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Behavioural and physiological responses of lumpfish (*Cyclopterus lumpus*) exposed to Atlantic salmon (*Salmo salar*) sensory cues

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Abstract

First interaction with carnivorous clients induces stress responses even in the boldest of cleaner fishes observed in nature. This is relevant for the expanding use of lumpfish in aquaculture, where stress could impede the species ability to interact with Atlantic salmon. The study investigated how naive lumpfish (34.3 g, S.D. ± 6.48) responded to different heterospecific cues including (1) exposure to water from a tank with Atlantic salmon ("Olfaction"), (2) salmon lifelike models ("Model") and (3) Atlantic salmon ("Live"). Experiments were repeated thrice, using duplicate tank replicates on each occasion (n = 36 per treatment). Behaviour was recorded 30 min before and 30 min after the introduction of each treatment. Responses measured included swimming activity, body colour and pigmentation, neurotransmitters, and plasma cortisol. Data demonstrated a significant increase in swimming activity upon introduction of Olfaction and Live salmon, but not from Models. After 30 min of interaction, swimming activity decreased toward levels observed in control groups. Body colour significantly increased in lumpfish exposed to Olfaction while body pigmentation significantly increased in both Olfaction and Model treatments. Neurotransmitters and plasma cortisol measurements did not differ between treatments and control, yet large individual variation was observed. Our findings revealed that lumpfish discriminated salmon cues,

whereas Olfaction induced the overall strongest behavioural and physiological responses. The study suggested that acute change in skin colour and pigmentation can be impacted by salmon interaction, yet deployment of naive juvenile lumpfish with small Atlantic salmon is preferable based on the overall mild stress responses.

Keywords

Lumpfish$_1$, Atlantic salmon$_2$, Sensory cues$_3$, Cleaner fish$_4$, Aquaculture$_5$.

1 Introduction

The use of cleaner fish is one of several strategies applied to combat ectoparasitic infestations in Atlantic salmon (*Salmo salar*) aquaculture (Blanco Gonzalez and de Boer, 2017; Powell et al., 2017). Infestations are primarily caused by the two sea lice copepods *Lepeophtheirus salmonis* and *Caligus elongatus* which have turmoiled the health and welfare status of farmed Atlantic salmon since the seventies (Brandal and Egidius, 1977). Additional consequences include the spread of sea lice into wild populations of salmonids and increased production costs (Costello, 2009; Pike, 1989; Torrissen et al., 2013). With an annual spend of more than 60 million cleaner fish in Norwegian aquaculture only (Norwegian Directorate of Fisheries, 2020), focus has shifted towards both welfare and ethical questions regarding the use of live animals for sea lice removal. For future use of cleaner fish, it has become crucial to unravel mortality causations, stress factors and health challenges (Geitung et al., 2020; Imsland et al., 2020; Overton et al., 2020). The lumpfish (*Cyclopterus lumpus*) is endemic to the North Atlantic Ocean and has a semi-pelagic strategy where juveniles hatch and live in the benthic zone prior to migration to offshore feeding areas (Davenport and Kjørsvik, 1986; Holst, 1993). Later, mature fish return inshore to spawn (Mitamura et al., 2012).

When used in aquaculture, the species graze ectoparasites in addition to other food items including pellets, crustaceans, and hydrozoans (Eliasen et al., 2018; Imsland et al., 2015). About 13 to 36 % of lumpfish are typically observed with sea lice in their stomach, which have been demonstrated to be sufficient to supress parasitic outbreaks in commercial scale salmon production (Eliasen et al., 2018; Imsland et al., 2018). Hatchery production of juvenile individuals and subsequent deployment into commercial-scale aquaculture with farmed salmon raise questions on how lumpfish cope with the transition regarding adaptation, stress, interspecific behaviour, habituation, and motivation to graze sea lice from a larger carnivorous fish. In comparison with other species of cleaner fish, observations of Bluestreak cleaner wrasse (*Labroides dimidiatus*) revealed that traits involving escape performance were

still present during early ontogeny, which elucidates the importance of predator caution even in obligate mutualistic cleaners (Gingins et al., 2017). A similar study on acute stress responses in lumpfish introduced to farmed Atlantic salmon resulted in comparable findings, where naive groups expressed increased swimming activity, predator avoidance and elevated cortisol concentrations in addition to strong indications of habituation after one month of duoculture with farmed Atlantic salmon (Staven et al., 2019). To further investigate innate threat detection in lumpfish towards Atlantic salmon, it was deemed necessary to separate the exposure to different salmon cues. Both olfaction and visual detection of a carnivorous and potential predator fish, are well known provokers of stress response in prey fish (Barcellos et al., 2007; Fischer et al., 2017; Lönnstedt and McCormick, 2011; McCormick and Manassa, 2008). Therefore, it has been suggested to study the gradual implementation of sensory cues and their possible functions for habituation of lumpfish prior to deployment at sea. While studying how lumpfish respond to Atlantic salmon predator sensory cues, it was considered important to implement measurements of neurotransmitters recently found to affect social interaction between cleaner fish and clients (de Abreu et al., 2020; Soares et al., 2016, 2017). When the cleaner fish Bluestreak cleaner wrasse faces different social and mutualistic contexts, changes in the activity of monoamine neurotransmitters occur. This include serotonin (5-hydroxytryptamine, 5-HT), which is a suggested regulator of behaviour linked to physical contact with clients, and dopamine (3,4-dihydroxyphenethylamine, DA), observed to affect cleaning engagement (de Abreu et al., 2018). Monoamine neurotransmitters are also involved in other functions in the vertebrate brain including stress coping (Backström and Winberg, 2017). The potential for further breeding programs on lumpfish could benefit from an elaboration of the function of these neurotransmitters during interaction with Atlantic salmon, whether as involved in stress responses (de Abreu et al., 2020; Gesto et al., 2013; Winberg et al., 1997) or cleaning behaviour (Paula et al., 2015; Soares et al., 2016). Lumpfish also express various colour and pigmented patterns during larval and juvenile stages and have previously been observed to regulate body pigmentation within minutes when background colours were switched from dark to light (Davenport and Thorsteinsson, 1989). In fish, the skin darkening, and colour change is related to mechanics of intracellular transport of pigment organelles in chromatophores (Nilsson Sköld et al., 2013). The regulative component for blue-green colouration in lumpfish is the antioxidant biliverdin, a metabolic breakdown product of haemoglobin (Davenport and Bradshaw, 1995; Mudge and Davenport, 1986). Skin darkening, and colour change have been observed in other aquaculture species when exposed to

production stress and could act as a potential welfare indicator for lumpfish used in salmon aquaculture (Ruane et al., 2005; Van der Salm et al., 2004; Van der Salm et al, 2006).

The study investigated the extent of the acute responses in naive lumpfish exposed to Atlantic salmon or salmon sensory cues. Treatments involved salmon Olfaction, salmon Models or Live Atlantic salmon as a positive control, in order to try to identify the most important sensory cues behind the physiological and behavioural response of naive lumpfish to the first encounter with their potential clients. We examined if treatment exposure influenced behavioural and physiological responses encompassing (1) swimming activity, (2) telencephalic neurotransmitters, (3) plasma cortisol levels and (4) body colour and pigmentation after the introduction of the different treatments.

2 Material and Methods

2.1 Ethical statement

Lumpfish and Atlantic salmon were handled with care based on the Norwegian law on Regulation of Animal Experimentation (FOR-1996-01-15-23). Research animals were accepted for experimental use by the Norwegian Food Safety Authority (FDU #17231). Personnel were certified with FELASA-C, developed by the Federation of European Laboratory Animal Science Association.

2.2 Research animals

2.2.1 Lumpfish

Milt and roe were collected from wild mature lumpfish captured in Troms and Finnmark county, Norway. From February 2018, lumpfish eggs were fertilized, hatched, and reared at Mørkvedbukta AS next to Mørkvedbukta Research Station, Nord University, Bodø, Norway. During the first two months, lumpfish were fed with Gemma Micro 150 and 300 (Skretting, Stavanger, Norway) and Gemma Wean Diamond, 0.5 mm (Skretting, Stavanger, Norway). In the next months and until departure from the hatchery to the research facilities in September, pellet size gradually increased from Gemma Diamond 0.8 mm, 1.5 mm, Silk 1.5 mm and 1.8 mm (Skretting, Stavanger, Norway) following feeding recommendations from Skretting AS. Rearing conditions showed daily monitored oxygen saturation from 90-100 % and a mean water temperature of 7.5 °C. All lumpfish were vaccinated with AMarine micro 4-2® (Pharmaq, Overhalla, Norway) and given 300-day degrees immunization before they were

transferred to Mørkvedbukta Research Station, Nord University, Norway. Light regime during rearing was 24:0 (summer signal).

2.2.2 Atlantic salmon

All domesticated Atlantic salmon originated from the Aquagen strain, hatched in March 2017, and later transferred to Mørkvedbukta Research Station. During rearing, fish were automatically fed (Arvo-Tec Oy, Finland) with Gemma diamond 150 (Skretting, Stavanger, Norway) three times daily for a daily total amount of 2 % biomass. Light regime during rearing was 24:0. Individuals randomly selected to the experiment (n = 24), had no visual or external deviations from good health.

2.3 Experimental preparation

Neither Atlantic salmon nor lumpfish had a history of interactions with other species prior to the experiment. At the research station, naive juvenile lumpfish and Atlantic salmon were acclimated to experimental conditions for four weeks in separate tanks (2 x 2 x 1 m) adjacent to the experimental room. Water flow was set to 500 l h^{-1}. Mean ± SD temperature and dissolved oxygen levels during the experiment were 8.5 ± 0.22 ˚C and 8.4 ± 0.31 mg l^{-1} O_2, respectively. Four days prior to experiments, lumpfish were sedated with 10.0 ml l^{-1} Benzoak Vet (ACD Pharmaceuticals, Leknes, Norway), tagged with Floy tag t-bars (Floy Tag and Mfg Inc, Seattle, USA) and photographed (see 2.6.1 for description). Each tag had a distinct colour so individual fish could be recognized during video recordings. The light regiment was 24:0. Automatic feeding continued during the research period as before with a 2 % biomass spread across daytime (08:00-16:00). Fish were kept undisturbed in the experimental tanks during the acclimation and experimental period.

2.4 Study design

A total number of 144 juvenile lumpfish with a mean ± SD weight of 34.3 ± 6.48 g were used in the experiment. Lumpfish were distributed in eight grey tank units (1 x 1 x 1 m) at a stocking density of six fish per tank. Experiments were repeated thrice with duplicated tanks on each occasion, giving n = 36 lumpfish for each treatment. Treatments were either (1) the introduction of two Atlantic salmon (184.1 ± 13.45 g), (2) two salmon Models type *3D Line Thru 15 cm* from Savage Gear ™ (same size as live fish, attached and moving with the water current in circles), (3) Atlantic salmon Olfaction (water connected to the flow meter from an adjacent tank with two Atlantic salmon) or (4) control tanks with no treatment. The tank visual shield lid was opened for both control and Olfaction groups, mirroring the introduction

of either Model or Live salmon in the other treatments. Each tank experiment was video recorded with a remotely started GoPro Hero 3^+ cameras (GoPro™, California, USA) placed centrally, 50 cm above each tank. The duration of the video was 1 h, from 30 min before to 30 min after the start of the treatment. After one hour of exposure to treatments, water flow was stopped and 5 mg l^{-1} Aquacalm™ (Western Chemical inc, Canada), containing metomidate hydrochloride, a blocker of cortisol synthesis, was added to the tank.

2.5 Data analysis

2.5.1 Behavioural data

Video recordings from experimental tanks with salmon Olfaction treatment and controls were analysed blindly (without knowing which treatment was applied), while recordings from tanks with salmon or Model fish treatments could not be analysed in such matters without acknowledging the specific treatment. Behavioural data included categorization of lumpfish swimming activity based on previous work (Imsland et al., 2014; Tully et al., 1996). During 60 min of continuous video recordings in each tank, individual lumpfish were identified and localized whereas their swimming activities were registered once every minute e.g., "1 min" corresponds to behaviour observed at the 60th second of the recording, "2 min" corresponds to behaviour observed at 120th second continuing until "60 min". The four distinguishable swimming activities were "attached", "hovering", "normal swimming" and "burst swimming" (Table 1). Distinguishable activities provided a rank score which were used to calculate and compare swimming activity among treatments and control (Staven et al., 2019).

2.5.2 Physiological data

Blood and brains were sampled within 5 min after the experiment was terminated. Blood was collected from the heart ventricle with 0.33 x 12.7 mm syringes (BD Micro-fine®) containing anticoagulating heparin and centrifuged at 6000 RPM (2000 x g) for 10 min in a Mini Star centrifuge (VWR™, UK). After centrifugation, plasma was separated with a pipette, transferred to a 1.8 ml Nunc Cryo Tube® and stored at -40 °C. Samples were later analysed for plasma cortisol measurements using Radioimmunoassay (Iversen et al., 1998). After blood sampling, fish were euthanized by spinal transection before the brain and the telencephalon was dissected out and stored on dry ice based on the method reported in Gesto et al., (2013). Concentrations of the neurotransmitters dopamine (DA), 3,4-dihydroxyphenylacetic acid, (DOPAC), serotonin (5-hydroxytryptamine, 5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were later quantified in the telencephalon by means of HPLC with electrochemical detection,

as previously described (Gesto et al., 2017). All lumpfish were juveniles, and with minimal gonadal development, sex was not determined.

2.6 Skin colour and pigmentation

2.6.1 Skin colour

To determine changes in skin pigmentation and colour intensity, each lumpfish was photographed four days before the experiment during tagging and photographed again after termination of the experiment. Lumpfish were positioned sideways in a white photo box next to a standard piece of blue tape for later colour corrections. The camera setup included a Canon 550d SLR camera (Canon, Tokyo, Japan), a Canon 20mm f/2.8 lens and an external Amaran Halo macro ring flashlight ™. Settings were pre-set with fixed ISO-800, 1/800 shutter speed and 5200 Kelvin and images were stored in RAW format. Images were analysed using ImageJ version 1.53e (NIH, Bethesda, MD, available at www.imagej.nih.gov/ij). A line was drawn around a lateral area where skin was most homogenous and without pigmentation (Fig. 1). The histogram function was used to determine the mean density values for the three primary colours red, green, and blue (mean R+G+B) within the defined area of the lumpfish skin. The method was repeated for the blue tape whereas the mean R+G+B values of the tape from all images were used for correction of the values for each lumpfish. Based on the Spearman's rank correlation between RGB for left and right side of each lumpfish (red = $P < 0.001$, Rs = 0.83, green = $P < 0.001$, Rs = 0.66, blue = $P < 0.001$, Rs = 0.47, n = 144), it was decided to use only the right lateral side.

2.6.2 Skin pigmentation

To quantify skin pigmentation, a line was drawn around the area of the whole fish before images were converted to 8 bit and the threshold setting adjusted to a pre-set value of 35 using the ImageJ tool function (Fig. 1). At this specific threshold, images would show melanophore rich areas along the dorsal crest and aggregated areas with pigmentation as black. The remaining areas of the skin would show as white. Next, skin pigmentation was calculated as the ratio of black versus white pixels withing the defined area. A ratio above 0.5 demonstrated that the number of pixels were more abundant compared to the number of white pixels.

2.7 Statistics

All statistical analyses were performed using R software™ R.3.2.2 (R Development Core Team, 2013). To compare variance among group means (difference in physiological measurements among treatment groups), a one-way ANOVA was selected. The appropriate

statistical tests used to cover the assumptions of a one-way ANOVA included a Shapiro-Wilk test (Shapiro and Wilk, 1965) for distribution of normality where the assumptions of normally distributed residuals were required, and a F-test for variance where implicit ANOVA assumptions required homogeneous variance. If assumptions were not met, a non-parametric Kruskal-Wallis test was used. Pearson correlation coefficient was used to evaluate linear relationships between physiological measurements. A significance level of $\alpha = 0.05$ was used for all tests. Mean swimming activity ± 95% confidence intervals were plotted for all time points.

3 Results

3.1 Behavioural analysis

3.1.1 Swimming activity for each treatment

Lumpfish mean swimming activity (± 95% confidence intervals) varied among the treatment groups Olfaction, Live and Model in comparison with control (Fig. 2). Swimming activity increased the most in the Olfaction treatment and confidence intervals did not overlap with control groups 9 out of 15 times. Live treatment did not overlap with control 2 out of 15 times. The least change in swimming activity was observed in the Model treatment in comparison with control. After exposure to the different treatments, swimming activity gradually decreased during the 30 min duration in all groups towards the mean activity level observed in control groups.

3.1.2 Swimming activity for each individual lumpfish

Individual swimming activity (sum of activity score after treatment introduction divided by the sum of activity before treatment introduction) significantly differed between treatments and control (Kruskal-Wallis test, H (3) = 32.91, $P < 0.001$). Pairwise comparison using Wilcoxon rank sum test revealed a significant difference between lumpfish exposed to Live treatment (Mdn = 1.47) and control (Mdn = 0.97) ($P < 0.001$). Lumpfish exposed to Olfaction (Mdn = 1.30) did also significantly differ from control ($P < 0.0001$) while lumpfish exposed to Model treatment (Mdn = 1.07) did not ($P = 0.17$) (Fig. 3).

3.2 Physiological analysis

3.2.1 Neurotransmitters and plasma cortisol measurements in treatment groups

Measurements of neurotransmitters and brain monoamines in lumpfish (n = 24) including serotonin (5HT) and the metabolite (5-HIAA) in addition to dopamine (DA) and the metabolite (DOPAC) did not differ between treatment groups and control groups (Table 2).

Using Pearson correlation test, significant positive correlations between 5HT and the metabolite 5-HIAA were observed in all treatment groups including Olfaction (r (22) = 0.79, $P < 0.001$), Models (r (22) = 0.67), $P < 0.001$) and Live Atlantic salmon (r (22) = 0.64, $P < 0.001$), but not in the control groups (r (22) = 0.27, $P = 0.18$). Mean measurements of plasma cortisol in treatments ranged from 60.2 to 64.2 nmol l^{-1} whereas mean levels in control was 46 nmol l^{-1}. Plasma cortisol did not differ between treatments and control (Table 2).

3.2.2 Skin colour and pigmentation

No difference was observed on lumpfish (n = 36) skin colour between groups prior to the experiment (one-way ANOVA, F (3,140) = 0.41, $P = 0.74$) (Fig. 4). After exposure to each treatment, a significant change on RGB skin colour was observed (one-way ANOVA, F (3, 136) = 3.17, $P = 0.02$), and the difference was significant between lumpfish exposed to Olfaction and the control groups (post hoc Tukey test, $P = 0.01$). Like colour, measurements of pigmentation yielded no significant variation prior to the experiment (one-way ANOVA, F (3, 136) = 0.58, $P = 0.62$) while treatments induced a significant difference in pigmentation (one-way ANOVA, F (3, 136) = 9.33, $P < 0.0001$). A post hoc Tukey test revealed significant increase in pigmentation in lumpfish exposed to Models ($P = 0.03$) and lumpfish exposed to Olfaction ($P < 0.0001$) in comparison with control groups (Fig. 4).

4 Discussion

The present study revealed for the first time how naive lumpfish, a facultative cleaner fish, responded to different sensory cues from a carnivorous client fish, in this case Atlantic salmon which is the current interspecific interaction of interest in aquaculture. The treatment most strongly affecting both behavioural and physiological responses was salmon Olfaction, which caused a significant acute increase in swimming activity (Fig. 2) and the overall activity level after introduction compared with activity level prior to the introduction (Fig. 3). Also, RGB skin colour and pigmentation ratio significantly increased in lumpfish exposed to the Olfaction treatment. In comparison, lumpfish exposed to the Live Atlantic salmon treatment showed a similar acute behavioural response and a significant overall higher activity level, but no change in RGB skin colour or pigmentation ratio. The treatment with least effect on lumpfish, was the Model treatment. Here, only pigmentation ratio increased in comparison with control. In addition, we found a significant positive relationship between serotonin and the metabolite 5-hydroxyindoleacetic acid in all treatment groups, but not in the control group. The strongest acute response in behaviour and body colour and pigmentation triggered

by salmon Olfaction, emphasize the importance of innate responses and risk assessment from chemical cues also in facultative cleaner fish (Berejikian et al., 2003; Brown, 2003; Dixson et al., 2010; Mitchell et al., 2012). In addition, the innate importance of predator recognition from olfaction is important in juvenile fish which spend early life inshore in a benthic environment with low visibility (Gerlai, 1993). Below, we discuss the results from both behavioural and physiological viewpoints in-depth.

4.1.1 Behavioural responses

In general, small prey fishes risk assesses potential predators and modify their behaviour within a given environment (Brown, 2003). The time framework of the experiment revealed modification through three clear behavioural phases: a basal swimming activity level prior to treatment introduction, an activation phase after treatment introduction and a recovery phase within 30 min. This coincided with the measurements of plasma cortisol (discussed in section 4.1.2.1), which suggest that the different treatment exposures induced mild stress responses. Even though cleaner fish act fearlessly when facing clients (Soares et al., 2012), behavioural escape performances are observed among other species of cleaner wrasses (Gingins et al., 2017) like what was observed during the first minutes after Olfaction or Live treatment introduction for lumpfish. Yet, swimming activity for lumpfish from both salmon Models and Live salmon treatment groups indicated that exposure to small ca. 184 g Atlantic salmon induced a similar response as previous results with exposure to larger ca. 1258 g Atlantic salmon (Staven et al., 2019). Thus, the size visualisation of the client itself was not the strongest cause of behavioural change, also observed in other teleost predator versus prey interactions (Tang et al., 2017). Prey fish perform various behavioural responses to predator presence, including fast escape or reduction in swimming activity (Barcellos et al., 2014; Stoks et al., 2003). A reduction in swimming activity produces fewer chemical traces and reduce hydrodynamic turbulence (Pohlmann et al., 2001), opposite to the overall increased activity level observed in lumpfish. This indicated that the purpose of a fast recovery from burst swimming activity was more like what has been observed in obligate Bluestreak wrasse with fast start escape performance during first interaction with a client fish (Gingins et al., 2017). While visual cues from the Model treatment induced the lowest response in behaviour, Olfaction treatment caused swimming activity to increase above other treatments. It is suggested that when lumpfish were unable to scale or inspect the potential risk, swimming activity increased as a response to the introduced chemical cues from Atlantic salmon (Laberge and Hara, 2001). Similar findings on the role of Olfaction in prey fish have been

observed on other species of teleost (Hartman and Abrahams, 2000; Holmes and McCormick, 2010).

4.1.2 Physiological responses

4.1.2.1 Plasma cortisol

Elevated plasma cortisol levels result from the activation of the hypothalamic-pituitary-interrenal (HPI) axis and the release of glucocorticoids into the blood stream, which promote metabolic adaptation to environmental stimuli (Wendelaar Bonga, 1997; Winberg et al., 2016). In the present study, lumpfish were exposed to different heterospecific salmon cues for 30 min, and an additional 30 min before physiological data were collected. When plasma cortisol is implemented as a measurement of stress in experimental work, timing of sampling is essential considering that cortisol in lumpfish gradually peak towards 1h after exposure to a stressor, before the concentration drops again (Iversen et al., 2014). Mean measurements of plasma cortisol in all treatment groups ranged from 60 to 64 nmol l^{-1} while the control revealed 30 % lower mean measurements of 46 nmol l^{-1}. In comparison, exposure to exhaustive exercise can increase measurements plasma cortisol in lumpfish to above 400 nmol l^{-1} (Hvas and Oppedal, 2019). The control group plasma cortisol levels, expected to fluctuate between 0-20 nmol l^{-1} (Hvas and Oppedal, 2019; Jørgensen et al., 2017), could be the result of an incomplete acclimatization to the movement to new experimental tanks. This was nevertheless accounted for with duplicate control groups during each of the three days of experimentation and the exact same protocol was used for the control groups as for the treatment groups, except the specific treatment exposures. Also, behavioural observations did not show increased mobilization in terms of swimming activity in the control tanks, as illustrated during the first 30 min (Fig. 2). A similar study on lumpfish in interaction with Atlantic salmon, only with salmon being five times larger in size, revealed increased mean cortisol levels to 115 nmol l^{-1} (Staven et al., 2019). This suggest that the size of a carnivorous client could have implications on the HPI-axis in lumpfish, even though the plasma cortisol levels measured in this study and in Staven et al. (2019) were remarkably lower compared to Hvas and Oppedal (2019).

4.1.2.2 Neurotransmitters

Both social behaviour and acute stress activate the dopaminergic and serotonergic systems whereas in this case, the two physiological responses intertwine for a naive cleaner fish interacting with a carnivorous client fish (Chaouloff, 2000; Messias et al., 2016; Paula et al., 2015). Among conspecifics, serotonin play a key role in individual stress coping styles and

life history traits (Winberg and Thörnqvist, 2016) while in interspecific interaction, increased levels of serotonergic activity have shown to motivate cleaning behaviour and cooperation among cleaner fish (Paula et al., 2015). Measurements of monoamines revealed strong variation among individuals of lumpfish in all treatment groups, including control groups. While the main interest for telencephalic levels of serotonin was to analyse its role on interspecific social behaviour in lumpfish, it was clear that conspecific interaction among the six lumpfish in each tank could enhance variation alone due to establishment of hierarchies (Cubitt et al., 2008; Loveland et al., 2014; Morandini et al., 2019). Stress is known to induce an increase in the serotonergic activity reflected in the 5HIAA/5HT ratio (Gesto et al., 2013). In the current study, the lack of differences in the ratio could be also explained by the high intragroup variability in terms of stress, as reflected by the cortisol data, which supports the view of the treatments as of mild stressors, as commented before.

4.1.3 Skin colour and pigmentation of lumpfish

Rapid change in colour and pigmentation caused by environmental stimulus, including stressors or social settings are not uncommon among fishes (Baker, 1993; Höglund et al., 2002; Nery and de Lauro Castrucci, 1997). For lumpfish, regulation of skin biliverdin and pigmentation have previously been suggested to function as crypsis for juveniles living in a benthic substratum and later during pelagic migration in upper surface waters (Davenport and Bradshaw, 1995; Moring, 1994). To our knowledge, increased colour and skin darkening from interspecific sensory input in lumpfish have not been documented before, which opens for new interpretations of the species stress responses and responses to social interaction. In addition, clinical observations on change in colour and darkening could be implemented as novel welfare indicators (Noble et al., 2019). With Atlantic salmon Olfaction inducing the strongest response, it is likely that more vivid colouration and a darker skin pigmentation were stress indicators coinciding with the acute increase in swimming activity observed.

4.1 Notes on the experimental setup

Practical limitations in the experimental room, associated with the requirement to connect adjacent water to deliver Atlantic salmon Olfaction to the experimental tanks, impeded switching the experimental groups between different tanks. This means that tanks were not completely randomized in the present study. The experimental room was nevertheless homogeneous in shape and interior and was left undisturbed during acclimation.

5 Conclusion

The objective of the study was to investigate how unexperienced naive lumpfish responded to heterospecific sensory cues from Atlantic salmon. In general, lumpfish in all treatments had a fast recovery phase and exhibited mild stress responses, which suggest quick habituation to salmon interspecific interaction. Olfaction treatment induced the strongest responses, which emphasizes the implication on the lack of visual recognition of potential risks in lumpfish. Results suggest that introducing lumpfish to aquaculture sea cages when farmed salmon are sized 100 - 200 g result in a low physiological stress response. Increased skin colour was observed in the Olfaction treatment, which indicates that mild stress induces body colour change in lumpfish. An additional increase in skin pigmentation was observed in Olfaction and Model treatments. As the colour analysis were based on pixel counts only, a thorough investigation on a cellular level is necessary to identify the mechanisms on the relationship between colour change, stress, and interspecific interaction. The future use of lumpfish in aquaculture require a thorough understanding of the species needs to provide good animal welfare, however, exposure to Atlantic salmon or salmon cues alone, have shown not to be a major stressor during the initial phase of interspecific interaction. Further, a long-term interaction between the two species should be investigated in regards of both behaviour and stress responses.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

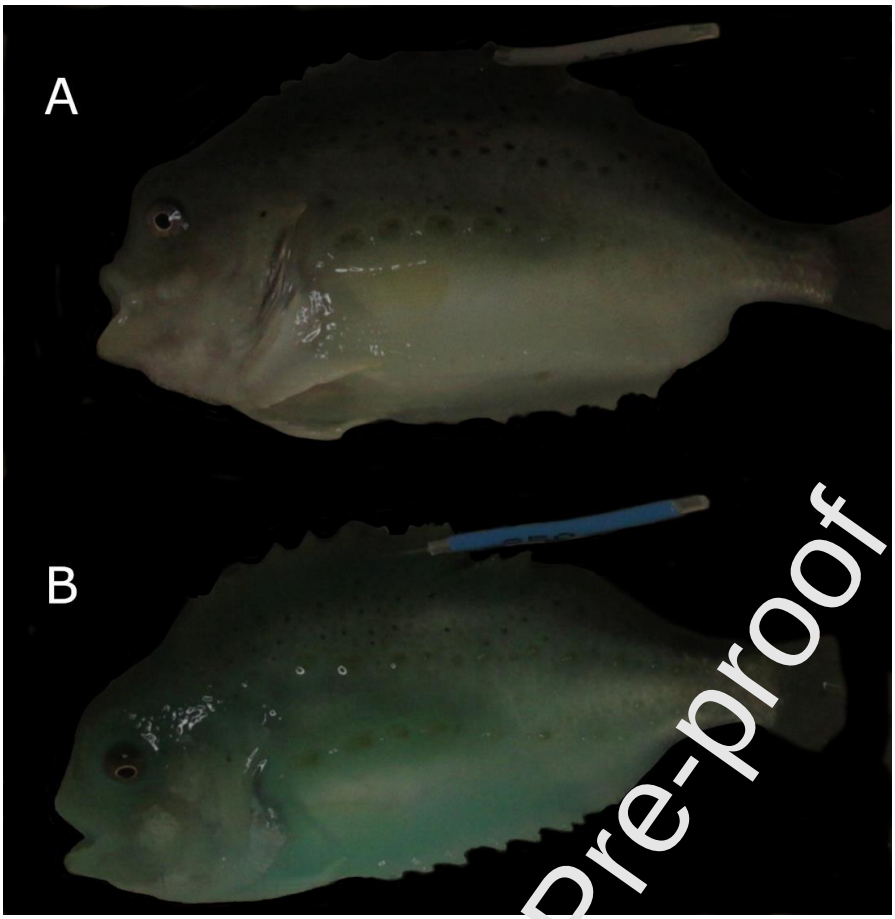

Figure A.1. Increase in skin colour observed in lumpfish after the experiment was terminated. Image "A" represent an individual with mean R+G+B = 224, which was the median value observed in control groups. Image "B" represents an individual with mean R+G+B = 254, which was the median value observed in lumpfish exposed to Atlantic salmon Olfaction. The difference in colour between Olfaction treatment and control groups was significant (post hoc Tukey test, $P < .01$). Data on colour and pigmentation are presented in Figure 4.

Figure 1. The upper image (A) shows the defined homogenous area used to calculate mean red, green and blue pixels (mean R+G+B), while the lower image (B) show the defined area used to calculate pigmentation as the ratio of black and white pixels (B:W). Both colour and pigmentation were measured using ImageJ.

Figure 2. Mean swimming activity from 6 replicates for each treatment with 95% confidence intervals. Means from each minute illustrates the change in swimming behaviour before treatments were introduced (first 30 min) and after the treatments were introduced (31-60 min). Treatments were A) Olfaction, B) Model and C) Live Atlantic salmon in addition to D) control.

Figure 3. Score sum of swimming activity for each individual lumpfish (n = 36) observed 30 min before and 30 min after interactions with the different treatments. A score above 1 implies an increase in activity level for each specific lumpfish based on categorization of swimming activities including (1) attached, (2) hovering, (3) normal swimming behaviour and (4) burst swimming.

Figure 4. Lumpfish skin colour (n = 36) measured in mean red, green, and blue (a) before and (b) after the experiment, and lumpfish skin pigmentation measured in mean ratio of black and white pixels (c) before and (d) after the experiment. Dissimilar letters indicates significant difference among treatments and control. Total duration of exposure in each treatment was 60 min. Images were analysed with ImageJ. See section 2.6.1 and 2.6.2 for detailed method description.

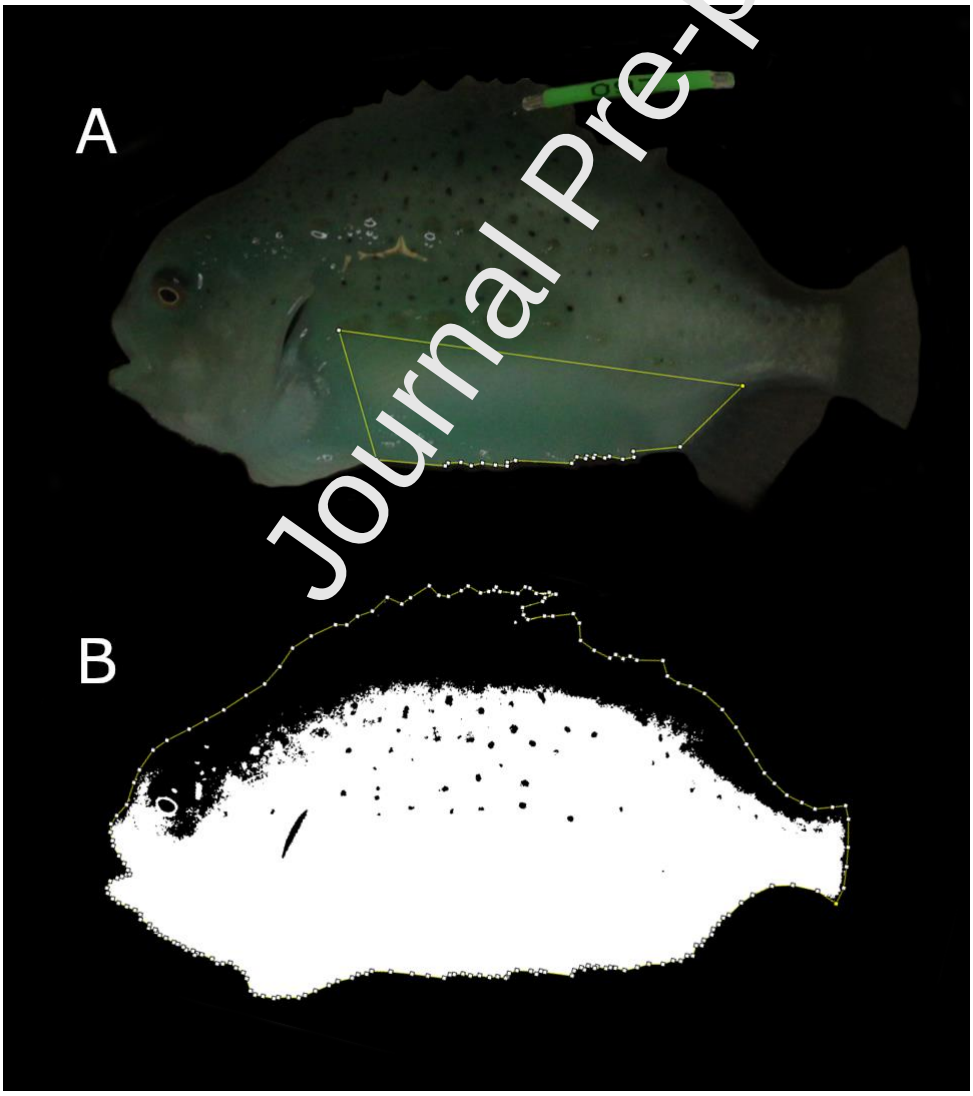

Figure 1. Staven et al.

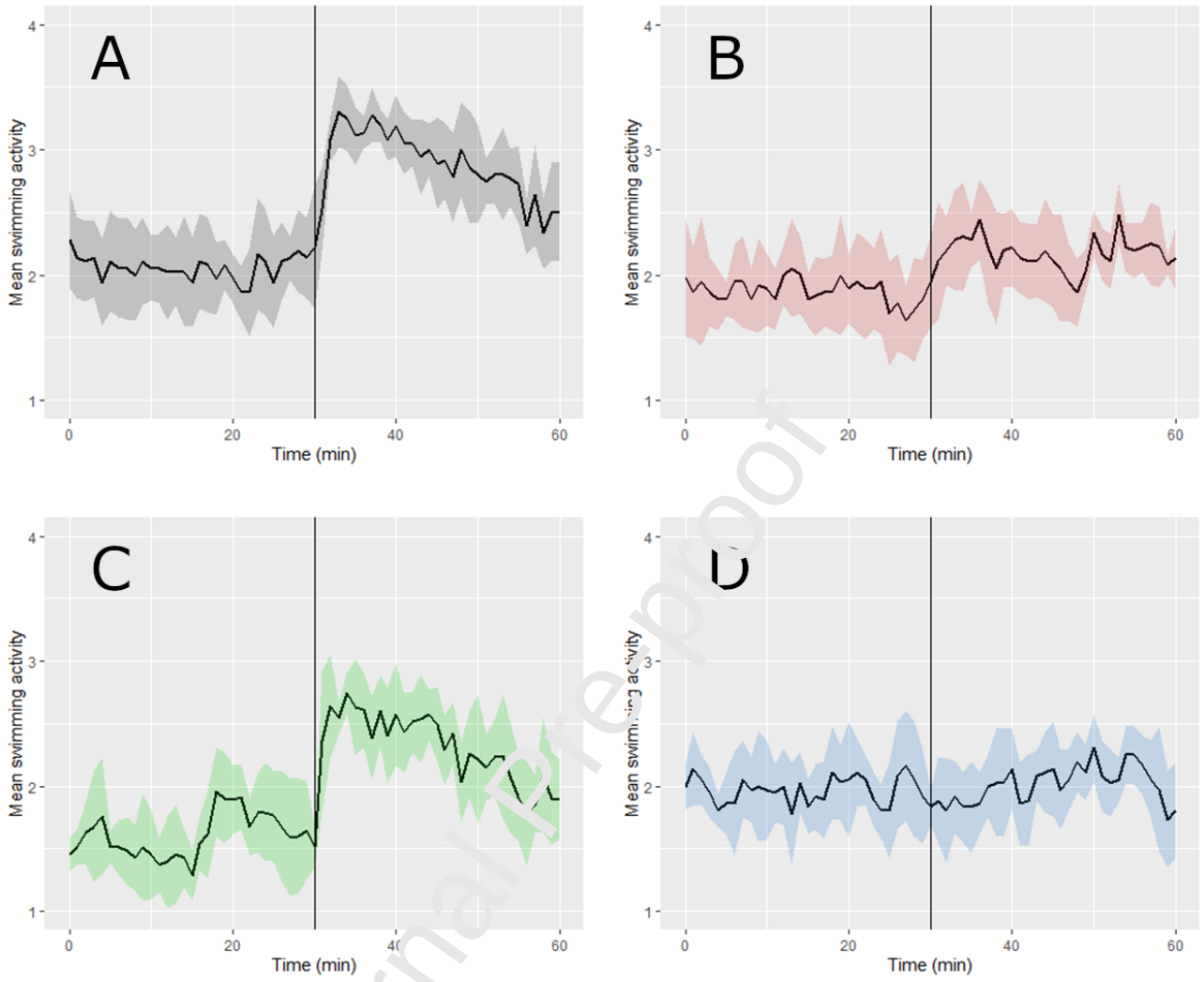

Figure 2. Staven et al.

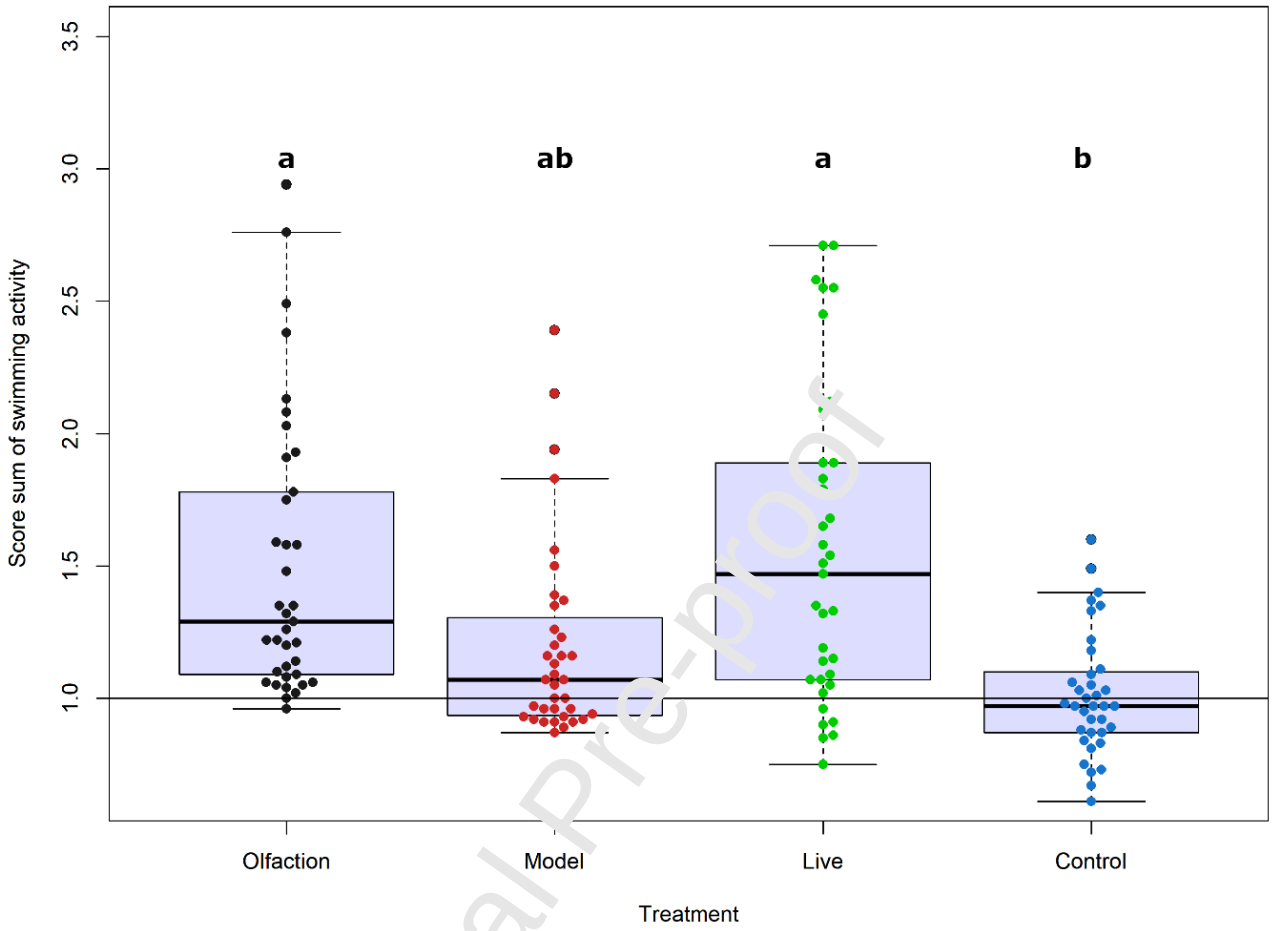

Figure 3. Staven et al.

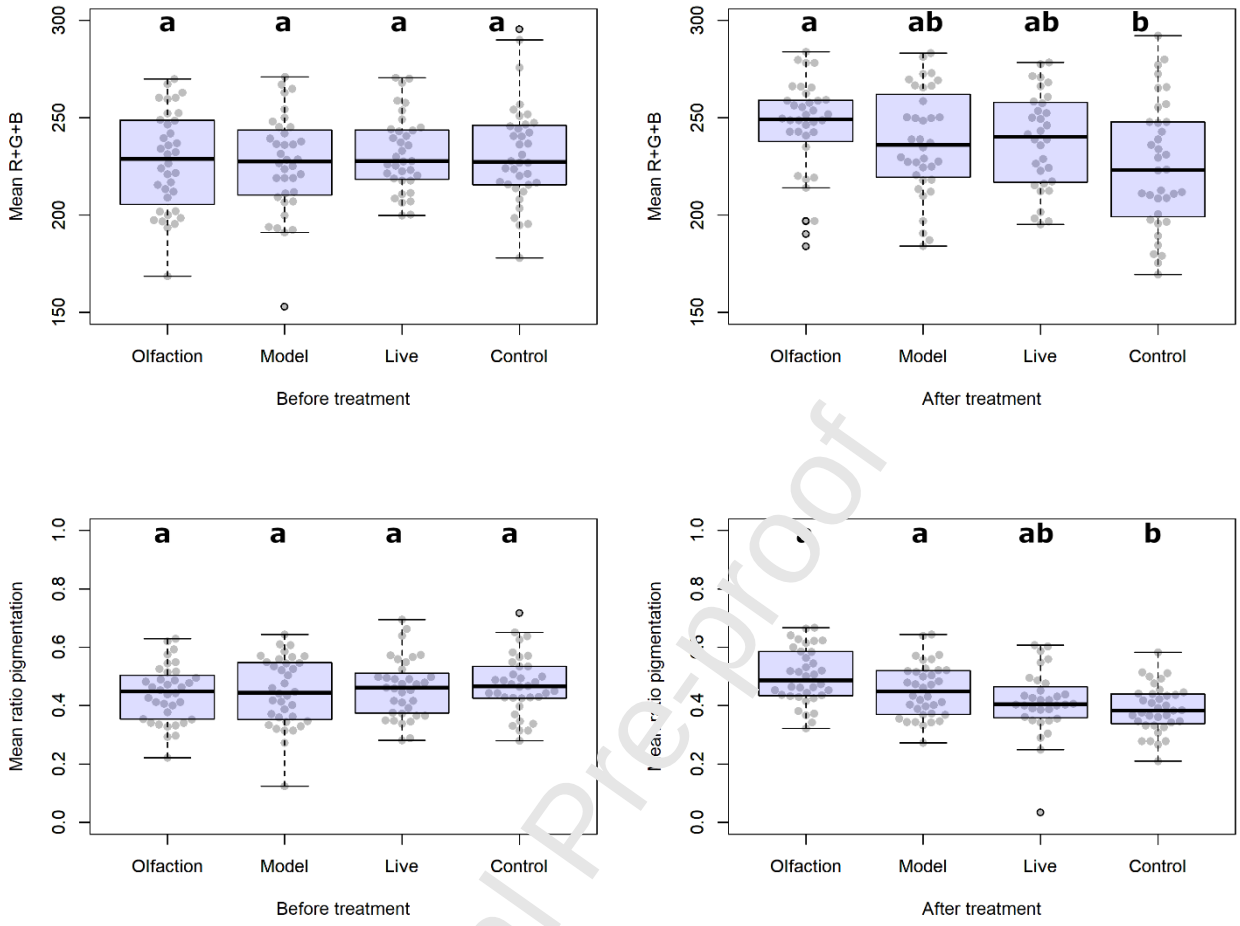

Figure 4. Staven et al.

Table 1. Classification of lumpfish swimming activity based on distinguishable locomotion.

Score	Swimming activity	Description
4	Burst swimming	Rapid acceleration
3	Normal swimming	Locomotion between hovering and burst swimming activity
2	Hovering	Hovering performance with no horizontal or vertical motion
1	Attached	Attached to substrate with sucker disc

Table 2. Measurements of neurotransmitters in the telencephalon of lumpfish (n = 24) and plasma cortisol levels from blood samples extracted after experiments were terminated. The neurotransmitters serotonin (5HT) and dopamine (DA) including the metabolites 5-hydroxyindoleacetic acid (HIAA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) were measured using HPLC while plasma cortisol was measured using Radioimmunoassay.

Compound	Control	Model	Live	Olfaction	Kruskal-Wallis rank sum test	*p*-value
5HT (ng/g)	649 ± 169.4	671 ± 288.9	642 ± 191.9	630 ± 207.4	H (3, 91) = 0.38	*P* = .94
5HIAA (ng/g)	17 ± 5.7	23 ± 18.3	18 ± 10.0	17 ± 6.8	H (3, 91) = 0.24	*P* = .97
5HIAA:5HT (%)	3 ± 1.1	4 ± 3.8	3 ± 1.2	3 ± 0.6	H (3, 91) = 0.99	*P* = .80
DA (ng/g)	141 ± 52.8	133 ± 45.6	150 ± 63.5	166 ± 58.7	H (3, 91) = 4.59	*P* = .20
DOPAC (ng/g)	15 ± 19.2	15 ± 24.4	10 ± 9.4	17 ± 17.4	H (3, 91) = 3.89	*P* = .27
DOPAC:DA	13 ± 21.1	12 ± 18.6	8 ± 8.4	11 ± 12.0	H (3, 91) = 4.83	*P* = .18
Cortisol (nmol l^{-1})	46 ± 42.3	60 ± 41.4	64 ± 43.8	61 ± 42.5	H (3, 91) = 4.98	*P* = .17

Highlights

- Lumpfish acute responses to salmon cues (live, olfaction and models) were studied.
- Olfaction and live salmon affect lumpfish swimming activity more than models.
- Olfaction and models impact skin colour and/or darkening.
- Atlantic salmon body size influence activation of the HPI axis in lumpfish.