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Behaviour, physiology and carotenoid pigmentation in Arctic charr *Salvelinus alpinus*

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The behaviour during an exploration task and the stress response to a confinement stress of Arctic charr *Salvelinus alpinus* were evaluated. Behaviour of individuals during 90 minutes of exploration was classified into high and low activity. High-activity individuals had higher plasma cortisol levels following stress compared to low-activity individuals. This indicates that high- and low- activity individuals correspondg to reactive and proactive stress coping style. Further, a pigmentation analysis showed that high-activity individuals had a higher number of carotenoid spots cm^{-2} than low-activity individuals. Thus, carotenoid pigmentation, as melanin pigmentation in other salmonids, could be linked to stress coping style in *S. alpinus*.

Keywords: activity: behaviour; confinement stress; stress coping style

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INTRODUCTION

Individual differences in animal behaviour have been shown during the last decades to be consistent across context and time and thus individuals express personality (Réale *et al.*, 2007). This has been described under several different synonyms including behavioural syndromes (Sih *et al.*, 2004), personality (Gosling, 2001), and temperament (Boissy, 1995). Another similar categorization of animals has been based on the different individual responses to stress. Individual stress responsiveness is shown in both behavioural and physiological differences which can be defined as stress-coping styles, and are generally divided into proactive and reactive coping style (Koolhaas *et al.*, 1999, 2007). Proactive animals behave more aggressively, are more active, and readily form routines compared to reactive animals. In response to a stressor, proactive animals have a lower hypothalamic–pituitary–adrenal (HPA) axis reactivity leading to a lower increase of glucocorticoids and a higher sympathetic activity leading to a higher increase of noradrenaline and adrenaline in blood compared to reactive animals.

In recent years, studies on teleost fish have established the existence of stress-coping styles in several different species such as halibut *Hippoglossus hippoglossus* (L. 1758) (Kristiansen & Fernö, 2007), Nile tilapia *Oreochromis niloticus* (L. 1758) (Barreto & Volpato, 2011) and Senegalese sole *Solea senegalensis* Kaup 1858 (Silva *et al.*, 2010). Additionally, salmonid species have also been examined thoroughly. For instance, in brown

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trout *Salmo trutta* L. 1758 individuals clustered into two separate stress-coping styles based on the physiological plasma levels of noradrenaline and adrenaline post confinement and behaviour during hypoxia (Brelin *et al.*, 2005). Similarly, in rainbow trout *Oncorhynchus mykiss* Walbaum 1792) aggression, dominance and the stress hormone cortisol differed between individuals and resulted in two distinct coping styles (Schjolden *et al.*, 2005a). Further, two selected strains of rainbow trout representing high (HR) and low responders (LR) with respect to cortisol release following a standardized confinement stress were developed and studied by Pottinger and Carrick (1999). Over a series of experiments the strains were shown to correspond to reactive and proactive stress coping strategy (Øverli *et al.*, 2007).

In a recent study of the HR and LR strains of *O. mykiss*, melanin-based pigmentation was negatively correlated with stress responsiveness, with the LR trout having more melanin spots thanthe HR trout (Kittilsen *et al.*, 2009). The same negative correlation between melanin-based spots and stress responsiveness was also found in Atlantic salmon *Salmo salar* L. 1758 (Kittilsen *et al.*, 2009). Thus, it appears that melanin pigmentation and stress responsiveness are coupled in salmonid species in general. The same pattern is apparent in vertebrates (Ducrest et al., 2008), with darker animals being less sensitive to stress. The physiological link between melanin pigmentation and stress responsiveness is that both are affected by proopiomelanincortin (POMC) (Ducrest *et al.*, 2008). In Arctic charr *Salvelinus alpinus* (L. 1758), however, the hypothesis that darker animals are more stress resilient does not appear to be valid. In interacting *S. alpinus*, the subordinates are darker and have higher plasma levels of the stress hormone cortisol (Höglund *et al.*, 2000, 2002). Furthermore, *S. alpinus* differ from other salmonid species in life history, social behaviour and pigmentation (Klemetsen *et al*., 2003). One of the most apparent differences is the carotenoid-based pigmentation in *S. alpinus*. In several studies of teleosts, carotenoid-based pigmentation and physical prowess have been positively linked (Nicoletto, 1991; Nicoletto & Kodric-Brown, 1999; Guderley & Couture, 2005; Kodric-Brown & Nicoletto, 2005). Based on this, an investigation was performed to see if stress-coping styles in *S. alpinus* are correlated to carotenoid-based pigmentation. Two hypotheses were formed: (1) behaviour and stress responsiveness are correlated in *S. alpinus* as in other salmonids and thus resulting in stress coping styles and (2) the resulting stress coping styles from hypothesis 1 are correlated (negatively or positively) with carotenoid-based pigmentation. Behaviour was monitored during an exploration task, plasma cortisol was measured after confinement stress and images analysed for carotenoid-based pigmentation.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS AND LOCATION

This study was carried out on 26 months old juvenile Arctic charr from 125 full sibling groups of the seventh generation of the Swedish *S. alpinus* breeding programme [Arctic superior, for details on the programme see Nilsson *et al.* (2010)], with a body mass (M_B) of 965.6 ± 352.8 g (mean ± S.D., n=60) and a fork length (L_F) of 371.6 ± 44.9 mm (mean ± S.D., n=60). Prior to the experiment, the *S. alpinus* were kept at the stocking facilities at the Fisheries Board Research Station in Kälarne, Sweden, and fed commercial pellets (Biomar, 4mm, www.biomar.com) at 1.0-1.5 % of body mass per day depending on temperature. The photoperiod was set to 10L:14D dark and temperature ranged from 2 to7 °C. The experiment was performed in May 2011.

EXPERIMENTAL PROTOCOL

At the start of the experiment, *S. alpinus* from the holding stock were anesthetized with tricaine methanesulfonate (MS-222, 0.15 g^{-1}), weighed and measured, and the left side of each *S. alpinus* was photographed using a Canon EOS 500D digital camera (www.canon.com) under ambient light kept constant throughout the session. *S. alpinus* were classified into one of three different size categories, small (591.9 ± 149.9 g, 322.0 ± 28.1 mm, n=19), medium (922.1 ± 76.1 g, 376.3 ± 12.3 mm, n=18), or large (1308.3 ± 256.8 g, 408.9 ± 32.1 mm, n=23), for the following experiments. Individual *S. alpinus* were moved to isolation overnight in an aquarium prepared for an open field test. The aquaria were of two different sizes (73 x 40 x 49 cm, 143 L; and 71 x 49.5 x 49 cm, 172 L) and supplied with water at ambient temperature from a nearby lake (also used for the holding stock) and the same photoperiod as in holding tanks (10L:14D). Each aquarium was separated into two compartments of equal size; one compartment was covered in black plastic, thus being dark, whereas the other compartment was exposed to light. The two compartments were connected by a opaque screen with a round opening (10 cm diameter) in the middle, covered by a sliding trapdoor. Initially, the *S. alpinus* were placed in the dark compartment. The second day, the trapdoor was removed and latency to enter the lit area and number of movements between the dark and the lit compartment (activity) was observed for 90 min. The observers were sitting motionless *c.* 2 m from the aquaria, and took care not to disturb the fish during the behavioural test. During the third day, *S. alpinus* were exposed to an acute confinement stress test. *S. alpinus* were placed in one of three different confinement aquaria based on size of individual *S. alpinus* (27.5 x 18 x 17 cm, filled with 3 l water for small size, n=19; 38 x 26 x 23.5 cm, filled with 4 l water for medium size, n=18; 42 x 31 x 25 cm, filled with 5 l water for large size, n=23). After 30 min, *S. alpinus* were sacrificed with a blow on the head, and blood was sampled. The blood was subsequently centrifuged, the plasma collected and stored at −70 °C until further analysis.

IMAGE ANALYSIS

Photographs were analysed for carotenoid-based pigmentation, *i.e.* light colouration, 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.). Each side, from gills to tail, was divided into eight separate segments based on the visual cues of fins, gills, and lateral line (Fig. 1). The number of spots per segment was counted using the plugin PointPicker (http://bigwww.epfl.ch/thevenaz/pointpicker/) by an observer not involved in the behavioural monitoring. The area for each segment was determined *via* rectangular and triangular areas using the existing scale tool in ImageJ, based on the known L_F of each individual. The end product used for analysis was number of spots cm^{-2}. This procedure was done twice with strongly correlated results.

RADIOIMMUNOASSAY

Plasma cortisol levels were analysed using a radioimmunoassay (Pottinger & Carrick, 2001). Each sample was run in a single assay with a minimal detection limit of 0.1 ng ml^{-1} and an intra-assay coefficient of variation (C:V) of 5.2%. The cortisol antibody (ABIN108346) was a rabbit polyclonal (www.antibodies-online.com).

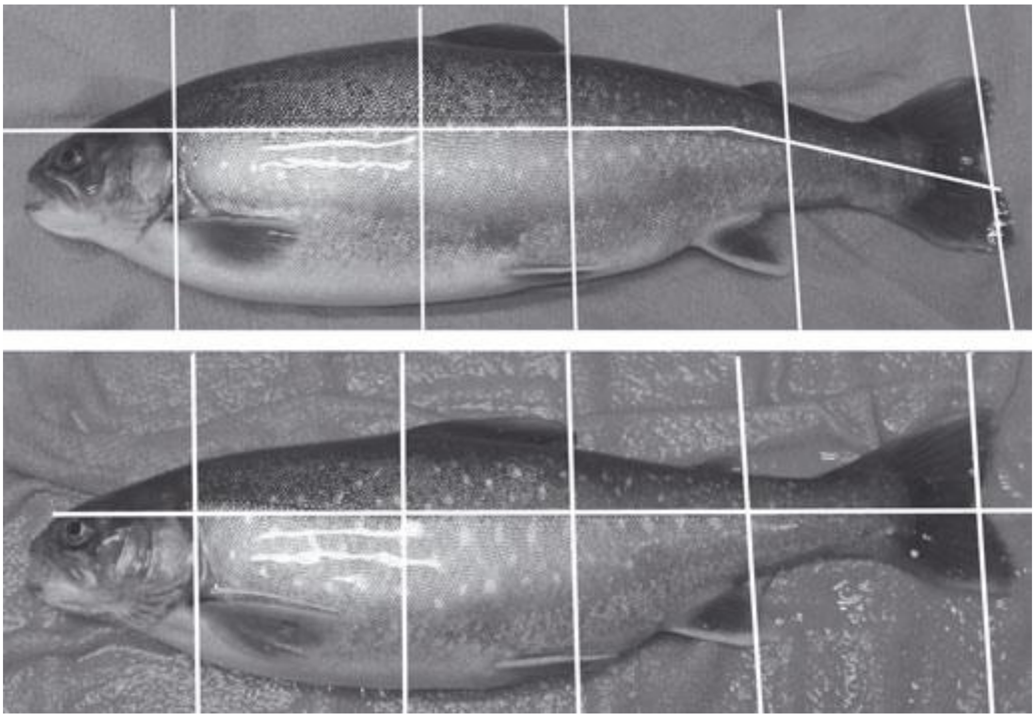

Fig. 1. Schematic representations of the areas that were used for the pigmentation counting. The top *Salvelinus* is representative of low pigmentation and the bottom *S. alpinus* is representative of high pigmentation.

STATISTICAL ANALYSIS

Two-tailed Spearman correlations were used to compare the aggregate as well as the individual eight areas of spots. Behavioural data (activity) was used to perform a classification process (TwoStep Cluster) dividing data into clusters. Following classification, Mann-Whitney *U*-test or Kruskal-Wallis comparisons were used to analyse all data, including the Fulton condition factor calculated by the formula $K = 100^* M_B L_F^{-3}$ and the different size classes. All statistics were performed in IBM SPSS Statistics 19 (IBM Corporation, www.ibm.com) and data are presented as mean ± S.E if not stated otherwise.

RESULTS

CAROTENOID PIGMENTATION

Carotenoid-based pigmentation, expressed as spots cm^{-2}, among the eight different areas were strongly positively correlated (Spearman correlation, all *P*-values<0.01; Table I). There was no difference between the size categories in the aggragate carotenoid-based pigmentation.

Table I. The Spearman correlation coefficients for number of spots cm−2 in each area and an aggregate for all the areas (areas 1–8), and the mean±s.e. for each area and the aggregate

Area	1	2	3	4	5	6	7	8	1–8	Spots cm^{-2}
1	1.00									0.74±0.15
2	0.69**	1.00								2.47±0.27
3	0.58**	0.73**	1.00							3.64±0.30
4	0.52**	0.67**	0.79**	1.00						4.21±0.41
5	0.45**	0.55**	0.49**	0.62**	1.00					2.18±0.14
6	0.42**	0.57**	0.70**	0.65**	0.71**	1.00				2.70±0.15
7	0.45**	0.62**	0.72**	0.74**	0.67**	0.80**	1.00			1.79±0.13
8	0.38**	0.41**	0.37**	0.66**	0.53**	0.44**	0.57**	1.00		2.36±0.26
1–8	0.64**	0.81**	0.84**	0.93**	0.74**	0.78**	0.84**	0.69**	1.00	2.51±0.18

**Significant at 0.01 level (Spearman correlation).

CLASSIFICATION OF DIFFERENT BEHAVIOURAL TYPES

Activity, based on number of movements between the dark and light compartments was used to classify behavioural types. This resulted in one high-activity group (6.46 ± 0.83 nr, n=13) and one low-activity group (1.09 ± 0.11 nr, n=47; Mann-Whitney U-test, $P<0.001$).

BEHAVIOUR, PHYSIOLOGY AND CAROTENOID-BASED PIGMENTATION IN HIGH ACTIVITY AND LOW ACTIVITY INDIVIDUALS

The two different classes based on activity were significantly different in behaviour, physiology, and pigmentation (Fig. 2). High-activity individuals had a lower latency to enter an open field area (Mann-Whitney U-test = 439.0, $P<0.05$) and higher plasma cortisol levels (Mann-Whitney U-test =185.0, $P<0.05$) than low-activity individuals. Further, high-activity individuals also had a significantly higher number of spots cm^{-2} (Mann-Whitney U-test =189.0, $P<0.05$) than low-activity individuals, and this difference was emphasized in area 2 (Mann-Whitney U-test =131.0, $P<0.01$).

The value of K ranged from 1.43 to 2.18, with no difference between high-activity and low-activity individuals within the size categories. The different size classes, however, differed in K (Kruskal-Wallis =10.682, d.f =2, $P<0.01$). The large individuals had higher K than both small (Kruskal-Wallis pair-wise comparison = - 15.721, $P<0.05$) and medium individuals (Kruskal-Wallis pair-wise comparison = -14.493, $P<0.05$).

DISCUSSION

BEHAVIOURAL CLASSIFICATION

In this study, *S. alpinus* were classified based on exploratory behaviour, thus creating groups of high- and low- activity individuals. These high- and low-activity individuals differed in behaviour, plasma cortisol and carotenoid skin pigmentation (*i.e.* number of spots). Behavioural differences between high- and low-activity individuals consisted of both exploration and latency to enter a novel area. This suggests that the behaviours are linked. Five different axes including shyness-boldness, exploration-avoidance, activity, sociability and aggressiveness, were suggested by Réale *et al.* (2007) that could be used for temperament, personality and behavioural syndrome studies. Using this terminology, latency to enter a novel area could be exploring. Swimming between compartments could also be exploring. A high general activity, however, could also lead to a shorter latency to enter a novel area as well as more swimming between compartments. In either case, behaviour could be used to determine at least one of these behavioural aspects of temperament, personality, and behavioural syndrome in individual fishes.

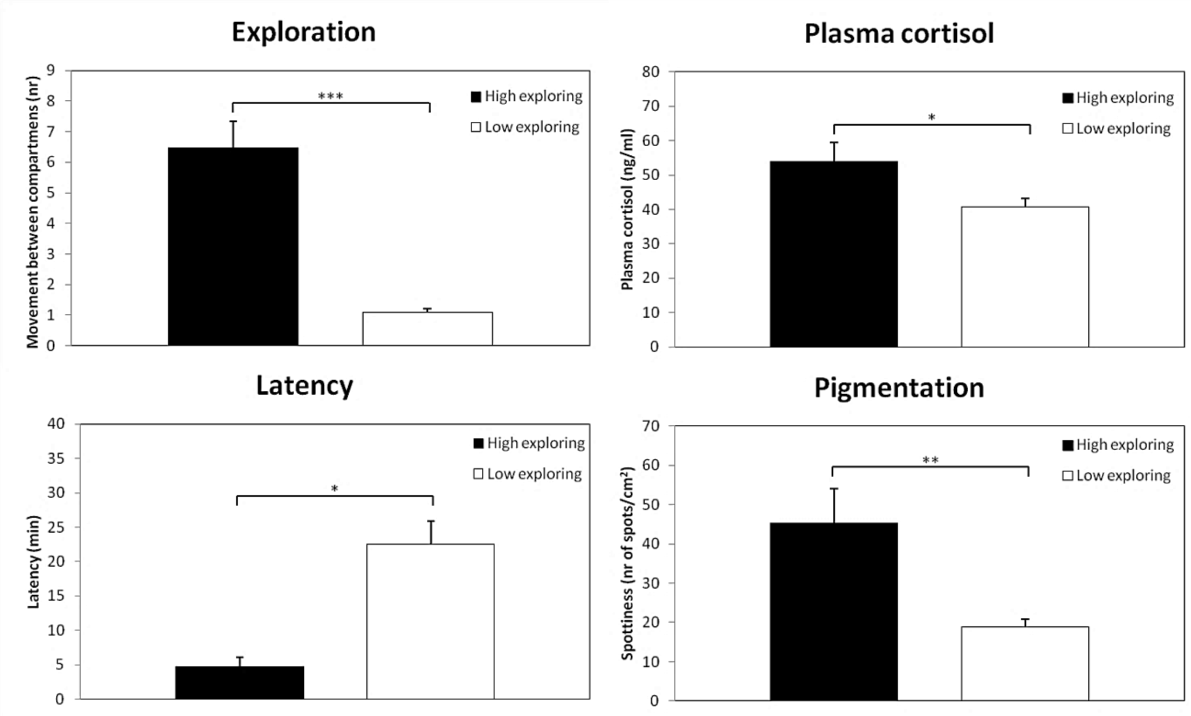

Fig. 2. The effect of classification into high-activity (*n* = 13) and low-activity (*n* = 47) individuals upon (a) exploration (movements), (b) latency to enter novel area, (c) plasma cortisol and (d) pigmenta- tion. Values are mean ± s.e. and asterisks indicate difference between high exploring and low exploring individuals (**P* < 0·05; ***P* < 0·01; ****P* < 0·001; Mann – Whitney *U* -test).

STRESS COPING STYLES

Behaviour coupled to stress responsiveness in animals has been termed stress-coping styles (Koolhaas *et al.*, 1999). In this study, a connection between behaviour and stress responsiveness was shown, with high-activity individuals having higher plasma cortisol levels following stress than low-activity individuals. Generally, high stress responsiveness is indicating a reactive stress coping style, *i.e.* individuals being less aggressive and less active (Koolhaas *et al.*, 1999). Previous studies in both *S. salar* and HR and LR *O. mykiss* have shown that high stress responsiveness leads to lower activity during acute stress (Kittilsen *et al.*, 2009; Backström *et al.*, 2011). In this study behaviour was not monitored during acute stress because *S. alpinus* had been acclimatized for 1 day before the exploration test. In an earlier study, LR *O. mykiss* were more active immediately after exposure to an open field test (during the first 3–5 min) and then settled down, whereas HR *O. mykiss* appeared to remain active (Schjolden *et al.*, 2005b). In this study, as the exploratory experiment was not an acute stressor, it appears likely that high-activity individuals were individuals remaining active over a longer time frame. Thus, it seems as high- and low-activity individuals appear to fit well with reactive and proactive coping style, respectively.

CAROTENOID-BASED PIGMENTATION

In this study, the carotenoid pigmentation appeared to be regularly distributed within each individual and a specific area could be used to predict the general pigmentation. Carotenoids are not synthesized in animals so they have to be obtained from diet. Ingested carotenoids have several functions including antioxidant activity, being the vitamin A precursor, and ornamental coloration (Svensson & Wong, 2011). The ornamental colouration of carotenoids as a signalling mechanism has been studied extensively (Svensson & Wong, 2011), and most of it has been concerned with sexual signalling. There have also been studies establishing a link between carotenoid pigmentation and physical prowess. For instance, in male *P. reticulata*, carotenoid pigmentation is positively correlated to swimming performance (Nicoletto, 1991). Male firemouth cichlids *Cichlasoma meeki* Brind 1918 fed a high carotenoid diet won more contest compared to males fed a low carotenoid diet (Evans & Norris, 1996). Similarly, in a dyadic contest between male three-spined stickleback *Gasterosteus aculeatus* L. 1758, losers with higher carotenoid levels than winners took a longer time to lose contests (Guderley & Couture, 2005). In zebra finches *Taeniopygia guttata*, carotenoid-supplemented diet enhanced flight performance (Blount & Matheson, 2006). All of these studies indicate that carotenoid coloration is coupled to better physical performance. Thus, the result that high activity individuals have more carotenoid spots than low activity individuals indicates that they have a better physical status. Additionally, it is worth noting that in this study *S. alpinus* were all fed the same diet, and thus there should be no difference in dietary provision of carotenoids.

CAROTENOIDS, STRESS COPING STYLES AND IMMUNE SYSTEM

In *S. salar* and *O. mykiss*, melanin skin pigmentation has been connected to stress responsiveness (Kittilsen *et al.*, 2009). In this study, a similar connection was noted between carotenoid skin pigmentation and stress responsiveness as well as behaviour, or stress coping style. Thus, it appears that carotenoid pigmentation in *S. alpinus* could be linked to stress-coping style. Furthermore, proactive juvenile *S. salar* have a higher number of melanin skin pigmentation and also harboured fewer female sea lice *Lepeophtheirus salmonis* carrying egg sacs than reactive *S. salar* with a low number of melanin skin pigmentation (Kittilsen *et al.*, 2012). Thus, a connection between immune system and stress-coping style appears to be evident by pigmentation analysis. As carotenoid involvement in immune system is similar to melanin in some ways (Griffith *et al.*, 2006), the observed differences in this study could possibly be linked to the immune system as well. This remains to be investigated.

Carotenoid pigmentation in *S. alpinus* could indicate more dietary carotenoid intake. All *S. alpinus*, however, were fed the same feed and K did not differ between high- and low-activity individuals. Therefore, the differences in pigmentation could either be differences in health status or some underlying difference in carotenoid metabolism. Specific tests for health status were not performed, but all individuals appeared healthy by visual inspection and their values of K supported this. Thus, following the same reasoning as for *S. salar*, where high pigmentation and low stress reaction indicated a better immune response, it is likely that individuals in this study with fewer pigment spots would have a better immune response. This implies a difference in usage of dietary intake of carotenoids, with some *S. alpinus* using more for the immune system whereas others use more for skin pigmentation.

In conclusion, this study indicates that behaviour and stress responsiveness are correlated in *S. alpinus*, indicating different stress coping styles. The different stress coping styles also appear to be linked to carotenoid-based pigmentation in *S. alpinus*. These interesting results warrant further investigation to clarify the underlying mechanisms driving this connection between behaviour and physiology.

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