Pink salmon (*Oncorhynchus gorbuscha*) osmoregulatory development plays a key role in sea louse (*Lepeophtheirus salmonis*) tolerance

M. Sackville, S. Tang, L. Nendick, A.P. Farrell, and C.J. Brauner

Abstract: Sea lice (*Lepeophtheirus salmonis*) of fish-farm origin have been implicated in reducing populations of pink salmon (*Oncorhynchus gorbuscha*) in British Columbia's Broughton Archipelago. Owing to the physically disruptive nature of louse attachment to fish skin in a hyperosmotic environment, we hypothesize that the impacts on fish performance are ionoregulatory in origin. Therefore, ionoregulatory status was measured in juvenile pink salmon artificially infected in the laboratory and naturally infected in the wild. Body [Na^+] of laboratory-infected fish (~1 week seawater (SW); 0.2–0.4 g) increased significantly by 12% with a single chalimus-4 louse, and by 23% with 2–3 chalimus-3 lice. Mortality over this 24-day trial was 2.4% for fish initially infected with 1–3 lice. Body [Na^+] for fish caught with natural infections (~4–12 weeks SW; 0.5–1.5 g) did not differ from uninfected controls. Combining data sets revealed a "no effect" threshold of 0.5 g for body [Na^+] of fish infected with one chalimus-4 louse. We propose that this size-related louse tolerance is associated with hypo-osmoregulatory development, adding to a previously suggested multifactorial mechanism based on epidermal and immune system development. We suggest management bodies consider this fish-mass threshold when planning to minimize risk to wild fish populations.

Résumé : Les poux de mer (*Lepeophtheirus salmonis*) provenant de pisciculturesont été impliqués dans la réduction des populations de saumons roses (*Oncorhynchus gorbuscha*) dans l'archipel de Broughton en Colombie-Britannique. À cause de la nature perturbatrice de type physique de la fixation des poux à la peau des poissons en milieu hyperosmotique, nous posons l'hypothèse selon laquelle les impacts sur la performance des poissons se font au niveau de la régulation ionique. Le statut d'ionorégulation a été mesuré chez de jeunes saumons roses infectés artificiellement au laboratoire et d'autres infectés naturellement dans le milieu. Le [Na^+] corporel des poissons infectés en laboratoire (~1 semaine en eau de mer (SW); 0,2–0,4 g) augmente significativement de 12 % en présence d'un seul pou de stade chalimus 4 et de 23 % avec 2–3 poux de stade chalimus 3. La mortalité durant ce test de 24 jours est de 2,4 % chez les poissons infectés initialement avec 1–3 poux. Le [Na^+] corporel des poissons capturés porteurs d'infections naturelles (~4–12 semaines en SW; 0,5–1,5 g) ne diffère pas de celui des témoins non infectés. Une combinaisons des ensembles de données indique l'existence d'un seuil « d'absence d'effet » de 0,5 g en ce qui a trait au [Na^+] corporel chez des poissons infectés avec un pou de stade chalimus 4. Nous croyons que cette tolérance aux poux reliée à la taille est associée au développement de l'hypo-osmorégulation, ce qui s'ajoute au mécanisme multifactoriel suggéré antérieurement basé sur le développement de l'épiderme et du système immunitaire. Nous suggérons aux agences de gestion de tenir compte de ce seuil de masse des poissons lorsqu'ils cherchent à minimiser le risque aux populations naturelles.

[Traduit par la Rédaction]

Introduction

The ectoparasitic salmon louse *Lepeophtheirus salmonis* has been recently implicated in pink salmon (*Oncorhynchus gorbuscha*) population declines and extinction predictions in British Columbia's Broughton Archipelago (Morton and Routledge 2005; Krkosek et al. 2007, 2009). Although these studies are compelling, major questions regarding parasite transmission and infection impacts on host physiology remain unresolved. The uncertainty has led to considerable debate as to whether sea lice, specifically of farm origin, are negatively impacting local pink salmon populations (Brooks and Stucchi 2006; Brooks and Jones 2008; Krkosek et al. 2008). A critical knowledge gap that this study aims to address is the level of louse infection required to sublethally impair the performance of the smallest out-migrating juvenile pink salmon, i.e., those weighing between 0.2 g and 2.0 g.

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Lice feed on host mucus, epidermal tissue, and blood (Kabata 1974; Brandal et al. 1976) as they grow in size and develop through four attached stages (termed chalimus 1–4) and three motile stages (termed pre-adult 1–2 and adult) on the fish's exterior surface (Johnson and Albright 1991; Tully 1992; Brooks 2005). As shown in 200 g Atlantic salmon and 90 g sea trout, this surface feeding can lead to significant increases in plasma electrolytes (up to 60%; Grimnes and Jakobsen 1996; Bjorn and Finstad 1997; Nolan et al. 1999). This hydromineral disruption is thought to result either directly from epidermal lesions that damage the external osmotic barrier, or indirectly from a stress-related increase in the osmorespiratory compromise (Gonzalez and McDonald 1992; Gonzalez and McDonald 1994; Wendelaar Bonga 1997). Regardless of origin, hydromineral imbalance is considered a major concern, as it can stunt growth (Brauer 1982; Folmar et al. 1982; McCormick et al. 1987), reduce swim performance (Brauner et al. 1992, 1994), and even cause death (Boeuf and Harache 1982).

Adult pink salmon are likely well equipped to cope with parasitism in the wild (~50 lice per fish; Beamish et al. 2005), but out-migrating juveniles are likely less prepared for a number of reasons. Foremost of these is that pink fry enter seawater as small as 0.2 g (Heard 1991), and are thus three orders of magnitude smaller than adult fish when first exposed to sea lice (Jones and Hargreaves 2007). Thus, their surface area to volume ratio is much higher, which is a major factor for ion and water exchange with the environment. Additionally, the epidermal (Jones et al. 2008*a*), immune (Johnson et al. 1982) and hypo-osmoregulatory (Grant et al. 2009) systems of pink salmon are all still developing during the first few months in seawater. Since these physiological characteristics are presumably critical to their resistance to and tolerance of infection with *L. salmonis*, juvenile pink salmon are likely particularly vulnerable to ectoparasites such as sea lice during their initially precocious out-migration.

Currently, only one study has quantified the sublethal effects of infection on out-migrating (0.2–3.0 g) pink salmon. One motile louse was shown to significantly reduce the swimming performance of fish with a body mass of less than 0.7 g (Nendick et al. 2011). Interestingly, this body-mass threshold for the impairment of swimming performance corresponds with the body mass when pink salmon complete hypo-osmoregulatory development (0.7–1.0 g; Grant et al. 2009), suggesting that the observed effects on swimming are perhaps linked to impaired hypo-osmoregulatory ability. Indeed, swimming performance was similarly reduced with elevated plasma ions in larger *O. kisutch* (8–20 g) following seawater entry (Randall and Brauner 1991; Brauner et al. 1992, 1994) and with larger *Salmo salar* (600 g) infected with 80 lice per fish (Wagner et al. 2003, 2004). Furthermore, louse-infected *S. salar* (200 g) have been shown to up-regulate gill Na^+/K^+-ATPase (NKA) activity by as much as 60% (Nolan et al. 1999), likely as an attempt to restore hydromineral balance. Based on these findings, we hypothesize that louse-induced performance reductions may be ionoregulatory in origin.

Thus, the objective of this study was to quantify the ionic disruptions and ionoregulatory compensations associated with ecologically relevant louse infection levels (1–3 *L. salmonis* per fish) in post-emergent juvenile pink salmon (0.2–1.5 g). Ionoregulatory status was measured in three separate series of fish: (*i*) laboratory-infected, naïve fish collected from the Glendale River (~0.2–0.4 g), (*ii*) naturally infected, ocean-caught fish collected from the Broughton Archipelago (~0.5–1.5 g), and (*iii*) uninfected, naïve fish subjected to various degrees of experimenter-induced epidermal abrasion (~0.7 g). Data for infected fish from the first two series were combined to determine a threshold fish mass for sublethal louse effects. This threshold could prove to be useful for fisheries managers when planning to minimize risk to wild populations.

Materials and methods

Research facility

All experiments were conducted at an autonomous field laboratory in the Broughton Archipelago, British Columbia, Canada, between March and July of 2008. Constructed on a float-house adjacent to Doctor Islets in Knight's Inlet, the laboratory location provided easy access to wild juvenile pink salmon throughout the entirety of their near-shore migration (transport time from collection areas never exceeded 4 h). Laboratory holding tanks were equipped with flow-through aerated seawater (SW) drawn from depth at 30 m to ensure stable temperature (7.0–8.5 °C) and salinity (32–34 ‰), and the facility's translucent canopy permitted a natural photoperiod throughout study duration. All fish were treated in accordance with the University of British Columbia Animal Care Committee and the Canadian Council on Animal Care.

Series 1: Controlled laboratory infection of river-caught fish

Experimental animals

River-caught fish

Approximately 2000 post-emergent pink fry were collected with rotary screw-traps from the Glendale River during their down-stream migration on 27 March 2008. Fish were immediately transported to the field laboratory where they were gradually exposed to full strength SW (34 ‰) over a period of 24 h. Initially at an approximate size of 25 mm and 0.2 g, pink fry were divided into four 100 L fibreglass holding tanks in which they were fed, and readily accepted, commercial trout chow twice daily (Bio-Vita starter feed; Bio-Oregon, Longview, Washington, USA). Glendale River was selected as a fish source because it is highly representative of the region's pink salmon productivity, accounting for more than 35% and 85% of total Broughton pink salmon in even and odd years, respectively (Brooks and Jones 2008). Out-migrating river caught (RC) fish were used for laboratory-based infections for two reasons: (*i*) they had no prior exposure to sea lice; and (*ii*) they represented the earliest and presumably most sensitive life-stage that could become infected in the wild given their precocious seawater entry.

Lepeophtheirus salmonis

Approximately 300 gravid female lice were collected from adult Atlantic salmon on 24 March 2008 during a commercial harvest at Marine Harvest Canada's Wicklow Point farm. Upon transport to the Doctor Islets laboratory, louse egg strings were incubated at 7 °C in four 4 L closed containers

filled with aerated SW (34 ‰). Active nauplii were filtered out every 2 days with 96 μm plankton mesh and placed in separate 2 L rearing chambers. Incubation water was changed every other day and nauplii monitored daily for copepodid development. Active copepodid densities were estimated by averaging counts from five 10 mL aliquots sampled from each rearing chamber. Infection was initiated when a copepodid:nauplii ratio of 3:1 was reached.

Infection protocol and results

Infection of pink salmon with *L. salmonis* was initiated on 3 April 2008 after RC fish had spent 1 week in SW. A total of 581 fish was divided into subgroups of ~75 and placed into 8 separate 11 L infection chambers (modified POS plastic totes; Rubbermaid). Each chamber was filled with 3 L of static aerated SW and approximately 1700 active copepodids, yielding a bath density of ~560 copepodids·L^{-1} and a copepodid:fish ratio of 23:1. Chamber dimensions were such that the infection bath depth was only 5 cm, minimizing fish–louse stratification and maximizing host–parasite interaction. Louse exposure was carried out in darkness for a total of 4 h, followed by a switch to flow-through SW to flush the infection bath. An additional 150 fish were subjected to an identical sham infection without lice and used as the control.

Approximately 48 h post-infection, all fish were lightly anaesthetized (0.05 g tricaine methanesulfonate (MS-222)·(L saltwater)$^{-1}$) and sorted according to louse load. Out of 581 fish, 511 were successfully infected with at least one louse (a prevalence of ~88%). Of these, 129 individuals (22%) were infected with 1 louse, 101 individuals (17%) with 2 lice, 99 individuals (17%) with 3 lice, and 182 individuals (31%) were infected with 4+ lice (up to 20 lice were present on some fish). Fish that remained uninfected following louse-exposure were not used. Following sorting, fish were placed in 10 separate 11 L holding tanks according to five categories of louse load (0, 1, 2, 3, and 4+ lice per fish; 2 replicate tanks per treatment). Each tank was equipped with flow-through SW (34 ‰, 7.0–8.5 °C), held no more than 75 fish and was supplied with commercial trout chow twice daily. The tanks with 4+ lice per fish were used only as a backup supply (see below).

Sampling protocol and constraints

Fish were sampled over the ensuing 24 days as sea lice matured towards adult stages. For the experiments described below, a total of 20 fish (10 for whole body [Na^+] and 10 for gill NKA activity) were sampled for each of four louse loads (0, 1, 2, and 3 lice per fish) at each of four development stages for an attached louse [chalimus stages 1 through 4, which represented 3, 7, 15, and 24 days post-infection (DPI), respectively]. All fish were examined by microscope at the time of sampling to determine the exact louse number, developmental stage and surface area of exposed subepidermal tissue. Subepidermal tissue exposure was quantified by overlaying a microscope slide equipped with a 1 mm × 1 mm grid on the louse wound. Subepidermal tissue in this case is defined as the flesh immediately beneath the scales and thin outer dermis.

As reported in recent laboratory studies (Jones et al. 2008*a*, 2008*b*), infected pink salmon fry experienced high rates of louse loss throughout trial duration. Consequently, the number of infected fish (especially those with more than one louse) declined over time. As a result, actual louse loads on fish did not always match the designated tank load from the initial fish sorting completed 48 h post-infection. On sampling days, fish were drawn randomly from all treatment tanks to find appropriate louse loads, which obviated a repeated sampling design. Furthermore, to compensate for louse loss, the back-up supply of the 4+ lice per fish group were resorted at 14 DPI (~chalimus-3 stage) and redistributed among the 1–3 lice per fish treatment tanks. A total of 99 fish were added to the sampling population (48 fish with 1 louse, 31 fish with 2 lice, and 20 fish with 3 lice). Even with these additions, infected fish numbers remained limited; hence, measurements of gill NKA activity were necessarily restricted to fish with 1–2 lice of chalimus stages 1–3, while measurements of whole body [Na^+] beyond the chalimus-3 stage were limited to fish with 1 louse.

As mentioned above, all reported louse loads correspond to infection levels at the time of sampling and not the initial sort completed 48 h post-infection. Owing to the high levels of louse loss, louse loads for some individuals would have been higher prior to sampling. Thus, the impacts of specific louse loads reported here would tend to overestimate rather than underestimate an impact for a given reported louse load.

Series 2: Natural infection of ocean-caught fish

Over 10 000 wild pink salmon fry were captured by beach or purse seine during their near-shore migration through the Broughton Archipelago between April and June of 2008. These ocean-caught (OC) fish were graded directly following collection, so that excess, uninfected fish could be released immediately, whereas louse-infected fish could be transported promptly to the field laboratory. Fish were permitted a 24-h recovery period in the laboratory, then lightly anaesthetized (0.05 mg MS-222·(L seawater)$^{-1}$; Syndel) for sorting according to louse number and developmental stage. After an additional 24-h recovery period, fish were sampled for assessments of whole body [Na^+] and (or) gill NKA activity, and the area of subepidermal tissue exposure was measured in both control and infected fish as in Series 1. To minimize the potential for confounding effects, fish were sampled to provide at least eight infected, similarly sized fish with a similar louse developmental stage that were collected at the same location on the same date. This sampling regime was designed to minimize any variation attributable to fish development and environmental experience. These criteria, combined with a low infection intensity among OC fish (total louse prevalence was roughly 10%, with an intensity of approximately 1), restricted analysis to fish infected with 1 attached chalimus stage louse. As with Series 1, assay priority was given to whole body [Na^+] analysis. Three size ranges of infected OC fish were obtained in sufficient number for analysis (~0.5 g, 1.0 g, and 1.5 g). A group of 10 uninfected, size-matched fish were used as a control for each range.

Series 3: Experimenter-induced epidermal abrasion of river-caught fish

In response to the low mortality and high louse loss observed in the laboratory infections of Series 1, low levels of infection and epidermal damage observed in the natural infections of Series 2 and a general lack of advanced infections in

both series, uninfected RC fish were subjected to a "worst case" scenario in an attempt to elicit negative effects thought to be associated with more severe louse infection. Because this series was conceived in response to observations in Series 1 and 2, our RC laboratory pink salmon population had already grown considerably. Therefore, the smallest fish size available was approximately 0.7 g.

To mimic mechanical skin damage inflicted by motile sea lice, the epidermis of uninfected RC fish acclimated to SW were subjected to various degrees of artificial abrasion following completion of Series 1. Using even pressure with a No. 2 scalpel blade, lightly anaesthetized fish (approximately 0.7 g and 50 mm in size) were subjected to the following: (*i*) 1 mm^2 scale removal, (*ii*) 1 mm^2 subepidermal tissue exposure, or (*iii*) 2 mm^2 subepidermal tissue exposure. The surface areas for abrasion were selected to match motile louse mouth sizes, and fish size corresponded to when wild fish might first encounter a motile sea louse in 2008. All lesions were located posterior to the dorsal fin on the lateral line, and abrasion time never exceeded 30 s. A sham group was abraded in an identical fashion with the scalpel handle to serve as a control. 1 mm^2 scale removal is defined as the removal of scales from an area of 1 mm^2; subepidermal tissue exposure is defined above (see Series 1).

Following abrasion, fish were held in duplicate tanks for each of the four treatment groups. Holding tanks, water conditions, and fish care were identical to those of Series 1. For whole body [Na^+] and gill NKA activity, a total of 20 fish from each treatment were sampled pre-abrasion, and at 1 and 5 days post-abrasion as described below. Gill NKA activity was not measured in the 2 mm^2 abrasion group, owing to the limited availability of fish.

Assays

Wet and dry mass, and total body [Na^+]

Fish were anaesthetized with a lethal dose of buffered MS-222 (0.8 g·(L seawater)$^{-1}$; Syndel Laboratories, Vancouver, British Columbia, Canada), rinsed in de-ionized water, blotted dry, and weighed for wet body mass. Individual fish were subsequently dried at 65 °C to a constant dry mass and digested in 1 mol·L^{-1} nitric acid (1 mL of acid for every 0.1 g of wet tissue mass). Digest supernatant was subsequently analyzed for Na^+ concentration ([Na^+]) using flame atomic absorption spectroscopy (Spectra AA-220FS; Varian, Mulgrave, Victoria, Australia). Total body [Na^+] is reported as μmol Na^+·(g wet mass)$^{-1}$.

Gill Na^+/K^+-ATPase activity (NKA)

Whole gills were removed from lethally anaesthetized fish (see above) and stored at –80 °C. A modified version of the method outlined by McCormick (1993) was used to determine gill NKA activity. Briefly, individual whole frozen gills were homogenized on ice in SEI buffer (250 mmol·L^{-1} sucrose, 10 mmol·L^{-1} EDTA, 50 mmol·L^{-1} imidazole; pH 7.3). Homogenates were centrifuged at 5000*g* for 1 min and the supernatant placed on ice. Supernatant ATPase activity was measured spectrophotometrically in the presence and absence of ouabain (1 mmol·L^{-1}), and taken as the difference between conditions. Protein concentration was measured using the bicinchoninic acid method (Sigma-Aldrich) with bovine serum albumin standards. NKA activity is reported as μmol ADP·(mg protein)$^{-1}$·h^{-1}.

Statistical analyses

Data are expressed as mean values ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) was used throughout to determine significant differences, and the Holm–Sidak post-hoc test was subsequently applied for pairwise comparisons. Second order polynomial regression lines were fitted to pooled data sets for infected and uninfected fish to estimate a threshold fish mass for louse effects. All data passed tests for normality and homogeneity of variance. All statistical analyses were conducted with Sigmastat (version 3.0, Systat Software Inc., San Jose, California, USA), and a significance level of $P < 0.05$ was used throughout.

Results

Series 1: Controlled laboratory infection of river-caught fish

Fish mortality, fish growth and epidermal damage by lice

Mortality among fish originally infected with 1–3 lice per fish was 2.4% (8 of 329 fish). Ninety-nine fish originally infected with 4 or more lice were added to the 1–3 lice per fish group at 14 DPI following a re-sort of infected fish (see methods). From the combined 428 fish, 25 died during the 24-day trial. Since there was no mortality among control fish, overall louse-induced mortality was 5.8%. However, of these 25 dead fish, 17 originated from the 4+ lice per fish group, which could have had an initial infection of as many as 20 lice per fish.

Control fish grew significantly from 0.224 ± 0.014 g to 0.358 ± 0.019 g over a 24-day period ($P < 0.001$, $t = 6.376$), yielding a specific growth rate (SGR) of 2.26 ± 0.66% body mass·day^{-1} (Table 1). Infected fish grew at a similar rate, with SGR ranging from 1.23% body mass·day^{-1} to 1.86% body mass·day^{-1}. Fish mass differed significantly from the control group at 7 DPI in the 2 lice per fish treatment ($P = 0.027$, $t = 2.241$; Table 1), but had recovered by 15 DPI.

Superficial, louse-inflicted epidermal damage was visible on host fish as early as 3 DPI (chalimus-1 stage) and subepidermal tissue was exposed by 15 DPI (chalimus-3 stage; Table 1) with just one louse per fish. The area of exposed subepidermal tissue significantly increased (3-fold) between 15 and 24 DPI in the 1 louse per fish treatment ($P = 0.18$, $t = 2.403$; Table 1), during which time lice developed from the chalimus-3 to chalimus-4 stage. No significant differences among louse loads were resolved ($P > 0.05$).

Total body [Na^+] and gill Na^+/K^+-ATPase

ANOVA revealed that both DPI and louse load had significant effects on total body [Na^+] ($P = 0.007$, $F = 3.671$ for DPI; $P < 0.001$, $F = 5.804$ for load) and on gill NKA activity ($P < 0.001$, $F = 27.745$ for DPI; $P < 0.001$, $F = 11.49$ for load) of RC fish. A statistically significant interaction between DPI and louse load was also found for both total body [Na^+] and gill NKA activity ($P < 0.001$ for each).

As expected of a normal pink salmon developmental trajectory in SW (see Grant et al. 2009), total body [Na^+] of control fish decreased steadily and significantly from

Table 1. Fish mass and quantified area of exposed sub-epidermal tissue in controlled laboratory infection of river-caught fish (Series 1).

DPI, stage	Specified damage	Control	1 Louse	2 Lice	3 Lice
Day 0	Mass (g)	0.224±0.014	NA	NA	NA
	Tissue damage (mm^2)	0			
Day 3, C1	Mass (g)	0.243±0.009	0.224±0.011	0.234±0.009	0.235±0.008
	Tissue damage (mm^2)	0	0	0	0
Day 7, C2	Mass (g)	0.272±0.011	0.254±0.016	0.231±0.015*	0.249±0.010
	Tissue damage (mm^2)	0	0	0	0
Day 14, C3	Mass (g)	0.283±0.010	0.313±0.015	0.279±0.019	0.259±0.014
	Tissue damage (mm^2)	0	0.10±0.05a	0.20±0.06ab	0.15±0.06ab
Day 24, C4	Mass (g)	0.358±0.019	0.336±0.031	NA	NA
	Tissue damage (mm^2)	0	0.30±0.08b		
Specific growth rate (%)		2.26±0.66 over 24 days	1.86±1.00 over 24 days	1.48±0.86 over 14 days	1.23±0.34 over 14 days

Note: Values are the mean ± SEM; sample size = 10; DPI, days post infection; C1–C4, chalimus developmental stages 1–4; NA, not available; * indicates significant differences from time-matched controls for fish mass; different letters indicate statistically significant differences from time-matched controls for tissue damage ($P \leq 0.05$). Specific growth rate is calculated as percent body weight per day.

81 $\mu mol \cdot g^{-1}$ at 0 DPI (1 week following SW transfer) to 73 $\mu mol \cdot g^{-1}$ by 24 DPI ($P = 0.006$, $t = 2.770$; Fig. 1*a*). Total body [Na^+] of fish infected with one louse did not differ significantly from control values until 24 DPI [chalimus-4 stage, Fig. 1*a*; $P = 0.01$, $t = 2.611$], when a 12% increase was observed. A larger and earlier increase in total body [Na^+] occurred in fish infected with two and three lice, where a 23% increase was observed by 15 DPI [chalimus-3 stage, Fig. 1*a*; $P < 0.001$, $t = 4.99$ for two lice; $P < 0.001$, $t = 4.348$ for three lice]. Thus, all three infection loads had a threshold louse developmental stage beyond which a significant ionic disturbance was triggered. This developmental threshold was advanced when fish hosted more than one louse (two lice triggered a greater ionic disturbance than one louse) but the effect was not necessarily additive (total body [Na^+] did not differ significantly between two and three lice per fish).

As expected of a normal developmental trajectory in SW, gill NKA activity of control fish increased steadily and significantly from 7 $\mu mol\ ADP \cdot mg^{-1} \cdot h^{-1}$ at 0 DPI to 11 $\mu mol\ ADP \cdot mg^{-1} \cdot h^{-1}$ by 15 DPI [Fig. 1*b*; $P < 0.001$, $t = 3.461$]. For infected fish, however, gill NKA activity was significantly depressed as early as 3 DPI with just one louse [chalimus-1 stage, Fig. 1*b*; $P < 0.001$, $t = 3.565$]. This approximately 40% depression in enzyme activity was maintained independent of louse load through 7 DPI (chalimus-2 stage). By 15 DPI (chalimus-3 stage), gill NKA activity had recovered to control levels in all treatments. Therefore, rather than showing a compensatory increase in association with elevated ionic loads, infected fish exhibited an initial reduction in gill NKA activity.

Series 2: Natural infection of ocean-caught fish

As with laboratory-infected fish (Series 1), subepidermal tissue was not exposed in infected OC fish prior to the chalimus-3 stage. Furthermore, subepidermal tissue was only exposed in the smallest size class of OC fish (0.474 ± 0.043 g).

A two-way ANOVA revealed that body mass had a significant effect on total body [Na^+] ($P < 0.001$, $F = 42.782$) and gill NKA activity ($P < 0.001$, $F = 10.216$) of OC fish. Louse load was shown to have no effect ($P = 0.627$, $F = 0.239$ for body [Na^+]; $P = 0.332$, $F = 0.957$ for gill NKA activity), and there was no significant interaction between body mass and louse load ($P = 0.332$).

The changes in total body [Na^+] and gill NKA activity with body mass of uninfected OC fish followed the same patterns as observed for control fish of Series 1. Over the fish mass ranging from ~0.5 g to ~1.5 g, total body [Na^+] decreased significantly from ~70 to ~50 $\mu mol \cdot g^{-1}$ [Fig. 2*a*; $P < 0.001$, $t = 5.997$]. Correspondingly, gill NKA activity increased significantly from ~10 to ~14 $\mu mol\ ADP \cdot mg^{-1} \cdot h^{-1}$ [Fig. 2*b*; $P = 0.016$, $t = 2.480$]. Total body [Na^+] and gill NKA activity of infected OC fish did not differ significantly from size-matched, uninfected OC fish (Fig. 2; $P > 0.05$).

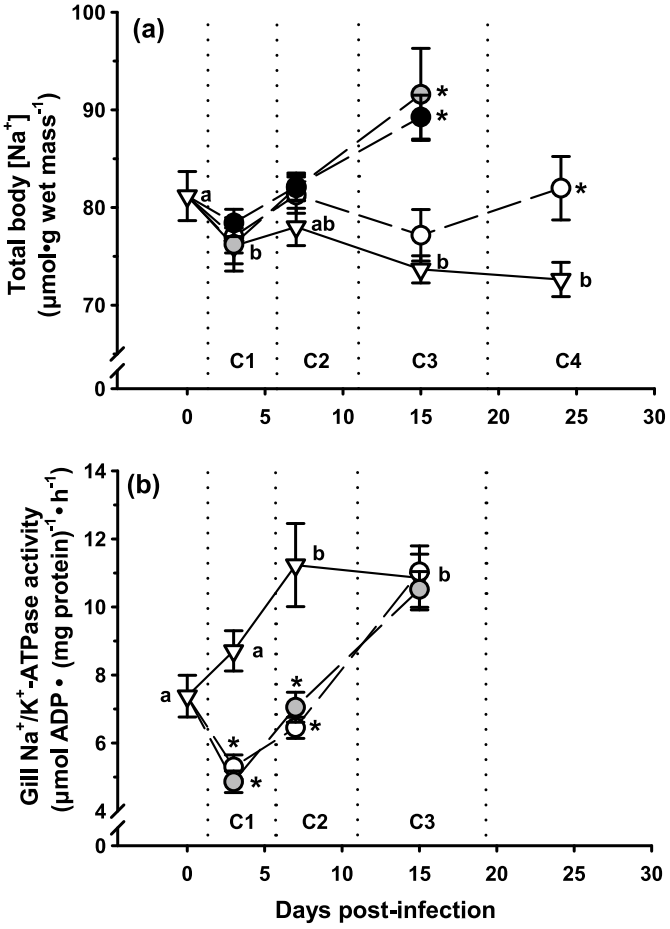
Series 3: Experimenter-induced epidermal abrasion of river-caught fish

The area of epidermal abrasion applied to RC fish exceeded the maximum area of louse-induced abrasion by nearly 10-fold (Series 1 and 2). Despite this excessive damage, neither total body [Na^+] nor gill NKA activity differed significantly from respective time-matched controls (Fig. 3; $P > 0.05$). Furthermore, total body [Na^+] and gill NKA activity were similar to size-matched, control fish in other experiments.

Estimated threshold for louse effect

The data sets for total body [Na^+] from laboratory (Series 1) and natural infections (Series 2) overlapped in terms of body mass and could be pooled to determine a body mass threshold for louse-induced ionoregulatory disturbance. Second order polynomial regression lines were fitted to the pooled data sets for uninfected$y = 84.6 - (41.7x) + (12.5x^2)$; $r^2 = 0.87$; $P < 0.001$; $F = 140$) and infected $y = 109.3 - (99.0x) + (43.8x^2)$; $r^2 = 0.89$; $P < 0.001$; $F = 84.5$) fish. Total body [Na^+] of fish infected with one chalimus-4 louse was significantly higher than that of uninfected fish below a body mass of 0.5 g (Fig. 4*a*), as clearly indicated by non-overlapping 95% confidence intervals. The observed difference in body [Na^+] between infected and uninfected fish below this threshold body mass of 0.5 g de-

Fig. 1. Total body [Na^+] relative to (*a*) fish wet mass and (*b*) gill Na^+/K^+-ATPase activity (*b*) of river-caught fish artificially infected with *L. salmonis* copepodids (triangles, control; white circles, one louse; grey circles, two lice; black circles = three lice). Fish were infected 1 week following transfer to seawater, and sampled over 24 days at times corresponding to each of the four chalimus developmental stages (C1–C4). The vertical broken lines represent approximate moulting events of lice as they develop from the copepodid through each of the chalimus stages (C1–C4). Data points represent mean ± SEM, $n = 10$. Asterisks indicate statistically significant differences from time-matched controls; different letters indicate statistically significant differences within the control group ($P \leq 0.05$).

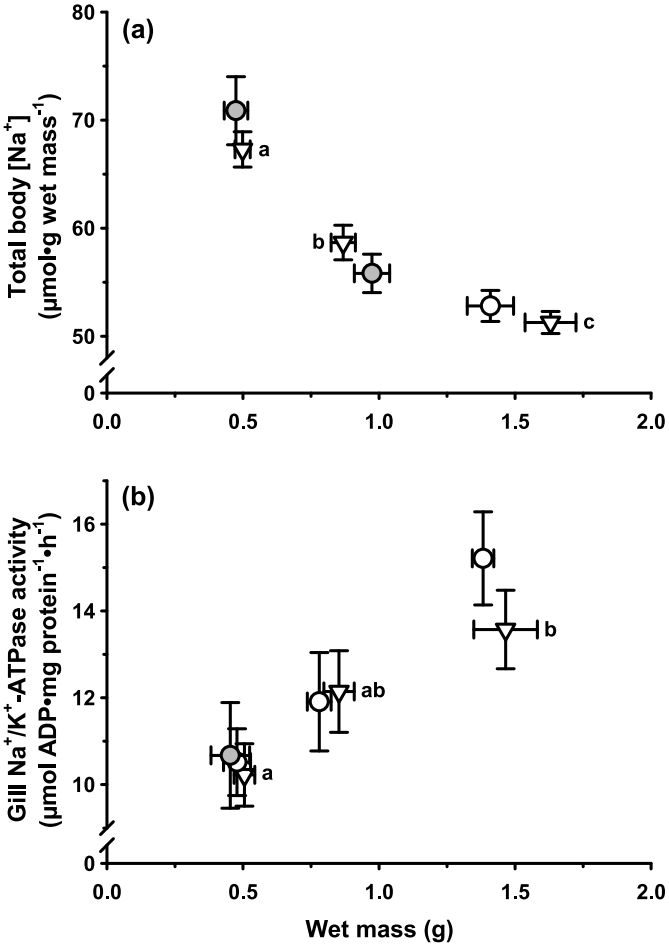


creased with increasing fish mass and gill NKA activity (Figs. 4*a* and 4*b*).

Discussion

Using ecologically relevant infection levels of 1–3 attached-stage lice per fish, we document some of the first sublethal physiological impacts of sea lice on post-emergent pink salmon. We conclude that beyond a threshold fish size of 0.5 g, one attached-stage louse (chalimus stages 1–4) no longer significantly disrupts hydromineral balance. Furthermore, we propose that this body mass threshold is linked to hypo-osmoregulatory development in juvenile pink salmon, which likely plays a key role in coping with the effects of epidermal damage and (or) stress incurred by sea louse infections.

Fig. 2. Total body [Na^+] relative to (*a*) fish wet mass and (*b*) gill Na^+/K^+-ATPase activity of ocean-caught fish naturally infected with one *L. salmonis* chalimus (triangles, control; white circles, chalimus stages 1 and 2; grey circles, chalimus stages 3 and 4). Data points represent mean ± SEM, $n = 10$. Values for infected fish do not differ significantly from size-matched controls; different letters indicate statistically significant differences within the control group ($P \leq 0.05$).



Results from the laboratory infection study clearly show that one louse at the chalimus-4 stage and two to three lice at the chalimus-3 stage are sufficient to significantly disturb total body Na^+ balance in pink salmon weighing less than 0.5 g. Because louse loads decreased over time in many fish, reported infection loads actually underestimated the infection history. Similarly, louse numbers for wild-infected OC fish also could have been greater prior to examination. Consequently, our measures of impact and the identified threshold, if anything, tend to overestimate rather than underestimate an impact for a given reported lice load.

We propose that this size-related louse tolerance is linked to pink salmon hypo-osmoregulatory development, adding to a previously suggested multifactorial mechanism based on epidermal and immune system development (Jones et al. 2008*a*). A primary proximal effect of louse infection is physical damage to the host epidermis (Kabata 1974; Brandal et al. 1976), which can potentially lead to hydromineral imbalance by disrupting the osmotic barrier and (or) inducing a stress response (Wendelaar Bonga 1997). While the immune

Fig. 3. Total body [Na^+] relative to (*a*) fish wet mass and (*b*) gill Na^+/K^+-ATPase activity of river-caught fish following various degrees of mechanical epidermal abrasion (triangles, control; white circles, 1 mm^2 de-scaling; grey circles, 1 mm^2 lesion; black circles, 2 mm^2 lesion). Fish were transferred to seawater 4.5 weeks prior to abrasion. Mean fish mass is 0.738 g ± 0.019 SEM. Data points represent mean ± SEM, $n = 10$; treatment groups did not differ significantly from control ($P \geq 0.05$).

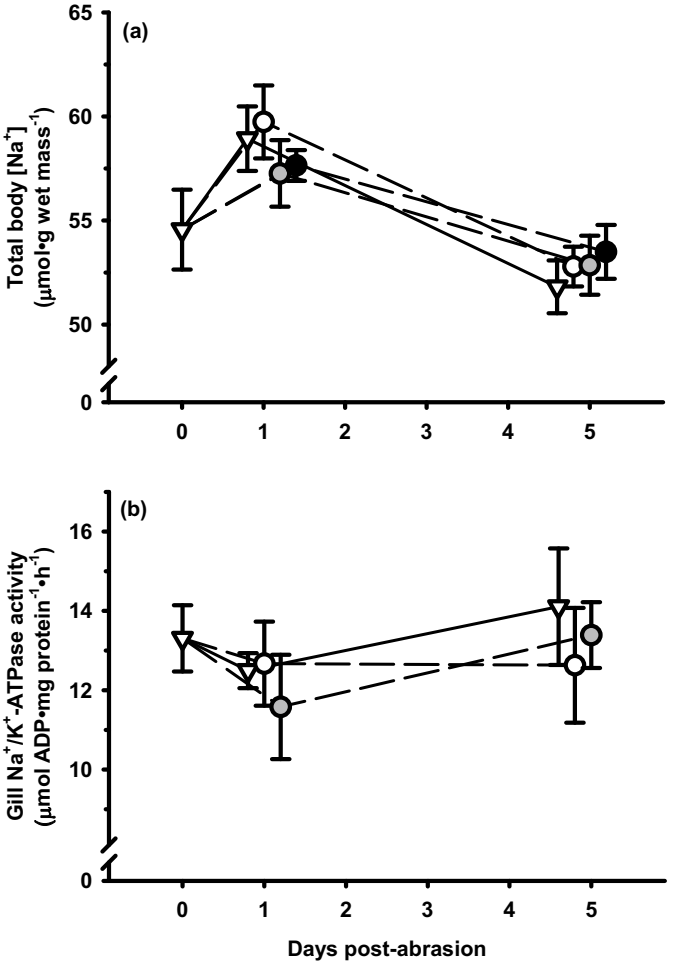
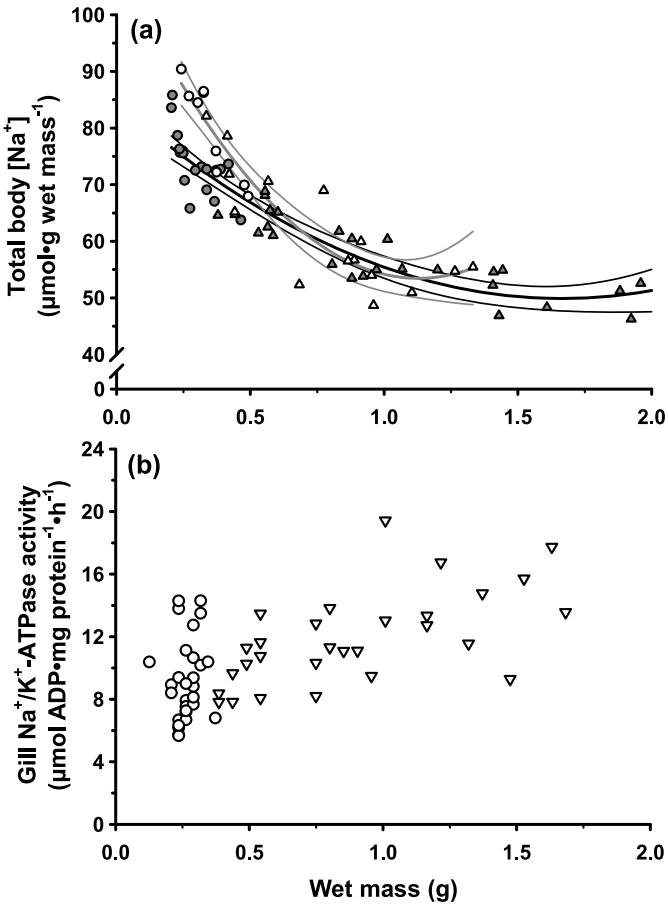


Fig. 4. Total body [Na^+] relative to (*a*) fish wet mass and (*b*) gill Na^+/K^+-ATPase activity of infected (shaded symbols) and uninfected (open symbols) fish. Circles represent individual river-caught fish from Series 1 infected in a laboratory setting, and triangles represent individual ocean-caught fish from Series 2 infected in the wild. All infected fish possess a load of one chalimus-4 louse. Regression lines are second order polynomials based on total uninfected (grey line; $y = 84.6 - (41.7x) + (12.5x^2)$; $r^2 = 0.87$; $P < 0.001$; $F = 140$) and infected (black line; $y = 109.3 - (99.0x) + (43.8x^2)$; $r^2 = 0.89$; $P < 0.001$; $F = 84.5$) fish. Fine lines represent 95% confidence intervals.



system and epidermis combat proximal effects of parasitism (Jones 2001; Jones et al. 2008*a*, 2008*b*), hypo-osmoregulatory ability enables fish to cope with the hydromineral consequences of any epidermal damage or attachment stress that might occur. The inverse relationship between total body Na^+ load and gill NKA activity of infected fish supports this notion, as does the absence of any ionic loading beyond attainment of maximum gill NKA activity. Hypo-osmoregulatory advanced fish even maintain Na^+ balance despite levels of skin abrasion 10-fold greater than those inflicted by lice. Although the nature of these two types of epidermal damage differs considerably, this result suggests larger-sized pink salmon may better tolerate at least the physical damage associated with louse loads beyond those explored here. Because epidermal damage for a given louse load decreases with increasing fish mass, this finding is also consistent with the proposed role of epidermal and immune system development in louse resistance (Jones et al. 2008*a*).

Louse-induced mortality over 24 days of small, laboratory-infected fish was 2.4% with an initial infection of 1–3 lice per fish, and 5.8% overall when fish previously infected with as many as 4–20 lice were re-sorted at 14 DPI into the 1–3 lice per fish group. Although low, this number could be much higher in the wild if sublethal ionic disturbances observed in the laboratory prove to affect survival in a natural setting. An associated study found reductions in pink salmon swimming performance to occur at infection thresholds similar to those identified here for hydromineral balance (Nendick et al. 2011), indicating that the ion load may indeed impart a performance cost. Negative impacts on fish growth, while not resolved here, may also occur in the wild where active foraging is required for feeding. To better understand the impacts on fish survival in the wild, studies such as those conducted here should be further paired with other surrogate measures of fitness. Lethality could also increase with louse development beyond that studied here, but so would louse

loss without re-infection. Clearly, fish growth, louse loss, and louse development are all important interactive factors in assessing the impacts of sea lice on juvenile pink salmon.

The mechanisms through which small juvenile pink salmon cope with a louse-induced ion load remain unclear. As in hydrominerally challenged rainbow trout, other areas of the body could buffer plasma and critical tissue osmolarity (e.g., Wood and Randall 1973*a*, 1973*b*; Bath and Eddy 1979) with associated costs to aerobic scope, energy stores, and (or) recipient buffer tissue function. Any of these impacts could explain the previously reported reductions in swimming ability (Nendick et al. 2011). Even the larger, more developed fish that maintained ion balance following infection and abrasion could be incurring substantial energetic costs. A simple and effective way to quantify the energetic costs associated with louse infection and any resulting ionoregulatory challenge would be to measure resting metabolic rate and metabolic scope in future studies.

Enhanced gill NKA activity of infected fish was predicted as a compensatory response to louse-induced hydromineral disturbance. However, neither louse-infected nor abraded fish showed this response. In fact, gill NKA activity was temporarily lower in laboratory-infected RC fish. Stress-related elevations in cortisol have been previously correlated with similar reductions in gill NKA activity of confined but uninfected chinook salmon (Strange et al. 1978), and louse-infected fish are known to exhibit stressful behaviours to rid themselves of lice (Webster et al. 2007). Therefore, the observed reduction in gill NKA activity could simply be the product of a generalized stress response (Wendelaar Bonga 1997). A stress response might also help explain the lack of ionic load in the epidermal abrasion experiments and the less than additive effects of more than one sea louse on ion balance in the laboratory infections. Nevertheless, an equally plausible explanation is that sea lice preferentially infect weaker and less developed fish. An unavoidable limitation to studying louse impacts is the inability to control which fish become infected and retain lice. As a result, infected fish could always represent a subset of the population more susceptible to parasitism. For example, such fish may have less-developed immune and epidermal systems, which may facilitate preferential louse attachment and retention (Jones et al. 2008*a*). If infected fish are relatively underdeveloped physiologically, hypo-osmoregulatory ability might also lag behind the population mean, thus potentially explaining the observed reduction in gill NKA activity. Experiments that sample those fish that reject parasites or resist initial infection would help clarify this issue, while providing further insight into the mechanisms underlying host susceptibility.

Although infected OC and RC fish overlapped in terms of body mass, and a louse-associated ionic load tended to decrease with increasing fish mass in both groups, a plausible explanation for the absence of significantly elevated total body [Na^+] in naturally infected OC fish (>0.5 g) is that fish with a louse-related ionic load simply fail to survive in the wild. This possibility cannot be discounted. OC control fish could have also been previously infected in the wild, resulting in an altered ionic status equal to infected OC fish. The latter possibility, however, can likely be discounted for two reasons. First, epidermal damage was not observed in OC control fish, and second, total body [Na^+] and gill NKA activity were similar to size-matched control fish in the other experiments. Pathogens and other nonlouse factors in the Broughton Archipelago could also be contributing to a sampling bias within the OC fish population. Further laboratory infections with bigger fish (>0.5 g) and more advanced louse stages in a controlled setting should help clarify this issue. Unfortunately, high rates of louse loss and low sea louse availability will remain challenges for controlled infection studies.

This study provides some of the first sublethal measures of physiological impact by sea lice on juvenile pink salmon in both a controlled laboratory setting and the wild. We clearly show that body mass and hypo-osmoregulatory development are important in tolerating the effects of sea louse parasitism. Furthermore, sublethal hydromineral disruption is shown to occur in fish less than 0.5 g infected with one attached-stage louse. This size threshold for sublethal louse effects is also consistent with that for swimming performance (0.7 g; Nendick et al. 2011) and infection resistance mechanisms (immunocompetence and epidermis; 0.5–0.7 g; Jones et al. 2008*a*). We hope that management groups will take this information into consideration when making decisions concerning the interaction between sea lice and juvenile pink salmon. We also recommend that these data be further integrated with surrogate measures of ecological fitness before more definitive conclusions regarding wild fish survival are made. Despite the strength of the observations made here, the consequences of louse infection in the wild remain difficult to quantify with any certainty.

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