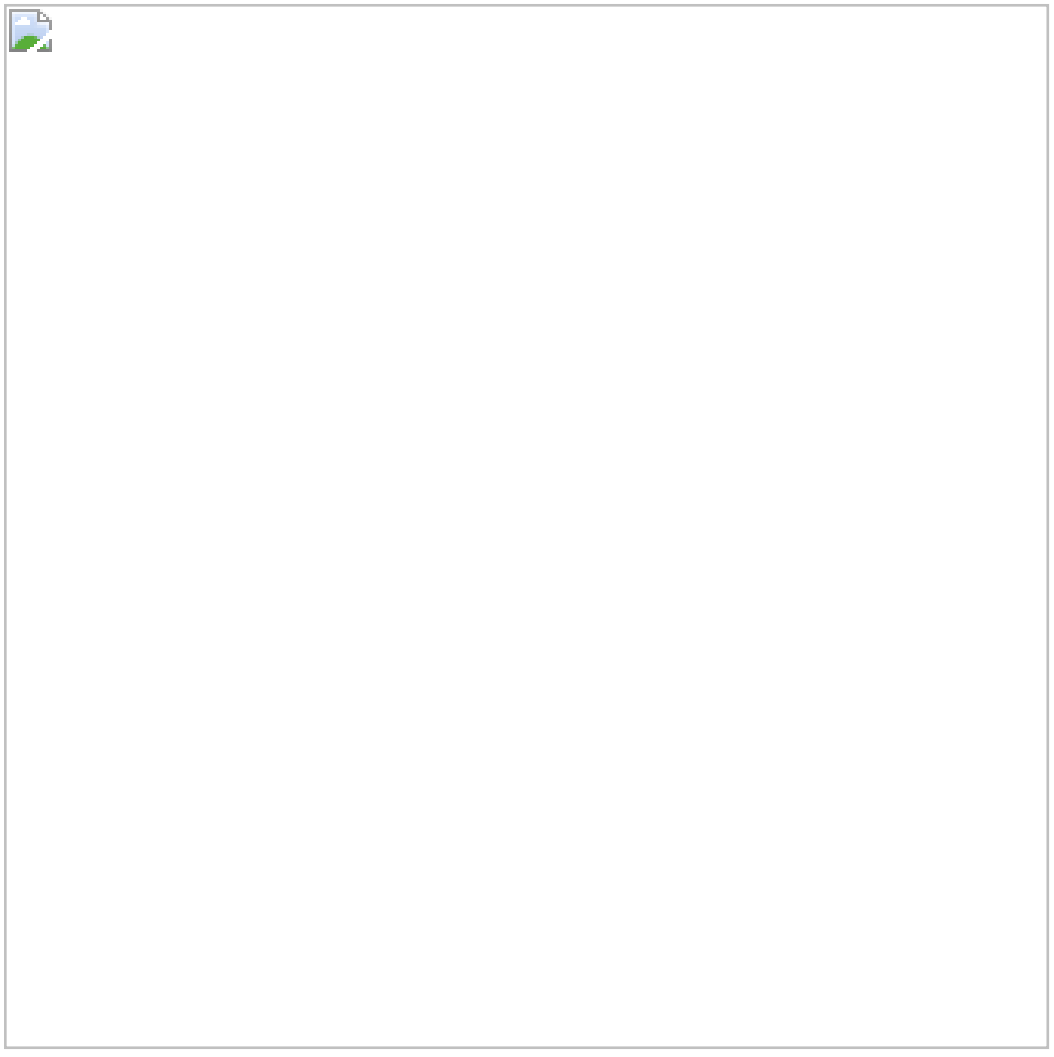

# Brain HSP70 mRNA Expression is Linked with Plasma Cortisol Levels in Goldfish (Carassius auratus) Exposed to a Potential Predator

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# Brain HSP70 mRNA Expression is Linked with Plasma Cortisol Levels in Goldfish (*Carassius auratus*) Exposed to a Potential Predator

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**ABSTRACT**—We previously found that when goldfish were exposed to a potential predator, bluegills, the goldfish experienced an increase in HSP70 mRNA expression in the brains and increased plasma cortisol levels. In the present study, we examined the potential causative relationship between HSP70 mRNA expression and plasma cortisol levels. Cortisol agonists (corticotropin releasing factor and cortisol) and antagonists (metyrapone and betamethasone) were used to modulate plasma cortisol levels. HSP70 mRNA expression and plasma cortisol levels were analyzed by Northern blotting and ELISA, respectively. Goldfish treated with the cortisol agonists showed marked increases in plasma cortisol levels and also in brain HSP70 mRNA expression. When goldfish were exposed to bluegills, plasma cortisol levels increased and HSP70 mRNA expression was enhanced after 6 hr. However, pre-treatment with the cortisol antagonists 24 hr prior to the exposure inhibited the enhancement as well as the increase in plasma cortisol levels. These results suggest that plasma cortisol plays a key role in the enhancement of brain HSP70 mRNA expression in goldfish stressed by exposure to bluegills.

**Key words**: brain HSP70, cortisol, predator-prey stress

## INTRODUCTION

Stress responses of organisms exposed to an unfavorable environment are expressed at behavioral, physiological, and biochemical levels. In fish, stress responses have been examined under various physico-chemical stressors. Wedemeyer (1997) reported that bacterial disease, fin erosion, and skeletal anomalies are induced by environmental stressors such as hyperthermia, handling, crowding, low oxygen, and elevated ammonia. Acidification and industrial pollutants in the ambient water also act as stressors, resulting in impairment of oogenesis and spermatogenesis (Jimenez and Stegeman, 1990; Pankhurst and Van Der Kraak, 1997).

Plasma cortisol is an excellent indicator of stress responses (Donaldson, 1981). For example, aluminium exposure increased plasma cortisol levels 200 fold in brown trout (Waring *et al*., 1996), and capture and handling increased plasma cortisol levels seven fold in coral trout (Frisch and Anderson, 2000). A rise in plasma cortisol levels is induced through the hypothalamus-pituitary-interrenal (HPI) axis. Stress stimulates the secretion of corticotropin-releasing factor (CRF) from the hypothalamus, which induces the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. Then, ACTH works on the head kidney to secrete cortisol into the blood (Mazeaud *et al*., 1977).

Another indicator of stress responses in fish is heat shock proteins (HSPs) which are induced by physico-chemical perturbations such as unfavorable temperature (Schmidt *et al*., 1998), toxic metals (Williams *et al*., 1996), hypoxia (Airaksinen *et al*., 1998), and hypertonicity (Smith *et al*., 1999). Kagawa and Mugiya (2000) found that psychogenic stress such as exposure of goldfish to a potential predator (bluegills) synchronously increased brain HSP70 mRNA expression and plasma cortisol levels. These results suggested that such enhancement in the brains was induced by HPI axis-dependent responses to the psychogenic stress, because the brains have cortisol receptors (Teitsma *et al*., 1998; Bernier *et al*., 1999). Recent studies (Iwama *et al*., 1999; Deane *et al*. 1999) reported that cortisol did not induce hepatic HSP70 expression in fish. On the other hand, Udelsman *et al*. (1994) reported that ACTH was related to HSP70 expression in the adrenal gland in rats. Thus, the

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cortisol-HSP70 relationship could be tissue specific. However, no information is available about the potential causative relationship between plasma cortisol levels and brain HSP70 expression in fish.

The present study was undertaken to elucidate whether stress responses through the HPI axis induce the enhancement of brain HSP70 expression. Goldfish were stressed by exposure to bluegills and plasma cortisol levels and brain HSP70 mRNA expression were analyzed. Cortisol agonists and antagonists were used to modulate plasma cortisol levels.

## MATERIALS AND METHODS

### Fish

Immature goldfish (*Carassius auratus*) weighing about 15 g and bluegills (*Lepomis macrochirus*) weighing about 150 g were selected from our laboratory stocks and used for experiments. Goldfish and bluegills were separately acclimated to experimental conditions in aerated 60-l glass tanks with filtration for 2 weeks before use. Throughout the acclimation and experimental periods, the fish were maintained at 20±0.5°C under LD 12:12 (light phase, 06:00-18:00 hr). Fish were fed carp food pellets once a day during the acclimation period but were starved on the day of the experiments. All experiments were carried out in the light phase.

### Cortisol secretion by agonists

CRF is known to stimulate ACTH release from the pituitary (Weld *et al*., 1987) and then to increase plasma cortisol levels in goldfish (De Pedro *et al*., 1997). Ovine CRF (Peptide Institute Inc.) was diluted in a saline (0.1 M NaCl and 1.8 mM $Na_2CO_3$) and intracerebroventricularly administered into goldfish anesthetized with 2-phenoxyethanol at a dose of 1 μg CRF / fish. CRF was administered through the central junction between the parietal and frontal bones using a 26S-needle connected to a 10-μl microsyringe (Hamilton) as described by De Pedro *et al*. (1993). The accuracy of administration into the third ventricle was preliminarily established by administering trypan blue and by confirming the position of the color in the ventricle. CRF-administered goldfish were sampled and analyzed for brain HSP70 mRNA expression and plasma cortisol levels after 2, 6, and 24 hr by Northern blotting and ELISA, respectively. Cortisol (Sigma) was diluted in dimethyl sulfoxide (DMSO)

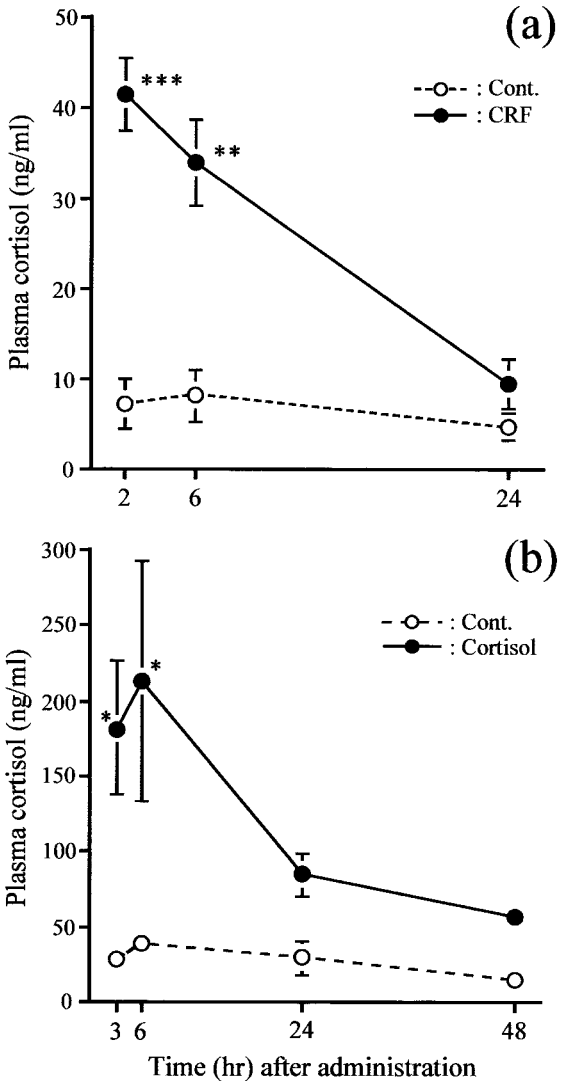


**Fig. 1.** Time course-related changes in plasma cortisol levels after administration of CRF (a) or cortisol (b) in goldfish. Data are presented as means ± SEM for three fish. *, ** and ***: p<0.05, 0.01, and 0.001 for each control, respectively.

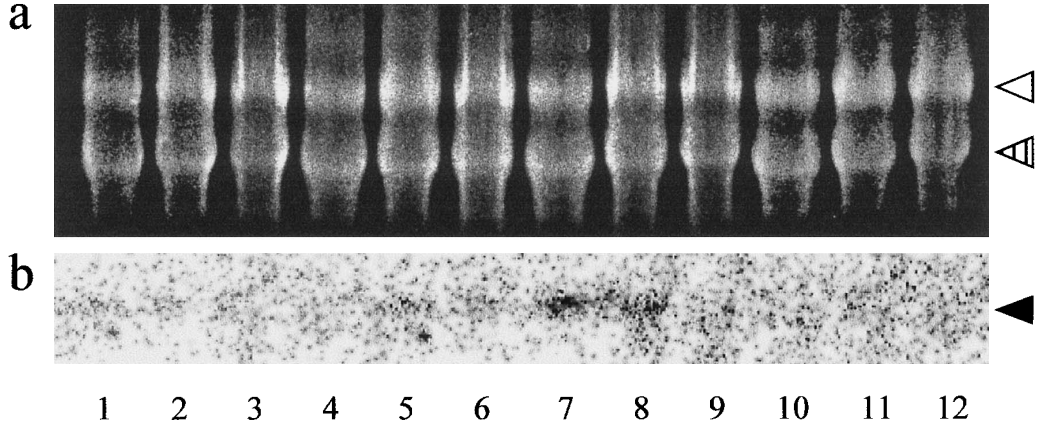


**Fig. 2.** Agarose gel (a) and Northern blot analyses (b) of HSP70mRNA (black arrowhead) in the brains of goldfish after administration of CRF. Open and hatched arrowheads show 28 S- and 18 S-rRNA, respectively. Lanes 1 and 2: control fish (2 hr) ; 3 and 4: experimental fish (2 hr) ; 5 and 6: control fish (6 hr) ; 7 and 8: experimental fish (6 hr) ; 9 and 10: control fish (24 hr) ; 11 and 12: experimental fish (24 hr).

and intraperitoneally administered at a dose of 8 μg/g-body weight. Cortisol-administered goldfish were also analyzed for plasma cortisol levels and HSP70 mRNA expression after 3, 6, 24, and 48 hr. The control groups of each experiment received the respective solvents.

**Inhibition of cortisol secretion by antagonists**

HSP70 mRNA induction by stress was examined under cortisol-blocked conditions using two cortisol antagonists. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, Aldrich) is an inhibitor for 11-β-hydroxylase which is involved in the biosynthesis of cortisol in the kidney (Fryer and Boudreault-Châteauvert, 1981). Betamethasone (Wako) is known to inhibit the secretion of CRF and ACTH and therefore of cortisol into the blood (Hawkins and Ball, 1973; Keller-Wood and Dallman, 1984). Metyrapone was diluted in a solution containing 2.7% methanol and 0.1 M NaCl and administered at a dose of 100 μg/g-body weight. Betamethasone was diluted in DMSO and intraperitoneally administered at a dose of 1 μg/g-body weight. The shams received the respective solvents only.

**Stress loading**

According to Fryer (1975) and Hopkins *et al.* (1995), betame–thasone and metyrapone blocked a stress-induced increase in plasma cortisol levels 24 hr after treatment in goldfish and toadfish. Thus in the present study, twenty-four hr after administration of the antagonists, goldfish were exposed to bluegills in the same protocol as described by Kagawa and Mugiya (2000). Briefly, six goldfish were acclimated in a 60-l single tank. Three of them were quickly removed for the control. Four bluegills were then transferred to the tank with three remaining goldfish and the mixed rearing was continued for 6 hr. Then the goldfish were quickly netted and sampled for plasma and the brains.

**Northern blot analyses**

RNA extraction and Northern hybridization were performed according to Kagawa and Mugiya (2000). Briefly, after electrophoresis of total RNA, the agarose gel was stained with ethidium bromide. RNA was transferred to a polyvinylidene difluoride membrane, prehybridized, and then hybridized with a $^{32}$P-labeled HSP70 probe. This probe was obtained by the reverse transcriptase-polymerase chain reaction (RT-PCR) from the brain total RNA of heat-shocked goldfish using an AMV RNA PCR kit (TaKaRa). The upstream primer was a 22-mer sense oligonucleotide (5'-GGATCATCAAT-GAGCCCACG-3') and the downstream primer was a 20-mer antisense oligonucleotide (5'-CTAGGGGTTCCAGGTCTTCG-3'). RT-PCR products were sequenced. The nucleotide sequences were confirmed to be identical to those reported for rainbow trout HSP70

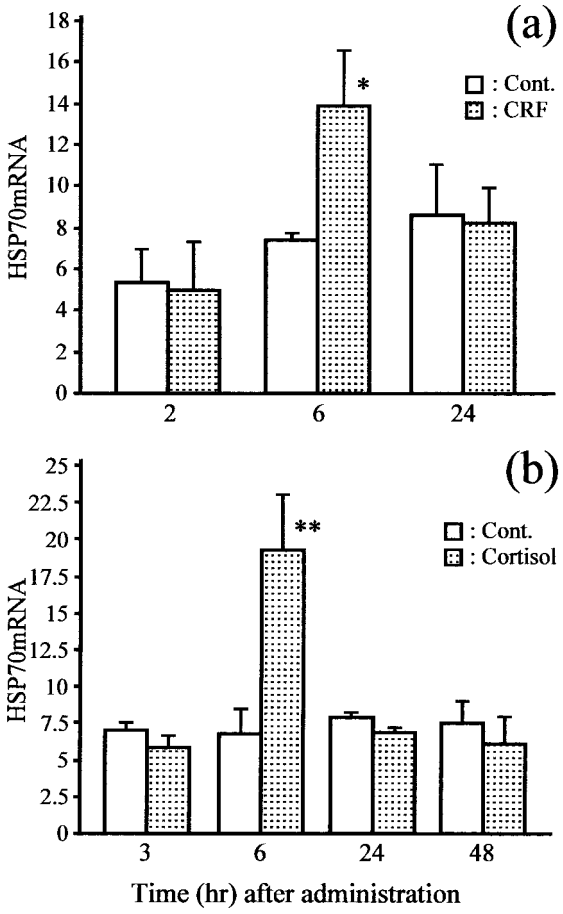


**Fig. 3.** HSP70 mRNA expression in the brains of goldfish after administration of CRF (a) or cortisol (b). Data are presented as means ± SEM for three fish. * and **: p<0.05 and p<0.01 for respective controls.

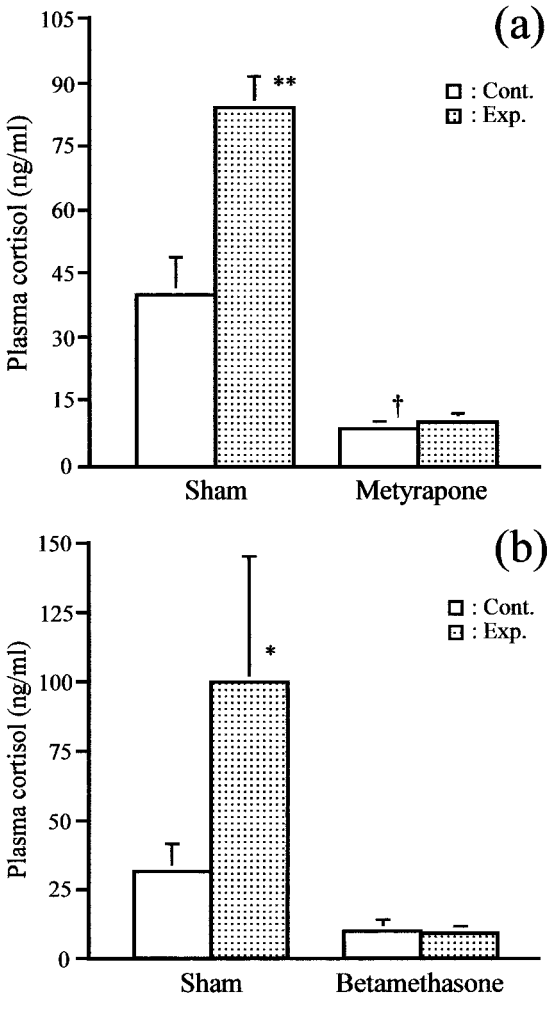


**Fig. 4.** Plasma cortisol levels in goldfish administered with metyrapone (a) or betamethasone (b) prior to exposure to bluegills. Data are presented as means ± SEM for three fish. * and **: p<0.05 and p<0.01 for each control, respectively. †: p<0.05 for sham control.

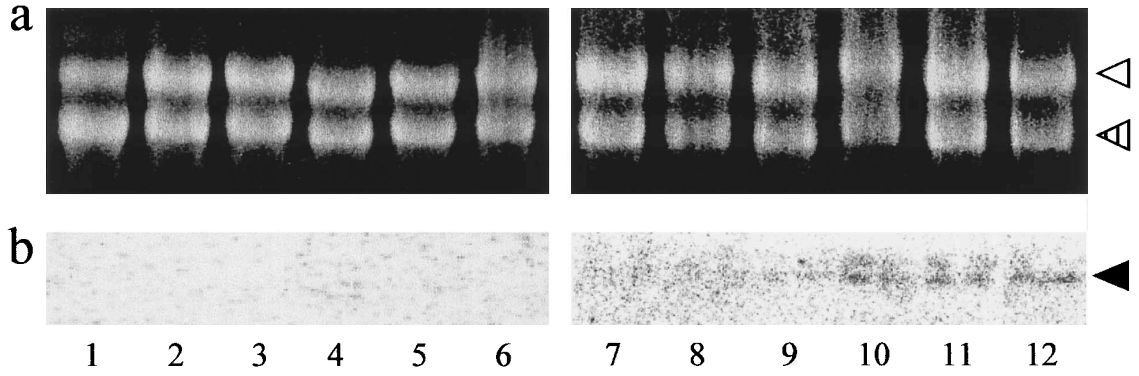


**Fig. 5.** Agarose gel (a) and Northern blot analyses (b) of HSP70mRNA (black arrowhead) in the brains of goldfish administered with metyrapone. Open and hatched arrowheads show 28 S- and 18 S-rRNA, respectively. Lanes 1–3: control fish (metyrapone without stress) ; 4–6: experimental fish (metyrapone with stress) ; 7–9: control fish (sham without stress) ; 10–12: experimental fish (sham with stress).

cDNA (Kothary, 1984).

The amount of HSP70 mRNA in the membrane was measured with $^{32}$P-stimulated luminescence using a BioImage System (BAS-2000, Fujix).

**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 700 × g for 20 min. Plasma cortisol levels were measured using ELISA plates (96 well-Maxisorp-immunoplate, Nunc) as described by Kagawa and Mugiya (2000).

**Statistical analyses**

Data were expressed as means ± SEM and analyzed by one way ANOVA followed by Fisher's PLSD test or two way ANOVA. Significance was accepted at $p<0.05$.

## RESULTS

### Cortisol agonist effects

The basal cortisol level in the control goldfish was about 10 ng/ml through the experimental periods. CRF administration, however, markedly increased the level to 40 ng/ml ($p<0.001$) after 2 hr and to 35 ng/ml ($p<0.01$) after 6 hr (Fig. 1a), followed by a decrease to the basal level after 24 hr. Cortisol administration also induced marked increases in plasma cortisol levels to 180 ng/ml ($p<0.05$) after 3 hr and to 210 ng/ml ($p<0.05$) after 6 hr (Fig. 1b), followed by decreases to 80 ng/ml and to 60 ng/ml after 24 and 48 hr, respectively. DMSO had no effect on the basal cortisol levels at any examination times.

The brains were analyzed for HSP70 mRNA expression 2, 6, and 24 hr after administration of CRF. In Northern blot analyses, HSP70 mRNA bands were clearly detected at about 2.4 kb and their expression seemed to be enhanced in the experimental samples after 6 hr (Fig. 2).

CRF administration induced a significant enhancement of HSP70 mRNA expression after 6 hr ($p<0.05$) (Fig. 3a), while the expression remained at the control level 2 and 24 hr after administration.

Cortisol administration also increased the expression of HSP70 mRNA in the brains after only 6 hr ($p<0.05$) (Fig. 3b). No changes in expression were found 3, 24, and 48 hr after administration.

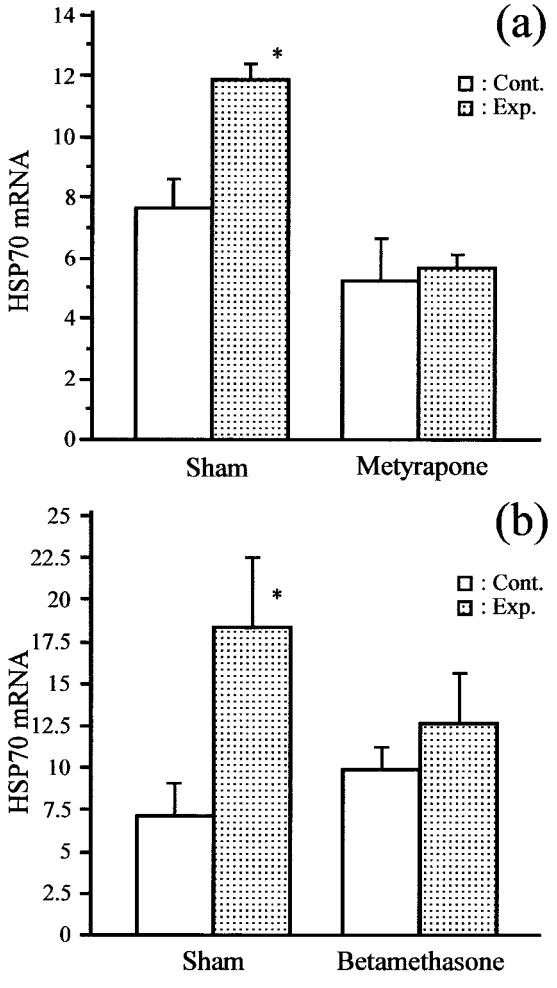


**Fig. 6.** HSP70 mRNA expression in the brains of goldfish administered with metyrapone (a) or betamethasone (b) prior to exposure to bluegills. Data are presented as means ± SEM for three fish. *: $p<0.05$ for respective controls.

**Cortisol antagonist effects**

When goldfish were exposed to bluegills, they panicked at first and seemed to be more active than usual with accelerated branchial movements until the end of the experiment. Goldfish were not physically attacked by bluegills and remained in a group keeping a distance from bluegills as far as we observed. The cortisol antagonists used did not seem to affect the fish behavior.

Plasma cortisol levels of the shams increased from 40 (basal level) to 80 ng/ml 6 hr after exposure to bluegills ($p<0.01$) (Fig. 4a). However, plasma cortisol levels remained unchanged after the exposure in the metyrapone-administered group. This treatment depressed the basal cortisol level from 38 to 8 ng/ml ($p<0.05$). In the betamethasone experiment, plasma cortisol levels increased from 30 (basal level) to 100 ng/ml ($p<0.05$) as a result of the predation-prey response in the shams (Fig. 4b). However, pre-treatment with betamethasone completely blocked this increase.

In the Northern blot analyses, HSP70 mRNA expression appeared to be enhanced in goldfish exposed to bluegills without metyrapone treatment (shams) (Fig. 5). Quantification of these bands revealed that this expression was significantly enhanced by the exposure ($p<0.05$) (Fig. 6a). However, pre-treatment with metyrapone reduced the enhanced expression of HSP70 mRNA to the control level. In the betamethasone experiment, the predator-prey stress induced a marked increase in HSP70 mRNA expression in the shams ($p<0.05$) (Fig. 6b), which was completely blocked by betamethasone.

## DISCUSSION

Stress responses in fish are expressed at several levels, from the physiological to the behavioral levels (Schreck, 1990). Increases in plasma cortisol levels and/or HSP70 expression are used as indicators of stress responses (Thomas, 1990; Donaldson, 1981). It is generally accepted that a stress-induced increase in plasma cortisol levels activates hepatic glyconeogenesis (Inui and Yokote, 1975; Chan *et al*., 1978) to cope with stress-induced energy demand (Barton and Schreck, 1987; Vijayan and Moon, 1994). On the other hand, stress-induced HSP70 is considered to function as “molecular chaperonage”, which stabilizes protein structure under stress conditions (Willer *et al*., 2000).

We previously reported that plasma cortisol levels increased simultaneously with or prior to an enhancement in induction of brain HSP70 mRNA in psychologically stressed goldfish (Kagawa and Mugiya, 2000). These results suggest that brain HSP70 expression was secondarily induced in response to an increase in plasma cortisol levels. To test this hypothesis, we modulated plasma cortisol levels by administering the cortisol agonists (CRF and cortisol) or the antagonists (betamethasone and metyrapone), and examined brain HSP70 mRNA expression.

Administration of the agonists resulted in increases in brain HSP70 mRNA expression as well as plasma cortisol levels without any stress. This is the first report showing that the enhancement of brain HSP70 mRNA was induced by an increase in plasma cortisol levels in fish. Similarly, Vanmuylder and Dourov (2000) showed that HSP70 mRNA expression in the rat thymus was significantly enhanced by cortisol administration.

To further confirm the cortisol-HSP70 mRNA expression relationship, the cortisol antagonists (betamethasone and metyrapone) were given to goldfish and then the predator-prey interaction stress was imposed on them. This stress increased plasma cortisol levels and brain HSP70 mRNA expression in the shams without the antagonist treatments. On the other hand, both antagonists completely inhibited such increases. It seems reasonable that the enhancement of HSP70 mRNA expression was suppressed by a decrease in plasma levels of cortisol, not CRF and/or ACTH, because the common action of both antagonists is to inhibit cortisol secretion, though their inhibition mechanisms are different (see Materials and Methods). Therefore, exposure of goldfish to bluegills imposed psychogenic stress on the goldfish and induced an elevation in plasma cortisol levels. Cortisol, at these higher levels, then functioned as a mother hormone for induction of HSP70 mRNA.

Deane *et al*. (1999) and Iwama *et al*. (1999) found that cortisol did not enhance hepatic HSP70 expression in sea bream or trout. Kagawa *et al*. (1999) and Kagawa and Mugiya (2000) found that the predator-prey stress enhanced HSP70 expression in the brains together with an increase in plasma cortisol levels in goldfish. In this case, however, the hepatopancreas was not affected for HSP70 expression. These results suggest that each tissue has its own interaction between cortisol and HSP70 induction.

Although the detailed mechanisms by which cortisol enhances brain HSP70 mRNA expression remain unclear, it is hypothesized that the high binding of cortisol to glucocorticoid receptor which needs the assistance of HSP70 results in a trimerization of heat shock factors and the trimers play an accelerative role in HSP70 transcription and induction in the brains (Sorger, 1991; Morimoto *et al*., 1992). In turn, HSP70 promotes the binding affinity of cortisol to the receptors (Hutchison *et al*., 1994).

In conclusion, we have suggested that increased plasma cortisol stimulates induction of brain HSP70 mRNA in goldfish stressed by exposure to bluegills.

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