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Do thyroid and testis modulate the effects of pineal and melatonin on haemopoietic variables in *Clarias batrachus*?

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Abstract. Involvement of pineal and its major hormone, melatonin, in the process of erythropoiesis in a freshwater catfish, *Clarias batrachus* has been investigated. The study was conducted during four phases, namely preparatory phase, spawning phase, postspawning phase and late postspawning phase of its annual reproductive cycle. During each phase a fish received either melatonin injections or subjected to pinealectomy. In addition, each fish in all the groups, received either iopanoic acid or cyproterone acetate or vehicle in the morning or late afternoon. Results clearly indicate that melatonin stimulates the rate of erythropoiesis in *Clarias batrachus*. It appears that the extent of stimulation depends upon the phase of the annual reproductive cycle. However, in general, the pineal- or melatonin-induced modulation of blood variables is gonad dependent and thyroid seems to play a time of the day dependent subtle role.

Keywords. Haemopoiesis; melatonin; pinealectomy; iopanoic acid; cyproterone acetate; catfish.

1. Introduction

In various vertebrates, thyroidal (Datta 1975; Thapliyal and Kaur 1976; Thapliyal 1980; Pati and Thapliyal 1984) and gonadal (James *et al* 1971; Shahidi 1973; Kaur and Thapliyal 1975; Robinzon and Rogers 1979) involvement in erythropoiesis have been well established. However, role of pineal in the regulation of erythropoiesis has been rarely reported (Pati and Gupta 1992; Shedpure and Pati 1993, 1995a). For the first time a stimulatory influence of melatonin on hemopoiesis in a finch has been demonstrated (Pati and Gupta 1992). Again the same phenomenon has been documented in *Clarias batrachus* (Shedpure and Pati 1993. 1995a). The former study in *C. batrachus* was conducted only during the spawning phase of its annual gonadal cycle involving several intermediary variables apart from the blood parameters. The latter study was performed during four different phases of the reproductive cycle and circannual responses of 8 physiologic variables, including circulating number of erythrocytes and haemoglobin concentration to melatonin treatment and pineal ablation were examined. Results of all these studies confirm that melatonin does play a key role in the process of haemopoiesis. However, this generalization could become more valid and universal only when more corroborative information would emerge from studies conducted on various other vertebrate species.

On the other hand the pineal-gonad relationship has been well documented (Reiter 1981; Pevet 1988; Creighton and Rudeen 1989). The antigonadotropic or antigonadal function of melatonin is probably the best known example of the above relationship in

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endothermic (Reiter 1980, 1981; Pevet 1988) as well as ectothermic (De Vlaming and Olcese 1981; Vollrath 1981; Pevet 1988; Joy and Khan 1991) vertebrates, including *C. batrachus* (Nayak and Singh 1988). Nonetheless, its effect has been shown to depend upon its dose, time and the route of administration and so also upon the reproductive state of the recipient (Nayak and Singh 1987a).

Identification and localization of thyrotropin release factor (TRF) in the pineal glands (White *et al* 1974) strengthen several other reports on pineal-thyroid associationship (Rowe 1971; Stebbins and Cohen 1973; Vriend 1983). In addition, in Indian freshwater catfishes, including *C. batrachus,* pineal and thyroid relationship has been documented (Nayak and Singh 1987a, b; Agha and Joy 1987). Thus, an obvious question arises: Does the regulatory influence of melatonin on erythropoiesis in *C. batrachus* (Shedpure and Pati 1993) depend on gonad and/or thyroid?

Therefore, the objective of the present study was to determine the effects of melatonin as well as pinealectomy on blood variables, viz, circulating red cell number and haemoglobin concentration as a function of time of the day and phase of the annual reproductive cycle of *C. batrachus.* Furthermore, attempts were also made to investigate if the above effects are thyroid and/or gonad dependent by using iopanoic acid (IOP)—a thyroid inhibitor or cyproterone acetate (CTA)—a potent antiandrogen, respectively, in conjunction with melatonin treatment or pineal extirpation.

2. Materials and methods

C. batrachus is a freshwater Indian catfish and breeds annually. Its reproductive cycle has been divided into four phases, such as preparatory phase (March-April), prespawnlng phase (May-June), spawning phase (July-August) and postspawning phase (September-February) (Nayak and Singh 1988).

In the preparatory phase of its gonadal cycle, ninety six live *C. batrachus* (body wt. 50–70 g) of mixed sexes were purchased locally in the month of March and kept in stock aquaria, for about 7–10 days for proper laboratory acclimatization. The animals were exposed to natural daylength and temperature prevailing at the time of experiment at Raipur (Lat. 21° 14'N; Long. 81° 38'E) and were fed *ad libitum.* The water inside the aquarium was renewed every alternate day.

Forty intact animals were randomly selected from the stock aquaria and were divided into six groups out of which four consisted of eight animals (4 males + 4 females) and the remaining two groups had males each. All the groups of fish received melatonin (100 µg/fish in 0·1 ml fish saline) on alternate day either at morning (05:30-06:30) or late afternoon (17:00-18:00). In order to resolve the thyroidal and/or gonadal dependency of melatonin *vis-a-vis* its efficacy on blood variables, in the present study two potent inhibitors were administered alongwith the melatonin. IOP, an inhibitor of peripheral deiodination of T_4 to T_3 (Leonard and Visser 1986) and CTA, a potent antiandrogen (Turner and Bagnara 1976) were used. Therefore, each group received either in the morning or late afternoon IOP (5 µg/fish in 0·1 ml fish saline) or CTA (5 µg/fish in 0·1ml olive oil) or vechile on alternate day, i.e., the day following the melatonin administration. Melatonin plus CTA administered groups had only male fishes. In total, each fish received 7 injections of melatonin (7 × 100 µg) +7 injections of vehicle or IOP (7 × 5 µg) or CTA (7 × 5 µg).

The dose of IOP and CTA was decided on the basis of a pilot study as well as studies conducted b^{y} various other workers (Leonard and Visser 1986; Galton 1989; Tews *et al*

Table 1. Design of experiment.

		Treatment†	
n	sex	First	Second
8	(4m4f)	Mlt/M	I/M
4	(4m)	Mlt/M	C/M
8	(4m4f)	Mlt/M	V/M
8	(4m4f)	Mlt/LA	I/LA
4	(4m)	Mlt/LA	C/LA
8	(4m4f)	Mlt/LA	V/LA
8	(4m4f)	None[a]	None[a]
8	(4m4f)	None[b]	None[b]
8	(4m4f)	Px[c]	I/M
4	(4m)	Px	C/M
8	(4m4f)	Px	V/M
8	(4m4f)	Px	I/LA
4	(4m)	Px	C/LA
8	(4m4f)	Px	V/LA

m, Male; f, female; Mlt, melatonin; Px, pinealectomized; M, morning; LA, late afternoon; I, iopanoic acid; C, cyproterone acetate; V, vehicle.

†$7 \times 2 = 14$ days duration.

[a,b]Received no treatment and sacrificed in the morning and late afternoon, respectively. They were considered as intact control groups. [c]Pinealectomy performed 1-week prior to the beginning of the experiment.

1988; Gupta *et al* 1989). The melatonin and IOP solutions were prepared by dissolving required amount of the hormone in a small volume of absolute ethanol and then diluting with fish saline for obtaining the desired strength. The CTA solution was prepared by dissolving the hormone in olive oil. Furthermore, two intact control groups were also included in the study each having 8 animals and designated as morning or late afternoon control groups. The design of experiment has been shown in table 1.

Forty animals were randomly selected and were divided into six groups. The pineal gland was removed surgically one week prior to the beginning of the experiment. The method of Nayak and Singh (1988) was slightly modified for performing the pinealectomy. These authors used a dental drill to make a hole (1 mm diameter) in the skull to remove the pineal. We skipped this step which is invariably associated with profuse bleeding and chances of damage of the other vital brain areas. In C. *batrachus* the pineal lies inside a shallow concavity called pineal window covered by a thin translucent skin having sparse melanophores (see Shedpure and Pati 1995b). The pineal was first exposed by cutting the skin flap over the window from three sides and folding it anteriorly. Thereafter, the pineal was removed by using surgical forceps. The pinealectomized animals did not receive melatonin but received vehicle or IOP or CTA under the identical protocol cited above.

Twentyfour hours after the last injection the fish were decapitated. Administration of melatonin and/or inhibitors at two different time of the day made it necessary to sacrifice half of the group of fishes in the early morning and the other half in the late

afternoon in order to maintain a time lag of 24 h between time of injection and decapitation. The blood was collected by caudal puncture and immediately processed for determining erythropoietic variables, viz., red cell number and haemoglobin concentration following the method suggested by Dacie and Lewis (1977). The red cells were counted by using an improved Neubauer counting chamber. Haemoglobin concentration was determined by employing acid-alkali method and Gibson and Harrison's standard was used to construct a standard curve (Dacie and Lewis 1977). Identical experimental protocols were adopted during the spawning phase (August), postspawning phase (October) and late postspawning phase (December) (see table 1).

2.1 *Statistical analyses*

In the present experiment, although the data were obtained separately for males and females for each treatment group, all the analyses were performed on pooled data. However, CTA-treated intact or pinealectomized groups consisted of males only. The pooling of the data was effected especially because statistically significant sex effect on either erythrocyte number or haemoglobin content could not be validated by ANOVA (sex effect: for RBC, F value = 0·753, D.F. = 1, 48, P value = 0·389, NS; for Hb, F value = 1·699, D.F. = 1,48, P value = 0·198, NS).

The data were analysed with the help of ANOVA (Snedecor and Cochran 1994). Means and Standard errors were computed, plotted as a function of the treatment and compared with the help of Student's t-test (Snedecor and Cochran 1994).

3. Results

Statistically significant effects of time of the day as well as phase of the reproductive cycle were obtained by ANOVA for circulating erythrocytes and haemoglobin content in the groups following pineal extirpation. However, in melatonin receiving animals the above effects could not be significantly validated for red cell number (table 2). Other first order and second order interaction effects are also shown in table 2.

Table 2. Analysis of variance summary for phase, time of injection and treatment[†] effects on red blood cell number and haemoglobin content in melatonin-treated and pinealectomized *C. batrachus.*

		F-value			
		Melatonin		Pinealectomy	
Source	df	RBC	Haemoglobin	RBC	Haemoglobin
Phase(P)	3	2·18	122·40**	8·30**	29·01**
Time(T)	1	0·77	10·33**	28·81**	10·47**
Treatment(Tr)	3	12·59**	85·94**	14·15**	17·86**
P × T	3	1·97	20·72**	3·04*	7·90**
P × Tr	9	5·07**	21·47**	7·10**	11·38**
T × Tr	3	10·16**	4·08**	6·96**	1·71**
P × T × Tr	9	4·58**	7·55**	7·73**	0·95

Sum square error df: 194, 193, 201, 199 respectively.
*,** $P < 0·05$; $P < 0·01$, respectively.
[†] Refer table 1.

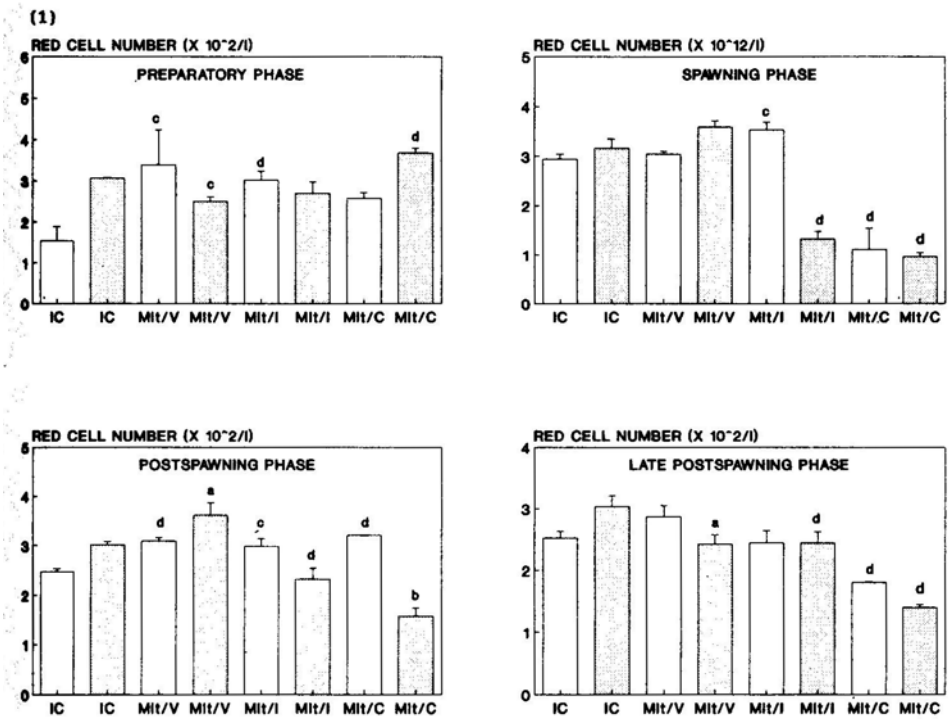

Figure 1

3.1 *Red cell number*

3.1a *Melatonin + vehicle:* A statistically significant stimulatory influence of early morning melatonin was validated only during the preparatory and postspawning phases although marginal but statistically insignificant increase in red blood cell number was witnessed during other two phases also (figure 1). In contrast, it signify-cantly depressed red cell number during preparatory and late postspawning phases if administered in the late afternoon (figure 1). However, melatonin injection at late afternoon elevated red cell number during spawning as well as postspawning phases (figure 1). The elevation witnessed during spawning phase was not statistically signifi-cant.

3.1b *Melatonin + IOP*: IOP when injected in the morning alongwith melatonin, it could not modulate the influence of the latter during all the phases of reproductive cycle except during spawning phase (figure 1). The modulation during the spawning phase was characterized by a statistically significant elevation in the number of circulating red blood cells. Interestingly, late afternoon administration of IOP caused statistically significant decline in circulating red cell number, irrespective of phase of the annual gonadal cycle, notwithstanding lack of statistical validation for its effect during the preparatory phase (figure 1).

3.1c *Melatonin + CTA:* Administration of CTA alongwith melatonin in the early morning while did not modulate the influence of the latter during preparatory as well

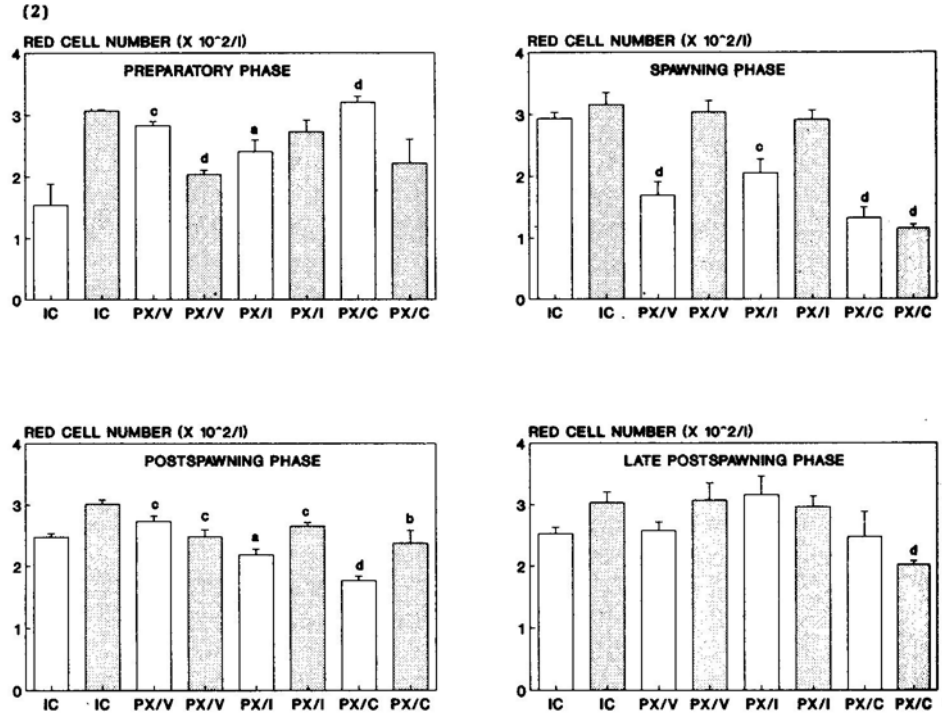

Figure 2

as postspawning phases, it reversed the melatonin-induced marginal increase in red cell number into a statistically significant sharp decline during spawning and late post-spawning phases (figure 1). However, CTA when injected alongwith melatonin in late afternoon, statistically significant decline in circulating red cell number was noticed during all the phases of annual gonadal cycle excepting the preparatory phase (figure 1).

3.1d *Pinealectomy + vehicle*: In the preparatory and postspawning phases the number of circulating erythrocytes was statistically significantly higher in pinealectomized + vehicle-injected fishes of early morning group. However, interestingly in the late afternoon identical treatments elicited an opposite effect during the above phases (figure 2). Furthermore, during the spawning phase the levels of circulating erythrocytes were found to be statistically significantly low in the pinealectomized + vehicle-injected early morning group (figure 2).

3.1e *Pinealectomy + IOP*: The treatment of IOP in pinealectomized animals in the early morning decreased the number of erythrocytes during spawning and postspawning phases but elevated the same during the preparatory phase. However, IOP when administered in the late afternoon decreased the red cell number, irrespective of the phase of the annual reproductive cycle. The decrement was statistically validated during the postspawning phase only (figure 2).

3.1f *Pinealectomy + CTA*: While administration of CTA in pinealectomized animals, in the early morning, significantly decreased the circulating erythrocytes during

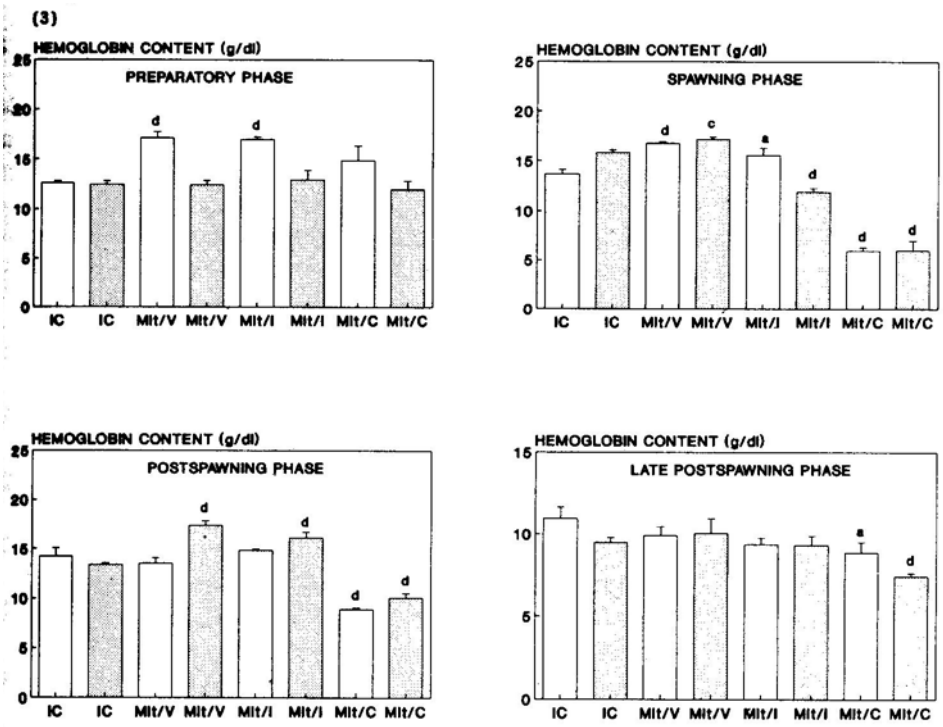

Figure 3

spawning and postspawning phases, it significantly increased the same during the preparatory phase. Interestingly, the CTA treatment in the late afternoon statistically significantly decline the red cell number, irrespective of phase of the annual gonadal cycle. However, the decline documented during preparatory phase was not statistically significant (figure 2).

3.2 *Haemoglobin content*

3.2a *Melatonin + vehicle*: Early morning melatonin elevated the level of haemoglobin content during preparatory and spawning phases with statistical validation. However, late afternoon melatonin treatment produced similar effect during the spawning and postspawning phases (figure 3).

3.2b *Melatonin + IOP*: Administration of IOP alongwith melatonin in the early morning could not alter the stimulatory effect of melatonin on haemoglobin content at least during preparatory and spawning phases. However, when IOP injected in the late afternoon, diverse responses were detected; a significant decrease during spawning phase and an increase during the postspawning phase (figure 3).

3.2c *Melatonin + CTA*: The treatment of CTA alongwith melatonin suppressed the level of haemoglobin content, irrespective of time of injection and phase of annual gonadal cycle, excluding the morning treatment during preparatory phase. However,

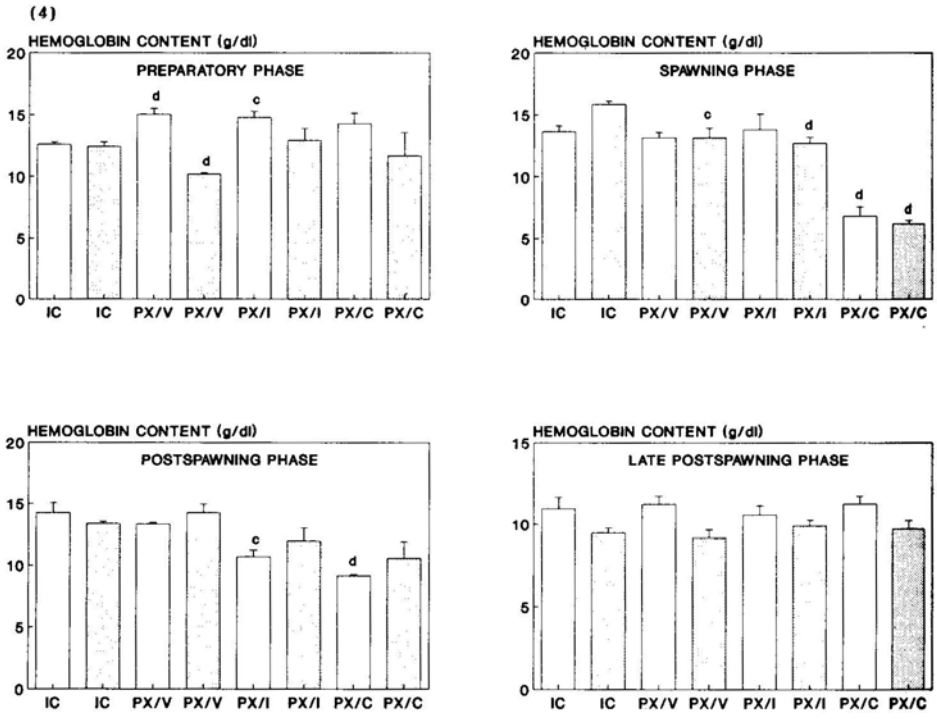

Figure 4.

Figures 1-4. Responsiveness of red cell number (**1, 2**) and haemoglobin content (**3, 4**) to various treatments along with melatonin (**1, 3**) and pinealectomy (**2, 4**) in *C. batrachus* during preparatory, spawning, post spawning and late postspawning phases of annual reproductive cycle. IC, intact control; Mlt, melatonin; V, vehicle; I, IOP; C, CTA; Px, pinealectomized. Open bar, morning shaded bar, late afternoon. ^{a,b,c,d}Differ from the corresponding time-qualified intact control group at . P <0·05, . P <0·02, . P <0·01 and . P <0·001, respectively.

the observed depression in the late afternoon during the preparatory phase was not statistically significant (figure 3).

3.2d *Pinealectomy + vehicle*: An elevated level of the haemoglobin content was noticed in the pinealectomized animals in the morning during the preparatory phase ($P < 0.001$). In contrast, significant decline in the haemoglobin content was detected during the preparatory as well as spawning phases in the late afternoon. However, during the other phases it had no significant effect (figure 4).

3.2e *Pinealectomy + IOP*: Early morning IOP administration in the pinealectomized animals raised the haemoglobin content during preparatory phase ($P < 0.01$) but a significant decrease was noticed during the postspawning phase. When IOP was treated in the late afternoon a singificant decline in the haemoglobin content was obtained only during the spawning phase (figure 4).

3.2f *Pinealectomy + CTA*: Administration of CTA in pinealectomized animals inhibited the process of haemoglobin synthesis, irrespective of time of injection during the spawning and postspawning phases. However, a significant decline could not be obtained for the late afternoon treatment group of postspawning phase (figure 4).

4. Discussion

Stimulatory role of melatonin on erythropoiesis has been recently established in the catfish, *C. batrachus* (Shedpure and Pati 1993, 1995a). Present results document that late afternoon melatonin is stimulatory for both the haemopoietic variables (RBC and haemoglobin during spawning and postspawning phases. However, early morning treatment of melatonin elevated the red cell number and haemoglobin content during preparatory and postspawning and preparatory and spawning phases, respectively. The above findings corroborate those reported earlier for *C. batrachus* (Shedpure and Pati 1993, 1995a).

It has been documented in *C. batrachus* that melatonin inhibits thyroid function, characterized by decline in both T_4 and T_3, when injected in the late afternoon (Nayak and Singh 1987a). Furthermore, in the same study conducted by Nayak and Singh (1987a) late afternoon administration of melatonin (100μg)has been shown to substanptially decrease the circulating levels of testosterone during prespawning phase. Therefore, in the present study, a reduction in circulating red cell number in the animals receiving melatonin + vehicle in late afternoon during preparatory and late postspawning phases could be imputed either to the thyroid inhibitory or gonado-inhibitory role of melatonin. However, there is no information if morning administration of melatonin would exert similar influence on thyroidal and gonadal activity over all the phases of annual reproductive cycle. Therefore, it is difficult at this moment to explain early morning melatonin-induced elevation in the red cell number and haemoglobin content in *C. batrachus* during preparatory and postspawning and preparatory and spawning phases, respectively.

It has been well documented that androgens stimulate the rate of erythropoiesis either directly, i.e., acting on bone marrow or indirectly by way of the kidney in various vertebrate species (James *et al* 1971; Kaur and Thapliyal 1975; Robinzon and Rogers 1979). Although the principal role of male hormone is in reproduction in mammals, it influences whole body and somatic tissue respiration in ectothermic vertebrates (Kaur and Thapliyal 1975; Thapliyal 1980). Furthermore, it has been reported that combination of testosterone and thyroxine is more effective than individual hormone in correcting anemia (Thapliyal *et al* 1982; Pati and Thapliyal 1984). In a nutshell, in *C. batrachus* melatonin has been shown to depress both circulating level of T_4, T_3 as well as testosterone when injected in the late afternoon during prespawning phase (Nayak and Singh 1987a).

Reproductive phase dependent as well as time of the day dependent erythropoietic response of *C. batrachus* to pinealectomy was detected in the present study. It has been well documented that pinealectomy produces a gonadal phase dependent effect on GSI, T_4, T_3 and testosterone in *C. batrachus* and in many other vertebrate species (Nayak and Singh 1987b; Agha and Joy 1987; Haldar-Misra and Thapliyal 1981). A different effect of pinealectomy observed on the same day, i.e., early morning effect vs late afternoon effect could be explained on the basis of an underlying biological clock mechanism relating to erythropoietic machinery. This speculation is based upon experimental evidence emerging from mammalian studies (Wide *et al* 1989; Haus *et al* 1983; Hrushesky *et al* 1992).

Erythropoiesis is known to be highly rhythmic on the circadian time scale with respect to serum levels of erythropoietin (Wide *et al* 1989), erythroid progenitor cell numbers, the resultant number of reticulocytes and mature erythrocytes (Haus *et al*

1983) and the susceptibility of red cell precursors to myelotoxic drugs (Laerum and Smaaland 1989) in mammals including humans (Hrushesky *et al* 1992). Haematopoietic rhythms in the present species is yet to be explored. However, in an ectothermic Indian garden lizard *Calotes versicolor,* a circadian stage dependent effect of mammalian urinary erythropoietin on blood morphology has already been demonstrated (Pati and Gupta 1991). It may be possible that at least during preparatory and postspawning phases pinealectomy failed to obliterate the circadian rhythm in haemopoiesis.

During the spawning phase, CTA drastically reduced the number of circulating red cell and haemoglobin concentration, irrespective of its time of administration both in melatonin-treated and pinealectomized fish. Similar results were obtained for pinealectomized fish during postspawning period and for melatonin-treated fish during late postspawning phase of the annual gonadal cycle. In the pinealectomized animals CTA could not produce any effect on blood variables during preparatory and late postspawning phases, excluding a lone increase in red cell number in morning group and a decline in haemoglobin content in the late afternoon group, during preparatory phase and postspawning phase, respectively. However, its role in the melatonin-treated group was found to be contradictory during both preparatory and postspawning phases of the annual gonadal cycle. During preparatory phase animals receiving melatonin alongwith CTA exhibited elevated circulating levels of red cell numbers only, irrespective of time of administration. In contrast, during postspawning phase CTA increased red cell number in the morning, decreased the same in the late afternoon and decreased haemoglobin content, irrespective of the time of the day. However, the results of CTA administration during spawning phase were extremely consistent in both melatonin-treated as well as in pinealectomized fish. The same was true for pinealectomized fish in postspawning phase and for melatonin-treated ones in the late postspawning phase. Could it be that the melatonin dependency on androgens in ameliorating erythropoietic activity is again circannual phase dependent?

The effects of IOP were found to be erratic in the melatonin co-treated groups. However, in general, melatonin + IOP-treated fishes had higher red cell number and haemoglobin content in the morning group as compared with that of the late afternoon group. IOP inhibits thyroidal activity by way of blocking the peripheral deiodination of T_4 into T_3. However, the blockage is effected by way of impairment of type-II 5' deiodinase (Silva *et al* 1982; Leonard and Visser 1986). In mammals, T_3 has been known to be the potent form and produces most of the well known thyroidal effects. However, this is questionable in case of lower vertebrates. Nonetheless, could it be that IOP is influencing some of the deiodinating enzymes in a circadian stage dependent manner? Perplexingly, in the pinealectomized animals specially during the spawning and postspawning phases IOP consistently decreased the blood variables. It produced no-effect during late postspawning phase and increase both red cell number and haemoglobin content during preparatory phase when given in the morning. In other words IOP failed to modulate the effects of pinealectomy in the morning, whereas it nullified the inhibitory effects of pineal removal during preparatory phase of the annual gonadal cycle. Some of the results are indeed difficult to explain. The IOP-induced results clearly suggest operation of an underlying clock mechanism with regards to its action.

It is speculated that pineal- or melatonin-induced modulation of blood variables is dependent on the gonads. However, thyroid appears to play subtle role, i.e., exerts circadian stage dependent modulation of the influence of pineal or melatonin on blood variables.

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