

Medaka as a model for seasonal plasticity: Photoperiod-mediated changes in behaviour, cognition, and hormones

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ARTICLE INFO

Keywords:

Fish brain
Fish models
Phenotypic plasticity
Seasonality
Stress research

ABSTRACT

Teleosts display the highest level of brain plasticity of all vertebrates. Yet we still know little about how seasonality affects fish behaviour and the underlying cognitive mechanisms since the common neurobehavioral fish models are native to tropical environments where seasonal variation is absent or reduced. The medaka, *Oryzias latipes*, which inhabits temperate zone habitats, represents a promising model in this context given its large phenotypic changes associated with seasonality and the possibility to induce seasonal plasticity by only manipulating photoperiod. Here, we report the first extended investigation of seasonal plasticity in medaka behaviour and cognition, as well as the potential underlying molecular mechanisms. We compared medaka exposed to summer photoperiod (16 h light:8 h dark) with medaka exposed to winter photoperiod (8 h light:16 h dark), and detected substantial differences. Medaka were more active and less social in summer photoperiod conditions, two effects that emerged in the second half of an open-field and a sociability test, respectively, and might be at least in part related to habituation to the testing apparatus. Moreover, the cognitive phenotype was significantly affected: in the early response to a social stimulus, brain functional lateralisation shifted between the two hemispheres under the two photoperiod conditions, and inhibitory and discrimination learning performance were reduced in summer conditions. Finally, the expression of genes encoding key pituitary hormones, *tsh β* and *gh*, and of the *tsh β* regulatory transcription factor *tef* in the brain was increased in summer photoperiod conditions. This work reveals remarkable behavioural and cognitive phenotypic plasticity in response to photoperiod in medaka, and suggests a potential regulatory role for the same hormones involved in seasonal plasticity of other vertebrates.

1. Introduction

In the last decade, teleosts have gained importance as models for research on the brain, behaviour, and cognition, from both mechanistic and evolutionary perspectives (e.g., Bshary and Brown, 2014; Kotrschal et al., 2013; Stewart et al., 2014). A field in which fish models have made notable contributions is neurobehavioral plasticity research. The fish brain displays extensive plasticity in response to environmental factors (reviewed in Ebbesson and Braithwaite, 2012) and in contrast to the mammalian brain, its neurogenesis extends throughout life (Zupanc, 2006). Not surprisingly, plasticity in fish behaviour and cognitive function has been described for a range of factors such as predation risk (Herczeg et al., 2016; Lucon-Xiccato et al., 2020a; Thomson et al., 2012), food availability (Kotrschal and Taborsky, 2010; Thomson et al.,

2012), social environment (Herczeg et al., 2016; Lucon-Xiccato et al., 2022), water flow (Bhat et al., 2015), and lighting conditions (Chapman et al., 2009; Ehlman et al., 2015). Such plasticity may have contributed to the success of this group, which includes more than half of the extant vertebrate species (Ravi and Venkatesh, 2018).

Despite the widespread evidence for enhanced plasticity, behavioural and cognitive changes due to seasonality are poorly understood in fish. Only a few observations in the field of aquaculture and a few laboratory experiments have suggested that behaviour such as activity and foraging (Lucas and Batley, 1996; Smith et al., 1993) and cognitive abilities such as spatial learning (Carbia and Brown, 2020) may vary across seasons. A potential reason for this gap in knowledge is that the fish species typically exploited as research models might not be appropriate to identify seasonal plasticity in behaviour and cognition. For

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example, the zebrafish, *Danio rerio*, the guppy, *Poecilia reticulata*, and African cichlids, which are among the most widely used models, are native to tropical habitats with very limited annual fluctuations in environmental conditions. Accordingly, they are not expected to display phenotypic plasticity in relation to seasons.

The medaka, *Oryzias latipes*, is particularly well suited for the study of seasonal plasticity. This fish species has recently gained importance as an animal model in several research fields, mostly due to the combination of powerful genetic tools it offers for the in-depth investigation of a range of biological mechanisms (Lin et al., 2016; Takeda and Shimada, 2010; Wittbrodt et al., 2002). The medaka is native to Eastern Asia, a temperate zone in which environmental conditions change significantly across the year. For example, the temperature to which medaka are exposed can vary between 4 and 40 °C (Hilgers and Schwarzer, 2019). Throughout this seasonal variation, medaka undergo distinct phenotypic changes related to the fact that reproduction occurs only during summer. For example, gonad maturation is triggered by exposure to summer conditions (Awaji and Hanyu, 1988; Koger et al., 1999). For the purpose of studying seasonal plasticity in fish, the medaka offers a further advantage. Several studies have indicated that the behavioural and physiological changes which occur between seasons can be triggered by simply manipulating the duration of the photoperiod (Awaji and Hanyu, 1989; Fujisawa et al., 2021; Ueda and Oishi, 1982; Urasaki, 1976). This marked response to photoperiod allows researchers to induce seasonal plasticity without the confounding effects of changes in temperature and food availability on metabolism and other physiological functions. Photoperiod-mediated plasticity of medaka behaviour and cognition may therefore provide an invaluable tool to study seasonal plasticity of vertebrates and its underlying mechanisms.

In this study, we have investigated behavioural, cognitive, and hormonal plasticity in response to photoperiod manipulation in the medaka. Subjects were exposed to one of two photoperiod treatments that mimic conditions experienced by medaka in its distribution range during summer (16 h light and 8 h dark) and winter (8 h light and 16 h dark). Several studies adopted this experimental design to examine physiological responses such as ovarian maturation, hepatic metabolism, and growth (Awaji and Hanyu, 1989; Davis et al., 2002; Fujisawa et al., 2021; Urasaki, 1976). The two photoperiods investigated have opposed biological meaning because medaka mate only in summer and reduce reproductive traits in winter (Awaji and Hanyu, 1988; Koger et al., 1999). After the photoperiod treatments, we subjected medaka to a range of cognitive/behavioural and molecular analysis.

For the behavioural analysis, we initially focused on novel environment responses using an open-field test, which is considered by most researchers as a reliable measure of behavioural traits such as activity, exploration, anxiety, and boldness in fish (Burns, 2008; Lucon-Xiccato et al., 2020b; Toms et al., 2010; Szaszkievicz et al., 2021). We then assayed sociability using a shoaling apparatus, testing the preference of subjects to swim close to a shoal of conspecifics (Cattelan et al., 2019; Mehlis et al., 2015). Regarding cognition, we first explored the effects on brain functional lateralisation, the tendency showed by animals to process information differentially between the two brain hemispheres (Rogers and Andrew, 2002). Cognitive lateralisation often results in asymmetrical responses to stimuli, and we assayed the medaka in their lateralised response to a visual social stimulus (Lucon-Xiccato et al., 2020d). We then studied medaka's inhibitory control, i.e. the cognitive function that allows withholding of behaviour, using a protocol in which the fish had to inhibit their tendency to feed on unreachable prey (Lucon-Xiccato et al., 2020c; Montalbano et al., 2020). Finally, we conducted two learning tests based on food conditioning: a colour discrimination learning task (Lucon-Xiccato et al., 2019; Roy et al., 2019) and a shape discrimination learning task (Lucon-Xiccato et al., 2019). We expected to detect increased activity in the medaka from the summer photoperiod in the open-field test (Yokota and Oishi, 1992). Given the absence of established results on seasonal plasticity of the remaining behavioural and cognitive traits in teleost fish, we adopted an

explorative approach.

Using qRT-PCR, we then measured the mRNA expression in brain extracts of two key pituitary hormones, *tsh β* (thyroid stimulating hormone β subunit) and *gh* (growth hormone), as well as the transcription factor *tef* (thyrotroph embryonic factor), which is a key regulator of *tsh β* gene transcription. Several studies in fish have shown that thyroid axis components, including TSH subunits, respond to environmental stimuli and that their levels change seasonally (Cowan et al., 2017; Cole and Volkoff, 2020). Furthermore, *tsh β* and *tef* are involved in the photoperiod sensing mechanism in mammals (Dardente et al., 2010). In fish, GH levels also change with seasons and are correlated with feeding behaviour (Björnsson, 1997; Jørgensen and Johnsen, 2014). Based on these comparative data, we predicted that we would observe higher levels of expression of the genes tested in the fish from the summer photoperiod treatment compared to those of the winter photoperiod treatment.

2. Materials and methods

2.1. Experimental fish

For all our experiments, we used a wild-type strain of medaka (isogenic inbred 'iCab' strain) bred in the laboratory at the University of Ferrara. The iCab strain is derived from the Southern Japanese population and is a medaka inbred strain commonly used for basic research (Furutani-Seiki et al., 2004; Loosli et al., 2000; López-Olmeda et al., 2021; Spivakov et al., 2014). All the subjects were adults at the time of testing (6–8 months). Before experiments, medaka were maintained under standard laboratory conditions: 200 L aquaria with air pumps and biological filters; temperature 28 ± 1 °C; feeding twice per day with live *Artemia salina* nauplii and dry food. The subjects were randomly selected among the stock population.

2.2. Photoperiod conditioning

To study the effect of seasonality on medaka, we exposed subjects to two treatments with different photoperiods (Awaji and Hanyu, 1989; Fujisawa et al., 2021; Ueda and Oishi, 1982; Urasaki, 1976). Half of the medaka were exposed to a photoperiod consisting of 8 h of dark and 16 h of light (summer photoperiod) and half of the medaka were exposed to a 16 h of dark and 8 h of light period (winter photoperiod). These photoperiods were administered to groups of 10 subjects maintained in 100 L aquaria with biological filters. We exposed 6 groups of fish to the summer photoperiod ($N = 60$ medaka) and 6 groups of fish to the winter photoperiod ($N = 60$ medaka). Three groups per condition were used in the analysis of behaviour, and the remaining groups were used in the analysis of cognition and gene expression. We isolated the aquaria from the external environment by covering them with black plastic panels. We also placed a black plastic lid over each aquarium, containing a white light-emitting diodes (LED) strip (Superlight Technology Co. Ltd., Shenzhen, China) for illumination. The light was turned on between 6 a. m. and 10 p.m. for the summer photoperiod and from 10 a.m. to 6 p.m. for the winter photoperiod. Medaka were fed twice per day as under maintenance conditions. Each week, the aquaria were cleaned and half of the water was exchanged with fresh aquarium water. The photoperiod conditioning lasted for 30 days to ensure full acclimatization to the respective photoperiods (Koger et al., 1999; López-Olmeda et al., 2021). After this period, the fish were tested in the following assays.

2.3. Analysis of behaviour

2.3.1. Behavioural test 1: novel environment response

In this test, we measured the behaviour of the subjects when exposed to a novel, unfamiliar environment (open field). We tested 20 medaka from the summer photoperiod treatment and 20 medaka from the winter photoperiod treatment in the open-field test. After collection from the treatment aquaria, each subject was introduced into a test tank with

white walls (40 × 40 cm, 15 cm height) filled with 8 cm of water (Fig. 1a). The experimental tank was illuminated by a white LED strip and the temperature of the water was kept constant (28 ± 1 °C). The subject was then left undisturbed for 30 min. To allow recording of subjects' behaviour, the experimental tank was set on a backlit IR LED table (λ > 980 nm). An infrared sensitive webcam (Monochrome GigE camera, Basler, Germany; resolution: 1280 × 1024) was placed 1 m above the tank to record the experiment. Finally, a computer running Ethovision XT software (Noldus Information Technology, The Netherlands) was connected with the camera to calculate the dependent variables used in the analysis. As measure of activity, the software recorded distance moved and time spent moving at least 1 body length per second. Additionally, the software recorded a measure of anxiety called thigmotaxis and computed as time spent within 1 body length from the edge of the arena (Fig. 1a).

A potential confounding factor for the application of the open-field test, and more generally for tests that are based on habituation to novelty, is that the behaviour of most teleost fish studied is not constant over the course of the testing period (Bell and Peeke, 2012; Bruzzone et al., 2020; Kotrschal et al., 2014; Lucon-Xiccato et al., 2014; Pazmino et al., 2020; Stewart et al., 2012; Wong et al., 2010). This temporal variability has also been reported for medaka (Lucon-Xiccato et al., 2020b; Matsunaga and Watanabe, 2010). For example, we previously demonstrated that medaka assayed with the open-field test exhibit a reduction in activity between 7 and 8 min after introduction into the arena (Lucon-Xiccato et al., 2020b). The main explanation for this effect is that due to habituation, the novel environment becomes progressively familiar for the fish as the test continues. This change in the perception of the environment in turn alters fish behaviour (Bell and Peeke, 2012; Matsunaga and Watanabe, 2010). Not considering this temporal variation when comparing experimental groups is problematic because it might prevent to detect a behavioural difference. For example, Lucon-Xiccato and Dadda (2016) compared male and female guppies, *Poecilia reticulata*, in a test measuring the response to a novel object. On average, the fish apparently did not explore the object and there was no difference between the two sexes in the time spent close to the stimulus. However, when the temporal dimension was included in the analyses, it was found that fish actually explored the object in specific periods of the test. Males approached the stimulus at the beginning of the test and then reduced this exploration behaviour while females initially avoided the stimulus and only in the second half of the test, did they approach it. It was therefore important to consider the contribution of differences in temporal variation in behaviour in our study. The precise time window of the behavioural changes is difficult to predict a priori because it might vary according to several factors such as the age, sex, experience, and genotype of the subjects (Lucon-Xiccato and Dadda, 2016; Lucon-Xiccato et al., 2020b; O'Neill et al., 2018). Considering that frequently, differences were observed between the first and the second halves of the experiment, we clustered all the variables collected in the open-field test into two phases, corresponding to the first half of the experiment (minutes 1–10) and the second half of the experiment (minutes 11–20). Analysis of the dependent variable collected with this repeated measures structure allowed us to consider treatment effects that were dependent on experimental time.

2.3.2. Behavioural test 2: sociability

In the sociability test, we presented the subjects with the choice to join a group of conspecifics or to stay alone, measuring their preference for the two options. The experimental tank was 20 × 60 cm (20 cm height) and filled with 10 cm of water (Fig. 1b) at 28 ± 1 °C. It was divided into 3 sectors: a central sector (30 cm), hosting the subject medaka, and two lateral sectors (15 cm each) to present the experimental choices. One lateral sector hosted a group of 5 stimulus medaka, collected from standard maintenance tanks maintained in an intermediate photoperiod condition (12 h:12 h). The second lateral sector was left empty, with no stimulus. Above each stimulus sector, we installed a

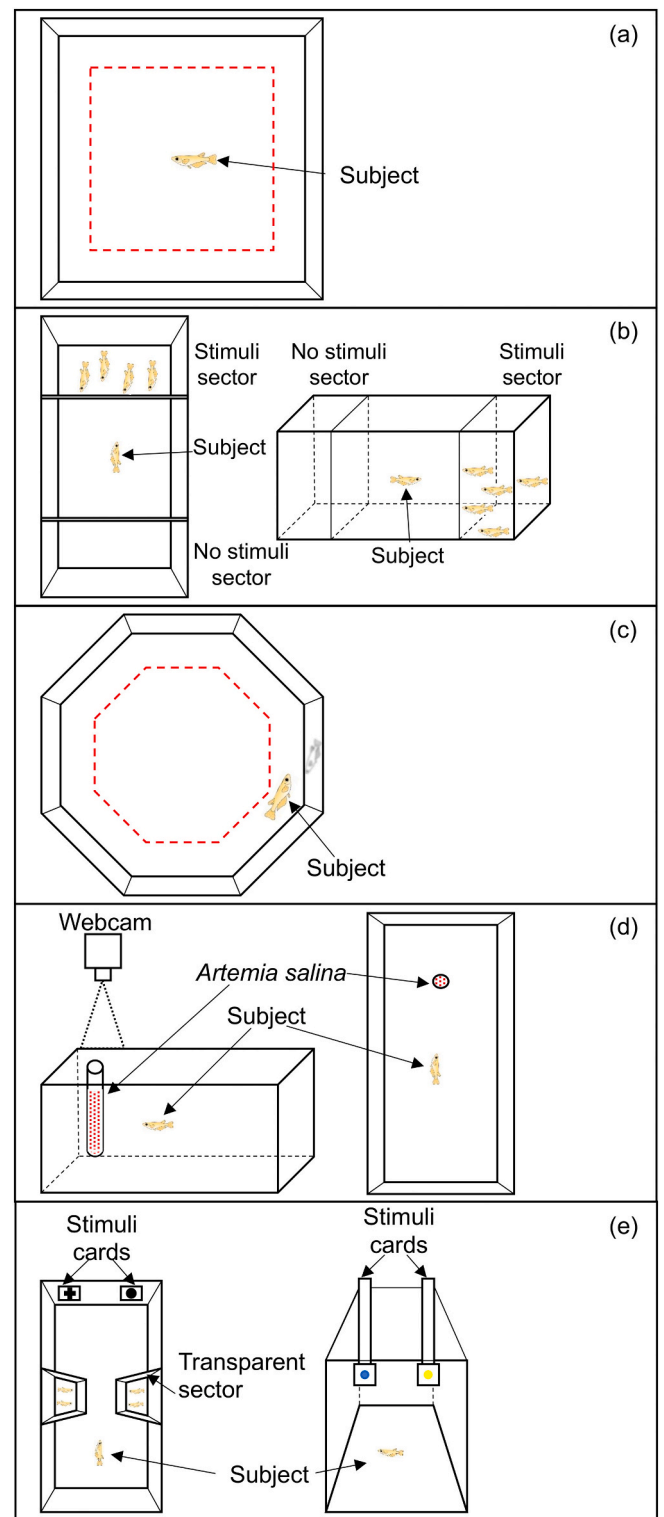


Fig. 1. Diagrams of our experimental set ups. (a) Open-field arena used to assess novel environment response; the dotted line represents the edge used to assess thigmotaxis. (b) Top (left) and side (right) view of the shoaling apparatus used to assess sociability. (c) Mirror apparatus used to assess brain functional lateralisation; the dotted line indicates the area in which subjects was considered to observe the mirror image of the apparatus used to assess inhibitory control. (d) Top (left) and side (right) view of the apparatus used in the colour and the shape discrimination learning experiments.

pair of fluorescent lamps. The experimental procedure consisted of releasing an experimental subject (collected from the treatment aquaria) into the centre of the shoaling apparatus, and then leaving it there undisturbed, free to interact with its conspecifics. We recorded the experiment for 20 min with a camera placed above the apparatus. To measure the behavioural variables, we performed an offline manual analysis. An experimenter played back the recordings on a computer and by using the software BORIS (<http://www.boris.unito.it>) and calculated the time spent by the subjects within 5 cm from each of the stimulus sectors. As reported for the open-field test, previous studies indicate that the behaviour of the subjects in the sociability test might vary over the course of the experiment (Dadda et al., 2007; Lucon-Xiccato et al., 2017; Miller and Gerlai, 2007). This is again attributable to the effect of habituation: once introduced into the testing apparatus, the social response of the fish is usually stronger because the environment is unfamiliar and they try to find protection in the context of their social group (Hager and Helfman, 1991); as the fish progressively become familiar with the testing apparatus, this social attraction is expected to decrease. Critically, temporal variability in social response has been shown to influence the conclusions of such studies (Lucon-Xiccato et al., 2017), requiring us to consider it in our experiment. We therefore assayed the sociability variable during two experimental phases (first and second half of the experiment), consistent with the design of our open-field test. In the experiment, we tested 20 medaka from the summer photoperiod treatment and 18 medaka from the winter photoperiod treatment.

2.4. Analysis of cognition

2.4.1. Cognitive test 1: brain functional lateralisation

To assess brain lateralisation, we studied asymmetric responses of individual fish to being confronted with a social stimulus. The lateralisation test took place in an octagonal tank with mirror walls, exploiting the fish's social response to their mirror image (Fig. 1c). Right hemisphere processing of the social visual information would cause the fish to observe the mirror image with the left eye and consequently to swim in a clockwise direction while trying to approach the mirror image. The opposite swimming direction would indicate left hemisphere processing of the stimulus. The mirrors of the apparatus were 17×15 cm each and were illuminated with a white LED strip placed 5 cm above. The apparatus contained 8 cm of water at 28 ± 1 °C. The experiment of each subject was recorded for 20 min with the Noldus set up described above. After testing, an experimenter scored the recordings to quantify the amount of time spent by each subject swimming in clockwise or anticlockwise directions within 1 body length of the mirrors using the software BORIS. As our lateralisation test is based on a social response, the behaviour of the fish was expected to vary over time as explained for the sociability test. This variation in lateralisation has already been reported in other fish species (Dadda et al., 2010a, 2010b). To ensure that this potential temporal change in lateralisation was considered, we collected the data in the first (minutes 1–10) and the second (minutes 11–20) phase of the experiment separately, obtaining a data structure analogous to that of our behavioural tests. This data was used to calculate a lateralisation index for each phase of the experiment using the following formula: $(\text{time spent swimming clockwise} - \text{time spent swimming anticlockwise}) / (\text{time spent swimming clockwise} + \text{time spent swimming anticlockwise})$. The lateralisation index ranged between -1 to $+1$, with negative values indicating preference for left hemisphere information processing and positive values indicating preference for right hemisphere information processing. The sample size was 16 medaka of the summer photoperiod treatment and 14 medaka of the winter photoperiod treatment.

2.4.2. Cognitive test 2: inhibitory control

In this test, we measured the ability of the subjects to withhold a particular behavioural response, namely their active feeding behaviour,

in the event that they are not able to access their food. The inhibitory control experiment was performed in plastic tanks (33×13 cm, 15 cm height; Fig. 1d) filled with 4 L of water kept at 28 ± 1 °C. The experimental tanks were provided with a plastic lid with a hole close to one of the short walls to permit feeding. White LED strips placed on the ceiling provided lighting with the treatment photoperiod of each subject. A back sector of the apparatus (5 cm) contained immature conspecifics as social companions.

Overall, the inhibitory control experiment lasted 4 days. The initial 3 days were part of a training phase; the inhibitory control testing took place on the fourth day. During the training phase, we fed the fish with dry food mixed with water for 3 days. The number of feeding events was increased across the 3 days: 2, 4 and 6 times per day, respectively. The food was delivered by means of a Pasteur pipette inserted into the hole of the lid. This protocol served to habituate the fish to receive food in correspondence with a specific area of the tank (i.e., under the hole in the lid).

After the training phase, the medaka underwent the testing phase. This consisted of a single trial that lasted 20 min. Instead of delivering food, we inserted a standard laboratory glass tube via the hole in the lid, with the tube suspended in the middle of the water column. Inside the tube, we placed 4 mL of a suspension of *Artemia salina* nauplii in water, which served as a prey stimulus for the medaka. With this procedure, subjects usually try to catch the prey inside the tube without success and slowly decrease their attack behaviour because the prey is not accessible. We recorded the behaviour of medaka with a webcam set 50 cm above the experimental tanks. The experimenter then played back the recording with reduced speed and counted the number of attacks directed towards the stimulus. Previous studies with this paradigm detected a substantial change in the number of attacks over the course of the testing time that indicated inhibitory control (Lucon-Xiccato et al., 2022; Montalbano et al., 2020). To consider this potential effect, which might differ between the experimental groups (e.g. Lucon-Xiccato et al., 2022), we collected these inhibitory control data in the same two experimental phases described for our previous tests (minutes 1–10 and 11–20 of testing). The sample size for this test was as follows: $N = 21$ medaka from the summer photoperiod treatment; $N = 18$ medaka from the winter photoperiod treatment.

2.4.3. Cognitive test 3: colour discrimination learning

In this experiment, we trained the fish to select a stimulus of a pre-determined colour to obtain a food reward. We performed the task in glass tanks (25×40 cm, 25 cm height) filled with 20 cm of water (temperature: 28 ± 1 °C) and enriched with gravel bottoms (Fig. 1e). Each tank hosted an individual subject for the entire experiment. The tank was provided with two sectors in the centre of the long walls. They were made with transparent plastic (10×5 cm) and hosted two immature conspecifics as social companions. Due to these small sectors, the central sector of the tank was shaped like an hourglass, with a narrow central corridor and two main sectors in correspondence with the short walls of the tank. The walls of the tank were covered with green plastic. The apparatus was illuminated by white LED strips in order to expose the tested fish to a light cycle corresponding to their adapted photoperiod treatment (winter or summer). The stimuli to be discriminated were a yellow spot and a blue spot (\varnothing 1.6 cm). They were made of plastic and glued onto a 4×4 cm white plastic card, which provided a homogenous background. The card was presented to the subjects by means of a transparent stick, which could be fixed to the short walls of the tanks to suspend the stimulus in the middle of the water column. Prior to the beginning of the experiment, we assigned a rewarded colour stimulus for each individual. Half of the subjects of each photoperiod treatment were assigned to the yellow stimulus and the other half to the blue stimulus.

The task procedure was composed of two consecutive phases: a training phase and a learning phase. The training phase lasted 2 days and consisted of a series of independent trials. On day 1 of the training

phase, we performed 8 trials. In each trial of day 1 of the training phase, we inserted the rewarded colour stimulus into the tank, against one of the short walls of the tank. When the medaka approached the stimulus, we gently released a food reward close to the card with the help of a Pasteur pipette. The food reward was a suspension of *A. salina* nauplii in water. The short wall of the tank in which we presented the stimulus was alternated across the trials. On day 2 of the training phase, we performed 12 trials. In each trial on day 2, we introduced both stimuli (rewarded and unrewarded colour), at the two corners of the short wall of the tank. We administered food to the medaka only when they approached the rewarded colour.

After the 2 days training phase, the subjects underwent the testing phase. Only data from this testing phase were used to assess the learning performance. The testing phase was composed of a non-fixed number of days, which was determined based on the performance of each subject (i.e. upon achieving a learning criterion; see below). In each day of the testing phase, we administered 12 trials: in each trial, we presented both stimuli to the subject and we provided the food reward only if the subject approached the correct colour stimulus at first. If the subjects approached the incorrect stimulus at first, we removed the cards and did not deliver the reward food. As in previous studies on fish (Baratti et al., 2021; Montalbano et al., 2022), we imposed a learning criterion consisting of 17 correct choices out of 24 over 2 consecutive days. This corresponded to a choice accuracy of 70 %, which is statistically significant ($\chi^2_1 = 4.167$, $P = 0.041$). When a subject achieved the learning criterion, the testing phase was terminated. The sample size for the colour discrimination learning task was 11 medaka of the summer photoperiod treatment and 12 medaka of the winter photoperiod treatment.

2.4.4. Cognitive test 4: shape discrimination learning

Since we found an interactive effect of the colour assigned as rewarded in the colour discrimination test, we ran a second learning experiment that did not involve colour stimuli, i.e., shape discrimination. The protocol and the apparatus that we used for the shape discrimination learning task was the same as in colour discrimination learning, but using a different stimulus card. We used white cards with a black shape on the centre: either a cross (1.5×1.5 cm) or a circle ($\varnothing 1.5$ cm). We tested 22 medaka, $N = 11$ per each photoperiod treatment. We used the same criterion to define successful learning of the task: namely, 17 correct choices out of 24 over 2 consecutive days.

2.5. Analysis of brain gene expression

Gene expression was measured in 10 medaka brains sampled from each experimental group. Fish were always sampled 3 h after lights on. After euthanasia, the whole brain of each subject was dissected under a stereomicroscope. We isolated total RNA using TRIzol™ reagent (ThermoFisher Scientific, Italy) following the manufacturer's instructions. We analysed the amount, quality, and composition of isolated RNA using BioSpec nano (Shimadzu Italia S.r.l., Italy). We then used DNase-treated RNA to perform cDNA synthesis in a final volume of 20 μ L using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories S.r.l., Italy). cDNA was PCR-amplified with the CFX Connect Real-Time PCR System using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories S.r.l., Italy). The thermal cycling conditions were as follows: 30 s of denaturation at 95 °C, followed by 40 cycles of a 15-s denaturation step at 95 °C and then by an annealing-elongation step for 30 s at 60 °C. After amplification, we performed a melting curve analysis to confirm amplicon specificity. All samples were analysed in triplicate. Gene-specific primers are indicated in Table 1. Finally, we calculated the relative expression levels of each sample by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *S18b* as a housekeeping gene (Cuesta et al., 2014).

Table 1
Primer sequences used in the RNA amplification.

Medaka gene	Primer sequence
<i>tshb</i> ; thyroid stimulating hormone, beta subunit	F-catccatattccaggccagag R-gcacaataggccaccaagt
<i>gh1</i> ; growth hormone1	F-ggaacaacgtcaactcagca R-tttgggtgagatttggttcc
<i>tefa</i> ; thyrotroph embryonic factor a	F-tgggacaaaaccattccta R-catcaggctactggagaca
<i>S18b</i> ; mrps18b; mitochondrial ribosomal protein S18B	F-tccccgagaaattccagcat R-ctctccgttagctctccag

2.6. Statistical analysis

Data were analysed using R statistical analysis software. All the statistical tests were two-tailed because of the lack of specific predictions in some of the experiments. We selected different models for each experiment in order to fit the structure and the distribution of the dependent variable.

2.6.1. Behavioural tests

Considering the need to account for the well-documented temporal variability in behaviour in our tests (e.g. Kotschal et al., 2014; Lucon-Xiccato et al., 2017), we collected two repeated behavioural measurements for each subject (in the first and second half of the test, respectively). This data structure prevented us from directly comparing the two treatments: an analysis of correlated samples without taking into account the correlation when computing the dispersion parameters would indeed represent pseudoreplication (Lazic, 2010). We therefore used models that included samples correlation. In particular, we fitted linear mixed-effects models (LMMs; *lmer* R function) with subject ID as random effect to deal with the repeated measurements. As independent variables, we fitted the photoperiod treatment (summer versus winter photoperiod) and the experimental phase (i.e. first half versus second half of the test). In these models, we expected to detect a significant effect of experiment phase as evidence of the predicted behavioural change over time. The impact of the photoperiod treatment was expected to result in either a main effect of treatment or an interaction effect between treatment and experimental phase, which would indicate behavioural differences in a specific phase of the experiment. For this latter situation, we additionally ran post-hoc *t*-tests by analysing the two experimental phases separately, thereby allowing us to determine in which experimental phase the effect emerged.

2.6.2. Cognitive tests

In the lateralisation experiment, the dependent variable was similar in structure to those of the behavioural tests (two measurements in the two experimental phases). Therefore, we applied the analysis previously described (i.e. LMM with treatment and experimental phase as fixed effects, and subject ID as random effect).

For the inhibitory control experiment, the dependent variable was a count of events with repeated measure structure (i.e. one data point per each half of the experiment) to account for the temporal change typically observed in this test (Montalbano et al., 2020). We therefore applied a generalised linear mixed-effects model (GLMM) with Poisson error structure. Besides the effect of photoperiod treatment, the model was fitted with the experimental phase and the subject ID (random effect) to handle the repeated measures. To account for the data distribution in the post-hoc test of this experiment, we used a general linear model with Poisson error structure.

For the two learning tests (colour and shape discrimination), the dependent variable was a single datapoint per subject (e.g. number of days to criterion in the learning experiments). Therefore, we used ANOVA fitted with photoperiod treatment and stimulus assigned as rewarded in the learning experiments as fixed effects.

2.6.3. Brain gene expression

Data of the gene expression analysis consisted of a single observation for the same subject. We analysed these data with two samples *t*-test to compare the means of the subjects from the two photoperiod treatments. Data were log-transformed data before running the analysis because of a right-skewed distribution.

2.7. Ethical note

Animal husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and National animal welfare standards (Tierschutzgesetz §11, Abs. 1, Nr. 1, AZ35-9185.64/BH, AZ 35-9185/G-179/13 for Germany; D.lgs. 26/2014, license 18/2017-TU, exp. procedures CB/01-2019 for Italy).

3. Results

3.1. Behavioural test 1: novel environment response

For the first parameter of activity recorded (distance moved during the open-field test), we found a significant effect of photoperiod

treatment modulated by experimental phase (LMM: $X_1^2 = 4.983$, $P = 0.026$). The effect of photoperiodic conditions was apparently related to the second half of the test, when medaka exposed to the summer photoperiod showed greater activity (Fig. 2a). However, evidence from the post-hoc tests was not significant (first half: $t_{38} = 0.712$, $P = 0.481$; second half: $t_{38} = 1.267$, $P = 0.213$). In the initial LMM, the main effect of experimental phase was also significant ($X_1^2 = 7.561$, $P = 0.006$), but the main effect of the photoperiodic condition was not significant ($X_1^2 = 0.116$, $P = 0.734$).

The second parameter of activity analysed, the time spent moving, revealed a pattern similar to that of distance moved. The LLM found a significant interaction between treatment and experimental phase ($X_1^2 = 6.022$, $P = 0.014$), a significant main effect of experimental phase ($X_1^2 = 5.177$, $P = 0.023$), and no significant main effect of treatment ($X_1^2 = 0.671$, $P = 0.413$). The post-hoc analysis to understand the significant interaction revealed that the two groups had similar activity in the first phase of the experiment ($t_{38} = 0.357$, $P = 0.723$); however, in the second phase of the experiment, the medaka from the winter photoperiod tended to spend less time moving ($t_{38} = 1.730$, $P = 0.092$; Fig. 2b).

For the parameter “time spent along the edges of the open-field arena” (thigmotaxis), we found no significant difference between the medaka exposed to the two different photoperiodic conditions (LMM:

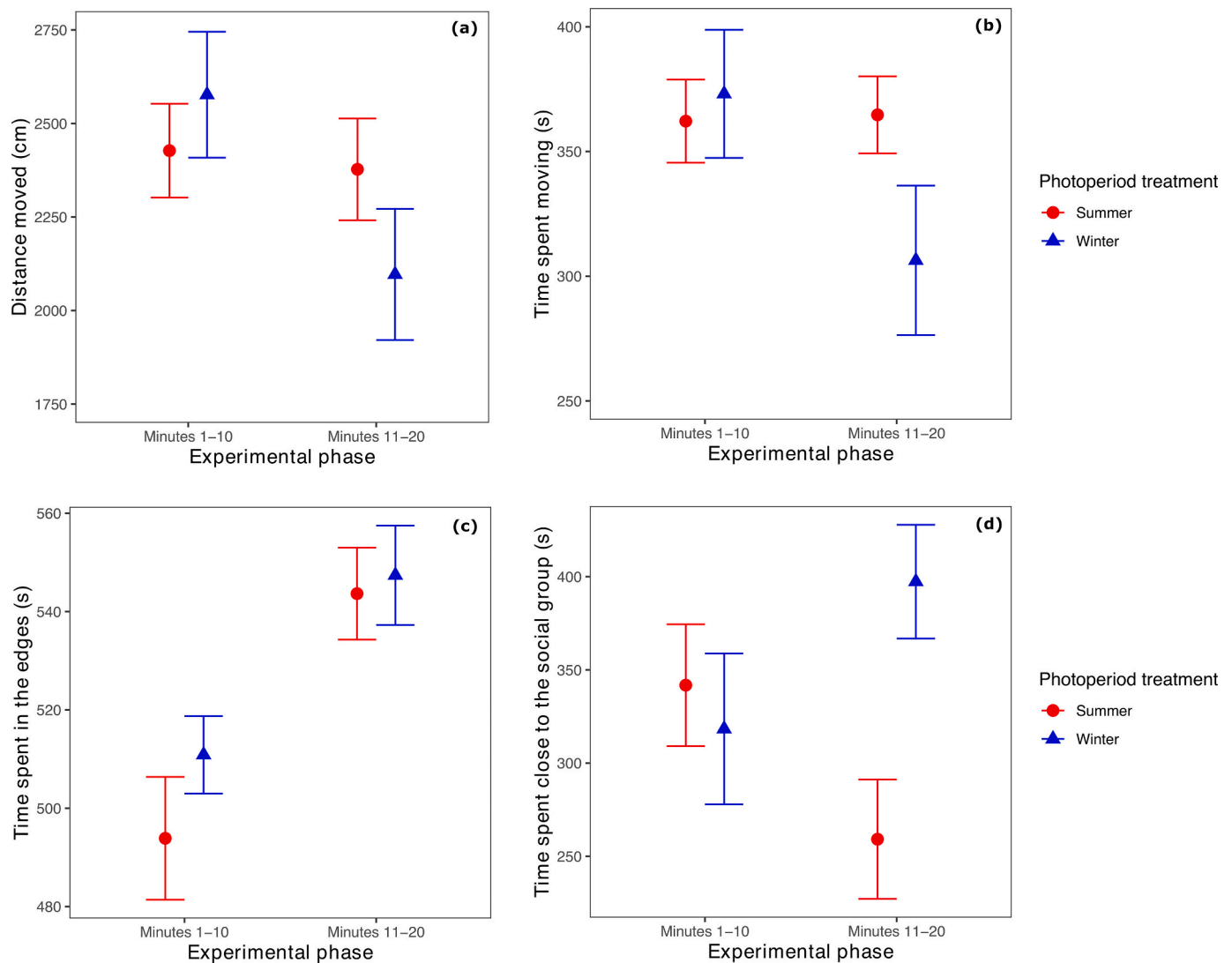


Fig. 2. Results of behavioural tests 1 and 2. (a) Distance moved in the open-field arena. (b) Time spent moving with 1 body length per second as threshold. (c) Time spent at the edges of the open-field arena. (d) Time spent close to the social group in the sociability test. Data points represent means and error bars represent standard errors.

treatment: $X_1^2 = 0.640$, $P = 0.424$; interaction treatment by experimental phase: $X_1^2 = 1.225$, $P = 0.268$; Fig. 2c). The main effect of experimental phase was significant ($X_1^2 = 51.994$, $P < 0.001$).

3.2. Behavioural test 2: sociability

Considering the entire testing time of the sociability test, the model of the time spent close to the conspecific shoal indicated that treatment marginally affected sociability (LMM, main effect of treatment: $X_1^2 = 2.724$, $P = 0.095$). The model term including the temporal variation (treatment by experimental phase interaction) revealed that the photoperiodic condition had a significant effect on sociability in relation to experimental phase ($X_1^2 = 5.588$, $P = 0.018$). At the beginning of the test, the medaka of both treatments showed similar attraction towards the stimulus shoal ($t_{35} = 0.453$, $P = 0.653$); thereafter, medaka exposed to the winter photoperiod spent more time close to the shoal compared to the medaka of the summer photoperiod treatment ($t_{35} = 3.092$, $P = 0.004$; Fig. 2c). The main effect of experimental phase was not significant ($X_1^2 = 0.033$, $P = 0.856$).

3.3. Analysis of cognition

3.3.1. Cognitive test 1: brain functional lateralisation

In the analysis of the lateralisation index, we found a significant effect of the photoperiodic conditions modulated by experimental phase (LMM: $X_1^2 = 10.203$, $P = 0.001$). In the first half of the test, medaka exposed to the summer photoperiod swam more often in a clockwise direction, which indicates left eye preference to observe the stimulus and therefore right hemisphere processing (post-hoc: $t_{32} = 2.699$, $P = 0.011$; Fig. 3a); the opposite lateralisation pattern was exhibited by medaka exposed to winter photoperiod (Fig. 3a). In the second half of the experiment, there was no difference between the lateralisation index of fish from the two photoperiod conditions ($t_{32} = 0.662$, $P = 0.513$). In the LMM, the main effect of experimental phase and the main effect of treatment were not significant ($X_1^2 = 1.527$, $P = 0.217$; $X_1^2 = 0.643$, $P = 0.423$, respectively).

3.3.2. Cognitive test 2: inhibitory control

The analysis of inhibitory control revealed a significant difference in the number of food “attacks” between the medaka from the two photoperiod treatments in relation to the experimental phase (GLMM: $X_1^2 = 23.684$, $P < 0.001$). This interaction was due to the fact that there

was no difference between the two photoperiod conditions in the first half of the experiment (GLM: $X_1^2 = 0.031$, $P = 0.861$), but the main effect of photoperiod condition was significant in the second half of the experiment (GLM: $X_1^2 = 0.293$, $P < 0.001$). This suggests that the medaka exposed to the winter photoperiodic conditions displayed a reduced number of attacks in the second half of the test (Fig. 3b) while in contrast, the medaka under summer photoperiodic conditions made continuous attempts to reach their prey, which is indicative of lower inhibition. In the initial GLMM model, the main effect of experimental phase was significant ($X_1^2 = 79.343$, $P < 0.001$) and the main effect of treatment was not significant ($X_1^2 = 0.293$, $P = 0.589$).

3.3.3. Cognitive test 3: colour discrimination learning

All the subjects achieved the learning criterion in the colour discrimination task. In analysis of the number of days necessary to reach the learning criterion, we found a significant effect of treatment modulated by the rewarded colours associated with each subject (ANOVA: $F_{1,19} = 4.389$, $P = 0.049$). Summer photoperiod treated medaka required more time to achieve the learning criterion, but only for those subjects which were assigned with blue as the rewarded colour (Fig. 4a). The main effect of rewarded colour was significant ($F_{1,19} = 5.297$, $P = 0.033$), and the main effect of photoperiodic condition was not significant ($F_{1,19} = 1.642$, $P = 0.209$).

3.3.4. Cognitive test 4: shape discrimination learning

All the subjects achieved the learning criterion in the shape discrimination task. The analysis of the number of days required to reach the learning criterion in the shape discrimination experiment revealed a significant main effect of the photoperiodic conditions (ANOVA: $F_{1,18} = 13.423$, $P = 0.002$), indicating slower learning rate in the medaka exposed to the summer photoperiod treatment (Fig. 4b). The main effect of rewarded stimulus and the interaction involving rewarded stimulus and treatment were not significant ($F_{1,18} = 2.453$, $P = 0.135$; $F_{1,18} = 2.453$, $P = 0.677$, respectively).

3.4. Analysis of brain gene expression

In the brain gene expression analysis, we found significant seasonal differences of expression levels for all mRNAs investigated (*t*-test: *tshβ*: $t_{11} = 2.632$, $P = 0.023$; *tef*: $t_{16} = 2.380$, $P = 0.030$; *gh1*: $t_{16} = 3.465$, $P = 0.003$). In all cases, the expression was higher for the medaka brain under summer photoperiod conditions (Fig. 5).

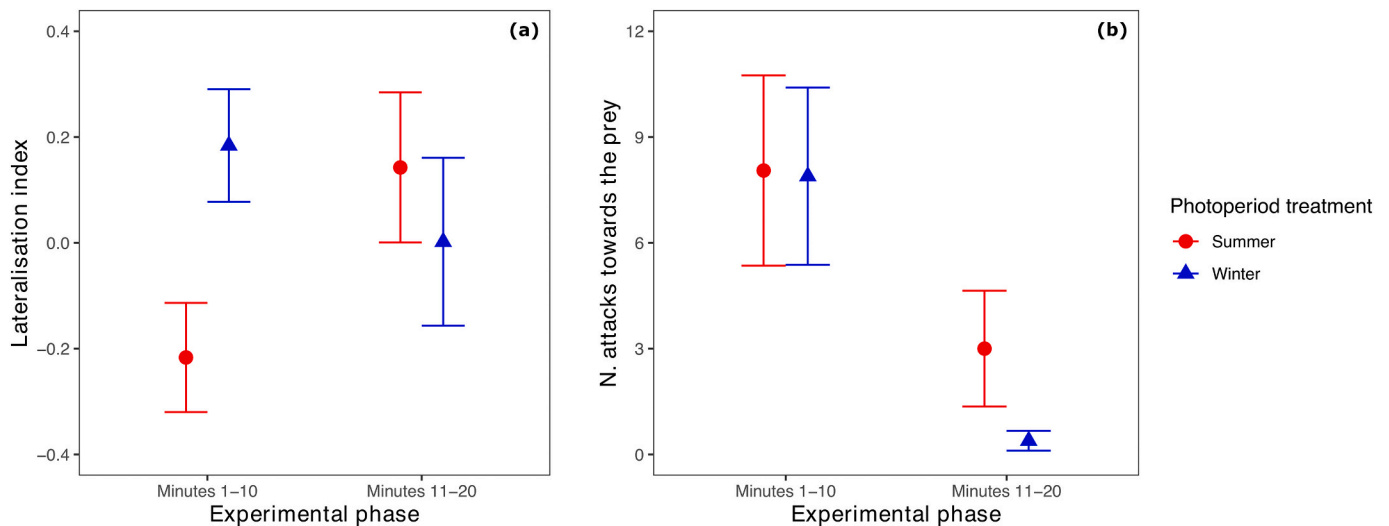


Fig. 3. Results of cognitive tests 1 and 2. (a) Cerebral lateralisation index indicating preference for processing social information with the right hemisphere (positive values) or left hemisphere (negative values). (b) Number of attacks in the inhibitory control task. Data points represent means and error bars represent standard errors.

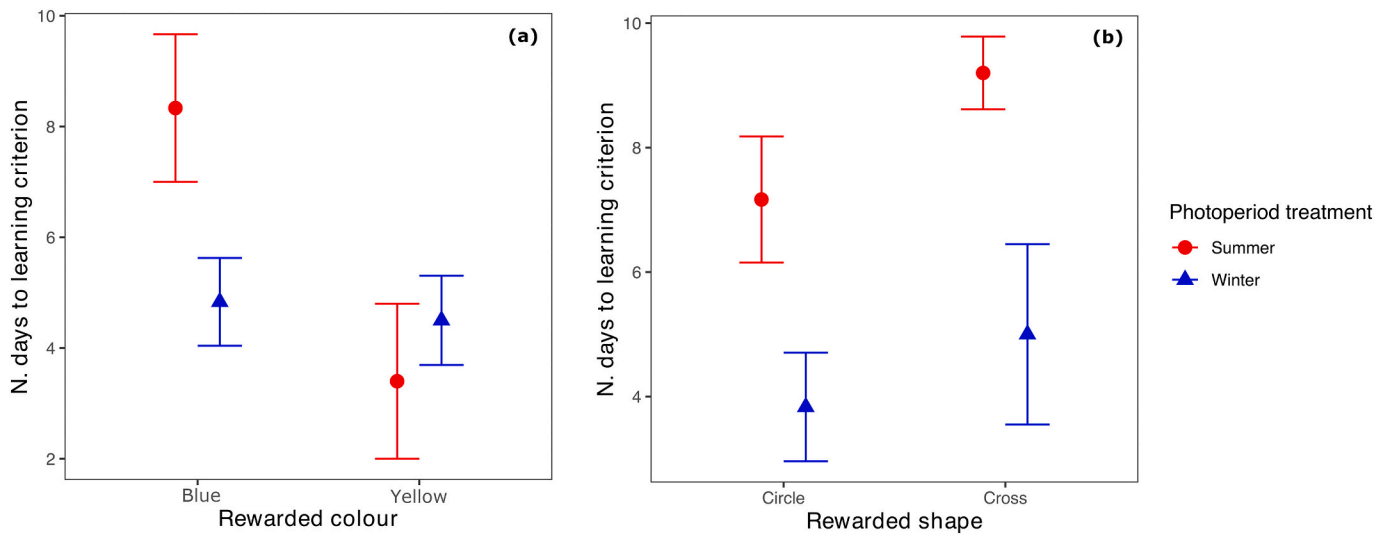


Fig. 4. Results of the discrimination learning tests (cognitive test 3 and 4). (a) Number of days required to achieve the learning criterion in the colour discrimination task, divided per rewarded colour assigned to the subjects. (b) Number of days required to achieve the learning criterion in the shape discrimination task, divided per rewarded shape assigned to the subjects. Data points represent means and error bars represent standard errors.

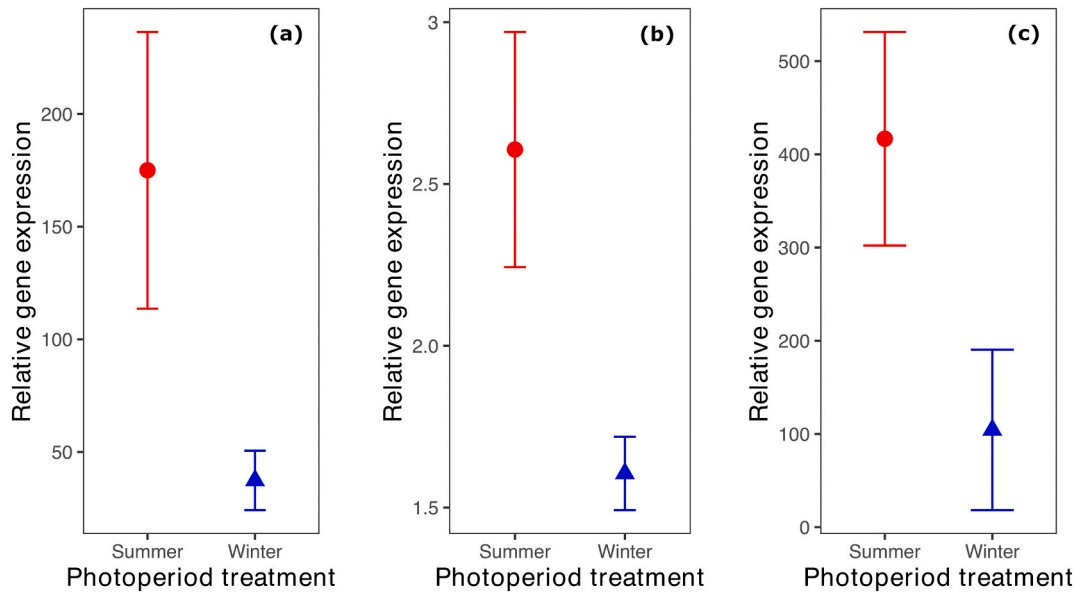


Fig. 5. Results of brain gene expression analysis. Relative expression of (a) *tshβ*, (b) *tef*, and (c) *gh1*. Data points represent means and error bars represent standard errors.

4. Discussion

In this study, we exposed medaka fish to two naturally relevant photoperiod conditions to investigate the effect of seasonal changes on behaviour and cognition. Specifically, we used a long photoperiod to simulate summer, during which reproduction occurs, and a short photoperiod for simulating winter. Our results show that the behavioural and cognitive phenotype of medaka plastically responds to the changes of photoperiod. Moreover, gene expression analysis reveals that the behavioural adaptations are accompanied by substantial changes in hormonal regulation between the different photoperiodic conditions.

The two behavioural tests conducted in our study were designed to analyse two key traits relevant for the fitness of the species and that are often adopted in applied research on fish models (e.g., Hong and Zha, 2019), namely the response to a novel environment and sociability. The novel environment test (open-field test) indicated a difference in activity

in terms of the distance moved and the time spent moving by medaka exposed to summer photoperiod compared to those exposed to the winter photoperiod. In both cases, the effect was mediated by the experimental phase. Graphical inspection (Fig. 2a and b) suggests that the effect was mostly due to medaka of the winter photoperiod showing reduced activity in the second half of the experiment, although the post-hoc tests failed to confirm this and only detected a marginal effect possibly due to lack of power. These parameters (distance moved and time spent moving) are mostly associated with activity although some studies associated it with forms of anxiety (Toms et al., 2010). A third behavioural parameter that we measured, namely the spatial preference for the edges of the novel environment (thigmotaxis), did not significantly vary between fish from the different photoperiods. Since thigmotaxis has been clearly associated with anxiety (Schnörr et al., 2012), we conclude that the downregulation of metabolic activity and other physiological traits due to the winter-like photoperiod were more likely

involved in the altered swimming activity detected with the variables distance moved and time spent moving (Fujisawa et al., 2021). In contrast to a previous study (Yokota and Oishi, 1992), in our experiments the temperature was kept constant. Therefore, the reduced activity is not the result of a lower water temperature during the winter period, and rather points towards the presence of a ‘winter’ behavioural phenotype induced by photoperiodic changes. It is worth noting that the temporal dimension of the difference in activity might indicate a role for habituation. Changes in activity in the open-field test are often associated with the fish becoming familiar with the novel environment and thereby reducing their exploration (e.g., O’Neill et al., 2018; Pazmino et al., 2020). From this perspective, it is possible that the winter photoperiod-treated medaka habituated faster to the open-field arena or reduced their exploration behaviour sooner.

Our second behavioural test indicated reduced sociability of medaka exposed to the summer photoperiod. Social aggregation in fish is associated with various activities such as defence from predators (Hager and Helfman, 1991) or improving foraging (Harpaz and Schneidman, 2020). Arguably, these activities assume a different relevance under different seasonal conditions (e.g., Harvey and Nakamoto, 2013), thereby representing a selective pressure for the evolution of behavioural plasticity in sociability (Johnsen and Hasler, 1977; Koizumi et al., 2017). In the case of medaka, high sociability during the winter season might be due to an increased need for predator avoidance, for example to counter-balance the costs of reduced activity or changed ecological parameters such as the presence of shelter (Fukuda et al., 2006; Fujisawa et al., 2021). However, to the best of our knowledge, the fitness significance of social attraction has not been fully described in this species making it difficult to interpret the significance of the observed plasticity. Alternatively, aggression in medaka, a behavioural trait usually negatively related to sociability (Kasper et al., 2019), has been associated with reproduction (Howard et al., 1998; Yamashita et al., 2020) and with other factors that vary with seasons such as food availability (Robb and Grant, 1998). Therefore, it is possible that the reduced sociability observed in the medaka adapted to the summer photoperiod is the consequence of an increase in aggression to cope with summer foraging and reproductive activities. Notably, the effect on sociability was observed during the second phase of the experiment. This is not unexpected because our protocol exploited the social attraction in response to a novel/unfamiliar environment (Lucon-Xiccato et al., 2017). As experimental time proceeds, the relative impact of the testing environment changes for the subjects, shifting progressively from unfamiliar to familiar, and this in turn alters the social response of the fish. We therefore speculate that habituation might be involved in the observed difference in sociability between medaka from the winter and the summer photoperiods, as proposed for the open-field test. More generally, our study emphasises the importance of incorporating temporal measures when analysing medaka behaviour.

Our cognitive experiments revealed substantial phenotypic variation according to photoperiod. Lateralisation is the tendency to split information processing between the two brain hemispheres, thus representing a mode of functional brain asymmetry which is commonly observed in vertebrates (Bisazza et al., 2000). We found that medaka exposed to the summer photoperiod preferentially used the left hemisphere for processing the image of a conspecific, at least in the first part of the experiment when the attraction towards the social stimulus was higher due to the unfamiliar testing environment. In the same time window, the medaka under winter photoperiod conditions showed the opposite hemisphere processing preference. Since we used a lateralisation test that was based on social interactions (Moscicki et al., 2011), the first explanation for the observed lateralisation plasticity is that it is linked to the seasonal plasticity in sociability observed in our previous experiment. However, the results of our lateralisation test may be considered as a proxy for a more general lateralisation phenotype because previous studies have revealed consistency across diverse lateralisation tests (Dadda et al., 2012). Therefore, the overall conclusion from our study is

that photoperiod generates major changes in functional lateralisation of the brain. Seasonal plasticity of lateralisation potentially has several explanations. A shift towards right-hemisphere processing has often been observed as a result of high stress levels (Rogers, 2010), a finding which also holds true in fish (Lucon-Xiccato et al., 2014). Medaka might therefore shift to right hemisphere control during winter to cope with more stressful environmental conditions. Lateralisation is also critical for foraging (Giljov et al., 2009) and mating (Torres-Dowdall et al., 2020); thus, the observed plasticity of lateralisation might be due to different occurrence of these activities between summer and winter. Alternatively, the plasticity of medaka lateralisation might be associated with seasonal variation in ecological parameters such as predation risk, vegetation abundance, or light exposure, which are all known to affect cerebral asymmetric functioning in anamniotes (Dadda and Bisazza, 2016; Dadda et al., 2010a, 2010b; Lucon-Xiccato et al., 2020a, 2020e).

The two remaining cognitive traits measured in this study (inhibition and learning) also showed variation across the photoperiod treatments. Specifically, exposure to summer photoperiod conditions was associated with lower inhibitory capacity. With our test, inhibition consisted of a reduction in attacks targeting unreachable prey over the course of the testing time (Lucon-Xiccato et al., 2022). The medaka from the summer photoperiod tried to reach the prey throughout the entire test, whereas those from the winter photoperiod inhibited this response after the first ten minutes of the experiment. The effect on inhibitory control might also be related to the known link between lateralisation and this ability (Lucon-Xiccato et al., 2020d). From an adaptive point of view, reduced inhibitory control might be advantageous during competition for food or mating. These activities mainly take place in the summer season for this species, thereby explaining the reduced inhibition observed in the subjects exposed to the summer photoperiod.

The final cognitive effect of the photoperiod that we observed was a reduced discrimination learning ability in medaka adapted to the summer photoperiod. The effect on discrimination learning also aligns well with our previous findings in this species (López-Olmeda et al., 2021). In that previous study, it was revealed that medaka, on average, reduce their learning performance during foraging in summer months, an effect that is thought to be related to hormonal changes or shifting interest towards mating. In support of this interpretation, the effect was mostly observed in males. It is worth noting that one of our learning experiments was based on colour discrimination and these results are difficult to interpret. Although, on average, medaka which experienced the summer photoperiod treatment took longer to learn colour discrimination, this effect was mediated by the rewarded stimulus assigned to each subject. In particular, summer photoperiod-treated medaka apparently learned much faster to select the yellow stimulus versus the blue stimulus. Such an effect is indicative of a seasonal shift in spontaneous colour preference (Roy et al., 2019), which is likely due to a reported change in the photoreceptor population in summer versus winter conditions (Shimmura et al., 2017). This finding is relevant for methodological purposes as it suggests caution in designing and interpreting colour learning experiments in medaka. Indeed, the results may vary according to the season and the lighting conditions.

Our gene expression analysis supports previous studies revealing that photoperiod treatment alters hormone production in medaka. The expression of both *tsh β* and *tefa* were upregulated in summer-adapted fish. It is known that in fish, the transcription factor TEF binds to D-box enhancer elements in the promoters of light-inducible genes (Vatine et al., 2009; Mracek et al., 2012). In mammals, TEF also binds to a D-box in the *tsh β* promoter and upregulates gene expression under long day conditions (Dardente et al., 2010). Further experiments will be required to evaluate whether seasonal variation of *tsh β* expression is directly light-driven by TEF via its regulation of D-box elements. It is also important to consider a possible role for deep brain photoreceptors in determining the phenotypic shift observed in medaka. Non-visual opsins are widely expressed in the fish brain (Sato and Ohuchi, 2021; Foulkes et al., 2016). Furthermore, the photoperiodic regulation of seasonal

reproduction in Japanese quail is mediated by deep brain photoreceptors expressing Opsin 5 that are located in the paraventricular organ and extend fibres to the pars tuberalis of the pituitary to translate lighting information into *tsh β* expression (Nakane et al., 2010). Finally, melatonin, a key hormone involved in the photoperiodic signalling pathway, may also play a role in the seasonal control of *tsh* expression. Indeed, melatonin exposure has been shown to reduce *tsh* mRNA levels in medaka pituitary organ culture (Kawabata-Sakata et al., 2020). GH is also a central mediator of seasonal changes in physiology and behaviour such as feeding, swimming, aggression, and anti-predator behaviour (Canosa et al., 2007). Since previous investigations in zebrafish have shown that GH overexpression has significant effects on the brain with an improvement in long-term memory (Studzinski et al., 2015), seasonal changes in the cognitive abilities of medaka could be due to altered expression of this pituitary hormone. Overall, these results reinforce the notion that seasonal changes in photoperiod alter the general hormonal phenotype of medaka. Hormonal modulation pathways have been described in vertebrates for both the behavioural (e.g., Killen et al., 2021; Yamashita et al., 2020) and cognitive traits (e.g., Riedstra et al., 2013; Rogers, 1974) described in this study. Therefore, hormonal changes resulting from changes in photoperiod may potentially underlie the plasticity that we have observed in medaka behaviour and cognition. Further insights into hormonal control might derive from studies exposing the medaka to intermediate photoperiods (i.e. spring-like and autumn-like conditions), corresponding to the biological phase in which the shift between summer and winter phenotype is expected to occur (Awaji and Hanyu, 1989).

In conclusion, our study reveals widespread behavioural and cognitive alterations in response to two critical photoperiod lengths in medaka, as well as accompanying changes in genes linked with hormonal regulation. This indicates considerable plasticity in these traits in natural populations of medaka, potentially mediated by a light-hormone regulatory axis. Thereby, our work paves the way for investigations of plasticity under other photoperiod conditions and for the use of this species as a model to explore the origins of seasonal behavioural diseases.

Funding

This work was supported by University of Ferrara [FAR2020 and FIR2020 grants to T.L.-X. and to C.B.] and by the Helmholtz Association [the Natural, Artificial and Cognitive Information Processing Programme to F.L. and N.S.F.].

Acknowledgements

We have no competing interests. We are grateful to Andrea Margutti for building the experimental apparatus, to Ines Cuesta for help with the qRT-PCR analysis and to Roberto Bianchi, Alex Zille, Marco Tomain, Jessica Visentin, and Valentina Siciliano for help in testing the fish.

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