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Protein synthesis is lowered while 20S proteasome activity is maintained following acclimation to low temperature in juvenile spotted wolffish (*Anarhichas minor* Olafsen)

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SUMMARY

The effects of temperature on protein metabolism have been studied mostly with respect to protein synthesis. Temperature generally has a parabolic effect on protein synthesis with a maximum rate being observed at optimal growth temperature. The effect of temperature on protein degradation is poorly understood. The 20S proteasome is mainly responsible for the degradation of short-lived and oxidatively modified proteins and has been recently identified as a potentially good proxy for protein degradation in fish. The aim of this experiment was to examine the relationships between the rate of protein synthesis, activity of the 20S proteasome, oxidative stress markers and antioxidant capacity in white muscle of juvenile spotted wolffish (*Anarhichas minor*) acclimated at three temperatures (4, 8 and 12°C). The rate of protein synthesis was lower at 4°C than at 8°C while it was intermediate at 12°C. Despite the decrease of protein synthesis at low temperature, the activity of 20S proteasome activity was maintained high in fish acclimated at lower temperature (4°C), reaching levels 130% of that of fish acclimated at 8°C when measured at a common temperature. The oxidative stress markers TBARS and protein-carbonyl content did not change among temperature groups, but reduced glutathione concentration was higher in cold-acclimated fish, suggesting a higher antioxidant capacity in this group. Our data suggest that lower growth rate in cold temperature results from both high 20S proteasome activity and a reduced rate of protein synthesis.

Key words: 20S proteasome, growth rate, protein synthesis, temperature.

INTRODUCTION

Protein turnover refers to the continuous degradation and renewal of intracellular proteins. These are hydrolyzed to their component amino acids and usually replaced by an equal amount of freshly synthesized protein (Hawkins, 1991). Positive growth occurs when the rate of synthesis surpasses the rate of degradation (Houlihan et al., 1995; Sugden and Fuller, 1991). Conversely, in the case of negative growth, protein degradation surpasses the rate of protein synthesis (Hawkins, 1991). Factors that have an effect on growth necessarily affect (directly or indirectly) protein synthesis, protein degradation or both. The rate of protein synthesis has received the most attention in the study of several intrinsic (e.g. species, mass, exercise, ontogenetic stage, nutritional condition, heterozygocity) and extrinsic factors (e.g. temperature, pollutants, oxygen levels and salinity) (for a review, see Carter and Houlihan, 2001; Fraser and Rogers, 2007). Although little is known about protein degradation, there is a strong indication that it is a very important process in the determination of growth rate (Dobly et al., 2004; Fraser and Rogers, 2007; Houlihan et al., 1995). For instance, in Atlantic wolffish (*Anarhichas lupus*), white muscle and whole-body fractional rates of protein synthesis (k_s; $\%\,day^{-1}$) were shown to increase linearly with temperature (McCarthy et al., 1999). However, the fractional rate of protein growth (k_g; $\%\,day^{-1}$) and protein retention efficiency (k_g/k_s) increased with temperature until they reached their optimal temperature and then decreased rapidly. As the upper thermal limit was approached, protein degradation increased while retention efficiency and growth decreased (Fraser and Rogers, 2007; McCarthy et al., 1999).

The inherent complexity of measuring protein degradation comes from the multiple pathways that are involved. Protein degradation is often estimated from the difference between protein synthesis and protein growth. This method, however, assumes that protein synthesis measured over a few hours is representative of growth rate measured over weeks. It also assumes that there is no change in tissue composition during the experiment. For now, however, it is the only method available to measure *in vivo* protein degradation (Fraser and Rogers, 2007). Several studies have focused on the activity of enzymes involved in protein degradation in fish experiencing muscle wasting conditions, such as food restriction, starvation (Guderley et al., 2003; Martin et al., 2002; Martin et al., 2001; Salem et al., 2007), migration and spawning in salmonids (Mommsen, 2004; Salem et al., 2006) that is also associated with starvation. In such situations, it is mainly the cathepsin and calpain systems that are responsible for protein mobilization as energetic substrate and/or as material for reproductive investment (Martin et al., 2002; Mommsen, 2004). To our knowledge, only one study has linked growth rate and the activity of one pathway of protein degradation in fish growing without food restriction or the complicated aspect of sexual maturation. In rainbow trout (*Oncorhynchus mykiss*), specific growth rate was negatively linked with the hepatic activity of 20S proteasome, and high proteasome activity was linked to decreased growth efficiency (Dobly et al., 2004). In a second study on starving rainbow trout, the activity of

20S proteasome was shown to decrease after 14 days of starvation in rainbow trout; most likely as a result of reduced protein turnover (Martin et al., 2002). These results indicate that the activity of 20S proteasome could be a good proxy to protein degradation in fish growing in 'normal' conditions.

The proteasome pathway is mainly responsible for degradation of oxidatively damaged proteins (Friguet, 2006; Perepechaeva et al., 2006; Poppek and Grune, 2006). This concept motivated us to assess if its activity is linked to the levels of markers of oxidative stress and the level of an antioxidant. Protein-carbonyls and thiobarbituric acid reactive substances (TBARS) were used as indicators of oxidative damage of protein and lipids, respectively. Reduced glutathione concentration was measured as an indicator of antioxidant capacity.

The aim of this study is to measure the impact of three temperatures (4, 8 and 12°C) on the rate of protein synthesis, activity of 20S proteasome and levels of oxidative stress markers and antioxidant in white muscle of juvenile spotted wolffish (*Anarhichas minor*), a close relative of Atlantic wolffish. The use of three acclimation temperatures within the species thermal range was utilized to generate variability in growth rate without introducing undue stress to fish. To our knowledge, there are no studies that have concurrently measured the rate of protein synthesis and 20S proteasome activity in an ectothermic animal.

MATERIALS AND METHODS

Growth trial

The growth trial and the measurement of protein synthetic rate were carried out in the aquaculture facilities of the Centre Aquacole Marin de Grande-Rivière (QC, Canada). The juvenile spotted wolffish used were the offspring of the domestic broodstock maintained at this facility since 1999. Prior to the experiment, fish were held at 8°C and fed to satiety according to established protocols. The growth trial took place during the period of April through May 2006 for 41 days using fish with a mean initial mass and length of 1.72±0.20 g and 5.86±0.25 cm, respectively. The fish were individually marked using visible implant elastomer (VIE, Northwest Marine Technology, Shaw Island, WA, USA) on the top of the head, and their initial mass and length were recorded before they were randomly transferred (12 fish per tank) into one of the three low-level rearing units (1.5 litre volume) supplied with oxygenated sea water (0.5 l min^{-1}) at 4°C, 8°C or 12°C (3.98±0.56°C; 8.09±0.5°C and 11.94±0.30°C) for the duration of the trial. Water temperature was controlled by mixing cold seawater with heated water in a mixing tank. Water depth was adjusted to 2 cm in order to facilitate feed ingestion (Strand et al., 1995). Fish were fed an adapted commercial feed (Gemma 1.0; Skretting, Bayside NB, Canada) to satiety daily for 41 days, and water quality was monitored daily. Fish were exposed to a 12 h:12 h L:D photoperiod, mean salinity was 29‰ and oxygen concentration was always over 80%.

Specific growth rate in mass was calculated using $SGR_m=[\log(M_f)-\log(M_0)]\times 100/t$, where M_f and M_0 are final and initial mass (g) and t is the length in days of the experiment. For SGR_l, M_f and M_0 were replaced by l_f and l_0, the final and initial length (cm), respectively. Fulton condition index (K) was calculated using, $K=M\times l^{-3}$, where M is fish weight and l is fish length (Ricker, 1975). Rates of protein synthesis, 20S proteasome activity, antioxidant and markers of oxidative stress were all measured on the same fish.

Protein synthesis

Protein synthetic rate was measured based on the principles of the flooding dose of radiolabelled phenylalanine method developed for rats (Garlick et al., 1980). In this experiment, we report the protein synthetic rate as the incorporation of nanomoles of phenylalanine per mg of protein per hour as expressed in other studies (Lewis and Driedzic, 2007; Treberg et al., 2005). Fish were starved for 24 h prior to experimentation. Each fish was injected intraperitoneally with 1 ml/100 g of the tracer solution. No anaesthetic was used during this procedure and the fish were immediately returned to their tank, thus protein synthetic rate is measured at acclimation temperature. The tracer solution was composed of 135 mmol l^{-1} phenylalanine containing L-[2,3,4,5,6-^{3}H]phenylalanine (GE Healthcare, Mississauga, ON, Canada) at a dosage of 1.85 MBq ml^{-1} in a buffered solution consisting of (in mmol l^{-1}) 150 NaCl, 5 KCl, 5 $NaPO_4$, 2 $CaCl_2$, 10 $NaHCO_3$, 2.0 Na_2HPO_4, 1.0 $MgSO_4$, 5 D-glucose, and 5.0 Hepes at pH 7.6 (Treberg et al., 2005). An incorporation period of about three hours was adopted following the results of McCarthy et al. on Atlantic wolffish in the same size and temperature range (McCarthy et al., 1999). Fish were thereafter killed by a blow to the head, and the peritoneal cavity was opened and thoroughly rinsed with distilled water. A sample of white muscle from the deep dorsolateral region was dissected (care was used not to sample red muscle), immediately frozen on dry ice and transported to the laboratories in Rimouski (QC, Canada) within 24 h after dissection to be stored at –80°C until further laboratory work. Frozen muscle samples were pulverized in liquid nitrogen using a stainless mortar and pestle, and samples of the resulting powder were stored in sealed cryogenic tubes at –80°C.

Tissue preparation and scintillation counting

Powdered muscle samples were homogenized in 10 volumes of ice-cold 0.2 mol l^{-1} perchloric acid (PCA) using a Heidolph Diax 900 homogenizer (3×10 s) and incubated for 10 min on ice prior to being centrifuged at 15 000 ***g*** for 5 min at 4°C (Thermo IEC Micromax RF benchtop centrifuge, Waltham, MA, USA). Supernatant (for free pool phenylalanine) was delicately removed, taking care not to include any lipid located on top of the microcentrifuge tube. The protein pellet was then washed three times by resuspending it in 1 ml of 0.2 mol l^{-1} PCA, incubating on ice for 10 min and centrifuging for 10 min at 15,000 ***g***. The protein pellet was then dissolved in 2 ml of 0.3 mol l^{-1} NaOH (less than 2 h at 37°C). The determination of labelled phenylalanine in proteins and in the free pool was conducted by mixing samples of the pellet dissolved in NaOH or PCA supernatant, respectively, in 10 ml ScintiVerse II (Fisher Scientific Canada, Ottawa, ON, Canada). This mixture was then counted in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Fullerton, CA, USA). The fluorometric method of McCaman and Robins (McCaman and Robins, 1962) was used for the determination of total phenylalanine in the free pool, allowing for the determination of free-pool phenylalanine specific activity. Briefly, phenylalanine forms a highly fluorescent compound with ninhydrin in the presence of L-leucyl-L-alanine after 2 h incubation at 60°C. Fluorescence was then measured in a Hitachi F-2500 spectrofluorometer (Hitachi High Technologies, San Jose, CA, USA) set at excitation 365 nm and emission 515 nm. A standard curve ranging from 0 to 120 pmol l^{-1} was prepared for each assay using 0.2 mol l^{-1} PCA as solvent. Protein synthesis was calculated from radioactivity in the protein bound pool divided by specific activity in the free pool. Data were normalized to tissue protein content. Protein concentration in the sample was determined with a standardized colorimetric assay (Bio-Rad, Mississauga, Canada).

20S proteasome assay

The chymotrypsin-like activity of 20S proteasome was assayed following Shibatani and Ward (Shibatani and Ward, 1995). The

powdered sample was homogenized in five volumes of lysis buffer using a Potter–Elvehjem (PTFE pestle and glass tube) and centrifuged at 20,000 ***g*** at 4°C for 1 h. The lysis buffer was composed of 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} β-mercaptoethanol in a 50 mmol l^{-1} Tris buffer (pH 8.0). The assay used the proteasome-specific synthetic substrate LLVY-AMC (Biomol International, Plymouth Meeting, PA, USA) and is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labelled substrate. Briefly, 50 µg of protein from the supernatant was incubated at 15°C with 40 µmol l^{-1} LLVY-AMC and 0.0475% SDS in 100 µl of 100 mmol l^{-1} Tris buffer (pH 8.0) for 30 min. The reaction was stopped with 300 µl of 1% SDS and 1 ml of 0.1 mol l^{-1} sodium borate (pH 9.1). Fluorescence was determined at excitation/emission wavelengths of 370/430 nm. A standard curve was prepared for each assay. Blanks were prepared by stopping the reaction prior to incubation, and parallel samples were supplemented with 50 µmol l^{-1} MG-115 and 50 µmol l^{-1} MG-132 (Biomol International), two potent inhibitors of the chymotrypsin-like activity of the proteasome. The inhibitor-sensitive activity is hereby reported as 20S proteasome activity using pmol AMC per hour per 50 µg protein as a unit. Protein concentration was determined as described for protein synthesis. The activity was linear for at least 60 min, and the assays never lasted more than 40 min (data not shown).

The *in vitro* thermosensitivity of the wolffish 20S proteasome chymotrypsin-like activity was evaluated by measuring the activity in samples as above but at different temperatures (4, 8, 16, 24, 32°C). Replicates of white muscle samples of five fish averaging 10 g and acclimated to 8°C were used. A linear model was fitted on the $\log_{10}$-transformed activities and used to convert the activities measured at 15°C to the fish's acclimation temperature. The Q_{10} was calculated using $Q_{10}=10^{(\text{slope}\times 10)}$.

Quantification of protein-bound carbonyls

Reactive oxygen species are the cause of many cellular damages, one of which is the oxidation of amino acid residues on proteins, forming protein carbonyls. The protein-carbonyl content was measured by the method of Levine et al. (Levine et al., 1990) with slight modifications. Briefly, powdered muscle samples were homogenized in ice-cold phosphate buffer saline (PBS; 3.2 mmol l^{-1} Na_2HPO_4, 0.5 mmol l^{-1} KH_2PO_4, 1.3 mmol l^{-1} KCl, 135 mmol l^{-1} NaCl, pH 7.4), and 50 µl of this homogenate was incubated with 500 µl of 10 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH)/2 mol l^{-1} HCl or 500 µl of 2 mol l^{-1} HCl (control) for 1 h. The tubes were vortexed every 10 min. The proteins were then precipitated by adding 500 µl of 20% (w/v) TCA (trichloroacetic acid). After an incubation on ice for 10 min, the tubes were centrifuged at 10,000 ***g*** for 10 min. To remove any unbound DNPH, the resulting pellet was washed with 1 ml of 10% TCA and then washed three times with 1 ml ethanol:ethyl acetate (1:1, v/v). After each wash, the supernatant was carefully aspirated and discarded. The final pellet was dissolved by incubating it in 500 µl of 6 mol l^{-1} guanidine hydrochloride at 37°C for 1 h. Absorbance at 370 nm was read on a Lambda 11 spectrophotometer (PerkinElmer, Woodbridge, Canada), and the molar absorption coefficient of 22,000 M^{-1} cm^{-1} was used to quantify the levels of protein-carbonyls. Protein concentration was determined as described for protein synthesis.

TBARS assay

Free radical damage to lipids results in the generation of malonedialdehyde (MDA), which reacts with thiobarbituric acid (TBA) to form a fluorescent compound. The thiobarbituric acid reactive substances (TBARS) were used as an index of lipid peroxidation in the white muscle samples. The assay was performed using a commercially available kit (Zeptometrix, Buffalo, NY, USA). The kit was used as recommended by the manufacturer except that the assay was downscaled to be performed in microtubes. Muscle samples were homogenized in 10 volumes of ice-cold PBS as above, and 20 µl was pipetted into labelled tubes, 20 µl of the supplied SDS solution was added, followed by 500 µl of the TBA reagent. The tubes were incubated at 95°C for 60 min along with a standard curve of MDA (0–4 nmol ml^{-1}). After cooling, the tubes were centrifuged at 10,000 ***g*** for 5 min, the fluorescence of the supernatant at excitation/emission wavelengths of 530/550 nm was recorded and the MDA equivalent concentration interpolated from the standard curve. The results are presented as the average of three replicates and expressed either in nmol g^{-1} tissue or nmol mg^{-1} protein.

Determination of reduced glutathione

Glutathione (GSH) is an intracellular low-molecular-mass thiol that plays a critical role in the cellular defence against oxidative stress. The GSH concentration was measured according to Kamencic et al. (Kamencic et al., 2000). Reduced glutathione is combined with monochlorobimane (mCB) by the enzyme glutathione-S-transferase to form a fluorescent GSH–mCB adduct that can be quantified by spectrofluorometry. Muscle samples were homogenized in 10 volumes of ice-cold PBS as above. The homogenate was incubated in triplicate for 30 min at room temperature with mCB and glutathione-S-transferase at a final concentration of 100 µmol l^{-1} and 1 U ml^{-1}, respectively. After centrifugation (5000 ***g***, 5 min) the fluorescence of the supernatant was recorded (excitation/emission: 380/470 nm) and the GSH concentration was extrapolated from a standard curve (0–100 µmol l^{-1}).

Statistical analysis

All data are presented as means ± 95% confidence interval. The GLM procedure of Systat 11 (Systat software, Chicago, IL, USA) was used to examine the effect of temperature on growth, protein synthesis, proteasome activity, protein-carbonyl, TBARS and glutathione. When a significant effect of temperature was detected, the multiple comparison test of Tukey was performed. Regression analysis was used to examine the relationship between protein synthesis or 20S proteasome activity and SGR. Finally, a multiple regression was used to analyse the combined effect of protein synthesis and 20S proteasome activity on SGR. Equality of variances was tested using Levene's test, and residual normality was tested using Kolmogorov–Smirnoff test with the correction of Lilliefors. All tests were two-tailed with a significance level of 0.05.

RESULTS

There were no mortalities during the time of the experiment. The final mass of the group acclimated at 4°C was significantly lower than the mass of the other two groups ($F_{2,28}$=17.93, P<0.001); the final masses were 3.44±0.51 g, 4.72±0.39 g and 4.89±0.79 g for fish acclimated at 4, 8 and 12°C, respectively. The final length was significantly different between the three groups ($F_{2,28}$=28.28, P<0.001); the final lengths were 6.9±0.4 mm, 7.6±0.3 mm and 8.0±0.4 mm for 4, 8 and 12°C, respectively. At 4°C, SGR_m was significantly lower than at 8 and 12°C and there was no significant difference between 8 and 12°C (Fig. 1A) ($F_{2,28}$=36.11, P<0.001). There was a strong trend for higher length increase at 12°C (Tuckey's P=0.06), which yielded a significantly lower condition index, i.e. fish that were more slender compared with fish acclimated at 4 and 8°C (Fig. 2) ($F_{2,28}$=8.96, P=0.001).

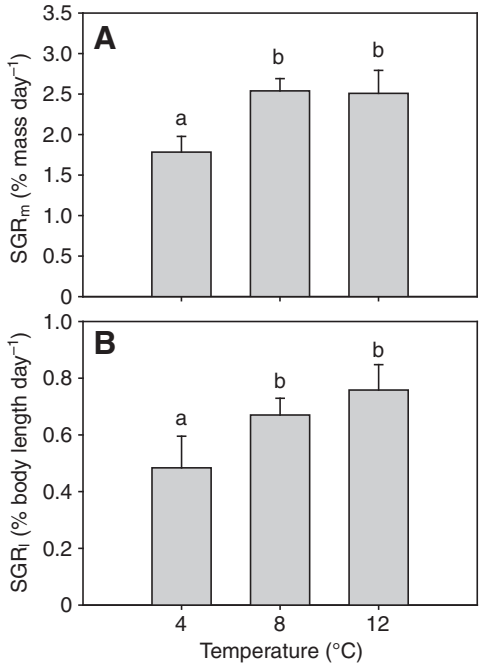

Fig. 1. The effect of temperature on specific growth rate (SGR) in (A) body mass per day and (B) body length per day of juvenile spotted wolffish. Values are means ± 95% CI (N=10); different letters indicate significant difference (P<0.05).

Protein synthesis

The protein synthetic activity was measured in white muscle at the end of the growth trial. The effect of water temperature on the rate of protein synthesis is presented in Fig. 3. At the lowest temperature (4°C), the rate of protein synthesis was significantly lower compared with the group acclimated at 8°C ($F_{2,23}$=3.150, P=0.026) while acclimation at 12°C led to an intermediate rate with no significant difference between 4 and 12°C groups (Fig. 3).

20S proteasome

The chymotrypsin-like activity of the 20S proteasome was affected by acclimation temperature. When measured at a common temperature, the activity in fish acclimated at 4°C was higher than in fish acclimated at 8°C, the latter also displaying a higher activity than those at 12°C (Fig. 4) ($F_{2,26}$=12.619, P<0.001). There was no

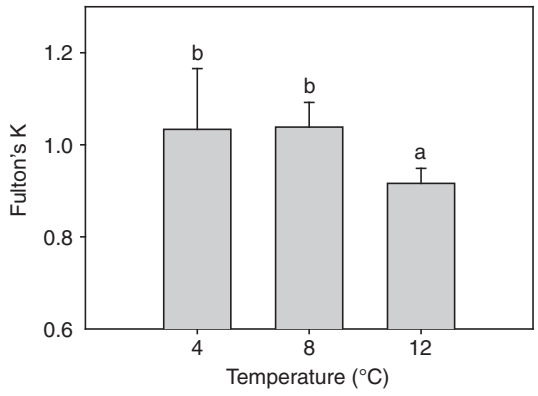

Fig. 2. Fulton's condition index of spotted wolffish growing at three temperatures. Values are means ± 95% CI (N=10); different letters indicate significant difference (P<0.05).

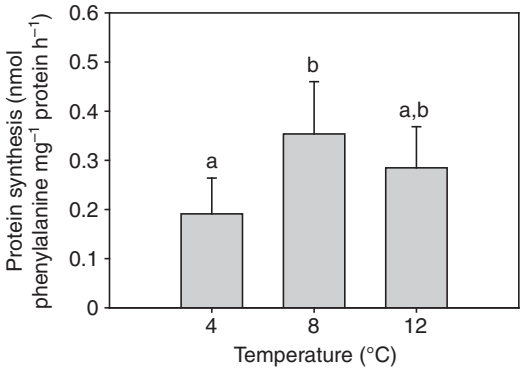

Fig. 3. Rate of protein synthesis expressed as incorporation of phenylalanine in white muscle proteins of juvenile spotted wolffish acclimated at three temperatures. Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05).

effect of acclimation temperature on the level of non-specific activity in the reactions supplemented with MG-115 and MG-132; the averages were 3.47±1.03, 3.52±0.51 and 2.51±0.61 pmol AMC h^{-1} 50 μg^{-1} protein for fish acclimated at 4, 8 and 12°C, respectively ($F_{2,26}$=2.196, P=0.12).

Catalytic rate of the chymotrypsin-like activity of 20S proteasome at physiological temperatures (Fig. 4, white bars) was estimated by first determining activity at different assay temperatures for homogenates from fish acclimated to 8°C. The relationship between proteasome activity and temperature, determined at temperatures ranging from 4°C to 32°C, was linear (Fig. 5). This reveals that nothing untoward with respect to thermal sensitivity is occurring over the temperature range of study. The expected activity at acclimation temperature was calculated from the activity measured at 15°C using the calculated Q_{10} of 1.33. When the activities were reported at their expected values at acclimation temperature, the direction of the differences remains the same but the group acclimated at 8°C was no longer significantly different from the extremes while the 4 and 12°C groups remained significantly different from each other (Fig. 4, open bars) ($F_{2,26}$=5.069, P=0.015).

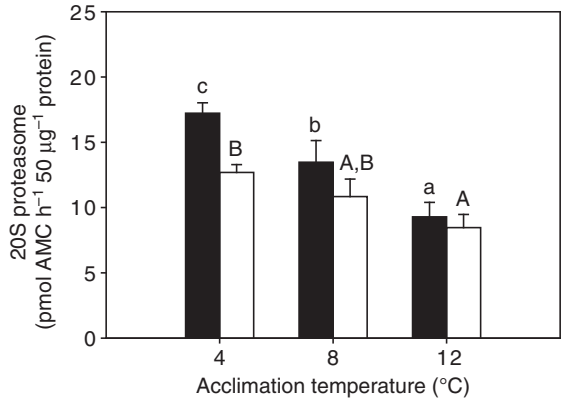

Fig. 4. Activity of 20S proteasome in white muscle of juvenile spotted wolffish acclimated at different temperatures. The black bars represent activity measured at 15°C while the open bars represent the activity calculated at acclimation temperature (see text for details). Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05).

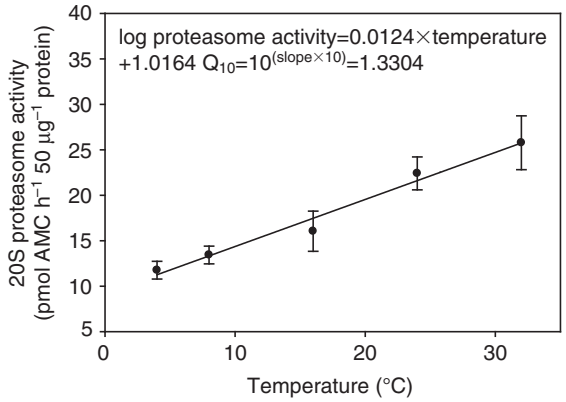

Fig. 5. Activity of 20S proteasome in relation to assay temperature in white muscle of spotted wolffish acclimated at 8°C. The top equation describes the linear relation between log-transformed proteasome 20S activity and temperature (r^2=0.680, P<0.001). The slope of this relationship was then used in the second equation to determine Q_{10}. Each data point represents the mean activity of five fish ± s.d.

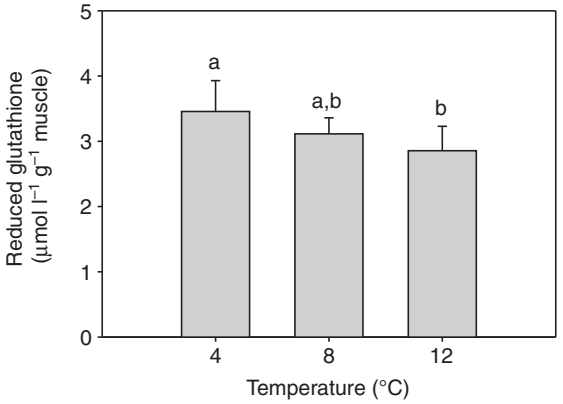

Fig. 6. White muscle concentration of reduced glutathione in juvenile spotted wolffish acclimated at three temperatures. Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05).

In an attempt to describe growth data using protein synthesis and 20S proteasome activity, simple and multiple regression analyses were performed on pooled groups. Growth rate was well described by protein synthesis according to the following equation: SGR_m=1.714+2.065×protein synthesis (r^2=0.343, P=0.001). The relationship between growth rate and 20S proteasome activity was best described by: SGR_m=2.926–0.048×proteasome (r^2=0.253, P=0.006). A multiple regression best described growth rate using both protein synthesis and 20S proteasome activity: SGR_m= 2.22+2.040×protein synthesis–0.039×proteasome (r^2=0.55, P<0.001).

Quantification of protein-bound carbonyls

Oxidative protein damages in the white muscle were measured as protein-bound carbonyls. There was no significant effect of acclimation temperature on protein-bound carbonyls ($F_{2,24}$=1.520, P=0.242). The protein-bound carbonyl content varied between 0.364 and 1.879 nmol mg^{-1} protein and averaged 1.074 nmol mg^{-1} protein.

Quantification of TBARS and reduced glutathione

Lipid peroxidation was measured as the content of TBARS. The TBARS concentrations varied between 0.087 and 0.263 µmol g^{-1} white muscle and averaged 0.154 µmol g^{-1} white muscle. There was no significant effect of temperature on TBARS content ($F_{2,25}$=1.50, P=0.245). Acclimation temperature had a significant effect on reduced glutathione (GSH) concentration (Fig. 6). Fish acclimated at 4°C had a higher concentration of GSH than those acclimated at 12°C while fish acclimated at 8°C had intermediate values ($F_{2,25}$=7.01, P=0.005).

DISCUSSION

Wolffishes possess several assets that make them useful as a model species for the study of relationships linking protein metabolism and growth. The effect of temperature on muscle fractional rate of protein synthesis was described in fish as a log-linear relationship for many species, and in wolffish, protein synthesis occurs at higher rates compared with other species (McCarthy et al., 1999). Wolffish also display very low spontaneous swimming activity and aggressive behaviour, allocating more energy to somatic growth than active metabolism (Le Francois et al., 2004; McCarthy et al., 1998; McCarthy et al., 1999; Savoie et al., 2008), which contributes to a reduced level of intraspecific growth variability. This, in turn, allows for a better assessment of the links between temperature, growth rate and protein metabolism measurements.

As expected, acclimation at the coldest temperature had a significant negative effect on growth rate, mean SGR_m being 70% of SGR_m of fish acclimated at 8°C. Fish acclimated at 12°C did not show significantly different growth rate when compared with the 8°C group, as found in Atlantic wolffish (McCarthy et al., 1998; McCarthy et al., 1999). However, Savoie et al. (Savoie et al., 2008), studying newly hatched spotted wolffish, reported highest growth rate at 12°C compared with 8°C during the first two weeks post-hatch, then a sharp decrease of growth rate at 12°C occurred. Our results are also in accordance with other published growth rates of juvenile spotted wolffish (Hansen and Falk-Petersen, 2002; Imsland et al., 2006a; Imsland et al., 2006b). The SGR_m presented here are, to our knowledge, the first published for spotted wolffish in the range of 2 to 5 g. The absence of mortality and generalized positive growth during the growth trial is indicative of the good health of the experimental fish but also shows that the selected experimental temperatures were well within the species thermal range. Acclimation to the highest temperature (12°C) seems to have an effect on the shape of fish, as suggested by the reduced Fulton's condition index (Fig. 2). This phenomenon might be indicative of the difficulty of retaining energy reserves at higher temperature due to higher maintenance costs. It might also be indicative of different thermosensitivity of growth rate and developmental processes.

Protein synthesis

In this study, the rate of protein synthesis was maximal at 8°C, lower at 4°C and intermediate at 12°C. At 12°C, the rate of amino acid incorporation tends to be lower than at 8°C. This contrasts with the results for Atlantic wolffish (McCarthy et al., 1999), where the fractional rate of protein synthesis increased linearly with temperature. In juvenile barramundi (*Lates calcarifer*), protein synthesis was shown to display an asymmetrical relation with temperature, as it tends to in the present study (Katersky and Carter, 2007). It has been suggested that, under satiation feeding, protein synthesis is maximal at optimum growth temperature (Carter and Houlihan, 2001; Loughna and Goldspink, 1985). This seems to be

the case in the present study as protein synthesis tends to be higher at 8°C, which is generally recognized as the optimal growth temperature of spotted wolffish (Foss et al., 2004; Hansen and Falk-Petersen, 2002; Imsland et al., 2006a; Imsland et al., 2006b).

Proteasome activity

The thermosensitivity of the chymotryptic-like activity of 20S proteasome was the same for the white muscle and liver (data not shown). The relatively low Q_{10} observed in spotted wolffish contrasts with findings of other studies, mostly on mammals, where reported Q_{10} are between 1.6 and 4 (Velickovska et al., 2005; Woods and Storey, 2005). The skeletal muscle chymotryptic-like activity of hibernating thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) has a lower Q_{10} than that of conspecific euthermic animals (1.6 *vs* 2.1) and is much lower than that of mouse (*Mus musculus*), which has a Q_{10} of 2.9 [calculated from fig. 3 of Woods and Storey (Woods and Storey, 2005)]. In general, Q_{10} increases at lower temperature, but this phenomenon was not observed in the range of temperature studied. This does not rule out the possibility of a very large Q_{10} between 4°C and lower temperatures but this is beyond the scope of this study. It is worth mentioning that, in the wild, spotted wolffish experience temperatures that range from –1 to 7°C (Barsukov, 1959). Consequently, it is not surprising that a low Q_{10} is measured even at cold temperatures. It is not clear whether wolffish 20S proteasome is adapted to be more efficient at cold temperature or if animals living at higher temperatures have evolved a protection mechanism that limits protein degradation during short-term decreases of temperature, such as in hibernating squirrels.

Protein degradation is a tightly regulated process (Hershko et al., 2000) and, in eukaryotic cells, three major protein degradation systems exist. The lysosomal system is composed of a vacuole-bound acidic environment where the cathepsins hydrolyze the proteins in a non-specific manner. The concentrations of the different cathepsins are under tight control (Aoki et al., 2000; Mommsen, 2004). The calpain system is composed of two calcium-activated proteolytic enzymes (the m-calpain and the μ-calpain) and their specific inhibitor (the calpastatin). This system is thought to be principally involved in the cleavage of cytoskeletal/membrane attachments and presumably signal transduction (Goll et al., 2003; Goll et al., 1998). The ubiquitin/proteasome system, examined here, is involved in the specific degradation of tagged proteins. Tagging of a protein for destruction is accomplished by covalent attachment of multiple ubiquitin moieties, a highly evolutionary conserved protein. Conjugation of ubiquitin to the protein is realized *via* a three-step cascade mechanism involving three classes of enzymes (E1, E2 and E3) that activate ubiquitin, transport it and attach it to substrate protein, respectively (Attaix et al., 2001). The 20S proteasome is composed of four stacked rings that form a barrel-like structure hosting proteolytic activities. Both ends of the 20S proteasome can be capped by a 19S regulatory particle that is responsible for substrate recognition and its translocation into the lumen for degradation (Attaix et al., 2001; Braun et al., 1999; Glickman and Ciechanover, 2002).

This is the first study to measure 20S proteasome activity in fish acclimated at different temperatures. There is a clear indication of thermal compensation of proteasome activity in fish acclimated at 4°C. Proteasome activity at 4°C was 130% higher than the activity level measured at 8°C, while in fish acclimated at 12°C, activity was 87% of that of 8°C (calculated using activities at acclimation temperature). The use of Q_{10} to calculate proteasome activity at acclimation temperature was preferred to measuring the enzyme activities at acclimation temperature for practical considerations. The effect of temperature on enzyme activity was linear in the studied range of temperature and there is no reason to believe that the fish express different isoforms of proteasome at different temperature. Therefore, the temperature sensitivity of the proteasome should be the same between acclimation groups and the calculated activities should represent reality. The assays were performed on tissues extracted at least 24 h after the last meal and the activities are likely to represent the basal rate of protein degradation in white muscle of juvenile spotted wolffish. Our results are in accordance with many transcriptomics studies that show an increase of the genes coding for 20S proteasome and/or ubiquitinating enzymes during acclimation to cold temperatures. In a study on gene expression level of annual killifish (*Austrofundulus limnaeus*) during acclimation to low and high temperature, Podrabsky and Somero found that a subunit of 26S proteasome was strongly upregulated during cold acclimation (Podrabsky and Somero, 2004). In the common carp (*Cyprinus carpio*), acclimation to cold temperature also led to upregulation of 21 genes involved in the ubiquitin–proteasome pathway (Gracey et al., 2004). Upregulation of genes of the proteasome pathway was also observed, along with an apparent increase in the ubiquitin-conjugated (Ub-conjugated) level of some proteins in common carp (McLean et al., 2007). Todgham et al. found a higher level of Ub-conjugated protein in Antarctic fish species than in fish inhabiting temperate waters of New Zealand (Todgham et al., 2007). The authors concluded that cold waters placed higher physiological constraints on maintaining proteins in their native state (Todgham et al., 2007). These authors also suggested that the accumulation of Ub-conjugated proteins could be a result of a lower efficiency of the proteasome at lower temperature. This interpretation is now challenged by our results, given the compensation of 20S proteasome activity at lower acclimation temperature. Our results, combined with those of Todgham et al. (Todgham et al., 2007), indicate that cold temperatures place an important physiological constraint on protein metabolism and that the ubiquitin–proteasome pathway seems to be upregulated in such conditions. It is noteworthy that the 20S proteasome activities reported in the present study reflect the capacity of degradation of a synthetic peptide and not the degradation of proteins *per se*. This is, for now, the closest we can get to protein degradation by the proteasome pathway.

Lower protein synthesis retention efficiency (PSRE) was observed in Atlantic wolffish at lower temperature (McCarthy et al., 1999). PSRE was also found to be lower at low temperature in juvenile barramundi (Katersky and Carter, 2007). Low PRSE indicates that a lower proportion of synthesized proteins is successfully retained for growth, in other words it is indicative of a high protein turnover and therefore of a low growth efficiency. Our results show that the proteasome pathway is a potentially significant component of the low PRSE generally observed at low temperature. It appears that, at temperatures slightly higher than the optimal, growth rate is maintained by a combination of skeletal growth and slightly reduced muscular activity of proteasome, as shown by the Fulton's K and proteasome 20S activity, respectively. Since the higher acclimation temperature was not high enough to produce a detrimental effect on growth rate, it is not possible to evaluate the role of proteasome in the case of acclimation to adversely high temperature. In their study on barramundi, Katersky and Carter also found that PRSE was maintained over a wide range of temperature at the higher end of the experimental temperatures tested (Katersky and Carter, 2007). To date, protein metabolism (synthesis, growth and degradation) has not been measured in animals acclimated at temperatures clearly above their optimal temperature. Such

information would provide further data to explore the relationships between protein metabolism and growth rate.

Protein turnover

A significant positive relationship was found between the rate of protein synthesis in white muscle and SGR_m while a negative relationship was found between 20S proteasome activity and SGR_m. A significant multiple regression described SGR_m by using protein synthesis and proteasome activity. As shown by the equation of this regression, white muscle 20S proteasome activity negatively impacts growth rate. Dobly et al. (Dobly et al., 2004) also found a negative relationship between growth rate and 20S proteasome activity in rainbow trout liver but not in white muscle. This is probably related to the different experimental approach they used to study the relationship between protein metabolism and growth rate and also to the sedentary swimming behaviour of spotted wolffish compared with rainbow trout. The multiple regression was computed on pooled fish without using temperature as a factor for two reasons; first, we wanted to avoid the use of too many parameters and their interactions. More importantly, since the selected acclimation temperatures were within the thermal range of spotted wolffish, it was considered as a mild treatment influencing growth rate in comparison to food restriction. Keeping in mind that during positive growth, protein turnover is equal to protein degradation (Hawkins, 1991), turnover appeared to be maintained high in slow-growing fish acclimated at lower temperature. Fish acclimated at the higher temperature maintained growth rate similar to the group acclimated at 8°C while having a slightly lower rate of protein synthesis and 20S proteasome activity.

Protein damage

Protein cold denaturation and/or protein misfolding have been suggested as causal factors of the high levels in Ub-conjugated protein content reported in cold-water-adapted fish species (Todgham et al., 2007). Although, as pointed out by these authors, millions of years of evolution in a cold environment should have led to adaptive modifications of proteins in order to maintain their stability under natural conditions. However, acclimation to cold temperature generally leads to increased mitochondrial capacity in white muscle (Guderley, 2004), and reactive oxygen species (ROS) are a byproduct of the mitochondrial respiration (Boveris and Chance, 1973). These ROS may damage all types of biological molecules but, because of their high relative importance in tissue composition, proteins are a major target (Dalle-Donne et al., 2003; Levine et al., 1990; Shacter, 2000). A generally recognized role of the proteasome pathway is the degradation of oxidatively damaged proteins. Oxidatively damaged proteins are tagged by enzymes of the ubiquitinating pathway, and proteasome activity is also known to be highly responsive to oxidative stress (Friguet, 2006; Perepechaeva et al., 2006; Poppek and Grune, 2006). A higher level of oxidative modification of proteins could perhaps explain the higher rate of protein degradation at cold temperature. In the present study, neither protein-carbonyl nor TBARS contents were higher at low temperature; however, GSH concentration was significantly higher at low temperature. Unfortunately, the method used for GSH assay did not allow for assessment of GSSG, making it impossible to estimate the total glutathione pool, its redox state and thus the antioxidant status of the fish. However, GSH concentration was shown to increase as a response to mild oxidative conditions (hyperoxia) in gills (Ritola et al., 2002a) and liver (Ritola et al., 2002b) of rainbow trout. In the North Sea eelpout (*Zoarces viviparous*), GSH levels were shown to increase following stressful exposure to low temperature (Heise et al., 2006). In the Antarctic eelpout (*Pachycara brachycephalum*), glutathione content is two to three times higher than in the North Sea eelpout, and the ratio GSSG/GSH of the Antarctic eelpout was among the highest reported in the literature. The authors argued that such a high ratio of GSSG/GSH and the high concentration of GSH were both characteristic traits of some polar fish, such that metabolic processes, adapted to function at low temperatures, are also able to operate in a more oxidized redox environment (Heise et al., 2007). In these studies, both GSH and GSSG were measured but, in all cases, the absolute content of GSH was increased following exposure to potentially stressful conditions. As such, the higher content of GSH in cold-acclimated spotted wolffish possibly indicates that the animal faces more oxidative conditions. The redox state of the glutathione pool following acclimation to different temperatures would definitely be more informative but the data obtained in this study present a valuable point of departure. Reduced glutathione was the only antioxidant investigated but it could well be that antioxidant enzymes are participating in ROS detoxification in cold-acclimated spotted wolffish. For instance, in common carp, along with the ubiquitin–proteasome pathway transcripts, the transcription levels of the antioxidant enzymes superoxide dismutase and glutathione-S-transferase were upregulated during cold acclimation (Gracey et al., 2004).

Conclusion

20S proteasome activity seems to be a good proxy for protein degradation at suboptimal temperature in spotted wolffish. The effect of low temperature on growth rate of juvenile spotted wolffish is attributed to the lower rate of protein synthesis and high proteasome activity resulting in lower protein retention efficiency and lower growth efficiency. High rate of protein degradation at low temperature is suggested as an adaptive response to keep the steady-state concentration of oxidatively modified proteins at an acceptable level. Further work on mitochondrial ROS generation and the antioxidant status of fish is nevertheless needed to strengthen this hypothesis. Adaptation to a colder environment could involve tradeoffs between increased mitochondrial capacity and lower protein retention efficiency due to higher oxidative damages incurred by proteins when ROS production is higher.

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