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Behaviour in Atlantic salmon confers protection against an ectoparasite

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ABSTRACT

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Altered behaviour is believed to be the first form of defense against parasite infection, through reducing opportunity for infection or deflecting parasites to sub-optimal sites on hosts. To determine whether the suite of behaviours fish exhibit deter or deflect infection, we tested for differences in salmon louse (*Lepeophtheirus salmonis*) infection level and attachment location on three groups of Atlantic salmon (*Salmo salar*) that were either normal, behaviorally modified via injection of ketamine hydrochloride, or sham-injected procedural controls. The frequency of behaviours differed between behaviourally modified and normal hosts, which resulted in 26 – 31% fewer lice in normal fish. Louse attachment locations were unaffected by behaviour. Jumping and motionless behaviours were more common and the frequency of burst swimming behaviour was reduced in non-manipulated fish, suggesting that these behaviours restrict parasite attachment success. Our results show that the suite of fine-scale behaviours that normal salmon display at the time of parasite encounter are associated with reduced infestation.

INTRODUCTION

The behaviour of animals is intrinsically linked with the multitude of parasites and infectious agents that permeate the environment, and host-parasite interactions drive the feedback to further develop resistant behaviours in hosts (Hart, 1990; Ezenwa *et al.*, 2016). As the first line of defence against parasites, host behaviours that limit exposure or parasite load will arise if the deleterious effects of infection are greater than the cost of displaying the behaviour (Barber *et al.*, 2000). Such behaviours are diverse, from fine-scale actions such as grooming or swatting, to broad-scale behaviours such as social interactions or spatial relocation (Hart, 1990; Moore, 2002; Hart, 2011). In fishes, behavioural resistance largely comes in the form of avoidance, whereby choices are made to evade or reject parasite-risky habitats, infected conspecifics, or sick prey (Kiesecker *et al.*, 1999; Barber & Rushbrook, 2008). Although anti-parasite behaviours are common in terrestrial and some aquatic species (Moore, 2002; Sears *et al.*, 2015), examples of behaviours to deter infection at a fine scale are few among fish species, with scraping and flashing, and employing the services of cleaner fish the predominant form of resistance post-infection (Barber *et al.*, 2000; Barber & Rushbrook, 2008). The range of behaviours may be narrower for fishes compared to terrestrial species, but the efficacy of behaviour in preventing infection plays an important role in susceptibility and transmission (Hawley *et al.*,

2011), and fish are unlikely to experience different strength of selection for fine-scale behavioural resistance. The low number of known examples of fine-scale behaviours in fish may be associated with the difficulty of conducting these experiments in the field, or linking behaviours with infection success of parasites.

Salmonids are a particularly well-studied group of host species, due to their social and economic value. The salmon louse *Lepeophtheirus salmonis* is an ectoparasite that specializes on salmonid hosts, and is economically significant due to amplification of its populations in Atlantic salmon aquaculture. The infective stage of the parasite is free-swimming, and infests the host in the marine environment. Once attached, the louse feeds on the mucous, skin, and blood of the host (Costello, 2006). Infestations of *L. salmonis* can be costly to the host at high densities of the mobile life-history stages, causing lesions and subsequently, secondary infections and osmoregulatory failure (Wagner *et al.*, 2008). A high parasite load lowers the fitness of the host during migration, affecting swimming performance and survival (Wagner *et al.*, 2003; Vollset *et al.*, 2015; Bui *et al.*, 2016), and so behaviours to reduce parasite number are expected. Indeed, salmonids prefer brackish depths early in their migration (Thorstad *et al.*, 2012), a behaviour thought to avoid freshwater-sensitive salmon lice copepodids (Wright *et al.*, 2016), which are absent in the brackish waters. Salmonids have little opportunity to remove parasites once they are attached, and thus the threat of infestation should drive the development of anti-parasite behaviours, particularly when low saline waters are inaccessible.

Evidence for behavioural responses of aquatic hosts post-infection are numerous (Barber *et al.*, 2000; Barber & Rushbrook, 2008), however, whether fine-scale behaviours prior to or at the point of infection confer protection remains unknown. If higher costs are associated with parasite attachment to certain body parts, deflective behaviours may also develop to influence the site of infection (e.g. away from the head, Sears *et al.*, 2013). The behavioural repertoire of salmon may provide an advantage over salmon lice and avert attachment away from costly locations on the host.

Here, we aimed to determine whether Atlantic salmon deter or deflect parasite attachment by displaying behaviours immediately prior to and at the point of infestation. To test the efficacy of behaviour in deterring infestation, we compared fish with normal and altered behavioural phenotypes. We exposed salmon that were

either injected with a chemical to modify their natural behaviours, sham injected with a biologically neutral adjuvant, or not injected, to infective salmon lice copepodids. We then recorded the number and location of attached copepodids in each treatment. We tested two predictions based on the hypothesis that the natural suite of host behaviours confers protection from infestation: (i) infestations should be higher in the treatment showing atypical behaviours compared to the natural behaviour treatment; and (ii) behavioural modification should alter the position of attached copepodids relative to the natural behaviour treatment.

MATERIALS AND METHODS

Creating treatments with different behaviours

To test if the normal behaviour of salmon in response to risk of infestation led to a degree of protection, we created treatments with different behavioural profiles, and measured parasite number and body attachment location after an infestation challenge where all groups were represented in a tank. We engineered three behavioural phenotypes: 1) behaviourally altered via intramuscular injection (behaviourally manipulated; BM); 2) a procedural control whereby fish were injected with the same volume of saline solution (procedural control; PC); and 3) a control treatment that did not receive an injection (non-manipulated; NM). Throughout the study, assessors of behaviour and parasite levels were blind to these treatment groups.

To create the behaviourally manipulated phenotype, we used a long-lasting sedative. Ketamine hydrochloride at sub-anaesthesia doses induces dissociative analgesia in fish, while maintaining normal ventilation and buoyancy (Neiffer & Stamper, 2009), and is appropriate when small groups of fish are to be sedated within a larger group (Graham & Iwama, 1990). Zebrafish exposed to ketamine remain behaviourally active (Riehl *et al.*, 2011), with a higher frequency of abnormal circling activity and hyperactivity to sensory stimuli, and a reduced stress response (Zakhary *et al.*, 2011). Using ketamine enabled us to partially sedate fish for the infestation period (60 minutes) with muted host responsiveness to parasite encounters, while fish maintained their normal buoyancy and respiration (Stage I of anaesthesia; Zahl *et al.*, 2012). The presence of intramuscular ketamine is unlikely to alter the chemical signals or immune response of the host in such a short time frame, and therefore host

attractiveness and response should remain the same to copepodids compared to control hosts.

Experimental fish

Atlantic salmon (AQUAGEN strain) were raised in tank facilities under a standard grow-out regime. Fish underwent smoltification 1.5 months prior to the experiment, and were housed in seawater tanks ($\varnothing = 3$ m, 5.3 m³) indoors at 12°C, identical to those used during the experiment. Salmon weight (mean $\pm$ SE = 139 $\pm$ 33g) did not differ among treatments (ANOVA: $F_{2,87} = 1.8$, $P > 0.1$). Salmon were kept in natural light cycles, and fed to a standard schedule. Feed was withheld 1 day prior to the infestation challenge.

Fish were hand-netted from their holding tanks into a smaller transfer tank (1 m³) with full sedation using tricaine methanesulfonate (Finquel 1 g/10 L). Treatments were applied to fish according to a random order, and sequentially assigned to treatment tanks. All fish were tagged adjacent to the dorsal fin with an anchor tag (11 cm, Hallprint Ltd, Australia) with unique colour combinations (e.g. Bui *et al.*, 2013). Fish in the sedated treatment ($N = 29$) were then injected into the dorso-posterior lateral muscle with a volume containing a 40 mg/kg dose of ketamine hydrochloride (in solution at 10 mg ketamine base millilitre; Ketalar™), calculated specifically for each individual according to their weight. Procedural control fish ($N=31$) underwent the same handling and intramuscular injection with a saline solution, whereas control fish ($N=30$) were not injected, but handled similarly. After being placed in the experimental tank, the group was monitored for full recovery before introducing the infective salmon lice copepodids. For each of the 5 experimental tank replicates, all three treatments were represented in the tank, equating to 15–21 individuals per tank. Numbers of fish in the groups, and therefore in the tanks, varied due to the pre-determined time frame in which fish could be processed, to standardise recovery time before infestation.

Experimental salmon lice

Adult female salmon lice were sourced from nearby salmon farms and transferred to a stock group of fish in tanks. When required, egg strings were harvested and incubated at 12°C for 10 days to produce the copepodids.

Infestation protocol

Infestation challenges were conducted on April 4, 16, and 24, 2014. The staggered days were due to the availability of infective copepodids. Immediately prior to the experiment, water level in a tank was dropped to 0.4 m depth (2.8 m³). Fish were then treated and added to the tank. After all fish had recovered from sedation with tricaine methanesulfonate, water inflow was reduced to 5–10 L/min, with a recirculating pump (110 L/min) creating low flow during the infestation period. When the tank environment had stabilised, infective copepodids were introduced. With a predicted infestation success rate of 30% (Bjørn & Finstad, 1998) and an intended infestation level of 10 lice per fish, 30 copepodids per fish were introduced. At the conclusion of the 60 min infestation period, water flow was returned to normal (120 L/min) and water levels rose back to 0.75 m depth.

After the infestation period, fish were left for ~30 h to allow the copepodids to settle. Fish were collected by hand after light sedation in the experimental tank, and placed into a smaller holding tank containing an overdose of anaesthesia. Infestation prevalence, intensity, and location of attachment of salmon lice were recorded for each individual, with the assessor blind to the treatment group. Areas of attachment on the host body were categorized following Bjørn and Finstad (1998) into: head (and operculum), gills, anterior-dorsal, anterior-ventral, posterior-dorsal, posterior-ventral, pectoral fin, ventral fin, dorsal fin, anal fin, and caudal fin.

Behavioural measures

During the 60 min infestation period, three submerged video cameras (GoPro™, California, USA) were affixed to the side of the experimental tank, with a wide-angle field of vision that captured ~90% of the experimental area. A separate camera attached above the tank looked downwards, covering 95% of the surface of the water. Recordings were made throughout the 60-minute infestation period, and viewed afterwards to document behaviours. Each fish was identified by its unique tag and the frequency of behaviours was recorded by a single observer for the first 45-minute period after copepodids were introduced, with the treatment blind to the observer. Behaviours after copepodids were introduced were categorized into frequency of

jumping/rolling at the surface, twitching, swimming bursts, and time spent motionless (Table I).

Statistical analyses

All statistical analyses were conducted in *R* version 3.1.0 (R Development Core Team, 2015). Infestation intensity was standardised to fish weight, and all analyses used lice g^{-1} . A two-way ANOVA compared the infestation intensities between treatments, with tank replication as a blocking factor. A post-hoc univariate test clarified which treatment groups differed.

The effect of all behaviours on the successful attachment of copepodids, across treatment groups, was determined with a linear mixed effect model. The model used behaviours as the predictor variables and lice intensity as the response variable, with tank as a random effect. The magnitude of behavioural responses exhibited during the infestation period was assessed with a MANOVA, comparing the effects of treatments (categorical predictor) on frequency of evasive behaviours – jumping, burst swimming, twitches, and proportion of time spent inactive (transformed using the inverse-logit function). Post-hoc univariate tests, with a Bonferroni correction, were used to determine which response variables differed. In addition, a discriminant analysis determined the nature of differentiation between treatments using their relationship with the behavioural response parameters.

Locations on the host where salmon lice copepodids attached were predicted to be concentrated on specific regions of the body and fins (Bjørn & Finstad, 1998). Therefore, planned comparisons were conducted for the proportion of lice on the head, ventral sides, dorsal fin and all ventral fins (pectoral, pelvic, anal). Proportions were transformed using the inverse-logit function and compared between groups using univariate tests, with a Bonferroni correction on our criterion for significance. For all analyses, parametric test assumptions (normality and homogeneity of variances) were evaluated using residual plots.

Animal Ethics

The experiment adhered to the regulations stipulated by the Norwegian Regulation on Animal Experimentation (Animal Ethics application ID: 6150).

RESULTS

Effect of behaviour on infestation intensity

The data satisfied the assumptions of all statistical tests. Behavioural responses modified subsequent infestation intensity in salmon, where NM and PC salmon acquired 31% and 26% fewer lice, respectively, compared to the BM treatment ($F_{6,83} = 8.6$, $P < 0.001$; Fig. 1). Infestation levels averaged 22 lice per fish for NM and PC treatments (0.17 lice g^{-1}), whereas BM fish acquired approximately 34 lice (0.24 lice g^{-1}). The post-hoc test confirmed that no difference in infestation levels existed between NM and PC groups (Tukey's HSD: $P = 0.9$), yet both groups differed from the BM group (Tukey's HSD: $P = 0.02$ and 0.01 for NM-BM and PC-BM, respectively; Fig. 1).

The effect of behaviours on the intensity of parasites acquired was significant for burst swimming ($t = 3.2$, $P = 0.002$) and time spent motionless ($t = -2.3$, $P = 0.02$), but not for jumping ($t = 1.6$, $P = 0.12$) or muscle twitches ($t = 0.7$, $P = 0.5$).

When parasites were introduced, NM and PC fish behaved similarly, however BM fish had a different behavioural profile. A MANOVA test that incorporated jumping, proportion of time inactive in the tank, and burst swimming and twitching frequency (Pillai's Trace $V = 0.5$, $F_{8,170} = 9.7$, $P < 0.001$) showed that behaviour differed among treatment groups. The BM group had approximately 6.5 times less surface activity (post-hoc univariate test with Bonferroni correction: $F_{2,87} = 9.7$, $P < 0.001$; Fig. 2a), spent 50% less time motionless ($F_{2,87} = 4.7$, $P = 0.01$; Fig. 2b) and displayed 1.9 to 2.8 times more bursts ($F_{2,87} = 17$, $P < 0.001$; Fig. 2c) compared to the two non-manipulated groups. Twitching did not differ among treatments ($F_{2,87} = 2.4$, $P = 0.10$; Fig. 2d).

Discriminant analysis revealed two discriminant functions explaining 96% and 4% of the variance, respectively. The first variate discriminated the BM treatment from NM and PC treatments, while its coefficients highlighted a large effect of inactivity ($b = 5.4$) with small contributions from jumping ($b = -0.13$), burst swimming ($b = 0.05$) and twitching ($b = -0.01$). The second function was less clear on discriminating between treatments, but produced similar coefficients for behaviours.

Effect of behaviour on parasite attachment location

Despite behavioural differences among behaviourally manipulated and non-manipulated groups, the distribution of lice attachment across the body did not differ among treatments (multiple comparisons with adjusted α criterion, all $P > 0.15$, Fig. 3).

DISCUSSION

Our results show that Atlantic salmon can deter salmon lice copepodid attachment through their behaviour. Clear differences in parasite load existed between behaviourally modified fish and those able to exhibit the normal repertoire of behaviours. Behavioural modification did not alter the attachment location of copepodids compared to the natural behaviour treatment. Normal salmon displayed greater frequencies of surface behaviours (jumping and rolling) and less swimming activity compared to behaviourally impaired individuals. This study retains high internal validity with the use of common garden replicate tanks, randomisation of fish groups and observers blind to the treatment when assessing behaviour and lice loads.

Potential mechanisms by which behaviour reduced infestation intensity

The combined behaviours exhibited by the alternative behavioural profile in manipulated salmon led to higher acquisition of parasites, however we cannot tease apart the effect of individual behaviours that drove this difference in parasite load. Instead, the purpose of this experiment was to determine whether natural behaviours conferred any protection against infestation by salmon lice and describe this behavioural repertoire. The differences in behaviour displayed in our experimental fish translated to reduced parasite loads in non-manipulated individuals, whereby jumping behaviour and inactivity contributed to resistance against infective lice. Burst swimming is theorised to also reduce successful attachment, however this behaviour was more pronounced in behaviourally manipulated fish.

Jumping behaviour, where the whole body bursts out of the water and becomes momentarily airborne, is thought to be a combination of physiological status and behaviour associated with their migration phase (Furevik *et al.*, 1993). In this study, jumping behaviour was 6 – 7 times higher in normal and procedural control fish than

behaviourally manipulated fish. While increased jumping in the behavioural profile correlated with fewer successful parasite attachments, we cannot determine whether jumping behaviour was causal or if it was a response to the sensation of being infested. Jumping increases after salmon become infested (Furevik *et al.*, 1993), yet whether this confers any advantage in removing early-stage copepodids or makes fish less likely to encounter free-swimming copepodids in the water column is unknown.

Activity levels in parasite-risky environments can affect infestation levels, with lower activity levels reducing possible encounters (Poulin *et al.*, 1991; Moore, 2002). *L. salmonis* detect hosts by sensing changes in water flow or mechanical disturbance that indicate a potential host swimming nearby (Heuch & Karlsen, 1997; Heuch *et al.*, 2007). Infestation susceptibility is influenced by fish swimming speed, with inactivity or slow swimming speeds leading to low infestations (Genna *et al.*, 2005). Similarly, we detected that in the behavioural profile that more inactive fish were less susceptible to parasite attachment. Reduced swimming activity likely leads to reduced encounters with infective copepodids, as hosts interact with a lower volume of water than if they were swimming (Poulin *et al.*, 1991). Our results produced a similar outcome to studies with behaviourally impaired tadpoles, whereby anaesthetised individuals with suppressed behaviours acquired 20–39% more parasites than unmanipulated tadpoles (Daly & Johnson, 2011; Sears *et al.*, 2013).

In contrast to jumping and inactivity levels that were markedly higher in non-manipulated fish, burst swimming events showed the opposite pattern. While increasing the encounter rate with parasites, burst swimming is likely to deny successful attachment of the parasite onto the host as copepodids have insufficient time to attach as they pass by the fish (e.g. Genna *et al.*, 2005). *L. salmonis* copepodids detect mechanical stimuli generated by potential hosts (Mordue Luntz & Birkett, 2009), and react to linear accelerations of the hydrodynamic flow derived from a fish swimming (Heuch & Karlsen, 1997). Copepodids exhibit swimming behaviours to situate themselves within the fish boundary layer where reduced micro-current flow allows them to attach to the host. If the host is swimming at high velocity, the chance of detection and attachment decreases for the parasite, as the fish boundary layer becomes thinner and the window for copepodids to respond is reduced (Mordue Luntz & Birkett, 2009). Increases in host swimming speed may also alter the flow field around the fish, hindering the settlement of copepodids in unsheltered areas

of the body (Bron *et al.*, 1991). While increased frequency of burst swimming in behaviourally modified fish could act to avoid infestation, it either did not in this experimental context or its effect was less than conferred by the other behaviours that were higher in the non-modified fish.

Although this study provides a narrow snapshot of behaviours during infestation, it relates to the broader context of domestication in host-parasite interactions. A wide range of human-induced change in aquatic systems alters the behaviours of animals (Tuomäinen & Candolin, 2011), and as these impacts become more pronounced, the capacity for hosts to deter parasites with their natural suite of behaviours may be limited. This is particularly the case with the rise of aquaculture, with over 1 billion Atlantic salmon now held globally at high densities in sea-cages, potentially restraining the ability or effectiveness of individuals to exhibit fine-scale anti-parasite behaviours (Price, 2002; Huntingford, 2004). As few viable options currently exist to mitigate disease in the oceans (Ward & Lafferty, 2004), understanding how anthropogenic changes alter fine-scale behaviours and host-parasite dynamics may facilitate a greater understanding of why parasite outbreaks occur.

Parasite attachment location

The location of ectoparasite attachment or endoparasite entry can drive the cost of infection in some host species, whereby high concentrations in vital areas (e.g. head or eyes) can be lethal. The cost of infection location can promote anti-parasite behaviours in the host that deter parasite attachment at optimal sites. For example, *Hyla femoralis* tadpoles have behaviours to prevent costly head infections from larval trematodes by deflecting the parasite towards the tail, resulting in better body mass preservation (Sears *et al.*, 2013).

Our results suggest that Atlantic salmon have experienced strong selection pressure for initial parasite attachment location and have not developed deflective behaviours. Behavioural phenotypes in this study were not associated with the location of copepodid attachment. Since the parasite largely exploits its host after moulting into the mobile stage, no selection pressure is likely. This is a logical result particularly as the parasite largely exploits its host after moulting into the mobile stage. Copepodid and chalimus stages incur a lesser physiological cost than the mobile pre-adult and adult lice stages, when feeding habits change and they move around the host for

feeding and sexual interaction (Jónsdóttir *et al.*, 1992; Todd *et al.*, 2000). The ability of pre-adult and adult stages to move around the body surface will render any initial host deflection behaviours ineffective.

Conclusions

The cost of infection is the largest driver for the development of anti-parasite or parasite avoidance behaviours (Barber *et al.*, 2000), and fitness reduction costs caused by *L. salmonis* are substantial (Wagner *et al.*, 2008). Fish allowed to exhibit natural behaviours had reduced infestation levels, which suggest these behaviours have an adaptive advantage and increase fitness. The effect sizes determined in the high infestation pressure tank environment may not necessarily translate to natural settings. The introduction of a single pulse of infective parasites to an environment with constrained water flow, external stimuli, and limited water volume contrasts to the natural exposure experienced by hosts. However, we aimed to capture host behaviours at the point of contact with copepodids, which may be more affected by group densities that restrict behaviours, and inflated by the high infestation pressure, compared to open waters. Further work is required to determine the external study validity of these results, yet this study provides early evidence for efficient anti-parasite behaviours in salmon using methods of high internal validity.

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TABLE I

Description of behaviours quantified during the infection period (45 min), and type of measure for the behaviour.

Behaviour	Description	Measure
Jumping & rolling	Jumps marked by upwards acceleration, breaking the surface and being momentarily airborne. Rolling behaviour is slower, with movements analogous to a surfacing whale (Furevik <i>et al.</i> , 1993; Korsøen <i>et al.</i> , 2012)	Frequency (per minute)
Bursts	The maximal speed achieved by a fish which cannot be maintained (Beamish, 1978), particularly marked by a push forward using the caudal fin. In this study, it was a high-energy burst from an individual's position, whether motionless or swimming slowly.	Frequency (per minute)
Twitches	A distinct contortion of the body along the midline following an 'S' shape, without resulting in movement away from the fish's original position	Frequency (per minute)
Inactivity	Time spent motionless, suspending themselves on their pectoral and pelvic fins against the tank bottom	Duration (proportion of observation period)

FIGURE CAPTIONS

Fig 1. The intensity of parasite infestation was different among three behavioural phenotypes of Atlantic salmon. Significantly more salmon lice were found on behaviourally manipulated (bm) compared to non-manipulated (nm) and procedural control (pc) individuals (mean $\pm$ SE; ANOVA: $P < 0.001$). Post hoc differences at $p < 0.05$ are indicated by letters above the bars.

Fig. 2. The manipulation of behavioural phenotype affected behavioural measures in Atlantic salmon. Behaviourally manipulated (bm) salmon exhibited (a) reduced frequency of jumping behaviour, (b) reduced levels of inactivity, (c) increased burst

swimming behaviours, and (d) a higher rate of twitching behaviour compared to the non-manipulated (nm) and procedural control (pc) salmon (mean $\pm$ SE; MANOVA: $P < 0.001$).

Fig. 3. Distribution of parasite attachment location as a proportion of the total parasite load in non-manipulated (white bars), procedural control (light grey bars) and behaviourally manipulated (dark grey bars) treatments. Abbreviations for body locations correspond to inset diagram: H = head, G = gills, A = anterior, P = posterior, D = dorsal, V = ventral, PF = pectoral fin, VF = ventral fin (labeled instead of pelvic fin), AF = anal fin, CF = caudal fin. Standard error bars are omitted for visual clarity. Body location diagram adapted from *Bjørn and Finstad (1998)*.

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