SPECIES DISCOVERY, EVOLUTION AND KLEPTOPLASTY IN MARINE MEIOFAUNAL FLATWORMS

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Abstract

Rhabdocoel flatworms are abundant members of marine meiofaunal communities worldwide, contributing to a reservoir of biodiversity that thrives between grains of sand. However, they are relatively understudied due to bias in meiofaunal sampling techniques and a lack of taxonomic expertise. Here, five species of neodalyellid rhabdocoels were discovered from intertidal habitats in British Columbia and characterised with molecular and morphological data: *Baicalellia solaris* n. sp., *Baicalellia daftpunka* n. sp., *Tamanawas kalipis* n. sp., *Pogaina paranygulgus* and *Baicalellia pusillus*. A molecular phylogenetic analysis using maximum likelihood and Bayesian inference on concatenated 18S and 28S rDNA sequences provided a framework for revising neodalyellid systematics and for inferring character evolution within the group. Kleptoplasty, the phenomenon by which one organism steals plastids from another, was discovered in the “solar panel worms” *B. solaris* and *P. paranygulgus*, representing just the second case in metazoans; kleptoplasty has only been described previously in sacoglossan sea slugs. Using a combination of light and electron microscopy, I demonstrated that plastids were intracellularly sequestered in the parenchymal tissue. DNA barcoding of partial *rbcL* sequences demonstrated that the plastids were stolen from raphid pennate diatoms, which was consistent with plastid ultrastucture. Measurements of oxygen consumption demonstrated that kleptoplasts remain functional for at least ten days after sequestration in *B. solaris* cells. Photosynthetic activity was of a similar magnitude to a dense chlorophyte culture, indicating that photosynthetically-fixed carbon enhanced survival in light-treated compared with dark-treated flatworms. Kleptoplasts ultimately lose function and are digested; therefore, heterotrophy is required to replenish healthy populations of kleptoplasts within the host tissue. The kleptoplasts might serve as a food store, providing sustenance when seasonal diatom blooms collapse. It cannot be determined whether kleptoplasty arose once in the common ancestor of *Pogaina* and *Baicalellia* or has evolved twice convergently.
**Lay Summary**

Rhabdocoel flatworms are abundant members of marine meiofaunal communities (animals less than 1mm in length) worldwide, contributing to a reservoir of biodiversity that thrives between grains of sand. However, they are understudied due to bias in sampling techniques. Here, five species of neodalyellid rhabdocoels were discovered from British Columbia and characterised with molecular and morphological data: *Baicalellia solaris* n. sp., *Baicalellia daftpunka* n. sp., *Tamanawas kalipis* n. sp., *Pogaina paranygulgus* and *Baicalellia pusillus*. A phylogenetic analysis allowed the revision of neodalyellid evolutionary relationships and inference of the evolution of traits within this group. Kleptoplasty, the phenomenon by which one organism steals plastids (photosynthetic machinery of the cell) from another, was discovered in the “solar panel worms” *B. solaris* and *P. paranygulgus*, representing just the second case in animals. Plastids were stolen from pennate diatoms, continued to photosynthesise and enhanced worm survival, but ultimately lost function and were digested.
Preface

All work presented in the current thesis is original work. The project was planned and designed with the help of Dr. Brian Leander and Dr. Niels Van Steenkiste. Field and laboratory work, including the collection of organisms, light microscopy, DNA extractions and sequencing and phylogenetic analysis were undertaken in collaboration with Niels. Laser scanning confocal micrographs were taken with the help of Dr. María Herranz. Oxygen evolution experiments and data analysis were carried out with the help of Joanna Bernhardt. Two publications, on which Niels and Brian are co-authors, are currently being prepared for submission to journals. The NSERC funding for the project and final interpretation of the thesis are owed to Brian.
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List of Abbreviations

BI – Bayesian inference
bp – base pair/s
DNA – deoxyribonucleic acid
Fig – figure
Figs – figures
hr – hour/s
LED – light emitting diode
LM – light microscopy
MgCl₂ – magnesium chloride
mg – milligrams
min – minute/s
mm – millimetres
nm – nanometers
n. comb. – new combination
n. g. – new genus
n. sp. – new species
p – p-distance
pp – posterior probability
rDNA – ribosomal DNA
PCR – polymerase chain reaction
RuBisCO – ribulose-1,5-bisphosphate carboxylase/oxygenase
sec – second/s
sp. – species
spp. – species (plural)
syn. – synonym
TEM – transmission electron microscopy
µL – microlitres
µm – micrometres
µM – micromoles
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Chapter 1: Introduction

1.1 Neodalyellid diversity and evolution

1.1.1 Meiofaunal flatworms

The Platyhelminthes consists of soft-bodied, acoelomate, unsegmented animals known as flatworms. While many parasitic flatworms (trematodes, cestodes and monogeneans) are well-studied due to their implications for human health, agri-/aquaculture and wildlife conservation, other, mostly free-living flatworms (turbellarians) have received less attention.

Among the Platyhelminthes, the Rhabdocoela is a species-rich order of turbellarians characterised by their small size (typically 0.5-1.5 mm long), presence of a bulbous pharynx and a simple, sac-like gut (Ehrenberg 1831). Rhabdocoels inhabit marine, limnic and limnoterrestrial environments, as well as having some symbiotic and parasitic forms (Jennings 1997; Van Steenkiste et al. 2013). Their small size classifies them as meiofaunal animals, generally defined as metazoans that can pass through a 0.5-mm mesh but is retained by 45-µm mesh (Valiela 2013), though the upper limit is often extended to 1 mm or larger (e.g. Schratzberger & Ingels 2017). Marine meiofaunal communities often live either interstitially between grains of sand, or epiphytically on macroalgae (Cannon 2004). They constitute a reservoir of biodiversity that is barely visible to the naked eye, but plays essential roles in ecosystem function, including nutrient cycling, secondary production and sediment transport (Giere 2008).

Meiofaunal animals are considered to be useful bioindicators of marine ecosystem health; their small size and direct development (lacking larval stages) results in a sessile lifestyle and the inability to escape habitats affected by human activities (Kennedy & Jacoby 1999). However, studying impacts on meiofaunal biodiversity requires delineation of present biodiversity in order to establish a baseline.

Meiofaunal communities are traditionally considered to be dominated by the Nematoda, roundworms which reportedly account for 80% of all individual animals on earth (Lorenzen
1994). However, flatworms are also extremely abundant in these communities; they have been ranked the second-richest lineage of meiofauna according to environmental sequence data (Fonseca et al. 2010), and their biomass in sandy habitats has been found to either equal or exceed that of nematodes (Martens & Schockaert 1986). Their low richness and abundance rankings in some studies likely reflects the fact that delicate flatworms are often damaged by sampling techniques, and must be alive or well-preserved in order to be identified. In addition to this, lack of taxonomic expertise has contributed to sampling bias against flatworms (Martens & Schockaert 1986); species identification is often laborious and based on complex reproductive structures.

1.1.2 Rhabdocoel morphology and systematics

While morphology of the stylet, a sclerotised structure of the male copulatory organ, is a robust character for species identification, diagnosis of taxa above species level has proved more difficult. The Rhabdocoela was formerly subdivided into the Dalyellioida, Typhloplanoida and Kalyptorhynchia, groups based on pharynx morphology and presence of a proboscis. However, molecular phylogenetic data found the former two taxa to be polyphyletic, and they were merged to form the Dalytyphloplanida, consisting of freshwater Limnotyphloplanida, and marine Thalassotyphloplanida and Neodalyellida (Willems et al. 2006; Van Steenkiste et al. 2013).

The Neodalyellida constitutes all marine species previously grouped within the polyphyletic Dalyellioida (rhabdocoels with no proboscis and a barrel-shaped “doliiform” pharynx), and two marine species that previously belonged to the Typhloplanoida (no proboscis and a rosulate pharynx). Most are free-living, though the Pterasticolidae (parasites of sea stars) and Umagillidae (parasites of sea cucumbers and sipunculids) also fall within this clade.

Though around 1,700 described species of rhabdocoels are known, only about 70 species are described from the Northeast Pacific Ocean (Van Steenkiste & Leander 2017). I sampled intertidal habitats in British Columbia, Canada in order to advance our understanding of neodalyellid diversity in this region. *Pogaina paranygulga*, *Baicalellia pusillus* n. comb.,
Baicalellia daftpunka n. sp., Baicalellia solaris n. sp. and Tamanawas kalipis n. sp. were discovered and described from these samples.

Van Steenkiste et al. (2013) included 30 lineages of neodalyellids in a phylogenetic analysis of the Dalytyphloplanida based on concatenated 18S and 28S rDNA sequences. With the inclusion of ribosomal sequences for nine new taxa, including the five species described here and four undescribed species, further insight into neodalyellid evolutionary relationships and character evolution is provided.

1.2 Kleptoplasty in rhabdocoels

1.2.1 Symbiosis

“Symbiosis” was coined as a biological term by mycologist Heinrich Anton de Bary (1879) while studying the association between fungi and photosynthetic microorganisms, which constitutes the chimeric entities known as lichens. The term was designated as “the living together of two unlike organisms”, a broad definition which encompasses an enormous variety of relationships between organisms across the tree of life.

Endosymbiosis is an intimate symbiotic relationship, in which one organism (the endosymbiont) lives physically inside another (the host) (Margulis 1981). The endosymbiont may be integrated into host cells (intracellularly) or reside outside of cells (extracellularly). In both scenarios, the endosymbiont benefits from shelter and/or nutrients derived from the host. If the relationship is mutualistic (rather than parasitic or commensal), the endosymbiont supplies nutrients in return, enhancing host fitness and often allowing it to occupy new environmental niches (Margulis & Chapman 2009).

Interest in endosymbiosis has heightened in recent years as novel microscopy and gene sequencing technologies have allowed us to investigate more associations involving single-celled organisms. In particular, our knowledge of the human microbiota has benefited from the Human
Microbiome Project, an initiative which sequenced the collective genome of microorganisms living on or inside humans (Human Microbiome Project Consortium 2012).

Another case where single-celled endosymbionts have an enormous impact on host health is the interaction between photosynthetic dinoflagellates (*Symbiodinium*) and their host corals. A popular endosymbiont of invertebrates, *Symbiodinium* species (spp.) provide photosynthetically-fixed carbon to almost all corals in the euphotic zone (Venn et al. 2008). However, if stressed by external factors such as unusually elevated temperatures, corals expel the dinoflagellates (Yong & Nicholls 1928) causing mass mortality known as coral bleaching events, with devastating consequences for the marine ecosystems they support (Baker et al. 2008). Knowledge of symbioses is therefore vital for our efforts to maintain healthy ecosystems.

### 1.2.2 Photosynthetic symbionts (photobionts) in animals

Single-celled algae, a broad term encompassing all single-celled photosynthetic eukaryotes plus photosynthetic blue-green cyanobacteria (Allaby 1992), occur as symbionts in a variety of metazoan hosts. Metazoan representatives from the Mollusca, Porifera, Cnidaria, Xenacoelomorpha, Platyhelminthes and Chordata, have adapted their physiologies in order to reap the benefits of an internal carbon and oxygen source derived from photosynthesis (Venn et al. 2008). This acquired phototrophy can allow some hosts to survive in oligotrophic or hypoxic environments (Smith & Douglas 1987).

Aside from *Symbiodinium*, well-studied photobionts in animals include cyanobacteria, which commonly inhabit shallow marine sponges (Lee et al. 2001), and chlorophyte (green) algae (*Chlorella*), which are widespread in freshwater invertebrates as diverse as sponges, cnidarians and flatworms (Venn et al. 2008). Additionally, the recent discovery of the chlorophyte *Oophila amblystomatis* living intracellularly in a vertebrate host (the spotted salamander *Ambystoma maculatum*) has challenged previous beliefs that such symbioses only exist in invertebrates, with generally simpler body plans and immune systems (Kerney et al. 2011).
1.2.3 Diatoms as photobionts

Diatoms are single-celled algae encased in a hard, silica cell wall known as a frustule. They belong to the Heterokonta, a group of eukaryotes proposed to have inherited plastids through endosymbiosis and subsequent reduction of a red alga (Cavalier-Smith 2002). The plastids possess the accessory pigment fucoxanthin, which gives diatoms a brownish colour (Round et al. 1990).

Diatoms are endosymbionts of other algae, including some dinoflagellates (Inagaki et al. 2000), rhodophytes (red algae) (Klochkova et al. 2014), and foraminiferans. The latter, a group of amoeboid single-celled eukaryotes, possess a variety of endosymbionts which is hypothesised to have promoted the evolution of enormous cell sizes in certain species (Lee & Hallock 1987), as large as 10 cm (Lamont 1998). However, reports of diatoms as animal endosymbionts are rare; the best known example is diatoms (Licmophora) that are freed from their frustules and live extracellularly in parenchymal tissues of the acoel worm Convoluta convoluta. The relationship is obligate for the worm, meaning the diatoms are essential for its survival (Apelt 1969). There are also a few accounts of diatoms inhabiting the mesohyl of sponges (e.g. Cox & Larkum 1983; Hamilton et al. 1997; Bavestrello et al. 2000).

1.2.4 Photobionts in rhabdocoels

Knowledge of photobionts in the Platyhelminthes is confined to the order Rhabdocoela. The freshwater genera Dalyellia, Typhloplana, Phaenocora, Castrada and Gieysztoria all have representatives that exhibit a Chlorella symbiosis (Douglas 1987; Young 1973; Braun 1885; Ruebush & Hayes 1939). This is best characterised in Dalyellia viridis and Typhloplana viridata, in which symbiont cells are stored near the periphery of the parenchyma and translocate photosynthetically-fixed carbon to their hosts (Douglas 1987). Marine species Baicalellia evelinae and all members of the genus Pogaina also harbour endosymbionts, described as “zooxanthellae” (Marcus 1946; Ax 2008). Zooxanthella is a general term used for an endosymbiotic alga, but the specific nature of these rhabdocoel symbionts has remained mysterious.
1.2.5 Kleptoplasty in sacoglossan sea slugs

While some species of nudibranch sea slugs (Gastropoda: Opisthobranchia: Nudibranchia) host dinoflagellates as endosymbionts (Wägele and Johnsen 2001), others have taken photosynthetic endosymbiosis a step further in what is arguably an ongoing evolutionary transition to photosynthetic animals. Several nudibranchs in the clade Sacoglossa ingest xanthophyte or chlorophyte algae, but rather than phagocytosing whole algal cells, they isolate and sequester just the plastids, a phenomenon known as kleptoplasty (Clark et al. 1990). The plastids are stored in the digestive epithelial cells that line the gut, and the rest of the algal contents is digested (Trench 1969).

This relationship has been thoroughly investigated since its discovery in the sea slug *Elysia atroviridis* (Kawaguti 1965). In some species of *Bosellia, Elysia, Plakobranchus* and *Thuridilla*, the kleptoplasts have been demonstrated to be photosynthetically active and provide the slugs with photosynthetically-fixed carbon (Händeler et al. 2009). This has been termed “functional kleptoplasty” (Clark et al. 1990) and has earned them a whirlwind of media attention and the title “solar-powered sea slugs” (Cruz et al. 2013).

Long-term retention, defined as maintenance of functional plastids for more than one month, has evolved at least four times within the Sacoglossa (Händeler et al. 2009). One species, *Elysia chlorotica*, is able to maintain kleptoplasts and survive heterotrophic starvation for over ten months (Green et al. 2000). Plastids are semi-autonomous organelles, possessing a reduced genome that encodes only a small percentage of the genes required for their function. This has led to much debate on how kleptoplasts are able to function for prolonged periods in the absence of the algal nuclear genome that encodes the majority of genes involved in plastid maintenance. Attempts to explain plastid longevity have included controversial reports of horizontal gene transfer from alga to sea slug (Pierce et al. 2007; 2011; Rumpho et al. 2008; 2009; Schwartz et al. 2010; 2014).

While kleptoplasty has been documented in single-celled eukaryotes including foraminiferans, dinoflagellates and ciliates (Stoecker et al. 2009), sacoglossans are the only animals capable of
kleptoplasty reported to date. Here the first evidence for kleptoplasty in rhabdocoels (Platyhelminthes) is presented. This represents only the second example of kleptoplasty within animals.

1.2.6 Kleptoplasty in rhabdocoels

Light, laser scanning confocal and transmission electron microscopy techniques were used to elucidate plastid ultrastructure and their location within the tissues of the solar panel worms *Pogaina paranygulgus* and *Baicalellia solaris* n. sp. The rbcL gene, which encodes the large subunit of RuBisCO, was used to identify the kleptoplasts. rbcL is present in the plastid genome and is a commonly-used barcode for identification of diatoms (Hamsher et al. 2011). The algal nuclei, along with their potential barcode genes, are digested by the worms, whereas the plastid-encoded location of the rbcL barcode made it appropriate for identification of the kleptoplasts. In order to determine the function of the kleptoplasts in *B. solaris*, starvation experiments were undertaken to find evidence for enhanced survival in light-treated versus dark-treated worms, implying kleptoplasts are functional and provide photosynthate to the worms. In addition, oxygen consumption was analysed to demonstrate that the kleptoplasts undergo photosynthesis while being maintained with the host tissues.
Chapter 2: Species discovery and evolution of neodalyellid flatworms from the Northeast Pacific Ocean

2.1 Synopsis

The advent of molecular phylogenetics has allowed for the reassessment of traditional rhabdocoel taxa that were inferred from comparative anatomy and ecological information. This has resulted in the recognition of the Neodalyellida, which consists of all marine species historically grouped within the polyphyletic Dalyellioida, and two marine species that previously belonged to the polyphyletic Typhloplanoidea (Willems et al. 2006; Van Steenkiste 2013). Most members of the Neodalyellida are free-living, although the Pterasticolidae (parasites of sea stars) and Umagillidae (parasites of sea cucumbers and sipunculids) also fall within this clade. I sampled intertidal habitats in British Columbia, Canada in order to increase our understanding of neodalyellid diversity in this region. This work allowed me to discover and characterise five neodalyellid species: *Pogaina paranygulgus*, *Baicalellia pusillus* n. comb., *Baicalellia daftpunka* n. sp., *Baicalellia solaris* n. sp. and *Tamanawas kalipis* n. sp. Nine new concatenated 18S and 28S rDNA sequences, including those extracted from the five described species and four undescribed species, provided further insight into neodalyellid evolutionary relationships and character evolution.

2.2 Methods

2.2.1 Collection and morphological examination of the taxa

Neodalyellids were collected from beaches, rocky intertidals and estuarine mudflats in British Columbia, Canada, during 2015-16. Specimens were isolated from sand and algae with the MgCl₂ decantation method, while the oxygen depletion method was used to extract animals from mud and finer sediments (Schockaert 1996). Specimens were studied alive with light and DIC microscopy and later mounted whole using lactophenol. Specimens intended for sectioning were fixed in marine Bouin’s solution, embedded in paraffin, serially sectioned (5 µm sections), and stained with Heidenhain’s iron haematoxylin, using erythrosin as counterstain. Live specimens,
whole mounts and histological sections were photographed using a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera. Differential interference contrast (DIC) optics were used to enhance contrast of images. Schematic diagrams were drawn using Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA). Measurements were taken from whole mounted and live specimens using Fiji v. 1.47 (Wayne Rasband, National Institutes of Health). All measurements were taken axially unless otherwise indicated. Holotypes will be deposited in the Swedish Museum of Natural History (SMNH, Stockholm, Sweden) and paratypes will remain in the Beaty Biodiversity Museum (BBM, University of British Columbia, Vancouver, Canada).

2.2.2 DNA extraction, amplification and sequencing

Genomic DNA was extracted from entire specimens frozen in a few µL of seawater with the Dneasy Blood & Tissue kit (Qiagen). Extractions followed manufacturer’s instructions except that: (1) the AE elution buffer was heated to 60°C before elution; and (2) DNA was eluted twice for every sample in reduced volumes of 60 µL and 30 µL respectively. For all five worm species, nearly-complete 18S (1633–1791 bp) and partial 28S rDNA (1293–1328 bp) were amplified, using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare) and the primers and thermocycling conditions listed in Table 1 (Appendix). Amplicons were visualised on 1.5% agarose gels stained with GelRed (Biotium) and enzymatically cleaned prior to sequencing with Illustra™ ExoProStar S (GE Healthcare). Clean amplicons were sequenced in 10 µL reactions using the amplification primers and internal sequencing primers (see Table 1, Appendix). Sequencing reactions contained 1 µL BigDye® Terminator (BDT) v3.1 (Applied Biosystems), 2 µL BDT buffer, 0.5 uM primer and 1–2 µL PCR product. Sequencing products were cleaned and run on an Applied Biosystems 3730S 48-capillary DNA analyzer by the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia. Resulting trace files were assembled into full sequences in Geneious v9.1.5 (Biomatters) and subjected to a BLAST search on the NCBI website (https://blast.ncbi.nlm.nih.gov) to verify taxonomic identity.
### 2.2.3 Phylogenetic analysis

New sequences were aligned with existing 18S and 28S neodalyellid rRNA sequences downloaded from Genbank (Table 2, Appendix) using the structural Q-INSI and E-INSI algorithms, respectively, in MAFFT (Katoh & Toh 2008). Two undescribed umagillids, three undescribed species of *Pogaina*, two undescribed solenopharyngids and three other undescribed neodalyellids collected in other sampling campaigns were included in the analysis to enhance its resolution, resulting in a total of 36 18S and 27 28S sequences. Taxa for the outgroup were selected based on current knowledge of neodalyellid relationships (Van Steenkiste et al. 2013). The 5′ and 3′ ends of the alignments were trimmed in Geneious v9.1.7 (www.geneious.com); Kearse et al., 2012). Ambiguous positions were selected with Aliscore v2.2 (Misof & Misof 2009) and removed with Alicut v2.3 (Kueck 2009).

Before running the phylogenetic analysis, best-fit partitioning schemes and models of molecular evolution for the concatenated dataset (18S + 28S) were recovered in PartitionFinder v.1.1.0 using a greedy search with PhyML and the Bayesian information criteria (BIC) (Lanfear et al., 2012). This resulted in two partitions corresponding with the 18S and 28S rDNA sequences and the GTR+GAMMA+I model for both partitions. Maximum likelihood (ML) and Bayesian analyses were done in RaxML v8.2.9 (Stamatakis, 2014) and MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) respectively, using XSEDE in the CIPRES Science Gateway v3.3 (https://phylo.org). Best scoring ML tree search and non-parametric bootstrapping (1,000 replicates) were performed under the recommended partition scheme and models of molecular evolution. Similarly, partitioned Bayesian analyses were done under the same substitution models, using default prior and meme settings, in two independent simultaneous runs for 10 million generations. Trees were sampled every 100th generation after a 25% burnin. LogL values and the average deviation of split frequencies were considered as convergence diagnostics. The remaining 75,000 trees were summarised in a 50% majority rule consensus tree. Nodes with maximum likelihood bootstrap support values <70% and Bayesian posterior probabilities <0.95 thresholds were collapsed using TreeGraph 2. The final concatenated tree was edited and bootstrap supports and posterior probabilities labelled using Adobe Illustrator.
2.3 Results

2.3.1 List of species
Neodalyellida Willems et al., 2006
   Provorticidae Beklemishev, 1927
   Provorticinae Luther, 1962
      *Tamanawas kalipis* n. g., n. sp.
Neokirgellinae Oswald et al., 2010
   *Pogaina paranygulgus* Karling, 1986
   *Baicalellia solaris* n. sp.
   *Baicalellia daftpunka* n. sp.
   *Baicalellia pusillus* (Luther, 1962) n. comb.

2.3.2 *Tamanawas kalipis* n. g., n. sp.

Localities
Macroalgae in rocky lower intertidal at Clover Point, Victoria, British Columbia, Canada
(48°24′1″N, 123°21′03″W; 09/02/2016): type locality.

Material
Observations on two live animals, histological sections of one animal and two whole mounts,
one whole mount designated holotype (SMNH, no. XXXX), the other paratype (BBM XXX).

Etymology
The genus name refers to its elusive nature, the species epithet to its occurrence in the intertidal.

DNA sequences
18S rDNA (GenBank accession #XXXX); 28S rDNA (GenBank accession #XXXX).
**Diagnosis**

*Tamanawas* n. g.: Provorticinae with eyes; doliiform pharynx; compact paired testes in first body half; ovovitellaria; long, muscular copulatory organ; coiled, tubiform male copulatory stylet.

Type species: *Tamanawas kalipis* n. sp.: species of *Tamanawas* with narrow, tubiform, 137–139 µm-long stylet forming one and a half coils with a diameter of 30–33 µm, ending in straight protrusion of 20–22 µm; afferent and efferent system consisting of female duct and sclerotised spermatic duct, respectively; syncytial seminal bursa with blind sclerotised duct; eyes with large lenses; short protrusions around pharynx opening.


**Description**

Animals about 0.3–0.6 mm long with rounded anterior end and slightly-tapering caudal end. Body is transparent with diatoms visible in the gut. The large doliiform pharynx (about one fifth of total body length) is situated in the first third of the body, directly posterior to the eyes with large 6 µm-long lenses (Fig 1A). Pharynx provided with circular and longitudinal muscles and has very short, hair-like protrusions around the wide proximal opening.

Short paired testes (Fig 1A) lie in the anterior body third, at each side of the pharynx. Long vasa deferentia leave the testes posteriorly and connect to the proximal end of the copulatory organ in the last third of the body. Paired, elongate vitellaria (Fig 1A) run dorsolaterally, beginning parallel to the middle of the pharynx. They merge with the paired ovaries at the posterior end of the body, thus forming ovovitellaria.

Long, sausage-shaped copulatory bulb (Fig 1B) surrounded by one thick spiral muscle layer. Extracapsular prostate glands enter the copulatory bulb proximally. Within the bulb, the intracapsular seminal vesicle is narrow and lies medial to the prostatic gland necks. Distal to the copulatory bulb, the ejaculatory duct and prostate glands enter the sclerotised stylet. The stylet
(Fig 1B,C,E) is a narrow tube of relatively consistent diameter that forms a spiral shape consisting of one and a half coils of length 137–139 µm (\( \bar{x} = 138; n = 2 \)), with widest point diameter of 30–33 µm (\( \bar{x} = 32; n = 2 \)). Coils encased within a muscular tube or sleeve. Stylet terminates in a straight protrusion that projects at about 90° to the coil, slightly thinner in diameter than the coil itself and measuring 20–22 µm (\( \bar{x} = 21; n = 2 \)). The protrusion enters the male atrium (Fig 1D), which is strongly sclerotised and forms folds that may be mistaken in whole mounts for projections of the stylet (Fig 1B). Male atrium connects to the common genital atrium (Fig 1D), which is surrounded by eosinophilic glands and contains glandular secretions.

Ovaries connected to the common genital atrium through an efferent and afferent system (Fig 1D). The latter consists of a syncytial seminal bursa positioned between the ovaries. The syncytial tissue appears in close contact with the ovaries. Its large central sperm vesicle is folded over on itself (Fig 1B,C) and surrounded by several smaller resorption vesicles (Fig 1B,D). It is sclerotised at the end where it connects to a curved, strongly-sclerotised spermatic duct, which runs to the distal part of the male atrium. Another blind-ending, sclerotised duct extends laterally from the central vesicle (Fig 1C,D). In the efferent system, female glands enter the female duct where the oviducts merge (Fig 1D). A protrusion (Fig 1D) of the common genital atrium or distal end of the female canal might function as a uterus. The epithelia of the genital atria appear to be syncytial.
**Fig 1.** *Tamanawas kalipis* n. sp. (A) Habitus of live animal. (B) Muscular male copulatory organ and syncytial seminal bursa with resorption vesicles. (C) Atrial organs of live animal. (D) Illustration of atrial organs based on serial sections. (E) Illustration of the male copulatory stylet.

Abbreviations: bd, blind duct; ca, common atrium; cg, caudal glands; cga, common genital atrium; co, copulatory organ; e, eye; eg, eosinophilic glands; epg, extracapsular prostate glands; fd, female duct; fg, female glands; gp, gonopore; ipg, intracapsular prostate glands; m, muscle; ma, male atrium; mco, male copulatory organ; ms, muscular sleeve; od, oviduct; ov, ovary; ph, pharynx; pr, protrusion; rv, resorption vesicle; sb, seminal bursa; sd, spermatic duct; sr, seminal receptacle; st, stylet; sv, seminal vesicle; t, testis; u, uterus; vd, vas deferens; vi, vitellarium.

Scale bars: A = 50µm; B = 200nm; C = 10 µm; D = 10 µm.
Discussion

*Tamanawas kalipis* n. sp. shares many morphological similarities with members of the provorticine genus *Vejdovskya*. These features include a doliiform pharynx, compact, paired testes lying close to the pharynx in the first body half, ovovitellaria, a long, muscular, male copulatory organ, and an elongate, tubiform copulatory stylet. A seminal bursa is reported for most species of *Vejdovskya*, often large in size as found in *T. kalipis* n. sp. (Ax 1956; Luther 1962; Ax 1997; Ax 1954; Ax 1950; Karling 1957).

The genus *Vejdovskya* has been diagnosed by a combination of characters, some of which are not present in all species. For instance, the position and size of the seminal vesicle and prostate glands in the copulatory bulb varies among species and two long tactile sensory cilia at the front end are not mentioned in the descriptions of *V. parapellucida* (Ax 1997) and *V. simrisiensis* (Karling 1957). Ax (2008) subdivided the genus into two groups. The first group consists of *V. ignava* Ax 1951, *V. simrisiensis* Karling 1957, *V. parapellucida* Ax 1997, *V. pellucida* Schultze 1851 and *V. mesostyla* Ax 1954, all of which lack eyes, have ovovitellaria in the second body half, and an elongate, non-spiralling stylet. The second group consists of *V. halileimonia* and *V. helictos*, which differ from the other species in this genus by the presence of eyes and a spiral stylet (Ax 2008). As such, the latter two species seem closely related to *T. kalipis* n. sp.

*V. halileimonia* has three to four large stylet coils (Ax 1960), which are similar in appearance to the one and a half coils observed in *T. kalipis* n. sp. The presence of a “muscular sleeve or cuff” that surrounds and follows the curves of the stylet is also shared by *T. kalipis* n. sp. While *V. halileimonia* also has elongate vitellaria, it diverges from *T. kalipis* n. sp. by having a connection between them, just distal to the pharynx, which is significantly smaller in size compared with the large *T. kalipis* n. sp. pharynx. Although a seminal bursa was not observed on live specimens of *V. halileimonia*, its presence cannot be excluded. *V. helictos* has a large pharynx relative to body size and a stylet formed by six weak spiral coils (Ax 1956). In addition, *V. helictos* also has a small, sclerotised, curved tube that Ax (1956) predicts connects to the bursa and is likely homologous with the sclerotised spermatic duct reported in *T. kalipis* n. sp. Attached to the sclerotised tube and close to the stylet, Ax (1956) also describes a weakly-sclerotised ring that he
predicts is part of a bursa in *V. helictos*. This is likely part of the sclerotised wall of the male genital atrium as in *T. kalipis* n. sp.

Because of the unique morphological similarities (eyes, spiral stylet, spermatic duct) between *V. helictos*, *V. halileimonia* and *T. kalipis* n. sp. and the distinct phylogenetic position of *T. kalipis* n. sp. (see Fig 6 and General discussion), the erection of a new genus, *Tamanawas*, and transferal *V. halileimonia* and *V. helictos* to this genus, is proposed. Further morphological details must be filled in for *T. halileimonia* n. comb. and *T. helictos* n. comb. to confirm similarities and homologies in the male and female genital system. For now, these three species differ in the number and extent of stylet coiling and the sclerotisation of the male genital atrium and spermatic duct (not observed in *T. halileimonia* n. comb.).

### 2.3.3  
*Pogaina paranygulgus* Karling, 1986

**New localities**
Estuarine mudflats in two locations: (1) Mud Bay Park, Surrey, BC (49°05′22.7″N 122°51′44.6″W; 28/10/2015); (2) Ladysmith, BC (49°00′56.3″N 123°50′35.7″W; 21/03/2016).

**Known distribution**
Northeast Pacific Ocean: California (Karling 1986).

**Material**
Observations on several live animals and 11 whole-mounted specimens, all designated paratypes (BBM, no. XXXX-XXXX).

**DNA sequences**
18S rDNA (GenBank accession #XXXX); 28S rDNA (GenBank accession #XXXX).

**Remarks**
Animals transparent and about 0.4–0.7 mm long. Internal anatomy in accordance with the description of Karling (1986). Small oviform pharynx situated directly posterior to the eyes (Fig
2A), which appear to have two lenses. Small tooth-like protrusions around the proximal pharynx opening (Fig 2C). Protrusions more numerous than “about 12” described by Karling (1986). Paired testes relatively short and situated laterally, directly posterior to the pharynx. Paired ovaries distinct from the paired vitellaria and situated laterally, posterior to copulatory organ. Brown plastids present throughout the parenchyma. Some are black and degraded.

Copulatory bulb (Fig 2D) situated posterior to the pharynx in the midsection of the animal taking the shape of a curved cylinder. The wall is formed by a thick layer of internal circular and external longitudinal muscles. Within the bulb, the seminal vesicle is proximal to the prostate vesicle. Extra-capsular prostatic glands are large and enter the copulatory bulb proximally. Gland necks merge with the copulatory duct inside the copulatory stylet. The stylet (Fig 2B,D) is a 74–104 µm-long curved funnel (\(\bar{x} = 85 \mu m; n = 4\)) with a 25–38 µm-wide proximal pore (\(\bar{x} = 30 \mu m; n = 4\)) and narrow 6–16 µm-wide distal pore (\(\bar{x} = 11 \mu m; n = 4\)). Male atrium (Fig 2D) fits loosely around the stylet. Copulatory bursa (Fig 2E) surrounded by a weakly-sclerotised basal membrane and a layer of circular muscle.

Previously described from Californian mudflats and tide pools (Karling 1986), this is the first report of *P. paranygulgus* in British Columbia.
Fig 2. *Pogaina paranygulgus*. (A) Habitus of live animal. (B) Stylet of a paratype. (C) Pharynx with protrusions (black arrow). (D) Copulatory organ of live animal. (E) Seminal bursa of live animal. Abbreviations: cb, copulatory bursa; co, copulatory organ; e, eye; epg, extracapsular prostate glands; ipg, intracapsular prostate glands; m, muscle; ma, male atrium; p, plastid; ph, pharynx; st, stylet; sv, seminal vesicle; vi, vitellaria. Scale bars: A = 50 µm; B = 20 µm; C = 10 µm; D = 20 µm; E = 10 µm.

2.3.4 *Baicalellia solaris* n. sp.

**Diagnosis**

Species of *Baicalellia* with tubiform, 22–35 µm-long stylet consisting of a 13–20 µm-wide straight tube that tapers distally at 65° into an asymmetrical funnel with flexible distal opening; plastids in the parenchyma; short pointed protrusions around pharynx opening; anastomosing testes posterior to pharynx.

**Localities**

Macroalgae in rocky lower intertidal at three locations: (1) Clover Point, Victoria, British Columbia, Canada (48°24′12″N, 123°21′03″W; 03/03/2016): type locality; (2) Little Wolf Beach, Calvert Island, British Columbia, Canada (51°39′53″N, 128°07′44″W; 20/05/2016), (3)
West Beach, Calvert Island, British Columbia, Canada (51°39′53″N, 128°07′44″W; 20/05/2016). Macroalgae on the dock of the marine station: Friday Harbor, San Juan Island, Washington, USA (48°32′42″N, 123°00′44″W; 10/08/2016).

**Material**
Observations on several live animals. Seven whole mounts, one of which designated as the holotype (SNMH, no. XXXX), the others paratypes (BBM, no. XXXX-XXXX).

**Etymology**
The species name refers to its symbiotic relationship with photosynthetic microalgae. Solaris (Lat.): of the sun.

**DNA sequences**
18S rDNA (GenBank accession #XXXX); 28S rDNA (GenBank accession #XXXX).

**Description**
Animals about 0.5–0.6 mm long with rounded anterior end and slightly tapering caudal end (Fig 3A). The body is transparent but appears spotted due to the presence of brown plastids in the parenchyma. Relatively small doliiform pharynx in the first third of the body directly posterior to the large lenticular eyes. Pharynx provided with circular and longitudinal muscles, and very small pointed protrusions around the proximal opening (Fig 3B).

Internal organisation similar to other species of the genus *Baicalellia* Nasonov, 1930 (see Nasonov 1930, 1932; Luther 1962; Ax 1995). Paired testes (Fig 3A) at each side reach from just posterior to the pharynx to the posterior third. They are connected by an anterior transverse commissure (Fig 3B), located just posterior to the pharynx. Vasa deferentia leave the testes posteriorly and can become widened to form extracapsular seminal vesicles. Paired vitellaria (Fig 3A) run dorsolaterally and produce vitellocytes that surround maturing egg cells in the paired ovaries at the posterior end of the body, thus forming ovovitellaria.
Copulatory bulb (Fig 3D) of the male copulatory organ consists of two halves. Its proximal half contains a very large intracapsular seminal vesicle receiving the vasa deferentia. About halfway down the bulb, just distal from the intracapsular vesicle, extracapsular prostate glands enter the copulatory bulb. The distal half of the copulatory bulb contains the ejaculatory duct and gland necks filled with prostate secretion. This half of the bulb is surrounded by two spiral muscle layers. Distal to the copulatory bulb, the ejaculatory duct and prostate secretion enter the weakly-sclerotised stylet (Fig 3D,E,F). The latter is an asymmetrical tube with no projections. From the base to the distal apex it measures 22–35 µm (\( \bar{x} = 28 \mu m; n = 2 \)). The proximal half is a 13–20 µm-wide (\( \bar{x} = 15 \mu m; n = 2 \)) straight tube with a thickened proximal rim. The distal half abruptly tapers into an asymmetrical funnel at an angle of about 65° to a narrow distal opening of 3–4 µm. The distal opening is extremely flexible and widens to the same width as the proximal part of the stylet (Fig 3D) to facilitate evacuation of sperm and prostate secretion into the male atrium. The latter fits tightly around the stylet and opens into a large common genital atrium.

Large copulatory bursa (Fig 3C) surrounded by thick circular muscle protrudes from the dorsal wall of the common genital atrium. Through a sphincter, the common genital atrium enters a short female canal. The latter connects to the ovovitelloducts and to a muscular syncytial seminal bursa with a thickened basal membrane. Sperm are visible in the seminal bursa. A uterus is absent.
Fig 3. *Baicalellia solaris* n. sp. (A) Habitus of live animal. (B) Proximal half of live animal, black arrow indicates pharyngeal protrusions. (C) Atrial organs of live animal. (D) Copulatory organ of live animal with stylet open wide. (E) Live animal with closed stylet. (F) Illustration of closed stylet. Abbreviations: cb, copulatory bursa; co, copulatory organ; e, eye; ipg, intracapsular prostate glands; ma, male atrium; ov, ovary; p, plastid; ph, pharynx; st, stylet; sv, seminal vesicle; t, testis; tc, testes commissure; vi, vitellarium. Scale bars: A = 50 µm; B = 50 µm; C = 30 µm; D = 20 µm; E = 20 µm; F = 10 µm; G = 5 µm.

2.3.5 *Baicalellia daftpunka* n. sp.

**Diagnosis**

Species of *Baicalellia* with 61–75 µm-long, upside-down helmet-shaped tubiform stylet with irregular rounded window and relatively wide distal opening; large pharynx with long tentacle-like protrusions; anastomosing testes in posterior body half.
Localities
Macroalgae in rocky lower intertidal, Clover Point, Victoria, British Columbia, Canada (48°24′12″N, 123°21′03″W; 02/09/2016): type locality.

Material
Observations on several live animals. Three whole mounts, one of which designated as the holotype (SNMH, no. XXXX), the others paratypes (BBM XXX).

Etymology
The species name refers to the helmet-shaped male copulatory stylet. Daftpunka: Daft Punk, an electronic music duo who wear helmets to conceal their identities while performing publicly.

DNA sequences
18S rDNA (GenBank accession #XXXX); 28S rDNA (GenBank accession #XXXX).

Description
Animals 0.5–0.8 mm long, transparent, and with rounded anterior end and slightly tapering caudal end. Directly posterior to the lenticular eyes is the large doliiform pharynx (Fig 4B), which has around 26 long, tentacle-like protrusions around the proximal opening and is provided with circular and longitudinal muscles. Diatoms are visible in the gut.

Organisation of ovovitellaria, vasa deferentia, prostate glands and intracapsular seminal vesicle (Fig 4C,D) as in B. solaris n. sp. Anastomosing paired testes in posterior half, lateral to the copulatory organ. The bulb of the latter is surrounded by two spiral muscle layers. The stylet (Fig 4E,F) is a 61–75 μm-long (x̄ = 69 μm; n = 2), asymmetrical, upside-down helmet-shaped tube consisting of the sclerotised wall of the male genital atrium. Distally the tube curves to a relatively wide opening, next to which there is an irregular rounded window. Sclerotisation is stronger on one side of this window creating the impression of a pincer-like extension.

A seminal bursa (Fig 4D) with vacuolar epithelium is present, but its connection to the rest of the female system could not be observed. A large copulatory bursa (Fig 4D) is present at the distal
end of the animal.

*Fig 4.* *Baicalellia daftpunka* n. sp. (A) Habitus of live animal. (B) Live animal, arrow indicates tentacles on pharynx. (C) Copulatory organ of live animal. (D) Atrial organs of live animal. (E-F) Stylet of the holotype. Abbreviations: cb, copulatory bursa; e, eye; epg, extracapsular prostate glands; ipg, intracapsular prostate glands; ph, pharynx; sm, spiral muscle; sb, seminal bursa; st, stylet; sv, seminal vesicle. Scale bars: A = 50 µm; B = 20 µm; C = 30 µm; D = 10 µm; E = 10 µm; F = 100 µm.

### 2.3.6 Discussion of new *Baicalellia* species

The general organisation of *B. solaris* n. sp. and *B. daftpunka* n. sp. strongly resembles members of the genus *Baicalellia* Nasanov, 1930, supporting their phylogenetic position based on molecular data (see *Fig 6* and General discussion). As is typical of *Baicalellia*, vitellaria are elongate and caudally join paired ovaries to form ovovitellaria, the gonopore is located in the
posterior body third, paired testes are connected by a transverse commissure, styles are tubiform and copulatory and seminal bursae are present. *B. groenlandica* has the most similar stylet to *B. solaris*, described as a slightly curved tube, though it differs by curving continuously from base to apex rather than only in the distal half, and the possession of a small, tooth like distal protrusion (Ax 1995). The helmet-shaped stylet of *B. daftpunka* is unlike those previously described for species of *Baicalellia*. The stronger sclerotisation on one side of its rounded, distal window gives the impression of a pincer like extension, such that the stylet may appear superficially similar to the stylet of *B. anchoragenesis* (Ax & Armonies 1990).

2.3.7  *Baicalellia pusillus* (Luther, 1962) n. comb.

syn. *Coronopharynx pusillus* Luther, 1962

**New locality**
Coarse sand and shell hash in lower intertidal, Departure Bay, Nanaimo, BC (49°11′43″N, 123°57′32″W; 11/10/2015).

**Known distribution**
Northeast Pacific Ocean: Oregon (Karling 1986), Gulf of Alaska (Ax & Armonies 1990); Baltic Sea (Luther 1962, 1963; Fenchel & Jansson 1966); North Sea (Tulp 1974; Armonies 1987); White Sea (Kotikova & Joffe 1988).

**Material**
Live animal observations and one whole mount designated paratype (BBM XXX).

**DNA sequences**
18S rDNA (GenBank accession #XXXX); 28S rDNA (GenBank accession #XXXX).

**Remarks**
Animals transparent and about 0.8 mm long, falling within the 0.5–1 mm length range of previously described Pacific specimens. Food visible in the gut (Fig 5A). Large doliiform
pharynx (Fig 5B) posterior to lenticular eyes with conspicuous crown of about 26 long pointed tentacles around the proximal opening. The tentacles appear to have small bristles on them (described by Luther 1962 as “flagellated brushes”), though this was not reported in the other Pacific forms.

Genital organisation consistent with the description of Luther (1962). Long, paired vitellaria reach from the pharynx to the paired ovaries, with which they form ovovitellaria (Fig 5A). This is consistent with the Alaskan (Ax & Armonies 1990) and Baltic (Luther 1962) specimens but differs from the Oregon morphotype (Karling 1986), which was reported to have distinct ovaries and vitellaria.

Paired testes not connected and located in posterior body third. Copulatory organ (Fig 5C) located between the testes and surrounded by spiral muscles. Prostate glands enter the seminal vesicle in the middle of the copulatory organ and long, thin gland necks are visible inside the stylet. The stylet (Fig 5C) is a cylindrical, 37 μm-long, slightly-tapering tube. It has a proximal, curved spur on one side, measuring 34 μm. This is longer than the spur on the Alaskan (22–23 μm) and Californian (17 μm) forms (Ax & Armonies 1990; Karling 1986). Previously described from estuarine habitats on the Oregon (Karling 1986) and Alaskan (Ax & Armonies 1990) coasts. Based on the results of the phylogenetic analysis (Fig 6) and morphological similarities to the genus *Baicalellia*, this species is transferred to *Baicalellia* (see General discussion).
Fig 5. *Baicalellia pusillus* n. comb. (A) Habitus of live animal. (B) Pharynx of live animal. (C) Copulatory organ of live animal. Abbreviations: cg, caudal glands; co, copulatory organ; e, eye; ipg, intracapsular prostate glands; ov, ovary; ph, pharynx; sm, spiral muscle; sp, spur; st, stylet; sv, seminal vesicle; t, testes; te, tentacle; v, vitellarium. Scale bars: A = 50 µm; B = 20 µm; C = 10 µm.

2.3.8 Molecular phylogenetic relationships

The phylogenetic analysis is based on the concatenated dataset including 36 taxa and 3077 bp. This consists of partial 18S (1845 bp) and 28S rDNA sequences (1232 bp) for 36 and 27 neodalyellid species, respectively. ML and Bayesian tree topologies were congruent and are summarised in Figure 6.

The outgroup includes four members of the monophyletic Solenopharyngidae, and the promesostomid *Einarella argillophyla*. The ingroup consists of two sister clades that are consistent with Van Steenkiste et al. (2013), but some relationships within them are updated: 1) Clade 1 includes the provorticine genera *Provortex* and *Vejdovskya* (Provorticidae), and the Umagillidae; the polytomy of these taxa is resolved here. Umagillidae and Provorticinae are sister taxa, and *T. kalipis* n. sp. falls basal to them; 2) Clade 2 includes the provorticid *Eldenia reducta* and several members of Neokirgellinae (Provorticidae), Pterastericolidae, and Graffillidae. *Baicalellia* sensu Nasonov is paraphyletic because of the position of *Baicalellia*.
beauchampi n. comb. (formerly Canetellia beauchampi) and Baicalellia pusillus n. comb. (formerly Coronopharynx pusillus). These species are now assigned to Baicalellia. The sister relationship of Baicalellia and Pterastericola recovered by Van Steenkiste et al. (2013) is supported, but the branching of Pogaina as basal to these taxa is not well supported in the analysis. Rather, the Baicalellia+Pterastericola group falls within a polytomy containing Pogaina, Provorticidae sp. and the group of graffillids Pseudograffilla arenicola, Graffilla buccinica, and Dalyellioida “houdini” sp. Support for a basal clade with Eldenia reducta, Bresslauilla relicta and Neodalyellidae spp. is found.
Fig 6. Bayesian majority-rule consensus tree of the concatenated 18S + 28S alignment. Symbols above and below branches indicate ML bootstrap values (bs) and Bayesian posterior probabilities (pp), respectively. Branches without symbols are fully supported, and unsupported branches (pp < 0.95, bs < 70) have been collapsed. Scale bar = number of substitutions per site. Taxa in bold have been sequenced for this study.
2.4 General discussion

The three main neodalyellid clades in Van Steenkiste et al. (2013), i.e., the outgroup Einarella+Solenopharyngidae clade, the Provorticinaceae+Umagillidae clade (clade 1), and a “mixed” clade (Graffillidae, Pterastericolidae, different provorticid genera and some undescribed taxa) (clade 2) are also recovered in this analysis.

2.4.1 Clade 1: Provorticinaceae and Umagillidae

The inclusion of *T. kalipis* n. sp. and two additional umagillids (*Anoplodium hymanae* and undescribed Umagillidae sp. 1) resolves the polytomy of the Provorticinaceae+Umagillidae clade of Van Steenkiste et al. (2013). The basal position of *T. kalipis* n. sp. to all umagillids and *Vejdovskya* + *Provortex*, suggests that a relatively long tubular stylet and the presence of an afferent and efferent system in the atrial organs are most likely plesiomorphic conditions. Umagillids typically have this double connection between the common atrium and the female system. This consists of a female duct (efferent) as in other neodalyellids, but also a vagina leading to a seminal bursa (afferent) that connects to the ovovitellarian junction, often via a seminal receptacle. Possibly, the spermatic duct of *T. kalipis* n. sp. is homologous with the umagillid vagina. In addition, sclerotisation is present in parts of the umagillid bursa and/or associated ducts, as seen in *T. kalipis* n. sp. though absent from the subfamily Collastominae (Cannon 1982).

A bursal valve, defined as a common plug at which two sclerotic ducts (leading from the vagina and to the seminal receptacle respectively) enter the bursa (Cannon 1987), is present in many members of the Umagillinae and was proposed by Cannon (1982) as the ancestral condition. This may be homologous with the point at which the spermatic duct and a second sclerotised duct enter a sclerotised part of the bursa in *T. kalipis* n. sp., though here the second duct appears blind rather than connecting to a seminal receptacle.

Several species of *Provortex* (*P. balticus*, *P. pallidus*, *P. tubiferus*, *P. karlingi* and *P. psammophilus* (Schultze 1851; Luther 1948; Ax 1951; Meixner 1938)) also have a double connection. Here the afferent system consists of a non-sclerotised spermatic duct connected to a
bursa. Although not reported, it may also be present in *Vejdovskya*. Only a large seminal bursa has been described in this genus (Ax 2008; Ax 1954; Karling 1957), but detailed reconstructions are currently lacking for all species. Umagillidae, *Provortex* and *V. ignava* all have a uterus, albeit generally more established in form than in *T. kalipis* n. sp. (Cannon 1982; Ax 2008). The absence of a sclerotised stylet in some umagillids (*Anoplodium* species, *Collastoma monorchis* and *Ozametra arbora* (Cannon 1982)) is most likely a secondary reduction, and eyes are lost in the endosymbiotic umagillids and all current species of *Vejdovskya*.

The separation of provorticid taxa Neokirgellinae and Provorticinae recovered by Van Steenkiste et al. (2013) is supported here, falling into clade 1 and 2 respectively. Their separation has been based on the proximal position of the seminal vesicle to distal prostate vesicle in Neokirgellinae (former Kirgisellinae) (Luther 1962), but this condition is also present in many Provorticine species. The double connection to the female system may be a synapomorphy that distinguishes clade 1 from clade 2.

### 2.4.2 Clade 2: Mixed clade

The inclusion of *B. solaris* n. sp., *B. daftpunka* n. sp., *B. pusillus* n. comb. (formerly *Coronopharynx pusillus*) and *B. canadensis* in this analysis provides an insight into relationships within *Baicalellia*. *B. pusillus* n. comb. and *B. beauchampi* n. comb. (formerly *Canetellia beauchampi*) fall within *Baicalellia*. This supports the observation by Ax (2008) that there is no adequate morphological justification for the monophyly of *Canetellia*.

Both *B. pusillus* n. comb. and *B. beauchampi* n. comb. share *Baicalellia*-like characters (paired, elongate ovovitellaria, paired testes, long, muscular copulatory organ, seminal and copulatory bursae, and posterior gonopore), with the exception that they lack a transverse commissure between the testes. *B. canadensis* (Ax & Armonies 1987) also lacks this connection. As such, the transverse commissure cannot be considered as a diagnostic character for the genus. Unconnected testes may represent multiple independent secondary losses of the anastomosing commissure, though it is possible that this character shows plasticity during the animal’s
development. This seems to be the case in rhabdocoel *Lurus evelinae*, whose paired testes are coalescing in some specimens and not others (Van Steenkiste et al. 2008).

*B. pusillus* n. comb. is sister to *B. daftpunka* n. sp., with which it shares long, tentacle-like protrusions around the pharynx opening. Such tentacles, which may have a sensory function, are also shared by *B. albicauda, B. nigrofasciata, B. baicali, B. posieti, B. evelinae* and *B. beauchampi* n. comb. (Nasonov 1930; Marcus 1946; Ax 1956), but are not reported in the remaining 12 *Baicalellia* species. Very short, tooth-like protrusions surround the opening of the *B. solaris* n. sp. pharynx (similar to those seen in some *Pogaina* species), but it is not possible to tell from the phylogeny which is the derived state.

Based on the phylogenetic position of *B. pusillus* n. comb. and *B. beauchampi* n. comb. and their morphological resemblance to species in the genus *Baicalellia, Canetellia* and the monotypic genus *Coronopharynx* are suppressed. The second described *Canetellia* species, *C. nana*, is not included in this study, but its limited description suggests it differs from *B. beauchampi* n. comb. only in its stylet morphology and vasa deferentia that leave the testes proximally rather than distally (Ax 2008).

The parasitic Pterasticolidae is the sister group to *Baicalellia*, but differs from species of *Baicalellia* in having unpaired gonads, a small, bean-shaped copulatory organ usually in the anterior body half, an ootype and sometimes pseudovagina. *Baicalellia*-like anastomosing testes could have been the plesiomorphic condition in the common ancestor of these taxa, which have become a single testis in pterasticolids. They also have several adaptations to a parasitic lifestyle: adhesive organs, no eyes and loss of cilia during their lifetimes (Cannon 1986).

The polytomy of *Pogaina*, the *Baicalellia+Pterasticola* sister group, Provorticidae sp. and the graffillid group *P. arenicola+G. buccicola+Dalellioida “Houdini”* sp. limits the discussion of evolution of traits in these taxa. The fact that pterasticolids, rather than *Pogaina*, fall as sister to *Baicalellia* is somewhat surprising, given that *Pogaina* and *Baicalellia* have many shared characters such that no single character can differentiate them. The presence of plastids (reported as “zooxanthellae”) in the parenchyma was the main diagnostic character to identify *Pogaina*
(Ax 1970). However, zooxanthellae are reported in *B. evelinae* and now plastids in *B. solaris*. As such, the two main characters that allowed *Pogaina* to be distinguished from *Baicalellia* (separate, non-anastomosing testes and zooxanthellae) are no longer valid.

*Baicalellia* and *Pogaina* species share the presence of a copulatory and seminal bursa. Two bursae are not reported for all species, but this likely reflects problems with identification and/or their absence in sexually immature specimens. Here “copulatory bursa” and “seminal bursa” are used *sensu* Van Steenkiste (2008) in an attempt to standardise the terminology. The copulatory bursa is a blind, usually muscular sack protruding from the common genital atrium, that likely serves to store a mate’s sperm. This organ has also been referred to as a “bursa copulatrix” (Nasonov 1930). The seminal bursa is a sack connected to the female system that may serve to resorb redundant sperm. It is often syncytial, contains spermatid vesicles and has also been referred to as “phagocytic organ”, “seminal receptacle” (Nasonov 1930), or “syncytial tissue” (Ax & Armonies 1990). Use of the term “seminal receptacle” is reserved for any additional sperm storage structures.
Chapter 3: Kleptoplasty in marine rhabdocoels (Platyhelminthes)

3.1 Synopsis

Algal endosymbionts are relatively widespread in metazoans, though knowledge of photobionts in the Platyhelminthes is limited to the Rhabdocoel. Some freshwater rhabdocoels harbour Chlorella symbionts (Douglas 1987; Young 1973; Braun 1885; Ruebush & Hayes 1939) and marine species Baicalella evelinae and all members of the genus Pogaina harbour “zooxanthellae” (Marcus 1946; Ax 2008), though the specific nature of these symbionts remains unknown.

Several nudibranch sea slugs in the clade Sacoglossa ingest either xanthophyte or chlorophyte algae, but rather than maintaining whole algal cells, they isolate and sequester just the plastids, a phenomenon known as kleptoplasty. In species capable of “functional kleptoplasty”, the sequestered plastids continue to photosynthesise and provide fixed carbon to their hosts (Clark et al. 1990).

Here I provide the first evidence for kleptoplasty in members of the Platyhelminthes, which represents only the second example of this phenomenon in animals. Light, laser scanning confocal and transmission electron microscopy were used to demonstrate the intracellular location and ultrastructure of the kleptoplasts in Pogaina paranygulgus and Baicalellia solaris. DNA barcoding of partial rbcL sequences demonstrated that the kleptoplasts were stolen from raphid pennate diatoms, though the relationship does not appear to be species-specific. In order to determine the function of the kleptoplasts in B. solaris, oxygen consumption was analysed to demonstrate that the kleptoplasts produced light-dependent oxygen for at least ten days. Starvation experiments provided evidence for enhanced survival in light-treated versus dark-treated worms, implying kleptoplasts supply photosynthate to the worms. Kleptoplasts are ultimately digested; thus heterotrophy is required to replenish healthy populations of kleptoplasts, but their digestion may provide sustenance when seasonal diatom blooms collapse.
3.2 Methods

3.2.1 Sample collection

*B. solaris* and *P. paranygulgus* specimens were sampled between March and October 2016. Macroalgae were collected from the rocky intertidal at Clover Point, Vancouver Island, BC, and Calvert Island, BC, from which worms were isolated using the MgCl$_2$ decantation method. Mud was collected from estuarine mudflats at Ladysmith and Mud Bay Park, BC, and worms were isolated using the oxygen depletion method (Schockaert 1996).

3.2.2 Histological observations using light microscopy

Specimens intended for sectioning were fixed in marine Bouin's solution, embedded in paraffin, serially sectioned (5 µm sections), and stained with Heidenhain's iron haematoxylin, using erythrosin as a counterstain. Histological sections and live worms mounted in seawater were photographed using a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera. Differential interference contrast (DIC) optics were used to enhance contrast of images.

3.2.3 Laser scanning confocal microscopy

One week-starved live specimens were mounted in seawater on glass slides, under coverslips elevated by modeling clay. Specimens were imaged using an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) at 20x magnification. Optical sections were taken of specimens excited at 635 nm.

3.2.4 Transmission electron microscopy

Isolated specimens were pipetted onto a coverslip coated with poly-L-lysine and fixed with 2.5% (v/v) glutaraldehyde in filtered seawater for 1 hr. Glutaraldehyde was drawn off using a pipette and specimens were post-fixed in 1% (w/v) osmium tetroxide with filtered seawater at 4°C for 1 hr. Specimens were rinsed with seawater and stuck to the coverslip using a large drop of low
melting point agar, then dehydrated through a graded ethanol series (30%, 50%, 75%, 85%, 90%, 95%, 100%, 100%) at 10 min each. Specimens were washed in 1:1 ethanol:acetone for 10 min and 100% acetone for 10 min. After infiltration with a 1:1 acetone-resin mixture for 10 min, specimens were embedded in EPON™ 812 Resin for 12 hr, after which the resin was polymerised at 65°C for 24 hr.

Specimens were cut from the resin using a fine razor and glued to a resin stub in the desired orientation for sectioning. Ultra-thin (45 nm) sections were cut using a DiATOME Ultracut diamond knife mounted on a Leica Ultracut-E microtome. Sections were placed on Formvar-coated copper grids and triple-stained with lead citrate for 10 min, uranyl acetate for 5 min, followed by lead citrate for 10 min. Stained grids were viewed under a Hitachi H7600 TEM with accelerating voltage of 80kV and photographed with an AMT XR50 CCD camera.

### 3.2.5 Image processing

Fiji v. 1.47 (Wayne Rasband, National Institutes of Health) was used to compile z-stack projections of confocal micrographs, crop and rotate images, adjust brightness and contrast, and add annotations and scale bars. Schematics and figure plates were prepared with Adobe Illustrator CS5 (Adobe Systems Incorporated, San Jose, CA).

### 3.2.6 Plastid DNA sequencing

DNA was extracted from *B. solaris* and *P. paranygulgus* specimens in a few µL of seawater with the DNeasy Blood & Tissue kit (Qiagen). Extractions followed manufacturer’s instructions except that: (a) the AE elution buffer was heated to 60 °C before elution; and (b) DNA was eluted twice for every sample, in reduced volumes of 60 µL and 30 µL respectively, to provide duplicates. Partial rbcL sequences were amplified using Illustra™ PuReTaq Ready-To-Go PCR beads (GE Healthcare), and the primers and thermocycling conditions listed in Table 1 (appendix). Amplicons were visualised on 1.5% agarose gels stained with GelRed™ (Biotium) and enzymatically cleaned prior to sequencing with Illustra™ ExoProStar S (GE Healthcare). Clean amplicons were sequenced in 10-µL reactions using the internal sequencing primers.
(Table 1). Sequencing reactions contained 1 µL BigDye® Terminator (BDT) v3.1 (Applied Biosystems), 2 µL BDT buffer, 0.5 µM primer and 1–2 µL PCR product. Sequencing products were cleaned and run on an Applied Biosystems 3730S 48-capillary DNA analyser by the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia. Resulting trace files were assembled into full sequences in Geneious v9.1.5 (Biomatters). Partial rbcL sequences were subjected to a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov) and an identification request in the Public Record Barcode Database on the BOLD website (http://www.boldsystems.org/index.php/).

3.2.7 Starvation experiments

40 freshly-collected *B. solaris* specimens in sterile filtered seawater were divided randomly into two treatments. Light treatment: 20 specimens were placed in a transparent Petri dish and incubated at 16°C on a 10:14 light-dark cycle. Dark treatment: 20 specimens were placed in an opaque black box and also incubated at 16°C. Seawater was refreshed and surviving individuals were counted every 7 days. Survival rates were calculated and visualised using Microsoft Excel. One specimen from each treatment was used to study loss of plastids over the starvation period. Light micrographs were taken every seven days using a Zeiss AxioPlan 2 microscope equipped with a Sony Alpha 6000 camera.

3.2.8 Optode experiments

Oxygen consumption measurements were undertaken to estimate photosynthetic rates. Oxygen saturation (O₂ mg/L) was measured using an SDR SensorDish Reader (PreSens, Regensburg, Germany) and sealed 24-chamber glass microplate with 0.2 mL wells (Loligo Systems, Copenhagen, Denmark). This system uses LED lights for non-invasive optical fluorescence oxygen sensing. *B. solaris* individuals were stored in sterile seawater and incubated at 16°C on a 14:10 light:dark cycle for ten days prior to the experiments. This starvation period ensured that no whole algal cells that could affect oxygen measurements remained in the gut. Worms were removed from the incubator 3 hr before the experiment and allowed to acclimatise to room temperature.
Microplate wells were sterilised with ethanol prior to use. Three wells each contained 25 *B. solaris* individuals (preliminary experiments found worms were too small to be placed in wells individually and produce a measurable signal), along with the sterile seawater medium that the specimens had previously been incubated in. Number of replicates was limited by the number of specimens available, as they are not cultivable and are difficult to find in abundance in the field. As a positive control, three replicate wells contained a culture of the marine chlorophyte *Tetraselmis tetrahele*, at a density of ~500,000 cells/mL. As a negative control, three replicate wells contained only sterile seawater. Remaining wells contained deionised water as an additional control.

Wells were overfilled, checked for air bubbles then sealed with transparent PCR film rather than the supplied well caps. This method was developed by Yaschenko *et al.* (2016) in response to the well caps preventing inspection for air bubbles and creating variable pressures that influence oxygen readings. The microplate was placed on the SDR reader and incubated in the dark for a 10-min acclimation period prior to illumination. A 90-min light treatment in light saturated conditions (light intensity = 120 µmol/m²/s) was provided by a NanoTech T5 Reflector light (Sun Blaster, Langley, Canada), followed by a 90-min dark treatment in which an opaque black box was placed over the wells. Oxygen measurements were made every 15 sec during the treatments. The experiment was conducted at room temperature, with the microplate placed in a water bath to maintain constant temperature. Specimens were observed at the end of the experiment to check they were still alive and healthy.

All data analysis was conducted using Rstudio v1.0.143 (Rstudio, Inc, Boston, MA). For analysis, 20-min optimal measurement intervals were chosen from each 90-min measurement period based on having the least temperature fluctuation (with temperature ranges of 0.05°C and 0.04°C for light and dark treatments respectively), in order to eliminate any confounding effect of temperature on oxygen saturation.

The rate of change of oxygen saturation (O₂ mg/L/hr) over the treatment intervals was calculated for each replicate using a linear regression. The average of the negative control slopes was subtracted from the experimental wells to correct for background microbial respiration. Gross
photosynthesis for each replicate was estimated using the formula:
Gross photosynthetic rate = net photosynthesis (oxygen evolution) + respiration. Mean and standard error were calculated and visualised on a bar plot.

3.3 Results

3.3.1 Kleptoplast ultrastructure and position within the host

Plastids were sequestered in the parenchyma (syn. mesenchyme) of *B. solaris* and *P. paranygulgus* specimens and were concentrated dorsally (Figs 7A,B; 8B). In healthy animals, plastids were present at high density, such that interstitial spaces between the organs were almost completely brown (Figs 7A,B; 8A,B). Plastids in worms starved for one week showed strong chlorophyll autofluorescence (Figs 7C; 8C). Plastids in a *P. paranygulgus* specimen starved for 10 weeks maintained strong autofluorescence, though plastids were smaller on average (Fig 8D).

The plastids were stored intracellularly, adjacent to host organelles, including nuclei and mitochondria with flat (lamellar) cristae (Figs 9B; 10A). Often multiple plastids were present in a single cell (Figs 9A,D; 10), closely associated with large lipid droplets. An oval-shaped pyrenoid was sometimes visible, depending on the plane of section (Figs 9A,D; 10B). In *B. solaris*, plastids had thylakoids formed from stacks of three lamellae, and a peripheral girdle lamella surrounded by three membranes (Fig 9C). Membrane resolution was not high enough to confirm the thylakoid ultrastructure in *P. paranygulgus* plastids. No dividing plastids were observed. Some degrading plastids, which appeared black in light micrographs (Fig 7A), were visible (Fig 9D). There was no evidence of either whole symbiont cells or other algal organelles (Figs 9, 10).

3.3.2 Molecular identification of the kleptoplasts

*rbcL* plastid gene sequences from five *B. solaris* specimens (four from Victoria and one from Calvert Island) were identical or had a one base pair difference (p-distance (p) = 0.99). Subjected to BLAST search, they matched most closely (p = 0.96) with species of the genus *Navicula*. Two
distinct sequences (p = 0.9), which matched most closely with species of the genera *Gyrosigma* (p = 0.94) and *Pleurosigma* (p = 0.97) respectively, were retrieved from five *P. paranygulgus* specimens from Mud Bay Park. Identical sequences, which matched most closely with *Gyrosigma* species (p = 0.97), were retrieved from five *P. paranygulgus* specimens from Ladysmith. *Navicula, Gyrosigma*, and *Pleurosigma* are all raphe-bearing pennate diatoms. *rbcL* sequences with multiple peaks were also acquired from some specimens in both species, indicating that the kleptoplasts within one host were derived from different lineages of pennate diatoms.

### 3.3.3 Kleptoplast photosynthetic activity in *B. solaris*

Survival rates in both treatments showed a decline over time, but were higher in light-treated versus dark-treated *B. solaris* specimens after two weeks of starvation. 10% of light-treated specimens survived more than 12 weeks of starvation, and no dark-treated specimens survived more than 8 weeks (Fig 11A). Individuals in both treatments showed loss of plastids over the starvation period, but this loss occurred faster in dark-treated specimens (as shown in two photographed specimens (Fig 12A-F). *B. solaris* replicates showed light-dependent oxygen evolution and a decline in oxygen saturation in the dark, allowing a calculation of mean gross photosynthesis (Fig 10B). Mean photosynthetic activity had a small standard error and was of similar magnitude to a control consisting of a dense chlorophyte phytoplankton culture (*T. tetrahele*).
Fig 7. (A–B) Light micrographs of live *B. solaris* specimens with brown kleptoplasts in the parenchyma between the organs (which appear transparent). e, eye; ph, pharynx; t, testis; vi, vitellarium. (A) Whole animal with some darker, degrading plastids. (B) Anterior half with healthy plastids. (C) Z-stack of confocal laser scanning micrographs showing autofluorescent plastids (green) densely packed within the parenchyma of *B. solaris*. Scale bars = 50 µm.
Fig 8. (A–B) Light micrographs of live *P. paranygulgus* specimens with brown kleptoplasts in the parenchyma between the organs (which appear transparent) taken at (A) a more ventral and (B) a more dorsal plane, demonstrating their dorsal concentration. e, eye; p, plastid; ph, pharynx. (C–D) Z-stack of confocal laser scanning micrographs showing autofluorescent plastids (green) densely packed within the parenchyma of *P. paranygulgus* in (C) a one week-starved and (D) 10 week-starved specimen, which maintained strong chlorophyll autofluorescence. Scale bars: A = 50 µm; B = 50 µm; C = 30 µm; D = 30 µm.
Fig 9. Transmission electron micrographs of *B. solaris*. (A) Epidermis with cilia (black arrowhead). Vacuoles (v) and rhabdites (r) are present apically. Lamina densa (white arrow) and lamina fibroreticularis (grey arrow) of the basement membrane separate the epidermis from underlying muscle layers (black arrow), parenchyma (pa) and plastids (p). (B) Parenchymal cell with nucleus (n), lipid droplet (li), large plastid (p) and mitochondria with lamellar cristae (white arrow). (C) Cell junction (white arrow) between two parenchymal cells, each with a plastid (p) in the cytoplasm. Three membranes (grey arrow) surround plastid girdle lamella (black arrow). (D) Parenchymal cell with four plastids (p) and many lipid droplets (li) in the cytoplasm (cy). Scale bars: A = 2 µm; B = 500 nm; C = 200 nm; D = 2 µm.
**Fig 10.** (A–B) Transmission electron micrographs of parenchymal cells in *P. paranygulgus* with plastids (p), lipid droplets (black arrows) and mitochondria with lamellar cristae (black arrowheads). Scale bars = 2 µm.

**Fig 11.** (A) Bar plot representing photosynthetic activity in 25 *B. solaris* specimens compared with the chlorophytic alga *T. tetrahele* (~500,000 cells/ml). Gross photosynthesis was calculated by summing the net photosynthesis and respiration rates. Data represent means ± standard error. (B) Bar plot showing survival rates (proportion of surviving individuals) of light-treated (*n* = 20) and dark-treated (*n* = 20) *B. solaris* specimens.
Fig 12. (A–C) Light micrographs of dark-treated *B. solaris* specimen showing the loss of plastids after a starvation period of (A) two weeks, (B) three weeks, (C) four weeks. (D–F) Light micrographs of light-treated *B. solaris* specimen showing loss of plastids after a starvation period of (D) two weeks, (E) three weeks, (F) four weeks. Scale bar = 100 µm.

3.4 Discussion

3.4.1 Diatom origins of the kleptoplasts

The identification of the kleptoplasts from previously-unsequenced raphe-bearing pennate diatoms is consistent with the empty pennate frustules observed in the gut of some freshly-sampled *B. solaris* and *P. paranygulgus* specimens.

The absence of a diatom sequence match above p = 0.97 reflects a paucity of available diatom sequences in public databases, in part due to difficulties in identification of diatom species. Species identification and the systematics of diatoms has relied upon subtle differences in frustule morphology (Evans et al. 2007), and in some cases, species differ only in reproductive
compatibility (Hamsher et al. 2011). Furthermore, there is no consensus on a single barcode gene for diatoms (MacGillivary and Kaczmarska 2011). While the partial rbcL barcode has been found to differentiate some pennate diatom species, a number of other barcodes are also in use, such as COI-5P, LSU D2/D3, UPA, ITS-1, ITS2, 18S and psbA (Hamsher et al. 2011; Amato et al. 2007; Hamilton et al. 2015); nevertheless, only the plastid-encoded barcodes rbcL and psbA are available for identifying kleptoplasts.

The partial rbcL sequences from kleptoplasts in B. solaris specimens have a maximum of 1 bp difference. However, it cannot be ascertained that these sequences represent a single species, given that rbcL sequences from different diatom species have been reported with as little as a 1 bp difference (e.g., Hamilton et al. 2015).

While the kleptoplasts from specimens of P. paranygulgus collected from Ladysmith had identical rbcL sequences, two distinct sequences were retrieved from P. paranygulgus specimens collected from Mud Bay Park. This, in addition to the acquisition of rbcL sequences with multiple peaks from all sampled locations, implies that kleptoplasts within a single host originate from more than one diatom species. Continued cloning of PCR products from the kleptoplasts of many more host specimens is required to determine the full breadth of sequence diversity present within these rhabdocoels. Feeding experiments could also investigate whether the worms have a preference for certain diatom species or whether the dominant sequences simply reflect the most seasonally abundant diatoms (which could be determined by environmental DNA sequencing).

The ultrastructure of the plastids support the molecular identification of their pennate diatom origin. Plastids appear brown, indicating presence of fucoxanthin as an accessory pigment, lamellae are formed by stacks of three thylakoids, and a girdle lamella encircles the periphery. All these features are characteristic of heterokont plastids (Bedoshvili et al. 2009).

While diatom plastids have four enveloping membranes (Round et al. 1990), our data show that only three membranes surround the kleptoplasts within B. solaris. The fourth, outermost plastid membrane is continuous with the rough endoplasmic reticulum and the nuclear envelope of the diatom, which is apparently removed during the process of plastid isolation from the diatom prey.
cells. It is likely that the outermost kleptoplast membrane is the third diatom plastid membrane rather than a host membrane derived from phagocytosis; if it were the latter, one would expect the ingested plastids to be degraded (rather than maintained) by phagosome-targeted enzymes.

3.4.2 Kleptoplast sequestration

Some flatworms, including the umagillid rhabdocoel *Wahlia macrostylifera*, sequester cnidocysts (stinging organelles) from their cnidarian prey in the epidermis and/or parenchyma. These so-called kleptocnidiae are selectively endocytosed by amoeboid gastrodermal phagocytes, which are able to migrate from the gastrodermis through the parenchyma, often delivering the cnidocysts to the dorsal epidermis where they can be utilised (Goodheart & Bely 2016). The flexibility of these “wandering cells” likely explains how kleptoplasts are also phagocytosed and transported from gut to parenchyma in rhabdocoels. The mechanism by which stolen organelles are selectively targeted by these cells for sequestration, thus evading digestion, remains unclear.

3.4.3 Kleptoplast function

The results demonstrate photosynthesis in 10 day-starved specimens, confirming functional kleptoplasty in *B. solaris*. The impressive magnitude of photosynthetic activity, which is comparable to a dense chlorophyte algal culture, could explain the enhanced survival rates found in light-treated compared with dark-treated starved specimens. These results suggest that kleptoplasts provide a fitness advantage to starved specimens by supplying fixed carbon, though carbon-tracing experiments must be undertaken to ascertain transport of photosynthate from the kleptoplasts to the host. If the kleptoplasts are naked within the cytoplasm (i.e, the third, outermost membrane is plastid-derived rather than host-derived), then it would be simpler for *B. solaris* cells to gain access to photosynthate.

The anatomy of the host worms facilitates photosynthesis in several ways; a transparent epidermis and a large surface area to volume ratio allow the kleptoplasts to receive sufficient sunlight. Worms appear to be negatively phototactic under high light intensities, which might be a photoprotective adaptation to reduce damage by reactive oxygen species produced by excess
irradiation on plastid photosystems. The shady macroalgal habitat of *B. solaris* provides an external mechanism for regulating the amount of sunlight reaching their tissues, and *P. paranygulgus* may find shade under grains of sediment.

Though photosynthesis-time experiments are yet to be performed, it is clear that photosynthesis is not sustained indefinitely. While healthy-looking kleptoplasts were observed in some worms starved up to 12 weeks, they show gradual degradation and disappearance over time. The abundance of lipid droplets present in kleptoplast-sequestering cells might reflect plastid degradation. The kleptoplasts must be routinely replenished via feeding for photosynthesis to be maintained.

As previously mentioned, plastids possess a reduced genome that is insufficient for autonomous plastid function. The plastid genome of the pennate diatom *Phaeodactylum tricornutum* encodes just 130 proteins (Oudot-Le Secq et al. 2007), and estimates of proteins necessary for plastid function vary from 1000-5000 (Rumpho et al. 2011). Hence, it is unsurprising that kleptoplasts lose function and ultimately degrade within the worms. Digestion of dysfunctional kleptoplasts would prevent the accumulation of reactive oxygen species from damaged photosystem proteins, and provide a lipid-rich food source.

This mixotrophic lifestyle might be an adaptation to periods of starvation through the reliance on diatoms as prey. Many diatom species show considerable seasonal variation in abundance (Round et al. 1990); thus kleptoplasts may serve as a food store that can be digested when diatom blooms collapse in summer and winter.

### 3.4.4 Prevalence and evolution of kleptoplasty in metazoans

Within metazoans, kleptoplasty has evolved convergently in neodalyellid rhabdoocels and sacoglossan sea slugs; both have flat, relatively simple body plans despite their phylogenetic distance. Within the Neodalyellida, it is unlikely that kleptoplasty is confined to the two species investigated here; the ability of *P. paranygulgus* and *B. solaris* to sequester kleptoplasts strongly suggests that the “zooxanthellae” reported in all other *Pogaina* species and *B. evelinae* are
actually plastids, rather than whole symbiont cells. Though their precise relationship is unresolved in the phylogenetic analysis, *Pogaina* and *Baicalellia* are clearly closely related; they share many morphological similarities and both belong to a polytomy within neodalyellid clade 2 (Chapter 2). As they are not sister genera, two evolutionary scenarios are possible: 1) kleptoplasty has evolved convergently in *Pogaina* and *B. solaris*; 2) kleptoplasty is a shared ancestral state that evolved in the last common ancestor of *Pogaina* and *Baicalellia*, with multiple losses in *Pterasticola* and many *Baicalellia* species.
Chapter 4: Conclusions

My sampling campaign of neodalyellids in British Columbia has contributed to knowledge of rhabdocoel species diversity in the Pacific Northeast, providing descriptions of three novel species and updates to two previously-described species. Nine new concatenated 18S/28S rDNA sequences from the five described species and four additional, undescribed species allowed me to improve inferences about the phylogenetic relationships within the Neodalyellida. These results have led to the following conclusions: (1) Confirmation of the paraphyly of the Provorticidae and Neokirgellinae; (2) a double connection to the female reproductive system may be a synapomorphy for clade 1; (3) Vejdosvky and Provorx (both Provorticinae) are sister genera; (4) the basal position of T. kalipis in clade 1 necessitates creation of a new genus Tamanawas n. gen., into which T. halileimonia n. comb. and T. helictos n. comb. are transferred based on shared characters; (5) Coronopharynx, Canetellia and Baicalellia cannot be differentiated based on morphological and DNA sequence data, resulting in suppression of the former two genera in favour of Baicalellia; (6) Baicalellia and Pogaina lack distinguishing diagnostic characters at the morphological level but are not sister genera within the molecular phylogenetic analysis.

As is evidenced by the polytomies in the analysis, many neodalyellid relationships remain uncertain. Increased sampling efforts will not only improve knowledge of the diversity and importance of rhabdocoels in meiofaunal communities, but also provide data to improve molecular phylogenetic inferences and to identify diagnostic characters at the genus level and above. Increased sampling of this group should also give more insight into the prevalence and evolution of kleptoplasty.

*B. solaris* and *P. paranygulgus* sequester the plastids of different lineages of raphid pennate diatom prey cells intracellularly within parenchymal cells. Continued DNA barcoding and cloning along with feeding experiments will help determine the relative diversity and specificity of the host-kleptoplast relationship. Plastids maintain photosynthetic function for at least 10 days in *B. solaris*, but feeding is required to maintain healthy plastids. Mixotrophy may be an adaptation to periodic nutrient limitation due to seasonal diatom fluctuations.
The discovery of kleptoplasty in meiofaunal rhabdocoels opens the door to a wealth of other investigations into the relationship between the hosts and their kleptoplasts. Identification of the precise diatom species origins of the kleptoplasts will allow the establishment of laboratory host cultures, providing the means for many more investigations into kleptoplast longevity and function. Measuring the change in photosynthetic activity in starved specimens over time will provide more information about how long functional kleptoplasts are maintained, and carbon-tracing experiments will confirm transfer of photosynthate from kleptoplast to the host. Transcriptomes sequenced at different stages during starvation and at different light-dark regimes could provide evidence for gene expression involved in the maintenance of kleptoplasts; these data plus a sequenced host genome could also demonstrate horizontal gene transfer of nucleus-encoded diatom genes to the nucleus of the host. Overall, knowledge of kleptoplasts in rhabdocoels contributes to our understanding of the ecological interactions within intertidal meiofaunal communities and represents only the second example of kleptoplasty within animals, the first example involving diatoms, and the first example involving the Platyhelminthes.
References


Kueck, P. 2009. ALICUT: a Perlscript which cuts ALISCORE identified RSS, version 2.0 edn.


## Appendix

Table 1. Amplification primers and internal sequencing primers used in this study.

<table>
<thead>
<tr>
<th></th>
<th>Forward primers (sequence)</th>
<th>Reverse primers (sequence)</th>
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1 95°C for 3m, touch down in 9 cycles (94°C for 30s, 60°C down to 56°C for 30s, 72°C for 1m30s), 31 cycles (94°C for 30s, 55°C for 30s, 72°C for 1m30s), 72°C for 5m

2 95°C for 5m10s, 36 cycles (94°C for 1m, 65°C for 1m, 72°C for 1m30s), 72°C for 5m

3 98°C for 1m, touch down in 9 cycles (98°C for 10s, 56°C down to 50°C for 20s, 72°C for 30s), 35 cycles (98°C for 10s, 49°C for 20s, 72°C for 30s), 72°C for 10m

4 35 cycles (95°C for 210s, 52°C for 30s, 72°C for 90s), 72°C for 10m
Table 2. 18S and 28S GenBank accession numbers, localities and geographic coordinates of the 39 taxa used in the phylogenetic analysis. Taxa in bold are new sequences for this study.

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