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Domperidone treatment advances onset of puberty in the viviparous mosquitofish *Gambusia affinis*

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Abstract

Background: Although dopamine (DA) exerts modulatory effect on reproduction in the majority of fishes, its role is not clearly understood in viviparous species. The aim of this investigation was to determine the influence of DA antagonist domperidone (DOM) on puberty in the viviparous species *Gambusia affinis*.

Results: Treatment of 1.5 or 4 mg DOM to 25 days post-hatching (DPH) juveniles for a period of 35 days resulted in dense aggregations of gonadotropin-releasing hormone (GnRH)-immunoreactive fibres in the proximal pars distalis (PPD) region of the pituitary gland in contrast to faintly seen fibres in treatment controls. In the ovary, the follicles belonging to stages I–IV did not differ significantly among different experimental groups. However, stage V (vitellogenic) follicles were completely absent in treatment controls in contrast to their presence in 1.5 or 4 mg DOM-treated fish. Besides, the diameter of stage V follicles was significantly higher in 1.5 mg DOM-treated fish compared with 4 mg DOM-treated fish.

Conclusions: The results suggest that DOM treatment triggers the follicular development and promotes the early onset of puberty, possibly by attenuating the dopaminergic inhibition on GnRH fibres, for the first time in a viviparous species.

Keywords: Dopamine, Domperidone, Mosquitofish, Puberty, Viviparity, Gonadotropin-releasing hormone

Background

Puberty is the transitional period between the immature juvenile stage and the reproductively mature adult stage in fish (Grumbach & Kaplan, 1990; Okuzawa, 2002). Although puberty onset is known to be controlled by activation of the hypothalamo-pituitary-gonad (HPG) axis and number of environmental factors (Dufour et al., 2010; Weltzien et al., 2004; Taranger et al., 2010), our knowledge on the endocrine regulation of puberty in lower vertebrates is very much limited (Holland, Hassin, & Zohar, 2000). Whereas some studies have examined the involvement of dopamine (DA) in the neuroendocrine control of puberty in juvenile fishes, these results are equivocal. For instance, gonadotropin-releasing hormone agonist (GnRHa) + testosterone induces puberty in males in the absence of DA antagonist treatment, but does not stimulate the reproductive system in the female juvenile rainbow trout *O. mykiss* (Crim & Evans, 1983). Similarly, DA does not affect puberty in the juvenile striped bass *Morone saxatilis* (Holland et al., 1998). In contrast, decrease in DA activity is observed in the hypothalamus at the time of puberty in the juvenile spadefish, *Chaetodipterus faber* (Marcano et al., 1995). Whereas removal of DA inhibition causes drastic increase in luteinizing hormone (LH) secretion as well as ovarian development in the prepubertal European eel *Anguilla anguilla* (Vidal et al., 2004), DA is shown to exert inhibitory effect on the onset of puberty in the grey mullet *Mugil cephalus* (Aizen et al., 2005). Since many species undergo either late puberty or completely fail to enter puberty under farming conditions (Taranger et al., 2010), understanding the endocrine regulation of puberty is essential for the effective broodstock management.

Evolution of viviparity involves several adaptations such as internal fertilization, gestation and parturition to facilitate the foetus development. *Gambusia affinis* is a

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viviparous teleost belonging into the family Poeciliidae. The placenta in this fish is formed within the follicle and the gestation is intrafollicular (Pandian, 2013). Whereas the female *G. affinis* are able to store sperm for extended periods of time (Farr, 1989), the intrafollicular gestation eliminates the process of ovulation (Uribe & Grier, 2011). While more studies are required to elucidate the role of DA in fish reproduction, currently, no information is available on the involvement of DA in puberty in viviparous species. Such studies will help to understand the evolutionary significance of dopaminergic involvement in the neuroendocrine regulation of reproduction in viviparous fish. Therefore, the objective of the present investigation is to determine the influence of DA on puberty in *G. affinis*.

Methods

Animals

Adult mosquitofish *G. affinis* collected from ponds in Karnatak University Campus, Dharwad, were brought to the laboratory, reared and acclimatized in 2 L freshwater aquaria (size, 30 × 30 × 16 cm; L × W × H) under natural photoperiod 11.75 ± 0.14. The fish were fed with commercial pellets (Taiyo pet feed, Chennai, India) daily twice. Each aquarium was provided with aerators and aquatic plants. The physicochemical conditions such as dissolved oxygen (DO), pH and water temperature were recorded daily and expressed as mean ± SE (DO, 6.24 ± 0.04; pH, 7.89 ± 0.36; temperature, 22.26 ± 0.40 °C).

Experimental procedure

For this experiment, the juveniles of 25 days post-hatching (DPH) from the stock, weighing 0.01–0.02 g were used. The experimental set up consisted of three groups (n = 20 in each group) with two replicates (n = 10 in each). The first group (treatment controls) were exposed to water consisting of 0.5 mg sodium metabisulphite and 0.5 mg NaCl/L, whereas those in the second and third groups were exposed to 1.5 and 4 mg DOM in water consisting of 0.5 mg sodium metabisulphite and 0.5 mg NaCl/L, respectively. Our pilot studies indicated that the juvenile spot (seen only in females) in *G. affinis* appears between 21 and 25 days. Based on this, 25 DPH female juveniles were carefully segregated and used for experimentation. All experimental fish were exposed to the treatment for a period of 35 days and euthanized on the 36th day. At autopsy, brains along with pituitaries were processed for GnRH immunocytochemistry,

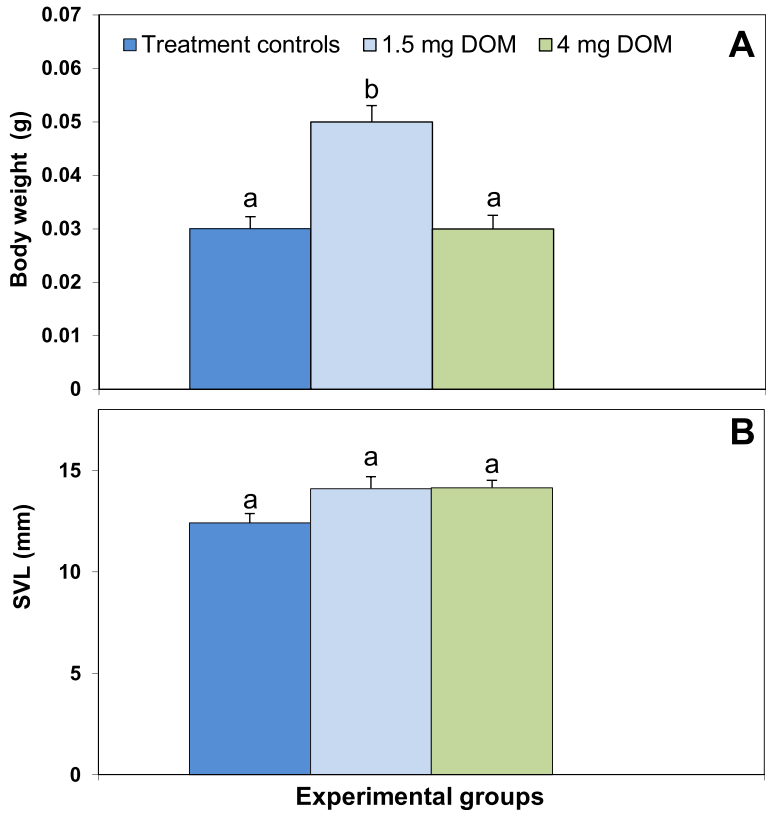

Fig. 1 **a** and **b** Effect of domperidone treatment on body weight (**a**) and snout-vent length (SVL) (**b**) in juvenile *G. affinis*. One-way ANOVA and Tukey's test: dissimilar superscripts indicate significant ($p < 0.05$) difference. Values are means ± SE

whereas the abdominal portion of the body consisting of the ovaries was fixed in Bouin's fluid and processed for histological studies.

Immunofluorescence labelling

The fish were anesthetized with 2-phenoxy-ethanol (Sigma, USA; 1:1500), killed by decapitation method, and the head region was immersed in the ice-cold paraformaldehyde fixative. Brains along with pituitaries were dissected quickly, kept in the same fixative overnight at 4 °C and cryoprotected subsequently in sucrose solution overnight. Transverse frozen sections were cut at 12 μm thickness using a cryostat (Leica CM 1510S; Wetzlar, Germany). The sections were washed in phosphate buffer saline (PBS, pH 7.45) and incubated in pre-soaking solution containing 2% BSA + 0.4% Triton X 100 in PBS followed by anti-GnRH polyclonal antibody produced in rabbit (G8294; Sigma, USA; 1:500) overnight. The sections were washed with PBS for 15 min and then incubated with Alexa Fluor 488 (1:200; Sigma, USA) for 2 h at room temperature in dark. The sections were washed in PBS for 15 min and mounted in vectashield (Vector laboratories Inc, USA). Control procedures for the specificity of the antibody comprised (1) omission of primary antibody, (2) replacement of primary antibody with 2% BSA and (3) omission of secondary antibody. These control procedures completely blocked the immunoreaction, confirming the specificity of the antibody. The photographs of the brain sections were captured using BX53 fluorescent microscope (Olympus, Japan), and brightness and contrast were adjusted using Photoshop CS5, version 12.0 (Adobe systems Inc. San Jose, CA, USA).

The ovarian histology

Five-micron-thick serial paraffin sections of the ovaries were cut using semi-automatic microtome (Leica, Wetzlar, Germany), stained with haematoxylin and eosin and used for the quantification of the follicles at different stages of the development according to the morphology and the size criteria as described earlier (Bhat & Ganesh, 2019). The follicular number and diameter were recorded using Q capture pro 7 image analysis software. The number of follicles belonging to each category was counted separately from the entire ovary and expressed as mean ± SE.

Statistical analysis

The significant differences among mean values of various parameters among different experimental groups were compared by one-way ANOVA followed by post hoc Tukey's test using SigmaStat, version 3.5 (Systat Software Inc. Chicago, IL, USA). For all differences, $p < 0.05$ was considered as statistically significant.

Results

There was a significant ($p < 0.001$) increase in the body weight of 1.5 mg DOM-treated fish compared with treatment controls; however, no such increase was observed in 4 mg DOM-treated fish (Fig. 1a). The SVL did not

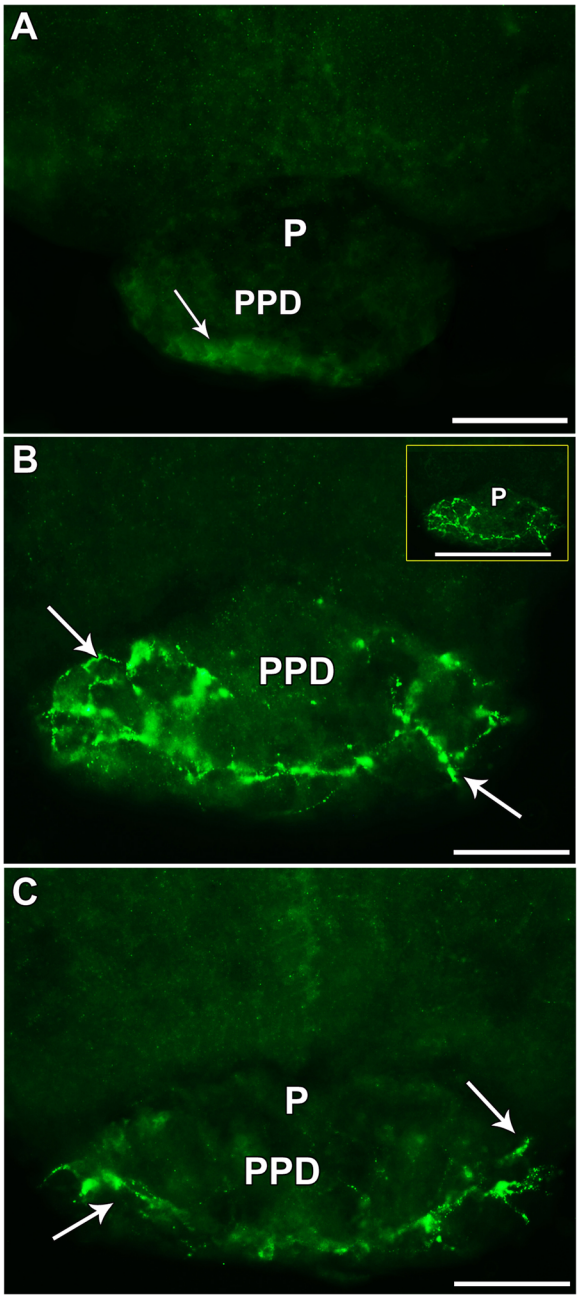

Fig. 2 a–c Photomicrographs of transverse sections through the proximal pars distalis (PPD) of the pituitary gland (P) in juvenile *G. affinis* showing GnRH-immunoreactive fibres (arrows) in treatment control (**a**), and 1.5 mg (**b**) and 4 mg DOM (**c**)-treated fish. Note the intensely immunoreactive GnRH fibres in DOM treated fish (**b, c**) compared to treatment controls (**a**). Alexa Fluor 488 labelled. Scale bar, 50 μm; Inset of figure b, 200 μm

show statistically significant difference among different experimental groups (Fig. 1b). The GnRH fibres were weakly immunoreactive in the PPD region of the pituitary of treatment controls, whereas intensely stained fibres were observed in the PPD region in DOM-treated juveniles (Fig. 2a–c). However, highest density of GnRH-immunoreactive fibres was observed in 1.5 mg DOM-treated fish compared with that of 4 mg DOM-treated fish group (Fig. 2b and c).

The ovary of treatment controls consisted of the follicles belonging to the stages I–IV, whereas the stage V follicles were completely absent (Fig. 3a and b). Although the treatment of DOM resulted in the appearance of the stage V follicles in the ovary (Fig. 3c–f), large yolk granules were found in 1.5 mg DOM-treated fish (Fig. 3c and d) compared with those of 4 mg DOM-treated fish (Fig. 3e and f). No significant differences were noticed in the mean numbers of stages I–IV follicles among different experimental groups (Fig. 4a–d). Whereas the stage V follicles were found in the ovary of fish treated with both doses of DOM in contrast to their absence in treatment controls, the number of these follicles was significantly ($p < 0.001$) higher in 1.5 mg DOM-treated fish compared with those of 4 mg DOM-treated fish (Fig. 4e). No significant differences were found in the mean diameters of stage I, III and IV follicles among different experimental groups (Fig. 5a, c and d). There was a significant ($p < 0.004$) increase in the mean diameter of stage II follicles in DOM-treated fish compared with treatment controls (Fig. 5b), whereas the mean diameter of stage V follicles was significantly higher ($p < 0.036$) in 1.5 mg DOM-treated fish compared with that of 4 mg DOM-treated fish (Fig. 5e). No atretic follicles were noticed in the ovary.

Discussion

In the present study, treatment of 1.5 mg DOM resulted in a significant increase in the mean body weight compared with those of treatment controls, whereas no significant difference was noticed in SVL among different experimental groups. The increase in the body weight might be related to increased weight of the ovary as shown by highest number of follicles in 1.5 mg DOM-treated fish compared with other groups.

In teleosts, several hypothalamic neuronal processes project into the pituitary forming an intricate association

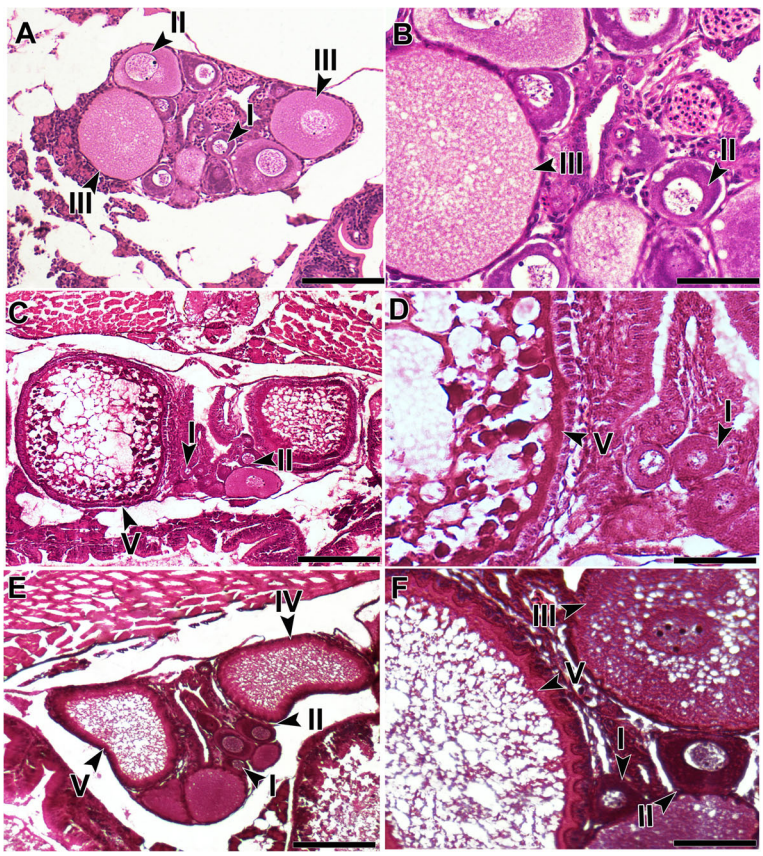

Fig. 3 a–f Photomicrographs showing cross sections of the ovary of juvenile *G. affinis* belonging to treatment control (**a** & **b**), and 1.5 mg (**c** & **d**) and 4 mg (**e** & **f**) DOM-treated groups. I, II, III, IV and V, stages of follicular development. Note the presence of stage V follicles in DOM treated fish (**c-f**) in contrast to their absence in treatment controls (**a**, **b**). Scale bar, **a**, **c** & **e**, 200 μm; **b**, **d** & **f**, 50 μm

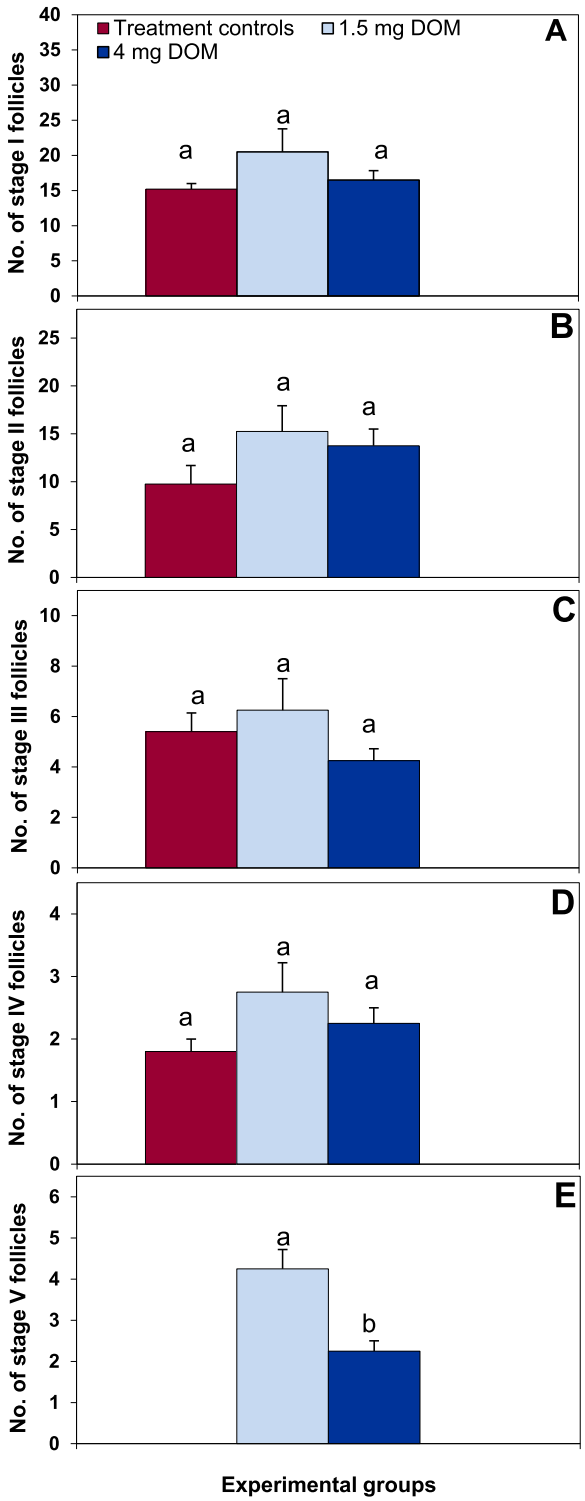

Fig. 4 a–e Effect of domperidone treatment on follicular development in juvenile *G. affinis*. One-way ANOVA and Tukey's test: groups with identical superscripts indicate no significance, whereas different superscripts indicate a significant ($p < 0.05$) difference. Values are means ± SE

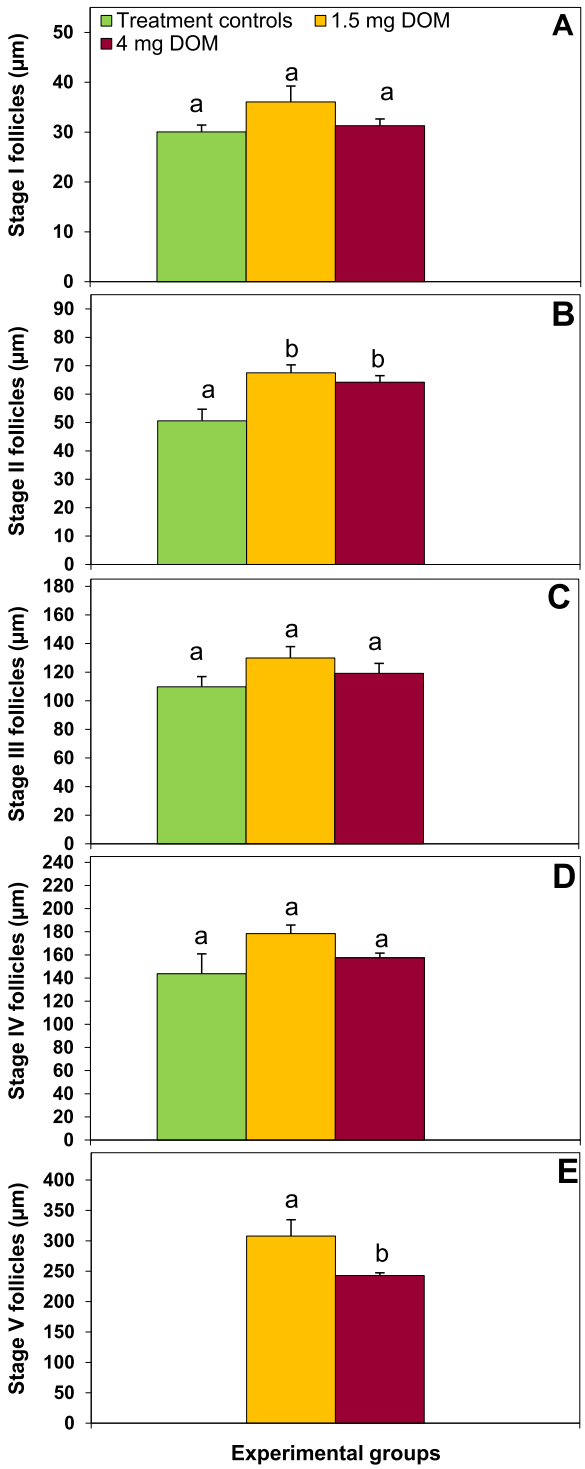

Fig. 5 a–e Effect of domperidone treatment on follicular diameter in juvenile *G. affinis*. One-way ANOVA and Tukey's test: groups with identical superscripts indicate no significance, whereas different superscripts indicate a significant ($p < 0.05$) difference. Values are means ± SE

with the adenohypophysis (Bernier et al., 2009). Hypothalamic release of GnRH stimulates the pituitary gonadotropic cells to release follicle stimulating hormone (FSH) and LH into the bloodstream (McCann et al., 2002; Saligaut et al., 1998; Zohar et al., 2010). Although most of the studies have suggested that DA acts primarily within the pituitary either indirectly by suppressing GnRH-releasing axons or directly by inhibiting the pituitary gonadotropes, a hypothalamic site for dopaminergic GnRH inhibition in fish was also implicated (Yu, Rosenblum, & Peter, 1991; Yu & Peter, 1992; Levavi-Sivan, Ofir, & Yaron, 1995; Van Goor, Goldberg, & Chang, 1998; Vacher et al., 2002). Recently, Bryant et al. (2016) have demonstrated that DA directly interacts with GnRH neurons within the hypothalamic compartment in the cichlid fish *Astatotilapia burtoni.* Indeed, previous study by Bhat and Ganesh (2017) has demonstrated the presence of tyrosine hydroxylase (a marker for DA) immunoreactive neurons or fibres in the preoptic area and the PPD region of the pituitary gland in *G. affinis*. On the other hand, Parhar, Ogawa and Sakuma (2004) observed increased expression of G-protein-coupled receptor 54 (GPR 54) concomitant with increased number of GnRH neurons and gonadal maturation in the male cichlid tilapia *Oreochromis niloticus*. In the present study, GnRH-immunoreactive fibres observed in the PPD region of the pituitary gland substantiates the regulatory influence of these fibres on gonadotropic hormone (GtH) secreting cells in *G. affinis*. Furthermore, increased network of densely accumulated GnRH-immunoreactive fibres in the PPD region of the pituitary gland in fish treated with DA receptor antagonist DOM compared with those of treatment controls suggests the possibility of blockade of inhibitory effect of DA either on GnRH neurons in the hypothalamus or its fibres within the pituitary gland, for the first time in a viviparous species.

The process of vitellogenesis is a prerequisite for the onset of puberty in fish (Holland et al., 1998; Okuzawa, 2002), whereas end point of vitellogenesis is characterized by deposition of large yolk granules and first ovulation (Wallace & Selman, 1990; Dufour et al., 2010). Although sexual maturation was succeeded following treatment with GnRHa alone or in combination with testosterone, but without a DA antagonist in males, the same treatment failed to stimulate the reproductive system of precocious female juvenile rainbow trout *O. mykiss* (Crim & Evans, 1983). Likewise, the addition of DA antagonist pimozide to GnRHa + testosterone treatment did not further influence the pituitary LH release, indicating that dopaminergic inhibition of LH release is absent in juvenile female striped bass (Holland et al., 1998). On the other hand, decrease in dopaminergic activity was observed at the time of onset of puberty in the juvenile spadefish *C. faber* (Marcano et al., 1995), whereas presence of GnRHa and pimozide was required to induce LH release and ovarian development leading to vitellogenesis in the immature European eel *A. anguilla* (Dufour et al., 1988; Vidal et al., 2004). Similarly, treatment of GnRHa alone or GnRHa + DOM to sexually immature red seabream *Pagrus major* resulted in vitellogenesis and ovulation in contrast to the absence of ovarian development in controls (Kumakura, Okuzawa, Gen, & Kagawa, 2003). In the present study, exposure of juveniles of 25 DPH to two doses of DOM for a period of 35 days resulted in appearance of vitellogenic follicles in the ovary as well as dense accumulations of GnRH-immunoreactive fibres in the PPD region of the pituitary gland in contrast to the faintly immunoreactive GnRH-fibres in treatment controls. Although stage V follicles with large yolk granules were generally found in the ovary following treatment with both doses of DOM, the number of these follicles was significantly higher concomitant with increased density of GnRH-immunoreactive fibres in 1.5 mg DOM-treated group compared with those of 4 mg DOM-treated juveniles. These results indicate that treatment of 1.5 mg DOM triggers the process of vitellogenesis and puberty in *G. affinis*. The present investigation for the first time reveals that the removal of dopaminergic inhibition promotes the onset of puberty without additional treatment of GnRHa in juveniles of viviparous species.

Conclusions

The results of the present investigation reveal that the blockade of dopaminergic inhibition triggers GnRH release into the pituitary gland and the promotes the ovarian follicular development leading to early onset of puberty in juveniles. It is suggested that the treatment of DOM without additional GnRHa and steroid hormones can be employed for inducing puberty in viviparous species.

Abbreviations

ANOVA: Analysis of variance; BSA: Bovine serum albumin; DA: Dopamine; DO: Dissolved oxygen; DOM: Domperidone; DPH: Days post-hatching; FSH: Follicle stimulating hormone; GnRH: Gonadotropin-releasing hormone; GnRHa: Gonadotropin-releasing hormone agonist; GPR 54: G-protein-coupled receptor 54; GtH: Gondaotropic hormone; HPG: Hypothalamo-pituitary-gonad; LH: Luteinizing hormone; NaCl: Sodium chloride; PBS: Phosphate-buffered saline; PPD: Proximal pars distalis; SVL: Snout-vent length

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Authors' contributions

SKB conducted the experiment and analyzed the data. CBG designed the experiment and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest
The authors declare that they have no competing interests.

Ethics approval and consent to participate
No human subjects are involved in the study. The experimental procedures were carried out in accordance with Institutional Animal Ethical Committee.

Consent for publication
Not applicable

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