Dominance and stress signaling of carotenoid pigmentation in Arctic charr (*Salvelinus alpinus*): Lateralization effects?

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Abstract
Social conflicts are usually solved by agonistic interactions where animals use cues to signal dominance or subordinance. Pigmentation change is a common cue used for signalling. In our study, the involvement of carotenoid-based pigmentation in signalling was investigated in juvenile Arctic charr (*Salvelinus alpinus*). Size-matched pairs were analysed for pigmentation both before and after being tested for competitive ability. We found that dominant individuals had fewer carotenoid-based spots, both on the right and the left side, as well as lower plasma cortisol levels compared to subordinate individuals. Further, the number of spots on both sides was positively associated with plasma cortisol levels. These results indicate that carotenoid-based pigmentation in Arctic charr signals dominance and stress coping style. Further, it also appears as if carotenoid-based pigmentation is lateralized in Arctic charr, and that the right side signals aggression and dominance whereas the left side signals stress responsiveness.

1. Introduction

In social animals conflicts concerning resources such as food, territory, and mate choice are common [1]. These social conflicts are often resolved by agonistic interactions. Agonistic interactions usually start with displays and then escalate into overt aggressive and violent behaviour until dominance has been established [1]. The dominant individuals typically maintain their dominance by continuous aggression which can lead to social stress, severe injuries, and even death for participants in the conflict [2]. However, many animals have evolved signals or cues to avoid prolonged and potentially harmful interactions. These signals or cues could be communicating dominance and subordinance. A common type of cue is pigmentation. For instance, in the lizard *Anolis carolinensis* eye spot darkening signals dominance [3-5], and eye darkening signals subordinance in several teleost fish [6-9].

Further, melanin-based pigmentation has in general been linked to behaviour in various vertebrates [10, 11]. For instance, melanin-based pigmentation co-varies positively with aggression in mammals [12] and birds [13-16]. There is also some evidence for a similar link in salmonids. In Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*), melanin-based skin spots are associated with behavioural and physiological stress responses [17]. Since stress responsiveness is commonly connected to aggression in accordance with stress coping style [18, 19], this suggests that densely spotted salmonids are more aggressive. However, in the salmonid Arctic charr (*Salvelinus alpinus*), the link between aggression and melanin-based pigmentation seems to be different with social subordinance inducing skin darkening [20].

There are inter-individual differences in both behavioural and physiological stress responses. These differences are typically consistent over context and time, and therefore form different stress coping styles. Generally, stress coping styles ranges between the proactive and reactive stress coping [18, 19]. For example, proactive animals respond with more aggression, higher general activity and a sympathetic activation (fight or flight reaction) when challenged by a conspecific individual, whereas reactive animals respond to the same challenge with immobility and a parasympathetic activation (conservation/withdrawal reaction). Therefore reactive animals, compared to proactive animals, have a higher elevation in glucocorticoid levels in response to stress [18, 19]. In recent years, consistent stress coping styles have been evident in several teleost species (see reviews by Schjolden and Winberg [21] and Øverli et al. [22]).

The asymmetrical specialization, or lateralization, is defined as one side being structurally different or performing different functions than the other side. Both behavioural and physiological lateralization is evident in vertebrates [23, 24]. For instance, aggression has been shown to be lateralized in teleost fish [25-27], and some evidence for behavioural lateralization parameters are involved in stress [24, 28].

Since Arctic charr differ ecologically from other salmonids [29] there could also be differences concerning the association between behaviour and pigmentation. One main difference between Arctic charr and other salmonids is the red-coloured ventral skin and abundance of light-coloured spots in charr [30]. This carotenoid-based pigmentation is

depending on diet [31, 32], and sexually mature charr have more carotenoid skin pigmentation than juvenile charr [33]. In this context, we recently reported that this carotenoid-based pigmentation in juvenile Arctic charr was linked to stress responsiveness, with individuals that had more spots also showed a higher stress response to a confinement stressor defined as a reactive stress coping style [34]. Therefore, we wanted to investigate if dominance is associated with the carotenoid-based pigmentation in juvenile Arctic charr. Two different hypotheses were tested using a dyadic interaction paradigm: 1) initial carotenoid-based pigmentation indicates dominance, and/or 2) carotenoid-based pigmentation after agonistic interactions differs between dominants and subordinates. In addition, we tested a method for an automated analysis of visible carotenoid-based pigmentation, that is, counting the number of light-coloured spots. Further, plasma cortisol and aggressive behaviour were also evaluated to investigate possible connections between pigmentation, behaviour and stress response as well as stress coping styles. Finally, pigmentation on both left and right side of each fish were evaluated for lateralization effects.

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. [35]], with an initial body mass of 150.2 ± 28.1 g (mean $\pm$ SD, N=106) and a fork length (L_F) of 24.2 ± 1.4 cm (mean $\pm$ SD, N=106). The fish were bred and kept in the stocking facilities at the Aquaculture Centre North in Kälarne, Sweden. Several months before the experiment, the fish were transported to Umeå Marine Research Station (UMF). The fish were kept in tanks supplied with running brackish water (3-4‰) from the Bothnian Bay with a temperature ranging between 5-10 °C and a photoperiod set to 12 h light/12 h dark.

2.2 Experimental protocol

At the start of the experiment, fish were randomly selected from the holding stock and photographed on both left and right side using a Canon EOS 500D digital camera. Fish were placed in a box with a transparent lid to be restrained during the photographing. The lighting was provided by two 650 W halogen lamps situated on the left and right side of the box at angles to avoid light reflections appearing in the photographs (see Figure 1A). Then the fish were anesthetized with tricaine methanesulfonate (MS-222, 0.15 g/l), weighed, measured, marked by a small cut in the caudal fin (either dorsally or ventrally), and put into social isolation. Individual compartments were created by separating experimental aquaria (170 L, 95 x 41 x 44 cm), into four equally-sized 42.5 L compartments with 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 aquaria. Each aquaria was continuously supplied with running aerated water as described above 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 fish were allowed to acclimate in isolation and their feeding behaviour was monitored for one week.
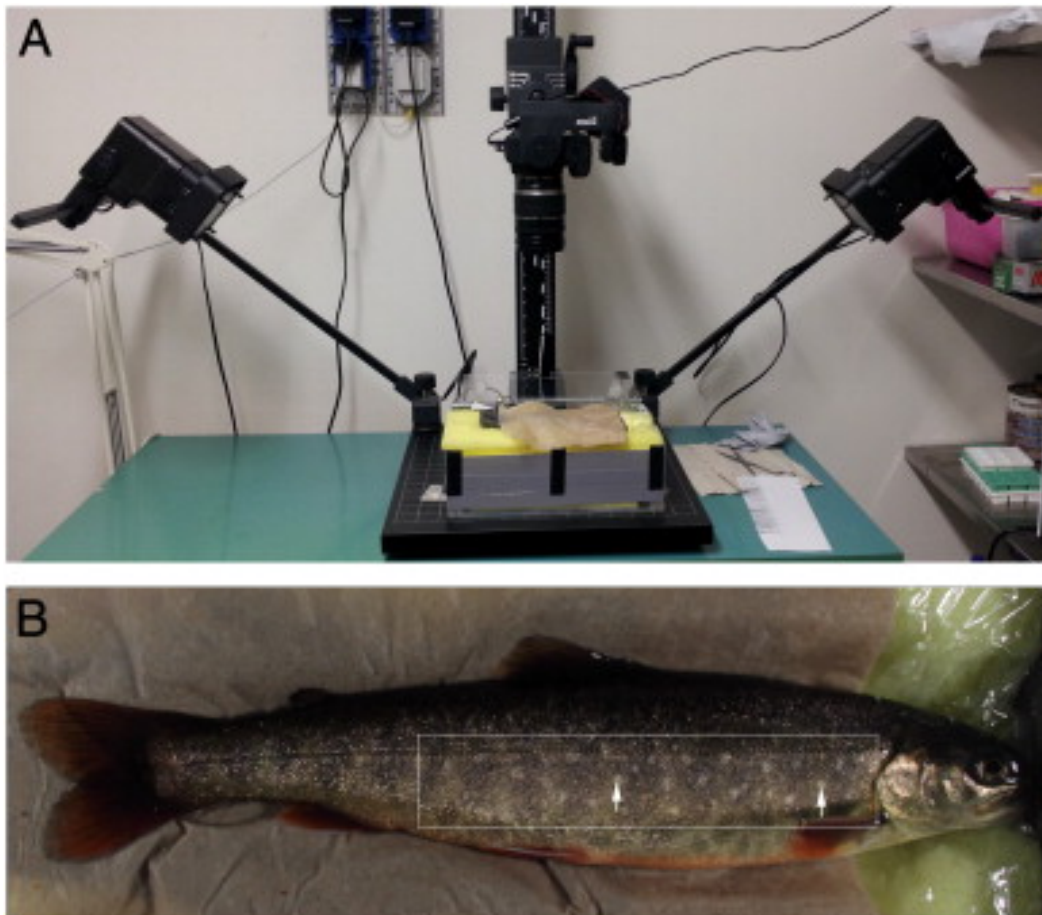
Fig. 1. (A) The set-up used for photographing individual fish. The set-up included a holder for the camera to keep the same distance, lamps for providing constant light, and a box for keeping the fish still. The box also included a SpyderCube™ used for setting white balance during the pigmentation analysis. (B) Photograph depicting the selection box used for carotenoid-based pigmentation analysis. Arrows indicates typical spots that were counted.

The dominance experiments were conducted on day 8, and were 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 hour and was recorded for later behavioural analysis. After 1 hour a clear dominant-subordinate relationship was established in all pairs except one, which was removed from further analyses. At the end of the 1 hour interaction period the fish were immediately taken from the aquaria and photographed (as described above). Afterwards the fish were sacrificed using a high dose of MS-222 (0.3 g/l), sampled for blood plasma via the caudal vein, and the sex was determined by visual inspection of gonads. The blood was subsequently centrifuged at 10 000 g for 10 min at 4 °C, the plasma was collected and stored at −20 °C until further analysis. In total 90 fish were used in the social interactions. In addition, 16 fish were exposed to the isolation procedure and then photographed after one week (day 8) without social interactions (control). The experiments were performed in May-June 2012. The methodology of this study was approved by the Umeå Animal Research Ethical Committee.

2.3 Image analysis and automated spot analysis evaluation

Photographs were analysed for carotenoid-based pigmentation, *i.e.* light coloration, with the free software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.). A rectangle (2 x 10 cm) was cut out of the photographs along the midline after the operculum (see Figure 1B). Number of spots in the rectangle in 27 randomly selected fish was counted manually at two separate occasions using the plugin PointPicker (http://bigwww.epfl.ch/thevenaz/pointpicker/) in ImageJ. The same rectangles were then used for testing an automated method. Tools provided in ImageJ were used to create an automated method. Briefly, the photograph was transformed into a black-white scale using the "Treshold" tool, the light patches were measured using "Set Measurements…", and finally analysing patches above and below a specific number of pixels using "Analyze Particles…", thus ending up with a specific number of spots per photograph.

2.4 Enzyme-linked immunosorbent assay

Plasma was analysed for cortisol using a commercial enzyme linked immunosorbent assay (ELISA) kit following the manufacturer's instruction (product # 402710, Neogen corporation, Lexington, USA). In brief, plasma was extracted 1:5 in ethyl acetate and the mixture was vortexed. The solvent was decanted and then evaporated in a vacuum concentrator. Before the ELISA procedure, it was dissolved in the extraction buffer included in the ELISA kit. Samples were run in duplicates. Cross reactivity of the kit for the main glucocorticoids was 100.0% for cortisol, 47.4% for prednisolone, 15.7% for cortisone, and 15.0% for 11-deoxycortisol. Each sample was run in duplicates during a single assay with an intra-assay coefficient of variation of 1.82%.

2.5 Behavioural observations

In our analysis of the 1 hour dyadic agonistic interactions the following was recorded per pair: (1) latency to first attack within the pair, (2) time until the dominant-subordinate relationship was settled after first attack, (3) time 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 after dominance), and finally individual (6) social rank obtained (the outcome of the fight for dominance) defined by position in the aquaria and general activity (see below).

2.6 Statistical analyses

The automated spot analysis was evaluated by testing the correlation between manual and automated spot counting with Pearson correlation test. To analyse the effects of the agonistic interactions and social rank on physiological and pigmentation parameters a repeated measures ANOVA was applied. Paired t-test was used to analyse individual differences in pigmentation between sides as well as the same side before and after social interactions. Behavioural data was analysed with a Mann-Whitney U-test. Further, correlations between behaviour, physiology and pigmentation were investigated using Spearman rank correlation test.

All statistics were performed in IBM SPSS Statistics 20 (IBM Corporation, New York, USA) and data are presented as mean ± SEM if not stated otherwise. Initial body mass (ANOVA, $F_{1,88}=0.001$, P=0.975) and body length (ANOVA, $F_{1,88}=0.028$, P=0.868), final body mass (ANOVA, $F_{1,88}=0.039$, P=0.843) and body length (ANOVA, $F_{1,88}=0.023$, P=0.879), sex (Mann–Whitney, P=0.182) and marking (cut on top or bottom of caudal fin) (Mann–Whitney, P=0.059) did not differ between dominant and subordinate individuals.

3. Results

3.1 Automated spot analysis
The two manual counts were both significantly correlated with the automated method (see Table 1). Thus the automated counting procedure was validated for the specific settings, and used for the rest of the photographical analysis throughout this experiment.

Table 1. The Pearson correlation coefficients for the number of carotenoid-based pigmentation spots in juvenile Arctic charr between three different counting methods. *P*-values indicate significant correlations between the different counting methods. N= 27

	1st manual count	2nd manual count	Automated count
1st manual count	1.00		
2nd manual count	0.76	1.00	
	P < 0.001		
Automated count	0.48	0.39	1.00
	P = 0.005	*P = 0.044*	

3.2 Behaviour
During the acclimation and observation of feeding behaviour all fish were eating, and during the agonistic interactions all pairs behaved aggressively and established a dominance hierarchy (see Table 2). Agonistic interactions typically started with displays that escalated into overt aggressive behaviour. The aggressive behaviour remained mutual with both individuals remaining approximately equally active in exchanging attacks and bites, until one individual stopped replying to attacks and dominance was established. For the remainder of the time, the subordinate usually took position close to the surface or in a corner of the aquaria and did not retaliate any aggressive acts. The dominant remained active and usually patrolled in the centre of the aquarium.

Several of the aggressive behavioural parameters were associated (see Table 2). For instance, attack rate prior to dominance was negatively associated with attack latency and time until dominance, and positively associated with attack rate after dominance. Further, attack latency was negatively associated with time after dominance.

Table 2. The Spearman correlation coefficients for aggressive behaviour and the mean ± SEM in juvenile Arctic charr. N=45 except in a where N= 16 due to omission of pairs where dominance was settled immediately

	Attack latency (min)	Time until dominance (min)	Time after dominance (min)	Attack rate prior dominance (no/min)	Attack rate after dominance (no/min)	$\bar{X} \pm$ SEM
Attack latency (min)	1.00					7.73 ± 1.13
Time until dominance (min)	0.06 **P** = 0.574	1.00				2.53 ± 0.60
Time after dominance (min)	− 0.58 **P < 0.001**	− 0.18 **P** = 0.090	1.00			51.79 ± 1.41
[a]Attack rate prior dominance (no/min)	− 0.42 **P = 0.017**	− 0.54 **P = 0.001**	0.24 **P** = 0.195	1.00		10.70 ± 1.55
Attack rate after dominance (no/min)	− 0.10 **P** = 0.360	− 0.03 **P** = 0.811	0.06 **P** = 0.592	0.39 **P = 0.026**	1.00	2.08 ± 0.17

3.3 Carotenoid-based pigmentation

3.3.1 Dominant-subordinate individuals

The carotenoid-based pigmentation in individuals before transfer to isolation did not differ between individuals subsequently becoming dominant and subordinate on either the right (repeated measures ANOVA, $F_{1,36}$=0.053, P=0.820; see Figure 2A) or the left side (repeated measures ANOVA, $F_{1,36}$=0.023, P=0.880; see Figure 2A). However, after the agonistic interactions dominant individuals had lower number of spots on the right side (repeated measures ANOVA, $F_{1,36}$=5.514, P=0.024; see Figure 2A) and the left side (repeated measures ANOVA, $F_{1,36}$=4,569, P=0.039; see Figure 2A) compared to subordinates.

The carotenoid pigmentation in individuals before transfer to isolation differed between right and left side (see Figure 2B). The right side had fewer spots than the left side (paired t-test, t_{89}=−2.607, P=0.011; Figure 2B) before transfer to isolation. However, this difference was not apparent after agonistic interactions (paired t-test, t_{73}=−1.301, P=0.197; Figure 2B). After agonistic interactions the number of spots was lower on the left (paired t-test, t_{73}=2.652, P=0.010; Figure 2B) and the right side (paired t-test, t_{73}=2.359, P=0.021; Figure 2B) compared to before isolation. Some differences were pronounced in the individuals becoming dominant. For instance, the right side having fewer spots than the left side (paired t-test, t_{44}=−2.621, P=0.012) before transfer to isolation, and after agonistic interactions number of spots was lower on the left (paired t-test, t_{36}=3.195, P=0.003) and the right (paired t-test, t_{36}=2.880, P=0.007) side compared to before isolation. Individuals becoming subordinate did

not change number of spots on either side following agonistic interactions nor did they differ in number of spots per side before transfer to isolation.
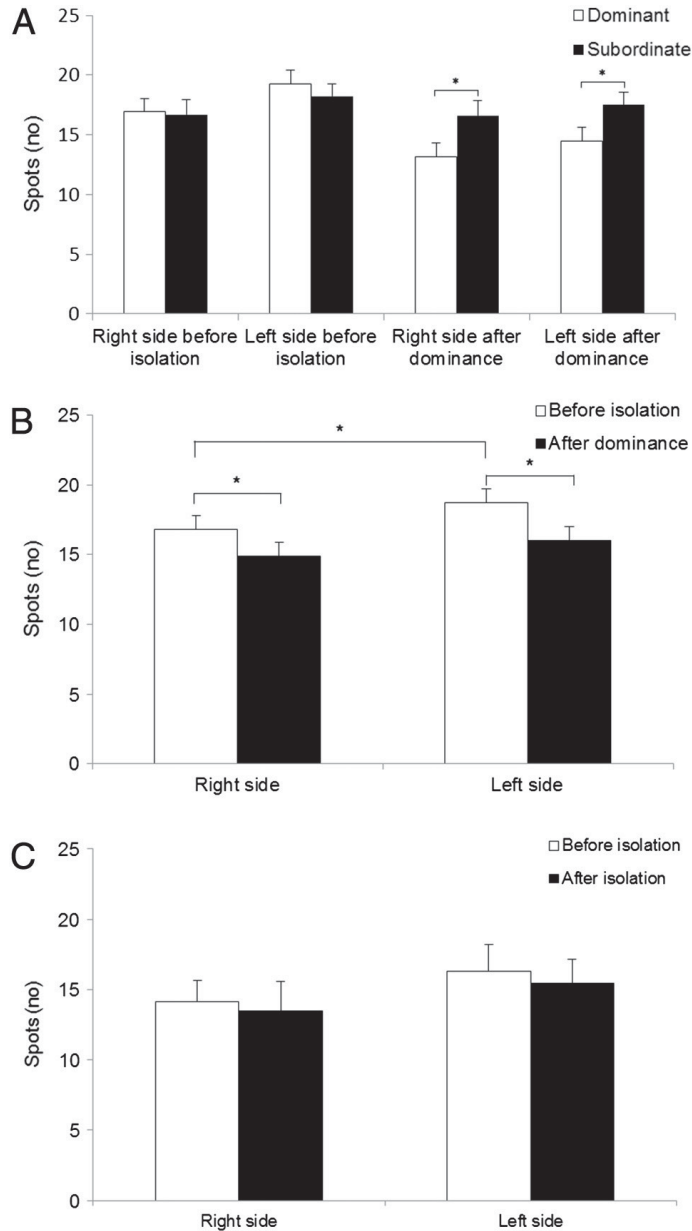

Fig. 1. Carotenoid-based spots in juvenile Arctic charr. Values are means ± SEM. (A) Number of spots on the right and left sides in socially interacting pairs. * Denotes difference in the same side before and after isolation between dominant and subordinate individuals ($P < 0.05$, repeated measures ANOVA). (B) Number of spots on the right and left sides of socially interacting pairs. * Denotes differences ($P < 0.05$, paired t-test). (C) Carotenoid-based spots in juvenile Arctic charr before isolation and after isolation
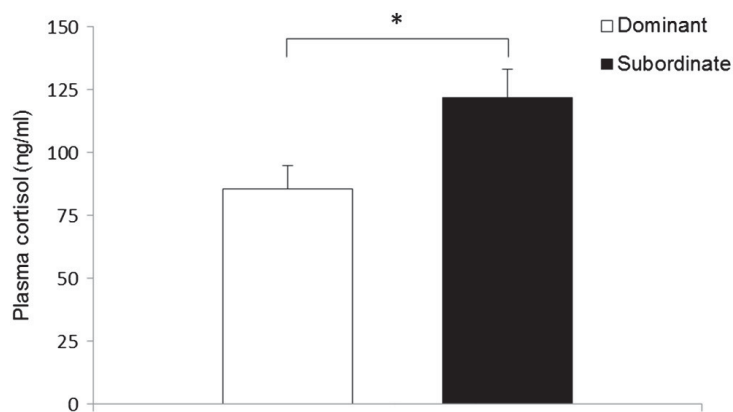
Fig. 3. Plasma cortisol concentrations in socially interacting juvenile Arctic charr. Values are means ± SEM. * Denotes difference between dominant and subordinate individuals (P < 0.05, repeated measures ANOVA).

The carotenoid-based spots were also correlated with behaviour and physiology. Number of spots both on the right and the left side after dominance was positively associated with plasma cortisol levels (right side, Spearman rank two-tailed, r_s=0.24, N=74, P=0.043; left side, Spearman rank two-tailed, r_s=0.32, N=74, P=0.006). When considering the social ranks separately, in subordinate charr number of spots on the left side after dominance was positively associated with plasma cortisol levels (Spearman rank two-tailed, r_s=0.39, N=37, P=0.016). No effects were seen on the right side. In dominant individuals no correlations between spots and cortisol levels were found.

3.3.2 Isolated individuals
The carotenoid-based pigmentation in individuals subjected to social isolation for one week did not change on either the right (paired t-test, t_{15}=0.271, P=0.790) or the left side (paired t-test, t_{15}=0.330, P=0.746)(Figure 2C). Further, there was no difference in number of spots between the right and left side before social isolation (paired t-test, t_{15}=-1.123, P=0.279) or after one week of social isolation (paired t-test, t_{15}=-1.048, P=0.311) (Figure 2C).

3.4 Plasma cortisol
The outcome of the agonistic interactions was reflected in the plasma cortisol levels. Dominant individuals had lower level of plasma cortisol than subordinate individuals (repeated measures ANOVA, $F_{1,36}$=13,379, P=0.001; see Figure 3).

4. Discussion

4.1 Carotenoid pigmentation and dominance
In this study we showed that dominance affected carotenoid-based pigmentation in Arctic charr, with dominant individuals having fewer spots after an agonistic interaction compared to subordinates. Thus it seems as if a low level of carotenoid-based pigmentation indicates

9

that dominance status had been achieved. However, in several earlier studies the evidence for carotenoid pigmentation affecting behaviour seems to be in the opposite direction, *i.e.* carotenoid pigmentation signals physical prowess and therefore would indicate a better competitive ability. For instance, in male guppy (*Poecilia reticulata*) carotenoid pigmentation is positively correlated to swimming performance [36] and boldness [37]. Further, in firemouth cichlids (*Cichlasoma meeki*), males fed on a diet enriched with carotenoids, thus having more carotenoid pigmentation, won more dyadic fights compared to males given a control diet [38]. In male three-spined stickleback (*Gasterosteus aculeatus*) fights lasted longer if losers had higher carotenoid levels than winners [39]. Our results are potentially opposing to these reports, but there are differences in methodologies and study animals that could explain to these differences. Earlier studies were performed in sexually mature males whereas our study was performed in male and female juveniles, so sexual maturity could be causes for the differences in results. Further, species differences in behaviour as well as carotenoid metabolism, *i.e.* where carotenoid is stored, could explain these discrepancies.

The difference in carotenoid spots between dominant and subordinate charr could be interpreted as a signal for communicating dominance. Since there was no difference between dominant and subordinate charr before isolation, it seems as if the carotenoid spots changed during the agonistic interactions and thereby signalled dominance. This hypothesis is further corroborated by the observation that Arctic charr isolated for one week showed no difference in carotenoid spots before and after isolation. However, there are other possible explanations. Earlier it has been shown that subordinate Arctic charr gets darker [20] and therefore the apparent difference in our study could be an effect of a better contrast in subordinate charr between spots and background pigmentation. However, the initial dominant carotenoid pigmentation did not differ from subordinate pigmentation, and thus it seems more likely that the subordinate charr did not change appearance, and the noted difference therefore stems from the change of appearance in dominant charr.. Therefore, it seems as carotenoid pigmentation can be linked to dominance in Arctic charr.

4.2 Carotenoid pigmentation, stress responsiveness, and stress coping style
Inter-individual differences in stress response, consistent across context and time, are found across the vertebrate species and referred to as stress coping styles [18, 19]. Typically, there is a gradient of stress coping styleswith the two extremes being the most proactive and reactive stress coping styles, respectively. Aggressiveness as well as a more active behaviour and routine formation are hallmarks of the proactive animals in comparison to the reactive animals [18, 19]. Further, proactive animals respond to stress with a lower reactivity of the hypothalamic-pituitary-adrenocortical (HPA) axis and parasympathetic nervous system, and a higher reactivity of the sympathetic nervous system than reactive animals [18, 19]. Stress coping styles have been reported across the vertebrate taxa including birds, mammals and teleost fish [19].

In this study we showed that dominant charr had fewer carotenoid spots as well as lower plasma cortisol levels than subordinates. Thus, the result fits well with an earlier report stating that proactive Arctic charr have fewer carotenoid spots than reactive individuals [34]. Further, as expected the stress response (plasma cortisol) was higher in subordinate charr

following previous reports [40-42]. Therefore, a reduction of spots could communicate an efficient stress coping. However, further studies are needed to elucidate if the reduction of spots is used as a signal and/or is an effect of becoming dominant.

4.3 Lateralization and behaviour

Asymmetrical specialization, or lateralization, of different behavioural or physiological parameters has been shown across the animal phyla [43-45]. For instance, several species direct more aggressive responses towards visual input from their left side including primates [46, 47], lizards [48], and teleost fish [49]. However, in some teleost fish the aggression is lateralized into the right visual field [25, 26]. Interestingly, our study showed that there was a stronger effect on the carotenoid pigmentation on the right side in dominant charr, and also before isolation there were fewer spots on the right side compared to the left side. Further, during agonistic interactions it seems as if a head-to-tail alignment is a common behaviour in fish [50]. Salmonids would typically circle each other head-to-tail during agonistic interactions (personal observation). Thus when circling clockwise, the right side would be more exposed to the right eye. This means that the right side would be the preferred side to use to signal dominance if the right eye is connected to aggression in Arctic charr as seen in other teleost fish (see above). Furthermore, our results also provide evidence that the left side would be more prone to show stress responsiveness. In subordinate charr, spots on the left side after dominance were positively associated with stress response. Together with our earlier report that left side is connected to stress coping style [34], this suggests that left side is signalling stress responsiveness in Arctic charr. Earlier reports have provided some evidence for behavioural lateralization in stress responses [24, 28], and that is mainly processed by the right brain hemisphere in mammals [24]. For instance, in sheep social isolation stress is reduced by familiar faces and leads to increased activity in the right hemisphere [51], and strongly lateralized mothers had higher stress response than weakly lateralized mothers following separation from their young [52]. Further, stress induced temperature elevation in the right ear, corresponding to the right hemisphere, has been reported in marmosets [53] and cats [54]. Chronic stress causes neural damage in the right hemisphere in rats [55], and in humans suffering of major depressive disorders there is also a right hemisphere balance [56]. Thus, there is some evidence for stress coping style being lateralized in mammals. Our results could also be interpreted as in line with these since the left side of the body is controlled by the right hemisphere.

4.4 Conclusion

Our study shows that carotenoid-based pigmentation is associated with dominance and stress in Arctic charr. Further, it seems as if there is a lateralization effect on carotenoid spots. However, further studies are needed to elucidate if and how lateralization affects the carotenoid-based pigmentation in Arctic charr.

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