Ultrastructure Study of the Stored Lipid Reserves in *Gyrodactylus gasterostei* (Monogenea) Using Confocal and Transmission Electron Microscopy

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Abstract

This study examines the distribution and depletion of stored lipids in *Gyrodactylus gasterostei* Gläser, 1974, migrating off its three-spine stickleback host *Gasterosteus aculeatus* L., with the prospect that it might prove informative for interpreting the biology of other gyrodactylids species more generally. Nonfeeding life cycle stages, such as the dispersal stages of parasites, are dependent for survival upon finite energy reserves gathered during feeding phases. Thus, those individuals with more limited reserves will die sooner and consequently have less time available to find a new host once detached. At this stage, the principal energy reserves in gyrodactylids are stored as large lipid droplets. Confocal laser scanning microscopy has been used to investigate the distribution of lipid droplets in *Gyrodactylus*, which have migrated off their fish host, testing the hypothesis that these droplets function as a proxy for the nutritional state. This study demonstrated that the lipid droplets were particularly associated with the gut and that there is a significant variability in the volume of stored lipid carried out by each individual. Transmission electron microscopy showed that gyrodactylids carry lipid droplets at all stages of their life cycle, including at release from the birth pore. It is likely that transferring worms requires stored energy reserves to survive in the event of failure to establish contact with a new host. These reserves could allow the parasite to survive without a host for several days.

Keywords: Ectoparasites, *Gasterosteus aculeatus*, microanalysis, microscopy, Monogenean

Introduction

Gyrodactylids are monogenean flukes with a direct life cycle, viviparous, and are capable of rapid multiplication. *Gyrodactylus* sp. is a freshwater monogenean, which is one of the most intensively studied ectoparasites of recent years, i.e. *Gyrodactylus salaris* on the Baltic strains of Atlantic salmon *Salmo salar* L., on which it generally causes no clinical disease. Infection of other strains of Atlantic salmon in Norway has resulted in high levels of juvenile salmon mortality and highly significant reductions in the population, causing significant epidemic disease in Norwegian salmon.[1-3]

Gyrodactylids lack a free swimming larval stage or oncomiracidium which is present in egg-laying monogeneans, but instead give birth to full-sized living individuals.

*Gyrodactylus* attach to fish by means of a terminal specialized attachment organ, the opisthaptor, which is equipped with two sharp, centrally positioned hooks called hamuli and an array of 16 peripherally distributed hooks. The parasite can also temporarily attach to its host by fixing its anterior extremity, the prohaptor, which consists primarily of two cephalic lobes, which produce a sticky secretion and the pharynx. When *Gyrodactylus* feeds, it inverts its pharynx through its mouth and releases a digestive solution containing proteolytic enzymes, i.e. proteases and lysozyme,[4] which act to break down the fish skin. Mucus and dissolved skin are then sucked into the gut. This feeding activity can result in small lesions in the fish skin,[5,6] and high numbers of lesions may cause

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death through disruption of normal osmoregulatory function. An experimental model with *Gyrodactylus gasterostei* and the host fish *Gasterosteus aculeatus* was used for the purposes of this study. This is a simple and successful system to examine the aspects of transmission of parasites from live to dead fish, since Harris[7] and recently Grano-Maldonado[8-12] employed this model on the biological and behavioral bases of host selection, gyrodactylid transfer during multiple fish transportation, influence of maturity in transmission routes; this information about parasite migration from the dead host has upgraded knowledge on the management of parasitic diseases.

The purpose of this research is to build up the current knowledge concerning gyrodactylids “transmission triggers,” although the key contribution of this article is to visualize their lipid dynamic, which can be achieved using photographic evidence with confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). Attempt to illustrate the role of lipid contain as a possible nutritional trigger for transmission factor in gyrodactylids.

**MATERIALS AND METHODS**

**Source of hosts and parasites**

A *Gyrodactylus gasterostei* /stickleback *Gasterosteus aculeatus* L. model was used for the purposes of this study. Twenty fish (3.57 cm ± 0.63) used for the study were collected from a settlement pond, feeding a commercial fish farm, situated on a branch of the River Allan near Stirling, Stirlingshire, Scotland (56° 06’ 37.77” N, 3° 58’ 25.25” W). Fish were transferred to an aquarium facility at the Institute of Aquaculture, University of Stirling, where they were held in 25 L black plastic tanks containing 10°C ± 1°C, aerated “home” stream water. Fish were fed *ad libitum* on a diet of frozen bloodworms (Gamma, Chorleywood, UK). The fish were allowed to settle for a minimum of 48 h following capture before experimentation. Feeding was stopped on the day prior to the start of all experiments to maintain water quality and reduce fish stress.

**Lipid content in migrating ectoparasites - *Gyrodactylus gasterostei***

For this experiment, we hypothesized that host transfer might be more favored in those parasites having higher energy/lipid reserves than nontransferring individuals. This experiment was designed to examine the lipid characteristics of *Gyrodactylus gasterostei* (*n* = 50) moving off dead hosts at 10°C. This experiment follows a previous experiment’s methods to evaluate the influence of the maturity of monogenean on host transfer using a parasite–host model.[8-12] Twenty individual sticklebacks were terminally euthanized with 0.01 M 2-phenoxyethanol (MERCK, Darmstadt, Germany) and placed in individual Petri dishes containing clean water at 10°C. Dead hosts were observed under an Olympus SZ30 stereomicroscope (Tokio, Japan) at different magnifications, with the time at which each gyrodactylid looped off the fish during 60 min being recorded. Worms detaching naturally from the host tissue within 60 min were then used for analysis.

Worms were carefully removed with a 200-µl pipette and were placed individually into 3-cm Petri dishes containing 5 ml of filtered (0.45 µm Minisart® Sartorius Stedim, Biotech, Leicestershire, UK) water taken from the same source as that used for fish maintenance and incubated at 10°C for 24, 48, and 72 h. Parasites used in this study sat stationary on the substrate of the Petri dish making occasional circular exploratory movements. No moribund or dead parasites were considered during the experiments.

Following incubation for the appropriate period of time, worms were fixed in 10% neutral-buffered formalin (NBF) at least 48 h prior to staining. The maturity status of worms was recorded using a compound microscope (Olympus BX51) under a × 100/100 immersion objective. The fish carcasses containing nonmigrated parasites were fixed in 10% NBF and employed as a control, and assessment of the maturity status of nonmigrated individuals was made to establish the overall population structure. Several features of the recovered parasites were assessed as follows: (i) an identification of species using the hard parts of the opisthaptor; (ii) four developmental states were recognized to describe the stage of maturation of each parasite; (1) no daughter and no male copulatory organ (MCO); (2) no MCO and a daughter present in *utero*; (3) MCO present but no daughter; and (4) both MCO present and a daughter present. A daughter/embryo was considered to be present if the rudimentary hard parts of the opisthaptor or attachment hooks were evident. The MCO was considered to be present only if the spines were clearly visible.[8,11] For taxonomical identification, each gyrodactylid was carefully mounted on triangular surgical needles (size 16, Barber of Sheffield, UK). Each specimen was then mounted on a glass slide in a drop of distilled water ensuring that the haptoral hooks were flat. The specimens were then stained and fixed *in situ* by the addition of a drop (~3 µl) of Malmberg’s fixative (ammonium picrate glycerin, saturated picric acid, and 100% glycerin) to the edge of the coverslip which was drawn under the coverslip by capillary action. The coverslip was then sealed with transparent nail vanish. The maturity and reproductive status of worms were recorded using a compound microscope (Olympus BX51) at × 100/100 immersion magnification. In addition, parasites were identified to species through morphological and morphometric analyses of the opisthaptorial hard parts. The prevalence was calculated by dividing the number of infected hosts with a particular parasite species by the total number of hosts of one species examined, expressed as a percentage. The average abundance was calculated by dividing the total number of individuals of a particular species of parasite by the total number of hosts of one species examined (both infected and uninfected) of gyrodactylids according to the procedures of Margolis *et al.*[13]

**Microscopy and analysis**

Following fixation in 10% NBF, the worms were stained with a lipid-specific stain green-fluorescent BODIPY™/FL® 505/513 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a, 4a-diaza-s-indacene; Molecular Probes, Eugene, OR, USA).
Worms were incubated in the dark for 1 h in 0.1% v/v solution of BODIPY™ in filtered water. Thereafter, worms were rinsed three times with filtered water and were then transferred to slides and mounted under a coverslip with a drop of water and sealed with transparent nail varnish. Worms were viewed using a Leica SP2 AOBS CLSM (Leica Microsystems AG, Wetzlar, Germany) coupled to a Leica DM IRE2 inverted microscope employing a ×20 glycerol-immersion lens to observe the lipid drops stained. Unstained worms were used as a negative control to assess autofluorescence. Confocal protocol setup is described as follows: SECTIONS 50, M 0.201, GAIN 504.1, OFFSET 0.4, GREEN 494-601 nm, ZOOM 1.5, IMAGE 1024 × 1024.

**Statistical analysis**

Normality and homogeneity tests were employed. A Pearson’s Chi-square test was used to detect the difference between the presence of MCO in the migrating and nonmigrating parasites.

**Image analysis software**

The image analysis was performed on serial images using Fiji-Win 32 (ver. 2011 Microsoft, Redmond, WA, USA) software which permits images taken with CLSM to be processed and analyzed as shown in Table 1.

**Transmission electron microscopy**

Individual worms were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and then postfixed in 1% osmium tetroxide. Specimens were then en bloc stained in 2% uranyl acetate and 30% acetone before dehydrating them through a 60–100% acetone series. Specimens were then embedded in Spurr resin and cut at 50–70 nm. TEM sections were first stained with 4% uranyl acetate in 50% ethanol and then Reynolds lead citrate. Sections were viewed on a Tecnai G2 Spirit BioTwin TEM.

**Results**

From twenty replicate trials, a total of 213 (46.9%) worms looped off the dead host; 88.7% (186) of these within the first 60 min following the death of the host. Of these migrating worms, 39.6% gave birth, accounting for 95.2% of all births observed during the experiments. Within each of these categories, the maturity and reproductive stages of gyroactylids were assessed to determine if these may be cues prompting their dispersal and their seeking out of a new host. The majority of parasites transferring off dead hosts were mature, i.e. 151 (70.9%) had an MCO present and 137 (64.6%) had a daughter in utero, while 28.3% of parasites which remained on the host were immature (no MCO) [Table 2] and the prevalence was 100% and the average abundance was 22.54 ± 5.56.

Lipid droplets in unstained (control) worms did not fluoresce [Figure 1], but once stained, a number of nutritional states (i.e. starved and fed) could be recognized [Figure 2], with significant differences (Kruskal–Wallis test = 9.8287, df = 3, P = 0.02008) in size, distribution, and number of lipid droplets evident [Figure 3]. The levels of lipid in worms that had abandoned dead hosts were followed over a period of time. The worms that were starved for 48 h contained more stored lipid than the control group [Figure 3], thus it may be the case that 87.5% (i.e. 14 worms) hold a daughter in uter us, this fact might increase the amount of lipids during

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**Table 1: Image analysis performed on serial images using Fiji-Win 32 (version 2011) software**

| Step | Operation performed in the main window | Function | Parameters used in this study | Observation |
|------|----------------------------------------|----------|-----------------------------|-------------|
| 1    | Image sequence                         | This command opens the image sequence |                             |             |
| 2    | Image properties                       | Calibrate image measurements           |                             |             |
| 3    | Analyze                                | Threshold                                   | Intensity threshold: 24     | Threshold lipid versus nonlipid |
|      | 3D objects counter                     | 3D select measurements                     | Volume                      |             |
|      | “3D OC”                                |                                       | Number of object voxel      |             |
|      |                                         |                                       | Integrated density          |             |
|      |                                         |                                       | SD gray value               |             |
|      |                                         |                                       | Minimum gray value          |             |
|      |                                         |                                       | Median gray value           |             |
|      |                                         |                                       | Maximum gray value          |             |

“3D viewer”: Reconstruct 3D image

SD: Standard deviation, 3D: Three dimensional

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**Figure 1:** Worms were picked off a host, stained with a lipid-specific stain, and viewed using a confocal laser scanning microscope. (a) Gyrodactyly in transmitted light and (b) negative control (no stain; lipids are not autofluorescent). Scale bar = 0.16 mm
the image analysis. Observations during the trial indicate that embryos have a maternally derived lipid store, but that the majority of newborn daughters (66%) die within 24 h if not fed. Serial confocal images taken through a worm starved for 48 h confirming that lipid staining is of material within cells positioned in the intestinal wall of the worm (arrowed) rather than of lipid in food items within the gut. This worm presents an empty uterus (star). Scale bar = 50 µm.

The data did not support the assumptions of ANOVA; therefore, a Kruskal–Wallis (nonparametric) test was applied to the data suggesting significant differences (Kruskal–Wallis test = 9.8287, df = 3, P = 0.02008) between the content lipid droplets in parasites starved at different times [Figure 3].

**Transmission electron microscopy**

To confirm the presence of lipid droplets, individual worms were fixed and studied using TEM. The luminal surface of the gut was observed to be highly microvillar, maximizing the surface area for absorptive functions [Figure 6]. Although small amounts of lipid were observed in the gut lumen [Figure 6], these were not substantial in the studied specimens. Clear evidence for the presence of lipid storage vesicles in the epithelium of the gut, however, was provided by these studies, confirming storage of lipid in the areas previously suggested by the CLSM study [Figures 2 and 4].
**Table 2: Maturity and reproductive status of worms migrating off dead hosts**

| Parasite reproductive status | A Nonmigrating parasites (n=240), n (%) | B Migrating parasites (n=213), n (%) | P (A vs. B) |
|-----------------------------|----------------------------------------|------------------------------------|------------|
| N MCO/ND                    | 36 (15.0)                              | 9 (4.2)                            | <0.001*** |
| N MCO/D                     | 136 (56.7)                             | 53 (24.9)                          | <0.001*** |
| MCO/ND                      | 25 (10.4)                              | 67 (31.5)                          | <0.001*** |
| MCO/D                       | 43 (17.9)                              | 84 (39.4)                          | <0.001*** |
| Total number without MCO    | 172 (71.7)                             | 62 (29.1)                          | <0.001*** |
| Total number with MCO       | 68 (28.3)                              | 151 (70.9)                         | <0.001*** |

***Significant P value (P<0.001). N: No presence, D: Daughter, MCO: Male copulatory organ

**DISCUSSION**

This study describes aspects of the nutritional status of *Gyrodactylus gasterostei* to its fish host, including the role of stored lipid status of migrating worms. The decision to leave a dead fish may reflect a given worm’s status in three ways. First, the worm’s reproductive and developmental status may inform the decision to abandon the host[7,11,12] and risky additional routes of transmission[9,11,12]. Second, recent ultrastructural studies have shown a detailed examination of the sensory structures that *Gyrodactylus* species employ to interpret both their host and ambient environments.[10] Third, there are no previous studies on the nutritional status of the worm, and this may either prompt or allow migration from the host. It is hypothesized here that the existence of a full gut or high stored reserves might favor decisions to leave the host.[14] Cooper et al. noted that detached starved parasites can abort their offspring (embryos) and that an interruption in nutrient flow to the embryo might have a significant impact on reproductive rate.[15] These authors elaborated on an initial study of cellular lipid content in a flagellated microalga *Chrysochromulina* sp. using fluorescent dye and confocal microscopy. Another study by Cook et al.[16] developed a technique allowing measurement of lipid reserves in copepods *Lepeophtheirus salmonis* (Kroyer, 1837) and by extension of chronological changes in lipid levels in small aquatic organisms. In this study, CLSM has been employed to quantify the number, size, and distribution of lipid droplets in each worm and their depletion with time. TEM was employed to localize the intestinal wall, showing the presence of lipid droplets’ storage vesicles in the underlying gut epithelium. Information about parasite migration from the dead host could be important and upgrade knowledge on the management of parasitic diseases. During this study, the results of the parasite migration from dead host suggested that worms with a developed MCO were more likely to leave the host.[11,12] The population remaining on the host was largely immature. This suggests that gyrodactylids that have given birth at least once are more likely to leave the host following host death in order to colonize new hosts, as an MCO appears after gyrodactylids have given birth for the first time (at an age of 24–30 h at 13°C for *G. gasterostei* according to a study by Harris, Grano-Maldonado, and Grano-Maldonado et al.[7,11,12]. The authors showed a significantly higher probability of parasites transferring in the groups with an MCO (penis) than those lacking an MCO, being a key factor during oral route transmission from dead to live fish in scavenging activities. The decision to leave a fish may also reflect the nutritional status of the worm, these perhaps requiring a filled gut or high stored reserves before leaving the host. Nonfeeding lifecycle stages, such as dispersal stages of parasites, are dependent for survival upon finite energy reserves gathered during previous feeding phases. Thus, those individuals with more limited reserves will die sooner and consequently have less time available to find a new host once detached. In many such stages, lipids represent the principal form of stored energy reserves, these often being stored as large droplets. Lipid studies in parasites are scarce; however, CLSM in gyrodactylids has been successfully used previously by El-Naggar et al.[17] to reveal the neuromusculature of *Macrogyrodactylus clarii*, a gill parasite of the Nile catfish *Clarias gariepinus*. In the present study, this microscopical tool was used to investigate and characterize the distribution of lipid droplets in *G. gasterostei* which have migrated off their fish host, using a working hypothesis that these droplets function as a proxy for nutritional state. There are few studies regarding the lipid content and their local body distribution in free-living aquatic organisms such as copepods[16] and marine microalgae.[15] The current research which focuses on gyrodactylids provides information on the localization of lipid droplets and how the number and volume of these change over increasing periods of starvation.

The work presented here has demonstrated that the majority of droplets were located within vesicles in the gut wall and that individuals were variable in the amount of stored lipid

**Figure 6:** Transmission electron microscopy micrographs of the intestine and surrounding tissue of *Gyrodactylus gasterostei*. (a) Intestinal wall showing the presence of absorptive microvilli (arrowhead), scale bar = 2 µm; (b) high magnification of the microvillous border; scale bar = 1 µm; (c), presence of lipid droplets (arrow) in the intestinal lumen, scale bar = 5 µm; (d), lipid storage vesicles in the underlying gut epithelium (arrow), scale bar = 2 µm
that they carry. It is likely that transferring worms requires a buffer of stored reserves to protect them against failure, this allowing survival off a host for several days. Clear observations in this study suggest that part of the stored lipid is derived from maternal reserves; this is also reported to occur in copepodids.\cite{16} In the current study, it is suggested that the lipid reserves passed from the mother to the embryo increase with the increasing developmental state of the daughter in uterus. However, the lipid reserves of individuals are exclusive and might differ between the organisms of the same species.\cite{16}

The limitation of this study was that the mortality rate of the parasites was not recorded until all parasites abandoned the dead fish and correlated to the volume of lipid droplets. However, it was clear that the presence of elevated lipid condition and MCO of the parasite was “favorable” for the transmission off the host.

For the gyrodactylids infecting 3-spine-sticklebacks, the importance of parasites remaining on the host and attaining an optimum nutritional status is crucial. While lipid consumption may be related with temperature and survival, detached Gyrodactylus alexanderi\cite{18} kept at 10°C had a mean survival of ~1.8 days. In a second example, Cable et al. showed that the survival of detached G. gasterostei depended on temperature and found that they could survive for a maximum of 101 h at 4°C but only 67 h at 15°C.\cite{14} As mortality was continuous during the first 60 h off the host at 15°C, these authors suggested that worms could survive until the extinction of energy reserves. However, in the present study, the worms kept at 10°C progressively decreased their lipid content over the 72 h experimental period, and the use of anesthetic (0.01 M 2-phenoxyethanol) did not affect the transmission of Gyrodactylus.\cite{12,19} Looking at the maximum survival of G. salaris remaining on a dead salmon host, the authors concluded that at 18°C, worms moving off the dead host survived for up to 27 h, while those remaining on the dead host benefited and survived for up to 72 h. During this study, no other gyrodactylid species (i.e. G. arcuatus) were found on the surface of the sticklebacks by confirming the absence of excretoary bladders and the shape of the ventral bar.\cite{7}

This technique using image analysis of laser scanning confocal microscope images (three dimensional [3D]) has been used to assess the distribution of lipid droplets in other aquatic organisms.\cite{15,16} The techniques used here for lipid measurement and evaluation in G. gasterostei can also be applied to other parasitic organisms, having the advantage of rapid preparation and observation of specimens and the production of lipid distribution map. The CLSM is used to detect and image structures stained using target-specific dyes. The approximate size and distribution of these structures, for example, lipid droplets, can then be determined from composite images reconstructed through multiple scans through the specimen. The use of the Fiji-image analysis software then permits the size and volume of each droplet to be calculated and the data exported in a format that permit subsequent statistical analysis. This study utilizes a novel approach to visualizing lipid content employed in the G. gasterostei/G. aculeatus infection model. The high variability in the lipid reserves between individuals means that a larger number of gyrodactylids in each nutritional state need to be examined to determine the distribution and use of lipids by each individual. Nevertheless, this study does suggest that the presence of high lipid reserves may encourage or facilitate the early migration/transmission of gyrodactylids.

The present study describes the lipid measurement in Gyrodactylus gasterostei where 3D reconstruction of lipid vesicles from confocal image stacks showed lipid vesicles distributed around the intestinal intestinal caeca. Serial images taken through the worm confirm that lipid staining is localized within cells positioned in the intestinal wall of the worm rather than localizing to lipid in food items within the gut. The number and volume of all lipid vesicles in each specimen were determined from stacked serial images using the Fiji-Win 32 image analysis program. This technique has the advantage of allowing rapid preparation and observation of specimens.

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Conflicts of interest

There are no conflicts of interest.

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