of paternity however, reduces the genetic variance of the offspring (Macías García and Gonzalez-Zuarth, 2005). Another trade-off is the

energetic cost to the female who has to support the metabolism of the brood (Wourms and Lombardi, 1992). One of the biggest constraints in viviparity is the greatly reduced fecundity because live-bearing species have smaller number of eggs than fish that fertilise externally (Meyer and Lydeard, 1993; Grier et al., 2005). Nevertheless, the disadvantages of viviparity seem to be outweighed by the increased offspring survival and thus viviparity and maternal inputs are thought to be selectively beneficial (Dulvy and Reynolds, 1997).

2.3 Viviparity – An Evolutionary Perspective

2.3.1 Basics for the Evolution of Viviparity

Several ecological factors might have led to the evolution of viviparity (Meyer and Lydeard, 1993). The transition from oviparity to viviparity will have been facilitated by numerous adaptations and each of the advantages must have outweighed the disadvantages (Wourms and Lombardi, 1992). Although the analysis of the evolution of viviparity in fish is complex due to the great variety of live-bearing species (Wourms and Lombardi, 1992), it is clear that some features were necessary for the evolution of piscine viviparity and must have preceded the evolution of viviparity (Meyer and Lydeard, 1993). One of those prerequisites for viviparity was the shift from external to internal fertilisation (Wourms and Lombardi, 1992; Meyer and Lydeard, 1993). Internal fertilisation evolved independently and several times in widely separated taxonomic groups and brought along various modifications in the reproductive system (Thibault and Schultz, 1978; Wourms, 1981). In the order of Cyprinodontiformes, for example, the three viviparous families of Anablepidae, Goodeidae and Poeciliidae each independently modified the male anal fin differently. The evolution of spermatozeugmata also seems to have evolved separately three times (Meyer and Lydeard, 1993).

In viviparous teleost females, the characteristics that led to the evolution of viviparity included the evolution of the ovary as a hollow organ and further modifications such that intra-ovarian gestation could occur (Uribe et al., 2005). In addition to egg retention, hormonal modification of the female and the offspring must have happened repeatedly and independently (Meyer and Lydeard, 1993) in order to maintain the developing embryos in the female's reproductive system during gestation (Wourms and Lombardi, 1992; Blackburn, 2005a). Unlike other vertebrates, many viviparous fish lack a placenta and appear to be entirely lecithotrophic (Wourms, 1981; Blackburn, 2005b). Piscine viviparity, however, involved a tendency to reduce the dependency on yolk reserves and to rely more on a continuing transfer of maternal nutrients during development (Wourms, 1981). Fish exhibit a variety of anatomical and physiological specialisations for maternal-embryonal nutrient transfer which involve both maternal and embryonic tissues and which must have evolved through different historical sequences

(Wourms, 1981; Wourms and Lombardi, 1992; Blackburn, 2005b). Moreover, the evolution of viviparity included modifications in osmoregulatory, excretory, respiratory, endocrinological and immunological processes that are common to the development of both oviparous and viviparous fish (Wourms, 1981, 2005; Wourms and Lombardi, 1992). Despite the great diversity in gonad morphology, the adaptations for viviparity do not arise from new cell types but rather from new functions of cells that are common in all fish (Grier et al., 2005).

2.3.2 Occurrence of Viviparity

Viviparity occurs in all classes of vertebrates except birds (Meyer and Lydeard, 1993) and first made its evolutionary appearance among fish (Wourms, 1981; Knight et al., 1985; Wourms and Lombardi, 1992). Fish are therefore – and they reveal the greatest diversity in the development of maternal-embryonal relationships – essential to the understanding of viviparity (Wourms and Lombardi, 1992). By comparing viviparous species with oviparous species to which they are presumed to be closely related, it is possible to gain insight into specialisations needed for viviparity or reasons for its evolution (Blackburn, 2005b). It appears that live-bearing must have originated independently on many different occasions as the widespread taxonomic distribution, the diversity of adaptations and the lack of correlation with particular environments seem to emphasize (Hoar, 1969; Wourms, 1981; Contreras-Balderas, 2005).

First appearance

Origins of viviparity in fish are widely scattered over geological times. (Blackburn, 2005a). The earliest known origin among chondrichthyans for which evidence is available is that of an extinct holocephalon species from the Carboniferous period (Lund, 1980) corresponding to around 380 million years ago (Blackburn, 2005a). In osteichthyans, the earliest known origins of viviparity are represented by fossils of a coelacanth species (Watson, 1927) and an actinopterygian species (Bürgin, 1990) both from the upper Triassic period, which dates back around 240 million years (Blackburn, 2005a). However, viviparity has also evolved much more recently in other cases. Contrary to proposals of Miller and Fitzsimons (1971) that Goodeidae could have separated as recently as in the Pleiocene, calibrated

molecular data suggest that the viviparous Goodeidae began diversifying during the mid to late Miocene (Webb, 1998) about 16.5 million (Webb et al., 2004) to 14.5 million years ago (Blackburn, 2005a). Evidently, piscine viviparity has a long evolutionary history (Blackburn, 2005a) and it appears that once it evolved from the egg-laying condition it is unlikely to revert back (Meyer and Lydeard, 1993).

Viviparity has mainly evolved at family and sub-family level, while viviparous clades are not closely related to one another but rather belong to separate sub-ordinal taxa in which oviparous species predominate (Blackburn, 2005a). Occurrence of both oviparity and viviparity within a taxon is evidence of independent evolutionary events (Wourms and Lombardi, 1992). Blackburn (2005a) states that viviparity evolved independently over 140 times in vertebrates, of which 29 origins (or ca. 20%) can be assigned to fish. Whereas there is no evidence of viviparity among jawless fish, it is the dominant mode of reproduction in cartilaginous fish (Wourms and Lombardi, 1992) and is present in only a minority of osteichthyan species (Wourms, 1981, 2005; Wourms and Lombardi, 1992; Meyer and Lydeard, 1993; Grier, 2005). Live-bearing appears to have independently evolved in 13 families of teleosts (see Fig 2.1 and Tab 2.2) (Knight et al., 1985; Wourms and Lombardi, 1992; Blackburn, 2005a) and thereof three times in the order of Cyprinodontiformes (Parenti, 1981, 2005; Wourms and Lombardi, 1992; Meyer and Lydeard, 1993; Iida et al., 2015)

Present occurrence

Viviparous teleosts distributed amongst these 13 families all have different lifestyles and appear in a wide variety of habitats (Blackburn, 2005a). It is difficult to correlate osteichthyan viviparity with specific geographical and ecological parameters, although there might be some environmental constraints. In contrast with lecithotrophic fish, matrotrophic species rely on a predictable nutrient supply (Wourms 1988).

2.3.3 Phylogeny of Viviparous Osteichthyans

Species are usually classified in a way that best represents their phylogenetic history. The first attempts to integrate classification with evolution were made in

the late 1930s and early 1940s. Since then barcoding of an approximately 665 bp long mtDNA region of the cytochrome oxidase subunit I gene (COI) has been proposed as a global bio-identification system for animals (Helfman et al., 2009). Although phylogenetic analyses helped define numerous convergent independent origins of viviparity (Blackburn, 2005a), phylogenetic patchiness of viviparity has made it difficult to understand both the evolution from oviparity to viviparity and the maternal-embryonal nutrient transfer (Dulvy and Reynolds, 1997). Blackburn (2005b) also ascertained difficulties in making evolutionary generalisations about viviparity across higher taxonomic boundaries, since vertebrates have apparently evolved viviparity and matrotrophy by different historical sequences and different anatomical and physiological specialisations. Nevertheless, phylogenetic analyses are used to test hypotheses about biological features, selective pressures, evolutionary sequences and constraints affecting the evolution of viviparity (Blackburn, 2005a).

The approximately 28,000 living fish species make up more than one half of the total recognised living vertebrate species. Since many groups of fish are expanding with newly described species and a few are decreasing because species are being synonymised (Nelson, 2006), the knowledge of the relationships of organisms increases and therefore their classification is subject to ongoing change (Helfman et al., 2009).

Teleosts

All 13 families of live-bearing teleosts share an anomaly amongst viviparous species, with gestation in these fish taking place in the ovary. In most viviparous teleosts gestation is intraluminal, but embryos develop in the follicles in a few species (see Tab 2.2) (Wourms, 2005). Thus far, the exact evolutionary process has not been resolved (Parenti, 2005). Phylogenetic hypotheses within these groups might be among the most studied (Parenti, 2005), however, their classification can be expected to alter continuously. In the following, the changing nature of phylogeny of viviparous teleosts is analysed, and a current state of relationships is given in the form of a phylogenetic tree in Fig 2.1.

Ophidiiformes, one of the five orders of viviparous teleosts, are currently under the superorder of Paracanthopterygii. Even though they were excluded from this

superorder, Nelson (2006) declared that further studies would be needed before major changes could be made with confidence. There is also a disagreement concerning the family of Parabrotulidae. While Nielsen et al. (1999) did not regard them as an ophidiiform family, Nelson (2006) suggested more research was required in order to give the parabrotulids a definite placement. Conflicting information further exists on viviparous fish under the superorder Acanthopterygii. Nelson (2006) found it impossible to make a comprehensive review due to many conflicting studies. Relationships in **Beloniformes**, another order of viviparous teleosts, have been reviewed by numerous authors. Although there is molecular evidence that the beloniform family of Hemiramphidae is paraphyletic (Nelson, 2006), Blackburn (2005a) and Parenti (2005) proposed that the Hemiramphidae belong to the Beloniformes. Classification within a third order of viviparous teleosts, the **Perciformes**, is unsettled and will likely be subject to change over time that will better reflect their evolutionary history. A fourth order of live-bearing teleosts are the **Scorpaeniformes**. Their placement is provisional and classifications of families extremely controversial (Nelson, 2006). It appears that there is phylogenetic evidence suggesting inclusion of all members of the scorpaeniform family of Comephoridae within another family under the same order. Further studies are required, however, to truly corroborate current views. Furthermore, there are differing opinions on the scorpaeniform family of Sebastidae. While their reorganisation as separate family from the Scorpaenidae might be premature (Love et al., 2002), it is provisionally accepted (Imamura, 2004). **Cyprinodontiformes** are the last of the viviparous orders of teleost fish. Despite disagreements on whether viviparity evolved once or multiple times in this order (Parenti, 1981; Blackburn, 2005a), unity of this group has been acknowledged, and its constituent members have remained unchanged over many years (Nelson, 2006). Distinctive reproductive features amongst species of this order are the marked sexual dimorphism, with males often being brightly coloured as well as the male testes which are of the lobular type, where spermatogonia are restricted to the distal termini of the lobules (Nelson, 2006). The cyprinodontiform family of Goodeidae further have a unique modification of the male anal fin and an internal muscular organ of apparent reproductive function in male (Fitzsimons, 1972).

Goodeidae

There are unifying reproductive characteristics that distinguish Goodeidae from other cyprinodontiform families, such as the above mentioned male features, the structure of the female ovary and the development of trophotaeniae in embryos (Fitzsimons, 1972). Nevertheless, Goodeidae have some reproductive features in common with Poeciliidae (Turner, 1933), another cyprinodontiform family. Turner (1933) first pointed out that Goodeidae and Poeciliidae were probably derived independently from oviparous Cyprinodontidae, he later indicated that the goodeids might have derived from an ancestor in which viviparity had already been established (Turner, 1937). Hubbs and Turner (1939) were eventually the first to conduct a comprehensive study of the live-bearing goodeids based on morphology related to viviparity. Founded on phylogenetic affinity, Parenti (1981) joined the oviparous Empetrichthynae and the viviparous Goodeinae (34 species within 16 genera, see Fig 2.1) as sister taxa which might contradict Turners (1937) hypothesis. Molecular (Meyer and Lydeard, 1993) and morphological phylogeny (Costa, 1998) subsequently supported Parentis theory. Webb and colleagues suggested in 2004 that the diversification of the Goodeinae might have been driven by intrinsic factors (such as sexual selection) and extrinsic factors, as these species occupy diverse ecological niches. The authors analysed the goodeid interrelationship based on sequence data from two mtDNA loci and the gene tree presented by them differed substantially from the hypotheses of relationships of previous workers. Most importantly, Webb et al., (2004) did not place *Ataenobius* in its own subfamily but considered it more closely related to the other goodein species and explained the only rudimentary presence of trophotaeniae as an evolutionary reversal. All other viviparous goodeids possess variously developed trophotaenial placentas (see chapter 2.4.3 (Trophotaenial Placenta)) (Nelson, 2006). Polyphyly of the two *Xenotoca* species *X. eiseni* and *X. melanosoma* (which are capable of interbreeding) with the third species *X. variata* (that is not capable of interbreeding with either of the other two) was observed previously by Fitzsimons (1972). Webb et al. (2004) therefore recommended reviewing the taxonomic status of *Xenotoca*.

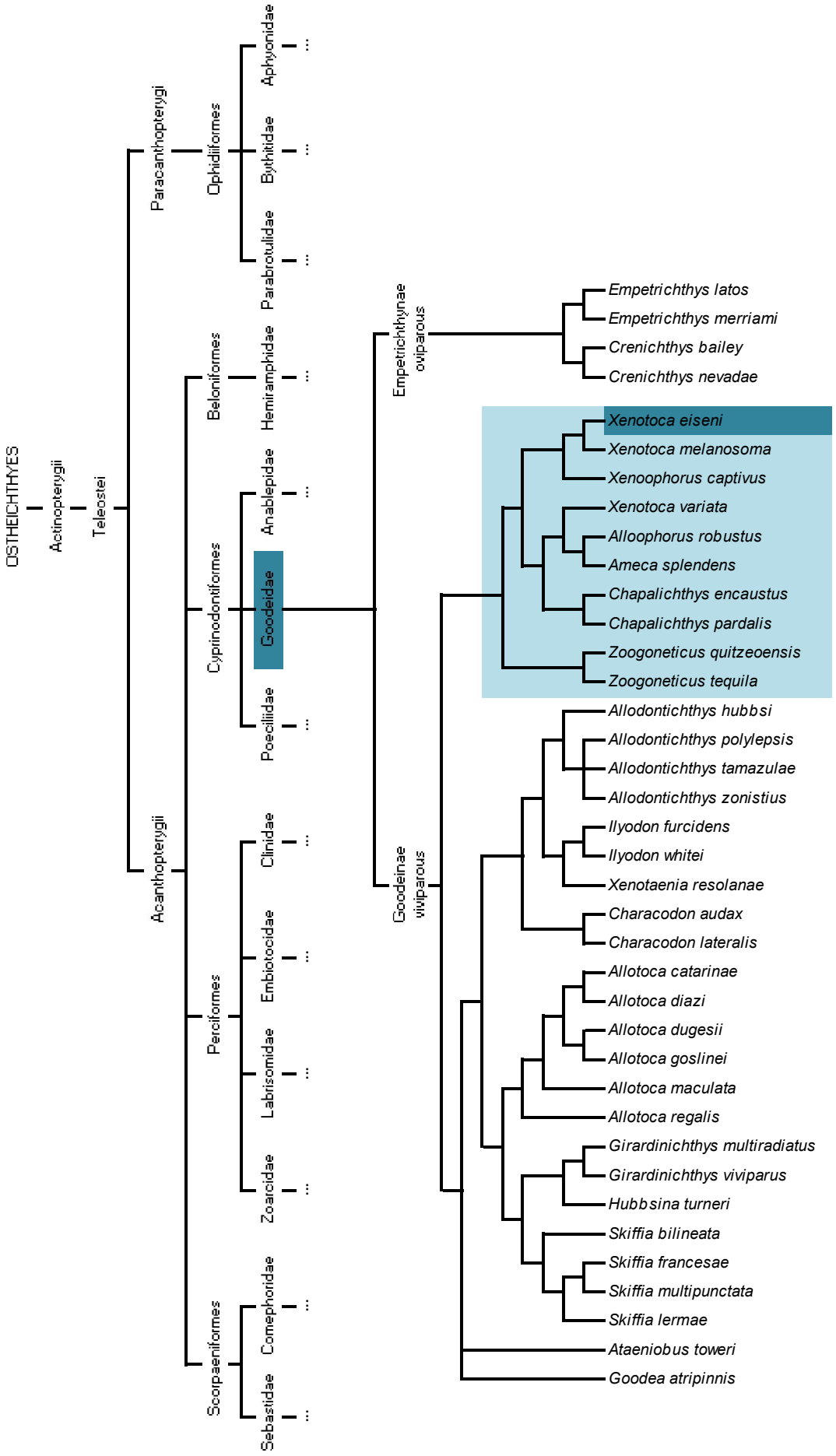


Fig 2.1: Phylogenetic tree of viviparous bony fish. Modified from Nelson (2006) and Webb et al. (2004).

Xenotoca eiseni

In viviparous teleosts, ovarian morphology is modified in a way that allows both egg production as well as internal fertilisation and gestation. (Uribe et al., 2005). The hollow ovary is enclosed within a capsule – which also forms a gonoduct – and the ovarian cavity is lined by the germinal epithelium (Wourms, 1981; Wourms and Lombardi, 1992). *Xenotoca eiseni* possess a typical goodeid ovary (Mendoza, 1965), which is characterised by its single elongated structure, a muscular wall and the division of the central lumen by a median septum (Mendoza, 1965, Uribe et al., 2005). Growing embryos of *X. eiseni* develop trophotaeniae (Uribe et al., 2005), an adaptation for the uptake of maternal nutrients that appears to have independently evolved in three teleost orders (Wourms and Lombardi, 1992; Wourms, 2005). Like other male goodeids, male *X. eiseni* lack a specialised copulatory organ but have a modified anal fin that helps with internal fertilisation (Bisazza, 1997). Another typical goodeid feature that can be observed in *X. eiseni* is the formation of spermatozeugmata (Grier et al., 2005).

Details on reproduction and developmental biology of *X. eiseni* will be given in the following chapter. An overview on live-bearing teleosts orders and their characteristic reproductive traits and number of viviparous members is summarised in Tab 2.2.

Tab 2.2: Teleost orders with viviparous fish. Classification according to Nelson (2006) and Eschmeyer and Fong (2015). The order of Ophidiiformes comprises of a total of 385 species (sp) within 100 genera (g) distributed among five families (f); numbers are 227 species within 36 genera amongst five families for Beloniformes, 1,013 species within 109 genera amongst ten families for Cyprinodontiformes, 10,033 species within 1,539 genera amongst 160 families for Perciformes and 1,477 species within 279 genera amongst 26 families for Scorpaeniformes (Nelson, 2006). Figures under "Viviparity" are number of viviparous genera (g) and species (sp) out of the total number of genera and species within according families, derived from Nelson (2006), unless otherwise stated. Other data relate to viviparous species only and are derived from Wourms (1981), complemented as noted.

Order	Family	Viviparity	Habitat	Mode of reproduction		Nutrients absorption	Gestation period	Female gonad	Male copulatory organ
Ophidiiformes									
	Aphyonidae	6*/6g 22*/22sp	marine[1]	intrafollicular retention, intraluminal	matrotroph	adelphophagy, ovigerous bulbs, absorptive finfolds, trophotaeniae[2]	unknown	paired ovary[1]	intromittent organ, spermatophores, exceptions exist[2]
	Bythitidae	17-19*/32*-37g 41-43*/96*-107sp	predominantly marine[1,2]	intrafollicular retention, intraluminal	matrotroph	adelphophagy, ovigerous bulbs, absorptive finfolds, trophotaeniae[2]	unknown	paired ovary[1]	intromittent organ, spermatophores, exceptions exist[2]
	Parabrotulidae	2/2g 2/3sp	marine	intraluminal[3]	matrotroph	three ribbon-like proctodeal extensions (similar to trophotaeniae[4])	unknown	paired ovary[1]	unknown
Beloniformes									
	Hemiramphidae	4/12g, 21/109sp	marine[1], fresh	intrafollicular[5], but mainly intraluminal[5]	from strict lecithotroph to unspecialised matrotroph[5]	lack of specialised trophic adaptations[5], oophagy and adelphophagy[5]	1-2 months, superfetation in some species	paired ovary	andropodium
Cyprinodontiformes									
	Anablepidae	2/3g 14/15sp	fresh, brakish, rarely coastal[1]	(prolonged) intrafollicular, intraluminal[6]	matrotroph	branchial placenta, follicular placenta with pericardial sac and hypertrophied hindgut	unknown	paired ovary, gonopore covered by a scale opening to the left or right	tubular gonopodium formed by anal fin rays incl. sperm duct[1] (bilateral asymmetry)
	Goodeidae	16/18g 34/38sp**	fresh	intraluminal	from strict lecithotroph (1sp) to specialised matrotroph	trophotaeniae (except for one species)	several weeks[7,8]	single median ovary[8]	andropodium (notched anal fin), spermatozeugmata (flagella at periphery)
	Poeciliidae	27/37g 225/304sp	fresh, brakish, rarely coastal[1]	prolonged intrafollicular	mostly lecithotroph and unspecialised matrotroph with a few specialised matrotroph	follicular placenta (in specialised matrotrophs), expanded pericardial sac	ca. 1 month, superfetation in some species	single ovary[9], sperm storage common	intromittent organ (gonopodium[1]), spermatozeugmata (flagella in centre)

Perciformes								
Clinidae	16/21g 60/75sp	marine	intrafollicular	matrotroph[10]	follicular placenta (uptake via dorsal fin, pericardial and yolk sac and later via mid- and hindgut	superfetation (up to 12 broods)	paired ovary[10]	fleshy intromittent organ[10]
Embiotocidae	13/13g 23/23sp	coastal marine, rarely fresh[1]	intraluminal	matrotroph	histotroph, sperm[6], dead embryos[6] (uptake via gills, fin epithelium, ingestion or hindgut)	6-12 months[6]	single median ovary, sperm storage (ca. 6 months)[6]	appendages and tubular system, spermatozeugmata
Labrisomidae	2/15g 21/105sp	marine	intrafollicular	lecithotroph[11]	n.a.[11]	superfetation[11]	paired ovary[11]	intromittent organ (modified genital papilla)[11]
Zoarcidae	1/46g 3/302sp	marine	intraluminal	matrotroph	enlarged hindgut with hypertrophied intestinal epithelium[12], suckling on follicles[12]	4-5 months[12,13,14]	single median ovary[14]	unknown
Scorpaeniformes†								
Comephoridae	1/1g 2/2sp	fresh	intraluminal	lecithotroph[15]	n.a.[15]	90-120 days	unknown	conical copulatory organ (hypertrophied urogenital papilla)[15]
Sebastidae††	5-7/5-7g†† >100/>100sp††	marine, rarely fresh[1]	prolonged intrafollicular, intraluminal[6]	from strict lecithotroph to unspecialised matrotroph[16]	lack of specialised trophic adaptations, oophagy and adelphophagy[16]	20-30 days, superfetation[16]	paired ovary[16], sperm storage (up to 6 months)[6,16]	unknown

* Nielsen et al. (1999)
** Webb et al. (2004)

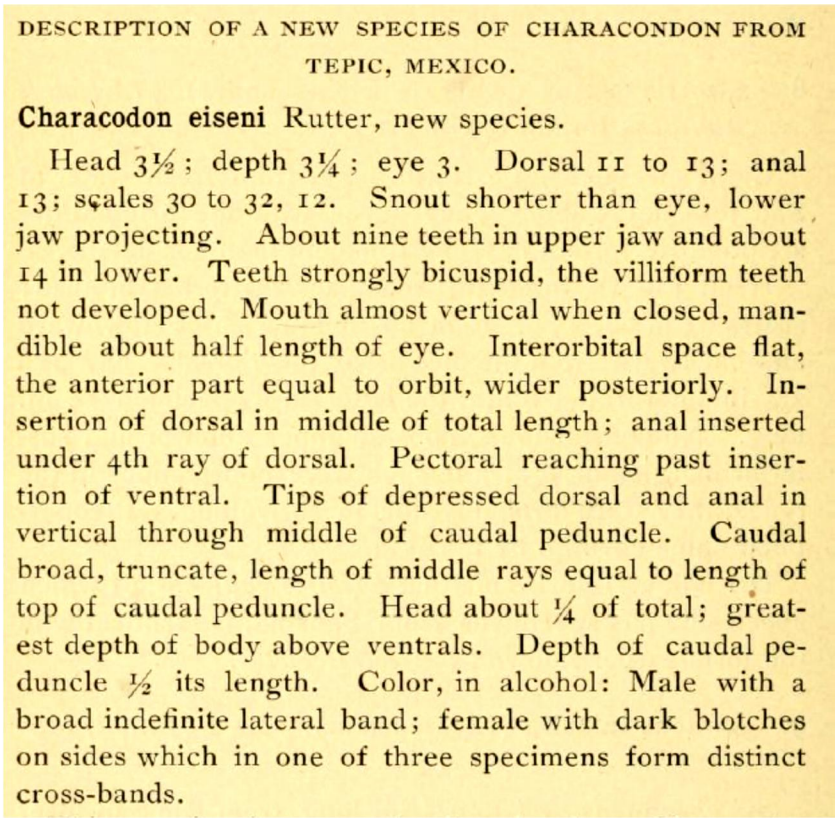
† Classification and placement in this order are very provisional (Nelson, 2006)
†† The reorganisation of the Sebastidae as separate family from the Scorpaenidae might be premature (Love et al., 2002) but is provisionally accepted (Imamura, 2004)

1 Nelson (2006)
2 Mead et al. (1964)
3 Wourms et al. (1988)
4 Turner (1936)
5 Meisner and Burns (1997)
6 Hoar (1969)
7 Schindler and de Vries (1987, 1988)
8 Schindler (2014)
9 Wootton and Smith (2014)
10 Fishelson and Gon (2009)
11 Rosenblatt and Taylor (1971)
12 Skov et al. (2010)
13 Kristofferson et al. (1973)
14 Korsgaard and Petersen (1979)
15 Chernyayev (1974)
16 Love et al. (2002)

2.4 Developmental Biology of *Xenotoca eiseni*

2.4.1 Derivation of *Xenotoca eiseni*

Originally, *Xenotoca eiseni* was described by Rutter as *Characodon eiseni* in 1896 (see Fig 2.2). He named the fish after the closely related species *Characodon variatus* (Bean, 1887) and Dr Gustav Eisen, who collected the species in 1894 from a branch of the Río Grande de Santiago at Tepic, Mexico.



DESCRIPTION OF A NEW SPECIES OF CHARACONDON FROM TEPIC, MEXICO.

Characodon eiseni Rutter, new species.

Head 3½; depth 3¼; eye 3. Dorsal 11 to 13; anal 13; scales 30 to 32, 12. Snout shorter than eye, lower jaw projecting. About nine teeth in upper jaw and about 14 in lower. Teeth strongly bicuspid, the villiform teeth not developed. Mouth almost vertical when closed, mandible about half length of eye. Interorbital space flat, the anterior part equal to orbit, wider posteriorly. Insertion of dorsal in middle of total length; anal inserted under 4th ray of dorsal. Pectoral reaching past insertion of ventral. Tips of depressed dorsal and anal in vertical through middle of caudal peduncle. Caudal broad, truncate, length of middle rays equal to length of top of caudal peduncle. Head about ¼ of total; greatest depth of body above ventrals. Depth of caudal peduncle ½ its length. Color, in alcohol: Male with a broad indefinite lateral band; female with dark blotches on sides which in one of three specimens form distinct cross-bands.

Fig 2.2: Original description by Rutter (1896) based on four specimens (No. 4999, L. S. Jr. Univ. Mus.) from a branch of the Río Grande de Santiago at Tepic (Mexico), the largest being 1¼ inches long.

Jordan and Everman (1896-1900) and Meek (1904) confirmed that *C. eiseni* and *C. variatus* were different species, although very closely related. In 1907, however, Regan stated that *C. eiseni* was synonymous to *C. variatus* and this was backed up by Hubbs (1926) after a re-examination. By reconstructing the classification of the order Cyprinodontes, Regan and Hubbs joined the genera *Zoogoneticus, Girardinichthys, Characodon, Chapalichthys, Goodea* and *Skiffia* into the family of Goodeidae basing it primarily upon the possession of a definite modification in the anal fin of the male (Turner, 1933). In 1939, Hubbs and Turner erected *Xenotoca* – from the Greek ξένος (strange) and τόκος (offspring) – as a new genus for *Characodon variatus* because this species differed from

Characodon lateralis in numerous internal as well as external features. The synonymy of *C. eiseni* and *C. variatus* was again corroborated and the species was renamed *Xenotoca variata* (Hubbs and Turner, 1939).

Although Meek (1902, 1904) identified criteria like viviparity, specialisation of the anal fin and geographic distribution and Hubbs and Turner (1939) recognised characteristics such as the ovarian structure and trophotaeniae, classifications of the fish of the family of Goodeidae were still largely based on features like jaws, teeth and length of intestine.

Mendoza (1965) doubted the validity and classification of *X. variata*. By comparing ovarian and trophotaenial characteristics, he found discrepancies between the synonymised species and distinguished again between *"Characodon" eiseni* and *Xenotoca variata*. This was confirmed by Fitzsimons (1972), who suggested reinstating the former species as *Xenotoca eiseni*. Fitzsimons results were based not only on ovarian anatomy and trophotaeniae but also on information on other morphological features as well as on ethology, karyotypes, courtship behaviour, hybridisation experiments and discrimination tests. Hereby, the distinctiveness of *Characodon* and *Xenotoca* could be confirmed and *Xenotoca* was found to consist of the three species: *X. variata*, *X. eiseni* and *X. melanosoma*.

An overview on the different classifications is given in Tab 2.3.

Tab 2.3: Classification according to different authors based on different characteristics

C. variatus* *C. eiseni	***C. variatus***	***X. variata***	***X. variata* „*C.*" *eiseni***	***X. variata* *X. eiseni***
Anatomical characters such as colour, type of jaws, teeth, length of intestine, scale numbers etc	Anal fin	Ovarian structure and types of trophotaeniae		Morphological features, karyotypes, courtship behaviour, hybridisation and discrimination tests
Jordan and Evermann (1896-1900) Meek (1902,1904)	Regan (1906-1908) Hubbs (1926)	Hubbs and Turner (1939)	Mendoza (1965)	Fitzsimons (1972)

Ecological distribution

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 et al., 2004; Contreras-Balderas, 2005; Dominguez-Dominguez et al., 2005). The plateau with altitudes between about 1,000 and 2,500 metres (Meek, 1902) forms an isolated biological area, where waterfalls and mountains form natural barriers to migration (Turner, 1933). *X. eiseni* occurs in a great variety of habitats ranging from warm to cold, 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). The species' centre of abundance 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 (see Fig 2.3) (Dominguez-Dominguez et al., 2005).

Fig 2.3: Map of Mexico. Approximate habitat of *X. eiseni* on the Central Mexican Plateau is highlighted in orange.

Key characteristics

Many goodein species are called splitfins (Nelson, 2006) and *Xenotoca eiseni* is also known as redtail splitfin (Fitzsimons, 1972). This name is derived from the secondary sexual characteristics of the male fish whose posterior half of the caudal peduncle is orange to red-orange (Fitzsimons, 1972) and whose anal fin has a notched anterior margin (see arrow in Fig 2.4 B) (Meyer and Lydeard, 1993). The latter sexual dimorphism, called andropodium (Meyer and Lydeard, 1993), is a modification in which the anterior rays of the anal fin in males are crowded, shortened and slightly separated from the rest of the fin (Miller and Fitzsimons, 1971; Nelson, 2006). Furthermore, the dorsal fin of male *X. eiseni* may become dusky with dark stripes (Fitzsimons, 1972). Females, on the other hand, have a prominent dark blotch on the lower abdomen that begins at the anal fin and extends forward about halfway to the base of the pelvic fin (Fitzsimons, 1972). The bodies of both genders are rather deep and somewhat compressed (Meek, 1904) with a standard length of 40.0 mm for males and 44.8 mm for females (Fitzsimons, 1972).

Goodeids are gonochoristic (Greven, 2013) and *X. eiseni* have a diploid chromosome number of 48 including six subtelocentrics and 42 telocentrics (Fitzsimons, 1972; Uyeno et al., 1983).

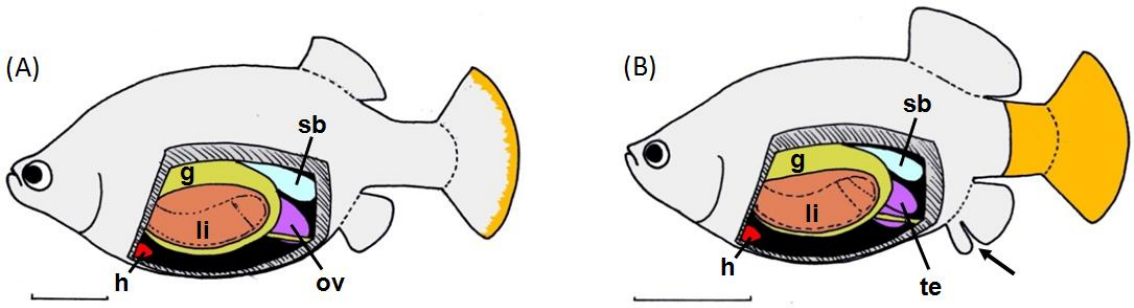


Fig 2.4: Rough anatomy of an adult female (A) and an adult male (B) *Xenotoca eiseni*. **Key:** g: gut, h: heart, li: liver, ov: ovary, sb: swim bladder, te: testes. Spleen, gallbladder and kidney are not shown in these pictures. Scale bars: 1 cm

2.4.2 Reproduction

Gonadal morphogenesis

Gonadal differentiation in teleost fish involves a modification in both germ and somatic cells which is revealed by mitotic and meiotic division of oogonia and spermatogonia as well as by specific anatomical formations in female and male gonads (Guerrero-Estévez and Moreno-Mendoza, 2012).

The goodein unpaired ovary resembles the gonad of other viviparous teleosts and differs from ovaries of oviparous teleosts and other vertebrate groups (Guerrero-Estévez and Moreno-Mendoza, 2012). The ovary of an adult viviparous goodeid is an unpaired spindle-shaped structure that is attached dorsally by a peritoneal mesentery (Mendoza, 1965; Uribe et al., 2005; Guerrero-Estévez and Moreno-Mendoza, 2012). *X. eiseni* possess a typical goodeid ovary, which has a muscular wall and a central lumen that is divided by a median septum (Turner, 1937; Hubbs and Turner, 1939; Mendoza, 1965; Uribe et al., 2005). In an averagely sized adult female, the ovary measures approximately two to three millimetres in diameter and the length of the ovary including the gonoduct varies between ten and 30 millimetres (Mendoza, 1965). The longitudinal septum extends the entire length of the ovary and divides it into two lateral halves (Mendoza, 1965; Lombardi and Wourms, 1985a). This median membrane is rather variable in structure and it may be complete or partially incomplete with one or more holes (fenestrae). Furthermore, the membrane can be branched and variably folded making its total width much greater than the diameter of the ovary (Mendoza, 1965; Lombardi and Wourms, 1985a; Fitzsimons, 1972). The internal ovarian wall is also extensively folded and projects into the lumen (Mendoza, 1941; Uribe et al., 2005).

Histologically, the ovarian wall consists of the following tissue layers: the serosa, formed by mesothelium and thin connective tissue; a thin layer of muscle (Uribe et al., 2005); a stroma, formed by collagenous vascularised connective tissue, and; a cuboidal germinal epithelium, which is separated from the stroma by a basement membrane (Mendoza, 1941; Uribe et al., 2005). Cells and tissue types are not created *de novo* but the germinal epithelium differs from all others in such a way that it is made up of both somatic and germ cells. Nests of oogonia are scattered within the germinal epithelium (Grier et al., 2005; Uribe et al., 2005) and

are located throughout the ovarian wall as well as in the septum (Mendoza, 1965; Uribe et al., 2005). Ovigerous tissue occurs more often in specific locations including the anterior half or third of the ovary, although it may extend all the way through most of the gonad in juvenile fish (Mendoza, 1965). Upon the initiation of meiosis and folliculogenesis, which is a continuous process (Grier et al., 2005), the ovary undergoes histological alteration (Guerrero-Estévez and Moreno-Mendoza, 2012). During folliculogenesis, oocytes become progressively surrounded by a single layer of epithelial cells, forming primary follicles (Uribe et al., 2005). The follicle that encloses an egg is usually squamous and is surrounded by a thin vascular connective tissue (theca). Mature eggs in *X. eiseni* attain a maximum size of 250 to 300 µm (Mendoza, 1965). As in other goodeids, the single basement membrane shared by the follicle and the germinal epithelium breaks at the time of ovulation (Grier et al., 2005), which is believed to take place after fertilisation (Wourms, 1981, 2005; Dulvy and Reynolds, 1997). After separation of follicle cells and epithelial cells of the germinal epithelium, the egg is voided into the ovarian lumen (Grier et al., 2005).

While the onset of meiosis in germ cells of the prospective ovaries marks the onset of differentiation in gonads of teleosts, testes remain histologically undifferentiated for a longer period of time and somatic differentiation precedes germ cell meiosis (Guerrero-Estévez and Moreno-Mendoza, 2012). Vertebrate testes are characterised by compartmentalisation, whether or not the reproductive mode is viviparous (Grier et al., 2005). While spermatogenesis takes part in the germinal compartment, somatic cells are involved in the formation of efferent ducts, spermatic ducts and of testicular lobules in testes (Nakamura et al., 1998; Rasmussen et al., 2006; Guerrero-Estévez and Moreno-Mendoza, 2012). The interstitial compartment further contains hormone-secreting Leydig cells (Grier et al., 2005).

Internally fertilising fish have evolved different morphological adaptations for the transfer of sperm into the female reproductive tract, such as genital papilla, neuronal modification coupled with evolution of coordinated behaviour, transport via modified anal fins, sperm packaging or the release of sperm bundles (Grier et al., 2005). As described earlier, un-encapsulated sperm bundles are called spermatozeugmata (Meyer and Lydeard, 1993). Spermatozeugmata is formed by

modified associations between (somatic) Sertoli cells and germ cells which have an altered function and morphology but are of the same cell types that are found in all fish testes (Grier et al., 2005). While spermatid flagella extend into the lumen of spermatozeugmata in some species (Meyer and Lydeard, 1993), they become associated with Sertoli cells in Goodeidae and form the surface of spermatozeugmata (Grier et al., 2005). Even though goodeids have an advanced form of viviparity, males lack a specialised copulatory organ, as their anal fin is not modified into a real intromittent device and cannot be inserted into the female genital opening (Greven and Brenner 2010). The modification of the anal fin, however, includes a crowding and shortening of the anterior six to seven fin rays which are separated from the rest of the fin by a notch. (Parenti, 1981; Bisazza, 1997). Furthermore, a muscular organ surrounding the vas deferens and urinary canal is believed to contract during copulation, ejecting spermatozeugmata (Dulvy and Reynolds, 1997) into a pocket formed by the anal fin (Parenti, 1981).

Internal fertilisation

During courtship, the male approaches the female, swinging its anal fin laterally towards the female (Greven and Brenner 2010). Internal fertilisation requires the partners to be exactly synchronised during copulation, which takes about two to five seconds (Bisazza, 1997). In order to introduce sperm into the female reproductive tract, male *X. eiseni* wrap the anterior lobe of their anal fin around the female's genital opening (Nelson, 1975), while the notch in the fin is placed near the anterior margin of the female's anal fin (Dulvy and Reynolds, 1997). While the urogenital openings of male and female come very close together, the male anal fin forms a pocket around the female genital orifice (Nelson, 1975; Dulvy and Reynolds, 1997) into which the male ejaculates spermatozeugmata with the help of contracting strong muscle fibres running between intestine and sperm duct (Greven and Brenner 2010).

According to Bisazza (1997), only females with mature eggs mate with males, whereas males seem to be unable to distinguish between ovulating and gravid females. Goodeid males are ready to mate for virtually all their adult lives, while females are receptive for a few days every two months only (Macías García and Gonzalez-Zuarth, 2005).

Intraluminal gestation

According to numerous authors, fertilisation in *X. eiseni* takes place within the follicle (Wourms, 1981, 2005; Dulvy and Reynolds, 1997, Uribe et al., 2005), where the embryos are retained for a short period only, before they are ovulated (Uribe et al., 2005; Wourms, 2005). Development continues in the ovarian lumen, where the egg remains encapsulated (Riehl and Greven 1993), while embryos absorb their small supply of yolk within the first third of gestation (Uribe et al., 2005). The egg envelope in *X. eiseni* is 1.2 µm thick, what lies within the normal range of viviparous teleosts, but is about ten times thinner than the envelopes of related oviparous species (Riehl and Greven 1993). Shortly before hatching, embryos develop externalised extension of their hindgut called trophotaeniae and henceforth, nourishment is provided by the ovarian lumen in a secreted form (Dominguez-Dominguez et al., 2005; Uribe et al., 2005; Iida et al., 2015).

Whereas the germinal epithelium remains relatively unchanged during the production of follicles, it undergoes remarkable changes throughout gestation (Grier et al., 2005). In gravid females, epithelial cells lining the ovarian cavity increase in height and become more glandular, connective tissue becomes swollen and the vascularity of the ovary increases as an extensive capillary network is built (Lombardi and Wourms, 1985a). With this modification, the germinal epithelium becomes involved in the transport and exchange of multiple metabolites from the maternal vascular system into the ovarian fluid (Uribe et al., 2005). As a result of maternally transferred nutrients, embryos undergo a 10 to 350 times increase in dry weight within a relatively short gestation period (Wourms, 2005).

After a regular gestation that ranges from six to eight weeks, 11 to 13 mm long embryos (Mendoza, 1965) are born at an advanced stage in which the gonads are already differentiated (Turner, 1933). Brood numbers vary from around 10 to 60, with only 4 to 5 in young mothers during first delivery. Roughly half of the original number of embryos is normally absorbed in the ovary during gestation (Turner, 1933).

2.4.3 Trophotaenial Placenta

In a trophotaenial placenta, the dynamic apposition of organs includes the modified ovarian lumen epithelium as maternal component and trophotaeniae as an embryonal structure (see Fig 2.7) (Wourms and Lombardi, 1992; Uribe et al., 2005; Wourms, 2005; Schindler, 2014). Nutrients are transported from the maternal vascular system to the ovarian fluid via the specialised ovarian epithelium (Uribe et al., 2005) and then taken up by the hatched embryos within the two chambered ovary (Schindler, 2014).

Inner ovarian epithelium and histotroph

In gravid female *Xenotoca eiseni*, the inner ovarian lining becomes extensively vascularised (Lombardi and Wourms, 1985a; Schindler, 2014). The well circularised connective tissue is covered by the inner ovarian epithelium (IOE), a simple cuboidal epithelium with morphological features for trans-epithelial transport of multiple metabolites (Uribe et al., 2005; Schindler, 2014). Micro- and macromolecules are transported from the maternal capillaries to the ovarian fluid by cells from the germinal epithelium. Some of these cells become thinner than 1 µm in late gestation in order to reduce the transport distance. Embryonic waste, on the other hand, is assumed to be transported in the opposite direction and subsequently removed via the maternal vascular system (Uribe et al., 2005).

Besides the vesicular transport of nutrients from maternal blood, the germinal epithelium also seems to be secreting histotroph into the ovarian cavity (Uribe et al., 2005). Schindler (2014) used electrophoretic profiles to compare the protein patterns of maternal blood serum and ovarian fluid in *X. eiseni* and found qualitatively similar band patterns between the two liquids. Analyses further revealed that the ovarian epithelial cells lack tight junctions during early and mid-gestation, permitting intercellular passage of blood derived proteins to the ovarian fluid (Schindler, 2005). Since the percentage of blood of the whole body mass is characteristically less in fish than in mammals, the volume of embryotroph that constantly has to be replenished is also rather small (Schindler, 2005).

Trophotaeniae

Goodeid embryos take up nutrients from the ovarian fluid through trophotaeniae (de la Vega-Salazar and Macías García, 2005), external structures that extend from the perianal region of an embryo (see Fig 2.5) (Wourms, 1981, 2005). While trophotaeniae characteristically consist of a simple surface epithelium surrounding a highly vascularised core of loose connective tissue, there is a considerable diversity in size, shape and number of appendages (Wourms, 1981; Uribe et al., 2005). *X. eiseni* typically have four basic processes of which any one can be reduced, absent or divided into branches (Fitzsimons, 1972).

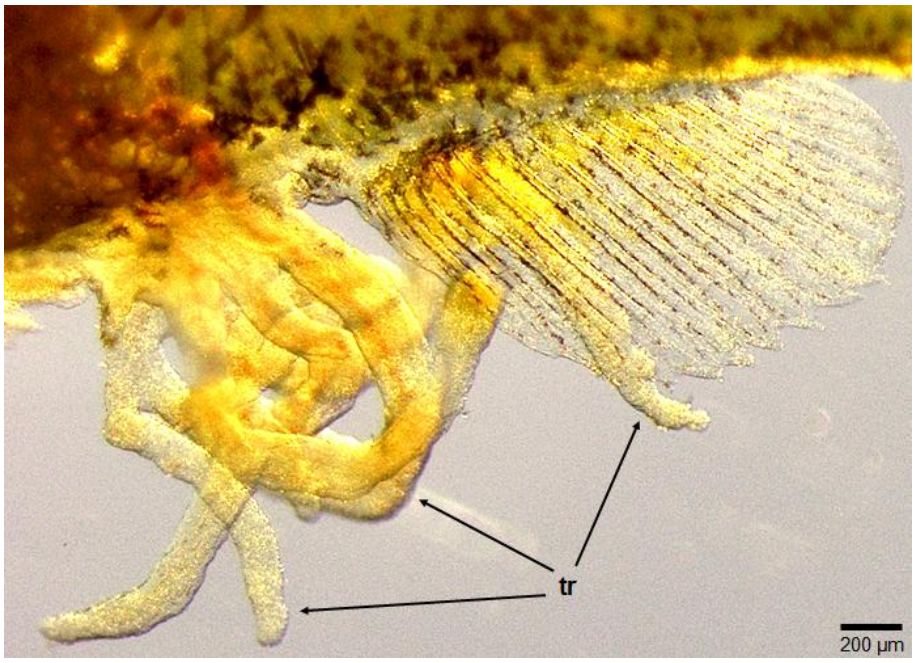


Fig 2.5: Trophotaeniae (tr) of a *Xenotoca eiseni* embryo.

There are two major types of trophotaeniae: rosette and ribbon (Wourms, 1981; Wourms and Lombardi, 1992; Uribe et al., 2005). The rosette type consists of short, blunt, flat, lobulated processes that are attached to the posterior end of the gut (Wourms and Lombardi, 1992; Uribe et al., 2005) and are irregular both in size and shape. Their stroma is made up of a spongy upper layer plus a dense basal layer and is surrounded by an external epithelium (Hubbs and Turner, 1939). Ribbon type on the other hand are long, thin processes with an amplified surface area for an increased nutrient transfer efficiency (Wourms and Lombardi, 1992; Uribe et al., 2005). The unsheathed subtype is pointed, flat, completely filled with stroma and represents the most primitive type. Sheathed ribbons, by contrast, are blunt and numerous, consist of a well vascularised spongy stroma

and an external epithelium with no primary tissue spaces (Hubbs and Turner, 1939). An overview on Goodeinae and their trophotaeniae types is given in Tab 2.4.

Tab 2.4: Different types of trophotaeniae in Goodeinae. Adapted from Hubbs and Turner (1939) and Wourms (1981), unless stated otherwise.

	Ribbon		**Rosette**
	sheathed	unsheathed	
Allodontichthys [1]			
Alloophorus	..	x	..
Allotoca	..	..	x
Ameca [2]	..	x	..
Ataeniobus [3]	..	..	..
Chapalichthys	..	x	..
Characodon	x	..	..
Girardinichthys	x	..	..
Goodea	..	..	x
Hubbsina [1]	x		..
Ilyodon	x	..	..
Skiffia	x	..	..
Xenoophorus	..	..	x
Xenotaenia [1]			
Xenotoca [2]	x	..	..
Zoogoneticus	..	x	..

[1] type or subtype unknown
[2] according to Mendoza (1965)
[3] no trophotaeniae

Trophotaeniae are embryological and evolutionary derivatives of the hindgut (Wourms and Lombardi, 1979, 1992; Wourms, 1981, 2005). Their development involves externalisation of the embryonic hindgut epithelium through growth-based dilation and evagination of the perianal lips (see Fig 2.6) (Schindler, 2014). The trophotaenial surface consists of both epidermal cells and microvillous cells, which are identical and continuous to intestinal brush border cells (Wourms and Lombardi, 1992; Wourms, 1981, 2005). These two cell origins imply that the trophotaenial blood supply is also derived both from the interstinal and the cutaneous circulation (Schindler, 2014). Trophotaeniae grow during embryogenesis and reach their maximum length in near-term embryos (Fitzsimons, 1972). Since they are becoming resorbed rather than being retained as functional organs, the invested energy into their development is eventually lost (Wourms, 2005).

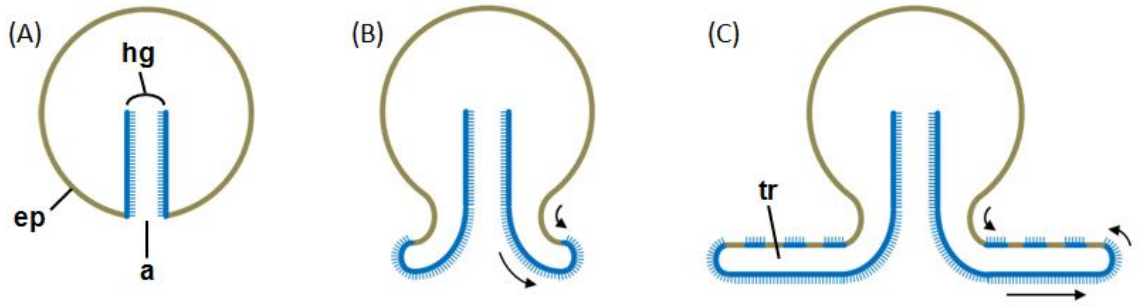


Fig 2.6: Schematic illustration of the development of trophotaeniae. Brush border cells of the hindgut are blue; cells of the body surface are brown. Arrows show the direction of growth. Modified from Lombardi and Wourms (1988) and Greven (2013). **Key:** a: anus, ep: epidermis, hg: hindgut, tr: trophotaeniae.

The two distinct epithelial cell types in trophotaeniae of *X. eiseni* are distributed unequally in a way that the smooth, squamous epithelium, which is equivalent to and continuous with the embryonic surface, is more likely to be found in the dorsal and lateral surfaces of trophotaeniae (Wourms et al., 1988). On the other hand, surface epithelium, which is derived from the hindgut and exhibits structural and functional characteristics of intestinal absorptive cells (Wourms and Lombardi, 1992), is predominantly present in ventral surfaces (Wourms et al., 1988). Surface epithelium cells are 8 to 10 µm high, have a cuboidal to low-columnar shape (Wourms et al., 1988) and possess an apical brush border (Wourms, 1981). A schematic illustration of a trophotaenial ribbon apposed to maternal tissue is given in Fig 2.7. Being virtually identical with neonatal mammalian intestinal absorptive cells (Wourms, 1981, 2005; Wourms and Lombardi, 1992), trophotaenial absorptive cells (TACs) possess an apical endocytic complex, lysosomes, phagocytic vacuoles and the standard cell organelles (Wourms, 1981). They are specialised either for transport of small molecules or endocytosis of macromolecules (Wourms, 2005) and show a great efficiency for taking up nutrients that are contained in histotroph (Uribe et al., 2005). Even at low concentrations, trophotaeniae are efficient in absorbing nutrients, which makes them well adapted to cases in which their mothers' food supplies are less optimal. As trophotaeniae are external structures and therefore not limited in their capacity to grow, this gives opportunity to increase the surface areas many fold as required to enhance nutrient absorption from the mother (Wourms, 2005).

Ingestion of macromolecules takes place in a highly specialised endocytic apparatus within trophotaenial absorptive cells (TACs, Schindler, 2005). There are two fundamental types of TACs, one of which was discovered in *X. eiseni* in

electron microscopic studies (Schindler, 2014). Its morphology is characterised by a prominent vacuolar apparatus indicative of high endocytic activity in the apical cytoplasm beneath the brush border. Schindler (2014) was able to demonstrate this endocytic activity by ingestion of unspecific tracer proteins in various species. He also revealed that the ingestion of various proteins or random copolymers conjugated to colloidal gold as well as radio-iodinated proteins by TACs satisfied the criteria of receptor-mediated endocytosis.

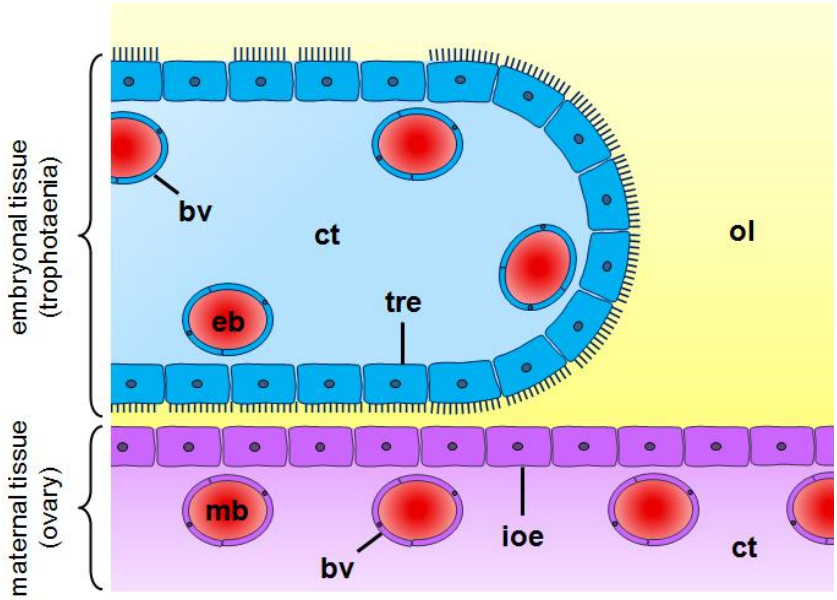


Fig 2.7: Schematic representation of the trophotaenial placenta in *X. eiseni*. Tip of a trophotaenial ribbon lying in direct apposition to the internal ovarian epithelium that lines the ovarian lumen. Modified from Lombardi (1985b). **Key**: bv: blood vessel, ct: connective tissue, eb: embryonal blood, ioe: inner ovarian epithelium, mb: maternal blood, ol: ovarian lumen, tre: trophotaenial epithelium.

The trophotaenial placenta is an advanced reproductive strategy found in most goodeid species. Its name is derived from the embryonal absorptive trophotaeniae which are apposed to the surface of the secretory inner ovarian epithelium. A trade-off of this system is the relatively small functional surface area and the long diffusion distance across the trophotaenial epithelium that would not allow for effective gas exchange. However, the epidermis in all but one viviparous goodeid embryo is in close contact with a dense meshed capillary plexus with a minimum diffusion distance less than one micrometre (Schindler, 2014). This apposition lasts until the end of the pregnancy and may be the site of gas exchange.

Relationships with the placenta systems in mammals

The purpose of the close apposition or fusion of parental and foetal tissues in placentas (Mossman, 1937) is to maximise physiological exchange, while minimising immunological rejection by the maternal immune system (Wooding and Burton, 2008). This task of interfacing between maternal and foetal blood at the same time as allowing for separation between the two circulatory systems (Enders and Blankenship, 1999; Schnorr and Kressin, 2011) has been solved through a range of various placental systems across different species.

Discrimination of placenta types can be made on different levels. Firstly, there are two different origins of foetal vessels that vascularise the embryonic membranes (Burton et al., 2006). They may either be derived from vessels covering the yolk sac, generating the choriovitelline placenta found in marsupials (Burton et al.,2006) or they might be associated with the allantois (a membrane formed by the hindgut) bringing about the mammalian chorioallantoic placenta (Enders and Blankenship, 1999; Burton et al., 2006). In chorioallantoic placentas, differences exist in the form of the maternal-embryonal contact zone. The simplest form is the “folded type”, where maternal and embryonal tissues are intertwined with primary folds. Secondary folds give rise to the “lamellar type” but if the folds are not complete and form branches instead, this is referred to as “trabecular type”. The more sophisticated tree-like structure that is also found in humans is called the “villous type”. The most common kind is the “labyrinth type”, where maternal and foetal tissues form a complex interlocking framework (Burton et al., 2006; Schnorr and Kressin, 2011).

Placenta systems may also be classified on the basis of tissue layers between maternal and embryonal circulatory system (Schnorr and Kressin, 2011). The outermost cell layer of the foetal tissue attaches to the maternal tissue and is called trophoblast or trophectoderm. Trophoblast cells may occur as mononuclear cells (cytotrophoblast) and as large multinucleate cells or layers (syncytiotrophoblast) (Enders and Blankenship, 1999). If all placental layers are retained, the trophoblast is apposed to the inner uterine epithelium and the placenta system is referred to as “epitheliochorial” (Fig 2.8 (A)) (Burton et al., 2006; Schnorr and Kressin, 2011). In some cases, foetal binucleate cells migrate and fuse with uterine epithelium cells, forming heterologous cells, creating a

placenta system called "synepitheliochorial" (Fig 2.8 (B)) (Burton et al., 2006). After destruction of the inner uterine epithelium, the trophoblast is directly adjoining to the maternal capillaries, which is classified as "endotheliochorial" placenta (Fig 2.8 (C)) (Burton et al., 2006; Schnorr and Kressin, 2011). Further invasion leads to erosion of the maternal vessels with the result that the trophoblast is in direct contact with the maternal blood. This so called "haemochorial" placenta occurs in three different variations (Fig 2.8 (D,E,F)), where the trophoblast is either one, two, or three layers thick (Burton et al., 2006; Schnorr and Kressin, 2011).

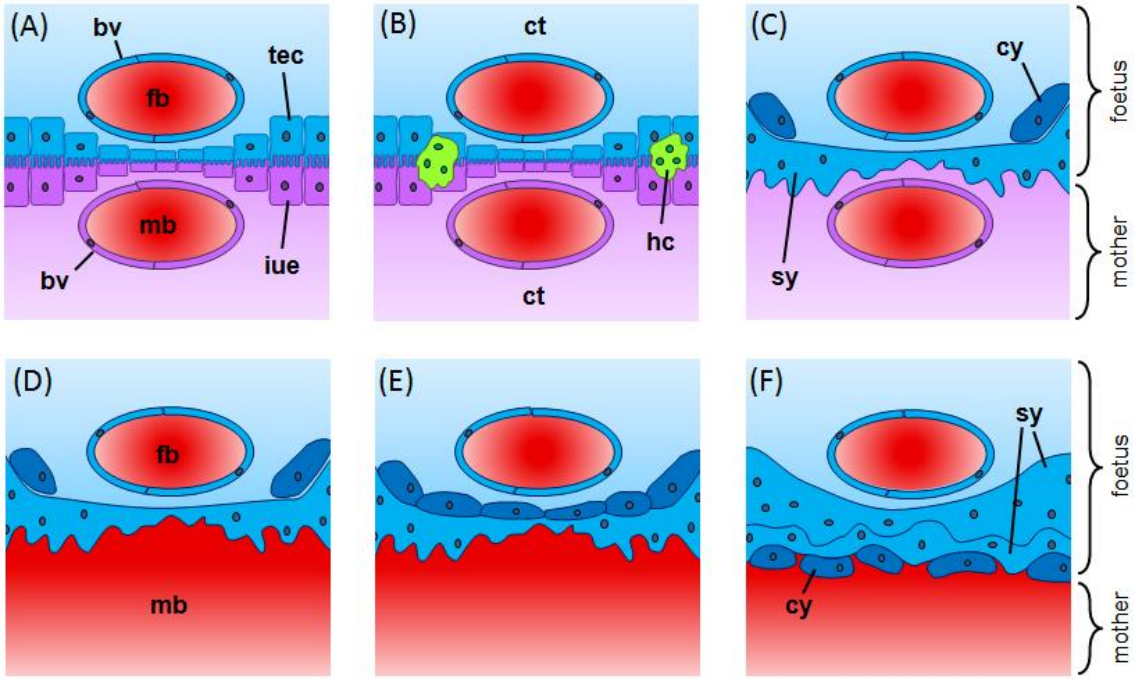


Fig 2.8: Schematic representation of different mammalian placenta types. **A:** Epitheliochorial type. **B:** Synepitheliochorial type. **C:** Endotheliochorial type. **D:** Haemomonochorial type. **E:** Haemodichorial type. **F:** Haemotrichorial type. Modified from Montiel et al. (2013) and Furukuwa et al. (2014). **Key**: bv: blood vessel, ct: connective tissue, cy: cytotrophoblast, fb: foetal blood, hc: heterologous cell, iue: inner uterine epithelium, mb: maternal blood, sy: syncytiotrophoblast, tec: trophectoderm.

Chorioallantoic placenta types have different numbers of cell layers separating maternal and embryonal blood streams (Furukawa et al., 2014). Although a small inter-haemal distance generally increases the rate at which molecules can transfer between maternal and foetal blood, their proximity is comparable across different placenta systems (Enders and Carter, 2004) as blood vessels on both sides are located very close to the surface epithelia so that the connective tissue does not play a major role in the diffusion path (Schnorr and Kressin, 2011).

There is, however, a greater difficulty for passage of materials between organisms in the epitheliochorial placenta, what in turn diminishes maternal immunological reactions. The incidence of passage of foetal cells into the maternal organism is still reduced in the endothelial type, whereas this chance becomes greater in the haemochorial placenta. Advantages of the haemochorial system on the other hand are the direct access to maternal blood for oxygen and carbon dioxide exchange as well as the facilitated transport of nutrients from the maternal blood via trophoblast receptors (Enders and Carter, 2004).

Species within the same family usually have similar placentas whereas structures begin to diverge at the ordinal rank with little correlation at higher levels of phylogeny (Enders and Carter, 2004; Burton et al., 2006). Main placenta types with some representative species are given in Tab 2.5.

Tab 2.5: Mammalian placenta types in different species. Modified from Enders and Carter (2004) and Schnorr and Kressin (2011)

Placenta type		**Layers**	**Species**
Epitheliochorial		4	Ruminants, pig, horse, whale
Endotheliochorial		3	Cat, dog, elephant, seal
Haemochorial	Monochorial	2	Human (primates), guinea pig
	Dichorial	3	Rabbit
	Trichorial	3-4	Mouse, rat (rodents)

Apart from the number of cell layers between maternal and foetal blood there are other factors that influence transport across the placenta, such as the activity and expression of specific transporter proteins (Lager and Powell, 2012; Furukawa et al., 2014). In humans, for example, there are more than 20 different amino acid transporters and glucose uptake is mediated by facilitated carrier-mediated diffusion through specific glucose transporter proteins (Lager and Powell, 2012). Transporter proteins like these form the placental barrier between the maternal and the embryonal circulations.

In order to assess the transfer of substances from the maternal to the foetal system, components of the inter-haemal barrier in individual species need to be taken into consideration as well as structures functioning at different gestational stages (Enders and Blankenship, 1999). Even though there might be differences in transit times due to the huge variety of mechanisms, Furukawa and co-workers stated in 2014 that the interspecies differences in the type of placenta do not play a dominant role in the placental transfer of most drugs.

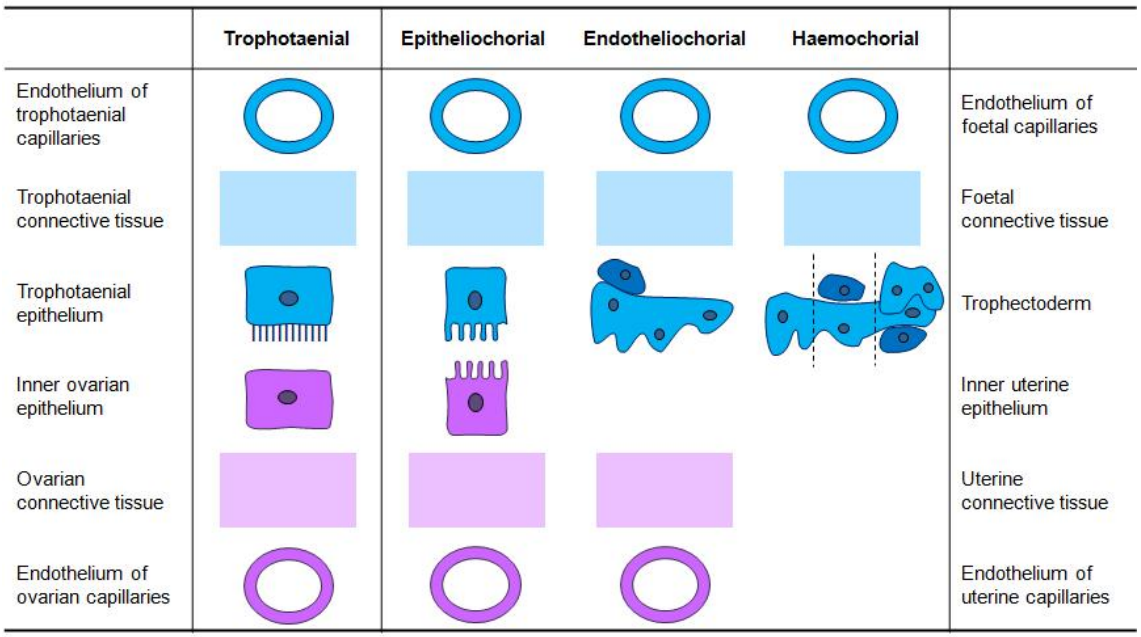
2.5 Viviparous Fish as Potential Models for Studies on Maternal Transfer of Pollutants

Early life stages tend to be amongst the most vulnerable to adverse effects of chemicals (Hutchinson et al. ,1998; Rasmussen et al., 2002; Brion et al., 2004; Hedman et al., 2011; Brande-Lavridsen et al., 2013), since concentrations of some pollutants are several orders of magnitude greater in embryos than in maternal tissue (de la Vega-Salazar at al., 1997; Webb et al., 2004). Regarding studies on maternal transfer of substances, the range of placental structures offer many possibilities of animal models. Thus far, however, models for studies on maternal transfer and effects of chemicals on developing early life stages have been focused on rats and rabbits (OECD, 2001). On the other hand, fish like medaka and zebrafish have become increasingly important in toxicology and pharmacology studies (Helfman et al., 2009). Viviparous fish species would therefore offer favourable models for maternal transfer studies in terms of their short generation times, high fecundity, larval anatomical features, ease of maintenance and cost-effectiveness. Principal biological processes affected by chemicals and the mechanisms of toxicants effects tend to be highly conserved across vertebrate species, further supporting fish models as an alternative for toxicological studies on maternal transfer (Ankley and Johnson, 2004). Finally, a gravid vivipaorus fish is able to carry a great number of embryos which all share the same environment and develop at the same rate.

In this regard, the live-bearing eelpout (*Zoarces viviparus*) has been used to study the maternal-embryonal trophic relationship in contaminant exposure (Rasmussen et al., 2002; Hedman et al., 2011; Brande-Lavridsen et al., 2013). Mature eelpout reproduce, however, once a year and gestation lasts four to five months (Rasmussen et al., 2002; Skov et al., 2010; Hedman et al., 2011; Brande-Lavridsen et al., 2013). Other viviparous fish, such as the goodeid species *Xenotoca eiseni,* may offer a new approach to examine maternal transfer of substances, since they have a much shorter gestation time of only a few weeks (Parenti, 2005; Skov et al., 2010).

In Tab 2.6, the trophotaenial placenta of *X. eiseni* is compared to mammalian placenta types.

Tab 2.6: Comparison of trophotaenial placenta type tissue layers to different mammalian placental systems. Connective tissues are shown for the sake of completion although they do not play a major role in the transfer of substances. The haemochorial type consists of three different subtypes what is illustrated with dashed lines in the trophectoderm layer.



The following chapters will focus on the investigation of the reproductive biology of *Xenotoca eiseni* with a view to its application as a model for maternal transfer studies of toxicants. Accordingly, studies on ontogeny and dynamics of the gonadal development as well as analyses on embryonal development including timing of sexual differentiation in *X. eiseni* will provide the underpinning for further examination on maternal transfer. Following this, exposure of pregnant females to 17α-ethinyloestradiol (EE2) will allow insight into maternal transfer of an environmental contaminant and its potential effects on developing embryos.

3 Materials and Methods

3.1 Chemicals

Benzocaine (ethyl-*p*-aminobenzoate), picric acid (1.3% in H_2O) and Paraplast® were purchased from Sigma Aldrich (Poole, UK). Formaldehyde (37-41%), ethanol (abs, analytical grade) and methylated industrial spirit (IMS 74 O.P.) were obtained from Fisher Chemicals (Loughborough, UK). Acetic acid glacial (~100%, AnalaR) was acquired from BHD Laboratory Supplies (Poole, UK). Histoclear II (alkyl hydrocarbon + orange terpenes) and Histomount (xylene, mixture of isomers) were purchased from National Diagnostics (Hessle Hull, UK). Harris hematoxylin non acidified (ethanol + aluminium ammonium sulphate) and eosin Y aqueous (0.5%) were acquired from Shandon (Cheshire, UK).

3.2 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 200 to 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. Fish were fed daily to satiation with a combination of gamma-irradiated brine shrimp (*Artemia salina*, Tropical Marine Center, Chorleywood, UK) and gamma-irradiated bloodworm (*Chironomidae*, Tropical Marine Center, Chorleywood, UK) and TetraMin flake food (Tetra GmbH, Melle, D). Young fish were fed with freshly hatched *Artemia* (cysts, from ZM Ltd., Hampshire, UK).

Adult fish were generally kept separated by gender and age. For breeding purposes, male and female fish of the same age (same size) were placed together (1:1 ratio) for three to ten days, then the sexes were separated again. In order to prevent filial cannibalism, gestating females close to parturition were transferred into small groups (two to three) to give birth. After parturition, adult female fish were placed back into stock tanks and broods of the same age were

held together until the expression of their secondary sexual characteristics allowed to separate them by gender.

3.3 Sampling Procedures

At incremental time periods (for details see following experimental chapters), fish were euthanized by terminal anaesthesia with benzocaine (50 mg/L, inhalative) followed by destruction of the brain. Protocols were carried out ethically in accordance with the UK Code of practice for the humane killing of animals under Schedule 1 of the Animals (Scientific Procedures) Act, 1986.

Fish were weighed and measured (total body length) in order to assess the condition factor ($K = 100 \times \text{weight (mg)} \times \text{length}^{-3}\ \text{(mm)}$). In experiments where no organs were removed, fish were fixed *in toto* in Bouin's solution for 1.5 to eight hours, depending on the size of the fish. A small abdominal incision in fish with a total body length of more than 3.0 cm was made to enable fixative penetration. After fixation, they were washed with and stored in 70% IMS at room temperature until further analysis.

3.4 Histology

After fixation in Bouin's solution, samples were stored in 70% IMS for at least a week before further processing. Fixed tissues were then dehydrated through a graded series of ethanol (see Tab 3.1) and embedded in tissue wax (Paraplast®) using a Shandon Citadel 2000 tissue processor. Tissues were cut into serial transverse or longitudinal sections at 3 to 5 µm, floated in a water bath, placed on glass slides and stained with haematoxylin and eosin (see Tab 3.2) using a Shandon Varistain 24-4 slide stainer. Finally, the slides were covered with a cover slip and examined by light microscopy.

Tab 3.1: Tissue dehydration steps, performed with a Shandon Citadel 2000 Tissue Processor

1	70% IMS	1.0 h	7	100% Ethanol	1.5 h
2	70% IMS	1.0 h	8	Histoclear	1.5 h
3	80% IMS	1.0 h	9	Histoclear	1.5 h
4	90% IMS	1.5 h	10	Histoclear	1.5 h
5	98% IMS	1.5 h	11	Tissue Wax	1.0 h
6	100% IMS	1.5 h	12	Tissue Wax	2.0 h

Tab 3.2: Tissue staining (H&E) using a Shandon Varistain 24-4 Slide Stainer

1	Histoclear	5 min
2	Histoclear	5 min
3	100% IMS	2 min
4	90% IMS	2 min
5	80% IMS	2 min
	Tap Water (running)	2 min
6	Harris Haematoxylin non-acidified	15 min
	Tap Water (running)	2 min
7	Acid Alcohol (0.5% HCl in 70% IMS)	5 sec
	Tap Water (running)	30 sec
8	70% IMS	30 sec
	Tap Water (running)	30 sec
9	Eosin Y Aqueous	15 sec
	Tap Water (running)	30 sec
10	80% IMS	30 sec
11	90% IMS	1 min
12	95% IMS	1 min
13	100% IMS	2 min
14	100% IMS	2 min
15	Histoclear	2 min
16	Histoclear	3 min

4 Ontogeny and Dynamics of the Gonadal Development in *Xenotoca eiseni*

4.1 Introduction

Xenotoca eiseni have a typical goodeid reproductive system (Mendoza, 1965), where females have a single hollow structured ovary and males in contrast have paired testes. Other viviparous features in the male include a modified anal fin and the production of spermatozeugmata – two vital adaptations for internal fertilisation (Bisazza, 1997; Grier et al., 2005). Anatomical characteristics of this special reproductive system have been described by numerous authors and they are detailed in chapter 2.4.2 (Reproduction) in this thesis. The literature review in chapter 2 describes viviparous reproduction in general with a subsequent focus on *X. eiseni*. Here the aim of this chapter was to develop detailed knowledge on the ontogeny of gonadal development of *X. eiseni*, which is essential for the potential application of this goodeid species as a model for studies on maternal transfer of environmental contaminants.

This chapter details the developmental structures and features of the gonads in the body of *X. eiseni* during development. Fish were studied from birth until six months old and examined at a series of different developmental time points. Histological processing was conducted to examine gonadal development and staging of gametes, and these were characterised based on cellular and morphological characteristics. Analysis of the size of fish and external secondary sex features was also undertaken to establish how growth dynamics relate to sex and sexual development and to determine when *X. eiseni* reach sexual maturity.

4.2 Materials and Methods

4.2.1 Fish

Ontogeny of gonad development was studied through sample collections from two separately maintained populations. Adult (3.5 to 4.5 months old) *Xenotoca eiseni* were derived from stocks at the University of Exeter (see chapter 3.2 Fish Maintenance). 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, were thereafter kept in glass aquaria (20 L) in groups of equal sizes and studied over a period of six months.

4.2.2 Sampling

Fish were checked daily for any visible signs of ill health and examined at incremental time periods during development (at 1 day, and 1, 2, 4, 8 and 12 weeks, and then 4, 5 and 6 months after birth) (see Tab 4.1). At each time point, all fish were measured for wet weight and total body length and for the period up to week eight, between 16 and 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 fish were selected to provide approximately 50% males and 50% females for histological processing.

4.2.3 Histology

The general methods for histological preparation are detailed in chapter 3.4 (Histology). Serial transverse 3 to 5 µm sections were cut through the entire length of the body of 16 fish for each sample collected. From the remaining fish prepared for histology, serial 3 to 5 µm lateral sections were taken. All whole body sections were examined for the presence of gonads to identify the sex and the stage of their sexual development and to determine the locational position of the gonads in the body.

Tab 4.1: Numbers of fish used at different sampling dates for different endpoints. A number of randomly sampled fish were sacrificed for gonadal histology at each sampling point. Body weight and total body length were measured for all remaining fish at the respective sampling dates. A total of four fish died during the 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.

	Weight and Length	**Histology**
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)

4.2.4 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, using GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

4.3 Results

4.3.1 Growth and Secondary Sexual Characteristics

At birth, *Xenotoca eiseni* embryos had a total body length of 13.5 ± 0.9 mm (mean ± SD) and a total body weight of 30.6 ± 7.1 mg. Their length and weight increased in a non-linear manner over time (see Fig 4.1). Up until week four, data for growth in male and female fish were not distinguishable as the sex could not be determined by external secondary sexual characteristics – this was possible only from week eight. At six months old, non-pregnant female fish were 43.0 ± 1.2 mm in length and weighed 1,800 ± 340 mg, whereas male fish were of an apparent smaller size, measuring 39.1 ± 1.7 mm and weighing 1,280 ± 200 mg. This apparent difference in size (length and weight) however, was not statistically significant. Obvious differences between male and female fish in their external appearances were clearly observed between four and eight weeks after birth (see Fig 4.2), denoted by the appearance of secondary sexual characteristics. The first external feature that distinguished males from females was a notched anal fin in males (see Fig 4.3), which first appeared between four and eight weeks of age. A few days to weeks later, the caudal fin and tail of male fish became orange in colour, which was the most obvious feature in their sexual dimorphism. More subtle differences were seen in the dorsal fin, which became duskier in colour in males. Eventually, the body shape and colouration were also clearly different between males and females from about two months of age. Males developed a hump behind their head that became more distinctive in older fish, and female fish had a dark blotch on their lower abdomen.

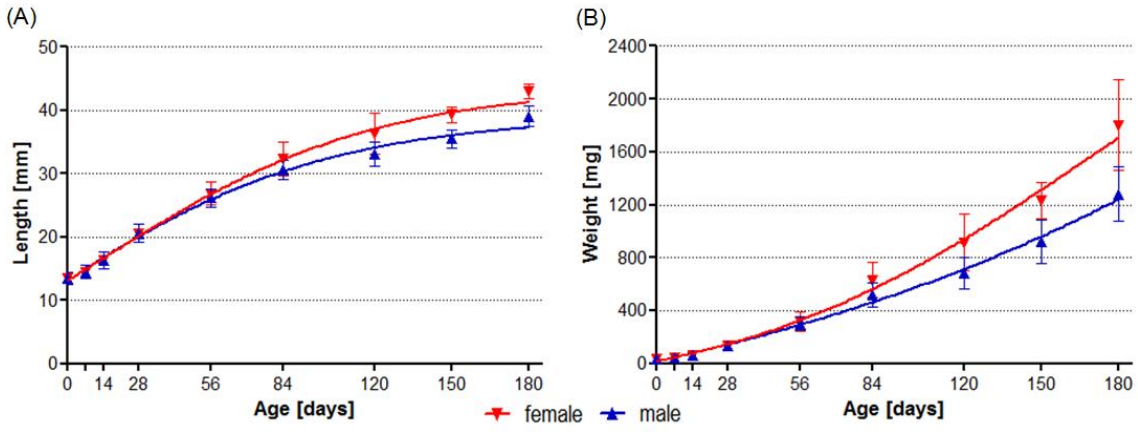


Fig 4.1: Growth data of *Xenotoca eiseni* from birth until the age of six months. Unpaired t-Tests revealed no significant differences between male and female growth. **A**: Total body length (P=0.6175). **B**: Body weight (pregnant fish excluded) (P=0.5297).

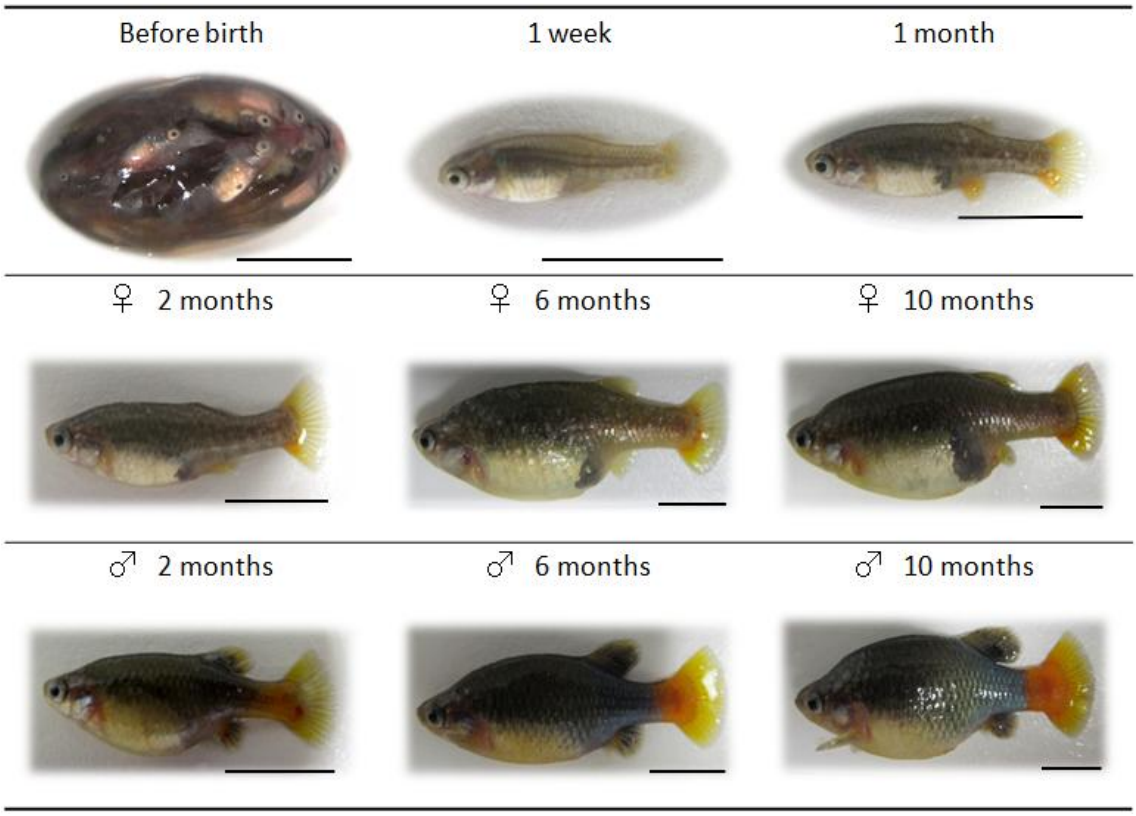


Fig 4.2: *Xenotoca eiseni* at different ages. After about a month the fish start to develop their secondary sex characteristics. Bars = 1 cm

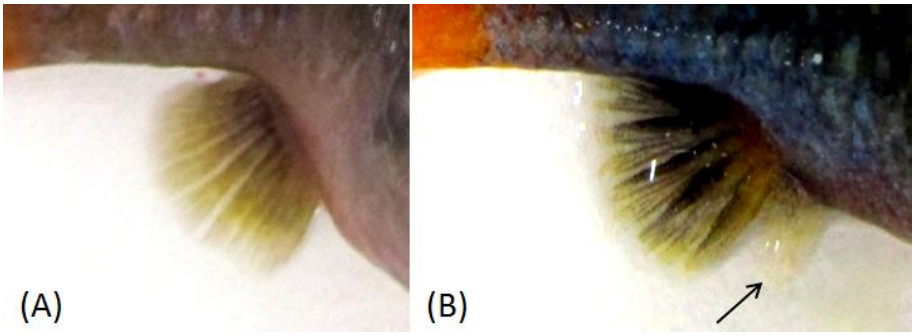


Fig 4.3: Anal fin of adult *Xenotoca eiseni*. **A**: Female anal fin. **B**: Male anal fin with notch and andropodium (arrow) at the anterior end.

4.3.2 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 was found to start at the anterior end at a position aligning with the start of the swim bladder, and extend through the length of the abdomen, ending at the genital pore (see Fig 4.4 and Fig 4.5). During the body sectioning process, the testis in adult males was found to extend from a position approximately halfway down the length of the swim bladder to the genital opening.

At birth, the gonads of *Xenotoca eiseni* were fully differentiated (Fig 4.5), although it was often not possible to histologically distinguish between the sexes at this time since the structures of ovary and testis were very similar in appearance (i.e. before the onset of gametogenesis). At two weeks, the structure of the gonads became more distinct (Fig 4.6). The female ovary was a single lobed structure, separated into two compartments by a highly folded median septum. Oogenesis was first observed between two and four weeks after birth and females reached full sexual maturity at approximately twelve weeks, when their total body length was at least 3 cm. Male fish had a two lobed testis joined at the anterior end and spermatogenesis started around four weeks after birth. Sperm bundles were first seen at between four and eight weeks.

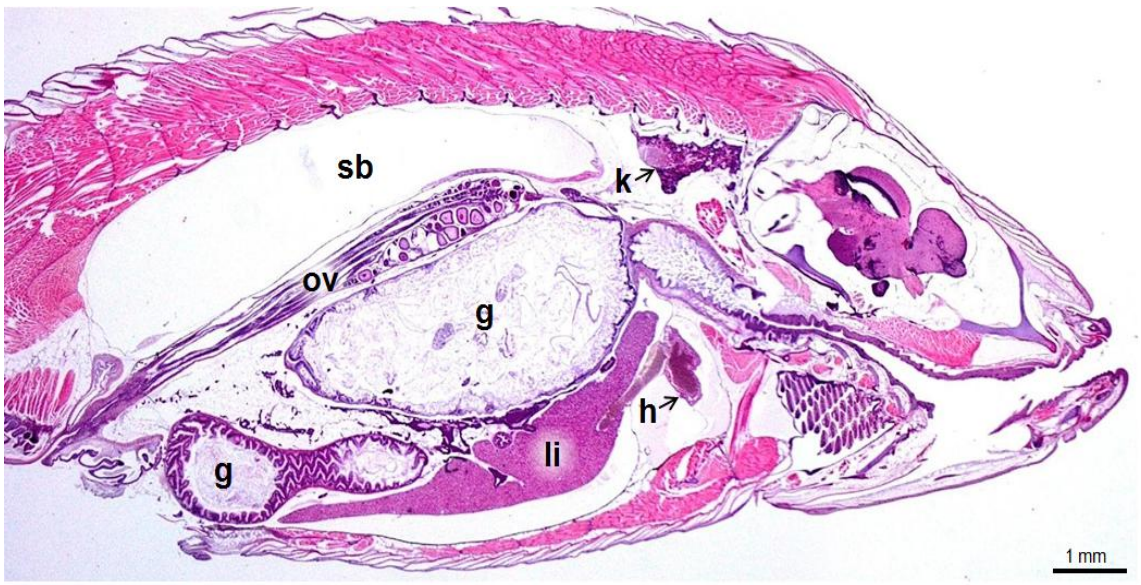


Fig 4.4: Lateral section of an adult (12 week old) female *Xenotoca eiseni* showing the position of the gonad.
Key: g: gut, h: heart, k: kidney, li: liver, ov: ovary (with oocytes at different stages), sb swim bladder.

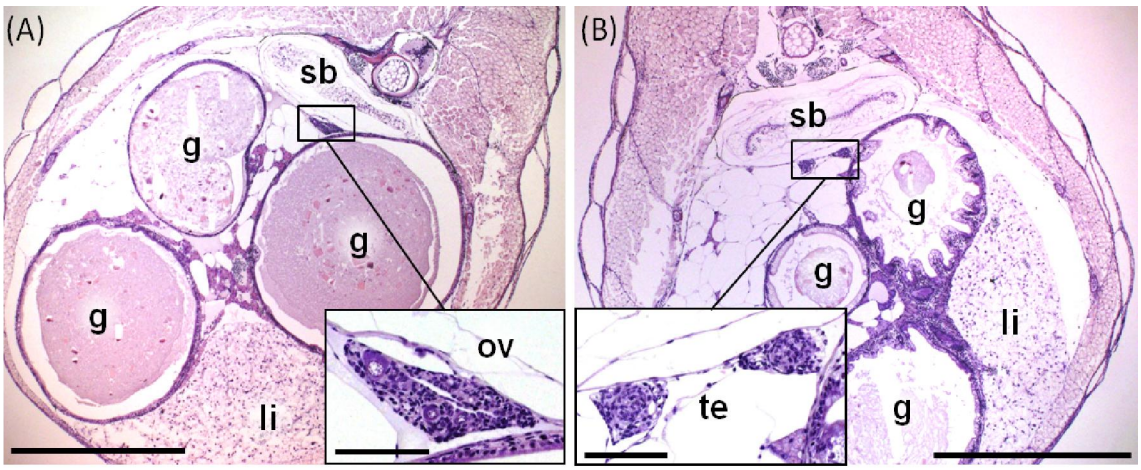
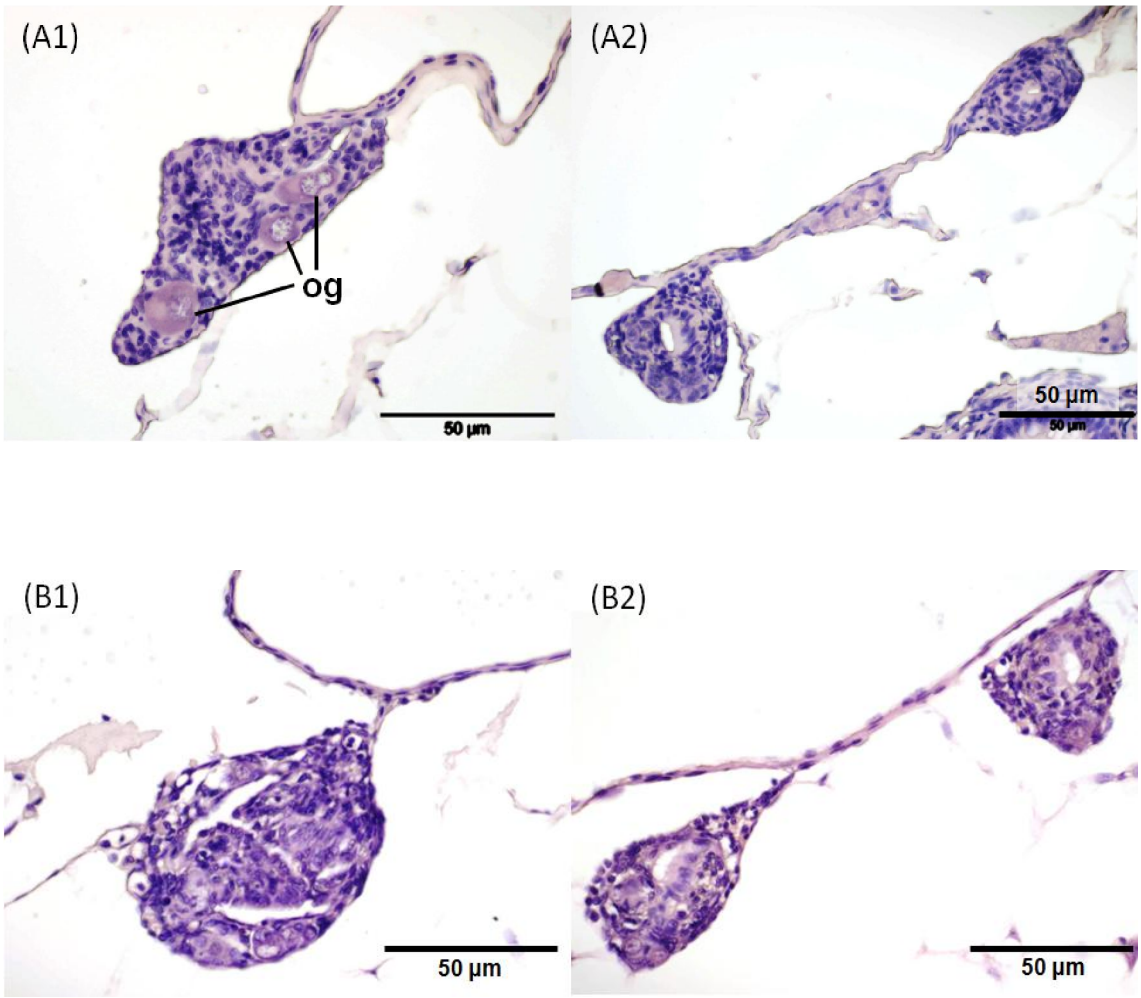
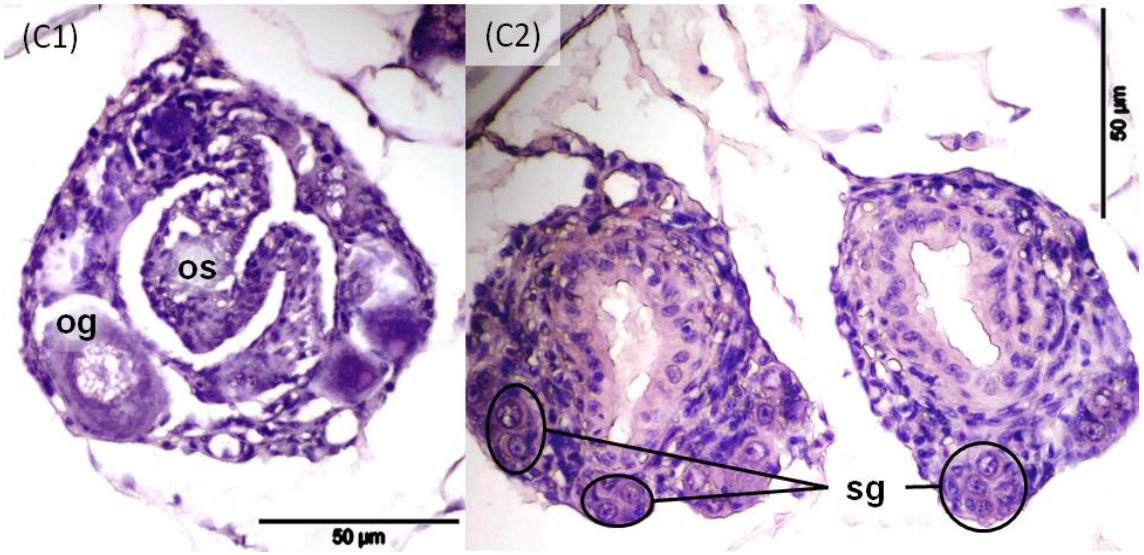
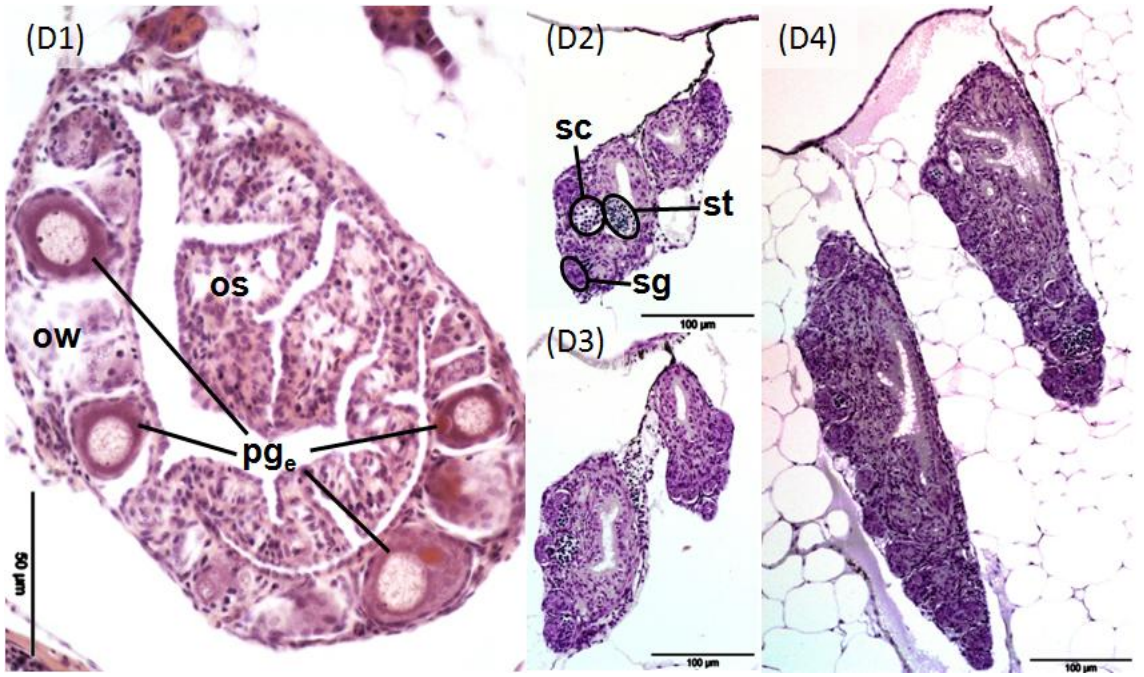


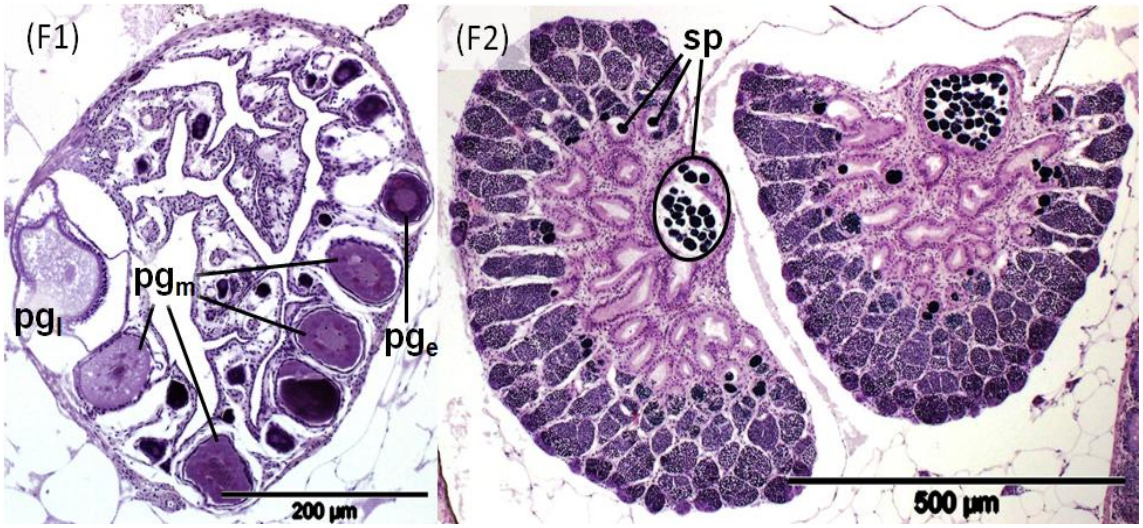
Fig 4.5: Transverse whole body sections of *Xenotoca eiseni* immediately after birth showing the position of the gonads and a more detailed view of these gonads (inset). **A**: Section of a female fish. **B**: Section of a male fish. **Key**: g: gut, li: liver, ov: ovary, sb swim bladder (not yet inflated), te: testis. Bars = 500 µm and 50 µm (insets).

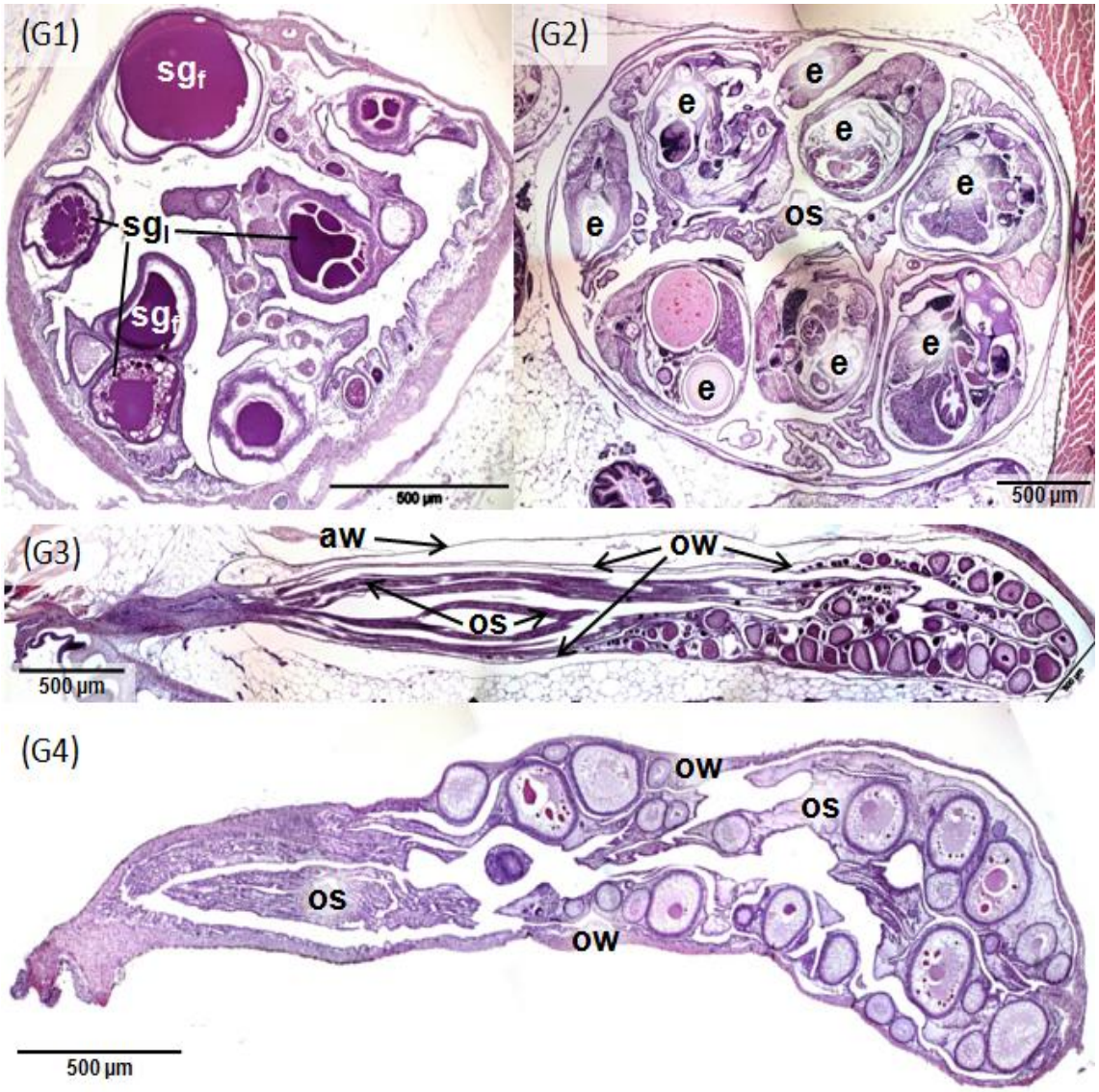



(C1)
os
og
50 µm
(C2)
50 µm
sg


(D1)
os
ow
pge
50 µm
(D2)
sc
st
sg
100 µm
(D3)
100 µm
(D4)
100 µm


(E1)
pge
pgm
100 µm
(E2)
sc
sp
sg
st
100 µm


(F1)
pg$_l$
pg$_m$
pg$_e$
200 µm
(F2)
sp
500 µm


(G1)
sg$_f$
sg$_l$
sg$_f$
500 µm
(G2)
e
os
500 µm
(G3)
aw
ow
os
500 µm
(G4)
ow
os
os
ow
500 µm

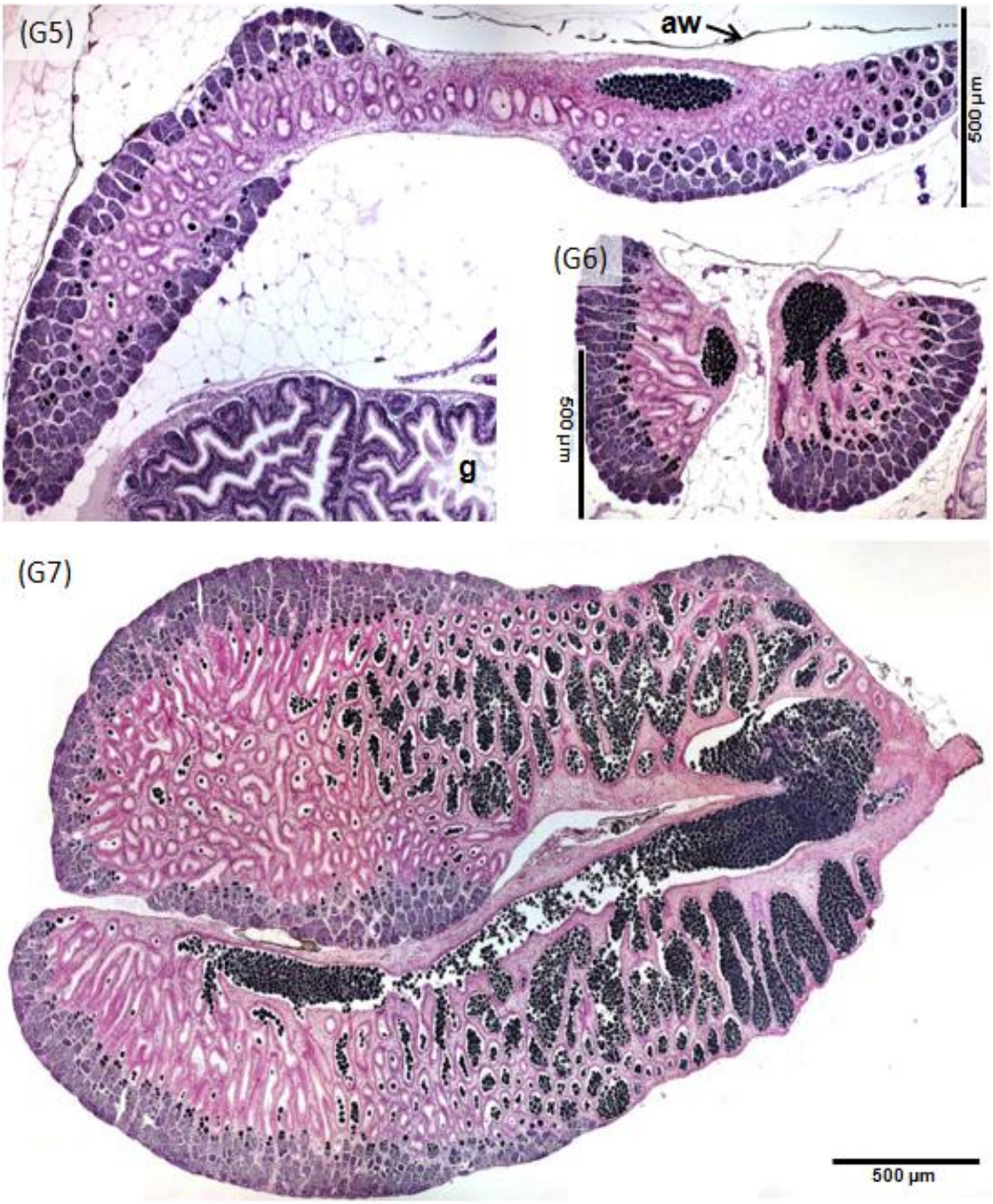


Fig 4.6: Sections of *Xenotoca eiseni* at different ages, focused on the gonads. **Female** fish: A1, B1, C1, D1, E1, F1, G1-4. **Male** fish: A2, B2, C2, D2-4, E2, F2, G5-7. **A1-2**: Transverse sections of one day old fish. **B1-2**: Transverse sections of one week old fish. **C1-2**: Transverse sections of two week old fish. **D1-4**: Transverse sections of four week old fish. **E1-2**: Transverse sections of eight week old fish. **F1-2**: Transverse sections of twelve week old fish. **G1**: Transverse section of an adult female fish. **G2**: Transverse section of a pregnant fish. **G3**: Lateral section of an adult female fish. **G4**: Lateral section of a mature ovary with oocytes at different stages. **G5**: Lateral section of an adult male fish. **G6**: Transverse section of an adult male fish. **G7**: Longitudinal section of mature testis. **Key**: aw: abdominal wall, e: embryo, g: gut, og: oogonium, 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, sgf: fully grown oocyte, sgl: late secondary growth oocyte, sp: sperm package, st: spermatid.

4.3.3 Ovary

The single lobed ovary of a mature non-gravid female was between 7 and 16 mm in length and between 0.5 and 2 mm in diameter, depending on the size of the female. The lumen was divided into two more or less equal lateral halves by a highly folded longitudinal septum. A three dimensional illustration of an ovary (without showing the oocytes) is given in Fig 4.7. 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, which opened to the exterior at the genital pore (see also Fig 4.6 (G3)).

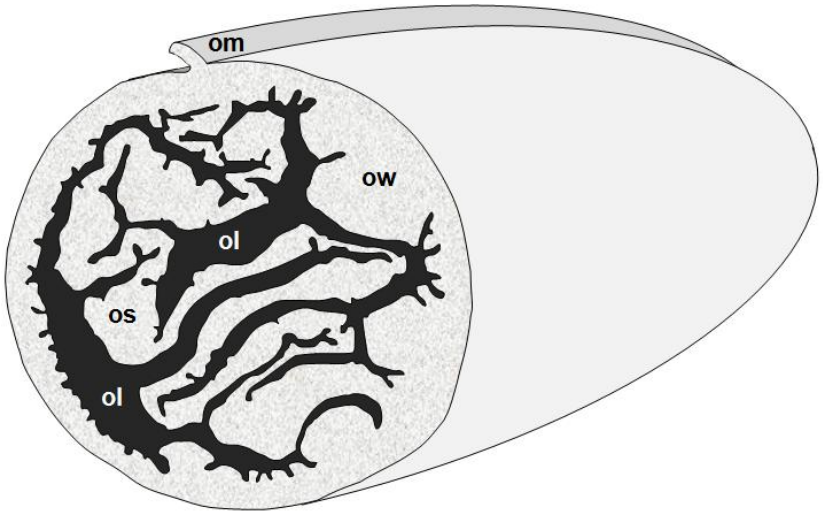


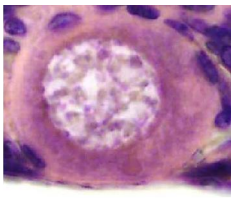
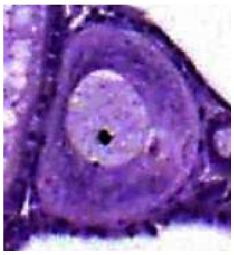
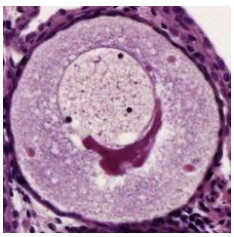
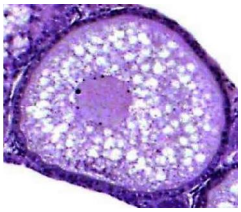
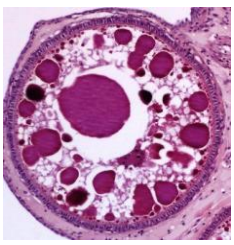
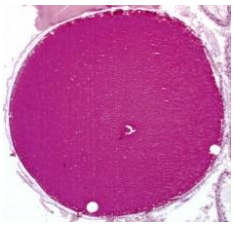
Fig 4.7: 3D scheme of a mature (non-gravid) ovary; oocytes not shown. **Key**: ol: ovarian lumen, om: ovarian mesentery, os: ovarian septum, ow: ovarian wall.

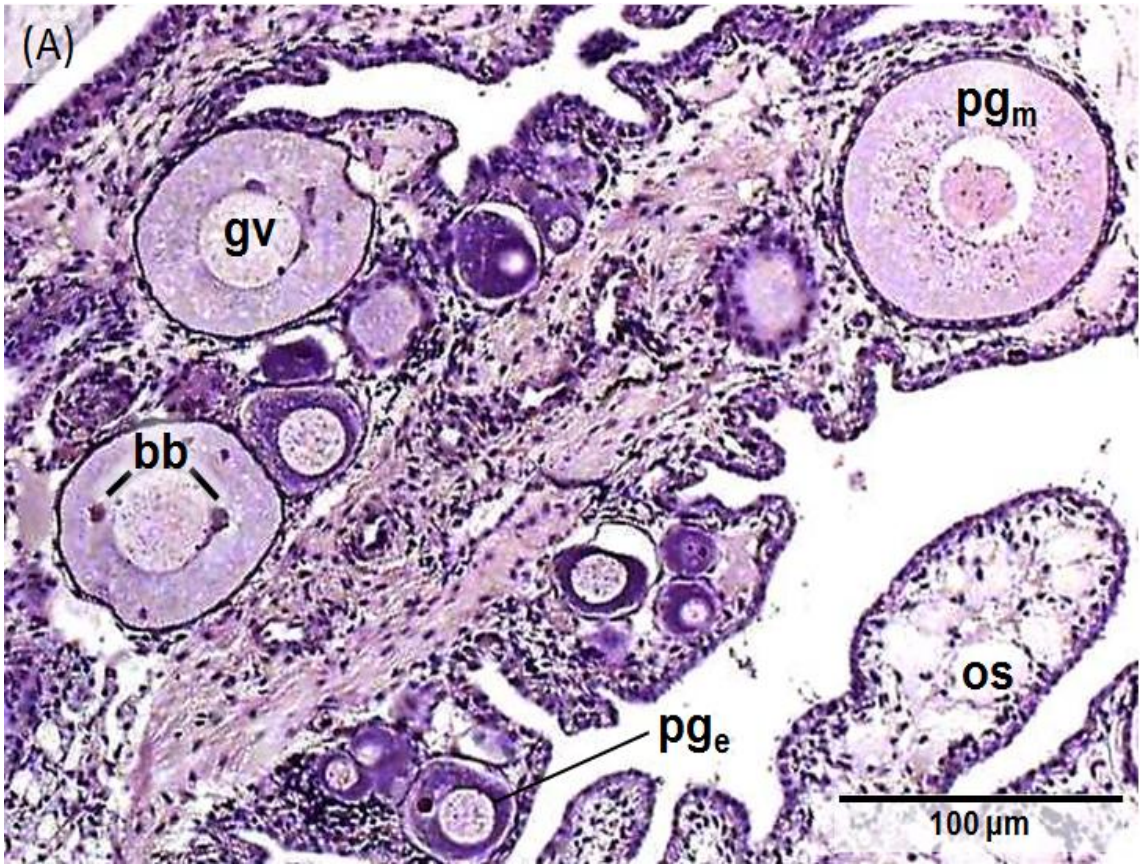
Oogenesis was a continuous process as was the maturation of the oocytes. Oocyte stages were determined according to Uribe and Grier (2011) and Uribe et al. (2012) and are listed in Tab 4.2. Primary growth comprised stages from the onset of oogenesis to oocytes containing oil droplets. In the first previtellogenetic stage, one or multiple nucleoli were visible. Oocyte diameters ranged from 23 to 58 µm with an average of 35 µm. Mid primary growth oocytes were characterised by the presence of balbiani bodies and their size ranged from 45 to 116 µm with an average diameter of 75 µm. There was an overlap of oocyte sizes for all consecutive stages, and staging was characterised based on the presence of specific cellular and morphological characteristics. The last period in the primary

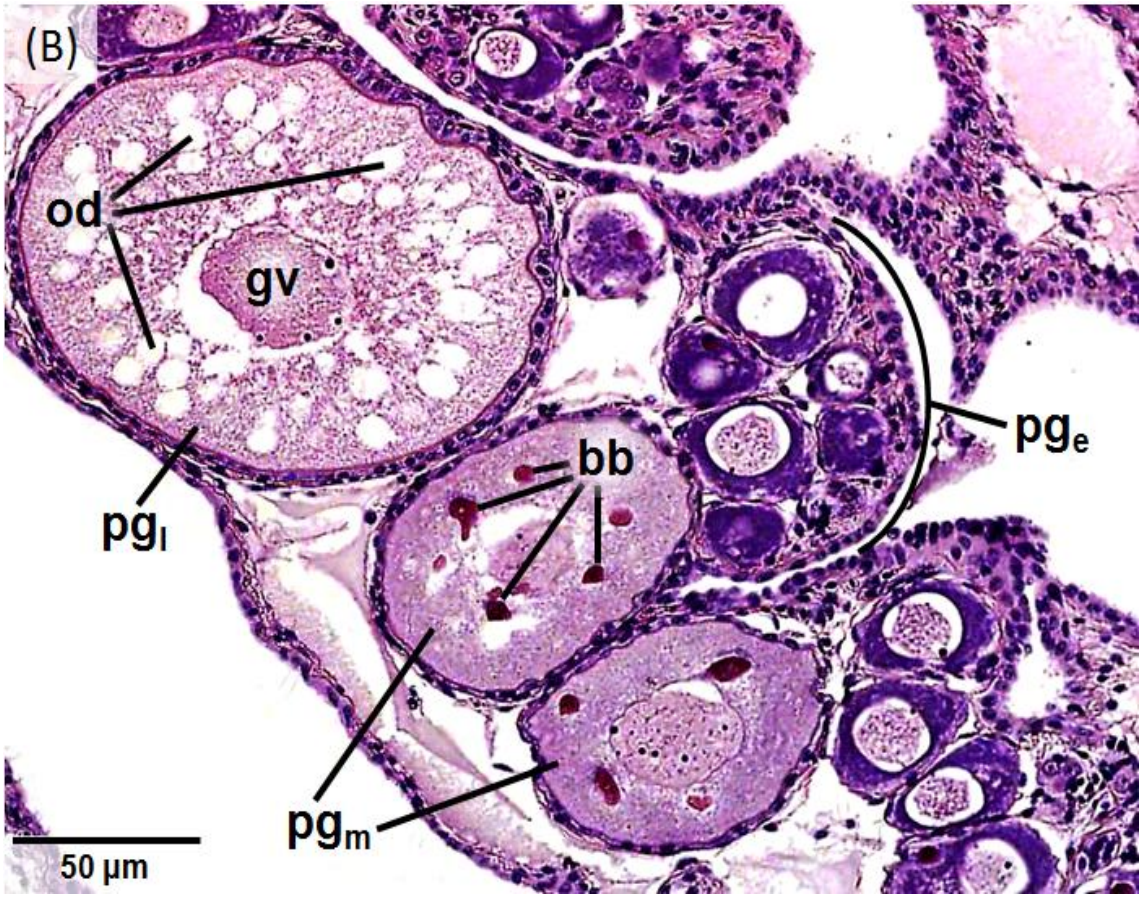
growth stage was characterised by the occurrence of large oil droplets. Oocytes at this stage had an average diameter of 150 µm with a range between 105 and 189 µm. Secondary growth was characterised by vitellogenesis and the generation (and thus inclusion) of yolk. During this process, the germinal vesicle migrated to the periphery of the oocyte and its surrounding somatic tissue including the follicle cells became clearly visible (see Fig 4.8). With the appearance of the first yolk globules, oocytes entered the early secondary growth stage and reached 165 to 232 µm in size (average 200 µm). During the late vitellogenic stage, yolk droplets fused 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. Oocytes at the late secondary growth stage measured between 206 and 359 µm (average 260 µm) in diameter. Fully grown oocytes had an average diameter of 380 µm, with a range between 234 and 506 µm. At this stage, female fish were ready to mate. When oocytes had not been fertilised, they were ovulated into the ovarian lumen where they underwent resorption.

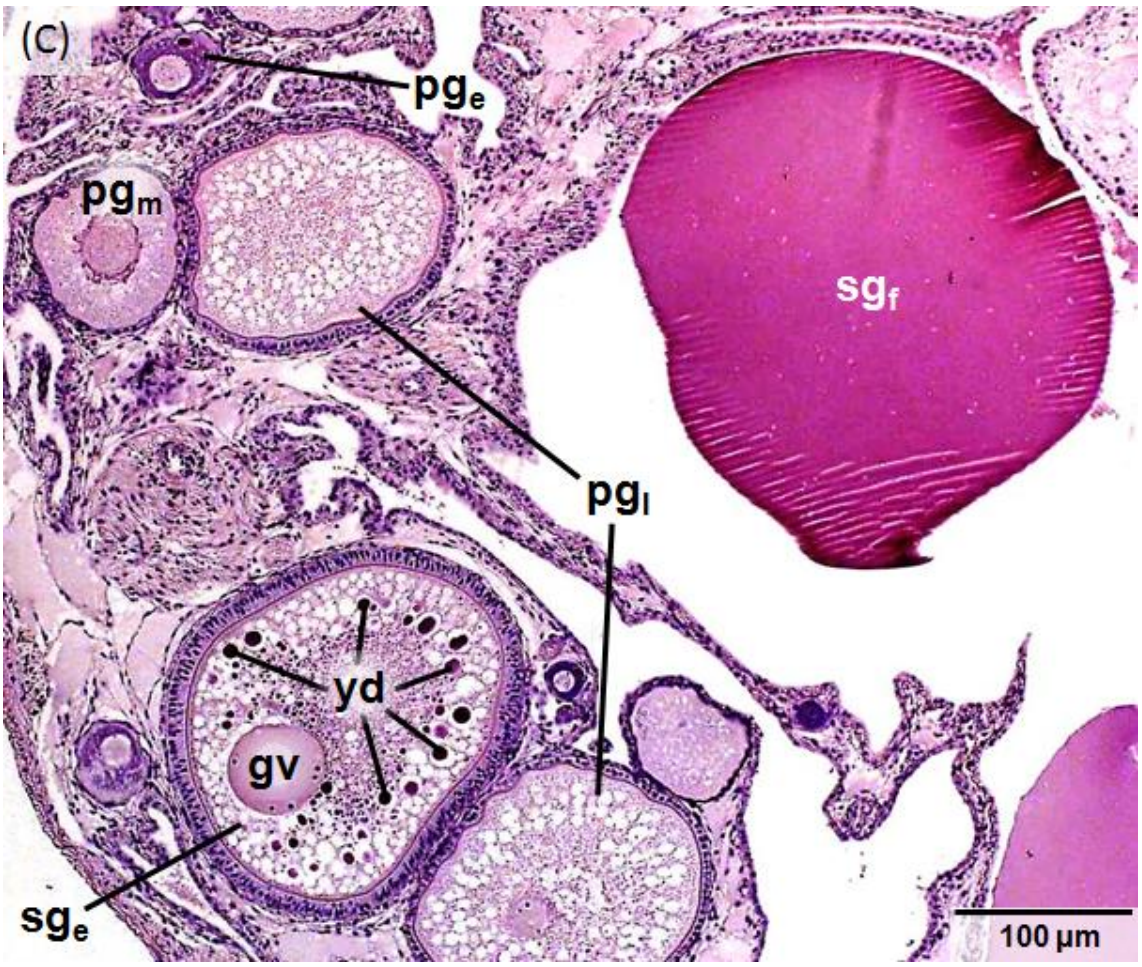
Analysis of oocytes in gravid and non-gravid ovaries identified high numbers of oocytes in stages 1 and 2, the former usually present in clusters (Fig 4.8). Exact numbers were not determined, as the sectional spacing of 60 µm was larger than the sizes of the oocytes at stages 0, 1 and 2. However, taking the probability of capturing an oocyte in a section into account ($P = 1 - (s-d)/s$, with s = sectional spacing and d = oocyte diameter) allowed for an estimation of the oocyte numbers ($x = n/P$, with n = number of oocytes counted and P = probability as above). These calculations gave numbers between 176 and 342 with an average of 243 for stage 1 oocytes and between 53 and 190 (average 87) for stage 2 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 around 5 to 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. Sections of ovaries containing oocytes at different developmental stages are illustrated in Fig 4.8.

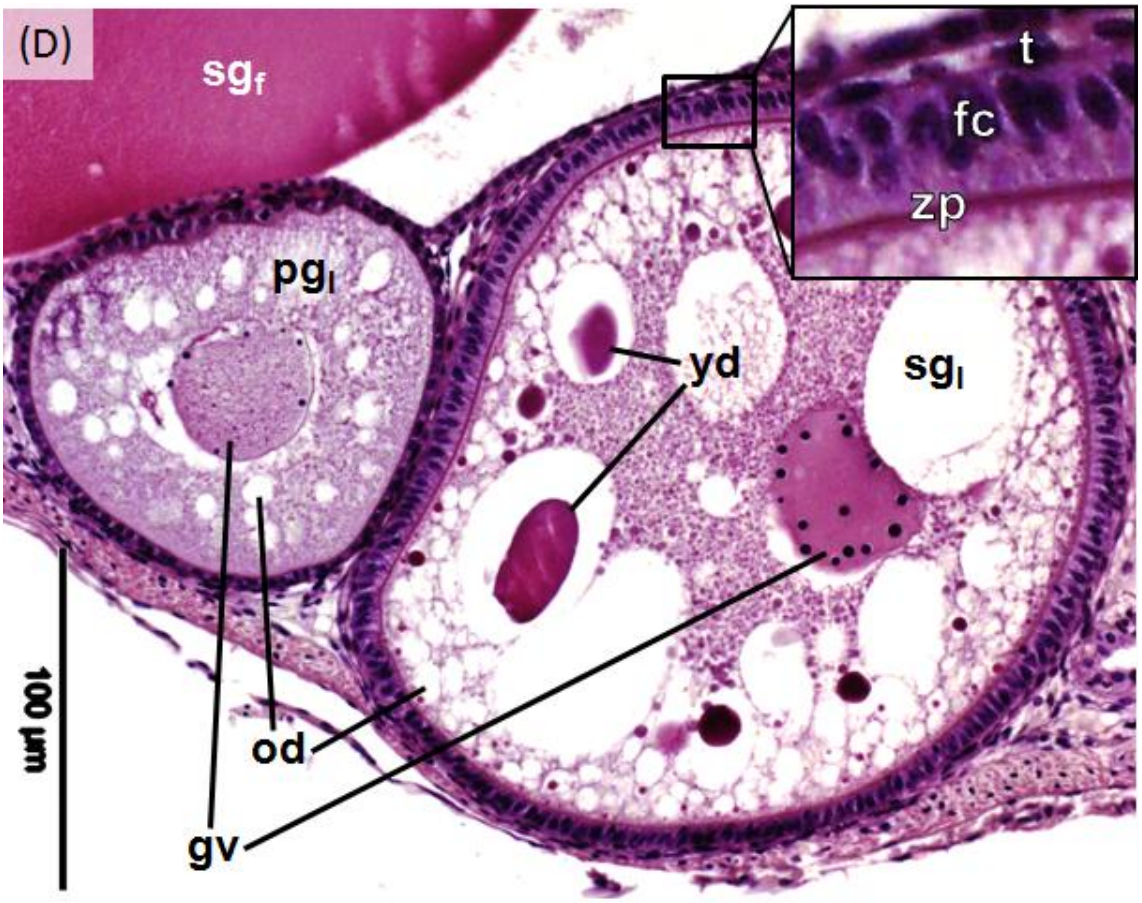
Tab 4.2: Oocyte stages in *Xenotoca eiseni*, modified from Uribe and Grier (2011) and Uribe et al. (2012).

	Stage		Characteristics	Picture	Average Oocyte Diameter
Oogonia	**0**		*Onset of Meiosis*		**≤25 µm**
Primary Growth = Previtellogenesis	**1**	Early Primary Growth	*One or Multiple Nucleoli*		**35 µm**
	2	Mid Primary Growth	*Multiple Nucleoli and Balbiani Bodies*		**75 µm**
	3	Late Primary Growth	*Oil Droplets and Cortical Alveoli*		**150 µm**
Secondary Growth = Vitellogenesis	**4**	Early Secondary Growth	*Small Yolk Droplets*		**200 µm**
	5	Late Secondary Growth	*Large Yolk Droplets*		**260 µm**
	6	Fully Grown Oocyte	*Filled with Yolk*		**380 µm**


(A)
gv
pgm
bb
os
pge
100 µm


(B)
od
gv
pge
bb
pgl
pgm
50 µm


(C)
pge
pgm
sgf
pgl
yd
gv
sge
100 µm


(D)
sgf
t
fc
zp
pgl
yd
sgl
od
gv
100 µm

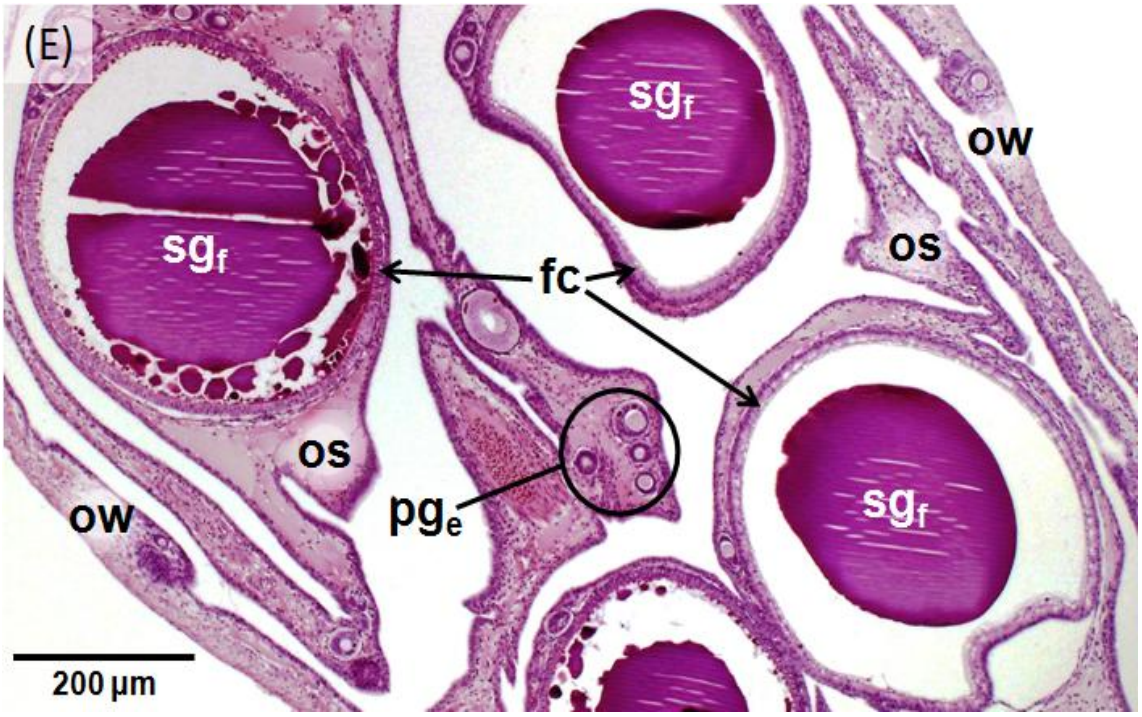
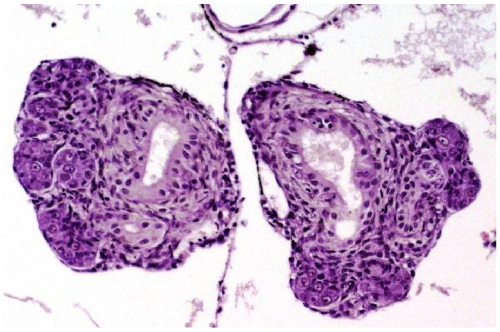
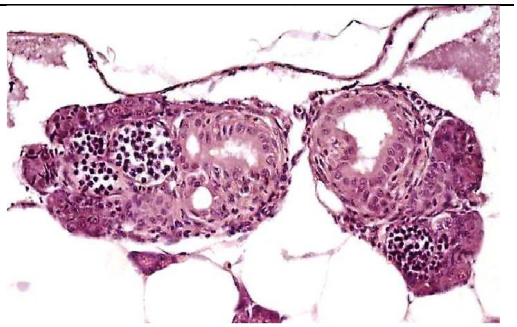
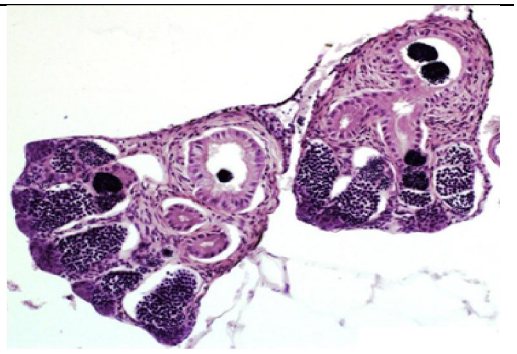


Fig 4.8: Histological sections of *Xenotoca eiseni* ovaries showing oocytes at different stages. **A**: Oocytes at early and mid-stages of primary growth. **B**: Oocytes at early, mid and late stages of primary growth. **C**: Oocytes at primary and secondary growth stages. **D**: Oocytes at primary and secondary growth stages with a magnification of the surrounding somatic tissue. **E**: Fully grown oocytes. Shrinkage of the yolk globule and therefore separation from the follicular cells has occurred most likely as a result of the histological fixation process. **Key**: bb: balbiani body, fc: follicle 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 oocyte, sgl: late secondary growth oocyte, t: theca cells, yd: yolk droplet, zp: zona pellucida.

4.3.4 Testis

Male *Xenotoca eiseni* had paired testis with a right and a left lobe joined together at the anterior end (see Fig 4.6 (G7)). The gonad of adult males was 3.6 to 8.6 mm long and the diameter of one of the lobes was 0.5 to 2.8 mm at its widest section. Shortly after birth, testicular tubes that ended blindly at the periphery of the gonad began to appear and four weeks later, spermatogenesis was initiated. Stage 0 was used to describe the testis with spermatogonia only. The onset of spermatogenesis defined the next stage of development (stage 1; Tab 4.3). The last stage of spermatogenesis was defined by the presence of sperm packages. These bundles are so called spermatozeugmata (see chapter 2.2.2 Internal Fertilisation) and are associations of thousands of sperm cells. In this form, sperm are stored in the testicular tube system.

Tab 4.3: Stages of spermatogenesis in *Xenotoca eiseni*.

	Stage	Description	Picture
No Spermatogenesis	**0**	*Spermatogonia*	
Start of Spermatogenesis	**1**	*Spermatocytes and Spermatids*	
Formation of Sperm Packages	**2**	*Sperm Packages*	

A transverse section of testis of an adult male (Fig 4.9) in high magnification revealed the process of spermatogenesis. Nests of spermatogonia at the periphery of the gonad synchronously underwent spermatogenesis and with progressing development migrated towards the centre of the gonad. During this process, spermatocytes, spermatids and sperm were present as clusters at the same developmental stage. These clusters were encapsulated by Sertoli cells, forming spermatocysts. The final step of spermatogenesis was the formation of sperm packages within these cysts and the release of these sperm bundles into the efferent duct system. Spermatozeugmata was stored in these tube systems that merged to form bilateral ducts. The bilateral ducts were finally joined to form a single sperm duct (Fig 4.10) that lead to the urogenital pore just anterior to the anal fin.

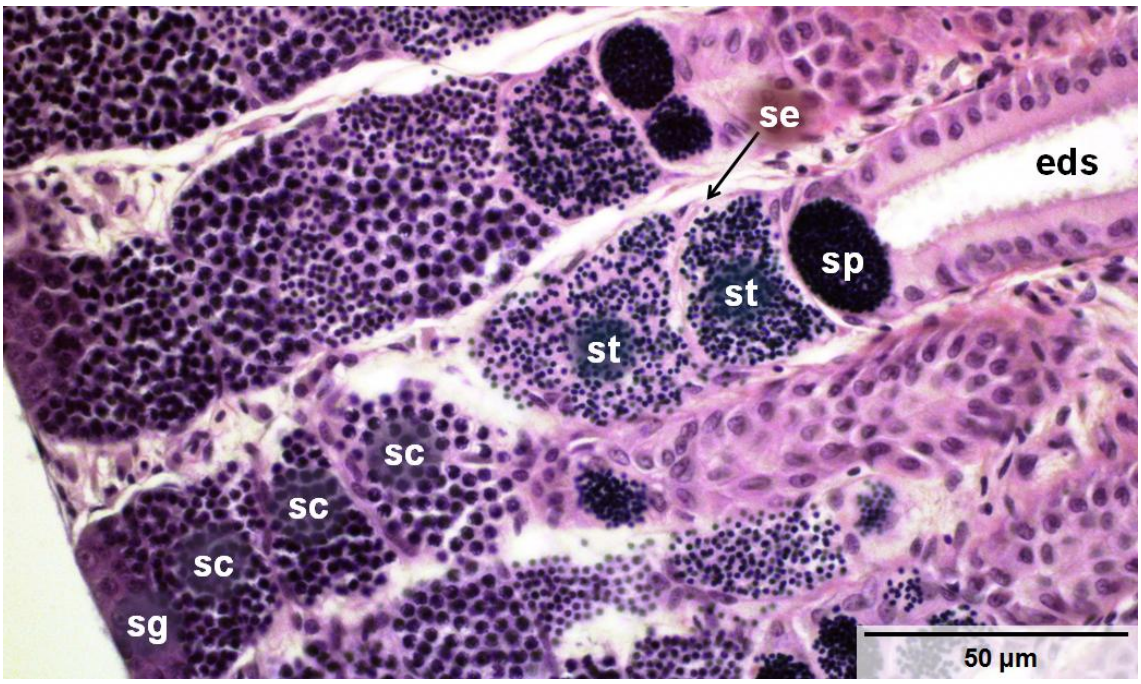


Fig 4.9: Transverse section of *Xenotoca eiseni* testis showing the process of spermatogenesis. **Key**: eds: efferent duct system, se: Sertoli cells, sc: spermatocyte, sg: spermatogonium, sp: sperm package, st: spermatid.

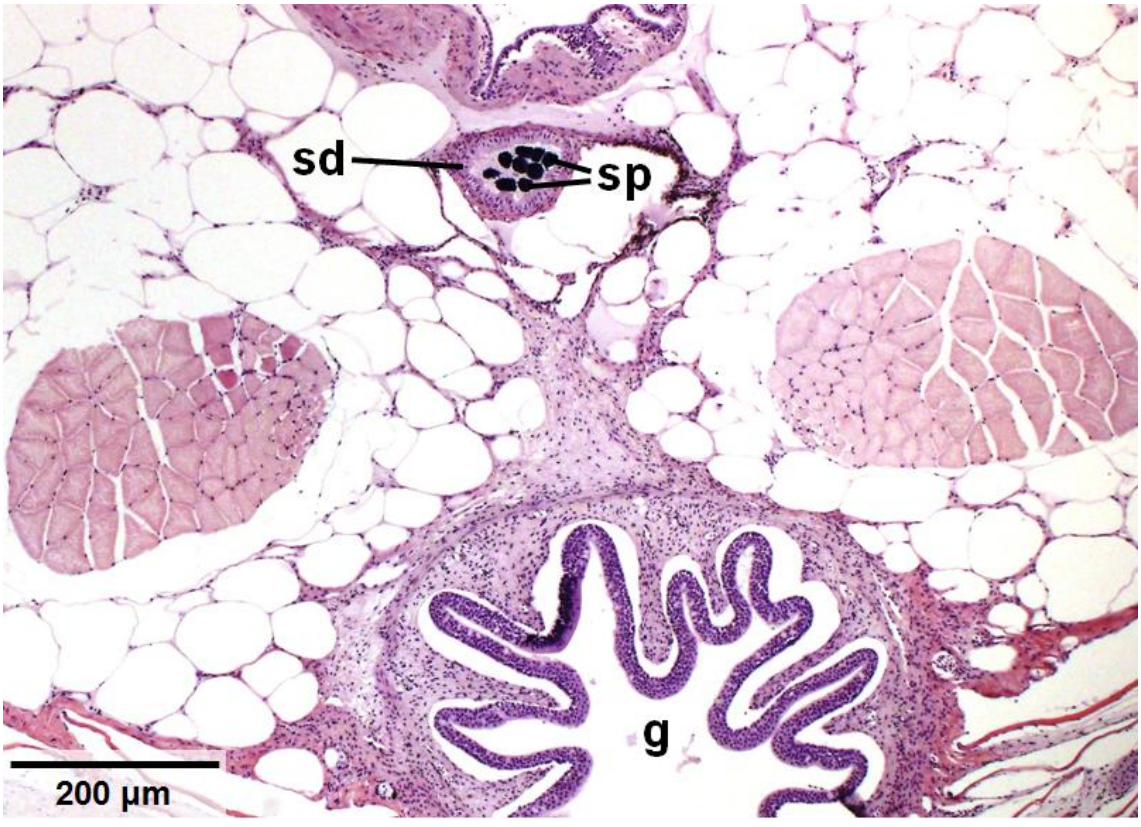


Fig 4.10: Transverse section of a male *Xenotoca eiseni* just anterior to the anus. **Key**: g: gut, sd: sperm duct, sp: sperm package.

4.4 Discussion

Gonads in *Xenotoca eiseni* are fully differentiated at birth, as occurs in other members of Goodeidae (Turner, 1933). Also typical for goodeids were the structures of both spindle shaped hollow single ovaries (Mendoza, 1965) and paired testes (Grier, et al., 1978; Greven, 2013). Male gonads are merged at the anterior end for about a quarter of their length, before they separate into two lobes. These fused anterior ends look similar to ovaries before the onset of gametogenesis, and this is why it is often not possible to determine gonadal sex histologically at birth.

Spermatogenesis starts approximately four weeks after birth. At this time, synchronous sperm development occurs in cysts and at the periphery of these cysts there are single layers of Sertoli cells, a distinctive feature for goodeid teleosts (Grier et al., 1978). Testes of *X. eiseni* comprise of a main duct with branched ramifications, again characteristic for goodeids (Nelson, 2006) and similar also in their morphology to poeciliid testicles (Grier et al., 1978). While the formation of spermatozeugmata is typical for both goodeids (Grier et al., 2005) and poeciliids (Nelson, 2006), the un-encapsulated sperm bundles differ between the two families (Grier et al, 1978) by the way in which sperm are orientated – in Goodeidae, flagella are located at the periphery of the sperm packages, whereas they are directed towards the centre in poeciliid sperm bundles (Nelson, 2006). Spermatozeugmata also occur in the perciform family of Embiotocidae, whereas species of the two ophidiiform families of Aphyonidae and Bythitidae form spermatophores (encapsulated sperm packages) (Nelson, 2006). The notched anal fin that functions as an andropodium in *X. eiseni* is typical and unique for 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 fertilisation (see “Male copulatory organ” in Tab 2.2 of chapter 2.3.3 Phylogeny of Viviparous Osteichthyans) (Nelson, 2006).

Female *X. eiseni* possess a hollow single median ovary, as is common not only for Goodeidae (Turner, 1933; Wourms, 1981; Wourms and Lombardi, 1992; Greven, 2013) but also for Poeciliidae, Embiotocidae and Zoarcidae (Nelson, 2006; Wootton and Smith, 2014). However, unlike poeciliid and embiotocid fish,

goodeids do not store sperm. Females of other viviparous teleost families normally have different ovarian structures, namely a paired gonad (see “Female gonad” Tab 2.2 of chapter 2.3.3 Phylogeny of Viviparous Osteichthyans) (Nelson, 2006). Regarding the average size of an ovary in a mature non-gravid female *X. eiseni*, Mendoza (1965) observed the same approximate range as was found in this study.

Oogenesis is first observed between two and four weeks after birth and female reach full sexual maturity when their total body length is at least 3 cm, at an age of around twelve weeks. Generally, fish show a wide span in their age for reproduction, ranging from sexual maturity at birth in some embiotocid fish (Helfman et al., 2009) to several years in zoarcid species (Hedman et al., 2011). Oocytes in mature ovaries comprise of different stages with earlier stages normally occurring in higher numbers. The average numbers of oocytes at the different stages of ovary development in *X. eiseni* are given in Tab 4.4. Most of the oocytes that are lost during their growth process are resorbed at a very early stage, just after the start of oogenesis. Once an oocyte has passed through previtellogenesis, the likelihood that it will reach full maturity is somewhere between 18 and 77%. Data on brood sizes (1-44, see chapters 5 and 6) imply that oocytes that reach the vitellogenic stages do not normally undergo degradation. Opposed to this, Turner (1933) stated that roughly half of the original number of embryos were normally absorbed during gestation.

Tab 4.4: Number of oocytes at different stages in *Xenotoca eiseni* as well as their relative incidence compared to the number of oocytes in the earliest stage and their preceding stage, respectively.

Oocyte Stage	Number	% of Previous Stage	% of Stage 1
Stage 1	243	100%	100%
Stage 2	87	36%	36%
Stage 3	57	66%	23%
Vitellogenic stages	5-44*	18-77%	2-18%

* highest number of embryos observed in another part of the study (see chapter 6)

In cases where oocytes are not fertilised, they undergo atresia. Atresia, the process of oocyte degeneration, occurs in many vertebrates and at different stages of oogenesis (Tyler and Sumpter, 1996, Uribe et al., 2006). While goodeid females have different average numbers of offspring, Uribe and colleagues (2006) observed atretic follicles in both pregnant and non-pregnant species and found that all of the non-fertilised mature oocytes underwent degradation. The fact that most of the fully grown oocytes in this study here were already in an atretic state suggests that mature oocytes only exist for a short period of time before they undergo degradation, unless fertilised. The time between the onset of oogenesis, which was first seen in two to four week old fish and a twelve weeks old sexually mature female fish implies that it takes about two months for an oocyte to go through the full process of oogenesis.

The staging of oocytes in the present study was modified from Uribe and Grier (2011) and Uribe et al. (2012). The former publication focuses on a viviparous poeciliid fish that forms the smallest oocytes amongst its family. Fully grown oocytes of this species have a size of 390 to 410 µm, a size that is just a little bigger than the average diameter of 380 µm attained by mature oocytes of *X. eiseni*. An obvious difference between the oocytes of these two species on the other hand, is the yolk content. The poeciliid fish studied by Uribe and Grier (2011) produces microlecithal oocytes, characterised by the little yolk globules at the periphery of the oocyte, which is mainly occupied by a large oil globule. In contrast, *X. eiseni* generate much more yolk, which indicates that embryos of *X. eiseni* have a larger yolk supply and therefore may not depend on maternal provisioning in early gestation. Oocytes filled with yolk are actually rather typical for egg-laying species. Accordingly, Uribe and colleagues (2012) found that mature eggs of two oviparous goodeid species were filled with a large yolk globule. As occurs in oocytes of *X. eiseni*, oil droplets were only found at the periphery of the ooplasm. The oocytes of the oviparous sister species of *X. eiseni*, however, were more than twice the diameter of oocytes of *X. eiseni*, corresponding to an eight-fold difference in volume.

The process of gestation and how long embryos of *X. eiseni* rely on their yolk supply before they are provided by maternally derived nutrients is addressed in the subsequent chapter of this thesis.

5 Embryogenesis and Gestation in *Xenotoca eiseni*

5.1 Introduction

Not much is known about fertilisation in goodeids. For example oocytes are believed to be fertilised within the follicle, but how sperm penetrate oocytes (as there is no acrosome) or how long spermatozeugmata are viable within an ovary have not been determined (Greven, 2013). Many assumptions made in the first half of the previous century on reproduction have been accepted without further investigation (Greven, 2013). Thus, goodeid reproduction and, most notably, embryogenesis and gestation in *Xenotoca eiseni* needs to be better understood to better assess their potential utility as a model organism for studies in ecotoxicology.

Viviparous teleosts have placental structures that vary between species (as discussed in chapter 2 and summarised in Tab 2.2). Two of the 13 viviparous fish families exhibit intrafollicular gestation (Nelson, 2006) of which one family is lecithotrophic (Rosenblatt and Taylor, 1971) and the other is matrotrophic (Fishelson and Gon, 2009). Embryos of six other families are retained in follicles for a prolonged period before they are released into the lumen of the ovary (Hoar, 1969; Meisner and Burns, 1997; Nelson, 2006). In the remaining five families – one of which belongs to the Goodeidae – gestation takes place mainly within the ovarian lumen (Wourms et al., 1988; Nelson, 2006), in which nutrients can be derived strictly by yolk supply or by means of maternal provisioning (Chernyayev, 1974; Meisner and Burns, 1997; Love et al., 2002; Nelson, 2006). Specialised uptake of nutrients in matrotroph species may involve suckling on follicles (Skov et al., 2010), absorption via gills, fins, pericardial and yolk sac (Nelson, 2006) or via hypertrophied gut cells. Furthermore, gut cells may become externalised and develop into ribbon like extensions, called trophotaeniae (see chapter 2). These structures can be found in all three ophidiiform viviparous families (Mead et al., 1964; Turner, 1936) as well as in 33 of 34 goodein species (Nelson, 2006). The point in time at which trophotaeniae start to develop during embryogenesis is not yet known, nor has the gestation period in viviparous Ophidiiformes been established. Gestation in Goodeinae is reported to take several weeks (Schindler

and de Vries, 1987, 1988 and Schindler 2014), while viviparous poeciliid fish are pregnant for approximately one month (Nelson, 2006). Some viviparous fish species of other orders are reported to have similar gestation periods, as for example hemiramphid (Nelson, 2006) or sebastid species (Love et al., 2002), whereas other fish may be pregnant for a significantly longer time. Gestation in Comephoridae takes three to four months (Nelson et al., 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).

This chapter applies histology to investigate embryonal development in *X. eiseni.*, presenting a comprehensive analysis of gestation and the ontogeny of embryo development. The study also investigates sex ratio and timing of sexual differentiation.

5.2 Materials and Methods

5.2.1 Fish

Studies on embryo development and gestation were performed using three separate groups of adult (five to nine months old) redtail splitfins (*Xenotoca eiseni*) from stocks held at the University of Exeter (see chapter 3.2 Fish Maintenance). A total of 153 male and 153 female fish were placed (1:1 ratio) in glass aquaria to initiate breeding. Male fish were removed after three or five days respectively and studies in the pregnant females were conducted over a period of six weeks.

5.2.2 Sampling

Fish were checked daily for any visible signs of ill health and examined at incremental time periods during gestation (2, 3 and 5 days, and 1, 2, 4 and 6 weeks after fertilisation) (see Tab 5.1). At each sampling time point, fish were measured for wet body weight and total body length and 6 to 7 fish (at days 2 to 5) or between 21 and 34 fish (at weeks 1 to 6) were randomly selected and sacrificed. Their ovaries were dissected out, weighed, measured and fixed in Bouin's solution for one to two hours before they were washed with and stored in 70% IMS, prior to histological processing.

Tab 5.1: Numbers of fish used at different sampling dates for different endpoints. A number of randomly selected fish was sacrificed for ovarian histology at each sampling point. Body weight and total body length were measured in all remaining fish at all sampling points.

	Weight and Length	Histology
Day 2	7 fish	7 fish
Day 3	7 fish	7 fish
Day 5	6 fish	6 fish
Week 1	133 fish	34 fish
Week 2	99 fish	33 fish
Week 4	66 fish	33 fish
Week 6	21 fish	21 fish

5.2.3 Histology

The general histological methods applied are detailed in chapter 3.4 (Histology). Serial transverse (49%) and lateral (51%) 3 µm sections were cut through the entire length of the gonad to investigate the structure of the gravid ovary and the stage of the embryonic development.

5.2.4 Statistics

Data on size related fecundity are presented as single replicates of 50 XY-pairs. Correlation between brood sizes and total body length of mother fish was tested for significance (two-tailed) using GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

5.3 Results

In the first group of 80 fish housed for three days in four groups of ten male and ten female fish breeding success was 25.0% (ten pregnant females). The second group comprised of 120 fish, which were divided into twelve smaller groups of five male and five female fish each and the male fish were again removed after three days. Four weeks after the initiation of breeding it became apparent that only one fish of the second group was pregnant, and therefore this study group was terminated and the remaining twelve fish were not examined further. In order to optimise the breeding success in the third group of 106 fish, male and female fish were housed for five days in groups of ten to eleven fish of each sex. Twenty eight of the 53 fish (52.8%) became pregnant.

In the study of the ontogeny of the sexual development (chapter 4) it was established that female *X. eiseni* reached sexual maturity at an age of approximately twelve weeks. It was however not possible to determine visually when an individual female was ready to mate. Furthermore, there was no external sign to indicate pregnancy. A gestating female could only be recognised as such three to four weeks after fertilisation by the swelling of her abdomen. The internal changes caused by developing oocytes became apparent upon dissection. Illustrated in Fig 5.1 (C) is a dissected ovary with large oocytes in late vitellogenic stages. Fig 5.1 (A) and (B) depict fully grown oocytes capable of being fertilised. Mature oocytes were fertilised within the ovary. Spermatozeugmata were found in an ovary one week after the initiation of a three day breeding period. This indicated that sperm were viable within the ovary for several days (Fig 5.2). No evidence was found for sperm storage. Fertilised eggs were always seen free in the ovarian lumen and it could therefore not be determined whether the fertilisation took place within the follicle or just after ovulation.

Embryos first developed within their egg envelope (see Fig 5.3 and Fig 5.4) during which time they were provided with nutrients via their yolk supply. In the first week of gestation, the yolk reserve did not decrease appreciably, but after the second week there was a faster rate of depletion. At that time, the pigmentation of developing embryos was visible through the ovarian wall. When dissected out of the ovary, it was possible to see the circulating blood in the developing embryos through the transparent egg envelope (Fig 5.4). At this time, some of the embryos

began to hatch within the ovary and their trophotaeniae had started to appear. This implied that the embryos started to draw on maternal provisioning at around two weeks after fertilisation.

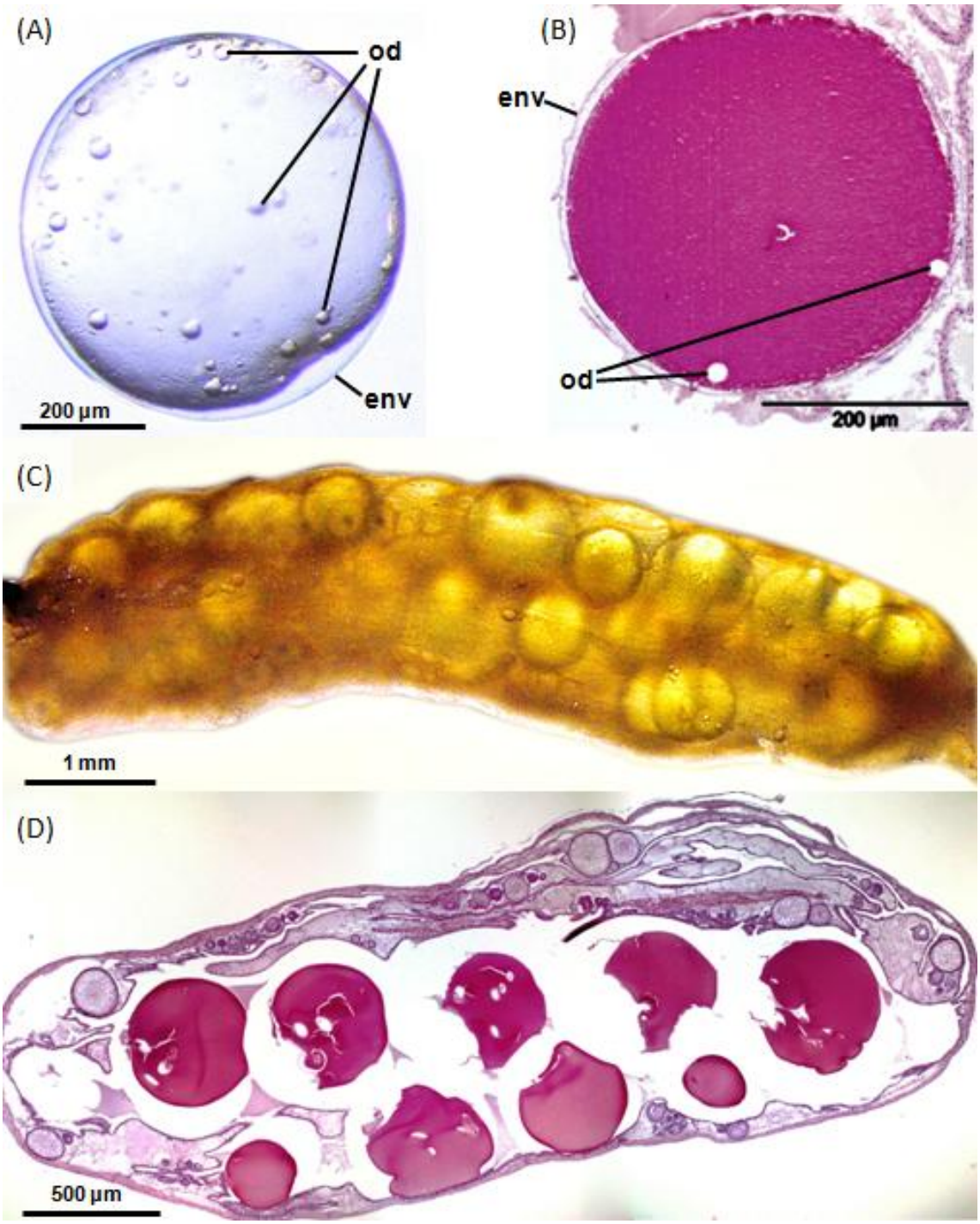


Fig 5.1: Mature oocytes of *Xenotoca eiseni*. **A**: Dissected oocyte filled with yolk, oil droplets at the periphery and transparent egg envelope. **B**: Histological section of a fully grown oocyte with oil droplets at the periphery. **C**: Ovary with semi-transparent ovarian wall that allows seeing oocytes at late vitellogenic stages. **D**: Lateral histological section of an ovary with disintegrating oocytes. **Key**: env: egg envelope, od: oil droplets.

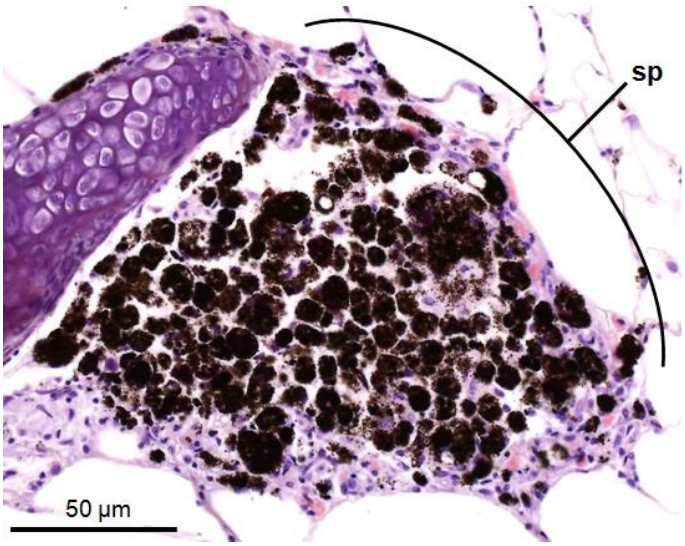


Fig 5.2: Transverse histological section of an ovary of *Xenotoca eiseni* one week after the initiation of a three day breeding period. **Key**: sp: spermatozeugmata.

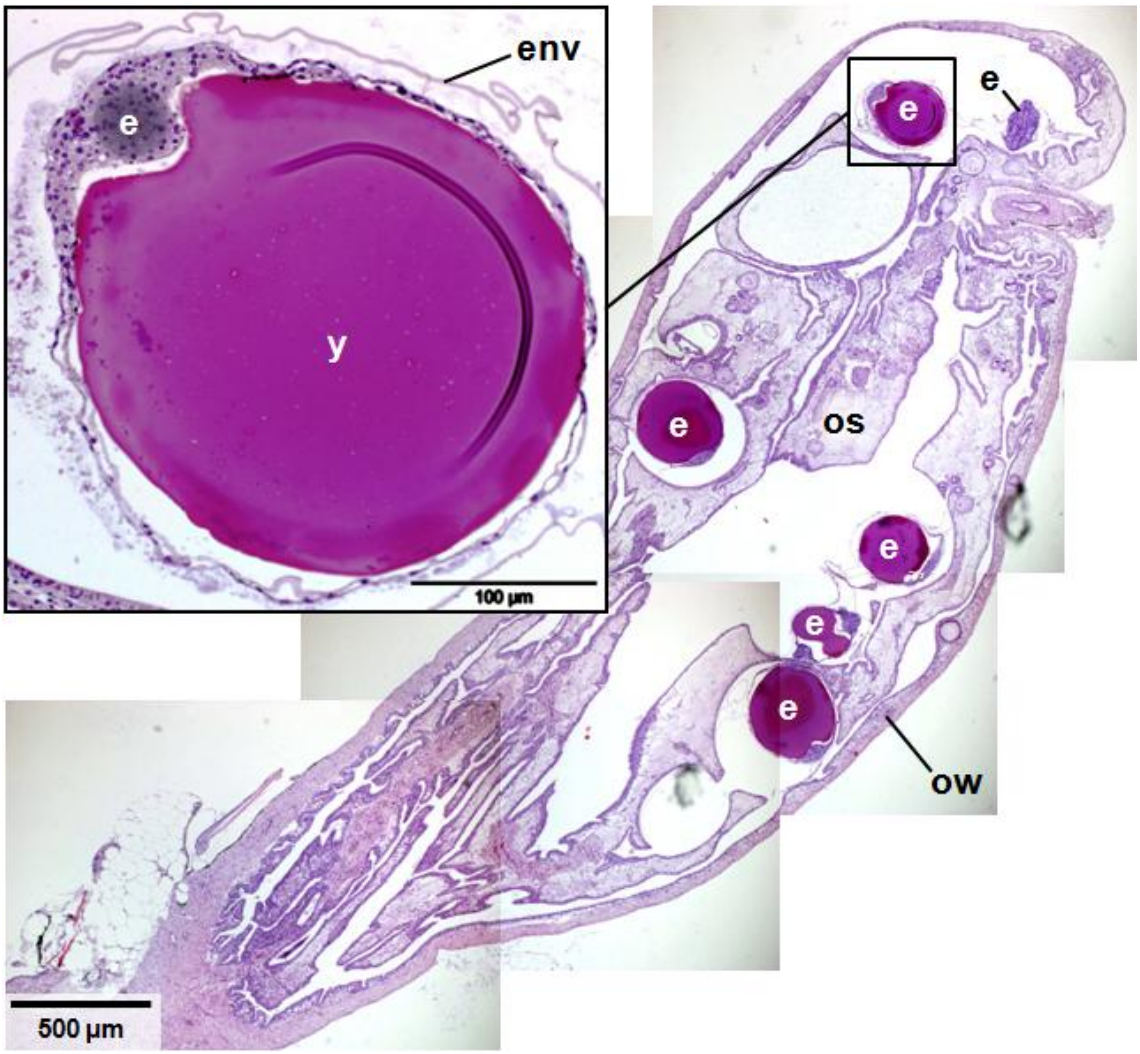
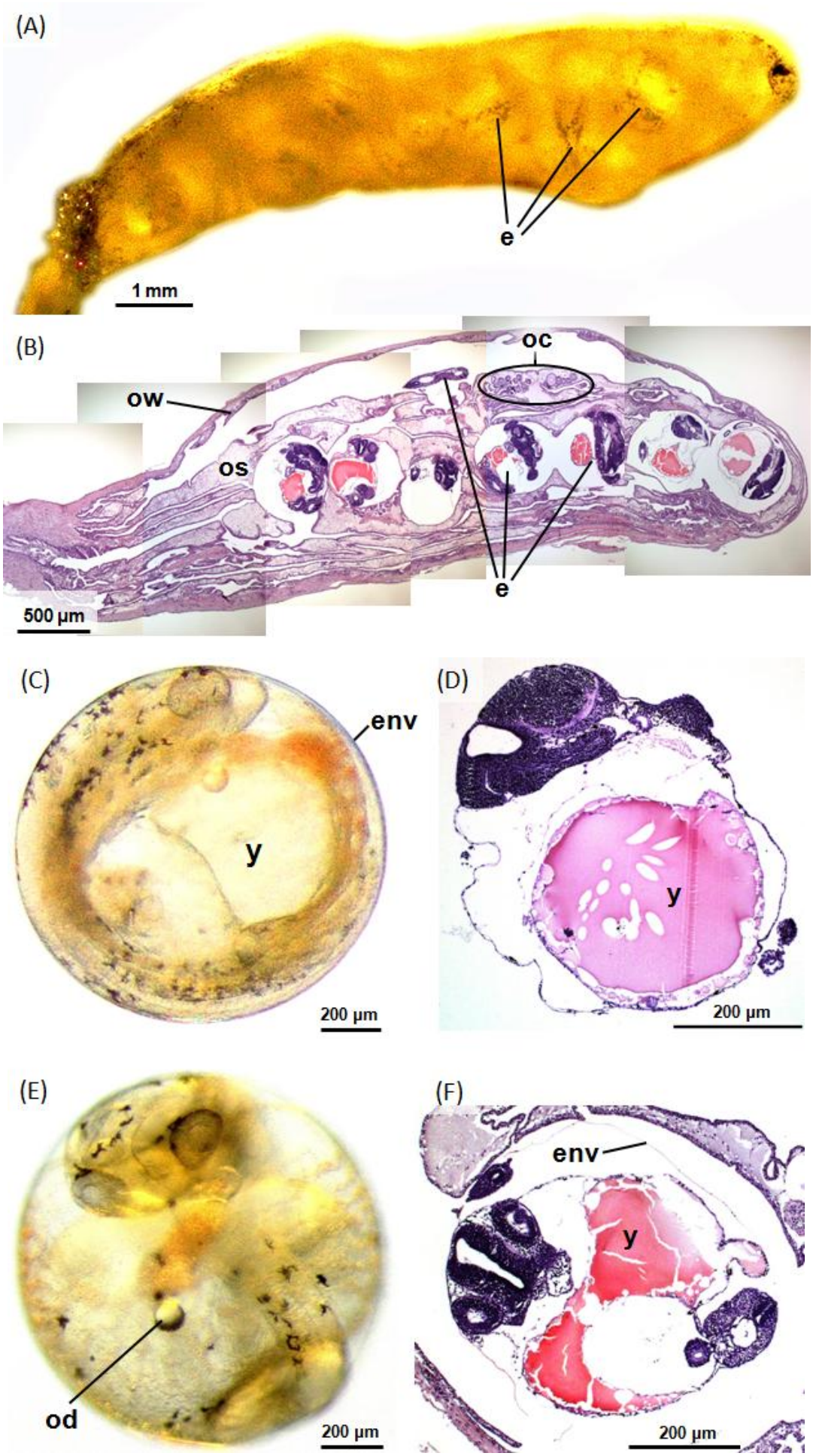


Fig 5.3: Lateral histological section of an ovary of *Xenotoca eiseni* one week after fertilisation. Embryos develop freely in the ovarian lumen but are still within the egg envelope. **Key**: e: embryo, env: egg envelope, os: ovarian septum, ow: ovarian wall, y: yolk.


(A)
e
1 mm
(B)
oc
ow
os
e
500 µm
(C)
env
y
200 µm
(D)
y
200 µm
(E)
od
200 µm
(F)
env
y
200 µm

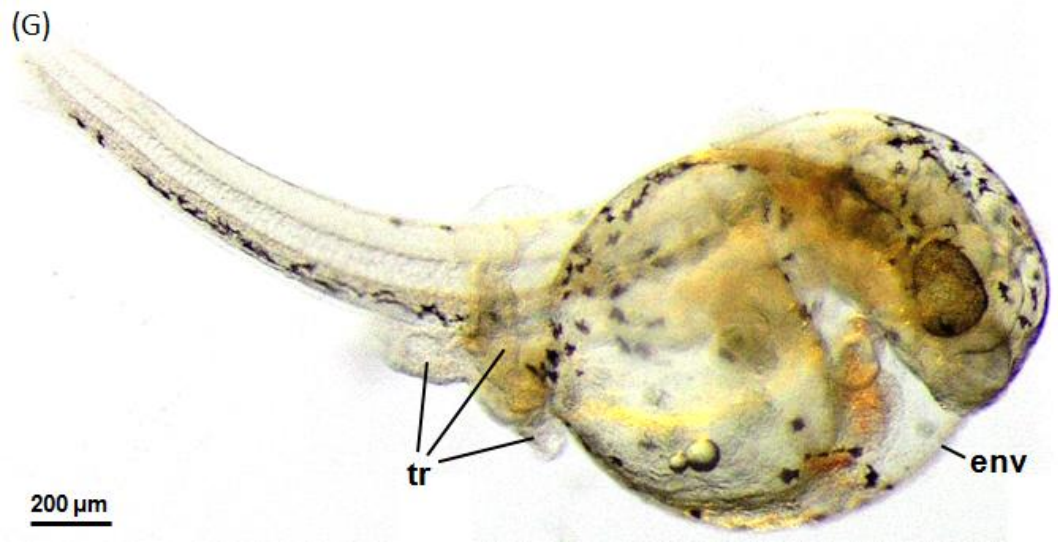


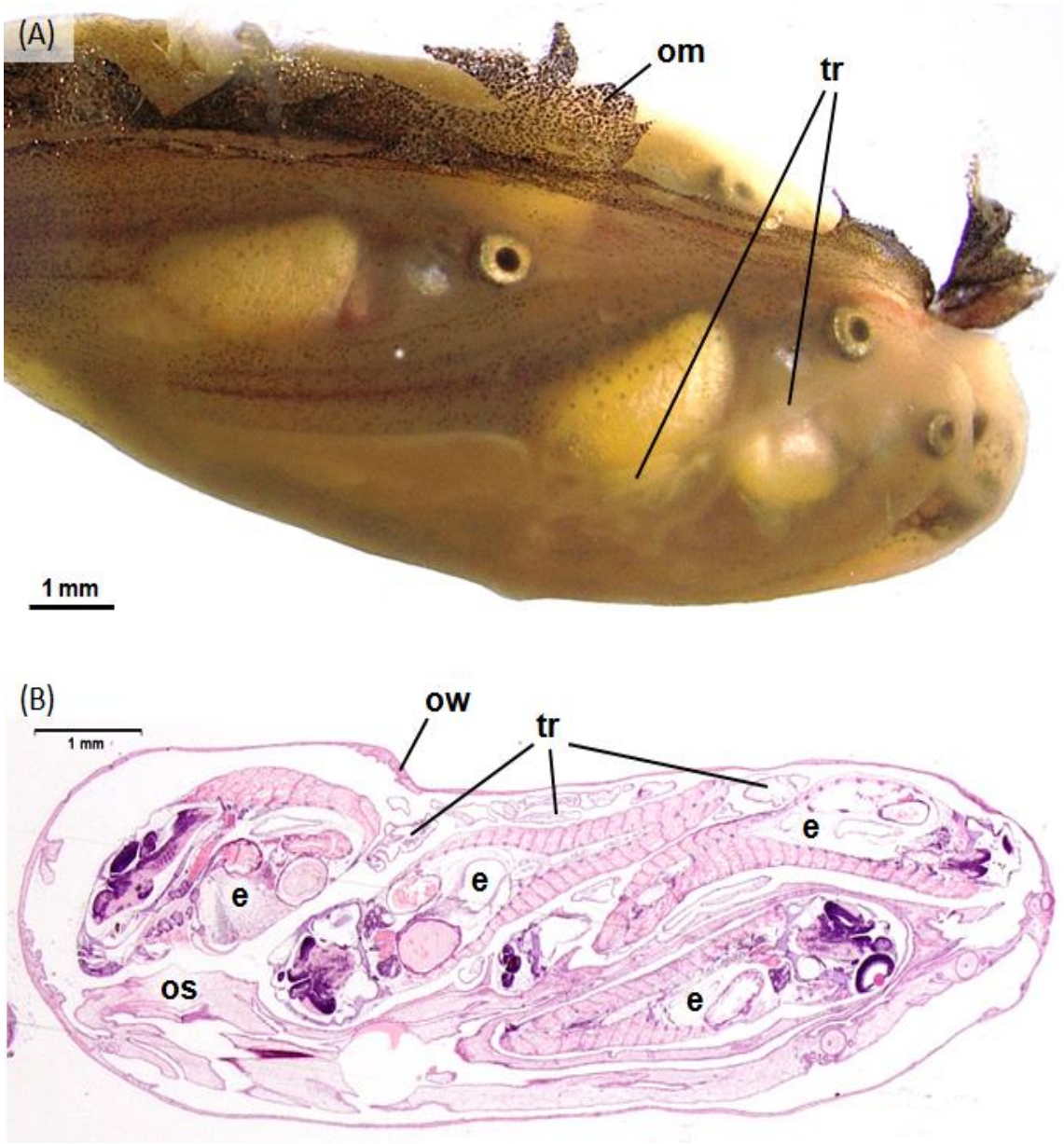
Fig 5.4: Ovaries and embryos of *Xenotoca eiseni* two weeks after fertilisation. **A**: Ovary with semi-transparent ovarian wall that allows seeing the pigmentation of developing embryos. **B**: Lateral histological section of an ovary with embryos. **C+E**: Dissected embryos in egg envelope. **D+F**: Histological sections of embryos. **G**: Dissected embryo in the process of hatching. Trophotaeniae are at the beginning of their growth. **Key**: e: embryo, env: egg envelope, oc: oocytes, od: oil droplets, os: ovarian septum, ow: ovarian wall, tr: trophotaeniae, y: yolk.

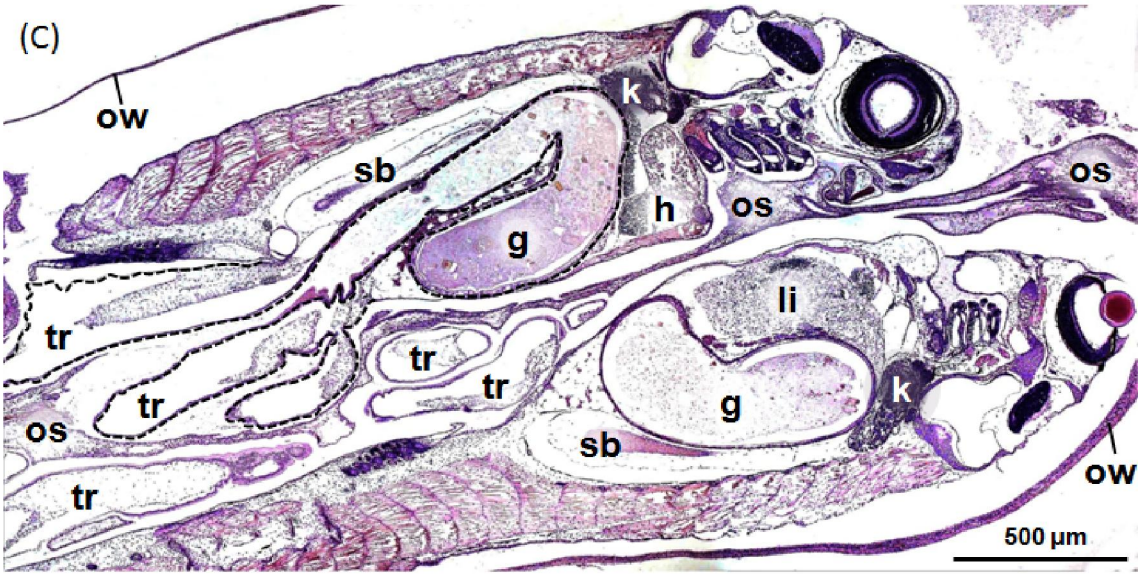
During the following two weeks, the yolk reserve became completely absorbed and trophotaeniae reached their maximum length. Embryos had also grown substantially and there was a very significant increase in brood volume, with a swelling of the abdomen of the gestating female. Four weeks into gestation, embryos had a total body length of approximately 10 mm (Fig 5.5 (F)) and were viable in water, if they were dissected out at this time point. Growth of the embryos caused the ovary to expand and the ovarian wall became thin and transparent (Fig 5.5 (A)). Nevertheless, the ovarian wall and the septum still showed folds into which some of the embryos were nestled (as shown in Fig 5.5 (C,E)).

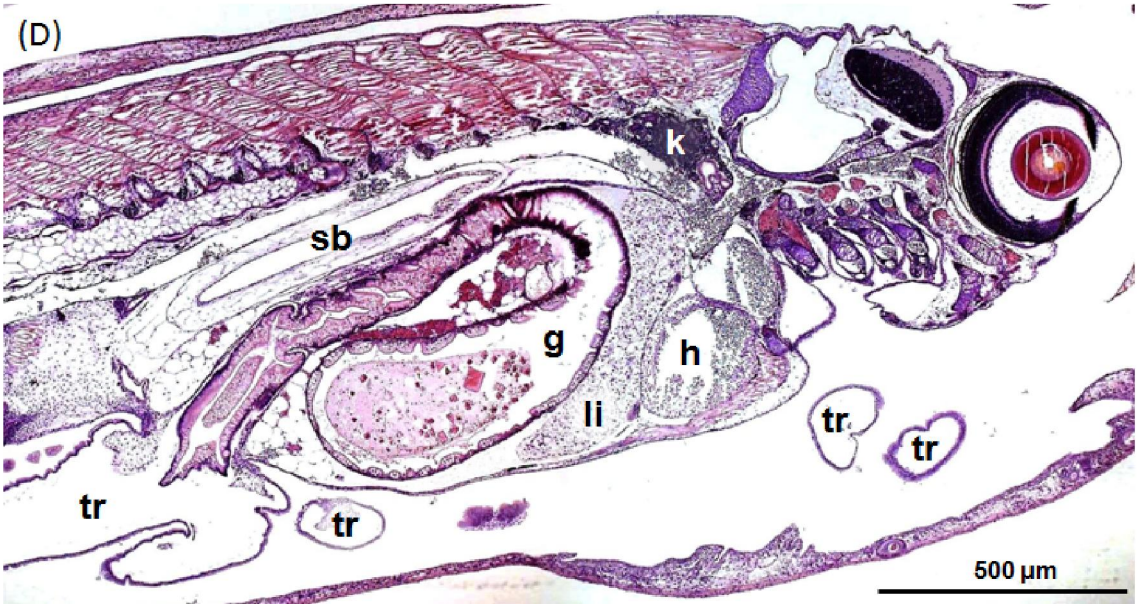
Embryos generally had four to six trophotaeniae with some of them ramified and some of them unbranched (Fig 5.5 (F)). These ribbon-like structures extended from the perianal region of the embryos (Fig 5.5 (C,D)) and consisted of a vascularised tissue core with surrounding epithelial cells. A detailed description of the trophotaenial placenta and nutrients exchange is given in chapter 2.4.3.

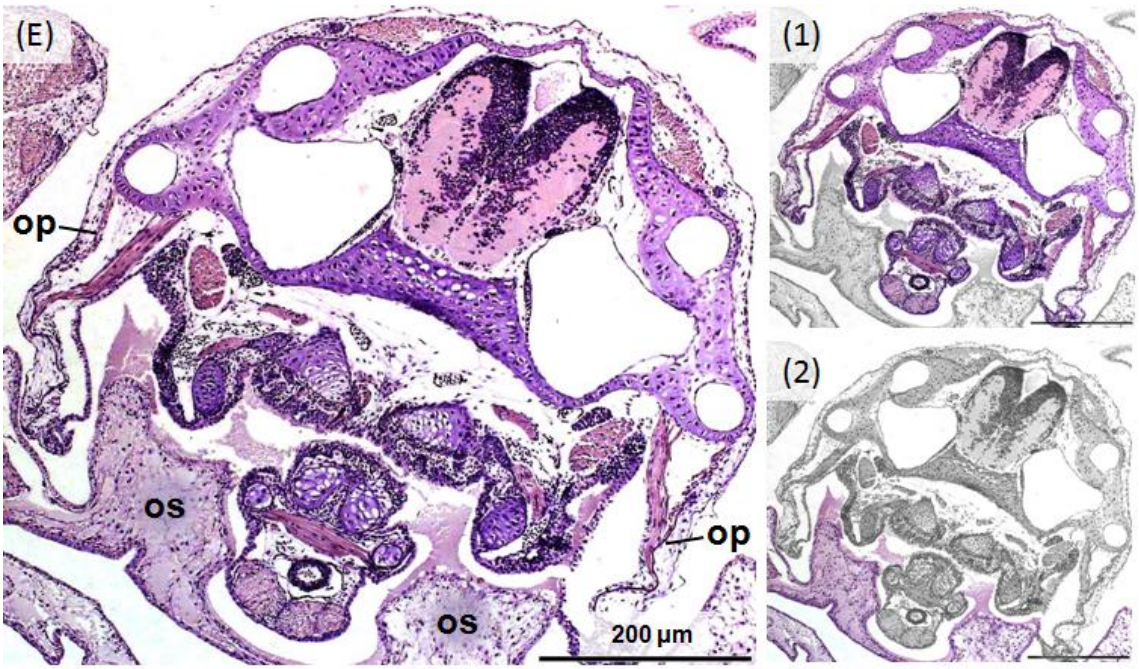
The embryos became tightly packed in the ovarian lumen as they developed for another two weeks. Generally, the embryos were aligned and orientated in the

same direction as their mother (Fig 5.6 (A,B)) although a few sometimes faced the opposite direction or lay askew within the ovary. Gestation normally took six weeks. At this time, the trophotaeniae were partly reabsorbed (Fig 5.6 (E)) and they then underwent complete regression within two weeks after birth. In some of the embryos, differentiated gonads were already visible below the swim bladder (Fig 5.6 (C,D)), although it was often not possible to determine the sex of the fish at that stage (see chapter 4).




(C)
ow
sb
k
h
os
os
g
li
tr
tr
tr
tr
k
os
g
sb
ow
tr
500 µm


(D)
k
sb
g
h
li
tr
tr
tr
tr
500 µm


(E)
op
os
op
os
200 µm
(1)
(2)

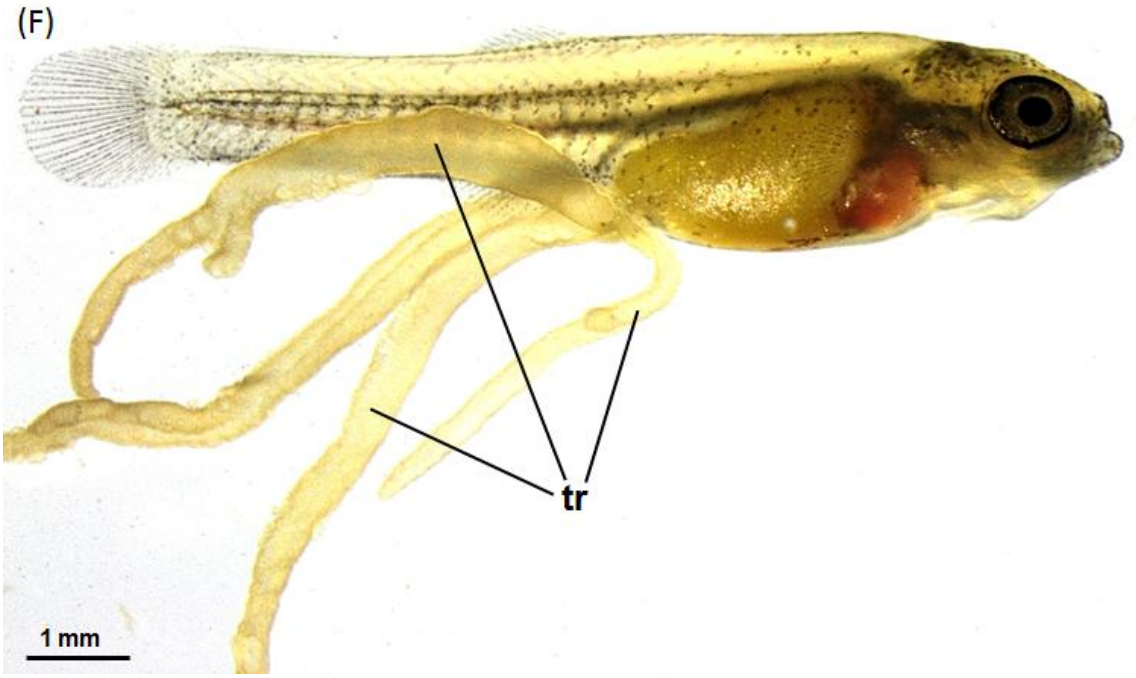
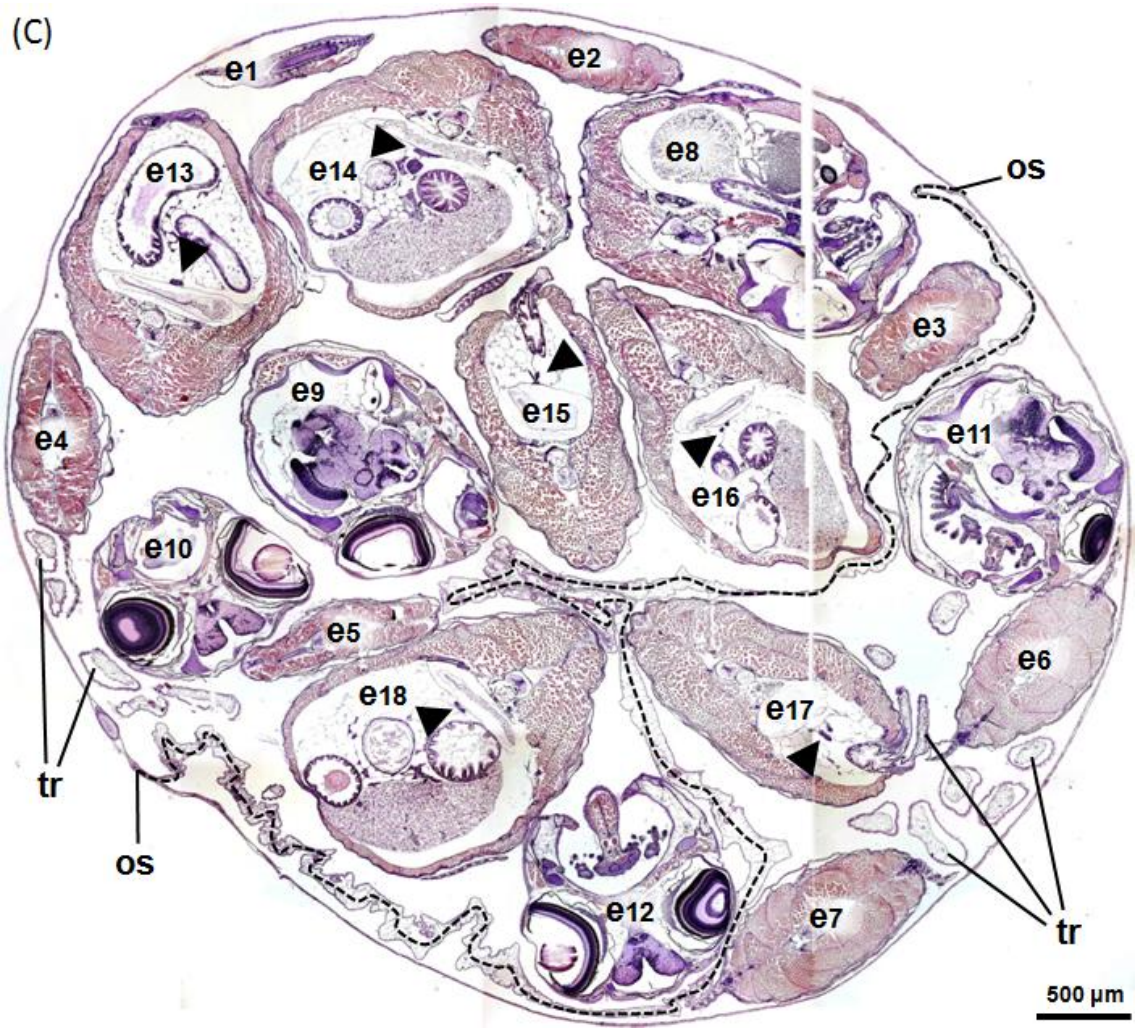
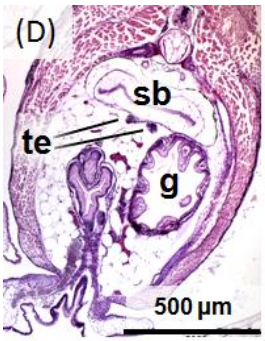


Fig 5.5: Ovaries and embryos of *Xenotoca eiseni* four weeks into gestation. **A**: Ovary with transparent ovarian wall that allows seeing the embryos and their trophotaeniae. **B**: Lateral histological section of an ovary with embryos. **C+D**: Lateral histological sections of embryos within an ovary showing clearly the connection between trophotaeniae and the embryonal gut (dashed line). **E**: Transverse histological section of an embryo within an ovary. The section was cut behind the embryo's eyes showing folds of the ovarian septum that migrated into the gill cavity of the embryo. The pictures on the right show highlighted parts of the embryo (1) and the ovarian septum (2) for reasons of better perceptibility. **F**: Dissected embryo with trophotaeniae at their maximum length. **Key**: e: embryo, g: gut, h: heart, k: kidney, li: liver, om: ovarian mesentery, op: operculum, os: ovarian septum, ow: ovarian wall, sb: swim bladder, tr: trophotaeniae.


(B)
os
1 mm
tr


(C)
e1
e2
e13
e14
e8
os
e3
e9
e15
e4
e11
e16
e10
e5
e6
e18
e17
tr
os
e12
e7
tr
500 µm


(D)
sb
te
g
500 µm


(E)
tr
1 mm

Fig 5.6: Ovaries and embryos of *Xenotoca eiseni* six weeks into gestation. **A**: Ovary with transparent ovarian wall that allows visualising of the embryos. **B**: Lateral histological section of an ovary with embryos. The dashed line highlights the folds of the ovarian septum. **C**: Transverse histological section of an ovary with 18 embryos. Seven embryos (e1-7) are cut through their tails, five embryos (e8-12) are cut through their heads and six embryos (e13-18) are cut through their abdomens. Of the last six embryos, two (e13-14) have presumptive female gonads (arrows), three (e15-17) have presumptive male gonads (arrows) and one (e18) has a not determinable gonad (arrow). The dashed line highlights the folds of the ovarian septum. **D**: Transverse histological section of an embryo within an ovary showing male gonads below the swim bladder and the connection between trophotaeniae and the embryonal gut. **E**: Dissected embryo with trophotaeniae already partly reabsorbed. **Key**: e: embryo, g: gut, os: ovarian septum, sb: swim bladder, te: testis, tr: trophotaeniae.

The duration of the birth process (Fig 5.7) was highly variable and depended, at least in part, on the brood size, which ranged between 1 and 27 in this part of the study. Females examined had a total body length of between 32 and 49 cm with smaller fish tending to produce fewer offspring than the larger fish. The significant correlation between size and fecundity is illustrated in Fig 5.8.

Fig 5.7: Female *Xenotoca eiseni* at parturition. **A**: 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. **B**: Mother fish giving birth to one of her embryos (e).

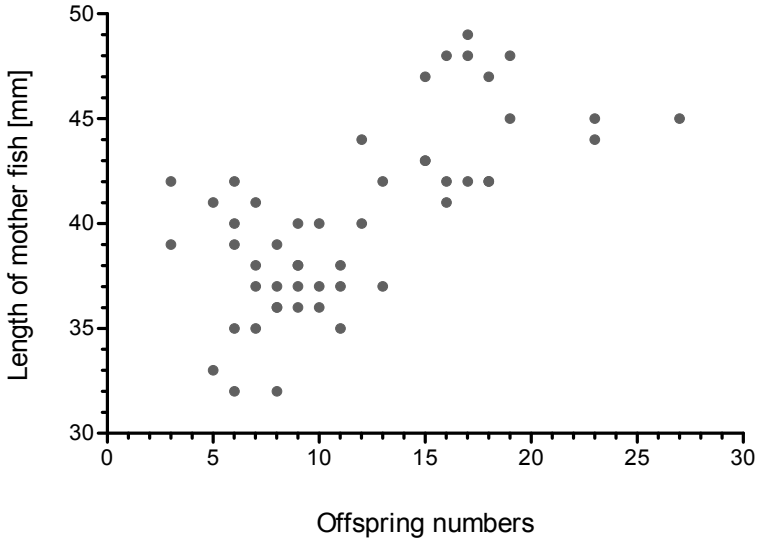


Fig 5.8: Brood sizes in relation to the total body length of the mother fish in *Xenotoca eiseni* showing size related fecundity. The correlation is significant with $P < 0.0001$.

When born, embryos were 13.5 ± 0.9 mm (mean ± SD) in length and weighed 30.6 ± 7.1 mg (see chapter 4). Development was synchronous both within broods and across different broods at the same stage of gestation. Occasionally however, there were one or two smaller or less developed embryos. Broods all comprised of both male and female embryos and the overall sex ratio was approximately 1:1 – exact numbers are given in the following chapter. 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.

5.4 Discussion

Housing small groups of young adult fish (five females and five males) for a short period of time (three days) resulted in very little breeding success. In larger groups of ten females and ten males each, housed for five days, breeding success improved to over 50%. Since there are no external signs to show if a female *Xenotoca eiseni* is impregnable and pregnancies only become visible after three weeks, fertilisation outcome is extremely difficult to predict. As results of this study indicate, fish that mature at a very young age are less likely to show high breeding success. On the other hand, prolonged housing periods increase the number of pregnant females, as one would expect. However, longer breeding periods also cause a wider range of embryonal stages, meaning that embryos examined at any one point could have difference in age of five days (assuming the fish were housed for five days). In proportion to a gestation period of a few weeks only, this might not be insignificant if studied fish are considered to be synchronised.

In case of successful insemination, fertilised eggs were always observed in the ovarian lumen. Whether or not fertilisation took place within the follicle could therefore not be determined in the present study. If eggs really are fertilised in their follicles, it is likely that this more or less coincides with ovulation, as Mendoza (1943) concluded in another goodeid species. Mendoza (1941) and Greven (2013) also determined that there was no sperm storage place in goodeids. The present study supports those findings, since spermatozeugmata were observed only in one female during the housing period and sperm packages were already shown to be undergoing disintegration (see Fig 5.2). The question of whether a female could possibly breed with multiple males is an intriguing one and genetic analyses would provide an answer to this question.

Progress in embryonal development between the first and second week and between the second and fourth week of gestation was considerable, while changes in the last third of gestation were less obvious. After the first two weeks of gestation, the yolk supply of the embryos was largely absorbed. Together with the presence of budding trophotaeniae, this implies that embryos start to depend on a continuous supply of maternal nutrients from that time. Unfortunately, it was not possible to analyse the composition of histotroph in this study, which

according to Schindler (2014) is composed of a similar protein mixture as found in the maternal blood serum, when examined by electrophoresis. Schindler (2014) also found that the trophotaenial uptake of proteins and random copolymers in goodeids can be both passive and specific (via receptor mediated processes).

The trophotaenial placenta of *X. eiseni* has been characterised by various authors, whereas a branchial placenta has not been mentioned before in association with *X. eiseni*. Uribe and colleagues (2014) stated that a branchial placenta had been observed in a few viviparous teleosts and for the first time they analysed the histological structure of the branchial placenta in the goodeid *Ilyodon whitei*. They described it as ovarian folds entering through the embryonic operculum into the branchial chamber, sometimes extending into the pharyngeal cavity. As illustrated in Fig 5.5 (E), this was also observed in some embryos of *X. eiseni*. If, or to what extent, the branchial placenta may supplement the trophotaenial placenta remains to be analysed. However, the main site of nutrient uptake in *X. eiseni* are the trophotaeniae. After their first appearance two weeks into gestation, they reach their maximum length at around four weeks of pregnancy and start to undergo regression during the last phase of gestation. According to Iida and colleagues (2015) trophotaenial regression is caused by apoptotic cell death. The group compared the process in which the epidermal cell layer and mesenchymal structures are lost while the vasculature remains intact to that of amphibian metamorphosis. Whether the process of apoptosis is triggered maternally or is regulated embryonically is not yet clear.

As stated above, trophotaeniae take up many substances non-specifically from the ovarian fluid. Therefore, any substance that is passed from the maternal system to the histotroph may end up in the embryonal system. In order to estimate what effect an exposure to a xenobiotic substance might have on the (sexual) development of *X. eiseni*, it is crucial to know how far the embryos' (sexual) organs are developed by the time they start to depend on maternal provisioning. The onset of gonadal development, nevertheless, could not be determined precisely in this study, as gonads were first histologically visible just before birth. Greven (2013) and other authors stated that the ovary starts to

develop as a two lobed structure that later fuses, which could neither be confirmed nor disproved in this study.

Females examined in this study had a total body length of between 32 and 49 cm. Smaller fish not only tended to produce fewer offspring than bigger fish, but also had a lower breeding success. It is therefore important to take this size related fecundity of the fish into consideration for further studies on gestating *X. eiseni*.

6 Exposure of Gravid *Xenotoca eiseni* to 17α-Ethinyloestradiol (EE2) and Potential Maternal Transfer to Developing Offspring

6.1 Introduction

Early life stages are likely to be amongst the most susceptible to adverse effects of toxicants (Hutchinson et al., 1998; Rasmussen et al., 2002; Brion et al., 2004; Hedman et al., 2011; Brande-Lavridsen et al., 2013) and models for studies on maternal transfer and effects of chemicals on developing early life stages have been developed for rats and rabbits (OECD, 2001). However, some fish species give birth to live young and offer arguably more favourable models for maternal transfer studies in terms of their short generation times, high fecundity, and other features including ease of maintenance and cost-effectiveness (see chapter 2.5).

Since principal biological processes affected by chemicals and the mechanisms of toxicants' effects tend to be highly conserved across vertebrate species, fish models offer an alternative for toxicological studies on maternal transfer (Ankley and Johnson, 2004). The life-bearing eelpout (*Zoarces viviparus*) has been used to study the maternal-embryonal trophic relationship in contaminant exposure (Rasmussen et al., 2002; Hedman et al., 2011; Brande-Lavridsen et al., 2013). Mature eelpout reproduce, however, once a year and gestation lasts four to five months (Rasmussen et al., 2002; Skov et al., 2010; Hedman et al., 2011; Brande-Lavridsen et al., 2013). Other viviparous fish, such as *Xenotoca eiseni,* may offer a more convenient fish model for studying maternal transfer of substances, as they have a much shorter gestation time of only a few weeks (Parenti, 2005; Skov et al., 2010).

Having established a detailed understanding on the reproductive biology including the ontogeny of sexual development and pregnancy of *X. eiseni* (chapters 4 and 5), here the fish model was applied in an exposure study with 17α-ethinyloestradiol (EE2) with a view to investigating its use for maternal transfer studies of toxicants. It was hypothesised that an exposure to EE2 would provide insight into the ability for maternal transfer of this synthetic oestrogen

through an analysis on the effects on sex partitioning and development in this species. It would also allow assessing for the relative sensitivity of this fish as a model species to EE2 compared with that for other fish species. Exposure to EE2 during the period of sexual differentiation has been shown to cause sex-reversal in gonochoristic offspring (Papoulias et al., 1999; Rasmussen et al., 2006). In *Z. viviparus* EE2 also induces the synthesis of vitellogenin (as it does in all other fish species studied) and this in turn causes disruption in the calcium distribution between maternal plasma and the histotroph in *Z. viviparus*, which then may affect growth and bone formation in the embryos (Korsgaard et al., 2002). The ability of a substance to cross the maternal-embryonal barrier is a prerequisite for it to have an adverse effect on development and sex ratio of embryos. Thus far, studies on a goodeid species (*G. multiradiatus*) revealed that pollutants not only crossed the said barrier but their concentrations were found to be up to several orders of magnitude higher in the embryos than in maternal tissue (de la Vega-Salazar at al., 1997). Korsgaard and co-workers (2002) studied the effects of different concentrations of EE2 in pregnant *Z. viviparus* over a period of three weeks and were able to demonstrate changes in the maternal-embryonal trophic relationship. Plasma levels of vitellogenin in the mother fish in that study were found to be higher in fish exposed to 5 ng EE2 /L (and higher) compared to vitellogenin concentrations in control fish. EE2 concentrations in sewage treatment effluent samples vary widely but have been reported at between 0.2 to 7.0 ng/L (Desbrow et al., 1998, Tyler et al., 1998). It was decided to expose *X. eiseni* to concentrations of EE2 between 1 and 5 ng EE2 /L, taking into account both effect concentrations in other fish species and those that are environmentally relevant.

In this study, 72 female fish were first housed with male fish before they were allocated to one of the following four exposure treatments: a water control, a solvent (ethanol) control, nominal EE2 concentrations of either 1 or 5 ng/L. Four weeks later, just prior to parturition, broods were dissected out of the mother fish and transferred to tanks containing fresh water only. Cardio- and hepato-somatic indices were calculated for mother fish and liver samples were later processed for mRNA extraction and analysis of vitellogenin expression. After another four weeks under non-exposure condition, offspring were analysed histologically in order to investigate their sex and stage of gonadal development.

6.2 Materials and Methods

6.2.1 Chemicals

17α-Ethinyloestradiol (EE2) was obtained from Sigma Aldrich (Poole, UK) and deuterated 17α-Ethinyloestradiol (EE2d4) was kindly supplied by the University of Sussex, UK. Methanol was purchased from Fisher Chemicals (Loughborough, UK). Other chemicals used for sampling and histology are listed under chapter 3.1 (Chemicals).

6.2.2 Fish

Naïve adult (seven months old) *Xenotoca eiseni* were obtained from stocks held at the University of Exeter (see chapter 3.2 Fish Maintenance). Before the exposure, male and female fish were pooled in six glass aquaria (50 L) – each holding twelve males and twelve females – under the same conditions as described under chapter 3.2. After a breeding period of ten days the male fish were removed from the aquaria and the female fish were left in the same aquaria for another four days before they were transferred to the exposure aquaria.

6.2.3 Experimental Setup

Exposure

The experimental system comprised of two EE2 treatments (nominal concentrations of 1 ng EE2 /L and 5 ng EE2 /L), a solvent (ethanol) control and a water control. Every treatment consisted of 18 plastic aquaria (1 L), each of them connected to a multi-channel pump that pumped the respective exposure water from a reservoir glass tank (30 L) at a flow rate of 10 L/d. The reservoir aquaria were filled via overflow from a flask into which reconstituted water (240 L/d) and stock solution (240 mL/d) were pumped and mixed (see experimental setup in Fig 6.1). EE2 was dissolved in ethanol (50 mg/L) from which the stock solutions (1 µg EE2 /L and 5 µg EE2 /L) were freshly prepared every week. The stock solution for the solvent control (300 µL/L) was also freshly made weekly. The experimental system was conditioned for five days before the start of the exposure.

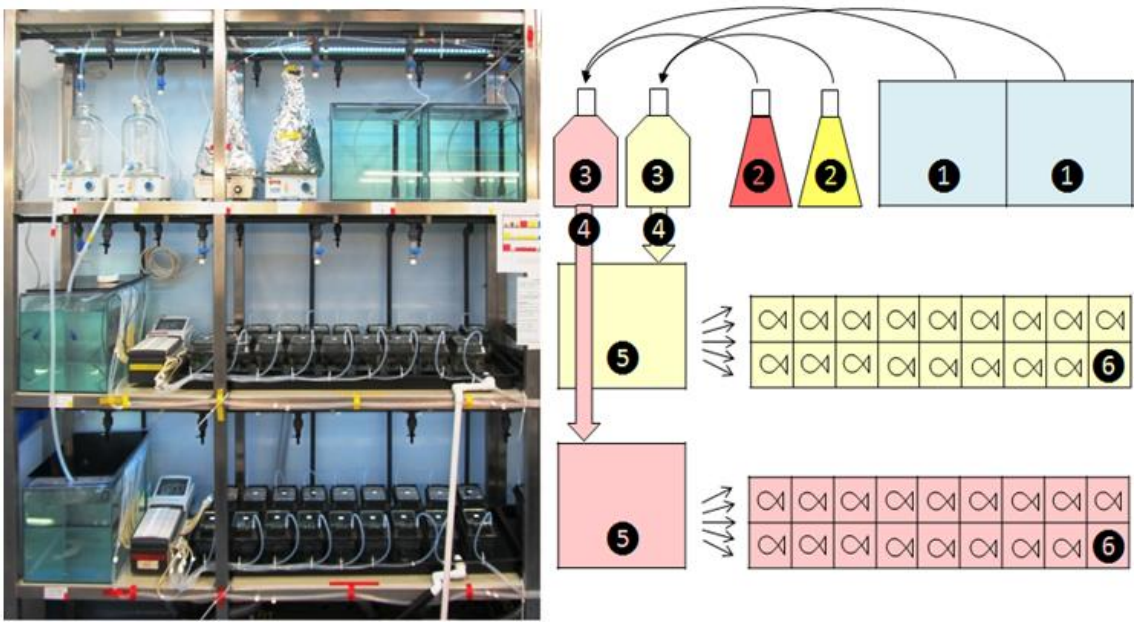


Fig 6.1: Experimental setup. **Left**: 1 ng EE2 /L and 5 ng EE2 /L treatments of the experimental system. **Right**: schematic representation of the picture on the left; (1) reservoir aquaria with constant inflow of reconstituted water, (2) 1 µg EE2 /L and 5 µg EE2 /L stock solutions, (3) flasks with overflow (mixing one part stock solution with 1000 parts water), (4) overflow into reservoir aquaria, (5) reservoir aquaria with exposure water (1 ng EE2 /L and 5 ng EE2 /L), (6) individual exposure aquaria (18 x 1 L per treatment) with tray for the collection of overflowing water from the individual aquaria and outlet; pumps: (1→3) 240 L/d, (2→3) 240 mL/d, (5→6) 18 x 10 L/d.

Two weeks after the initial housing of male and female fish, the female fish were weighed, measured (total body length) and transferred into individual 1 L plastic aquaria, where they were exposed to the treatments detailed above for a period of four weeks, i.e. the rest of the gestation period. Fish were checked twice daily for any visible signs of ill health and feeding behaviour. They were fed to satiation with gamma-irradiated bloodworm (*Chironomidae*, Tropical Marine Center, Chorleywood, UK) and TetraMin flake food (Tetra GmbH, Melle, D). Aquaria were siphoned (100 - 150 mL) every second day to remove food remains and faeces.

Offspring

Embryos were dissected out just before parturition and transferred to plastic aquaria (1 L) containing fresh water only. They were held there for four weeks and fed with freshly hatched *Artemia* (cysts from ZM Ltd., Hampshire, UK). Aquaria were siphoned (100 - 150 mL) once a week and the flow-rate of the reconstituted water was approximately 10 L/day per aquarium. Broods were kept separately in equally sized groups of nine to ten fish. In cases where remaining

fish formed a smaller group, they were excluded from the later developmental analysis due to possible different growth rates in smaller groups in same tank sizes.

6.2.4 Analysis of EE2 in Water Samples

Solid phase extraction (SPE)

Water samples (2.5 L) were taken from the reservoir aquaria (inlet) of each treatment at the onset of the exposure and every week thereafter in order to determine the actual exposure concentration of EE2. Samples from the outlet were also taken after the first and the third week of the exposure to assess any potential loss of EE2 due to adherence to the experimental system (see Tab 6.1). The samples were all stabilised with 1% acetic acid and 5% methanol and stored at 4 °C until the next steps of the analysis. Within 24 hours, 20 µL EE2d4 (0.5 µg/mL) were added to 1 L duplicates of each sample as internal standard. All samples were then pre-filtered through glass wool and a filter paper (Whatman No. 1, Maidstone, UK) in order to remove particulates. Subsequently, the filtrates were extracted through Oasis HLB (6 mL, 500 mg sorbent, 60 µm particle size) solid reverse phase extraction cartridges (Waters, Manchester, UK) which were previously conditioned with 10 mL methanol and 10 mL distilled water. The maximum flow rate was set to 10 mL/min using a gentle vacuum. After the extraction, the cartridges were washed with 10 mL distilled water and then dried with air, sealed with parafilm, wrapped in aluminium foil and stored at -80 °C until further analysis.

High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS)

Extraction cartridges were sent to the University of Sussex, were the elution was performed with 10 mL methanol. Extracts were dried, reconstituted in 100 µL water/acetonitrile (7:3, v/v) and passed through 0.22 µm centrifuge filters. HPLC-MS/MS analyses were carried out using a Waters Alliance 2695 HPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Kinetex C18 column (2.6 µm, 4.6 × 100 mm) (Phenomenex, Macclesfield,

UK). The injection volume was 20 µL and mobile phase solvents were water (A) and acetonitrile (B) with 0.05% ammonium hydroxide in each. Separation was achieved at 20 °C using a flow rate of 0.25 mL/min with the following gradient: (A:B) 70:30 to 0:100 in 13 min; 0:100 for 5 min, then return to the initial condition at 19 min and equilibration for 6 min. Retention times were 13.05 min for EE2 and 13.01 min for EE2d4. Ionisation and fragmentation settings were as reported in Labadie et al. (2007): MS/MS was performed in the Multiple Reaction Mode (MRM) using ESI in the negative mode. For EE2 and EE2d4, two characteristic fragmentations of the deprotonated molecular ion $[M-H]^-$ were monitored (295>145 and 295>159 for EE2 and 299>147 and 299>161 for EE2d4). The first and most abundant one was used for quantitation, while the second one was used as a qualifier. Collision energies were set at 40 eV and 45 eV for EE2 and EE2d4 respectively and cone voltage was 50 V for both compounds. Other parameters were optimised as follows: capillary voltage −3.1 kV, extractor voltage 4V, multiplier voltage 650V, source temperature 120 °C and desolvation temperature 400 °C. Argon was used as collision gas (P collision cell: 3×10^{-3} mbar), while nitrogen was used as both nebulising (100 L/h) and desolvation gas (600 L/h). Mass calibration of the spectrometer was performed with sodium iodide. Data were acquired using MassLynx 4.1 and identification of analytes and surrogates was performed by comparing the retention times and the MS signals in the samples with those observed with standard solutions. Quantification was carried out by calculating the response factor of EE2 to EE2d4. Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio of native to deuterated. A five point calibration curve ($R^2 > 0.99$) covered the range from 50 to 2,000 pg (injected onto column) for EE2, which was within the linear range of the instrument.

6.2.5 Sampling

Female fish

After the four week exposure period, all female fish were sacrificed, weighed and measured (total body length). The ovaries of gravid and non-gravid females were dissected out and weighed. Embryos were immediately dissected out of gravid ovaries, counted and transferred to aquaria containing freshwater only, and the

empty ovaries were weighed again to assess the brood weight. In order to calculate the cardio- and the hepato-somatic indices (CSI, HSI), hearts and livers were dissected out and weighed. Livers were immediately flash frozen in liquid nitrogen and stored at -80 °C for subsequent mRNA extraction.

Offspring

With the aim of examining the (sexual) development of the offspring, offspring were kept for four weeks under non-exposure conditions before they were sacrificed, weighed, measured (total body length) and prepared for gonadal histology (as described in chapter 3.3 Sampling Procedures).

Tab 6.1: Overview of fish transfer and sampling dates. **Key**: f: female fish, m: male fish

	day	**fish transfer**	**water samples**		**female fish**		**offspring**	
			in	out	length + weight	heart + liver	brood-weight	length, weight, histology
Breeding	0	housing f + m						
	10	removal m						
	140	transfer f	x		x			
Exposure	7		x	x				
	14		x					
	21		x	x				
	280	transfer offspring	x		x	x	x	
Offspring	28							x

6.2.6 Histology

General histological methods are described in chapter 3.4 (Histology). Serial transverse 3 µm sections were cut through the abdominal region of the body and whole body sections were examined for the presence of gonads to identify the sex and the stage of their development.

6.2.7 RNA Extraction and Reverse Transcription

Total RNA was extracted from liver tissue using the RNeasy Mini Kit (Qiagen, Crawley, UK) including a DNase treatment according to the manufacturer's instruction. The RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop (Thermo Scientific) and the RNA quality was verified by $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios. Extracted RNA samples were stored at -80 °C until further use.

The reverse transcription was carried out using 1 µg total RNA, random hexamers (0.4 µM), dNTPs (2 mM each), 5xMMLV RT buffer (with 2 mM DTT incorporated) and MMLV reverse transcriptase (Promega, Southampton, UK). RNA and hexamers were incubated at 70 °C for 5 min and then chilled on ice before the rest was added and the mixture was incubated at 37 °C for 1 hour. The cDNA samples were then stored at -20 °C until further analysis.

6.2.8 PCR Analysis, Gel Electrophoresis and DNA Extraction

No housekeeping gene sequence for *Xenotoca eiseni* was available and therefore a search for sequences of 18S ribosomal RNA of related species was conducted using the NCBI GenBank database. Partial sequences from *Gambusia holbrooki* (GenBank accession number FJ710842), *Kryptolebias marmoratus* (FJ438821), *Cyprinodon variegatus* (EF431912) and *Fundulus heteroclitus* (M91180) – all species within the same order as *X. eiseni* (Cyprinodontiformes) – were aligned using ClustalW2. Based on this alignment, different primers were designed with Beacon Designer 7.2 software (Premier Biosoft International, Palo Alto, CA, USA) and purchased from Eurofins MWG Operon (Ebersberg, Germany).

Different PCR analyses were performed for all primer pairs in 20 µL reaction volumes using 1 µL template cDNA, forward and reverse primers (0.2 µM each), dNTPs (0.2 mM each), 5xGoTaq reaction buffer (1.5 mM $MgCl_2$) and GoTaq Polymerase (0.5 u / 20 µL) (Promega, Southampton, UK). The polymerase was activated by an initial denaturation at 95 °C for 2 min followed by 15 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for

1 min and 20 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min. After the final extension at 72 °C for 5 min the reaction mix was allowed to soak at 4 °C for 30 min.

Subsequently, the PCR products were separated by agarose (1.5%) gel electrophoresis using a 100 bp ladder as reference. Obtained DNA fragments of expected size were then excised from the gel and extracted using NucleoSpin® Extract II Kits (Macherey-Nagel) according to the manufacturer's protocol. The purified DNA was sequenced by Eurofins MWG Operon (Ebersberg, Germany) and finally verified by sequence alignment using BLASTn.

6.2.9 RT-qPCR

Sequences of *vtg A* (EU761161), *vtg B* (EU761162) and *vtg C* (EU761163) for *Xenotoca eiseni* were available in the NCBI GenBank database. Specific primers for all vtg and the 18S sequences were designed with Beacon Designer 7.2 software (Premier Biosoft International, Palo Alto, CA, USA) and purchased from Eurofins MWG Operon (Ebersberg, Germany). Primer pair annealing temperatures were optimised using cDNA on a temperature-gradient program which was run in duplicates according to Filby and Tyler (2005): 95 °C for 15 min followed by 40 cycles of 95 °C for 10 sec and Ta for 20 sec, where Ta were gradient temperatures between 55 °C and 62 °C. In order to validate the primer pairs, the detection range, the linearity and the amplification efficiency were determined using 5 dilutions of a 10-fold dilution series of cDNA that was run in triplicates under following qPCR conditions (Filby and Tyler, 2005): 95 °C for 15 min followed by 40 cycles of 95 °C for 10 sec and Ta for 20 sec (where Ta were the optimised annealing temperatures, see Tab 6.2) with a subsequent melt curve analysis to assess primer specificity. Standard curves were calculated by plotting the threshold cycle (C(t)) against the logarithm of the cDNA dilution, whereby slopes of between -3.19 and -3.50 with linear correlations (R^2) of ≥ 0.997 were converted to efficiencies (*E*) of 1.93 to 2.06 (corresponding to 93.0 % - 106.0 %) with the following equation: $E = 10^{(-1/slope)}$ (Rasmussen, 2001).

Hepatic expression of *vtg* mRNA was determined by quantitative real-time PCR using the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc., CA, USA). Amplifications were performed in 96-well clear plates in triplicate 15 µL reaction volumes using 0.75 µL template cDNA, forward and reverse primers (0.25 µM each) and 7.5 µL 2xSYBR green mix (ABgene, Epsom, UK). In addition to a no reverse transcript control (NTC) a pooled cDNA sample was run on every plate to assess inter assay variability. The RT-qPCR started with an initial denaturation at 95 °C for 2 min followed by 40 cycles of 95 °C for 10 sec and Ta for 20 sec (where Ta were the optimised annealing temperatures) with a subsequent melt curve analysis. Details on primer sequences, annealing temperatures (Ta) and efficiencies are shown in Tab 6.2.

Relative expression levels (*RE*) of *vtg A*, *vtg B* and *vtg C* were calculated according to Filby and Tyler (2005) using the equation $RE = (E_{18S})^{C(t)\ 18S} / (E_{vtg})^{C(t)\ vtg}$, whereby *E* is the qPCR amplification efficiency for the specified gene, 18S is the housekeeping gene which was measured in each sample and was used for the normalisation and *vtg* stands for either *vtg A*, *vtg B* or *vtg C*.

Tab 6.2: Overview of RT-qPCR parameters

Gene	Accession no	Primer direction	Primer sequence (5'-3')	Position	Ta (°C)	Efficiency
vtg A	EU761161	sense	TGTCTGCCCTATCAACTTTCG	220	61.5	93.0 %
		antisense	GGATGTGGTAGCCGTTTCTC	333		
vtg B	EU761161	sense	GAGATGGAGGTTAAGGTTGGAG	3,112	61.0	95.0 %
		antisense	GAAGATGAGGAGCGGTTGC	3,257		
vtg C	EU761161	sense	CCTGATGTCGGGTTACTCTTTG	2,316	61.0	99.8 %
		antisense	ATGGATGCGGCTGTCACG	2,447		
18S	n/a	sense	ACCCTCACAGCGTTCAG	1,583	60.5	106.0 %
		antisense	GGACACAAGAGCCATCG	1,704		

6.2.10 Statistics

Data of the water chemistry are presented as mean ± SD and were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. Results

for weight, length, condition factor and vitellogenin expression of mother fish are illustrated as mean ± SD. They were tested by using two-way (RM) ANOVA with Bonferroni's post-test. For CSI and HSI of mother fish as well as for weight, length and condition of offspring results are shown as mean ± SD. Data were compared using one-way ANOVA followed by Tukey's multiple comparison test. The same analyses were applied to the data of offspring numbers and weights, which are presented as box and whiskers (min-max). Viability and sex cell stage data are shown as stacked bars, the latter being tested using one-way ANOVA with Bonferroni's post-test. Results met the assumption of approximate normality and equality of variance and differences were considered statistically significant at $P \leq 0.05$. All data were analysed using GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

6.3 Results

6.3.1 Water Chemistry

Water analysis showed consistent data for EE2 concentrations of in- and outlet in all treatments and across all sampling dates (Fig 6.2 (A)). This illustrates consistent exposure concentrations with no significant loss of EE2 in the exposure tanks. Results were therefore combined for the individual tanks within treatments (Fig 6.2 (B)). The mean measured concentrations for the 1 and 5 ng EE2 /L nominals were 0.9 ± 0.2 ng EE2 /L (mean ± SD, n = 13) and 3.4 ± 0.4 ng EE2 /L (n = 14) respectively. Traces of EE2 were sometimes, but not consistently, detected in both water and solvent controls. These levels however were always at least 5.5-fold lower than for the lowest EE2 exposure. The nominal limit of detection (LOD) was < 0.1 ng EE2 /L.

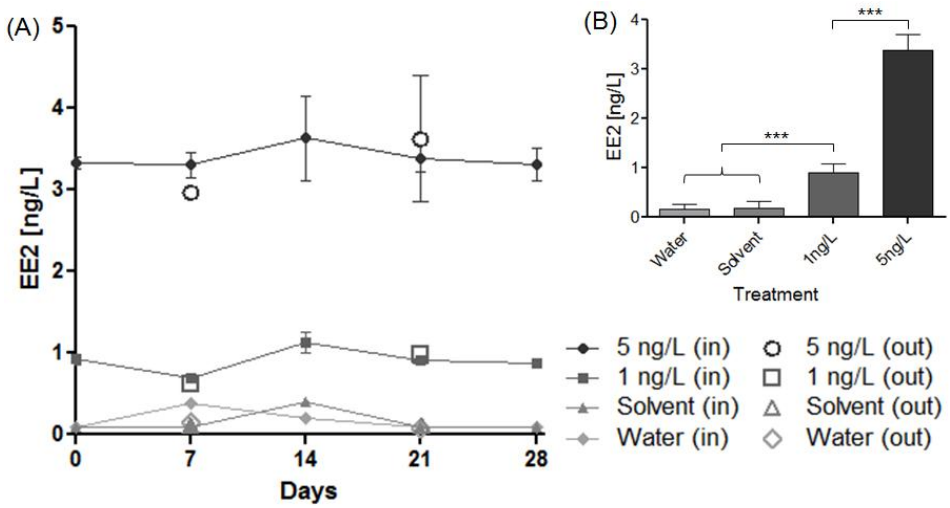


Fig 6.2: Water chemistry. **A**: Overview on all results of every sampling date and from in- and outlet separately, shown as mean of duplicates ± SD. **B**: Summary: all data of respective treatments are combined and shown as mean + SD. Water and solvent: n = 12, 1 ng/L: n = 13, 5 ng/L: n = 14. **Key**: ******* = P ≤ 0.001 (extremely significant).

6.3.2 Mother Fish

Total body length, weight and the condition factor (K = 100 x weight (mg) x length^{-3} (mm)) of mother fish were compared between treatments both before and after exposure. There were no differences between treatments before the exposure or between treatments after exposure in non-pregnant fish. Results for the pregnant fish, however, showed that after the exposure, these parameters varied between treatments (Fig 6.3). A significant difference (P ≤ 0.05) occurred in total body

length between pregnant fish exposed to 1 and 5 ng EE2 /L (Fig 6.3 (A2)). Moreover, significant differences (P ≤ 0.001) in weight and condition of pregnant fish occurred between the 1 ng EE2 /L treatment and all the other treatments (Fig 6.3 (B2,C2)). Comparing these three endpoints before and after exposure within treatments, there were significant increases (P ≤ 0.01) in weight and condition factor in all pregnant females, with the exception of those exposed to 1 ng EE2 /L (Fig 6.3 (B2,C2)). Females showed an increase in total body length during the exposure, except for the pregnant females of the water and the 1 ng/L treatments (Fig 6.3 (A1,A2)). Non-pregnant fish showed a significant increase in weight with the exception of the fish exposed to 1 ng EE2 /L (Fig 6.3 (B1)). Finally, there was a significant loss in condition in all non-pregnant females during the exposure.

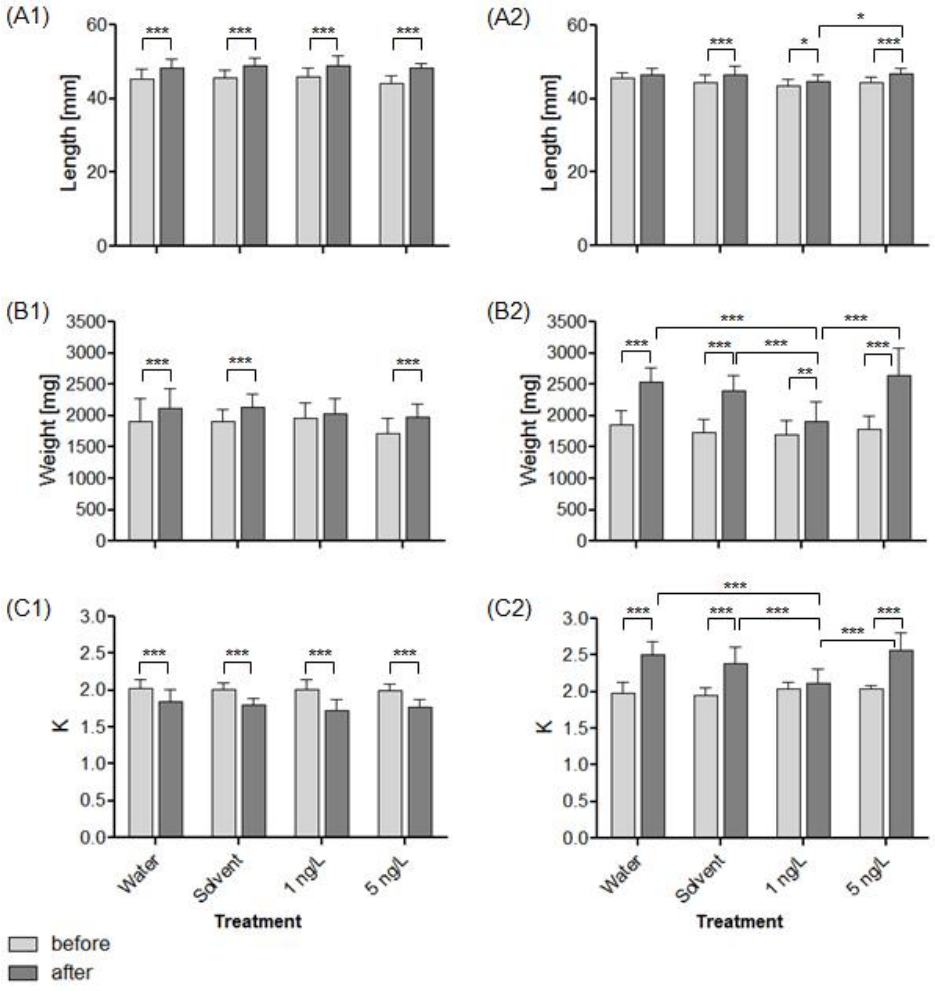


Fig 6.3: Condition of mother fish. **A1, B1, C1**: Total body length, weight and condition factor (K = 100 x weight (mg) x length^{-3} (mm)) of non-pregnant fish. **A2, B2, C2**: Total body length, weight and condition factor (K) of pregnant fish. **Key**: * = P ≤ 0.05 (significant), ** = P ≤ 0.01 (very significant), *** = P ≤ 0.001 (extremely significant).

The analysis of heart and liver weight, expressed as cardio- and hepato-somatic indices (CSI and HSI) (Fig 6.4) revealed no differences between fish of different treatments with the exceptions of the CSI between the water and solvent controls in non-pregnant fish.

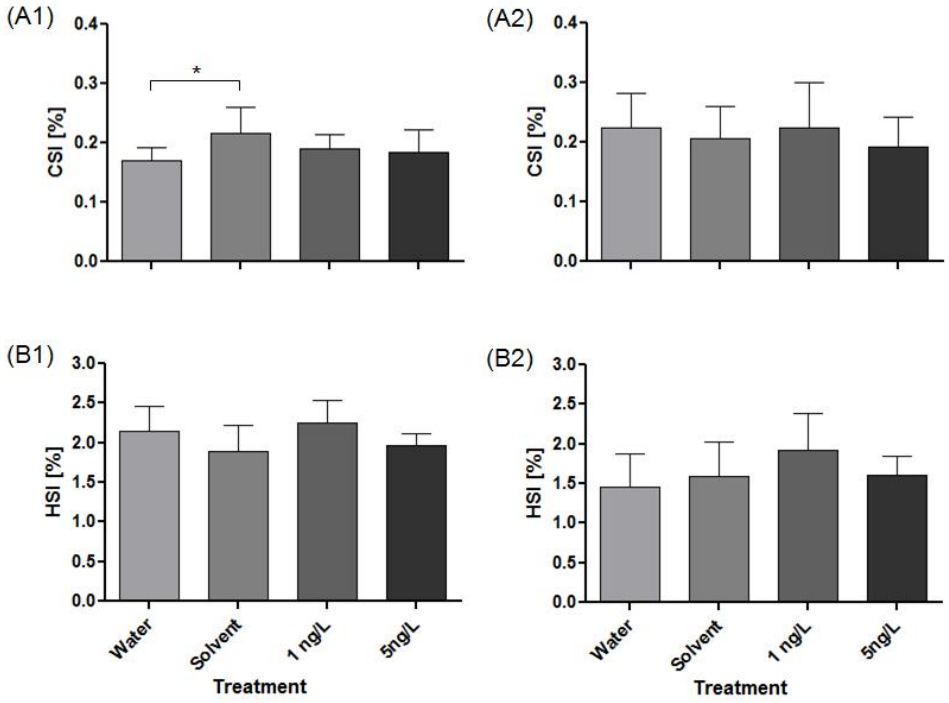


Fig 6.4: Cardio- (CSI) and hepato-somatic (HSI) indices of mother fish. **A1, B1**: Non-pregnant fish. **A2, B2**: Pregnant fish. **Key**: * = P ≤ 0.05 (significant).

In seeking to obtain the 18S sequence for *Xenotoca eiseni*, two of the four tested primer pairs yielded a PCR product and these were therefore combined to get a longer DNA fragment. The DNA section of the 18S gene obtained was 602 bp long and its sequence is shown in Fig 6.5. This 18S gene was subsequently used as internal control to normalise the quantitative PCR data for vitellogenins A, B and C.

Data of the change in vitellogenin expression for the water and the solvent control treatments were compared, whereby no significant differences were identified. Since the data for the solvent control were more consistent, they were subsequently used to compare the results of the other treatments to. The normalised results for non-pregnant fish, shown in Fig 6.6 (A1) (mean ± SEM),

revealed no change in the expression level in any of the vitellogenin genes in all treatments. Noteworthy, however, is that vitellogenin C showed a tendency for an up-regulation in non-pregnant fish exposed to 5 ng EE2 /L. In pregnant fish (Fig 6.6 (A2)), vitellogenin B was significantly elevated in fish exposed to 5 ng EE2 /L compared with the other treatments. Vitellogenin B in these fish was also significantly higher expressed than vitellogenin A and C. Compared with pregnant fish in the control treatments, pregnant females exposed to the nominal concentration of 5 ng EE2 /L exhibited fold changes in vitellogenin expression of 3.44 ± 1.62 (*vtg A*), 9.50 ± 3.85 (*vtg B*) and 2.41 ± 0.69 (*vtg C*) respectively.

```
Xenotoca eiseni 18S mRNA, partial sequence
ATCAGTTATGGTTCCTTTGATCGCTCTACCGTTACTTGGATAACTGTGGCAATTCTAGAGCTAATAC
ATGCAAACGAGCGCTGACCTTCGGGGATGCGTGCATTTATCAGACCCAAAACCCATGCGGGGTGCTC
CCCGTGGGCGCCCCGGCCGCTTTGGTGACTCTAGATAACCTCGAGCCGATCGCTGGCCCTCCGTGGC
GGCGACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTACGCTACGTGCCTACCATGGTGA
CCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAA
GGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGACTCGGGGAGGTAGTGACGAAAAATAACAATAC
AGGACTCTTTCGAGGCCCTGTAATTGGAATGAGTACACTTTAAATCCTTTAACGAGGATCCATTGGA
GGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATCTTAAAGTTGCTGCAGT
TAAAAAGCTCGTAGTTGGATCTCGGGATCGAGCTGACGGTCCGCCGCGAGGCGAGCTACCGTCGCC
```

Fig 6.5: Partial sequence of 18S mRNA of *Xenotoca eiseni*.

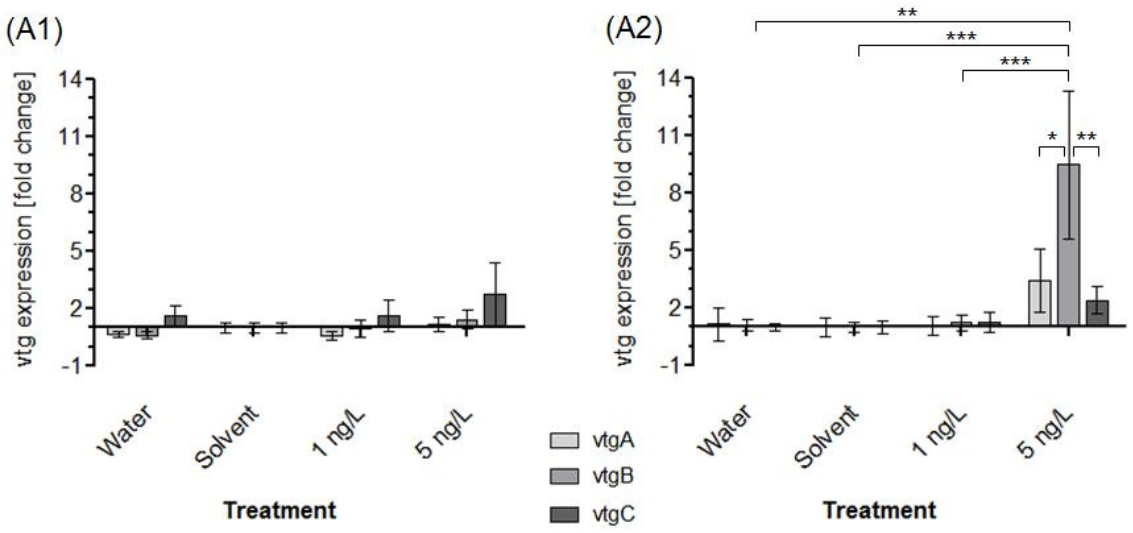


Fig 6.6: Change in vitellogenin expression of mother fish in reference to the solvent control treatment, normalised with 18S. **A1**: Non-pregnant fish. Water control: n (*vtg A*) = 11, n (*vtg B*) = 10, n (*vtg C*) = 6; solvent control: n (*vtg A*) = 7, n (*vtg B*) = 8, n (*vtg C*) = 6; 1 ng EE2 /L: n (*vtg A*) = 5, n (*vtg B*) = 6, n (*vtg C*) = 4; 5 ng EE2 /L: n (*vtg A*) = 8, n (*vtg B*) = 8, n (*vtg C*) = 5. **A2**: Pregnant fish. Water control: n (*vtg A*) = 5, n (*vtg B*) = 4, n (*vtg C*) = 5; solvent control: n (*vtg A*) = 7, n (*vtg B*) = 5, n (*vtg C*) = 7; 1 ng EE2 /L: n (*vtg A*) = 8, n (*vtg B*) = 7, n (*vtg C*) = 8; 5 ng EE2 /L: n (*vtg A*) = 6, n (*vtg B*) = 7, n (*vtg C*) = 5. **Key**: * = P ≤ 0.05 (significant), ** = P ≤ 0.01 (very significant), *** = P ≤ 0.001 (extremely significant).

Brood sizes did not vary significantly across the different treatments (Fig 6.7 (B)). There were significant differences in brood weight (Fig 6.7 (A)), however, which was also reflected in the average offspring weight (Fig 6.7 (C)). Broods of fish exposed to a nominal concentration of 1 ng EE2 /L weighed significantly less than broods of all the other treatments. The only difference in brood weight amongst the other treatments was observed between the solvent control and the 5 ng EE2 /L. Dividing the brood weight by the number of offspring allowed calculating for the average individual offspring weight. Again, fish of the 1 ng EE2 /L treatment had offspring with a lower average weight than the ones from the solvent control and the 5 ng EE2 /L treatment. One fish exposed to the nominal concentration of 1 ng EE2 /L had one offspring only, which is shown as an outlier in Fig 6.7 (A,B,C). In the controls, there was a significant negative correlation between brood size and average offspring weight (Fig 6.7 (D)).

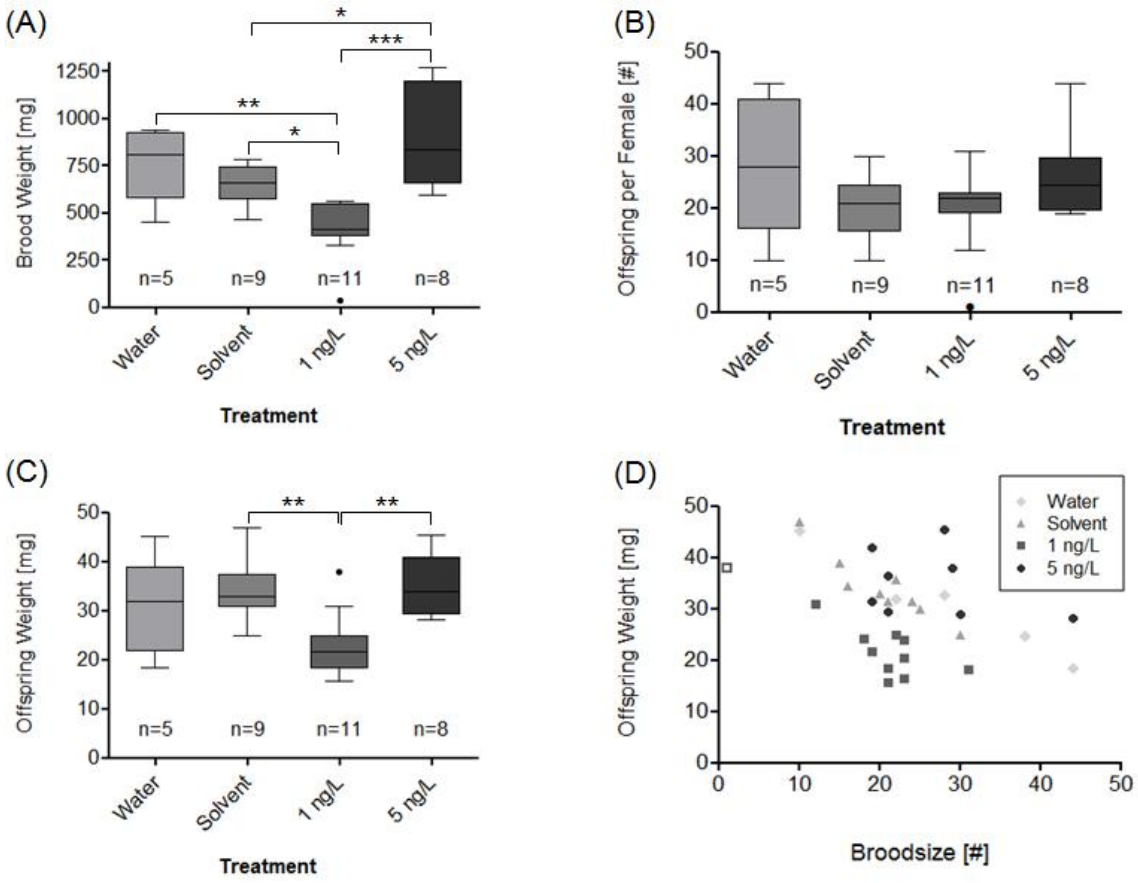


Fig 6.7: Offspring numbers and brood weights. **A**: Total brood weight per female. **B**: Total number of offspring per female. **C**: Average offspring weight calculated from brood weight divided by offspring number. **D**: Correlation of offspring weight versus brood size. **Key**: n = total number of pregnant females per treatment, * = P ≤ 0.05 (significant), ** = P ≤ 0.01 (very significant), *** = P ≤ 0.001 (extremely significant), • = outlier.

There were five pregnant fish in the water control, which collectively produced 142 embryos (Tab 6.3). Three of them were dead at birth, another five died within 24 hours after birth. Nine of the solvent control fish were pregnant with a collective total of 183 embryos of which two were born dead and seven died within 24 hours of birth. Twelve fish of the 1 ng EE2 /L treatment were pregnant, of which one carried dead embryos only: this was not considered to be a treatment related effect and as it was not consistent with any of the other females, it was considered an outlier and excluded from further analysis. The remaining eleven fish had a total collective offspring number of 214 of which 16 were born dead and 17 died within the following 24 hours. In the 5 ng EE2 /L treatment, there were eight pregnant females which carried 211 embryos. One of them was born dead and eleven died within 24h of birth. A summary of viability and survivorship is shown in Fig 6.8.

Some of the fish that were alive at birth but died within 24 hours were less developed than their siblings (Fig 6.9 (B)) or exhibited deformations. The most common abnormality observed was a bent tail (Fig 6.9 (C)). This restricted young fish in their mobility or made movement altogether impossible.

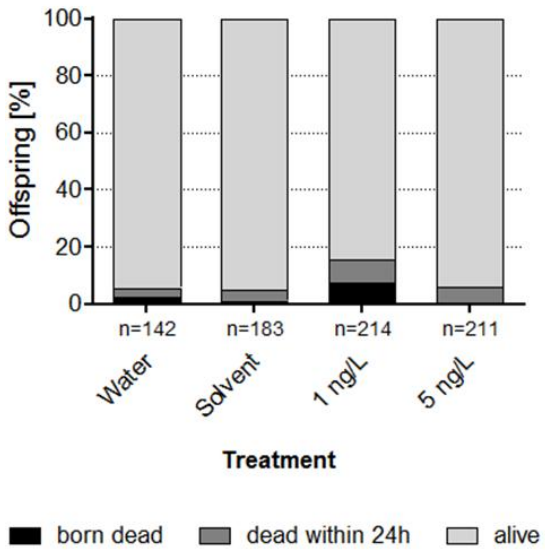


Fig 6.8: Viability and survivorship of the offspring normalised to 100%. **Key**: n = total number of offspring per treatment. See also Tab 6.3.

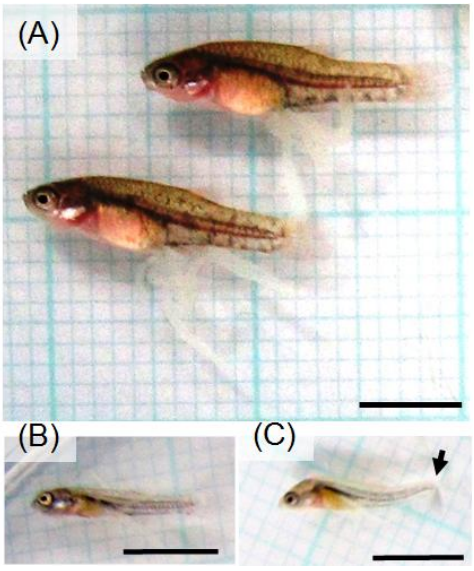


Fig 6.9: Deformities. **A**: Normal sized fish. **B**: Less developed fish. **C**: Less developed fish with bent tail (arrow). Bars: 5 mm.

There were five less developed fish in females of the water control, corresponding to 3.6% of all water control offspring that were alive at birth. In the solvent treatment, there was only one fish with a bent tail (0.6%). Eight young of the 1 ng EE2 /L offspring were underdeveloped and two had bent tails, accounting for a total proportion of 5.0% deformities. In the 5 ng EE2 /L treatment, 5.2% of the offspring exhibited abnormalities – three fish were underdeveloped and eight fish had bent tails. Overall, we would conclude that there was a tendency for more deformities and developmental abnormalities in the females in the EE2 treatments.

6.3.3 Offspring

The majority of the offspring that lived for 24 hours after birth survived the rest of the non-exposure period (see Tab 6.3). There were, nonetheless, 25 fish that died within the first week – five from the broods from the water control and the 5 ng EE2 /L treatment respectively, four from the solvent control and eleven from the 1 ng EE2 /L treatment. Four weeks after birth, a total of 129 offspring from the water control, 170 young of the solvent control and the 1 ng EE2 /L treatment respectively, and 194 offspring from the 5 ng EE2 /L treatment were examined for sex ratio.

Not all broods could be divided into groups of nine to ten fish; therefore, there were a few tanks with a smaller number of fish. Since the growth and development rate of fish in smaller groups might have been different, in turn possibly affecting rates of sexual development (and even gonadal sex itself), these fish were excluded from the analysis on the status of gonadal development and from size comparisons (total body lengths and weight). Total body length of broods from the 1 ng EE2 /L treatment was lower than the body length of all other broods (Fig 6.10 (A)). The same was observed for the average weight (Fig 6.10 (B)) of the offspring from the fish exposed to the nominal concentration of 1 ng EE2 /L. They also had a significantly lower condition factor (Fig 6.10 (C)) than the offspring from the solvent control and water control. There were no differences in any of the body size or condition factors for the offspring of the 5 ng EE2 /L treatment compared with the water and the solvent control.

Tab 6.3: Overview of offspring numbers

Treatment	Total offspring	Born dead	Dead within 24 hours	Transferred to non-exposure tanks	Dead within first week	Examined for sex ratio	Examined for weight, length and sexual development
Water	142	3	5	134	5	129	124
Solvent	183	2	7	174	4	170	155
1 ng/L	214	16	17	181	11	170	166
5 ng/L	211	1	11	199	5	194	194
Total	**750**	**22**	**40**	**688**	**25**	**663**	**639**

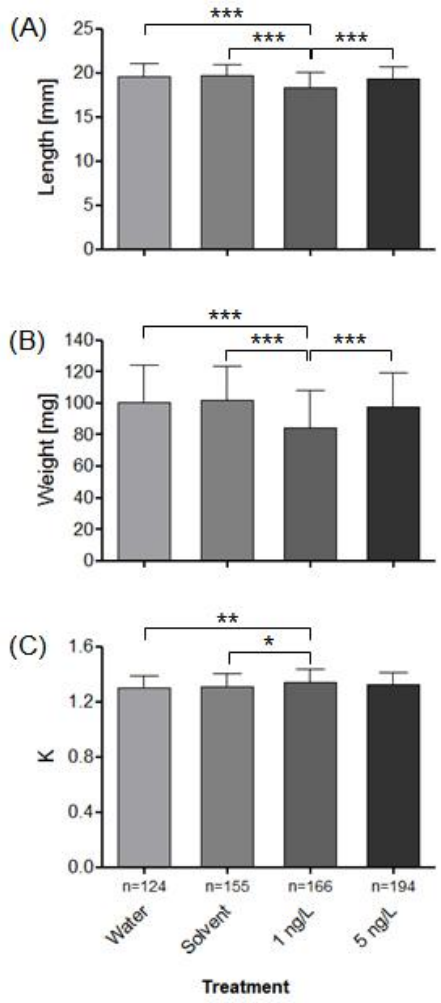


Fig 6.10: Condition of offspring 4 weeks after birth. **A**: Total body length. **B**: Weight. **C**: Condition factor (K = 100 x weight (mg) x length^{-3} (mm)). **Key**: * = P ≤ 0.05 (significant), ** = P ≤ 0.01 (very significant), *** = P ≤ 0.001 (extremely significant).

All broods were of mixed sexes. Across all treatments, sex ratios varied from 20%:80% to 68%:32% (m:f) (see Fig 6.11 (A)). There were no obvious differences between the four treatments and the overall sex rations were 49%:51% in the water control offspring, 47%:53% in both the solvent control and the 1 ng EE2 /L treatment, and 52%:48% in the offspring of the 5 ng EE2 /L treatment (Fig 6.11 (B)).

Status of development of both male and female gonads was evaluated using the staging system described in chapter 4 (Tab 4.2 and Tab 4.3). Male gonads were either in stage 0 (before the onset of spermatogenesis) or in stage 1 (process of spermatogenesis apparent but no formation of sperm packages at that point). Oocytes in

females were also between stage 0 (onset of meiosis) and stage 1 (early primary growth). In order to compare the state of development across treatments, average stages were determined and presented in Tab 6.4. There were no significant differences between the treatments for both sexes.

Tab 6.4: Status of development of male and female gonads in four week old offspring.

Treatment	male	female
Water	0.66±0.26	1.00±0.00
Solvent	0.71±0.16	1.00±0.00
1 ng/L	0.62±0.24	0.99±0.03
5 ng/L	0.64±0.22	0.99±0.04

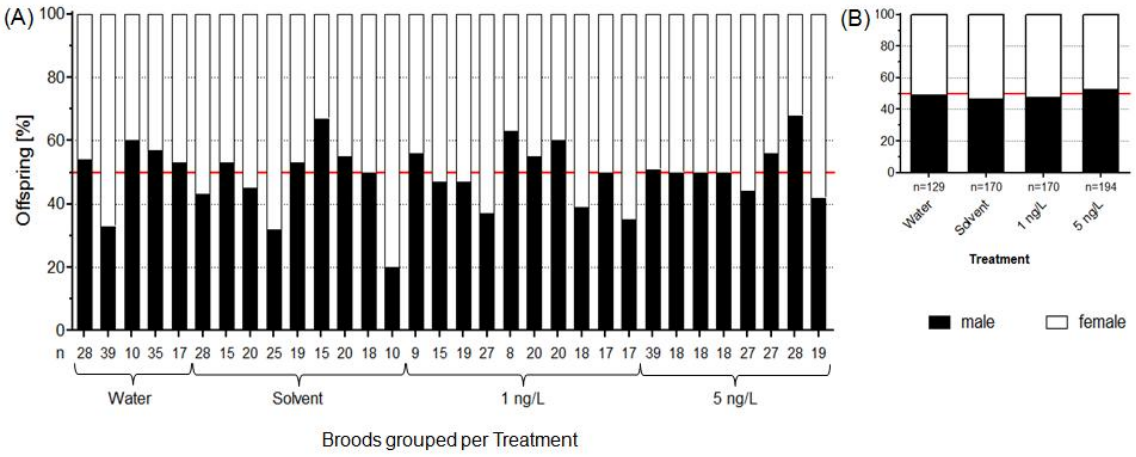


Fig 6.11: Sex ratio of offspring 4 weeks after birth. **A**: Overview on gender proportions in each brood normalised to 100%. **B**: Summarised data for each treatment. **Key**: n = total number of offspring per column; red line = 50%.

6.4 Discussion

Comparing the EE2 concentrations in the water of the inlet and outlet indicated that – owing to the preconditioning of the exposure system – there was no loss of EE2 during the exposure period. The measured concentration of the lower treatment was 0.9 ± 0.2 ng EE2 /L and thus consistent with the nominal concentration of 1 ng EE2 /L. For the higher treatment the analysed concentration of 3.4 ± 0.4 ng EE2 /L was below the nominal concentration of 5 ng EE2 /L, yet still significantly above the lower concentration.

There was no difference in weight, length or condition of fish amongst all treatments before the exposure, showing a non-biased distribution of fish between treatments. Non-pregnant fish did not show any differences in weight, length or condition after the exposure either, indicating that an exposure to up to 3.4 ± 0.4 ng EE2 /L did not have an effect on these endpoints in non-pregnant females. Even though all fish increased in weight and size during the period of exposure, there was a significant loss in condition in all non-pregnant females. Since this effect occurred in all treatments, it cannot be accounted for by the exposure to EE2. It might rather be associated with potential stress fish suffered from handling or from being held in individual small tanks, although hepato- and cardio-somatic indices showed no major signs of stress (albeit these parameters were analysed after the exposure only and there was no way of knowing whether they had changed over time).

No data on relative hepatic vitellogenin expression in *X. eiseni* have been published before. In this study, the fold changes in the relative expression of vitellogenins A, B and C were analysed. Up-regulation was found in fish exposed to 5 ng EE2 /L only. In non-pregnant females exposed to the higher EE2 concentration, there was a trend to an up-regulation of *vtg C* and pregnant females in the same treatment showed a significant up-regulation of *vtg B* and a tendency also for an up-regulation of *vtg A* and *vtg C*. The finding that an up-regulation mainly occurred in pregnant fish may be related to lower vitellogenin levels in pregnant fish which would make them more susceptible to an EE2 exposure. This assumption was confirmed by the comparison of vitellogenin levels in fish of the control treatments. Vitellogenins were expressed at 47.7 -

81.5 (*vtg A*), 35.7 - 63.2 (*vtg B*) and 1.1 - 1.8 (*vtg C*) lower levels in pregnant females compared with in non-pregnant females.

In fish exposed to 1 ng EE2 /L the data for the various endpoints differed compared with that for all other fish. Non-pregnant fish of this treatment, for example, showed only a slight weight increase during the exposure, while all the other non-pregnant fish gained weight significantly. The same was observed for the pregnant fish. Furthermore, pregnant fish of aforementioned treatment had a minimally higher condition factor after the exposure compared to before the exposure, which was significantly different from all the other pregnant fish. A considerable weight gain leading to a higher condition factor is expected in pregnant individuals. The fact that the weight of the pregnant fish exposed to 1 ng EE2 /L turned out to be significantly below the average of the weight of the fish of all other treatments can be explained by the lower brood weight (compare Fig 6.3 (B2) and Fig 6.7 (A)). Broods of fish exposed to 1 ng EE2 /L weighed significantly less than all the other broods. Since there was no difference in brood sizes amongst treatments, the lower brood weight has to be attributed to a lower average offspring weight. As the breeding period before the exposure lasted for ten days, it is possible that the fish transferred to the 1 ng EE2 /L tanks were on average less progressed in gestation than the fish transferred to the other tanks, which would explain the lower weight. This, however, does not explain the differences in viability and survivorship, which, again, was different in the 1 ng EE2 /L treatment compared to all the other treatments.

At four weeks of age, offspring of fish exposed to 1 ng EE2 /L weighed significantly less than all the offspring in all other treatments. They were also significantly smaller in total body length and had a lower condition factor. Sex ratio and status of development was similar to those of the other treatments, however. It could not be clarified whether the smaller sizes of the four week old offspring was simply related to their smaller size and lower weight at birth or if it was due to an effect of the exposure of their mother fish to 1 ng EE2 /L. In the latter case, however, an even more distinct effect on the offspring of the fish exposed to 5 ng EE2 /L would be expected.

In conclusion it can be said that an exposure of pregnant *X. eiseni* to environmentally relevant concentrations of EE2 does not adversely affect offspring development. Being that the expression level of vitellogenin increased approximately ten times in mother fish exposed to the higher treatment concentration it could be assumed that the offspring was not exposed to an EE2 concentration high enough to cause an effect. This implies that EE2 may not get (completely) across the placental barrier or that it may not affect *X. eiseni* embryos. Furthermore, if the programming determining sex takes place earlier during gestation in *X. eiseni*, the window of sensitivity to estrogenic effects might have been missed in this study. In the assumption that fish exposed to 1 ng EE2 /L were less progressed in their gestation than in the pregnant fish of the other treatments, this may explain why these offspring differed from those in the other treatments, since these might have been the only embryos exposed in an earlier (and sensitive) window. It is also not known whether effects on gonadal development in *X. eiseni* are permanent or if potential changes in their sexual organs might have changed (e.g. reverted) during the four week period after the exposure.

In order to monitor embryogenesis of *X. eiseni* in a more continuous way, embryos could be dissected shortly after fertilisation and be investigated *in vitro*. This would allow for monitoring at closer increments in early development, since a smaller number of embryos (instead of whole broods) could be analysed at one time and therefore allowing for more and nearer sampling points. Continuous monitoring would provide a better understanding of the timing and process of gonadal differentiation and development. In a further step, embryos could be exposed *in vitro*. This would not only be easier to handle than an exposure of pregnant females but it would also enable testing a wider range of EE2 concentrations without having to sacrifice as many adult fish. Since there is nothing known yet about endocrinology of *X. eiseni*, a direct exposure of embryos would help determining the potential effect of EE2 on (sexual) development in this species. Starting the exposure at different time points of development could furthermore make it possible to find the most harmful window within ontogenesis. Subsequent investigations involving an exposure of pregnant females to higher EE2 concentrations than in this study here might, in combination with data from embryo exposures, shed more light on maternal transfer of EE2.

7 General Discussion and Conclusions

Results obtained within this thesis are discussed in detail in chapters 4, 5 and 6. This chapter highlights and discusses some of the most significant overall findings, drawing some conclusions and an outlook for future studies on *Xenotoca eiseni.*

7.1 Reproductive Processes

The analysis of the gonads in *X. eiseni* confirmed that the developmental processes are typical for goodeid species and as reviewed in chapter 2. The study on the ontogeny and dynamics of gonadal development revealed that in male *X. eiseni* spermatogenesis starts approximately four weeks after birth and spermatozeugmata are first observed at eight weeks. In female *X. eiseni*, oogenesis is initiated at between two and four weeks after birth and full sexual maturity is reached at an age of approximately twelve weeks. Oocytes in ovaries of mature females include different stages with earlier stages normally occurring in higher numbers. It takes about two months for an oocyte to go through the full process of oogenesis, after which the mature oocyte soon undergoes atresia, unless fertilised. Fertilisation happens shortly after breeding and, as common for goodeid species, sperm storage or superfetation do not take place.

Mature oocytes have an average diameter of 380 µm and are completely filled with yolk. Compared to other viviparous species, this is a large yolk supply, which indicates that developing embryos do not depend on maternal provisioning in early gestation. This assumption is further supported by the finding that embryos start to develop trophotaeniae after two weeks of gestation. Trophotaeniae then grow to their maximum length within the next two weeks, before they begin to regress in the last phase of gestation. A trophotaenial placenta is common in Ophidiiformes and Cyprinodontiformes and occurs in almost all viviparous goodeids. While this placenta type has been described in *X. eiseni* by various authors, the study on embryogenesis and gestation in this thesis identified the presence of a branchial placenta in a number of embryos for the first time. It

remains to be further examined, however, to what extent the branchial placenta may complement the trophotaenial placenta in *X. eiseni*.

For the studies on reproductive processes in *X. eiseni*, results in this thesis indicate that there is a positive correlation between size and fecundity, with smaller (or younger) fish also tending to be less successful in breeding. Different housing periods and diversely sized breeding groups further allowed the conclusions that groups of at least ten females and ten males housed for five days attain the highest breeding success of over 50%, with brood sizes normally ranging from 3 to 44. Since there are no external signs to show if a female *X. eiseni* has been impregnated, adopting a prolonged period of housing males with females is a sensible precaution to best achieve a higher breeding success. On the downside of this approach, however, females may be less synchronised in their pregnancy and this has implications where the desire is in fact to have synchronously developing offspring between the females for any particular study.

In summary, *X. eiseni* possess a common viviparous breeding system amongst goodeids. Their gestation time is six weeks only and breeding is possible at any time of year with an inter breeding interval of under two months. Studies in this thesis demonstrate that *X. eiseni* appear to breed most successfully in larger groups, where more than half of the females turned out to be pregnant. This is a valuable lesson for the production of pregnant females.

7.2 *Xenotoca eiseni* as Model for Studies on Maternal Transfer

After the detailed analysis of the developmental biology of *X. eiseni*, gravid females were exposed to the widely documented environmental contaminant 17α-ethinyloestradiol (EE2). A four-week exposure to up to 3.4 ± 0.4 ng EE2 /L did not have an effect on morphological endpoints in mother fish nor were there any effects on somatic growth, gonadal development or sex partitioning in the developing offspring. Nevertheless, the female fish were responsive to EE2, showing an up-regulation in the expression of hepatic vitellogenin genes. There was a trend to an up-regulation of *vtg C* in non-pregnant females and a significant

up-regulation of *vtg B,* as well as a tendency for an up-regulation of *vtg A* and *vtg C* in pregnant females. The different responses of gravid and non-gravid females to the oestrogen exposure may relate to the naturally lower vitellogenin expression in gestating females. In controls (not treated with EE2) the normal level of vitellogenin expression in non-pregnant female *X. eiseni* was more than 80 times higher than the vitellogenin expression in pregnant females.

In exposed gravid females, there was a 10-fold up-regulation of *vtg B* for an exposure to 3.4 ng EE2 /L (measured concentration). In some other species, for a similar exposure regime responses can be as high as 10,000-fold (for *vtg* transcripts) which indicates that *X. eiseni* may not be especially sensitive to oestrogenic compounds.

Based on studies by Schindler (2014), it is expected that substances will pass from the maternal system to the embryonal system via histotroph and thus affect the offspring's (sexual) development. Even though the embryo is undoubtedly the most vulnerable stage to effects of endocrine disrupters, the outcome of the exposure study supports the hypothesis that *X. eiseni* is not especially sensitive to oestrogens as indicated by no effects of EE2 on gonadal development or sex partitioning. It is possible that maternal transfer of EE2 (and other toxicants) may be highly regulated however, and detailed studies quantifying the uptake of EE2 into developing young is warranted to determine this.

The studies presented overall in this thesis suggests that *X. eiseni* would be a suitable candidate as a new model for studies on maternal transfer in ecotoxicology. This viviparous fish offers the advantages of rapid development, fecundity, ease of maintenance and relatively short assay length. However, further work is required to elucidate more about the sensitivity of this species to toxicants and to better establish the mechanisms of maternal transfer for toxicants. In this endeavour, further investigations are now being conducted by colleagues at the University of Exeter to assess the contaminant transfer into the developing offspring in pregnant female *X. eiseni*. Initial studies indicate that silver readily crosses the maternal-embryonal barrier (personal correspondence).

8 Acknowledgement

First and foremost I would like to thank my supervisor, Prof Charles Tyler from the University of Exeter, for the opportunity he gave me to study this fascinating viviparous fish species. I also greatly appreciate his valuable guidance and the encouragement he extended to me throughout my project. I am further grateful to Dr Anke Lange from the University of Exeter for her great support with the molecular works and for answering my numerous questions. Besides, I am thankful for the help of Dr Arthur David from the University of Sussex with the solid phase extraction. He also kindly eluted the extraction cartridges, conducted the EE2 analysis and therefore wrote the HPLC-MS/MS part in the water analysis chapter (6.2.4). Many thanks also to Victoria Jennings, Eliane Bastos, Jan Shears and Steven Cooper from the University of Exeter for their help with sampling and technical support. Finally, a thank you to both Dr Armin Zenker and Tom Chant for reading my manuscript.

9 References

Ankley GT, Johnson RD. **2004**. Small Fish Models for Identifying and Assessing the Effects of Endocrine-Disrupting Chemicals. ILAR J 45 (4): 469-483.

Bean TH. **1887**. Descriptions of five New Species of Fishes sent by Prof. A. Dugès from the Province of Guanajuato, Mexico. Proc US Nat Mus 10: 370-375.

Bisazza A. **1997**. Sexual Selection Constrained by Internal Fertilization in the Livebearing Fish *Xenotoca eiseni*. Anim Behav 54: 1347-1355.

Blackburn DG. **1992**. Convergent Evolution of Viviparity, Matrotrophy, and Specializations for Fetal Nutrition in Reptiles and Other Vertebrates. Amer Zool 32: 313-321.

Blackburn DG. **2005a**. Evolutionary Origins of Viviparity in Fishes. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 303-317.

Blackburn DG. **2005b**. Amniote Perspectives on the Evolutionary Origins of Viviparity and Placentation. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 319-340.

Brande-Lavridsen N, Korsgaard B, Dahllöf I, Strand J, Tairova Z, Bjerregaard P. **2013**. Abnormalities in Eelpout *Zoarces viviparous* upon Chemical Exposure. Mar Environ Res 92: 87-94.

Brion F, Tyler CR, Palazzi X, Laillet B, Porcher JM, Garric J, Flammarion P. **2004**. Impacts of 17β-Estradiol, Including Environmentally Relevant Concentrations, on Reproduction after Exposure during Embryo-Larval-, Juvenile- and Adult-Life Stages in Zebrafish (*Danio rerio*). Aquat Toxicol 68: 193-217.

Bürgin T. **1990**. Reproduction in Middle Triassic actinopterygians; complex fin structures and evidence of viviparity in fossil fishes. Zool J Linn Soc 100: 379-391.

Burton GJ, Kaufmann P, Huppertz B. **2006**. Anatomy and Genesis of the Placenta. In: Neill JD (ed) Knobil and Neill's Physiology of Reproduction (Volume 1). Third Edition. Acad Press, Amsterdam, 189-243.

Cazan AM, Klerks PL. **2014**. Evidence of Maternal Copper and Cadmium Transfer in two Live-Bearing Fish Species. Ecotoxicology 23: 1774-1783.

Cazan AM, Klerks PL. **2015**. Effects on Life History Variables and Population Dynamics Following Maternal Metal Exposure in the Live-Bearing Fish *Gambusia affinis*. Ecotoxicology 24: 626-635.

Chernyayev ZA. **1974**. Morphological and Ecological Features of the Reproduction and Development of the “Big Golomyanka” or Baikal Oil-Fish (*Comephorus baicalensis*). J Ichthyol 14: 856-867.

Contreras-Balderas S. **2005**. Fish Viviparity: Diversity, Biogeography, and Conservation. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 31-39.

Costa WJEM. **1998**. Phylogeny and Classification of the Cyprinodontiformes (Euteleostei: Atherinomorpha): a Reappraisal. In: Malabarba LR, Reis RE, Vari RP, Lucena ZMS, Lucena CAS (eds) Phylogeny and Classification of Neotropical Fishes (Part 6). Edipucrs, Porto Alegre, Brazil, 537-560.

de la Vega-Salazar MY, Martínez Tabche L, Macías García C. **1997**. Bioaccumulation of Methyl Parathion and its Toxicology in several Species of the Freshwater Community in Ignacio Ramirez Dam in Mexico. Ecotox Environ Safe 38: 53-62.

de la Vega-Salazar MY, Macías García C. **2005**. Principal Factors in the Decline of the Mexican Endemic Viviparous Fishes (Goodeinae: Goodeidae). In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 505-513.

Denslow ND, Chow MC, Kroll KJ, Green L. **1999**. Vitellogenin as a Biomarker of Exposure for Estrogen or Estrogen Mimics. Ecotoxicology 8 (5): 385-398.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. **1998**. Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening. Environ Sci Technol 32 (11): 1549-1558.

Dominguez-Dominguez O, Mercado-Silva N, Lyons J, Grier HJ. **2005**. The Viviparous Goodeid Species. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 525-569.

Dulvy NK, Reynolds JD. **1997**. Evolutionary Transitions among Egg-Laying, Live-Bearing and Maternal Inputs in Sharks and Rays. P Roy Soc Lond B Bio 264: 1309-1315.

Enders AC, Blankenship TN. **1999**. Comparative Placental Structure. Adv Drug Deliv Rev 38 (1): 3-15.

Enders AC, Carter AM. **2004**. What Can Comparative Studies of Placental Structure Tell Us? – A Review. Placenta 25, Suppl A: S3-9.

Eschmeyer WN, Fong JD. **2015**. Catalog of Fishes – Online Version: http://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp, updated on 7th January 2015, retrieved on 20th January 2015.

Filby AL, Tyler CR. **2005**. Molecular Characterization of Estrogen Receptors 1, 2a, and 2b and Their Tissue and Ontogenic Expression Profiles in Fathead Minnow (*Pimephales promelas*). Biol Reprod 73: 648-662.

Filby AL, Tyler CR. **2007**. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. BMC Mol Biol 8: 10.

Fishelson L, Gon O. **2009**. Comparison of the Ovaries and Oogenesis of some Australian and South African Viviparid Clinid Fishes (Clinidae, Blennioidei, Percifromes). Environ Biol Fish 86: 527-540.

Fitzsimons JM. **1972**. Revision of Two Genera of Goodeid Fishes (Cyprinodontiformes, Osteichthyes) from the Mexican Plateau. Copeia 4: 728-756.

Furukawa S, Kuroda Y, Sugiyama A. **2014**. A Comparison of the Histological Structure of the Placenta in Experimental Animals. J Toxicol Pathol 27 (1): 11-18.

Greven H, Brenner M. **2010**. How to Copulate without an Intromittent Organ: the External Genital Structures and Mating Behaviour of *Xenotoca eiseni* (Goodeidae). In: Uribe MC, Grier HJ (eds) Viviparous Fishes II. New Life Publications, Homestead, Florida, 446-450.

Greven H. **2013**. Zur Fortpflanzungsbiologie von Hochlandkärpflingen. In: Kempkes M, Köck M, Stawikowski R (eds) Beiträge zur Biologie und zum Artenschutz der Hochlandkärpflinge: Goodeidae. Edition 1. Westarp Wissenschaften, Hohenwarsleben, 176-216.

Grier HJ, Fitzsimons JM, Linton JR. **1978**. Structure and Ultrastructure of the Testis and Sperm Formation in Goodeid Teleost. J Morphol 156: 419-438.

Grier HJ, Uribe MC, Parenti LR, De la Rosa-Cruz G. **2005**. Fecundity, the Germinal Epithelium, and Folliculogenesis in Viviparous Fishes. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 191-216.

Guerrero-Estévez S, Moreno-Mendoza N. **2012**. Gonadal Morphogenesis and Sex Differentiation in the Viviparous Fish *Chapalichthys encaustus* (Teleostei, Cyprinodontiformes, Goodeidae). J Fish Biol 80 (3): 572-594.

Hedman JE, Rüdel H, Gercken J, Bergek S, Strand J, Quack M, Appelberg M, Förlin L, Tuvikene A, Bignert A. **2011**. Eelpout (*Zoarces viviparus*) in Marine Environmental Monitoring. Mar Pollut Bull 62: 2015-2029.

Helfman GS, Collette BB, Facey DE, Bowen BW. **2009**. The Diversity of Fishes: Biology, Evolution and Ecology. Second Edition. Wiley-Blackwell, Chichester, UK, 736p.

Hoar WS. **1969**. Reproduction – III. Viviparity and Gestation. In: Hoar WS, Randall DJ (eds) Fish Physiology, Volume III – Reproduction and Growth; Bioluminescence, Pigments, and Poisons. Acad Press, New York, 20-40.

Hubbs CL. **1926**. Studies of the Fishes of the Order Cyprinodontes. VI. Material for a Revision of the American Genera and Species. Misc Publ Mus Zool, Univ Mich 16: 1-87.

Hubbs CL, Turner CL. **1939**. Studies of the Fishes of the Order Cyprinodontes. XVI. A Revision of the Goodeidae. Misc Publ Mus Zool, Univ Mich 42: 1-80.

Hutchinson TH, Solbé J, Kloepper-Sams PJ. **1998**. Analysis of the ECETOC Aquatic Toxicity (EAT) Database III – Comparative Toxicity of Chemical Substances to Different Life Stages of Aquatic Organisms. Chemosphere 36 (1): 129-142.

Iida A, Nishimaki T, Sehara-Fujisawa A. **2015**. Prenatal Regression of the Trophotaenial Placenta in a Viviparous Fish, *Xenotoca eiseni*. Sci Rep 5: 7855 (1-5).

Imamura H. **2004**. Phylogenetic Relationships and New Classification of the Superfamily Scorpaeniodea (Actinopterygii: Perciformes). Spec Divers 9: 1-36.

Jordan DS, Evermann BW. **1896-1900**. The Fishes of North and Middle America: A Descriptive Catalogue of the Species of Fish-Like Vertebrates found in the Waters of North America, North of the Isthmus of Panama. Bull U.S. Nat Mus 47 (4 parts): 3313p.

Knight FM, Lombardi J, Wourms JP, Burns JR. **1985**. Follicular Placenta and Embryonic Growth of the Viviparous Four-Eyed Fish (*Anableps*). J Morphol 185: 131-142.

Korsgaard B, Petersen I. **1979**. Vitellogenin, Lipid and Carbohydrate Metabolism during Vitellogenesis and Pregnancy, and after Hormonal Induction in the Blenny *Zoarces viviparus* (L.). Comp Biochem Physiol 63B: 245-251.

Korsgaard B, Andreassen TK, Rasmussen TH. **2002**. Effects of an Environmental Estrogen, 17α-Ethinyl-Estradiol, on the Maternal-Fetal Trophic Relationship on the Eelpout *Zoarces viviparous* (L). Mar Environ Res 54: 735-739.

Kristoffersson R, Broberg S, Pekkarinen M. **1973**. Histology and Physiology of Embryotrophe Formation, Embryonic Nutrition and Growth in the Eelpout, *Zoarces viviparus* (L.). Ann Zool Fennici 10: 467-477.

Labadie P, Hill EM. **2007**. Analysis of Estrogens in River Sediments by Liquid Chromatography–Electrospray Ionisation Mass Spectrometry Comparison of Tandem Mass Spectrometry and Time-of-Flight Mass Spectrometry. J Chromatogr A 1441 (2): 174-181.

Lager S, Powell TL. **2012**. Regulation of Nutrient Transport across the Placenta. J Pregnancy 2012: 179827.

Lange A, Katsu Y, Miyagawa S, Ogino Y, Urushitani H, Kobayashi T, Hirai T, Shears JA, Nagae M, Yamamoto J, Ohnishi Y, Oka T, Tatarazako N, Ohta Y, Tyler CR, Iguchi T. **2012**. Comparative Responsiveness to Natural and Synthetic Estrogens of Fish Species Commonly used in the Laboratory and Field Monitoring. Aquat Toxicol 109: 250-258.

Lombardi J, Wourms JP. **1985a**. The Trophotaenial Placenta of a Viviparous Goodeid Fish. I. Ultrastructure of the Internal Ovarian Epithelium, the Maternal Component. J Morphol 184: 277-292.

Lombardi J, Wourms JP. **1985b**. The Trophotaenial Placenta of a Viviparous Goodeid Fish. II. Ultrastructure of Trophotaeniae, the Embryonic Component. J Morphol 184: 293-309.

Love MS, Yoklavich M, Thorsteinson LK. **2002**. The Rockfishes of the Northeast Pacific. University of California Press, Berkeley and Los Angeles, 404p.

Lund R. **1980**. Viviparity and Intrauterine Feeding in a New Holocephalan Fish from the Lower Carboniferous of Montana. Science 209: 697-699.
Macías García C, Gonzalez-Zuarth CA. **2005**. Reproductive Behavior of Viviparous Fish and Intersexual Conflict. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 289-302.
Mead GW, Bertelsen E, Cohen DM. **1964**. Reproduction among Deep-Sea Fishes. Deep Sea Res 11: 569-596.
Meek SE. **1902**. A Contribution to the Ichthyology of Mexico. Field Columbian Mus Publ 65 Zool Ser III: 63-128.
Meek SE. **1904**. The Fresh-Water Fishes of Mexico North of the Isthmus of Tehuantepec. Field Columbian Mus Publ 93 Zool Ser V: 1-252.
Meisner AD, Burns JR. **1997**. Viviparity in the Halfbeak Genera *Dermogenys* and *Nomorhamphus* (Teleostei: Hemiramphidae). J Morphol 234: 295-317.
Meisner AD. **2005**. Male Modifications Associated with Insemination in Teleosts. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 165-190.
Mendoza G. **1941**. The Reproductive Cycle of the Viviparous Teleost, *Neotoca bilineata*, a Member of the Family Goodeidae. III. The Germ Cell Cycle. Biol Bull 81 (1): 70-79.
Mendoza G. **1943**. The Reproductive Cycle of the Viviparous Teleost, *Neotoca bilineata*, a Member of the Family Goodeidae. IV. The Germinal Tissue. Biol Bull 84 (1): 87-97.
Mendoza G. **1965**. The Ovary and Anal Processes of "*Characodon*" *eiseni*, a Viviparous Cyprinodont Teleost from Mexico. Biol Bull 129 (2): 303-315.
Meyer A, Lydeard C. **1993**. The Evolution of Copulatory Organs, Internal Fertilization, Placentae and Viviparity in Killifishes (Cyprinodontiformes) Inferred from a DNA Phylogeny of the Tyrosine Kinase Gene *X-src*. Proc Biol Sci 254: 153-162.
Miller RR, Fitzsimons JM. **1971**. *Ameca splendens*, a New Genus and Species of Goodeid Fish from Western Mexico, with Remarks on the Classification of the Goodeidae. Copeia 1: 1-13.
Mossman HW. **1937**. Comparative Morphogenesis of the Fetal Membranes and Accessory Uterine Structures. Contrib Embryol Carnegie Inst 26: 129-246.
Nakamura M, Kobayashi T, Chang X-T, Nagahama Y. **1998**. Gonadal Sex Differentiation in Teleost Fish. J Exp Zool 281: 362-372.
Nelson GG. **1975**. Anatomy of the Male Urogenital Organs of *Goodea atripinnis* and *Characodon lateralis* (Atheriniformes: Cyprinodontoidei), and *G. atripinnis* Courtship. Copeia 1975 (3): 475-482.
Nelson JS. **2006**. Fishes of the World. Fourth Edition. John Wiley & Sons, Hoboken, New Jersey, 624p.
Nielsen JG, Cohen DM, Markle DF, Robins CR. **1999**. Ophidiiform Fishes of the World (Order Ophidiiformes) – An Annotated and Illustrated Catalogue of Pearlfishes, Cusk-Eels, Brotulas and other Ophidiiform Fishes known to Date. FAO Fisheries Synopsis 18 (125): 178p.
OECD – Organisation for Economic Cooperation and Development. **2001**. Guidelines for Testing of Chemicals. Test No. 414: Prenatal Development Toxicity Study.
Papoulias DM, Noltie DB, Tillitt DE. **1999**. An *in vivo* Model Fish System to test Chemical Effects on Sexual Differentiation and Development: Exposure to Ethinyl Estradiol. Aquat Toxicol 48: 37-50.
Parenti LR. **1981**. A Phylogenetic and Biogeographic Analysis of Cyprinodontiform Fishes (Teleostei, Atherinomorpha). B Am Mus Nat Hist 168 (4): 335-557.
Parenti LR. **2005**. The Phylogeny of Atherinomorphs: Evolution of a Novel Fish Reproductive System. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 13-30.

Rasmussen R. **2001**. Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K (eds) Rapid Cycle Real-Time PCR, Methods and Applications. Springer Press, Heidelberg, 21-34.

Rasmussen TH, Andreassen TK, Pedersen SN, Van der Ven LTM, Bjerregaard P, Korsgaard B. **2002**. Effects of Waterborne Exposure of Octylphenol and Oestrogen on Pregnant Viviparous Eelpout (*Zoarces viviparus*) and her Embryos *in ovario*. J Exp Biol 205: 3857-3876.

Rasmussen TH, Jespersen A, Korsgaard B. **2006**. Gonadal Morphogenesis and Sex Differentiation in Intraovarian Embryos of the Viviparous Fish *Zoarces viviparus* (Teleostei, Perciformes, Zoarcidae): A Histological and Ultrastructural Study. J Morphol 267: 1032-1047.

Regan CT. **1906-1908**. Pisces. Biologia Centrali-Americana. Taylor and Francis, London, 1-203.

Riehl R, Greven H. **1993**. Fine Structure of Egg Envelopes in some Viviparous Goodeid Fishes, with Comments on the Relation of Envelope Thinness to Viviparity. Can J Zool 71: 91-97.

Rosa-Molinar E. **2005**. Edwin S. Goodrich's Theory of Transposition Revisited: The Shift to a Sexually Dimorphic Axial Formulae and Nervous System in a Poeciliine Fish. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 59-70.

Rosenblatt RH, Taylor LR Jr **1971**. The Pacific Species of the Clinid Fish Tribe Starksiini. Pacific Sci 25: 436-463.

Rutter C. **1896**. Notes on Fresh Water Fishes of the Pacific Slope of North America. Proc Calif Acad Sci 6 (2): 245-267.

Schindler JF, de Vries U. **1987**. Maternal-Embryonic Relationships in the Goodeid Teleost, *Xenoophorus captivus*. Embryonic Structural Adaptations to Viviparity. Cell Tissue Res 247: 325-338.

Schindler JF, de Vries U. **1988**. Maternal-Embryonic Relationships in the Goodeid Teleost, *Xenoophorus captivus*. The Vacuolar Apparatus in Trophotaenial Absorptive Cells and its Role in Macromolecular Transport. Cell Tissue Res. 253: 115-128.

Schindler JF. **2005**. Digestion of Embryotrophic Proteins in Goodeid Teleosts. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 263-272.

Schindler JF. **2014**. Structure and Function of Placental Exchange Surfaces in Goodeid Fishes (Teleostei: Atheriniformes). J Morphol doi:10.1002/jmor. 20292.

Schnorr B, Kressin M. **2011**. Embryologie der Haustiere. 6., aktualisierte Auflage. Enke Verlag, Stuttgart, 288p.

Skov PV, Steffensen JF, Sørensen TF, Qvortrup K. **2010**. Embryonic Suckling and Maternal Specializations in the Live-Bearing Teleost *Zoarces viviparus*. J Exp Mar Biol Ecol 395: 120-127.

Sumpter JP, Jobling S. **1995**. Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment. Environ Health Perspect 103 (Suppl 7): 173-178.

Thibault RE, Schultz RJ. **1978**. Reproductive Adaptations among Viviparous Fishes (Cyprinodontifomres: Poeciliidae). Evolution 32 (2): 320-333.

Turner CL. **1933**. Viviparity Superimposed upon Ovo-Viviparity in the Goodeidae, a Family of Cyprinodont Teleost Fishes of the Mexican Plateau. J Morphol 55 (2): 207-251.

Turner CL. **1936**. The Absorptive Processes in the Embryos of *Parabrotula dentiens*, a Viviparous Deep-Sea Brotulid Fish. J Morphol 59 (2): 313-321.

Turner CL. **1937**. The Trophotaeniae of the Goodeidae, a Family of Viviparous Cyprinodont Fishes. J Morphol 61 (3): 495-523.

Tyler CR, Sumpter JP. **1996**. Oocyte Growth and Development in Teleosts. Rev Fish Biol Fisher 6: 287-311.

Tyler CR, Jobling S, Sumpter JP. **1998**. Endocrine Disruption in Wildlife: A Critical Review of the Evidence. Crit Rev Toxicol 28 (4): 319-361.

Uribe MC, De la Rosa-Cruz G, García-Alarcón A. **2005**. The Ovary of Viviparous Teleosts. Morphological Differences between the Ovaries of *Goodea atripinnis* and *Ilyodon whitei* (Goodeidae). In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 215-235.

Uribe MC, De la Rosa-Cruz G, García-Alarcón A, Guerrero-Estévez S, Aguilar-Morales M. **2006**. Histological features of atretic stages of the ovarian follicles of two viviparous teleost species: *Ilyodon whitei* (Meek, 1904) and *Goodea atripinnis* (Jordan, 1880) (Goodeidae). Hidrobiológica 16 (1): 67-73.

Uribe MC, Grier HJ. **2011**. Oogenesis of Microlecithal Oocytes in the Viviparous Teleost *Heterandria formosa*. J Morphol 272: 241-257.

Uribe MC, Grier HJ, Parenti LR. **2012**. Ovarian Structure and Oogenesis of the Oviparous Goodeids *Crenichthys baileyi* (Gilbert, 1893) and *Empetrichthys latos* Miller, 1948 (Teleostei, Cyprinodontiformes). J Morphol 273: 371-387.

Uribe MC, De la Rosa-Cruz G, García-Alarcón A. **2014**. Branchial Placenta in the Viviparous Teleost *Ilyodon whitei* (Goodeidae). J Morphol 275: 1406-1417.

Uyeno T, Miller RR, Fitzsimons JM. **1983**. Karyology of the Cyprinodontoid Fishes of the Mexican Family Goodeidae. Copeia 1983 (2): 497-510.

Watson DMS. **1927**. The Reproduction of the Cœlacanth Fish, *Undina*. Proc Zool Soc Lond 97 (2): 453-457.

Webb SA. **1998**. A Phylogenetic Analysis of the Goodeidae (Teleostei: Cyprinodontiformes). PhD thesis, University of Michigan.

Webb SA, Graves JA, Macías García C, Magurran AE, Foighil DO, Ritchie MG. **2004**. Molecular Phylogeny of the Livebearing Goodeidae (Cyprinodontiformes). Mol Phylogenet Evol 30: 527-544.

Wooding FBP, Burton GJ. **2008**. Comparative Placentation: Structures, Functions and Evolution. Springer-Verlag, Berlin Heidelberg, D, 301p.

Wootton RJ, Smith C. **2014**. Reproductive Biology of Teleost Fishes. Wiley-Blackwell, Chichester, UK, 496p.

Wourms JP. **1981**. Viviparity: The Maternal-Fetal Relationship in Fishes. Amer Zool 21: 473-515.

Wourms JP, Grove BD, Lombardi J. **1988**. The Maternal-Embryonic Relationship in Viviparous Fishes. In: Hoar WS, Randall DJ (eds) Fish Physiology, Volume XIB – Viviparity and Posthatching Juveniles. Acad Press, San Diego, 1-134.

Wourms JP, Lombardi J. **1992**. Reflections on the Evolution of Piscine Viviparity. Amer Zool 32: 276-293.

Wourms JP. **2005**. Functional Morphology, Development, and Evolution of Trophotaeniae. In: Uribe MC, Grier HJ (eds) Viviparous Fishes. New Life Publications, Mexico, 237-262.