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Exposure of Goldfish (*Carassius auratus*) to Bluegills (*Lepomis macrochirus*) Enhances Expression of Stress Protein 70 mRNA in the Brains and Increases Plasma Cortisol Levels

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ABSTRACT—The effect of psychological stress on HSP70 mRNA in the brains and plasma cortisol levels in goldfish was examined. Stress was induced by exposure to a predator (bluegills). HSP70 mRNA and cortisol were determined by Northern blotting and ELISA, respectively. Goldfish exposed to four predators in the same tank without a partition showed marked increases in HSP70 mRNA and cortisol levels 6 hr and 12 hr after commencement of exposure. When goldfish were separated from bluegills with a net partition, HSP70 mRNA expression was enhanced after 6 hr, and returned to the control level after 12 hr. Plasma cortisol levels increased after 2 hr, and returned to the control level after 6 hr. When goldfish were placed in a transparent tank around which bluegills were swimming, HSP70 mRNA expression and cortisol levels increased after 6 hr and 12 hr. Goldfish exposed to water circulating through a tank with bluegills showed no sign of changes in HSP70 mRNA expression or cortisol levels. These results suggest that psychological stress enhanced HSP70 mRNA expression in the brains and increased plasma cortisol levels via visual perception.

INTRODUCTION

Exposure of fish to an unfavorable environment imposes varying amounts of stress. Stress responses are expressed at several levels, including the biochemical, physiological, behavioral, and performance levels (Schreck, 1990). Plasma cortisol levels have been used as an excellent indicator of stress (Donaldson, 1981). For example, handling increased plasma cortisol levels 8 fold in rainbow trout (Vijayan and Moon, 1992) and aluminium exposure increased plasma cortisol levels 200 fold in brown trout (Waring *et al.*, 1996).

A set of proteins termed stress proteins or heat shock proteins (HSPs) were shown to be induced by physico-chemical stressors such as temperature (Dyer *et al.*, 1990), metals (Levinson *et al.*, 1980), hypoxia (Sciandra *et al.*, 1984), and osmolarity (Sheikh-Hamad *et al.*, 1994). HSP70 is the most widely studied HSP, and its expression has been studied at the mRNA level (Airaksinen, 1998). Despite these studies, little is known about what is involved in psychological stress in fish.

Psychological stimuli were generally shown to lead to changes at the molecular and biochemical levels (Mazeaud *et al.*, 1977), and this might induce HSP70. In a previous study (Kagawa *et al.*, 1999), we reported the enhancement of HSP70 in the brains of goldfish exposed to a predator (bluegills) and suggested that visual perception plays a primary role in this response. HSP70 expression by this stress should be further analyzed at the gene level. Plasma cortisol level also should be examined as an indicator of psychological stress.

The purpose of the present study is to develop the idea that fish perceive psychological stress via vision by measuring multiple indicators. We exposed goldfish to bluegills and examined the induction of HSP70 mRNA in the brains by Northern blotting and plasma cortisol levels by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Both sexes of immature goldfish (*Carassius auratus*) weighing 18.7±2.1g (mean and standard deviation) were selected from our laboratory stocks and used for the following experiments. Bluegills (*Lepomis macrochirus*) weighing 109.7±19.8 g were obtained from the moat of Gryoukaku Park in Hakodate and Lake Biwa in Shiga Prefecture. Goldfish and bluegills were separately acclimated to experimental conditions in aerated 40-l plastic or 60-l glass tanks with a filtration for 2 weeks before use. Throughout the acclimation and experimental periods, they were maintained at 20±0.5°C under LD 12:12 (light phase, 06:00–18:00 hr). Fish were fed carp food pellets at 17:00 hr during the acclimation period but were starved on the day of the

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experiments. All experiments were carried out in the light phase.

Experimental protocol

Goldfish were exposed to bluegills in four ways (Exps. 1–4) for two different time periods (6 and 12 hr). In addition, another experimental period of 2 hr was tested in Exp. 2. In each case, the goldfish were quickly netted and sampled for HSP70 mRNA analyses and plasma cortisol assay. Exps. 1–4 were repeated 2 or 3 times each and the brains from 8 or 9 goldfish were pooled for a single RNA sample.

Exp. 1: Six goldfish were acclimated in a 60-l single tank. Three of them were removed and sampled for a control. Four bluegills were then transferred to the tank with the 3 remaining goldfish. The mixed rearing was continued for the allotted time (6 hr in one trial and 12 hr in the second) and then the goldfish were sampled.

Exp. 2: The 60-l tank was divided in half by a net with a mesh size of 1 × 1 cm and 3 goldfish were placed in each compartment. After acclimation, 3 fish in one compartment were removed for a control and 4 bluegills were transferred to the empty compartment. Both groups of fish were maintained in this condition for 2, 6 or 12 hr and then the goldfish were sampled.

Exp. 3: Four goldfish were acclimated in a 40-l transparent plastic tank set at the corner of a larger opaque container (285 l). Four bluegills were then transferred to the container. Thus the goldfish were exposed to the bluegills from three sides (two lateral and bottom sides). Both of fish were maintained in this condition for 6 or 12 hr and then the goldfish were sampled. Goldfish reared with other goldfish instead of the bluegills were used as a control.

Exp. 4: Six goldfish and 4 bluegills were acclimated in separate 60-l tanks covered with black-painted boards on all sides except the top and the bottom. Three goldfish were then removed and sampled for a control. The ambient water in both tanks was then mixed by circulating it constantly at a rate of 10 l per min. The remaining goldfish were sampled after 6 or 12 hr after commencement of the circulation.

RNA extraction

Total RNA was extracted from the brains of goldfish using an RNA extraction kit, ISOGEN (Nippon Gene) according to the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). After homogenization of the brains in ISOGEN, the homogenate was centrifuged at 15,000 rpm for 30 min. Chloroform was added to the supernatant and the mixture was centrifuged at 15,000 rpm for 30 min. Then, 2-propanol was added to the supernatant and the mixture was centrifuged at 15,000 rpm for 30 min. The pellet was washed in 80% ethanol, lysed with water as a total RNA sample, and stored at –75°C for Northern blot analyses.

RT-PCR for HSP70 cDNA

Goldfish were heat-shocked by direct transfer from 20°C to 35°C and total RNA (including HSP70 mRNA) was extracted from the brains after 6 hr as described above.

The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using an AMV RNA PCR kit (TaKaRa) by a DNA thermal cycler (Perkin Elmer). Primers were chosen so as to include partial sequences of the rainbow trout HSP70 cDNA (Kothary, 1984). The upstream primer was a 22-mer sense oligonucleotide (5'-CCCTGCCTACTTCAACGATTCA-3') and the downstream primer was a 20-mer antisense oligonucleotide (5'-CCCTGCCTACTTCAACGATTCA-3'). Total RNA (1μg) was reverse transcribed at 94°C for 3 min and then PCR amplifications were made for 38 cycles (94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min). 487 bp RT-PCR products were analyzed on 2% agarose gel (Fig. 1) and confirmed to be identical to those reported for rainbow trout HSP70 cDNA (Kothary, 1984) using a DNA sequencer (Model-373A, Applied Biosystems).

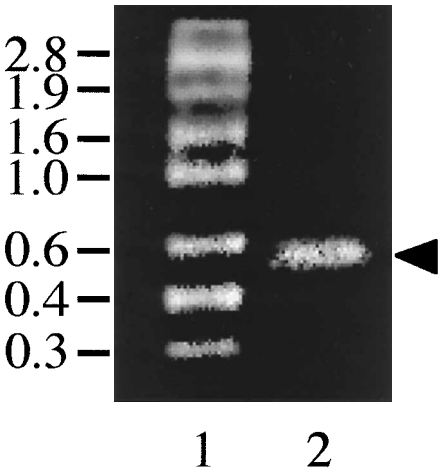


Fig. 1. Agarose gel electrophoresis of RT-PCR product from the brains of heat-shocked goldfish. Lane 1: DNA size markers (kb); lane 2: RT-PCR product (487 bp, arrowhead).

Northern blotting

Total RNA concentrations were quantified spectrophotometrically. Twenty μg of each RNA sample were mixed with 10% 3-morpholinopropane sulfonic acid (MOPS) buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), pH 7.0), 16.25% formaldehyde, and 50% formamide, and then were denatured at 65°C for 5 min. After mixing with a 1/10 volume of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue), samples were applied to each lane of 1% agarose-formaldehyde gel. After 1 hr electrophoresis at 40 mA, the gel was stained with ethidium bromide for 25 min and bleached for 25 min with 200 mM sodium acetate solution (pH 4.0). After electrophoresis, RNA was transferred overnight to polyvinylidene difluoride membranes (Millipore). The transfer buffer was 20×saline sodium citrate (SSC: 333 mM NaCl, 333 mM sodium citrate, pH 7.0). The completion of transfer was confirmed by ultraviolet transillumination. After being dried and heated at 80°C for 2 hr, the membranes were prehybridized with 10×SSC containing 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 10% sodium dodecyl sulfate (SDS), 0.2% bovine serum albumin, and 0.1 mg/ml calf thymus DNA at 65°C for 2 hr. They were hybridized overnight under the above conditions with a random-primed ^{32}P-labeled HSP70 probe (100,000 cpm/ml). The membranes were washed in 1 × SSC containing 0.1% SDS for 30 min. The amount of HSP70 mRNA in the membrane was measured by autoradiography using a Bio Image System (BAS2000, Fujix).

All glassware and solutions were treated with 0.1% diethylpyrocarbonate and baked or autoclaved before use.

ELISA for cortisol

Goldfish were bled by cutting off the caudal peduncle and blood was collected into heparinized capillary tubes. Plasma was separated by centrifugation at 4,000 rpm for 20 min and stored at –80°C for cortisol analyses.

Plasma cortisol levels were measured according to the method of Barry (1993) and Lewis (1986). Briefly, ELISA plates (96 wells-Maxisorp-immunoplate, Nunc) were coated with rabbit antiserum against cortisol-3-carboxymethyloxime(CMO) -bovine serum albumin (BSA) in coating buffer (0.035 M sodium bicarbonate, 0.015 M sodium carbonate, pH 9.6). After coating, each well was washed 4 times with washing buffer (phosphate buffered saline, PBS: 0.05 M NaH_2PO_4, 0.15 M NaCl, pH 7.4, containing 0.1% Tween20). After the plates were blocked with assay buffer (PBS containing 0.1% Tween20 and 0.1% BSA), either cortisol standards (Sigma) or plasma samples diluted in cortisol-3-CMO-horseradish peroxidase (HRP) (CosmoBio)

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were added. The standards contained 0–1000 ng/ml cortisol. Two wells received cortisol-3-CMO-HRP only and were served as the maximum binding wells. After the plates were washed, substrate solution (50 mM Na_2HPO_4, 25 M citric acid, pH 5.0, containing 0.04% o-phenylenediamine and 0.03% H_2O_2) was added and the plates were incubated for 20 min. The substrate reaction was stopped by adding 1.25 M sulfuric acid.

Absorbance (B) of plasma and standard samples was measured at 492 nm with a microplate reader (Corona). The absorbance of the maximum binding wells was served as the zero standard (Bo) in determining the percent bound (B/Bo).

Plasma cortisol concentrations were calculated using a standard curve established by plotting B/Bo against cortisol concentrations.

Statistical analyses

Data were expressed as means ± SEM and analyzed by one way ANOVA followed by Bonferroni/Dunn test. Significance was accepted at $p<0.05$.

RESULTS

Fish behavior

Fish behavior was essentially the same as described in the previous study (Kagawa *et al.*, 1999). When bluegills were

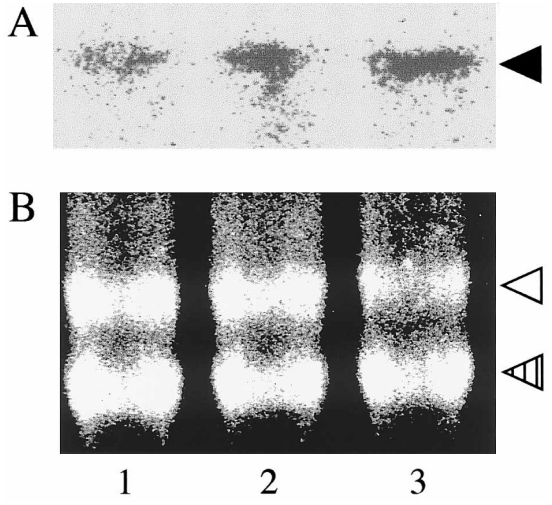


Fig. 2. Northern blot analyses (A) of HSP70 mRNA (2.4 kb, arrowhead) in the brains of goldfish from Exp. 1. B shows 28 S- (open arrowhead) and 18 S-rRNA (hatched arrowhead). Lane 1: control fish; lane 2: experimental fish (6 hr); lane 3: experimental fish (12 hr).

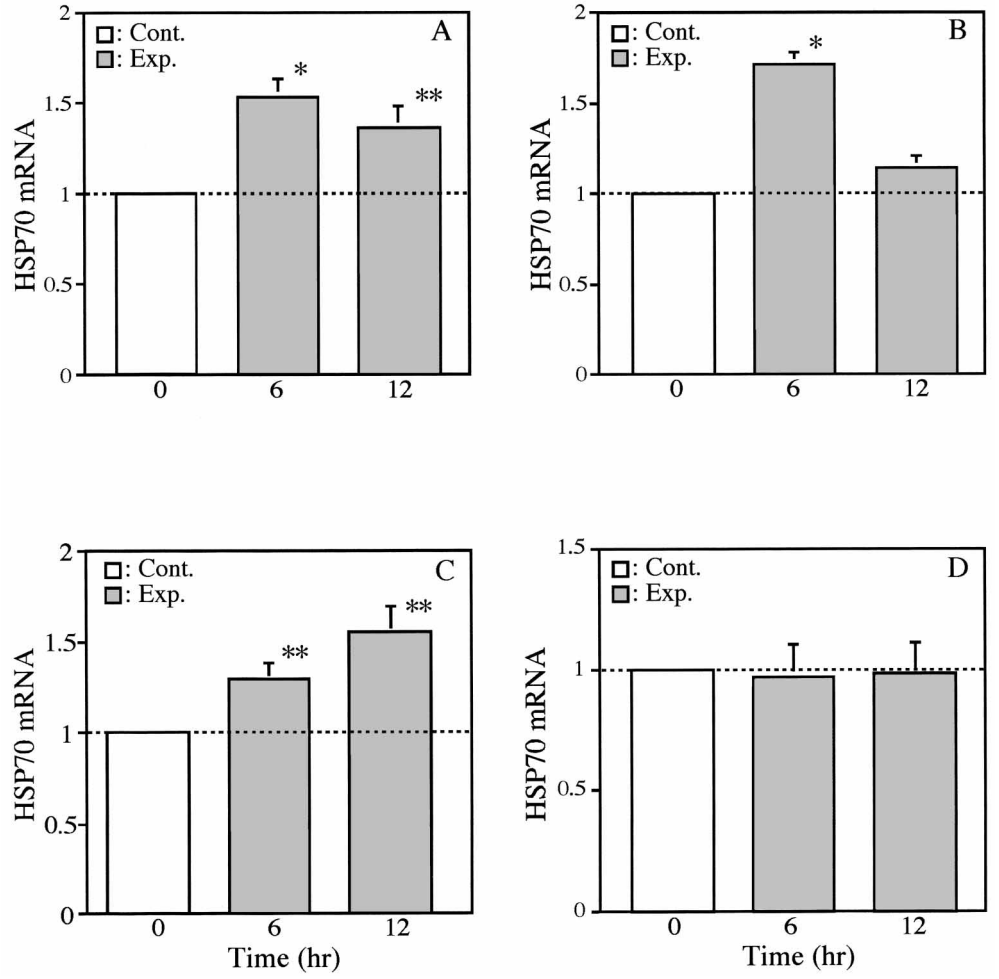


Fig. 3. HSP70 mRNA expression in the brains of goldfish from Exps. 1 (A), 2 (B), 3 (C), and 4 (D). Each experiment was repeated 3 times and the brains from 8 or 9 fish were pooled for a single analysis. This series of experiments was repeated 3 times for statistical analyses. Results are presented as relative to control values (means±SEM for 3 determinations with 24 or 27 fish). * and **: $p<0.01$ and $p<0.05$ for each control, respectively.

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transferred to the tank with goldfish (Exp. 1), the goldfish panicked and swam randomly and more actively than usual for the first 1 hr. Their branchial movement seemed accelerated. Then they remained in a group keeping a distance of about 10 cm from the bluegills through the experimental period. The branchial movement seemed normal at the end. The bluegills calmly swam near the bottom and did not attack the goldfish throughout observation. In the experiment with the net partition (Exp. 2), goldfish always kept a distance from the side of bluegills until about 6 hr after being placed together. Thereafter, they behaved as usual, sometimes approaching the net. In the experiment with the transparent tank (Exp. 3), goldfish were in much the same behavior as that in Exp. 1. They made a group near the upper and kept a distance from the side of bluegills through the experimental period. In the water circulation experiment (Exp. 4), goldfish calmly swam as in the acclimation period through the 12-hr experimental period.

HSP70 mRNA expression

The brains of goldfish were analyzed for HSP70 mRNA 6 and 12 hr after being placed together with the bluegills in a single tank without a partition (Exp. 1). In Northern blot analyses, HSP70 mRNA bands were detected at about 2.4 kb in all samples from the control and experimental fish (Fig. 2). The level of HSP70 mRNA expression was quantified by autoradiography and expressed as ratios of experimental to control levels, because the control levels were almost the same throughout Exps. 1–4. HSP70 mRNA significantly increased in the brains of the experimental fish after 6 ($p<0.01$) and 12 hr ($p<0.05$), compared with the control level (Fig. 3A). Goldfish exposed to bluegills through the net partition (Exp. 2) also showed a marked increase in HSP70 mRNA after 6 hr ($p<0.01$, Fig. 3B). However, this level returned to the control level after 12 hr. When goldfish were placed in the transparent tank around which bluegills were swimming (Exp. 3), the expression of HSP70 mRNA was enhanced after 6 and 12 hr, compared with the control level (Fig. 3C). The level of HSP70 mRNA expression remained unchanged at both examination times (Fig. 3D) in the brains of goldfish exposed to the ambient water circulating through the tank of bluegills (Exp. 4).

Plasma cortisol levels

Goldfish directly exposed to bluegills (Exp. 1) showed marked increases in plasma cortisol levels from 30 ng/ml (basal level) to more than 200 ng/ml after 6 and 12 hr ($p<0.01$, Fig. 4A). In the experiment with the net partition (Exp. 2), plasma

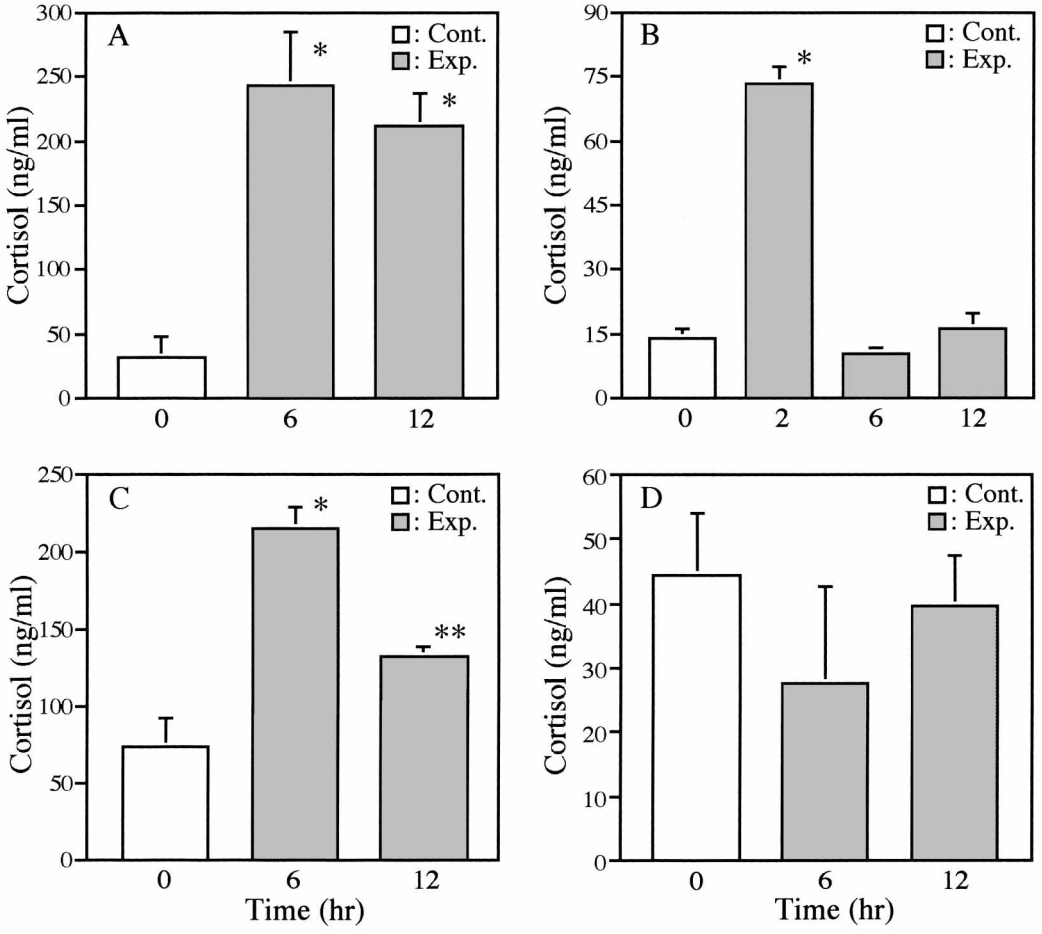


Fig. 4. Plasma cortisol levels in goldfish from Exps. 1 (A), 2 (B), 3 (C), and 4 (D). Data are presented as means ± SEM for 3 fish. * and **: $p<0.01$ and $p<0.05$ for each control, respectively.

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cortisol levels increased to 75 ng/ml after 2 hr ($p<0.01$, Fig. 4B), but this level returned to the basal level after 6 and 12 hr. Plasma cortisol levels also increased to 210 ng/ml ($p<0.01$) after 6 hr and to 120 ng/ml ($p<0.05$) after 12 hr (Fig. 4C) in goldfish exposed to bluegills through the transparent tank (Exp. 3). In the experiment in which goldfish were exposed to bluegills through the ambient water (Exp. 4), plasma cortisol levels showed no difference between the experimental and control fish at either examination time (Fig. 4D).

DISCUSSION

Fish are exposed to various stressors in the environment. Enhancement of HSP70 expression and an increase in plasma cortisol levels have been used as biochemical indicators of stress responses. (Thomas, 1990; Donaldson, 1981). HSP70 expression was enhanced by stressors such as heat shock (Iwama *et al*., 1998), toxicants (Sanders *et al*., 1995), and bacterial infection (Forsyth *et al*., 1997). Plasma cortisol levels were also elevated by toxicants (Waring *et al*., 1996), handling (Vijayan and Moon, 1992), and heat shock (Strange *et al*., 1977). These stressors rather belong to physico-chemical categories. We have very little information as to what is involved in responses to psychological stress in fish. Kagawa *et al*. (1999) stressed goldfish psychologically by exposing them to a predator, bluegills, and found the enhancement of HSP70 in the brains of the goldfish. In the present study, we analyzed HSP70 mRNA in the brains and plasma cortisol levels in four experimental conditions including the same experimental regime as used in the previous study (Kagawa *et al*., 1999). This is the first report showing that exposure to a predator (psychological stress) enhanced HSP70 mRNA expression in the brains and plasma cortisol levels in fish.

Because handling induces stress responses in fish (Pickering and Pottinger, 1989), handling effects should be discriminated from psychological stress in the present study. Sumpter *et al*. (1986) found that plasma cortisol levels did not change after 2-min handling, but rose significantly after 5-min handling in rainbow trout. Three min-handling stress did not affect hepatic HSP70 expression in rainbow trout (Vijayan *et al*., 1997). Considering these results and the fact that it took no more than 2 min to collect blood samples in the present study, increases in blood cortisol levels and HSP70 mRNA expression in the brains are attributable to the psychological stress induced by bluegills. Actually, HSP70 expression in the brains of the control goldfish, i. e., goldfish that were exposed to goldfish instead of bluegills, remained unchanged in the previous study (Kagawa *et al*., 1999).

A significant enhancement of HSP70 mRNA occurred 6 and 12 hr after exposure in Exp. 1 (mixed rearing in a single tank without a partition), 6 hr in Exp. 2 (mixed rearing with a net partition), and 6 and 12 hr in Exp. 3 (rearing in a transparent tank). No enhancement was found at any examination time in Exp. 4 (rearing in two separate tanks with water circulating through the tanks). These results except Exp. 3 are quite similar to those described for HSP70 expression (Kagawa *et al*., 1999), supporting, at the gene level, the previous conclusion that fish primarily perceive stress psychologically via vision, which then leads to enhancement of HSP70 in the brains. The results in Exp. 3 strengthened this conclusion.

When goldfish were exposed to bluegills through the net partition (Exp. 2), HSP70 mRNA expression was enhanced 6 hr and returned to the control level 12 hr after exposure. However, goldfish exposed to the predators from the inside of the transparent tank showed the enhanced expression of HSP70 mRNA through an experimental period of 12 hr (Exp. 3). This is probably explained by the following. Goldfish may become acclimatized to the experimental condition in an exposure to the predators from only one side until 12 hr in Exp. 2, while they might be continuously stressed when exposed from three sides in Exp. 3. The behavior of goldfish mentioned in the results supports this possibility.

Although the causative relationship between cortisol and HSP70 is not clear, the present psychological stress induced marked increases in plasma cortisol levels. The response of cortisol was similar to that of HSP70 mRNA except 6 hr after exposure in Exp. 2, in which HSP70 mRNA expression was enhanced, while cortisol levels remained unchanged. It can be argued that exposure of goldfish to bluegills induced an elevation in plasma cortisol levels, but the cortisol levels returned to the basal level before 6 hr, because plasma cortisol has a half-life of 4 hr in coho salmon (Redding *et al*., 1984). Actually, handling- and pollutant-induced increases in plasma cortisol levels returned to control levels within 4 hr in roach (Pottinger *et al*., 1999) and 6 hr in striped mullet (Thomas *et al*., 1980). An increase in plasma cortisol levels after 2 hr in Exp. 2 supports this idea.

In conclusion, HSP70 mRNA expression in the brains was enhanced by exposing goldfish to bluegills. Plasma cortisol levels were also responded to the stress.

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