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Determining the age of individual *Lepeophtheirus salmonis* (Krøyer, 1837) copepodids by measuring stored lipid volume; proof of principle

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Summary

Confocal microscopy has facilitated measurement of stained lipid volume in *Lepeophtheirus salmonis* copepodid larvae. Quantity of lipid, location and morphology of vesicles may allow an estimate of age and viability

The salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837), is an ectoparasitic marine copepod principally parasitizing salmonids. In Scotland this parasite mainly affects farmed and wild Atlantic salmon (*Salmo salar* L., 1758) and sea trout (*Salmo trutta trutta* L., 1758). Infection of cultured salmon by sea lice constitutes a major fish health and welfare concern and imposes a considerable economic burden upon the salmonid culture industry (Costello, 2009). In addition, where management of infection fails, reservoirs of lice from farmed fish have the potential to increase infection pressures upon wild fish stocks. Gravid adult females of *L. salmonis* produce paired egg sacs, attached to the genital segment by a complex hook-eye mechanism (Schram, 2000), with each sac containing up to 865 uniseriate eggs separated by membranes (Heuch *et al.*, 2000; Charmantier & Charmantier-Daures, 2001; Yonge 1946). Embryos hatch directly into the water column as free-swimming nauplius I larvae which moult to a near-identical nauplius II stage. Both nauplius stages are non-feeding and wholly planktonic. The nauplius II moults to a host-seeking copepodid stage, which attaches to the fish host via hooked antennae. These three free-swimming larval stages are entirely lecithotrophic, relying for energy upon maternally derived lipid reserves stored in the cells of the developing gut and ultimately in large lipid vesicles within the gut epithelium

(Bron *et al.*, 1993). Feeding on host tissue starts immediately following attachment to the host and continues following the moult to a permanently attached chalimus stage secured by a cuticle-derived frontal filament. (Bron *et al.*, 1991).

Whilst many authors have attempted to construct infection models for salmon lice infecting farmed and wild fish, the fate of larval stages in open water remains somewhat of a black box due to difficulties capturing and aging larval stages, a problem shared in some respects by those studying free-living copepod species. The method described here attempts to construct a standard curve of depleting lipid reserves in individual, free-living copepodids against time. If this were achieved, and appropriate curves constructed taking into account, e.g. environmental variables such as temperature, then lipids in animals of unknown age could be compared against such a curve, allowing estimation of the age of planktonic copepodids and thus, with knowledge of local hydrodynamics and sea temperatures, identification of probable sources of infection. Access to such information would allow improved modelling of larval movements and infection parameters based on real-world data.

Gravid female salmon lice were collected from a wild fish netting station near Montrose, Angus, Scotland, U.K. They were transported to the Marine Scotland Marine Laboratory in Aberdeen, U.K. (formerly Fisheries Research Services) for culture. Lice with egg strings attached were maintained individually in aerated beakers containing sea water collected near the sampling site. The beakers were placed in an incubator (Model 303, LMS Ltd., Sevenoaks, U.K.), set to $12^{\circ}\text{C} \pm 2^{\circ}\text{C}$ to reflect initial capture conditions, and were inspected regularly. Hatching usually occurred overnight, and following hatching the culture was filtered through 500 μm and 150 μm woven stainless steel wire screens (Glenammer Engineering Ltd., Ayrshire, U.K.). The fraction containing hatched nauplius I larvae was retained at 150 μm and was

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collected. Sibling progeny from individual females were kept together but separate from those from other females, with subsequent larval culture carried at 12°C as described above. Surviving larvae developed and the majority had moulted through nauplius II to the copepodid stage by day 7. Collection of samples was made daily from day 7. Thirty living copepodids were collected from each beaker and stored individually in 2 mL cryogenic vials containing 20% formal saline (8% formaldehyde). For three cohorts sufficient numbers survived to allow sampling up to day 22 post-hatch and the cohort with the highest number of survivors was employed for the present study. The results reported here comprise a sample set of 54 copepodids arising from a single egg batch produced by one adult female, and represent scanned volumes from eight copepodids taken at each of nine time points.

In order to visualize storage lipids, individual animals were stained with the fluorescent non-polar lipid stain BODIPY (BODIPY® 505/515, D-3921 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, Molecular Probes, Invitrogen, CA, U.S.A.) and were examined using confocal laser scanning microscopy (Fig. 1). The following protocol was employed for staining, confocal laser scanning microscopy observation and lipid quantitation. BODIPY was prepared as per the manufacturer's instructions: 10 mg was dissolved in 5 mL dimethyl sulfoxide (Sigma-Aldrich Company Ltd, Poole, Dorset, U.K.) to give a 2 mg mL⁻¹ stock solution. This was aliquoted into 10 µL sub-samples which were stored at -20°C. For use, a single 10 µL aliquot was added to 1 mL water to give a working solution of 80 µm. Individually, animals were removed from storage vials and placed into a small dish, rinsed with distilled water, then drained. Excess liquid was removed with a pipette. Stain solution at working strength was added to dishes containing individual specimens. Each dish was covered and placed in the dark for 30 min to allow staining to occur. The specimen

Fig. 1. Photomicrograph showing vesicles within the wall of the midgut epithelium of a *L. salmonis* copepodid; paraffin processed, parasagittal section. C, external cuticle; L, lumen; M, muscle; Sg, sub-oesophageal ganglion and V, vessicle. Stain haematoxylin and eosin. Scale bar = 10 µm.

Fig. 2. Inverted greyscale confocal 3D reconstruction: The lipid is stained with BODIPY and shows as black bodies within the gut sides. Total length = 125 µm.

was then rinsed $\times 3$ with distilled water into a glass bottomed Petri dish (35 mm glass based dish, glass 12 mm diameter, catalogue #393 I-035, Iwaki, Japan). The Petri dish was transferred to the stage of an inverted confocal laser scanning microscope (TCS SP2 AOBS, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). The specimen was orientated to lie on its dorsal surface (i.e. dorsum nearest objective lens) and the image aligned to the longitudinal axis of the copepodid by scan-rotation. An image stack (xyz) for 3D reconstruction was then captured, with a standard area being selected for scanning (Fig. 2). To ensure consistency between scans, a landmark was employed to define the anterior edge of the scanning region, comprising the margin of the dorsal boundary of the first proximally visible abdominal segment. The area of interest was scanned to a standard depth of 50 µm from the dorsal surface of each animal, with the scan region being a 512 $\times$ 512 pixel square centred along the medial line of the abdomen. A zoom setting of 6 $\times$ on a 20 $\times$ glycerol immersion objective provided a scan window of x and $y = 0.244$ µm; $z = 0.505$ µm for each optical section. This translates into a study stack volume of sides 125 µm and 50 µm deep. Image stacks were loaded into Imagepro version 6 with 3D Constructor (MediaCybernetics, Bethesda, Maryland, U.S.A.) for 3D reconstruction and volume measurement.

The mean values for volume of lipid present in the scanned region of the copepodid gut show an increasing trend from day 7 post-hatch to day 10 post-hatch (Table 1). From day 12 to day 20 a downward trend in the means was observed. Low levels of lipid were consistently achieved after day 16. Results from this current study can only divide copepodids into three groups, namely (1) early copepodids, which often show the large undifferentiated lipid mass found in the gut lumen, as seen in nauplius stages; (2) mid-life copepodids which show lipid stored within vesicles in the midgut epithelium, which may be the most active animals and those with mature infective capabilities and (3) late copepodids with low reserves of lipid, which may be less capable of infection.

Table 1. Table of the mean volumes of lipid stained in the selected abdominal region of *Lepeophtheirus salmonis* copepodid larvae in relation to days post-hatch (1 pL = 10^{-12} L).

Days post-hatch	Mean lipid volume (pL)	Standard error $n = 8$
7	133.4	34.1
9	287.6	44.7
10	605.2	138.0
12	298.3	59.8
14	328.7	64.5
15	172.6	86.0
16	40.0	18.8
18	14.2	11.6
20	7.3	6.7

Discussion

The techniques employed in this study have successfully demonstrated changes in lipid levels and distribution in copepodid larvae of *L. salmonis*. There has been no previous consensus on the relationship between physiological and chronological age for this species; however, previous authors have noted both a maturation of infective capability with time following the moult from nauplius I to copepodid and an overall decline in energy reserves expressed in terms of C:H:N ratios (Tucker *et al.*, 2000). The chronological changes of lipid levels and distribution have not been previously studied in caligid copepods and this is therefore the first study to present observations of variation of these parameters with time. This said, a number of experimental parameters will have affected the observed results, including the potential effects of a physically constraining laboratory culture system on lipid consumption, which is likely to limit 'normal' swimming behaviour in planktonic stages. The water temperatures, reflecting those observed in the field at the time of capture, will also have affected the rate and possibly the pattern of lipid usage. Observations in this study suggest that lipid storage in nauplius larvae, derived from maternal reserves, occurs in the area that will become the lumen of the gut in the copepodid. In the current study, the earliest copepodid stages observed show low lipid levels in the scanned volume, the majority of reserves being held in the cephalothoracic anterior midgut lumen, this moving to vesicles within the midgut epithelium and then becoming exhausted over the period of the study. The exact form of naupliar lipid storage remains to be determined; however, this study demonstrates that, during the early copepodid stage following the second naupliar moult, stored lipid in the area that will become the gut lumen moves to lipid vesicles in the gut epithelium. This occurs either through lipid transport across the apical surface of midgut epithelial cells or alternatively through differentiation of naupliar lipid storage

Fig. 3. One plane from a low resolution frontal scan of a *L. salmonis* copepodid: The lipid is stained with BODIPY and is showing as green bodies within the gut. The bright channel has been overlain. Scale bar = 200 μ m.

cells occupying the future copepodid midgut lumen to become part of the gut epithelial cell population (Fig. 3). Storage of lipid in vesicles within the gut epithelial cells has been previously reported for this species by Bron *et al.* (1993). Lipid levels and distribution do not solely affect availability of energy for the copepodid but, as for many aquatic animals and plants, affect buoyancy characteristics, which in turn affect energy costs for host location and therefore the temporal window during which the copepodid is maximally infective. Despite the variability observed in the current study for progeny of a single adult female, given wider observations on a larger number of specimens, the reported technique may allow the age of an animal to be estimated with sufficient resolution to provide useful data. For a body of water for which the temperature, tidal excursion, currents and wind effects are known, it may be possible to estimate from such data, for larvae captured at a particular point, their probable origin. For *L. salmonis* and other parasitic species, such data has particular utility in modelling infection parameters and may assist management of these pathogens in both wild and farmed host contexts.

This study has provided proof of principle for a novel technique for measuring lipid storage reserves in individuals and hence potentially chronological changes in lipid levels and distribution in small aquatic organisms. The method is simple and could be transferred to other species and to the measurement of other metabolites and structures.

Advantages of this technique include the ability to measure individual organisms, the employment of cheap, readily available reagents and the fact that the technique may be used for fresh or formalin-fixed specimens. The major limitations of the technique include the time taken to perform a series of scans, the depth of scan penetration and the cost and availability of the technology and trained operators to run it. As the technology matures, confocal scan speeds continue to increase, and the use of dual-photon confocal microscopy, whilst expensive, allows much better sample penetration depth. In any case, sample penetration depth ceases to be problematical with smaller organisms as the whole specimen may be imaged. As confocal and allied technologies become cheaper and more widely available, a wider range of applications can be expected. In addition, applications of new deep focus and deconvolution algorithms to standard light and fluorescent microscopy may open these techniques to a wider user base.

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