



Research report

Social stress effects on pigmentation and monoamines in Arctic charr



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HIGHLIGHTS

- Social stress induces differences in carotenoid spots of Arctic charr.
- Carotenoid spots are associated with behavioural stress responses in Arctic charr.
- Monoaminergic activity is to some extent associated with carotenoid spots in Arctic charr.

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ABSTRACT

Pigmentation often signals status and in general melanin-based pigmentation is indicative of aggression and stress resilience in vertebrates. This is evident in the salmonids Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) where more melanin spotted individuals are more stress resilient. However, in the salmonid Arctic charr (*Salvelinus alpinus*) it seems as if it is carotenoid-based pigmentation that signals aggression and stress resilience. In our study, social stress effects on carotenoid-based spots, and behavioural and physiological stress responses were investigated. Socially stressed individuals have more spots, and behavioural stress responses were associated with spots. Some of the results concerning physiological stress responses, such as plasma cortisol levels and monoaminergic activity, are associated with spottiness. Further, the earlier proposed lateralization of spots, with left side connected to stress responsiveness and right side to aggression, is to some extent validated although not conclusively. In conclusion, this study provides further evidence that more stressed charr have more carotenoid spots, and for the first time monoaminergic activity is shown to be connected with carotenoid pigmentation.

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1. Introduction

Animal's visual appearance often signals status or condition [1], and in vertebrates the two pigment groups of carotenoids and melanins cause this colour variation. This is often evident in agonistic interactions where aggression, dominance, and/or subordination can be signalled with colours. For instance, eye darkening signals dominance in lizards [2,3], whereas eye darkening signals subordination in several teleost fish [4–8]. Further, melanin-based pigmentation correlates positively with aggression in birds [9–11] and mammals [12]. Similarly, some evidence for correlations

between carotenoid pigmentation and aggression is also reported. Male firemouth cichlids (*Cichlasoma meeki*) that obtained more carotenoid pigmentation via feed enrichment won more dyadic fights compared to controls [13]. Fights lasted longer in male three-spined stickleback (*Gasterosteus aculeatus*) if losers had higher carotenoid levels than winners [14].

Interestingly, the more melanised animals that typically are more aggressive [15,16] also seem to be more stress resilient [15]. Similar results have also been reported in teleost fish. In the salmonids Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), fish with more permanent melanin-based skin spots have a lower physiological stress response than the fish with few spots [17]. However, in the Arctic charr (*Salvelinus alpinus*) pigmentation is different compared to most salmonids [18] and social subordination induces a transient skin darkening [19,20] similar to other salmonids [21,22]. Thus, in Arctic charr melanisation does not seem to be indicative of stress resilience. Instead, a recent study reported that the carotenoid pigmentation in Arctic

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charr could be used as an indicator of physiological stress response [23]. Additionally, social subordinate charr have more carotenoid spots compared to dominant individuals [24]. Therefore, carotenoid pigmentation, rather than melanin-based pigmentation, seems to be associated with both aggression and stress in Arctic charr.

There are both behavioural and physiological responses following social stress. The behavioural responses following defeat include inhibited aggression [25]. For the physiological responses, there is an increase of glucocorticoids as well as monoaminergic activity changes [25]. Therefore, the monoaminergic activity can also be used as a stress indicator [26,27]. Monoaminergic activity also regulates aggression [26] and there are interactions between the regulation of stress and aggression [27]. Thus it seems likely that pigmentation will be associated with monoaminergic activity as well.

Further, both aggression and stress have been suggested to be lateralized, that is, one side being different from the other side in function or structure [28,29]. For instance, aggression has been shown to be lateralized in teleost fish [30–33] and mammals [34–38]. In addition, some behaviour during stress seems to be lateralized [29,39]. Recently it was suggested that the carotenoid spots in Arctic charr are lateralized, with the right side connected with aggression and the left side connected with stress responsiveness [24].

Based on these earlier results, we investigated how social stress in Arctic charr affected behavioural and physiological stress responses, including brain monoamines, and carotenoid-based pigmentation, and if there was any lateralization effects. Specifically, three different hypotheses were tested using social stress: (1) stressor affects number of carotenoid spots, (2) behavioural and physiological responses during stress can be coupled to number of spots before and/or after stressor, and (3) there is a lateralized effect of spots associated with behaviour and/or physiology.

2. Material and methods

2.1. Experimental animals and location

This study was carried out on 1 year old juvenile Arctic charr from the 7th generation of the Swedish Arctic charr breeding programme (Arctic superior, for details on the programme see Nilsson et al. [40]). Several months before the experiment, the fish were transported to Umeå Marine Research Station (UMF). At UMF, the fish were kept in tanks supplied with running brackish water (3–4‰) from the Bothnian Bay with a temperature ranging between 5 and 10 °C and a photoperiod set to 12 h light/12 h dark. The experiments were performed in May–June 2012 and the methodology was approved by the Umeå Animal Research Ethical Committee.

2.2. Social stress experiment

At the start of the experiment (day 1) fish (body mass: 149.8 ± 28.5 g, fork length (L_F): 24.3 ± 1.5 cm, mean \pm SD, $N = 32$), randomly selected from stock, were photographed on both sides using a Canon EOS 500D digital camera in a setup providing constant bright light. During the photographing, fish were restrained in a box with a transparent lid (see Backström et al. [24]), and after the photographs were taken the fish were anaesthetized with Tricaine methanesulfonate (MS-222, 0.15 g/L), weighed and measured. This procedure with anaesthetics following photographs instead of before, which would be easier and maybe less stressful for the fish, was based on that anaesthetics induce spots in Arctic charr [41]. The fish were marked by a small cut in the caudal fin, either dorsally or ventrally, and put into social isolation in individual compartments. Individual compartments were created

by separating experimental aquaria (170 L, $95 \times 41 \times 44$ cm) into four equally-sized 42.5 L compartments using removable dark PVC walls. Two neighbouring fish were matched for mass (asymmetries in mass within pairs was less than 5%) thus creating two pairs per aquarium. The fish were allowed to acclimate for one week during which they were hand-fed commercial pellets. Each aquarium was continuously supplied with running water from the Bothnian Bay (5–10 °C) and the light/dark regimen was set to 12 h light/12 h dark (light on at 06.00 and light off at 18.00 h).

The dominance experiments were conducted on day 8, and started by removing the PVC walls separating each pair of fish. The fish were allowed to interact in their size-matched pairs, and the interaction lasted for 1 h and was recorded by a camcorder for later behavioural analysis. After 1 h a clear dominant-subordinate relationship was established in all pairs, and the fish were immediately taken from the aquaria and photographed (as described above). After the photographs were taken, fish were sacrificed in a high dose of MS-222 (0.30 g/L). Blood was immediately sampled via a heparinized syringe from the caudal vein, then the fish was decapitated and the brain dissected out. Sex was determined by visual inspection of gonads. The blood was subsequently centrifuged, the plasma collected and stored at -20°C until further analysis. The brains were divided into the easily identified telencephalon, cerebellum, optic tectum and brainstem (including hypothalamus) and were stored at -80°C until further analysis.

2.3. Image analysis

Photographs were analysed as described and validated earlier [24]. Briefly, carotenoid-based spots were counted systematically in a rectangle (2 cm \times 10 cm) on each side of the fish using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2012.).

2.4. Assays

Plasma was analysed for cortisol using a commercial enzyme linked immunosorbent assay (ELISA) kit (product no. 402710, Neogen corporation, Lexington, USA). Each sample was run in duplicates during a single assay with an intra-assay coefficient of variation of 1.82%.

Tissue levels of serotonin (5-hydroxytryptamine; 5-HT) and the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and norepinephrine (NE) were analysed in telencephalon, cerebellum, optic tectum and brainstem samples using high performance liquid chromatography with electrochemical detection (HPLC–EC) as described by Höglund et al. [19]. The concentrations were standardized against the weight of the brain tissues. The ratios of 5-HIAA to 5-HT concentration and DOPAC to DA concentration were calculated and used as an index of serotonergic and dopaminergic activity, respectively.

2.5. Behavioural observations

In our analysis of the 1 h dyadic agonistic interactions the following was recorded per pair by one observer (TB): (1) latency to first attack within the pair, (2) time until the dominant-subordinate relationship was settled after first attack, (3) time remaining of the hour after the dominant-subordinate relationship was settled, (4) the number of aggressive acts per minute performed prior to the dominant-subordinate relationship was settled (attack rate prior dominance), (5) the number of aggressive acts per minute performed by dominant individual after the dominant-subordinate relationship was settled (attack rate post dominance), and finally

Table 1

The number of carotenoid spots in dominant and subordinate Arctic charr after social stress.

Side	Rank	Day 1	Day 8
Left	Dominant	17.81 ± 7.45 (16)	14.53 ± 5.62 (15)*
	Subordinate	19.31 ± 6.13 (16)	19.00 ± 6.55 (15)
Right	Dominant	15.75 ± 6.67 (16)	10.60 ± 4.76 (15)*
	Subordinate	16.62 ± 6.97 (16)	16.40 ± 6.22 (15)

Values are mean ± S.D. (N).

* Indicates that dominant individuals have significantly lower numbers of spots compared to subordinate individuals on the same day ($P < 0.05$, repeated measures ANOVA).

(6) individual social rank obtained (the outcome of the fight for dominance) defined by position in the aquaria and general activity.

2.6. Statistical analyses

Normally distributed data were analysed using ANOVA or repeated measures ANOVA with LSD post hoc tests. Gender did not affect probability to become dominant (Mann–Whitney U -test, $P = 1.00$), and was thus not analysed in further detail. Further, correlations between behaviour, physiology and pigmentation were investigated using either Pearson correlation test if data were normally distributed or Spearman rank correlation test if they were not. All statistics were performed in IBM SPSS Statistics 20 (IBM Corporation, New York, USA) or the graphical user interface R Commander for the free software R [42]. Data are presented as mean ± SEM if not stated otherwise.

3. Results

3.1. Carotenoid pigmentation

The carotenoid-based pigmentation (see Table 1) in individuals before transfer to isolation (day 1) did not differ between individuals subsequently becoming dominant and subordinate on either the right side (repeated measures ANOVA, $F_{1,14} = 0.133$, $P = 0.721$) or the left side (repeated measures ANOVA, $F_{1,14} = 0.966$, $P = 0.342$). However, after the agonistic interactions (day 8) dominant individuals had lower number of spots both on the right (repeated measures ANOVA, $F_{1,14} = 7.402$, $P = 0.017$) and the left side (repeated measures ANOVA, $F_{1,14} = 4.931$, $P = 0.043$) compared to subordinate individuals. This seems to be an effect of number of spots decreasing in dominant individuals whereas in subordinate individuals the number of spots remains the same.

Further, on the same day left and right side spots were positively associated both before isolation (day 1) (Pearson correlation, $r = 0.623$, $P = 0.010$, $N = 16$) and post dominance (day 8) (Pearson correlation, $r = 0.577$, $P = 0.024$, $N = 15$) in dominant individuals. No correlations were seen in subordinate individuals (before isolation; Pearson correlation, $r = 0.356$, $P = 0.176$, $N = 16$; post dominance; Pearson correlation, $r = 0.386$, $P = 0.155$, $N = 15$).

3.2. Behaviour

During the acclimation all fish were eating, and during the agonistic interactions all pairs behaved aggressively and established a dominance hierarchy. The agonistic interactions started with displays that escalated into overt aggressive behaviour. At first, the aggressive behaviour remained mutual with both individuals exchanging attacks and bites until one individual stopped replying to attacks. Then dominance was established, and the subordinate usually took position close to the surface or in a corner of the aquaria and did not retaliate against any aggressive acts, whereas the

dominant remained active and usually patrolled in the centre of the aquarium.

Behaviour during the interactions could be associated with carotenoid pigmentation in both dominant and subordinate individuals. In dominant individuals the attack frequency post dominance was positively correlated with spots on the right side post dominance (Spearman correlation, $r_s = 0.630$, $P = 0.012$, $N = 15$), and in subordinate individuals the latency to first interaction was negatively correlated with spots on the left side post dominance (Spearman correlation, $r_s = -0.645$, $P = 0.009$, $N = 15$).

3.3. Physiological parameters

The outcome of the agonistic interactions was reflected in the plasma cortisol levels. Plasma cortisol levels were lower (repeated measures ANOVA, $F_{1,15} = 9.149$, $P = 0.009$) in dominant individuals (99.4 ± 14.8 ng/mL; $N = 16$) compared to subordinate individuals (135.5 ± 16.3 ng/mL; $N = 16$). Similarly, the outcome also affected some of the monoamines and their metabolites (see Table 2). Dominant individuals had lower concentrations of 5-HIAA in the brain stem (repeated measures ANOVA, $F_{1,15} = 5.928$, $P = 0.028$) and 5-HT in the optic tectum (repeated measures ANOVA, $F_{1,13} = 5.193$, $P = 0.040$), and higher concentrations of NE in the telencephalon (repeated measures ANOVA, $F_{1,14} = 5.736$, $P = 0.031$) compared to subordinate individuals.

Associations between monoamines and carotenoid pigmentation were evident in both dominant and subordinate individuals. After the dominance test, 5-HIAA/5-HT in the telencephalon was positively correlated to spots on the left side in dominant individuals (Pearson correlation, $r = 0.597$, $P = 0.024$, $N = 14$). Further, DA in the optic tectum was negatively correlated with spots on the right side in dominant individuals (Pearson correlation, $r = -0.535$, $P = 0.049$, $N = 14$). In subordinate individuals, 5-HIAA (Pearson correlation, $r = -0.685$, $P = 0.007$, $N = 14$) and DA (Spearman correlation, $r_s = -0.664$, $P = 0.010$, $N = 14$) in the optic tectum were negatively correlated to spots on the left side, and DOPAC/DA (Spearman correlation, $r_s = 0.588$, $P = 0.027$, $N = 14$) in the optic tectum were positively correlated to spots on the left side.

Additionally, plasma cortisol was positively correlated to attack frequency post dominance in dominant individuals (Spearman correlation, $r_s = 0.574$, $P = 0.020$, $N = 16$).

4. Discussion

4.1. Stress and carotenoid pigmentation

In our study we present further evidence for an association between carotenoid pigmentation and stress responsiveness in Arctic charr. Subordinate individuals had more spots and higher plasma cortisol levels than dominant individuals as previously reported [24]. In addition, we found that the dominant individuals had lower 5-HIAA levels in the brainstem and 5-HT in the optic tectum, and higher NE in the telencephalon than subordinate individuals. The monoaminergic activity can also be used as a stress indicator [26,27], and is typically measured by the metabolite or the ratio of the metabolite to the parent monoamine [43]. Thus, dominant individuals had lower serotonergic activity similar to several earlier studies [19,44]. The difference in NE is difficult to interpret, since the metabolite was not measured.

4.2. Behavioural and physiological stress responses and their connection to carotenoid pigmentation

Behaviour during stress was connected to carotenoid pigmentation after social stress. Dominant individuals with more spots had higher attack frequency post dominance. The reason for this could

Table 2
Concentrations of NE, DOPAC, DA, 5-HIAA and 5-HT, and DOPAC/DA and 5-HIAA/5-HT ratios in the brainstem, cerebellum, optic tectum, and telencephalon of dominant and subordinate Arctic charr.

Brain part	Rank	NE (ng/g)	DOPAC (ng/g)	DA (ng/g)	DOPAC/DA $\times 10^{-3}$	5-HIAA (ng/g)	5-HT (ng/g)	5-HIAA/5-HT $\times 10^{-3}$
Brain stem	Dominant	70 \pm 21 (16)	24 \pm 22 (16)	81 \pm 94 (16)	398 \pm 252 (16)	4 \pm 1 (16)*	37 \pm 15 (16)	109 \pm 33 (16)
	Subordinate	72 \pm 19 (16)	28 \pm 38 (16)	79 \pm 101 (16)	418 \pm 274 (16)	5 \pm 1 (16)	40 \pm 9 (16)	124 \pm 42 (16)
Cerebellum	Dominant	35 \pm 45 (16)	53 \pm 70 (14)	n.d.	n.d.	n.d.	n.d.	n.d.
	Subordinate	23 \pm 8 (16)	50 \pm 46 (16)	n.d.	n.d.	n.d.	n.d.	n.d.
Optic tectum	Dominant	61 \pm 25 (16)	46 \pm 48 (16)	33 \pm 69 (15)	4050 \pm 2848 (15)	4 \pm 1 (15)	21 \pm 10 (15)*	179 \pm 43 (15)
	Subordinate	56 \pm 17 (15)	43 \pm 60 (15)	13 \pm 13 (15)	4117 \pm 2652 (15)	4 \pm 1 (15)	25 \pm 10 (15)	181 \pm 63 (15)
Telencephalon	Dominant	113 \pm 43 (16)*	40 \pm 54 (15)	49 \pm 64 (15)	1026 \pm 738 (15)	8 \pm 3 (15)	49 \pm 16 (15)	173 \pm 44 (15)
	Subordinate	93 \pm 23 (16)	32 \pm 21 (16)	41 \pm 49 (16)	1548 \pm 1728 (16)	7 \pm 2 (16)	44 \pm 15 (16)	178 \pm 69 (16)

Values are mean \pm S.D. (N). n.d. indicates that monoamines were not detected or too few to be analysed further.

* Indicates that levels in dominant individuals differs significantly from subordinate individuals in the specific brain part ($P < 0.05$, repeated measures ANOVA).

be that the less assertive dominants are using this as a stress coping mechanism. Interestingly, displaced aggression has been suggested as a stress coping mechanism in teleost fish [45,46]. Displaced aggression means that aggression is directed at another individual instead of the source. This interpretation is corroborated by plasma cortisol being positively correlated to attack frequency post dominance in dominants in our study.

Similar to the behavioural stress responses, several of the physiological stress responses were also connected to carotenoid spots, and for the first time monoaminergic activity is connected to carotenoid pigmentation. Serotonergic activity in the telencephalon was positively correlated to spots in dominants following the normal predictions with higher serotonergic activity in stressed individuals [26,27]. However, serotonergic activity in the optic tectum was negatively correlated with spots in subordinate individuals and thus not in line with predictions. This difference between stress levels could depend on several different mechanisms. The most likely explanation is that the different brain parts could be more or less involved in the stress response as shown in earlier studies [44,47], and therefore these differences could be site specific. Further, in subordinate individuals dopaminergic activity in optic tectum was positively correlated to spots. Typically dopaminergic activity is connected to increased activity and aggression [27]. However, dopaminergic suppression of aggression has also been reported [48]. Thus, DA in subordinate individuals could be involved in suppressing aggression.

4.3. Lateralization and carotenoid pigmentation

In a previous study it was suggested that the carotenoid spots were lateralized [24], with the left side being a cue for stress responsiveness and the right side a cue for aggressiveness. In this study of social stress, there were several interesting results also indicating lateralized responses consistent with this hypothesis. For instance, the start of interactions were negatively correlated with left side spots. This means that individuals with longer time of stress had more spots. Further, attack frequency post dominance was positively correlated with right side spots indicating an association between the right side and aggression.

Concerning physiological lateralization, serotonergic activity in the telencephalon was positively associated with left side spots in dominant individuals. Since increased serotonergic activity is associated with stress response (see above), this follows predictions. Further, dopaminergic activity in the optic tectum is positively correlated to left side spots in subordinate individuals. These results are consistent with the hypothesis that left side carotenoid pigmentation is connected to stress responsiveness [24]. However, the opposite pattern was evident in serotonergic activity in the optic tectum of subordinate individuals, with 5-HIAA being negatively correlated to spots on the left side. This discrepancy could be dependent on the temporal difference. For instance, temporal

differences in serotonergic and dopaminergic activity following stress were recently reported by Gesto et al. [49]. Following a handling (chasing) stress, both the acute stress response (up to 15 min) and the recovery after acute stress (up to 8 h) showed differences in serotonergic and dopaminergic activity in hypothalamus and telencephalon [49]. Thus temporal differences could be in part explanatory for our diverging results. Especially since the pairs were allowed to interact for one hour and not a specific time post dominance, meaning that there are temporal differences present.

Additionally, dopaminergic activity in the optic tectum was negatively correlated with spots on the right side in dominant individuals. Thus there are some connections between right side spots and the monoaminergic systems. Since dopaminergic activity usually is associated with aggression and activity [27], and dominant individuals with fewer spots were less aggressive post dominance (see above), this further corroborates the connection between right side spots and aggression. However, dopaminergic activity in optic tectum was positively correlated to spots on the left side in subordinate individuals, and was discussed as being involved in suppression of aggression (see above).

5. Conclusion

In conclusion, this study presents further evidence that more stressed charr have more carotenoid spots. This is further corroborated by links to both behavioural and physiological stress responses, with monoamines being investigated for the first time. Finally, several of the behavioural and physiological responses seem to be associated with a lateralized carotenoid pigmentation.

Authors' contributions

TB and MH designed and performed the study. TB carried out cortisol and monoamine analysis, and drafted the manuscript. EB, JN and CM participated in design of the study. SW helped with monoamine analysis. All authors participated in revising the manuscript and gave final approval for publication.

Competing interests

We have no competing interests.

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