



Sex-selective Predation by Threespine Sticklebacks on Sea Lice: A Novel Cleaning Behaviour

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Abstract

Cleaning interactions have been described in a wide range of fish species and other taxa. We observed a novel cleaning behaviour during a study of the transmission dynamics of sea lice (*Lepeophtheirus salmonis*) between juvenile pink salmon (*Oncorhynchus gorbuscha*) and threespine sticklebacks (*Gasterosteus aculeatus*) in the Broughton Archipelago, British Columbia, Canada. Experiments showed that sticklebacks significantly reduced the number of sea lice on individual juvenile salmon. Adult female lice were preferentially consumed by sticklebacks, and gravid female lice also experienced egg string cropping. Overall, 76% of gravid female lice experienced either consumption, egg string cropping, or both by sticklebacks. This preference by sticklebacks for female parasites may stem from female lice being larger than males and the added nutritional value of egg strings on gravid females. Cleaning by sticklebacks can potentially have an impact on sea louse populations on wild juvenile salmon.

Introduction

Cleaning symbioses are interspecific, mutualistic interactions in which a cleaner receives some or all of its food in the form of ectoparasites from a client, who benefits in the form of a reduction in this parasite burden (Limbaugh 1961). These relationships are widespread in nature, especially among coral reef fishes, where this behaviour has evolved independently many times in distantly related taxa (Côté 2000). These interactions are usually presumed to be mutualistic, although it has not always been possible to demonstrate this empirically. While some studies have failed to show significant, direct benefits to client populations (Grutter 1997a; Cheney & Côté 2003) or a reduction in parasite load (Grutter 1996), others have found that cleaning interactions can reduce parasite loads as much as 4.5-fold in a relatively short time (Grutter 1999). This apparent benefit to clients is further supported in nature, as more

heavily parasitized clients are known to visit cleaning stations more frequently than their conspecifics (Arnal et al. 2001).

The importance of cleaning, however, goes beyond reducing numbers of parasites on clients. Selective feeding on certain age/size classes of parasites by cleaners can alter the demographic structure of the parasite population. Preferential consumption of larger parasites can skew the parasite population size structure towards smaller body size and result in decreased biomass of parasites on client fish, even if parasite numbers are unaffected (Gorlick et al. 1987). For example, gnathiid parasites found in the diet of the cleaner wrasse *Labroides dimidiatus* include a greater proportion of large individuals than do the parasites found on the body of the client – again indicating size-selective predation by the cleaner (Grutter 1997b). Opposite trends have also been seen in cleaners preferring smaller prey, which is likely because of throat-width limitations (Grutter &

Lester 2002). Impacts of cleaning interactions, therefore, extend beyond the commonly evaluated criterion of parasite numbers.

We have discovered a cleaning interaction in the Broughton Archipelago, British Columbia, Canada, which appears to involve mutualistic benefits. The system involves a massive outmigration of juvenile pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon. These are semelparous species that spawn in the fall in coastal streams. In the spring, the juvenile fish migrate to sea shortly after emerging from eggs. Pink salmon have a 2-yr life cycle, whereas chum salmon typically spawn at age 4 or 5. Juvenile salmon of these species have been found to possess elevated sea louse (*Lepeophtheirus salmonis*) parasite loads when they migrate near fish farms (Morton et al. 2004; Krkosek et al. 2005a), and infection patterns indicate farmed salmon as the main source of infection on these juveniles (Krkosek et al. 2006). *Lepeophtheirus salmonis* is an ectoparasitic copepod infecting salmonids throughout the northern hemisphere. Shortly after hatching from egg strings carried by females, the infective copepodid lice parasitize a host and are stationary through four chalimus lifestages. Upon reaching pre-adult and adult lifestages, lice become motile and can move on and between hosts (Johnson & Albright 1991). The artificially high parasite intensities on wild salmon associated with salmon farming can have dramatic negative health impacts on wild fish and fish populations (Morton & Routledge 2005; Krkosek et al. 2006, 2007).

Investigation into sea louse transmission dynamics in the region in 2006 and 2007 revealed a novel cleaning relationship between threespine sticklebacks (*Gasterosteus aculeatus*) (cleaner) and juvenile pink salmon (client). The objective of this paper is to present results from experiments that test for size- or stage-selective cleaning by sticklebacks and quantify the effect of cleaning on ectoparasite load of juvenile pink salmon.

Methods

Wild fish were collected from the nearshore environment in the Broughton Archipelago, BC, between 1 April and 30 June, in 2006 and 2007, during the main outmigration of juvenile salmon from local streams. Juvenile pink salmon were collected by beach seine net, and three spine sticklebacks were collected with beach seines or minnow traps. Fish were housed at the Salmon Coast Field Station (50°44'42.02"N; 126°29'57.42"W) on Gilford Island

within the archipelago in stock tanks with flow-through seawater (28–33%; 9–13°C) and exposed to natural light cycles until needed for trials (0–48 hours) and were fed regularly, 3–4 times per day. All sea lice present on fish were the result of natural infestation.

Individual trials were performed in 2006 and 2007, while group trials took place only in 2007. The aim of individual trials was to understand details of the cleaning behaviour, including predictors of cleaning interactions. Group trials were used to verify the results of the individual trials in a larger arena, as both fish species exhibit shoaling behaviour.

Individual Trials

Juvenile salmon for individual trials were selected haphazardly from the stock tanks subject to the criterion of being infested by at least one motile (pre-adult or adult) louse. Each juvenile salmon was caught carefully using a small, soft, fine-meshed dip net and placed into a rectangular (L35 × W20 × D20 cm) plastic aquarium with a single stickleback, also chosen haphazardly from the stickleback stock tank. Louse load on these salmon was assessed after placement in the experimental aquaria as sea lice can be dislodged during transfer. Accuracy of the parasite enumeration was confirmed by close inspection of all fish at the conclusion of the trials.

We created one treatment and two control set-ups. The treatment aquaria allowed unrestricted movement and interaction between the stickleback and infested salmon. The 2006 control set-up contained one naturally infested salmon and one clean salmon, to confirm that any effect on lice was because of the presence of the stickleback (see Fig. 2 for the experimental set-up). For the 2007 control set-up, each aquarium was divided into three sections using 6.35 mm mesh that would allow for passage of motile lice, but not of fish. This is important, as motile *L. salmonis* are able to switch hosts (Ritchie 1997) and, thus, could be consumed free-swimming in the water column. In these 2007 control aquaria, a stickleback was placed in one end and an infested juvenile salmon in the other. The empty middle section ensured that no cleaning could occur across a single mesh layer.

All aquaria were placed in a flow-through seawater bath to buffer water temperature changes. No food was provided during most trials; however, a subset of trials (N = 12) was conducted with food supplementation to control for any effect of

starvation over the 24-h period. Freeze-dried krill were provided to the food-supplemented sticklebacks while they were held prior to the start of the trial and then every 2 h during the daylight hours of the trial.

Trials took place in sets of 8–12 tanks curtained off to prevent disturbance and on an artificial light cycle of 16 h light to 8 h dark. Trials were run for 24 h, with regular monitoring at 6, 12, and 24 h. All observations were made by the primary author to ensure observer reliability. Observations and video of cleaning behaviour were taken from separate tanks that were not included in the trials to ensure that conditions were identical in all experimental aquaria. At each monitoring time, juvenile salmon were inspected from above and the absence of any of the lice identified at the beginning of the trial was noted. At the end of the trial, the fork length and body depth of each juvenile salmon were measured, and louse lifestage and sex were confirmed using methodology described by Krkosek et al. (2005b). Sticklebacks were similarly measured for total length and body depth. External gape width was also measured using electronic calipers at the base of a closed mouth, where the upper and lower jaws meet (the posterior edge of the maxillae).

If lice were missing from a salmon at the conclusion of the 24-h trial period, the aquarium was thoroughly inspected. If no lice were found, it was assumed that the parasite had been ingested. In these cases, the stickleback was euthanized by a blow to the head, and stomach contents were examined to confirm that a cleaning event had taken place. The treatment experiment was replicated 45 times in 2006 (with 30 control replicates), and 110 times in 2007 (with 42 control replicates) (see

Table 1a for sample sizes and lice age/sex distribution for individual trials).

Group Trials

For group trials, a flow-through trough (L55 × W55 × D55 cm) was separated into three sections, each containing approximately 120 l of seawater. Movement of fish and lice was not possible between sections, thus each section served as an independent, experimental arena for group trials. Methods used to select and transfer juvenile salmon and sticklebacks were as described earlier.

Each treatment group consisted of 10 naturally infested juvenile salmon and 10 sticklebacks. Salmon were transferred to the experimental tanks in small containers of seawater to avoid dislodging lice. While in these containers, lifestage and sex of each motile louse were identified. Each control group consisted of 10 naturally infected juvenile salmon and 10 uninfected salmon. The sticklebacks and uninfected salmon were measured prior to each trial, as the uninfected salmon would otherwise be indistinguishable from any cleaned salmon at the conclusion of the trial. The fish were then left for 24 h. At the completion of the trials, all fish were measured and louse load, lifestage and sex recorded as in the individual trials. This experiment was repeated 13 times in 2007 (see Table 1b for sample sizes and lice characteristics).

Analysis

The proportion of lice of all lifestages consumed was not significantly different between 2006 (35.4%; $N = 79$) and 2007 (37.4%; $N = 163$) ($\chi^2 = 0.09$,

Table 1: Summary lice characteristics in treatment and control groups for (a) individual trials in 2006 and 2007, and (b) 2007 group trials

(a) Individual trials in 2006 and 2007									
Year		No. of trials	Total No. of lice	Adult female lice	Adult male lice	Pre-adult female lice	Pre-adult males	Pre-adult unknown sex	No. of trials with lice lost
2006	Treatment	45	79	49	19	2	2	7	17
	Control	30	38	18	17	0	1	2	0
2007	Treatment	110	163	77	51	13	11	11	48
	Control	42	62	10	28	14	7	3	3

(b) 2007 group trials						
Year		No. of trials	Total No. of lice	Adult female lice	Adult male lice	No. of trials with lice lost
2007	Treatment	13	162	97	65	13
	Control	7	86	49	37	1

df = 1, $p = 0.76$) in the individual trials; therefore, these 2 yr of results are combined for analysis. Control trials in 2007 showed background levels of 4.8% (individual trials, $N = 62$ lice) and 1.2% (group trials, $N = 86$ lice) lice lost; thus, treatment louse loss is corrected by these amounts for both experimental set-ups. A generalized linear model with binomial error distribution was used to examine the influence of potential morphometric predictors of cleaning behaviour: stickleback total length, salmon fork length, body depth of both species, stickleback external gape width, salmon louse load, and stickleback/salmon length ratio. A maximal model with all predictors was built, and non-significant predictors were sequentially removed based on analysis of deviance until a minimum adequate model (i.e. only significant predictors) remained. Analysis was performed in R v 2.3. All p -values are two-tailed because of non-directional hypotheses.

Results

Cleaning Behaviour

Video documentation and direct observations show clear consumption by sticklebacks of lice and/or their egg strings directly from the skin of salmon (see still images from video in Fig. 1; see supplementary electronic information). There is no obvious posing (i.e. immobile posture with fins held erect) by the client to solicit cleaning; however, when approached by a stickleback, salmon did not exhibit avoidance behaviour. During a cleaning interaction, the stickleback positions itself perpendicular to the salmon and approaches the sea louse. The stickleback grabs the louse's genital complex, which is unattached to the host. The salmon can be dragged through the water during this process, indicating a great deal of force exerted by the stickleback. On several occasions, sticklebacks cropped or completely consumed the egg strings of gravid female lice without attacking the body of the louse itself. Cleaning is quantified below.

Cleaning Trials

A greater proportion of trials showed lice consumed from juvenile salmon when allowed direct interaction with sticklebacks (treatment) than when no interaction was permitted (2007 controls) in all individual ($\chi^2 = 23.43$, df = 1, $p < 0.001$) and group trials ($\chi^2 = 19.86$, df = 1, $p < 0.001$) (Fig. 2a, b). The proportions of consumed lice were not significantly



Fig. 1: Still images of sticklebacks engaging in removal of sea lice (top) and sea lice egg string cropping (bottom) behaviours (taken from video ©Twyla Roscovich; See supplementary electronic information).

different between individual (31.8%; $N = 242$ lice) and group (24.7%; $N = 162$ lice) trials ($\chi^2 = 2.40$, df = 1, $p = 0.12$). Cleaning activity resulted in a significant decrease in the mean louse load on juvenile salmon in both the individual trials (mean \pm 1SE; before cleaning: 1.56 ± 0.07 lice per salmon; after cleaning: 0.99 ± 0.08 lice per salmon; paired-sample $t_{154} = 8.79$, $p < 0.001$) and the group trials (mean \pm 1SE; before cleaning: 1.25 ± 0.02 lice per salmon; after cleaning: 0.92 ± 0.07 lice per salmon; paired-sample $t_{12} = 4.68$, $p = 0.001$). In those trials where cleaning took place, the louse load was decreased by an average of 91%. Cleaning took place within the first 6 h in 60% of the 65 individual trials in which cleaning occurred. Stomach content analysis confirmed cleaning behaviour in all cases. The supplementation of food had no effect on the proportion (41.7%) of trials ($N = 12$) where cleaning took place when compared to non-supplemented trials ($\chi^2 = 0.01$, df = 1, $p = 0.93$).

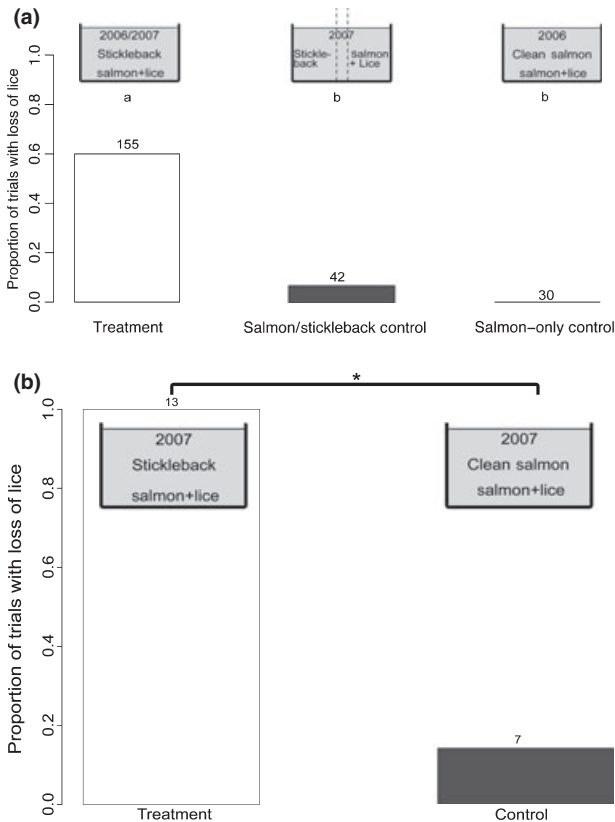


Fig. 2: Proportion of trials in which at least one louse was lost in (a) the individual and (b) the group trials. Numbers above columns indicate sample sizes; letters above columns indicate significant differences between proportions; asterisk in (b) indicates a significant difference. Vignettes illustrate experimental set-up.

Stickleback:salmon length ratio was the only significant predictor of cleaning (GLM; Δ deviance = 9.039, $df = 1$, $p = 0.003$).

Female-Biased Consumption by Sticklebacks

Sticklebacks consumed a significantly greater proportion of adult female lice than adult male lice in both the 2006 ($\chi^2 = 5.78$, $df = 1$, $p = 0.02$) and 2007 ($\chi^2 = 5.28$, $df = 1$, $p = 0.02$) individual trials (Fig. 3a). There was no difference in the sex ratio of lice consumed between the 2 yr ($\chi^2 = 1.671$, $df = 1$, $p = 0.20$). Similar sex-biased consumption of lice was seen in the group trials (Fig. 3b), although it fell short of statistical significance ($\chi^2 = 3.15$, $df = 1$, $p = 0.08$).

In addition to sex-selective predation by cleaning sticklebacks on adult female lice, there was further cleaning pressure exerted on gravid female lice observed in the 2007 individual trials (2006 trials did

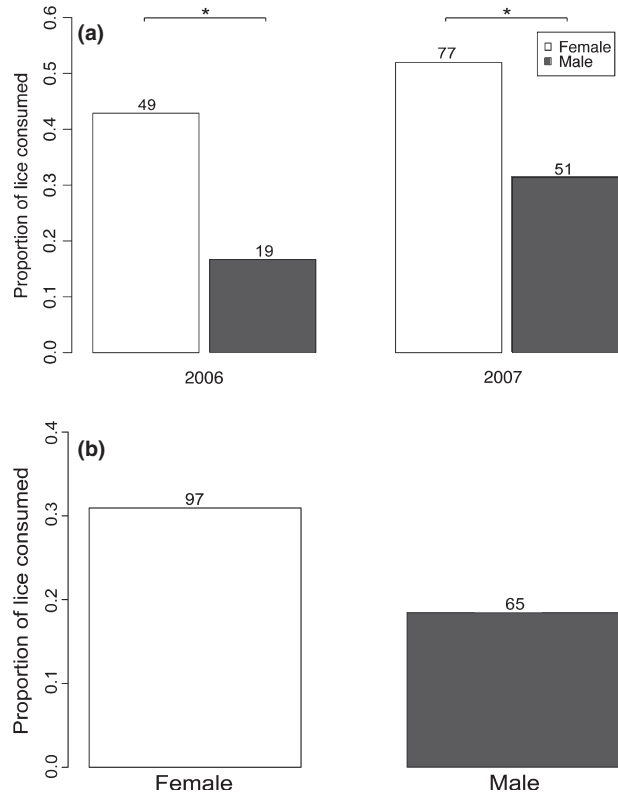


Fig. 3: Proportion of adult female lice on juvenile pink salmon that were consumed by sticklebacks in (a) individual and (b) group trials. Numbers above columns indicate sample sizes; asterisks indicate significant differences.

not include gravid females). Egg string cropping occurred on 29.4% of the gravid female lice available ($N = 17$) when sticklebacks were present, but never on gravid females ($N = 10$) in the control groups with uninfected salmon. Gravid female lice (47.1%; $N = 17$) were not more likely to be consumed by sticklebacks than non-gravid females (45.0%; $N = 60$) ($\chi^2 = 1.33$, $df = 1$, $p = 0.38$); however, when combined with egg string cropping, a significantly greater proportion of gravid females (76.5%) than non-gravid females experienced at least one impact of cleaning ($\chi^2 = 5.26$, $df = 1$, $p = 0.02$) (Fig. 4).

Discussion

The size- and sex- selective nature of this predation by sticklebacks advances the current understanding of how cleaning behaviour can impact parasite populations and benefit the client. Drastic sexual dimorphism seen in sea lice elicits key predatory behaviours that may drive these population-level changes.

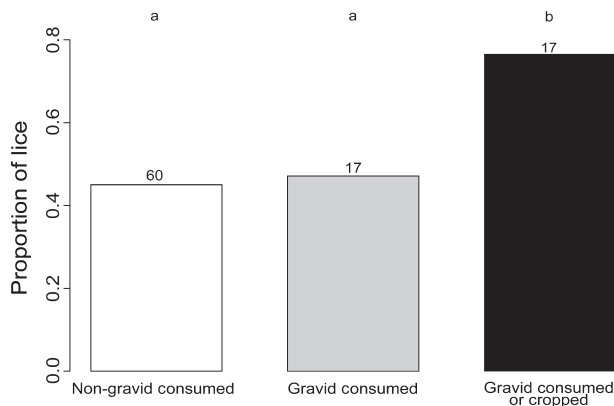


Fig. 4: Proportion of female lice experiencing various types of cleaning behaviour in 2007 individual trials. Numbers above columns indicate sample sizes; a and b indicate significant differences between proportions.

Novel Cleaning Behaviour

Sticklebacks are primarily visual predators feeding mostly on three invertebrate groups, including copepods (which include *L. salmonis*), cladocerans, and ostracods (Wootton 1984). It is thus an easy step to the consumption of conspicuous ectoparasites from juvenile salmon, which are known to be seasonally sympatric with sticklebacks in the Broughton Archipelago, as they are regularly caught together in seine nets during months of salmon outmigration (Jones et al. 2006; B. Connors, pers. comm.; C. J. C. Losos, pers. obs.). Because contact with juvenile salmon infested with adult *L. salmonis* may be irregular and seasonal, cleaning may be an opportunistic behaviour resulting from the elevated parasite loads documented on juvenile salmon during this time of sympatry (Morton et al. 2004; Krkosek et al. 2005a). Similar temporal variation in diet has been noted in the cleaner wrasse, *L. dimidiatus*, which consumes more parasitic gnathiid copepods during certain months of high parasite abundance (Grutter 1997c). Threespine sticklebacks are also known to exhibit temporal changes in their diet to reflect the seasonal availability of prey items in the environment (Wootton 1984).

The interspecific cleaning behaviour described here is the first known instance of its kind in the family Gasterosteidae or on the temperate Pacific coast of North America, widening both the taxonomic and geographical distribution of known cleaner fish (Côté 2000). There are several lines of evidence that suggest the cleaning interactions studied here are not artefacts of confinement in aquaria. First, similar levels of cleaning behaviour were observed in two different experimental set-ups,

including individual trials in small tanks and group trials in much larger ones. Second, the presence or absence of food did not influence the cleaning behaviour of the sticklebacks, and most of the sea lice were consumed in the first 6 h of the 24 h trials, suggesting that the behaviour was not the result of starvation prior to or during the trials. Finally, juvenile salmon do not avoid the interaction, indicating that this is not a coercive relationship, whereby sticklebacks take advantage of the salmon's inability to flee while in the experimental tanks.

The absence of any formal posing behaviour by juvenile salmon could be a result of a newly developed interaction that has been facilitated by the recently elevated parasite levels on juvenile salmon in the region (Morton et al. 2004; Krkosek et al. 2005a). Posing behaviour in established tropical reef cleaning relationships is characterized by a deliberate, conspicuous body position adopted by the client that can increase the likelihood of being cleaned but is not essential for cleaning to take place (Côté et al. 1998). In this study system, posing may not be necessary because of the conspicuous nature of these parasites.

Effects of Cleaning

In addition to sticklebacks consuming nearly 40% of the total available lice, a significant proportion of these were the adult female parasites. Removal of female lice may be particularly beneficial to juvenile salmon, as female lice are 2–3 times larger in mass than males and may have correspondingly greater negative impacts on their host. The reasons for the selection of adult females by sticklebacks are likely threefold. First, the larger size of adult female lice may make them more conspicuous to visually foraging sticklebacks. Second, females have greatly reduced motility compared to males because of their larger size and *L. salmonis*' behavioural strategy of male-biased host switching, which makes them less able to avoid predation (Connors et al. 2008). Finally, the larger females may also offer a greater nutritional benefit per cleaning event than males that may outweigh any cost of increased handling time associated with this larger prey (Winfield & Townsend 1983). This nutritional benefit could be enhanced by the presence of egg strings, which may explain the elevated cleaning pressure exhibited towards gravid females.

Preferential consumption of females (and their egg strings) by sticklebacks removes the most reproductively important individuals from the louse population. Similar size-selective pressure on sea lice has

also been documented on Atlantic salmon by cleaner wrasse (Treasurer 1994; Tully et al. 1996). For ovigerous (egg-carrying, as opposed to egg-broadcasting) non-parasitic copepods, the presence of egg masses is generally associated with increased risk of predation from visually foraging planktivorous fishes (Hairston et al. 1983; Bollens & Frost 1991a,b), including sticklebacks (Vuorinen et al. 1983). This selective predation by planktivores contributes not only to increased mortality but also a potential decrease in reproduction – a phenomenon that can amplify the effect of such predators (Gliwicz 1994). The combined pressure of consumption and egg string cropping on 76.5% of the gravid female lice in our test population has implications for the impact that cleaning behaviour, assuming it occurs in the wild, may have on both the louse population and the population of juvenile salmon subjected to these elevated parasite levels.

In individual trials in 2007, 4.8% of lice were lost in the control groups in which sticklebacks and salmon could not interact directly. Of these three lice lost, two were adult males and the other an early pre-adult whose sex could not be identified. Stomach content analysis confirmed that the sticklebacks had consumed these lice, which could only have occurred after they had left their salmonid host and strayed onto the stickleback side of the aquaria. The number of lice consumed in this manner was very small, and the sex bias is consistent with sex-biased host switching behaviour previously observed in lice (Ritchie 1997; Hull et al. 1998; Connors et al. 2008), although a very small sample size limits the strength of this connection. The consumption of free-swimming lice by sticklebacks also occurred readily when lice were offered as a food item in unrelated trials (C. J. C. Losos, pers. obs.). The consumption of male lice in this manner implies their consumption in the wild, which could be tested with a field study of stickleback dietary composition.

In 2008, we opportunistically examined the stomach contents of three spine sticklebacks that were captured with juvenile salmonids by other researchers as a by-product of a sampling scheme aimed at other sea louse research in the Broughton Archipelago. The sticklebacks were killed humanely and preserved in 95% ethanol for later examination. None of the 42 adult stickleback stomach contents included identifiable sea lice. Although the prevalence and intensities of sea louse infections on juvenile salmon captured in the same nets were not recorded in a manner that allowed a direct match to the sticklebacks that were caught with them, typical

prevalence on juvenile pink salmon at the time was >50%, with more than 10% motile lice. We emphasize that this was a preliminary test; further sampling is needed, designed specifically to determine the occurrence and magnitude of cleaning in the wild, with a larger sample size of sticklebacks and direct counts of all life stages of sea lice on co-occurring juvenile salmon. Variables such as time of cohabitation, composition of louse load, and species of lice present should also be considered.

Cleaning by sticklebacks in experimental enclosures significantly reduced the parasite loads of juvenile salmon. This can have important benefits to salmon, as significant mortality of juvenile salmon in this region has been seen at louse abundance levels at or below that used in our experiments (Krkosek et al. 2006). Small juvenile salmon can sometimes succumb to infestation by a single louse (Morton & Routledge 2005). Thus, removal of even one louse can have important survival implications for juvenile salmon, and the average reduction of louse load by 91% during cleaning interactions creates a strong selective pressure to reduce individual parasite loads for these fish. Conditions such as these have led to the evolution of honest cleaning relationships in established, stable systems (Freckleton & Côté 2003). Unlike the large client-to-cleaner size ratios commonly seen in established tropical cleaning relationships (Grutter et al. 2005), the high cost of parasitism on small juvenile salmon may be driving the large cleaner-to-client ratio seen in our tests. In addition, high parasite loads on clients can motivate cleaning interactions regardless of body size (Arnal et al. 2001). The Broughton Archipelago, however, is in a state of flux because of anthropogenic disturbance and the unnaturally high levels of parasites on juvenile salmon (which are now declining as farm management practices improve). Nonetheless, the strong selective pressure on juvenile salmon, combined with the nutritional benefit to sticklebacks, creates a set of environmental characteristics that lend themselves to the establishment of cleaning relationships.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video S1. Cleaning Behaviour Movie – video of a stickleback grabbing and attempting to dislodge an adult female louse from a juvenile pink salmon.

Video S2. Egg String Cropping Movie – video of a stickleback consuming the two protruding eggstrings from a gravid adult female louse.

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