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Molecular quantification of parasitic sea louse larvae depends on species and life stage

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Abstract

Sea lice cause substantial economic and environmental harm to Norway's aquaculture industry and wild salmonid populations. Rapid, accurate quantification of lice larval densities in coastal waters remains the greatest bottleneck for providing empirical data on infestation risk within wild salmon habitats and aquaculture production regions. We evaluated the capability of droplet digital PCR (ddPCR) as an absolute quantification method for the planktonic stages of two parasitic louse species, *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (von Nordman). Results demonstrated linear relationships between the DNA quantity measured and the number of spiked larvae for both species and life stages. However, *L. salmonis* contained a significantly greater number of DNA copies than *C. elongatus* individuals and for *C. elongatus*, nauplii displayed a significantly higher number of DNA copies than copepodids. Our results suggest that ddPCR can effectively enumerate louse larvae, but interpreting ddPCR results differ between the two louse species. Obtaining larval abundance estimates from marine plankton samples will depend on the nauplii to copepodid ratio for *C. elongatus*, but not for *L. salmonis*.

KEYWORDS
Caligus, copepodid, ddPCR, eDNA, *Lepeophtheirus*

1 | INTRODUCTION

Sea lice are common marine ectoparasites that infest a wide diversity of fish globally. Two prominent species in the North Atlantic, *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (von Nordmann), are well-known for their impacts on mariculture species and wild fish populations. In particular, *L. salmonis* currently represents the greatest challenge to the Norwegian salmon farming industry due to financial impact, environmental sustainability and fish welfare (Dempster et al., 2021; Karlsen et al., 2023; Stene et al., 2022; Taranger et al., 2015). *L. salmonis* is a native ectoparasite of wild Atlantic salmon (*Salmo salar*, L.), sea trout (*Salmo trutta*, L.) and Arctic charr (*Salvelinus alpinus*, L.) but has increased in population size due to the increased scale of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*, Walbaum) aquaculture operations in Norway over the last 60 years (Finstad et al., 2021; Heuch et al., 2005). *C. elongatus* is another parasitic louse species infesting salmonids but has a more diverse host range and offshore distribution (Jackson et al., 2000). In recent years, *C. elongatus* infestations on farmed and wild fish have increased in frequency, making these parasites a growing concern to the aquaculture industry and other ocean stakeholders (Øines et al., 2006). The industry is burdened with financial losses of nearly 5 billion NOK each year, in part from prophylaxis and treatments against louse, as well as mortality and growth reduction in infested fish (Abolofia et al., 2017).

Salmon producers have also come under public scrutiny and government regulation under the 'traffic light system' to reduce the spread of louse and therefore lessen the health risks to wild salmonids (Taranger et al., 2015; Vollset et al., 2018).

The two louse species are morphologically and developmentally similar, including three planktonic larval life stages. Their life begins with hatching from egg strings carried by the adult female, where they immediately enter the planktonic environment and drift as nauplii. After moulting into a second nauplii stage and finally a copepodid, the larvae must find a host to attach to and begin feeding as they will starve if no host is found (Samsing et al., 2016). Physiologically, *L. salmonis* and *C. elongatus* diverge in size during their development, as both hatch to nauplii stages at nearly the same length (500 μm vs. 450 μm), but *L. salmonis* mature up to twice the length of *C. elongatus* (Piasecki, 1996; Schram, 1993).

A large increase in the populations of both parasite species, and in particular *L. salmonis*, is evident from mandatory nation-wide weekly counts of lice attached to farmed fish in Norway. There is, however, little evidence of how lice larval abundance and distribution outside the fish farms changes over time. Even with a presumed increase in larval abundance in regions with intensive aquaculture production, *L. salmonis* larvae are rarely detected in mesoplankton samples at densities above one larva per 10 m^3 except in the immediate vicinity of an active salmon farming site (á Norði et al., 2015; Jevne et al., 2021; Nelson et al., 2018). Due to the extremely low density of larval lice in the planktonic community and the high monetary cost of identifying plankton from bulk samples by microscopic means, assessment of lice larval abundances through time and space have remained challenging to implement on a large scale (Bui et al., 2021; McBeath et al., 2006).

Molecular methods have been proposed as a cost-effective solution for directly measuring sea louse larval quantity in plankton and water samples collected at fish farms or the open sea (Bui et al., 2021; Krolicka et al., 2022). To date, these methods have only been utilized for relative quantification of *L. salmonis* (Bui et al., 2021; Krolicka et al., 2022; McBeath et al., 2006; Peters et al., 2018; Turon et al., 2022), where the measured DNA quantities generated by qPCR and metabarcoding cannot be accurately converted to an exact biomass or number of individuals. Current molecular methodologies also lack the capability to distinguish between infective copepodid and non-infective nauplii life stages of sea lice, limiting their use to studies where total larval abundance is of interest (Byrne et al., 2018). Variability in DNA copy number between the life stages of *L. salmonis* or *C. elongatus* or even between the two species also remains unknown. For *C. elongatus*, no quantification experiments have been performed to determine whether molecular methods can measure their absolute or relative abundance and no in vitro tests have taken place to confirm that the genetic marker can independently distinguish *C. elongatus* from *L. salmonis*.

Droplet digital PCR (ddPCR), a technology combining microfluidics and Poisson statistics, can provide absolute quantification of nucleic acids using genetic markers (Hindson et al., 2011). In a comparative study of abundance estimation in controlled samples, including ddPCR, qPCR, light microscopy and fluorescence microscopy, Bui et al. (2021) found that the ddPCR technology was best able to detect and enumerate the number of *L. salmonis* copepodid spiked plankton samples with comparable accuracy to the current benchmark method, light microscopy. However, their study only included one stage (copepodids) and one species, so the variation in DNA-copy number between life stages and species are still unknown. Therefore, we investigated this variation using ddPCR technology and experimental spiking of larval numbers for two species and two stages.

In this study, we use cultured sea lice larvae in a spiking experiment to determine whether DNA quantification measured by ddPCR differs across species, stages and spiking densities.

2 | MATERIALS AND METHODS

2.1 | Lice culturing

Gravid female *L. salmonis* and *C. elongatus* were collected from infested Atlantic salmon from aquaculture facilities in Sulfjord, Trøndelag, Norway in November 2019. These lice were separated by species and their egg strings were harvested for culturing at NTNU SeaLab (https://www.ntnu.no/sealab). The larvae produced were introduced to adult Atlantic salmon in laboratory aquariums and allowed to reach maturity and begin reproducing in order to generate a continuous stock of egg strings and larvae for experimental work (Norwegian Food Safety Authority - FOTS ID 15366).

In June 2021 when fertilized egg strings planned for this experiment formed on the female lice, host fish were sedated with a dose of MS222 (0.005 g/L) and the female lice were gently removed. Egg string pairs were then disconnected from the louse and placed in individual incubation wells. Harvested egg string pairs were incubated at 10°C in individual 35 mL mesh-bottom wells with a mild flow-through current of sand-filtered sea water at 3.5 mL/min. Wells were checked multiple times per day to mark their hatching date. After hatching, half of the larvae of each species were permitted to develop for 3 days and half for 7 days, ensuring that they moulted into the nauplii II and copepodid stages before being collected and preserved.

2.2 | Collection, preservation and DNA extraction

Lice larvae were collected live by pipette from the water's surface and checked for life stage before being preserved in 96% EtOH and stored at −20°C. Once approximately 250 individuals of each stage and species were preserved, counting plates were used to separate preserved larvae into triplicate samples of 1, 5, 10, 20 and 30 individuals preserved in 35 mL of 96% EtOH. Samples were then homogenized in 50 mL Matrix D tubes (MP Biomedicals, containing 1.4 mm ceramic beads) at 4.0 rpm for 40 s using a FastPrep-24 homogenizer (MP Biomedicals) in a total volume of 35 mL 96% saline EtOH. Three subsamples of 500 μL were transferred from each sample to 2 mL Eppendorf tubes and dried in a heating cabinet at 56°C for 4–6 h.

TABLE 1 Results of three linear mixed-effect analyses (LMMs with ML) of ddPCR detected DNA copies in (a) *Lepeophtheirus salmonis* and *Caligus elongatus*-spiked samples, (b) only *L. salmonis* samples and (c) only *C. elongatus* samples.

Reference	Factor	Estimate	SE	*t*-Value	*p*-Value
(a)					
L. salmonis	(Intercept)	−0.81	2.13	−0.38	.704
	Species (*C. elongatus*)	1.30	3.00	0.44	.666
	Lice number	**2.54**	**0.13**	**19.44**	**<.001**
	Lice number × Species	**−1.30**	**0.18**	**−7.17**	**<.001**
(b)					
L. salmonis nauplii	(Intercept)	−2.31	2.85	−0.81	.424
	Life stage (copepodid)	2.88	3.01	0.96	.346
	Lice number	**2.55**	**0.15**	**17.38**	**<.001**
(c)					
C. elongatus nauplii	(Intercept)	−0.69	1.34	−0.51	.611
	Life stage (copepodid)	2.36	1.90	1.24	.224
	Lice number	**1.60**	**0.08**	**20.14**	**<.001**
	Lice number × Life stage	**−0.71**	**0.11**	**−6.35**	**<.001**

Note: Significant individual and interactive effects in bold.

Subsamples had 500 μL ATL buffer (Qiagen) and 125 μL proteinase K (Qiagen) added and were then vortexed and incubated at 56°C overnight. DNA extraction was performed the following day using DNeasy Blood and Tissue Kit (Qiagen) and eluted in 200 μL AE buffer (Qiagen).

2.3 | ddPCR and target DNA copy estimation

DNA concentration for each subsample was measured using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad) using species-specific assays for *L. salmonis* and *C. elongatus* (McBeath et al., 2006) and included a VIC-labelled and a FAM-labelled TaqMan MGB Probe (McBeath et al., 2006). In a total reaction volume of 22 μL, ddPCRs consisted of primers of both *L. salmonis* and *C. elongatus* forward and reverse primer in a final concentration of 0.9 μM each, FAM-labelled probe (*C. elongatus*) and VIC-labelled probe in a final concentration of 0.25 μL, 10 μL ddPCR Supermix for Probes (No dUTP; Bio-Rad Laboratories), dH_2O and 1 μL of extracted DNA. Both assays were run for all samples to check for potential cross-contamination.

Droplets were generated using the AutoDG instrument (Bio-Rad) and PCR amplification was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 58°C for 1 min, final denaturation step at 98°C for 10 min and a hold at 4°C. PCR plates were transferred to a QX200 Droplet Reader (Bio-Rad Laboratories) for automatic detection of fluorescent signal in the droplets. QX Manager Standard Edition v1.2 (Bio-Rad) was used to separate positive and negative droplets and generate an estimated concentration of the target DNA sequence (DNA_{conc}) in ng/μL. The number of target DNA copies (TDC) in each replicate were then calculated by:

$$\text{TDC (millions)} = \frac{20 \times \text{DNA}_{\text{conc}} \times \text{Vol}_{\text{Elution}} \times \text{Vol}_{\text{Sample}}}{\text{Vol}_{\text{template}} \times \text{Vol}_{\text{extDNA}} \times \text{Vol}_{\text{Subsample}} \times 1{,}000{,}000}$$

For wells with less than three positive droplets or less than 6000 overall accepted droplets, estimated values were converted to N/A (Dobnik et al., 2015). To obtain an estimate of the DNA copies detected per louse, TDC was divided by the number of spiked lice in each sample.

To examine the possibility of PCR inhibition due to oversaturation of target DNA, an additional round of ddPCR amplification and quantification was performed on a 10× dilution of all DNA extracts.

2.4 | Statistical analysis

Statistical analyses were performed and visualized in R v4.2.2 (R Core Team, 2022) using the tidyverse package (Wickham et al., 2019). A linear mixed effects model (LMM) was used to test the individual and interactive effects of species and number of spiked lice on TDC across all samples (lme4::lmer; Bates et al., 2015). Two subsequent LMMs were created using subsets of the data by species and tested the effects of stage and lice number on *L. salmonis* and *C. elongatus* samples separately. All models accounted for the random effect of triplicate subsamples within each sample replicate and were fitted using the maximum likelihood estimation method. Outlier data were detected by visual assessment of residual plots. Of the 90 *L. salmonis* subsamples run, two replicates of the nauplii and four of the copepodids were identified by residual plots as containing a greatly reduced TDC value compared with the other samples of their same stage and spiking densities. All four of the outlying copepodid samples occurred at the highest spiking density (30) and three of the four were the triplicate subsamples of a single sample. This sample was assumed to have been mishandled and was therefore excluded from all statistical models and TDC per louse calculations. The remaining outliers were left in the data set to ensure normal subsampling variance was fully accounted for. No outliers were detected in the *C. elongatus* data set.

3 | RESULTS

From the samples containing *L. salmonis* nauplii and copepodids, ddPCR detected similar amounts of DNA per louse across both larval stages, an average of ~2.33 million copies per nauplii (SD = 0.84M) and ~2.48 million copies per copepodid (SD = 0.76M). The *C. elongatus* samples contained considerably less DNA per louse than *L. salmonis*, with a mean value of ~1.51 million copies detected per nauplii (SD = 0.29M) and only ~1.03 million per copepodid (SD = 0.37M).

The first linear mixed effects model similarly indicated that there was a significant interactive effect between the number of lice spiked and the louse species ($p < .001$) on the response variable, TDC. Samples containing *L. salmonis* larvae were predicted to increase by ~2.54 million TDC for each spiked louse present while each *C. elongatus* larvae corresponded to an ~1.24 million TDC increase (Table 1a). In the model pertaining to *L. salmonis* samples only, no significance was detected in the interaction of stage and lice number ($p = .719$), so life stage did not impact the number of DNA copies detected and therefore this interaction was removed to simplify the model. In the simplified model, the effect of lice number on TDC remained significant ($p < .001$) and the effect size remained similar at ~2.55 million TDC per *L. salmonis* larvae (Table 1b). The final LMM detected a significant interactive effect between life stage and number of spiked lice for *C. elongatus* ($p < .001$) indicating that the extracted DNA from each copepodid contained an estimated 710,000 less COI gene copies per louse than the nauplii (Table 1c).

The three outliers detected in the single *L. salmonis* sample were examined alongside the results of the second ddPCR of 10× diluted samples to check for PCR inhibition as a potential cause. These samples as well as the remainder of 10× dilution data set returned TDC values which did not differ significantly from those generated by the first ddPCR analysis, indicating no inhibition.

Across all 180 ddPCR replicates, one subsample from a *C. elongatus*-spiked sample with 10 copepodids indicated cross-amplification, as 36 of 19,300 droplets were detected positive for the *L. salmonis* target gene. This was extrapolated to a detection of ~615,000 non-target DNA copies of the *L. salmonis*-specific COI sequence in the bulk 35 mL sample. No other subsamples from this sample had positive droplets detected for *L. salmonis* and target DNA copies detected using the *C. elongatus*-specific primer and probes returned results in the expected range. Three additional PCR replicates displayed a single positive droplet detected for the non-target species, but an estimate of DNA copies was not calculated due to the minimum droplet cut off value.

TABLE 2 Linear regression formula and conditional R^2 generated by individual LMMs for each life stage of each species calculated by DNA copies in response to spiked lice number with subsamples as a random factor.

Group	Formula	R^2
L. salmonis nauplii	$Y = 2.50\,x - 1.67$	.86
L. salmonis copepodids	$Y = 2.61\,x - 0.13$	.90
C. elongatus nauplii	$Y = 1.60\,x - 0.69$	.91
C. elongatus copepodids	$Y = 0.89\,x + 1.67$	.78

4 | DISCUSSION

This experiment validates the use of ddPCR to accurately and reproducibly estimate the number of *L. salmonis* larvae present in a marine plankton sample regardless of larval stage. The pipeline used here has been shown to be highly accurate quantifying *L. salmonis* copepodid abundance from spiked plankton samples (Bui et al., 2021) and should in practice share a similar accuracy in quantifying nauplii or communities of mixed stage larvae. However, our results also indicate that this quantification method cannot produce an equally precise estimate for *C. elongatus* individuals if the ratio of nauplii to copepodids is unknown, because the number of DNA copies of three copepodids equals that of approximately two nauplii.

By using the ddPCR gene quantification method to calculate TDC in samples ranging from low to high densities of each louse species, we confirmed that the number of DNA copies detected increased with the number of spiked lice. The results clearly indicated linear relationships between density of spiked lice and the number of TDC detected for both species, though the slope differed greatly between them (Table 1a, Figure 1). Specifically within the *C. elongatus* samples, a significant interactive effect detected between stage and lice number revealed a difference in relative TDC abundance between the two larval life stages of *C. elongatus*. This becomes particularly clear as the density of spiked lice increases.

4.1 | Differences between species

After calculating the mean TDC per individual louse in each sample, we observed that DNA copy number per larvae in *L. salmonis* was twice that of *C. elongatus*, with 2.40M versus 1.27M DNA copies respectively. While the large difference in COI DNA copy number between these two closely related species was unexpected, the result has little to no implications on the outcomes of ddPCR quantification as each species can be detected individually in each sample by the separate genetic species-specific primers.

The difference in DNA copy count exposes a divergence in COI gene numbers between these two closely related species. While we did not measure the size or mass of the individual larvae analysed, the descriptions of *L. salmonis* and *C. elongatus* stages by Schram (1993) and Piasecki (1996) indicate that *L. salmonis* larvae are only ~10% greater in length than *C. elongatus* larvae until attachment to a host and sustained feeding occurs. However, differences in the ecological niches of these two species may be responsible for some of the discrepancy in their DNA abundance. Ershova et al. (2021) suggested that the high lipid content of calanoid copepods might be the reason for their low relative read abundance in metabarcoded samples compared to the biomass of those species as calculated by light microscopy. Similarly, lipid content may differ greatly between *L. salmonis* and *C. elongatus*

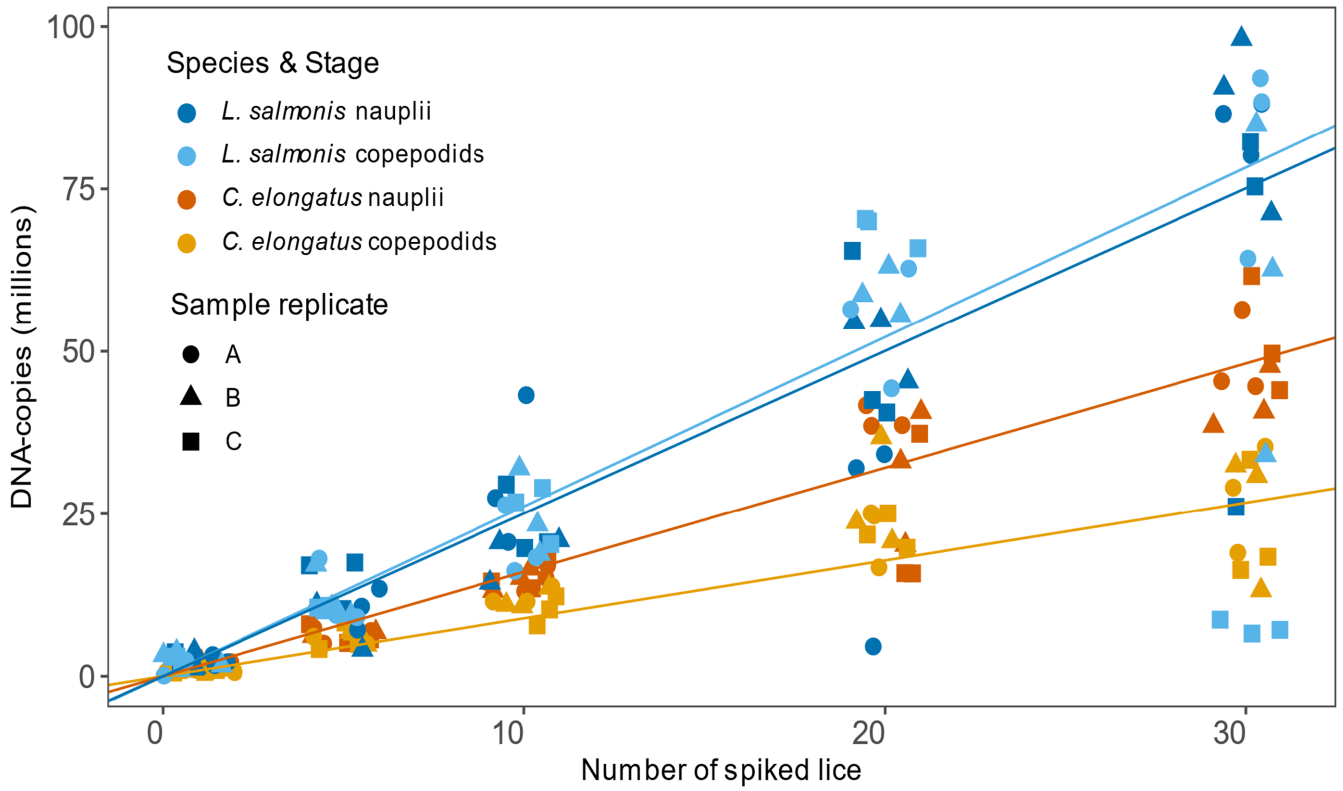

FIGURE 1 The number of *Lepeophtheirus salmonis* and *Caligus elongatus* DNA copies detected in each sample as a function of the number of spiked lice. Points in each horizontal grouping contain the same number of spiked individuals. Colour indicates the species and life stage of the louse larva spiked in each sample. Points of the same colour and shape indicate triplicate subsample replicates taken after homogenization. Regression lines correspond to the linear mixed model predictions for each species' life stages. Formulas and R^2 values for each line are listed in Table 2. The three light blue squares in the bottom right corner represent the sample that was excluded from the statistical analysis.

larvae based on potential differences in energetics of the two species. Furthermore, relative mtDNA abundance has been linked to mitochondria abundance and metabolic traits in humans and livestock and the differences we detected in the mtDNA copy amounts between *C. elongatus* and *L. salmonis* may indicate a difference between the resting metabolism or metabolic potential of the two louse species (Bai et al., 2020; Ganel et al., 2021; Gibbons et al., 2014). As a specialist to salmonids, *L. salmonis* are relatively rarely occurring and mostly inhabit surface waters, whereas *C. elongatus* is widely considered a generalist and has been recorded parasitizing over 80 teleosts and elasmobranchs within the North Atlantic, including both pelagic and demersal species (Kabata, 1979; Øines & Heuch, 2007). Therefore, it may be worth considering that variation in larval host-seeking behaviour causes a difference in energetic needs for larvae of the two species. It is also documented that *C. elongatus* copepodids are capable of surviving >4 weeks in water salinities of at least 25 ppt (Andersen, 2006), compared to ~2 weeks for *L. salmonis* copepodids (Samsing et al., 2016) which could indicate a higher ratio of lipid stores and lower metabolic activity in larvae of *C. elongatus* compared to *L. salmonis*.

Due to the COI gene fragment's frequent use as a universal primer for eukaryotes, the difference in DNA abundance we discovered between these two species is relevant for those considering attempts at absolute and relative quantification of sea lice and other marine eukaryotes using DNA metabarcoding methods. As suggested by Ji et al. (2019), establishing baseline TDC per individual or per gram of tissue is beneficial when estimating the biomass or abundance of a specific species of interest rather than only quantification of DNA copies.

4.2 | Differences between life stages

Within *L. salmonis*, model predictions indicated that TDC per louse values were consistent across both stages, with the means of the two life stages differing by only 160 k copies (~6%) of the overall mean. This marginal increase in TDC could be expected based on the similarly marginal increase in length between nauplii II and copepodid life stages (Schram, 1993). Even though no growth due to feeding could have occurred in the culturing wells, consumption of lipid energy stores in *L. salmonis* nauplii are assumed to fuel the morphological changes that take place during moulting from nauplii to copepodid as well as the burst swimming needed to maintain buoyancy (Tucker et al., 2000). The minimal TDC difference between *L. salmonis* life stages was not considered significant by the statistical model. Therefore, we conclude that ddPCR quantification method has the capability to estimate *L. salmonis* larvae and that the ratio of nauplii to copepodid larval stages in each sample has little to no effect on the subsequent estimate of individuals.

In contrast to *L. salmonis*, the model predicted a ~44% loss of DNA copies from nauplii to copepodid for *C. elongatus* and the calculated mean TDC per louse for these stages showed a decrease of almost one third (1.51M → 1.03M) of the DNA copies. This distinct difference in target DNA copy number between *C. elongatus* nauplii and copepodids hampers the accuracy of the ddPCR quantification method when used on plankton samples containing an unknown ratio of copepodids to nauplii. Therefore, we suggest that quantification of *C. elongatus* is less certain and could either include an approximate estimate based on the average DNA copy number of the two stages, or a range where two estimates based on each stage are reported. Doing so allows for better density estimation of this widespread parasite that often displays clustered and unpredictable distributions.

The scale of change we observed in the gene copy number between just 3 and 7 days post-hatch in *C. elongatus* larvae was unexpected and runs counter to both the slight increase of DNA copies in developing *L. salmonis* larvae we detected, and to an assumed increase in size between the nauplii and copepodid stages of *C. elongatus* (Piasecki, 1996). The cause for this decrease is unknown, but potential sources of influence on DNA copy number may include differences in metabolism necessitated by moulting and new tissue development, active swimming frequency or other behavioural changes between the two life stages. Based upon the knowledge that mtDNA copy number is associated with mitochondrial abundance or biogenesis (Gibbons et al., 2014), the decrease observed between these two life stages could be the result of reduced metabolic activity due to a halting of further cellular or tissue development between completion of the moult to copepodid and attachment to a host after which feeding occurs. Survivorship of the copepodid life stage of *C. elongatus* has been observed for over 4 weeks in laboratory incubators (Andersen, 2006) and adult stages also have capability to endure many days without a host (Stine Østerhus, unpublished observations), indicating that *C. elongatus* individuals may have evolved mechanisms to reduce their metabolic rate and remain infective through periods of fasting. The knowledge gaps on basic biology and behaviour of these early life stages of *C. elongatus* are particularly abundant, but as more information about them is acquired an explanation for this inconsistency and a better epidemiological understanding of this species may be revealed.

4.3 | Methodological challenges

The outlier TDC values observed in samples with the highest densities of *L. salmonis* larvae present some challenges to be solved in the method's future development. The three subsamples excluded from the analysis belonged to a 30 copepodid sample replicate that seemed to have issues that may have resulted from incomplete homogenization or PCR saturation. The other extremely low TDC values for *L. salmonis* were observed in one additional subsample from a copepodid sample and one subsample from each of two different nauplii samples. Since these outliers were only found in the highest density spiking groups, PCR oversaturation was controlled against with a second run at 10× dilution. Those samples returned similar results as the original run and therefore there is no evidence of inhibition due to oversaturation of target DNA during the PCR amplification. This confirms the capability of the molecular component of the ddPCR methodology for quantifying louse DNA abundance within controlled samples of this density range, but we could not rule out the potential for incomplete homogenization of some samples being the cause of these extremely low TDC values. Niu et al. (2022) tested four matrix bead-lysing protocols on a variety of benthic eDNA samples and found that bead size and material can have a significant impact on the performance of homogenization and DNA extraction, especially in species with tough cell walls or in samples with fine sediments. The chitin exoskeleton of the louse may also have to capability to partially withstand the physical lysing protocols used in this experiment and perhaps other dense organic and inorganic materials found in wild plankton samples provide similar difficulties. Thus, there remains a need for further optimization of lysing and extraction protocols on plankton samples with a diverse array of physical and biological compositions to increase the precision of this method for its future applications. In the meantime, continued sample and subsample replication is essential to measure environmental stochasticity and to control for variance present after homogenization. Further, to control for inhibition and other factors which may affect amplification and the number of DNA copies detected per louse in future plankton samples, systematic calibrations by spiking and reanalysing samples with additional larvae and/or previously extracted pure louse DNA can be implemented.

Regarding cross-amplification between the two species, only a single subsample from one of the *C. elongatus* spiked samples displayed potential signs of this. The sample was falsely projected to have ~one fourth of an *L. salmonis* larvae based upon the predictive model described above. This could be interpreted as potential cross-amplification of *C. elongatus* DNA with the *L. salmonis* primers and Taqman probe, but in the opinion of the authors, this is more likely due to contamination of that sample during the harvesting, counting and sample preservation process, which took place within the same workspace as the *L. salmonis* counting and preservation.

When looking across all louse enumeration methods currently in use, quantification of larvae by ddPCR appears the most capable of assessing planktonic louse populations on a large scale. In a blind comparison of six such methods, Bui et al. (2021) found that ddPCR most comparably measures the abundance of *L. salmonis* individuals alongside the benchmark method, light microscopy. The continued improvements to automation of DNA purification and PCR protocols reduce chances for human-introduced error while simultaneously increasing the sample processing speed and reducing cost (Ransom et al., 2020; Wetterstrand, 2023), all of which further qualify the ddPCR method for expanded use in measuring planktonic louse abundance and other marine pathogen monitoring. Use of plankton sampling and ddPCR quantification in this manner would provide a much-needed verification step for salmon louse risk models, which are currently the only parameter being used to generate management advice to the traffic light system for Norwegian aquaculture production (Vollset et al., 2018).

In summary, gathering accurate information on the abundance and distribution of sea lice larvae over time and space is essential for determining infestation risk for migrating wild salmonids and farmed Atlantic salmon. Molecular methods such as ddPCR show considerable promise for detecting and quantifying *L. salmonis* larvae directly from bulk plankton samples, and our findings demonstrate that the application of this methodology produces accurate estimates for this species across a broad range of densities. Additionally, to gain a better understanding of *C. elongatus* interactions with wild and farmed salmon, approximating a larval abundance from plankton samples would be possible with the ddPCR method despite the stage-specific differences in DNA copy numbers. Plankton sampling and ddPCR quantification have the potential to provide verifications of modelled estimates of lice abundances in locations with high uncertainty or contested data quality and could even provide a replacement for ethically challenging practices such as salmon smolt cage use, which is currently used to measure infestation risk. With further field testing and sufficient correlation to the above-mentioned methods, ddPCR generated lice abundances from plankton samples could prove a valuable addition to the national salmon lice monitoring scheme and provide hard data about lice epidemiology to policymakers during decision making processes of Norway's 'traffic light system'.

AUTHOR CONTRIBUTIONS

Nathan E. Mertz: Investigation; writing – original draft; writing – review and editing; formal analysis; methodology; visualization; project administration. **Rachel A. Paterson:** Writing – review and editing; supervision; formal analysis. **Bengt Finstad:** Supervision; writing – review and editing; funding acquisition; conceptualization. **Hege Brandsegg:** Writing – review and editing; investigation; methodology. **Ida Pernille Øystese Andersskog:** Writing – review and editing; investigation; formal analysis; methodology. **Frode Fossøy:** Writing – review and editing; conceptualization; methodology; supervision; funding acquisition; project administration; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are stored in NINAGENs database and will be freely available upon request.

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