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# The Acclimation of European Sea Bass (*Dicentrarchus labrax*) to Temperature: Behavioural and Neurochemical Responses

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## Abstract

Studies on fish behavioural and neurophysiological responses to water temperature change may contribute to an improved understanding of the ecological consequences of global warming. We investigated behavioural and neurochemical responses to water temperature in European sea bass (*Dicentrarchus labrax*) acclimated to three temperatures (18, 22 and 28°C). After 21 d of acclimation, three groups of 25 fish each were exposed to four behavioural challenges (foraging, olfactory, aversive and mirror tests). The expression of choline acetyltransferase (ChAT) was then analysed by Western blotting in CNS homogenates (from a subset of the same fish) as a marker for cholinergic system activity. In both foraging and olfactory tests, fish acclimated to 28°C exhibited significantly higher arousal responses than fish acclimated to lower temperatures. All specimens showed fright behaviour in the aversive test, but the latency of the escape response was significantly less in the fish at 28°C. Finally, the highest mirror responsiveness was exhibited by the fish acclimated to 22°C. As in the case of cholinergic neurotransmission, significantly higher ChAT levels were detected in the telencephalon, diencephalon, cerebellum and spinal cord of fish acclimated to 22 or 28°C in comparison with those maintained at 18°C. Lower ChAT levels were detected in the mesencephalon (optic tectum) at 22 and 28°C than at 18°C. These data indicate that neuronal functions are affected by water temperature. Increases or decreases in ChAT expression can be related to the functional modulation of brain and spinal cord centres involved in behavioural responses to temperature change. Overall, the results of this study suggest that the environmental temperature level influences behaviour and CNS neurochemistry in the European sea bass.

## Introduction

An increase in temperature is thought to be one of the principal effects of climate change (IPCC 2007). Indeed, an increase of a few degrees in the water temperature modifies the structure and functionality of sea and estuarine communities, affecting the abundance and the geographical distribution of living organisms (Kankaala et al. 2002; Cairns et al. 2005; Hiddink & ter Hofstede 2008; Daufresne 2009), primarily in highly vulnerable environments such as lagoons and coastal wetlands (Eisenreich 2005).

Water temperature is a crucial factor for all aquatic ectothermic species, including fish (Atkinson 1996; Jobling 1996; Manciocco et al. 2014). Indeed, thermal acclimatisation can affect fish growth, reproduction,

spontaneous activity, feeding behaviour and metabolism (Pörtner 2002).

*Dicentrarchus labrax* is a eurythermal and euryhaline species that spends its early life in transitional environments (i.e. lagoons) before the adults move to the sea and then return to estuarine waters for reproduction (Blaber et al. 1989; Pickett & Pawson 1994). Several studies have analysed the impact of water temperature on different aspects of sea bass physiology and biochemistry, such as growth (Vinagre et al. 2009), morphology (Sfakianakis et al. 2006), metabolism (Person-Le Ruyet et al. 2004), protein utilisation (Peres & Oliva-Teles 1999), sex determination (Saillant et al. 2002; Navarro-Martin et al. 2011) and swimming speed (Koumoundouros et al. 2002; Claireaux et al. 2006). In this regard, it was reported that sea bass juveniles cease growing at 11–15°C and grow more rapidly at 22–25°C, with upper and lower thermal limits at 2–3°C and 30–32°C, respectively (Barnabé 1991). Moreover, positive correlations have been found between temperature, food absorption and growth rate in the same species (Russell et al. 1996; Person-Le Ruyet et al. 2004).

In natural environments, fish migrate towards regions with a more suitable temperature. However, this study may have ecological relevance because the experiments performed allowed us to assess the effect of water temperature variations on behaviour and neurochemistry. From a global warming perspective, ethological studies strictly associated with neurochemical analyses under controlled laboratory conditions appear necessary to obtain detailed information on the species-specific coping styles affected by the temperature modifications, particularly in terms of the effects of temperature variations on social, life-threatening and feeding behaviours.

Nevertheless, although data are relatively abundant in the literature, the effects of water warming on behaviour and CNS activity have not been investigated in relevant commercial species such as the European sea bass. Recent experiments performed by our group on wild European sea bass juveniles (standard length 3–4 cm) captured from the Venetian lagoon have indicated that thermal acclimation may influence several quantitative components of the anti-predator behaviour directed towards a live fish and an aerial stimulus. Moreover, a Western blot (WB) analysis performed on the same specimens showed that the level of ChAT expression is lower in the brains of fish acclimated to 26°C than in those at 18°C (Malavasi et al. 2013). These results indicated that sea bass juveniles from natural lagoons are sensitive to thermal regimes. This finding should be confirmed in different fish stocks.

The aim of this study was to further investigate the behavioural and neurochemical responses to temperature change of European sea bass juveniles (s.l. 10 cm) from a commercial hatchery. The greater size of the fishes used in the present work allowed us to dissect the main brain regions, thus obtaining a more accurate topographic analysis of ChAT expression. Moreover, the behavioural scoring included exploratory and social traits not previously described (Malavasi et al. 2013).

The acclimation temperatures used in this study were based on Barnabé (1991), who reported the 21–25°C interval as the optimal growth range for the sea bass. The three selected temperatures were situated slightly below (18°C), within (22°C) and above (28°C) the best thermal range. Temperature-acclimated fish were subjected to four behavioural challenges (foraging, olfactory, aversive and mirror test), and their responsiveness was compared. The expectation was that fish at the highest temperature would be more responsive to experimental challenges than fish at lower temperatures and would show a higher level of exploratory behaviour during the olfactory, aversive and mirror tests and more feeding events in the foraging test. Indeed, an increased interest in food is expected in ectothermic animals as a response to an enhanced metabolic rate due to high temperature (Cossins & Bowler 1987).

As a marker for CNS activity, the expression of choline acetyltransferase (ChAT) was investigated, as ChAT is reportedly the enzyme responsible for ACh synthesis in cholinergic neurons (Eckenstein et al. 1981). According to the literature, thermal acclimation affects ChAT expression and activity in both vertebrate (Hebb et al. 1972; Poli et al. 1997; Malavasi et al. 2013) and invertebrate neurons (Salvaterra & McCaman 1985; Tajima & Salvaterra 1992; Takagawa & Salvaterra 1996). Moreover, experiments on goldfish have demonstrated that ACh is directly involved in behavioural thermoregulation (Crawshaw & Wollmuth 1992), which is the main mechanism of thermal regulation in fish. In this study, ChAT neurochemistry was analysed with WB testing in the brain and spinal cord.

## Materials and Methods

### Fish Collection and Maintenance

A total of 216 juvenile sea bass of mean standard length 10 ± 1 (Standard error, SE) cm were collected

from a commercial hatchery between Mar. and Nov. 2012 (Panittica Pugliese, Brindisi, Italy). According to Giffard-Mena et al. (2011), this size corresponds to fish that are approximately 2 yr old. Upon arrival at the laboratory, the fish were randomly allocated to 180 L (100 × 40 × 50 cm) indoor well-aerated glass aquaria (12 fish per tank, designated 'home tank') supplied with recirculated and filtered seawater (300 l/hr), with salinity averaging 15 ± 1 (SE) ‰ and a temperature of 19 ± 1 (SE) °C, replicating the housing conditions of the hatchery that provided the fish. The same chemical–physical conditions experienced by fish in the source hatchery were maintained to reduce the potential stress level of the fish. During the quarantine period (1 mo long), a procedure directed against external skin parasites was applied for 1 wk in the form of a disinfecting agent (Oidimol Dajana Pet, Brno, Czech Republic) at a concentration of 0.2 ml/l as indicated by the manufacturer's directions for use. To our knowledge, no data are available to suggest that such a procedure could affect the neurochemical variables examined in this study. Furthermore, on the basis of data from the literature, the salinity was gradually reduced to 5 ± 2 (SE) ‰ during the quarantine period to minimise the risk of spread of pathogens (Chang & Plumb 1996). The fish were maintained under an artificial photoperiod (12:12 light/dark cycle) and fed daily by hand with commercial dry pellets (2% of body mass) (Aqualim, France). To ensure good water quality, a constant flow of filtered water (300 l/hr) was maintained with internal filter systems in each aquarium, faeces and the remaining food items were removed from the animal tanks at least three times per week, salinity was checked with a hand-held refractometer and pH, and changes in the concentrations of nitrites and nitrates were monitored in all aquaria. During the tank-cleaning operations, a water exchange of approximately 20–30% per week was performed to restore the correct volume of water and maintain its chemical–physical parameters.

The mortality rate averaged 20% for all tanks during the quarantine period, primarily due to the presence of pre-existing parasitic organisms. Specifically, two or three fish died in each tank.

Moreover, two fish died during the experimental period (see 'Experimental Procedure'). All procedures of maintenance complied with both the requirements for the care and accommodation of fish stated in Annex III, Section B of Directive 2010/63/EU (EU 2010) and Recommendation 2007/526/EC on guidelines for the care of animals used for experimental and other scientific purposes (EC 2007).

At the end of the behavioural tests, to minimise the sacrifice of animals, a total of 38 subjects were captured haphazardly with a hand net and were euthanised for neurochemical analysis. The remaining animals were kept in tanks under stock conditions (19°C) and eventually used in other experiments.

### Experimental Procedure

The experimental temperatures of 18, 22 and 28°C were achieved, in 1, 3 and 9 d, respectively, by gradually changing the water temperature in the home tanks by 1°C per day beginning from the initial temperature of 19°C. When these temperatures were reached, the experimental period began. All fish were kept at the experimental temperatures for 21 d (designated 'thermal treatment' below). Six aquaria were used for each experimental temperature. The water temperature was checked twice daily (10:00 and 17:00 local time) and showed a mean standard error of ±1°C relative to the desired value. Opaque panels were located between tanks to prevent visual contact between fish in different tanks.

At the end of the 21-d-long thermal treatment, four behavioural challenges (foraging, olfactory, aversive and mirror test) were presented to the subjects (150 in total). For each trial, two fish were captured haphazardly by a hand net and moved from the home tanks to the behavioural tanks (61 l, indoor, 53.5 × 40.0 × 36.5 cm). In a pilot study, persistent freezing behaviour was observed in singly housed subjects. Conversely, swimming activity normally occurred when two fish were captured and then housed together in the behavioural tanks. For this reason, pairs of animals to be used in the behavioural tests (N = 25) were moved for each trial to the behavioural tanks (for a total of 50 subjects for each single thermal treatment). The behavioural tanks were identical in water temperature and salinity to the home tanks. Sanyo video cameras (VPC-GH1, Vietnam) were placed approximately 50 cm in front of the behavioural tanks 24 hr before the beginning of the behavioural challenges. This procedure permitted animals to become familiar with the camera. The position of the cameras allowed video coverage of the entire surface of the tanks. Video recording always began 2 min before the start of the behavioural tests. The cameras were turned on without sudden movements, and no evidence of stress was observed (for example, no change in swimming movements was ever observed). The fish were used to the presence of human beings close to the tanks (e.g. for routine cleaning and feeding procedures).

The four behavioural challenges were performed in sequence, with a time interval of 30 min between two consecutive tests. On the basis of behavioural observations conducted during the pilot study, a 30 min long intertest interval was found to be an appropriate period of time to allow the fish to resume normal swimming activity after each of the four behavioural challenges presented.

In all four tests, the experimenter placed herself behind a panel located near the experimental tanks and proceeded to introduce the objects used in the tests. The fish never showed any changes in activity that could be attributed to fear of the experimenter or surprise.

To enhance the interest of the fish in the food items presented in the foraging and olfactory tests, no food was provided during a period of 24 hr before the day of the experiment.

### Foraging Test

The goal of this test was to assess the effects of temperature change on foraging behaviour. The ability to forage was tested using frozen larvae of *Chironomus salinarius* (mean length 1 cm). These larvae were selected for the experiment because they are commonly used as feeding enrichment in recreational aquaria and are, thus, easily available in large quantities. As prior experience plays a role in the foraging behaviour of certain fish species (Brown et al. 2003), a swarm of frozen larvae was released three times (once weekly) in all the home tanks during the 21 d of thermal treatment. At the start of the trial, a swarm of larvae (approximately 30 larvae) was delivered to a side of the behavioural tank by a syringe. Fish behaviour was video-recorded for 5 min soon after the release of the food. The behaviours considered were as follows: 'first biting', defined as the first bite given by the fish to food item; 'feeding', catching and/or eating a food item as a result of darting movements; 'fin raising', rapid and repeated erection and lowering of dorsal fins; and 'fast swimming', rapid and darting movements across the behavioural tank (Table 1).

**Table 1:** Description of the behavioural patterns considered (Pickett & Pawson 1994)

| Test | Behaviour | Description |
|---|---|---|
| Foraging | Feeding | Catching and eating food item from below by darting movement |
| | Fin raising | Fast and repeated erection and lowering of dorsal fins |
| | Fast swimming | Fast darting movement across the tank |
| Olfactive | Swimming close to the cue | Fish presence in a 8 $cm^2$ around the cue |
| | Contact with the cue | Touching and knocking the cue by the mouth |
| | Fin raising | Fast and repeated erection and lowering of dorsal fins |
| Aversive | Escape response | Darting away following the fallen down object |
| | Swimming close to the object | Fish presence in a 8 $cm^2$ around object |
| | Contact with the object | Touching the object by the mouth |
| | Inactivity | Staying motionless through the water column with fin movements |
| | Freezing | Staying motionless on the bottom without fin movements |
| Mirror | Contact with the mirror | Touching and knocking the mirror by the mouth |
| | C-start reaction | Showing a C-shape bending of the body towards right or left in front of mirror. |
| | Arousal | Fast and repeated erection and lowering of first dorsal fin, zigzag swimming, quickly buccal pumping, directed at and within 8 cm from the mirror |

### Olfactory Test

Fish rely on olfaction for food detection, recognition and selection (Atema 1980; Hara 1993). Indeed, previous studies on sea bass have reported that the addition of an attractive amino acid mixture to the diet enhanced voluntary food intake, weight gain and protein utilisation by this species (Mackie & Mitchell 1982; Dias et al. 1997), supporting the role of olfaction as a key sense for foraging in *D. labrax*.

In this test, the olfactory cue was represented by *Sarcophaga carnaria* larvae, which are commonly used as live bait by anglers. Ten *Sarcophaga carnaria* larvae were put into a tea infuser and immersed at middle height in the water column on one side of the 'behavioural tank'. Fish gained experience with both the empty tea infuser and the tea infuser containing *S. carnaria* as a result of the introduction of these objects to the home tanks three times (one time weekly during the 21 d of thermal treatment) to avoid any effect that the novelty of the stimulus might have on fish behaviour. The duration of video recording was 10 min. The measured behaviours were as follows: 'swimming close to the cue', defined as the presence of the fish in an 8 cm square around the olfactory stimulus; 'contact with the cue', the action of touching and knocking the cue with the mouth or the side of the body; and 'fin raising', the rapid and repeated erection and lowering of the dorsal fins (Table 1). To score the behaviour 'swimming close to

the cue', a 64 $cm^2$ area was defined around the stimulus by attaching a transparent film inscribed with vertical and horizontal lines to the front wall of the behavioural tank. Swimming activity was measured by counting the number of entries in the square area and recording the time spent within that area.

### Aversive Test

The aim of this test was to reproduce the water movement and vibrations due to the immersion of a potentially dangerous element (e.g. fishing net, float, avian predator) to investigate the response to an aversive stimulus. The stimulus chosen was a rubber ball 4 cm in diameter thrown into the water from a shelf located 0.5 m over the experimental tanks. The experimenter (AT) waited to throw the ball until both fish were stationary under the shelf. Fish behaviour was recorded for 10 min after the aversive stimulation. The behaviours analysed were as follows: 'escape response', defined as an escape with darting movements following the impact of the ball; 'swimming close to the object', the presence of the fish in an 8 cm square area around the ball; 'contact with the object', touching the ball with the mouth or the side of the body; 'inactivity', lack of motion in the water column and performance only of fin movements; and 'freezing', lying motionless on the tank bottom with no fin movements (see Table 1). The 'escape response' was recorded soon after the aversive stimulation. All other behaviours were recorded within a 64 cm square area that was defined around the plastic ball by attaching a transparent film ruled with vertical and horizontal lines to the front wall of the behavioural tank. Swimming activity was measured by counting the number of entries and recording the time spent by the focal fish within the square area.

### Mirror Test

Mirror images are often used to investigate fish predator inspection (Milinski 1987), but a social response to this stimulus has also been observed (Meliska et al. 1980; Miklosi et al. 1998). Furthermore, many fish display a systematic preferential use of one eye, and their laterally placed eyes influence their everyday behaviour (Tsubokawa et al. 2009).

Our goal was to measure the responsiveness of *D. labrax* to its mirror image. A mirror was carefully placed outside the behavioural tank along the short side, and the fish were then recorded for 5 min. Measured behaviours were the following: 'contact with the mirror', defined as touching and knocking the side of the mirror with the mouth; latency to contact, estimated as the elapsed time between the presentation of the mirror and the first contact with it; 'C-start right reaction', bending the body towards the right in front of the mirror side and making a C-shaped movement; 'C-start left reaction', bending the body towards the left in front of the mirror side and making a C-shaped movement; 'arousal', rapid and repeated erection and lowering of dorsal fins associated with zigzag swimming and/or rapid buccal pumping, all behaviours directed within a distance of 2 cm from the mirror (see Table 1).

### Behavioural Data Analysis

Tank effects were minimised by keeping all aquaria in identical care regime and macro- and micro-environment. Then, statistical analysis to investigate tank effects was conducted within each temperature and no effect was found (for details, see below).

The experimental unit was the individual. All experimental fish were tested out of their home tanks, isolated from the social group, and the behaviour of only one fish (called the focal subject) for each pair of the tested animals was analysed (total subjects: N = 75; subjects per temperature: N = 25). This procedure guaranteed the independence of data. The first animal that moved was defined as the focal subject. The second individual acted as a companion, and its behaviour was not analysed. The recognition of the focal subject occurred on the basis of slight body differences (e.g. the size, colour and shape of the caudal fin). The video recordings were replayed and analysed using Noldus Observer recording software (Noldus, Wageningen, The Netherlands) on a laptop (Asus K52F).

A total of 16 variables were considered across four different behavioural tests, with four behaviours measured for each test on average. In the olfactory test, 'swimming close to the cue' and 'contact with the cue' were analysed both separately and together (named 'interest in the cue'). In the aversive test, 'swimming close to the object' and 'contact with the object' were analysed both separately and together (named 'interest in the object').

The data did not obey the assumptions of normality and homoscedasticity needed for parametric statistics. Therefore, a Kruskal–Wallis test was performed, and a Mann–Whitney *U*-test was used for *post hoc* comparisons. The Bonferroni–Holm correction was applied for multiple comparisons and not for measured variables (Perneger 1998). The Bonferroni–Holm correction was preferred to the

Bonferroni method, as an analysis of *post hoc* comparisons with a Student's unpaired *t*-test has shown that statistically significant differences vanished after the application of a Bonferroni correction. Therefore, this method is considered overly conservative (Aickin & Gensler 1996). Data analyses were performed with Statview 5.0 (SAS Institute Inc.). The significance level used for the tests was 0.05.

### Sample Collection and Analysis for ChAT Neurochemistry

A total of 38 fish haphazardly chosen from those previously subjected to the behavioural tests were used for neurochemical analyses (Table 2). The number of fish sampled for these analyses was restricted to minimise the sacrifice of animals.

Thirty-six fish (12 for each temperature) were analysed with the WB method to evaluate ChAT expression in the CNS. Two additional fish acclimated to 18 ± 1 (SE) °C were processed for ChAT immunofluorescence to verify the specificity of the polyclonal anti-ChAT antibody used in the WB.

For the WB analysis, fish were euthanised with an overdose of 2-phenoxyethanol (8 ml/l), their weight and standard length were measured, and their brain and spinal cord were then rapidly dissected out. Brain samples from 18 specimens (six for each temperature) were processed as a whole, whereas brains from 18 other specimens (six for each temperature) were further subdivided into samples corresponding to the five main brain regions: telencephalon (including olfactory bulbs and preoptic region), diencephalon, mesencephalon (midbrain optic lobes), cerebellum and brainstem (Fig. 5a). Tissues were placed in RNA Later (Ambion, Austin, TX) at −70°C until they were processed.

In the WB analysis, pools of tissues from three animals were processed. Samples were homogenised and analysed in triplicate following the protocol previously described in Malavasi et al. (2013). In the present study, 50 μg of protein was loaded in each lane and separated in 8% SDS-polyacrylamide gels (SDS-PAGE). ChAT detection was achieved by the same ChAT polyclonal antibody (Cat.# AB144P, Lot.# NG1752017; Millipore, Temecula, CA, USA, dilution 1:3000) previously used successfully in a WB analysis performed on the whole-brain homogenates of European sea bass juveniles (Malavasi et al. 2013). Normalisation was performed against b-actin (actin antibody at 1:3000, Santa Cruz Biotechnology, Dallas, TX, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH antibody at 1:2500, Abcam, Cambridge, UK) expression. In the controls, the primary antibody was omitted.

The reactivity of the ChAT antibody was verified by immunofluorescence in brain sections from fish acclimated to 18 ± 1 (SE) °C. Fish were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). The brain was dissected out, post-fixed in PFA for 24 hr and cryoprotected in PBS containing 30% sucrose at 4°C for at least 36 hr. Samples were successively embedded in O.C.T. compound (Tissue-Tek II, Quiagen, Italy), frozen and cut into 20-μm transverse sections in a cryostat. Consecutive serial sections collected on microscope slides and sections containing the midbrain tectum were selected and processed for immunofluorescence. Briefly, sections were permeabilised with PBS plus 0.5% Triton-X-100 (PBST), blocked with 3% bovine serum albumin in PBST for 30 min and successively incubated with the anti-ChAT antibody (diluted 1:50) overnight at 4°C. Sections were then incubated for 1 hr at room temperature with a CY3-conjugated anti-goat antibody (diluted 1:50, Sigma-Aldrich, St. Louis, MO, USA). After several washes with PBS, sections were mounted on slides, coverslipped and observed with a fluorescence microscope. In the controls, the primary antibody was omitted.

**Table 2:** Number of fishes analysed

| | Behavioural test | | Neurochemical analysis | | | | | |
|---|---|---|---|---|---|---|---|---|
| Acclimation temperature | N. of fishes tested | N. of fishes analysed | N. of fishes analysed | Method of analysis | Whole-brain analysis (pool n. 1) | Whole-brain analysis (pool n. 2) | Brain regions analysis (pool n. 1) | Brain regions analysis (pool n.2) |
| 18°C | 50 | 25 | 2 | IHC | | | | |
| | | | 12 | WB | 3 | 3 | 3 | 3 |
| 22°C | 50 | 25 | 12 | WB | 3 | 3 | 3 | 3 |
| 28°C | 50 | 25 | 12 | WB | 3 | 3 | 3 | 3 |
| Total | 150 | 75 | 38 | | | | | |

### Ethical Note

All experiments have been performed in accordance with Directive 2010/63/EU (European Union (EU) 2010) with Italian administrative order DM 256/2012 of the Italian Ministry of Health. The experiment was designed to minimise the impact on the welfare of animals by including a filter system whose physical structure was used as cover by the fish and by replicating the chemical–physical conditions experienced by fish in the hatchery on arrival at the laboratory. The number of animals tested at each temperature was chosen to comply with the reduction principle but, at the same time, to recognise interindividual variability and to guarantee the scientific objectives of the experiment. A pilot study was previously conducted to refine the methodology and to minimise stressful conditions during the experimental challenges (for details, see the paragraph 'Experimental procedure') (see Russell & Burch 1959). In particular, fish euthanasia was performed by an overdose of 2-phenoxyethanol. This chemical is used for anaesthesia in freshwater fish research for routine work because of its rapid effect and rapid recovery time if exposure to the drug is not overly long (Idler et al. 1961; Sehdev et al. 1963). The data available in the literature primarily refer to the concentrations of 2-phenoxyethanol used for fish anaesthesia and not for euthanasia. In a pilot study, we tested five different concentrations (1, 2, 4, 6 and 8 ml/l) of 2-phenoxyethanol to induce rapid euthanasia in fish. The concentration of 8 ml/l was the most effective in inducing rapid euthanasia, and no strong behavioural reactions were observed.

## Results

A total of 150 fishes acclimated for 21 d at 18, 22 and 28°C were subjected to the behavioural tests. The behaviour of 75 subjects was then statistically analysed. Among these fish, a subsample of 38 (14 acclimated at 18°C, 12 at 22°C and 12 at 28°C) were haphazardly captured with a hand net, euthanised, measured and used for neurochemical analyses (Table 3). Both weight and standard length across-temperature differences were statistically significant (weight: $H_2 = 16.\ 615$, $p = 0.0002$; standard length: $H_2 = 11.577$, $p = 0.0031$), *post hoc* comparisons showed the mean fish standard length at 18°C differing from that at 28°C but not from that at 22°C. The fish acclimated at 22 and 28°C showed an increase in average weight of 1.3 and 1.7 times, respectively, and an increase in average standard length of 1.1 and 1.2 times, respectively, compared with those acclimated at 18°C.

**Table 3:** Weight and standard length (s.l.) of fish killed for immunofluorescence and Western blot analysis at the end of the acclimation period

| | 18°C | | 22°C | | 28°C | |
|---|---|---|---|---|---|---|
| | Weight (g) | s.l. (cm) | Weight (g) | s.l. (cm) | Weight (g) | s.l. (cm) |
| 1 | 21.3 | 10.2 | 30.7 | 12.0 | 23.9 | 11.0 |
| 2 | 20.2 | 10.5 | 18.9 | 10.5 | 35.7 | 12.5 |
| 3 | 22.2 | 11.0 | 32.8 | 12.0 | 29.1 | 11.0 |
| 4 | 19.5 | 10.5 | 26.5 | 11.5 | 45.8 | 13.5 |
| 5 | 14.5 | 10.0 | 30.0 | 12.0 | 25.6 | 11.0 |
| 6 | 15.2 | 10.0 | 30.3 | 12.0 | 48.2 | 13.5 |
| 7 | 9.8 | 9.0 | 33.5 | 12.5 | 33.1 | 12.5 |
| 8 | 17.0 | 10.2 | 27.2 | 12.0 | 30.7 | 12.0 |
| 9 | 31.1 | 12.0 | 26.6 | 11.0 | 42.3 | 13.3 |
| 10 | 26.1 | 11.5 | 23.5 | 11.0 | 24.8 | 11.0 |
| 11 | 23.8 | 11.5 | 19.0 | 9.5 | 40.7 | 13.0 |
| 12 | 23.4 | 11.0 | 19.8 | 10.0 | 39.0 | 13.0 |
| 13 | 27.7 | 12.0 | | | | |
| 14 | 18.5 | 11.0 | | | | |

### Foraging Test

No temperature effect was found for latency to 'first bite' and in frequency of 'feeding' although the fish at 28°C showed a slight increase in the number of feeding events (19, on average) compared with the fish at 18 and 22°C (15–16, on average). 'Fin raising' behaviour was significantly more prevalent in fish acclimated to 28 ± 1 (SE) °C than in those at 18 ± 1 (SE) °C ($H_2 = 9.690$, $p = 0.0079$) (Fig. 1a). Furthermore, the fish acclimated to the highest temperature spent more time in 'fast swimming' than individuals at the intermediate and lowest temperatures ($H_2 = 11.366$, $p = 0.0034$) (Fig. 1b). No tank effect within each experimental temperature was found (T 18: $H_2 = 6.946$, $p = 0.2247$; T 22: $H_2 = 8.829$, $p = 0.1161$; T 28: $H_2 = 10.191$, $p = 0.0700$).

### Olfactory Test

No temperature effect was found on the frequencies and durations of 'swimming close to the cue', 'contact with the cue' and 'interest in the cue' behaviour. However, a slight decrease in the time spent in the area around the cue was detected in the fish acclimated to the lower temperatures. In contrast, a temperature effect on the number of 'fin raising' events was found ($H_2 = 12.861$, $p = 0.0016$) in fish at 28 ± 1 (SE) °C. The levels displayed at this temperature were significantly higher than those observed at 18 ± 1 (SE) °C

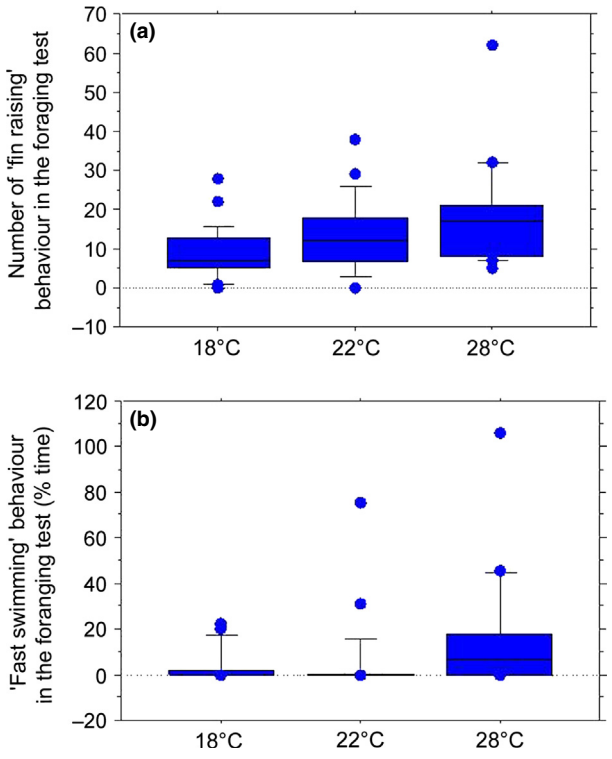


**Fig. 1:** Box plots summarising the distribution of the number of 'fin raising' behaviours (* 18°C vs. 28°C) (a) and the percentage time spent in 'fast swimming' behaviour (** 22°C vs. 28°C, * 18°C vs. 28°C) (b) in European sea bass relative to the three temperature treatments during the foraging test (N = 25). Each box plot contains a central line representing the median; the box itself delineates 25–75% of the data; the upper whisker represents the 90th percentile; the lower whisker represents the 10th percentile; and open circles represent outlying data points.*p ≤ 0.05, **p ≤ 0.01.

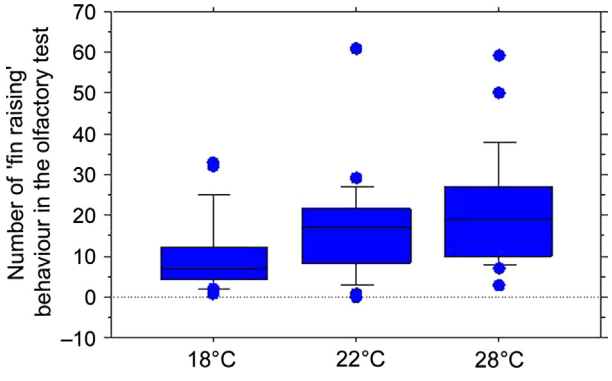


**Fig. 2:** Box plots summarising the distribution of the number of 'fin raising' behaviours (** 18°C vs. 28°C, * 18°C vs. 22°C) in European sea bass relative to the three temperature treatments during the olfactory test (N = 25). Each box plot contains a central line representing the median; the box itself delineates 25–75% of the data; the upper whisker represents the 90th percentile; the lower whisker represents the 10th percentile; and open circles represent outlying data points. *p ≤ 0.05, **p ≤ 0.01.

(Fig. 2). A tendency for this behaviour to occur more frequently at 22°C than at 18°C was also observed ($U$ = 129.5, tied p-value = 0.0309, not significant following Bonferroni–Holm correction). No tank effect within each experimental temperature was found (T 18: $H_2$ = 5.215, p = 0.3902; T 22: $H_2$ = 7.463, p = 0.1884; T 28: $H_2$ = 4.840, p = 0.4357).

### Aversive Test

Approximately 96% of the fish acclimated to 18 ± 1 (SE) °C (24) and 100% of those at 28 ± 1 (SE) °C responded to the aversive stimulus applied at the middle of the tank, in contrast to 76% of the fish at 22 ± 1(SE) °C (19). The remaining fish could not be stimulated, as they remained motionless near the tank wall for the entire duration of the test session. As a result, AT did not throw the ball in this case. These fish were excluded from the statistical analysis. The fish at 28°C escaped more rapidly than the fish at 22°C ($H_2$ = 11.278, p = 0.0036) (Fig. 3). Temperature effects were not found either in the frequencies or in the durations of 'swimming close to the object', 'contact with the object', 'interest in the object' and 'freezing' (data not shown). In general, all fish spent a small percentage of time (on average, 1.6%) in the area with the object and never touched it. However, 'freezing' behaviour was rarely observed (on average, 2.7%). After exposure to the experimental stimulus, fish acclimated to 28 ± 1(SE) °C showed a tendency to remain inactive in the water column for more time than did fish acclimated to the other two temperatures ($U$ = 192.0, tied p-value = 0.0190 not significant after

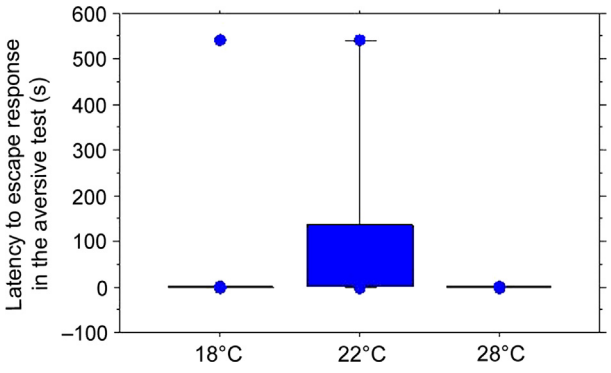


**Fig. 3:** Box plots summarising the distribution of the latency to escape response (** 22°C vs. 28°C) in European sea bass relative to the three temperature treatments during the aversive test (N = 25). Each box plot contains a central line representing the median; the box itself delineates 25–75% of the data; the upper whisker represents the 90th percentile; the lower whisker represents the 10th percentile; and open circles represent outlying data points. *p ≤ 0.05, **p ≤ 0.01.

the correction of Bonferroni–Holm). No tank effect within each experimental temperature was found (T 18: $H_2 = 0.799$ $p = 0.9771$; T 22: $H_2 = 5.769$, $p = 0.3293$; T 28: $H_2 = 2.828$, $p = 0.7265$).

### Mirror Test

In general, the fish acclimated to the intermediate temperature directed more behaviour to the mirror than the fish in the other two groups. A significant higher latency was measured in 'contact with the mirror' behaviour performed by the fish acclimated to 28 ± 1°C than in those at 18 ± 1 (SE) °C or 22 ± 1 (SE) °C ($H_2 = 7.263$, $p = 0.0265$) (Fig. 4a). Fish at 22 ± 1 (SE) °C displayed significantly more frequent 'contact with the mirror' than fish at 28 ± 1 (SE) °C ($H_2 = 8.319$, $p = 0.0156$) (Fig. 4b). Overall, the frequency of 'C-start reaction' differed among the three

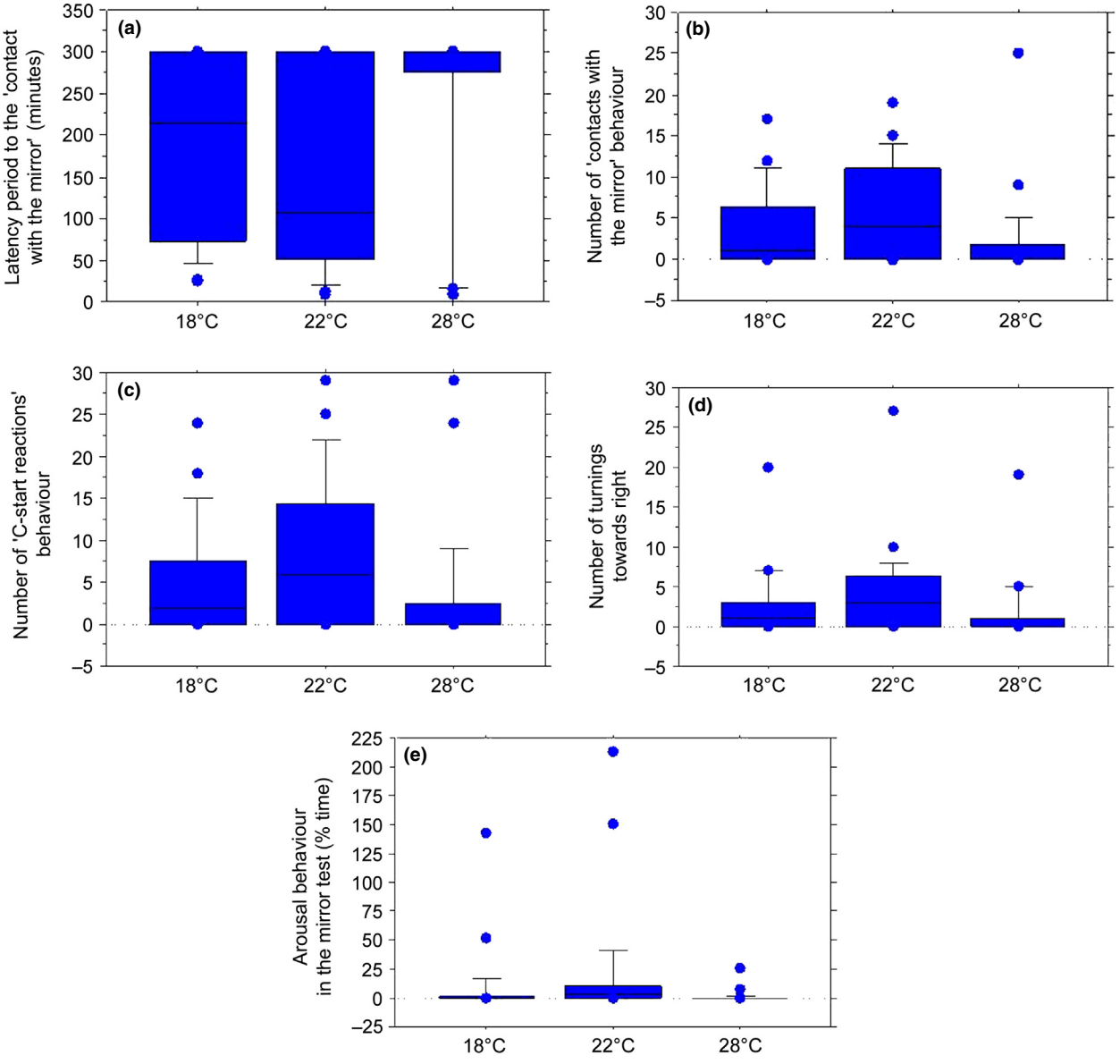


**Fig. 4:** Box plots summarising the distribution of latency to contact with the mirror (* 22°C vs. 28°C, 18°C vs. 28°C) (a), the number of 'contact with the mirror' behaviours (** 22°C vs. 28°C) (b), the 'C-start reaction' behaviour (* 22°C vs. 28°C) (c), the number of turns towards the right relative to the mirror (* 22°C vs. 28°C) (d) and the percentage time spent in 'arousal' behaviour (** 22°C vs. 28°C) (e) in European sea bass relative to the three temperature treatments during the mirror test (N = 25). Each box plot contains a central line representing the median; the box itself delineates 25–75% of the data; the upper whisker represents the 90th percentile; the lower whisker represents the 10th percentile; and open circles represent outlying data points. *$p \leq 0.05$, **$p \leq 0.01$.

temperatures ($H_2 = 6.544$, $p = 0.0379$), with fish acclimated to 22 ± 1 (SE) °C showing significantly higher values than individuals at 28 ± 1°C (Fig. 4c). Furthermore, a temperature effect on the number of turns towards the right was found ($H_2 = 8.262$, $p = 0.0161$), with fish at 18 ± 1 (SE) °C and 22 ± 1 (SE) °C turning towards the right in 51% and 50%, respectively, of the cases of 'C-start reaction' behaviour, whereas individuals at 28 ± 1 (SE) °C turned towards the right in approximately 40% of the cases (Fig. 4d). The mean number of 'C-start reaction' behaviours directed towards the right or towards the left did not differ within temperatures, even if fish acclimated to 22 ± 1°C showed higher values than individuals at 28 ± 1°C.

The fish acclimated to 22 ± 1 (SE) °C spent significantly more time displaying 'arousal' behaviour in the proximity of the mirror ($H_2 = 10.135$, $p = 0.0063$), whereas the fish at 28 ± 1°C showed the smallest amount of time displaying this behaviour (Fig. 4e).

No tank effect within each experimental temperature was found (T 18: $H_2 = 3.650$, $p = 0.6009$; T 22: $H_2 = 1.507$, $p = 0.9123$; T 28: $H_2 = 1.830$, $p = 0.8721$).

### Analysis of ChAT Expression

A total of 38 fish (14 specimens acclimated at 18°C, 12 at 22°C and 12 at 28°C) captured haphazardly with a hand net from those previously subjected to behavioural tests were euthanised, measured and used for neurochemical analyses.

ChAT levels were analysed in protein homogenates from the brain and spinal cord of fish previously subjected to behavioural tests. This analysis used a polyclonal antibody against human ChAT. The same antibody has been used successfully in a WB analysis performed on the whole-brain homogenates of European sea bass juveniles (Malavasi et al. 2013). Its cellular reactivity was verified by immunofluorescence on brain sections of the midbrain tectum, where densely packed unipolar neurons with apical dendrites (arrowheads) were stained in the periventricular stratum (arrows) of the laminated tectum (Fig. 5b,c). These cells display ChAT immunoreactivity in all teleosts studied to date, including trout (Pérez et al. 2000), zebrafish (Clemente et al. 2004) and goldfish (Zottoli et al. 1987; Giraldez-Perez et al. 2009). Thus, the mammalian polyclonal antibody is able to cross-react with sea bass ChAT.

A WB analysis of regional ChAT expression from fish acclimated at 18 ± 1 (SE) °C revealed that the polyclonal antibody labelled an intense ChAT immunoreactive band at approximately 70–75 kDa (Fig. 5d). A quantitative evaluation of the immunolabelled bands showed that the ChAT band was more intense in the mesencephalon (midbrain/optic tectum), the diencephalon and the brainstem followed by the cerebellum, the spinal cord and the telencephalon homogenates. In particular, the highest ChAT level was detected in the optic tectum and the lowest in the telencephalon, with a an eightfold difference between the highest and lowest levels (Fig. 5d). The comparison of brain ChAT expression in the whole-brain homogenates from fish acclimated to 18 ± 1, 22 ± 1 and 28 ± 1 (SE) °C revealed higher ChAT levels at 22 ± 1°C and 28 ± 1 (SE) °C than in 18 ± 1 (SE) °C (Fig. 5e). Regional ChAT expression at the three temperatures revealed a significant increase in the telencephalon (a), diencephalon (b), cerebellum (d) and the spinal cord (f) at 22 ± 1 (SE) °C and 28 ± 1°C relative to 18 ± 1°C (Fig. 5 f-g). Conversely, a decrease in ChAT expression levels was detected in the optic tectum at 22 ± 1 (SE) °C and 28 ± 1°C relative to 18 ± 1°C.

## Discussion

Present findings indicate that thermal conditions influence both behaviour and central ChAT neurochemistry in the European sea bass. After 3 wk at 18, 22 and 28°C, feeding behaviour was not affected by temperature, whereas fin raising and fast swimming were clearly affected by thermal conditions. Following the temperature acclimation period at 28 ± 1°C, fish showed higher values of these two behaviours compared with fish maintained at lower temperatures in the presence of living prey and olfactory cues. Furthermore, fin raising events and the time spent in rapid swimming increased linearly across the three temperatures (28°C > 22°C > 18°C). Pickett & Pawson (1994) noted these two behaviours as foraging-linked behavioural patterns, representing a response indicating an awareness of the presence of food in *D. labrax*. Indeed, high temperatures have been found to be correlated with increases in food demand in sea bass (Claireaux & Lagardere 1999; Peres & Oliva-Teles 1999; Person-Le Ruyet et al. 2004). Higher body growth in fish at 28°C could also have affected their behaviour in presence of food, even if no temperature effect on food consumption and/or on latency to feed in the foraging test was detected in this study. Furthermore, it should be noted that this increase of size has been measured only in a sub-sample of animals. The occurrence of an increased temperature

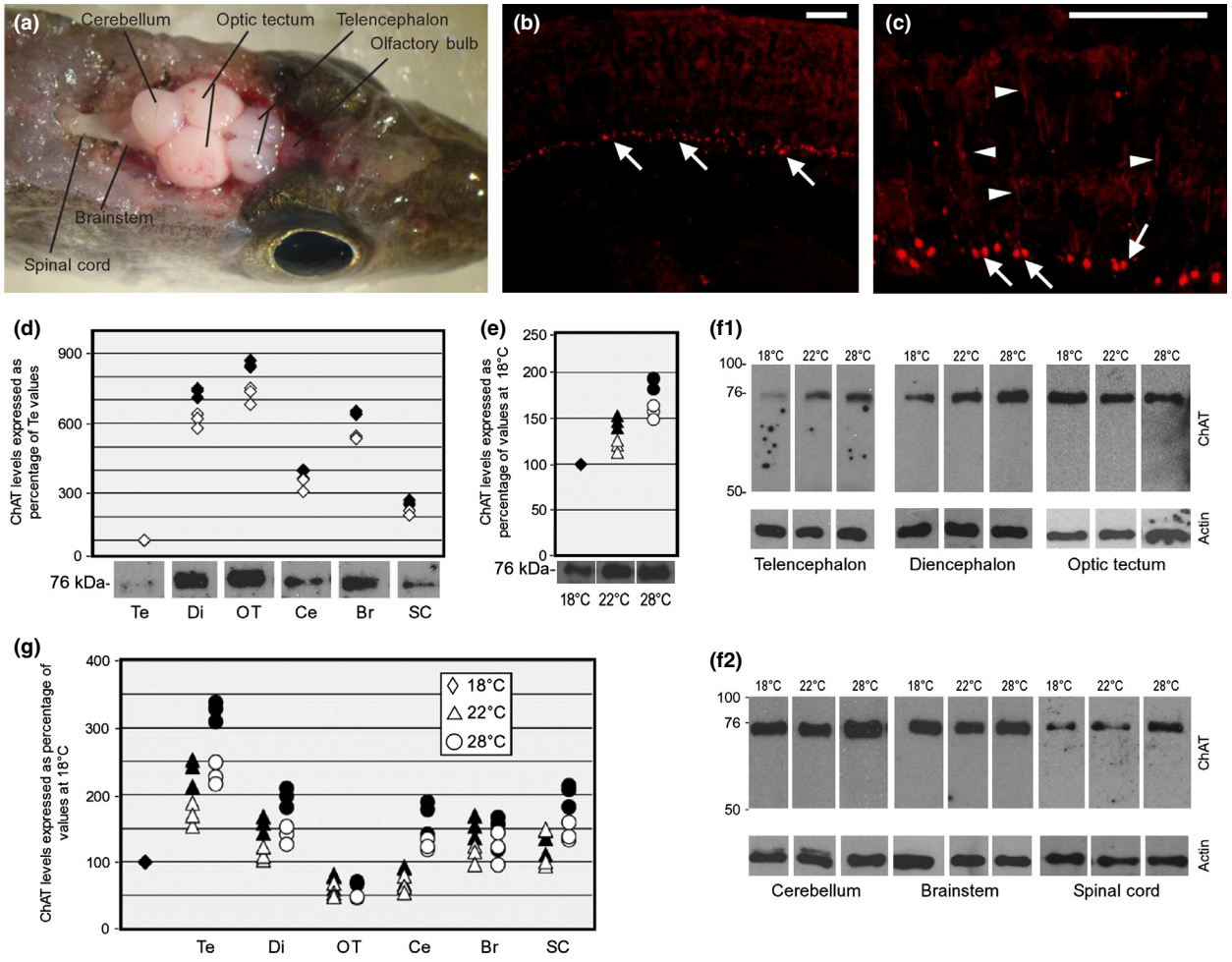


**Fig. 5:** ChAT expression in the brain and spinal cord of fish acclimated to different temperatures. Brain and spinal cord regions analysed with the Western blot method are shown in a freshly dissected fish (a). (b, c) ChAT immunofluorescence performed on 20–μm-thick frozen sections of the mid-brain optic tectum. Arrows point to the unipolar neurons of the periventricular stratum and arrowheads to their apical processes. Bar = 100 μm. (d) ChAT expression levels analysed with the Western blot method in telencephalon (Te), diencephalon (Di), optic tectum (OT), cerebellum (Ce), brainstem (Br) and spinal cord (SC) of specimens acclimated to 18°C (lower panel). Quantification of ChAT levels by normalisation on actin levels (upper panel). For each encephalic region, two pools of tissues from three fish were analysed in triplicate. Single observations are reported. White and black rhombuses discriminate data on the two different pools of tissues. (e) ChAT expression levels in the whole-brain homogenates of specimens acclimated to 18 ± 1 (SE) °C, 22 ± 1°C and 28 ± 1°C (lower panel). Quantification of ChAT levels by normalisation on GAPDH levels (upper panel). For each temperature, two pools of whole brains from three fishes were analysed in triplicate. Single data are reported as rhombuses (18°C), triangles (22°C) and circles (28°C). White and black colours discriminate data on the two different pools of tissues. (f) Comparison of ChAT levels in the main encephalic regions and spinal cord of fish acclimated to given temperatures (18 ± 1 (SE) °C, 22 ± 1°C, 28 ± 1°C). (g) Quantification of ChAT protein expression levels normalised on actin levels. For each encephalic region, two pools of tissues from three fishes were analysed in triplicate. Single data are reported as rhombuses (18°C), triangles (22°C) and circles (28°C). White and black colours discriminate data on the two different pools of tissues.

dependence of overall activity may also serve to explain our observations.

In this context, the more rapid escape response performed by fish at 28°C can be explained by a generalised high responsiveness to the environment. However, all fish, independent of the temperature at which they were housed, showed a fright response to the ball thrown into the tanks, confirming the flight response as an innate behavioural component of their defence behaviour.

In this study, the response to a mirror image was used to investigate temperature-dependent social responses in the sea bass. Responses of fish to mirrors have been observed in several different contexts, including predator avoidance (Milinski 1987; Bisazza et al. 1999) and agonistic encounters (Meliska et al.

1980; Bronstein 1985). Furthermore, fish recognise unusual characteristics of the mirror image in comparison with a real fish image, showing a differential brain response that may reflect a cognitive distinction involving fear experience (Desjardins & Fernald 2010). Moreover, C-start movements have been described during hatching and feeding (Canfield & Rose 1993). The relatively high latency periods observed in this study suggest that the fish showed a cautious approach to the mirror at both the lowest and the highest temperature tested. The fish at the intermediate temperature (22 ± 1 (SE) °C) displayed major interest in the mirror test, interacting with the stimulus more rapidly and for a longer time than at the extreme temperatures of the tested range, thus exhibiting C-start reactions and the highest arousal levels. Accordingly, zigzag swimming, buccal pumping and dorsal fin movements around and towards the mirror were primarily found in the fish maintained at 22°C. This observation indicated a general state of excitement resulting from the stimulus and most likely associated with agonistic behaviour (Pickett & Pawson 1994). Responses such as gill erection and bites directed towards the mirror have also been interpreted in terms of aggressive motivation (Meliska et al. 1980). Unfortunately, the distension of the opercula, sometimes associated with agonistic posturing in the sea bass (Pickett & Pawson 1994), could not be measured in the current study because the movements of the fish did not allow this pattern to be followed for the entire duration of the test. Several studies have observed optimal growth rates and feed conversion efficiency in the sea bass at 22°C relative to cooler (16°C) and warmer (29°C) waters (Gardeur et al. 2001; Pichavant et al. 2001; Person-Le Ruyet et al. 2004). According to these studies, the mirror responsiveness shown by fish at 22°C may represent an indirect measurement of good health status in terms of a strong propensity for social interaction, including aggressive/fear displays. The results of the WB analysis demonstrated that temperature acclimation involves region-specific modulation of ChAT expression. In particular, a general increase in ChAT expression was found in most brain and spinal regions of the fish acclimated to 22°C and 28 ± 1°C relative to the fish acclimated at 18 ± 1°C. The opposite pattern was observed in the midbrain optic tectum. This finding suggests that ChAT levels are not directly related to the metabolic rate but are specifically modulated in different brain centres.

In the present study, the variation of ChAT expression was analysed as a valuable marker for neuronal activity in the CNS. Therefore, ChAT expression must not be understood as an indirect measure of the amount of ACh in the brain vesicles because the concentration of this neurotransmitter depends on its synthesis by ChAT and its degradation by AChE. However, given the behavioural results, the almost generalised increase of ChAT protein expression in the brain and spinal cord homogenates might be related to the large amount of ACh required by cranial and spinal motoneurons to regulate the improved motor performances observed in fish acclimated to high temperatures in comparison with fish acclimated at 18°C.

ChAT levels also increase in the forebrain (telencephalon and diencephalon) in response to thermal conditions. Because the first response of fish to thermal changes is typically behavioural and involves migration to a more thermostable microenvironment, the increased ChAT expression in the forebrain may be related to the neuroendocrine activation of behavioural thermoregulation. In this context, experiments in goldfish have demonstrated that behavioural thermoregulation is mediated by ACh and that the amount of ACh in the preoptic region and the basal telencephalon can affect the ambient temperature selected by the fish (Crawshaw & Wollmuth 1992). The increased ChAT expression found in the cerebellum in the current study is in agreement with the increase in ChAT activity found in goldfish acclimated from 5 to 22°C and 35°C (Poli et al. 1997). In contrast to all other brain regions, the optic tectum showed a decrease in ChAT expression in the sea bass acclimated to high temperatures. This finding suggests that ChAT levels are not merely related to the metabolic rate but are differentially modulated in the brain centres. The midbrain tectum is involved in visual processing, and it is also one of the main sensorimotor integration centres of the teleostean CNS (Meek & Nieuwenhuys 1998). The decrease in ChAT levels in this region could be responsible for changes in the coordination of movements.

Studies from our group have recently demonstrated that ChAT expression decreases in the whole-brain homogenates of wild juvenile sea bass (s.l. 3–4 cm) acclimated at 26°C relative to those acclimated at 18°C (Malavasi et al. 2013). The discrepancy in ChAT expression between wild sea bass juveniles (s.l. 3–4 cm) and juveniles (s.l. 10 cm) from a commercial hatchery could be related to the difference in ages and/or to the genetic variability of the populations analysed. In fact, cholinergic systems may fulfil different roles in response to environmental changes occurring at different life stages.

However, the differences in ChAT regulation following thermal changes could arise from putative genetic differences between wild juvenile sea bass captured in the Venetian Lagoon and juveniles provided by a commercial hatchery. Nevertheless, both experiments provide evidence that the activity of cholinergic neurons is modulated in response to external temperature. Different sea bass populations of the same age should be examined to clarify the role of genetic and developmental factors in the response of the cholinergic system to thermal changes.

Fish maintained at 28°C showed more rapid growth than fish maintained at 22°C and 18°C, as expected, although these data were obtained from a sub-sample from each experimental group. Body size is known to affect aggressive and competitive behaviour if food resources are severely limited (Knights 1987; Dugatkin & Reeve 1998). In the present study, the scarcity of food was not tested as experimental condition and the size-dependent competitive behaviour could not to be considered because fish pairs were similar in body size within each thermal treatment. Aggressive behaviour was indirectly considered in the mirror test because the fish's own image induced a response. Even in this case, no evidence was found that body size affected the observed behaviour. Finally, no data in the literature suggest or support an effect of body size on ChAT expression levels. Nevertheless, such effects cannot be excluded on the basis of the present experiments. Future studies of groups of different-sized fish will be aimed at obtaining a specific clarification of the effect of body size on the behaviour of thermally acclimated fish.

Overall, the present findings suggest that water temperature exerts an impact on certain aspects of both behaviour and CNS neurochemistry in juvenile European sea bass. In the present experiments, we exposed the fish to 3 wk of thermal acclimation. This experimental condition should be viewed as 'long-term' rather than 'short-term' acclimation, which, instead, involves acute responses of membranes/molecules within minutes or hours.

The present results point to putative effects of physical parameters such as water temperature on CNS activity and fish behaviour. Given the commercial value of European sea bass and the extensive systems of farming in which it is involved, these effects need to be monitored. Our data may be helpful in the context of aquaculture, for example net pen systems, in which animals cannot migrate to cooler water.

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## Conflict of Interest

All of the authors declare no conflict of interest.

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