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SKIN DARKENING, A POTENTIAL SOCIAL SIGNAL IN SUBORDINATE ARCTIC CHARR (*SALVELINUS ALPINUS*): THE REGULATORY ROLE OF BRAIN MONOAMINES AND PRO-OPIOMELANOCORTIN-DERIVED PEPTIDES

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Summary

Arctic charr were allowed to interact in groups of three for 5 days. Skin darkness was quantified by measuring the mean brightness of individual fish before and after social interaction. Brain levels of monoamines and monoamine metabolites and plasma concentrations of cortisol, adrenocorticotropic hormone (ACTH), *N*-acetyl-β-endorphin and α-melanocyte-stimulating hormone (α-MSH) were analysed. The results show that social subordination resulted in a significant skin darkening. Furthermore, plasma concentrations of α-MSH, ACTH and cortisol were elevated in subordinates, and these fish also displayed elevated levels of 5-hydroxyindoleacetic acid (5-HIAA) in the telencephalon. The ratio of [5-HIAA] to serotonin [5-HT] was increased in several brain areas. In addition, the ratio of 3-methoxy-4-hydroxyphenylglycol (MHPG) to norepinephrine (NE) concentrations was significantly increased in the optic tectum of subordinate fish. Skin darkness following social interaction showed a significant positive correlation with plasma levels of α-MSH. Plasma levels of ACTH and α-MSH were both positively correlated with that of cortisol. Brain [5-HIAA]/[5-HT] ratios were positively correlated with circulating plasma levels of ACTH, and a similar positive correlation was seen between [MHPG]/[NE] ratios in the optic tectum and plasma levels of ACTH, α-MSH and *N*-acetyl-β-endorphin. In contrast, hypothalamic [MHPG]/[NE] ratios displayed a negative correlation with plasma α-MSH concentrations. The present study demonstrates that social stress induces skin darkening in Arctic charr and that this effect could be mediated by a stress-induced increase in the levels of α-MSH in the circulation. Furthermore, the results suggest that 5-HT and NE in the central nervous system could be factors regulating the pituitary release of ACTH and α-MSH.

Key words: salmonid, Arctic charr, *Salvelinus alpinus*, monoamine, pro-opiomelanocortin-derived peptides, brain, skin darkening, social signal.

Introduction

Animals often announce their fighting ability and aggressive state using a complex collection of cues, including visual, auditory and olfactory stimuli as well as actual physical contact. In fish, colour patterns often seem to play a rather specific role in the control of agnostic behaviour (Huntingford and Turner, 1987). Social subordination has been reported to be coupled to a darker body colour in several species of salmonid fish, e.g. rainbow trout (*Oncorhynchus mykiss*, Abbot et al., 1985) and Atlantic salmon (*Salmo salar*, O'Connor et al., 1999). It has been suggested that the darker body coloration of subordinates may act as an important social signal in salmonids and that darker body colour may reduce aggression from dominant individuals (Abbot et al., 1985; O'Connor et al., 1999). However, the effects of social interaction on skin colour have never been quantified, and the neuroendocrine mechanisms involved in socially induced colour changes in fish are largely unknown.

In socially organised teleosts, as in many other vertebrates, subordinate individuals are subjected to chronic stress induced by a general lack of control as a result of aggressive acts from individuals of higher social rank (for a review, see Winberg and Nilsson, 1993). Sustained social stress leads to chronic activation of the hypothalamic–pituitary–interrenal (HPI) axis [the teleost homologue of the mammalian hypothalamic–pituitary–adrenal (HPA) axis], resulting in a sustained elevation of plasma cortisol levels (Winberg and Lepage, 1998). Interestingly, the peptide adrenocorticotropic hormone (ACTH), which is considered to be the main factor for pituitary

control of interrenal cortisol release, also has a dispersing effect on chromatophores, causing skin darkening (Fujii and Oshima, 1986). The skin darkening effect of ACTH may be related to its structural similarity with α-melanocyte-stimulating hormone (α-MSH), another pituitary peptide well known for inducing skin darkening (Fujii and Oshima, 1986). Both ACTH and α-MSH are synthesised from the same prohormone, pro-opiomelanocortin (POMC) (Hadley 1992). α-MSH is also able to stimulate interrenal cortisol release (Balm et al., 1995). Furthermore, β-endorphin, another peptide originating from POMC, appears to act in synergism with α-MSH, stimulating cortisol release in tilapia (*Oreochromis mossambicus*) (Balm et al., 1995). Some, but apparently not all, stressors generate a general rise in plasma ACTH, α-MSH and β-endorphin levels (Sumpter, 1997). Winberg and Lepage (1998) reported that the expression of POMC mRNA was elevated in the pituitary of subordinate rainbow trout, suggesting elevated synthesis and possibly release of POMC-derived peptides. However, the effect of social stress on the actual pituitary release of POMC-derived peptides in teleost fish is still unknown.

It has been suggested that the central monoaminergic systems take part in the regulation of the release of the POMC-derived peptides from the pituitary. Several studies have shown that serotonin (5-hydroxytryptamine, 5-HT) has a stimulatory effect on ACTH release in mammals (Dinan, 1996). Treatment with 8-hydroxy-2-*N*-propylamino-tetralin (8-OH-DPAT), a specific 5-HT_{1A} agonist, stimulates cortisol release in cannulated rainbow trout in a dose-dependent manner, suggesting a further stimulatory action of 5-HT on the teleost HPI axis (Winberg et al., 1997). Furthermore, it has been suggested that 5-HT acts as an α-MSH-releasing factor in lower vertebrates (Olivereau et al., 1980). In addition, socially subordinate fish usually display pronounced behavioural inhibition, i.e. suppressed aggression, feeding and locomotion, effects that are probably mediated in part by the central 5-HT system (Winberg and Nilsson, 1993; Øverli et al., 1998).

The brain catecholamines dopamine (DA) and norepinephrine (NE) are known to have inhibitory effects on the release of α-MSH from the pituitary (Bentley, 1998). There are also some studies indicating that the brain DA system facilitates intraspecific aggressive behaviour (Winberg and Nilsson, 1993). It has been shown that treatment with 3,4-dihydroxyphenylalanine (L-DOPA), the immediate precursor of DA, increased the likelihood of a juvenile Arctic charr (*Salvelinus alpinus*) becoming dominant over a size-matched conspecific in staged fights (Winberg and Nilsson, 1992). In the weakly electric fish *Apteronotus leptorhynchus*, it has been demonstrated that the central NE system is involved in inter-male aggression (Maler and Ellis, 1987). Many studies have measured a relationship between behaviour and brain monoaminergic activity. However, socially induced effects on behaviour and central monoaminergic activity have never been related to plasma levels of POMC-related peptides and skin colour.

In the present study, the effects of social interaction on behaviour, brain monoaminergic activity, plasma levels of POMC-related peptides (ACTH, α-MSH and *N*-acetyl-β-endorphin) and cortisol and skin colour were examined in juvenile Arctic charr.

Materials and methods

Animals

The fish were 2-year-old offspring of Arctic charr (*Salvelinus alpinus* L.) caught in lake Hornavan, Lapland, Sweden, and weighed 83.7±13.7 g (mean ± S.D., *N*=36). Fish were kept indoors at the Evolutionary Biology Centre, Uppsala University, at a density of 300 fish m^{-3}, in a grey-coloured holding tank continuously supplied with Uppsala tap water (8–10 °C, 1.5 l min^{-1}) for more than 1 year before the experiments. The light/dark regime was continuously and automatically adjusted to conditions at a latitude of 51°N. The fish were hand-fed with commercial trout pellets (Ewos ST40, Astra-Ewos Sweden) at 1–2 % of their body mass per day.

Behavioural observations

Behavioural observations were made in six glass aquaria (1000 mm×300 mm×500 mm) continuously supplied with aerated water. The bottoms of the aquaria were white, and light was provided by two fluorescent tubes (20 W, warm white) placed 250 mm above the water surface. Each aquarium was divided into three 33 l chambers by removable black plastic walls. Fish were tagged using small clips in the caudal fin and isolated in individual chambers within the observation aquaria. In this way, the fish were kept visually isolated for 3 weeks before the experiment in an attempt to reduce the effects of previous tank colour and social experience. After the isolation period, groups consisting of three fish (within-group mass deviation <5 %) were formed by gently removing the plastic walls that had kept them separated. Experimental fish were allowed to interact for 5 days. Nine fish were kept visually isolated throughout the experiment and served as controls.

Aggressive acts performed and received by individual fish were counted during two daily observation sessions of 5 min each, at 10:00 and 16:00 h. Three types of aggressive acts were counted: attack, charge and bite. The first observation was performed 30 min after placing the fish in groups and the last on day 4, the day before terminating the experiment. The fish were ranked as 1 (dominant), 2 and 3 from the number of aggressive acts performed and received, using a dominance matrix (Martin and Bateson, 1986).

Since the number of observation aquaria was limited, the experiment was performed in two consecutive rounds, the second following immediately after the first. The first round consisted of three controls and five groups of socially interacting fish, and the second round of six controls and four groups of socially interacting fish.

Skin pigmentation measurements

Skin pigmentation, quantified as the darkness of the skin, was measured by placing the fish in a plastic box with a

transparent cover. The box was filled with foam rubber, which immobilised the fish against the transparent cover. The fish were filmed with a video camera through the plastic cover under constant light conditions. Thereafter, the film was analysed using an image-analysis program (Scion Image, based on NIH image for Macintosh modified for windows, by Wayne Rasband, NIH, Betheseda, MD, USA). Skin darkness was measured on a linear black/white scale on which 0 corresponds to white and 255 to black. A grey scale with 11 standard measurement points ranging from 0 to 250 with a step value of 25 was attached to the transparent cover and used for calibration between measurements. The time taken for the pigment-measuring procedure, from netting to the completion of measurements, was approximately 40 s. Skin pigmentation measurements were performed 24 h prior to and immediately after 5 days of social interaction. On these two occasions, skin pigmentation measurements were also performed on isolated controls (time 1 and time 2 controls, respectively).

Blood and brain tissue sampling

Following the final measurement of skin pigmentation, the fish was anaesthetised (500 mg l^{-1} ethyl-*m*-aminobenzoate methanesulphonate) and blood (approximately 1 ml) was collected from the caudal vasculature using a syringe pretreated with 1.5 mg of EDTA. Blood samples were rapidly transferred to Eppendorf tubes containing aprotinin (Sigma, A1153, 3000 i.u. ml^{-1} blood) and were centrifuged at 1500 ***g*** for 10 min at 4 °C. Following centrifugation, the blood plasma was separated, divided into samples, frozen on dry ice and stored at −80 °C. The time from first disturbance (netting of the first fish in the group) to obtaining a blood sample from the last fish (fish 3) in the group was less than 3 min in all cases. Following blood sampling, the fish was killed by decapitation, and the brain was rapidly removed (within 2 min) and divided into telencephalon (excluding olfactory bulbs), hypothalamus (excluding the pituitary gland), optic tectum, cerebellum and brain stem (including the medulla and part of the spinal cord). Each brain part was wrapped in aluminium foil, frozen in liquid nitrogen and stored at −80 °C.

Assays

The frozen brain samples were homogenised in 4 % (w/v) ice-cold perchloric acid (PCA) containing 0.2 % EDTA and 40 ng ml^{-1} epinine (deoxyepinephrine, the internal standard), using a Potter–Elvehjem homogenizer (optic lobes, cerebellum and brain stem) or an MSE 100 W ultrasonic disintegrator (telencephalon and hypothalamus).

5-HT, 5-HIAA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC, a major DA metabolite), NE and 3-methoxy-4-hydroxyphenylglycol (MHPG, a major NE metabolite) were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent-delivery system (CostaMetric II, LDC, USA), an autoinjector (Midas, Spark, Holland), a reverse-phase column (4.6 mm×100 mm, Hichrom, C18, 3.5 μm) kept at 40 °C and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at oxidising potentials of +320 mV and +450 mV. A conditioning electrode with a potential of +40 mV was employed before the analytical electrodes to oxidise any contaminants. The mobile phase consisted of 75 mmol l^{-1} sodium phosphate, 0.7 mmol l^{-1} octane sulphonic acid and 10 μmol l^{-1} EDTA in deionized (resistance 18.2 MΩ) water containing 10 % methanol brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, the Czech Republic).

As a result of the presence of interfering unidentified peaks in the chromatogram, we were unable to quantify DOPAC in the telencephalon and optic tectum. For the same reason, we were also unable to quantify MHPG in the telencephalon.

Blood samples were assayed for cortisol, ACTH, α-MSH and *N*-acetyl-β-endorphin. Cortisol analysis was performed directly on Arctic charr plasma without extraction using a validated radioimmunoassay (RIA) modified from Olsen et al. (1992), as described by Winberg and Lepage (1998). Plasma concentrations of ACTH were determined by RIA, as described by Balm and Pottinger (1993) and Balm et al. (1994). α-MSH and *N*-acetyl-β-endorphin concentrations in the plasma samples were quantified by validated RIAs following the method of Balm et al. (1995).

Statistical analyses

All data are presented as means ± standard error of the mean (S.E.M.). Plasma concentrations of α-MSH, ACTH, *N*-acetyl-β-endorphin and cortisol, brain levels of monoamines and monoamine metabolites and ratios of monoamine metabolite to parent monoamine concentrations (i.e. [5-HIAA]/[5-HT], [DOPAC]/[DA] and [MHPG]/[NE]) were subjected to multiple analysis of variance (MANOVA) with social rank as the dependent factor. In the case of the skin colour data, a repeated-measures MANOVA was performed. If significant effects were indicated by variance analysis, the Tukey honest significant difference (HSD) test for unequal *N* (Spjotvoll/Stoline test) was used to investigate differences between fish of different social rank and differences between interacting fish and controls.

To investigate the relationships between [5-HIAA]/[5-HT], [DOPAC]/[DA] and [MHPG]/[NE] in different brain parts (telencephalon, hypothalamus, optic tectum and brain stem) and plasma levels of α-MSH, ACTH and *N*-acetyl-β-endorphin, linear regression analysis was performed. In addition, stepwise multiple regression analyses were performed to investigate correlations between plasma concentrations of POMC-derived peptides (α-MSH, ACTH and *N*-acetyl-β-endorphin), circulating cortisol levels and skin darkness following social interaction. To fulfil the assumption of normal distribution, data on plasma concentrations of ACTH and cortisol were $\log_{10}$-transformed, whereas the [5-HIAA/5-HT], [DOPAC/DA] and [MHPG/NE] ratios were subjected to arcsine transformation. All statistical analyses were performed using Statistica 5.1 (StatSoft Inc.) software.

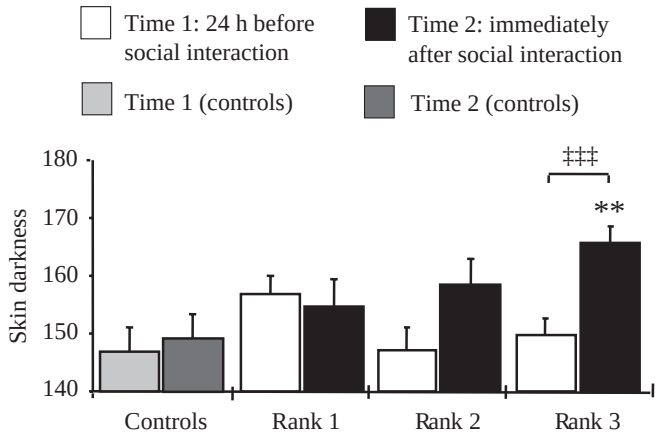

Fig. 1. Skin darkness of Arctic charr occupying different positions in a dominance hierarchy, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Measurements were performed on a linear grey scale, on which 0 is white and 255 is black, before and after 5 days of social interaction. Controls are fish that were kept visually isolated. Values are means + S.E.M. from nine fish of social rank 1, four fish of social rank 2 and 14 fish of social rank 3 (see Materials and methods for details). An asterisk indicates a significant difference from visually isolated controls at time 2, and a double dagger indicates a significant difference between times 1 and 2. $^{**}P<0.01$; ‡‡‡$P<0.001$.

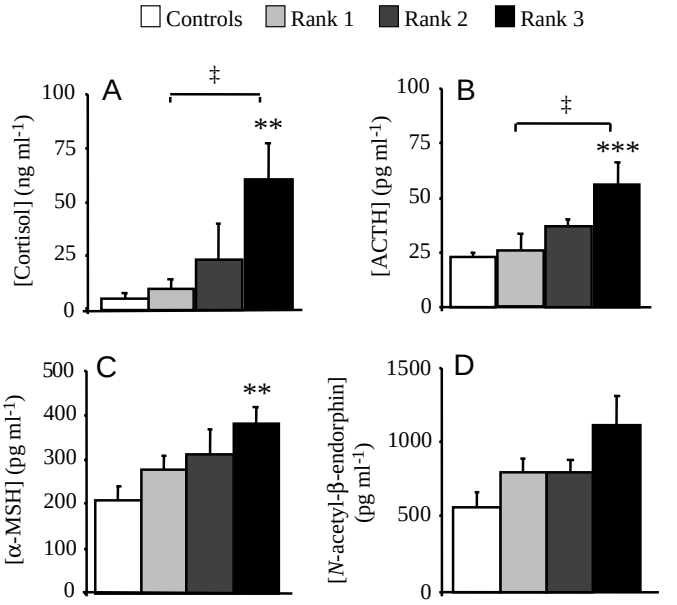

Fig. 2. Plasma concentrations of cortisol (A), adrenocorticotropic hormone (ACTH) (B), α-melanocyte-stimulating hormone (α-MSH) (C) and *N*-acetyl-β-endorphin (D) in Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. Values are means + S.E.M. from nine fish of social rank 1, four fish of social rank 2, 14 fish of social rank 3 and nine controls. An asterisk indicates a significant difference from visually isolated controls, and a double dagger indicates a significant difference between social ranks. ‡$P<0.05$, $^{**}P<0.01$, $^{**}P<0.001$.

Results

Behaviour

In four out of nine groups, the fish could be ranked into three categories (social rank 1, 2 or 3) on the basis of the number of aggressive acts performed and received by individual group members. Dominant fish (social rank 1) were highly aggressive, performing 31.0±5.9 aggressive acts per 10 min (mean ± S.E.M., N=9), and usually moved directly above the bottom, close to the centre of the aquarium, often biting and nipping subordinate fish (social ranks 2 and 3). Fish of social rank 2 were clearly less aggressive, performing 7.2±2.2 aggressive acts per 10 min (mean ± S.E.M., N=4) and were less active than dominant fish, but moved fairly freely in the aquarium. Fish of social rank 3, in contrast, stayed inactive close to the surface, often close to the walls or in a corner of the aquarium, and were never observed to perform any aggressive acts. In five of the groups, the dominant fish performed all the aggressive acts observed, and in these groups both subordinates were classified as social rank 3.

Effects of social rank on skin darkness

The level of skin darkness of isolated controls, and of fish of different social status before and after 5 days of social interaction, is presented in Fig. 1. There were no significant differences in skin colour between the groups before social interaction ($F_{3.32}$=1.93, P=0.145, repeated-measures MANOVA). However, social interaction had a significant effect on skin colour ($F_{3.32}$=6.23, P=0.0019) and resulted in a significant skin darkening in fish of social rank 3 (P=0.0009). Moreover, following, social interaction, fish of social rank 3 were significantly darker than controls (P=0.048).

Effects of social rank on plasma levels of cortisol, ACTH, α-MSH and N*-acetyl-β-endorphin*

Plasma levels of cortisol ($F_{3.31}$=6.82, P=0.0012), ACTH ($F_{3.31}$=4.77, P=0.0076) and α-MSH ($F_{3.30}$=6.82, P=0.0012) were all significantly affected by social interaction (Fig. 2A–C). In general, the effects on plasma concentrations of cortisol, ACTH and α-MSH followed the same pattern as that of skin darkness, with fish of social rank 3 showing the highest levels. In fish of social rank 3, plasma levels of cortisol (P=0.0029), ACTH (P=0.045) and α-MSH (P=0.011) were all significantly elevated compared with controls. Moreover, in fish of social rank 3, the plasma levels of cortisol (P=0.023) and ACTH (P=0.029) was also significantly higher than those of dominant fish (Fig. 2A,B). However, social interaction had no significant effects on plasma concentrations of *N*-acetyl-β-endorphin (Fig. 2D).

Effects of social rank on brain monoaminergic activity

As expected, social interaction had significant effects on brain [5-HIAA]/[5-HT] ratios (Fig. 3A–C). Specifically, significant effects of social interaction on [5-HIAA]/[5-HT] ratios were observed in the telecephalon ($F_{3.31}$=5.35,

Table 1. *Effects of social rank on the concentrations of monoamines and monoamine metabolites, and the ratios of concentrations of monoamine metabolite to parent monoamine neurotransmitter, in Arctic charr*

Rank	[DA] (ng g^{-1})	[DOPAC] (ng g^{-1})	[NE] (ng g^{-1})	[MHPG] (ng g^{-1})	[5-HT] (ng g^{-1})	[5-HIAA] (ng g^{-1})	[DOPAC]/[DA] ×10^{-3}	[MHPG/NE] ×10^{-3}	[5-HIAA]/[5-HT] ×10^{-3}
Cerebellum									
1	498±14.4	ND	1610±94.1	223±19.1	ND	ND	–	140±0.015	–
2	448±18.4	ND	1440±389	167±54.3	ND	ND	–	117±0.031	–
3	492±10.2	ND	1640±138	533±310	ND	ND	–	322±0.180	–
Control	552±56.4	ND	1500±109	217±10.6	ND	ND	–	153±0.015	–
Telencephalon									
1	291±39.6	ND	3480±347	ND	655±63.5	228±19.7	–	–	367±25.9
2	272±52.6	ND	3780±557	ND	645±134	221±17.1	–	–	377±53.9
3	241±24.7	ND	3460±302	ND	678±39.0	276±18.6*	–	–	408±15.0***
Control	248±42.3	ND	3030±287	ND	614±71.8	158±4.93	–	–	279±27.6
Optic tectum									
1	109±12.2	ND	1053±106	8.74±0.745	190±21.7	85.0±10.1	–	8.70±0.824	452±28.6
2	116±15.2	ND	1147±130	7.98±1.31	196±16.3	82.6±5.31	–	6.94±0.736	424±9.55
3	109±16.0	ND	1157±177	12.4±1.55	206±29.6	91.1±13.5	–	11.3±0.813*	449±18.9
Control	106±13.9	ND	1090±118	8.42±1.55	189±24.3	63.9±7.02	–	7.60±0.966	364±36.7
Brain stem									
1	227±23.7	19.8±1.15	790±28.3	33.7±1.79	307±18.3	102±8.58	93.3±12.7	43.1±2.95	333±20.9
2	228±23.3	20.4±1.86	827±94.2	35.6±3.76	294±33.1	114±14.0	92.4±5.30	43.7±3.78	391±35.2
3	199±11.6	18.5±0.79	740±25.8	34.0±1.92	271±9.97	102±6.09	96.3±6.46	45.8±2.19	381±25.3**
Control	230±13.0	19.7±2.94	763±26.5	29.7±26.5	287±13.9	76.0±5.25	85.0±9.36	39.0±9.36	266±18.0
Hypothalamus									
1	2480±104	7.11±1.49	1830±90.2	315±79	1840±237	196±23.6	2.75±0.560	187±41.3	114±12,5
2	1610±691	3.25±2.16	1420±624	260±125	1450±603	155±71.7	1.75±0.606	221±55.2	114±18.0
3	2220±256	10.6±3.77	1640±209	268±45.0	1520±214	243±32.9	4.18±1.17	159±11.7	171±11.0**
Control	2070±400	5.14±1.26	1570±286	349±73.1	1660±383	165±40.7	2.50±0.366	265±36.5	106±17.8

Concentrations are ng g^{-1} brain tissue.

Fish were occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated.

Values are means ± S.E.M. for nine controls, nine fish of social rank 1, four fish of social rank 2 and 14 fish of social rank 3.

Asterisks indicate a significant difference compared with controls: *P<0.05, **P<0.01, ***P<0.001.

ND, not detectable; DA, dopamine, DOPAC, 3,4-dihydroxyphenylacetic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

P=0.0044), brain stem ($F_{3.31}$=5,49, P=0.0038) and hypothalamus ($F_{3.31}$=5.10, P=0.0055). Fish of social rank 3 displayed significantly elevated [5-HIAA]/[5-HT] ratios in the telencephalon (P=0.069), brain stem (P=0.0011) and hypothalamus (P=0.004) compared with controls (Fig. 3A–C). Moreover, telencephalic 5-HIAA levels were significantly affected by social interaction ($F_{3,31}$=7.70, P<0.0006), fish of social rank 3 displaying significantly elevated telencephalic 5-HIAA levels compared with controls (P=0.0006; Table 1). However, no effects were observed on 5-HT levels in any of the brain areas analysed (Table 1).

The [MHPG]/[NE] ratio in the optic tectum was also significantly affected by social interaction ($F_{3.29}$=4.74, P=0.00821), and in this part of the brain fish of social rank 3 displayed significantly higher [MHPG]/[NE] ratios than controls (P=0.045) (Fig. 3D).

Social interaction had no significant effects on brain levels of NE, MHPG, DA or DOPAC, and [DOPAC]/[DA] ratios did not differ between socially interacting fish and controls or between fish of different social rank (Table 1).

In the cerebellum, 5-HT, 5-HIAA and DOPAC concentrations were below the level of detection, and no significant effects were observed on DA, NE or MHPG levels, nor on the [MHPG]/[NE] ratio (Table 1).

Correlations between plasma concentrations of ACTH, α-MSH, N-*acetyl-β-endorphin and skin darkness*

Skin darkness quantified following 5 days of social interaction showed a positive correlation with plasma concentrations of α-MSH (Fig. 4). The multiple regression analysis indicated that α-MSH was the only POMC-derived peptide having a significant effect on skin darkness, explaining 25 % of the variance ($F_{2,31}$=6.62, P=0.004) (Table 2; Fig. 4).

Table 2. *Results of step-wise multiple linear regression analysis with plasma concentrations of cortisol and skin darkness as dependent variables and plasma concentrations of α-MSH,* N*-acetyl-β-endorphin and ACTH as independent variables*

Dependent variable	Independent variable	*F*	d.f.	Adjusted r^2	*P*	β
log[cortisol]		**22.24**	**2,31**	**0.56**	**0.00001**	
	[α-MSH]				0.015	0.40
	[*N*-acetyl-β-endorphin]				NS	
	log[ACTH]				0.0002	0.64
Skin darkness		**6.62**	**2,31**	**0.25**	**0.00403**	
	[α-MSH]				0.019	0.41
	[*N*-acetyl-β-endorphin]				NS	
	log[ACTH]				NS	

The *P* values given in bold type stand for the total probability of the model, other *P* values stand for the contribution of each independent variable to the model.

NS, not significant; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotrophic hormone.

Correlations between plasma concentrations of ACTH, α-MSH, N*-acetyl-β-endorphin and cortisol*

Again using a multiple regression analysis, we observed that plasma levels of ACTH and α-MSH both showed significant positive correlations with plasma cortisol concentrations (Fig. 5A,B). Together, plasma levels of these two peptides explained 56 % of the variance ($F_{2,31}$=22.2, P<0.00001) (Table 2; Fig. 5A,B).

Correlations between brain [monoamine metabolite]/[monoamine] ratios and plasma levels of ACTH, α-MSH and N*-acetyl-β-endorphin*

Plasma concentration of ACTH was positively correlated with [5-HIAA]/[5-HT] in the telencephalon (r=0.38, P=0.024), hypothalamus (r=0.39, P=0.020), optic tectum (r=0.40, P=0.021) and brain stem (r=0.35, P=0.0036) (Table 3).

Positive correlations were also observed between brain [MHPG]/[NE] ratios and plasma levels of POMC peptides (Table 3). Specifically, [MHPG]/[NE] ratios in the optic tectum showed significant positive correlations with α-MSH (r=0.40, P=0.024), *N*-acetyl-β-endorphin (r=0.57, P=0.0056) and ACTH (r=0.73, P=0.00002) concentrations (Fig. 6). Furthermore, [MHPG]/[NE] ratios in the brain stem displayed a significant positive correlation with plasma levels of ACTH (r=0.35, P=0.038). Hypothalamic [MHPG]/[NE], however, showed a significant negative correlation with plasma α-MSH concentrations (r=–0.37, P=0.036) (Fig. 7; Table 3).

However, no significant correlations were observed between [DOPAC]/[DA] ratios in any of the brain parts analysed and plasma levels of any POMC-derived peptides.

Discussion

The present study is the first to quantify socially induced effects on the skin colour in a teleost fish, the Arctic charr (Salmonidae). Our results show that social subordination induces darkening of the skin in this species. Furthermore, skin darkening appears to be mediated by factors that are also involved in the neuroendocrine stress response.

Social subordination is stressful and is known to affect brain monoaminergic activity (Winberg and Nilsson, 1993). Monoamine neurotransmitters, including both catecholamines and 5-HT, also take part in the regulation of the pituitary release of ACTH and α-MSH, peptides that have been reported to cause skin darkening (Bentley, 1998).

In the present study, we used the ratio [metabolite]/[parent monoamine] as an index of brain monoaminergic activity. This

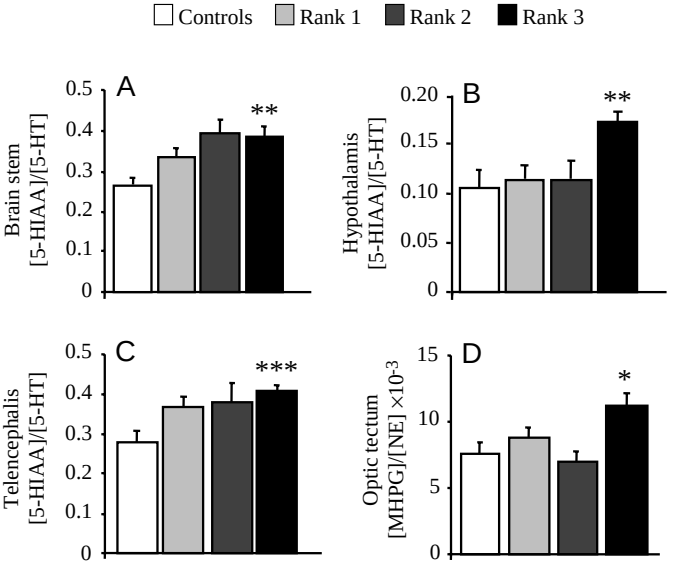

Fig. 3. The [5-hydroxyindoleacetic acid]/[5-hydroxytryptamine] ([5-HIAA]/[5-HT]) ratios in the brain stem (A), hypothalamus (B) and telencephalon (C) and the [3-methoxy-4-hydroxyphenylglycol]/[norepinephrine] ([MHPG]/[NE]) ratio in the optic tectum (D) of Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. Values are means + S.E.M. from nine fish of social rank 1, four fish of social rank 2, 14 fish of social rank 3 and nine controls. An asterisk indicates a significant difference from visually isolated controls. **P*<0.05, ***P*<0.01, ****P*<0.001.

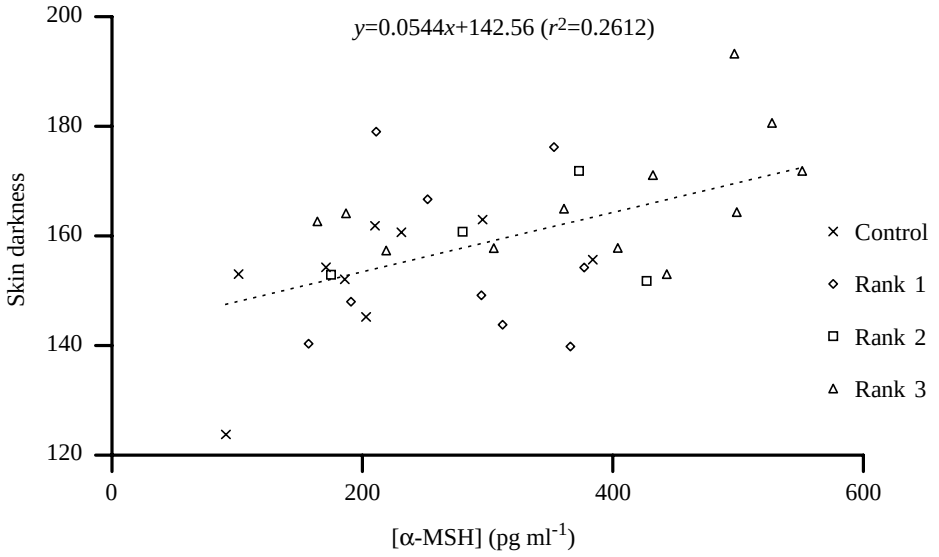

Fig. 4. The relationship between plasma concentrations of α-melanocyte-stimulating hormone (α-MSH) and skin darkness of Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. Skin darkness was measured on a linear grey scale, on which 0 is white and 255 is black.

is a more direct index of monoaminergic activity than brain levels of monoamine metabolites *per se*, since variance related to tissue sampling and differences related to total levels of the parent monoamine and its metabolite are reduced (Shannon et al., 1986). The subordinate fish showed a general elevation of brain [5-HIAA]/[5-HT] ratios, which is in good accordance with previous studies (Winberg and Nilsson, 1993). We were also able to quantify levels of the NE metabolite MHPG in the brain, and the results show that fish of social rank 3 displayed an elevation of [MHPG]/[NE] ratios in the optic tectum, suggesting an increased NE activity in this part of the brain of subordinate fish. In general, the elevation of the [5-HIAA]/[5-HT] and [MHPG]/[NE] ratios tended to be reflected by an increase in 5-HIAA and MHPG concentrations, even though only the effect on telencephalic 5-HIAA levels reached the level of statistical significance.

Little is known about the role of the central NE system in the regulation of agonistic behaviour and stress reactions in teleost fish. In mammals, stress is known to activate the brain NE system (for a review, see Stanford, 1993), and some results suggest that the central NE system could have a stimulatory effect on agonistic behaviour in fish and lizards. For instance, in the weakly electric fish *Apteronotus leptorhynchus*, intracranial injection of NE stimulates inter-male aggressive signalling (Maler and Ellis, 1987), and an elevation of brain [MHPG]/[NE] ratios has been observed in lizards defending a territory (Matter et al., 1998). In addition, McIntyre et al. (1979) reported that dominant rainbow trout had lower NE concentrations in the brain than subordinate conspecifics, a result that is difficult to interpret since brain levels of NE metabolites were not analysed.

The elevation of [MHPG]/[NE] ratios in the optic tectum of subordinate fish (rank 3) observed in the present study is

Table 3. *Linear regression analysis of relationships between [MHPG]/[NE] and [5-HIAA]/[5-HT] ratios in different brain areas and plasma concentrations of α-MSH, N-acetyl-β-endorphin and ACTH*

	Brain stem		Hypothalamus		Optic tectum		Telencephalon	
	[MHPG]/[NE]	[5-HIAA]/[5HT]	[MHPG]/[NE]	[5-HIAA]/[5-HT]	[MHPG]/[NE]	[5-HIAA]/[5-HT]	[MHPG]/[NE]	[5-HIAA]/[5-HT]
[α-MSH]								
r			–0.37		0.40			NS
P	NS	NS	0.036	NS	0.024	NS	NS	
[*N*-acetyl-β-endorphin]								
r						NS	NS	NS
P	NS	NS	NS	NS	0.57			
[ACTH]								
r	0.35	0.35	NS	0.39	0.73	0.4	NS	0.39
P	0.038	0.0036		0.020	0.00002	0.021		0.024

Pearson *r*- and *P*-values are given.

NS, not significant; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone.

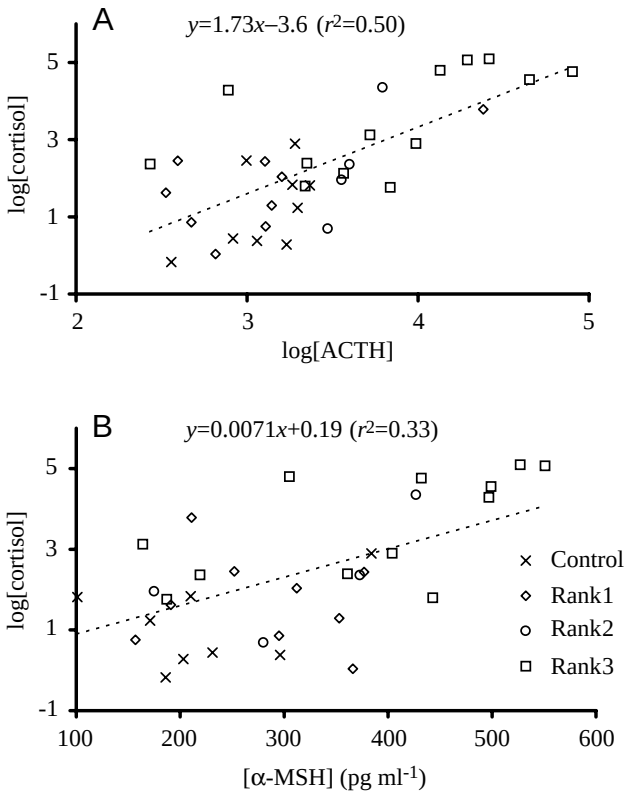

Fig. 5. The relationships between plasma concentrations of adrenocorticotropic hormone (ACTH) (pg ml^{-1}) and cortisol (ng ml^{-1}) (A) and between α-melanocyte-stimulating hormone (α-MSH) (pg ml^{-1}) and cortisol (ng ml^{-1}) (B) in Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. To fulfil the assumption of normal distribution, plasma concentrations of ACTH and cortisol were log_{10}-transformed prior to regression analysis.

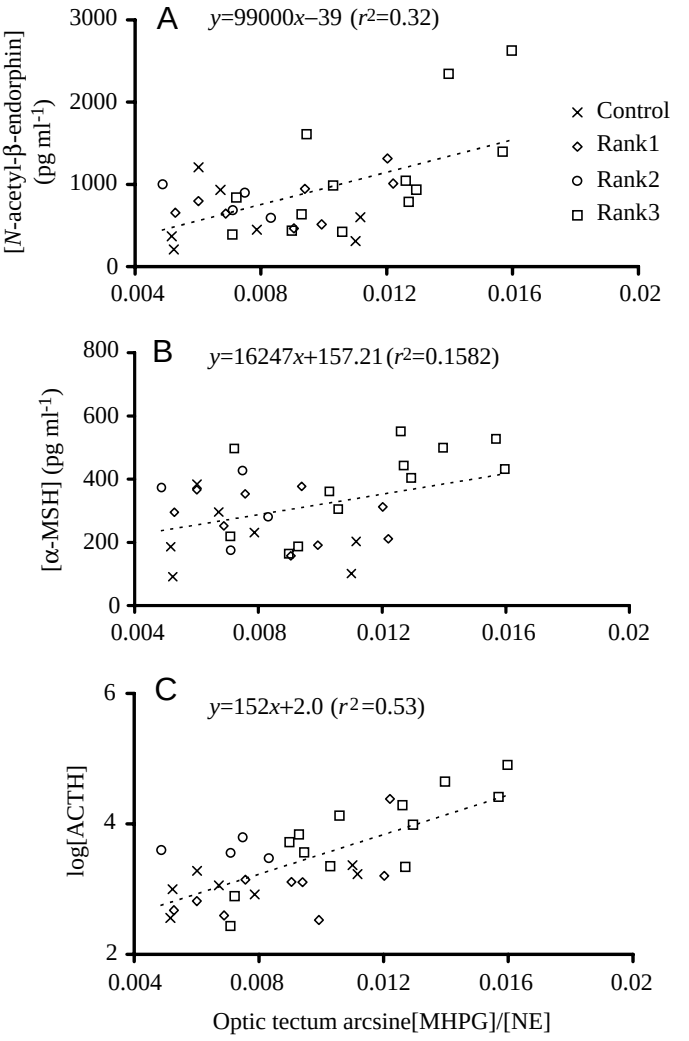

Fig. 6. The relationships between and [3-methoxy-4-hydroxyphenylglycol]/[norepinephrine] ([MHPG]/[NE]) ratios in the optic tectum and plasma concentrations of *N*-acetyl-β-endorphin (A), α-melanocyte-stimulating hormone (α-MSH) (B) and adrenocorticotropic hormone (ACTH) (C) in Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. To fulfil the assumption of normal distribution, [MHPG]/[NE] ratios were arcsine-transformed and ACTH values log_{10}-transformed prior to regression analysis.

probably to be related to stress since these individuals were not observed to perform any aggressive acts. Subordinate animals are subjected to chronic social stress, and fish of social rank 3 also displayed elevated brain [5-HIAA]/[5-HT] ratios as well as an increase in plasma concentrations of cortisol and ACTH. The fact that [MHPG]/[NE] ratios in the optic tectum showed a strong positive correlation with plasma levels of ACTH further supports the suggestion that the elevation of [MHPG]/[NE] ratios seen in the optic tectum of subordinate Arctic charr is related to stress. Moreover, Øverli et al. (1999) reported elevated brain [MHPG]/[NE] ratios and a positive correlation between brain [MHPG]/[NE] ratios and plasma cortisol levels in subordinate rainbow trout.

It has been suggested that the brain 5-HT system stimulates the HPI axis in salmonid fish (Winberg and Lepage, 1998; Winberg et al., 1997), a suggestion that is supported by the results of the present study, which imply a positive relationship between brain 5-HT activity and plasma levels of ACTH. The 5-HT system of mammals stimulates the HPA axis activity by inducing the release of corticotropin-releasing hormone (CRH) which, in turn, activates pituitary corticotrophes (Chaouloff, 1993; Dinan, 1996). In addition, there are results suggesting that 5-HT may stimulate ACTH release directly from the mammalian pituitary (Dinan, 1996). However, in teleosts, it is not clear whether the major 5-HT cell-body-containing nuclei of the brain, the raphe nucleus and the paraventricular organ, project to the pituitary (for a review, see Kah et al., 1993).

The mechanism controlling the release of α-MSH from the pituitary pars intermedia is multifactorial and not fully understood (Bentley, 1998). There is evidence to suggest that pituitary α-MSH is under inhibitory control by DA and NE

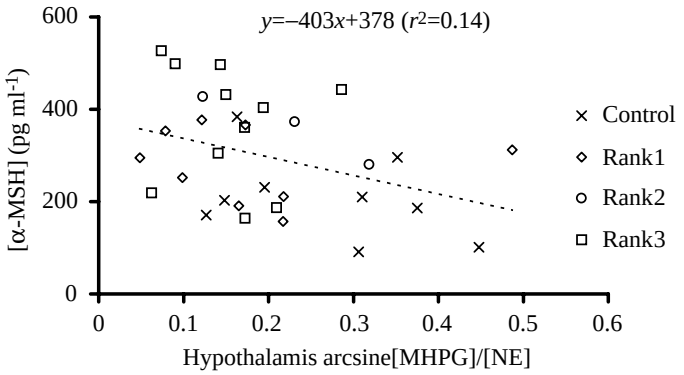

Fig. 7. The relationship between hypothalamic [3-methoxy-4-hydroxyphenylglycol]/[norepinephrine] ([MHPG]/[NE]) ratios and plasma concentrations of α-melanocyte-stimulating hormone (α-MSH) (pg ml^{-1}) in Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the dominant and social rank 3 the most subordinate fish in a group. Controls are fish that were kept visually isolated. To fulfil the assumption of normal distribution, [MHPG]/[NE] ratios were arcsine-transformed prior to regression analysis.

(Bentley, 1998). The inhibitory effects of these two catecholamines on pituitary α-MSH release seem to be evolutionarily well conserved, since they have been demonstrated in species that are as phylogenetically distant as rats and dogfish (Bentley, 1998). In the present study, we observed a negative correlation between hypothalamic [MHPG]/[NE] and plasma levels of α-MSH. However, hypothalamic [DOPAC]/[DA] ratios showed no significant correlation with plasma concentrations of α-MSH.

Subordinate fish displayed an elevation of hypothalamic [5-HIAA]/[5-HT] ratios and a general rise in the plasma content of ACTH and α-MSH, suggesting that 5-HT might play a role in the regulation of the release of POMC-derived peptides of both melanotrophic and corticotrophic origin. Skin darkening, a known effect of α-MSH, has been observed following injections of 5-HT or 5-hydroxytryptophan (5-HTP), the precursor of 5-HT in goldfish (*Carassius auratus*, Olivereau et al., 1980), lizards (*Anolis caroliensis*, Levitin, 1980) and frogs (*Xenopus laevis*, Olivereau et al., 1980). The finding that treatment with 5-HT, as well as with 5-HTP, caused degranulation of pars intermedia cells in goldfish (Olivereau et al., 1980) further supports a role for 5-HT as an α-MSH-releasing factor, at least in non-mammalian vertebrates. Alternatively, 5-HT inhibits DA activity, thereby releasing DA inhibition of the pituitary α-MSH release. Goudreau et al. (1994) demonstrated that the stimulating effect of 5-HT on α-MSH release could be mediated by 5-HT-induced suppression of DA activity in the rat pituitary. Moreover, treatment with MK-212 (a 5-HT receptor agonist) induced α-MSH release *in vivo* in rats, an effect that was abolished by apomorphine (a DA receptor agonist), suggesting a direct antagonism between DA and 5-HT (Carr et al., 1991). Administration of 5-HT precursors also generates pituitary release of other POMC-derived peptides such as β-endorphin and β-lipotropin, in rats (Sapun-Malcom et al., 1983). Sapun-Malcom et al. (1986) demonstrated that DA exerted inhibitory control of circulating β-endorphin levels *in vivo* in rats, indicating an antagonism between 5-HT and DA even in the control of β-endorphin release.

The results from the present study do not support an antagonistic effect between 5-HT and DA on α-MSH release in the Arctic charr. However, the negative relationship between hypothalamic [MHPG]/[NE] ratios and plasma concentrations of α-MSH in combination with the higher [5-HIAA]/[5-HT] ratio in the hypothalamus of subordinates could indicate an antagonistic effect of NE and 5-HT on pituitary α-MSH release. The monoaminergic control of pituitary release of POMC-derived peptides in fish should be investigated further by measuring the actual pituitary monoaminergic activity and the pituitary content and release of POMC-derived peptides.

Winberg and Lepage (1998) reported a sustained up-regulation of pituitary POMC mRNA expression in subordinate rainbow trout, an effect that appeared to be caused mainly by elevated expression of POMC mRNA in melanotrophes of the pituitary neurointermediate lobe. In the present study, we observed elevated plasma levels of both ACTH and α-MSH in subordinate fish. Taken together, these results clearly suggest that social stress results in an elevation of the production and release of POMC-derived peptides of both corticotrophic and melanotrophic origin.

Activation of the pituitary corticotrophes and an elevation of circulating plasma levels of ACTH seems to be a general response to all stressors (Sumpter, 1997). In contrast, the effects on pituitary melanotrophes and on circulating plasma levels of α-MSH and β-endorphin seem to depend on the nature and/or the intensity of the stressor (Wendelaar Bonga et al., 1995). For instance, handling and confinement stress in combination with a thermal shock induced a rise in plasma concentrations of β-endorphin and α-MSH in brown trout (*Salmo trutta*) (Sumpter et al., 1985). Similarly, restraint stress caused an elevation of α-MSH levels in rainbow trout (Sumpter et al., 1986), as did exposure to acidified water in tilapia (Lamers et al., 1991). However, other types and/or combinations of stressors may not affect plasma levels of α-MSH and β-endorphin or may even reduce plasma concentrations of these peptides (Balm and Pottinger, 1995).

In the present study, the plasma cortisol levels display a similar pattern to those of ACTH, α-MSH and skin colour, with fish of social rank 3 showing the highest plasma cortisol concentrations. The multiple linear regression analysis showed that plasma levels of ACTH and α-MSH were both positively correlated with the concentration of cortisol in plasma, suggesting the possibility that ACTH and α-MSH had a combined stimulatory effect on interrenal cortisol release. ACTH is known to play a central role in the control of corticosteroid secretion, and other POMC-derived peptides stimulate interrenal cortisol release. Lamers et al. (1992) demonstrated that α-MSH stimulates cortisol release *in vitro* in tilapia. The finding that oral administration of cortisol decreased the activity of the α-MSH cells 10-fold suggests that

α-MSH release is under negative feedback control by cortisol (Lamers et al., 1994). The results from the present study lend further support to the hypothesis that products from the melanotrophes may have a corticotrophic action in teleost fish (Balm et al., 1995; Wendelaar Bonga et al., 1995).

In teleost fish, colour changes can be mediated by both humoral and neural mechanisms, as well as by a combination of these two mechanisms (Bentley, 1998). α-MSH is known to initiate skin darkening in poikilotherm vertebrates by inducing chromatophore dispersion (physiological colour changes) and/or by increasing the number of chromatophores (morphological colour changes; Bentley, 1998). In the present study, increased levels of α-MSH in plasma were positively correlated with skin darkening, suggesting a humoral involvement in socially induced colour changes even though the involvement of neural mechanisms could not be excluded. It is not clear whether α-MSH induces skin darkening by physiological or morphological colour change in salmonids. Rodrigues and Sumpter (1984) suggested that α-MSH may only induce morphological colour change, since there is a considerable time lag between the rise in plasma α-MSH levels and the actual colour changes in brown trout adapting to a darker background colour.

Melanin-concentrating hormone (MCH) is another peptide that could be involved in mediating socially induced effects on skin colour in salmonid fish. The release of MCH is affected by stress; MCH interacts with other factors of the neuroendocrine stress response and affects skin colour in teleost fish. The effect of MCH is to lighten the skin and, overall, MCH seems to have effects antagonistic to those of α-MSH (for reviews, see Baker, 1993; Balm and Gröneveld, 1998). Unfortunately, the blood volume obtained from the fish used in the present experiment did not allow us to quantify plasma levels of MCH along with plasma concentrations of POMC-derived peptides and cortisol. Other factors affecting skin colour are circulating levels of NE and epinephrine (Fujii and Oshima, 1986).

The results of the present study suggest that skin darkening in subordinates could be mediated by a stress-induced elevation of plasma α-MSH levels. It appears that stress experienced by subordinate individuals initially results from losing fights, but that later on, when the level of overt aggression declines, it is more related to being constantly threatened simply by the presence of the dominant fish (Winberg and Lepage, 1998). Subordinate fish continue to display an activation of the HPI axis and the brain 5-HT system even in established dominance hierarchies at a time when the frequency of received overt aggressive acts falls to a low and constant level (Winberg and Nilsson, 1993; Winberg and Lepage, 1998). The fact that skin darkening and the elevation of plasma α-MSH levels was present after 5 days of social interaction suggests that these effects could be part of the response to chronic social stress in subordinate Arctic charr. If so, skin darkening could well act as a social signal announcing submission to reduce costly and meaningless confrontations in an established hierarchy, the subordinate fish staying dark as long as the stressor (i.e. the dominant individual) is present. However, it should be acknowledged that skin darkening could also provide a cryptic coloration, thus making the subordinate fish less visible, or it could be simply a side effect of HPI axis activation. Clearly, further studies are needed to clarify whether skin darkening of subordinate Arctic charr acts as a social signal.

In conclusion, the results from the present study demonstrate that social stress induces skin darkening in Arctic charr and that this effect may well be mediated by a stress-induced elevation of plasma α-MSH levels even though MCH could not be excluded as a factor contributing to this effect. The results also suggest that hypothalamic NE could be an important factor in the regulation of pituitary α-MSH release in Arctic charr. In addition, together with previous studies, our work suggests that hypothalamic 5-HT has an excitatory effect on the HPI axis, probably by stimulating hypothalamic CRH release to the pituitary. Furthermore, a strong positive correlation was demonstrated between [MHPG]/[NE] ratios in the optic tectum and plasma levels of ACTH, suggesting that the central NE system is involved in the neuroendocrine stress response by regulating the HPI axis.

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