Received: 5 October 2020 | Accepted: 25 November 2020
DOI: 10.1111/jfb.14633

BRIEF COMMUNICATION

JOURNAL OF FISH BIOLOGY fsbi

Proof of concept: visual categorization of carotenoid pigmentation in Arctic charr (*Salvelinus alpinus* L) can predict stress response

Tobias Backström[1] | Eva Brännäs[2] | Jan Nilsson[2] | Hanna Carlberg[2] | Kajsa Johansson[2] | Carin Magnhagen[2]

[1]Institute of Integrated Natural Sciences, University Koblenz-Landau, Koblenz, Universitätsstraße 1, Koblenz, Germany

[2]Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, Umeå, Sweden

Correspondence
Tobias Backström, Institute of Integrated Natural Sciences, University Koblenz-Landau, Koblenz, Universitätsstraße 1, DE-56070 Koblenz, Germany.
Email: tbackstrom@uni-koblenz.de

Funding information
This study was financed by the Swedish Research Council Formas (Dnr 2011-888).

Abstract

Carotenoid pigmentation in *Salvelinus alpinus* has been connected to stress responsiveness in earlier studies. This has, however, only been tested with time-consuming image analysis from photos. Here, we used quick visual categorization of carotenoid pigmentation to investigate the stress responsiveness of the extreme groups. The visually selected charr were then exposed to a net restraint stressor. Arctic charr with few spots also had a lower stress responsiveness compared to charr with many spots. Thus, visual selection could be used as a simple method within aquaculture.

KEYWORDS
aquaculture, Arctic superior, carotenoid, net restraint stressor, pigmentation

Stress and stress-related behaviour are problematic in farmed animals since they could lead to lowered productivity and reduced welfare (*The EFSA Journal*, 2009). Therefore, studies concerning management methods to reduce stress are of both financial and ethical interest. This includes studies of fish in aquaculture and social stress. The general stress response of teleost fish is well studied (Wendelaar Bonga, 1997), and teleost fish (as other vertebrates) typically vary within species and populations in their stress responsiveness (Conrad *et al*., 2011; Øverli *et al*., 2005; Schjolden & Winberg, 2007). Stress responsiveness also impacts several other health and welfare issues in fish (Sneddon *et al*., 2016). For instance, in Nile tilapia (*Oreochromis niloticus* L.) it has been shown that males with lower stress responsiveness had higher sperm motility and sperm density than males with higher stress responsiveness (Manliclic & Vera Cruz, 2017), which may affect productivity. Animal pigmentation is often an indication of various aspects such as body condition, health and immune system [reviewed by Ducrest *et al*. (2008) and Svensson and Wong (2011)], and could potentially be used to identify individual state in any of these aspects. In several earlier studies we found a correlation between carotenoid-based pigmentation and stress responsiveness in Arctic charr (*Salvelinus alpinus* L), for instance *Salvelinus alpinus* with lower stress responsiveness had fewer carotenoid spots compared to charr with higher stress responsiveness (Backström *et al*., 2014). *Salvelinus alpinus* is a territorial fish at the juvenile stage and thus forms social hierarchies. When size-matched in pairs for agonistic interactions, the dominant charrs had fewer spots and lower plasma cortisol levels (stress responsiveness) than the subordinant charrs (Backström *et al*., 2015a). The carotenoid pigmentation has also shown to be heritable in a study with over 100 families of *Salvelinus alpinus* (Nilsson *et al*., 2016).

Of further interest is that the carotenoid spots seem to be dynamic and thereby quickly change during stress. Specifically, the spots appear to change within 1 h of agonistic interactions (Backström *et al*., 2015a), and anaesthetic treatments appeared to induce spots within 2 min (Backström *et al*., 2015b). These changes seem to be more dynamic on the right side in comparison to the left side during a net restraint stressor, where the ratio of number of spots after/before test ranged from 0.9 to 1.2 on the left side compared to 0.6 to 2.3 on the right side (Backström *et al*., 2016).

Thus, it seems that it could be possible to utilize carotenoid pigmentation as a tool for selection of *Salvelinus alpinus* with diverging stress responsiveness. Earlier studies analysed spottiness using photographs, which is too time-consuming to be used in breeding programs of *Salvelinus alpinus*. Here, we test whether a simple visual assessment of pigmentation could be used to categorize individuals according to stress response. This would provide a more practical method to use within aquaculture. Therefore, we investigated if *Salvelinus alpinus* visually divided into two extremes related to carotenoid spottiness would differ in response to a net restraint stressor test. Specifically, one hypothesis was tested: visual selection based on carotenoid number of spots could predict stress responsiveness (proof of concept). We evaluated behaviour during a net restraint stressor, measuring cortisol and carotenoid spots after the stressor.

This study was carried out at the Aquaculture Centre North in Kälarne, Sweden on 19 May 2014 using *Salvelinus alpinus* from the seventh generation of the Swedish Arctic charr breeding programme [Arctic superior, for details on the programme see Nilsson *et al*. (2010)]. The 1-year-old juvenile *Salvelinus alpinus* were bred and kept in the stocking facilities in tanks (10 m^3). Tanks were supplied with running water at natural temperatures (5–6°C) from the nearby lake Ansjön and kept under a photoperiod period of 12 h dark/12 h light. The *Salvelinus alpinus* were fed continuously by automatic feeders. The *Salvelinus alpinus* used in this study had a fork length (L_F) of 21.2 ± 1.5 cm (mean ± S.D., n = 32) and a body mass of 125.0 ± 26.9 g (mean ± S.D., n = 32).

One week prior to the experiment, *Salvelinus alpinus* were selected based on carotenoid pigmentation on the left side, where the pigmentation has been found to be more stable than on the right side (Backström *et al*., 2016). Selection was done by two observers that estimated the number of spots on a three-scaled grade (few, intermediate and many). Fish that were judged to have few or many spots (the extremes, see Figure 1 for an example) by both observers were transferred to two separate tanks (1 m^3) until the experiment started (n = 20 per tank).

FIGURE 1 Illustration of the difference in pigmentation between *Salvelinus alpinus* with few spots (upper photograph) and many spots (lower photograph)

At the start of the experiment, one fish from each tank (few or many spots) was randomly selected to be stressed simultaneously. The stressor was net restraint followed by confinement, with some modifications, as described by Magnhagen *et al*. (2015). Briefly, the fish were filmed during the 1 min net restraint stressor (see below) and were then confined in buckets (10 l) for 30 min between 17.30 and 20.00. After confinement fish were photographed on both the right and left sides using a digital camera (Canon EOS 500D, Tokyo, Japan) as described earlier (Backström *et al*., 2015a). The fish were then anaesthetised using tricaine methanesulfonate (0.15 g l^{-1}), weighed, measured, sampled for blood *via* the caudal vein and then sacrificed. Gender was assigned by ocular inspection of gonads. Subsequently, the blood was centrifuged at 10000*g* for 5 min, and the plasma was collected and stored until further analysis at −20°C.

Plasma cortisol was analysed with a commercial enzyme-linked immunosorbent assay (ELISA) kit, used according to the manufacturer's instructions (product # 402710, Neogen Corporation, Lexington, USA). In brief, plasma was extracted and vortexed 1:5 in ethyl acetate. The solvent was decanted, evaporated in a vacuum concentrator and dissolved in the extraction buffer included in the ELISA kit. Cross-reactivity 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 duplicate during a single assay with an intra-assay coefficient of variation of 0.87%.

Photographs of each side of the individuals were manually analysed for carotenoid-based pigmentation in a 2 × 10 cm rectangle [see Backström *et al*. (2015a)]. Unaware of grouping, an observer counted all photographs at three separate times. The mean score per photograph was used.

The percentage of time trying to escape from the net, struggling, was analysed from the video recordings from the net restraint stressor played at half speed. The analysis was done by an observer unaware of groupings.

All statistics were performed in the graphical user interface R Commander for the free software R (Fox, 2005) or IBM SPSS Statistics 20 (IBM Corporation, www.ibm.com). Two-tailed unpaired t-tests were used to analyse if fork length and body mass differed between groups. Gender differences between groups were tested with χ^2-test. One tailed paired *t*-tests were used to analyse differences between pairs (that were tested simultaneously) in number of spots, struggling and plasma cortisol based on our visual selection of spottiness and assumption that fish with fewer spots would be less stressed, and hence have a lower struggling activity and plasma cortisol levels. We did not get any blood from one fish and therefore only have plasma cortisol data for 31 fish. Data are presented as mean ± S.D. if not stated otherwise. Fork length (*t*-test, P = 0.136; few spots 20.8 ± 1.2 cm, n = 16; many spots 21.6 ± 1.8, n = 16), body mass (*t*-test, P = 0.311; few spots 120.1 ± 23.9 g, n = 16; many spots 130.0 ± 29.8, n = 16) and gender (χ^2 test, P = 0.710; few spots 10 males, 6 females; many spots 11 males, 5 females) did not differ between groups. The methods used here were approved by the Umeå Animal Research Ethical Committee.

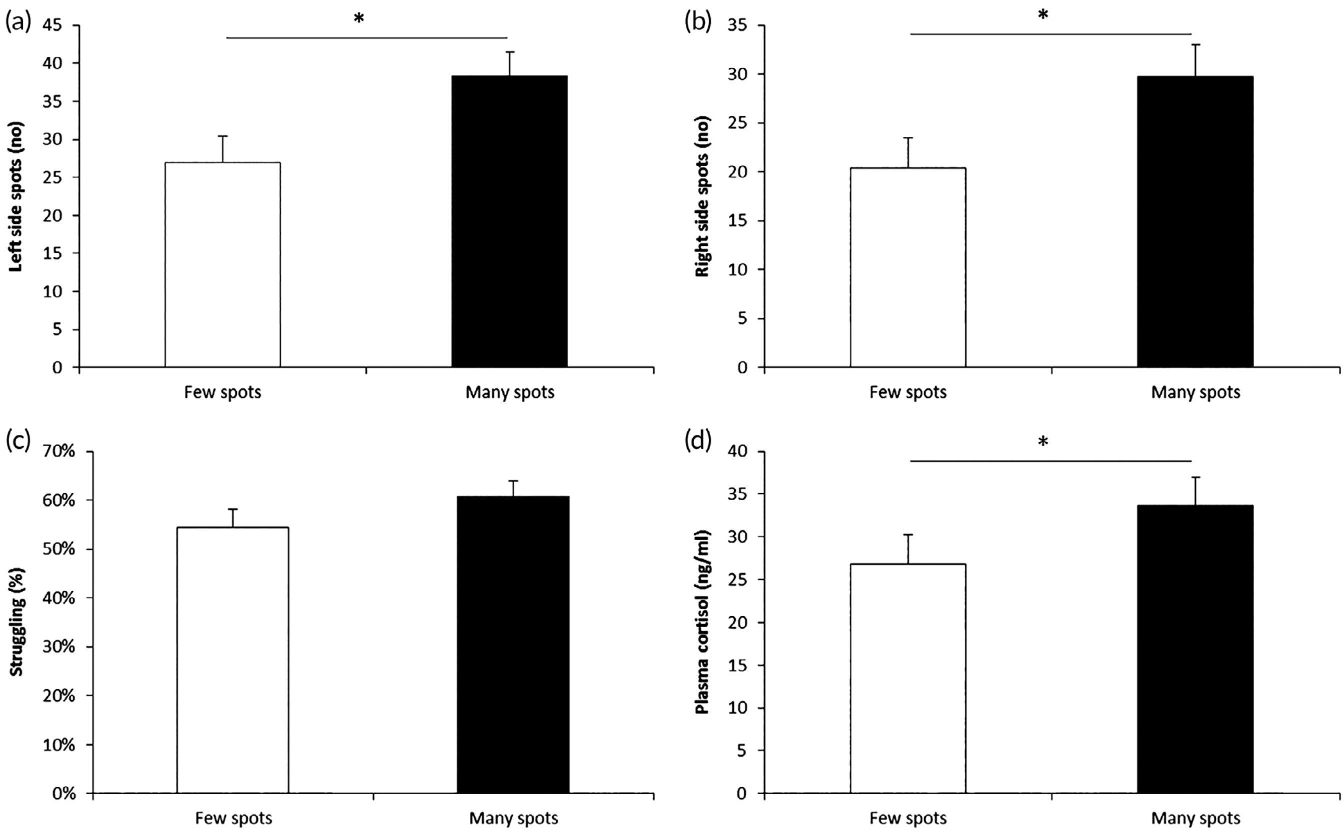

FIGURE 2 Measurements in the few spots and many spots groups of *Salvelinus alpinus* subjected to a net restraint test. (a) Number of spots on the left side after net restraint. (b) Number of spots on the right side after net restraint. (c) Percentage of time struggling during net restraint. (d) Plasma cortisol levels 30 min after net restraint. Values are mean ± S.E.M. and asterisks indicate a difference between the groups (one-tailed paired *t*-test, $P < 0.05$)

The groups differed in the number of carotenoid spots, with the group few spots having a lower number of spots on both the left (one-tailed paired *t*-test, $P = 0.004$; few spots 26.9 ± 14.0, $n = 16$; many spots 38.4 ± 12.4, $n = 16$) and right (one-tailed paired *t*-test, $P = 0.041$; few spots 20.4 ± 12.4, $n = 16$; many spots 29.8 ± 12.9, $n = 16$) sides compared to the group with many spots (see Figure 2a and b).

Fish tried to escape by struggling vigorously during the net restraint stressor. There was no statistical difference in struggling between the groups few and many spots (one-tailed paired *t*-test, $P = 0.109$; Figure 2c; few spots 54.5 ± 14.8%, $n = 16$; many spots 60.7 ± 13.0%, $n = 16$). However, plasma cortisol levels after the net restraint stressor differed (one-tailed paired *t*-test, $P = 0.047$; Figure 2d; few spots 26.9 ± 13.0 ng ml^{-1}, $n = 15$; many spots 33.7 ± 13.1 ng ml^{-1}, $n = 16$), with the group few spots having lower levels than the fish with many spots.

In this study we provide proof of the concept that visual selection based on carotenoid pigmentation can predict stress responsiveness in *Salvelinus alpinus*. While an overlap in number of spots after the test appears to exist, this is largely attributed to group selection being based on whole fish (Figure 1) while statistical quantification was done using a subsection of the fish. This further strengthens earlier research showing correlation between carotenoid spots and stress responsiveness in *Salvelinus alpinus* (Backström *et al*., 2014, 2015a, b, 2015c, 2016, 2017). Taken together with the reports concerning the heritability of the pigmentation (Nilsson *et al*., 2016) as well as the stability over time within individuals (Brännäs *et al*., 2016), this means that the visual selection of carotenoid pigmentation could be used as a quick and simple method within aquaculture to select for stress responsiveness (with the aim of decreasing stress in farmed fish).

We could not see any differences in behaviour during the net restraint stressor between the two groups. This was somewhat surprising based on an earlier study showing a negative correlation between plasma cortisol and struggling in *Salvelinus alpinus* (Magnhagen *et al*., 2015). This difference could be attributed to methodological differences between the studies. Magnhagen *et al*. (2015) used a 30 min confinement stressor in a smaller container directly following the net restraint stressor, while we used several buckets to keep track of the fish and had no intent to stress them further. The mean for plasma cortisol was also somewhat higher in Magnhagen *et al*. (2015).

In conclusion, in this study we show visual selection based on carotenoid pigmentation in *Salvelinus alpinus* does indicate stress responsiveness. Thus this could be used as a simple method within aquaculture to select divergent stress responders. However, pigmentation/stress response connections may be species specific and should be investigated for each species of interest. Further research is also needed to explore the long-term effects of this selection.

ACKNOWLEDGEMENTS

The experimental methods used in this study were approved by the Swedish University of Agricultural Sciences Ethical Committee, and it was financed by the Swedish Research Council Formas (Dnr 2011-888).

CONTRIBUTIONS

TB, EB, JN and CM planned the research. TB, EB, JN, HC and CM performed the experiment. KJ analysed images and CM analysed behaviour. TB and CM analysed data and wrote the paper. All authors contributed significantly to the manuscript and gave final approval for publication.

ACKNOWLEDGEMENTS

Open access funding enabled and organized by Projekt DEAL.

ORCID

Tobias Backström https://orcid.org/0000-0003-1984-9533

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How to cite this article: Backström T, Brännäs E, Nilsson J, Carlberg H, Johansson K, Magnhagen C. Proof of concept: visual categorization of carotenoid pigmentation in Arctic charr (*Salvelinus alpinus* L) can predict stress response. *J Fish Biol*. 2021;98:1192–1195. https://doi.org/10.1111/jfb.14633