

Interaction studies on lumpfish exposed to Atlantic salmon: behavioural observations and the underlying physiological and neurobiological mechanisms

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FACULTY OF BIOSCIENCES AND AQUACULTURE

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behavioural observations and the underlying
physiological and neurobiological mechanisms

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Preface

The following thesis was submitted in fulfilment of the requirements for the Degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA) under Nord University, Bodø, Norway. Original research included in the dissertation was completed during a four-year industrial PhD-project (NAERINGSPHD) funded by The Research Council of Norway (CleanWel ES610697) in collaboration with Aqua Kompetanse AS and Nord University.

The project team consisted of the following members:

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Per Anders Andersen, Cand. real, Aqua Kompetanse AS: mentor

Kim Halvorsen, PhD, Institute of Marine Research: mentor

Alex Lindseth, MD, Aqua Kompetanse AS: project leader



Fredrik Ribsskog Staven

Flatanger, Feb 1st, 2022

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While I have spent a considerable amount of the last two years in my home office due to the circumstances, I never felt alone. Thanks to colleagues and friends the wheels have kept turning. I am also incredibly lucky to be surrounded by a wonderful family full of life and enthusiasm.

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And so, today my world it smiles. Your hand in mine, we walk the miles.

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- Paper II **Staven, F. R.**, Nordeide, J. T., Gesto, M., Andersen, P., Patel, D. M., Kristensen, T. (2021). Behavioural and physiological responses of lumpfish (*Cyclopterus lumpus*) exposed to Atlantic salmon (*Salmo salar*) sensory cues. Aquaculture, 544: 737066
- Paper III **Staven, F. R.**, Gesto, M., Iversen, M. H., Andersen, P., Patel, D. M., Nordeide, J. T., Kristensen, T. (2022). Cohabitation with Atlantic salmon (*Salmo salar*) affects brain neuromodulators but not welfare indicators in lumpfish (*Cyclopterus lumpus*). Front Physiol [Accepted 27.01.2022]

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Authors' note

Reading Niko Tinbergen's work on social behaviour really inspired me, especially the part involving hours, days, and sometimes months spent observing groups of animals. Today we have access to more simple ways of collecting data. Also, modern technology has opened new doors into the knowledge of underlying mechanisms that affect or regulate behaviour, even if the basal method of observing animals is still necessary to decipher the conundrum of patterns in life. The complexity of the evolutionary machinery is mind-blowing and sometimes hard to grasp. It is a fascinating theatre to witness, especially the acts on the unfoldment of life, and now, the anthropocentric impact on nature. Everything we know of that is alive, sentient or not, share room with us inside this tiny biosphere that protects us from the vastness. Unfortunately, humanity do not manage to protect diversity and animal integrity from itself. Research on animal behaviour has provided us with answers, and new clues regarding our own species. Even in our very distant fish ancestors we find remarkable similarities and homologous structures that can be used as models in human science and medicine. A deeper understanding of shared ancestry with living creatures of the sea could be the key to unlock better welfare and dignity for other species. While this dissertation dives into the complex world of cleaner fish, I hope the reader find interest in the beauty of cooperation in a broader perspective.

Summary

Cleaner fish include multiple species known to remove ectoparasites from other species of fish, namely “clients”. Due to challenges with ectoparasitic epidemics in aquaculture, the natural concept of delousing was implemented as a method already in the eighties before escalating during the last fifteen years. To avoid exploitation of wild fish stocks of cleaner fish, it has become more common to hatch and rear certain species, including lumpfish (*Cyclopterus lumpus*) and ballan wrasse (*Labrus bergylta*). This could open for selective breeding on preferable traits to improve both health and cleaning efficacy. While a natural environment provides cleaner fish with the opportunity to choose which relationship to favour in terms of client preferences and interaction, cleaner fish used in aquaculture are constrained within a confined environment with Atlantic salmon (*Salmo salar*) exclusively. This clarifies the importance to investigate behavioural and physiological responses and the question of habituation to novel heterospecific interactions. Today, the most used cleaner fish in Atlantic salmon farming is the lumpfish. A cleaning symbiosis between lumpfish and Atlantic salmon has yet to be documented in nature. Thus, the baseline of cleaner fish and client interaction is solely studied in laboratory or aquaculture settings. Based on how different species of cleaner fish cope with clients, including carnivorous species, the project aimed to investigate how lumpfish responded to interaction with Atlantic salmon.

In **paper I**, we studied lumpfish collected from Atlantic salmon sea cages to investigate how the species was affected by commercial scale aquaculture. A comparison of experienced lumpfish, collected after 30 days of interspecific interaction with Atlantic salmon, with naive lumpfish with no earlier interaction with Atlantic salmon, revealed significant changes in both physiological and behavioural parameters. Naive individuals with no earlier experience with Atlantic salmon showed elevated plasma cortisol levels upon first exposure while experienced lumpfish did not. In addition, behaviour was significantly different in terms of increased swimming activity and avoidance among

naive lumpfish. These findings were indications of non-associative learning known as habituation. It was necessary to repeat interspecific interaction in a controlled environment to remove variables from a full-scale study design and test specific acute responses from salmon sensory signals. In addition to behaviour and physiological stress responses, we investigated the effect on neurotransmitters relevant to both stress and social behaviour in addition to change in body colour and pigmentation.

In **paper II**, a significant acute increase in swimming activity was observed in lumpfish exposed to both live Atlantic salmon and salmon olfaction cues before recovering toward basal levels within 30 minutes. In addition, exposure to salmon olfaction caused body colour and pigmentation to significantly increase, while salmon models, which induced the lowest overall behavioural responses, had a significant impact on skin pigmentation. Measurements of neurotransmitters in treatments did not deviate from control, yet a statistically significant positive correlation between serotonin (5-HT) and the metabolite 5-hydroxyindoleacetic acid (5-HIAA) was observed in all treatments apart from the control groups. Plasma cortisol was on average 25 % higher in all treatments compared to control, but not significantly different. Overall, the study showed a mild stress response to salmon sensory cues, including live Atlantic salmon. The main difference between **paper I** and **paper II** was the size of the client used (on average 1,200 g and 180 g, respectively). While swimming activity increased in lumpfish in both studies, only the larger Atlantic salmon interaction significantly affected the HPI-axis. This emphasised a size dependent risk assessment in lumpfish.

In **paper III**, lumpfish were exposed to the same treatment protocol, but prolonged over 6 weeks of interaction. The aim was to investigate long-term effects on behaviour and physiological responses, including neurotransmitters. Like the observed indications on habituation in **paper I**, the long-term effect on neurotransmitters revealed lower concentrations of both dopamine and noradrenaline in the brain of lumpfish exposed to Atlantic salmon. These changes, combined with no negative impact on growth or health scores in lumpfish, suggested a novel finding of habituation

of neurotransmitters in lumpfish through interaction with Atlantic salmon alone. It is concluded that lumpfish brain neurotransmitter activity is affected by client interaction, and the implications might be relevant in the first step to performing cleaner fish behaviour and removing ectoparasites from Atlantic salmon. With no negative long-term effects on welfare parameters including growth, operational welfare indicators, stress, and colour, the three papers coincide and suggest that lumpfish as a species have the capacity to change both behaviour and physiological responses to the presence of Atlantic salmon. The welfare of lumpfish was not impeded by heterospecific interaction or salmon sensory cues under the conditions provided in the experiments. A better understanding of the species basal needs is still crucial to improve its use in aquaculture.

Sammendrag (Norwegian)

Rensefisk er et fellesbegrep for arter som fjerner ektoparasitter fra andre fiskearter, referert til som «klienter». På grunn av utfordringer med lakselus i oppdrettsnæringen, ble bruken av rensefisk innført allerede på åttitallet, og eskalerte fra og med 2007. For å unngå overfiske av ville fiskebestander har det i dag blitt mer vanlig å oppdrette visse arter av rensefisk, inkludert rognkjeks (*Cyclopterus lumpus*) og berggylt (*Labrus bergylta*). Dette åpner for muligheten til selektiv avl på foretrukne egenskaper som kan forbedre både helse og effektivitet som lusespiser. Mens et naturlig miljø gir rensefisk mulighet til å velge hvilket forhold som skal favoriseres når det gjelder klientpreferanser og interaksjon, er rensefisk brukt i oppdrettsnæringen begrenset innenfor et gitt miljø med Atlantisk laks (*Salmo salar*). Dette tydeliggjør viktigheten av å undersøke atferdsmessige og fysiologiske responser, og spesielt spørsmålet om tilvenning. Den vanligste arten brukt som rensefisk i lakseoppdrett er rognkjeks. Rensefisk-symbiose mellom rognkjeks og Atlantisk laks er ennå ikke dokumentert i naturen. Samspillet mellom de to artene er derfor utelukkende studert i laboratorie- eller akvakulturmiljøer. Basert på hvordan ulike arter av rensefisk samhandler med klienter, inkludert predatorer, hadde doktorgradsavhandlingen som hensikt å undersøke hvordan rognkjeks påvirkes av sosial interaksjon med Atlantisk laks.

I artikkel I ble «erfaren» rognkjeks hentet ut fra kommersielle merder etter 30 dager sammen med oppdrettslaks for å undersøke mulige grader av tilvenning. Erfaren rognkjeks ble senere reintrodusert med oppdrettslaks i karforsøk. Kontrollgruppen besto av «naiv» rognkjeks som ikke hadde tidligere erfaring med oppdrettslaks. Studiet fant betydelige endringer i både atferdsmessige og fysiologiske parametere. Mens målinger av stresshormonet plasmakortisol økte signifikant hos naiv rognkjeks, var tilsvarende målinger hos erfaren rognkjeks generelt lave. I tillegg var atferd signifikant forskjellig i form av økt svømmeaktivitet og distansering til oppdrettslaks blant naive rognkjeks. Disse funnene indikerer at interaksjon med oppdrettslaks i kommersielle merder fører til ikke-assosiativ læring, kjent som tilvenning. Videre var behovet for å

gjenskape studiet i et kontrollert miljø nødvendig for å fjerne variabler som oppstår i et fullskala studiedesign. Dette gjorde det mulig å kartlegge effekten av spesifikke sensoriske faktorer fra oppdrettslaks, inkludert lukt og visuelt inntrykk. I tillegg til atferd og fysiologisk stressresponser ble neurotransmittere, relevante for både stress og sosial atferd, undersøkt. Endring i kroppsfarge og pigmentering ble også kartlagt.

I artikkel II ble en signifikant akutt økning i svømmeaktivitet observert hos rognkjeks utsatt for både levende oppdrettslaks og lakselukt, før aktivitetsnivået gradvis avtok. I tillegg førte eksponering for lakselukt til at kroppsfarge og pigmentering økte. Eksponering for laksemodeller ga lite endring i atferd, men hadde en betydelig innvirkning på hudpigmentering. Målinger av neurotransmittere i alle behandlinger (luft, modell og levende oppdrettslaks) avvok ikke fra kontrollgruppen. Det ble likevel observert en signifikant positiv korrelasjon mellom serotonin (5-HT) og metabolitten 5-hydroxyindoleddisyre (5-HIAA) i alle behandlinger bortsett fra i kontrollgruppene. Plasmakortisol var gjennomgående 25 % høyere i alle behandlinger sammenlignet med kontroll, men ikke signifikant forskjellig. Samlet sett viste studien en mild stressrespons på laksens sensoriske signaler, inkludert levende laks. Hovedforskjellen mellom artikkel I og artikkel II var størrelsen på oppdrettslaks brukt (i gjennomsnitt henholdsvis 1200 g og 180 g). Mens svømmeaktiviteten økte hos rognkjeks i begge studiene, var det bare interaksjonen med større atlantisk laks som påvirket HPI-aksen signifikant. Dette indikerer at rognkjeks gjør en risikovurdering basert på størrelsen på klienten.

I artikkel III ble rognkjeks utsatt for tilsvarende behandlingsprotokoll som i artikkel II (luft, visuelt eller levende laks), men forlenget over 6 uker med interaksjon. Hensikten var å undersøke langtidseffekt på atferd og fysiologiske responser, inkludert neurotransmittere. I likhet med artikkel I, ble det observert indikasjoner på tilvenning, men i form av lavere konsentrasjoner av både dopamin og noradrenalin i hjernen hos rognkjeks eksponert for laks. Disse endringene, kombinert med ingen negativ innvirkning på vekst eller velferds-score hos rognkjeks, antyder et nytt funn av tilvenning av neurotransmittere i rognkjeks gjennom interaksjon med laks. Det

konkluderes med at neurotransmitter påvirkes av interaksjon med klienter, og at dette kan være relevant i forbindelse med atferds knyttet til selve fjerningen av ektoparasitter fra Atlantisk laks. Uten negative langtidseffekter på velferdsparametere inkludert vekst, velferds-score, stress og farge, er det mye som tyder på at rognkjeks som art har kapasitet til å endre både atferd og fysiologiske responser i interaksjon med Atlantisk laks. Velferd ble ikke påvirket av heterospesifikk interaksjon eller laksens sensoriske signaler under forholdene gitt i eksperimentene. En bedre forståelse av artens basale behov er fortsatt avgjørende for å forbedre bruken i kommersiell akvakultur.

Introduction

Our planet is the only known system where life exists and interacts with itself as evolved biological creatures in multifaceted shapes and sizes. Here, the behavioural ecologist aims to study such interactions, whether at individual or population scale, or within communities with multiple species (Bronstein, 1994). Whenever species share an environment, there is a slight chance that a symbiotic relationship will develop. In general, symbiosis comprises two or multiple organisms living together where one, or both participants benefit from the relationship (de Bary, 1879, Oulhen et al., 2016). The three classical outcomes of symbiosis are:

- Parasitism (+/-) – or predation, where one organism gains benefits while the other suffers
- Commensalism (+/0) – where one organism benefits while the other remains unaffected
- Mutualism (+/+) – interaction or a series of interactions between individuals belonging to different species that yields, on average, direct fitness benefits to all participants (Bshary, 2021)

Mutualistic relationships have evolved through the competitive drive of natural selection on successful and innovative traits (Darwin, 1859). Such traits can over time make two organisms dependent on one another (Leigh, 2010). A dependency that requires the other species to survive and reproduce is termed obligate, while a partly dependent relationship is termed facultative (Grutter and Feeney, 2016). Studies on symbiotic relationships require observations and systematic mapping of behaviour and should be seen in context of both ultimate and proximate questions. While ultimate questions are based on evolution and adaptation, proximate questions seek to understand the mechanisms involved (Tinbergen, 1963). Tinbergen highlighted the important link between quantitative measurements and behavioural observations. Observations left alone are susceptible to bias and is why animal interaction must be

studied specifically, unambiguously and from multiple angles (e.g., behavioural, and physiological mechanisms) (Wynne, 2004).

Cleaner symbiosis

A thoroughly documented symbiosis in the animal kingdom is the mutualistic cleaner symbiosis. Cleaner symbiosis involves a *cleaner* that removes parasites and/or dead skin from another individual, often referred to as the *client* (Limbaugh, 1961, Losey, 1972). Cleaning symbiosis occurs among both terrestrial and marine animals, whereas most cleaners belong to the ray-finned fish class which comprises more than 200 species (Palmer et al., 2019, Vaughan et al., 2017, Weeks, 2000). Marine cleaner fish have been studied from multiple scientific perspectives, especially the bluestreak cleaner wrasse (*Labroides dimidiatus*), as later discussed. Within behavioural ecology, the game theory - or the theory of social situations - are calculations of mathematical models that rely on decision making in animals, such as involvement in cooperation, or symbiotic relationships (Axelrod and Hamilton, 1981). Trivers (1971) applied cooperative approaches from game theory on the relationship between cleaner fish and parasitised client fish. While most research suggests that cleaning symbiosis is a mutualistic relationship (Cheney and Côté, 2001, Bshary, 2021, Bshary and Grutter, 2006), Trivers (1971) named the relationship mutualistic selfishness, or reciprocal altruism, which in terms is also a relationship that favours both parts, yet the returning favours can be extended in time. Today, most research commonly refers to cleaning symbiosis as mutualistic, especially within the topic of cleaner fish (Bshary, 2021).

Cleaner fish

Cleaner fish feed obligately, or facultatively, on ectoparasites and dead skin from other fish species, namely the clients (Côté, 2000, Grutter, 1999). They are usually smaller in size in comparison with the client, and the client can be both herbivorous and piscivorous (Soares et al., 2007). In coral reef ecosystems, cleaner fish can play a key role in regulating ectoparasitic pressure on whole populations of clients (Clague et al.,

2011, Waldie et al., 2011). Cleaner fish have different strategies when it comes to interaction with their clients, including working as singletons, in pairs, or in larger groups (Bshary et al., 2008, Tebbich et al., 2002). Such groups are often observed at coral reef cleaner stations that clients revisit when the ectoparasitic load is increasing and a treatment is necessary (Waldie et al., 2011). While cleaner fish are commonly studied in coral reefs in the Pacific Ocean and the Indian Ocean, multiple species are observed all around the globe, and different species from different families seem to have evolved cleaner fish behaviour several times (Baliga and Law, 2016). Some cleaner fish have strong obligate interspecific relationships with their clients (Feder, 1966). Others consume ectoparasites during certain stages of ontogeny or as part of more general diet (Imsland et al., 2015). Cleaner fish are one of the most studied group of animals when it comes to mutualistic cooperation, which in general is rarely observed among vertebrates (Bronstein, 1994). Most species are found in benthic complex environments which require good spatial memory and cognitive performance (Ebbesson and Braithwaite, 2012), and some species are known to outperform both chimpanzees and orangutans in foraging tasks including delayed rewards, which is used as an indicator for fast learning and long memory (Salwiczek et al., 2012). Without the relief of ectoparasitic loads due to absence of cleaner fish, clients could suffer from secondary infections, increased plasma cortisol values and behavioural changes (Grutter, 1999, Soares et al., 2011). While clients depend on the services provided by cleaner fish, they must also distinguish between fish that take advantage of the client's behaviour to cheat (Stephens, 1996). This includes species that mimic cleaner fish morphology (Côté and Cheney, 2005) and cleaner fish that deceive by nipping and eating client epidermal mucus (Grutter and Bshary, 2003). Among studies on mutualism versus anti-cheater behaviour, Ferriere et al. (2002) suggested that both phenotypes could coexist, and that individuals which expressed mutualistic behaviour would have a favourable competitive superiority if the number of cheaters within the population increased.

One of the most prominent strategies cleaner fish uses to assess ectoparasites from potentially dangerous clients is termed tactile behaviour. Tactile behaviour involves swimming patterns and client interaction through gentle touches along the dorsal area of the client (Bshary and Würth, 2001). This behaviour has the potential to reduce physiological stress levels in clients, which was suggested to reduce aggression against the inspecting cleaner fish (Soares et al., 2011). With a thorough understanding of cleaner fish mutualism from a behavioural perspective, recent studies have investigated the underlying proximate physiological and neurobiological mechanisms (Soares, 2017). To grasp how the brain regulates cleaner fish behaviour, it is necessary to identify the anatomy of the teleost brain regions and their different involvements in social behaviour, stress and learning functions.

Behaviour and physiology

Brain function

During early embryonic stages of the neural tube, actinopterygians and non-actinopterygians have almost completely opposite morphological development (Braford, 1995). While the non-actinopterygian telencephalon, which is the anterior forebrain, undergoes an evagination with centred ventricles, the actinopterygian telencephalon develops in eversion (Rodríguez et al., 2002). Historically, the difference in morphology leads to induced complexities in recognising homologous structures between the two groups (Braford, 1995). In addition, the conventional model of morphogenesis is not completely uniform among all teleost, and the laterally directed outfolding has shown to vary from species to species (Folgueira et al., 2012). Despite these cytoarchitectural differences, phylogenetic analysis (Wullimann and Rink, 2002) and studies on lesions of the subpallial (Alunni et al., 2004) and pallial division (Kage et al., 2004) have revealed indications of homologous structures (Figure 1). The lateral pallium has been suggested as a homologue to the mammalian hippocampus (Portavella et al., 2004) while the medial pallium - more specifically the dorsomedial portion of the pallium - has been suggested as a homologue to the mammalian

lateral/basolateral amygdala while the supracommissural and postcommissural subpallia are the suggested candidates for the extended central amygdala, as reviewed by (Maximino et al., 2013).

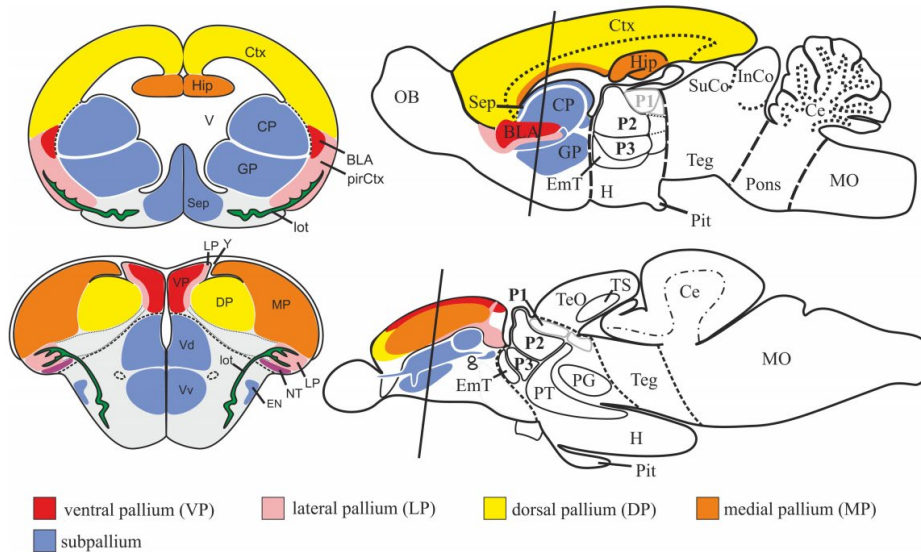


Figure 1 Coronal brain slice illustration comparing topological correspondences in the telencephalon observed in a rodent (above) and teleostan (below). The figure is used with permission from Mueller (2012).

In mammals, both the hippocampus and the amygdala have elevated synaptic activity during the expression of behaviour related to avoidance, e.g., escape performance, deflection or hiding (Izquierdo et al., 1997). In comparison, observations on goldfish during conditioned avoidance learning showed comparable traits (Portavella et al., 2004). Lateral pallial lesions affected the spacial memory of the avoidance responses, while lesions of the medial pallium impaired the emotional memory of fear (Portavella et al., 2004), similar to amygdaloid acute responses, referred to as “fight or flight” responses (Gökdemir et al., 2018). There is an overall progression on the understanding of the resemblances of cytoarchitecture in the actinopterygian and non-actinopterygian regions of the telencephalon. Alterations in emotional and social

behaviour as well as species specific capabilities to learn and memorise occur in the telencephalon of fish (Brown, 2006), which indicate that specialised systems of memory have been conserved throughout vertebrate evolution in these areas of the brain. In cleaner fish, learning and memorising of client interaction is necessary to both recognise hostile clients as well as the reoccurring “good” clients. Both size difference and the piscivorous nature of a client emphasise the necessity of a plastic ability to override common innate fear or flight responses. Such plastic change is observed among organisms that coexists with predators and is usually referred to as habituation.

Habituation

Habituation is a non-associative type of learning developing as an animal ceases to respond to an external stimulus which has no direct consequence for the animal itself (Rankin et al., 2009). The other type of non-associative learning is sensitisation, which causes an increased response opposite to habituation (Gerlai, 2020). Habituation is considered a basic and highly conserved form of learning, occurring within both short-term and long-term time perspectives (Rankin et al., 2009, Glanzman, 2009). Human experience habituation in everyday life situations which protects us against constant activation of the stress responses from environmental input (McDiarmid et al., 2019). Habituation is involved throughout ontogeny, from the plastic shaping of an infant’s brain or as therapeutic treatments of phobias in adults (Jeffrey and Cohen, 1971, Veltman et al., 2004). These external stimuli typically originate from emotional annoyance, while studies on habituation in fish are commonly linked to what we can observe as behavioural and physiological changes involved to sort out non-threatening factors. In contrast, being constantly vigilant and alerted is costly as physiological stress responses eventually cause a release and turnover of stored energy to fuel the organism (Wendelaar Bonga, 1997). As an adaptation to environmental risk assessment, involving sorting out the higher risks over the lower risks, animals go through a non-associative process with neurological plastic change in synaptic responses from sensory cues. While habituation can be determined through “simple” behavioural descriptions and function, e.g., reduction in tactile responses in an animal,

the underlying neurobiological mechanisms observed at a molecular level culminate to multiple factors occurring in the synaptic cleft between two neurons (McDiarmid et al., 2019). Recently, Randlett et al. (2019) highlighted that multiple circuit functions could be involved during habituation in zebrafish (*Danio rerio*) and revealed how habituation can be affected by molecular mechanisms involving both the dopaminergic and serotonergic systems. With today's technology and more sophisticated scientific tools, it has become clear that habituation is also indeed more sophisticated than previously determined (Dissegna et al., 2021). Habituation on a neurophysiological scale can provide insightful information on evolution and the natural selection of traits in mutualistic relationships.

Neurophysiological influence on cleaning behaviour

Neurotransmission is the basis of neuronal communication and is critical for normal brain development and function (Horzmann and Freeman, 2016). Neuronal communication includes both neurotransmitters and neuropeptides, which differ in that neurotransmitters are fast acting communicators occurring at axon terminals of presynaptic neurons while neuropeptides are slow acting communicators that are found all over the neuron (Liu et al., 2011). Neurotransmitters involved in emotional behaviours are the biogenic amines which include, in addition to many other biogenic substances, the catecholamines dopamine, adrenaline, noradrenaline and the indoleamine serotonin (Purves et al., 2001). In fish, these signalling molecules have been studied in context of social interaction (Miczek et al., 2002), reproduction (MOORE, 1992), stress (Øverli et al., 2005), dominance (Lepage et al., 2005), learning (Soares et al., 2016), memory (Messias et al., 2016a), feeding (de Pedro et al., 1998), arousal (Panula, 2010) and circadian rhythms (Burns et al., 2003). Within research on cleaner fish, the interest of biogenic amines has increased extensively during the last decade, as summarized in Soares (2017). Cleaner fish have highly developed social skills which are used to access ectoparasites, skin and nutrient rich mucus from clients. Recent studies have investigated the role of dopamine and serotonin in the bluestreak cleaner wrasse (*Labroides dimidiatus*). Dopamine is activated during social rewards,

and acts as a teaching signal linking the reward together with a specific environmental cue and enabling future prediction when the same cue reoccurs (Schultz, 2002). Pharmacological injections of dopamine receptor agonists improved the learning capacity during cue discrimination tasks in bluestreak cleaner wrasse (Messias et al., 2016b). Blocking the same receptors had implications on cleaning behaviour where individuals would spend more time negotiating through expression of physical contact with the client fish (Messias et al., 2016a). Also, the familiarization of a client would affect the behaviour of the cleaner fish when dopamine receptors were pharmacologically disrupted (Soares et al., 2017). Serotonin, which is involved in coordination of responses to social stress (Backström and Winberg, 2017) is also one of many regulators of cleaner fish cooperative behaviour. An injection with the serotonin 1A antagonist receptor, caused a delay in learning performances due to serotonin's role in inhibiting fear (Soares et al., 2016). The effect neurotransmitters have on cleaner fish and fish in general is conserved across the animal kingdom, and it is not unlikely that biogenic amines have similar functions in other species of cleaner fish. It was also of interest to study the relationship between neurotransmitters and habituation to initiate a novel research approach in a cleaner fish species relevant for the fish farm industry. What links neurotransmitters, habituation and brain functions together are the stress responses.

Stress physiology in fish

Wendelaar Bonga (1997) defines stress as “a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli”. Selye (1976) was broader in the definition of stress and emphasised that both pleasant and unpleasant stimuli can induce stress responses of different strengths. Homeostasis is the self-regulating predefined steady state of which internal conditions are maintained at a level that reflect the well-being of the animal (Cannon, 1929, Chrousos, 2009). In fish, stress in the context of social interaction usually starts after perception of intra- or interspecific cues in the surrounding environment, e.g., visual, olfactory, hydrodynamic, or auditory

cues (Brown and Magnavacca, 2003, Brown et al., 2000, Popper et al., 2019, Tuttle et al., 2019). The signal pathways activate both the hypothalamic-pituitary-interrenal (HPI) axis and the release of chromaffin tissue catecholamines enhancing circulation of glucose and oxygen, respectively, in preparation for a potential workload increase (Reid et al., 1998, Wendelaar Bonga, 1997). Such work is often interpreted in a detrimental context during lifesaving risk assessment, as discussed in both Barton (2002) and Mommsen et al. (1999). Nonetheless, HPI-axis activation is important in regulating other potential adaptive tasks including diurnal rhythm in fish (Lorenzi et al., 2008), osmoregulation (McCormick, 1995) or proactiveness in cleaner fish (Soares et al., 2012).

These examples, and descriptions mentioned above, are accommodated within the definition of primary stress responses, which occur during the first seconds, or minutes, after the initiation of a stressful encounter (Reid et al., 1998, Wendelaar Bonga, 1997). The secondary responses involve multiple factors which can have implications on the immune system, cellular ion levels in plasma and tissue and cause metabolic changes (Barton, 2002). When an animal, including both mammalian and non-mammalian, is exposed to repeated stressors, where the timespan in between is shorter than the time necessary to re-establish homeostasis, the outcome is a potential chronic state of stress. Chronic stress can have negative effects on overall physiological and behavioural mechanisms, including reduction in growth, reproduction, cognition, and potential survivability. Chronic stress is often referred to as type 2 allostatic overload, while preliminary stress responses associated with acute stress is referred to as type 1 allostatic overload (Schreck, 2010, McEwen and Wingfield, 2003). It is not always easy to classify based on the two categories as fish can exhibit individual variation to stressors (Vindas et al., 2017, Øverli et al., 2007). Also, more than one stressor can overlap at a given time, sequentially or in series (Schreck, 2010, Schreck, 2000).

Lumpfish

Ecology and biology

The lumpfish (*Cyclopterus lumpus*) is a cottoid teleost belonging to the family of Cyclopteridae and the order scorpaeniformes (Davenport and Thorsteinsson, 1990). The species is endemic to the North Atlantic, and adults are commonly observed in the upper 50-60 metres of the epipelagic zone associated with Atlantic water temperatures above 3 °C (Blacker, 1974, Eriksen et al., 2014). Genetic differentiation suggests five distinct groups located in the West, Mid-, and East Atlantic in addition to the English Channel and the Baltic Sea (Whittaker et al., 2018). Adults have sexually dimorphic growth where females are larger in both length and weight compared to males of the same age. The life cycle entails females seeking out a benthic spot (e.g., solid rock substrates) guarded by a single male to spawn (Davenport, 1985). During spawning, multiple females can attach their batches of eggs to the substrate which the male then fertilises and defends until hatching occurs (Goulet et al., 1986). Newly hatched juveniles continue to stay near shore for an uncertain amount of time, previously observed for at least the first few months latent in tidal pools (Moring, 2001), before migrating into pelagic seas to feed and mature (Kennedy et al., 2015). Juveniles have been observed attached to debris or floating seaweed, which can move individuals far off the spawning area and into pelagic waters (Ingólfsson, 2000).

Lumpfish have morphological adaptations to both open water swimming and life in a complex shore environment. While a ball-shaped body face hydrodynamic drawbacks, its height and width in addition to pointy skin tubercles make lumpfish a challenging meal. After hatching, juvenile fish can quickly attach to the substrate with a pelvic suction organ, or sucker disc evolved from the pelvic fins (Budney and Hall, 2010). The sucker disc provides a stationary resting state, which is useful during early ontogeny in a coastal environment of kelp and rocky substratum with strong currents (Davenport and Thorsteinsson, 1990). Davenport and Thorsteinsson (1990) also observed that individuals would move along a surface with the sucker disc merely touching the

substrate, adjusting their position without unnecessary swimming activity. Another distinctive feature is the lack of a swim bladder (Davenport and Kjørsvik, 1986). Considering that lumpfish can migrate over long distances, the advantage of neutral buoyancy is apparent. Instead of using a gas-filled swim bladder, the lumpfish maintains buoyancy with a dorsal crest containing a low-density subcutaneous jelly. In addition, a cartilaginous skeleton helps provide a neutral state of weightlessness with a density like that of seawater (Davenport and Kjørsvik, 1986). The skin is rough without scales and has horizontally organised lines of tubercles. The skin colouration, blood plasma colour and colouration of internal muscles or subcutaneous jelly can vary between young individuals (Davenport and Bradshaw, 1995). Especially phenotypic skin colour can be anything from brown, orange, blue or green with melanophore cells creating cryptic patterns. When reaching a size of 20-30 grams, lumpfish become more unison blue or blue green regardless of sex. During maturation, the male turns vivid red while the female remains blue (Davenport and Thorsteinsson, 1989). The blue-green colouration derives from the biochemical regulation of heme catabolism into biliverdin and the variation in colour have been suggested as a functional role in crypsis through colour changes based on substrate hue and colour (Davenport and Thorsteinsson, 1989).

A closer look on the nervous system of lumpfish revealed the lack of Mauthner cells (Hale, 2000). This specialised pair of nervous cell clusters found in the hindbrain of most fish is strongly involved in the startle flight response (Eaton et al., 1977, Korn and Faber, 2005). Still, lumpfish can express rapid escape responses, especially observed early in ontogeny, which suggests the existence of homologous structures with similar features as the Mauthner cells (Hale, 2000). Even with morphological adaptations to both benthic and pelagic life, multiple physiological responses show characteristics of a moderately active benthic species (Hvas et al., 2018). This includes a low aerobic scope, which is the difference observed between maximum and minimum aerobic metabolism (Fry, 1947) and a relatively low maximal sustainable swimming performance of 1.3-1.7 body lengths s^{-1} (Hvas et al., 2018). An additional important

aspect of environmental adaptations is the lumpfish intolerance for low oxygen saturation. Jørgensen et al. (2017) observed increased mortality rates on oxygen saturations close to 55 %, and an overall cortisol elevation, associated with disruption of the HPI-axis feedback system (Wendelaar Bonga, 1997), at 69 to 55 % saturation. Remen et al. (2022) observed similar elevations in plasma cortisol when two groups of lumpfish (15 and 60 g) were exposed to moderate, acute hypoxia (63 % O₂) at two different temperatures (5 and 12 °C). Considering that oxygen saturations can drop rapidly during summer in an intertidal environment, the benefit from the sucker disc is indeed favourable, providing a resting state and less aerobic or anaerobic effort. Lumpfish are tolerant to lower temperatures with an optimal temperature ranging from 16 to 8 °C, with larger and mature fish preferring the lowest temperatures on the scale (Hvas et al., 2018, Jonassen et al., 2018, Nytrø et al., 2014). Temperatures below 4 °C and above 18 °C have proven to increase mortality rates in confined tank experiments (Hvas et al., 2018, Imsland et al., 2018).

While physiological responses to environmental factors have been published in increasing numbers, research on lumpfish natural behaviour is scarce, and mostly related to spawning behaviour and migration (Davenport, 1985, Goulet, 1986, Holst, 1993, Ingolfsson and Kristjansson, 2002, Mitamura et al., 2012). Whether lumpfish perform cleaning behaviour and cleaning services in the ecosystem is uncertain. As for now, no such documentation exists, and observations of cleaning behaviour with Atlantic salmon origin from experiments in sea cages or experimental tanks (Imsland et al., 2014c, Imsland et al., 2019, Leclercq et al., 2018).



Figure 2 The lumpfish (*Cyclopterus lumpus*). © Fredrik Staven.

Lumpfish in aquaculture

Human interest and exploitation of lumpfish as a resource started with caviar production (Paradis et al., 1975, Holst, 1993). The chain of events that eventually led to an annual production of nearly 43 million lumpfish in 2019 in Norwegian aquaculture (Directorate of Fisheries, 2021) escalated after a peculiar observation at a fish farm site. During the late nineties, juvenile lumpfish were observed entering and swimming inside a salmon sea cage in Northern Norway (Anonymous, 1998). Upon inspection, the lumpfish stomach contents included ectoparasites (Willumsen, 2001). Later, F₁ generations of hatched and reared lumpfish, originating from wild parental brood fish, were assessed in duoculture with farmed Atlantic salmon. Cleaning efficacy was first reported in small-scale studies in Gildeskål, Norway (Imslund et al., 2014a, Imslund et al., 2014b, Imslund et al., 2014c, Imslund et al., 2015) and later in full commercial scale sea cages in both Norway and the Faroe Islands (Eliassen et al., 2018, Imslund et al., 2018).

In all experiments mentioned above, lumpfish reduced or delayed epidemic eruptions of ectoparasites. Nonetheless, individual variation and group variations were reported based on both investigations of stomach contents and differences in infestation rates between sea cages with and without lumpfish. According to data from commercial sea cages, Eliassen et al. (2018) recorded sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*) in 13.5% of 5,511 lumpfish examined, while Imsland et al. (2018) found a significant reduction in sea lice at different ecdysis life stages (chalimus, preadult, adult) compared to control groups. A later review article published by Overton et al. (2020) summarised 11 studies on the effect of cleaner fish (including both lumpfish and wrasses) in Atlantic salmon interaction. The authors concluded that there was overall promising evidence on the effect. Yet, more research and replications in commercial scale was suggested necessary to form a better conclusion on the effect lumpfish has on sea lice removal. Based on two commercial-scale studies, there is an agreement that sea lice consumption is related to lumpfish size, suggesting that sea lice count per lumpfish reduces when body weight increases (Eliassen et al., 2018, Imsland et al., 2016). However, this is only one of multiple variables that has or might have an impact on behaviour related to sea lice consumption.

With the lack of basal knowledge on the natural behaviour of lumpfish in the wild it is indeed difficult to know what one should expect when deciphering behavioural patterns in a confined aquaculture environment. A recent study on lumpfish personality revealed different behavioural strategies when exposed to a novel test object, and later to Atlantic salmon (Whittaker et al., 2021). While individual variation among conspecifics is common in teleosts, the implications of cleaner fish used in food production opens for further domestication based upon desired traits such as cleaning efficacy, pathological and stress resilience. With smaller individuals (40-140 g) being effective cleaners (Imsland et al., 2021), selection from genetically distinctive groups with slower growth could be of interest to the industry (Whittaker et al., 2018).

The main challenge related to the use of lumpfish is high mortality rates. A survey conducted in Norway in 2013, based on five fish farm sites and data from 79,000 lumpfish, reported in average 48% mortality during the Atlantic salmon production period (Imsland et al., 2020). In 2019, another survey based on interviews with 228 personnel from different fish farms estimated that lumpfish mortality was 10-20% after one month, 20-40% after three months and 60-80% during the whole production cycle (Størkersen and Amundsen, 2019). The Norwegian Food Safety Authority completed a two-year campaign to investigate the welfare of cleaner used in fish farming and the report concluded that if no actions were taken, the future use of cleaner fish is indefensible due to welfare and ethical reasons (Norwegian Food Safety Authority, 2020). In comparison, the average mortality in farmed Atlantic salmon was 15% in 2020 (Sommerset et al., 2021). The lumpfish is susceptible for different pathogens whereas atypical furunculosis was the most common disease observed at 51 fish farm sites in 2020 (Sommerset et al., 2021).

Since 2015 and the escalation on the use of lumpfish in aquaculture, demands for better housekeeping and welfare have emerged (Johannesen et al., 2018, Noble et al., 2019, Imsland et al., 2020, Gutierrez Rabadan et al., 2021, Geitung et al., 2020). The meaning of animal welfare is often up for debate, due to questions of definition and objectiveness. A general guideline is that animal welfare involves the freedom from hunger, thirst, discomfort, pain, injury, disease, fear, and distress (Farmed Animal Welfare Council, 1996). As with other animals used in food production, minor stress and disturbance have positive effects on factors such as growth, disease, and expression of normal behaviour (e.g., sea lice grazing) (Ashley, 2007). For lumpfish used in aquaculture, another challenge is how the species cope with environmental changes, especially when transferred from hatchery tanks with conspecifics only, to commercial sea cages (Powell et al., 2017). These environmental changes include rapid transition to various temperatures, currents, depths, feeding habits and the presence of a hundred thousand, or more, Atlantic salmon. While farmed Atlantic salmon is fed

upon saturation with pellet feed, a wild Atlantic salmon is nonetheless strictly piscivorous (Jacobsen and Hansen, 2001).

During a production cycle in aquaculture, a farmed Atlantic salmon reaches weights of typically 4 to 6 kg, accounting for a 200 to 300 times higher body weight compared to a 20 g lumpfish, which suggests that certain risk assessments could be involved. To approach a better understanding of lumpfish responses to client heterospecific interaction, it is necessary to evaluate what has been observed among other cleaner fish species in search of homologous patterns of behaviour and underlying physiological mechanisms. The first obvious comparison would be other species of cleaner fish, namely wrasses, used in Atlantic salmon farming. Unfortunately, no such data on stress responses from client interaction exist, which is noteworthy considering that wrasses have been used for the purpose for over three decades. Thus, the main source of comparable examples of heterospecific stress responses exists among cleaner fish in the wild. Here, most species appear unaffected upon observation, e.g., when removing parasites inside a predator's mouth cavity (Losey, 1979). Nonetheless, cleaner gobies with no previous historic interaction with a predator client did show elevations of plasma cortisol (Soares et al., 2012). Innate predator recognition and antipredator decision making are strong selective features in most prey animals. Nonetheless, the behavioural execution following an activation of the HPI-axis in the cleaner groupies, did instead increase predator interaction, quite opposite of the normal freeze or flight responses observed in prey fish exposed to predators (Soares et al., 2012). This emphasises the importance to investigate the plastic ability in the lumpfish to cope with the presence of Atlantic salmon.

Main objectives

This is a doctoral dissertation in collaboration between the Research Council of Norway, Nord University and Aqua Kompetanse AS. The intended outcome of the collaboration was to interpret and implement relevant findings into better practice in the aquaculture industry. The use of lumpfish in aquaculture has gradually led to increased focus on animal welfare. To improve the conditions and life in captivity for lumpfish, a major gamechanger may thus be necessary. This involves a better understanding of the underlying ability to cohabit with Atlantic salmon, and at the same time investigate the sole effect on behavioural and physiological responses from Atlantic salmon interaction alone. Without such basal knowledge, it is difficult to interpret the effects from other stressors that lumpfish are exposed to during a production cycle in commercial production at sea.

The main objective was to study how lumpfish adapt and tolerate a life in cohabitation with Atlantic salmon and the role of stress, habituation, acute- and long-term effects on behaviour and physiology. Below is a list of specific objectives the dissertation aimed to investigate:

1. How cohabitation with Atlantic salmon in commercial sea cages affected behaviour and stress responses in lumpfish, compared to naive individuals **(paper I)**
2. The effect from acute exposure to Atlantic salmon or salmon sensory cues **(paper II)**
3. How long-term cohabitation with Atlantic salmon affected stress and welfare parameters **(paper III)**
4. The role of brain neuromodulators in lumpfish during social interaction **(paper II and paper III)**
5. The effect on skin colouration and pigmentation in lumpfish from acute and long-term interaction with Atlantic salmon **(paper II and paper III)**

Material and methods

A total of three experiments were conducted as part of the thesis. Detailed descriptions for each specific experiment are given in the manuscripts (**paper I, II and III**). The following chapter presents the material and methods involved. This includes methods and techniques which were modified to fit the experiments and thus requires a more thorough description. Peer reviewed and published methods used, including high performance liquid chromatography (HPLC) of neurotransmitters and radioimmunoassay (RIA) of plasma cortisol, are described in the respective manuscripts. It is emphasised that **paper I** included data from an earlier research project conducted in 2016 (Staven, 2017). The other two experiments, described in **paper II** and **paper III**, were conducted in 2018. Common features for all three experiments were the use of lumpfish and Atlantic salmon studied in controlled tank experiments.

Study location

The study location of the experiment described in **paper I** was the barge fleet on the fish farm Raudøya (64°21'59.5" N, 10°26'40.9" E) in Osen, Trøndelag, Norway. The study location of the experiment described in **paper II** and **paper III** was Mørkvedbukta Research Station (67°16'41.9" N, 14°33'26.9" E) in Bodø, Norway. The main justification for the geographic spread was the difference in study design. In **paper I**, we aimed to investigate a group of lumpfish which had cohabited with Atlantic salmon in a commercial scale, namely "experienced" lumpfish, while **paper II** and **paper III** investigated "naive" lumpfish only, with no previous history of interaction with Atlantic salmon. The latter group could thus be collected directly from a local hatchery both in Trøndelag (Namdal Rensefisk AS) and in Bodø (Mørkvedbukta AS). Transferring experienced lumpfish, on the other hand, from a fish farm into a research facility increases the risk of disease transfer and was thus avoided by conducting the experiment at the fish farm location instead.

Experimental analysis

Behaviour

To understand why an animal behaves the way it does, observing behaviour and developing ethograms is essential at its core (Tinbergen, 1963). While fish behaviour sometimes seems balletic with nimble movements in a three-dimensional environment, patterns of phenotypic behaviours come to the fore when observed over time.

This requires both systematic mapping and often time-consuming analysis. There are several methods to study fish behaviour in a confined tank experiment, and it is sometimes challenging to choose the best suitable way to record sample points and to choose proper sample intervals (Martin and Bateson, 2018). The aim of the behavioural analysis was to standardise a method to distinguish different swimming activities. The choice of method was based on an ethogram previously used on wrasse (Tully et al., 1996) and lumpfish (Imsland et al., 2014c) in Atlantic salmon farming. By adding scores (1-4) based on activity levels (attached, hovering, swimming and burst swimming, respectively), it was possible to calculate the activity level in the tank in a conservative perspective, with minimal risk in misinterpreting expressed behaviours. While both **paper I** and **paper II** hypothesised the short-term effect both in relation to habituation (**paper I**) and acute stress responses (**paper I and II**), it was decided to record behaviour in a brief period prior to and after exposure to the specific treatment. In **paper I**, lumpfish behaviour was mapped every minute (more specific at the 60th second, the 120th second, and so forth) ten minutes before and ten minutes after Atlantic salmon were added to the experimental tank. In **paper II** the timeframe was extended to thirty minutes before and until thirty minutes after the introduction of Atlantic salmon or salmon sensory cues. The reason for the extension was to collect more data on behaviour before and after exposure and generate a longer window of interaction to the different treatments so physiological responses could be comparable to previous published work on neurotransmitters in fish (Gesto et al., 2013). In **paper I**, the

experiment produced a total number of 3,600 individual recordings (10 lumpfish times 18 repeated trials times 20 minutes of behavioural recordings), while in **paper II** this produced a total number of 8640 recordings (6 lumpfish times 24 trials times 60 minutes of behavioural recordings). In **paper III**, behavioural analysis was also assessed. Due to unsynchronised feeding during the video recording window, lumpfish behaviour was biased as lumpfish in the tanks with feeding at that point showed a persistent change in behaviour.

Analysis of skin colouration and pigmentation

In **paper II** and **paper III**, ImageJ software (Schindelin et al., 2015) was used to measure the mean levels of red, blue, and green (RGB) pixels and the ratio of black and white pixels (B:W ratio) in lumpfish skin. Data collected came from standardised images captured of each lumpfish before and after the different treatments. Both techniques were based on similar methodological approaches used on other species of fish or within human medicine (Amundsen et al., 2015, Eckmann et al., 2017, Houde, 1981, Touchon and Warkentin, 2008, Villafuerte and Negro, 1998). The methods were adjusted to fit the morphology of lumpfish and the experimental design. To assess RGB measurements, an area located at the lower lateral side was used. This area was the most homogeneous in skin structure and had little interference with melanophores or tubercles (Figure 3A). Davenport and Bradshaw (1995) used a ventral area posterior to the sucker disc to measure colouration, mainly because of the intention of the study design which involved measurements of colour change from the substrate underneath the lumpfish.

Like RGB measurements and its effect from interaction with Atlantic salmon, we investigated if skin pigmentation could be analysed using the same tools, e.g., images captured before and after and the software ImageJ. While RGB analysis involved a histogram where mean levels of each colour were counted as pixels within the defined area, skin darkening analysis had a different approach. The whole lateral view of the lumpfish was used, mainly because of the variation in melanophore dispersal in the

skin and an accumulation of pigmental darkening towards the dorsal area. By applying a standardised adjustment of the threshold level in ImageJ, set to 35, the already dark melanophore skin pattern would appear as dark pixels. Using binary 8-bit type image adjustments, all other pixels outside the threshold range appeared white (Figure 3B). Next, the dark and white pixel counts were used to estimate the ratio of black pigmentation in the skin.

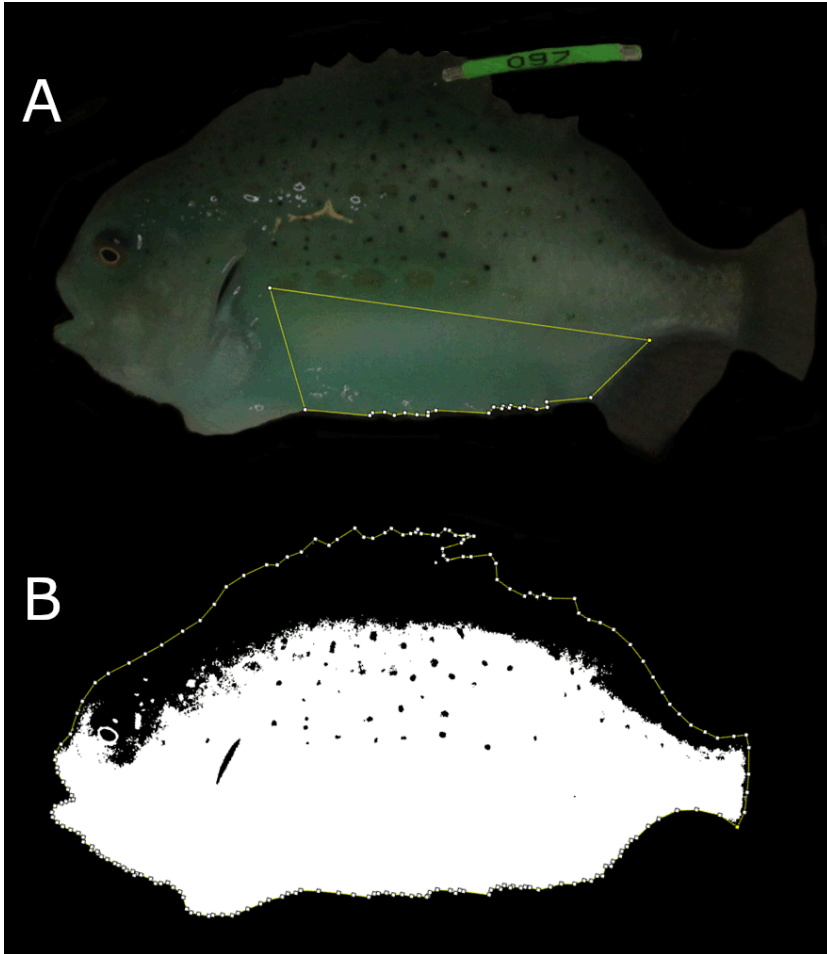


Figure 3 The upper image (A) shows the defined homogenous area used to calculate mean red, green and blue pixels (mean R+G+B), while the lower image (B) shows the defined area used to calculate pigmentation as the ratio of black and white pixels (B:W). Both colour and pigmentation were measured using ImageJ. Image is from **paper II**.

Summary of papers and abstract

Paper I

Staven, F. R., Nordeide, J. T., Imsland, A. K., Andersen, P., Iversen, N. S., and Kristensen, T. (2019). Is Habituation measurable in lumpfish *Cyclopterus lumpus* when used as cleaner fish in Atlantic salmon *Salmo salar* Aquaculture? *Front Vet Sci*, 6: 227. doi:10.3389/fvets.2019.00227

Abstract

To investigate how lumpfish interact in Atlantic salmon aquaculture, physiological stress responses and changes in behaviour were analysed in experienced and naive lumpfish. Experienced lumpfish (30.2 ± 7.93 g, mean \pm SD) coexisted with a commercial scale production unit of Atlantic salmon (1258.5 ± 152.12 g) for 30 to 60 days, while naive lumpfish (38.2 ± 12.37 g) were kept with conspecifics only. Ten trials from each background were assessed. For each trial, ten lumpfish were tagged and transferred to a video monitored experimental tank (2 x 2 x 0.7 m). In each trial, swimming behaviour was mapped for all lumpfish every 60 seconds in 20 minutes, ten minutes before and ten minutes after the introduction of four Atlantic salmon. Naive lumpfish expressed significantly increased burst swimming activity and maintained longer interspecific distance to Atlantic salmon in comparison with experienced fish. In addition, mean plasma cortisol levels were significantly elevated in naive fish after exposure to Atlantic salmon. We argue that naive lumpfish expressed innate physiological and behavioural stress responses during first encounter with Atlantic salmon, while reduced responses in experienced individuals indicated habituation. The effect from behavioural and physiological stress in newly deployed naive lumpfish - before and during habituation - should be taken account for when lumpfish are introduced in commercial sea cages to improve welfare for the species. In addition, we suggest that habituation could be applicable during the rearing phase to moderate the transition from a simple tank environment with conspecifics only to interspecies interaction with Atlantic salmon in sea cages.

Paper II

Staven, F. R., Nordeide, J. T., Gesto, M., Andersen, P., Patel, D. M., Kristensen, T. (2021). Behavioural and physiological responses of lumpfish (*Cyclopterus lumpus*) exposed to Atlantic salmon (*Salmo salar*) sensory cues. *Aquaculture*, 544: 737066. doi: 10.1016/j.aquaculture.2021.737066

Abstract

First interaction with carnivorous clients induces stress responses even in the boldest of cleaner fish 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. \pm 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 could be affected by salmon interaction, yet deployment of naive juvenile lumpfish with small Atlantic salmon is preferable based on the overall mild stress responses.

Paper III

Staven, F. R., Gesto, M., Iversen, M.H., Andersen, P., Patel, D. M., Nordeide, J. T., Kristensen, T. (2021). Cohabitation with Atlantic salmon (*Salmo salar*) affects brain neuromodulators but not welfare indicators in lumpfish (*Cyclopterus lumpus*). *Front Physiol*, [Accepted 21 Jan 2021]

Abstract

Lumpfish are utilized to combat ectoparasitic epidemics in salmon farming. Research gaps on both cleaning behaviour and client preferences in a natural environment, emphasizes the need to investigate the physiological impacts on lumpfish during cohabitation with carnivorous Atlantic salmon. Lumpfish (39.9 g, S.D \pm 8.98) were arranged in duplicate tanks (n = 40 per treatment) and exposed to Live Atlantic salmon (245.7 g, S.D \pm 25.05), salmon Olfaction or lifelike salmon Models for 6 weeks. Growth and health scores were measured every second week. In addition, the final sampling included measurements of neuromodulators, body colour and plasma cortisol. A stimulation and suppression test of the hypothalamic-pituitary-interrenal (HPI) axis was used for chronic stress assessment. Results showed that growth, health scores and body colour remained unaffected by treatments. Significant reductions in levels of brain dopamine and norepinephrine were observed in Live compared to Control. Plasma cortisol was low in all treatments, while the stimulation and suppression test of the HPI-axis revealed no indications of chronic stress. This study presents novel findings on the impact on neuromodulators from Atlantic salmon interaction in the lumpfish brain. We argue that the downregulation of dopamine and norepinephrine indicate plastic adjustments to cohabitation with no negative effect on the species. This is in accordance with no observed deviations in welfare measurements, including growth, health scores, body colour and stress. We conclude that exposure to salmon or salmon cues did not impact the welfare of the species in our laboratory setup, and that neuromodulators are affected by heterospecific interaction.

General discussion

Overview

This thesis aimed to identify innate responses and habituation in lumpfish when exposed to Atlantic salmon or salmon sensory cues. During commercial use in sea cages, lumpfish are subjected to handling and unavoidable stressors that can affect, and potentially activate the hypothalamic-pituitary interrenal (HPI) axis (Wendelaar Bonga, 1997). One such factor is the interaction with piscivorous Atlantic salmon, yet few studies have focused on the physiological and behavioural effects of interaction with Atlantic salmon alone.

While all three scientific papers assessed the treatment effects on lumpfish, each paper had a different approach. The experimental work of the thesis followed a timeline initiated with the investigation of general behavioural and physiological responses in association with habituation (**paper I**), to the study of acute physiological and behavioural responses from specific salmon sensory cues (**paper II**). In comparison with **paper II**, **paper III** did assess the long-term effect of exposure to Atlantic salmon or salmon sensory cues. **Paper III** opened for the possibility to survey the effect on welfare indicators, including operational welfare indicators (OWIs) and growth performance. Further, **paper III** included measurements of neuromodulators and their involvement in social behaviour and responses to different salmon sensory cues. Based on field observations in **paper I**, new questions came to mind. This involved the role of skin pigmentation and darkening, and its relationship to external stimulus. While previous studies of colour change in lumpfish were related to sexual ornamentation or surface colour adaptation (Davenport and Thorsteinsson, 1989, Davenport and Bradshaw, 1995), **paper II** and **paper III** assessed the effect from heterospecific interaction and exposure to salmon sensory cues. In the next subchapters, the different parameters involved in all three papers are discussed.

Predator adaptation, behaviour, and habituation

Naive lumpfish revealed strong behavioural responses in swimming activity and avoidance during the first encounter with large Atlantic salmon (**paper I**) and smaller Atlantic salmon, in addition to salmon olfactory cues (**paper II**). Experienced fish revealed a weaker acute response upon reintroduction (**paper I**). The behavioural acute responses were as expected from the perspective of innate predator recognition in fish (Berejikian et al., 2003, Oulton et al., 2013). Lumpfish as a prey species have two fundamental optional strategies available to cope with potential predator fish. These strategies are known as behavioural modification, which involves escape and avoidance, or morphological defences which are the bodily structures that reduce the likelihood of becoming someone's meal (Abrahams, 1995). A cross section of the lumpfish body reveals a pentagon shape with a flat ventral side and an angled dorsal roof. The skin has diverse colour patterns and contains multiple horizontal lines of skin tubercles. Structure, cryptic colouration, and pointy tubercles suggest that the main strategy of the species is morphological defences against predators (Chivers et al., 2007). This is important to consider when lumpfish behaviour is studied as there is no data on lumpfish interaction with predators or carnivorous fish in nature. Prey fish with morphological defences express little behavioural modification in terms of escape and avoidance (Abrahams, 1995), which coincides with the reduction in swimming activity observed after a brief period of Atlantic salmon interaction, as described in **paper I** and **paper II**.

Nonetheless, the selective force against predators could have affected the innate stress responses of lumpfish over the timespan in which the two species have coexisted in the same environment. These responses were both measured as behavioural change but also HPI-axis activation and elevations of plasma cortisol. Such innate responses, especially in naive juvenile individuals, have been observed among other species of cleaner fish (Soares et al., 2012, Gingins et al., 2017). The observations of different behavioural swimming activity based on the lumpfish's earlier history of heterospecific

interaction suggest that innate responses are still present in an acute phase (**paper I** and **paper II**) and diminish after a period of interaction (**paper III** and in the experienced lumpfish used in **paper I**).

Scoring and generalisation of swimming activity based on four conservative categories (attached, hovering, normal swimming and burst swimming) is challenging since lumpfish can switch between mobile escape performance (flight) and stationary strategies (freeze). The sucker disc provides lumpfish with the ability to quickly attach to a substrate which adds a passive behavioural tactic to their portfolio (Davenport and Thorsteinsson, 1990). Thus, exposure to potential risks and stress-inducing external stimuli can have different strategic outcomes. While some individuals remain attached after the exposure to Atlantic salmon or salmon cues, the overall picture revealed that most lumpfish increased their swimming activity. These individuals would detach from the tank surface, or depart from a hovering state, and become vigilant after the introduction of the different treatments. These results demonstrate that there is not one specific response to a potential stressor, which coincides with individual variation observed among actinopterygians (Brown and Bibost, 2014, Rupia et al., 2016).

Studies involving the question of habituation in other species of fish do not paint a clear picture of the potential outcome of exposure to repeated stressors in lumpfish. While other species such as catfish (*Rhamdia quelen*) and Senegalese sole (*Solea senegalensis*) were not susceptible to habituation (Barcellos et al., 2006, Conde-Sieira et al., 2018, Koakoski et al., 2013), Atlantic salmon, Eurasian perch (*Perca fluviatilis*) and common roach (*Rutilus rutilus*) did habituate to external stressors (Johansson et al., 2016, Madaro et al., 2016). The variation observed among different life history strategies, either as a sluggish or a fast-swimming fish, has an impact on the species' adaptation to repeated stressors. While the thesis provides novel insight into aspects of habituation to Atlantic salmon, experienced lumpfish in **paper I** were also exposed to other variables, since these individuals were collected from a commercial sea cage prior to the experiment. In addition, **paper III** did not use the same method of

reintroducing Atlantic salmon as in **paper I**. While both behavioural and physiological parameters, in a simplified matter, revealed weaker responses over time or upon salmon reintroduction, the underlying biomechanical complexity of habituation was not investigated in the study. Neither should these data be used as an overall justification to expose lumpfish to other stressors without precautions, due to the risk of type 2 allostatic overload. In **paper II**, the salmon olfactory treatment induced an acute behavioural response in lumpfish, in comparison with visual cues from salmon models. The strong innate response in naive individuals would support previous work on how predator recognition evolves among prey and predators living in the same environment (Gall and Mathis, 2010). While a prey fish uses olfactory cues to recognise and generalise predators (Ferrari et al., 2007), the initial acute response in lumpfish seemed to gradually dissipate through cognitive adaptations. With the loss of behavioural information during the long-term study in **paper III**, we cannot conclude at which time point habituation is measurable in physiological and behavioural change, only that an effect is present after thirty days of interaction with Atlantic salmon at sea, or after six weeks in a tank environment and that welfare is not impeded, except during the first minutes of cohabitation.

Physiological aspects of stress responses

To investigate heterospecific stress responses in lumpfish, measurements of circulating plasma cortisol were assessed. Plasma cortisol is the main corticosteroid involved in the primary stress response in fish (Wendelaar Bonga, 1997). While most fish share common HPI-axis physiological structure, the response itself, measured as plasma cortisol concentrations, can vary among individuals (Vindas et al., 2017), populations (Brown et al., 2005) and species (Mommsen et al., 1999). This also applies to the difference in previously mentioned life strategies, where slow-moving species with crypsis may express such adaptations to keep energy expenditure low (Vijayan and Moon, 1994). A species such as the lumpfish, with its morphological defences, shows a weaker stress response in comparison with a species that might depend on speed

and higher energy expenditure. When comparing plasma cortisol measured one hour post stress test in different fish species, Iversen et al. (2015) observed concentrations of 200 nM in lumpfish, which was similar to Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). In contrast, Atlantic salmon and ballan wrasse (*Labrus bergylta*) had concentrations at 625 nM and 750 nM, respectively. In **paper I** and **paper II**, measurements of cortisol sampled one hour after introduction of the different heterospecific treatments revealed elevations in the blood plasma where the highest mean values occurred among naive lumpfish exposed to Atlantic salmon (>1200g). Here, mean levels among all naive fish was 115 nmol L⁻¹ while mean level among all experienced lumpfish was 58 nmol L⁻¹. In addition, no naive lumpfish had plasma cortisol levels below 15 nmol L⁻¹, while 27 of 100 experienced fish had levels below the detection limit of 1.68 nmol L⁻¹.

While measurements of plasma cortisol in lumpfish entails a narrower range in comparison with other species, the results revealed a significant difference between the two groups, which suggests that the method is applicable. In **paper II**, exposure to small Atlantic salmon (<200g) and salmon sensory cues caused plasma cortisol levels to elevate to 60-64 nmol L⁻¹, while mean level in the control group was 45 nmol L⁻¹. In comparison with (Iversen et al., 2015) and (Hvas et al., 2018), which involved tapping out the tank water until the dorsal crest/fin was aerated and through exhaustive exercise, respectively, measurements suggested that treatment exposure did not elicit a strong response. In **paper III**, the long-term interaction with the same sensory cues and Atlantic salmon of the same size as **paper II**, had minor impacts on plasma cortisol levels. Surprisingly, the lowest measurements were observed in lumpfish cohabiting with Atlantic salmon (3 nmol L⁻¹), while model salmon and olfaction measurements were 11 and 12 nmol L⁻¹, respectively. The control group had mean levels of 26 nmol L⁻¹. It should be noted that these measurements did not differ significantly and varied within a range observed in control groups in other tank experiments (Hvas and Oppedal, 2019, Jørgensen et al., 2017). The stimulation and suppression test of the HPI-axis showed no indication of chronic stress in these treatments, which coincided

with growth performance, operational welfare indicators and the findings in **paper I** and **paper II**.

Cleaner fish neurochemistry

The thesis involved analysis of dopaminergic and serotonergic activity in the lumpfish telencephalon (**paper II**) and dopaminergic and serotonergic activity, in addition to measurement of noradrenaline, in left brain hemispheres (**paper III**). The aim was to assess biogenic amines due to their role in cognitive functions (Broglia et al., 2003) including social engagement (Abreu et al., 2018). In the study on long-term interaction, right brain hemispheres were assigned to a different study on transcriptome analysis not involved in this work. Nevertheless, it was decided to use remaining half brains and investigate the effect from Atlantic salmon interaction on neurotransmitters in the brain hemisphere regardless of the limitations it caused on data comparison between **paper II** and **paper III**.

Recent findings have revealed how cleaner fish cognitive performance in social settings is regulated by certain biogenic amines (de Abreu et al., 2020, de Abreu et al., 2018, Messias et al., 2016a, Messias et al., 2016b, Soares et al., 2017). Both serotonin and dopamine impact learning and memory in the bluestreak cleaner wrasses (Messias et al., 2016b, Soares et al., 2016) and the serotonergic system is also involved in social behaviour and regulation of stress responses in other species of fish (Winberg and Thörnqvist, 2016). The acute effect on lumpfish from Atlantic salmon interaction or salmon sensory cues did not impact the concentrations of biogenic amines in the telencephalon (**paper II**). While significant positive correlations were observed between serotonin and its metabolite in all treatments, but not in the control groups, the concentrations of either quantity alone did not deviate between treatments and control. Large individual variation was observed in terms of serotonergic activity also in the control group. As discussed in **paper II**, this could be a result of the formation of social hierarchies in the tank environment (Cubitt et al., 2008, Loveland et al., 2014, Morandini et al., 2019). Also, the fact that lumpfish originated from wild captured

parents could help explain some of the overall variations observed. The concentrations of serotonin and its metabolite measured in the telencephalon (**paper II**) accounted for half of the concentrations measured in left brain hemispheres (**paper III**). While such a comparison should be interpreted with caution due to the use of different brain regions, they provide the first measurements of biogenic amines in the lumpfish brain, and in the telencephalon, during a resting state and in a social context.

Noradrenaline is mostly associated with acute stress responses (Reid et al., 1998). Importantly, inter- and intraspecies variation do occur, for instance as lower means of noradrenalin and signal strength in the HPI-axis in proactive individuals (Alfonso et al., 2020). In **paper III**, both noradrenaline and dopamine mean measurements were lower when lumpfish cohabited with Atlantic salmon for six weeks. While the underlying mechanisms for these observations were not determined, the lower concentrations were most likely a result of heterospecific interaction. In **paper I**, experienced lumpfish showed indications of habituation of the HPI-axis upon reintroduction with Atlantic salmon. The link between acute stress and release of noradrenaline is well documented (Reid et al., 1998), and a reduction in both dopamine and noradrenaline concentrations could be a similar indication of habituation only at a neuronal level. Nonetheless, neither **paper II** or **paper III** involved reintroduction with Atlantic salmon and it cannot be determined that the observed effect was a cause of habituation alone. Interestingly, elevated concentration of noradrenaline can increase the overall time spent hiding or avoiding risks (Blazevic et al., 2020), and the significant decrease observed in lumpfish could have implications on the likelihood of heterospecific interaction, and eventually sea lice grazing.

Skin colouration and pigmentation

Earlier observations of lumpfish used in aquaculture show variation in visual skin colour, and the thesis aimed to measure the effect from Atlantic salmon interaction. Both **paper I** and **paper II** revealed that such interactions impact the HPI-axis, neurotransmitters, and behaviour, and would provide a common setting that lumpfish

are put into during duoculture. Considering that lumpfish can quickly change colour based on environmental changes, e.g., substrate crypsis (Davenport and Bradshaw, 1995), it was of importance to measure the effect from salmon cues. While the underlying biochemical mechanism were not studied, any visual change in skin colour would be a straightforward and non-invasive indicator of stress. This method could apply a novel clinical measurement in the toolbox of operational welfare indicators (Gutierrez Rabadan et al., 2021, Imsland et al., 2020, Noble et al., 2019). The regulation of blue-green colouration in lumpfish has previously been associated to be a by-product of heme catabolism, named biliverdin (Davenport and Bradshaw, 1995, Mudge and Davenport, 1986). Among other species of marine fish, biliverdin is also the suggested explanation for blue-green skin and plasma colouration, yet the ecological or physiological aspects of the role of biliverdin is ambiguous and not unison among the different species studied (Fang and Bada, 1990).

The relationship between skin darkening and disruption of the resting state has previously been seen in other marine fish. Skin darkening has been observed in relationship with elevated cortisol levels of larval Senegalese sole (*Solea senegalensis*) (Ruane et al., 2005) and red porgy (*Pagrus pagrus*) (Van der Salm et al., 2004). Selective breeding on salmonid strains based on HPI-axis responsiveness, including Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), showed that individuals with lower stress responses had increased numbers of black skin spots while individuals with higher stress responses had fewer numbers of black skin spots (Kittilsen et al., 2009). Also, Aspengren et al. (2003) found that elevations of noradrenaline, associated with acute stress responses (Reid et al., 1998), caused melanosome to aggregate in Atlantic cod (*Gadus morhua*) dorsal fin monolayers. Overall, the importance of morphological change in darkening in response to behavioural and physiological changes was relevant, as lumpfish were introduced to different stressors and measured by means of behaviour, physiological stress and neuromodulators associated with stress and/or social interaction.

Concluding remarks

Conclusion

The research presented in the following thesis reveals new insights on how lumpfish respond, interact and habituate to interspecific interaction with Atlantic salmon. The main objective was to study behavioural responses and the underlying physiological mechanisms involved over a series of three **papers**. **Paper I** focused on the effect from cohabitation in full-scale sea cages and the innate responses in naive fish. **Paper II** focused on the acute phase of interaction in addition to the solely responses from specific salmon sensory cues, while **paper III** focused on the long-term effect and welfare implications from Atlantic salmon interaction or the different sensory cues. Based on complementary observations on swimming activity in **paper I** and **paper II**, it is concluded that naive lumpfish show innate responses to the presence of Atlantic salmon seen as increased swimming activity and distancing. These behavioural responses diminished with time and were not observed in the case where lumpfish had cohabitated with Atlantic salmon in a commercial sea cage for thirty days (**paper I**). In **paper II**, exposure to different sensory cues revealed that salmon olfaction affected swimming activity more than exposure to live Atlantic salmon and salmon visual models. While a visual inspection of the live Atlantic salmon provided cognitive information about the relatively small size and gape limitation of the client, olfaction alone did not. This suggest that lumpfish depend on multiple cues as part of a risk assessment and reflects innate evolutionary responses to Atlantic salmon olfaction due to shared habitat. Within the time scope of acute stress, a detectable change in skin colouration and hue can be observed. Effects on the HPI-axis coincided with behavioural responses. Plasma cortisol was elevated in naive fish during first exposure to large Atlantic salmon but did not differ from control in experienced lumpfish or during interaction with smaller Atlantic salmon. Long-term interaction did not instigate a type 2 allostatic overload (chronic stress response) which was in accordance with both good growth performance and no negative impact on operational welfare

indicators (**paper III**). The thesis presents novel findings on the impact on neuromodulators from interspecific interaction. While acute responses affected the serotonergic system, long-term interaction altered brain regulation of both dopamine and noradrenaline. It is concluded that these data provide a deeper understanding of the underlying neurobiological implication on lumpfish biology. It is concluded that lumpfish have innate acute responses to Atlantic salmon cues which diminish through plastic change and habituation. Indications of habituation is observed in behaviour, change in HPI-axis, brain neurotransmitters and skin colouration. The overall conclusion is that Atlantic salmon as a single variable has little negative influence on juvenile lumpfish, considering the plastic changes observed in behavioural and physiological factors involved in the study.

Future perspectives

The thesis describes how lumpfish interact with Atlantic salmon. Yet, this is only one of many variables present in a commercial sea cage. Future studies should focus on other stressors in commercial aquaculture and their impact on lumpfish welfare. Reducing the overall load of stress factors could have a positive influence on cleaning efficacy and disease development in lumpfish. The link between stress and immunology should gain more attention especially in association with seasonal variations in abiotic factors. Both controlled tank experiments and commercial scale experiments have been conducted in increasing numbers, which can supply the industry with a new understanding on how to facilitate optimal conditions for lumpfish in duoculture. The future use of cleaner fish will depend on whether the aquaculture industry is able to reduce lumpfish mortality to a number acceptable from governmental, public, and ethical aspects. This will require that the ongoing collaboration between research and hands-on aquaculture expertise is maintained.

Practical implications

The concept of an industrial Ph.D. involves the contribution of research into practical use. This includes information which could be beneficial for the future understanding of lumpfish in aquaculture. The thesis involves research mostly from a laboratory environment, yet certain aspects of social interaction and general behavioural and physiological findings could be applicable in commercial scale use. Suggestions based on the thesis are listed below:

- Deploying lumpfish with larger Atlantic salmon (1000-2000 g) might induce stronger acute stress responses in lumpfish compared to small Atlantic salmon (100-200 g).
- Expected duration of habituation should be accounted for prior to deployment in a sea cage.
- Habituation could delay cleaning efficacy. Expect three to four weeks before cleaning efficacy is optimal.
- Individual variation in stress responses and social behaviour could be useful during selective breeding on future families of domesticated lumpfish.
- Change in colouration and pigmentation from external novel cues could be applied as a non-invasive clinical indicator of internal stress levels in lumpfish.
- Burst swimming activity related to acute stress responses is an indicator of acute stress.
- Growth performance observed while in duoculture under controlled conditions provides a basal growth curve, which can be used to compare growth performance in commercial scale sea pens.
- Change in neuromodulators is impacted by external stimuli, both as acute stress and probably as habituation, and could be used in future studies on social interaction in lumpfish.

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Paper I

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Is Habituation Measurable in Lumpfish *Cyclopterus lumpus* When Used as Cleaner Fish in Atlantic Salmon *Salmo salar* Aquaculture?

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To investigate how lumpfish interact in Atlantic salmon aquaculture, physiological stress responses and changes in behaviour were analysed in experienced and naive lumpfish. Experienced lumpfish (30.2 ± 7.93 g, mean \pm SD) coexisted with a commercial scale production unit of Atlantic salmon (1258.5 ± 152.12 g) for 30 to 60 days, while naive lumpfish (38.2 ± 12.37 g) were kept with conspecifics only. Ten trials from each background were tested. For each trial, 10 lumpfish were tagged and transferred to a video monitored experimental tank ($2 \times 2 \times 0.7$ m). In each trial, swimming behaviour was mapped for all lumpfish every 60 s in 20 min, 10 min before, and 10 min after the introduction of four Atlantic salmon. Naive lumpfish expressed significantly increased burst swimming activity and maintained longer interspecific distance to Atlantic salmon in comparison with experienced fish. In addition, mean plasma cortisol levels were significantly elevated in naive fish after exposure to Atlantic salmon. We argue that naive lumpfish expressed innate physiological and behavioural stress responses during first encounter with Atlantic salmon, while reduced responses in experienced individuals indicated habituation. The effect from behavioural and physiological stress in newly deployed naive lumpfish—before and during habituation—should be taken account for when lumpfish are introduced in commercial sea cages to improve welfare for the species. In addition, we suggest that habituation could be applicable during the rearing phase to moderate the transition from a simple tank environment with conspecifics only to interspecies interaction with Atlantic salmon in sea cages.

Keywords: lumpfish, Atlantic salmon, interaction, stress, habituation

INTRODUCTION

The main limitation for further growth in Norwegian aquaculture of Atlantic salmon is the ectoparasitic sea lice copepods *Lepeophtheirus salmonis* Krøyer, 1837 and *Caligus elongatus* von Nordmann 1832. Sea lice have a negative impact on animal welfare, economic costs, public reputation, and on wild populations of salmonids (1–3). A complete method for sea lice removal has remained yet to be discovered, and the aquaculture industry has been dependent on a toolbox with multiple treatment methods, some more successful than others (4). In contrast to chemotherapeutic

and mechanical treatments, the use of cleaner fish is a considerably less stressful and a more sustainable delousing method concerning mortality and welfare of the farmed Atlantic salmon (5–7). On the other hand, animal welfare of cleaner fish has become a new moral challenge for the industry, as uncontrolled loss of cleaner fish, assumed dead during the production period of Atlantic salmon could be considered unethical use of live animals.

Cleaner fish are usually small fish species from different taxa that remove ectoparasites, dead skin or mucus from a larger host “client” fish (8–10). The interspecific relationship resembles a unique mutualistic interaction that benefits both parts. The cleaner collects an ectoparasitic food item from a potential killer, while the client is relieved from parasitic pressure without falling for the temptation of eating the cleaner fish (9, 11). In Norway, four endemic species of cleaner wrasses have been captured and implemented in aquaculture (12–16). In recent years, the lumpfish (or lumpsucker) *Cyclopterus lumpus* Linnaeus, 1758 has been systematically tested as a possible sea lice grazer (17–19), especially at cold sea water temperatures, where the use of wrasse is ineffective (20). Studies conducted in Northern Norway found significantly reduced sea lice infestations on Atlantic salmon when reared together with 5, 10, or 15% lumpfish, with removal of up to 93–97% adult female stages of sea lice in comparison with control groups (17–19, 21). From 2012 to 2016, the production of hatched, reared, and deployed juvenile lumpfish increased from a few thousands to 30 million, which by 2019 made it the second most reared fish species in Norway (22).

During the transition from hatchery to sea cages, lumpfish are put through a series of stressors, including the introduction to Atlantic salmon. While domesticated Atlantic salmon are presumed uninterested in other food items than pellets, their carnivorous nature include consumption of fish of similar size as juvenile lumpfish (23). Nature selects swiftly upon individuals with deficient innate escape performance during predatory encounters, and even obligate cleaner fish, including bluestreak cleaner wrasse *Labroides dimidiatus* Valenciennes, 1839 express such performance when interacting with predatory clients, especially during first encounter (24). Prey fishes first encounter with carnivores triggers behavioural rapid burst swimming (25–27) and predatory avoidance (28–30). First encounters may also lead to physiological responses with increased plasma cortisol levels which promote an increased energy budget from plasma glucose to use for escape and overall vigilance (31, 32). In other teleosts, exposure to stressors which cannot be avoided, has previously revealed behavioural changes in place preference, locomotion style and swim speed (33), as well as impacts on cognitive decision-making, which could result in less time spent on feeding and resting (29, 34), and in the case for aquaculture cleaner fish, less time spend grazing sea lice. Repeated stressors have also shown to desensitize fish and moderate neuroendocrine responses to similar stressors (35), commonly referred to as habituation (36). Habituation, measured as desensitized neuroendocrine responses, for example reduced cortisol secretion during repeated predatory interactions, has previously been demonstrated in model species zebrafish *Danio rerio* Hamilton, 1822 (37). Habituation is considered adaptive

when the individual cease to respond to repeated stimuli that has no direct consequence (38). In the light of an increased use of lumpfish in aquaculture, information about the species ability to cope and adapt to the presence of Atlantic salmon is of interest, and available for investigation.

The aim of the study was to examine how lumpfish with experience from living in an open sea cage with farmed Atlantic salmon for 30–60 days expressed measurable changes in behaviour and physiological responses compared to first encounter for naive lumpfish. This emphasises a relatively broad question whether behavioural and physiological responses are affected by interactions with client fish, in this case Atlantic salmon. It was hypothesized that experienced lumpfish would express habituation when reintroduced with Atlantic salmon measured as (1) lower swimming activity, (2) shorter interspecies distance, and (3) lower plasma cortisol levels compared to naive lumpfish.

MATERIALS AND METHODS

Ethical Statement

The study was conducted according to the Animal Welfare Act (LOV-2009-06-19-97) and the Norwegian law on Regulation of Animal Experimentation (FOR-1996-01-15-23). Handling of live fish was managed by personnel with FELASA-C course, based on the policies by the Federation of European Laboratory Animal Science Association. Lumpfish and Atlantic salmon used during the field experiment were assigned to project FDU 7835, accepted by the Norwegian Food Safety Authority under the regulation of the Research Animal Act (FOR-2015-06-18-761).

Lumpfish

All lumpfish used during this study originated from gametes extracted from sexually mature wild individuals (7 males and 24 females) caught with gill nets in Flatanger (64°30′20.4″N, 10°50′40.8″E), Norway in September 2015. Fertilized eggs were distributed between Namdal Rensefisk in Flatanger and Nordland Rensefisk at Lovund (66°22′01.1″N, 12°22′36.4″E). At Namdal Rensefisk, incubation started during the 1st week of October, while at Lovund, incubation started during the 1st week of November. Larvae hatched from November 15 at Namdal Rensefisk and from December 18 at Lovund Rensefisk. At both locations, feeding consisted of dry feed pellets. During the first 2 months, all 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, lumpfish were gradually fed with Gemma Diamond 0.8, 1.5, Silk 1.5, and 1.8 mm (Skretting, Stavanger, Norway) following feeding recommendations from Skretting AS. All lumpfish were reared in circular green tanks measuring 2.5 m³ and later moved to 5.5 m³ tanks during the last month prior to departure. At both locations, daily monitored oxygen saturation was above 80%. At Lovund Rensefisk, mean water temperature was 8.0°C, S.D. ± 0.95, with T_{max} 9.5°C and T_{min} 7.5°C. At Namdal Rensefisk, mean water temperature was 7.4°C, S.D. ± 0.95, with T_{max} 9.6°C, and T_{min} 6.3°C. All lumpfish, both at Nordland rensesk and Namdal rensesk, were manually

vaccinated with AMarine micro 4-2[®] (Pharmaq, Overhalla, Norway) during the 1st week of June.

Atlantic Salmon

All Atlantic salmon originated from the AquaGen strain, hatched and reared at Flatanger Settefisk, Trøndelag, Norway. Smolt were deployed in October 2015 and reared in sea cages, 135 m in diameter, until the experiment started at Raudøya (64°21'59.5" N, 10°26'40.9" E) on August 2, 2016. Atlantic salmon ($n = 80$) later selected to the experiment, had a mean weight of 1258.5 g, S.D. \pm 152.12 and were collected from sea cage number 4, thus unfamiliar with experienced lumpfish used in the experiment.

Pre-experiment Preparations for Experienced Lumpfish

Lumpfish referred to as experienced lumpfish, were transported on June 5 from Nordland Rensefisk to Raudøya and deployed in sea cage 5. On arrival, a random sample of experienced lumpfish ($n = 30$) had a mean weight 30.2 g, S.D. \pm 2.50. Experienced lumpfish coexisted with Atlantic salmon from June 5 and until the first trial was conducted on August 2, 2016. Each sea cage had cleaner fish shelters preinstalled, 8 m deep spread along 30 m of rope. Experienced lumpfish were fed daily with 2% of total biomass with Lumpfish Grower 2.0 mm pellets (Biomar, Karmøy, Norway).

Pre-experiment Preparations for Naive Lumpfish

Naive lumpfish ($n = 100$) were transported from Namdal Rensefisk to Raudøya and kept in a $2 \times 2 \times 1.5$ m tank at the feed barge, 200 m northwest from sea cage 4, from July 27 and throughout the trial period until September 6. Naive fish had no previous experience with Atlantic salmon. On arrival, naive fish had a mean weight of 38.2 g, S.D. \pm 3.91. Naive lumpfish were fed daily with 2% of total biomass with Lumpfish Grower 2.0 mm pellets (Biomar, Karmøy, Norway). A Metabo[®] 24 V immersion pump (Metabo, Nürtingen, Germany) provided 7,000 l/h unfiltered seawater from 3 m depth. Environmental data were logged every tenth min using a SD 204 CTD (SAIV, Bergen, Norway). From July 27 to September 3, mean dissolved oxygen was 7.05 mg/L. Mean water temperature was 14.8°C with T_{\max} 16.17°C, and T_{\min} 13.54°C. Mean conductivity was 40.47 uS/cm and mean fluorescence was 5.71 ug/L.

Experimental Design

From August 2 and until September 3 in 2016, 20th trials were conducted, including 10 trials with interaction between experienced lumpfish and Atlantic salmon, and 10 trials with interaction between naive lumpfish and Atlantic salmon. Each trial consisted of 10 new lumpfish and four new Atlantic salmon, and no fish was included in more than one trial, which makes each trial a true replicate. Each trial was recorded at the feed barge in a green $2 \times 2 \times 0.7$ m experimental tank with a tarpaulin attached 2 m above to give even light in the tank. To reduce diurnal variation in hormonal outputs (39), all experimental activity was carried out at daytime between 12:00 p.m. and 14:00 p.m. Experienced lumpfish were collected from sea cage

5, while naive fish were collected from the nearby tank at the feed barge. Experienced and naive lumpfish were kept in different environments prior to the experiment due to practical limitations and reason that all sea cages were in full production, accommodated by farmed salmon. Prior to each trial, all 10 lumpfish were lightly sedated with 0.1 ml l⁻¹ Benzoak Vet (ACD Pharmaceuticals, Leknes, Norway) and tagged with numbered Petersons discs to identify each fish during video recordings. After tagging, fish were taken off feeding and acclimated for 48 h before the trial was ready for video recording of behaviour and measurements of physiological stress responses.

Video Recordings

Trials were video recorded with GoPro Hero 3+ cameras (GoPro[™], California, USA) attached in each corner of the experimental tank, in addition to a camera above the tank. Video was recorded in Full HD at 60 frames per second with ultra-wide field of view in the tank, and medium field of view above the tank. Ten minutes prior to filming, water circulation was stopped to increase visibility in the tank. Next, cameras were synchronously started with a GoPro Wi-Fi remote to record behaviour among conspecifics of lumpfish in the tank. After 10 min, four Atlantic salmon were carefully added to the tank by hand net from behind a cover. To avoid burst swimming after handling, Atlantic salmon were lightly sedated with 0.05 ml l⁻¹ dosage of Benzoak Vet (ACD Pharmaceuticals, Leknes, Norway) for 5 min. The video recordings continued for 10 min to observe the behavioural interaction between lumpfish and Atlantic salmon. A total of 20 min was video recorded during each trial, in addition to 25 min of delay before each trial was ended. This was done to ensure that cortisol reached closer to peak level after 45 min from introduced stressor, as previously observed by Iversen et al. (40). A dosage of 3 mg l⁻¹ Aqua calm[®] (Western Chemical Inc., Canada) with cortisol blocking properties, was added to the experimental tank. After 5 min, lumpfish were hand netted and humanely euthanized with a blow to the head. Oxygen and temperature were logged in the experimental tank after each trial using a SD 204 CTD (SAIV, Bergen, Norway). From 20 trials, mean oxygen was 7.68 mg/L and mean water temperature was 14.7°C.

Data Collection

Physiological Data

Blood from euthanized lumpfish was collected after each trial. Blood was collected from the heart ventricle using a 0.33 \times 12.7 mm syringe (BD Micro-fine[®]) containing anticoagulating heparin before weight and length was registered. Blood were put in Eppendorf tubes and centrifuged at 6,000 rpm (rounds per minute) for 5 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 -30°C . Samples were later analysed for plasma cortisol measurements at Nord University in Bodø, Norway using Radioimmunoassay, previously described by Iversen et al. (41). As a tracer, [1, 2, 6, 7, -3H] Cortisol (Amersham plc, Oslo, Norway), treated with 250 mCi (9.25 MBq) and diluted in 25 mL of absolute alcohol (Activity of about 10 mCi/mL) was used. Hydrocortisone (H

4001, Sigma-Aldrich, Oslo, Norway) was applied to produce a standard range from 0 to 137.5 nmol/L. The antibody was obtained from Sheep Anti-Cortisol, code: S020 (Guildhay Ltd, Surrey, UK). Samples were incubated at 1–2°C for 24 h before centrifuged with a Heraeus sepatech Omnifuge 2.ORS radius 154 mm, rotor 3,360. Antibody-antigen complex was counted in a scintillation counter type Packard Tri Carb 1900 TR. The sensitivity in the assay was 1.68 nmol/L. Samples under “detection limit” were set equal to the sensitivity of the assay. Intra-assay was below 10% and inter-assay was 12.5% at 80 nmol/L. NSB ranged from 2.1 to 4.8% of the total activity. Previously executed recovery tests at the laboratory of Nord University gave the following results: Measurement of 4, 17, 34, and 69 nmol/L radiolabelled cortisol with added plasma, showed a recovery of 90, 94, 96, and 95%.

Behavioural Data

In the experimental tank, swimming activity was registered once every minute for each lumpfish, 10 min before and 10 min after the introduction of Atlantic salmon. Swimming activity was categorized based on previous work by Tully et al. (16) and Inmsland et al. (19), with distinguishable locomotion separated into scores (Table 1). Interspecific distance between each lumpfish and the nearest Atlantic salmon was measured using ImageJ2 (42) on still photos from video of the experimental tank in 2D perspective. A line calibrated against the 20 × 20 cm grids in the bottom of the experimental tank was drawn from between the eyes of the lumpfish to between the eyes of the nearest Atlantic salmon.

Statistics

All statistical work was computed with R software™ R.3.2.2 (43). A chi-square test was used to compare observed changes in counts of swimming activity between trials of naive and trials of experienced lumpfish. A Shapiro–Wilk test (44) was used to test normality of distributions for both physiological and behavioural data. For comparison of plasma cortisol levels, a Student's *t*-test was used when normality and Levene's *F*-test assumption of homogeneity in variances between populations were met. A Wilcoxon rank sum test was used on non-parametric independent data of naive and experienced trials when these assumptions were not met. A significance level of $\alpha = 0.05$ was used.

TABLE 1 | Classification of lumpfish swimming activity based on distinguishable locomotion.

Score	Swimming activity	Description
4	Burst	Rapid acceleration in any direction
3	Normal	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

RESULTS

Swimming Activity

Counts of the swimming activities “burst” and “hovering”—but not “normal” and “attached”—differed significantly between naive and experienced lumpfish (Table 2). First trial with naive lumpfish and first trial with experienced lumpfish were not included in behavioural analysis due to low visibility in the experimental tank. Main alteration was observed in trials ($n = 9$) with naive fish, where counts of “burst” swimming activity, increased from 9 to 229 after the introduction of Atlantic salmon in the experimental tank, compared to from 5 to 27 in trials ($n = 9$) with experienced lumpfish. Moreover, counts of “hovering” decreased in naive lumpfish, whereas counts of “hovering” increased in experienced fish from before to after Atlantic salmon were introduced (Table 2).

Mean (\pm 95% confidence intervals) swimming activity observed every minute from trials of naive ($n = 9$) and experienced ($n = 9$) lumpfish revealed lower means at all observations among naive trials prior to Atlantic salmon introduction and higher means after, in comparison with experienced trials (Figure 1). Mean swimming activity in naive fish increased from the last observation before (10 min), to the first observation after (11 min) after the introduction of Atlantic salmon (Figure 1). (Wilcoxon rank sum test, $W = 65$, $p = 0.033$), and confidence intervals did not overlap at 18 and 20 min into the experiment (Figure 1) while overlapping on the remaining.

Interspecies Distance

Naive compared to experienced lumpfish tended to keep longer distance to Atlantic salmon (Figure 2). Mean values with 95% confidence intervals did not overlap at 14 and 17 min and overlapping on the remaining minutes. A significant difference was observed after pooling means of interspecific distance from trials ($n = 9$) of naive and trials ($n = 9$) of experienced lumpfish (Wilcoxon rank sum test, $W = 79$, $p = 0.028$).

Plasma Cortisol

Plasma cortisol levels in naive lumpfish trials accounted for 8 of the top 10 highest median and mean values out of 20 trials of naive and experienced lumpfish (Figure 3). Moreover, trials ($n = 10$) of naive lumpfish had significantly

TABLE 2 | Counts of swimming activity among replicates ($n = 9$) of naive and replicates ($n = 9$) of experienced lumpfish.

Swimming activity	Naive		Experienced		Chi-Square values		
	Before	After	Before	After	χ^2	df	<i>P</i> -value
Burst	9	229	5	27	8.047	1	0.005
Normal	526	403	606	507	0.967	1	0.325
Hovering	180	129	149	234	25.673	1	<0.001
Attached	185	139	130	112	0.641	1	0.423

Counts were done of all 10 lumpfish every min in each trial, 10 min prior to introduction of Atlantic salmon in the experimental tank and 10 min after the introduction. Difference in swimming activity before and after the introduction of Atlantic salmon in both naive and experienced lumpfish, was tested using Chi-square tests.

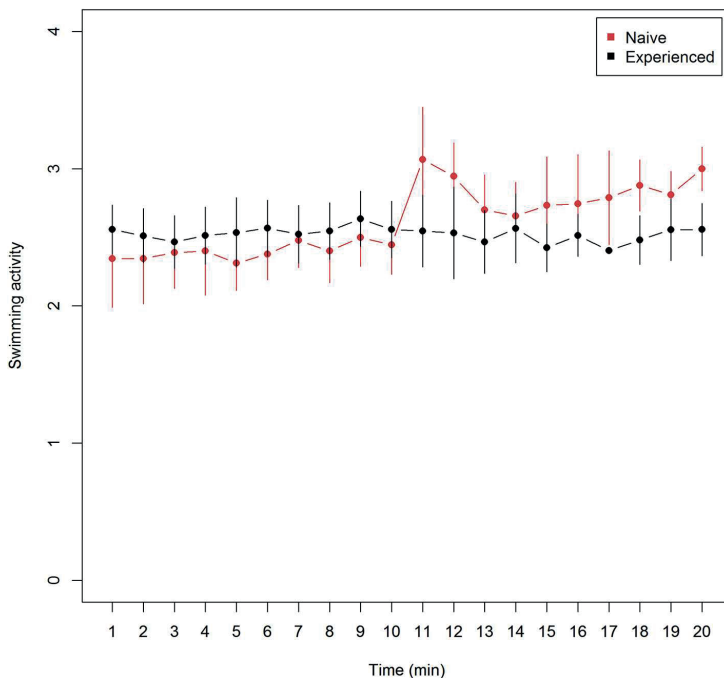


FIGURE 1 | Swimming activity (mean \pm 95% confidence intervals) for naive ($n = 9$) and experienced ($n = 9$) trials of lumpfish during 20 min of video observations. Swimming activity include mean ranked observations from the 4 categories (1) attached, (2) hovering, (3) normal swimming, and (4) burst swimming while time show the duration of video recordings – 10 min without and 10 min with Atlantic salmon interaction. The Atlantic salmon were added to the tank between 10 and 11 min. While mean values of swimming activity among experienced lumpfish remained unchanged, mean values of naive swimming activity increased significantly at 11 min (Two-sided Wilcoxon rank sum test, $W = 65$, $p < 0.033$).

higher cortisol values in comparison with trials ($n = 10$) of experienced lumpfish after calculating the mean from each of the 10 trials from each of the two groups of lumpfish (Student's t -test, $t = 3.67$, $p = 0.001$, d.f. = 18).

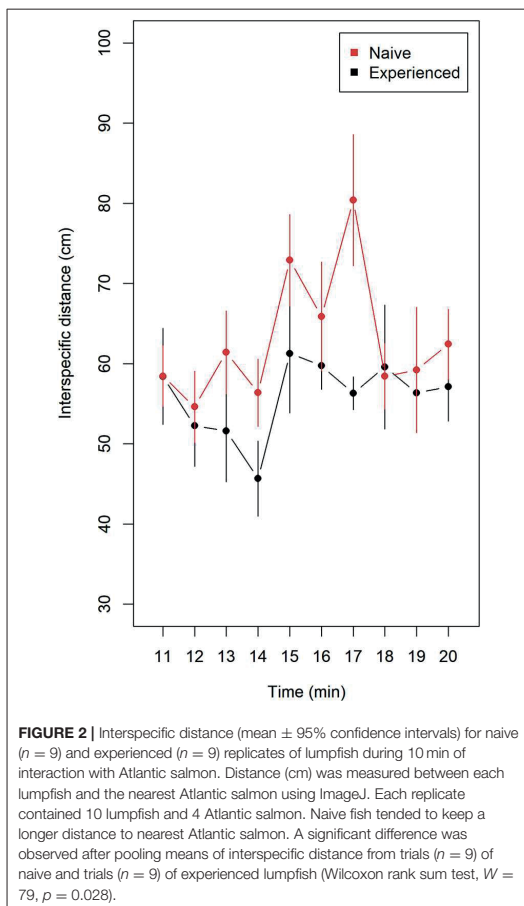
DISCUSSION

Swimming Activity

Naive lumpfish increased burst swimming activity and interspecies distance to Atlantic salmon, revealed that innate escape behaviour is present and lasting during first interaction, even if lumpfish is considered a facultative cleaner fish which graze ectoparasitic sea lice in salmon aquaculture. This increased vigilance during first encounter with a client fish is similar to observations of obligate cleaner fish (24, 45). Thus, the process of habituating to stressors in an unfamiliar environment with Atlantic salmon before ideal grazing of sea lice occur, require additional learning through perception and repeated interactions to memorize and separate harmless stimuli from harmful ones (46). Prey fish escape behaviour from predators is in many ways a most fundamental defensive adaptation to

avoid death and increase individual fitness (47). In nature, juvenile lumpfish have been observed to quickly detach from substrates and flee when a predator fish approached (48), which coincided with observation on naive fish swimming behaviour in the current study. A rapid innate escape response from environmental stressors has also been observed in larval lumpfish, and increased swimming activity as a measurement of escape behaviour is without difficulty applied to the species (26, 49).

Escape behaviour and burst swimming activity are both energetically costly (50) and affects the overall fitness regarding time spent on other tasks (51). In nature, lumpfish are exposed to different stimuli that trigger antipredator behaviours at daily basis, and the ability to habituate and quickly learn how to distinguish danger from safety is essential to adapt and cope in a changing and complex environment (52, 53). Experienced lumpfish that coexisted with Atlantic salmon did not significantly change their swimming activity from the moment of reintroduction in the experimental tank, and the previous experience from interspecies interaction in commercial sea cages appeared to have impacted their behaviour, to the presence



of salmon. Predator recognition from sensory cues, including vision, the lateral line system and olfaction, require experience through the ontogeny of fish (54–56). Yet, many species of fish have appeared to be predisposed to certain visual predator cues including shape, colour and size of the body as well as mouth structure (53, 57, 58). When considering the origin of escape behaviour in lumpfish, it should be noticed that the obligate cleaner fish bluestreak cleaner wrasse *L. dimidiatus* have shown to express similar fast-start escape behaviour during hit and runs after intentionally consuming nutrient rich mucus from the skin of a client fish, before being chased away (24). It is uncertain if lumpfish sometimes cheats and eat mucus from Atlantic salmon epidermal skin, and observations on such behaviour would only partly explain why, as ectoparasites are only fractions of the species food types (21) in comparison to obligate cleaner fish (9).

Physiological Stress Response

In trials with naive lumpfish, plasma cortisol measurements were similar to measurements observed after exposing lumpfish to crowding stress, by draining the tank environment (40), and after exhaustive chasing of lumpfish, also in a tank environment (59). Iversen et al. (40) found that plasma cortisol elevated to 200 nmol l^{-1} 1 h after exposure the stressor, while Hvas et al. (59) found mean plasma cortisol elevations of $150.9 \text{ nmol l}^{-1}$. The present study observed a mean value of $114.94 \text{ nmol l}^{-1}$ 45 min after the introduction of Atlantic salmon in trials with naive fish. These values interrelated with the time perspective, which indicated that naive lumpfish showed a strong primary stress response due to the presence of Atlantic salmon. Publications on habituation in lumpfish are absent, yet similar studies on other species have observed habituation in prey zebrafish *D. rerio* after repeated interactions for 5 days with predatory cichlids *Parachromis managuensis* Günther, 1867 (37), using different methods of exposure and length of the stressor. Other species including Eurasian perch *Perca fluviatilis* Linnaeus, 1758 and rainbow trout *Oncorhynchus mykiss* Walbaum, 1792 showed habituation to mechanical stress after 8 weeks (60). In contrast, studies on catfish *Rhamdia quelen* Quoy and Gaimard, 1824 did not observe habituation after 3 weeks of exposure to mechanical stress (61). Thus, habituation to predatory presence in a time perspective, should be considered species specific. One might predict that cleaner fish and other species that partly include ectoparasites in their diet, have shorter habituation to predatory client fish, or share comparable behavioural and physiological traits that distinguish them from pure predator vs. prey interaction. The use of cortisol as a stress marker has limitations that should be taken into account when interpreting measurements of lumpfish tested in the following experiment. All measurements were done during afternoon, from 12:00 p.m. to 14:00 p.m. to avoid sampling bias. Thus, measured elevation of plasma cortisol might have looked different if trials had been conducted at a different time during the day or the season (62). In addition, Ellis et al. (62) reviewed cortisol in relation to fish welfare, and highlighted limitations on dose- and context dependency when analyzing cortisol responses in different fish species. In fish farming, stress is both multifactorial and unavoidable and the observed stress responses in naive lumpfish to Atlantic salmon should be accounted for as one such stressor when adding up all factors.

Experimental Setup

In aquaculture, lumpfish are commonly deployed in sea cages with Atlantic salmon, while in this study the sequence of fish added to the tank was contrary with lumpfish already present when Atlantic salmon were added to the experimental tank. The study design made it possible to measure stress responses from Atlantic salmon introduction only, thus avoiding disruptive stressors including transportation and handling. Sea cages provided a larger and deeper water volume and exposed experienced fish to other stimuli that could have influenced how these individuals responded during the experiment. Thus, we cannot exclude the possibility that experience—other than

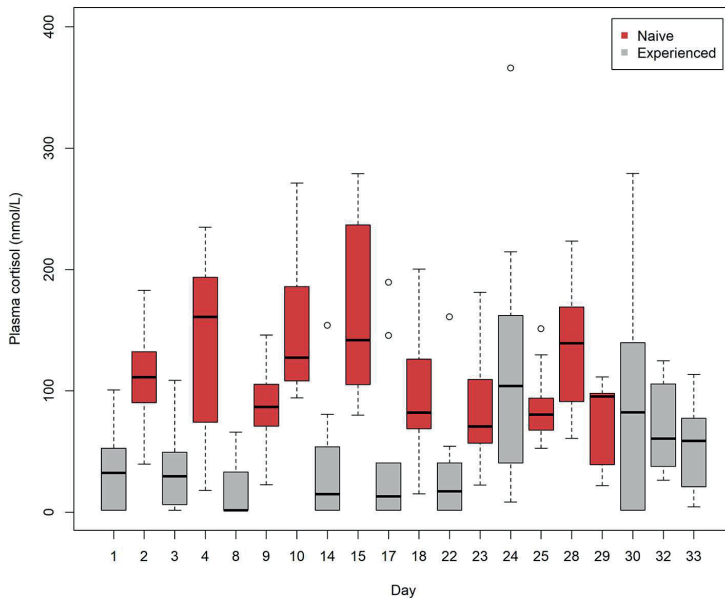


FIGURE 3 | A Box-and-Whiskers plot showing plasma cortisol levels in 10 trials of naive lumpfish and 10 trials of experienced lumpfish after interacting with Atlantic salmon. Trials ($n = 10$) of naive lumpfish had significantly elevated cortisol levels in comparison with trials ($n = 10$) of experienced lumpfish after calculating the mean from each of the 10 trials from each of the two groups of lumpfish (Student's t -test, $t = 3.67$, $p = 0.001$, d.f. = 18). Trials were conducted from August 2 to September 3. Plasma cortisol (nmol/l) was measured using radioimmunoassay (RIA).

exposure to predatory salmon—prior to the experimental testing, caused the difference in behaviour and stress between the two groups. However, the results showed that naive (kept in a tank) and not experienced lumpfish (kept in a sea cage) demonstrated increased stress (swimming behaviour, interspecies distance and cortisol levels) after the introduction of Atlantic salmon. This is the opposite of expected if the measured differences in stress level were a result of different experience (tank vs. cage), since the experimental trials were run in tanks, and not in cages. Moreover, the experiment revealed minimal differences in swimming behaviour between naive and experienced lumpfish during the first 10 min before salmon were introduced into the experimental tank, and the difference occurred the first minute after the introduction of salmon. These findings suggested that Atlantic salmon—and not the variation in environment (tank vs. cage)—caused a different response between the lumpfish groups.

CONCLUSION

The following study suggest three measurements of habituation in experienced lumpfish after interactions with Atlantic salmon in commercial scale sea cages. In the experimental tank, analysis of naive lumpfish exposed to Atlantic salmon revealed increased swimming activity, increased interspecies distance, and elevated

plasma cortisol concentrations. In comparison, experienced lumpfish showed no change in swimming activity when reintroduced to Atlantic salmon, shorter interspecies distance, and additional desensitized physiological stress responses depicted from significant lower plasma cortisol levels. Innate predatory stress responses in naive lumpfish are likely to influence their behaviour during first interactions with farmed Atlantic salmon, and the length of the habituation period should be taken account for when lumpfish are introduced to commercial use to improve welfare for the species. Further studies will focus on social behaviour in lumpfish, stress induced from specific predator sensory cues, and how knowledge on habituation could become more applicable in the aquaculture industry.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was conducted according to the Animal Welfare Act (LOV-2009-06-19-97) and the Norwegian law on Regulation of Animal Experimentation (FOR-1996-01-15-23). Handling of live

fish was managed by personnel with FELASA-C course, based on the policies by the Federation of European Laboratory Animal Science Association. Lumpfish and Atlantic salmon used during the field experiment were assigned to project FDU 7835, accepted by the Norwegian Food Safety Authority under the regulation of the Research Animal Act (FOR-2015-06-18-761).

AUTHOR CONTRIBUTIONS

This work was part of a Ph.D. titled Habituation and learning in lumpsuckers during interaction with Atlantic salmon, with focus on behaviour and physiology involving the main author. All

co-authors collaborated with the preparation of the manuscript and are fully responsible for its contents.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper II

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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. \pm 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 towards 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.

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 Egildus, 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 Kjorsvik, 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 (Eliassen 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 suppress parasitic outbreaks in commercial scale salmon production (Eliassen et al., 2018; Imsland et al., 2018). Hatchery production of juvenile individuals and subsequent deployment into commercial-scale aquaculture with farmed salmon

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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 to 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 ($2 \times 2 \times 1$ m) adjacent to the experimental room. Water flow was set to 500 l h^{-1} . Mean \pm SD temperature and dissolved oxygen levels during the experiment were 8.5 ± 0.22 °C and $8.4 \pm 0.31 \text{ mg l}^{-1} \text{ O}_2$, 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 \pm SD weight of 34.3 ± 6.48 g were used in the experiment. Lumpfish were distributed in eight grey tank units ($1 \times 1 \times 1$ 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 (Immsland 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 0.33×12.7 mm syringes (BD Micro-fine®) containing anticoagulating heparin and centrifuged at 6000 RPM (2000 xg) 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

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

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 20 mm 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 1.53c (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 = $P < 0.001$, $R_s = 0.83$, green = $P < 0.001$, $R_s = 0.66$, blue = $P < 0.001$, $R_s = 0.47$, $n = 144$), 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

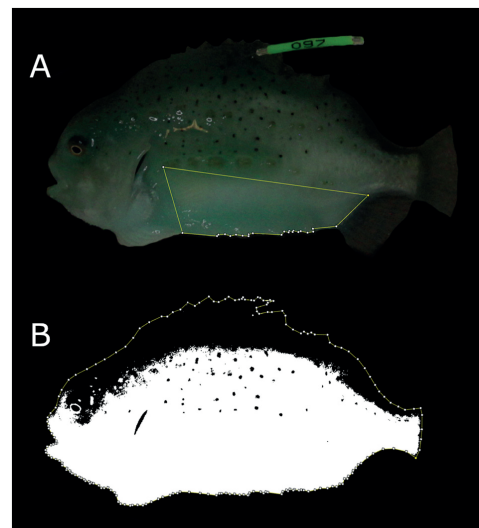


Fig. 1. The upper image (A) shows the defined homogenous area used to calculate mean red, green and blue pixels (mean R + G + B), while the lower image (B) show the defined area used to calculate pigmentation as the ratio of black and white pixels (B:W). Both colour and pigmentation were measured using ImageJ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pigmentation was calculated as the ratio of black versus white pixels within 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 $\pm 95\%$ 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 ($\pm 95\%$ 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

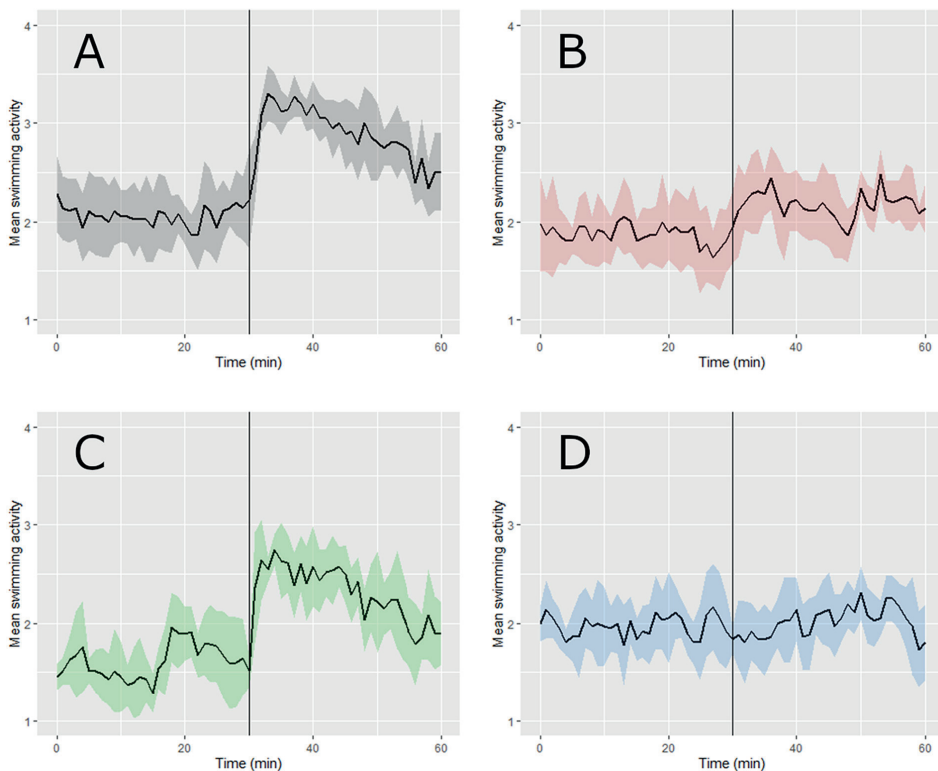


Fig. 2. Mean swimming activity from 6 replicates for each treatment with 95% confidence intervals. Means from each minute illustrates the change in swimming behaviour before treatments were introduced (first 30 min) and after the treatments were introduced (31–60 min). Treatments were A) Olfaction, B)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

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

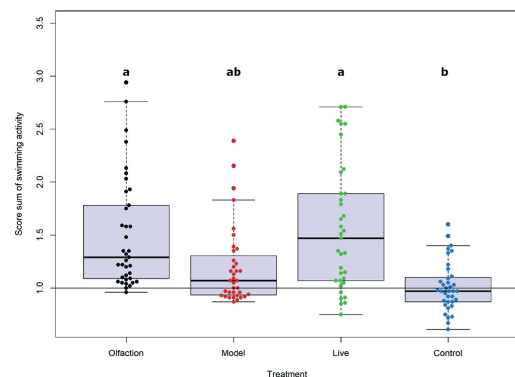


Fig. 3. Score sum of swimming activity for each individual lumpfish ($n = 36$) observed 30 min before and 30 min after interactions with the different treatments. A score above 1 implies an increase in activity level for each specific lumpfish based on categorization of swimming activities including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

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)	$P = 0.38$
5HIAA (ng/g)	17 ± 5.7	23 ± 18.3	18 ± 10.0	17 ± 6.8	H (3, 91)	$P = 0.24$
5HIAA:5HT (%)	3 ± 1.1	4 ± 3.8	3 ± 1.2	3 ± 0.6	H (3, 91)	$P = 0.99$
DA (ng/g)	141 ± 52.8	133 ± 45.6	150 ± 63.5	166 ± 58.7	H (3, 91)	$P = 4.59$
DOPAC (ng/g)	15 ± 19.2	15 ± 24.4	10 ± 9.4	17 ± 17.4	H (3, 91)	$P = 3.89$
DOPAC:DA	13 ± 21.1	12 ± 18.6	8 ± 8.4	11 ± 12.0	H (3, 91)	$P = 4.83$
Cortisol (nmol l^{-1})	46 ± 42.3	60 ± 41.4	64 ± 43.8	61 ± 42.5	H (3, 91)	$P = 4.98$

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; Dixon 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

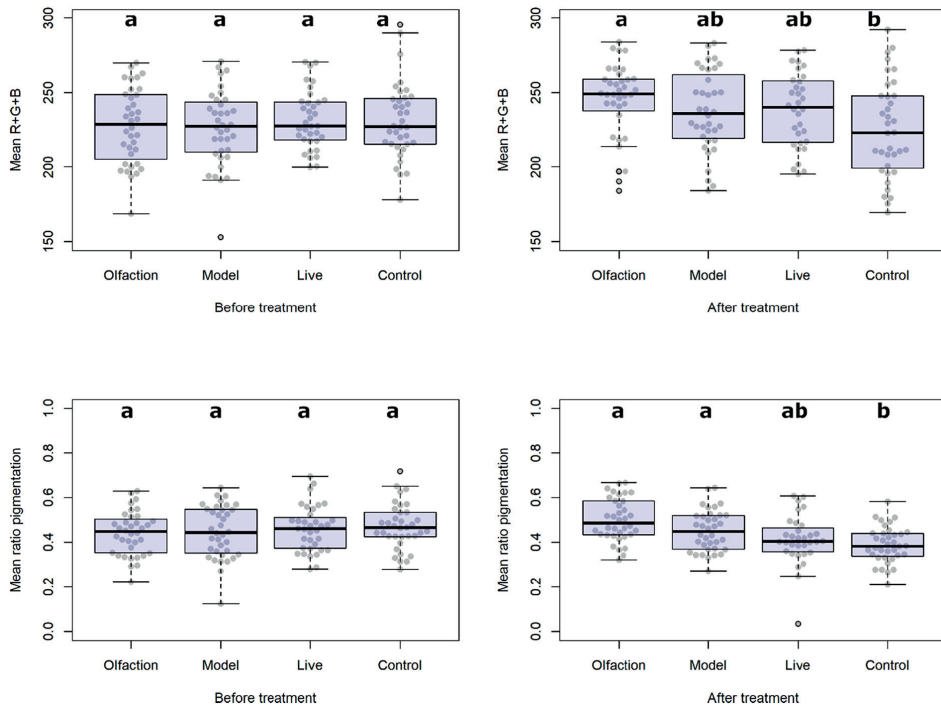


Fig. 4. Lumpfish skin colour ($n = 36$) measured in mean red, green, and blue (a) before and (b) after the experiment, and lumpfish skin pigmentation measured in mean ratio of black and white pixels (c) before and (d) after the experiment. Dissimilar letters indicates significant difference among treatments 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 1 h 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 and 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.

4.2. 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.

Declaration of Competing Interest

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

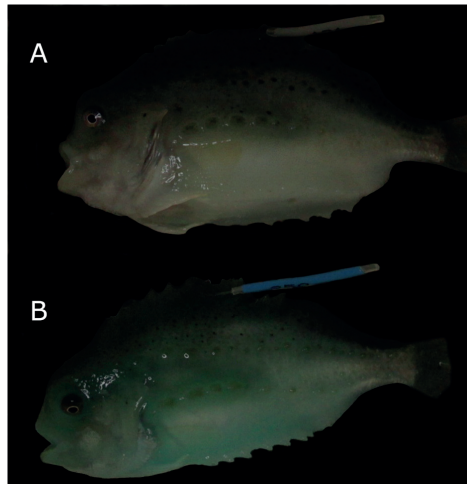


Fig. A.1. Increase in skin colour observed in lumpfish after the experiment was terminated. Image “A” represent an individual with mean $R + G + B = 224$, which was the median value observed in control groups. Image “B” represents an individual with mean $R + G + B = 254$, which was the median value observed in lumpfish exposed to Atlantic salmon Olfaction. The difference in colour between Olfaction treatment and control groups was significant (post hoc Tukey test, $P = 0.01$). Data on colour and pigmentation are presented in Fig. 4

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Paper III

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1 **Cohabitation with Atlantic salmon (*Salmo salar*) affects brain**
2 **neuromodulators but not welfare indicators in lumpfish (*Cyclopterus***
3 ***lumpus*)**
4

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13 **Keywords:** Lumpfish₁, Atlantic salmon₂, Cleaner fish₃, Aquaculture₄, Welfare₅,
14 Neuromodulators₆, Habituation₇

15 **Abstract**

16 Lumpfish are utilized to combat ectoparasitic epidemics in salmon farming. Research gaps on both
17 cleaning behavior and client preferences in a natural environment, emphasizes the need to investigate
18 the physiological impacts on lumpfish during cohabitation with piscivorous Atlantic salmon. Lumpfish
19 (39.9 g, S.D ± 8.98) were arranged in duplicate tanks (n = 40 per treatment) and exposed to Live
20 Atlantic salmon (245.7 g, S.D ± 25.05), salmon Olfaction or lifelike salmon Models for 6 weeks.
21 Growth and health scores were measured every second week. In addition, the final sampling included
22 measurements of neuromodulators, body color and plasma cortisol. A stimulation and suppression test
23 of the hypothalamic-pituitary-interrenal (HPI) axis was used for chronic stress assessment. Results
24 showed that growth, health scores and body color remained unaffected by treatments. Significant
25 reductions in levels of brain dopamine and norepinephrine were observed in Live compared to
26 Control. Plasma cortisol was low in all treatments, while the stimulation and suppression test of the
27 HPI axis revealed no indications of chronic stress. This study presents novel findings on the impact on
28 neuromodulators from Atlantic salmon interaction in the lumpfish brain. We argue that the
29 downregulation of dopamine and norepinephrine indicate plastic adjustments to cohabitation with no
30 negative effect on the species. This is in accordance with no observed deviations in welfare
31 measurements, including growth, health scores, body color and stress. We conclude that exposure to
32 salmon or salmon cues did not impact the welfare of the species in our laboratory setup, and that
33 neuromodulators are affected by heterospecific interaction.

34 **1 Introduction**

35 The presence of predators induces multiple behavioral and physiological responses in prey
36 animals. Among teleost fish, such responses involve freezing, fight or flight; each strategy or
37 a mix of strategies aimed at enhancing the likelihood for survival (Eaton et al., 1977; Clinchy
38 et al., 2013; Rupia et al., 2016). Recognition of potential predators can be inherited and entails
39 adapted systems for both sensory detection and defensive responses to external threats
40 (Brown and Godin, 1999; Berejikian et al., 2003; Brown, 2003). Both olfaction and vision are
41 essential sensory detection systems in aquatic prey fish, due to variable turbidity and light
42 conditions in the environment (Chivers and Smith, 1998; Ferrari et al., 2010). For a prey fish,
43 the time spent on high alert is time lost on fitness-related activities such as foraging and
44 reproduction (Schreck, 2010). As a counter mechanism, habituation and threat-sensitive
45 predator avoidance occur through plastic cognitive development when physiological and
46 behavioral mechanisms are altered to no longer respond to a repeated non-specific risk
47 (Brown et al., 2006; Rankin et al., 2009). A textbook example of adaptive plasticity to
48 predators is observed among cleaner fish. Cleaner fish are specialized in the removal of
49 ectoparasites from other fish, here called clients. Natural selection on habituation to client
50 interactions have earned cleaner fish unique access to both parasite food items and nipping of
51 nutrient rich skin mucus, delicately balancing mutualism over parasitism (Grutter, 1999;
52 Bshary, 2001; Gingsin et al., 2017). The behavioral approach sometimes involves cleaner fish
53 signaling to the client fish, who respond with a non-aggressive behavior before parasites are
54 removed (Grutter, 2004). In a cleaner fish - client fish interaction, the importance of
55 habituation might thus seem apparent. To access ectoparasites, cleaners can approach much
56 larger predator clients and perform behaviors most perilous, such as entering the clients
57 mouth cavity in search of food items (Brown and Godin, 1999; Bshary and Würth, 2001).

58 In Atlantic salmon (*Salmo salar*) aquaculture, certain wrasse species (Labridae) and lumpfish
59 (*Cyclopterus lumpus*) are utilized as cleaner fish to combat ectoparasites (Bjordal, 1990;
60 Imsland et al., 2014a; Blanco Gonzalez and de Boer, 2017). Failing to control epidemics of
61 the sea lice copepod *Lepheophtheirus salmonis* have both economic and environmental
62 consequences (Costello, 2009; Torrissen et al., 2013; Øverli et al., 2014). Today, one of the
63 most common species of cleaner fish used in Atlantic salmon farming is lumpfish (Powell et
64 al., 2017). Studies in commercial large-scale sea cages with Atlantic salmon, revealed
65 significant reductions in sea lice numbers when juvenile lumpfish were added, where 13-36 %
66 of the lumpfish stock had sea lice in their stomach (Eliassen et al., 2018; Imsland et al., 2018).

67 Why sea lice grazing on Atlantic salmon is not homogeneous among all lumpfish individuals
68 is uncertain, but variation between family strains have been observed (Imsland et al., 2016).
69 Ontogeny is also relevant for sea lice consumption, where small individuals (20-50g) have
70 been observed with a higher total count of sea lice in their stomach (Imsland et al., 2016;
71 Eliassen et al., 2018). With individual variation in behavior, it is thus relevant to assess
72 physiological mechanisms involved in social situations.

73 The main challenges of using lumpfish in aquaculture are high mortality rates and the
74 maintenance of good welfare during the commercial sea phase together with Atlantic salmon.
75 It is necessary to investigate each part of the puzzle related to increased mortality in the
76 myriad of stressors capable of impeding the resting state of lumpfish. One such stressor is
77 social interaction with Atlantic salmon and the ability to habituate to the presence of a larger,
78 potentially predatory, species. Whether lumpfish has been documented to be predated upon by
79 Atlantic salmon in a commercial fish farm is uncertain. Nonetheless, Imsland et al., (2014b)
80 observed no antagonistic behavior during cohabitation with the two species. While cleaner-
81 client behavioral interactions have been documented for decades in aquaculture (Bjordal,
82 1990), including almost ten years of research on lumpfish (Imsland et al., 2014a), knowledge
83 gaps still exist on the underlying mechanisms that regulate sea lice grazing. Recently, the
84 neurobiological, and neuro-molecular mechanisms involved in interspecific interaction have
85 been investigated in other cleaner fish species (Soares, 2017; Ramírez-Calero et al., 2021).
86 These novel approaches have accentuated the role of neuromodulators in social contexts
87 (Soares et al., 2012, 2017; Paula et al., 2015; de Abreu et al., 2018, 2020). Neurotransmitters
88 of relevance are dopamine, which influence Bluestreak wrasse (*Labroides dimidiatus*)
89 learning capacity in cooperative investment, and time spent in physical contact with clients
90 (Messias et al., 2016a, b; Soares et al., 2017). Serotonin has multiple roles including
91 regulation of HPI activity in teleost fish, cleaner fish social stress, cooperation, and learning
92 (Winberg et al., 1997; Paula et al., 2015; Soares et al., 2016; Winberg and Thörnqvist, 2016;
93 Backström and Winberg, 2017). A third neurotransmitter, associated with acute stress in
94 multiple species of fish, as reviewed by Gamperl et al. (1994) is norepinephrine. The role of
95 norepinephrine in association with cleaner fish behavior and physiological change to client
96 interaction is nonetheless undetermined.

97 Individual variation in wild parental brood fish during rearing, and a breeding program in
98 early development, both emphasize the importance of investigating lumpfish responses to
99 Atlantic salmon. While acute responses in lumpfish exposed to Atlantic salmon or salmon

100 sensory cues have been investigated to evaluate behavioral and physiological plasticity
101 (Staven et al., 2019, 2021), the following study aimed to investigate how lumpfish responded
102 to the presence of Atlantic salmon or salmon sensory cues over a period of 6 weeks. We
103 measured physiological parameters relevant to stress and habituation including (1) growth, (2)
104 health score assessment, (3) body color, (4) neurotransmitters and (5) HPI axis responses.

105 **2 Material and methods**

106 **2.1 Ethical statement**

107 Use of research animals was accepted for experimental purposes by the Norwegian Food
108 Safety Authority (FDU #17231). Animals were carefully handled based on the Norwegian law
109 on Regulation of Animal Experimentation (FOR-1996-01-15-23). Personnel involved were
110 certified with FELASA-C, developed by the Federation of European Laboratory Animal
111 Science Association. The experiment was conducted based on the three R's and the ARRIVE
112 guidelines (Kilkenny et al., 2010).

113 **2.2 Research animals and tagging**

114 2.2.1 Lumpfish

115 Lumpfish used in the study originated from wild mature lumpfish captured in Troms and
116 Finnmark county, Norway. Milt and roe were collected, fertilized, hatched, and reared from
117 February 2018 at Mørkvedbukta AS. Lumpfish were fed with pellets based on standard
118 recommendations given by a commercial feed producer (Skretting, Stavanger, Norway). All
119 lumpfish were vaccinated with AMarine micro 4-2® (Pharmaq, Overhalla, Norway) and
120 given 300 day-degrees immunization before they were transferred to the Mørkvedbukta
121 research station. Lumpfish used were juvenile individuals with low gonadal development,
122 indicating no maturation. Lumpfish were tagged 1 week prior to experiment start with Floy
123 tag t-bars (Floy Tag and Mfg Inc, Seattle, USA). Tags were attached in the dorsal crest using
124 a t-bar pistol after fish had been anesthetized with 100 mg l⁻¹ benzocaine (ACD
125 Pharmaceuticals, Leknes, Norway) based on recommendations in Skår et al. (2017).

126 2.2.2 Atlantic salmon

127 Atlantic salmon used in the study were produced from an eleventh generation Aquagen strain,
128 hatched at Sundsfjord Smolt AS in Nordland, Norway. Smolts were transported to
129 Mørkvedbukta research station in Bodø during the spring of 2017. At the station, feeding was
130 conducted in same manners as later during the experiment. This included the use of automatic
131 feeders (Arvo-Tec Oy, Finland) containing Gemma diamond 150 (Skretting, Stavanger,

132 Norway) split across three dosages adding up to a daily amount of 2 % biomass. A total of 8
133 Atlantic salmon (246.7 g, S.D \pm 25.50) were used in the experiment and had no previous
134 experience with lumpfish.

135 **2.3 Experimental preparation**

136 On the first day of the experiment (30 October 2018), 160 lumpfish (39.7 g, S.D \pm 8.99) were
137 morphologically measured (see section 2.5.1), photographed (see section 2.5.2) and health
138 assessed (see section 2.5.3). Next, lumpfish were randomly distributed in eight grey tank units
139 (1 x 1 x 1 m, 480 L) with lids to avoid visual disturbance. With three different treatments in
140 addition to a control group, the distribution of lumpfish were 20 individuals per tank with two
141 tanks per treatment (n = 40). During the 6-week experimental period, the water flow in each
142 tank was 500 L h⁻¹ while mean \pm SD oxygen saturation and water temperature from all 8 tanks
143 was 91.6 \pm 1.84 % and 7.5 \pm 0.07 °C, respectively. The photoperiod during the experiment
144 was 24:0. Fish were fed 2 % of their biomass separated into three meals served 08:00, 11:00
145 and 14:00 with 30 doses of pellets for each meal (Arvo-Tec Oy, Finland). Spilled feed and
146 feces were removed once a week, but not within 4 days prior to sampling dates to avoid
147 disturbance. Every second week, each individual lumpfish was morphologically measured and
148 assessed for health scores. To avoid stress during handling, fish were quickly hand netted
149 from the experimental tank to a 30 L tank with 5 mg L⁻¹ of cortisol blocking metomidate
150 (Aquacalm™, Western chemical Inc, Canada). This concentration causes rapid anaesthesia
151 and stops further increase of plasma cortisol (Iversen et al., 2003, 2014). Fish were moved
152 back to their respective tanks after the measurements were done.

153 **2.4 Treatments**

154 The different treatments in the duplicate tanks were named “Live”, “Model” and “Olfaction”.
155 The duration of the treatments was 6 weeks. Two remaining tanks (“Control”) contained
156 lumpfish exposed to no specific treatment. The Live treatment included cohabitation with 2
157 Atlantic salmon per tank. Model treatments involved 2 silicone salmonid models (*3D Line*
158 *Thru 15 cm*, Savage Gear™, USA). Models were the same size as the initial size of Atlantic
159 salmon used in the Live treatment. Each salmon model was attached to a nylon string and a
160 swivel above the tank, which caused the models to move with the water current in the surface
161 center area of the tank during the entire experiment. In the Olfaction treatment, the water
162 input was connected to an adjacent tank containing 4 Atlantic salmon. Without disturbing
163 flow or environmental parameters, the duplicate tanks were provided with a stable influx of
164 water representing the presence of Atlantic salmon without any visual cues.

165 **2.5 Data analysis**

166 2.5.1 Growth

167 Weight (W) was measured with 0.1 g accuracy, while length (L) was measured to the nearest
168 0.1 cm, one fish at the time. Specific growth rates (SGR % day⁻¹) were calculated using the
169 formulae developed by Houde and Schekter (1981):

170 $SGR = (e^g - 1) * 100$

171 where $g = (\ln(W_2) - \ln(W_1)) / (t_2 - t_1)$. W_2 refers to weight on the last day of measurement for the
172 respective period, while W_1 refers to weight on the first day. t_2 and t_1 refer to the time period
173 described as number of days.

174 Formula for condition factor (K) was:

175 $K = 100 * W / L^3$

176 It was decided to use SGR and K-factor in the present study to compare findings with
177 previous studies on lumpfish involving growth (Nytro et al., 2014; Jørgensen et al., 2017;
178 Imsland et al., 2018, 2019).

179 2.5.2 Skin coloration

180 Skin color analysis of lumpfish epidermal skin was assessed using methods described in
181 Staven et al. (2021). Each lumpfish was photographed, and later analyzed for pixel counts
182 within a defined area of the lateral side of the fish. Mean values of each of the three primary
183 colors red (R), green (G), and blue (B) were measured using the histogram function in
184 ImageJ, version 1.53e (NIH, Bethesda, MD, available at www.imagej.nih.gov/ij). Eventually,
185 the mean value for each of the three colors were added together, referred to as mean R+G+B.

186 2.5.3 Health assessment

187 External health was scored based on health assessment criteria specifically developed for
188 lumpfish (Imsland et al., 2020). This involved scoring (from 0-4) body conditions, tail fins,
189 other fins, deformities, cataract, eye ulceration and the condition factor (K). For each
190 parameter, a lower score indicated less deviations from normal health conditions. Individual
191 scores were summated and the mean score per treatment calculated. Mean welfare score from
192 0-11 suggested “no to minimal deterioration”, while mean welfare score from 11-16
193 suggested “higher incidence of compromised health”. A score above 16 indicated “evidence
194 of further extensive health deterioration”. For a detailed scoring description, see Imsland et al.
195 (2020).

196 2.5.4 Blood and brain sampling for plasma cortisol and neurotransmitters
197 Blood and brains were sampled after 6 weeks of experiment. To avoid activation of the HPI-
198 axis during the final handling, water supply was stopped and 5 mg L⁻¹ metomidate
199 (Aquacalm™, Western Chemical Inc, Canada) quickly added to one tank at the time.
200 Amnesia (hypnosis) occurred within 2 minutes in lumpfish. Next, fish were removed from the
201 tank with a hand net before blood was sampled from the caudal vessel using 0.33 x 12.7 mm
202 syringes (BD Micro-fine®). Anticoagulating heparin (Leo Pharma, Sweden) was added to the
203 syringes by pulling the plunger out and back again into its initial position, thus leaving a small
204 amount of heparin in the dead space of the syringe (approximately 0.08 mL). Samples were
205 centrifuged at 5000 RPM (2000 x g) for 10 minutes in a Mini Star centrifuge (VWR™, UK).
206 After centrifugation, the plasma was separated with a pipette, transferred to a 1.8 mL Nunc
207 Cryo Tube® and stored at -40 °C. Plasma cortisol levels were later analyzed using
208 radioimmunoassay, based on methods described in Iversen et al., (1998). Next, fish were
209 euthanized by spinal transection and gills were cut. Left hemisphere brains were removed,
210 separated along the sagittal plane, and stored on dry ice, while right hemisphere brains were
211 designated for other experiments and not included in the study. Concentrations of
212 norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid, (DOPAC), serotonin
213 (5-hydroxytryptamine, 5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in left hemisphere
214 brain tissue were later quantified by means of HPLC with electrochemical detection as
215 previously described (Alfonso et al., 2019).

216 2.5.5 Stimulation and suppression test of HPI axis

217 To investigate treatment impact on the HPI-axis feedback system, a stimulation and
218 suppression test using adrenocorticotrophic hormone (ACTH) and dexamethasone (DEX)
219 respectively, was conducted in accordance with the previous study by Pottinger and Carrick
220 (2001), with some minor modifications as described in Iversen and Eliassen (2014).

221 3 Statistics

222 All statistical tests were performed using the R software™ R.3.2.2 (R Development Core
223 Team, 2013) at a 95 % level of significance. Data were tested for normality using the Shapiro-
224 Wilk test (Shapiro and Wilk, 1965) while variance was tested using Levene's F-test
225 (Gastwirth et al., 2009). Treatments and control were analyzed with a one-way ANOVA test.
226 When assumptions of normality or homoscedasticity were not met, a non-parametric Kruskal-
227 Wallis test were used instead. If significant differences occurred, a post hoc test was used to
228 compare treatments and control with one another. A Tukey test was used after one-way

229 ANOVA, while a Dunn test was used after a Kruskal-Wallis test. Results were presented
230 using mean values \pm standard deviation (mean \pm S.D). Figures were graphically depicted as
231 bar graphs with means and 95 % confidence intervals, or as boxplots with a median central
232 line and whiskers covering 95 % of values, while separate dots indicated outliers. Letters were
233 used to illustrate significant differences from post hoc tests, while no letters indicated no
234 significant difference from the ANOVA or Kruskal-Wallis tests. From 20 lumpfish in each
235 experimental tank, 12 were used for real time measurements of plasma cortisol, including 6
236 lumpfish also used for measurements of brain neurotransmitters. The remaining 8 in each tank
237 were used for stimulation and suppression test of HPI axis.

238 **4 Results**

239 **4.1 Morphometric parameters**

240 4.1.1 Growth and condition of lumpfish

241 Initial measurements of mean weight did not differ between treatments and control ($F(3,157)$
242 $= 0.950, p = 0.44$). During the experiment, specific growth rates did not differ between
243 treatments after 2 weeks ($K(158) = 159, p = 0.467$), 4 weeks ($K(158) = 159, p = 0.485$), 6
244 weeks ($K(158) = 159, p = 0.485$) or overall ($K(160) = 160, p = 0.485$; Figure 1). Also, the
245 condition factors (K) measured after experimental termination in Control (3.93 ± 0.44), Live
246 (3.93 ± 0.34), Models (4.01 ± 0.44) and Olfaction (3.91 ± 0.48) did not significantly differ (F
247 ($3,157$) $= 0.388, p = 0.76$).

248 4.1.2 Mortality and health score assessments

249 No mortality was observed during the experiment. Health score assessments done after the
250 experiment was terminated, revealed no visible damage (score = 0) for mouth, skin, gills, fins,
251 or eyes in all fish, with exception of a single fish in the Live treatment, that had a skin score
252 of 1. Skin penetration in the dorsal crest from Floy tag t-bars did not cause wounds. Mean
253 condition factor (K) for each treatment, including Control, were within the score range of 3.5
254 – 4.5, which was defined as in “moderate condition”, giving a health score of “1”.

255 4.1.3 Skin coloration

256 Skin coloration measured on the first day of experiment did not differ when comparing mean
257 R+G+B in treatments and control ($F(3,85) = 1.35, p = 0.261$; Figure 2A). Similarly, the 6
258 weeks of cohabitation did not induce any change in mean R+G+B measurements between
259 treatments and control ($F(3,85) = 0.727, p = 0.539$; Figure 2B).

260 **4.2 Neurotransmitters**

261 4.2.1 Dopamine

262 The levels of dopamine (DA) in treatments and control were significantly different ($F(3, 44)$
263 $= 3.97, p = 0.013$) and a post hoc comparison revealed significantly lower concentrations ($p =$
264 0.010) in Live (92.7 ± 42.60 ng/g) compared to Control (140.2 ± 27.26 ng/g). The
265 dopaminergic activity index (the DOPAC:DA ratio) was significantly different between
266 treatments and control ($H(3) = 9.67, p = 0.021$), and a post hoc comparison revealed
267 significant increase ($p = 0.002$) of the DOPAC:DA ratio in the Live treatment (DOPAC:DA =
268 $13.0 \% \pm 7.58$) compared to the Control (DOPAC:DA = $7.0 \% \pm 2.70$). Levels of the
269 metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) did not differ between treatments and
270 control ($F(3,44) = 0.21, p = 0.889$; Figure 3A,B,C).

271 4.2.2 Serotonin

272 No difference between treatments and control were observed for serotonin (5-HT) ($F(3,44) =$
273 $1.26, p = 0.3$), the serotonin metabolite (5HIAA) ($F(3,44) = 1.03, p = 0.387$) or the
274 serotonergic activity index (5HIAA:5-HT) ($F(3,44) = 0.94, p = 0.425$) (Figure 3D,E,F).

275 4.2.3 Norepinephrine

276 A significant difference in levels of norepinephrine (NA) was observed between treatments
277 and control ($F(3,44) = 3.08, p = 0.036$; Figure 3G), and a post hoc test showed that the
278 difference ($p = 0.021$) occurred between Live (455.8 ± 159.49) and Control (624.4 ± 170.64).

279 **4.3 HPI axis responses**

280 4.3.1 Basal levels

281 Basal levels of plasma cortisol concentrations were similar between lumpfish ($n = 24$) in
282 treatments and control ($H(3) = 6.23, p = 0.1$). Mean \pm SD plasma cortisol for the treatments
283 were lowest in Live (3.0 ± 3.92 nmol L⁻¹) and highest in Control (26.6 ± 48.11 nmol L⁻¹),
284 while Model and Olfaction were 11.7 ± 49.24 nmol L⁻¹ and 12.1 ± 35.15 nmol L⁻¹
285 respectively (Figure 4).

286 4.3.2 Stimulation and suppression test of HPI axis

287 The remaining lumpfish ($n = 16$) per treatment that were used to evaluate the stimulation and
288 suppression of the HPA axis showed no significant difference in plasma cortisol levels after
289 phosphate-saline injection (PBS) ($H(3) = 7.11, p = 0.07$), while injection with
290 adrenocorticotrophic hormone (ACTH) revealed different responses between treatments and
291 control ($H(3) = 11.51, p = 0.009$). A post hoc test found significant differences between

292 Model ($76.7 \pm 118.40 \text{ nmol L}^{-1}$) and Live ($14.7 \pm 18.60 \text{ nmol L}^{-1}$) ($p = 0.006$), Model and
293 Olfaction ($8.0 \pm 6.43 \text{ nmol L}^{-1}$) ($p = 0.001$) and Olfaction and Control ($30.6 \pm 26.73 \text{ nmol L}^{-1}$)
294 ($p = 0.017$).

295 **5 Discussion**

296 The study evaluated physiological effects in lumpfish exposed to Atlantic salmon or to
297 different salmon sensory cues over a duration of 6 weeks. Live, Model and Olfaction
298 treatments induced no change in growth, body coloration, health assessments or basal levels
299 of plasma cortisol. Brain analysis revealed significant changes in concentrations of
300 neurotransmitters observed in lumpfish cohabiting with Live Atlantic salmon. Both dopamine
301 and norepinephrine concentrations were significantly lower in the Live treatment compared to
302 Control. The stimulation and suppression test of the HPI axis found oversensitivity from the
303 ACTH injection in one lumpfish from the Model treatment, while the Live and Olfaction
304 treatments did not show any indications of oversensitivity. The present findings coincide with
305 previously observed physiological acute impacts on lumpfish exposed to the same, or similar
306 treatment protocols, and adds novel perspectives on how the species coexist over longer time
307 periods without impeding the resting state or the welfare of the species (Staven et al., 2019,
308 2021). Below, we elaborate each specific investigated parameter.

309 **5.1 Growth**

310 In aquaculture, specific growth rate (SGR) and the condition factor (K) can be used as
311 indicators on the wellbeing of farmed fish (North et al., 2006; Calabrese et al., 2017),
312 Deviations from a normal resting state can alter the metabolic scope and energy expenditure
313 in fish due to the relocation of energy surpluses (e.g., gluconeogenic effect of corticosteroids)
314 (Barton and Iwama, 1991). In lumpfish, SGR has been previously studied in relationship with
315 temperature and fish size (Nyrtrø et al., 2014), families (Imsland et al., 2021), diet types
316 (Imsland et al., 2019), water exchange and oxygen saturation (Jørgensen et al., 2017). In the
317 present study we investigated if predator cues could impact lumpfish to an extent where
318 growth was affected. After 6 weeks of interaction, lumpfish showed no change in SGR or
319 condition factors (K) from salmon sensory cues or cohabitation with Atlantic salmon in
320 comparison with the Control. In other prey fish, or fish relatively small, the presence of
321 predatory cues caused reduced growth performance (Strand et al., 2007; Sunardi et al., 2007)
322 or impairments on the capacity of prey fish to obtain and preserve energy resources (Lima and
323 Dill, 1990). When lumpfish are utilized in aquaculture, the transition from rearing conditions
324 with conspecifics only to interaction with large Atlantic salmon induces elevated levels of

325 plasma cortisol, increased swimming activity and avoidance behavior (Staven et al., 2019). A
326 follow up study revealed that swimming activity rapidly increased upon exposure to salmon
327 cues before dropping again within the first hour of interaction (Staven et al., 2021). In
328 accordance with both Staven et al. (2019) and Staven et al. (2021), salmon cues can initiate
329 moderate acute stress responses, while these responses diminished during a prolonged
330 interaction and did not affect growth. Overall SGR in treatments were approximately 0.6
331 points lower in comparison with Nytrø et al. (2014). Both studies had similar light regimes,
332 temperatures and oxygen saturations, yet Nytrø et al. (2014) conducted an optimal feeding
333 trial with more lumpfish per tank and lower initial individual variation in weight. This shows
334 that SGR is a relatively stable measurement of expected growth in a tank experiment and thus
335 a good welfare indicator based on the given environment. The overall importance of our
336 findings on SGR and condition factor was the limited effect from Atlantic salmon or salmon
337 cues alone.

338 **5.2 Skin coloration**

339 Lumpfish express variation in skin coloration both early in ontogeny and after maturation
340 (Davenport and Thorsteinsson, 1989; Davenport and Bradshaw, 1995). While skin coloration
341 and pigmentation have previously been observed to alter based on background color and hue
342 (Davenport and Bradshaw, 1995), acute changes within an hour of interaction were previously
343 detected in both parameters when juvenile lumpfish were exposed to Atlantic salmon
344 olfaction (Staven et al., 2021). In the current study, lumpfish color (measured as mean R + G
345 + B) did not differ between any of the three treatments compared to Control. Acute stress has
346 been associated with mediation and aggregation of melanosomes inside melanophore cells in
347 fish, regulating both color and darkening (Fujii, 2000; Aspengren et al., 2003; Nilsson Sköld
348 et al., 2013). Given that mechanisms of color change require energy, the physiological process
349 of habituation to salmon cues would cause an overall reduction in unnecessary energy
350 expenditure in lumpfish. Overall, the long-term interaction with salmon cues revealed a novel
351 understanding of skin coloration change from social interaction and suggests that lumpfish
352 induce little visual alterations when the resting state is not disrupted, while acute stress
353 responses can increase both darkening and the vividness of the skin (Staven et al., 2021).

354 **5.3 Health assessment and mortality**

355 Health scores were assessed before, during and after the experiment based on methods
356 described in Imsland et al. (2020). The experiment did not cause any deterioration in health
357 score from interaction with the different salmon cues in comparison to the health scores

358 observed in Control. This is the first experiment to measure the effect on health for lumpfish
359 in interaction with Atlantic salmon or exposure to salmon sensory cues in a confined tank
360 experiment. Previous studies on health scores in lumpfish were conducted in commercial
361 scale sea pens together with Atlantic salmon and were not designed to measure solely the
362 effect from salmon interaction or salmon cues (Imsland et al., 2020; Gutierrez Rabadan et al.,
363 2021). In general, the little negative impacts on health scores were in accordance with the
364 physiological measurements. With no clinical or physiological indications of negative impacts
365 from treatments, zero mortalities in lumpfish were as expected.

366 **5.4 Neurotransmitters**

367 Monoaminergic neuromodulators have important roles in the regulation of different
368 physiological and behavioral processes, both in vertebrates and invertebrates. This includes
369 learning and memory, wakefulness and arousal, stress responses, social interactions and
370 aggression, among others (Swallow et al., 2016; Ranjbar-Slamloo and Fazlali, 2020). Recent
371 studies have shown that brain monoamines might be important in regulating interspecific
372 patterns of behavior such as those between cleaner fish and their clients (Soares 2017; de
373 Abreu et al., 2018). In our study, the 6-week cohabitation with Live salmon induced a
374 decrease in the levels of catecholamines, dopamine and norepinephrine in the brain of the
375 lumpfish. Both compounds share a biosynthetic route from the amino acid tyrosine and seem
376 to functionally overlap to a certain extent in vertebrates (Ranjbar-Slamloo and Fazlali, 2020).
377 The dopaminergic system in fish is known to be involved in processes such as locomotor
378 activity, learning, motivation, reward, reproduction, stress and social behavior including
379 aggression, dominance, and interspecific interactions, as observed between cleaner and client
380 fish (Summers and Winberg, 2006; Gesto et al., 2013; Soares, 2017; Ganesh, 2021). Among
381 those functions, both stress-related factors and the social interaction with the introduced
382 salmon might have been involved in the observed changes in the lumpfish brain. Furthermore,
383 based on the results of this and a previous study (Staven et al., 2021), the social factor was
384 likely the most important. Social factors were considered the most relevant because stress
385 related alterations in the brain monoaminergic systems are usually more prominent in the
386 serotonergic than in the dopaminergic system, both in situations of acute and prolonged stress
387 (Øverli et al., 2001; Gesto et al., 2013; Conde-Sieira et al., 2018; López-Patiño et al., 2021),
388 and no alterations were found in the serotonergic system in this study. Furthermore, our
389 previous study (Staven et al., 2021) showed that both Live and Olfaction treatment were
390 equally able to acutely modify the swimming activity of the lumpfish (in a kind of “startling”

391 response), while only the Live treatment significantly affected the lumpfish brain in the
392 current study. Even if the observed brain alterations could be the result of social factors
393 related to the presence of salmon, the precise physiological significance of the reduced levels
394 of both dopamine and norepinephrine in the lumpfish exposed to live salmon are difficult to
395 interpret. Reduced levels of the parent catecholamines could be the result of a reduced
396 synthesis rate, a sustained higher release and subsequent oxidation, or both. In the Live group,
397 despite the reduction in dopamine, the DOPAC levels remained unaltered, thus resulting in a
398 higher DOPAC/DA turnover ratio. The higher turnover ratio suggests that DAergic neurons
399 had a higher firing rate in the Live group. Acute changes in the dopamine system have been
400 observed before in specific brain areas upon acute exposure to stress or social interaction
401 (Gesto et al., 2013; Teles et al., 2013; de Abreu et al., 2018b). However, the comparison of
402 the current data with those studies is complex since we did not perform a brain region-specific
403 analysis. Furthermore, prolonged exposure to the same events might result in different
404 alterations, since the brain will have time to finely modulate the release of neurotransmitters
405 to adjust to the new situation by using different feedback mechanisms (Best et al., 2009,
406 2010). In addition, prolonged exposure periods would include potential effects of other brain
407 processes related to memory, learning, and motivation/reward mechanisms to adjust fish
408 behavior to the new situation. In this regard, it can be even questioned whether the turnover
409 ratios (metabolite/amine) are still good estimators of neuron firing rates, since other factors
410 could be having a relevant effect on the levels of the metabolite; for example, alterations in
411 the monoamine oxidase activity cannot be ruled out (Higuchi et al., 2017, 2019).
412 Interestingly, a long-term increase in overall brain catecholaminergic activity was observed in
413 gilthead seabream after early life exposure to stress for 2 weeks (Vindas et al., 2018). In that
414 study, however, the DAergic ratio was increased in the brain because of increased levels of
415 the metabolite DOPAC, and not because of a decrease in dopamine amounts, so the
416 mechanism behind the alterations is likely different. Together with the lack of differences in
417 terms of growth performance and health scores, brain data suggest that lumpfish were able to
418 cope with the treatments, and only the Live group required some adjustment in the brain
419 pathways involved in behavioral regulation. The fact that dopamine adjustments were
420 observed only in Live and not in the Model or Olfaction treatments, demonstrate that single
421 cues were not having a relevant effect on the lumpfish brain in the long term, also supporting
422 the view that dopamine alterations were likely the result of interspecific social contact.

423 **5.5 Effects on the HPI axis**

424 In mammals, several changes in the HPI-axis have been documented during a chronic stress
425 state. This includes weight loss, proliferation of the corticotrope cells in the anterior pituitary,
426 inhibition of ACTH synthesis by cortisol, reduction of the feedback effect of glucocorticoid
427 receptor agonists on ACTH release, increase of the size of the adrenal glands and of the
428 response of the adrenals to ACTH (Mormède et al., 2007). This resetting of the HPI axis at a
429 new level of activity (set points), that Selye (1975) described as the stage of resistance, is also
430 known as allostasis (McEwen, 1998; McEwen and Wingfield, 2003; Goymann and Wingfield,
431 2004; Wingfield, 2005). Different approaches can be used to detect these changes as
432 described above, including stimulation tests (activating corticotropic releasing hormone,
433 vasopressin, ACTH and insulin-induced hypoglycemia) that measure the relative sensitivity of
434 the pituitary and the interrenal cells, and the use of an inhibition test utilizing dexamethasone
435 (DEX) to demonstrate the reduced efficiency of the negative feedback by corticosteroids
436 (Mormède et al., 2007). In this experiment, the treatment exposure induced variations in mean
437 concentrations of plasma cortisol after administration of a weight-adjusted dose of ACTH. At
438 first observation, lumpfish exposed to Models revealed oversensitivity to administration of a
439 weight-adjusted dose of ACTH compared to the other treatments. Data unveiled a single
440 outlier (strong stress response of 366.85 nM), which increased mean plasma cortisol level of
441 the Model treatment from 18.34 to 32.86 nM. While removal of the outlier would have
442 impacted the statistical outcome, resulting in no significant difference between Model,
443 Olfaction and Live treatments, individual variation in stress responses in fish should be
444 accounted for. This is due to the reality of different behavioral strategies observed between
445 individuals within the same species referred to as bold or shy, proactive, or reactive, or as
446 differences in personalities (Schjolden and Winberg, 2007; Toms et al., 2010; Yuan et al.,
447 2018). In lumpfish, differences in personality have recently been observed when individuals
448 were exposed to novel objects and later introduced to Atlantic salmon (Whittaker et al., 2021).
449 Variation in stress responses in lumpfish during novel heterospecific interactions could be
450 associated with why only certain lumpfish graze sea lice when deployed in net pens with
451 Atlantic salmon, especially in the context of neurotransmitters associated with cooperative
452 establishment and social behavior. Overall, few studies on ACTH sensitivity have been done
453 on fish. However, Pottinger and Carrick (2001) showed that two strains of rainbow trout
454 selected for high (HR) and low (LR) responsiveness to a standard crowding test had different
455 responsiveness to a weight-adjusted dose of ACTH. The LR strain had significant lower

456 production of plasma cortisol compared to the HR strain. In domesticated mammals, an
457 injection of ACTH has shown an increased cortisol response in animals reared in poor
458 conditions or subjected to repeated stressors. Similarly, Iversen and Eliassen (2014) showed
459 that salmon that were stressed daily over a 4-week period became oversensitive to ACTH,
460 regardless of whether the fish were vaccinated before or after applied stressor. One can only
461 speculate how the Model treatment triggered the HPI axis in a single individual. However, the
462 models used were novel, and the introduction of novel objects is a common method used to
463 distinguish different individual responses in fish (Castanheira et al., 2013; White et al., 2013;
464 Whittaker et al., 2021). Exposure to Models were not enough to elicit at full blown allostasis
465 type 2 response, referred to as chronic stress, recognized by an oversensitive ACTH axis
466 linked with non-functioning negative feedback (DEX) as described by Iversen and Eliassen
467 (2014). Plasma cortisol measured in lumpfish sampled from the experimental tanks after 6
468 weeks of treatment exposure revealed no significant difference between the different
469 treatments and the control. Still, a tendency of reduced plasma cortisol levels in the Live
470 treatment was observed, similar what was seen as reduced concentrations of norepinephrine
471 and dopamine after coexisting with Atlantic salmon. The combination of the stimulation and
472 suppression test together with overall low basal levels of plasma cortisol suggested that
473 treatments and interaction with Atlantic salmon induced a plastic change in lumpfish where
474 the threshold of activation of the HPI-axis was downregulated.

475 **6 Conclusion**

476 We investigated how lumpfish responded to the presence of carnivorous Atlantic salmon or
477 salmon sensory cues through analysis of multiple physiological parameters relevant to
478 welfare, social interaction, and stress during a 6-week tank experiment. Results showed that
479 growth, health scores and skin coloration remained unaltered. The stimulation and
480 suppression test of HPI-axis and the overall low plasma cortisol levels suggested no indication
481 of allostasis type 2 chronic stress or acute stress, respectively. Interaction with live salmon
482 induced alterations in the brain of the lumpfish, which revealed reduced levels of brain
483 catecholamines, namely norepinephrine and dopamine. The cause and function of these
484 alterations are not completely understood but could be part of a necessary process to adjust
485 lumpfish behavior to cohabiting with free-roaming salmon. Lumpfish behavior was not
486 assessed during the experiment, and a future focus on potential relationships between altered
487 neurotransmission and behavioral adjustment would be necessary to test if that was the case.
488 The novel findings on the plastic change in neuromodulators could be utilized in future work

489 identifying targets for breeding selection, considering the importance of their role in social
490 interaction in other species of cleaner fish. Overall, this study showed that lumpfish were not
491 negatively impacted by cohabitation or exposure to salmon cues, which suggested that welfare
492 disruption in commercial production is probably related to a combination of Atlantic salmon
493 exposure with other stressors.

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802 **9 Figure captions**

803 Figure 1. Specific growth rate (SGR % day⁻¹) in lumpfish (n = 40 per treatment) during 6
804 weeks of interaction with Live, Model or Olfaction treatments or Control. Mean
805 measurements with 95 % confidence interval error bars revealed no difference between
806 treatments measured every second week, including an overall measurement comparing initial
807 weight with endpoint weight.

808 Figure 2. Lumpfish skin color (n = 24) measured as mean Red + Green + Blue pixels (A)
809 before and (B) after treatment exposure. Mean R + G + B did not deviate between treatments
810 before or between treatments after the experiment. Lumpfish were reared in experimental
811 tanks with Live Atlantic salmon or different salmon sensory cues (Olfaction and Model).
812 Boxplots describe median values with quantiles and outliers.

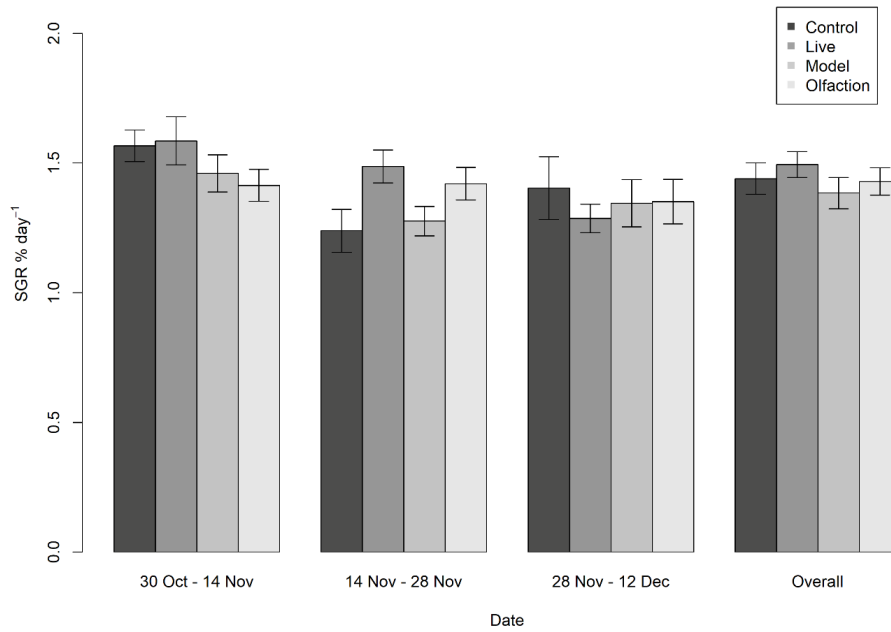
813 Figure 3. The levels of neurotransmitters (ng g⁻¹) in left hemisphere of brains from lumpfish
814 (n = 12) exposed to Live, Model or Olfaction treatments in addition to a Control.
815 Neurotransmitters include (A) DA (dopamine), (B) DOPAC (3,4-dihydroxyphenylacetic
816 acid), (C) the DOPAC:DA turnover ratio, (D) 5HT (serotonin), (E) 5HIAA (5-
817 hydroxyindoleacetic acid), (F) the 5HIAA:5HT turnover ratio, and (G) NE (Norepinephrine).
818 Treatments that share a letter were not significantly different ($p < 0.05$).

819 Figure 4. Mean basal plasma cortisol (nM) levels with 95 % confidence intervals in lumpfish
820 (n = 24) after 6 weeks of treatment exposure. Treatments involved interaction with 2 Atlantic
821 salmon (Live), 2 salmon models (Model) or salmon smell from a tank containing 4 Atlantic
822 salmon (Olfaction).

823 Figure 5. Mean plasma cortisol (nM) levels with 95 % confidence intervals in lumpfish (n =
824 16) 2h post intraperitoneal injection with either 0.5 mL kg⁻¹ phosphate-buffered saline (PBS)
825 or 45 µg mL⁻¹ adrenocorticotrophic hormone (ACTH). 24h prior to sampling, lumpfish were
826 injected with 1 mg kg⁻¹ dexamethasone in ethanol: PBS; 1:3; 1 µg L⁻¹ (DEX) and relocated to
827 dark tanks with the same environmental conditions as during the experiment. Treatments
828 involved interaction with Live Atlantic salmon, salmon Models or salmon smell from a tank
829 containing 4 Atlantic salmon. Treatments that share a letter were not significantly different (p
830 < 0.05).

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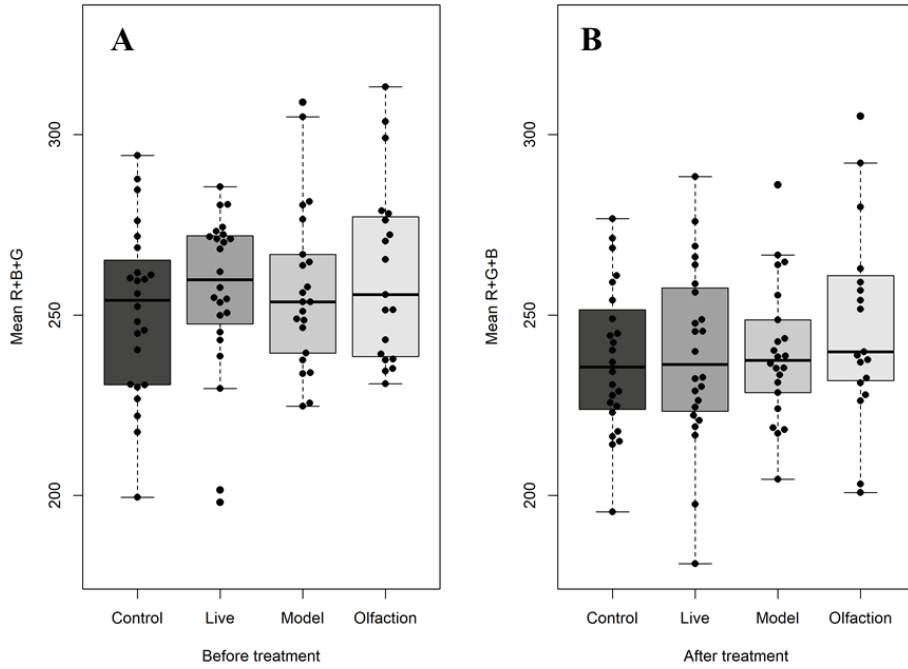
832 **10 Figures**



833

834 Figure 1. Staven et al. 2022

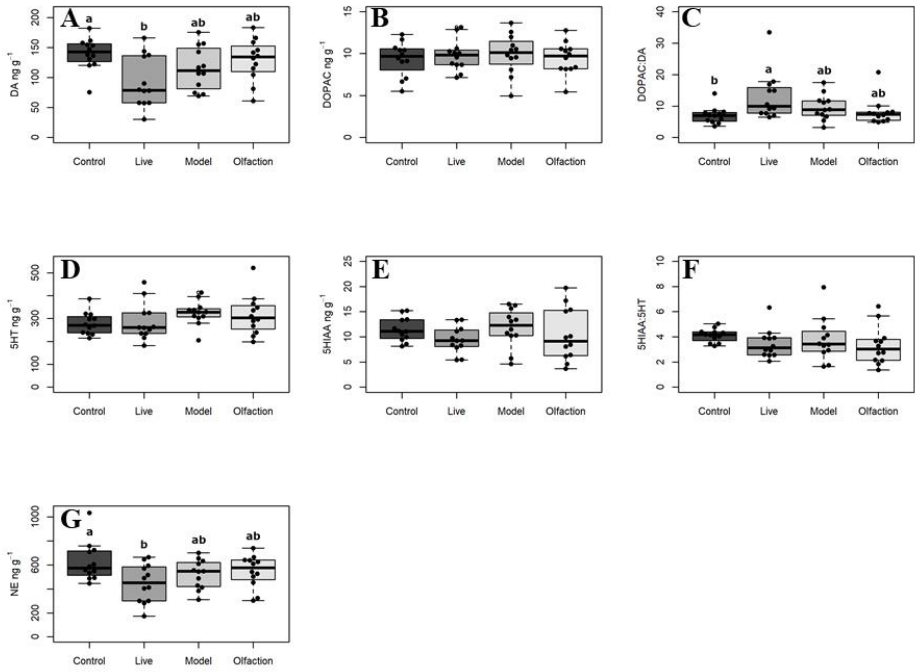
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837 Figure 2. Staven et al. 2022

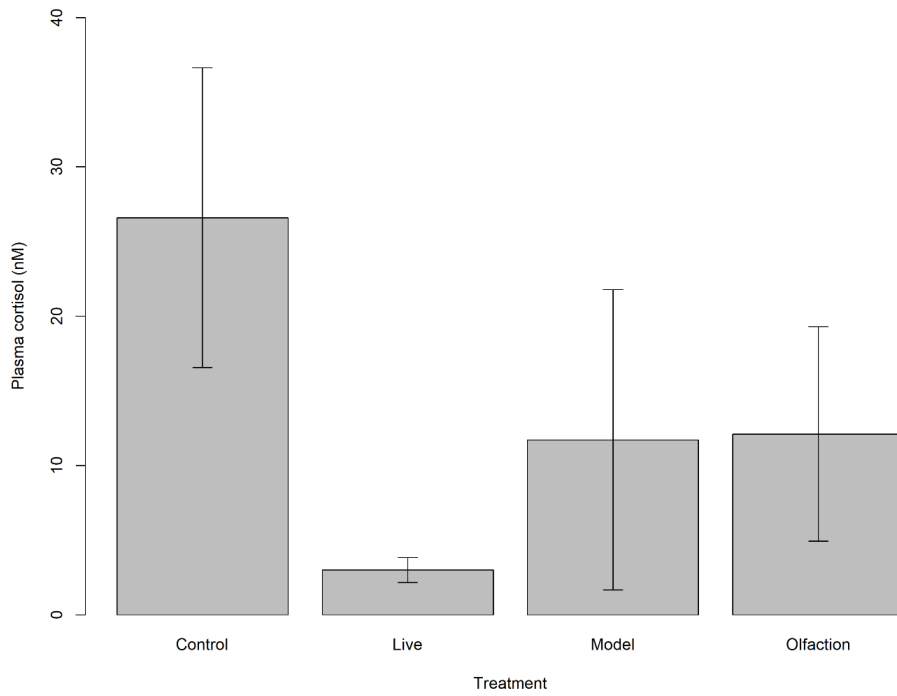
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840 Figure 3. Staven et al. 2022

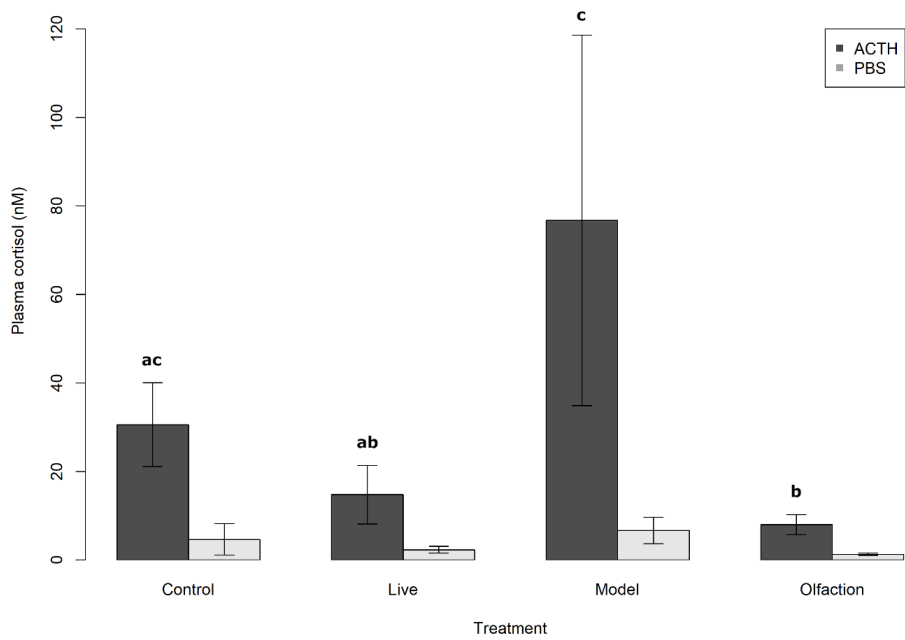
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843 Figure 4. Staven et al. 2022

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846 Figure 5. Staven et al. 2022

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Relationship status? It's complicated. Especially when it comes to lumpfish and their craving for farmed Atlantic salmon sea lice. These species could benefit from each other's company, as the lumpfish gets a snack, and the salmon is relieved from the itchy congregation on its back. Behind what we observe, a complex cognitive machinery is running on high gear. Interacting with salmon involves risk-taking, and most smaller fish species are in general equipped with innate fear to predator cues. In aquaculture, the lumpfish suffer from high mortality, so how can we ensure that stress from interacting with thousands of salmon is not impeding the welfare of the species? In addition, is the lumpfish capable of habituation, and can we measure it? In this thesis we present research on how behaviour and physiology in lumpfish changed in response to salmon or salmon sensory cues. Our findings revealed that lumpfish have innate stress responses to salmon, but only during an acute initial phase. Lumpfish changed its behaviour, activated the stress responses and neurochemistry related to social interaction and altered body colouration and pigmentation. Over time in cohabitation, these responses diminished while welfare parameters revealed no negative effect on the species.