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Gonad Differentiation in Guppy Fish and Testing Strategies for the Production of Sterile and All-Female Offspring

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“Either grant me the bliss of the ignorant or give me the strength to bear the knowledge.”

Elif Shafak, in: The Bastard of Istanbul

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ABSTRACT

The guppy (*Poecilia reticulata*) is a fresh water fish originated from the American tropics but distributed worldwide because of the ornamental fish trade. It is an ovoviviparous multiple breeder and even a single pregnant female is capable of establishing new populations. As a complement to the use of insecticides, it has been introduced in ponds as a biological control against mosquitos. The danger that the introduced fish might become an invasive species makes sterile fish (still unavailable) look like an exciting idea. Several studies have been done concerning the reproductive biology of the guppy and the modulation of its sex distribution, but the early development has been neglected. In accordance, we are aiming both to describe better the gonadal development and to model the guppy sex differentiation. Here we tested the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the first three days after previous parturition, described as the window for germ cells migration, in 10 female guppies while keeping other 10 as control; kept in individual 10 L aquaria at 26 ºC, with a 14:10 photoperiod. Along the 3-days of exposure to GABA (1 mg/L) whole water changes were made with fresh solutions. The new progeny was sampled at 5 dab (days after birth), half for growth and half for histology. After embedding in paraffin, fish were serially sectioned at 4 µm thick (ventral-dorsal direction). A second trial used a 6 days of exposure to GABA (2 mg/L) followed by a 15-day exposure to 17α-ethynylestradiol (EE2, 10 ng/L) and the same histological procedure. The guppy gonadal development begins before birth and continues in some fishes until 5 dab. When individuals are sufficiently differentiated, we estimated by stereology that the ratio between the gonad volumes is 4 times larger for females than for males in both ages. Up until 60 dab there was no significant difference between the body weights of females and males. At 0 dab, 40% of the gonads were found to be undifferentiated and in some particular cases even at 5 dab there were guppies with undifferentiated gonads. The protocol tested in this work for inducing fish sterility, by exploring the disruption of the known GABA effect in the regulation of germ cell development, was not successful. The theoretic feminizing hormone EE2, administered via bath immersion, showed no effect in either the gonad or the BW (body weight). Further studies should focus on tuning the GABA exposure window, increasing EE2 dosage and capturing more stages of the gonadal development. Ultimately, this work offer new histological and stereological data on the guppy gonad formation and is a baseline for further assays to model the species sex differentiation.

RESUMO

O guppy (*Poecilia reticulata*) é um peixe ovovivíparo de água doce dos trópicos da América Latina, distribuído mundialmente graças ao comércio de peixes ornamentais. Foi introduzido em lagos, numa tentativa de controlo biológico contra mosquitos, em alternativa a métodos químicos. O perigo é que os peixes introduzidos se tornem invasivos pelo que a esterilização (ainda não desenvolvida) parece uma ideia que merece ser investigada. Vários estudos têm sido feitos sobre a sua biologia reprodutiva na tentativa de modular a razão entre os sexos à nascença, mas as primeiras fases de desenvolvimento têm sido negligenciadas. O objetivo deste trabalho é descrever o desenvolvimento da gónada e modelar a diferenciação desta. Um dos compostos testados é o ácido gama-aminobutírico (GABA) nos primeiros três dias após o primeiro parto, que é a altura descrita para a migração das células germinativas. Este teste é feito em 10 guppies fêmeas, mantendo outras 10 como controlo, em aquários individuais de 10 L a 26 ºC, com um fotoperíodo 14:10. Ao longo dos 3 dias de exposição ao GABA (1 mg/L) as mudanças de água foram feitas diariamente. A amostragem da descendência deu-se 5 dias após o nascimento. Metade dos animais foram selecionados para crescimento e metade sacrificados e preparados para análise histológica. Após a inclusão em parafina, os peixes foram seccionados a 4 µm de espessura (direção ventral-dorsal). Foi realizada uma segunda experiência com 6 dias de exposição ao GABA (2 mg/L), seguidos por uma exposição de 15 dias a 17α-etinilestradiol (EE2, 10 ng/L) e utilizando o mesmo processo histológico. O desenvolvimento da gonada começa antes do nascimento até 5 dab (dias pós-parto), cada peixe começa a diferenciação com uma dessincronização do desenvolvimento. Quando os indivíduos estão suficientemente diferenciados, a relação entre os volumes das gónadas é 4 vezes maior para as fêmeas do que para os machos. Até 60 dab não houve diferenças significativas entre os pesos das fêmeas e dos machos. Aos 0 dab 40% das gónadas foram encontradas num estado indiferenciado e, em alguns casos, até a 5 dab havia guppies com gónadas indiferenciadas. Os protocolos testados neste trabalho para induzir esterilidade neste peixe, usando o efeito do GABA na regulação do desenvolvimento de células germinativas revelaram-se insuficientes. A hormona feminizante EE2, administrada através de imersão em banho, não mostrou nenhum efeito em qualquer gónada ou no BW (peso corporal). Novos estudos devem centrar-se na melhor janela de exposição do GABA, aumentar a administração de EE2 e estudar mais etapas do desenvolvimento da gónada. Este estudo mostra novos dados histológicos e estereológicos da gónada do guppy, constituindo uma base para modelação experimental da sua diferenciação sexual.

ABBREVIATIONS LIST

A - Area
As – Area of the Section
BW – Body Weight
C – Celsius
CTRL - Control
CV – Coefficient of Variation
dab – Days After Birth
dpf – Days Post Fertilization
E2 - 17β-Estradiol
EE2 – 17α-Ethynylestradiol
g - Gram
GABA – Gamma-aminobutyric Acid
GnRH – Gonadotropin-Releasing Hormones
GV – Gonad Volume
i.e. – Id Est
L - Litre
m – Meter
mg – Milligram
mL – Millilitre
mm – Millimetre
ng - Nanogram
PGC – Primordial Germ Cells
PLGA – Poly(lactic-co-glycolic acid)
PPM – Parts Per Milion
SD – Standard Deviation
SDF-1 – Stromal Derived Growth Factor
V – Volume
Vs – Volume of the Section
Vt – Total Volume of the Structure
WHO – World Health Organization
µg – Microgram
µm – Micrometre
µL – Microlitre

Chapter I – Introduction

I.1 Guppy: From Central-South America to the World

The guppy, *Poecilia reticulata* (Peters 1859) is a larvivorous fish native to the tropics of the American continent, and consequently a prolific breeder in warm waters; the Trinidad population is even often used as a scientific model for population genetics, behavioural and evolutionary questions (Seghers, Shaw, & Carvalho, 1995; Templeton & Shriner, 2004). It is a sturdy species, easily bred in captivity, with sexual dimorphism with and a variety of bright colours (Figure 1), that it especially evident in the males (Seghers et al., 1995). It has been distributed throughout the world because of the ornamental fish trade (Cheong, 1996).

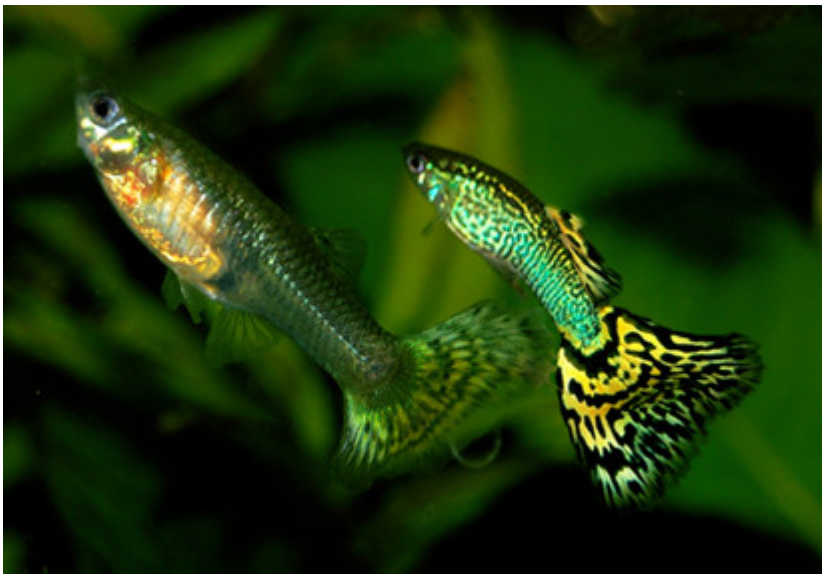

Figure 1: Evidence of the sexual dimorphism between female (left) and male (right) adult guppies. Author: Marrabbio2 in Wikimedia Commons [en.wikipedia.org/wiki/Guppy, access date 20th of September 2016].

Because the sexual dimorphism makes the male guppy more valuable, research on redirecting sex differentiation has been focused on the production of all-male stocks, mostly using hormones, as it will be discussed further on (Basavaraja, Chandrashekhara, & Ahamad, 2014).

On its natural habitat, the species breeds all over the year, except in the winter months, and reaches the peak of its reproductive activity in July. Guppies are ovoviviparous and multiple breeders with a mean egg diameter of 1.02 mm and a fecundity of 40 to 89 eggs per grams of body weight. Gestation periods range from 25 to 35 days. At the time of birth, when the fry is born the body part that

comes out first is the tail. The number of fry per brood goes from 12 to 60 and newborn are transparent or blackish, with slender bodies, developed jaws with a mouth, and fully capable of swimming, eating or displaying evasive behaviour. Sexual maturity is attained at 8 to 10 weeks of age and full body size is achieved in six months (Shahjahan, Ahmed, Begum, & Rashid, 2014).

The sexual behaviour of the guppy females goes through cyclical changes in receptivity in non-virgin females, in which ovarian hormones play an important role (Liley, 1972), with maximum receptiveness shortly after parturition (Liley, 1966). Virgin females are highly receptive when first exposed to courting males but receptiveness diminishes with repetitive exposures (Liley & Wishlow, 1974). As such, short-term incremental effects of courtship may have a role in regulating female receptivity in a way to terminate sexual responsiveness once insemination has occurred several times.

Female fecundity and offspring growth is a family trait, though larger females at the time of mating produce larger broods, but the size at parturition is not relevant. Brood size is inversely proportional to the body size of the neonates and brood retention time is associated with female fecundity as well as offspring growth. Selection may work against the gestation time with an increase in growth and number of offspring (Karino & Ikeuchi, 2011).

Female guppies not only are internally fertilized, but they also have the ability to storage male sperm (Gasparini, Kelley, & Evans, 2014); because older sperm exhibits significant reduction in velocity when compared with younger sperm of the same male, polyandry may be an effective strategy for females to minimize the probability of being fertilized by older sperm.

Despite showing a synchronous development in a single batch of embryos during a reproductive cycle, evidence of asynchronous fertilization has been found by observing an asynchronous growth in the yolking oocytes (Martyn, Weigel, & Dreyer, 2006).

When it comes to food and feeding behaviour the guppy is an opportunistic feeder with a major component of algae in its diet. Males feed at lower food densities and eat more per peck than females of alike weight, though females have extended feeding periods when compared to males, also ingestion per peck increases with size as well as the capacity to exploit varied food sources; sexual differences in reproductive performance also play a role (Dussault & Kramer, 1981). Guppies have been reported to eat benthic invertebrates, dried food (Rose, 1959), zooplankton (Davis, 1968), flies (Murdoch, Avery, & Smyth, 1975), and even

(cannibalistically) their own fry (Rose, 1959). However, it has quite a remarkable aptitude to eat mosquito larvae (Awoyemi, Uwafili, Izegaegbe & Fadeyi, 2014).

In Central Java, Indonesia, a demonstration of the ability of the guppy to decrease the population of the malaria vector, the mosquito *Anopheles aconitus*, was made with a result of a decrease of 99.7% of the population in rice fields (Nalim & Tribuwono, 1987). Furthermore, the rate of consumption was calculated to be 119.4 larva/fish/24 hours, which meant that 2 guppies/m^2 would be enough to obtain a suppression of the anopheline larvae (Nalim & Tribuwono, 1987).

Since 1937, the guppy has been introduced as an agent for controlling mosquito populations in several countries from Asia, Pacific, Africa, and Europe (Jordan, 2013) in an effort to diminish the use of insecticides, by coupling these with biological solutions for larval control (Chandra, Bhattacharjee, Chatterjee, & Ghosh, 2008). Since females have extended feeding periods when compared to males and due to their bigger size, ingest a bigger amount of food per peck (Dussault & Kramer, 1981), these might be the preferable sex to apply to plague control, since it is possible to modulate the entire brood phenotypic sex, as it will be mentioned further on.

The World Health Organization (WHO) has pointed out the need for the control of mosquito populations as a means to fight viruses like Zika, highlighting the importance of integrated approaches to combat all the stages of development, including the introduction of larvae-devouring fish with El Salvador as an example (WHO, 2016).

Having in mind the reproductive effectiveness of the guppy it is important to be aware of the negative impact that its introduction in foreign habitats may have. In fact, it is universally considered a harmful invasive species (Deacon et al. 2014); indeed, the introduction of a single fish can lead to a thriving population, explicitly the introduction of a pregnant female (Deacon, Ramnarine, & Magurran, 2011). But it is not only the species' prolific nature that matters, for example, one way the guppy can harm an endemic population is by harassing females of other species, as observed by Valero, Garcia, and Magurran (2008) with *Skiffia bilineata* in Central Mexico, an endangered ovoviviparous goodeid. Male guppies attempted to court and force copulation with the females of this species even if female guppies were in excess.

I.2 Guppy: Gonadal Differentiation

The original fish model for sex differentiation, and most current fish studies, is the zebrafish (*Danio renio*), an oviparous fish whose gonadal tissue begins its development as an embryonic ovary. Differentiation occurs between 25 and 45 days post fertilization (dpf) into mature ovary or testis (Takahashi, 1977), although there is recent evidence that in the male juvenile zebrafish two processes of gonadal development are in place since not all males develop the initial ovarian tissue (Luzio et al., 2015).

In the zebrafish, gonadal differentiation is influenced by environmental influence like water temperature and contaminants. Exogenous sex steroids administered during the sex determination window can influence its outcome, suggesting that they may have a role in assigning male or female gonad differentiation, which can also be controlled by neuroendocrine and gonadal factors, such as the pituitary gland hormones gonadotropins (Devlin & Nagahama, 2002). High temperature accelerates zebrafish growth rate, gonad differentiation and maturation, with low temperature producing the opposite effect (Luzio, Monteiro, Rocha, Fontaínhas-Fernandes, & Coimbra, 2016). 17α-Ethinylestradiol (EE2) also enhances zebrafish growth in both genders and skewed the sex ratio slightly towards females (Luzio et al., 2016).

The gonadal differentiation was described in detail in a species closely resembling the guppy, *i.e.*, the mosquitofish (*Gambusia affinis*) (Koya, Fujita, Niki, Ishihara, & Miyama, 2003). In this species, the primordial germ cells appeared in the sub-endodermal space of the embryo 14 days before birth, migrated to the dorsal mesentery to form a pair of genital ridges 12 days before birth and finally formed the gonadal primordia 10 days before birth.

Two days before birth, all the gonadal primordia of the mosquitofish are differentiated into ovaries and half of them differentiate to testis just after birth. Following birth, there is an enlargement of the oocytes in the females and the somatic cells go from the hilar region to the inner portion in the males. Fusion of the ovaries happens at 5 dab, resulting in a single ovary at 10 dab with the oocytes beginning vitellogenesis at 100 dab and maturation after 110 dab. In the males, spermatogenic cells form cysts at 20 dab and begin meiosis at 70 dab. Sexual maturation happens first in males (Koya et al., 2003).

The guppy gonads form as two units (Dildine, 1936; Goodrich, Dee, Flynn, & Mercer, 1934), and all embryonic gonads are said to go through an ovarian phase,

which means that males have a period of hermaphroditism (Figure 2). Visible sex differentiation is reported to occur shortly before birth when half the embryos undergo sex reversal to testis. By the time the young are born, their gonads are definitely differentiated and with the expected sex ratio at 1:1. According with Dildine (1936), at a time of the ovarian phase the main criteria for differentiation between sexes is the larger size of the female gonads. The same that the presence of synaptic and pycnotic oocytes in the same gonad indicates that a mix mechanism is in place in the early, hermaphroditic testis. The ovarian cavity and the fusion of the two testis occurs in the few days after birth.

During early testis development, spermatogenesis is initiated under the control of the pituitary and then it is maintained by androgens produced in the testicular interstitial (Leydig) cells (Schulz et al., 2010). To prove these mechanisms in guppies, juveniles were hypophysectomised and this resulted first in the inhibition of the development of gametogenesis and of the Leydig cells, and this then led to undifferentiated secondary sex characters. Administration of methyl testosterone reversed these effects by stimulating the differentiation of the sperm ducts and secondary sex characters but had no effect on the spermatogenesis or on the differentiation of the Leydig cells (Pandey, 1969).

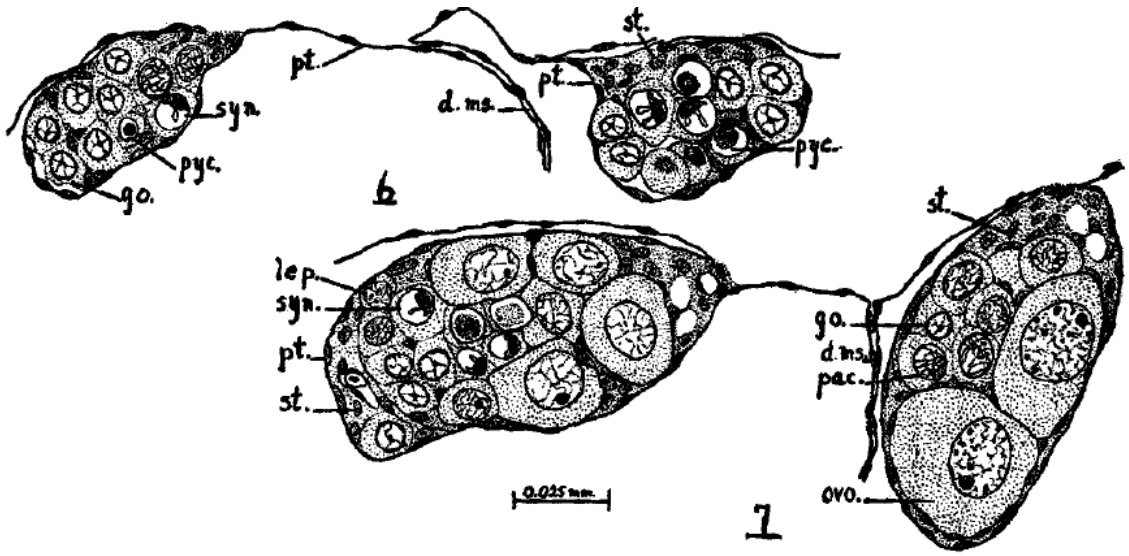

Figure 2: Illustration of early hermaphroditism of the testis in the guppy by Dildine (1936). By the time the fish are born, its gonads are differentiated. Author's abbreviations: d.ms, dorsal mesentery; go, gonium; lep, leptotene; ovo, ovocyte; pt, peritoneum; pyc, pyknotic cell; st, stroma; syn, synizesis.

The next Chapter approaches the sex modulation of the guppy by exposing the pregnant female in a key time window to feminizing or masculinizing

hormones. These studies covering the last two weeks of gestation have identified this period as the one when sex differentiation occurs (Takahashi, 1975a).

In the first stages of growth (Arisaka & Hamai, 1975), not counting newborn fry, the body length of the males is said to be slightly longer than that of females between the first and the eight weeks; although it did not differ statistically. During the nine to ten weeks of age, this relation is inverted and the female grows larger than the male, the typical dimorphism described for this species. Regarding the anal fin, it is situated in an anterior position in the males when compared to the female throughout the immature period since birth, but this is considered a difficult way to discriminate pre-adult guppies in regards to sex.

I.3 Guppy: Masculinization and Feminization Strategies

To enable the masculinization of broods, the synthetic androgens 19-nor-ethynyltestosterone and 17α-ethynyltestosterone were administered to gravid females 5 to 24 days prior to parturition, and succeeded in their predicted purpose (Kavumpurath & Pandian, 1993a). By the contrary, 9(11)-dimethyltestosterone failed in inducing a 100% rate of sex reversal and the highest dose produced a similar effect, but significantly increased mortality of gravid females due to obstructed parturition. The same study concluded that the natural steroid androstenedione was the most potent androgen that ensured masculinization with maximum survival and functional equality when administered in the same time window.

Methyltestosterone is an androgen that was used to reverse the gonadal sex of the female guppy with a treatment of 400 µg/g of feed performed 13 to 15 days before birth (Takahashi, 1975b). It induced a male-type of aggregation of somatic cells in the newly born that still had developing oocytes. These cells then developed into definite male germ cells within 20 days after birth (Takahashi, 1975b). As long as there is masculinization of the somatic elements in the embryo, the masculinization of the germinal elements seems certain. Indeed, a treatment of the gravid guppies with 3-4 mg/L of Methyltestosterone for only 24 hours successfully caused functional masculinization (Dzwillo, 1966).

Norethindrone has also been used for this purpose. Exposing both young fry and breeders caused a dose dependent increased percentage of males, with oral administrations of 75 ppm for 40 days (in the feed) or 100 ppm for 30 days to first feeding fry, yielding 100% of males (Basavaraja et al., 2014). In the same study,

administration to brooders before parturition and the resultant fry also produced an all-male population.

One of the most recent methods is the use of letrozole (a non-steroidal aromatase inhibitor) loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles during the sex differentiation period for complete phenotypical masculinization of the offspring. The ratio of males increased with dose and duration of treatment, and a lesser dose of nano-encapsulated letrozole-loaded PLGA nanoparticles was needed than the “naked" letrozole drug delivery (Joshi, Ghode, & Gore, 2015).

Functional feminization of the guppy has been less tackled, but is not completely overlooked either. 17α-Ethinylestradiol (EE2) at a concentration of 125 µg/g in the food for 30 dab has been proven effective in the induction of 100% endocrine sex reversal (with no male progeny) for a functional feminization (irreversible in the later stages of growth). A larger dose (150 µg/g) and an extended treatment (30 and 40 days) although also effective, caused atypical reproductive functions, infertility or an unstable production of broods with a scantiness in the number of young born. Dienestrol, a synthetic oestrogen, at a dosage of 100 µg/g in the food, and a treatment length of 40 days, did not induce a successful sex reversal (Takahashi, 1975a).

The production of functional feminization can also be used to obtain an YY female guppy, as the first step to a program for the mass production of an YY male broodstock (Kavumpurath & Pandian, 1993b). In such experiments, the oestrogens 17β-estradiol (E2), diethylstilbestrol and EE2 all succeeded in the birth of a 100% female broodstock, or the induction of endocrine sex reversal, while 3-benzoate failed to do so; the compounds were administered to gravid females in the feed, via steroid-supplemented food, for 5 to 24 days prior to parturition.

Feminization by exposure of guppies to EE2 during the gonadal development via bath immersion has produced contradictory evidences to this date. An example is that Kinnberg, Korsgaard, & Bjerregaard (2003) found no sign that EE2 affects gonadal development at a concentration of 0.85 µg/L, but Toft and Baatrup (2003) found a statistically significant female bias in the sex ratio with a concentration of 0.5 µg/L.

I.4 Production of Sterile Fish

In accordance with Nico and Walsh (2011), the production of sterile fish is an important tool for commercial aquaculture for two main reasons:

i) It can potentiate growth;
ii) It is able to prevent escapes from being an environmental danger.

It is also a way to use as an eradication tool that is sensible to a specific species and in being so does not harm the native species or the ecosystem (Nico & Walsh, 2011).

As to being a bonus for production is that sterile fish, since they do not go through the process of gonadal maturation that requires great amounts of energy, are animals that may have an increase in weight by almost 50% and lose less mass after evisceration, which means they have more edible meat (Ali & Rao, 1989).

In what concerns environmental danger, sterile fish can be a safety measure since a non-sterile fish escaping from an aquaculture, especially artificially selected fishes or even transgenic ones, can pose a serious ecological threat (Muir & Howard, 1999). The worst threat is that the aquaculture selection may result in a viability disadvantage, but also a mating benefit, which means that the artificially selected genes will spread with a reduced viability of offspring and finally lead to the local extinction of both natural and artificial groups. There is also a biological risk of invasion and impacts on the habitat if the species is non-native (Daga et al., 2016).

Another use given to sterile fish is the control of invasive fishes with triploids in order to reduce the hatching success of a species in an area with a technique called sterile-male-release (Bergstedt et al., 2003), or Trojan Y (Thresher et al., 2014). In such strategy, sterile males are released in the wild to compete with the fertile population and cause a decline in the population.

In aquaculture, the main strategy used to produce sterile fish is chromosome manipulation by producing triploids that have an enhanced growth (Arai, 2001). However, new technologies are emerging that focus on the elimination of the germ cells. A containment strategy that allows for a sterile offspring and at the same time leaves the parent sterile — by using a transgenic approach to the disruption of the migration of the primordial germ cells — has been achieved in zebrafish (Zhang et al., 2015). Another recent example is a protocol based on non-transgenic gene silencing, by a bath immersion technology of zebrafish embryos immediately after fertilization, which offered 100% sterile progeny up to adulthood, via disruption of primordial germ cell migration and channelling differentiation into somatic cells (Wong & Zohar, 2015a).

Gonadotropin releasing hormone (GnRH) neurons play a key role in vertebrate reproduction during development and throughout life since they are components of the hypothalamus-pituitary-gonadal axis controlling the release of the reproductive hormones (Casoni et al., 2012). Initially emerging in the embryo in the olfactory/nasal placodes, those cells later migrate into the brain; through the cribriform plate when present. According to current knowledge (Bhattacharyya et al., 2008; Cancedda, Fiumelli, Chen, & Poo, 2007), from a scarce number of models, this migration is regulated by two extracellular cues, which are the stromal derived growth factor (SDF-1) and the gamma-aminobutyric acid (GABA). Whereas SDF-1 activates a hyperpolarization of the signalling pathway by a change in potassium and accelerates the migration, GABA depolarizes the signalling pathway via changes in chloride, and slows down the migration. A representation of the regulatory mechanism is offered in Figure 3.

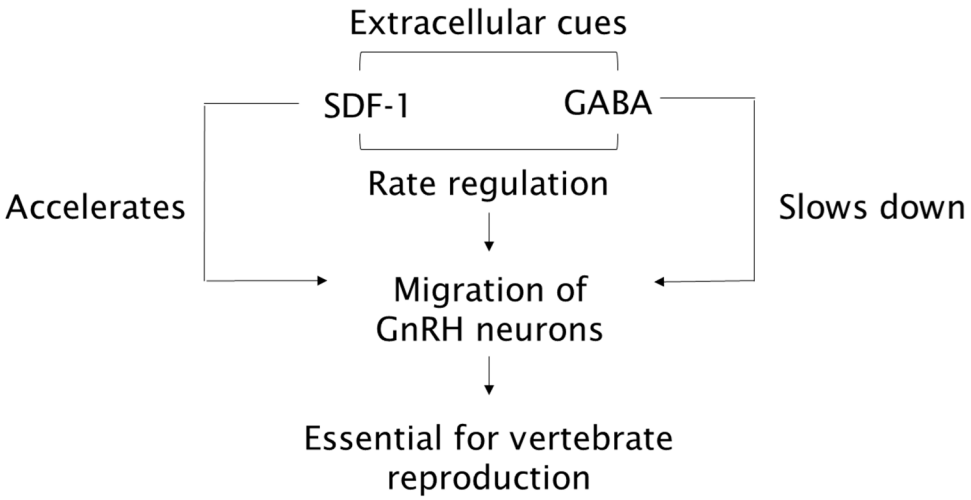

Figure 3: Gamma-aminobutyric acid (GABA), along with stromal derived growth factor (SDF-1), is part of a duo of extracellular cues that regulate the rate of the migration of the gonadotropin-releasing hormone (GnRH) neurons, which are essential for vertebrate reproduction as regulators of gonadotropins. SDF-1 accelerates neural migration and GABA slows it down; a balance between the two not only controls the speed but also ensures that the neurons migrate in a linear direction and not at random.

Because they exert opposite effects on the speed of the cell movement, GABA and SDF-1 promote a linear movement thus holding a tight control in the speed and direction of the migratory pathway of the GnRH neurones (Casoni et al., 2012). The acceleration promoted by SDF-1 is different from the one caused by blocking the GABA signal, which causes an increase in random movement while SDF-1 facilitates directional movement (Casoni et al., 2012).

A gradient of SDF-1 also provides directional input in the migration of the primordial germ cells to the gonadal tissue. Interrupting this signal pathway by creating a transgenic line in zebrafish that expresses SDF-1 everywhere has been shown to be an efficient way to disturb the germ cell migration to such an extent that it results in sterile fish (Wong & Collodi, 2013).

Due to the lack of knowledge still apparent in the signalling pathways of SDF-1 and GABA, there is a great need to screen compounds identified as blockers, antagonists, agonists or disrupters of this system that can successfully lead to an effective, practical, and efficient production of sterile broods (Zohar, Gothilf, & Wray, 2005). In zebrafish, PGC migration happens within 24 hours after fertilization and this is the window proposed for testing in this species (Wong & Zohar, 2015b). The concept should be adapted considering the more opportune time windows for every species of interest.

I.5 Main Objectives

Considering that there are caveats and contradictions in the description of the guppy sex and gonadal differentiations, and bearing in mind the need to improve the environmental safety of the species when used as a biological control of mosquito populations, namely using sterile fish, ideally females (better mosquito eaters), we devised the following goals for the present Dissertation:

- To better describe and illustrate the guppy gonadal development;
- To conceive and test hypothesis driven protocols for inducing fish sterility, namely exploring the GABA potential to disrupt germ cell development;
- To try to model the guppy sex differentiation towards a female phenotype, by exploring the feminizing effects of EE2 at critical times of development.

Chapter II – Materials and Methods

II.1 Fish and Husbandry

Adult guppies originated from Israel were imported and acquired via a local commercial pet fish shop; upon arrival to the laboratory, an hour of acclimatization was performed in which the temperature was adjusted to that of the quarantine tank, and the transporting water was slowly replaced by the one from the in-house system. The fish were kept in quarantine for at least one week before the placement in the experimental set-up.

For experiments, the adult female guppies were kept in individual ten litre aquaria, inside standard ovoviviparous breeding floating boxes; with a bottom grid trap to prevent the mother from eating the offspring. Temperature was kept at 26 ± 1 ºC under a photoperiod of 14:10 (light:dark cycle). Water quality parameters (NO_3, NO_2, and NH_3/NH_4) were checked regularly (every other day) with commercial kits (PRODAC), to guarantee that they were kept in optimal ranges for the species. The pH was checked with a benchtop meter (WTW). In addition, partial water exchanges were made once weekly, along with a general cleaning up of the aquaria. The guppies were fed one to two times a day, with Tropical Fish Flakes (PRODAC). A stock of females and males was kept at a density of ten individuals per ten litre aquaria, with the same husbandry conditions as described before.

In every experiment, after parturition, the juveniles were immediately separated from the mother and left to grow until 5 days of age. At this time, half were collected for histological study and the other half was put into a communal aquarium, in similar conditions as the adults, for growth until having two months of age. Then, they were sacrificed and used for histological analysis of the gonads.

II.2 List of Chemicals and of Equipment for Histological Processing

The chemicals for the exposure were GABA (CAS 56122) with a purity over 99% and a molar mass of 103.12 g/mol; and EE2 (CAS 57636) with a purity over 98% and a molar mass of 296.40 g/mol both by SIGMA-ALDRICH Co.

A list of the chemicals either used as solvents for the tested compounds or in the histological procedures is presented in Table 1.

For processing the biological pieces for histology, the tissue processor Leica TP1020 was used, as well as the embedding device Leica EG1140H and the rotary

microtome Leica RM2255. Sections were collected in microscope slides (KLINIPATH), extended with a GFL 1052 water bath, and mounted under cover slips (MEDITE), using Coverquick 2000 (VWR) as mounting medium.

Pictures of the sections were taken using an OLYMPUS DP21 camera coupled to an OLYMPUS BX50 microscope.

Table 1: List of the chemicals used in this work, their purity and manufacturer.

Compound	Purity	Manufacture
Ethylic Alcohol	96%	Manuel Vieira & Cª (Irmão), Sucrs., Lda
Ethylic Alcohol	99.9%	Manuel Vieira & Cª (Irmão), Sucrs., Lda
Ethylene Glycol Monophenyl Ether	≥ 99.0%	Merck KGaA
Bouin's Solution	-	VWR International S.A.S
Xylene	96%	VWR International S.A.S
Histoplast Pelletised Paraffin Wax	-	Thermo Scientific
Mayer's Hemalum Solution	-	Merck KGaA
Eosin Y (yellowish) (C.I. 45380)	-	Merck KGaA

II.3 Experimental Design

The data presented in this Dissertation was collected in two independent and consecutive experiments, named for practical purposes as A and B.

Experiment A was made up of two conditions, control (CTRL) and GABA exposure (GABA) – in which the compound was dissolved in the water – each with ten replicas (one experimental unit equivalent to one aquarium) distributed with the set-up presented in Figure 4. After randomly assigning the first square, the "black aquaria" (see Figure 4) were the control group in Experience A and solvent control in Experience B; the "white tanks" were for GABA exposure in experience A and EE2 in Experience B (more details below).

In each aquarium, a breeding box was placed with a female kept there until its first parturition.

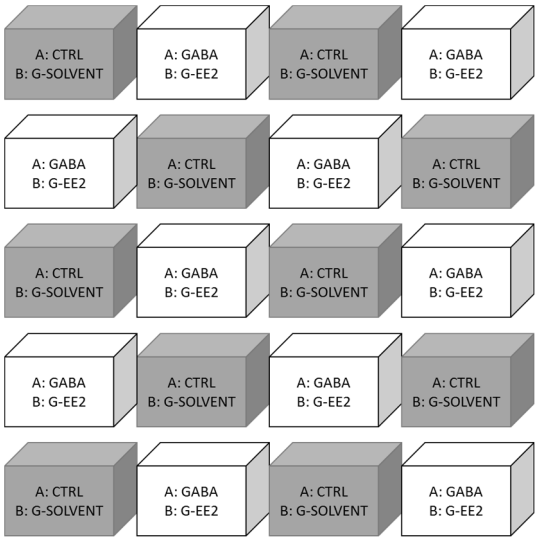

Figure 4*:* Layout used for systematically distribution of the twenty experimental units (aquariums) between the two conditions of exposure in each experiment.

As seen in the schematization on Figure 5, the first parturition is considered day 0, being the fry removed. In order to ensure fertilisation for the next generation, the female was exposed to a male in a 24 hours period between day 0 and 1. The GABA exposure began at day 1, with a concentration of 1 mg/L, and lasted until day 4 (included), with daily whole water exchanges and renovation of the GABA concentration. After the exposure, two complete water exchanges (without GABA) were performed on days 4 and 5 to ensure a zero GABA concentration, and the husbandry procedure mentioned before (**II.1**) was followed until the second parturition. Because of preliminary data (presented in **Chapter III**), a decision was made to extend the sampling until the juveniles were 5 days of age. At this point, half the progeny was sampled for histological procedures and the other half was placed in a communal aquarium so they could grow until two months of age, at which point they followed the described histological protocol (**II.4**).

In the ten replicas for the control condition, the same exact husbandry process was followed (including water exchanges after the first parturition and cohabitation with one male) but no GABA was added to the water.

In experiment B, the same set-up was used, but this time for exposure to both GABA and EE2, consecutively. Because preliminary results showed no obvious malformations after exposure to GABA, in trial B the concentration and duration of the treatment were extended to 2 mg/L for 6 days after the first parturition, specifically from day 1 to 7. At day 15, each replicate fish were exposed to 10 ng/L

of EE2 until second parturition (a stock solution of 10 mg/L of EE2 dissolved in ethanol 99% was beforehand prepared in order to dissolve 10 µL of it in the 10 L aquaria). Another ten replicates were exposed to the solvent for EE2, i.e., ethanol at a final concentration of 0.0001%. The ten replicates that served as control solvent were subjected to the same protocol as described afore, but instead of EE2 dissolved in ethanol, only 10 µL of ethanol were dissolved in the water (10 L).

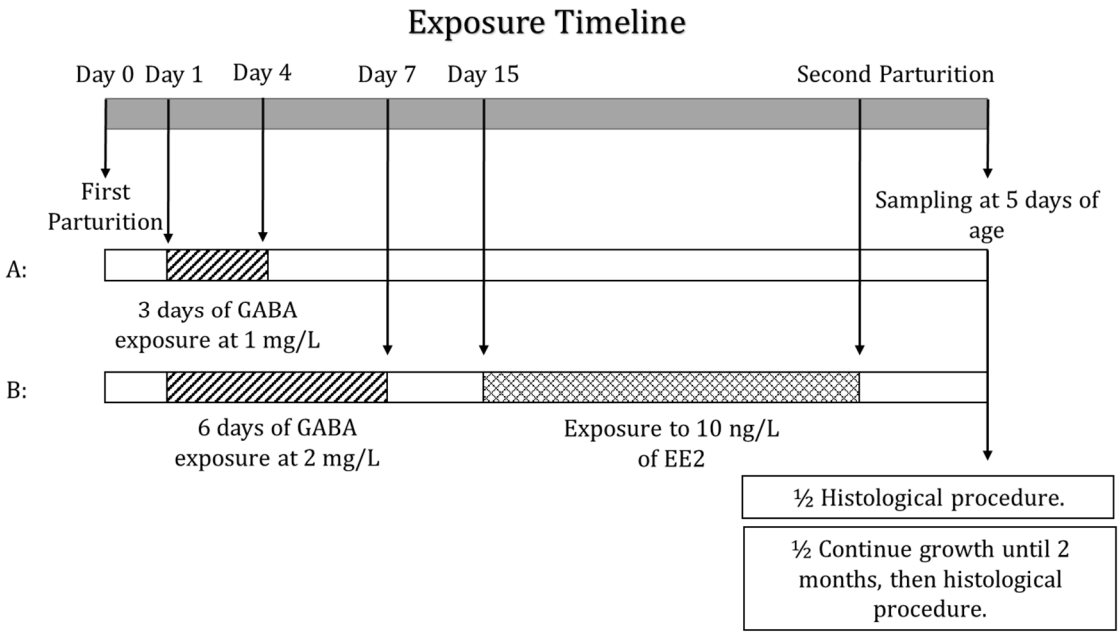

Figure 5: Simplified representation of the design and timeline of experiments A and B. Both start with the female guppy first parturition (day 0) and follow gestation until the second parturition, when an additional 5 days are required before sampling for histological procedure and growth. In experiment A, there is a GABA exposure of 1 mg/L during 3 days after the first parturition. Experiment B begins with the first 6 days of exposure to GABA at 2 mg/L and at day 15 starts the exposure to 10 ng/L of EE2 until the second parturition. Blank and solvent controls are omitted from the illustration.

Following the procedures for experiment A, in the assay B also 5 days after birth were given for the fish to grow before the sampling. At this time, half of the juveniles were sampled for histology and the other half remained in the set-up so they could grow until 60 days (2 months) of age.

II.4 Necropsy and Histology

In order to observe the samples with brightfield microscopy, tissue processing was needed in advance. The process began with euthanizing the fish (the juveniles) by an overdose of ethylene glycol monophenyl ether, at a

concentration of 1 µL/mL. After confirming that there was no opercular movement and there was a loss of all reflexes, the tiny animals were then placed in 2 mL of Bouin's fixative solution; a mixture composed of picric acid (which also served as a mildly decalcifying agent), formaldehyde and glacial acetic acid (a protein denaturant).

After the fixation for 24 hours, it followed the impregnation of the samples with a solid medium for sectioning, done with an automated tissue processor (Figure 6A). This replaces water for paraffin by first dehydrating the fixed pieces with an ascending series of alcohol (70, 95, and 100%), followed by their clearing (replacement of the alcohol by xylene), and finally by their embedding, in which the xylene is replaced by paraffin. The whole protocol is presented in Annex A.

After having the sample embedded in paraffin (with the embedding device, Figure 6B), followed the sectioning using a rotary microtome (Figure 6C), where 4 µm thick sections were produced; in a ventral-dorsal direction of the fish. The sections were placed on a glass microscope slide and extension was made using a water bath at 55 ºC. Before the staining routine, the slides were placed for at least 2 hours in an incubator at 60ºC.

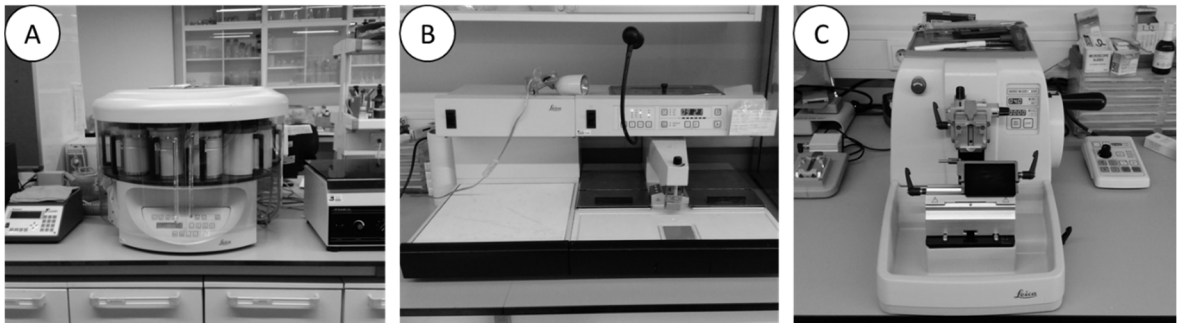

Figure 6: Tissue processor Leica TP1020 (A), embedding device Leica EG1140H (B), and rotary microtome Leica RM2255 (C).

The slides with the sections proceeded to a routine staining which started with a dewaxing process that removes the paraffin in excess with xylene. Because the dyes used in the staining are aqueous, the tissue had to be hydrated in a series of descending alcohols (100, 95 and 70%). The dyes used are Mayer's haematoxylin for purple nuclear staining and eosin for cytoplasm and fibres; together they show the general structural design of the tissues. After staining, a dehydration process was repeated with an increasing series of alcohol and the tissue sections were then cleared with xylene. Mounting was done by placing mounting medium and a coverslip above it. The complete protocol is presented in Annex B.

II.5 Analysis of the Gonad Differentiation

By carefully analysing the literature (Dildine, 1936; Goodrich et al., 1934), and particularly how the authors described the different stages of gonadal differentiation, a preliminary classification method was designed with the purpose of assigning a sex to each individual fish according to the gonad morphology and this is described in Table 2.

Table 2: Classification of the guppy gonad morphology, from the undifferentiated ovary to the differentiation of ovary and testis, based on Dildine (1936) and on Goodrich et al. (1934).

Type	Description
Undifferentiated Gonad	Undifferentiated, oogonia are still present.
Early Ovary Gonad	Early ovary, most cells are either oogonia or oocytes.
Early Testis Gonad	Early testis, most cells are spermatogonia.
Ovary	Completely differentiated ovary. Oocytes larger than 20 µm of diameter.
Testis	Completely differentiated testis.

II.6 Stereological Analysis

The volume of the juvenile guppy gonad was estimated using the so-called Cavalieri's principle (Cavalieri, 1635). Adapted to histology (Howard & Reed, 2004), it consists in the preparation of a series of sections, as thin as possible, of the structure to be measured. The areas of the anatomical target of interest are then measured in all (or in a subsample of) the sections. Although in stereology the measurement of the areas is usually done with a point grid, herein it was more practical to determine the area of each gonad in a section (A) by using digital images and with the program Image J (calibrated for magnification). The areas of each gonad were summed across all sections, and the result was multiplied by 4 µm (the nominal section thickness), for getting an estimation of the organ volume (V):

$$V\,(\text{gonad}) = 4 \times \Sigma A\,(\text{gonad})$$

II.7 Statistical Analysis

The statistical analysis was performed with the programs Past 3.12 (Hammer, Harper, & Ryan, 2001) and STATISTICA 13.1 (Dell), and with the Website for Statistical Computation (VassarStats.net). The latter was used solely for comparing proportions with either the chi-squared or Fisher's exact probability tests. Logarithmic transformations were done when variables did not meet the assumptions of parametric tests. In every test, the significance level was set at 0.05.

According to the exact experiment and data set in analysis, the tests performed with Past were: One-Way ANOVA, Levene's test for homogeneity, Welch F test in case of unequal variances, Shapiro-Wilk's W test for normality, Tukey's pairwise test, Kruskal-Wallis ANOVA on ranks (used for non-parametric data sets), and Mann-Whitney's (post-hoc) pairwise test with p-values corrected by the sequential Bonferroni approach. For the One-Way ANOVA, whenever there was no homoscedasticity, the Welch F test was used instead, and whenever there was no homogeneity and no homoscedasticity, Kruskal-Wallis was done. In post-hoc analyses after a significant One-Way or Kruskal-Wallis ANOVA, when there was no homogeneity or homoscedasticity, the Bonferroni corrected Mann-Whitney was the test of choice. Anyway, the parametric and non-parametric approaches provided the same set of significant differences. Since Past 3.12 does not offer post-hoc analyses for Two-Way ANOVA, this test and subsequent procedures were made with the STATISTICA. In the absence of a consensual non-parametric alternative for the parametric Two-Way ANOVA, even in the few cases where we could not statistically confirm both normality and homoscedasticity these were assumed to occur. This is regarded an acceptable approach due to the general good robustness of the parametric tests from departures of those two assumptions (McKillup, 2011; Rasch & Guiard, 2004; Schmider, Ziegler, Danay, Beyer, & Bühner, 2010).

Chapter III – Results

III.1 Gonadal Differentiation in Guppy from Unexposed Mothers

Body Weight

The body weight (BW) with age (as assessed in the progeny of non-exposed mothers) is presented in Figure 7. At 0 dab the mean body weight, presented as mean (standard deviation), was 19 (11) mg, with a coefficient of variation (CV) of 56%. At 5 dab the mean body weight was 14 (7) mg, with a CV of 46% and finally, at 60 dab the mean was 35 (22) mg with a CV of 63%.

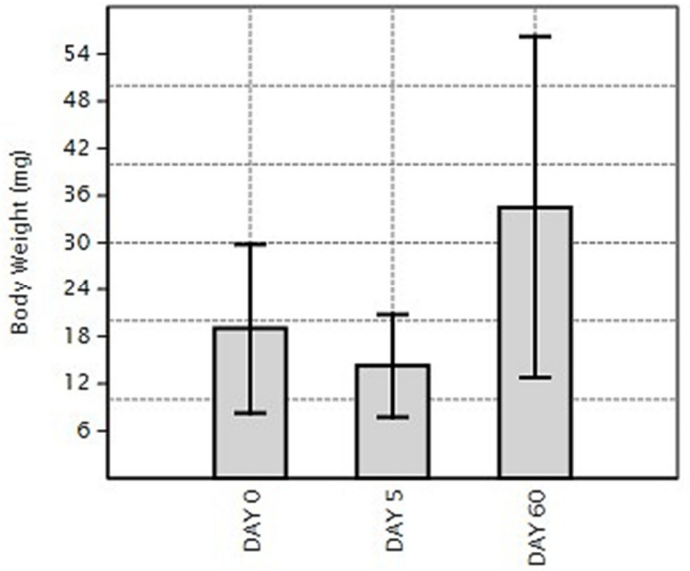

Figure 7: The body weight (mg) with age (days after birth). The bars represent the standard deviation. Day 0, n = 24; Day 5, n = 21; Day 60, n = 20.

As to the statistical analysis, the One-Way ANOVA test for equal means between groups had a p-value of 7.488×10^{-6}, which means there is a significant difference between the BW of 0, 5 and 60 dab. Despite the fact there is no homoscedasticity (Levene's test; p = 0.0004), the Welch F test (p = 0.0012) backed the ANOVA. The Tukey's test revealed significant differences between 60 and 0 dab (p = 0.0002), and between 60 and 5 dab (p = 0.0001). Accordingly, the Kruskal-Wallis test for equal medians rejected the null hypothesis ($p = 4.424 \times 10^{-6}$) and the Bonferroni corrected Mann-Whitney test also gave significant differences between 60 and 0 dab (8.736×10^{-5}) and between 60 and 5 dab (2.957×10^{-6}).

Table 3 presents these same results but segregated per sex. At 0 dab some individuals were still too undifferentiated to be possible to make an accurate visual evaluation of the sex, and so they were considered a group apart.

Table 3: Body weight (mg) distribution according with sex, from the juveniles collected from the control group of experiment A.

	Days of Age						
	0			5		60	
	♂ (n = 6)	♀ (n = 8)	[?] (n = 10)	♂ (n = 6)	♀ (n = 15)	♂ (n = 9)	♀ (n = 11)
Mean	18	20	13	14	14	32	37
SD	13	9	7	6	7	18	25
CV	74%	46%	54%	40%	49%	58%	68%

[?] – Undifferentiated fish at 0 dab.

When splitting the data by sexes, the "n" per group decreased, and, contrarily to the global One-Way ANOVA, the Two-Way ANOVA analysis of BW failed to show significant differences according to the fish age ($p = 0.1390$). In addition, the analysis did not evidence significant differences between males and females ($p = 0.7739$) and no effect of interaction between age and sex ($p = 0.9240$). The CVs show that there is a very high biological variability among animals (at least up to 60 days of age).

Gonad Volume

The evolution of the gonad volume (GV) from 0 to 5 dab is presented in Table 4. To test the significance of the increase in the GV between the ages of 0 and 5 dab, a Two-Way ANOVA test for means was performed considering age and sex. In order to achieve homogeneity of variances, a logarithmic transformation was performed (Levene's test: $p = 0.1648$). The Two-Way ANOVA gives no significant interaction between the two variables ($p=0.1066$), although there is a significant difference in the mean BW between ages ($p=0.0050$) and sexes ($p=0.0000$). Using the Tukey's test for an analysis for unequal N, it was determined that there is a significant difference between the means of GV of males and females at 0 dab; the females of 0 and 5 dab and between the males and females at 5 dab.

As to the variability of the GV, as evidenced by the values of CVs for each age and type of gonad (Table 4), there is an increase with age and in both sexes. In spite of this, the volume ratio ovary:testis at each age is kept stable (around 4).

Table 4: Gonad volume (10^3 μm^3), for females and males, and volume ratio ovary: testis (ratio O:T). Volumes given as mean, SD, and CV.

	0 dab		5 dab	
	Ovary (n = 8)	Testis (n = 6)	Ovary (n = 15)	Testis (n = 6)
Mean	1242	318	1786	442
SD	415	61	874	299
CV	33%	19%	49%	68%
Ratio O:T	3.9		4.0	

At the end of this work, it was possible to measure the GV of four additional guppies at 10 dab, one male and three females. Facing the low "n", particularly for males, we opted to make an additional exploratory analysis including the new data (plotted in the Figures 8 and 9). (These results do not have the corresponding BW due to data loss for fish at 10 dab.)

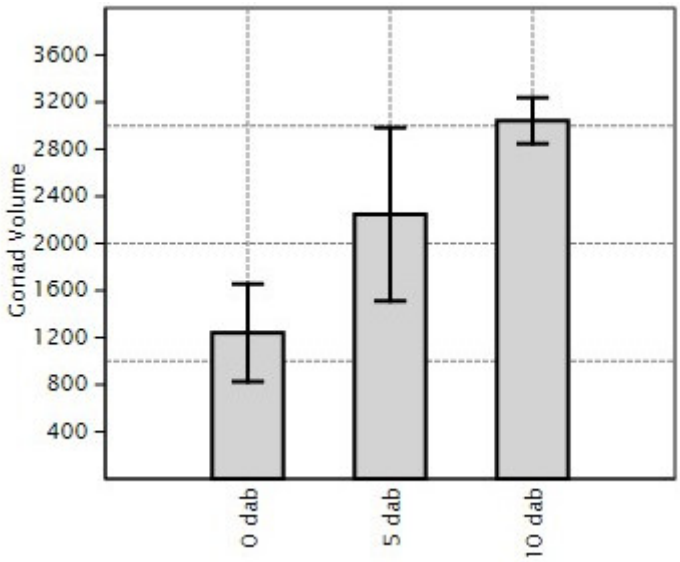

Figure 8: Mean gonad volume (10^3 μm^3) with age, in females at 0, 5 and 10 dab. Bars represent standard deviations.

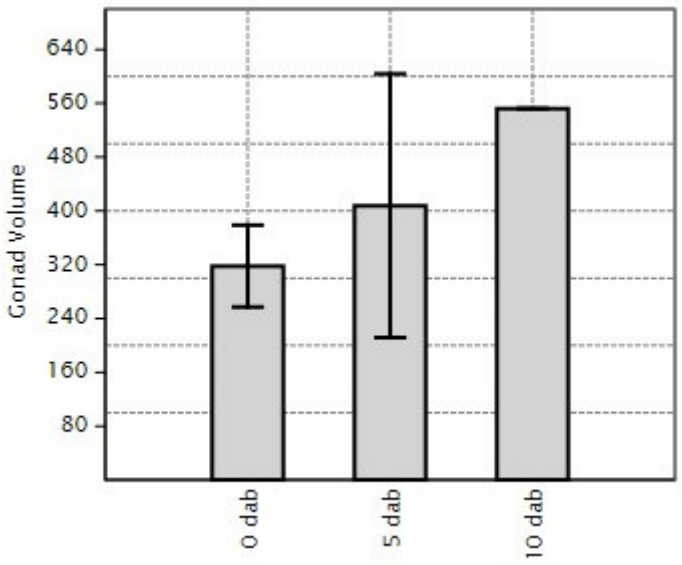

Figure 9: Mean gonad volume (10^3 µm^3) with age, in males at 0, 5 and 10 dab. Bars represent standard deviations.

The addition of the preliminary results of the age of 10 dab, that were also transformed with a logarithmic function in order to have an homogeneity of variances, added to the tendency seen before. There is a significant difference between the GV means when it comes to age ($p = 0.0012$) and sex ($p = 0.0000$) and no interaction exists between the two variables. There is a significant difference between the GV of males and females at all ages and a significant difference was observed in the females between the three ages (0, 5 and 10).

Histological Study

The serial sectioning of the gonads provided the opportunity to undertake a descriptive histological observation and collect a diversified set of representative images of the gonadal differentiation, from 0 dab to 2 month-old young adults. In this section, we will offer the imagery, correlating the qualitative aspect with the specific quantitative parameters of the gonad under illustration.

The Figures 10 and 11 show undifferentiated gonads at 0 dab, with the phenotype of ovary-like organs containing immature early oocytes. The gonad in Figure 10 has a volume of 1793 x 10^3 µm^3 in a fish with a BW of 18.5 mg. Figure 11 is of a gonad with a volume of 779 x 10^3 µm^3 from a BW of 18.1 mg.

Figure 12 is an example of a 0 dab female with a GV of 1454 x 10^3 µm^3 and a BW of 13.4 mg. The fish developing true ovaries start to present several primary oocytes in chromatin nucleolar oocytes and even perinucleolar stages. A male of the same age is shown in Figure 13, corresponding to a fish with a GV of 735 x 10^3

μm^3 and a BW of 15.0 mg. In the latter animal, the contingent of stromal cells is increased and cells organize in clusters, forming spermatogonia cysts.

At 5 dab, a few undifferentiated fish were found (in the sense that a definitive diagnosis as male or female could not be made), but they are considered particular cases. One example of one such fish is given in Figure 14, with a GV of 1340 x 10^3 μm^3. In this specific animal, the presence of acid (eosinophilic) staining bodies/cells may indicate oocyte degeneration and consequently a process of differentiation towards testis.

Examples for female and male gonads of fish with 5 dab are given in Figures 15 and 16, respectively. The ovary in Figure 15 has a GV of 1897 x $10^3 \mu m^3$ and a BW of 5.6 mg, while the testis in Figure 16 has a GV of 759 x $10^3 \mu m^3$ and a BW of 9.5 mg. At this age, primary oocytes in perinucleolar stage have clearly increased sizes and appear much more frequently. On the other hand, males have numerous and much better-defined spermatogonia cysts along the testis.

At 10 dab, undifferentiated ovary-like gonads were not found. An example of an ovary is given in Figure 17 (from a fish with a GV of 2975 x 10^3 μm^3) and of a testis in Figure 18 (from a fish with a GV of 552 x 10^3 μm^3). In females, the overall increase in the oocyte size continues, in line with the incrementing ovarian volume.

Examples of ovaries and testis at 60 dab are given in Figures 19 and 20 and finally, examples of ovaries and testis of seemingly fertile adult guppies are given in Figures 21 and 22. Females at 60 dab show not only oogonia and initial primary oocytes, but also frequent oocytes in cortical alveoli stage and beyond. As to the males, germ cell cysts contain from spermatogonia to (at least) spermatids. Adults exhibit the normal excepted structure, displaying all developmental stages of the respective gametes. The males depict the restricted spermatogonial testis type, with increasingly mature lobules being seen at testis axis (Figure 21).

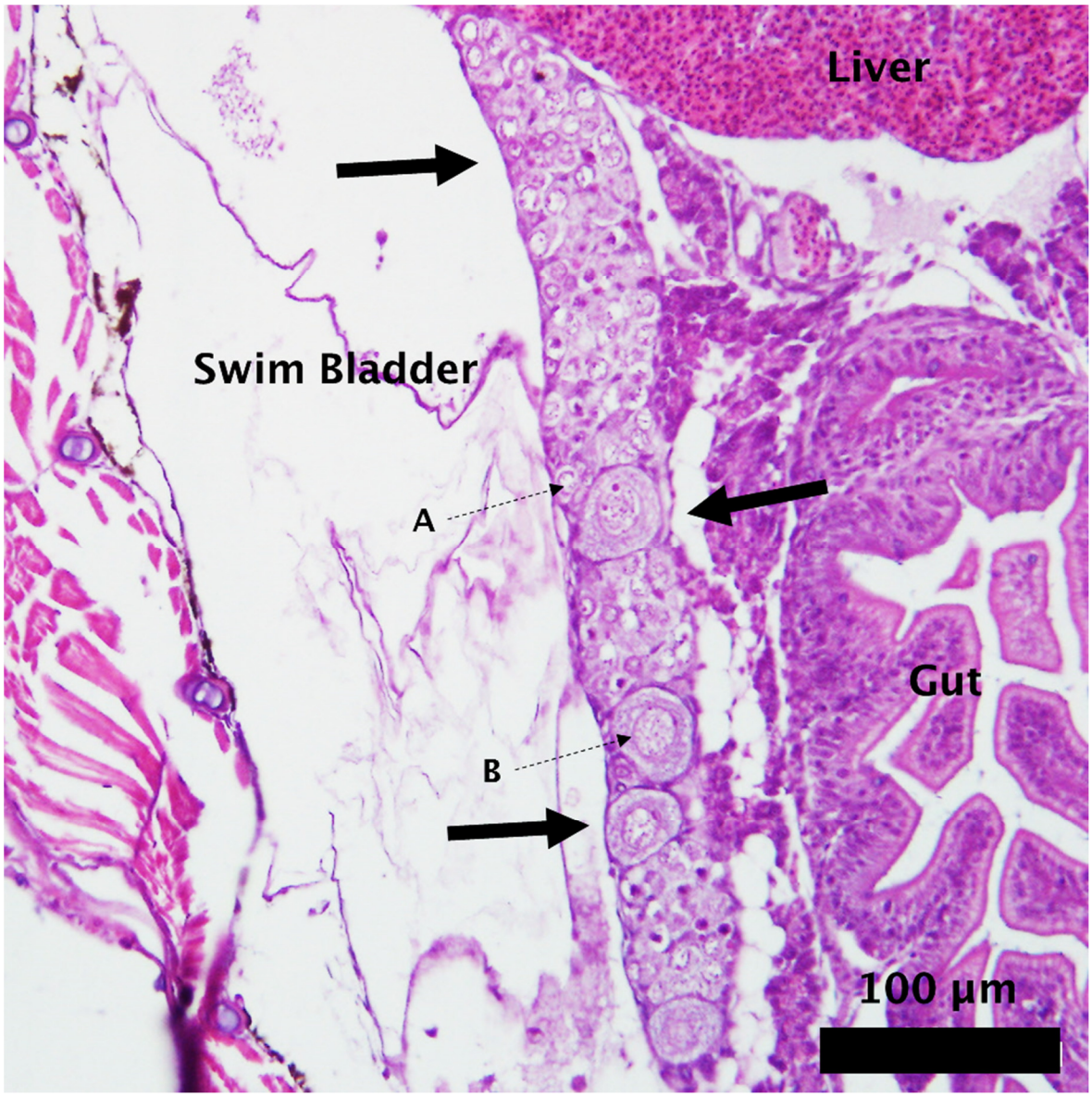

Figure 10: A guppy's undifferentiated ovary-like gonad (arrows), at 0 days of age. The gonad is elongated and regular in profile. The smaller round to oval cells with scant cytoplasm are correspondent to oogonia and early primary oocytes (A). They exist both at the periphery and at the inner parts of the gonad. There are also a few chromatin nucleolar oocytes evolving to perinucleolar (B), bigger cells characterized by the larger nucleus with one or already more noticeable nucleoli.

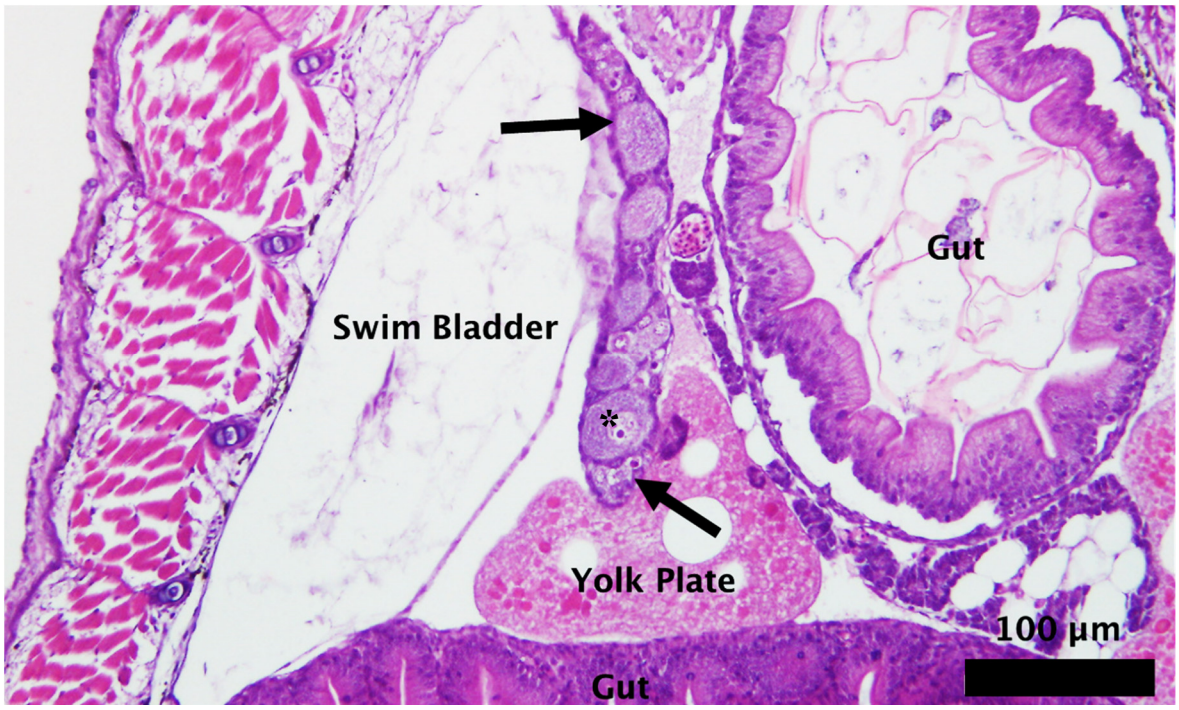

Figure 11: A guppy's undifferentiated gonad (arrows) at 0 days of age. A few oogonia exist (the cells with scant cytoplasm). They occur throughout the gonad without a visible pattern. Most of the volume is occupied with chromatin nucleolar oocytes, the larger cells with big nucleus and one nucleolus (*).

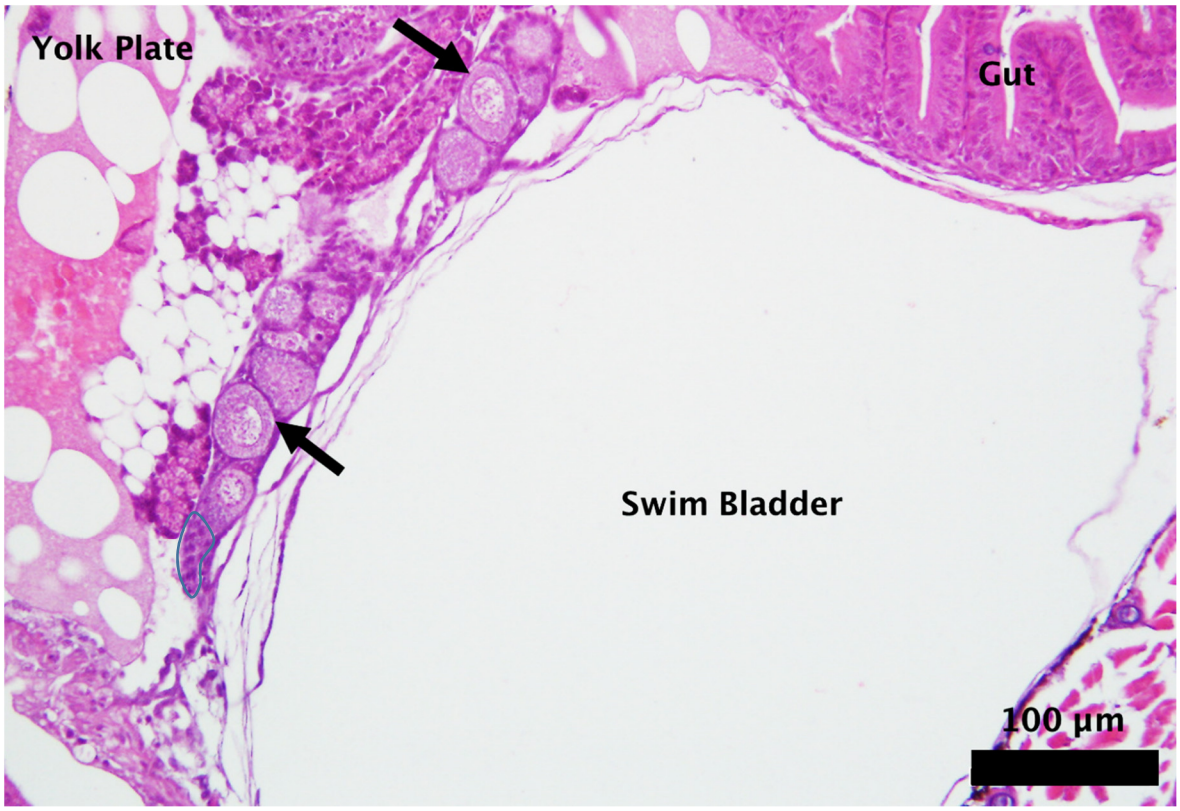

Figure 12: A guppy's female gonad (arrows) at 0 days of age, with an elongated shape, with very few oogonia (encircled), small cells with scarce cytoplasm. The majority of the gametes are in the stage of chromatin nucleolar oocytes, appearing as larger cells with a relatively big nucleus bearing one-to-few small nucleoli.

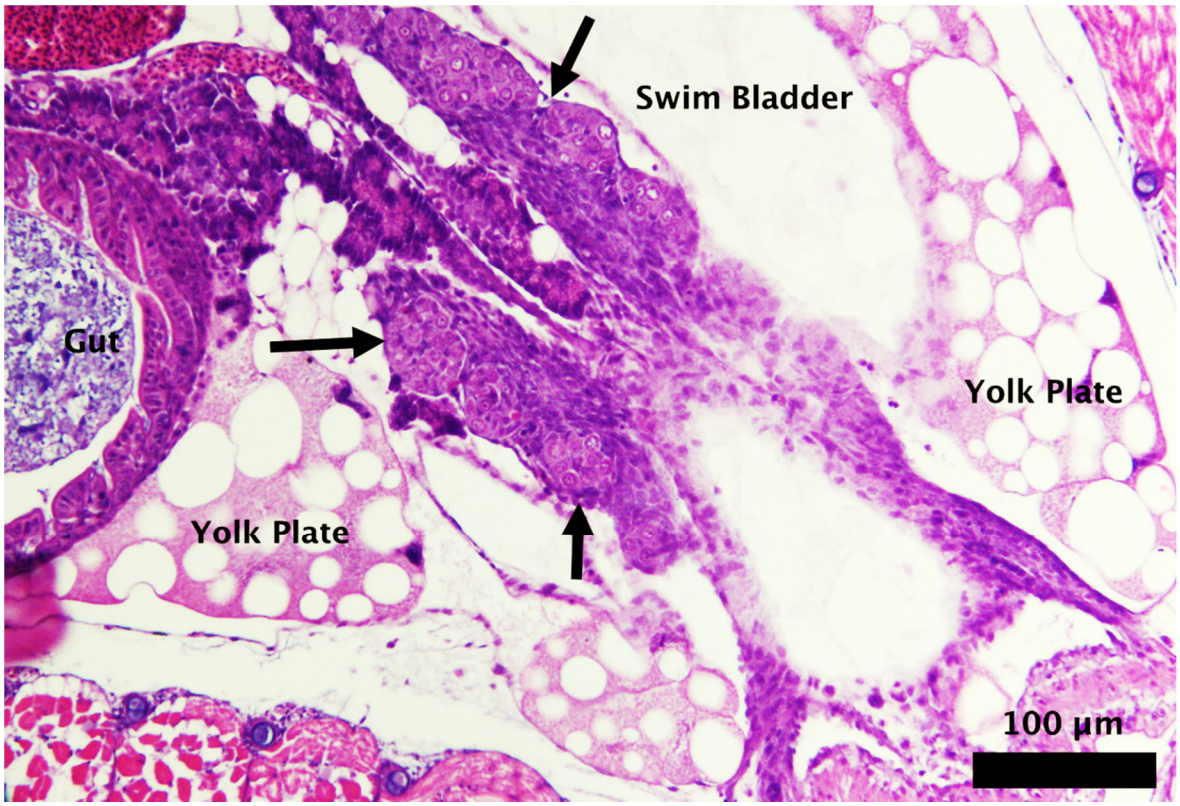

Figure 13: A guppy's male gonad (arrows) at 0 days of age, with germ cell cysts already developed in a gonad that is segregated into two units. All germ cells are spermatogonia, appearing as small roundish cells with scarce cytoplasm.

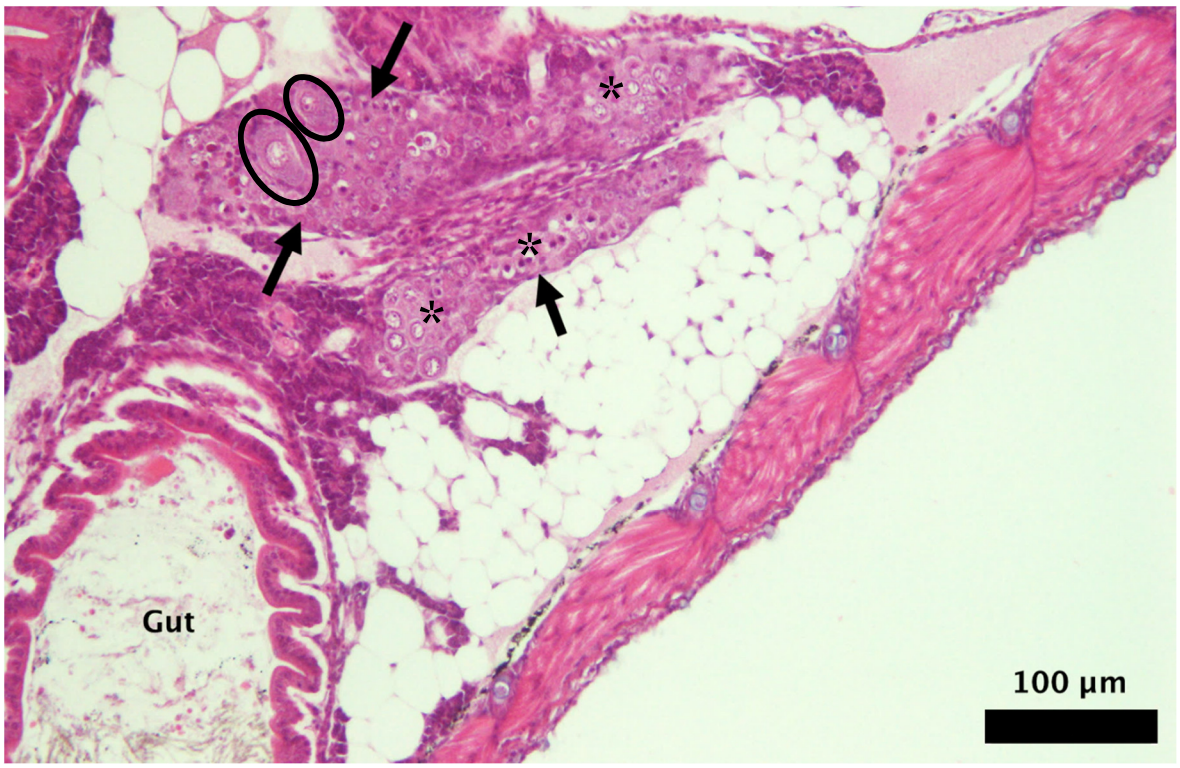

Figure 14: A guppy's undifferentiated gonad (arrows) at 5 days of age. The organ is elongated and rich in evolving oogonia (*). These appear as small round to oval cells with scant cytoplasm, present both at the periphery and at the inner parts of the gonad. There are also some primary oocytes in the chromatin nucleolar stage (ellipses), showing a relatively large nucleus with a small nucleolus.

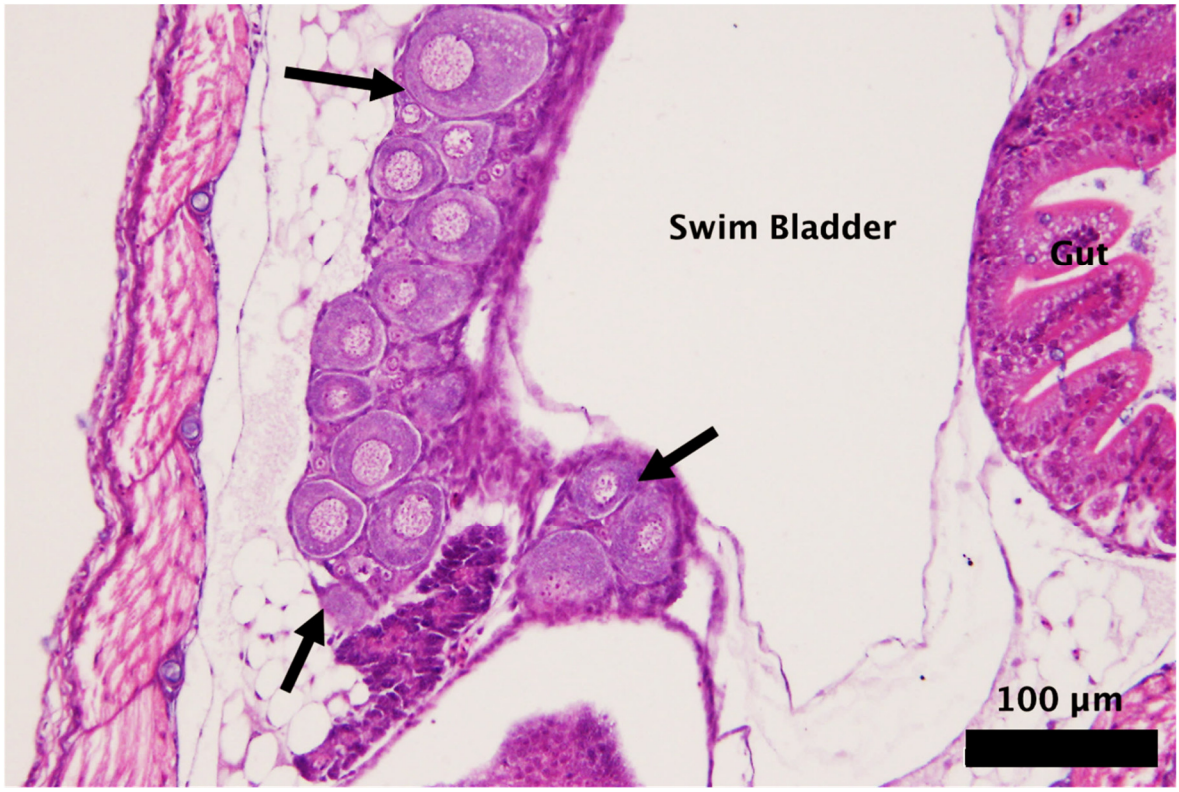

Figure 15: A guppy's female gonad (arrows) at 5 days of age. Almost all the germ cells are perinucleolar stage oocytes, the bigger cells with the large nucleus with small nucleoli circumscribing the nuclear periphery. A few oogonia are present.

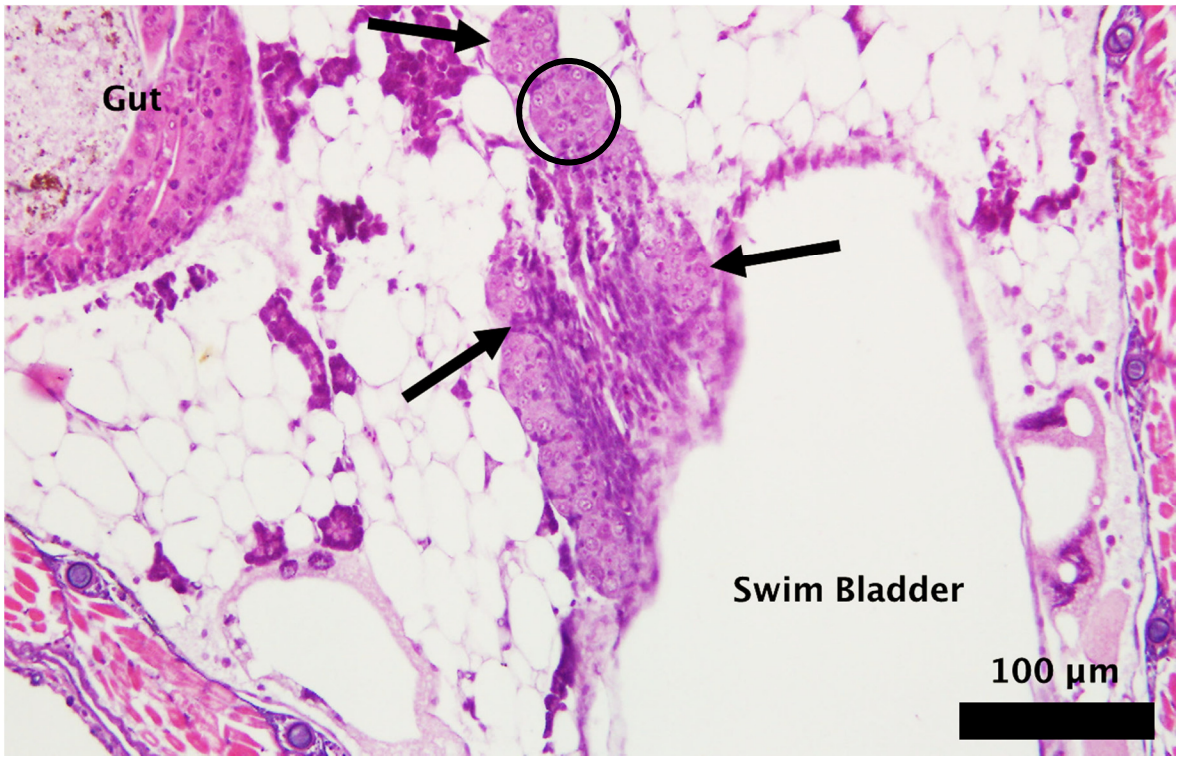

Figure 16: A guppy's male gonad (arrows) at 5 days of age. The testes are still segregated into two units. All the germ cells are spermatogonia, looking as small cells with scanty cytoplasm, already forming the roundish spermatocysts (circle).

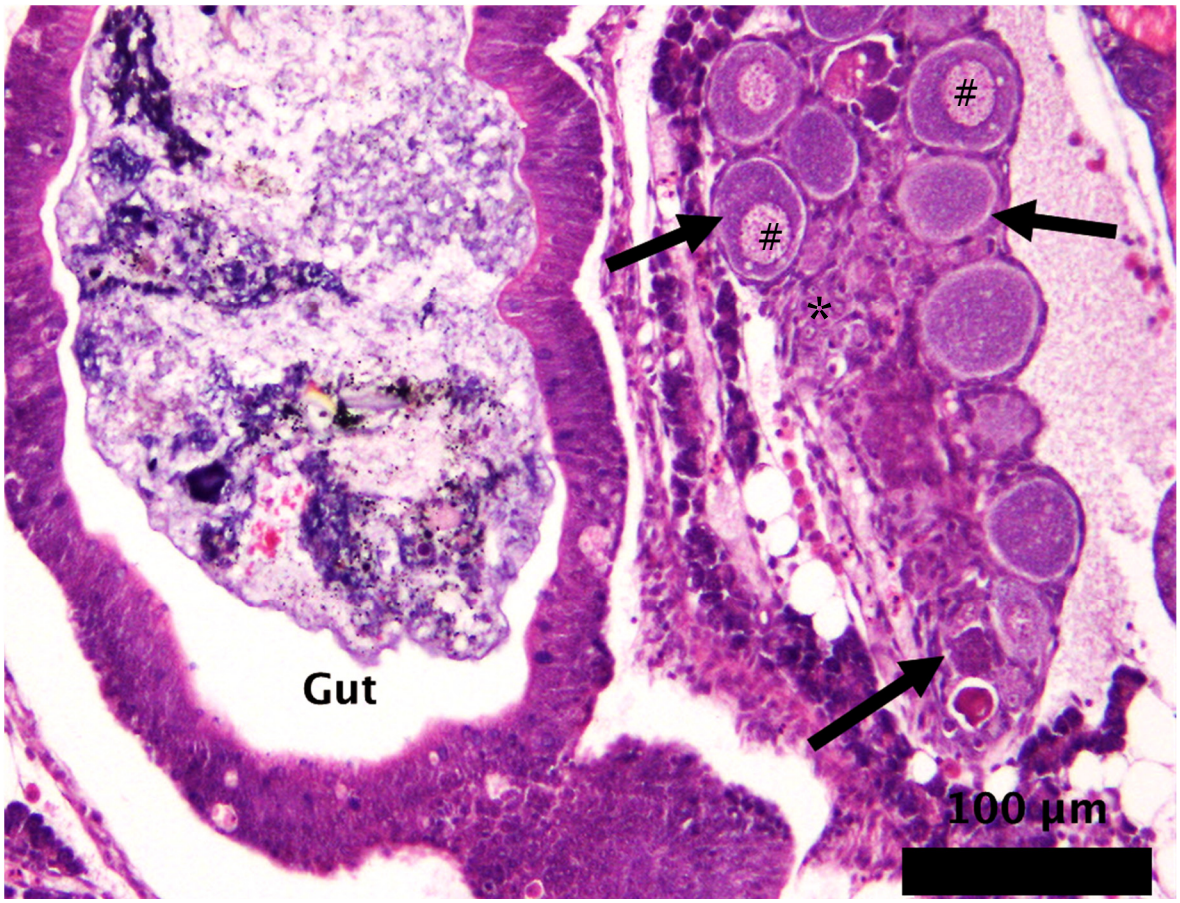

Figure 17: A guppy's ovary (arrows) at 10 days of age. There are scattered oogonia, the replicative pool that will continue to divide into adulthood, evidenced as the smaller cells with a large nucleus and minimal amount of cytoplasm (*). There are also chromatin nucleolar oocytes, large cells with large nucleus, in "transit" to the next stage, the perinuclear oocyte, with multiple nucleoli located and the periphery of the nucleus and with the cytoplasm becoming uniformly basophilic (#).

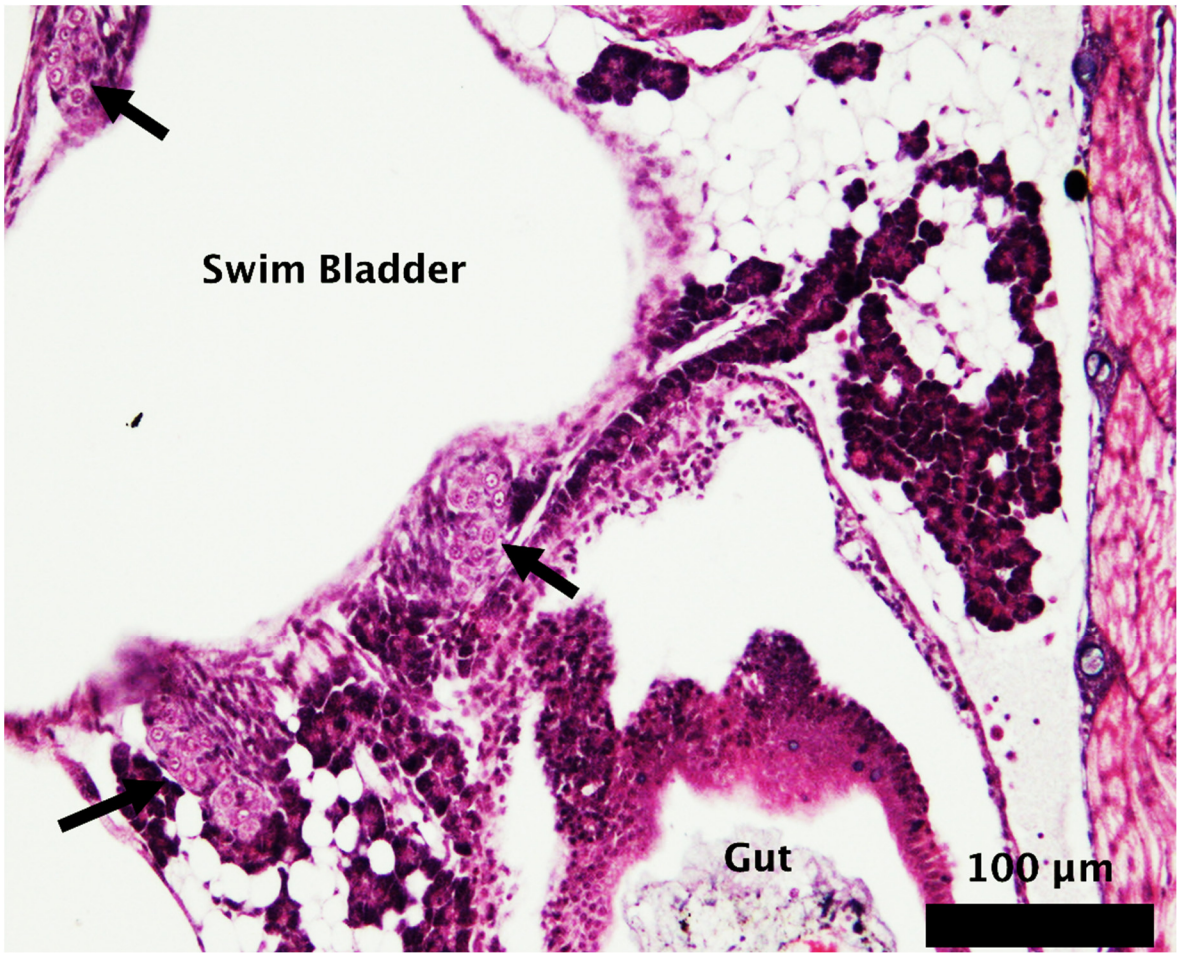

Figure 18: A guppy's testis (arrows) at 10 days of age, with testicular primordia still segregated into two units, "embracing" the swim bladder. All germ cells are clusters of spermatogonia, looking as small roundish cells with scarce cytoplasm.

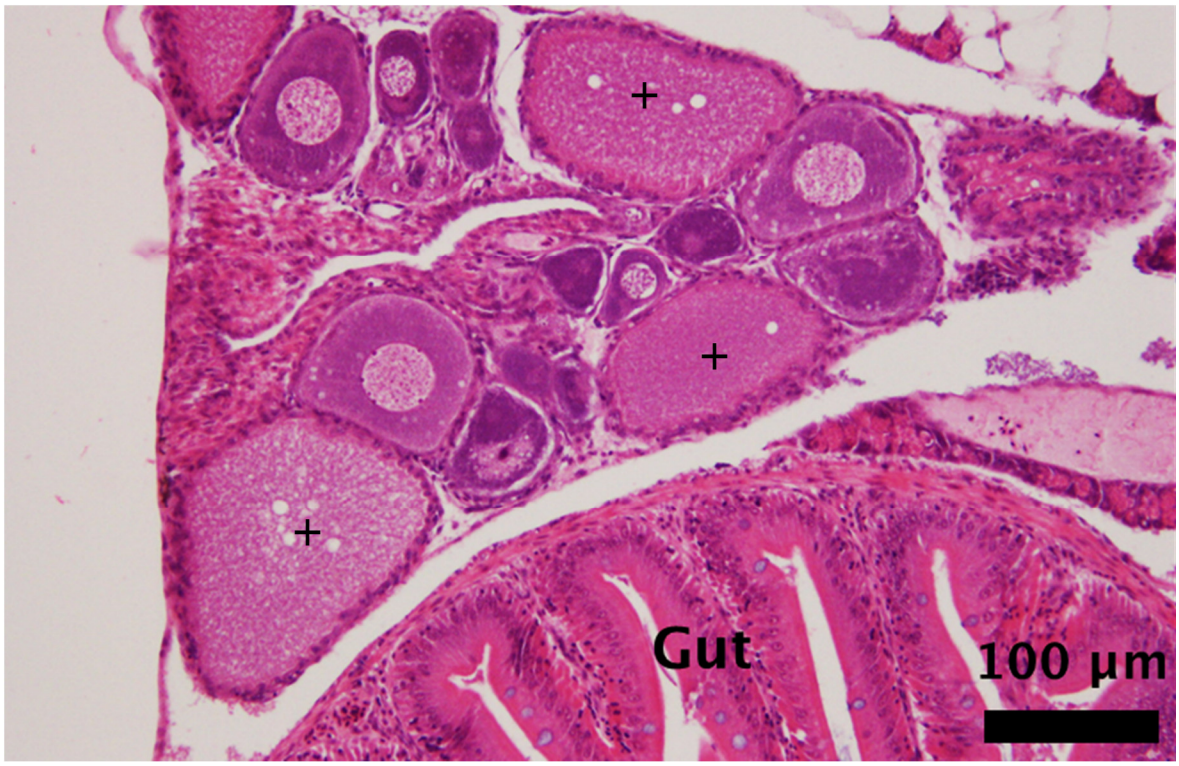

Figure 19: A guppy's ovaries at 60 days of age, already unified, with germ cells ranging from oogonia (small, with relatively large nucleus), to chromatin nucleolar oocytes (larger than oogonia, and with a single large nucleolus), perinuclear oocytes (increased nucleus size, with multiple nucleoli at the periphery) and growing cortical alveolar oocytes (+) (characterized by the presence of cortical alveoli).

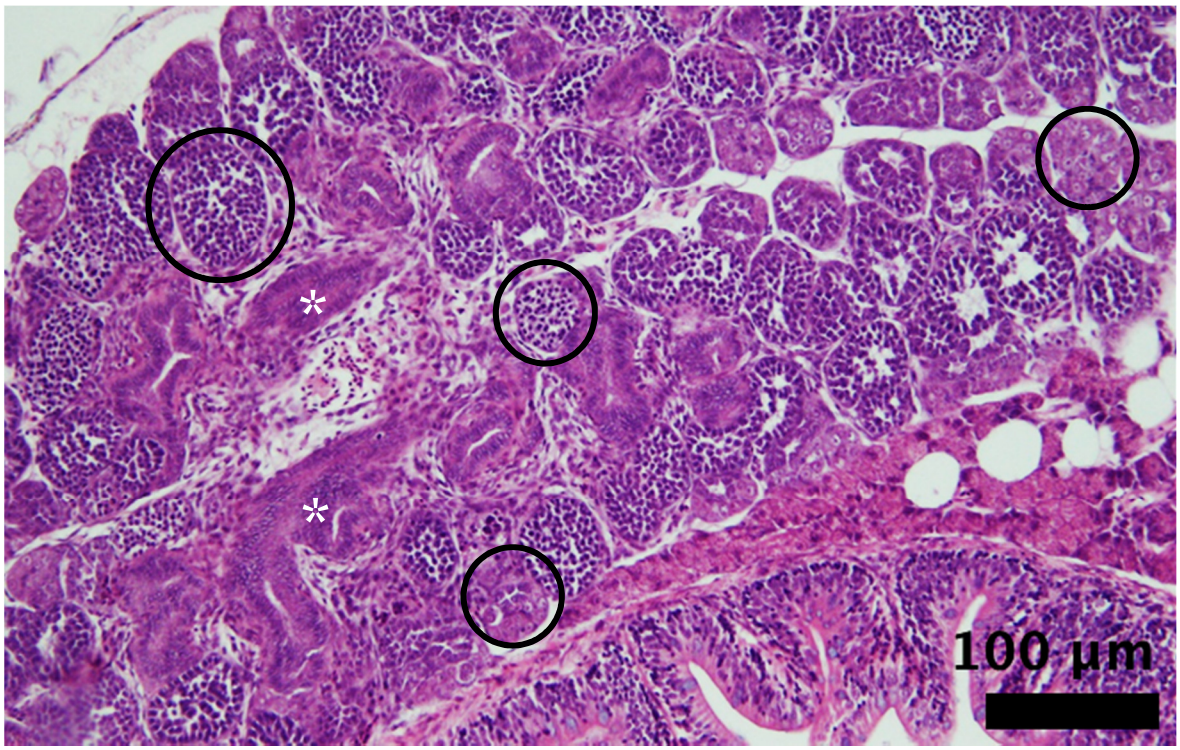

Figure 20: A guppy's testis at 60 days of age, with spermatocysts (circles) (germ cells lined by Sertoli cells) beginning to mature from the periphery towards the centre of the organ, getting closer to central sperm ducts (*) of the unified gonad.

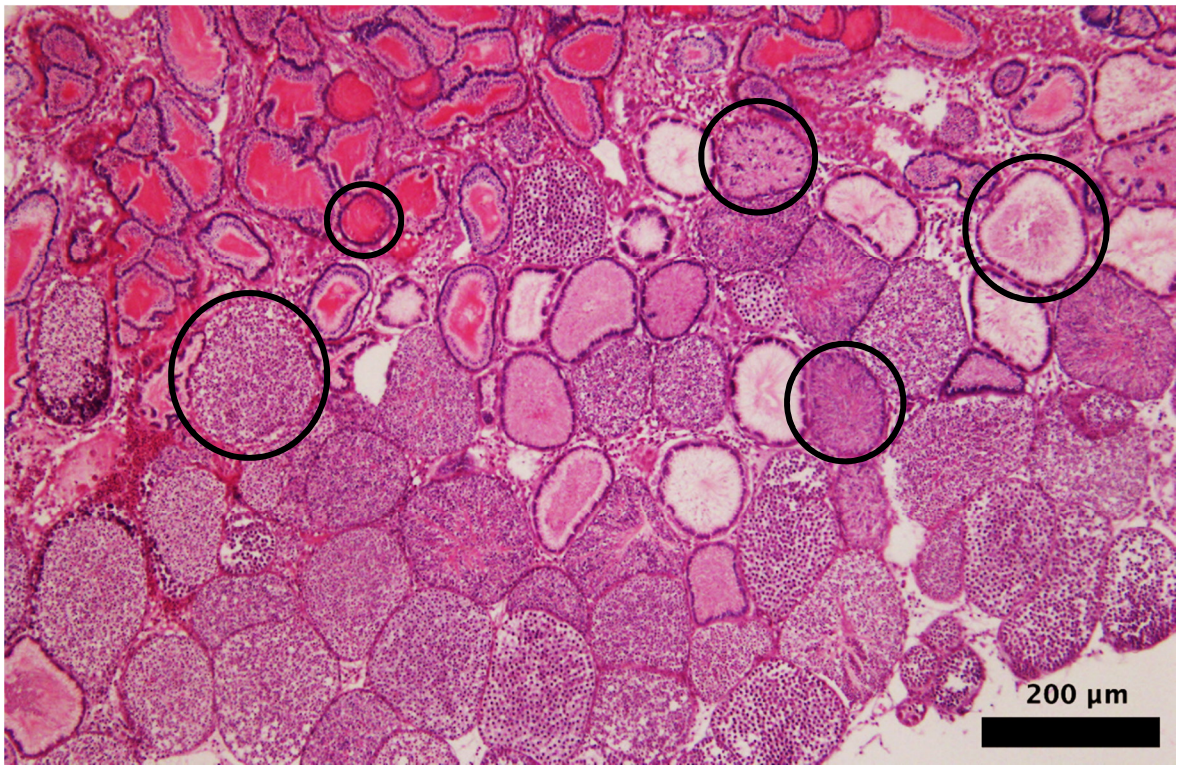

Figure 21: Example of the testis of an apparently fertile adult guppy. The centripetal maturational kinetics of the spermatogenesis within the spermatocysts (encircled) is evident. The more central part of the testis is at the image top, appearing with an orange tone due to the presence of late spermatocytes/mature sperm.

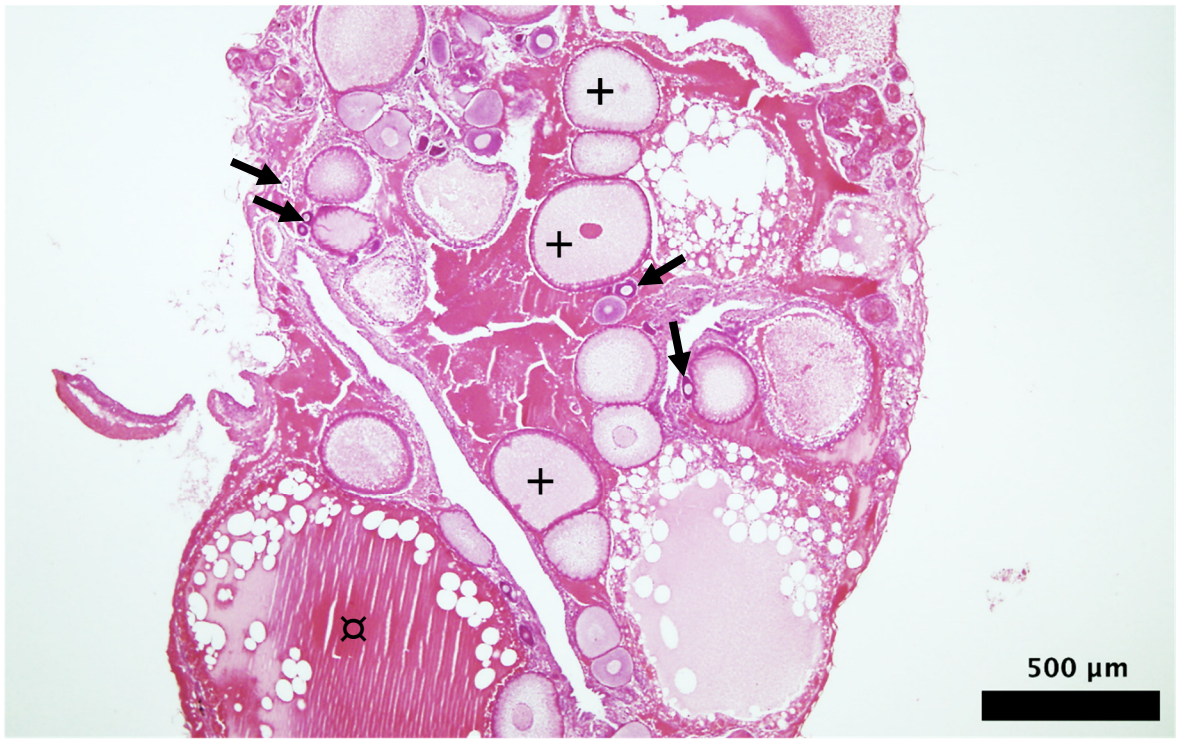

Figure 22: Example of an ovary of a seemingly fertile adult guppy. Oogonia mitosis happens throughout the fish life and oocyte maturation is continuous. Therefore, all the germ cell stages are present, from oogonia to the mature oocytes (¤). At this magnification, oocytes in chromatin nucleolar and perinucleolar stages are hardly seen (arrows). Many advanced cortical alveoli oocytes are easily spotted (+).

III.2 Exposure to GABA

Body Weight

Regarding experiment A, in which pregnant guppies where exposed to GABA against a negative CTRL, the BW data sets of the progeny (5 dab) are summarized in Table 5. All the animals were sexed according with the earlier defined and illustrated criteria.

Table 5: Mean, SD, and CV of the body weight (mg) of guppies aged 5 dab, generated in the two treatments: exposure to GABA and negative CTRL.

	GABA		CTRL	
	♀ (n = 6)	♂ (n = 6)	♀ (n = 3)	♂ (n = 3)
Mean	10.4	11.0	15.2	13.5
SD	3.2	2.8	3.5	5.3
CV	31%	25%	23%	39%

The Two-Way ANOVA suggested the existence of a marginally significant difference between GABA and CTRL as to the BW ($p = 0.0521$), with the CTRL leaning towards a greater BW, but there were no differences as to variable sex ($p = 0.9205$), and there was no interaction between age and sex ($p = 0.5103$) too. A post-hoc Tukey test did not confirm the difference between GABA and CTRL. In fact, by analysing the pairwise comparisons, the lower p-value (0.2442) was between the females exposed to GABA and the females of the control group. Variability was high in every group, with the CVs varying from ≈ 20 to 40%.

Gonad Volume

Data for the GV measured in guppies of 5 dab of age, from the GABA and CTRL groups, and for experiment A, are presented in Table 6. To test if groups differ significantly, a Two-Way ANOVA was performed. There are no significant differences between the means of the GV regarding different treatments ($p = 0.1028$), but there are differences when it comes to the factor sex ($p = 0.0000$), with ovaries being heavier. No interaction exists between the two variables at stake ($p = 0.8022$). The CRTL group evidences a much higher variability, which seems

not related with the variability of the BW, as seen in Table 5, where the four groups are not much different as to the range of values of the respective CV.

Table 6: Mean, SD, and CV of the GV (10^3 μm^3) of guppies aged 5 dab, generated in the two treatments: exposure to GABA and negative CTRL.

	GABA		CTRL	
	Ovary (n = 9)	Testis (n = 10)	Ovary (n = 8)	Testis (n = 7)
Mean	1412.3	356.2	1517.1	482.3
SD	385.3	84.9	694.0	299.2
CV	27%	24%	46%	62%

Male to Female Ratio

The proportions of male and female progeny obtained for each treatment, at 5 dab, are presented in Table 7.

Table 7: Male to female total number and relative proportion (%) of the guppy progeny for each treatment, *i.e.*, from females exposed to GABA *vs* CTRL.

	Females		Males	
	n	**%**	**n**	**%**
GABA	24	41	35	59
CTRL	36	57	27	43

According with the Fisher's Exact Probability Test, the proportions of males and females do not differ significantly between the two experimental groups ($p = 0.0739$); the same result was obtained using a Yates corrected Chi-Square test.

Histological Study

Examples of microphotographs for the control condition have already been presented in the last chapter (Figures 15 and 16). Examples of microphotographs of the progeny gonads after exposure to GABA are now given below. Figure 23 is of an ovary with a GV of 1428 x 10^3 μm^3 of a fish with a BW of 9.0 mg, and Figure 24 of a testis with a GV of 452 x 10^3 μm^3 of a fish with a BW of 8.8 mg.

Microphotographs of gonads of the guppies' exposed to GABA during embryonic development, and sampled at 60 days of age, were also taken and are presented in Figures 25 (ovary) and 26 (testis).

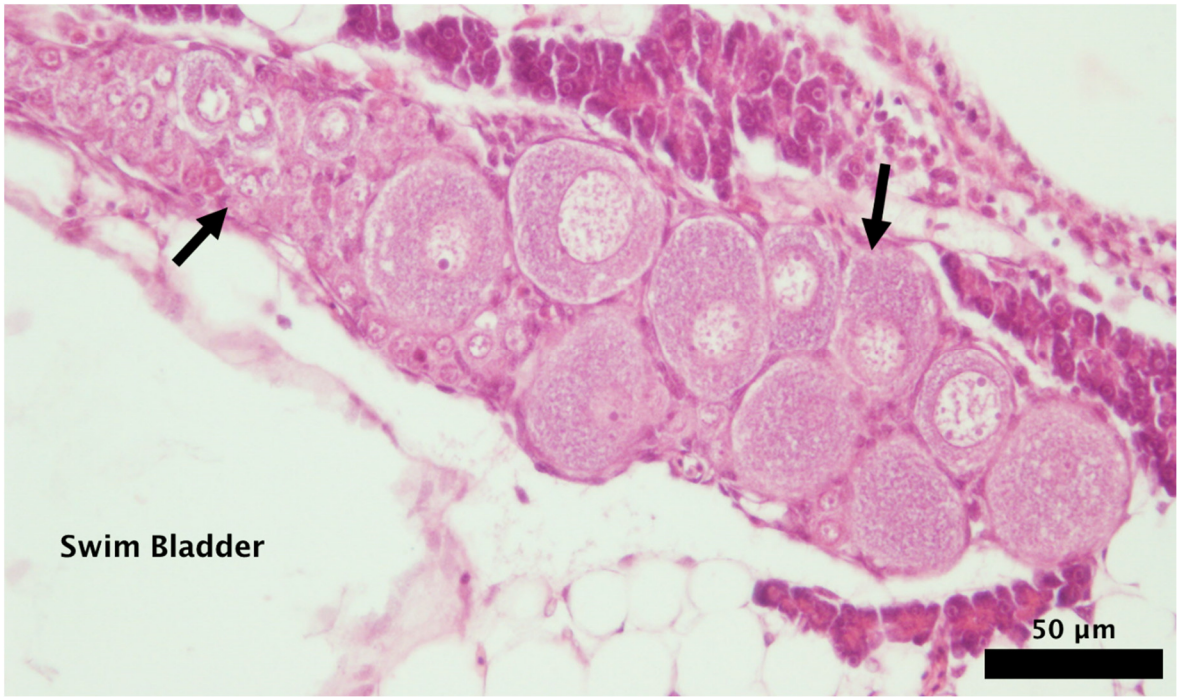

Figure 23: A guppy's female elongated gonad after exposure to GABA (arrows) at 5 days of age, bearing oogonia, chromatin nucleolar and perinucleolar oocytes.

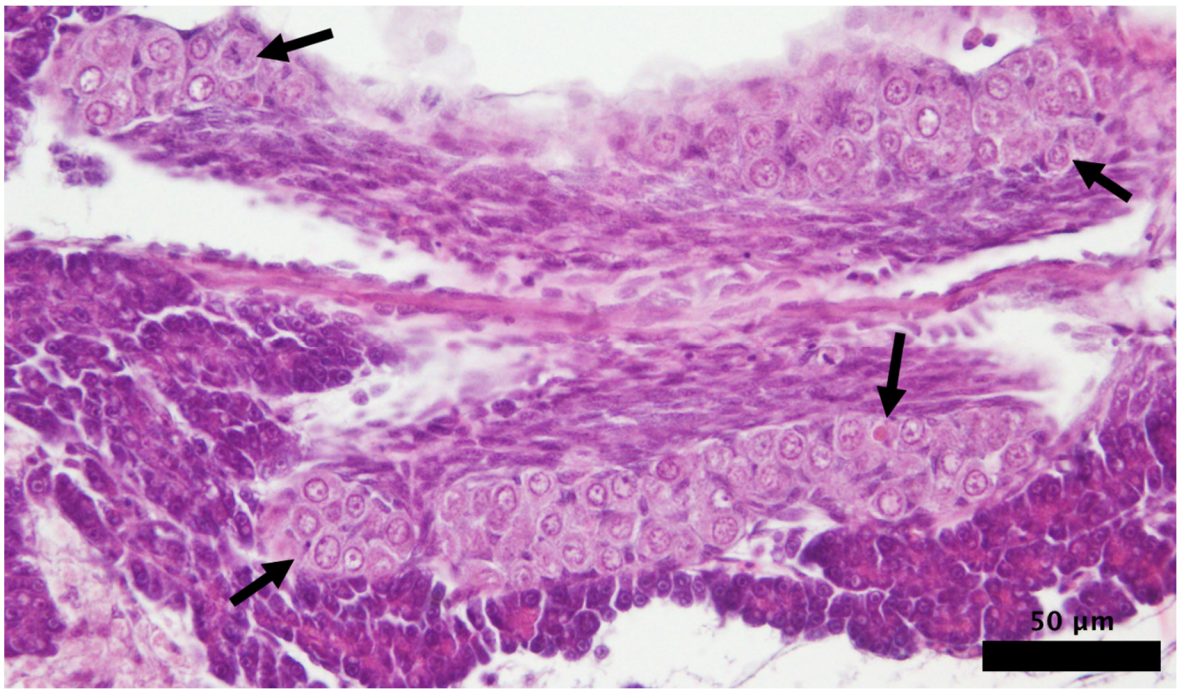

Figure 24: Guppy male gonad after exposure to GABA (arrows) at 5 days of age, with two elongated units within which only spermatogonia germ cells are present.

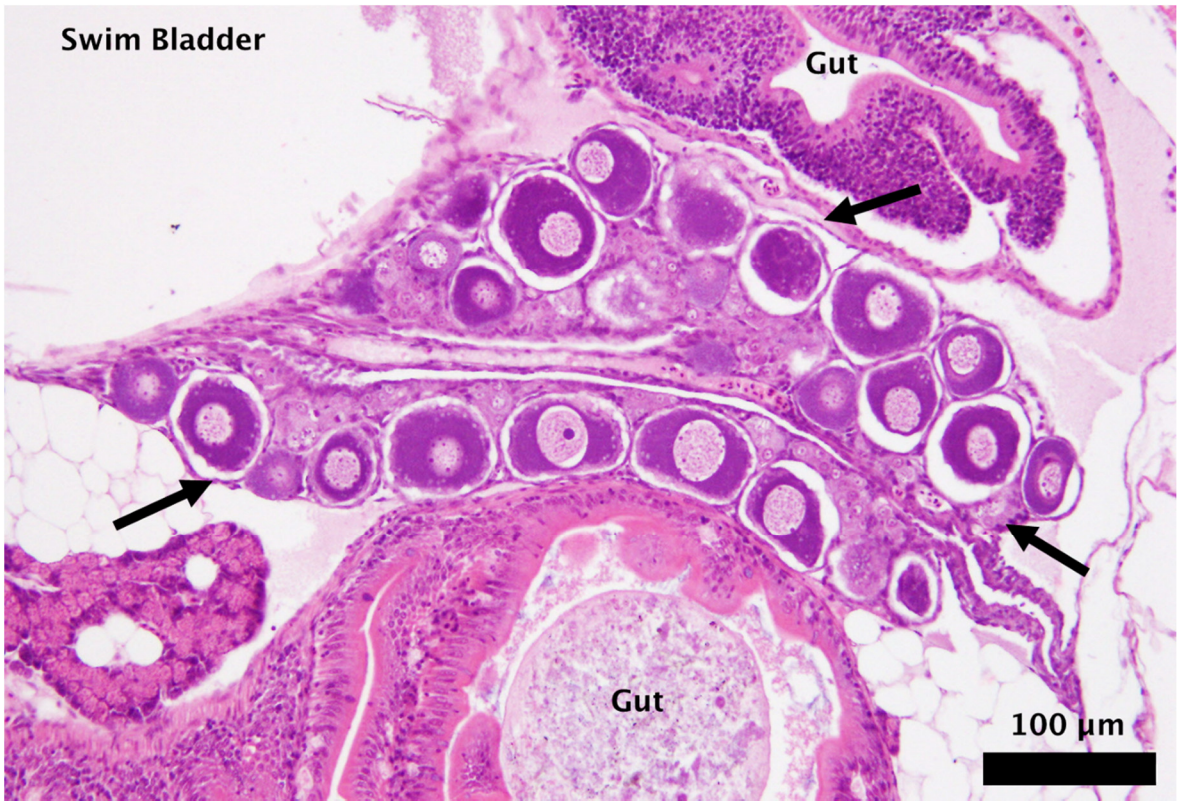

Figure 25: Guppy female gonad after exposure to GABA (arrows), at 60 days of age, already united in a single organ, with an elongated shape, where the oviduct is visible. Germ cells stages range from oogonia to early cortical alveolar oocytes.

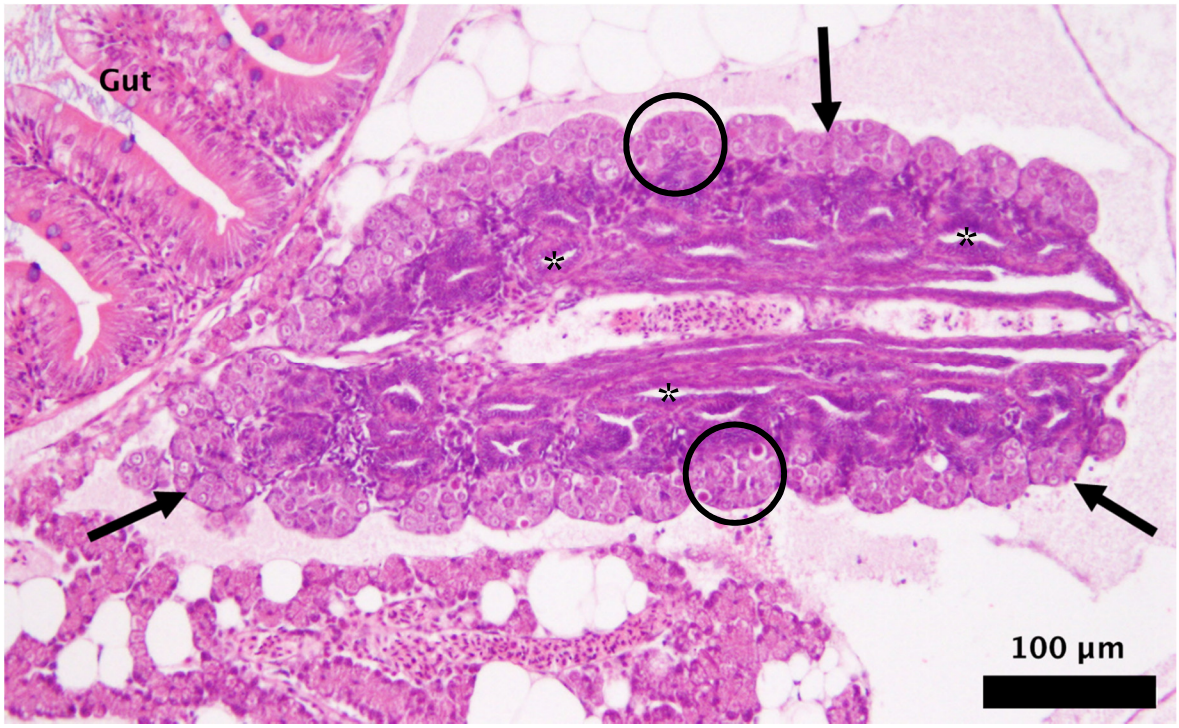

Figure 26: Guppy male gonad after exposure to GABA (arrows) at 60 days of age. A single (merged) testicular structure is observed, with sperm ducts (*) already formed, and the periphery-to-centre maturation progress of the spermatocysts (encircled) starts to establish.

III.3 Exposure to GABA and EE2

Body Weight

The BW data of the progeny from experiment B, in which a paired exposure to GABA and EE2 was performed, are given in Table 8, segregated according to sex. The G-EE2 refers to the condition in which EE2 was dissolved in ethanol and administered after an initial exposure to GABA. The G-SOLVENT refers to the solvent control that repeats this process but without EE2.

Table 8: Guppies body weight means (mg), with values for SD and CV of the guppies exposed to EE2 dissolved in ethanol after an initial exposure to GABA (G-EE2), and solvent control after an initial exposure to GABA (G-SOLVENT), segregated according to sex.

	G-EE2		G-SOLVENT	
	♀ (n = 3)	♂ (n = 3)	♀ (n = 3)	♂ (n = 2)
Mean	12.6	15.2	14.3	10.8
SD	0.8	5.0	1.4	3.3
CV	6%	33%	10%	30%

To compare the values for the BW between the sexes and the two treatments, a Two-Way ANOVA was performed. The Levene's test showed that there is no homogeneity of variances in this sample ($p = 0.0242$), even after logarithmic transformation ($p=0.0168$). Anyway, the ANOVA showed no significant differences between the average BW of the two conditions ($p = 0.5152$), no significant differences between the means of males and females ($p = 0.6244$) and no interaction between the variables sex and experimental condition ($p = 0.1763$).

Gonad Volume

To compare the GV between the sexes and the two treatments, which the results are given in Table 9, a Two-Way ANOVA was used. The analysis was made with non-transformed data facing the results of normality tests and because the Levene's test for the homogeneity of means confirmed that these were so ($p = 0.0867$). The ANOVA showed that there are no significant differences between the means of the GV of regarding the effect of the different treatments ($p = 0.4627$), but there are significant differences when it comes to the variable sex, with the females having more voluminous gonads ($p = 0.0114$); in accordance with results

presented in an earlier Chapter. There is no interaction between the variables treatment and sex ($p = 0.4619$).

Table 9: Guppies gonad volume means (10^3 μm^3), with values for standard deviation (SD) and coefficient of variation (CV), of the guppies exposed to EE2 dissolved in ethanol and after an initial exposure to GABA (G-EE2), and solvent control after an initial exposure to GABA (G-SOLVENT), segregated according to sex.

	G-EE2		**G-SOLVENT**	
	Ovary (n = 3)	Testis (n = 3)	Ovary (n = 3)	Testis (n =2)
Mean	1556	454	2210	453
SD	1080	146	680	71
CV	69%	32%	31%	16%

Male to Female Ratio

The proportions of males and females obtained for each condition are presented in Table 10.

Table 10: Male to female total number and relative proportion (%) of the guppy progeny for each treatment, *i.e.*, from females exposed to EE2 dissolved in ethanol and after an initial exposure to GABA (G-EE2), and solvent control after an initial exposure to GABA (G-SOLVENT).

	Females		**Males**	
	n	%	n	%
G-EE2	6	40	9	60
G-SOLVENT	8	44	10	56

According with the Fisher's Exact Probability Test, the proportions of males and females do not differ significantly between the two experimental groups ($p = 0.5390$). The same result was found using a Yates corrected Chi-Square test.

Histological Study

An example of an ovary at 5 days of age after an exposure to EE2 dissolved in ethanol, after the initial exposure to GABA (the G-EE2 group) is given in Figure 27. The estimated GV was 2746x10^3 µm^3 from a fish with a BW of 15.5 mg. A testis from the same experimental conditions, with a GV of 569x10^3 µm^3 from a fish with a BW of 17.4 is represented in Figure 28. Figure 29 offers an example of an ovary from a fish subjected to the solvent control condition. The gonad was collected at 5 days of age, and had a GV of 2844x10^3 µm^3 in a fish with a BW of 15.5 mg. Figure 30 depicts a testis with a GV of 478x10^3 µm^3, from a control fish with a BW of 5.2 mg.

An example of an ovary at 60 dab from progeny of females from the G-EE2 group is given in Figure 31. A testis in the same conditions is represented in Figure 32. For the G-SOLVENT group, one example of an ovary at 60 dab is given in Figure 33 and of a testis in Figure 38.

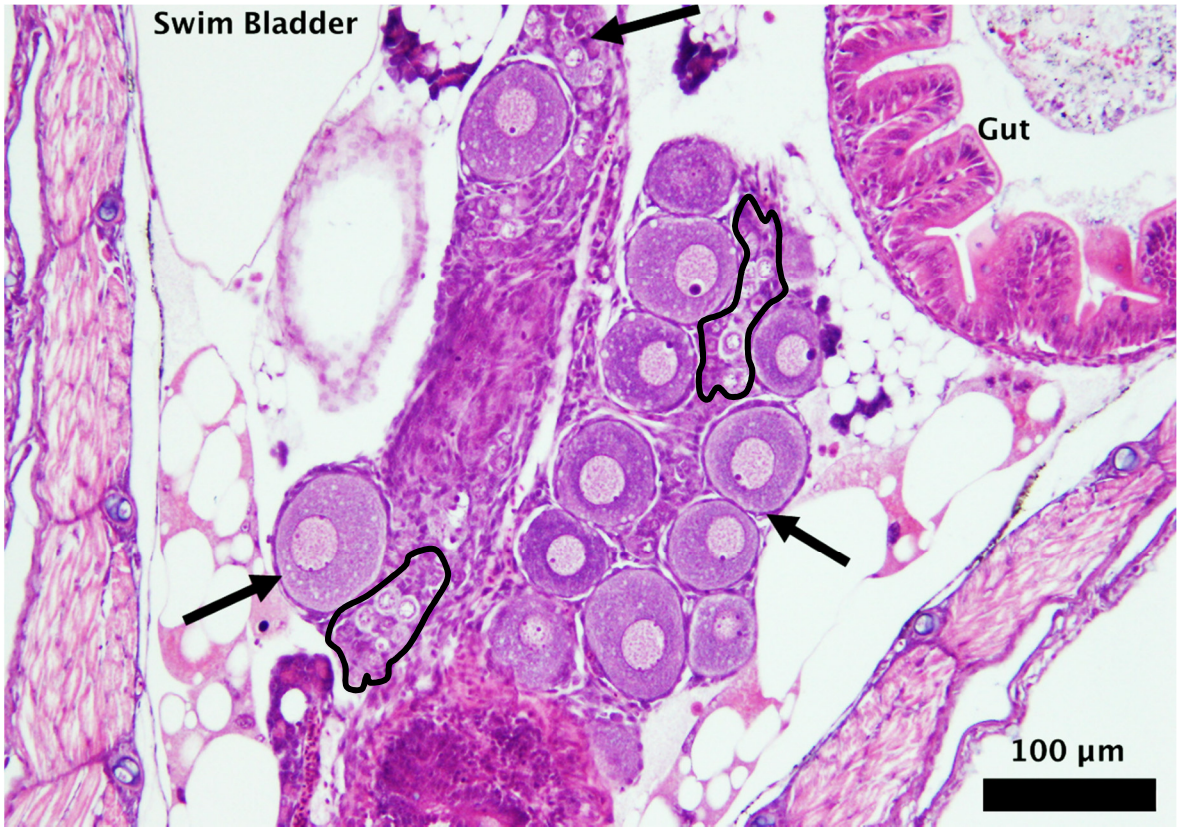

Figure 27: An ovary of a female guppy exposed to EE2 dissolved in ethanol and after an initial exposure to GABA (arrows), at 5 days of age. Most germ cells are growing chromatin nucleolus and peripherally perinucleolar oocytes, the bigger cells with the large nucleus and several small peripherally located nucleoli. A few much smaller germ cells (oogonia and early oocytes) are present (delimited areas).

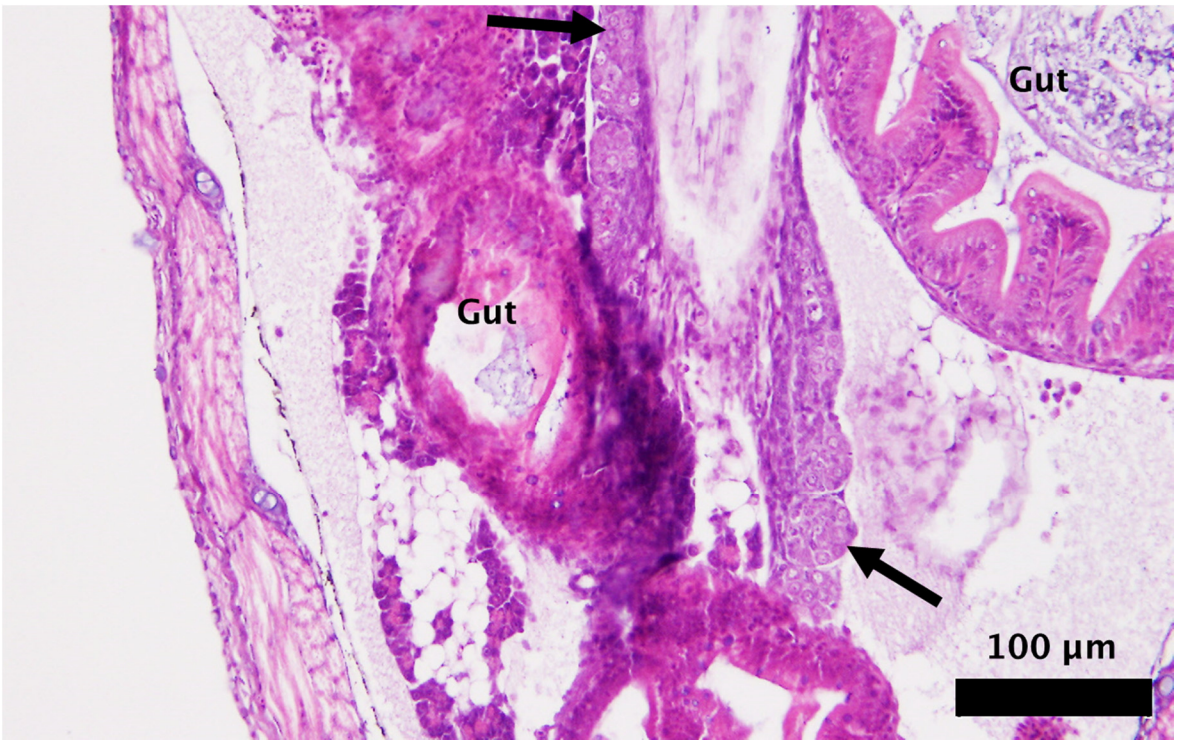

Figure 28: Early male gonads (arrows), from a fish exposed to EE2 after an initial exposure to GABA, at 5 days of age. All germ cells are spermatogonia, looking as small cells with scarce cytoplasm, organized in cysts.

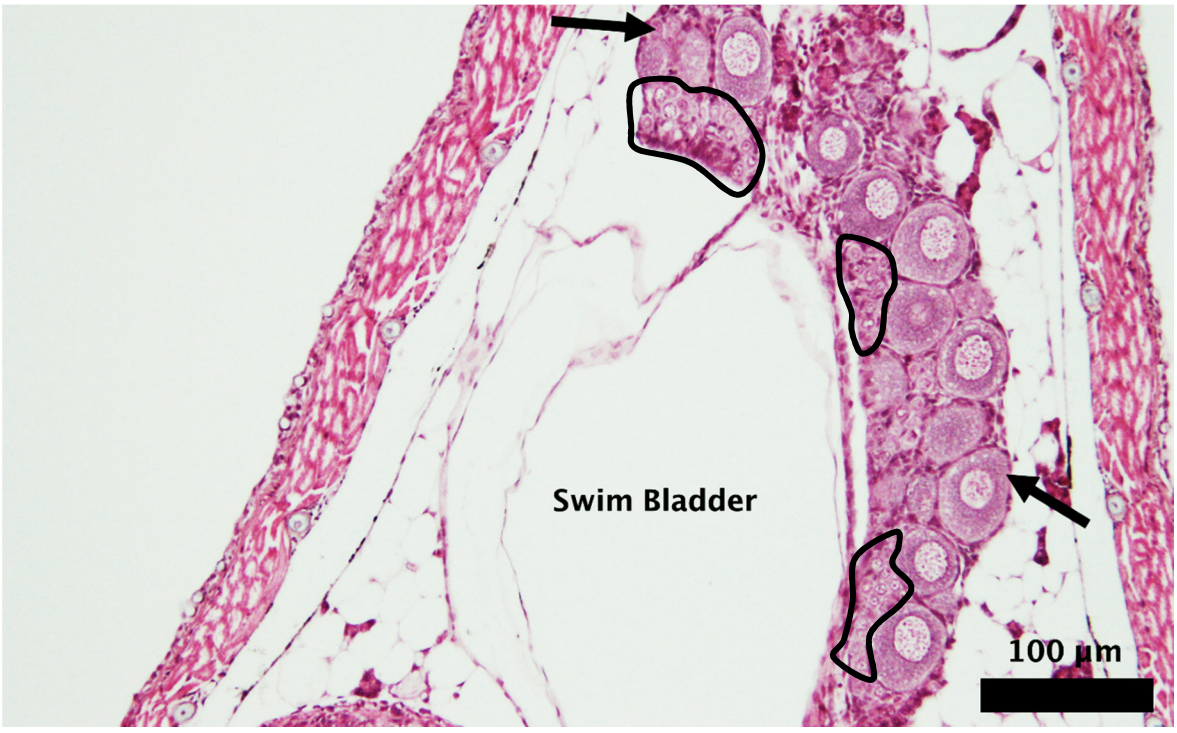

Figure 29: Guppy female gonad (arrows) from a control fish, exposed to solvent after an initial exposure to GABA, at 5 days of age. The ovary is elongated, with a plethora of growing perinucleolar oocytes, the bigger cells with large nucleus and small nucleoli. Groups of oogonia and smaller early oocytes) are present (circumscribed areas).

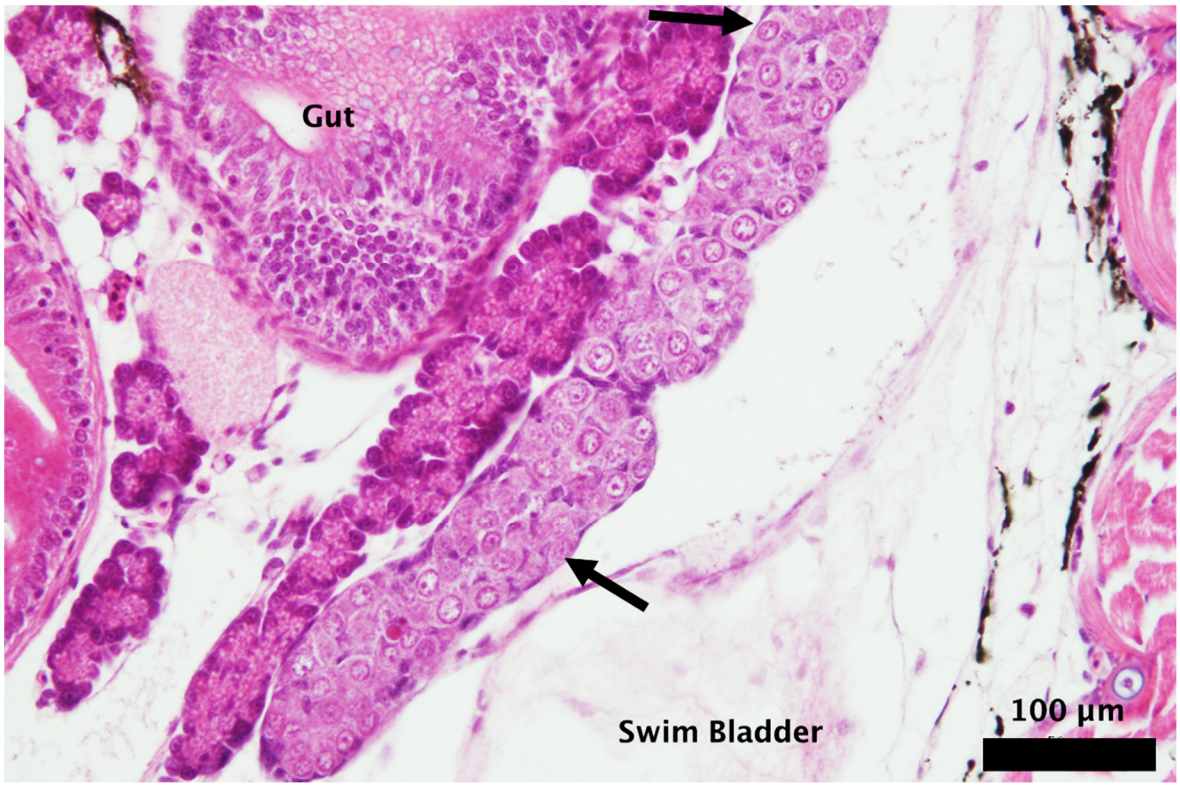

Figure 30: Guppy male gonad (arrows) from a fish of the solvent control group, after an initial exposure to GABA, at 5 days of age. The early testis is elongated, and all the small cells with a high nuclear-to-cytoplasm ratio are spermatogonia.

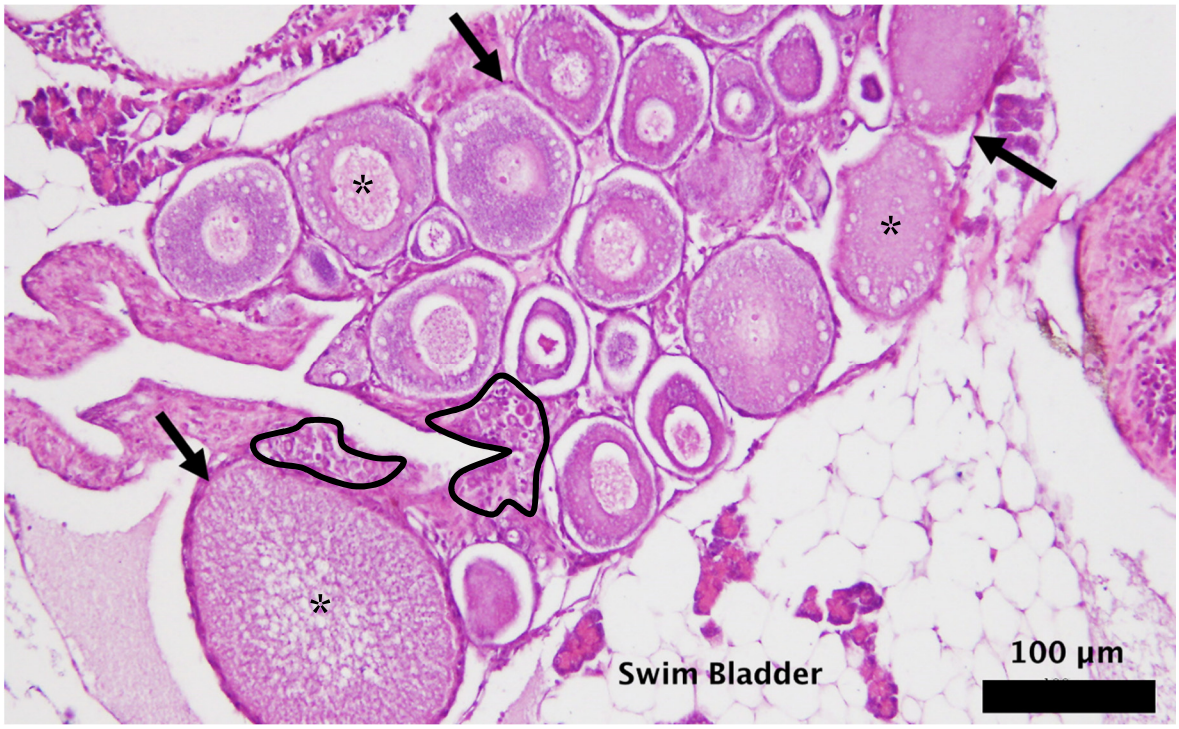

Figure 31: Ovary of a guppy exposed to EE2, after an initial exposure to GABA, at 60 days of age. Arrows point to external boundaries. The organ is unified, having germ cells ranging from oogonia/initial oocytes (circumscribed areas) to growing cortical alveolar oocytes (*).

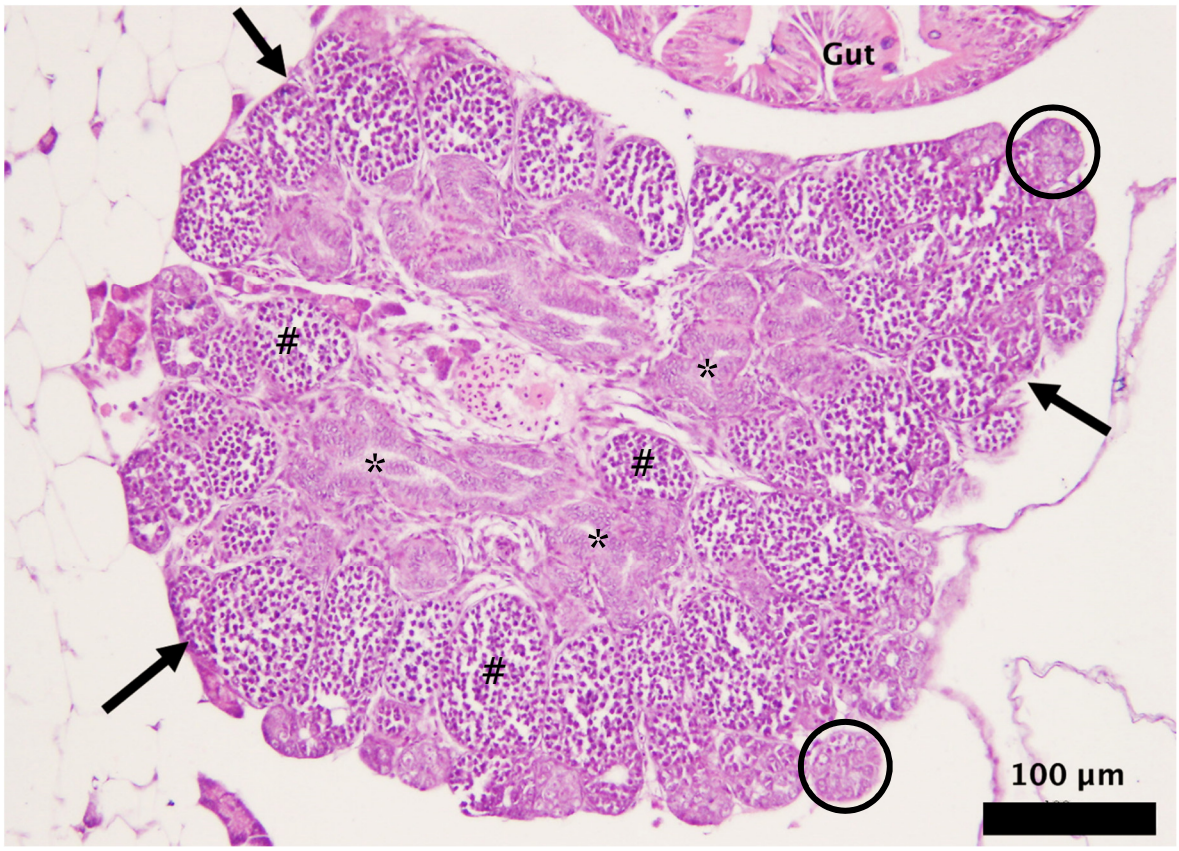

Figure 32: Testis from a guppy exposed to EE2, after an initial exposure to GABA at 60 days of age. Arrows point to external boundaries. The spermatocysts mature centripetally, and so those with earlier germ cells stages are more peripheral (circles) than those more at the centre (#), getting closer to sperm ducts (*).

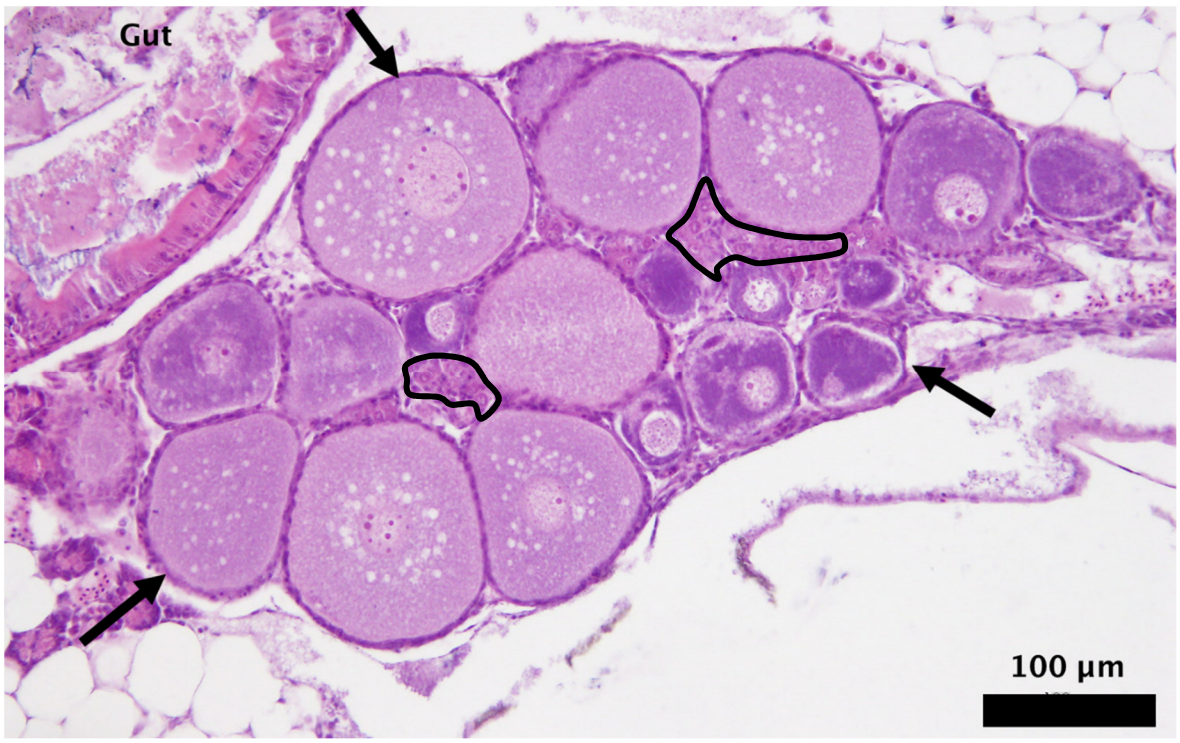

Figure 33: Ovary (arrows) from a guppy of the control group, exposed to ethanol, after an initial exposure to GABA, at 60 days of age. The gonad is unified. Germ cells vary from oogonia (within bounded zones) to cortical alveolar oocytes (*).

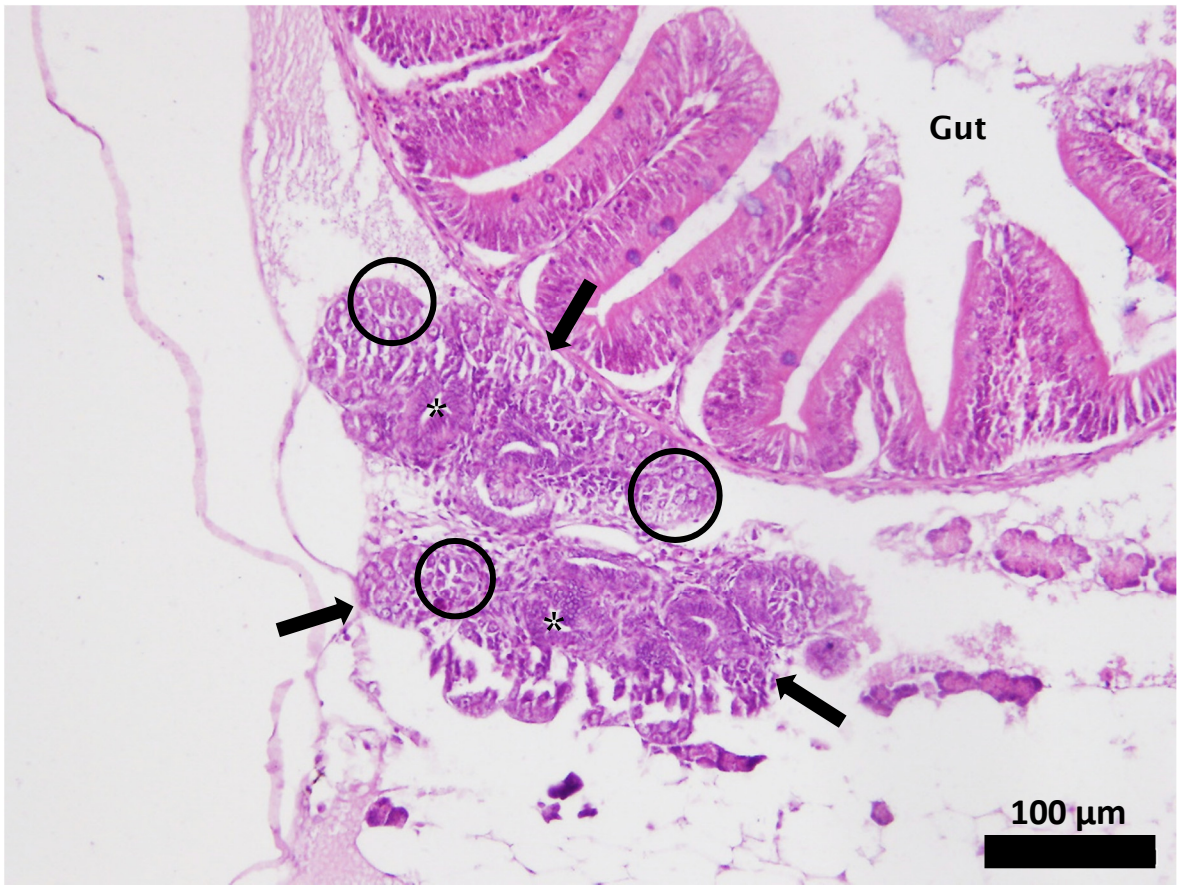

Figure 34: Testis from a guppy of the control group, exposed to ethanol, after an initial exposure to GABA, at 60 days of age. Arrows point to external boundaries. The early-paired gonads are already merged into the single testis. The spermatocysts (circles) are maturing centripetally, towards the developing sperm ducts (*).

Chapter IV – Discussion

IV.1 Revisiting Early Growth and Gonadal Differentiation in the Guppy

The results regarding the BW at birth, 5 dab and 60 dab suggest a trend — although statistical significance could not be proved — for a loss of weight between 0 and 5 dab, which occurred in parallel with the absorption of the remaining yolk sac. Likely, our study lacks sufficient power to uncover a significant difference. Our idea is corroborated by the histological study, showing that yolk is still present at 0 days of age (Figures 10-13), but rarely at 5 dab (Figures 14-16).

Another finding here is that the only significant difference in BW is seen between the ages of 5 and 60 dab, corresponding to a period where the guppies start growing. According to Shahjahan et al. (2014) females become larger in length at 28 dab, which agrees with our data in what concerns expecting a greater mass at 60 dab. However, no significant differences between the BW of males and females were found at any of the ages in which it was measured. Examples in the literature claim that the guppies can be segregated according to sex from an early age (Pierson, 1981), but at birth many of the sexual secondary characters have yet to be developed (Shahjahan et al., 2014), and in this work no evidence was found that there was a significant difference in the size of the juveniles.

It is also interesting to note that the CV of the BW is high at any age with a maximum of 74% in males at 0 dab and a minimum of 40% in males of 5 dab. This is a clear illustration of an asynchronous growth, most likely, or at least in part, a consequence of the asynchronous fertilization that happens in this species (Shahjahan et al., 2014). Even though the average BW does not differ for males and females at any age, at 0 dab it is found that the individuals that were left uncharacterized, due to lack of sufficient gonad differentiation, are also the ones that have a clear delay in growth, with a mean BW of just 13 mg. At the same age, females have a mean BW of 20 mg and males of 18 mg. This makes us think that the "delay" in gonadal differentiation seen in some fry is due not only to gonadal events but is likely associated to the overall fish growth at the very earliest age. This hypothesis is worth exploring mechanistically in the future.

The main criteria for differentiating early ovaries from early testis in the guppy is by visually analysing the types of germ cells present, since all gonads go through an ovarian phase (Dildine, 1936; Goodrich et al., 1934). Thus, oogonia and chromatin nucleolar oocytes (i.e., germ cells with large nucleus with a unique big nucleolus and granular cytoplasm) should be present in undifferentiated gonads or

early ovaries. The lack of chromatin nucleolar oocytes indicates that the early stage of development being seen is in fact spermatogonia and that the gonad is no longer a juvenile ovary, but is what can be rated as early testis. If the germ cells are mainly chromatin nucleolus (or nucleolar) oocytes, and some of these have begun the process of expansion to become perinucleolar oocytes then there is no doubt that the gonad is an early ovary. Here we applied such criteria, which worked from 5 dab on, but not always at 0 dab, where some new-born fish still displayed indistinct gonads. Anyway, this variability agrees with the idea that onset timing and extent of the early ovary may vary substantially among the young juvenile fish, as for example seen in zebrafish (Wang, Bartfai, Sleptsova-Freidrich, & Orban, 2007).

As to the gonad size, here measured by the volume, we found that the organ has a significant development in the evaluated age period (0 to 10 dab), with differences between both sexes, but with no interaction between age and sex. Since the increase in BW is only statistically significant between 5 and 60 dab and the development of the gonad is significant since birth, between 0 and 5 dab, it is possible that the gonadal development in this period is somewhat independent of the fish general growth.

The high CV seen in both BW and GV, in males and females, is in our view justifiable by the desynchronization of development and growth expected for this species. Indeed, there is asynchronous fertilization even in the same batch of embryos, meaning these should have slightly different ages post-fertilization, despite having the same age post birth (Martyn et al., 2006).

It is interesting to note that the ratio of the volumes of ovary and testis is consistently 4, which means that at least up to 5 dab the ovary is on average four times bigger than a testis, suggesting there is a quite strictly regulated process of anatomical dimorphism. This helped to characterize the gonads that left some doubts after observation — like the one showed in Figure 10 — that was left uncharacterized due to the perceived lack of a sufficient number of oocytes, but that had a GV of 1793×10^3 μm^3, and so it is unmistakably an early ovary. Figure 11, however, presents an example of gonads that even after volume determination (GV = 779×10^3 μm^3) the doubt remained, since it is just 2.3 times smaller than an average ovary and 1.8 times bigger than an ordinary testis. In the particular case depicted in Figure 11, looking at the cell types, it is possible to point out oogonia and chromatin nucleolar oocytes. Although these are typically ovarian, more or less misleadingly similar type of cells may be present to some extent in undeveloped males.

This is a species reported to have a period of embryonic hermaphroditism, in which the gonads of the embryo go through an ovarian phase before said to be differentiating into testis at birth (Dildine, 1936; Goodrich et al., 1934). Therefore, it makes sense that oogonia and chromatin nucleolar oocytes are both present at an age when gonadal differentiation is still happening. Despite the latter is a process that according to the mentioned literature should be either complete, or nearly so, at birth, it was surprising in our study to find that in many larvae differentiation is still rather unfinished. Undifferentiated fish were detected here as late as 5 dab, as seen in Figure 14, in which chromatin nucleolar oocytes are a minority among the cell types and unequivocal characterization was only accomplished after determining the GV of $1340x10^3$ μm^3, a volume range only found in females.

The evolution of the gonad development, in its general terms, follows the already made descriptions (Dildine, 1936; Goodrich et al., 1934), but the timings did not match with what was observed in the classic studies. As observed here, the gonads form as two units and all embryonic gonads go through an ovarian phase, however, visible sex differentiation did not happen shortly before birth in all of the fishes, and in some, it took as long as 5 dab to be fully concluded. The ovarian cavity and the fusion of the two testis has also been confirmed, but while this was earlier claimed to occur in the few days after birth, here it was not observed in individuals at 10 dab and it was only seen in a minority of the individuals of 60 dab. Our data fit better with the observations of Koya et al. (2003). These authors determined key timings for the mosquitofish, a species closely resembling the guppy, describing that 5 dab is just the beginning of ovarian fusion which lasts at least up to 10 dab, where single ovaries were seen. Therefore, both the guppy and the mosquitofish have a time window of over 5 dab for the early ovaries to merge.

Considering the time of publication of the papers regarding the guppy sex differentiation, it is possible that the incongruity between those works and our data is derived from genetic traits due to the artificial selection that this species has seen been subjected to, or from factors dependent on strains. Indeed, female fecundity and offspring growth is a family trait (Karino & Ikeuchi, 2011). Therefore, we warn that generalizations for the species should be viewed now with care and that the issue calls for more studies with guppies from various sources.

IV.2 Exposure to Gonad Development Disrupting Compounds

There are still few works regarding GABA exposure, and even less in fishes. Anyway, some interesting facts are known for quite some time. For example, it is known for some time that chronic GABA exposure of cerebral cortical cultured neurons induced a down-regulation of the GABA receptor system (Mehta & Ticku, 1992). In a toxicological context, a study showed that benzodiazepines, pharmaceutical drugs for human psychological disorders that enhance the GABA signal, alter the behaviour – increasing agitation and decreasing social interaction – and increase the feeding rate of wild European perch (*Perca fluviatilis*) (Brodin, Fick, Jonsson, & Klaminder, 2013). In rockfish (*Sebastes diploproa*) high anxiety levels have been reported due to the environmental drop in ocean pH that alters the regulation of GABA receptors (Hamilton, Holcombe, & Tresguerres, 2014).

Herein, in experiment A, there were no significant differences between the GABA exposure and the control group, but they existed between the sexes. The latter are due to sex-specific morphological differences and not to the exposure. No differences were also found in the sex ratio and no obvious histological evidence was obtained of any effect of the GABA exposure, although both sex ratio and BW show marginal no-significance, with values close to 0.05. In view of this scenario, more studies must be done to refine the appropriate concentration of GABA in order for it to have a disrupting effect. A caveat of our study is that we did not measure the real concentration of GABA in the water so to confirm if there is a difference in relation to the nominal concentration. However, facing the high water solubility of GABA and stability, to the point of being used for instance as an effective spray to protect some plants from environmental stress (Li, Peng, & Huang, 2016), the idea that GABA would be totally degraded and/or would not be up taken (at least to some extent) by the exposed guppy females is unrealistic. Anyway, facing the no-effect seen in our present study, in future ones we recommend that the GABA real concentration in the water should be measured chemically.

In the second trial, experiment B, the pregnant female guppies were exposed to EE2 after a previous exposure to GABA, and in the solvent control group this procedure was repeated but the vehicle (ethanol) was dissolved in the aquarium water without EE2. GABA concentration was doubled, along with the exposure time in an effort to trigger the dysregulation not found in the prior experiment A.

There were no significant differences in BW between the guppies exposed to EE2 and the control solvent, and no significant difference between sexes too. There were no significant differences in the GV of the group exposed to EE2 and the solvent control group. There was a significant difference between the sexes but no interaction between sex and treatment. Kinnberg et al. (2003) had similar results with a higher concentration. Indeed, in the latter study, guppy embryos were exposed to E2 (0.85 µg/L) or to the industrial xenoestrogen octylphenol (26 µg/L) via the mother fish, but no statistically significant impacts were seen in BW, length, gonopodium index and sex distribution of the offspring. Herein, we also obtained a balanced male to female ratio of 1:1 in all the tested conditions.

Facing the range of concentrations of EE2 tested in the literature and that were proved effective in feminizing fish when given at the right window of sex differentiation, we expected that our progeny would be skewed towards females. The most logical explanation for the lack of such effect is that EE2 did not reach the embryos in sufficient amounts and/or time so to influence the differentiation. In this vein, our study absolutely backs the hypothesis advanced by Kinnberg et al. (2003), according to which environmental concentrations of E2 can reach the guppy embryos but are insufficient to promote their feminization. This might be due to a mechanism of "protection of the eggs and embryo" inside the mother. It is worth recalling that in the guppy both the fertilization and gestation are intrafollicular (Venkatesh et al., 1992). In this reproductive strategy, the fully developed embryos are ovulated at the end of gestation just prior to parturition, and while E2 is produced in vitellogenic follicles, no single sex-steroid is involved in maintaining gestation in the guppy (Venkatesh et al., 1992). Moreover, once absorbed by pregnant females, and in view of what we know from fishes, and particularly zebrafish, circulating E2 will be subjected to cytochrome P450-mediated metabolism and transformed in various metabolites (Scornaienchi, Thornton, Willett, & Wilson, 2010). This phenomenon may contribute to a certain "protection" of embryos against the influence of waterborne xenobiotics, including sex-steroids, contacting a pregnant guppy. Finally, there are evidences and statements that the Poeciliidae tend to be less sensitive to oestrogens then species from other families, as illustrated by a comparatively higher resistance to sex reversal by the established xenoestrogens (Toft & Baatrup, 2003), even when compared to other livebearers, such as the mosquitofish (Drèze, Monod, Cravedi, Biagianti-Risbourg, & Le Gac, 2000; Gray & Metcalfe, 1997). Considering the state-of-the-art, we do

not know much about eventual differential impacts of EE2 and E2 in the guppy, therefore, more refined studies are justified.

In conclusion, GABA exposure, via the mother and at the concentrations tested, seems to have at most a marginal diminishing effect in the growth of the guppy in the first 5 dab, and this effect occurs independently of the sex of the fish. It is interesting to note that this effect was not observed in experiment B, in which the GABA concentration was doubled and applied to twice as long. The lack of any significant result in experiment B leads to the conclusion that EE2 is ineffective. However, because EE2 has been proven to be effective and further considering that we worked in the right time window of sex differentiation (Kavumpurath & Pandian, 1993b; Takahashi, 1975a), our results *per se* would suggest that a greater concentration of EE2 might be needed in order for the effect to be noticeable. However, this is not straightforward, because exposure route also seems to have a major influence in the guppy. Indeed, whereas Kinnberg et al. (2003) using the above cited high concentration of waterborne EE2 and observed no impacts on the progeny, Kavumpurath and Pandian (1993b) earlier tested a dietary exposure of a high dose of EE2 (400 µg/g food) and elicited a 100% female progeny. The state-of-the-art calls for studies that "play" with route, dose and dosage.

Chapter V – Conclusive Remarks

The guppy gonadal development begins before birth. The anlage of the final gonad begins as two distinctive anatomical organs with an ovarian phenotype, in which cells go from oogonia as far as chromatin nuclear oocytes before final sex differentiation. At some time before birth, throughout it and until 5 dab, each individual begins sex differentiation, either continuing it by a true ovarian development, with the enlargement and multiplication of oocytes, or by emergence of spermatogonia in parallel to death (apoptosis) of oocytes. This differentiation can happen at different times for dissimilar fishes and even in the same batch of embryos. Divergence can start with an asynchronous fertilization, and its descriptive aspects are visible at light bright field microscopy. In the guppy, the finding that ovary-to-testis transition is not made for all the fish at birth is controversial, backing some previous authors and contracting others that reported a clear gonadal definition for all fish at the time of birth; our study do not support the latter view.

In guppies, the gonad is located near the swim bladder, gut, and remains of the yolk sac when present. In the more developed individuals at 5 dab, the beginning of the unification of the two primordial gonads into one can be observed.

At 10 dab, some gonads have already been unified and at 60 dab there are only a few ovaries still in a dual morphology. However, the testis shows a delay in the unification and development when comparing to the ultimate result of the adult, i.e., a fully developed fertile gonad present in a single structure.

Using design-based stereology (based on the Cavalieri's principle), it was possible to quantify the volume of the early ovaries and early testis. At 0 dab an ovary has a mean volume (not corrected for paraffin shrinkage) of 1242 x 10^3 μm^3 (SD = 415 x 10^3 μm^3) and this volume increases to 1786 x 10^3 μm^3 (SD = 874 x 10^3 μm^3) at 5 dab. In males, the testis begins with an average volume of 318 (SD = 61 x 10^3 μm^3) at 0 dab, and growths to about 442 (SD = 299 x 10^3 μm^3) at 5 dab. When the individuals are appropriately differentiated, the ratio between the gonad volumes is exactly 4 times larger for females of both ages.

At 0 dab, many gonads were found to be undifferentiated and in some particular cases even 5 dab there were guppies with undifferentiated gonads. However, the variability of the guppy development seems to be due to not only deviations of the time of fertilization of a given brood, but also deviations between strains and wild versus ornamental fishes, since many of the measurements in this

work did not match the references published. Up until 60 dab there were no significant differences between the body weights of the females and the males.

The protocol tested here, in the Experiment A, for inducing fish sterility, by relying on the potential disruptive effect GABA on the formation of the gonadotropin-releasing hormone system during early development, and consequently of the regulation of germ cell development, proved insufficient. At a concentration of 1 mg/L administered via bath immersion of females during the first 3 days after the parturition, a decrease in the BW had a marginal non-significance and well as a disruption in the sex ratio with p-values of ≈ 0.05. Nevertheless, this modest consequence disappeared when the concentration was increased to 2 mg/L for 6 days after the first parturition, in Experiment B. These results made us suggest that the next phase should focus not in an increase in dosage, but in tuning out the key concentration (and eventually delivery mode) for obtaining the desirable effect. Testing lower quantities and different timings should be a priority, since the sex differentiation window in the guppy varies with strain and might be larger than that described for other species. In fact, even in very well studied species, particularly in zebrafish, unforeseen new facts continue to emerge as to sex differentiation.

Here, in the Experiment B, which tried to bias the sex differentiation mechanism of the guppy towards a female phenotype, recurring to the feminizing potential of the hormone EE2, administered via bath immersion of the mother, we found no effect in either the gonad or the BW. Under certain experimental conditions (high dose in the food), EE2 is known to be capable of inducing total feminization in the gonad differentiation of a guppy's progeny, promoting feminization. Accordingly, future studies should test higher water concentrations of EE2, since it is seems that with a bath immersion technique and at environmentally relevant doses, the offspring is "shielded" by the mother from the compound effects.

Despite the experiments A and B could not support our hypotheses, they allow us to discard some scenarios and equate new ones in the future, while offering new qualitative and quantitative data on the guppy gonad differentiation.

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Note: In-text citations and reference list based on the 6th edition of the Publication Manual of the American Psychological Association (APA).

Annex A – Protocol of Tissue Processing for Histology

Protocol for tissue processing after fixation for 24 hours. Each of the 11 steps has a duration of 30 minutes, as programed in the automated tissue processor:

1. Alcohol 70%
2. Alcohol 95%
3. Alcohol 95%
4. Alcohol 100%
5. Alcohol 100%
6. Alcohol 100%
7. Alcohol/Xylene (1:1)
8. Xylene
9. Xylene
10. Paraffin
11. Paraffin

Annex B – Protocol of Haematoxylin-Eosin Staining

Protocol for the staining routine of the slides with the sections after extension and 24 hours to dry in the incubator:

Dewaxing:

1. 10 minutes in xylene
2. 10 minutes in xylene

Hydration:

1. 5 minutes in Alcohol 100%
2. 5 minutes in Alcohol 95%
3. 5 minutes in Alcohol 70%
4. 5 minutes in flowing water

Staining:

1. 2 minutes in Mayer's Hemalum Solution
2. 5 minutes in flowing water
3. Quick dip in alcohol acid (differentiation solution)
4. 5 minutes in flowing water
5. 1 minute in aqueous 1% (1g/100 mL) Eosin Y solution
6. Quick dip in flowing water
7. Quick dip in Alcohol 100%
8. Quick dip in Alcohol 100%
9. Quick dip in Alcohol 100%
10. Xylene
11. Xylene
12. Mounting with DPX

Solutions:

A. 1% Eosin Y Solution: In 100 mL in distilled water, dissolve 1 g of Eosin Y.

B. Differentiation Solution: Add to an alcohol 80% solution (800 mL of 99.9% alcohol to 200 mL of distilled water), 5 mL of 37% hydrochloric acid and make up to 1 L of alcohol 80%.

Gonad Differentiation in Guppy Fish and Testing Strategies for the Production of Sterile and All-Female Offspring

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