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Oocytes Development in the Fry of Blue Gourami, *Trichogaster trichopterus*

Gad Degani [1,2]

[1]MIGAL – Galilee Technology Center, P.O. Box 831, KiryatShmona 10200, Israel
[2]School of Science and Technology, Tel-Hai Academic College, Galilee, Israel

**Corresponding Author*

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Abstract: In this study, we examined the first period of ovarian development as well as the levels of Estradiol-17β (E_2) and gonadal CYP19 (bgCYP19a) in blue gourami, *Trichogaster trichopterus*. Samples of larvae were taken 38, 55 and 80 days after hatching for histological examination. In the ovary of 38 days larvae, the number of oocytes and ovary size were small. On day 80 after hatching, the number of oocytes increased. On day 38 after hatching, all the oocytes were in the perinuclear stage. This stage did not change on day 80, but very few oocytes did change. In several oocytes, there is brining of granulose continuous vitelline which indicates the start of vitellogenesis. The mRNA expression of the bgCYP19a gene was detected by using real-time PCR (TPCR) in the tissues of 38- and 80-days larvae after hatching. The level decreased insignificantly from day 38 to day 80. The E_2 increased significantly in larvae at the age of 80 days compared to larvae at the age of 38 days.

Keywords: Estradiol-17β (E_2), Gonadal CYP19 (bgCYP19a), Blue gourami, Vitellogenesis

Introduction

The increasing economic importance of aquarium fish has encouraged studies of their reproductive physiology. Blue gourami (*Trichogaster trichopterus*) is a multispawning, asynchronic and male-dependent fish (Degani, 1993). Blue gourami belongs to the order Perciformes and suborder Labyrinthici (characterized by the presence of an air-filled breathing cavity, the labyrinth located above the gills under the operculum), and to the family Anabantidae. The Anabantidae family contains 16 genera and about 50 species. It is distributed throughout most of southern Asia, India and central Africa, and is native to Malaysia and Thailand (Forselius, 1975). It gets its name from its basic blue coloring. The gourami has a touch of silver on its belly and even a slash of green on the tips of its gill covers and fins. This native blue gourami also has two large black spots on its sides, with the eye making up the third spot

(hence the name three-spot) (Degani, 2001). The labyrinth is a circular spot of much wrinkled tissue that offers more surface area for oxygen intake. This cavity supplements the breathing function, since it is well suited to gaseous interchange (Degani, 2001). Because of these accessory organs, Anabantidae fishes can survive in water with very little oxygen content (Forselius, 1975; Degani, 2001). The males often become territorial and very protective of the bubble nest. As the female prepares to lay her eggs, the male wraps himself around her, catches the eggs in his mouth and spits out the eggs into the nest (Degani, 1989, 1993).

The blue gourami fish offers the great advantage in that the later stages of oogenesis can be controlled. In females, ovarian development starts at three months of age, and vitellogenesis is completed at five months in fish maintained in dense populations, at which stage the female is ready to reproduce. Oocyte maturation (OM) and ovulation (OEV) take place only when the female is isolated with a male. This clear differentiation between vitellogenic and maturational stages makes this species a good model for the study of hormonal control of oogenesis (Jackson *et al*., 1994). The oogenesis of mature female blue gourami is described in detail (Jackson *et al*., 1994) and the hormones controlling oogenesis have been studied in detail along the brain pituitary gonadaxis (BPG-axis). In blue gourami, relative mRNA levels of Kisspeptin2 (Kiss2) and Kiss receptors (GPR54 or Kiss2r, Kiss1r) were determined in brains excised from females before vitellogenesis (previtellogenic) (PV) and during vitellogenesis (VT) in mature females (Degani *et al*. 2017a). The differences found between Kiss2 and Kiss2r in juvenile and mature females were statistically significant. There was no significant difference between PV and VT in the mRNA levels of Kiss1r. In comparing the transcription of Kiss1r to Kiss2r and Kiss2 mRNA levels, we found significant differences in transcription levels in both juvenile and mature females between the genes, and proposed a quality model showing the Kiss2 mechanism involved in regulating the main states of oogenesis VT (Degani *et al*., 2017a). Thus, Kiss1 controls the hypothalamus pituitary gland (HPG) axis, acts on the caudal hypothalamus and seems to affect receptors of the gonadotropin-releasing hormone (GnRH) (Servili *et al*., 2011 ; Shahjahan *et al*., 2014). The GnRH1 controls VT, and GnRH3 regulates OM and OEV (Levy *et al*., 2009; Levy and Degani, 2011) in blue gourami by affecting gonadotropins (GTH), the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) (Jackson *et al*., 1999). The GTH hormones control oogenesis by various steroids in blue gourami (Degani, 2016, 2017). FSH controls 17β-estradiol (E_2). Testosterone (T) increased during VT at a relatively high level in the plasma (Degani, 1990; Degani *et al*., 1994) and 17α,20β-dihydroxy-4-pregnen-3-one (17, 20 P) increased during OM and OEV in blue gourami (Degani and Boker, 1992; Degani, 2017).

Although several studies exist on oogenesis in mature female blue gourami, the period of gonadal development before the maturation of ovarian development has been studied meagerly. This is a very critical period of gonadal differentiation and well described in different fish (Tzchori *et al*.,

2004; Hurvitz *et al*., 2007; Yaron and Levavi-Sivan, 2011) and is controlled by various genes and steroids. In blue gourami, the gonad cytochrome P450 aromatase affects gonadal differentiation in the ovary (Ezagouri *et al*., 2008) similar to the other fish (Tzchori *et al*., 2004).

The aim of the present study is to examine the critical period when gonads differentiate to the ovary and describe the beginning of ovarian development in the fry of blue gourami.

Materials and Methods

Fish maintenance and growth

Female and male blue gourami (*Trichogaster trichopterus*), maintained and bred at MIGAL laboratories in northern Israel, were used in this study. The fish were grown in containers measuring 2 x2 x0.5 m at a temperature of 27C and under a light regime of 12h light: 12h darkness (Degani, 2014). The fish were fed artificial diet (45% protein,7% fat) supplemented by live food (*Artemia salina*). Breeding and larvae growth were described previously in detail (Degani, 1989, 2001; Degani and Gur, 1992).

Several days after the larvae hatched from the eggs, the fish were fed twice a day with a mix of larvae dry food with yeast (52% protein and 5.8% fat (Miloubar, Oshrat, Industry, Israel). After one month, the fry were fed only dry food (48% protein and 6% fat), about 5% of their body weight, and 3% of mixed meat was added (77% turkey heart, 13% chicken liver, 1% vitamins and minerals, and 3% tubifex).

The larvae of blue gourami were collected five days after hatching and every two days for two months for whole body analysis and histological examination during the 80 days of growth. The larvae were sampled on different days after hatching (38, 55 and 80 days). For sampling, each fish was anesthetized in a clove oil bath (0.25 mg/l), and length was recorded using binoculars. All of the larvae bodies (n=7) at different ages (38, 55 and 80 days) were used for expression studies and were stored in RNALater buffer (AmbionInc., Austin, TX). Total RNA was extracted from RNALater stored in tissues using the RNeasy_Total RNA Kit (QIAGEN, Alameda,CA) according to manufacturer's recommendations (Ezagouri *et al*., 2008).

Real-time PCR ovary aromatase (CYP19a)

Estradiol-17β (E_2)

The real-time PCR ovary aromatase of blue gourami (bgCYP19a) was carried out as described in detail by Ezagouri *et al.* (2008). cDNA cloning was made of all larvae bgCYP19a, sequence, assembly and analysis, as described by Ezagouri *et al.* (2008). Total RNA was extracted from freshly excised blue gourami larvae. First-strand cDNAs were synthesized from 2 lg of each total RNA sample larvae using the Superscript System (Invitrogen, Carlsbad, CA). The single-strand cDNAs were used to amplify, clone and sequence cDNA internal fragments using gene-specific primers for bgCYP19a (Ezagouri *et al.*, 2008). The gene-specific primers for the cloning of bgCYP19a cDNA internal fragments were designed according to conserved DNA sequences and identified by multiple sequence alignment of several larvae aromatase cDNAs (Ezagouri *et al*., 2008).

In order to compare the levels of bgCYP19a mRNAs in larvae, their relative abundance was normalized with an endogenous reference, the rRNA of the 18S subunit, using the comparative threshold cycle (CT) method (Livak and Schmittgen, 2001). This method was validated using serial dilutions (0.5, 0.10, 0.01, 0.02 and 0.005) of cDNA preparations from the larvae body (for CYP19a). The amplification efficiencies of each target mRNA and 18S rRNA were compared by plotting DCT versus log (template) (Muller *et al*., 2002; Ezagouri *et al*., 2008). Estradiol-17β (E_2) was determined by specific ELISA (Hurvitz *et al*., 2005).

Histological examination

The histological preparations of larvae during growth and gonadal development were made as previously described (Jackson *et al*., 1994; Jackson *et al*., 2005), with modifications to study the larvae histology. The larvae were aestheticized with 0.2 g/l ethyl m-amino benzoate methane sulfomate (MS222). The larvae and juveniles were then transferred for 48h in 8% formic acid. The samples were examined using a light microscope, incubated at different ethanol concentrations, washed with 70% for 1h, twice with 96% for 2h (after 1h added fresh solution) and with 100% for 3h (after every 1h changed with fresh solutions). The samples were then incubated in Xylene (Frutarom, Haifa, Israel) and each washed twice for 45 minutes. To penetrate paraffin into the tissue, the samples were incubated overnight in a mixture of 1:1 paraffin xylene at 60C. One day later, the samples were washed twice (one hour each) with 100% paraffin and incubated overnight at 60C.

The larvae were embedded in paraffin and glycol methacrylate. Thin paraffin sections (6 μm) were cut using Microtome (Minnesota, USA GMI, Ramsey Biocut 2030 Microtome) and stained with Mallory trichrome or PAS (Jackson *et al*., 1994).

Results

The gonadal development of juvenile females is presented in Figures 1-3. In Figure 1, the histology of juvenile females at the age of 38 days and a length of 11 mm is depicted. Before this age after hatching, it is very difficult to identify the gonads in a light microscopic study.

In the larvae at 38 days, it is possible to identify the ovary just after differentiation with primary oocytes at perinuclear stages, kidney, gut, liver and muscle (Fig. 1).

In female larvae at the age of 55 days and a length of 22 mm, the number of oocytes increased at the perinuclear stage and the diameter was larger than 50 μm (Fig. 2). In female larvae at the age of 80 days and a length of 45 mm, it is possible to recognize several oocytes at the brining of granulose continuous vitellogenin (Fig. 3).

The mRNA expression of the bgCYP19a gene was investigated in larval tissues at 38 and 80 days after hatching using RTPCR and are shown in Figure 4. The abundance of bgCYP19a transcripts indicated that the gene was expressed at both ages as indicated. The mRNA expression of bgCYP19a at 38 and 80 days was relatively high in the larvae at these ages; the difference was not significant at the different stages (Fig. 4). The E_2 level of larval tissues at the age of 38 days was significantly lower than in the larvae at the age of 80 days (Fig. 5).

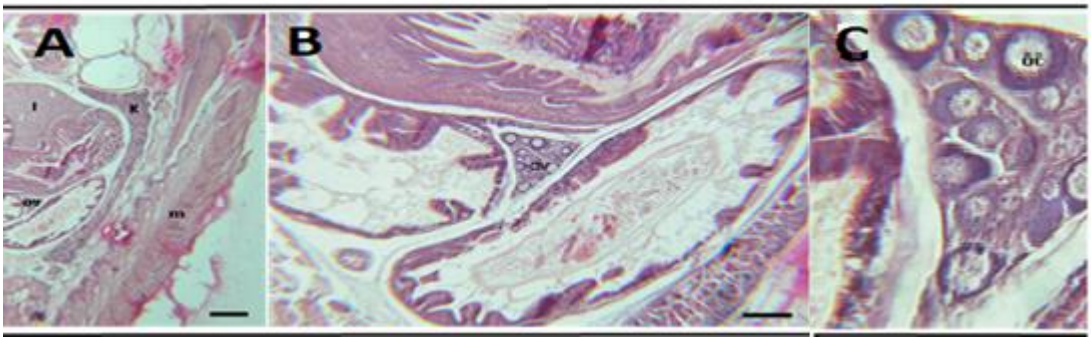

Figure 1: Histological section of a juvenile female with a standard length of 11mm (38 days). (A) ov, ovary; k, kidney; g, gut; l, liver; m, muscle. Bar =190µm. (B) ov, ovary. Bar = 71µm. (C) Oocyte (OC) at the perinuclear stage. Bar = 20µm.

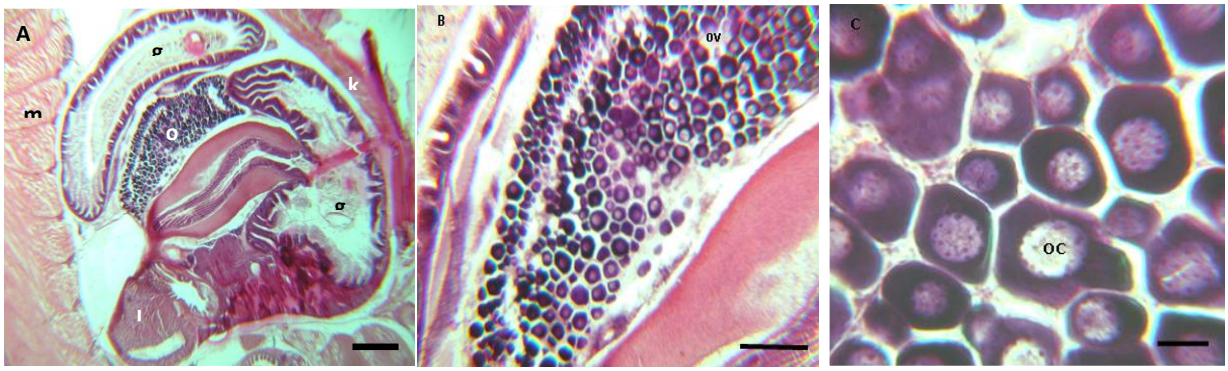

Figure 2: Histological section of a juvenile female with a standard length of 22mm (55 days). (A) ov, ovary; k, kidney; g, gut; l, liver; m, muscle. Bar = 320µm. (B) ov, ovary. Bar = 121µm. C) Oocyte (OC) at the perinuclear stage. Bar = 20µm.

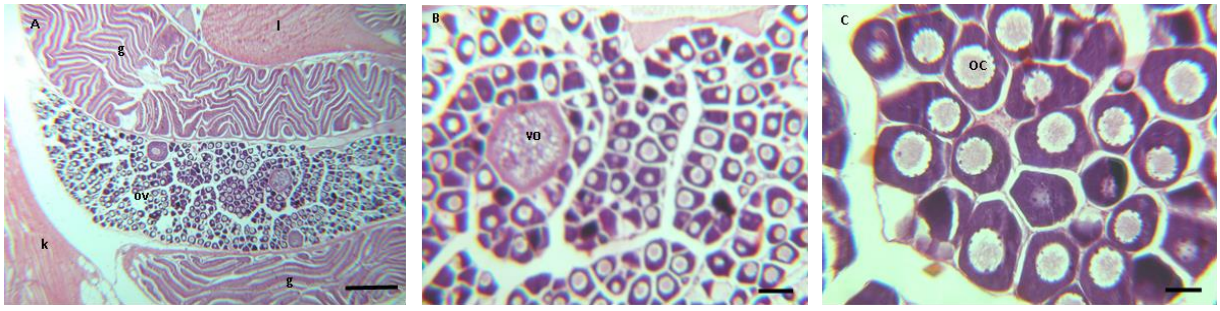

Figure 3: Histological section of a female larvae with a standard length of 45mm (80 days). (A) ov, ovary; k, kidney; g, gut; l, liver. Bar = 360µm. (B) vo, vitallogenic oocyte. Bar = 45µm. (C) Oocyte (OC) at the perinuclear stage. Bar = 20µm.

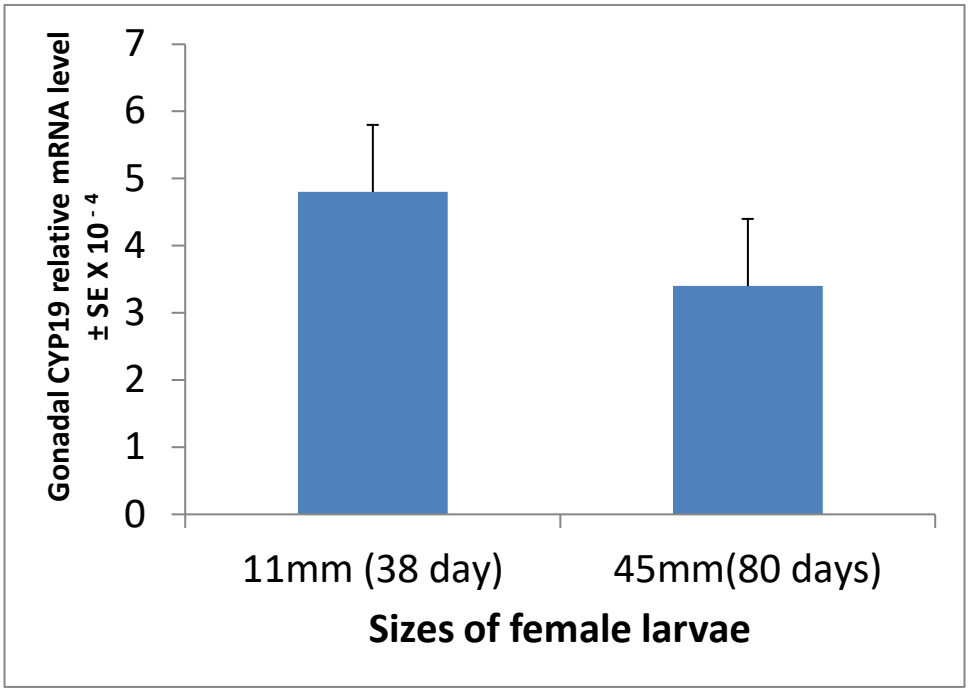

Figure 4: Gonadal bgCYP19a mRNA level (mRNA/18 S)(mean+SE) in blue gourami larvae

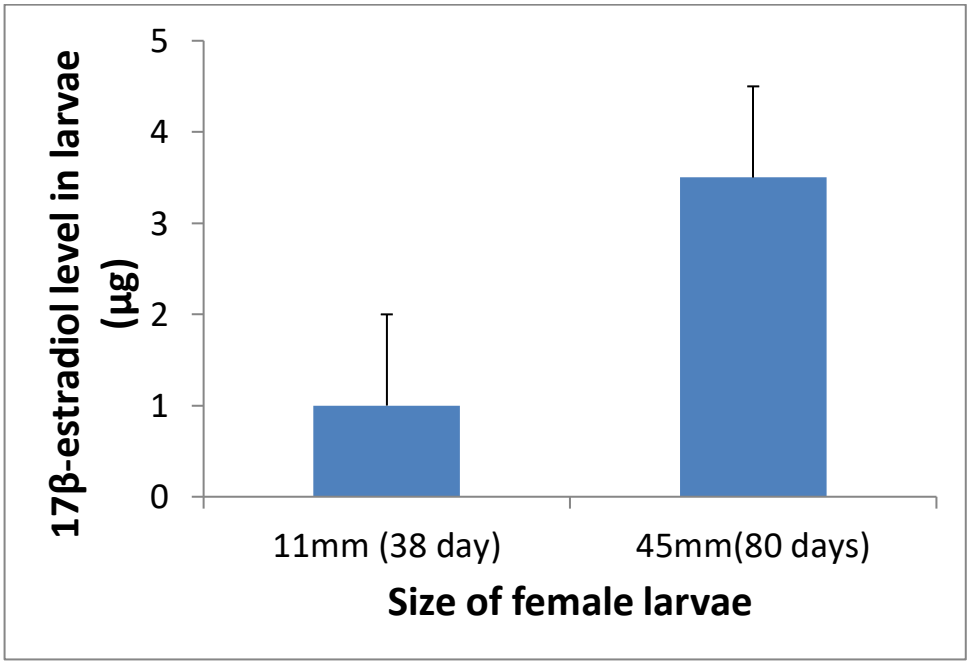

Figure 5:17β-estradiol (E_2) level in larvae. Each histogram represents the average of five independent measurements (mean+SE)

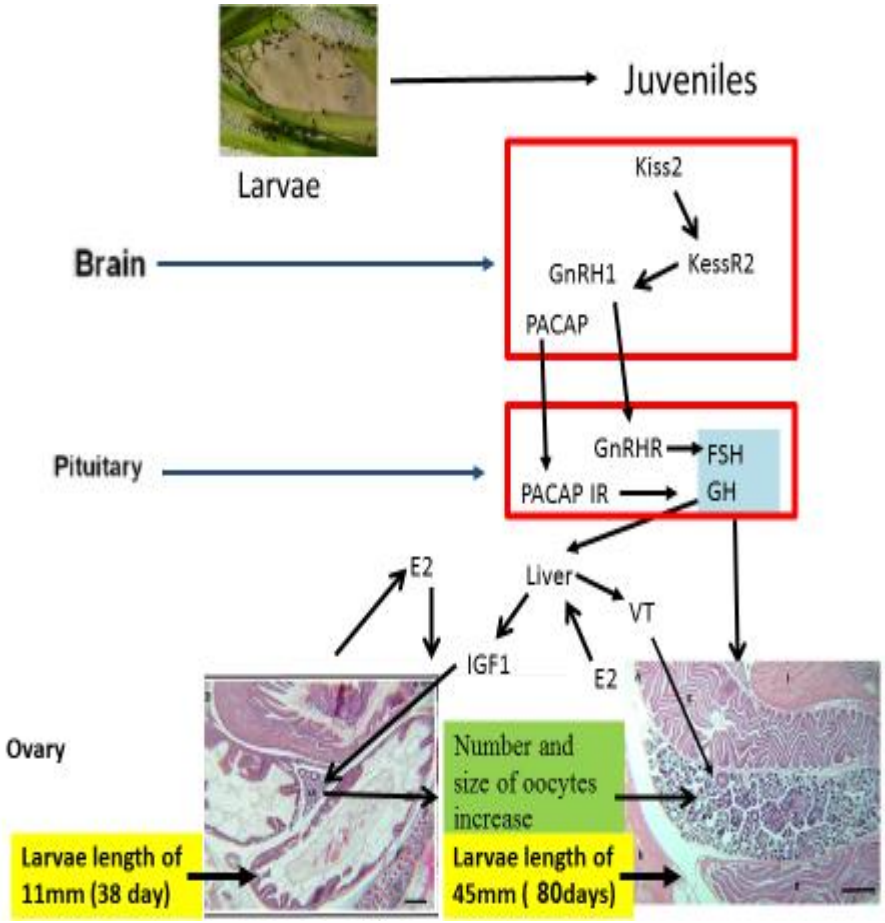

Figure 6: A proposed quality model showing the hormone involved in ovarian development before vitellogenensis. Abbreviations: II receptor (GnRH IIR), pituitary adenylate cyclase-activating polypeptide type I receptor (PACAP IR), and G-Protein coupled receptor 54 (Kisspeptin-54 (GPR54, KissR2), Kisspeptin-2 (Kiss2),17β-estradiol (E_2), follicle-stimulating hormone (FSH), growth hormone (GH), insulin-like growth factor 1 (IGF1), vitellogenin (VT) mechanism involved in regulating oocyte develop before vitellogenesis in blue gourami. The model is based on the present study and Degani (1990, 1993), Degani and Boker (1992), Jackson *et al*. (1994), Degani *et al*. (1994, 1995, 1999, 2017 a), Mananos *et al*. (1997), Levy and Degani (2011, 2012)

Discussion

In the present study, the ovarian development is described in the larvae of blue gourami hatched from eggs at various stages. The larvae of blue gourami were grown in optimal conditions at 27C. At the age of 38 days, only small ovaries were detected before vitellogenesis, and at the age of 80 days, this is just before this process started. The vitellogenesis period has been described in numerous studies. It is of special interest in fish having economic value in order to study the control of reproduction in those species (Yaron and Levavi-Sivan, 2011). During this period, the steroids controlled by FSH affect gonad differentiation (Degani, 2017). However, this critical period varies in different fish species (Degani, 2016). In the present study the bgCYP19a mRNA level was high before vitellogenesis, and decreased slightly when vitellogenesis started. This result is in agreement with Ezagouri *et al.* (2008) who described the effect of bgCYP19a during vitellogenesis in blue gourami. The bgCYP19a mRNA level was high at vitellogenesis and very low at advanced vitellogenesis (Ezagouri *et al*., 2008). The explanation for this result is given by Ezagouri *et al.* (2008), whereby in advanced vitellogenesis, the process stopped and waited until maturation, which was dependent on male behavior (Degani, 1993). On the other hand, the E_2 was very low in ovaries of larvae after 38 days of growth, as noticed in the present study, and increased at 80 days of growth just before vitellogenesis started. This result is also in agreement with reports by Degani (1990). The transcription of Kisspeptin2 (Kiss2) and Kiss receptors (GPR54 or Kiss2r, Kiss1r) in the brain of blue gourami (Degani *et al*., 2017a), together with a study on GnRH1, 2 and 3 (Levy and Degani, 2012), showed an effect of FH and LH in controlling oogenesis (Degani, 2016) with steroids. A proposed quality model showing the Kiss2 mechanism involved in regulating oogenesis in the BPG-axis in blue gourami affected vitellogenesis (Degani *et al.*, 2017a) strengthens the results of the present study. The process of sex differentiation in blue gourami was less clear before the female ovary appears. In other fish, CYP19 is the main enzyme affecting the gonad to differentiate to the female (Tzchori *et al.*, 2004). CYP19a expression is higher in females than in males (Ezagouri *et al*., 2008), therefore CYP19a is apparently involved in sex differentiation in females. In the present study, CYP19a was detected in the larvae at both 38 and 80 days, but the difference was not significant. Therefore, we suggest that this enzyme, which changes testosterone to E_2 is active just before the start of vitellogenesis. E_2 is significantly higher in larvae at the age of 80 days compared to 38 days, and at 80 days, several oocytes are at the beginning stage of vitellogenesis. Another very important finding is that the number of oocytes found in the pre-vitellogenesis stage increased in larvae from 38 days to 80 days. This finding in blue gourami might be explained by the interaction between the BPG-axis including kiss, GnRH, FSH, LH (Levy and Degani, 2012; Degani, 2016; Degani *et al*., 2017a) and steroids. The situation of larvae growth and gonad development before vitellogenenin (VT) synthesis and accumulation in the oocytes is more complex due to the fact that not only is the BPG-axis involved but the

somatic-axis has an important effect. The somatic-axis, which includes PACAP (Levy and Degani, 2011), GH (Goldberg *et al*., 2004) and IGF 1 (Degani, 2014), is described in blue gourami and is involved in oogenesis in this species. This interaction was found not only in blue gourami but also in other fish, e.g. Russian sturgeon (*Acipenser gueldenstaedtii*) (Hurvitz *et al*., 2005, 2007; Yom Din *et al.*, 2008, 2016; Degani *et al*., 2017a, b), European eel (*Anguilla anguilla*) (Degani *et al*., 2003; Tzchori *et al*., 2004), as well as in other fish (Yaron and Levavi-Sivan, 2011). We propose the quality model describing ovarian development before vitellogenesisis (Fig. 6).

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