



Melatonin-mediated effects on killifish reproductive axis

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ABSTRACT

The aim of this study was to investigate the melatonin-mediated effects upon the neuroendocrine axis of the brackish killifish (*Fundulus heteroclitus*), a suitable experimental model to study reproductive events. The ability of melatonin to enhance reproductive capacity (fecundity, embryo survival and hatching rate) inducing the transcriptional activity of gonadotropin releasing hormone (*gnrh*), luteinizing hormone receptor (*lhr*) and melatonin receptor (*mtnr*) was investigated in adult females. Moreover, the melatonin-mediated enhancement of killifish sperm motility and velocity was found consistent with higher fecundity of melatonin-exposed fishes. As a further extent, Fourier Transform Infrared (FT-IR) microspectroscopy evidenced a reduction of lipid unsaturation level on isolated spermatozoa from treated males. Moreover, the reduction of *mtnr* gene expression during embryo development and lower biometric parameters documented in the larvae from melatonin-exposed parents suggest that melatonin acts as a hormonal mediator able to transfer the environmental signal to oocytes and then to embryos as inheritance of adaptive environmental changes. These results support the positive role of melatonin on killifish reproduction and its role as a maternal factor on embryo and larval development.

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1. Introduction

In all vertebrates, the photo-neuro-endocrine structure involved in environmental stimuli perception is the pineal organ and, because photoperiod is the most reliable cue signalling the changing seasons, interpretation and transduction of this message are one of the most essential events in the regulation of reproduction and thus in the progress of life cycle (Maitra and Chatteraj, 2007).

The Kiss system, under the photoperiodic control of the pineal melatonin, acts on the hypothalamus working as central processor and neuroendocrine conduit for conveying the environmental information onto brain reproductive centre, the gonadotropin releasing hormone *gnrh* neurones. Thus, during puberty or during annual recrudescence, the *gnrh*-dependent gonadotropins (*fsh*, follicle stimulating hormone; *lh*, luteinizing hormone) released from the anterior pituitary gland regulate in turn the steroid-signalling pathway involved in the gonadal biosynthesis and metabolism of steroid hormones. The latter, in turn, mediate the physiological processes

that regulate oogenesis and spermatogenesis (Shahab et al., 2005; Filby et al., 2008).

The environmental message by which animals regulate their life cycle is translated into a rhythmic endocrine signal, the pineal indoleamine (N-acetyl-5-methoxytryptamine) melatonin, exclusively secreted at night. This hormone plays a central role in transmitting day-length information to the whole organism, and particularly to the neuroendocrine-gonadal axis (Malpaux et al., 2001), therefore, animals adjust their bio-clock/bio-calendar with their reproductive activities for the best time for spawning (Hofman, 2004; Carnevali et al., 2010) to ensure a better survival of the new progeny.

Melatonin is a highly conserved molecule and its presence can be traced in all evolutionary life forms from the simplest bacterium to the human being. A primitive and primary function of melatonin was to protect against oxidative stress. Later in evolution, melatonin produced by the pineal gland evolved to be a chemical signal of dark/light, to mediate seasonal physiological functions, immunostimulation and other receptor-mediated functions in multicellular organisms, such as body weight and energy balance (Piccinetti et al., 2010; Tan et al., 2010).

In all vertebrates, melatonin exerts its biological effects via two specific G protein-coupled seven transmembrane-spanning domain receptors: *mt1* (*mel1a*) and *mt2* (*mel1b*), all these receptors are expressed both singly and together in various areas of the central

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nervous system and in peripheral organs. An additional form *mt3* (*mel1c*) is expressed only in extrapineal tissues of non-mammalian species, such as the kidney, brain, heart, lung, intestine, muscle, brown adipose tissue and eye (Pandi-Perumal et al., 2008; Ikegami et al., 2009).

The link between photoperiod, and thus melatonin, and the regulation of reproductive functions are still little explored in teleosts (Falcón et al., 2007; Carnevali et al., 2011; Lombardo et al., 2012) as opposed to higher vertebrates (mainly mammals) where clear links between reproduction and melatonin have been reported (Arendt, 1998). However, its molecular mechanisms of action (gonadotropic or anti-gonadotropic) are still not well comprehended and the results differ between species (Falcón et al., 2007; Tamura et al., 2009; Carnevali et al., 2010, 2011). This is because these studies used different experimental procedures (the time of the year at which the experiments were done was crucial), different species or, within a same species, animals of different sex and historical status. However, evidence is now coming to light that indicates melatonin mediates the effects of photoperiod on several neuroendocrine and behavioural functions. Depending on the species (long-day/short day seasonal breeders), melatonin may induce or inhibit the Kiss-HPG-reproductive axis, thus, interruption of the neural pathways anywhere between the suprachiasmatic nuclei and the pineal, turns this gland non-functional in terms of its capacity to alter the reproductive system (Kauffman et al., 2007; Roa et al., 2008; Castellano et al., 2009; Ansel et al., 2010).

Considering this discrepancy in the melatonin effects on vertebrate reproduction and despite the increasing interest in its physiological role in reproduction (Tamura et al., 2009), the aim of the current study was to prove the melatonin-mediated effects upon *Fundulus heteroclitus* reproduction. In this light, the effects of melatonin administration on gametes quality were investigated by reproductive performance and sperm motility parameters assessment. More in detail, the effects of melatonin treatment, at central (brain) and peripheral level (ovary), were analysed taking into account *mtnr* as well as genes codifying for key factors involved in the regulation of gametogenesis and thus in the reproductive axis, such as *gnrh* and *lhr*.

In addition, sperm motility and the antioxidant effects in the sperm of melatonin-exposed fishes were also studied together with the expression of *mtnr* gene during embryo development and larval growth from melatonin-treated parents.

2. Materials and methods

2.1. Animals

The mummichog *F. heteroclitus* (Cyprinodontidae) or killifish is a euryhaline oviparous teleost that in nature displays a semi-lunar spawning cycles synchronized with new and full moon tides. Such a semi-lunar pattern of reproduction is free-running under proper laboratory conditions and can be monitored by daily egg collection or regular ovarian sampling. The fish utilized in this study didn't display a semilunar spawning rhythm. Mature killifish, *F. heteroclitus* L. TL = 70–90 mm were maintained in our facility under controlled condition (24 °C; 14 L/10 D, 30‰) and fed *ad libitum* with commercial diet (Tetramin®, Tetra, Melle, Germany) twice a day. In addition, the spawning tanks were equipped with a vertical plastic screen (5 mm diameter) since these fishes tend to spawn against the screen (Hsiao et al., 1994). Procedures were performed in accordance with the Guidelines on the handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW) and with the Italian animal welfare legislation (D.L. 116/92).

2.2. Melatonin exposure

Two experimental groups were set up with a sex ratio of 3 females:2 males, a control group (CTRL) and a treated group (MEL)

exposed via water, for 8 days, to 1 µM melatonin (Sigma Aldrich Co Milan, Italy) following Zhdanova et al. (2008). The experiment was repeated three times. During the treatment, melatonin was added daily at 11 a.m. and the concentration in the tank water was maintained by renewing water every 24 h in each tank.

2.3. Killifish reproductive performance assay

To evaluate the effects of melatonin on *F. heteroclitus* reproductive activity, daily spawning assessment during the 8 days treatment was performed at 10 a.m. by siphoning out the spawning tank bottom. The fertilized eggs from each experimental group were then deposited on a piece of blotting paper in a 90 mm plastic Petri dish moistened with 30‰ artificial seawater and incubated, at room temperature (24 °C) for 8 days; thus, at the end of the embryo development (8 dpf) embryo survival and number of hatched embryo were assessed.

At the end of the experiment, females were sacrificed with a lethal overdose of anaesthesia [500 mg L⁻¹ MS-222 (3-aminobenzoic acid ethyl ester) buffered to pH 7.4] and the brain and ovary were sampled and kept at -80 °C for future molecular analyses. Sperm samples, obtained by stripping live males, were utilized for sperm motility and FT-IR assays. During embryo incubation period, 6 embryos from each experimental group were sampled at 3 (stage 24: Prominent finbud), 6 (stage 29: circulation in pectoral fin), 8 (stage 31: formation of swim bladder) dpf and at 0 (stage 32: hatching) dph (day post-hatching) (Oppenheimer, 1937) for future molecular analyses.

2.4. Killifish progeny biometric parameters assessment

To assess the potential effects of melatonin treatment on the progeny of treated broodstocks, 50 ± 2 larvae (9 replicates each), hatched from CTRL and MEL groups were reared in 5 L tanks for a period of 30 days. Both groups were maintained in the same rearing condition as the broodstocks and fed with *Artemia salina* nauplii (at 9 a.m.) and commercial dry diet (Tetramin®, Tetra, Melle, Germany) (at 5 p.m.). Larval survival was monitored during the experiment and larval biometrical parameters, such as BW (mg) and TL (mm), were recorded during sampling at 0 (start), 7, 14 and 21 (end) dph. At each sampling, 10 larvae from each experimental group were captured by netting and sacrificed by a lethal overdose of anaesthesia [200 mg L⁻¹ MS-222 (3-aminobenzoic acid ethyl ester) buffered to pH 7.4], dried on blotting paper and weighed using an analytical balance accurate to 0.1 mg (OHAUS Explorer E11140). Subsequently, larvae were measured in length using a sliding micrometer microscope (STEMI 2000).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from embryos *in toto* and from the adult brain and ovary samples with RNeasy Mini Kit (Qiagen) and eluted in 15 µL of RNase-free water. Final RNA concentrations were determined by a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. Total RNA was treated with DNase (10 UI at 37 °C for 10 min, MBI Fermentas), and a total amount of 1 µg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad).

2.6. Real time PCR

Specific amplification primers were designed using PCR designer software PRIMER3 (<http://frodo.wi.mit.edu/primer3/>). The sequences of primers used at a final concentration of 200 nM were reported in Table 1. Triplicate PCR reactions were carried out for each sample analysed. After real-time condition optimization, PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad).

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