Seasonal effects of melatonin on ovary and plasma gonadotropin and vitellogenin levels in intact and pinealectomized catfish, *Clarias batrachus* (Linn)

Juthika Ghosh & Panchanan Nath
Department of Zoology, Visva Bharati University, Santiniketan 731 235, India
Received 14 June 2004; revised 19 November 2004

Effects of daily administration of melatonin for 15 days were evaluated with respect to ovarian activities and plasma gonadotropin (GtH II) and vitellogenin (Vg) levels in intact (INT) and pinealectomized (Px) female catfish, *C. batrachus*, during preparatory (April), prespawning (May and June), spawning (July) and post-spawning (September) periods. Px (saline control groups) caused a stimulatory effect during preparatory (with respect to Vg synthesis and incorporation) and prespawning (with respect to Vg synthesis) periods whereas no effect was observed during spawning and post-spawning periods with respect to the reproductive parameters studied. During April, melatonin-treatment significantly decreased plasma GtH II levels and percentage of vitellogenic oocytes without any significant changes in plasma Vg levels and gonadosomatic index (GSI). During early prespawning period, in May, 50μg melatonin brought about a significant reduction in plasma GtH II levels in INT group, whereas 100μg caused a decrease in all parameters; on the other hand, in Px groups both dose levels proved to be inhibitory. In June (late prespawning period) melatonin-treatment could not bring about any change in GSI and plasma Vg levels compared to the control groups regardless of Px but plasma GtH II and mean number of yolky oocytes were significantly reduced in melatonin-treated INT group. During spawning period (July) melatonin inhibited the GSI, mean number of yolky oocytes and plasma GtH II levels without affecting plasma Vg levels. In September (post-spawning period), melatonin did inhibit both GSI and plasma GtH II levels. The results, thus, indicate that melatonin showed variable effects (inhibitory and/or no effect) to GSI, mean number of yolky oocytes and plasma Vg levels but a consistent inhibition of plasma GtH II levels indicating that melatonin may control the reproduction by blocking the GtH II release from the pituitary via affecting the hypothalano-hypophysial axis.

**Keywords:** Catfish, Gonadotropin, Melatonin, Ovary, Vitellogenin, Yolky oocytes

In fish, seasonality is a common phenomenon with respect to many physiological processes like growth, feeding behaviour and reproduction. Among these, although reproduction seems to be controlled by the endogenous annual parameters like gonadotropin and steroid hormones, seasonal changes in photo-thermal regimes are the main environmental cues involved in the synchronization of such rhythms. It is well established that the seasonal reproduction is mediated via the functioning of the hypothalamo-hypophysial-gonadal axis and pineal gland, through melatonin secretion, plays important role in the functioning of such axis. As reported by Reiter, melatonin functions as a clock and calendar providing two kinds of temporal information to animals (here it is fish), such as the daily variations of melatonin with maximal production during the scotophase of the light-dark (LD) cycle may indicate an internal signal of the specific time of the day, while the duration of the dark phase increase would provide information of the length of the photoperiod and hence about the season (see Garcia-Allegue et al. Further, the temperature variations influence the amplitude of the melatonin rhythms and contribute to seasonal changes in melatonin levels. Therefore, it seems that in nature at different time of the year a particular combination of photoperiod and temperature is probably used by the pineal to provide precise information regarding calendar time, so much so that the reproductive activities are properly regulated by melatonin.

A general idea is that daily injections of melatonin attenuate ovarian development induced by long-day conditions. However, as rightly pointed out by Ekstrom and Missel, systematic studies of the effects of melatonin during different seasons are scarce. In female catfish, *Heteropneustes fossilis*, melatonin exerts an antigonadotrophic effect during the prespawning and spawning periods and that may be mediated by the hypothalamic serotonergic system.
In contrast, according to Garg, melatonin does not affect ovarian activity or Vg levels expressed as alkali-labile phosphorus during the preparatory and prespawning periods, whereas Px does so during the preparatory and post-spawning periods only.

The catfish, *Clarias batrachus* (Linn), is a seasonal breeder and breeds during the monsoon period. Its annual ovarian cycle has been divided into four periods i.e. preparatory (Feb-Apr), pre-spawning (May-Jun), spawning (Jul-Aug) and post-spawning (Sep-Jan). In nature, the ovarian weight increases rapidly with the formation of yolky oocytes, which is caused by hepatic synthesis of Vg and its incorporation into the developing oocytes during prespawning-spawning periods, resulting in peak ovarian growth during prespawning-spawning periods, Px causes progonadal, anti gonadal and no effect in the female catfish exposed to different photoperiodic regimes during different reproductive phases and hence the effect of Px may possibly be due to lack of melatonin, the pineal hormone. A circannual rhythm of plasma melatonin levels was well documented in the catfish and its amplitude was high during early preparatory and low during prespawning phase. Therefore, it is clear that melatonin may be behaving differently during different reproductive phases of the catfish. Keeping this in view attempts have been made, in the present study, to see the effects of melatonin administration on ovarian growth and plasma GtH II and Vg level in INT and Px female catfish, *C. batrachus*, during different time periods of its annual ovarian activity.

**Materials and Methods**

*Collection and care of fishes*—Sexually mature female catfish, *C. batrachus*, (body weight range: 50-100 g) were collected around Santiniketan (Lat. 23°41'30"N and Long. 87°30'47"E) and acclimatized for 7 days in the laboratory under natural photoperiod and temperature before the commencement of experiments during different months, such as April (preparatory period), May and June (prespawning period), July (spawning period) and September (post-spawning period) of its annual ovarian cycle. Water in the aquarium was replenished daily with tap water and procaine penicillin (1: 1000) was added to aquarium water to prevent skin infection. Laboratory-made food containing rice bran, oil cake, wheat flour, soyabean powder (5 mg in 5 g of feed) and chopped goat liver was provided *ad libitum* during acclimation and the tenure of experiments.

*Experimental design*—Five experiments were conducted at different reproductive phases, preparatory (April), pre-spawning (May and June), spawning (July) and post-spawning (September) of the catfish, *C. batrachus*.

During each experimental month, fishes were collected in the first week, sexed and females were maintained as described above. A group of female catfish was segregated and pinealectomized a day before the commencement of hormone treatment.

*Pinealectomy and hormone treatment*—Pinealectomy (Px) of the experimental catfish was performed one day before the commencement of each experiment. During Px each catfish was wrapped in a napkin and a sharp incision was made on the skin covering the pineal fontanelle to form a 'v' shaped flap, which was then folded posteriorly to expose the pineal area. The membranous connective tissue covering that region was cut along the edge. The pineal vesicle along with a major portion of pineal stalk was extirpated by gently pulling out with the help of a pair of fine forceps; thereafter the fontanelle was cleaned thoroughly with a cotton ball soaked in 70% ethanol. The whole process was carried out under a dissecting binocular microscope. Fish showing any remnants of pineal tissue or any obvious damage to the underlying olfactory nerves and tracts were discarded. After surgery, the skin flap was gently pressed back into its position and the fish was returned to aquarium water. The wound was healed within one week and survival rate was more than 90% till the end of each experiment. At the time of autopsy the pineal area was again examined under magnification. No regeneration or incomplete surgery was found in any of the Px fish. The catfish, which served as intact (INT) groups, were not sham operated because of the fact that earlier studies from our laboratory (unpublished data) and from the findings made by Garg revealed that no significant difference in GSI and plasma alkali-labile phosphorus levels was noticed between the intact and sham operated groups in similar studies performed in Indian catfish.

Melatonin (Sigma Chemical Co. USA) was dissolved first in ethanol (50-100 μl) and the solution was made up to desired concentrations (50 μg and/or 100 μg/0.1 ml) with 0.6% sodium chloride solution and kept in dark bottles. Fresh solutions were prepared every week. Melatonin was administered in
both Px and INT female catfish at the dose level of 50 µg and/or 100 µg/fish/day for 15 days; saline (0.6% sodium chloride) was injected at the same volume (0.1ml) to separate groups of Px and INT catfish to serve as controls. Five females were sacrificed on the day of commencement of hormone treatment to serve as initial control.

Sampling and evaluation—The experimental fish were autopsied following the last day of injections. At the time of autopsy, each fish was weighed to the nearest gram on a salter balance; bled by caudal puncture and plasma was separated by centrifugation (1500 g for 10 min at 4°C). Plasma samples were diluted (1:1 and 1:10) with PBS (10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl), and stored at −30°C until their use in Vg and GtH II estimation by respective ELISAs. After the blood collection, fish were killed by decapitation; ovaries were dissected out, weighed to the nearest milligram on an electric single pan balance (Monopan Industries, India) and then fixed in aqueous Bouin’s fixative (24 hr) for histological observations or counting of yolky oocytes.

Histological studies—Ovary of each fish (wherever necessary) was cut at 7 µm and stained with hematoxylin-eosin to identify the different stages of oogenesis, which were characterized and identified in the ovaries of catfish, *C. batrachus*, with the help of Leitz microprojector (MP3, Mr 1644, PZO, Warszawa, Poland). The different stages of oocytes are as follows.

(a) Oogonia and stage I (SI) primary oocytes, which are non-yolky oocytes and present in the ovary throughout the annual reproductive cycle. The yolk nucleus (YN) is, sometimes, present in the cytoplasm of stage I primary oocytes. All these types of oocytes were categorized as stage I (SI) primary oocytes; (b) Stage II (SII) oocytes, which are characterized by the presence of cortical alveoli (CA) in the cytoplasm indicating the onset of vitellogenesis; (c) Stage III (SIII) oocytes, which are either the fully formed yolky oocytes or the ooplasm containing plenty of yolk vesicles (see Fig. 2C); (d) Atretic follicles (AF), which are present just after spawning and were characterized by the liquefaction of yolk material in the cytoplasm. AF may be present throughout the reproductive cycle. In the present study, the post-ovulatory follicles were also considered as AF as many have described such follicles as AF (see Guraya14). From the ovarian sections the different stages of oogenesis were counted under the Leitz microprojector and expressed in percentage.

Oocyte count—Yolky oocytes were counted (whenever necessary) from the fixed ovaries by gravimetric method19. Each ovary was weighed to the nearest milligram and then a piece was cut and weighed, and yolky oocytes were separated and counted manually and then calculated per 100 g body weight basis [(no. of yolky oocytes in the ovaries/body wt) × 100]

Assay of plasma Vg and GtH II—Plasma Vg from the experimental fish was estimated with the help of catfish Vg ELISA as described by Nath and Maitra20 and expressed as µg/ml plasma.

Plasma GtH II of the experimental fish was estimated by developing a heterogenous Gth-II ELISA (unpublished data) using common carp GtH II and its antibodies, which were kindly supplied by Dr. R.E. Peter, Biological Sciences, University of Alberta, C W 223 Biological Sciences Building, Edmonston, Canada T6G2E9. Briefly, for the assay, microplates were coated with 20 ng/well of carp GtH II in 200 µl of carbonate-bicarbonate buffer (pH 9.6), incubated overnight at 4°C, washed with PBS-Twin-20 (PBS-T) pH 7.4 and blocked with 200 µl/well of hypophysectomized catfish serum (Hypox CFS). Standard common carp GtH II (0.078 to 40 ng/ml) or serial dilutions of plasma samples (1:10 or more) were incubated with 1:25000 anti-GtH II antisera (diluted 1:25000 with PBS-T-hypox CFS) in microtubes for 14 hr at 4°C. Samples were transferred in triplicate to the precoated microplates, incubated for 3 hr at room temperature, and washed with PBS-T. The immobilized GtH II-antibody complex was detected with anti-rabbit IgG conjugated to horseradish peroxidase purchased from Sigma Chemical, USA (Batch No. A-9169, Lot 58H4837) (diluted 1:4000 in PBS-T-Hypox-CFS). Colour development was achieved by the addition of orthophenylene diamine (10 mg OPD in 25 ml of 0.1 M citrate-phosphate buffer, pH 5, containing 3 µl of hydrogen peroxide) to the plate (200 µl/well) and incubated for 30 min in dark at room temperature. Reaction was stopped with 2N H2SO4 (50 µl/well) and OD was read at 492 nm using an Anthos 2001 microplate reader. Under these conditions the lower limit of detection of Common carp GtH II was 78 pg/ml (95% binding). The intra and inter assay coefficients of variance (CV) range from 2 to 8% (n=6) and 1.45 to 10% (n=12), respectively. In this
carp-GtH II ELISA, plasma from male and female and pituitary extracts of catfish, C. batrachus, at the tested dilutions showed parallelism to the common carp GtH-II standard curve (unpublished observations) and therefore, the heterogenous GtH II ELISA has been used to estimate catfish GtH II in experimental fishes.

Analysis of data—For comparison of data, all ovarian weight and number of yolky oocytes (wherever applicable) were calculated on 100 g body wt basis. The number of stage I, II and III oocytes were expressed as percentage and the plasma Vg and GtH II levels were expressed as nanogram or microgram/ml plasma. P values between the control and experimental groups were calculated by Student’s ‘t’ test.3

Results

Preparatory period (April)—Figure 1 shows the effect of 15-day treatment of 50 μg melatonin in INT and Px female catfish during late preparatory period, when vitellogenesis has started and vitellogenic oocytes (Stage II) appeared in the ovary (Fig. 2A). Ovarian weight increased significantly in all the experimental groups compared to that of the initial control. Regardless of surgery, no significant changes were noticed in ovarian weight of melatonin-treated groups. However, Px caused a significant increase in plasma Vg level compared to that of the initial control. Melatonin treatment decreased the plasma GtH II levels only in INT group with a simultaneous increase in plasma Vg levels (not significant due to high standard error) compared to the INT control group. The histological observations of ovaries revealed that Px caused the appearance of stage III oocytes, besides the stage II vitellogenic oocytes in the female catfish (Fig. 2C). Melatonin treatment did not affect vitellogenesis in both INT and Px groups although the percentage of vitellogenic oocytes is less compared to that of the saline control groups. (See Fig 1 and 2 D-E).

Prespawning period (May-June)—Two separate experiments were conducted in May and June and the results are presented in Figs 3 and 4, respectively. In May, two dosages (50 and 100 μg) of melatonin were used to evaluate their effects. Since this period is the vitellogenic period the ovary contained yolky oocytes and plasma levels of Vg (600±20 μg/ml) and GtH II (14±0.9 ng/ml) were high in the catfish (Fig. 3). After 15 days of saline treatment there was a significant increase in ovarian weight without having any significant change in mean number of yolky oocytes and plasma levels of Vg and GtH II over the initial control values. Melatonin (50 μg) treatment resulted in a significant reduction in plasma GtH II without affecting the GSI (gonadosomatic index), number of yolky oocytes and plasma Vg levels in INT catfish whereas in Px group a significant decrease was noticed in GSI and plasma Vg levels in comparison to those of saline control groups. At 100 μg dose level regardless of surgery, significant decrease was observed in plasma Vg and GtH II levels without any significant changes in the GSI and mean number of yolky oocytes over the saline control values (Fig. 3).

In June, the GSI was high and the ovary contained a large number of yolky oocytes coinciding with higher level of plasma Vg and low GtH II level
Fig. 2—Photomicrographs of transverse sections of ovary of female catfish, *C. batrachus*, injected daily for 15 days with 50 µg/fish/day melatonin, during the preparatory period (April). Haematoxylin-eosin ×13.2. Note that vitellogenic oocytes (S II and S III) are present in the ovaries of (A) initial control (IC), (B) intact control (INTC), (C) Pinealectomized control (PxC), (D) Intact melatonin treated (INTM) and (E) Pinealectomized melatonin treated (PxM).
Fifteen-day of saline treatment showed a decreasing trend in all parameters except plasma GtH II levels than those of initial control. After 15 days of melatonin treatment no significant changes were noticed in GSI and plasma Vg levels although plasma GtH II levels tend to decrease in both INT and Px groups. A significant reduction in mean number of yolky oocytes and plasma GtH II levels was observed in the melatonin-treated INT group (Fig. 4).

Spawning period (July)—In the initial control group, the GSI became 4.85±0.8 and the percentage of yolky oocytes was reduced (18.5±0.01) although plasma Vg (1297±354 μg/ml) and GtH II (13.6±0.5 ng/ml) levels were high. After 15 days of saline treatment, the GSI, percentage of stage III oocytes, plasma Vg and GtH II levels were significantly reduced without any change in the percentage of stage II oocytes over the initial control values. Melatonin-treatment showed a decrease in GSI, stage III oocytes and plasma GtH II levels regardless of surgery over their respective controls. However, no significant change was noticed in plasma Vg levels in treated groups (Fig. 5).

Post-spawning period (September)—In the initial control group the ovary was regressed and contained only oogonia and stage I primary oocytes and Vg was not detected in plasma. However, GtH II was present in plasma. Fifteen-day melatonin treatment resulted in a decrease in GSI as well as plasma GtH II levels regardless of surgery over their respective controls (Fig. 6).

Discussion

The data indicate that melatonin administration during preparatory, pre-spawning, spawning and post-spawning periods in INT and/or Px female catfish, C. batrachus, maintained under natural photoperiod and temperature in the laboratory either retarded or showed no effect on ovarian activity with...
simultaneous changes in plasma levels of GtH II and Vg, which is responsible for the conversion of non yolky oocytes into yolky ones.

During preparatory period, melatonin treatment reduced the plasma GtH II levels and the percentage of vitellogenic oocytes (stage II and III) in the ovaries without affecting the GSI and plasma Vg levels. These observations indicate that melatonin may be affecting the hypothalamo-hypophyosal axis so much so that it inhibits the release of pituitary GtH II that is responsible for the incorporation of Vg into oocytes. There are many reports providing evidence that melatonin controls gonadal growth by influencing this axis\textsuperscript{122}. The lack of effect of melatonin on GSI and plasma Vg levels is in agreement with the findings of Garg\textsuperscript{14} in the catfish, \textit{Heteropneustes fossilis} and this suggests that melatonin does not directly affect the gonad, a hypothesis that was already suggested in carp by Popek \textit{et al}\textsuperscript{23}. This is further confirmed from the observations of ovarian histology in the present study that yolky oocytes were not affected as no atretic follicles were seen (see Fig. 2) after melatonin treatment. However, the percentage of vitellogenic oocytes were reduced compared to the control group, further suggesting that incorporation of Vg into oocytes may have been blocked and the event is generally triggered by GtH and thus indicate no direct action of melatonin at gonads rather it may affect either at the level of hypothalamus or pituitary as shown in carp\textsuperscript{23,24}.

During pre-spawning period (May) regardless of pinealectomy melatonin inhibited ovarian activity coincident with a lowering of plasma GtH II and Vg levels and is in agreement with the findings of Sundararaj and Keshavanath\textsuperscript{19}, who suggested that in \textit{H. fossilis} melatonin inhibited gonadal growth but not the number of gonadotrophs in pituitary, indicating a reduction in the release of GtH. The present findings further suggest that melatonin may be involved in blocking the release of pituitary GtH II into circulation, which, in turn, affect the synthesis and secretion of ovarian estradiol-17β, the steroid hormone, responsible for the synthesis and secretion of hepatic Vg. Popek \textit{et al.}\textsuperscript{27} demonstrated that Px reduced the plasma estradiol-17β levels in mature carp. At this point, it is to be noted that neither Px nor melatonin implants caused any effect on basal or estradiol-17β-stimulated estrogen receptor and Vg mRNA in the liver of rainbow trout as demonstrated

![Fig. 5](image-url)

**Fig. 5**—Effect of melatonin (50 μg/fish/day) on ovary, plasma GtH II and Vg levels in INT and Px catfish, \textit{C. batrachus}, during spawning period (July). Abbreviation as in Fig 1. The mean body weights from left to right were 58.75, 51, 57, 51 and 46 g. \(P\) values were calculated according to student’s ‘t’ test between IC and INTC for GSI: \(P<0.001\), plasma Vg: \(P<0.02\), GtH II: \(P<0.001\), Stage I: \(P<0.01\), Stage III: \(P<0.001\); INT and INTM for GtH II: \(P<0.001\); PxC and PxM for GSI \(P<0.001\), GtH II: \(P<0.05\); Stage III: \(P<0.05\).

![Fig. 6](image-url)

**Fig. 6**—Effect of melatonin (50 μg/fish/day) on ovary, plasma GtH II and Vg levels in INT and Px catfish, \textit{C. batrachus}, during early postspawning period (September). Abbreviation as in Fig 1. The mean body weights from left to right were 24, 25, 20.5, 19 and 20 g. \(P\) values were calculated according to student’s ‘t’ test between IC and INTC for GSI: \(P<0.005\), GtH II: \(P<0.02\); INT and INTM for GSI: \(P<0.025\); PxC and PxM for GtH II: \(P<0.001\).
by Mazurais et al. Therefore, the hypothesis stands that melatonin may control the reproduction via operating on hypothalamic-hypophysial axis.

In June, the late prespawning period, melatonin effect was not prominent with respect to different reproductive parameters studied and this may be due to the fact that the ovary had attained its maximal size during this period and made the detection of any effects difficult as suggested by Garg in the catfish, *H. fossilis*, where Px did not show any effect during prespawning period. However, regardless of surgery melatonin inhibited the plasma GH II levels indicating again that melatonin may be affecting the hypothalamo-hypophysial axis for controlling the release of pituitary GH II as indicated earlier.

In spawning period under laboratory photo-thermal conditions if gravid females were simply maintained for 15 days or more there occurred a decrease in ovarian weight along with the number of yolky oocytes resulting in regression (unpublished data). This may be due to the fact that fishes at this time wait for the proper environmental conditions (such as the rainfall in India) so that they could spawn but in laboratory such favourable conditions could not be achieved and therefore, they undergo regression. However, during such situation single injection of either mammalian gonadotropin (LH, hCG) or fish gonadotropin (SG-G100, Carp GtH) into the catfish causes ovulation, which depends on the physiological condition of the ovary (see Sundararaj, 1981)\textsuperscript{27}. Even during this period, melatonin-treatment reduced the GSI and plasma GtH II levels indicating further the inhibitory action of melatonin. During post-spawing period the catfish is generally having a regressed ovary containing only oogonia and stage I primary oocytes, even then melatonin inhibited the GSI and plasma GtH II levels indicating the inhibitory role.

The antigonadal effects of melatonin injections observed in the catfish during the different phases of reproductive cycle indicate mild inhibition during preparatory to strong inhibition during prespawning and spawning periods. To have a conclusive report on melatonin’s effect further experiments are required to be conducted during other months of the annual reproductive cycle of the catfish.

In this study it was observed that Px (control groups) caused a stimulatory effect and melatonin acts as inhibitory in the Px catfish during preparatory and prespawning period whereas during spawning and postspawning periods Px was seemed to be ineffective although melatonin was inhibitory with respect to ovarian growth and plasma Vg levels. The stimulatory and inhibitory effects of pineal / melatonin, have been well established in many teleost fishes.

As pointed out by Ekstorm and Meissl\textsuperscript{2} that in long day breeder the pineal appears to be inhibitory in short photoperiod and stimulatory in long photoperiod. The catfish is a long day breeder and Px was found to be stimulatory during preparatory period (April, LD cycle: 12:12) and prespawning period (May-June: LD cycle: 13:14:11:10) and melatonin treatment was inhibitory with respect to plasma GtH II and/or Vg levels and percentage of yolky oocytes (stage II and III) in the ovaries. These findings thus indicate that pineal may be producing some other substances, which may possibly control the gonadal growth. To substantiate the above statement the work of Senthilkumaran and Joy\textsuperscript{28} could enlighten the fact that variations in hypothalamic content of 5-HT (serotonin) and MAO (monoamine oxidase) during different reproductive phases of catfish, *Heteropneustes fossilis*, suggest their involvement in the control of gonadotropin secretion, and cue the hypothalamic-hypophyseal system to initiate reproductive activity. Further, a correlation has already been established in *Heteropneustes fossilis* between 5-HT and GSI, which is positive at long photoperiod and negative at short photoperiod and thereby suggesting the photoperiodic effects on gonadal activities may be mediated via hypothalamic serotonergic system. Therefore, it is assumed that a similar system may be operating in this catfish, *C. batrachus*. However, at this juncture the action of melatonin cannot be ruled out in this respect.

If the pineal is controlling fish reproduction via melatonin then why melatonin administration inhibits the ovarian function? At present we are unable to answer but could suggest that melatonin may indirectly be doing this job possibly by affecting the hypothalamus resulting in the blocking of the release of gonadotropin releasing hormone (GnRH), which, in turn, controls the release of GtH from pituitary.

**Acknowledgement**

The study was supported by the UGC-sponsored DSA program to Department of Zoology, Visva Bharati and by the ICAR Grant.
References

10. Sundararaj B I & Keshavananth P, Effects of melatonin and prolactin treatments on the hypothalamic-ovarian system in the catfish, Heteropneustes fossilis (Bloch), Gen Comp Endocrinol, 29 (1976) 84.