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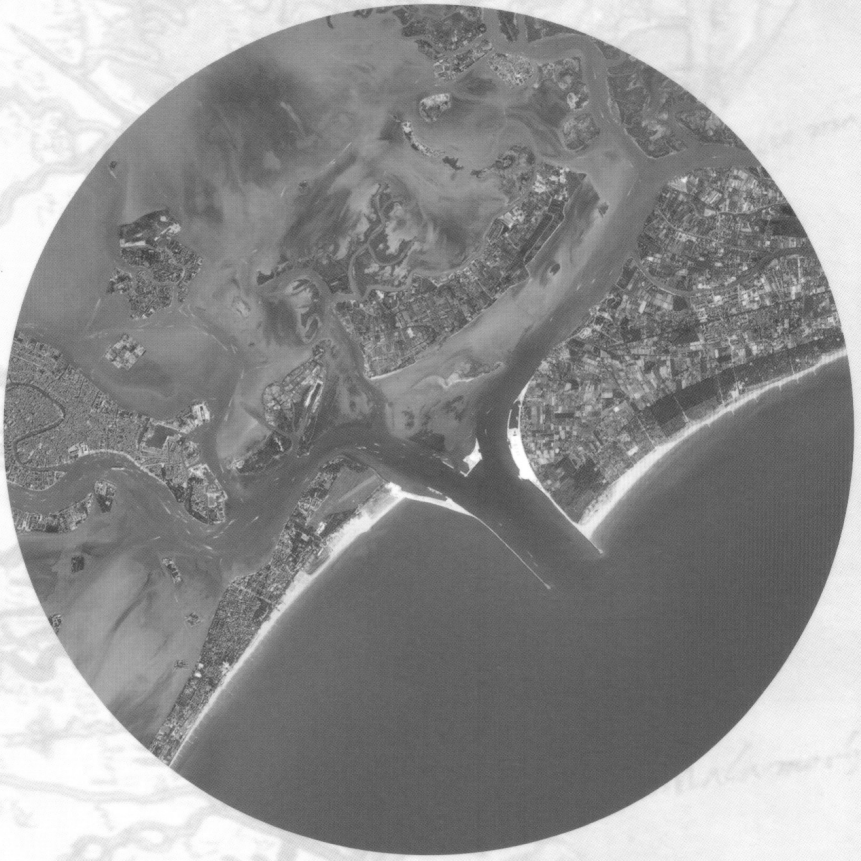

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BEHAVIOURAL AND PHYSIOLOGICAL EFFECTS OF TEMPERATURE INCREASE ON EUROPEAN SEA BASS (*Dicentratchus labrax L.*) AND GILTHEAD SEABREAM (*Sparus aurata L.*)

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Riassunto

Nell'ambito del progetto "Analisi neurochimica e comportamentale degli effetti del cambiamento climatico e conseguente aumento della temperatura sull'attività del sistema nervoso centrale nelle specie ittiche di interesse alieutica quali spigola *(Dicentratchus labrax)* e orata (*Sparus aurata*)", sono stati svolti esperimenti condotti in condizioni di laboratorio controllate su esemplari giovanili e post-larvali di spigola. In questo lavoro vengono presentati alcuni dei risultati finora ottenti riguardanti l'attività di nuoto, il comportamento affiliativo e quello alimentare. Vengono inoltre riportati i risultati di esperimenti di western blot e di immunofluorescenza effettuati per indagare l'effetto della temperatura sui sistemi colinergici e nitrergici di esemplari giovanili.

L'attività di nuoto e il comportamento affiliativo sono stati studiati in giovanili (10-15 cm di lunghezza standard) esposti per un periodo di 21 giorni a due differenti temperature (18 °C e 22 °C) quando posti in presenza di prede (*Chironomus salinarius)*, di uno stimolo olfattivo e di uno avverso. Inoltre, gli effetti della temperatura (tre trattamenti a 19, 22, e 26 °C) e della densità di prede (nauplii di *Artemia*, due trattamenti, bassa vs alta densità) sono stati testati sul comportamento alimentare di fasi post-larvali di spigola (13-20 mm di lunghezza standard). I risultati mostrano che dopo 21 giorni di alta temperatura gli animali modificano la loro attività di nuoto, passando da una locomozione esibita prevalentmente sul fondo della vasca a un utilizzo più omogeneo dell'ambiente. Inoltre, gli individui giovanili mantenuti a 22 °C, quando posti in presenza di uno stimolo olfattivo di tipo alimentare, mostrano un'attività esplorativa di gruppo maggiore di quelli mantenuti alla temperatura più bassa. Diversamente, la temperatura non risulta avere effetto nell'esibizione del comportamento affiliativo in risposta a uno stimolo avverso. Per quanto riguarda le fasi post-larvali, i risultati mostrano che gli animali aggiustano le loro tattiche alimentari tanto in relazione alla temperatura quanto alla densità di prede, riducendo l'attività predatoria alle

basse temperature e alte densità, e aumentando l'attività predatoria alle alte temperature, a scapito dell'attività di nuoto.

Le analisi di western blot effettuate sugli omogenati del cervello degli stessi giovanili osservati nei test comportamentali, dimostrano che gli enzimi colina acetiltransferasi (ChAT) e ossido nitrico sintasi neuronale (nNOS/NOS1) sono espressi con un pattern simile a 18°C e a 22°C. Ad entrambe le temperature ne la proteina da shock termico HSP70 ne l'isoforma inducibile dell'ossido nitrico sintasi (iNOS/NOS2) sono espresse. Questi risultati suggeriscono che l'aumento della temperatura testato non determina effetti da stress individuabili a livello cellulare.

Abstract

In regard to the research project "Analisi neurochimica e comportamentale degli effetti del cambiamento climatico e conseguente aumento della temperatura sull'attività del sistema nervoso centrale nelle specie ittiche di interesse alieutica quali spigola *(Dicentratchus labrax)* e orata (*Sparus aurata*)", experiments under laboratory conditions on juveniles (10-15 cm, Standard Length) and post-larval stages (13-20 mm, Standard Length) of European sea bass were carried out. We present preliminary results on swimming activity, affiliative and feeding behaviours. Moreover, results of western blot and immunofluorescence analysis on the effects of the increased temperature on cholinergic and nitrergic systems of juveniles are reported.

Behavioural tests such as prey presence, olfactive cue and object introduction were proposed to juveniles maintained at 18 and 22 °C over a period of 21 days to investigate the effects of temperature on swimming activity and affiliative behavior. Furthermore, effects of increased temperatures (19, 22, 26 °C) and prey density (*Artemia* nauplii) were tested on feeding behaviour of post-larval stages. Results showed that juveniles changed their swimming activity with a reduction of the time spent on bottom after 21 days at the upper temperature and the presence of peer groups in the area with olfactive cue resulted to be higher at 22 than 18 °C. Conversely, affiliative behaviour did not affect from temperature in response to object presence. Post-larval stages adjusted their feeding tactics in relation to both temperature and density, reducing predatory attacks at low temperatures and high density, and increasing predatory attacks at higher temperatures, at the expense of swimming activity.

Western blot analysis performed on the brain homogenates of the juveniles observed in behaviour tests, demonstrated that both the enzymes choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS/NOS1) are expressed with a similar pattern at both 18°C and 22°C. Neither the heat-shock protein HSP70 nor the inducible isoform of nitric oxide synthase (iNOS/NOS2) were expressed at both temperatures. These results suggested that the tested increased temperature does not induce detectable stressor effects at the cellular level.

1. Introduction

The increase of temperature is thought to be one of the main effects of climate change (IPCC, 2007) and highly unpredictable environments such as lagoons and coastal wetlands are recognized to be particularly affected by these changes (Eisenreich, 2005). One of effects of the projected increase of temperature is the influence on physical, chemical and biological properties of aquatic ecosystems, with predominantly adverse impacts on many animal and plant species, community composition and water quality (IPCC, 2007). Based on this evidence, the research project "Analisi neurochimica e comportamentale degli effetti del cambiamento climatico e conseguente aumento della temperatura sull'attività del sistema nervoso centrale nelle specie ittiche di interesse alieutica spigola *(Dicentratchus labrax* L.) e orata (*Sparus aurata* L.)" (Progetto Corila-Regione Veneto) provides an experimental approach to the analysis of effects that the raising temperature may have on behavior and physiology of commercially important fish species. In particular, the European sea bass was selected as study model species, to extend then the results and conclusions also to the gilthead sea bream . The European sea bass is slightly more sensitive to higher temperatures than the gilthead sea bream, constituting a more conservative model to test the effects of increased temperature. The juvenile stages of the European sea bass are a valuable biological resource in the Northern Adriatic lagoons, including the Venice lagoon, where they are subjected to specialized forms of aquaculture and fisheries.

The combination of behavioral studies with neurochemical and neuromorphological investigations allows to correlate the sub-cellular and cellular responses to temperature increase and to their possible ecological consequences due to potential alterations of behaviors significantly affecting the individual fitness. The experimental control of temperature allows to simulate the potential warming of some degrees, as it is thought to occur in shallow sea basins and wetlands, such as the Venice lagoon.

In this extended abstract we present preliminary results of some experiments carried out in the framework of the project, regarding i) the effect of temperature and prey density on feeding behaviour of post-larval stages of *D. labrax, ii)* the effects of temperature on swimming activity and affiliative behaviour of juveniles *D. labrax* iii) the effects of the increased temperature on cholinergic and nitrergic systems.

2. Materials and Methods

2.1 Effects of increased temperature on feeding behaviour of post-larval stages of the European sea bass D. labrax (L.) *in relation to prey density*

The effects of increasing temperature, in relation to prey density, was tested on the feeding behaviour of post-larval stages of the European sea bass *Dicentrarchus labrax* L. under controlled laboratory conditions. Feeding behaviour was analysed in relation to three temperature treatments (19, 22 and

26 ° C) and two prey density treatment (high vs low density treatment). *Artemia* nauplii at 4 days from hatching were used as living prey. Post-larval stages of European sea bass from hatchery origin (13-20 mm of Standard Length) were assigned to three thermal treatments, allocating them into three different communal tanks (50 specimens for each tank) of 200 L capacity, where the acclimatization process was started. Room temperature was maintained at 19 ° C and all fish were initially maintained at this base-line temperature. In two of the tanks, temperature was then gradually raised of 1 °C/day to reach 22 and 26 °C in each tank respectively, whereas in the reaming tank the base-line temperature of 19 °C was not further changed. Once treatment temperatures were reached, fish were subjected to an acclimatization period of 21 days, before the beginning of experiments. Experiments were conducted in small glass tanks of about 3 l capacity, illumined by a suspended 60 W lamp and filled with the same water of the acclimatization tanks. Before the beginning of the experiments, larvae were subjected to a starvation period of 12 h, to standardise the motivational hungry level. Fish were randomly captured from the acclimatization tanks and transferred singly into the small experimental tank. After a period of 10 minutes of acclimatization, the experiment trials could start by releasing Artemia nauplii into the tank by means of a pipette. Depending on the prey density treatment assigned (low: d1 and high: d2), a solution of nauplii was introduced into the tank to reach a prey density of 400 nauplii/L (d1) and 1400 nauplii/L (d2), respectively. By means of a digital video-camera focused on the fish, the behaviour of the experimental specimens was recorded for about 10 minutes, starting from the release of the nauplii into the tank. The video-recording of behaviours allowed then to measure the main MAPs (Modal action Patterns, according with Brown 1986, and Georgalas et al. 2007) characterising the larval behaviour. In the present work, the results related to two behavioural variables are reported: "normal swimming", that is the "ordinary" swimming activity performed with apparently constant speed and sustained by rapid movements of the caudal fin (expressed as percent time on the total recording time, %), and the feeding attacks, that is the lunge associated to prey ingestion and mastication (expressed as frequency, n/min). Results were analysed by non-parametric statistics.

2.2 Effects of increased temperature on swimming activity of juveniles of the European sea bass Dicentrarchus labrax (L.) *in relation to prey presence*

The effects of increasing temperature, in relation to prey presence, was tested on the swimming activity of juveniles of the European sea bass under controlled laboratory conditions. Swimming activity was analysed in relation to two temperature treatments (18 and 22°C) in presence of living prey *Chironomus salinarius*. Sixty juveniles of European sea bass (10-15 cm of Standard Lenght, hatched and reared at the farm facility of Panittica Pugliese, BR Italy) were assigned to 2 thermal treatments, allocating them into six different communal tanks (10 specimens for each tank, with 3 replicates for each temperature treatments) of 180 l capacity. All fish where initially maintained at the temperature present in the farm facility, equal to 20°C. In six experimental

tanks, temperature was then gradually raised or decreased of 1°C/day to reach 18 and 22 °C, respectively. The temperature of 18 °C represented the base-line condition. Fish were subjected to an acclimatization period of 21 days. After 24 hours that the treatment temperatures were reached, a solution of *Chironomus salinarus* was introduced into each tank by means of a pipette, to reach a prey density of 30 *C. salinarus*/180l. This procedure was replicated at the day 7, 14 and 21. Before the beginning of the experiments, juveniles were subjected to a starvation period of 24 h, to standardise the motivational hungry level. By means of digital video-camera focused on the tank, the behaviour of the specimens was recorded for about 5 min., starting from the release of the *C. salinarus* into the tank. The video-recording and the use of specific software package (Observer 3.0, NOLDUS 1991) allowed then to measure the swimming behaviour, as well as the feeding attacks. In the present abstract, the results related to two swimming variables on day 1 and 21 are reported: "swimming on the bottom", that is, the presence of 3 or more individuals on the bottom of tank (expressed as time spent on the bottom, s) and "swimming on the top" performed with presence of 3 or more individuals on the top of tank (expressed as time spent on the top, s). Results were analysed by non-parametric statistics.

2.3 Effects of increased temperature on affilliative behaviour of juveniles of D. labrax (L.) *in relation to olfactive and aversive stimulus*

Fish maintained in the experimental conditions described above were subjected to olfactive cue and aversive stimulus challenges to investigate the effects of the increasing of temperature on affiliative behaviour. After 24 hours from the reaching of treatments temperatures, and then at 7, 14 and 21 day, the two challenges were proposed to individuals.

The olfactive cue test was performed using five larvae of *Sarcophaga carnaria* inserted into a holed steel tin can and dipped in a side of tank. By means of digital video-camera focused on the tank, the behaviour of the specimens was recorded for about 10 min, starting from the dipping of larvae into the tank. The video-recording and the use of specific software package (Observer 3.0, NOLDUS 1991) allowed then to measure behaviours such as "cohesiveness around cue", "contact with cue", "excitatory events". In the present work, the results related to "cohesiveness around cue" in the day 1 and 21 are reported. This behaviour is defined as the presence of three or more fish in proximity of olfactive cue and expressed as frequency (events/min).

With regard to aversive stimulus challenge, a black ball (4 cm diameter) was thrown into the tank. Few seconds before the ball dropping, a video recording started and went on 10 min. By video recording, behaviours such as "flight response", "contact with object", "cohesiveness around object", "excitatory events" were measured. In the present abstract, the results related to "cohesiveness around object" in the day 1 and 21 are reported. This behaviour is defined as the presence of 3 or more fish in proximity of object and expressed as frequency (events/min). All results were analysed by non-parametric statistics.

2.4 Samples collection for western blot and immunofluorescence analysis

In western blot and immunofluorescence experiments were analyzed the same juveniles studied in the behaviour tests described above. At the end of the observations, samples were anesthetized with 2-phenoxyethanol (0,3 ml/L) and decapitated for western blot analysis or transcardially perfused for immunofluorescence experiments.

2.4.1 Western blot experiments

Brains (N=4) were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 % Triton X100, 5 mM NaF, 1 mM Na3VO4, 10 mM β-glycerolphosphate, 1 mM PMSF) containing protease inhibitors (Roche, Germany) and the particulate matter was removed by centrifugation at 10.000g for 20 min. Rat brain was used as positive control. The protein concentration was determined by the Lowry method (1951). For electrophoresis analysis, the samples were boiled in a Sample Buffer for 5 min to denature the proteins. Then, 100 µg of proteins was loaded in each lane and separated in 8 % SDS-polyacrylamide gels (SDS-PAGE) according to Laemmli (1970). After electrophoresis, gels were immunoblotted on nitrocellulose paper (Hybond C+ Extra, GE Healthcare, UK). Membranes were stained with Ponceau S to confirm transfer of proteins and succesively immunolabelled with the following antibodies raised against mammalian proteins: anti NOS 1 C-terminus (Santa Cruz Biotechnology, U.S.A.) diluted 1:500, anti NOS 2 (Santa Cruz Biotechnology, U.S.A.) diluted 1:500, anti ChAT (Millipore, USA) diluted 1:1000, anti HSP 70 (Santa Cruz Biotechnology, U.S.A.) diluted 1:200 and anti actin diluted 1:600 (Santa Cruz Biotechnology, U.S.A.).

Detection was performed using the ECL Plus system (GE Healthcare, UK). In electrophoresis experiments, the molecular weight marker "Wide range (6.5–205 kDa)" (Sigma-Aldrich, U.S.A.) was used.

2.4.2 Immunofluorescence analysis

Fish were transcardially perfused with 4% paraformaldehyde (PFA), the brain was dissected out, post-fixed in PFA for 24 hours, and cryoprotected in PBS containing 30% sucrose for 48 hours. Thereafter, samples were embedded in O.C.T. compound (Tissue-Tek II, Quiagen, Italy), frozen and cut in a cryostat into 10-20 µm transverse sections. Consecutive serial sections were collected on microscope slides coated with chromalum gelatin and processed for immunofluorescence. Briefly, sections were permeabilized with PBS plus 0.5% Triton-X-100 (PBST), blocked with 3% Bovin serum albumine in PBST for 30 min. and succesively incubated with a goat anti ChAT antibody (diluted 1:50) overnight at 4°C. The sections were then incubated for 1 h at room temperature with a CY3-conjugated anti-goat antibody (diluted 1:50). After several washes with PBS, the sections were mounted on slides coverslipped and observed with a fluorescence microscope.

3. Results and discussion

3.1 Effects of increased temperature on feeding behaviour of post-larval stages of D. labrax (L.) *in relation to prey density*

Within the low density treatment (d1), no statistically significant effect of temperature was detected (Kruskal-Wallis test, $p>0.05$). By contrast, within the high density treatment (d2), there were statistically significantly differences across temperatures in terms of both percent swimming activity and attack frequency (Figure 1, a and b).

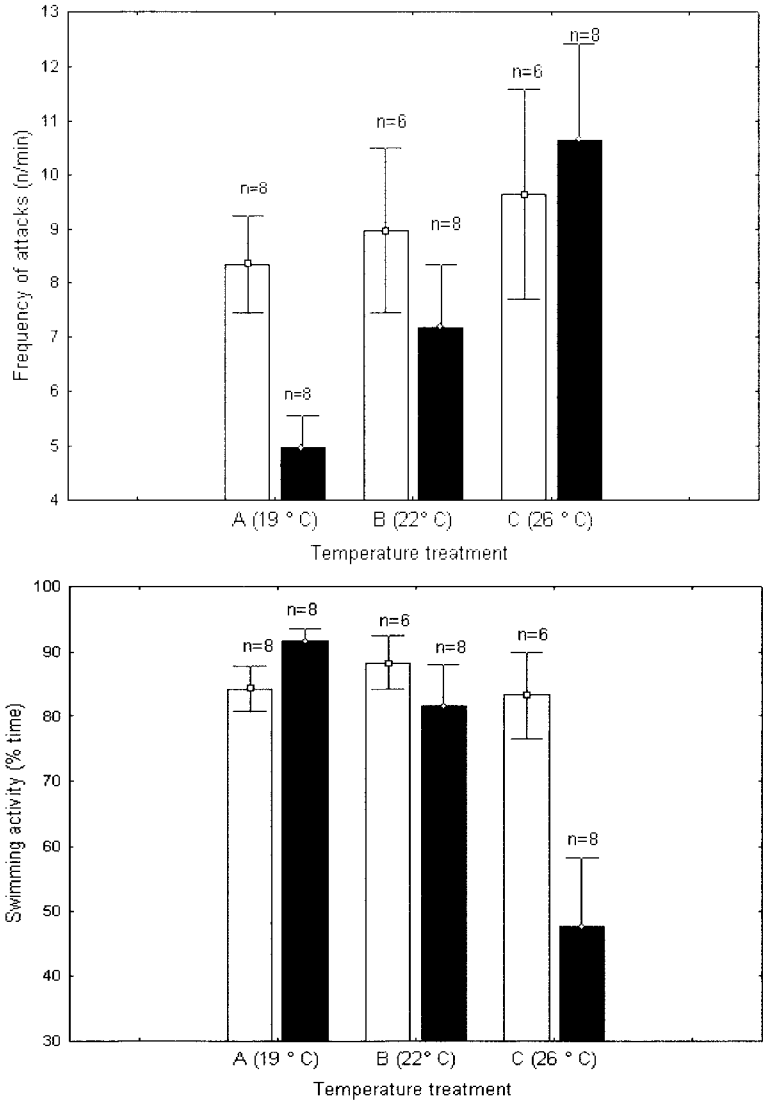

Fig. 1. Frequency of feeding attacks (a) and percent time of swimming activity (b) in the post-larval European sea bass as regards three temperature treatments and two density treatments (white bars= low density, d1=400 Artemia nauplii/l; black bars= high density, 1400 Artemia nauplii/l). Numbers on the bars refer to the number of replicates conducted for each treatment.

Percent of swimming activity decreased significantly with increasing temperature, whereas frequency of attacks showed an opposite pattern, with the number of attacks increasing at higher temperatures. Within the base-line temperature of 19 °C, the frequency of attacks was higher in the high-density treatment than in the low density treatment (Mann-Whitney Test, $p< 0.05$), whereas within the highest temperature treatment (26 °C), the swimming activity was significantly lower in the low-density treatment than in the high-density treatment.

Body size of fish was not significantly different between density treatments whereas experimental fish were significantly larger in the highest temperature treatment (26 °C) with respect to intermediate and low temperatures (Kruskal-Wallis test, $p< 0.05$), and this was likely to be the consequence of a higher growth rate in the warmer tank.

These results suggest that the feeding strategy is modulated as a response to the combined effect of temperature and prey density with fish changing their behaviour especially at the extremes of the temperature range, and at higher prey density. The reduction of feeding rate at the base-line temperature of 19°C seems to suggest that the so called confusion effect is acting: fish attack less frequently the prey, to probably be more vigilant in terms of anti-predator responses. On the other hand, higher densities were able to induce higher feeding rate as temperature increased, at the expense of swimming activity at 26 ° C. This higher tendency to perform more attacks with increasing prey density could enhance growth at the expense of antipredator response, with potentially negative consequence on individual fitness.

3.2 Effects of increased temperature on swimming activity of juveniles of the European sea bass in relation to prey presence

With regard to "swimming on the bottom" and "swimming on the top", no statistically significant effect of temperature was detected between 18 and 22 °C on day 1 (Mann-Whitney Test, MWT, $p>0.05$, respectively). By contrast, there was statistically significantly difference across temperatures in terms of time spent on the bottom of tank during the day 21 (MWT, $p<0.05$). In particular, initially all fish mainly used the bottom of tanks, probably as response to an anti-predator strategy (Pickett and Pawson, 1994). Then, fish maintained at higher temperature shifted their swimming activity, balancing through the whole tank and including the top (Figure 2, a and b). This result can be likely the consequence of a higher metabolic demand and feeding rate at 22°C as response to the faster growing of animals (Barnabé, 1991), that drives fish to swim across the whole tank to search prey, with potentially negative consequences on individual fitness, being more easily vulnerable by aerial predator. Nevertheless, it should also noted that high temperature influences the oxygen availability (Taylor et al., 1997) could finally affect the swimming pattern of animals.

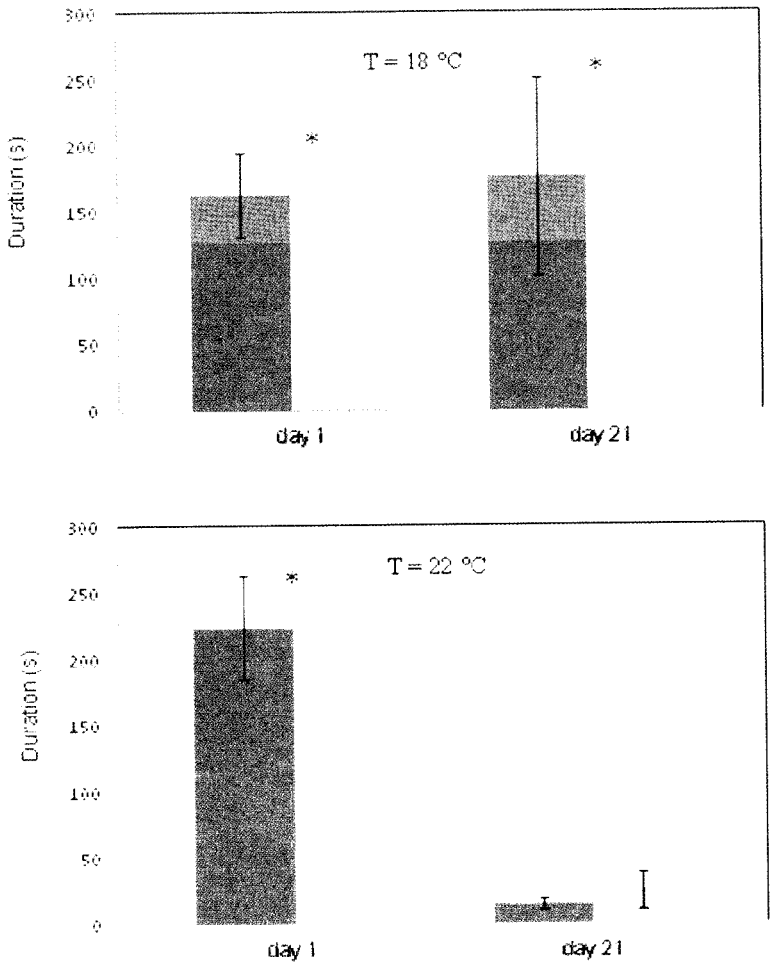

Fig. 2. Time spent (± S.E.) on the bottom or top of tanks (black grey bars= bottom; light grey bars= top) from three or more individuals at 18 °C (a) and 22 °C (b) during the acclimatization period. Three replicates were conducted for each temperature.

3.3 Effects of increased temperature on affilliative behaviour of juveniles of D. labrax (L.) *in relation to olfactive and aversive stimulus*

Statistically significant effect of temperature for behaviour "cohesiveness around cue" during the day 21 was found (MWT, $p<0.05$). Fish maintained at 22 °C over 21 days established shoal to approach olfactive cue more frequently than those at 18 °C (Figure 3a). By contrast, in presence of aversive stimulus no statistically significant effect of temperature was found (MWT, $p>0.05$), even if after 21 day of temperature treatments a trend with fish maintained at 18 °C more interested in shoaling to explore object was present (Fig. 3b). These results seem to suggest an influence of temperature on affiliative behaviour of juveniles of *D. labrax* in response to feeding stimulus. In accordance with results described above, higher explorative activity seems to be present to

satisfy higher feeding rate associated with a warmer temperature. Instead, prolonged periods of upper temperature did not influence the response to negative stimulus. Higher frequency of shoaling from fish at 18 °C in correspondence of object during 21th day may be due to higher presence of individuals on bottom of tank where object is located. In conclusions these preliminary results show an effect of high temperature on changes in the feeding strategy, whereas no significant impact of temperature seems to be as respect to anti-predatory response.

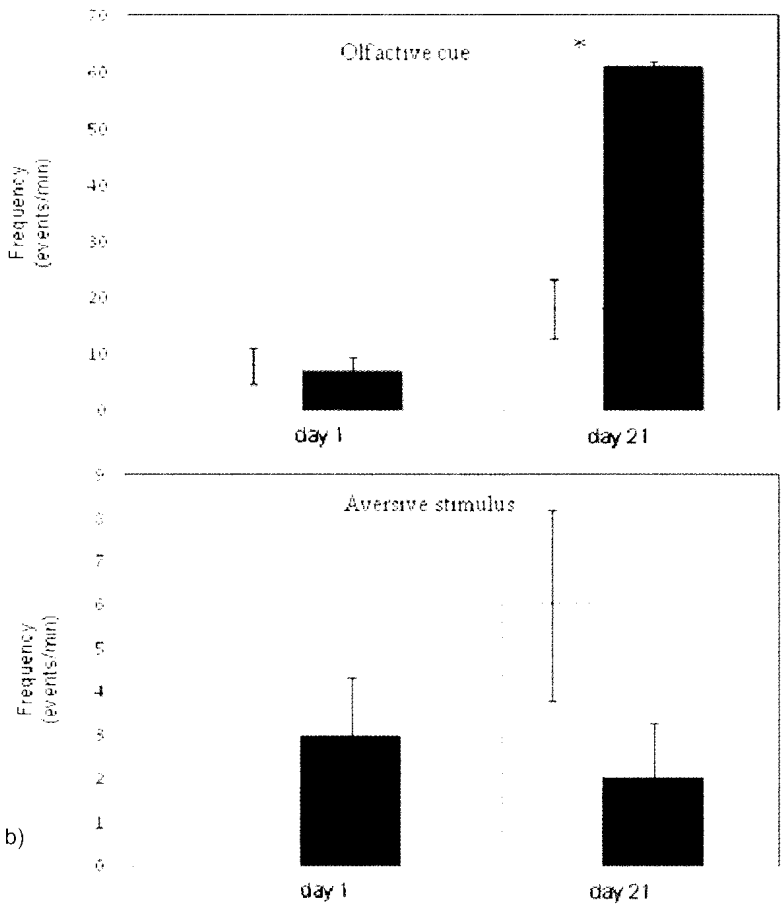

Fig. 3. Frequency (± S.E.) of visits in the area with olfactive cue (a) and object (b) of three or more individuals as regards two temperature treatments (white bars= 18 °C; black bars= 22 °C) during the acclimatization period. Three replicates were conducted for each temperature.

3.4 Effects of increased temperature on the expression of ChAT and NOS1

To investigate the cholinergic system, we have used antibodies against the enzyme ChAT, which catalyzes the one-step synthesis of the neurotransmitter acetylcholine (ACh) in the cytoplasm of cholinergic neurons. The effects of thermal acclimation on nitrergic system were investigated by antibodies against

the NO- producing neuronal nitric oxide synthase (nNOS/NOS1), that is expressed in the brain of most vertebrates, including fish. ACh is co-localized with NO in the fish brain (Giraldez-Perez et al., 2009) and a cross-talk between the two enzymatic pathways was suggested since the activation of ACh muscarinic receptor may influence the endogenous NO production in mammals (Marquez-Ruiz et al., 2007). However, NO production must be finely regulated since NO may be responsible for oxidative and nitrosative damages at the cellular level (Stamler, 1994).

The expression of ChAT and NOS1 were compared in brain homogenates from fish acclimed to 18° and 22°C by Western blot. Immunoblots performed with a polyclonal anti-ChAT antiserum revealed the presence of two bands with a slightly higher and lower molecular weight than the rat brain ChAT (68-70 kDa) used as control (Fig.4 C).

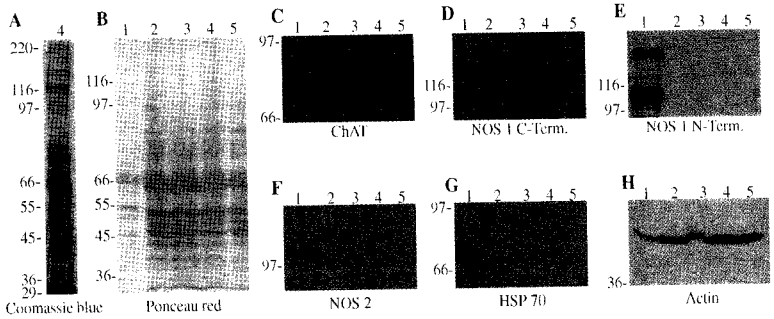

Fig. 4. Western blot analysis of protein homogenates of rat brain (lane 1, as a positive control) and of the brain of European sea bass specimen acclimated at 18°C (lanes 2 and 3) and at 22°C (lanes 4 and 5). Coomassie blue (A) and Ponceau red (B) staining of brain proteins. Immunoblotting cross-reactivity with different antibodies raised against mammalian proteins (C-H). Polyclonal anti ChAT antibody (C), polyclonal antibodies against the C-terminus (D) and N-terminus (E) of nNOS, polyclonal anti NOS2 antibody (F), monoclonal anti HSP 70 antibody (G) and monoclonal anti actin antibody (H). Polyacrylammide gel concentration: 8%.

The immunoreaction product increased as a function of the amount of protein (not shown) and these results indicated that the antibodies specifically recognized fish ChAT proteins. The two bands were detected at both T0 and T1. Thus, ChAT expression in the sea bass brain showed a temperature-insensitive pattern at the temperatures tested. The two protein bands could represent two different ChAT isoforms that are specifically expressed in this species. Indeed, a similar Western blot analysis revealed a single band around 68-72 KDa in the goldfish (Giraldez-Perez et al., 2009). The expression of NOS1 was analyzed by two different polyclonal antibodies raised against the C-terminus or the N-terminus of this enzyme (Fig. 4 D and E). A single band around 155 kDa was detected by the antibody against the C-terminus (Fig. 4 D) at both T0 and T1, whereas no bands were detected by the antibody against the N-terminus (Fig. 4 E). A similar positive band was found in the rat brain homogenates used as control. These results demonstrated that sea bass NOS1 molecules are expressed in the brain at both temperatures. They also suggested that the C-terminus of sea bass NOS1 is evolutionary conserved more than the N-terminus, which was not recognized by heterologous antibodies.

The distribution of cholinergic neurons was analyzed by ChAT immunofluorescence in the brain of a specimen acclimed to 22°C. ChAT

positive cells were widely distributed in the brain vesicles, with the exception of the telencephalon, where they were only detected in the paired olfactory bulbs (Fig 5 A). ChAT immunoreactive (ChAT-ir) cells were localized in the preoptic region of the diencephalon, the optic tectum (Fig 5 B, C), the tegmentum of the mesencephalon (Fig 5 D), the cerebellum (Fig 5 E), the rostral (Fig 5 F) and caudal (Fig 5 G, H) medulla oblongata. Within the optic tectum, the only ChAT-ir neurons appeared in the periventricular layer (Fig. 5 B).

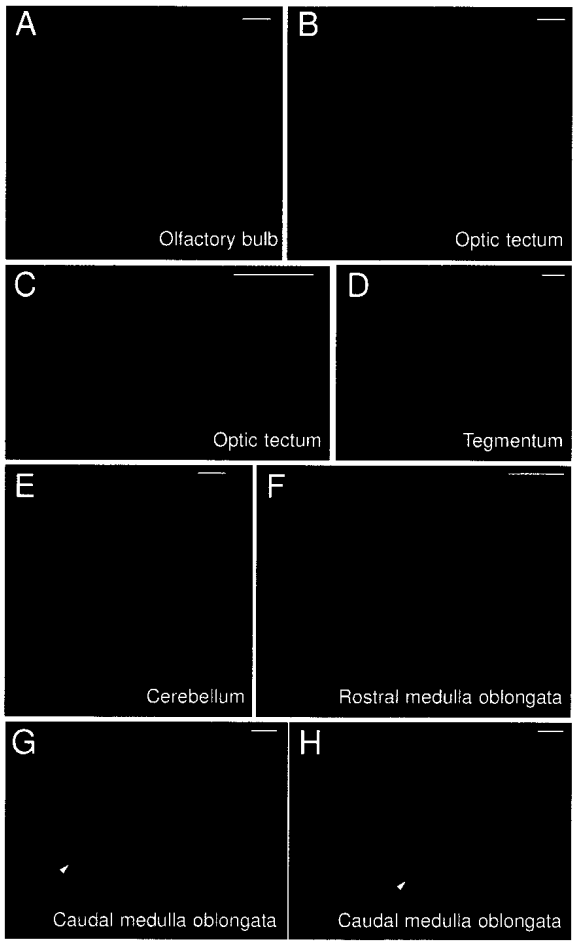

Fig. 5. Distribution of ChAT immunoreactivity in the brain of European sea bass acclimated at 22°C. A: ChAT positive cells in the olfactory bulb. B and C: photomicrographs of the optic tectum showing ChAT positive cells in the periventricular stratum; D: immunolabelled cells in the the tegmentum of the mesencephalon; E: ChAT positive cells can be observed in the valvula cerebelli; F-H: distribution of ChAT positive cells in the rostral (F) and caudal (G, H) part of medulla oblongata. The white arrowheads in G and H indicate the same cell at different magnification. Scale bar= 50 um expet for G (200 um).

Positive cells showed pyriform somata and one apical dendrite oriented perpendicular to the lamination. This thick dendrite ramified in the superficial fibrous layer which was also positive (Fig. 5 C). This type of cholinergic neurons were identified in the optic tectum of different species and considered a well-conserved feature among all groups of teleosts (Clemente et al., 2004). ChAT immunoreactivity is widely distributed in the sea bass tegmentum, in the oculomotor (III) and trochlear (IV) nuclei (Fig. 5 D) as well as in the rostral

medulla oblongata, where motoneurons of the trigeminal (V), abducens (VI) and facial motor nuclei are cholinergic (Fig. 5 F). In the caudal medulla oblongata, motoneurons of the glossopharyngeal (IX) and vagal (X) motor nuclei also appeared ChAT immunoreactive (Fig. 5 G,H). In the sea bass cerebellum, ChAT-ir cells were only observed in the valvula cerebelli (Fig. 5 E).

3.5 Effects of increased temperature on the expression of HSP and NOS2

We have also examined the expression of the heat shock protein HSP70 and the inducible NOS2 by Western blot to evaluate the effects of the increased temperature on molecular markers of the cellular stress response. Western blotting experiments performed by polyclonal NOS2 or HSP 70 antibodies did not reveal the expression of the iNOS isoform or the heat shock protein in the brain homogenates of fish acclimed to both temperatures (Fig. 4 F). These results suggested that the increased temperature did not cause a cellular stress sufficient to induce the expression of HSP 70, a molecular marker of thermal stress, and of the inducible isoform of NOS in the brain of European sea bass.

Conclusions

Although the present results are still preliminary, they suggest that different aspects of the behavioural repertoire of the study species, the European sea bass *Dicentrarchus labrax* L., could be significantly changed by increased temperatures, with potential consequences on individual fitness. Further experiments will allow to better understand the behavioural impairment determined by temperature increases and the relationships between the neurochemical and the behavioural level.

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