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Photoperiodic modulation of reproductive physiology and behaviour in the cichlid fish *Cichlasoma dimerus*

Ana Fiszbein, Maximiliano Cánepa, Graciela Rey Vázquez, Cristina Maggese, Matías Pandolfi *

Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (C1428EHA) Buenos Aires, Argentina

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ABSTRACT

Timing of breeding to an optimal season is a requirement for a successful reproductive outcome in seasonally breeding species. Photoperiodic signals are capable of modifying the reproductive behaviour and reproductive systems in several vertebrate species. The cichlid fish *Cichlasoma dimerus* shows highly organized breeding activities and different social status. The aim of this study is to test whether *C. dimerus* reproductive behaviour (male aggressive behaviour and female choice) and reproductive physiology (GnRH3 morphometric parameters, pituitary hormones content and organ-somatic indexes) are modulated by photoperiod. Before spawning, dominant pairs were isolated and kept in opposite tanks of 20 l for one week, so they could see each other but not physically interact. Afterwards, a group was exposed for four weeks to a short photoperiod (8 h light:16 h dark) (short photoperiod exposed animals: SP) while another group was exposed to a long photoperiod (14 h light:10 h dark) (long photoperiod exposed animals: LP). Temperature was maintained constant. Behavioural experiments showed that male aggression related to territory selection and its defence is reduced in SP males. Further, SP females were never chosen. At the brain level we demonstrated that GnRH3 neuronal optical density of staining was reduced. Finally, at the pituitary level we showed that SP males showed low levels of β-LH, PRL and GH in the pituitary, and that SP females showed no significant differences in the pituitary content of any hormone. Taken all together these results suggest that in *C. dimerus* the photoperiod is a relevant environmental cue related to reproductive behaviour and physiology.

1. Introduction

It is widely documented that the timing of reproduction is controlled by external signals such as seasonal changes in day-length or photoperiod, chemosensory cues from con-specifics, food availability and temperature. Timing of breeding to an optimal season is a requirement for a successful reproductive outcome, and many organisms evolved to use changes in photoperiod as the primary signal that coordinates the breeding season [1,2]. Photoperiodic signals are capable of modifying the excitability of GnRH neurons which are the command cells in the central nervous system that control reproductive physiology and behaviour in all vertebrates studied to date [3]. Photoperiod alters morphological characteristics of both GnRH neurons and glia that surround GnRH-neuron axon terminalis in Japanese quail [4]. It was also shown that photoperiod is capable to alter synaptic inputs to GnRH neurons in sheep [5]. In male masu salmon it was shown that photoperiod influences the expression of GnRH mRNA [6]. Recently, in the atheriniform fish pejerrey *Odontesthes bonariensis* it was shown that the increase in day length but not on temperature triggers the maturation of pejerrey females after the winter period of gonadal rest [7]. The manipulation of the photoperiod is currently being used in aquaculture to induce maturation, control spawning and stimulate growth in different species [8–10]. The pineal organ, which is a component of the circadian system, is influenced by the light/dark cycle and it is involved in the control of circadian and circannual rhythms in vertebrates [11,12]. The pineal gland secretes melatonin (N-acetyl-5-methoxytryptamine) exclusively at night and it has been shown in fish that this hormone, acting on different elements of the hypothalamic–pituitary–gonadal axis, provides a crucial endocrine signal to influence reproductive activity [13].

The South American cichlid fish *Cichlasoma dimerus* is a freshwater species that adapts easily to captivity and spawns with high frequency during 6 months of the year (October–April), providing an appropriate model for reproductive and developmental studies. It is important to remark that these animals breed under long photoperiodic conditions (about 13–14 h of light/day). As many cichlids do, *C. dimerus* has highly organized breeding activities that can be observed in the laboratory. A few days after fish have been transferred to a new aquarium, territories are progressively established and defended by a dominant male that later will choose a female which would become aggressive during the reproductive period [14]. The dominant pair

* Corresponding author.
E-mail address: pandolfi@bg.fcen.uba.ar (M. Pandolfi).

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will defend the prospective spawning site (usually a flat stone) and will start to display stereotyped prespawning activities, such as jerking and quivering, digging in the gravel, and nipping off and nibbling at the spawning substrate. The other individuals will remain subordinated in the tank showing a submissive behaviour. The existence of different social status is associated with variations on the physiological parameters that they present [15].

Three GnRH variants were expressed in this species: GnRH1 and GnRH3 neuronal populations were located in the ventral forebrain showing an overlapped distribution pattern with projections that contribute to the pituitary innervation and the GnRH2 population was restricted to the midbrain tegmentum without projecting axons to the pituitary [16]. Most of the focus on GnRH neurons has been, and continues to be, on the population of cells located in the preoptic area-hypothalamus (GnRH1) that control the pituitary–gonadal axis and the number and scope of the studies on GnRH2 and GnRH3 neurons have been very limited [6,14]. GnRH2 has been suggested to play a role as a facilitator of sexual behaviour under energy-restricted conditions in musk shrews and mice [17]. GnRH3 neurons are always associated with the olfactory bulbs and they project axons to the retina and pineal organ which suggest a role in light and photoperiodic behavioural and physiological responses [16,18]. In male tilapia *Oreochromis niloticus* it was shown that GnRH3-immunoneutralization significantly decreased nest-building ability, nest size and aggressive behaviour [19]. Those results provided evidence that GnRH3 is a potent neuromodulator of reproductive behaviours.

The aim of this study is to test whether *C. dimerus* reproductive behaviour (male aggressive behaviour and female choice) and reproductive physiology (GnRH3 morphometric parameters, pituitary hormones content and organ-somatic indexes) are modulated by photoperiod.

2. Materials and methods

2.1. Animals

Adult animals were collected from Esteros del Riachuelo, Corrientes, Argentina (27°25′S 58°15′W). Fish were collected from the wild mainly in early spring (October). The photoperiod in the zone of collection was about 13 h light:11 h dark in that month. In all cases animals were acclimatized to laboratory conditions for at least two months: photoperiod (14 h light:10 h dark) and temperature (25 ± 2 °C) in order to reduce the impact of seasonal effects. Aquaria were well aerated, provided with external filtration and fish were fed daily with commercial pellets.

2.2. Experimental design

Thirty-seven adult fish (21 males and 16 females) were used in the study. Total length and weight (expressed as mean ± standard error) were 13 ± 1.6 cm and 47.8 ± 12.2 g for males and 11.1 ± 1.6 cm and 37.3 ± 10 g for females. Animals were kept in groups of eight in communitarian tanks of 150 l, in order to allow the establishment of dominant pairs (Fig. 1a). When adults are placed in community tanks, after a couple of hours it is possible to define three different kinds of social status: Territorial–Dominant male (defines a territory and then chooses a female for reproduction), Reproductive Aggressive female (that is chosen by the Territorial–Dominant male), Territorial–noDominant fish [15]. Before spawning, dominant pairs were isolated and male and female were kept in opposite tanks of 20 l for one week, so they could see each other but not physically interact. Afterwards, pairs were exposed for four weeks to a short photoperiod (8 h light:16 h dark) (short photoperiod exposed animals: SP) (Fig. 1b) or kept in the original long photoperiod (14 h light:10 h dark) (long photoperiod exposed animals: LP) (Fig. 1c). Temperature (25 ± 2 °C) was maintained constant in both cases. Principles of laboratory animal care were followed in all cases (NIH publication no. 85-23, revised in 1985).

2.2.1. Experiment 1: Two kinds of behavioural experiments were performed

a) Comparison of aggressive behaviour between a LP and a SP males (Fig. 2a): Experiments for studying territory competition in a neutral tank were performed using adult animals that were collected from the wild in Esteros del Riachuelo (Corrientes, Argentina) and acclimatized to laboratory conditions for at least two months: photoperiod (14 h light:10 h dark) and temperature (25 ± 2 °C) which mimic natural environment conditions. After

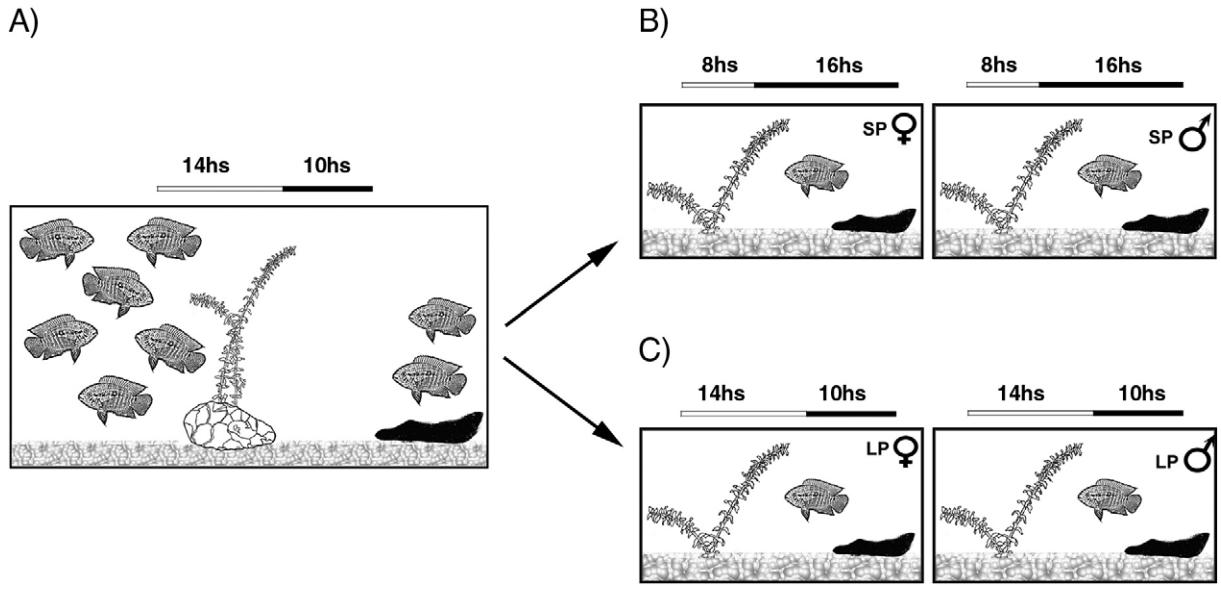

Fig. 1. Experimental design of the photoperiod experiment. A) Animals ($n = 37$) were kept in groups of eight in communitarian tanks of 150 l with aquatic plants and stones, in order to allow the establishment of dominant pairs. Before spawning, dominant pairs were isolated and male and female were kept in opposite tanks of 20 l for one week, so they could see each other but not physically interact. Afterwards, pairs were exposed for four weeks to: B) a short photoperiod (8 h light:16 h dark) (short photoperiod exposed animals: SP) or C) long photoperiod (14 h light:10 h dark) (long photoperiod exposed animals: LP). Temperature (25 ± 2 °C) was maintained constant in both cases.

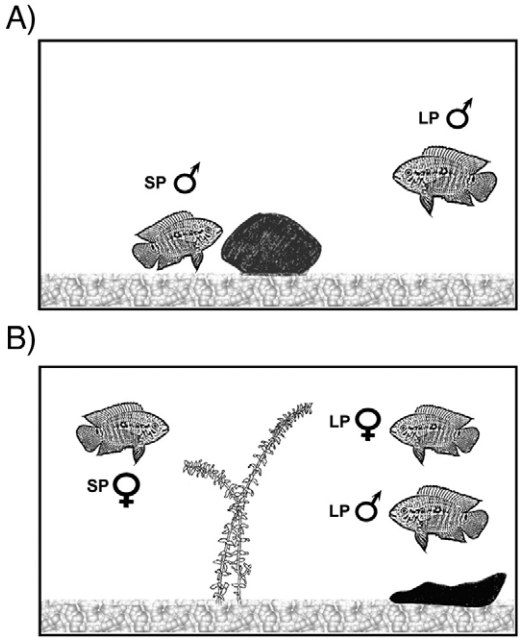

Fig. 2. Behavioural experiments. A) A long (LP) and a short (SP) photoperiod exposed males were placed for no more than 2 h in a neutral 20 l tank that had gravel and a medium size rock in the middle in order to compare their aggressive behaviour ($N=5$). B) A LP male was placed in a neutral tank with a LP and a SP photoperiod exposed females (neither of them previously chosen by the male as a mate) to analyse which of the females would be selected for reproduction ($N=5$).

this acclimatising they were exposed to different photoperiods (LP and SP). Subordinate status was previously determined by repeated observations of submissive behaviours by the focal fish toward a dominant individual of the same sex [15]. Male fish were always originated from different tanks to avoid effects of familiarity and previous interactions, and each fish was only used once. LP and SP males were placed together in a neutral tank (50 L) with 2 cm of gravel and a big stone in the middle. As dominance may be influenced by male size in cichlids we always used animals of similar length and weight for the experiment. Trials lasted for 120 min and fish start showing physical interactions 22 ± 7 min after being placed together. All interactions and aggressive behaviours were registered by observers blind to the identities of the fish. Aggressive interactions were quantified considering two kinds of contact aggression: bites (one fish bites another fish mainly in the fins and lateral parts of the body) and mouth holding (one fish and another lockjaws and push against one another). Trials were always conducted between 13:00 and 15:00 h to control for daily variation in sexual hormones and behaviour. Winner status was assigned to the fish that performed more aggressive interactions. Looser status was assigned to the fish that received most numerous contact–aggressive interactions. Body coloration patterns and territory holding were also registered ($n=5$).

b) Effects of the photoperiod on the conditions for female selection (Fig. 2b): a LP male was placed in a neutral tank with a LP and a SP female (neither of them previously chosen by the male as a mate) to analyse which of the females would be selected for reproduction ($n=5$). The following criteria was followed for considering a female "selected" by the male: when the LP male, the LP female and the SP female were transferred to a new aquarium, the spawning territory was quickly established and defended by the male. These male would attack other males and females. The selected female will not be attacked and will acquire a green-brown dark coloration specially darkened in the opercular zone and the ventral part of the head.

2.2.2. Experiment 2: Effect of photoperiod in the brain–pituitary–gonadal axis

Another group of 6 males and 6 females were individually exposed to LP and SP conditions. These animals were just sacrificed after four weeks of exposure to LP or SP in order to evaluate and compare their haematological parameters, pituitary hormones content and the morphometry of GnRH3 neurons. Animals were anesthetized by immersion in a 0.1% benzocaine solution. Before sacrifice by decapitation, blood samples were taken to measure haematological parameters. Gonads, liver and spleen were weighted and fixed. Gonado-somatic, hepato-somatic and spleen-somatic indexes were calculated (i.e: GSI = [gonadal weight/fish weight]*100). Pituitaries and brains were processed as is described below. It is important to remark that blood and tissue sampling were made at the same time of the day (between 13:00 and 15:00) since hormone levels can vary widely throughout the day.

2.3. Haematological parameters

In order to analyze animals' health and study their variation in different photoperiods, several haematological parameters were determined. Once anesthetized, the peripheral blood was collected by puncture of the caudal vein with a heparin-coated 25 gauge × 0.5 in needle, attached to a 1 ml syringe. Careful netting and handling was implemented to minimize stress. In all cases no more that 5 min passed before netting and sampling. Within the first 2 h after each extraction, the blood samples were processed for determination of the total erythrocyte count (RBC) using a Neubauer hemocytometer [20]. Hematocrite value was determined by the standard microhematocrit method, and expressed in percentage. Duplicate blood samples were loaded into standard heparinized capillary tubes, spun in a micro-hematocrit centrifuge at 12000 rpm for 5 min and measured on a microcapillary reader. Later hemoglobin in erythrocytes was determined using the cyanmethaemoglobin method (hemogloWiener reactive, Wiener Lab.). Prior to reading the absorbance, hemoglobin test samples were centrifuged at 3500 rpm for 5 min in order to remove dispersed nuclear material. The following indices: mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and mean corpuscular volume were calculated according to Siverd (1964).

2.4. Immunohistochemical analysis of GnRH3 neurons (experiment 2 fish)

After dissection, 12 brains were fixed in Bouin solution for 24 h at 4 °C, dehydrated and embedded in Paraplast. Samples were completely sectioned coronally at 15 µm intervals and sections were mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher, Wash). Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4), treated for 30 min with PBS containing 5% nonfat dry milk, incubated for 16 h at 4 °C with a 1:2000 dilution of LRH13 antibody (a monoclonal antibody which recognizes the three GnRH isoforms from this species). This antibody was generously gifted by Dr K. Wakabayashi (Zoological Institute-Graduate School of Science-University of Tokyo-Japan). The specificity of this antibody was previously demonstrated in this species by preadsortion tests and all the proper negative and positive controls [21]. After incubation with the primary antibody, sections were washed in PBS, and then incubated for 45 min in a biotinylated anti-mouse IgG following manufacturer instructions (Dako, CSA Amplification Kit). In order to amplify the signal, sections were afterwards incubated in streptavidin, tyramide and peroxidase-conjugated streptavidin (Dako) for 30 min each. After three washes in PBS, peroxidase activity was visualized with 0.1% 3.3′-diaminobenzidine (DAB) in TRIS buffer (pH 7.6) and 0.03% H2O2. Sections were lightly counterstained with hematoxylin, mounted, examined with a NIKON microphot FX microscope and digitally photographed (Nikon,

Table 1
Antisera used in the Western blot analysis and estimated molecular weight of *C. dimerus* pituitary hormones.

Antisera	Dilution	Source	Donated from	Detected ir-band/s
anti-PRL	1:3000	*Oncorhynchus keta*	Dr Hiroshi Kawauchi[a]	23 kDa
anti-GH	1:3000	*Oncorhynchus keta*	Dr Hiroshi Kawauchi[a]	25.5 kDa
anti-SL	1:2000	*Sparus aurata*	Dr Antonio Astola[b]	32 & 28 kDa
anti-βFSH	1:1000	*Fundulus heteroclitus*	Dr Akio Shimizu[c]	19 &15 kDa
anti-βLH	1:2000	*Fundulus heteroclitus*	Dr Akio Shimizu[c]	24 kDa

[a] Dr Hiroshi Kawauchi (School of Fisheries Sciences, Kitasato University, Iwate, Japan).
[b] Dr Antonio Astola (Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Cádiz, Spain).
[c] Dr Akio Shimizu (National Research Institute of Fisheries Science. Fisheries Research Agency. Kanazawa, Yokohama. Japan).

Coolpix 4500). As it may be difficult to compare staining intensity among tissues reacted at different times, representatives of males or females of both photoperiod conditions were included in each batch of IHC reactions to further control for staining differences.

From the total number of immunoreactive GnRH3 cells in each brain, 10 of them were randomly selected for measurement of the average nuclear diameter, nuclear area (Image Pro-Plus) and optical density of staining (Image Gauge version 3.12). To reduce variability in immunocytochemical results, the conditions of the immunocytochemical reactions were controlled and were kept homogeneous on all parameters. Slides were coded so that the observer was blind to the respective treatments.

2.5. Western blot analysis of pituitary hormones content (experiment 2 fish)

In order to semiquantify pituitary content of hormones related to GnRH and reproduction [prolactin (PRL), growth hormone (GH), β-follicle-stimulating hormone (β-FSH), β-luteinizing hormone (β-LH) and somatolactin (SL)], an analysis with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot was performed. The hormones were detected using heterologous antisera, the specificity of which had been previously tested in this species [16,22–24] (Table 1). After dissection each pituitary was homogenized in 100 µl of Tris–HCl buffer 50 mM, pH 7.4, with 1 µl of protease inhibitor cocktail (Sigma, St Louis, Mo.). Each homogenate was centrifuged at 12000 g for 10 min and supernatants were collected. 9 µl of each homogenate (15 µg of protein) with 3 µl of loading buffer (120 mM Tris–HCl pH 6.8, 3% dodecylsulfate, 10% glycerol, 1% β-mercaptoethanol) were heated at 100 °C for 5 min and loaded into the SDS-PAGE. After electrophoresis, proteins and molecular markers (SeeBlue Plus2 PreStained Standard; Invitrogen) were transferred to a nitrocellulose membrane (Amersham Biosciences) for 60 min at 4 °C and 75 V. Then, membranes were washed in TBST pH 7.5 and blocked with TBST containing 3% nonfat dry milk overnight. Later, membranes were incubated for 3 h at room temperature (RT) with different primary antisera (Table 1) and washed in TBST afterwards. Membranes were then incubated with a biotinylated anti-rabbit IgG (Sigma-Aldrich) (1:1000) for 1 h at RT, washed again and finally incubated with a streptavidin complex conjugated to alkaline phosphatase (Sigma-Aldrich) (1:2000) for 45 min at RT. After washing, the reaction was visualized using an alkaline phosphatase developing kit (BCIP/NBT, Vector Blue, Dako). Finally, membranes were dried, digitized and optical density semiquantification was performed using Image Gauge version 3.12 (Fuji Photo Film) software. In order to avoid a possible loading error in the SDS-PAGE, pituitary hormone content was semiquantified by densitometric analysis and normalized to the optical density obtained for α-tubuline.

2.6. Statistical analysis

Statistical analysis of Western blot data was performed by using an analysis of variance (ANOVA). Statistical analysis of data pertaining to nuclear area and optical density of GnRH neurons was performed using a two-way nested analysis of variance. Statistical analysis of organ-somatic index was performed by using student test. Data met all the assumptions required to perform ANOVA and student *t* tests. Data from male aggressive behaviour assay were analysed by a Wilcoxon matched pairs test. All data are presented as mean ± SEM. Statistical significance was established at the $p<0.05$ level. The Statistica 7.0 Software (Statsoft, Inc) was used for analysis.

3. Results

3.1. Effect of photoperiod on male aggresive behaviour

LP males demonstrated, in all cases and in less than an hour, a stronger territorial and more aggressive behaviour than SP males. Soon after being placed in the same tank (within the first 20 min), LP males performed the first attack biting SP males side part of the body. LP males performed more aggressive interactions than SP males ($p=0.043$) (Fig. 3). In all cases animals showed mouth holding during the experiment which is a very aggressive interaction that was observed in this species especially when two male fish met for the first time in a new environment. In most cases, SP males did not respond and they exhibited behavioural inhibition such as decreased locomotor activity and stayed hidden behind the stone or in the top surface of the aquaria. Winner status was always related to the fish that keeps most of the territory and that posses a colourful body pattern (green-blue-yellow-black rays). Looser status was always related to the fish that, at the end of the trial, posses a dark-homogeneous body colour.

3.2. Effect of photoperiod on female selection

In all cases, a few hours after the beginning of the experiment, SP females were driven to the upper part of the tank by the attacks of LP males and females. The first attacks were performed by LP male over SP female side part of the body. Afterwards, LP female attacked SP female while the not-chosen female hid among the plants. The

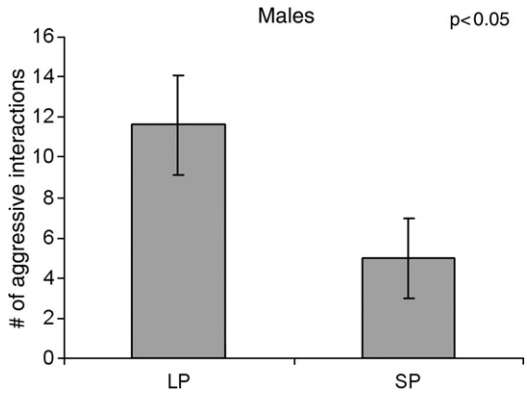

Fig. 3. Number of male aggressive interactions between a LP and a SP animal. LP males demonstrated more aggressive interactions compared to SP males ($p<0.05$). The aggressive interactions are the pooled number of bites and mouth holding behaviours per 120 min.

established pair colonized the whole tank and kept on attacking the SP female all experiment long. LP female was selected by LP male for reproduction in all cases ($n=5$). The following criterion was followed for considering a female "selected": the selected female will not be attacked by the male and will acquire a green-brown dark coloration specially darkened in the opercular zone and the ventral part of the head.

3.3. Haematological parameters

Values were compared to those previously described in this species [25], showing no significant variations in short and long photoperiods. Hematocrite (%) values were 37 ± 2 (LP male); 32 ± 5 (SP male); 34 ± 4 (LP female); 34 ± 2 (SP female). Haemoglobin concentration HB (g/dl) values were 11 ± 3.4 (LP male); 12.8 ± 0.9 (SP male); 11.2 ± 2.5 (LP female); 12.4 ± 1.7 (SP female). No animals showed abnormal values so that all of them were considered healthy and used for the corresponding experiment.

3.4. Indexes

Males exposed to LP showed a higher hepatosomatic index than SP males (2.7% vs 1.5%, $p=0.031$). Gonadosomatic and spleensomatic indexes did not show significant differences (Fig. 4a). Females from both photoperiodic conditions did not show significant differences in any of the indexes (Fig. 4b).

3.5. GnRH3 neurons

Using immunohistochemistry, neuronal nuclear area and relative optical density of staining of ir-GnRH3 cell bodies were examined. While nuclear area showed no significant differences between LP and SP animals, optical density of staining was higher in LP than in SP animals The analyses were performed between males and females separately (males: 152 ± 4.5 a.u. vs 117 ± 4.8 a.u. $p=0.0059$; females: 133.23 ± 7.09 a.u. vs 108.20 ± 4.63 a.u. $p=0.042$) (Fig. 5).

3.6. Pituitary hormones content

The relative optical density of ir-PRL, GH, β-FSH, β-LH and SL bands (normalized to α-tubuline) was measured in order to study whether the photoperiod influences pituitary content of these hormones. The analysis showed that LP males had significantly higher pituitary β-LH, PRL and GH levels than SP males: β-LH (385 ± 34 a.u. vs 98 ± 11.3 a.u. $p=0.007$, $n=3$), PRL (305 ± 131 a.u. vs 113.5 ± 24 a.u. $p=0.04$, $n=3$) and GH (427 ± 66.5 a.u. vs 109 ± 25 a.u. $p=0.043$, $n=3$) (Fig. 6), while no significant differences were detected in β-FSH and SL pituitary content. In the case of the females, no significant differences were detected in the pituitary content of any measured hormone ($N=6$) (Fig. 7).

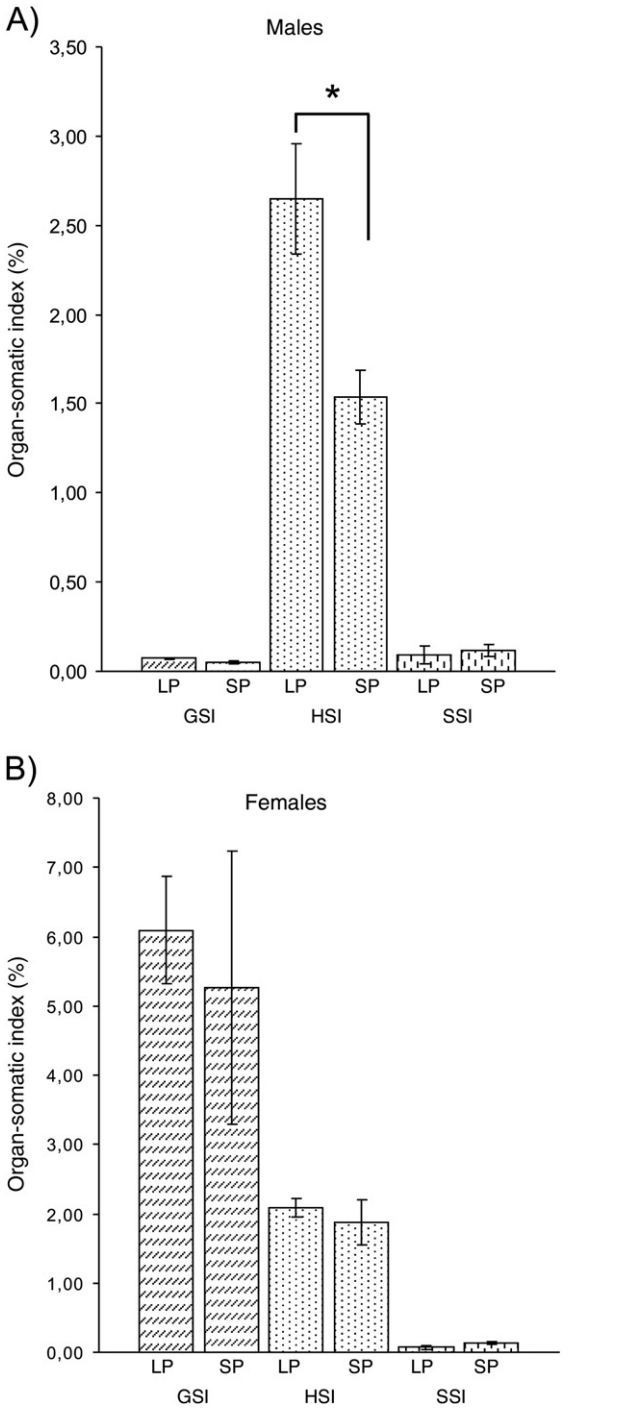

Fig. 4. Organ-somatic indexes of males and females after a 4 week exposure to long or short photoperiods ($n=6$) (LP and SP, respectively). A) Males showed a significant higher hepatosomatic index when expose to LP ($p<0.01$). B) Females did not show any significant difference in their indexes.

4. Discussion

Environmental cues such as photoperiod and temperature can have important effects on the timing of gametogenesis, vitellogenesis and maturation in fish [26]. As in many cichlids, *C. dimerus* has highly organized breeding activities that can be observed under laboratory conditions. After several years of close observation we realized that this species has a very sophisticated social system. For this reason, we studied the effect of different photoperiods (with a constant temperature) not only on some neuroendocrine parameters but on reproductive behaviour as well. It is important to remark that the LP condition mimics the natural breeding period for these species.

One of the characteristic reproductive behaviours in this species is the aggressive interaction between males which leads to the selection of a territory before choosing a reproductive female that will also become aggressive afterwards. When LP males were matched with SP males, the first ones became dominant in less than an hour. This assumption was supported by the quantitative analysis of aggressive interactions and by observing that the LP males always initiated the attacks. When GnRH3 nuclear area and diameter of LP and SP males were analyzed no significant differences were observed. Interestingly, LP males showed a higher GnRH3 neuronal optical density of staining. In the African cichlid tilapia *O. niloticus* it was clearly shown that immunoneutralization of GnRH3 inhibited nest-building and aggressive behaviours [19]. Also, in other non-

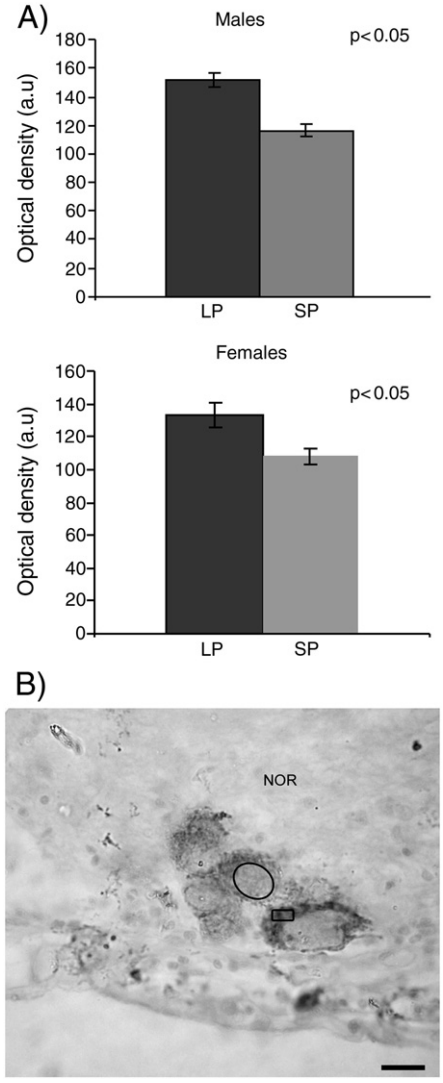

Fig. 5. Optical density of GnRH3 cells in the Nucleus olfacto retinalis (NOR) ($N=6$). A) Long photoperiod exposed males and females (LP) had a major optical density than short photoperiod exposed males and females (SP). ($p<0.05$; Student's t-test). B) Photomicrograph of the stained GnRH3 neurons. Examples of measured nuclear area (circle) and the optical density (square) were indicated. Bar = 10 μm.

mammalian vertebrate species a neuromodulatory role for GnRH3 was demonstrated [27–29]. Particularly, in adult males of *C. dimerus* we hypothesized that a short photoperiod induced an inhibition in male aggression and this occurs with a lower GnRH3 synthesis and release. This assumption is supported by the optical density of staining that reduced in SP males GnRH3 cell bodies. The reduced GnRH optical density in SP fish may be also a negative effect of cortisol on GnRH levels. This assumption is based on the fact that SP fish may be more stressed and have higher cortisol levels because they went from a 2 month LP holding condition into a SP condition. The possibility of an effect because of a photoperiodic change rather than because of photoperiod length should be also considered.

After the dominant male beats other males and establishes a territory it will select a reproductive female. In order to study the effect of photoperiod on this selection a dominant male was placed with a LP and a SP female. The SP female was attacked immediately by the male and then by the LP female in all cases. We hypothesized then that short photoperiods inhibit female reproductive systems, behaviour and probably pheromone release too, increasing the dominant male aggression. When GnRH3 nuclear area and diameter of LP and SP females were analyzed, no significant differences were observed. But,

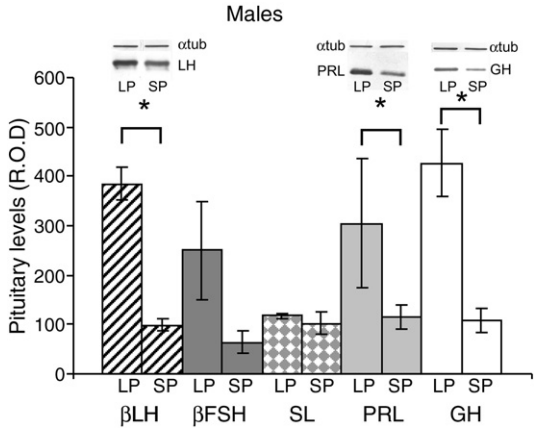

Fig. 6. Semiquantitative analysis of pituitary hormone content from long (14:10) and short (8:16) photoperiod exposed males (LP and SP) ($n=6$). Values are expressed in arbitrary units (a.u.) as means ± SEM and normalized to α-tubuline. ROD = relative optical density. A major pituitary β-LH, PRL and GH content was observed in LP males compared to SP males ($p<0.05$). Representative Western blot image for males PRL, β-LH and GH ir-bands compared to the α-tubuline band. No significant differences were observed in β-FSH and SL pituitary content between LP and SP males.

as in males, LP females showed a higher GnRH3 neuronal optical density of staining. In the dwarf gourami *Colisa lalia* no sexual differences were found in the physiological or morphological characteristics of GnRH neurones, including the expression pattern of different GnRH receptors [30]. By the other hand, in pejerrey *Odontesthes bonariensis* females it was also shown that GnRH1, GnRH2 and GnRH3 increased their expression levels when females were exposed to a long photoperiod, suggesting that all of them may be involved in reproduction [7]. Recently it was clearly demonstrated in the medaka that social cues from conspecifics alter electrical activity of GnRH3 neurons via visual signals [31]. The GnRH3 population in *C. dimerus* formed a tight cluster, so it is difficult to estimate the individual cell area. That is why we only considered nuclear area/diameter and cytoplasmatic optical density of staining as morphometric estimators of GnRH3 cell activity.

Behavioural observations in our experimental design showed that in spite of seeing the female in the tank besides males drastically reduced their locomotor activity remaining quiet in the bottom of the aquarium. This was more noticeable from the second week in short photoperiod when males get a pale body color and a reduction in their food intake. This was reflected by a reduction in the hepatosomatic index of SP males. By the other hand, SP females got a dark skin color, a very active locomotor and feeding activity when compared to LP

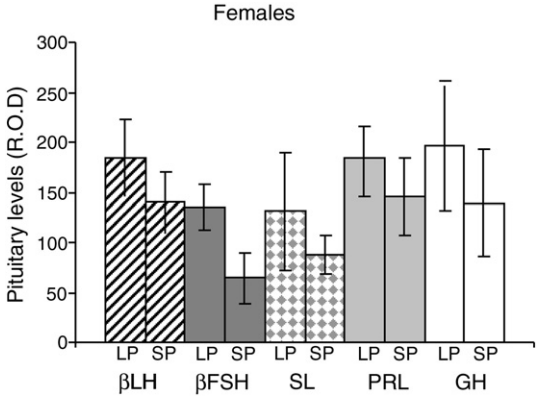

Fig. 7. Semiquantitative analysis of pituitary hormone content from long (14:10) and short (8:16) photoperiod exposed females (LP and SP) ($n=6$). Values are expressed in arbitrary units (a.u.) as means ± SEM and normalized to α-tubuline. ROD = relative optical density. No significant differences were observed in the pituitary content of any of the measured hormones.

ones. No differences were observed between LP and SP animals' spleen and gonadosomatic indexes. There is no data available about gonadal regression in the resting season of this species, but it is probably that low temperatures combined with short photoperiods result in gonadal regression. Nevertheless, in the pejerrey adult female it was clearly demonstrated that the increase in day length but not on temperature, triggers the maturation after the winter period of gonadal rest [7]. Future studies modifying not only photoperiod but also temperature should be performed in *C. dimerus*.

Haematological indexes are important parameters for the evaluation of fish physiological status. Their changes depend on the fish species, age, the cycle of sexual maturity and health condition [32]. These parameters can provide substantial diagnostic information once reference values are established under standardized conditions, which is the case of *C. dimerus* [25]. As several items of our experimental design may alter animals health (ie: isolation, aggressive interactions) several haematological parameters were measured in order to establish if they vary with the different photoperiods and in order to discard not healthy animals for behavioural and physiological analysis. The evaluated haematological parameters were not significantly different in males and females exposed to LP and SP. Hemoglobin concentration in this experiment showed higher values when compared to those previously reported in this species [25]. This could be explained because in this work animals were isolated and in the previous one they were grouped.

Our results clearly demonstrate that *C. dimerus* SP males showed less β-LH pituitary content. Future molecular studies will be performed in order to elucidate changes in pituitary hormone mRNA expression. The use of Western blot for semiquantitative analysis should be viewed with caution if the differences in concentrations are low since the analysis may not be sensitive enough, but this was not the case because the differences in relative optical density of the corresponding ir-band were high in most cases. The higher levels of gene expression of β-GtHs in pejerrey females and threespine stickleback *Gasterosteus aculeatus* kept under long photoperiod are consistent with *C. dimerus* β-GtHs pituitary content in LP and SP [7,33].

It was also shown that in *C. dimerus* LP males had significant higher pituitary levels of PRL (about 2-fold) than SP males. This is consistent with studies in *Oncorhynchus mykiss* where it was demonstrated that melatonin, strongly related to photoperiod, inhibits pituitary PRL secretion [34]. *C. dimerus* SP males had been in the dark for 16 hs/day, and this probably result in a higher release of melatonin that may be reducing pituitary prolactin content when compared to LP males that were just 10 hs/day in the dark. Pituitary PRL gene expression in *Cyprinus carpio* males is deeply influenced by seasonal acclimatization [35]. In the same species, it has been shown that photoperiod constitutes a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription [36]. Recently, it was shown in *Sparus aurata* that PRL gene and protein expressions were increased during spring and autumn [37].

It was also shown in *C. dimerus* that LP males had significant higher pituitary levels of GH (about 3.5-fold) than SP males. In *S. aurata* a decrease in GH gene and protein expression was observed during autumn when the photoperiod is starting to get reduced [37], the highest plasma GH concentration has been found from spring to early summer [38] and the lowest values were observed in winter [39]. This higher GH pituitary content in *C. dimerus* LP males is also related to a higher feeding as was reported in other teleost species [40,41]. In *C. carpio,* GH pituitary gene and protein expressions were affected by seasonal variation, with the greatest increase during summer [42]. In *O. mykiss*, it was shown that melatonin regulates the increase in GH secretion [32].

It is probable that females have some changes in their reproductive axis that were not detected with the used techniques or the selected parameters because LP males were able to detect and select them in spite of that. It may be possible that a visual coloration clue is involved because females showed at least three different body coloration patterns. Prespawning females, postspawning females and females taking care of larvae have different body color patterns. We also could not discard the existence of olfactory clues and the influence of temperature that, in the case of this experiment, was maintained constant indicating that photoperiod is not the sole cue used for seasonal reproductive readiness in this species.

In summary, at the behavioural level we showed that male aggression related to territory selection and its defence is reduced in SP exposed animals and that females exposed to SP are never chosen by males and also received strong aggressive interactions. At the brain level we demonstrated that GnRH3 neuronal optical density of staining is reduced in SP exposed males and females. Finally, at the pituitary level we showed that SP males showed less β-LH, PRL and GH pituitary content when exposed to SP and females in SP showed no significant differences in their pituitary content for any of the five measured hormones. Taken all together these results suggest that in *C. dimerus* the photoperiod is a relevant environmental cue related to reproductive behaviour and physiology. Further studies will be necessary to understand the interaction of temperature and photoperiod on *C. dimerus* reproduction at protein and genomic levels.

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