**REVIEW**

# Evaluating the potential for sea lice to evolve freshwater tolerance as a consequence of freshwater treatments in salmon aquaculture

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ABSTRACT: Increasing usage of non-medicinal methods (NMMs) to control sea louse infestations on salmon farms has raised questions about whether sea lice may be able to evolve tolerance of NMMs. Of particular concern is the potential for sea lice to evolve freshwater tolerance as a result of freshwater treatments. Wild trout and some juvenile salmonids swim into freshwater to control infestations and regain ionic balance after disruption by sea lice; freshwater tolerance would compromise this potentially adaptive behavior. Here we evaluated the potential for freshwater tolerance to evolve in the sea louse *Lepeophtheirus salmonis*. When exposed to low-salinity water, parasitic stages of sea lice are able to osmoregulate through the host, while larval planktonic stages are not. Transcriptomic work suggests that sea lice mount a costly polygenic stress response when exposed to brackish water. The population structure of sea lice is panmictic in both the Pacific and Atlantic, making it conducive to rapid evolutionary responses. It is unknown how much heritable genetic variation these panmictic populations have for freshwater treatments. While usage of freshwater treatments on wellboats is increasing, it is unclear whether the freshwater itself is a strong selective force; during the freshwater exposure, sea lice can die from physical disruption during pumping and filtration on the wellboat. Future studies are advised to quantify the heritable variation in freshwater tolerance in sea louse populations, characterize mechanisms for freshwater tolerance in planktonic and attached sea lice, and assess the risk of freshwater tolerance evolution under different management strategies.

KEY WORDS: *Lepeophtheirus salmonis* · Non-medicinal method · Osmoregulation · Salinity · Artificial selection · Ectoparasite · Copepod

## 1. INTRODUCTION

Pest control is a major challenge in high-intensity farming. High densities of single species are susceptible to density-dependent infestations (Lafferty et al. 2015). Use of integrated pest management (IPM) strategies are increasing as 'traditional' chemical control strategies are compromised by the evolution of resistance and with concerns about impacts of chemotherapeutants on human health and non-target species (Bale et al. 2008). While IPM can facilitate processes that naturally control pest populations,

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these methods require evaluation; for example, increasing exposure to environments that control pests in the wild may artificially select for increased environmental tolerance, potentially widening the ecological niche that the pest inhabits.

In salmonid aquaculture, the success of IPM to control sea lice, which are prevalent parasites, has high stakes for both farmed and wild fish. On salmon farms, infestations of the sea louse *Lepeophtheirus salmonis* are costly to control, and many of the commonly used chemotherapeutants are controversial, expensive, and decreasing in effectiveness due to the evolution of chemotherapeutant resistance in sea lice (Aaen et al. 2015, Abolofia et al. 2017). For example, in 2011, sea lice (*L. salmonis*) cost the Norwegian salmon industry an estimated US \$436 million (Abolofia et al. 2017). Sea louse infestations on wild salmonids are associated with spillover and spillback of sea lice to and from farmed fish and have been correlated with declining returns in some stocks (Thorstad et al. 2015, Groner et al. 2016a, Vollset et al. 2018). These factors have led to an increased demand for alternate treatments, also called non-medicinal methods (NMMs). The past decade has seen a substantial increase in the use NMMs to control sea lice, including stocking salmon farms with sea louse predators (so-called 'cleaner fish') and/or exposing salmon to salinities or temperatures that sea lice cannot tolerate (Hjeltnes et al. 2019).

As the use of NMMs (freshwater, warm water, brushes, flushing, etc.) for sea louse infestations on farmed salmonids increases, so do concerns about the potential for evolving tolerance. This is particularly concerning for freshwater treatments because freshwater exposure is a method that wild trout use to self-control infestations. Some salmonids, such as sea trout and juvenile pink salmon, travel into freshwater to shed parasitic sea lice and regain ionic and osmotic balance, which can be disrupted by sea lice (Birkeland & Jakobsen 1997, Bjørn et al. 2001, Heuch et al. 2002, Webster et al. 2007, Halttunen et al. 2018). Wild sea trout have weak physiological defenses against sea lice and so are more reliant on behavioral controls such as changing habitat (Bui et al. 2018). When infected, they can remain for more than a month in freshwater (Bjørn et al. 2001). This behavior comes at a cost of reduced growth and/or mortality (Birkeland & Jakobsen 1997), although this is presumably less costly than maintaining an infection (Wells et al. 2007). If this behavior becomes less effective due to artificial selection for freshwater tolerance in sea lice, or if control of sea lice on farms is compromised because sea lice have evolved toler-

ance to treatments, these wild salmonid species would be even more susceptible to the deleterious effects of infestations on behavior and health.

Concerns about the evolution of freshwater tolerance in sea lice are driven by several observations. First, sea lice have evolved resistance to numerous chemotherapeutants over a relatively short time span (< 20 yr) (Aaen et al. 2015). This suggests that the strong and frequent selective pressure imposed by repeated exposure to sea louse control methods can yield a strong evolutionary response on a short timescale. Secondly, higher tolerance to low salinity (~15 psu) was observed in 1 (of 12) genetic families of *L. salmonis* generated from 2 locations (6 families generated per location, Ljungfeldt et al. 2017). If this tolerance turns out to be heritable, the raw genetic or epigenetic material for the evolution of tolerance to less saline (if not fresh) water exists; the evolution of tolerance will not depend upon the stochastic occurrence of beneficial mutations. Given the deleterious role that freshwater tolerance could have for sea louse control in wild fish, further investigation into this process is paramount.

Here, we evaluated the potential for freshwater tolerance to evolve in *L. salmonis*, the most problematic sea louse species in the northern hemisphere (Aaen et al. 2015, Thorstad et al. 2015, Groner et al. 2016b, Abolofia et al. 2017, Vollset et al. 2018) (Table 1). We review sea louse biology, including the physiological mechanisms for freshwater tolerance and their genetic components in sea lice and related taxa, and the population genetic structure of sea lice. We also describe the various methods for applying freshwater treatments and consider the potential influence of differing management strategies on salmon farms to contribute to resistance evolution. We identify knowledge gaps related to these topics and provide parameters which could potentially inform a risk analysis for freshwater tolerance evolution in sea lice, given the continued use of freshwater treatments.

## 2. REVIEW

### 2.1. Sea louse biology and farming techniques

The biology of sea lice (family Caligadae) has been thoroughly reviewed (Boxaspen 2006, Costello 2006). Briefly, these parasitic copepods occur in all salmon-inhabited marine waters of the northern hemisphere. They are attached to salmonid hosts as copepodid, chalimus, pre-adult and adult stages.

Table 1. Mechanisms affecting the evolution of freshwater tolerance, state of knowledge, and relevant literature

| Mechanism | State of knowledge | Relevant literature |
|---|---|---|
| Heritable variation in freshwater tolerance | Lab-based evidence for tolerance of brackish water based on 2 populations (Section 2.5) | Ljungfeldt et al. (2017) |
| Population genetic structure | Weak population structure in Atlantic sea lice on wild fish (Section 2.6) | Glover et al. (2011), McEwan et al. (2015) |
| Fitness trade-offs associated with freshwater tolerance | Freshwater tolerance slows development and reduces salt-water tolerance in the non-parasitic copepod *Eurytemora affinis* (Section 2.5) | Lee & Petersen (2003), Lee et al. (2007) |
| | Sea louse transcriptome suggests that tolerating less saline water is energetically demanding (Section 2.4) | Sutherland et al. (2012) |
| Selection for increased tolerance of freshwater | Unclear whether freshwater is the main cause of sea louse death in wellboat treatments (Section 2.2) | Reynolds (2013), Oppedal et al. (2017) |

Reproduction is sexual, with high fecundity and short generation times (~30 d at 16°C to 110 d at 4°C) (Groner et al. 2014). Female sea lice carry eggs in 2 egg-strings which can contain between 50 and 1000 eggs per string, depending upon environmental conditions, and parasite age and condition (reviewed by Brooker et al. 2018). Once hatched, the non-feeding larval stages (nauplii and then copepodids) are planktonic for several weeks, after which they must find a host or die as a result of depleted endogenous energy supplies. Where hydrodynamic conditions permit, planktonic sea lice have been found congregating at river mouths, particularly during spring when salmon smolts are outmigrating (Costelloe et al. 1998, McKibben & Hay 2004). The potential exposure to low-salinity water in these locations requires passive vertical migration to avoid low-salinity seawater, and laboratory studies suggest that planktonic sea lice can actively orient towards the halocline (Heuch 1995). Successful attachment to a host is dependent upon host densities and hydrodynamic conditions, which can disperse infectious stages more than 30 km (Johnsen et al. 2016, Cantrell et al. 2018).

While sea lice have been observed for centuries to parasitize salmonids (Berland & Margolis 1983), increased infestation intensities are associated with high densities of salmonids in aquaculture (Jansen et al. 2012) as well as, in some places, spillover and spillback of sea lice between farmed salmon and wild migratory salmonids (Krkošek 2017). Methods for controlling sea lice on salmon farms are multi-faceted. In addition to chemotherapeutant usage when sea lice levels exceed density thresholds or approach maximum allowable values, farms often co-stock sea pens with predators of sea lice, such as various wrasse species or lumpfish. At a regional level, farms coordinate

fallowing and stocking sea pens to disrupt the exponential growth of established sea lice (reviewed by Groner et al. 2016b). The recent increase in the use of NMMs reflects a strong push to develop alternative methods for sea louse control. For example, in Norway, there has been an overall decrease in the rate at which pens are treated each year. This accompanies a proportional increase in the use of NMMs, including freshwater and warm water treatments, prophylactic skirts, snorkels, deep-water lights and deep-water feeding to attract salmon away from surface waters (Stien et al. 2012, Oppedal et al. 2017, Wright et al. 2017). These changes coincide with a shift towards targeted treatments of selected pens instead of treating all the pens on a farm (Hjeltnes et al. 2019; Fig. 1). Freshwater treatments contributed 5.7 and 5.0% of the total registered NMM treatments in 2017 and 2018, respectively (Hjeltnes et al. 2019). In addition to freshwater and warm water treatments and prophylactic skirts, other methods in development include novel chemotherapeutants (Poley et al. 2018) and selective breeding for sea louse resistant Atlantic salmon (Gharbi et al. 2015).

## 2.2. Freshwater treatments

Positive correlations between sea surface salinity and the prevalence and incidence of sea lice on farmed and wild salmon have been found in western Canada, Chile and Norway (e.g. Heuch et al. 2002, Kristoffersen et al. 2013, Rogers et al. 2013, Rees et al. 2015). Therefore, it is not surprising that, as resistance to chemotherapeutants has rendered these methods to control sea lice less effective, the industry has focused on freshwater treatments as an alternative.
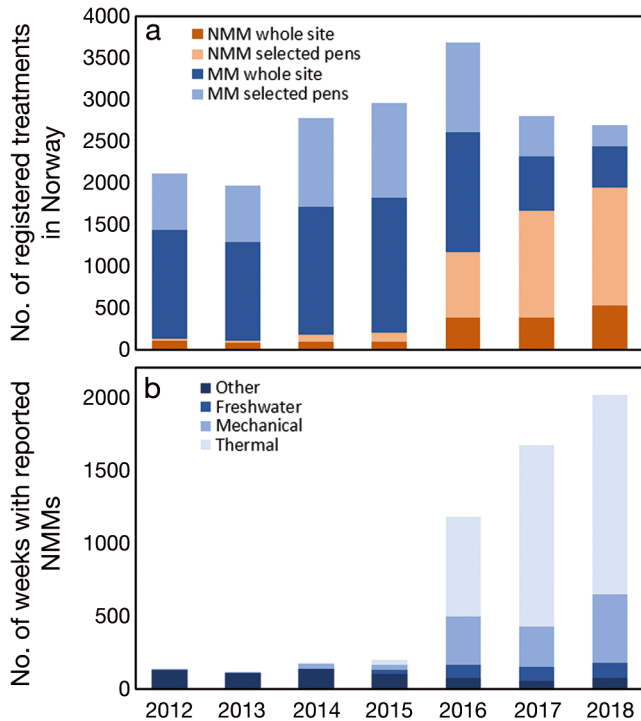
A variety of techniques for applying freshwater treatments have been tested in salmon aquaculture, particularly in Norway. Two types of freshwater treatments are being used (Fig. 2). Freshwater baths on wellboats into which salmon are placed for fixed time periods are the main freshwater treatment for sea lice control. This strategy targets only the parasitic stages of sea lice (i.e. copepodid, chalimus, preadult and adult sea lice). The second technique is the use of freshwater caps in snorkel pens. The freshwater caps are designed to target amoebic gill disease caused by *Neoparamoeba perurens*, but they do have the additional effect of exposing attached sea lice to freshwater when salmon swim through the freshwater cap. The methods differ in terms of the length of exposure to freshwater, whether or not they use filters to remove sea lice that detach during freshwater exposure, and the potential for different life stages of sea lice to be exposed to freshwater.

Freshwater exposure in wellboats is by far the most frequently used technique for delousing fish with freshwater. When this method is adopted, fish are seined within the net pen and

pumped over a dewatering unit to remove the seawater. They are then held in freshwater for 5 to 8 h, before being pumped back over the dewatering unit (in order to reuse the freshwater) and returned to the open sea pen. The relatively short freshwater exposure does not cause the attached sea lice to die, but it will often cause them to detach. For this reason, the freshwater is filtered to avoid the possibility of detached lice resettling on fish. The newest wellboats use filters of 100 µm (S. Gaasø pers. comm.), which is fine enough to collect all parasitic stages. Freshwater in wellboats may be reused for up to 4 treatments (S. Gaasø pers. comm.). The exact method depends upon the equipment available; some of the older wellboats do not have dewatering units and the filter type may vary. Wellboat treatments can be highly effective; Reynolds (2013) found reductions ranging from 92 to 100% in sea louse burdens on
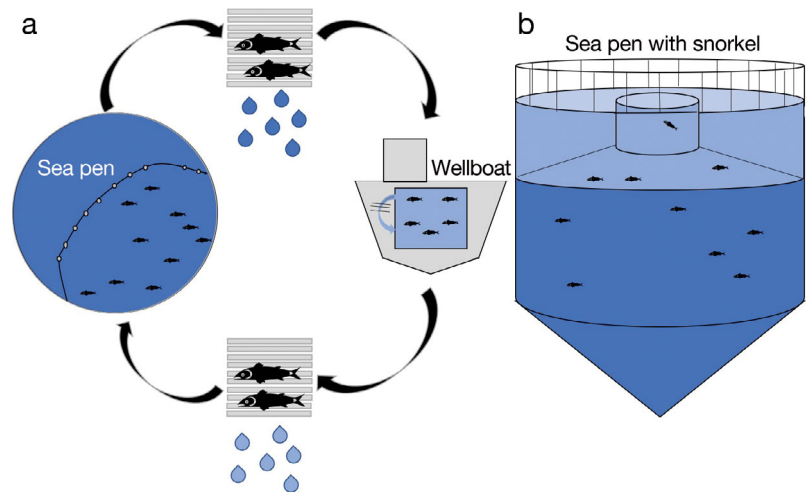


Fig. 2. Common methods for applying freshwater treatments on salmon farms. (a) Salmon are removed from the sea pen, transferred briefly to a dewatering area and then into a wellboat where they are exposed to freshwater (light blue). After 6–8 h, salmon are dewatered (in order to reuse the freshwater) and returned to the sea pen. The freshwater is filtered to remove any detached sea lice before it is pumped out of the wells. (b) Salmon are in a snorkel pen where they must pass through a freshwater layer to gulp air in order to fill their swim bladder. The snorkel has a higher density of mesh designed to keep sea lice, which occupy the top few meters of the water column, from entering the net pen

Atlantic salmon. Much of this is likely due to mechanical disruption rather that from the freshwater treatment itself. Evidence for this was presented in the same study, as a wellboat treatment simply using saltwater yielded a 50% reduction in sea lice.

Snorkel pens consist of net pens submerged several meters below the surface with a cylindrical 'snorkel' that extends to the surface. The snorkel is enclosed with a sea louse impermeable mesh, and the area inside is filled with freshwater, which floats on top of the seawater due to its lower density. While lower dissolved oxygen levels in the snorkel and the more limited swimming area generally keep the salmon in the larger net pen, they must enter the snorkel in order to reach the surface and gulp air, a behavior which is necessary for maintaining their air bladder equilibrium (Saunders 1965). Snorkel pens control sea lice through multiple mechanisms. The main mechanism is by placing salmon at depths where infective sea louse stages are less likely to occur (Oppedal et al. 2017). Secondly, when salmon surface into the 'snorkels' they are obligated to swim through freshwater, which may reduce sea louse loads further, although the extent of this exposure is short. Salmon lice are likely regularly exposed to lower-salinity water with these techniques, similar to exposures they would experience naturally in fjords with freshwater layers. Testing and optimization of snorkel pens is ongoing (Stien et al. 2016, Oppedal et al. 2017, Wright et al. 2017, 2018); however 2 Norwegian companies (Bremnes Seashore AS and Sinkaberg-Hansen) are using them (F. Oppedal pers. comm.).

*In situ* freshwater treatments have also been tested on sea pens by containing the treatment water with a tarpaulin skirt that is wrapped around the pen. This method is rarely used in practice because there is no mechanism to collect or filter sea lice that become detached during this operation to prevent them from later reattaching (G. Ritchie pers. comm.).

From an evolutionary perspective, the method used to apply freshwater treatments will influence the selection pressure experienced by the sea louse. Wellboats apply a selective force that causes parasitic stages to detach from the host either due to the freshwater itself or due to the mechanical disruption that occurs during dewatering and transfer of the fish into the wellboat. These detached sea lice will die during filtration of the freshwater. Due to the method of moving salmon onto the wellboats, planktonic stages of sea lice are unlikely to be exposed to freshwater in wellboats. Planktonic stages

may be exposed to freshwater caps in snorkel pens, while motile sea lice will be exposed for short durations when salmon enter the snorkel. Whether this causes detachment of sea lice is unknown. Short-term exposures to freshwater are unlikely to be lethal for attached stages of sea lice, but they may be lethal to pelagic stages (Dalesman 2003, Connors et al. 2008, Wright et al. 2016).

## 2.3. Sea lice and salinity

As with other marine invertebrates, environmental conditions influence sea louse population dynamics (Groner et al. 2016a). Temperature and salinity, in particular, have been identified as major drivers of sea louse population dynamics (Heuch et al. 2002, Arriagada et al. 2016, Groner et al. 2016a, Rittenhouse et al. 2016, Harte et al. 2017). Typical of many marine ectotherms, increasing temperatures are associated with faster development, with temperature limits ranging between around 3 and 21°C (Stien et al. 2005, Samsing et al. 2016). Reproductive output shifts from many small eggs to fewer larger eggs at the upper and lower thermal tolerance limits for sea lice (Samsing et al. 2016).

Sea lice are frequently exposed to water with varying salinities. They are often found in fjords and near river mouths where haloclines can form as a result of freshwater input. Pelagic sea lice are able to adjust their position in the water column to avoid water with lower salinities (Heuch 1995). As part of their host-seeking behavior, copepodids congregate at haloclines near river mouths during salmon migrations (Brooks 2005). In contrast, attached sea lice experience the salinities that their host is swimming in, unless they detach.

Numerous laboratory studies have demonstrated that salinity can influence development, behavior, infestation success and survival of sea lice (Table 2). Salinity, in contrast to temperature, acts more as a threshold, although the effect depends upon the developmental stage at which the exposure occurs. Planktonic larval stages are vulnerable to salinities of less than ~15 psu and experience mortality after several hours (reviewed by Groner et al. 2016b). In contrast, attached stages of sea lice are able to osmoregulate through consumption of the host mucus and, in experiments, have been found to survive for a week or more at salinities as low as 7 psu (Connors et al. 2008, Wright et al. 2016). While adults, including gravid adult females, can tolerate freshwater for several days, their eggs will not survive. In a labora-

tory study, hatching did not occur below 20 psu (Johnson & Albright 1991). Field studies indicate that reductions in fecundity due to salinity can substantially decrease sea louse populations in British Columbia, Canada (Arriagada et al. 2016).

## 2.4. Mechanisms of freshwater tolerance

Most marine invertebrates are isosmotic (also called osmoconformers), passively conforming to the osmolality of the surrounding seawater. In contrast, many invertebrates living in fluctuating salinities, such as estuaries, are hyper-hypo-osmoregulators; they are isosmotic across intermediate salinities (15–30 psu), hyper-osmotic at lower salinities (so that hemolymph osmolality is higher than that of ambient water) and hypo-osmotic at higher salinities (hemolymph osmolality is lower than ambient water) (Pequeux 1995, Lee et al. 2012). Attached sea lice are osmoconformers in full-strength seawater and hyper-osmotic at lower salinity (Hahnenkamp & Fyhn 1985). Free-swimming stages cannot maintain their osmolality in lower-salinity waters and die within hours of exposure, while parasitic stages can acclimate to lower-salinity water (over 1–3 d) and then maintain their osmolality for days. Maintenance of osmolality is likely facilitated by borrowing ions from the host to replace those lost to the environment and by burrowing into the mucosal layer of the host in order to reduce the surface area exposed to seawater (Hahnenkamp & Fyhn 1985, Sievers et al. 2019).

In crustaceans, active regulation of osmolality occurs through transport of ions in specialized gill cells and possibly in the gut epithelium and excretory glands (Pequeux 1995, Charmantier 1998). In the free-living copepod *Eurytemora affinis*, freshwater invasion has been associated with increased hemolymph osmolality (hyper-osmoregulation). Laboratory experiments and field surveys suggest that this is due to increased ion transport capacity in specialized tissue in the swimming legs that have increased activity and expression of the ion-motive transport enzymes V-type $H^+$ ATPase and Na/K-ATPase (Lee et al. 2011, 2012, Johnson et al. 2014).

More energy is required to maintain hemolymph osmolality that differs from the surrounding environment. In a laboratory experiment, transcriptional changes in copepodid sea lice across a salinity gradient suggested costly osmoregulatory strategies are employed (Sutherland et al. 2012). Differential regulation of genes in response to lower salinity exposures was associated with apoptosis, chaperones and the cuticle, and included the genes: *26S proteasome non-ATPase regulatory subunit 6* (*psmd6*), *26S proteasome non-ATPase regulatory subunit 4* (*psmd4*), *proteasome subunit beta type-3* (*psmb3*), *60-kDa heat shock protein* (*HSP60*), *heat shock 70 kDa protein cognate 4* (*Hsc-70*), p*rogrammed cell death protein 4*, *cuticle protein*, *cuticle protein CP14.6* and *chitin bind 4* (Sutherland et al. 2012). One must be careful placing too much weight on genome annotations such as these; in many instances within *L. salmonis*, no functional annotation has been con-

Table 2. Equations from Groner et al. (2016a) characterizing the effects of salinity (*S*) on *Lepeophtheirus salmonis* mortality (*M*) and attachment success (*C*), and the data sources from which these equations were modeled. The first 3 equations characterize the proportion of individuals that will die or find a host while they are in that life stage, while the fourth equation describes a daily mortality rate

| Parameter | Equation | Data source |
|---|---|---|
| Egg mortality (proportion of eggs) | $M_{egg} = \dfrac{1}{1 + \left(\dfrac{S}{20.8}\right)^{13.98}}$ | Johnson & Albright (1991) |
| Copepodid mortality (proportion of copepodids) | $M_{copepodid} = \dfrac{1}{1 + \left(\dfrac{S}{19.1 \pm 1.8}\right)^{7.1 \pm 1.3}}$ | Dalesman (2003) |
| Copepodid attachment success (proportion) | $C_{attachment} = 1 - \dfrac{1}{1 + \left(\dfrac{S}{21.2}\right)^{5.8}}$ | Bricknell et al. (2006) |
| Mortality of motile lice (daily rate) | $M_{motile} = 1 - \{1 - e^{[(-0.23 \pm 0.03)-S]}\}^{24}$ | Connors et al. (2008) |

ducted. However, genes coding for proteasome (*psmd4*, *psmd6*, *psmb3*) can have important functions in the ubiquitin-proteasome system, and dysregulation can have clinical significance through reducing proteolytic activity and misfolding of proteins. Heat shock proteins (such as HSP60, Hsc-70) ensure proper folding and a chaperone function for many proteins. This protective role of HSPs can essentially act in an anti-apoptotic manner, and prevent programmed cell death (Lanneau et al. 2008). These markers, along with impacts in cuticle and chitin binding proteins, would suggest osmoregulatory stress in these lice, likely causing cell death and cuticle/chitin damage. Altered regulation of these pathways is likely energetically expensive, particularly for non-feeding larval stages (Sutherland et al. 2012). Upregulation of HSPs, which can also be important for 'hardening' an individual to future instances of a stressful condition, has been found in response to lower-salinity water by both Sutherland et al. (2012) and Borchel et al. (2018), although in both cases, only a few HSPs were upregulated and to a relatively low degree compared to a simultaneously conducted thermal challenge.

Sutherland et al. (2012) and Borchel et al. (2018) used different subspecies of *Lepeophtheirus salmonis*, namely *L. salmonis oncorhynchi* (Pacific) and *L. salmonis salmonis* (Atlantic), respectively, showing that, despite significant environmental and evolutionary pressures linked to phenotypic differences between these subspecies, salinity stress responses were conserved. Going one step further, Borchel et al. (2018) noted that 2 of the HSPs expressed by *L. salmonis salmonis* copepodids in response to salinity stress were also induced following exposure to the anti-louse treatment hydrogen peroxide. Poley et al. (2017) also showed that HSc70 and HSP60 were induced in *L. salmonis oncorhynchi* copepodids infected with the microsporidian *Facilispora margolisi* and exposed to the anti-louse treatment emamectin benzoate. Other known pathways for freshwater tolerance in crustaceans include reduced cuticular permeability, ability to excrete urine that is more dilute than hemolymph and adaptation to lower hemolymph osmolality relative to stenohaline species (Pequeux 1995). It is unknown which, if any, of these strategies are used by sea lice.

Costs of osmotic and ionic regulation are likely greater at early developmental stages as a result of a greater surface area:volume ratio. Indeed, in some crustaceans, larval stages have different osmolality than adults (Charmantier 1998). In sea lice, pelagic stages are much more susceptible to lower salinities (Johnson & Albright 1991, Dalesman 2003, Bricknell et al. 2006, Groner et al. 2016a), though how much of this is due to surface area:volume ratios as opposed to the lack of host-mediated osmoregulation (which benefits attached stages) is unknown. Environmental factors, particularly temperature, have been found to alter the capacity of crustaceans to osmoregulate (reviewed by Pequeux 1995); however, this is not well understood in euryhaline copepods. Molting may also alter freshwater susceptibility, although, again, this is not well explored in sea lice or even copepods in general. In American lobster *Homarus americanus*, pronounced hyper-osmotic regulation precedes ecdysis, while isoosmotic regulation occurs post-molt (Charmantier et al. 1988).

## 2.5. Heritability of freshwater tolerance

For tolerance to evolve in response to freshwater treatments, there must be heritable genetic or epigenetic variation in freshwater tolerance. In a common garden experiment in which F2 generation pedigreed sea lice were exposed to 15 psu salinity, Ljungfeldt et al. (2017) found preliminary evidence for heritable genetic variation in low-salinity tolerance. This experiment used F2 offspring from sea lice sourced from 2 locations: a site with full-salinity seawater (>30 ppt) and a fjordic site with lower and more variable salinity (13−30 ppt). Counterintuitively, those sea lice that were collected from high-salinity environments had greater survival at low salinity; however, there was no high-salinity treatment in this study that could be used to calculate baseline survival for each family. Given the caveats associated with this experiment, general conclusions are not possible. Research is required to characterize the extent of genetic variation in freshwater tolerance and, if found, the mechanisms associated with freshwater tolerance.

Phenotypic plasticity could also increase survival of copepods in freshwater environments. Indeed, the ability to adapt physiologically in response to environmental cues (in this case salinity) could benefit species exposed to a variety of environmental conditions. Phenotypic plasticity in response to variable salinities has not been investigated in parasitic copepods; however, Lee & Petersen (2002, 2003) explored this question in *E. affinis*, which has invaded freshwater environments at least 5 times (Lee & Bell 1999). They found no evidence of a within-generation plastic response to freshwater; exposure of embryos to freshwater did not increase survival post-metamor-

phosis. Further work exploring transgenerational plasticity (epigenetic) effects on freshwater tolerance could be interesting and relevant in the context of freshwater tolerance in sea lice.

Several other species of marine copepods have also evolved freshwater tolerance. Transport in ship ballast water has facilitated 2 coastal species, *Acartia tonsa* and *Oithona davisae*, to adapt to low-salinity estuarine habitats in the Black Sea (Svetlichny & Hubareva 2014), demonstrating the critical role of human actions in freshwater invasions (Lee & Bell 1999). While those examples do not include parasitic species, Hairston & Bohonak (1998) suggested that 2 life history strategies, diapause and parasitism, likely facilitate freshwater invasions by providing mechanisms for coping with salinity variance (i.e. osmoregulation through the host or suspending development until conditions improve). Parasitism of anadromous fish can expose sea lice to a variety of fresh, brackish and saline environments (Boxshall & Jaume 2000). Indeed, within the order Siphonostomatoida, which contains the family Caligidae (to which *L. salmonis* belongs), all instances of freshwater invasion occur with parasites. These include the numerous species of *Salmincola*, which parasitize a variety of fish genera including anadromous acipenserids and salmonids. There is a single freshwater parasite within the Caligidae, *Caligus lacustris,* and a single monotypic genus (*Dichelesthium*) that infects members of the family Acipenseridae, the sturgeons (Boxshall & Jaume 2000). Similar to *L. salmonis*, *D. oblongum* can be found on Atlantic sturgeon more than 2 wk after they have returned to freshwater rivers (M. D. Fast pers. obs.). Copepodids of this species also exhibit poor survival after exposure to salinities less than 20 ppt (M. D. Fast pers. obs). In summary, freshwater tolerance has evolved both in parasitic species and as a result of anthropogenic exposures. Both scenarios are relevant to the case of sea lice.

While selection may favor a specific trait, trade-offs in the form of negative genetic correlations between a trait that increases fitness and one that decreases fitness can hinder evolutionary processes. The relative cost of the trade-off to the cost of the selective agent will dictate how constraining it is. In many cases, trade-offs may be context-dependent, only occurring in specific environments (Roff & Fairbairn 2007). Selection to decrease trade-offs may reduce these costs over time, although this likely depends on the genetic architecture of the genetic correlation (i.e. pleiotropy or linkage disequilibrium) (Roff & Fairbairn 2007). Due to the metabolic cost of osmoregulation, trade-offs associated with fresh-

water tolerance are likely. For example, freshwater-adapted *E. affinis* copepods have decreased saltwater tolerance and slower development (Lee & Petersen 2003, Lee et al. 2007). Consistent and widespread transcriptional changes, particularly chaperone and catabolic processes in copepodid *L. salmonis* (Pacific) in response to salinities below 28 psu and apoptosis at 25–26 psu, suggest that these responses are energetically demanding and impact cellular integrity, a situation which may be particularly stressful for lecithotrophic stages (Sutherland et al. 2012).

## 2.6. Influence of metapopulation structure

The response to selection is influenced by the metapopulation structure of sea lice. This influences the rate at which beneficial genes spread and the probability that a beneficial mutation could be lost to drift. Genetic evidence supports the idea of weak population structure among sea lice. Evaluation of microsatellites of *L. salmonis* across 12 farms distributed across the North Atlantic provides support for a single panmictic population, with subtle differentiation between locations ($F_{ST}$ < 0.002) (Glover et al. 2011). Machine-learning algorithms combined with IIb-RAD (reduced representation library sequencing) genomic data have since been used to fingerprint distinct sea lice subpopulations in the eastern North Atlantic and have also found weak population structure ($F_{ST}$ < 0.02) (Jacobs et al. 2018). In the North Pacific, analyses of single-nucleotide polymorphisms and microsatellites from sea lice collected in a wide range of locations also supports the idea that sea lice are panmictic (Messmer et al. 2011).

The panmictic population structure of sea lice, combined with their relatively short generation time, can lead to fast evolutionary responses on large spatial scales. *L. salmonis* have evolved resistance to numerous chemotherapeutants over a relatively short time span (<20 yr) (Besnier et al. 2014, Aaen et al. 2015). Use of the organophosphate azamethiphos (beginning in 1992) caused rapid selection for a resistant allele (*Phe326Tyr*) that is now found across the North Atlantic in sea lice collected on both wild and farmed salmonids (Fjørtoft et al. 2017, Kaur et al. 2017). Genomic data suggest that panmixia in sea lice contributed to a selective sweep for resistance to the commonly used chemotherapeutant emamectin benzoate in *L. salmonis salmonis.* Within 11 yr of the found in pesticide′s introduction, linkage groups associated with pesticide resistance were found in popu-

lations across the north Atlantic (Besnier et al. 2014). The structure of the genomic data suggests that the resistance evolved in a single location and spread rapidly throughout the Atlantic (Besnier et al. 2014). The genomic signature of an additional putative selective sweep due to selection by an organophosphate chemotherapeutant was also evident in this dataset (Besnier et al. 2014). Collectively, these results suggest that, should freshwater tolerance evolve in sea lice at a single site, connectivity among populations could facilitate a rapid selective sweep.

### 2.7. Strategies to avoid tolerance evolution

Management strategies can be used to reduce evolutionary responses to freshwater treatments. In general, the application of fewer treatments will reduce selection for tolerance. Data from terrestrial systems and mathematical models of aquaculture systems suggest that combination treatments, which kill pests using multiple mechanisms, have more effective control and result in a slower rate of resistance (or tolerance) evolution than applying a single treatment (REX Consortium 2013, McEwan et al. 2016). This likely occurs during wellboat treatments as a result of physical disruption of the sea lice followed by filtration of the water (Reynolds 2013, Powell et al. 2015). Other models suggest that the evolution of resistance to chemotherapeutants on salmon farms is slowed when the farms are in proximity to wild salmon pop-

ulations, as occurs on the west coast of Canada, because they maintain a population that is never exposed to the selective agent and can dilute the artificially selected gene pool (Murray 2011, McEwan et al. 2015). However, this idea will need to be re-evaluated in the case of freshwater treatments because some infested salmonids, such as resident juveniles, may swim in and out of freshwater (Webster et al. 2007).

### 3. CONCLUSIONS

Several criteria must be met for artificial selection to result in the evolution of freshwater tolerance in sea lice populations (Fig. 3, Table 1). These include: (1) heritable genetic variation for freshwater tolerance, (2) metapopulation structure and size that are conducive to evolution (3) weak to nonexistent fitness trade-offs associated with the trait and (4) strong selection pressure for the trait in question.

Our review suggests there is preliminary evidence for variation in tolerance of less saline water (criterion 1). However, the single study investigating this topic (Ljungfeldt et al. 2017) is inconclusive and this should be a priority for future research.

There is strong evidence that the metapopulation of sea lice is conducive to rapid evolution (criterion 2). As has been demonstrated with chemotherapeutant resistance, the panmictic population structure of sea lice and their short generation times are con-
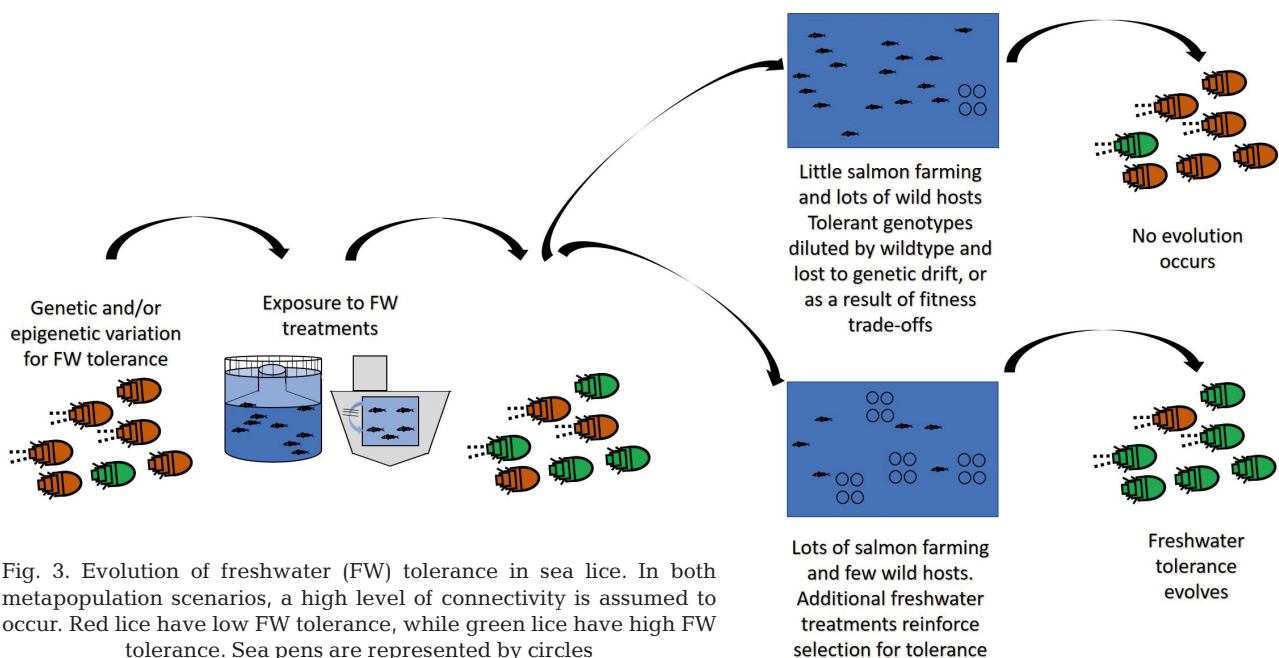


Fig. 3. Evolution of freshwater (FW) tolerance in sea lice. In both metapopulation scenarios, a high level of connectivity is assumed to occur. Red lice have low FW tolerance, while green lice have high FW tolerance. Sea pens are represented by circles

ducive to rapid selective sweeps of beneficial genes.

Less is known about potential fitness costs of freshwater tolerance (criterion 3); the high energetic demands of hyper-osmoregulation and the presence of catabolic processes in sea lice exposed to more brackish water suggest that strong physiological and energetic barriers may lead to fitness trade-offs in freshwater-tolerant sea lice (Sutherland et al. 2012). This may explain why freshwater tolerance has not evolved in sea lice already, since freshwater exposures can occur naturally when they parasitize wild sea trout or juvenile salmonids.

The amount of selective pressure that sea lice experience for freshwater tolerance (i.e. freshwater exposure) depends upon the location of the sea lice. Sea lice parasitizing wild trout that retreat into freshwater streams and rivers may certainly experience sustained selective pressure for freshwater tolerance. This may be an important selective pressure in the Pacific, where wild salmonids far outnumber farmed salmonids, but not in the Atlantic Ocean, where the opposite is true. In the Atlantic, the more relevant question is whether freshwater treatments place a strong enough selection pressure to cause populations to evolve freshwater tolerance. Wellboats, which are the main mechanisms for freshwater treatments, only target parasitic stages, and the mechanism for death is through filtration of detached sea lice or mechanical disruption of sea lice during transport (Powell et al. 2015). The freshwater exposures do not occur for a long enough time to be a cause of death in attached lice, which can osmoregulate through the host. Snorkel pens are effective at reducing sea louse populations because they limit transmission by placing salmon deeper in the water than sea lice typically go. Thus, in both cases freshwater exposure is not likely to be the only or the main selective pressure. On the other hand, the relative use of freshwater treatments is increasing in Norway, with 102 reported uses in 2018 compared to only 1 in 2014.

It is unclear whether the resulting selection is for freshwater tolerance in the stages that experience the selection (i.e. attached stages, which osmoregulate through the host), or if it will increase freshwater tolerance in planktonic larval stages (which do not osmoregulate through the host). A better understanding of the mechanisms that dictate freshwater tolerance in both stages is necessary to answer this question. Recent studies on the mechanisms of freshwater tolerance in *E. affinis,* as well as the transcriptomics of sea lice exposed to low salinities, can provide a starting point for further investigation into mechanisms of freshwater tolerance in various stages

of sea lice (Lee & Petersen 2003, Lee et al. 2003, 2011, 2012, Sutherland et al. 2012). Sea lice transcriptomic studies suggest that generic and costly polygenic stress responses may be associated with freshwater exposure (Sutherland et al. 2012). If this is the case, it may be worth investigating if exposure to other stressors, such as thermal treatments, may select for the same alleles that increase freshwater tolerance.

Despite being a well-studied marine parasite (Groner et al. 2016b), numerous research gaps hinder our understanding of evolutionary processes in sea lice. Research is needed to characterize the extent of variation in and the mechanisms associated with freshwater tolerance across spatial scales and life stages. Additional topics include the influence of gradual versus abrupt exposure to freshwater, differences in freshwater tolerance between Pacific and Atlantic sea lice and the influence of multiple freshwater exposures.

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