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### Effects of short- and long-term thermal stress in perch (*Perca fluviatilis*) determined through fluctuating asymmetry and HSP70 expression

Livia Lucentini <sup>a</sup>; Massimo Lorenzoni <sup>b</sup>; Fausto Panara <sup>a</sup>; Mario Mearelli <sup>b</sup>

<sup>a</sup> Dipartimento di Biologia Cellulare e Molecolare, Università degli Studi di Perugia, Perugia, Italy <sup>b</sup>

Dipartimento di Biologia Animale ed Ecologia, Università degli Studi di Perugia, Perugia, Italy

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# Effects of short- and long-term thermal stress in perch (*Perca fluviatilis*) determined through fluctuating asymmetry and HSP70 expression

LIVIA LUCENTINI

Dipartimento di Biologia Cellulare e Molecolare,  
Università degli Studi di Perugia,  
via Pascoli, I-06123 Perugia (Italy)  
e-mail: livial@tin.it

MASSIMO LORENZONI

Dipartimento di Biologia Animale ed Ecologia,  
Università degli Studi di Perugia,  
via Pascoli, I-06123 Perugia (Italy)

FAUSTO PANARA

Dipartimento di Biologia Cellulare e Molecolare,  
Università degli Studi di Perugia,  
via Pascoli, I-06123 Perugia (Italy)

MARIO MEARELLI

Dipartimento di Biologia Animale ed Ecologia,  
Università degli Studi di Perugia,  
via Pascoli, I-06123 Perugia (Italy)

## ABSTRACT

The effect of short- and long-term exposure to temperature ranges near the optimal developmental values of perch (*Perca fluviatilis*) during pre- and post-natal development was studied. A fertilized egg raft was divided and placed in four tanks kept at 10°, 17°, 20° C and variable temperature (10° to 20° C), respectively. Short-time effects of temperature were investigated analyzing fluctuating asymmetry by measuring three meristic and seven morphometric bilateral characters. Perches reared below 17° C or at variable temperature were less asymmetric than those reared at 20° C. Perches reared at 10° C showed a non-homogeneous trend of the characters analysed. Long-term effects were investigated by studying the liver and skeletal muscle 70 kDa heat shock proteins (HSP/HSC70) three and six months after hatching. Two HSP isoforms, HSC73 (73 kDa) and HSP72 (72 kDa), were detected in perch liver by immunoblotting. HSC73 was present at the two developmental stages and at all temperature conditions, while HSP72 was detected only at the variable temperature and 20° C. The muscle tissue always showed the HSC73 form. Our data suggest that below 20° C only some morphological characters were affected, whereas the 20° C temperature and, presumably, higher ones, seemed to have both short- and long-term effects.

**KEY WORDS:** Fluctuating asymmetry - 70 kDa heat-shock protein - Developmental stability - Thermal stress - Breeding - *Perca fluviatilis*.

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## INTRODUCTION

Mechanisms regulating the fine tuning of development and reproduction of wild-type phenotype are not well understood. The relative fitness of a given genotype is therefore a function of the fidelity with which its genome is able to control development (Wood, 1997). Any perturbation of development which deviates from wild-type phenotype is likely to cause stress with a potential reduction of fitness (Orr & Coyne, 1992; Møller & Gangestad, 1999). The most frequent form of stress in animal populations is chronic sublethal stress. Previous studies on aquatic organisms showed that developmental stability can be affected by environmental stress such as water temperature variation (Lucentini *et al.*, 1997; Campbell *et al.*, 1998; Savage & Hogart, 1999). In fact, water temperature is considered one of the most important factors controlling fish growth and the response of a fish to temperature change may vary according to its biological condition (Love, 1970; Guma's, 1978a; Melard *et al.*, 1995). It may, for example, lead to more subtle influences on the development, detected as differences between the right and left sides (R-L) of normally bilaterally symmetrical organisms, also known as fluctuating asymmetry (FA) (Leary & Allendorf, 1989; Parson, 1990; Palmer, 1994). Fluctuating asymmetry differs both from directional asymmetry and antisymmetry and has been used in evolutionary biology as a measure of developmental homeostasis, since the same genome controls the development of the left and right sides of a bilaterally symmetrical organism. Deviation from symmetry can be used to measure genetic or environmental short-time exposure perturbations (Palmer & Strobeck, 1986; Leary & Allendorf, 1989). Furthermore, it is known that all organisms express or synthesize a protein family in response to several environmental stresses. These proteins, called heat shock proteins (HSPs), play a protective role in cells exposed to stress although they are also constitutively expressed (heat shock cognates, HSCs) under normal conditions (Craig *et al.*, 1994). Heat shock proteins constitute an abundant family whose main classes including HSP25, -60, -70, -80, -90 and -110 members (Gething & Sambrook, 1992; Becker & Craig, 1994). A major role of HSP proteins is to act as molecular chaperones, thus forming complexes with a variety of cellular proteins (Schlesinger, 1990). The HSP70 family is the most conserved and is found in response to stress in all organisms from bacteria to humans, including aquatic organisms (Sanders, 1993; Kharouna-Renier & Zer, 1999). Two cytosolic members of the HSP70 family present in mammalian tissues are the heat-inducible HSP70 (Mr 72,000) and the constitutively expressed HSC70 (Mr 73,000) (Becker & Craig, 1994). Although the presence of constitutive and inducible HSPs in various fish species has been documented (for the literature see Smith *et al.*, 1999), the main reports concern studies of HSPs in relation to exposure to various pollutants or in response to thermal shock induced by high tempera-

ture treatment for a short time. The effect of long-term exposure to temperature ranges near the optimal developmental values for a given organism was not well understood.

The aim of the present paper was to study perch (*Perca fluviatilis* Linnaeus, 1758) growth and to identify environmental conditions influencing normal development of this species in Italy. Perch lives in inland, fresh, preferably stagnant, waters containing at least 3 ml/l of oxygen. Spawning occurs in spring at a water temperature ranging from 0° to 14° C (Franzoi *et al.*, 1991). The temperature range for embryonic perch growth is 6–22° C (Sandstrom *et al.*, 1997), the optimal being 12–16° C (Hoestlandt, 1980). Perch was selected owing to its diffusion in Italian inland waters (Gandolfi *et al.*, 1991; Gandolfi & Zerunian, 1987) and to the consistent knowledge on growth and fluctuating asymmetry deriving from many researches and communications (Lorenzoni *et al.*, 1993; Lucentini *et al.*, 1997; Ostbye *et al.*, 1997). In the present paper, we report results concerning the effects of different temperature regimes on perches reared from a single egg raft and exposed to four different controlled thermal stresses.

## MATERIALS AND METHODS

### *Animals and thermal treatment*

An egg raft of perch was taken at Lake Piediluco (Central Italy) at least two hours after natural spawning and fertilization and divided into four portions; these were ventilated and supported by a plastic-coated wire net, to prevent anoxia and mycoses (Sandstrom *et al.*, 1997). Each portion was placed in a small tank (15 l) floating in a larger one (500 l) to control thermoregulation to various temperature regimes. Thus, one tank was kept at 17° C, another at a lower temperature (10° C); and yet another at a higher temperature (20° C), whereas in the last one the daily temperature varied from 10° to 20° C using a combined system of refrigerator and heater connected with timers and a graphical restitution thermometer. After hatching and yolk reabsorption, the artificial feeding of the fries was started gradually according to the different development of perch larvae in the four tanks (Meyer & Wahl, 1997; Tamazouzt *et al.*, 1998). Two hundred and sixty eight specimens, including those dead and preserved, were employed to evaluate growth and FA. Three (July) and six (October) months after hatching, HSP/HSC70 in liver and skeletal muscle tissue was analysed in some samples. On the whole, 59 samples from the 20° C, 108 from the variable temperature, 73 from the 17° C and 28 from the 10° C tanks, were analysed.

### *Fluctuating asymmetry analysis*

To define FA, seven morphometric (pectoral and ventral fin length, pre-pectoral fin distance, pre-ventral fin distance, pectoral-ventral fin distance, postorbital distance, head length) and three meristic characters (lateral line scales, pectoral and ventral fin rays) on the left and right side of each fish, were measured. They were selected on the basis of a previous study (Lucentini *et al.*, 1997), and because they could be easily and accurately measured even in such small specimens. Data were transformed into asymmetry values using the formula: asymmetry value equal to right-side values minus left side values ( $d = R - L$ ). Total morphometric and meristic indexes and global parameters of asymmetry, were calculated adding asymmetry values of all characters for the four populations separately. Growth values and FA data of the four

groups were compared; the comparison between sexes was not made since the specimens age did not allow sex determination and because previous studies had demonstrated that in perch there are no differences either of growth or FA between sexes (Lucentini *et al.*, 1997).

### *Statistical analysis*

Growth was measured applying the length-weight regression analysis on data desegregated according to the tank examined. Covariance analysis (ANCOVA) was used to detect the differences among these regressions. Characters were described through global descriptive statistics for both sides of the body. For each meristic and morphometric trait, the linear regression between  $d$ -value and total length was estimated in order to exclude that larger fish exhibited greater asymmetry either as a consequence of their size (Oxnevad *et al.*, 1995; Ostbye *et al.*, 1997) or because of measurement errors due to larger size. The analysis of fluctuating asymmetry required the exclusion of alternative hypotheses: directional asymmetry through the sign test and antisymmetry through kurtosis analysis. The normality of absolute values distributions was tested using Shapiro-Wilk's test; since they were not normal, the non-parametric Kruskal-Wallis test was used to compare groups for each character and for total morphometric indexes (Palmer & Strobeck, 1986; Oxnevad *et al.*, 1995) and Bonferroni's test (Oxnevad *et al.*, 1995) to rank by means of the asymmetry level. Differences among values of this test were assayed with analysis of variance (ANOVA).

### *Western-blot analysis of perch liver and skeletal muscle HSP/HSC70*

Liver and muscle specimens were taken from fish three and six months after hatching and stored at -80° C until analysed. They were homogenized in PBS (10 mM  $\text{KH}_2\text{PO}_4$ , 10 mM  $\text{Na}_2\text{HPO}_4$ , 0.9% NaCl, pH 7.4) containing an antiprotease cocktail (Sigma). After centrifugation (28,000  $g$  for 30 min at 4° C) the supernatant was immediately treated with 1:1 Laemmli sample buffer (2 $\times$ ) (Laemmli, 1970) containing 1% SDS (sodium dodecyl sulphate). Proteins were determined (Lowry *et al.*, 1954) and aliquots of each sample containing the same protein amount (20  $\mu\text{g}$ ) were loaded on a 10% polyacrylamide gel and separated by electrophoresis in the presence of 0.1% SDS (SDS-PAGE). After the run, the protein bands were electroblotted on nitrocellulose membrane and processed for western-blotting analysis (Towbin *et al.*, 1979). Nitrocellulose sheets were treated overnight with the anti HSP70 monoclonal antibody (mAb) (SIGMA, clone BRM-22) diluted at a 1:2,500 ratio. After several washes with TBS (20 mM Tris, 0.9% NaCl, pH 7.2), the sheets were treated with a goat anti-mouse secondary antibody linked to alkaline phosphatase (Bio-Rad) diluted at a 1:5,000 ratio. The following standards were used for molecular weight calibration in SDS-PAGE: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.4 kDa). Data analysis was referred to at least three independent experiments.

## RESULTS AND DISCUSSION

Adaptation to environmental stress is essential for the survival of all living organisms. For instance, poikilothermal animals are strongly affected by water temperature fluctuations in their environment, and therefore must possess compensatory biochemical mechanisms. In particular, developing fishes are strictly dependent on water temperature variations whose marked effects are evident at both biochemical and morphological lev-

TABLE I - Descriptive statistics and results of Shapiro-Wilk's test ( $W$ , test value) for seven morphometric and three meristic characters of perch. Minimum (Min), Maximum (Max) are expressed in mm for morphometric and in absolute values for meristic ones. Mean  $\pm$  SD is also reported.

Character	Min-Max	Mean $\pm$ SD	W	P
Opercular distance	0.58-3.50	1.30 $\pm$ 0.64	0.80	0.00
Orbital distance	0.34-2.00	0.77 $\pm$ 0.36	0.89	0.00
Pre-ventral distance	0.17-4.10	1.55 $\pm$ 0.75	0.59	0.00
Pre-pectoral distance	0.01-3.40	1.31 $\pm$ 0.62	0.70	0.00
Pectoral-ventral distance	0.10-0.74	0.22 $\pm$ 0.08	0.93	0.00
Pectoral fin length	0.25-2.30	0.70 $\pm$ 0.40	0.75	0.00
Ventral fin length	0.24-2.10	0.71 $\pm$ 0.41	0.79	0.00
Scales	38-65	49.50 $\pm$ 4.44	0.94	0.00
Pectoral fin rays	7-16	13.62 $\pm$ 1.75	0.86	0.00
Ventral fin rays	5-6	5.99 $\pm$ 0.00	0.06	0.00

els (Seddon, 1997; Watabe *et al.*, 1997; Campbell *et al.*, 1998). This investigation concerned the effect of short-term and long-term exposure to different temperatures of developing perch through the measurement of FA and HSP/HSC70 isoforms in liver and muscle tissues.

Overall results for perches grown under different thermal conditions showed a non-homogeneous or comparable growth. ANCOVA analysis demonstrated that length-weight regressions (10° C:  $y = 0.03x^{2.45}$ ; 20° C:  $y = 0.02x^{2.77}$ ; 17° C:  $y = 0.02x^{2.71}$ ; variable:  $y = 0.01x^{2.95}$ ) presented significant differences among groups ( $F_{4, 1060} = 9.48$ ) and that a single model cannot describe them ( $F_{3, 1060} = 7.81$ ).

Fluctuating asymmetry analysis was carried out by measuring a set of morphometric and meristic traits listed in Table I. Correlations between meristic ( $0.03 < R^2$

$< 0.04$ ) and morphometric ( $0.00 < R^2 < 0.14$ ) characters with total length were not significant and no standardization was necessary, thus indicating that asymmetry levels were not due to measurement errors. The number of pectoral fin rays, constantly equal to six, tended to be the most canalised character, since it usually responds less to an input of genetic and environmental origin (Leary *et al.*, 1992; Lucentini *et al.*, 1997). Ventral fin length showed directional asymmetry and was excluded in subsequent analyses. No character was platykurtic, i.e. none demonstrated antisymmetry with the graphical method of normal probability plot and the analysis of kurtosis (not reported). Shapiro-Wilk's test indicated that no character distributions were normal (Table I). The Kruskal-Wallis test showed which differences between the thermal conditions were statistically significant for the nine characters and the total indexes (Table II). Subsequently, tanks were ranked according to the asymmetry level of characters using Bonferroni's multiple range test that pointed to different metabolic responses in the four groups. Analysis of variance confirmed significant differences among Bonferroni's means relating to the four groups ( $F = 2.91$ ,  $P = 0.82$ ). Our data did not show a correlation between FA and increasing temperature, as recently reported for *Asellus aquaticus* L. (Savage & Hogart, 1999). The Bonferroni ranking demonstrated that characters had a different trend, presumably correlated to developmental homeostasis. These data, coupled with the others on growth, showed that the 20° C temperature is the most stressful environment for hatching and growth, as confirmed by a clearly greater asymmetric level of the total morphometric index (0.32) compared to the others (mean value = 0.20). This may be due to the nearness of this temperature to the value of 22° C, considered the maximum value compatible with ontogenesis of perch (Guma's, 1978b). Ranking also showed that at 10° C characters

TABLE II - Results of Kruskal-Wallis test ( $H$ , test value) for the evaluation of statistically significative differences among tanks and values of the Bonferroni test for ranking tanks on the basis of asymmetry values

Character	Kruskal-Wallis test		Bonferroni value			
	H	P	10° C	Variable	17° C	20° C
Opercular distance	10.63	0.01	0.03	0.03	0.02	0.05
Orbital distance	11.74	0.00	0.06	0.03	0.02	0.06
Pectoral-ventral distance	0.53	0.91	0.01	0.01	0.02	0.03
Lateral line	2.08	0.55	0.33	1.96	1.58	1.98
Pre-ventral length	5.41	0.14	0.01	0.04	0.07	0.07
Pre-pectoral length	8.72	0.03	0.05	0.03	0.04	0.06
Ventral fin length	5.63	0.13	0.04	0.03	0.02	0.04
Pectoral fin length	0.49	0.92	0.06	0.04	0.03	0.04
Pectoral fin rays	3.49	0.32	0.58	0.61	0.28	0.83
Total meristic index	2.73	0.43	0.54	0.54	0.51	0.71
Total morphometric index	11.44	0.01	0.28	0.20	0.23	0.25

have a non-homogeneous trend, possibly due to very slow development exposing fishes to prolonged stress, although 10° C is near the natural rearing temperature. Therefore, it is possible that only some characters develop rightly. However, our data indicated lower asymmetry levels in perches reared at variable temperature, therefore they differ from those reported for coho salmon (Campbell *et al.*, 1998).

For the analysis of long-term exposure to different temperatures, we assayed the expression of the 70 kDa HSP family using the western-blotting technique stained with an anti HSP70 mAb which recognizes both HSP/HSC70 in mammals.

In perch liver and muscle, a 73 kDa (HSC73) protein band was detectable at all developmental stages and examined temperatures (Fig. 1). A new 72 kDa band (HSP72) appeared in liver but not in the muscle extract when the analysed samples were obtained from fishes grown at different temperatures (Fig. 1). Three months after hatching, the HSP72 band was present only in specimens reared at variable temperatures and 17° C. At six months, the HSP72 was clearly evident in the variable and 20° C tanks. Muscle specimens showed only the HSC73 protein band at all developmental stages and examined temperatures (Fig. 1). Results suggest that prolonged variable temperatures and 20° C exert stress conditions in perches, as revealed by the presence of inducible HSP72 protein, whereas lower temperatures have no apparent effect on HSP72 expression.

Our data agree with those recently published on Atlantic salmon (*Salmo salar*) in which the HSP65 and HSC66 homologues of mammalian HSPs 72/73 were found and characterized (Smith *et al.*, 1999). Previous data reported that in the eurythermal teleost *Fundulus heteroclitus* (Koban *et al.*, 1991), high levels of HSP70 were detected at a temperature lower than that of normal development. Those authors, however, did not distinguish between HSP and HSC70 isoforms.

Our findings demonstrate that thermal conditions are a source of variability having monitorable influence on perch ontogenesis; in addition, they are consistent with

previous works indicating that analysis of homeostasis can be a measure of environmental stress. In conclusion, the exposure to different temperature regimes, near the optimum for perch growth, causes both short-term and long-term effects on developing perch, as evidenced by FA and HSP/HSC70 measurement, respectively.

Our data suggest that below 20° C only some morphological characters were affected, whereas 20° C and, presumably, higher temperature exposure had both short- and long-term effects. Moreover, we observed that a single model describing short- and long-term exposure effects cannot be detected. However, the relative ease of breeding in this species may be a valid tool for estimating controlled environmental stress influence, not only of thermal origin, and a valid informational basis for studies on wild populations.

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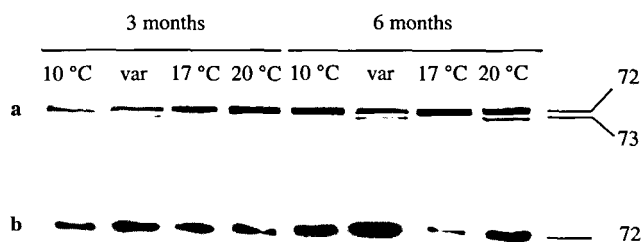


Fig. 1 - Western blot analysis of liver (a) and muscle (b) extract at three and six months after hatching and at different temperature (10° C, variable, 17° C, and 20° C). Nitrocellulose filters were incubated in an anti-HSP70 antibody after SDS-PAGE in 10% polyacrylamide and then incubated in a secondary antibody linked to an alkaline phosphatase (Bio-Rad). Molecular mass (in kDa) is reported on the right-hand edge of the figure.

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