International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

ISSN: 2454-3055

Evidence of a Reproduction-Related Function for Brine Kisspeptin2 and its Receptors in Fish *Trichogaster trichopterus*

Degani Gad[1,2*], Alon Amir[1,3], Stoler Aviad[4] and Bercovich Dani[1,4]

[1] School of Science and Technology, Tel-Hai Academic College, Upper Galilee 12210, Israel.
[2] MIGAL–Galilee Technology Center, KiryatShmona 11016, Israel
[3] Ma'abarot fish farm, KibutzMa'abarot, Israel.
[4] GGA (Galilee Genetic Analysis lab) Israel.

**Corresponding Author*

Received: 6th October 2017
Accepted: 27th October 2017

Abstract: This paper describes the transcription of Kisspeptin2 (Kiss2) and Kiss receptors (GPR54 or Kiss2r, Kiss1r) in blue gourami (*Trichogaster trichopterus*), which belongs to the suborder Anabantoidei and family Osphronemidae. A low degree of identity cDNA acid sequences was found in homologous partial sequences Kiss2 and Kiss1r from a number of fish species as compared to the blue gourami sequence, however, the variation in Kiss2r and other fish species is much lower. In order to determine whether the Kiss2, Kiss1r and Kiss2r genes are expressed in the brain of females, total RNA were reverse-transcribed using qPCR amplification in comparison to the Beta-Actin gene. Relative mRNA levels of Kiss2, Kiss1r and Kiss2r were determined in brains excised from females before vitellogenesis (previtellogenic) (PV) and during vitellogenesis (VT) in mature females. The differences found between Kiss2, Kiss2r, 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. We proposed a quality model showing the Kiss2 mechanism involved in regulating vitellogenesis in blue gourami.

Keywords: Kisspeptin2, Kiss receptors, Blue gourami, Vitellogenesis, Gonads

Introduction

In teleosts, as in other vertebrates, Kisspeptin has recently received considerable attention as a potential key player in the neuroendocrine control indirectly of reproduction (Oakley *et al.*, 2009). Kisspeptin is a member of the RFamide peptide family. Originally identified as a metastasis suppressor in mammals, the Kiss1 gene produces several peptides called Kisspeptins in mammals. Kisspeptin-54 and its endogenous variants, Kisspeptin-14, Kisspeptin-13 and Kisspeptin-10, are

generated by proteolytic cleavage of the Kisspeptin precursor derived from the Kiss1 gene. They share a common core 10 amino acid sequence Kisspeptin-10 at their C-terminal, which allows them to bind to their cognate G-protein coupled receptor GPR54 or Kiss1r (Muir *et al*., 2001; Servili *et al*., 2011). Thus, Kiss1 controls the hypothalamus pituitary gland (HPG) axis, acts on the caudal hypothalamus and seem to affect receptors of the gonadotropin-releasing hormone (GnRH) (Servili *et al*., 2011; Shahjahan *et al*., 2014). It controls the release of pituitary gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), which in turn control gametogenesis (Yaron and Levavi-Sivan, 2011) by steroids (Degani, 1990; Degani and Boker, 1992). Studies on Kisspeptins in teleosts have shown some variation of their involvement in reproduction; more detailed studies are required due to the relatively large systematic class and high variations of hormones involved in reproduction. The brain of zebrafish (*Danio rerio*), one of the most intensively studied model fish, has two kiss genes, Kiss1 and Kiss2, and two kiss receptors (GPR54), kiss1r and kiss2r (Lee *et al*., 2009; Servili *et al*., 2011) similar to other fish such as lamprey (*Petromyzon marinus*)(Lee *et al*., 2009), medaka (*Oryzias latipes*)(Kitahashi *et al*., 2009), gold fish (*Carassius auratus*)(Huang *et al*., 2009) and sea bass (*Morone saxatilis*)(Espigares *et al*., 2017).

Blue gourami (*Trichogaster trichopterus*) belongs to the Labyrinthici suborder (characterized by the presence of an air-filled breathing cavity (the labyrinth is located above the gills under the operculum) and the Anabantidae family (Degani, 2001). It serves as a useful model for studying the role of endocrine regulation on reproduction since it is a multi-spawning and male-dependent, with asynchronic ovary development (Jackson *et al*., 1994). Both environments and pheromones (Becker *et al*., 1992; Degani and Schreibman, 1993) affect hormonal control of reproduction, oogenesis (Degani, 2016) and spermatogenesis (Degani, 2015). Thus, each stage of its gonadal development can be controlled and examined separately in the laboratory (Degani, 1993; Jackson *et al*., 1999; Degani, 2001). The secretion and gene expression pattern of βFSH, βLH growth hormone (GH) (Degani, 2016), as well as sex steroid secretion during gonadal development in male and female blue gourami have been previously reported (Degani and Boker, 1992 ; Becker *et al*., 1992 ; Degani *et al*., 1994). In blue gourami, the mRNA levels of GnRH1, GnRH2 and GnRH3 (Levy and Degani, 2012) are measured during the oogenesis and are correlated to FSH and LH (Jackson *et al*., 1999) transcription. The interaction of these hormones along the hypothalamus, pituitary and gonad axis (HPG) was suggested (Degani, 2016). Pituitary adenylate cyclase-activating polypeptide (PACAP) cDNA sequence measured mRNA expression profiles of PACAP in blue gourami during different states of reproduction, and the role of PACAP in regulating pituitary hormone transcription was examined (Levy and Degani, 2011; Levy *et al*., 2011). In this study, we examined the brain peptide Kiss2 and two kiss receptors (GPR54), kiss1R and kiss2R, involved in reproduction during vitellogenesis in a teleost blue gourami (*Trichogaster trichopterus*). More specifically, we examined if the brain hormone mRNA level would differ in transcription between juvenile and adult

females of Kiss2 and two kiss receptors (GPR54), kiss1r and kiss2r.

Materials and Methods

Fish and sampling procedure

Blue gourami fish (*T. trichopterus*), which were maintained and bred on the Ma'abarot fish farm on Kibbutz Ma'abarot, Israel, were used in this study. The mean body weights (BW) of the mature females were 6.64±0.55 g and of the juvenile females, 0.96±0.13 g; the difference between two groups was significant ($p<0.05$, t-test). Investigations were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. The fish were grown in containers (2×2×0.5 m) at a temperature of 27 C under a light regime of 12-h light/12-h darkness (Jackson *et al.*, 1999) and fed artificial diet (45% protein, 7% fat) supplemented by live food (*Artemia salina*).

Brains were collected from females at different stages of gonadal development (juvenile and mature) (Fig.1). The samples were taken and cDNA was prepared from each sample for further study, as previously described (Levy and Degani, 2011). For the specific study of gene expression in the brain, 2-month-old females (BW 2.6±0.57 g) at various stages of pre-vitellogenesis (PV) (Fig. 2) of gonadal development and 4-month-old females at high vitellogenesis (HV) (BW 4.6±0.57 g) (Jackson *et al.*, 1994) were used (Fig. 2). The fish were anesthetized with 0.03% tricaine methane sulfonate (MS222, Sigma-Aldrich), and their fork length and body weight were measured. To examine the Kiss1 and 2, and kiss1r and kiss2r mRNA levels, brains were removed (Levy and Degani, 2011) after decapitation and sectioned sagittally along the hemi-brains and frozen in tubes (1.5ml) with RNAlater at -25 C for analysis (Fig.1).

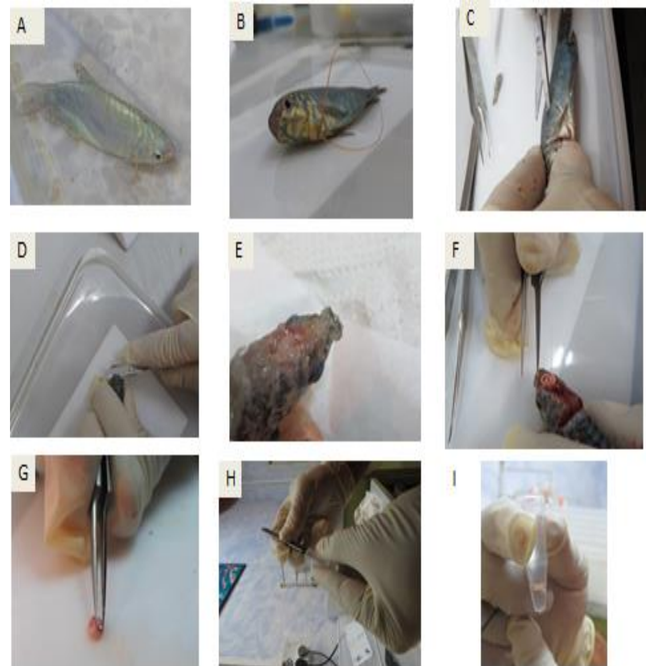


Fig. 1. The fish were anesthetized, the brains were removed after decapitation and the brains were sectioned sagittally along the hemi-brains, frozen in tubes 1.5 ml with RNAlater at -25 C for analysis. **A**: female, **B**: anaesthetized, **C**: open the female and examine ovary development, **D**: remove the top-side brain, **E**: the top-side brain in the head, **F and G**: remove the brain, **H and I**: insert the brain into the tube with RNAlater.

Histological analysis

Gonadal samples were fixed in Bouin's fluid and subsequently processed for light microscopy. Paraffin sections of 6mm were stained with hematoxylin and eosin, as previously described (Jackson *et al.*, 1994) (Fig.2).

RNA extraction and cDNA synthesis

Total RNA was extracted from freshly excised whole brains of females. The brain was removed and soaked in RNAlater (ThermoFisher, Waltham, Massachusetts, USA) at 4 C for 20 h. The brains were trimmed to prepare diencephalon/midbrain for Kiss1,

Kiss2, Kiss1r and Kiss2r examined in this study. Brains were frozen immediately with liquid nitrogen and stored at -80 C until the

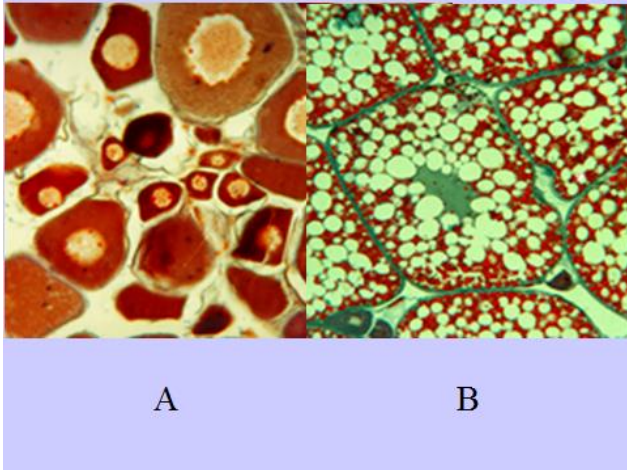


Fig. 2. Females divided into two groups according to gonadal development stage. A: Young females before vitellogenesis (pre-vitellogenesis), B:Females at advanced stages of vitellogenesis (high vitellogenesis).

extraction of total RNA (RNeasy® Mini, Qiagen) using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. First-strand cDNA was synthesized by the QuantiTect Reverse Transcription Kit (Qiagen) from 0.5 to 2 mg total RNA according to the manufacturer's recommendations (Levy and Degani, 2011). The sequences obtained from the cloned fragments were used to design additional gene-specific primers for the 3' and 5' rapid amplification of cDNA ends (RACE)-PCR (Table 1). Sequencing of the cloned cDNA was carried out. At least three independent clones were sequenced in each case. The sequences (Table 1) of each cDNA were assembled using the GAP4 software package (Bonfield *et al.*, 1995), and sequence analyses were carried out by the Genetics Computer Group (GCG) Version 11.1 software package Accelrys Inc. (San Diego, CA, 2006). The position of the signal-peptide cleavage site was determined with the SignalP V1.1 program (Nielsen *et al.*, 1997), and multiple sequence alignments and phylogenetic cluster analysis were done by using the ClustalX computer program.

Real-time PCR

cDNA levels of Kiss1, Kiss2, Kiss1r and Kiss2r from the brains of individual gourami in the primary culture of dispersed brain cells were measured with NanoDrop® ND-1000 (Thermo Scientific). The relative abundance of mRNA was normalized with B-actin as a reference gene according to previous studies (Pfaffl, 2001), and mRNA expiration was calculated (Muller *et al.*, 2002; Pfaffl, 2004). The relative amount of each gene was calculated using the formula $2^{-\Delta Ct}$, where ΔCT corresponds to the difference between the CT measured for each target gene and that determined for B-acti RNA. To validate this method, serial dilutions were prepared from a brain cDNA sample, and the efficiencies of gene amplifications were compared by plotting ΔCT versus log (template) according to the method of Muller *et al.* (2002). Different primers were used to amplify the genes Kiss2, Kiss1r and Kiss2r in various species (Blue bass, Puffer fish, Medaka and zebrafish; Table 1). Linear regressions of the plots showing R^2 values of 0.99 were further considered for use in the present study.

Statistical analysis

Data are presented as mean ± SE. The significance of the differences in fish in the experimental groups was examined by t-test. The significant difference among the mRNA levels was determined using one-way ANOVA (F). Differences were considered statistically significant at $p<0.05$.

Results

The deduced partial sequences of this gene in blue gourami Kiss2 (Table 2) were compared with homologous sequences from a number of other fish species (Fig. 3). There was a low

Table 1. Nucleotide sequences of primers for brain Kiss1, Kiss2, Kiss1r and Kess2r genes of blue gourami cyclase-activating expression by reverse-transcribed PCR and for real-time PCR

Species	Gene	Synthesis direction	5'-3' sequence
Zebrafish	Kiss1Receptor	Forward	TCCGTTCAGAAGCACTGTGG
Zebrafish	Kiss1Receptor	Reverse	TATTTCCACCTTCGGTGCTC
Zebrafish	Kiss2 receptor	Forward	GTCATTAAAAACCAGCAGATGAAGAC
Zebrafish	Kiss2 receptor	Reverse	GTGGTGCACACAGACAGAGCCA
Medaka	Kiss2	Forward	GGTTGTGCTCGTGCTGTGC
Medaka	Kiss2	Reverse	CAGAGTCGTCCTCGCTCCTG

Table 2. Partial cDNA sequences of this gene Kiss 2, Kiss 2r and Kiss 1r of blue gourami were used to compare with homologous sequences from other fish species (Figs. 3, 4 and 5)

	Trichogaster trichopterus sequences
Kiss2	CTCCGCTGTTGTGTCTCGCKCGGGGAGCGCYGGCCTGTGAATCTYCCCTCCTGGTGGTGG ATTCAGCTCTWGCAWTAWCGGGAGGCAGSAACGMCCGAGCTSGGTGCTMCCGTGGGGGGC ATTTYCYGTTTGWGGCCCGACTCGAAAGCCCRACCCAGGTAKGYGGACTTGKTGCMCAGC TGTGAKGGGCTYTMACWYTAAGMGAAMTGSACACAGGRSGTTRWGTACKGAGAACAYKGA CTCATAGARGGWSRTCAAGAACAGKACAATGACGGAGCGAGGACGACTYTGGTGACTTTG GCTTCATGTTTGGAGGCAACAGACAGCAACAGGACAGAAACATTCCTAGAGGAAATGACA TAGTGCTAGACCTGGAGGTCACCCTAGAAGAGGTGTATTCTGGGAACTTTGTGGAGGTTG TACGTAACAAACCCATAGCCAAAGAAGCTCCTGGCAAGAGGAAGTGTAACTGCAGACAGG AGCGAGGACGACTCTG
Kiss2r	ATTTATGTCGCCCCCACTTTGCACGCACGCACACACAGARRAATCGYTGTGGCTSTGTST GTGTGCACCACAG
Kiss1r	CTWWCRRRCMSSWASCWTGGCYTTMRRGGMCGCMACGCCGAGATMAGTTTGTGGCTGCAGAGGAGCGCAACAACAAT

degree of identity on both nucleotide and amino acid sequences of cDNA compared to the blue gourami sequence and the respective fish sequence (Figs. 3A, B).

A higher degree of identity in cDNA was found between blue gourami kiss2r compared to kiss2r belonging to other species of fishes, presented in Figs. 4 A and B than Kiss2.

The deduced sequences of this gene in the blue gourami kiss 1r were compared with homologous sequences from a number of other fish species kiss1r (Fig. 5). There was a low degree of identity on both nucleotide and amino acid sequences between the blue gourami sequence and the respective fish sequence of species (Figs. 5).

In order to determine whether the Kiss1, Kiss2, Kiss1r and Kiss2r genes are expressed in the brains of females, total RNA were reverse-transcribed using qPCR amplification curves in comparison to Beta-Actin gene. Amplification curves represented cDNA detected in samples treated for ALDOA siRNA (Fig. 6).

Kiss1 was not identified clearly using these methods, and the results from the dissociation and qPCR amplification in the brain need more detailed study in order to verify if Kiss1 is present and expressed. The expression of B-actin was also measured in order to provide internal control. Amplification of actin in brain samples gave a clear expression (Fig. 6), indicating that there was no degradation of RNA in the preparation of brain RNA samples. Relative mRNA levels of Kiss2, Kiss1r and Kiss2r were determined in brains excised from females before vitellogenesis (juveniles in pre-vitellogenesis) (PV), and advanced vitellogenesis (VT) in mature females (Fig. 2). The differences were found between the Kiss2, Kiss2r, juvenile and mature females, were statistically significant ($p<0.05$, t-test)(Fig. 7).

Nevertheless, the mean of PV mRNA levels was higher than females with HV oocytes, and the difference was statistically significant (Fig. 7). Similar results were obtained in the transcription of Kiss2r in that mRNA levels were higher in juvenile females (Fig. 8). No significant difference was found between the Kiss1rmRNA levels of the mature reproductive fish (HV) and the juveniles (PV) (Fig. 9). In comparing the expression mRNA of Kiss1r to Kiss2r and Kiss2, the mRNA level was found to be significant in both juvenile and mature females ($p<0.05$, ANOVA and t-test) among those genes.

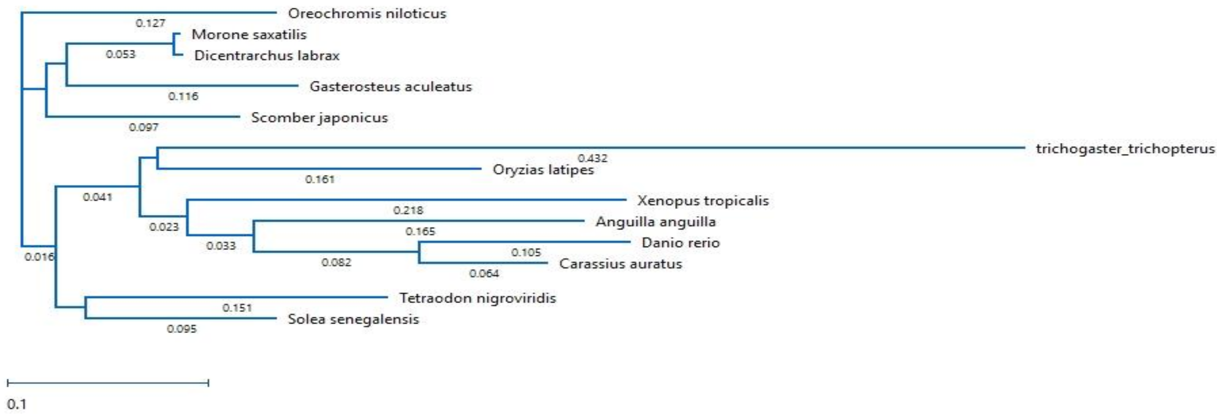
Discussion

In the present study, we showed the expression of three genes-- Kiss2, Kiss1r and Kiss2r, in the brains of female blue gourami during vitellogenesis. This stage is the main stage of oogenesis in fish (Yaron and Levavi-Sivan, 2011). The results support the hypothesis that Kiss2 affects Kiss2r involved in control reproduction. These results are supported by other studies in various fish (Oakley *et al*., 2009). However, the results from various fish species are not uniform. The number of Kiss peptins and their receptors existing in the brain of fish and involved in reproduction differs and is not always clear (Lee *et al*., 2009; Pasquier *et al*., 2014).

In fish, for example, in the brain of zebrafish, two kiss genes, Kiss1 and Kiss2, and two kiss receptors (GPR54), Kiss1r and Kiss2r, were described (Lee *et al*., 2009; Servili *et al*., 2011). The kiss neurosecretory hormones were also studied in other fish

(Table 2) such as lamprey- *Petromyzon marinus* (Lee *et al.*, 2009), medaka- *Oryzias latipes* (Kitahashi *et al.*, 2009), gold fish- *Carassius auratus* (Huang *et al.*, 2009) and sea bass -- *Dicentrarchus labrax* (Espigares *et al.*, 2017) (Table 6).

A



B

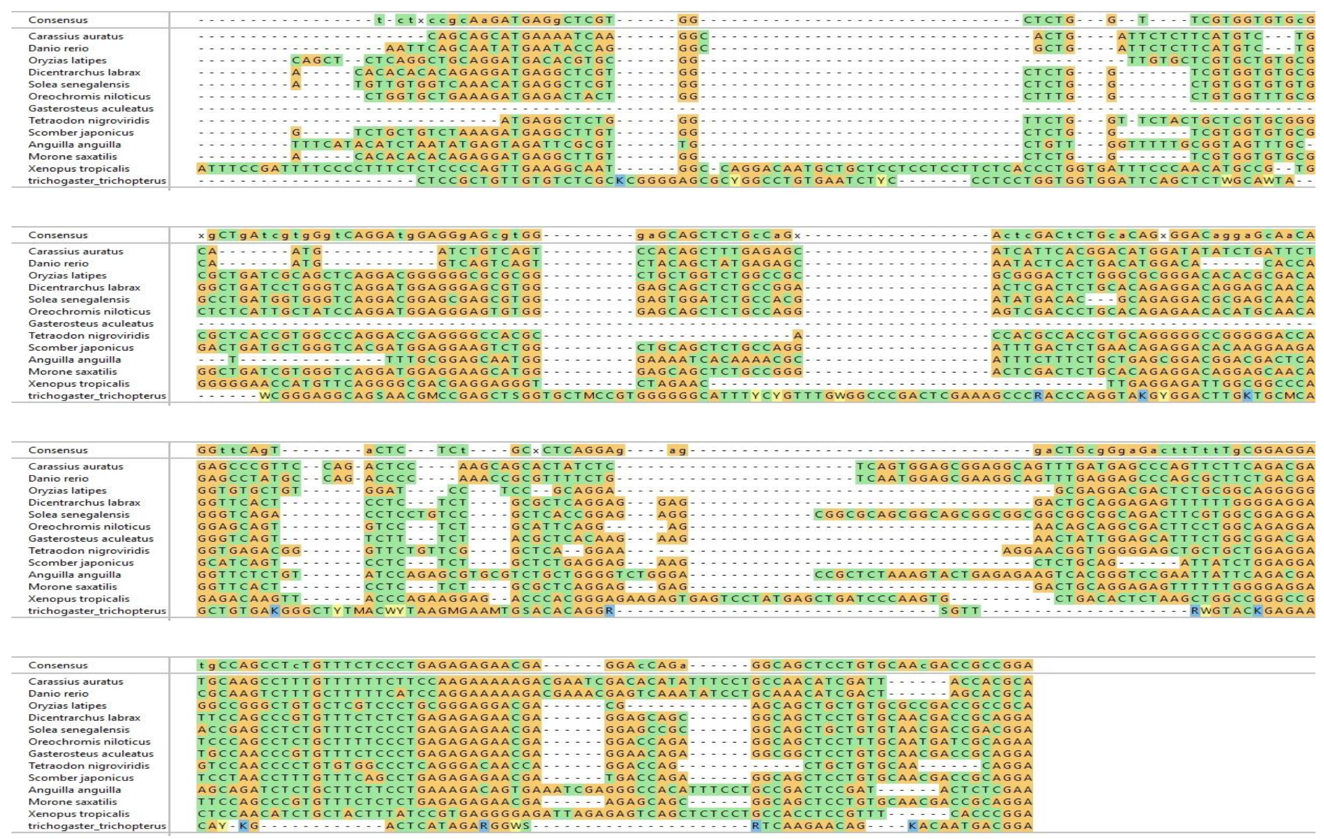


Fig. 3 A. Phylogenetic tree showing the relationship between fish Kiss2 cDNA sequences. The tree was generated using DNA Star MegAlign PRO MAFFT. *Trichogaster trichopterus* sequence was obtained from the present study. All other sequences were obtained from NCBI Gene bank according to the accession numbers provided in Tables 2 and 3. The nucleotide and deduced amino-acid sequences of cDNA are represented in Fig 3B.

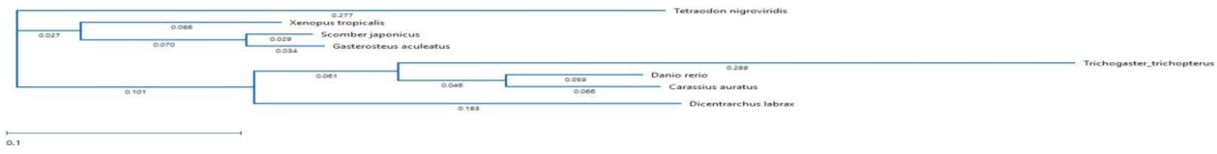
Table 3. Kiss2 mRNA sequences used for alignment and phylogenetic tree

Order	Species	Accession no.
Cypriniformes	*Carassius auratus*	GQ141877.1
Cypriniformes	*Danio rerio*	NM_001142585.1
Beloniformes	*Oryzias latipes*	NM_001160441.1
Perciformes	*Dicentrarchus_labrax*	FJ008915.1
Perciformes	*Oreochromis niloticus*	NM_001279468.1
Perciformes	*Scomber japonicus*	GU731673.1
Perciformes	*Morone saxatilis*	GU351865.1
Perciformes	*Trichogaster trichopterus*	According to this study.
Pleuronectiformes	*Solea senegalensis*	HM116743.1
Gasterosteiformes	*Gasterosteus aculeatus*	KT202354.1
Tetraodontiformes	*Tetraodon nigroviridis*	KT202353.1
Anguilliformes	*Anguilla anguilla*	LT844561.1
Anura	*Xenopus tropicalis*	NM_001162860.1

Table 4. Kiss2r cDNA sequences used for alignment and for the phylogenetic tree

Order	Species	Accession no.
Cypriniformes	*Carassius auratus*	FJ465140.1
Cypriniformes	*Danio rerio*	EU047918.1
Perciformes	*Dicentrarchus_labrax*	JN202446.1
Perciformes	*Scomber japonicus*	JX982323.1
Perciformes	*Trichogaster trichopterus*	According to this study.
Gasterosteiformes	*Gasterosteus aculeatus*	KT261496.1
Tetraodontiformes	*Tetraodon nigroviridis*	KT261495.1
Anura	*Xenopus tropicalis*	NM_001171825.1

A



B

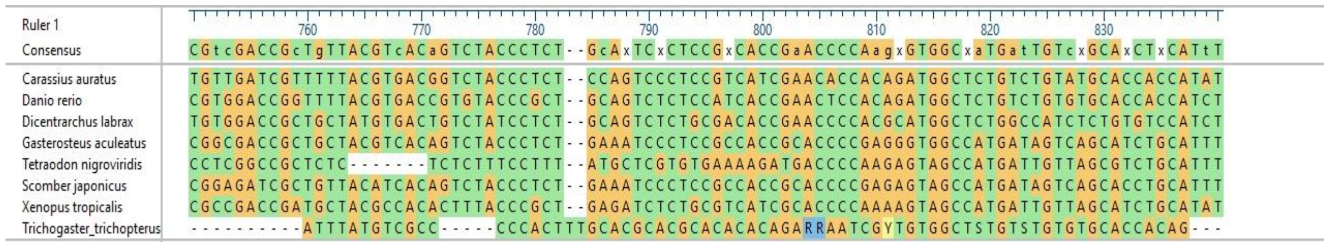
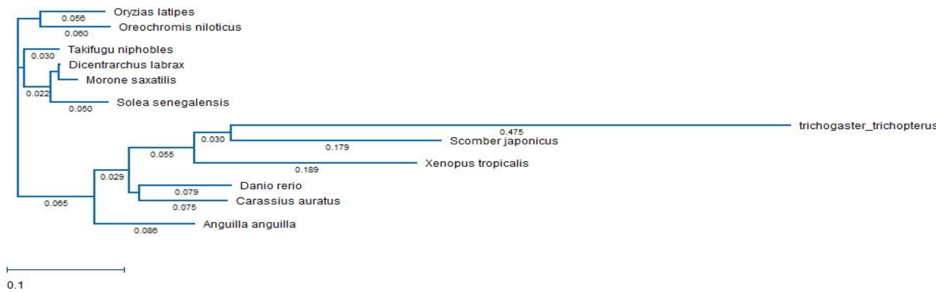


Fig. 4 Phylogenetic tree showing the relationship between vertebrate Kiss2 Receptor amino acid sequences. The tree was generated by maximum Clustal Wusing DNAStar WI Megalign software. All sequences were obtained from NCBI, Gene bank. Sequence alignment was conducted using DNA Star MegAlign Pro Clustal Omega. *Trichogaster trichopterus* sequence was obtained from the present study. All the other sequences were obtained from the NCBI Gene bank according to the accession numbers provided in Table 4.

A



B

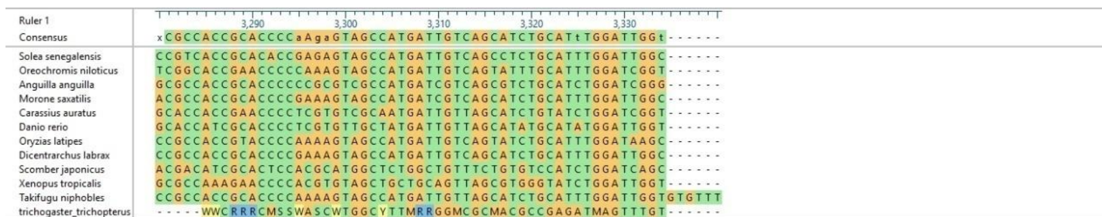


Fig. 5. Phylogenetic tree showing the relationship between vertebrate Kiss1r cDNA sequences. The tree was generated using DNA Star MegAlign PRO MAFFT. *Trichogaster trichopterus* sequence was obtained from the present study. All other sequences were obtained from the NCBI Gene bank according to the accession numbers provided in Table 5. The units of branch length represent nucleotide substitutions per site

Table 5. Kiss1r mRNA sequences used for alignment and phylogenetic tree construction

Order	Species	Accession no.
Cypriniformes	*Carassius auratus*	FJ465139.1
Cypriniformes	*Danio rerio*	NM_001105679.2
Pleuronectiformes	*Solea senegalensis*	EU136710.1
Perciformes	*Oreochromis niloticus*	AB162143.1
Perciformes	*Morone saxatilis*	GU351869.1
Perciformes	*Dicentrarchus_labrax*	JQ839286.1
Perciformes	*Scomber japonicus*	JX982322.1
Perciformes	*Trichogaster trichopterus*	According to this study.
Anguilliformes	*Anguilla anguilla*	FR667382.1
Beloniformes	*Oryzias latipes*	XM_004079431.3
Tetraodontiformes	*Takifugu niphobles*	AB548356.1
Anura	*Xenopus tropicalis*	NM_001170514.1

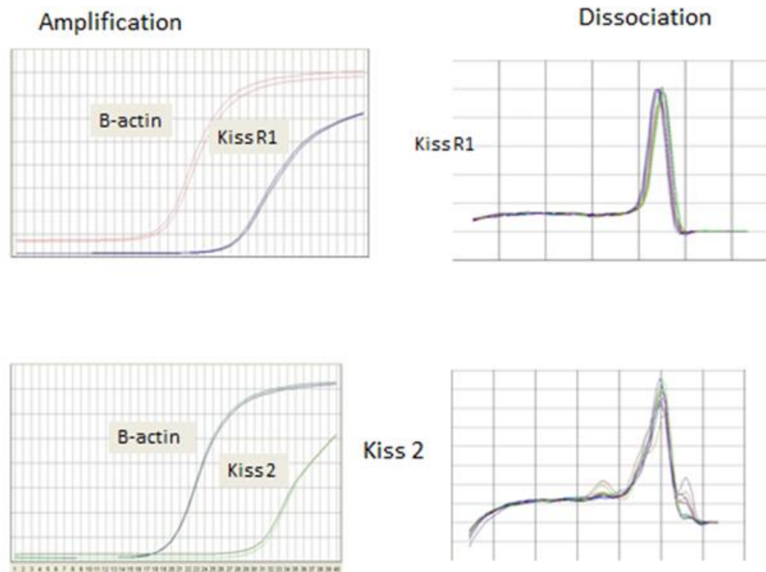


Fig. 6. qPCR results for KissR1 and Kiss2 genes in comparison to the Beta-Actin gene. The dissociation was in cycle 36. Kiss1R (Kiss1r F1 -TCCGTTCAGAAGCACTGTGG- Kiss1r R1-TATTTCCACCTTCGGTGCTC-). The dissociation was in cycle 25. Kiss2, Zebrafish primers (Kiss2 1F: GTCATTAAAAACCAGCAGATGAAGAC, Kiss2r1 R GTGGTGCACACAGACAGAGCCA).

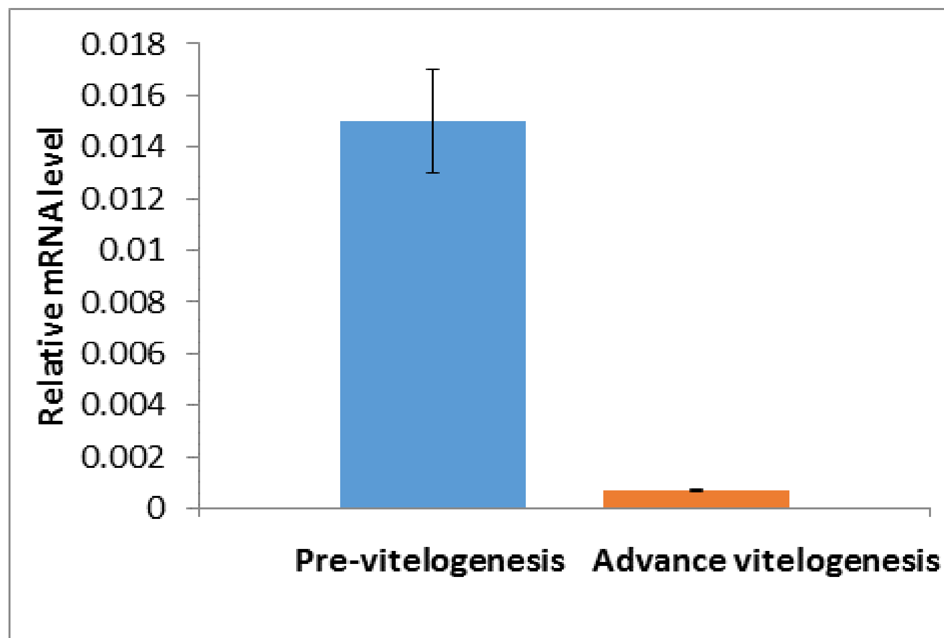


Fig. 7. Relative mRNA levels of Kiss2 in the brains of blue gourami females at different stages of oogenesis: pre-vitellogenesis (PV) and high vitellogenesis (HV). Total brain RNA was reverse-transcribed, and the resulting cDNA was used in quantitative real-time PCR (Fig.6). The relative amount of Kiss2 mRNA was normalized to that of Beta-actin using the CT cycle method, where 2KDCT reflects the relative amount of Kiss2 transcription (Muller *et al.*, 2002). Each histogram represents the average of independent measurements (mean ± SE). Significant differences between mRNA levels were found ($p<0.05$, by ANOVA, t-test).

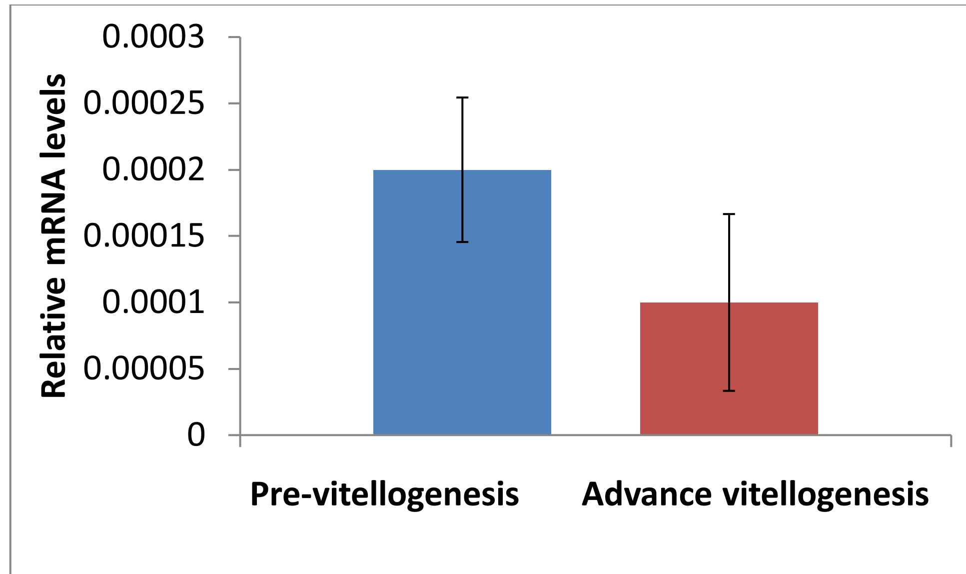


Fig. 8. Relative mRNA levels of Kiss2r in brains of blue gourami females at different stages of oogenesis: pre-vitellogenesis (PV) and high vitellogenesis (HV). Total brain RNA was reverse transcribed and the resulting cDNA was used in quantitative real-time PCR (Fig. 6). The relative amount of Kiss2r mRNA was normalized to that of Beta-actin using the CT cycle method, where 2KDCT reflects the relative amount of Kiss2r transcription (Muller *et al*., 2002). Each histogram represents the average of independent measurements (mean±SE). No significant differences between mRNA levels were found ($p>0.05$, t- test).

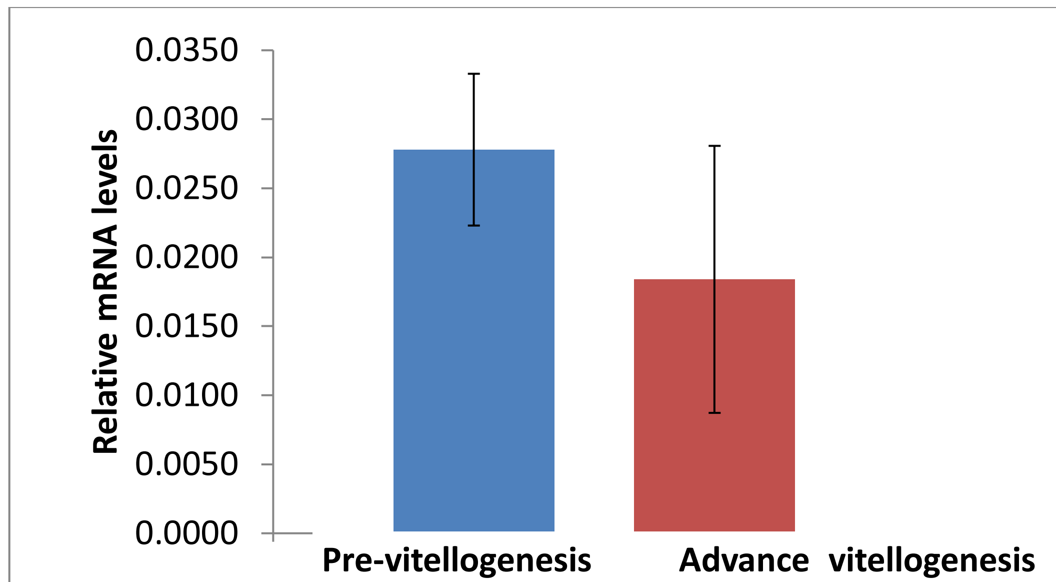


Fig. 9. Relative mRNA levels of Kiss1r in brains of blue gourami females at different stages of oogenesis: pre-vitellogenesis (PV) and high vitellogenesis (HV). Total brain RNA was reverse transcribed, and the resulting cDNA was used in quantitative real-time PCR (Fig. 6). The relative amount of Kiss1r mRNA was normalized to that of actin using the CT cycle method, where 2KDCT reflects the relative amount of Kiss1r transcription (Muller *et al*., 2002). Each histogram represents the average of independent measurements (mean± SE). No significant differences between mRNA levels were found ($p>0.05$, ANOVA, Bonferroni post-hoc test).

Table 6. The kiss neurosecretory hormones and their receptors studied in various fish

Species	Kiss1	Kiss2	Kiss1r	Kiss2r	References
Zebra fish *Danio rerio*	+	+	+	+	Ogawa *et al.*, 2014
Chub mackerel (*Scomber japonicus*)	+	+	-	-	Mechaly *et al.*, 2013
Striped bass (*Morone saxatilis*)	+	+	+	+	Mechaly *et al.*, 2013
Senegalese sole *(Solea senegalensis)*	-	+	+	+	Mechaly *et al.*, 2009, 2013
Medaka (*Oryzias latipes*)	+	+	+	+	Kanda, 2012
European Sea Bass *(Dicentrarchus labrax*)	+	+	+	+	Escobar *et al.*, 2013
Grass puffer (*Takifugu niphobles*)	-	+	-	+	Tena-Sempere *et al.*, 2012
Three-spined stickleback (*Gasterosteus aculeatus)*	-	+	+	+	Tena-Sempere *et al.*, 2012
European eel (*Anguilla anguilla*)	+	+	+	+	Tena-Sempere *et al.*, 2012
Tetraodon (*Tetraodon nigroviridis*)	-	+	-	+	Tena-Sempere *et al.*, 2012
Goldfish (*Carassius auratus*)	+	+	+	+	Tena-Sempere *et al.*, 2012
Blue gourami (*Trichopodus trichopterus*)	-	+	+	+	According to this study

In blue gourami, pheromones have a chemical (Becker *et al*., 1992; Degani and Schreibman, 1993) signal effect on oogenesis via the HPG-axis. Pheromones (steroid glucoronides) that are dissolved in water, as described in fish and blue gourami, and detected by gas chromatography-mass spectrometry (GCMS), the radioimmunoassay method (RIA) and thin-layer chromatography(TLC), are found in gonads and in water in which the fish were maintained (Degani and Schreibman, 1993; Ogawa *et al*., 2014). The pheromones of blue gourami males affect the female's brain hormones and gonadotropins, which control VTL and OMT. Pheromones affect female BPG and oogenesis (Degani, 2016). Our hypothesis is that pheromones and environmental factors affect Kiss2, Kiss1r and Kiss2r expression, which are described in the present study, as well as GnRH, which controls gonadotropins (FSH and LH)(Levy *et al*., 2011; Levy and Degani, 2011; Levy and Degani, 2012).

The hypothesis supported by the present study indicates that blue gourami Kiss2 and Kiss2r expression during vitellogenesis and GnRH involved in oogenesis control (Levy and Degani, 2012). The highest levels of FSH mRNA were found in specimens classified as high vitellogenic (Jackson *et al*., 1999), and in females, GnRH1 may be involved in the final vitellogenesis stage by FSH and growth hormone (GH) (Levy and Degani, 2012). In grass puffer, temperature affects sexual maturation through the control of Kisspeptin1 and Kisspeptin2 receptor, GnRH and GTH subunit gene expression during the spawning season (Shahjahan *et al*., 2010). In zebrafish, Kiss2-expressing neurons are found in the dorsal and ventral hypothalamus (Pasquier *et al*., 2014). Kiss1-expressing cells project only to the inter-peduncular and raphe nuclei, and strongly express the kiss1r receptor. In the present study, different expression by Kiss1r and Kiss2r and Kiss 2 during vitellogenesis was also found. Kiss1r is not sharply different in terms of its expression between PV and HV, so we will not prove for certain that this Kiss1r is involved in vitellogenesis in blue gourami. Our suggested explanation for these results in blue gourami is that in PV, just before vitellogenesis starts and is controlled by Kiss2, Kiss2r, GnRH1, FSH and E (Fig. 8), the mRNA of Kiss2 and Kiss2r transcription might synthesize these hormones before secretion. These hormones will control vitellogenesis in the HPG axis (Jackson et al., 1999; Levy and Degani, 2012; Degani, 2016). In other words, the blue gourami is multi-

spawning and male-dependent, having an asynchronic ovary development (Degani, 1993). This situation might explain the low mRNA level in HV compared to PV in blue gourami.

In zebrafish, Kiss2-expressing cells are mostly present in the dorsal and ventral hypothalamus, and project widely onto the sub-pallium, the preoptic area, the thalamus, the ventral and caudal hypothalamus, and the mesencephalon. In zebrafish, all these regions strongly expressed the Kiss2r messengers. Kiss2 fibers profusely innervate the ventral forebrain and notably make close apposition with GnRH3 neurons (Pasquier *et al.*, 2014). Estradiol treatment to juvenile fish causes an increase in Kiss2 and Kiss2r expression. These results are in agreement with the present study, which found that both Kiss2 and Kiss2r expression are high in juveniles just before the vitellogenesis start and low in mature female blue gourami occurring at the end of vitellogenesis, which is well known to be controlled by estrogen (Degani, 1994, 2001, 2016). Levi and Degani (2012) suggested a model that brain hormone, gonadotropin-releasing hormone GnRHs (GnRH1, GnRH2 and GnRH3) and pituitary adenylate cyclase-activating polypeptide (PACAP) are

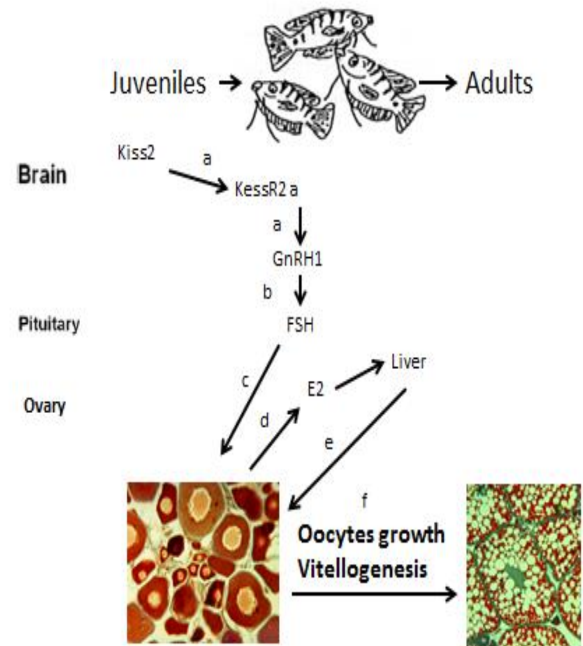


Fig. 10. A proposed quality model showing the Kiss2 mechanism involved in regulating vitellogenesis in blue gourami. **a**: present study, **b**: Levy and Degani, 2011, 2012; Degani *et al.*, 1995, **c**: Degani and Boker, 1992; Degani, 1993; Mananos *et al.*, 1997; Degani *et al.*, 1999 , **d**: Degani, 1990; Degani and Boker, 1992; Degani *et al.*, 1994, **e**: Degani *et al.*, 1994 ; Jackson *et al.*, 1994, **f**: Degani, 1993,1990; Jackson *et al.*, 1994

involved in oogenesis in blue gourami based on studies of hormone control reproduction of blue gourami. The contribution of the present study is that Kiss2 and the Kiss2r receptors might transfer the signal to GnRH1, which influence the release of these hormones involved in the control of oogenesis. However, more studies are needed in order to prove this hypothesis. We propose a model for the control of vitellogenesis by brain, adding the Kiss2 and its receptors in blue gourami (Fig. 10).

References

Becker D, Galili N and Degani G. (1992) GCNS-identified steroids and steroids goucoronides in gonads and holding water in gonads and holding water of *Trichogaster tricopterus* (anabantidea, Pallas 1770). Comp. Biochem. Physiol. 103B:15-19.

Bonfield JK and Smith KRS. (1995). A new DNA sequence assembly program. . Nucleic Acids Res. 23: 4992-4999.

Degani G. (1990) The effects of human chorionic gonadotropin on steroid changes in *Trichogaster trichopterus.* Comp. Biochem. Physiol. 96A: 525-528.

Degani G. (1993) The effect of sexual behavior on oocyte development and steroid changes in *Trichogaster trichopterus.* Copeia 4: 1091-1096.

Degani G. (1994) Effect of gonadotropin and steroids on vitellogenesis during ovarian development in *Trichogaster trichopterus*. J. Aquacult. Trop. 9: 15-23.

Degani G. (2001) Blue gourami (*Trichogaster trichopterus*): Model for labyrinth fish. Laser Pages Publishing, Israel,1-134.

Degani G. (2015) 11-ketotesterone (KT-11), estradiol estradiol (E_2) level and cytochrome P450 (bgcyp19a) transcription in the testis of male blue gourami (*Trichogaster trichopterus*) Intern. J. Sci. Res. 4: 641-643.

Degani G. (2016) Oogenesis control in multi-spawning blue gourami (*Trichogaster trichopterus*) as a model for the anabantidae family. Intern. J. Sci. Res. 5:179-184.

Degani G and Boker R. (1992) Sensitivity to maturation inducing steroids and gonadotropin in the oocytes of blue gourami *Trichogaster trichopterus* (anabantidae, Pallas, 1770). Gen. Comp. Endinocrinol. 85:430-439.

Degani G and Schreibman MP. (1993) Pheronome of male blue gourami and its effect on vitellogenesis, steroidogenesis and cells in pituitary of female. J. Fish Biol. 43: 475-485.

Degani G, Gal E and Vaya J. (1994) *In vitro* biosynthesis of steroids in ovary of female *Trichogaster trichopterus* (Pallas, 1770). Comp. Biochem. Physiol. 109: 715-723.

Degani G, Jackson K, Dosoretz C and Plotzky Y. (1999) Molecular DNA variation in *Salamandra infraimmaculata* from different habitats. Israel J. Zool. 44: 239-246.

Degani G, Jackson K and Marmelstein G. (1995) The effect of LHRH analogue on sex steroid profiles in female *Trichogaster trichopterus* (Anabantidae, Pallas). J. Aquacult. Tropic. 10: 297-307.

Escobar S, Servili A, Espigares F, Gueguen MM, Brocal I, Felip A and Kah O. (2013) Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. PLoS One 8: e70177.

Espigares F, Rocha A, Gómez A, Carrillo M, Zanuy S. (2017) Photoperiod modulates the reproductive axis of European sea bass through regulation of kiss1 and GnRH2 neuronal expression. Gen. Comp. Endocrinol. 240: 35-45.

Huang T, Huang X, LuD W, Zhu P, Shi Y, Cheng CH, Liu X and Lin H. (2009) Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*). J. Endocrinol. 201: 407–441.

Jackson K, Abraham M and Degani G. (1994) Oocyte maturation triggered by the male in a tropical fish *Trichogaster trichopterus.* J. Morphol. 220: 1-9.

Jackson K, Goldberg G, Ofir R, Abraham M and Degani G. (1999) Blue gourami (*Trichogaster trichopterus*) gonadotropic beta subunits (I & II) cDNA sequences and expression during oogenesis. J. Mol. Endocrinol. 23: 177-187.

Kanda S and Yoshitaka O. (2012) Evolutionary insights into the steroid sensitive kiss1 and kiss2 neurons in the vertebrate brain. Frontiers Endocrinology 3: 28. doi: 10.3389/fendo.2012.00028

Kitahashi T, Ogawa S and Parihar IS. (2009) Cloning and expression of kiss2 in the zebrafish and medaka. Endocrinology 150: 821–831.

Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang JI, Osugi T, Otaki N, Sunakawa Y, Kim K, Vaudry H, Kwon HB, Seong JY and Tsutsui K. (2009) Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology 150: 2837–2846.

Levy G and Degani G. (2011) Evidence for a reproduction-related function of pituitary adenylate cyclase-activating polypeptide-related peptide (prp) in an anabantidae fish. J. Mol. Endocrinol. 46: 1-11.

Levy G and Degani G. (2012) Involvement of GnRH, PACAP and PRP in the reproduction of blue gourami females (*Trichogaster trichopterus*). J. Mol. Neurosci. 48: 9730-9738.

Levy G, David D and Degani G. (2011) Effect of environmental temperature on growth- and reproduction-related hormone gene expression in the female Blue gourami (*Trichogaster trichopterus*). Comp. Biochem. Physiol. Part A 160: 381–389.

Mananos E, Zohar Y and Degani G. (1997) The relationship between gonadotropin and sexual behavior of male *Trichogaster trichopterus* (Pallas). Indian J. Fish. 44: 239-246.

Mechaly AS, Vinas J andPiferrer F. (2009) Identification of two isoforms of the kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*). Biol. Reprod. 80: 60-69.

Mechaly AS, Viñas J and Piferrer F. (2013) The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in pleuronectiformes. Gen. Comp. Endocrinol. 188: 258-268.

Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ and Calamari A. (2001) AXOR12, a novel human G protein-coupled receptor, activated by the peptide kiss-1. J. Biol. Chem. 276: 28969–28975.

Muller PY, Janovjak H, Miserez AR and Dobbie Z. (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32: 1372-1379.

Nielsen H, Engelbrecht J, Brunak S and von Heijne G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10: 1-6.

Oakley AE, Clifton DK and Steiner RA. (2009) Kisspeptin signaling in the brain. Endocrinol. Rev. Esp. Herp. 30: 713–743.

Ogawa S, Nathan FM and Parhar IS. (2014) Habenular kisspeptin modulates fear in the Zebrafish. Proc. National Acad. Sci. 111: 3841-3846.

Pasquier J, Kamech N, Lafont A, H. V, Rousseau K and Dufour S. (2014) Molecular evolution of GPCRs kisspeptin/kisspeptin receptors. J. Molec. Endocrinol. 52: 101-117.

Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: 2002-2007.

Pfaffl MW. (2004) Quantification strategies in real-time pcr. A-Z of quantitative PCR (Ed.: SA Bustin (International University Line (IUL) La Jolla, CA, USA 3: 87-112.

Servili A, Le Page Y, Leprince J, Caraty A, Escobar S, Parhar IS, Seong JY, Vaudry H and Kah O. (2011) Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of Zebrafish. Endocrinology 152: 1527–1540.

Shahjahan M, Kitahashi T and Parhar IS. (2014) Central pathways integrating metabolism and reproduction in teleosts. Frontiers Endocrinology 5: 1-17.

Shahjahan M, Motohashi E, Doi H and Ando H. (2010) Elevation of kiss2 and its receptor gene expression in the brain and pituitary of grass puffer during the spawning season. Gen. Comp. Endocrinol. 169: 48–57.

Tena-Sempere M, Felip A, Gómez A, Zanuy S and Carrillo M. (2012) Comparative insights of the kisspeptin/kisspeptin receptor system: Lessons from non-mammalian vertebrates. Gen. Comp. Endocrinol. 175: 234-243.

Yaron Z and Levavi-Sivan B. (2011) Endocrine regulation of fish reproduction. In: Farrell A.P., (ed.), San Diego: Encyclopedia of Fish Physiology: From Genome to Environment 2: 1500–1508.