Seasonal progression of embryo size and lipid reserves in sea lice *Lepeophtheirus salmonis* collected from salmon farms

Emma Y. Taccardi[1,2,*], Ian R. Bricknell[1,2], Heather J. Hamlin[1,2]

[1]School of Marine Sciences, University of Maine, Orono, ME 04469, USA
[2]Aquaculture Research Institute, University of Maine, Orono, ME 04469, USA

ABSTRACT: Sea lice *Lepeophtheirus salmonis* are marine copepods that are the primary parasitic threat to Atlantic salmon *Salmo salar* aquaculture. Prior to infecting a host, *L. salmonis* embryos and larvae rely entirely on maternally derived lipid reserves, offering a unique lens for investigation of energetic trade-offs and reproductive investment. In the current study, we combined histology and image processing to assess *L. salmonis* embryo size, number of lipid droplets per egg, and lipid area across monthly collections (2018–2019) from *S. salar* farms in Maine, USA. Results indicate consistent embryo areas from season to season, peak lipid metrics in May, and minima in lipid quantities from October–December. Therefore, gravid females appear to invest the highest lipid levels in their embryos under biologically favorable conditions, when future larvae may thrive in the plankton and infection typically begins to surge on farms. In contrast, maternal lice likely allocate proportionately more energy into metabolizing their own lipid stores for vertical migration and survival through the winter. A detailed understanding of seasonal lipid reserves is fundamental for the improvement of infection models. These indicators at the earliest developmental stage partially encode recruitment of subsequent planktonic larvae, enabling unique forecasting potential to inform pest management on salmon farms.

KEY WORDS: Atlantic salmon · Aquaculture · Copepod · Egg · Energy reserve · Histology

1. INTRODUCTION

The salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) is a parasitic copepod whose infestations comprise the greatest economic constraint to Atlantic salmon *Salmo salar* aquaculture, making it the most widely studied species of sea lice. Its annual cost to the global industry was estimated at US $480 million in 2006 (Costello 2009) and is currently around $1 billion (Brooker et al. 2018, Thompson et al. 2019), predominantly due to treatment expenses and production losses (Costello 2009, Murray & Salama 2016, Abolofia et al. 2017). With prolonged attachment and feeding, *L. salmonis* causes external damage,

reduced fish growth, and increased susceptibility to secondary infections, which are often the cause of host mortality (Mustafa et al. 2001, Boxaspen 2006, Costello 2006).

With each reproductive cycle, gravid female sea lice extrude paired egg strings, each containing approximately 100–1000 eggs (Costello 1993) that hatch as planktonic nauplius larvae and survive off of maternally derived lipid reserves (Cook et al. 2010). Nauplii mature through 2 stages before molting into infective copepodids, which must seek and attach to a host before endogenous energy stores expire, and then actively feed through the mobile adult stages (Boxaspen 2006, Hamre et al. 2009). Planktonic de-

pletion of maternally derived reserves varies by temperature, with documented survival windows of 2–8 d (Johnson & Albright 1991) and 9–17 d (Gravil 1996), as well as reduced infectivity by 7 d old copepodids at summer and winter temperatures (Tucker et al. 2000).

Sea lice abatement has been met with limited success, due primarily to their biological aptitude for overcoming anti-parasitic tactics, both host- and human-induced (Torrissen et al. 2013). Therefore, there is an urgent need to increase our understanding of sea lice biology, especially as it relates to ecological resilience. In particular, the physiological mechanisms that underlie egg quality warrant attention, given the large reproductive capacity in paired egg strings (Boxaspen 2006, Hamre et al. 2009). Epidemiological models play a proactive and practical role in integrated pest management, yet the condition of *L. salmonis* embryos, how it may be indicated, and how it ultimately influences infection capability remains a gap in our knowledge (Jones & Johnson 2015, Brooker et al. 2018).

The accumulation of large amounts of lipid is a widespread strategy for energy storage among zooplankton, and is perhaps one of the most important metrics of egg quality (Pond et al. 1996, Alonzo et al. 2001). Energy content is critical in predicting the quality of future planktonic larvae, and thus infection potential, as lipid levels in eggs influence hatching success and naupliar survival (Pond et al. 1996, Tucker et al. 2000, Alonzo et al. 2001), and govern both the longevity and viability of copepodids prior to and during infection (Tucker et al. 2000, Boxaspen 2006, Cook et al. 2010, Gonçalves et al. 2014, Khan et al. 2017, Skern-Mauritzen et al. 2020). Lipid droplets additionally play key roles in reproduction, development, buoyancy, and migration (Lee et al. 2006, Cook et al. 2010). Because of its diverse functions, lipid content exerts considerable influence on the complex energy budgets and trade-offs that vary throughout progressing life histories. Egg-bearing copepods divert energy resources toward reproduction and feeding, as they undergo minimal somatic growth (Kiørboe et al. 1985); conversely, embryos and larval stages allocate more resources toward rapid growth and ultimately host location.

Although the energy budgets of free-living copepods are frequently studied due to their well-defined roles in food webs, the dynamics of their parasitic relatives remain ambiguous. Within the research that has addressed lipid stores in sea lice, most studies have placed the infective copepodid and naupliar stages at the forefront (Tucker et al. 2000, Cook et al.

2010, Thompson et al. 2019, Skern-Mauritzen et al. 2020), and have documented these dynamics solely under conditions that are biologically favorable for sea lice development without incorporating phenological influences. Maternal investments must balance a variety of variables, including egg size and number, and the energy reserves that are allocated to each. Previous studies have demonstrated that sea lice egg size and viability are reduced at lower temperatures (Ritchie et al. 1993, Heuch et al. 2000, Costello 2006); however, these investigations represent *L. salmonis* from Europe and Canada. Average sea temperatures in Europe are generally higher than those in Maine, USA, year-round, which typically range from about 0–10°C (Larsen 2004). These differences may encourage a range of physiological strategies that are unique among geographies, highlighting the importance of sharing novel data from this region. To our knowledge, we present the first study of lipids in *L. salmonis* embryos throughout progressing seasons, each of which presents environmental parameters that may directly influence embryo quality and/or influence maternal allocation of energy reserves.

Obligate lecithotrophy, in which the embryo receives no maternal investment post-extrusion, provides a unique opportunity to investigate energetics in response to environmental factors, as organisms cannot compensate for metabolic changes through consumption of additional items (Thompson et al. 2019). Furthermore, lack of mobility at the egg stage enables quantification of energy reserves that are not reduced by swimming or other behaviors, creating a closed system that does not gain or lose reserves through confounding activity. In the current study, we investigated embryo size and lipid profiles of sea lice on monthly and seasonal scales. These data and their implications regarding reproductive success may contribute to more biologically accurate models of larval dispersal at the earliest phase, enabling considerable forecasting to inform more proactive management strategies on salmon farms.

2. MATERIALS AND METHODS

2.1. Sea lice collection

Sea lice *Lepeophtheirus salmonis* were collected on a monthly basis from Machiasport and Eastport, Maine, in 2018 and 2019 (Fig. 1, see Table 1). Gravid female *L. salmonis* were removed with forceps from farmed Atlantic salmon *Salmo salar*, and sea lice
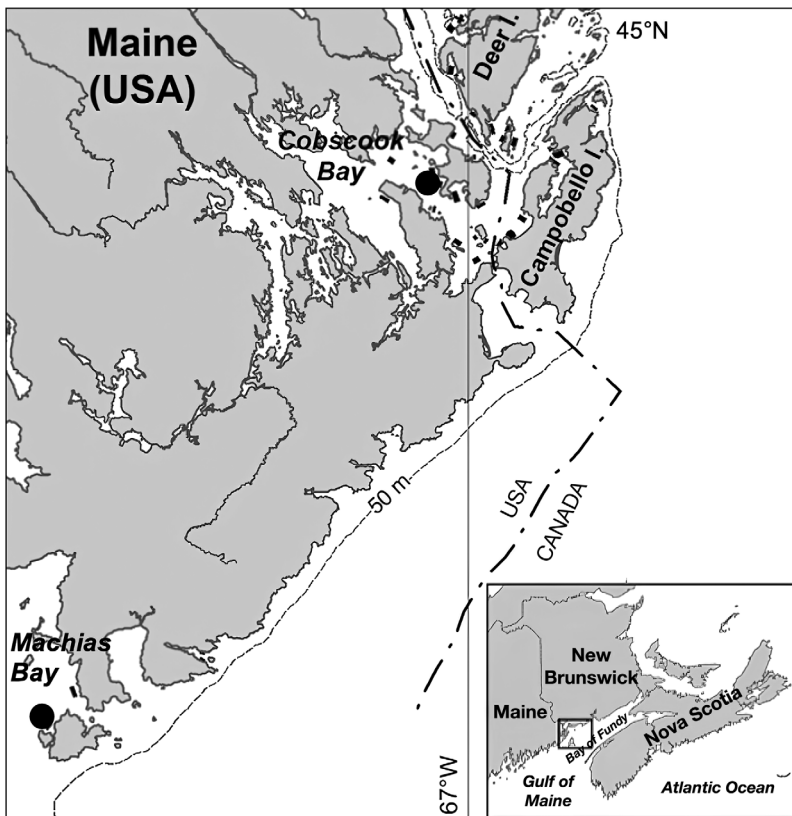
Fig. 1. Sea lice collection points (black circles) in Eastport (Cobscook Bay) and Machiasport (Machias Bay), Maine. Adapted from Chang et al. (2014)

were stored in natural seawater on ice during transport to the University of Maine. After arriving at the laboratory, egg strings were carefully detached from females. Light-colored egg strings were chosen in order to target early development, as sea lice embryos become increasingly pigmented with maturity, and color is a presumptive yet reliable indicator of developmental stage (Hamre et al. 2009).

2.2. Histology

Upon detachment, egg strings were immediately fixed in Davidson's solution for 24 h and then transferred to 70% ethanol for storage until further processing. Samples were dehydrated in a graduated ethanol series, cleared with CitriSolv (Decon Laboratories), and embedded in paraffin in a vacuum oven (Isotemp 281A; Fisher Scientific). Blocks were trimmed and sectioned at 6 μm using a rotary microtome (Shandon Finesse 325; Thermo Scientific). Approximately 10–15 eggs were analyzed for each egg string (see Table 1). Slides were dried on a slide warmer set at 60°C for 24 h prior to staining. Slides

were stained using a modified Masson's protocol (Presnell et al. 1997). Micrographs were captured at 100× using a Nikon Eclipse E200 light microscope and a C-mount digital camera (MU1803; AmScope).

2.3. Lipid quantification

2.3.1. Embryo and lipid standards

Embryos that were ellipsoid and displayed an unbroken cuticle were used in the analyses. Fertilized eggs were classified as Stage A or B according to presence or absence of substantial nucleated tissue, as an indication of developmental progress. The first 5 eggs at proximal and distal ends of egg strings were excluded, as these often exhibit abnormal lengths and shapes. Samples that displayed artefacts of sectioning within the body of the egg were also removed from analysis.

Throughout histological processing, lipids themselves largely dissolve from samples; it is generally assumed that the remaining vacant, ovular spaces are proxies for previously existing droplets (Pastorinho et al. 2003, Messick et al. 2004); therefore, lipid droplets were measured by proxy. Droplets became increasingly difficult to quantify in embryos that were farther along in development; therefore, only Stage A embryos were analyzed. Lipid droplets that were a minimum size of 10 μm^2 were measured due to tracing accuracy and visibility.

2.3.2. Image processing

Embryo images were processed in Fiji (ImageJ) software. Contrast was optimized, and a scale was set for each image. For each fertilized egg, we measured length (μm), width (μm) at the middle of its length, number of lipid droplets, and areas (μm^2) of individual lipid droplets. Embryo area was calculated assuming an ellipsoid shape using the following formula: Area = π (length / 2) × (width / 2). Due to the range of shapes, area of each lipid droplet was manually measured using the 'freehand selection' tool.

2.4. Statistical analysis

All data were analyzed using statistical packages in JMP v.14.0 from SAS. Means (±SE) were calculated for embryo areas and lipid quantities across embryos to statistically compare individual egg strings, representing individual female lice. Lipid area egg^{-1} data were arcsine transformed for analysis in order to normalize and stabilize variance (Snedecor & Cochran 1967). Paired egg strings were largely uniform in embryo size and lipid quantities throughout preliminary analysis; therefore, we sampled one of the 2 strings per gravid female to avoid pseudo-replication. In addition, we did not find consistent differences in the majority of metrics between sampling sites. For monthly and seasonal analyses, we ran Bartlett's and Shapiro-Wilk tests for equal variances and normality, respectively, and conducted 1-way ANOVAs with Tukey's HSD post hoc tests. Welch's correction was applied when the assumption for homogeneity of variance was not met. The alpha level was set at 0.05 in all cases. Seasons were defined as spring: May 2019; summer: June–August 2019; fall: October–November 2018 and 2019; and winter: December 2018.

3. RESULTS

3.1. Embryo size

Mean embryo area remained consistent across months and seasons. However, lengths of fertilized eggs in May and June were longer than that in the preceding October (p = 0.006

and 0.02, respectively). Embryos were generally longer in warmer months compared to colder months (Figs. 2 & 3). Within collections, there was a relatively high level of variability in embryo size across egg strings.

3.2. Number of lipid droplets

Fertilized eggs in May contained a higher number of lipid droplets than those in the previous October (p = 0.04), with an average difference of 12 droplets (Table 1). More broadly, spring 2019 had a higher number of lipid stores compared to fall 2018 (p =
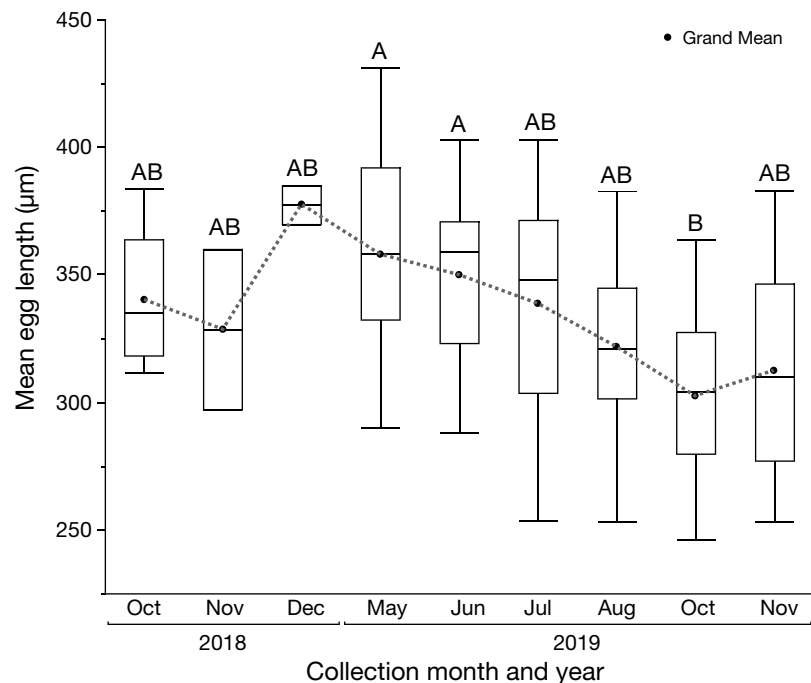

Fig. 2. Mean length (μm) of *Lepeophtheirus salmonis* embryos. Standard error is presented when sample sizes allow. Different letters indicate significant differences (p < 0.05) between months

Table 1. Date, site information, and mean (+SE) measurements from fertilized eggs of sea lice collected from commercial Atlantic salmon. Each egg string corresponds to an individual gravid female louse

| Date collected (mm.dd.yy)	Site	Egg strings (n)	Eggs (n)	Egg area	No. of lipid (μm^2)	Lipid droplet droplets	Lipid area egg^{-1} area (μm^2)
10.30.18	Machiasport	7	10 (2.8)	13109 (787)	15 (1.5)	2682 (180)	0.21 (0.02)
11.05.18	Eastport	2	18 (14)	14114 (2057)	16 (6.5)	2327 (634)	0.16 (0.02)
12.05.18	Eastport	2	7 (3.5)	13178 (474)	16 (2.5)	2769 (87)	0.21 (0.00)
05.02.19	Machiasport	14	14 (2.5)	14598 (656)	27 (2.7)	4792 (465)	0.32 (0.02)
06.19.19	Machiasport	17	19 (4.5)	14099 (399)	22 (1.7)	3474 (210)	0.25 (0.02)
07.10.19	Machiasport	29	7 (1.0)	13288 (434)	21 (1.3)	3287 (236)	0.25 (0.01)
08.13.19	Machiasport	24	6 (1.3)	12686 (357)	23 (1.7)	3022 (206)	0.24 (0.01)
10.18.19	Eastport	12	9 (2.3)	13216 (714)	25 (2.5)	3547 (311)	0.27 (0.02)
11.15.19	Eastport	9	10 (2.2)	12189 (688)	22 (3.3)	2915 (420)	0.23 (0.03)
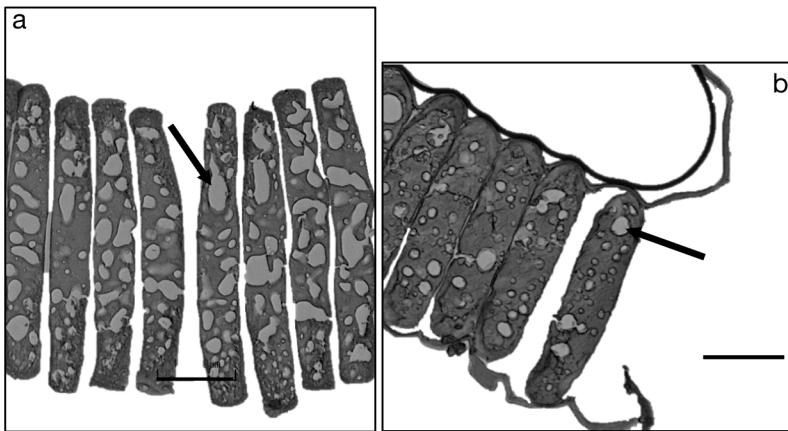
Fig. 3. Histological sections of sea lice eggs from (a) May 2019 and (b) November 2019. Arrows point to exemplary proxies for lipid droplets. For both micrographs, scale bar = 100 μm; magnification = 100×

0.006). Numbers of lipid stores remained similar throughout the fall and late winter of 2018, as well as the summer through fall of 2019.

3.3. Lipid area

Individual lipid droplet areas were highly variable and average values did not adequately represent collective egg strings; therefore, total lipid droplet areas were compared among embryos. Lipid area was greatest in May and differed from that of the preceding October, as well as the following summer months and November (Fig. 4). In May, lipid stores comprised approximately one-third of the total embryo area, which was double the lipid area egg^{-1} in November 2018 (Table 1). Lipid areas did not differ between any respective months in fall 2018 and 2019. Spring lipid areas were higher than those in the previous fall (p < 0.0001), as well as the subsequent summer (p < 0.0001) and fall (p = 0.0008).

When each embryo area was factored in, transformed lipid area egg^{-1} displayed the same pattern as in Fig. 4. However, lipid area egg^{-1} in May did not differ from that in June nor November 2019. Lipid area egg^{-1} in the spring was greater than that of fall 2018, summer, and fall 2019 (p = 0.0009, 0.002, and 0.04, respectively).

4. DISCUSSION

As the first study to profile seasonal lipid droplet quantities in sea lice *Lepeophtheirus salmonis* embryos, our data highlight peaks in all examined lipid metrics in May, as well as consistent minima throughout the preceding fall and winter. The timing of these indicators of high reproductive investment from maternal sea lice coincides with the beginning of typically observed infection surges on salmon farms. Specifically, under biologically favorable conditions that begin to manifest in the spring, most notably featuring temperature increases, gravid females appear to invest the highest level of lipid stores in their growing oocytes.

Conversely, given reduced lipid quantities in embryos throughout the late fall and winter, adult females may retain and metabolize their own lipids to survive harsher conditions leading up to the spring. In addition to providing nourishment, lipid stores play a key role in buoyancy, which may be particularly crucial when lice are conjectured to overwinter at depth under warmer temperatures (Johnsen et al. 2014, á Norði et al. 2015). Unlike a
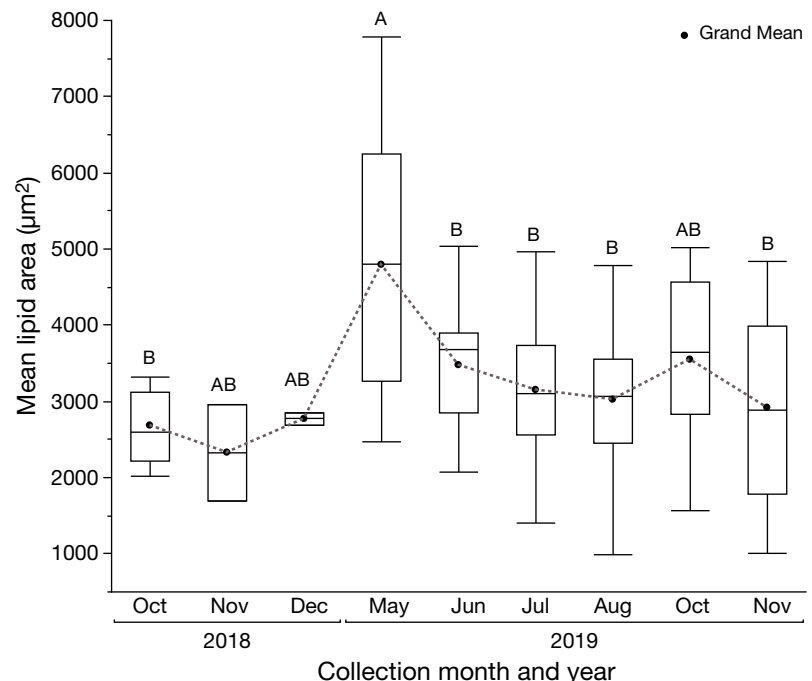

Fig. 4. Mean lipid area (μm^2) of sea lice embryos. Standard error is presented when sample sizes allow. Different letters indicate significant differences (p < 0.05) between months

variety of other copepod species, *L. salmonis* lipid reserves predominantly comprise denser triacylglycerides rather than more buoyant wax esters (Lee 1975, Hagen 1999, Tucker et al. 2000), which may aid in downward migration. Maternal *L. salmonis* may likewise utilize her own lipid stores to locate a suitable host and/or regenerate gonads during the winter period (Halvorsen 2015). Alternatively, sea lice may use all of their food sources for growth and general maintenance in the winter rather than store them as lipid reserves to begin with, as demonstrated by other zooplankton (Lee et al. 2006). Lipid droplets are one of multiple sources of energy to sea lice, as lipid reserves themselves exist in a variety of forms. Structural phospholipids, for example, contribute additional energy and may follow a different seasonal cycle based on sea louse growth. Additionally, although lipids constitute the major energy source for embryonic development in oviparous animals (Khan et al. 2018), proteins, carbohydrates, and other key components further aid in fueling larval reserves.

In most cases, summer lipid metrics resembled those in the fall more so than those in the spring, further highlighting the month of May. Although local sea lice infection prevalence and intensity generally remain high throughout the summer, the boost in reproductive investment in the spring appears to adequately support embryonic development through the following months, avoiding the need for a second wave of distinguished lipid allocation to the fertilized eggs. This peak in transferred lipid reserves provides sustenance for nauplii and copepodids, allowing larvae to remain in the plankton longer before attaching to a host for active nourishment (Costello 2006) — particularly throughout months that present optimal conditions for sea lice development (Boxaspen 2006, Jones & Johnson 2015, Samsing et al. 2016).

Our egg length conclusions are in partial agreement with previous evidence that spring embryos tend to be larger than those in colder seasons (Heuch et al. 2000, Costello 2006). In the current study, *L. salmonis* embryos were longer than those in early fall 2019 and did not differ from those in late 2018. Furthermore, with the incorporation of embryo width, embryo area did not differ across collection months, and 3-dimensional analysis of egg size would prove useful in future investigations.

Lipid droplets in sea lice embryos are distributed in a range of patterns, with a relatively high level of variability across egg strings. A portion of the embryos in our study contained 1 or 2 large droplets that were located centrally and/or anteriorly or pos-teriorly, while others featured many small droplets that were evenly dispersed. Assessing intrapopulation variability in lipid quantities and studying at the level of the individual provide insight on population fitness (Narcy et al. 2009), and these methods are applicable to other lecithotrophic copepods. Due to the relatively high variability across individuals in the current study, future research may be conducted at a finer temporal scale and will ideally incorporate greater sample sizes across collections. Additionally, future studies may compare statistical approaches that refine hypothesis testing, as both monthly and seasonal scales functioned as explanatory variables of the current exploratory study. Lastly, although preliminary analysis did not reveal differences in lipid quantities between collections in the beginning and middle of June 2019, the effects of varying water parameters warrant investigation, as both temperature and photoperiod, likely among others, influence the reproductive output of sea lice (Ritchie et al. 1993).

The detailed mechanism behind lipid transfer from maternal *L. salmonis* to its embryos remains unclear. In a laboratory strain of sea lice, maternally derived lipids in nauplii were found in the form of droplets in yolk (Khan et al. 2018). However, *in situ* females may modify their lipid stores and/or directly transfer them to oocytes (Pond et al. 1996) according to environmental factors and individual condition. In laboratory settings, adult female *L. salmonis* are able to continuously extrude up to 11 pairs of egg strings after fertilization (Hamre et al. 2009). Generation number likely affects maternal trade-offs, as females in later reproductive cycles are expected to invest all of their lipid stores into their embryos prior to death (Halvorsen 2015). In addition, complementary measures of reproductive success, such as the number of fertilized eggs produced per batch, hatching and survival rates through the infective copepodid stage, and other measures of egg viability, warrant further attention (Kiørboe et al. 1988, Ianora et al. 1992, Poulet et al. 1995).

An improved understanding of embryo condition is critical for proactive sea lice management, particularly given the large reproductive potential in paired egg strings (Boxaspen 2006) and thus recruitment prospect (Halvorsen 2015). Lipid reserves are central indicators of embryo and larval condition, as they are finite and obligate nutritional resources prior to host attachment. While many recent epidemiological models enhance global aims for the practical management of sea lice (Johnsen et al. 2014, Rittenhouse et al. 2016, Salama et al. 2016, Sandvik et al. 2016,

Samsing et al. 2017), they require a suite of biological inputs that are ideally evidenced *in vivo*. Although the effects of seasonal parameters are consistently applied across projected development as a whole, the influence of specific indicators that can be rapidly detected, primarily featuring lipid quantities, has yet to be applied. These lipid dynamics play a chief role in partially encoding population fitness at the earliest life stages, providing a unique opportunity for substantial forecasting. Altogether, a refined biological understanding of *L. salmonis* begins at the egg stage, which is often overlooked relative to planktonic larval phases. With more comprehensive knowledge of energy budgets and condition across all life stages, particularly in the context of a changing environment, the increased efficacy of infection models will promote sustainable salmon aquaculture.

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