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Histophagous ciliate *Pseudocollinia brintoni* and bacterial assemblage interaction with krill *Nyctiphanes simplex*. I. Transmission process

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ABSTRACT: Histophagous ciliates of the genus *Pseudocollinia* cause epizootic events that kill adult female krill (Euphausiacea), but their mode of transmission is unknown. We compared 16S rRNA sequences of bacterial strains isolated from stomachs of healthy krill *Nyctiphanes simplex* specimens with sequences of bacterial isolates and sequences of natural bacterial communities from the hemocoel of *N. simplex* specimens infected with *P. brintoni* to determine possible transmission pathways. All *P. brintoni* endoparasitic life stages and the transmission tomite stage (outside the host) were associated with bacterial assemblages. 16S rRNA sequences from isolated bacterial strains showed that *Photobacterium* spp. and *Pseudoalteromonas* spp. were dominant members of the bacterial assemblages during all life phases of *P. brintoni* and potential pathobionts. They were apparently unaffected by the krill's immune system or the histophagous activity of *P. brintoni*. However, other bacterial strains were found only in certain *P. brintoni* life phases, indicating that as the infection progressed, microhabitat conditions and microbial interactions may have become unfavorable for some strains of bacteria. Trophic infection is the most parsimonious explanation for how *P. brintoni* infects krill. We estimated *N. simplex* vulnerability to *P. brintoni* infection during more than three-fourths of their life span, infecting mostly adult females. The ciliates have relatively high prevalence levels (albeit at <10% of sampled stations) and a short life cycle (estimated <7 d). Histophagous ciliate–krill interactions may occur in other krill species, particularly those that form dense swarms and attain high population densities that potentially enhance trophic transmission and allow completion of the *Pseudocollinia* spp. life cycle.

KEY WORDS: *Collinia* · Histophagous · Apostome ciliates · Euphausiacea · Parasite–host association · Gulf of California

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INTRODUCTION

Although parasites may represent ~40% of species diversity on Earth (Dobson et al. 2008) and attain relatively large biomass in marine ecosystems (Lafferty et al. 2006), the current prevailing perception is that parasitoids, i.e. parasites that must kill their host to complete their life cycle, are rare and have little influence in pelagic marine population hosts. This perspective is gradually changing with the discovery of dinoflagellates that kill and play a major role in controlling phytoplankton blooms (Peacock et al. 2014) and apostome histophagous ciliates that cause epizootias of krill (Gómez-Gutiérrez et al. 2003). Most research on parasitoids that annihilate marine zooplankton, in contrast with those of terrestrial habitats, have mostly focused on taxonomic concerns, rather than their ecology, parasite–host interactions, and transmission and infection mechanisms. To reduce our knowledge gap, we experimentally tested the hypothesis of a trophic infection mechanism of the apostome ciliate *Pseudocollinia brintoni* Gómez-Gutiérrez et al., 2012, an endoparasitic histophagous ciliate of the subtropical krill *Nyctiphanes simplex* Hansen, 1910 (Order Euphausiacea) occurring in the Gulf of California.

Two types of parasitoids are currently known that infect and without exception kill krill: (1) dinoflagellates of the genus *Chytriodinium* that kill eggs of *Meganyctiphanes norvegica* (M. Sars, 1857), as discovered by Dogiel (1906) and detected only in 3 other studies, although this parasitoid is likely not specific to krill (Cachon & Cachon 1968, Daugbjerg et al. 2000, Gómez-Gutiérrez et al. 2009); and (2) apostome histophagous ciliates of the genus *Collinia*, discovered 80 yr later (Capriulo & Small 1986, Capriulo et al. 1991, Gómez-Gutiérrez et al. 2003, 2006). Ciliates of the genus *Collinia* that infect krill were later transferred to the new genus *Pseudocollinia* (Gómez-Gutiérrez et al. 2012a), which at present includes 4 species: *P. beringensis* (Capriulo & Small, 1986); *P. oregonensis* (Gómez-Gutiérrez et al., 2006); *P. brintoni* Gómez-Gutiérrez et al., 2012; and *P. similis* Lynn et al., 2014. This genus may also include unidentified endoparasitic apostome ciliates infecting *Thysanoessa inermis* (Krøyer, 1846) in the northeastern part of the Atlantic Ocean (Kulka & Corey 1984) and *Euphausia superba* Dana, 1850 in the Antarctic Sea (Stankovic & Rakusa-Suszczewski 1996).

Because parasitoids were originally discovered and the term was coined from terrestrial parasite–host arthropods (obligate parasite killers that, like parasitic castrators, have a relatively large size or biomass [3–50%] relative to their hosts, and have a density-independent relationship with their hosts [or a few parasitoid larvae in a host will develop and kill the host]), there currently exists the debate whether *Pseudocollinia* ciliates are parasitoids or micro-parasites (parasites that cause a density-dependent pathology, and the unit of epidemiological interest is the host, not the density of the parasite). In nature, there exists a broad continuum in trophic strategies that challenge assumptions about where one type of inter-specific association ends and another begins in parasite–host interactions (with overlap of distinct criteria) (Parmentier & Michel 2013). Observational evidence, collected since 2000 (Gómez-Gutiérrez et al. 2003, 2006, 2012a, Lynn et al. 2014), indicates that *Pseudocollinia* ciliates are parasitoids. The argument is that (1) they actively cause death of their obligate hosts to continue their life cycle, (2) they progressively transform virtually all host biomass into ciliate biomass during the infection period (feeding by osmotrophy as obligate histophagous ciliates), (3) they have a long relative duration of the host–parasite association sensu Parmentier & Michel (2013), (4) they first infect the hemocoel and obtain energy from the host gonad, considering that sterilization of the host is an ante mortem event for a parasitoid (Lafferty & Kuris 2002, 2009, Parmentier & Michel 2013), and (5) although microparasites can cause death of the host if intensity becomes high (density-dependent pathology), it is clear that, if the host dies, the microparasites may also die or decrease their probability of survival. For simplicity, we will mostly refer to *Pseudocollinia* ciliates as histophagous ciliates that kill their host to complete their life cycle. We are convinced that *Pseudocollinia* ciliates fit well with the general concept of parasitoids (Gómez-Gutiérrez et al. 2003, 2006, 2012a).

Although *Pseudocollinia* ciliates have relatively short life cycles (<7 d), high reproduction rates by palintomy, and transform virtually the entire biomass of infected krill into parasite biomass (Gómez-Gutiérrez et al. 2003, 2006), little research has been conducted to understand the transmission of the parasite (Gómez-Gutiérrez et al. 2012a). Recent efforts to understand the immune system of *E. superba*, such as melanized nodules (Miwa et al. 2008), immune gene expression (Seear et al. 2012), and effects of antimicrobial polypeptides (Zhao et al. 2013), could be useful to understand the response of krill to specific epibionts, pathogens, parasites, and parasitoids.

The first study to report bacteria associated with *Pseudocollinia* infections in krill proposed that *N. simplex* became infected when krill ate *Pseudocollinia*-encysted phoronts, likely adhering to filaments that resemble 'marine snow' (Gómez-Gutiérrez et al.

2012a). Gómez-Gutiérrez et al. (2006, 2012a) and Lynn et al. (2014) rejected the idea of transmission via penetration of the cuticle (as occurs in *Synophrya* infections) and vertical or sexual transmission as the mechanism for *Pseudocollinia* infection because to date no larvae or juvenile krill have been found infected with *Pseudocollinia* ciliates. Exposure of healthy krill to parasitoid ciliates in shipboard experiments also failed to produce infections, suggesting that experimental incubation cannot reproduce the necessary natural conditions of the epipelagic habitat. For this reason, we designed an alternative indirect methodological approach to test for a trophic infection mechanism by comparing the bacterial community from the stomachs of healthy *N. simplex* specimens and the bacteria associated with the cells of *P. brintoni* at all life stages, including those in the krill hemocoel, and identifying bacterial strains using molecular methods. Our goal was to match sequences of 16S rRNA of bacterial strains and natural bacterial communities from the stomachs of healthy krill to use as tags to follow and test the potential trophic infection pathway of *Pseudocollinia* parasitoid ciliates. This is a novel methodological approach that can be used to investigate transmission pathways of other endoparasitic species infecting marine invertebrates.

MATERIALS AND METHODS

Sampling krill

Hydroacoustic surveys, using the SIMRAD split beam (120 kHz frequency), were used to locate aggregations of krill during oceanographic cruises in the Gulf of California in January 2007. Sampling locations of zooplankton are shown in Tremblay et al. (2010) and Gómez-Gutiérrez et al. (2012b); zooplankton was collected during 12 oceanographic cruises carried out between 2004 and 2012 (see Table 1). Methods for collecting krill and ciliates in the Gulf of California were previously described by Gómez-Gutiérrez et al. (2012a,b). *Pseudocollinia*-infected *Nyctiphanes simplex* were analyzed alive (for bacterial study) and preserved in 96% non-denatured ethanol for further bacterial genetic analyses, or frozen for biochemical analyses.

Bacterial strains and molecular operational taxonomic units of 16S rRNA sequences

To test for *Pseudocollinia* trophic infection and to identify changes in bacterial assemblages during the ciliate life cycle, 16S rRNA sequences were obtained by 2 methods.

The first was culture-dependent, where 32 bacterial strains were retrieved from *N. simplex.* Seven bacterial strains were retrieved from the stomachs of healthy *N. simplex* (and 2 from healthy *Nematoscelis difficilis* specimens; both were used as standards for comparison) and 23 from krill infected with *Pseudocollinia*. We also collected 2 samples of bacteria associated with *Pseudocollinia* ciliates outside the host and 1 free-swimming filamentous form. The complete krill stomach or 500 µl of *Pseudocollinia*-infected tissue were immersed in 1 ml of a half-concentration formulation of artificial seawater (Baumann & Baumann 1981). An aliquot of 0.1 ml was used for serial dilutions from the stomach and hemocoel of healthy and infected *N. simplex* and spread on plates with marine agar 2216 (Difco) to cultivate bacteria. Plates were incubated at 20 ± 2.6°C for 48 h; the colonies of bacteria were then counted. Colonies were randomly selected and transferred to fresh medium to obtain axenic (pure) cultures of bacteria. Discrete and distinctive colonial morphologies were isolated and cryopreserved in liquid nitrogen. DNA was extracted (DNeasy Tissue and Blood Kit, Qiagen) to obtain DNA from pure (axenic) bacterial strains and from infected krill tissue. PCR amplifications were performed with a thermal cycler (MJ Mini, Bio-Rad Laboratories). The oligonucleotide primers 341F+GC and 907R were applied to selectively amplify bacteria 16S rRNA segments (up to 465 bp) from environmental DNA of infected krill tissues. PCR amplification of 16S rRNA followed protocols of Muyzer et al. (1998) and López-Cortés et al. (2008). The PCR products that contained the 16S rRNA alleles were separated using denaturing gradient gel electrophoresis (DGGE); their bands were excised and re-amplified for sequencing. Another set of oligonucleotides, viz. Primer A, positions 8–27, and Primer B, positions 1541–1518 (*Escherichia coli* numbering; Giovannoni 1991), was used to amplify bacterial 16S rRNA segments (up to 1500 bp) from isolates of bacterial strains. The PCR reaction mixture was prepared according to López-Cortés et al. (2008). The thermal cycle parameters were modified from Giovannoni (1991), starting with denaturation for 3 min at 94°C; then 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; and extension at 72°C for 7 min.

The second method was a culture-independent approach, where 16S rRNA sequences were retrieved from DGGE band amplification to define molecular operational taxonomic units (MOTUs); DGGE was

constructed using DNA extraction from tissues of 3 *N. simplex* in an early infection state, 4 in a late infection stage, 1 free-swimming ciliate, and 1 filamentous phase; 30 µl of extract with a mean (±SD) concentration of 496 ± 48 ng μl^{-1} was applied to individual lanes (1 mm wide) of a 6% polyacrylamide gel containing a 30–70% chemical gradient of denaturants. For the standard marker, we used an amplicon mixture of pure bacterial strains: *Pseudoalteromonas* sp. strain 10Xb1; *Kytococcus* sp. strain 9X-22; *Shewanella* sp. strain 54Xa1, and *Alteromonas* sp. strain 42Xb4. The mixture was applied in the middle and borders of the gel, using 5 µl at an average concentration of 459 ± 42 ng μl^{-1} of PCR products from each strain of bacteria. The gel was electrophoresed for 16 h at 100 V in the Dcode universal mutation detection system (Bio-Rad) submerged in a tank containing 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA; pH 8.3) that was maintained at a constant temperature of 60°C (López-Cortés et al. 2008). Subsequently, detection was done using the silver staining method (Bassam et al. 1991). DNA bands in the gel were documented with a scanner (Powerlook 2100XL, UMAX Technologies). Well-separated bands were carefully excised and used for re-amplification and sequencing. DNA elution from bands was performed, using the crush-and-soak method (Sambrook & Russell 2006). Re-amplification was performed with primers 341F and 907R, designed by Muyzer et al. (1998). Macrogen provided all sequencing reactions. The bands were sequenced in both directions.

DGGE community analysis and images were processed with Gelcompar II 4.0 software (Applied Maths). A similarity matrix was created using the Dice Similarity Coefficient and the band patterns were clustered, using the unweighed pair group method with arithmetic mean, assuming that each band represents a MOTU and that the band intensity is a proxy of its relative abundance. The Shannon Diversity Index, maximum diversity, evenness, and dominance of each bacterial community were calculated.

Bacterial phylogenetic reconstruction

The 16S rRNA sequences were edited with Chromas Lite 2.01 software (Technelysium) and, using BLAST software, they were compared with the GenBank database to find similar sequences for assignment of genera (Altschul et al. 1997). All sequences were aligned using NAST software (DeSantis et al. 2006). The weighted neighbor-joining tree-building algorithm generated a phylogenetic tree, using the Ribosomal Database Project software (RDP II; http://rdp.cme.msu.edu/treebuilder/treeing.spr; Bruno et al. 2000). *P. brintoni* and the bacteria infecting the *N. simplex* hemocoel and surface of the host (forming filaments) were observed with a scanning electron microscope (SEM; S-3000N, Hitachi High-Technologies) using standard methods (Gómez-Gutiérrez et al. 2003, 2006, 2012a).

RESULTS

We found 62 *Nyctiphanes simplex* specimens infected with *Pseudocollinia brintoni* after analyzing 257 zooplankton samples collected at night (mean of 21 zooplankton collections per cruise). Parasitized krill were detected in 6.2% of the zooplankton collections. From March 2010 to February 2014 (last 5 oceanographic cruises in the Gulf of California), no *N. simplex* or other krill species were parasitized with *P. brintoni* (Table 1). The current perception of the *Pseudocollinia* life cycle (infection route and the associated bacterial assemblage detected by scanning electron microscopy) was first demonstrated by Gómez-Gutiérrez et al. (2006, 2012a). These data are not repeated here. However, because intensity of *Pseudocollinia* ciliates has never been measured, here we estimated, using a flow cytometer, that a krill female (22.4 mm total length, TL) in the advanced stage of infection had >8500 *Pseudocollinia* cells in the hemocoel. This is a conservative estimate because it was not possible to remove all ciliate cells from the krill body. However, it is a preliminary estimate of the magnitude of intensity of this histophagous ciliate–krill interaction. SEM micrographs showed that *Pseudocollinia*-infected tissues of *N. simplex* had abundant rod-shaped bacteria (0.7 µm diameter × 1.5 µm length), which were associated with all stages of the *Pseudocollinia* life cycle (sampled with a micropipette directly from the hemocoel of a live, infected krill; Fig. 1). We used those bacterial assemblages as biological tags to infer how *Pseudocollinia* ciliates entered the hemocoel, comparing bacteria detected in the stomach of healthy krill versus bacteria obtained from the hemocoel of krill infected with ciliates.

Bacterial assemblages to infer the mechanism of *P. brintoni* infection

Bacterial community components associated with krill yielded 12 identifiable genera, using 16S rRNA

Table 1. Proportion of oceanographic stations and average values (in parentheses) where *Nyctiphanes simplex* was infected with the apostome ciliate *Pseudocollinia brintoni* along the southwestern coast of the Baja California Peninsula near Bahía Magdalena (BAMA cruises) and in the Gulf of California (GOLCA cruises). Zooplankton samples were collected with non-quantitative plankton nets at night. Bacterial strain observations and bacteria cultures were done only during the January 2007 cruise

Oceanographic cruise period	Region	No. of stations sampled	Stations with infected krill		No. of infected krill collected
			No.	Percentage	
16 Mar–2 Apr 2004	BAMA	32	3	9.4	5
29 Jun–16 Jul 2004	BAMA	16	2	12.5	5
1–18 Dec 2004	BAMA	35	6	17.1	39
15 Nov–5 Dec 2005	GOLCA	24	1	4.2	1
12–31 Jan 2007	GOLCA	35	4	11.4	4
17 Jul–2 Aug 2007	GOLCA	25	2	8.0	5
11–2 Mar 2010	GOLCA	16	1	12.5	3
24 Sep–8 Oct 2010	GOLCA	18	0	0.0	0
24 Nov–11 Dec 2011	GOLCA	13	0	0.0	0
15–31 Aug 2012	GOLCA	14	0	0.0	0
13–27 Jun 2013	GOLCA	15	0	0.0	0
5–18 Feb 2014	GOLCA	14	0	0.0	0
Total (average)		257 (21)	19	(6.2)	62

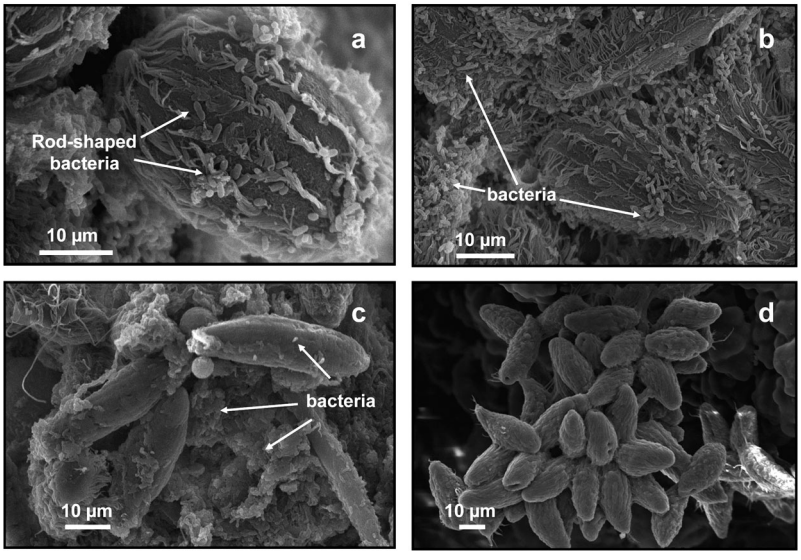


Fig. 1. Scanning electron microscope images showing dense rod-shaped bacteria on the surface of *Pseudocollinia brintoni* cells at 4 ciliate life stages in the krill host *Nyctiphanes simplex*: (a) trophont stage (early infection) in the hemocoel, (b) tomont stage (late infection) in the hemocoel, (c) encysted phoront stage forming bacteria–ciliate filaments under shipboard laboratory conditions, and (d) ciliates forming the mucilaginous filament, apparently with low density of bacteria, suggesting that ciliates are principally responsible for filament formation

from cultures. Several sequences of uncultivable bacteria strains and other sequences were retrieved by culture-independent approaches as indicated by MOTUs (Fig. 2a,b). Bacterial MOTUs in stomachs of healthy krill included *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*. The *Gammaproteobacteria*, mainly strains of *Photobacterium* spp. and *Pseudoalteromonas* spp., dominated the assemblage, proliferating during all *P. brintoni* life phases. Other bacteria from the stomachs of healthy individuals, such as *Roseobacter* (*Alphaproteobacteria*), *Shewanella* (*Gammaproteobacteria*), *Microbacterium*, *Dietzia*, and *Kytococcus* (*Actinobacteria*) were not detected in hemocoels infected with *P. brintoni*. This indicates either that conditions in the hemocoel are unfavorable for proliferation of these strains or that histophagous ciliates can feed selectively on those bacteria (Fig. 2c,d). Assemblages in stomachs of healthy krill were modified by histophagous activity of *P. brintoni*, both in the hemocoel and when ciliates were outside the host (Fig. 2c,d). Thus, other bacteria replaced several bacterial strains or MOTUs, probably better adapted to the new conditions in the hemocoel of infected krill.

The phylogenetic trees including 16S rRNA sequences of all strains and MOTUs found in the subtropical krill *N. simplex* (healthy and infected) and healthy subarctic transitional *Nematoscelis difficilis* (stomach contents treated as controls) are shown in Fig. 3. The sequences of strains and those from the excised DGGE bands were deposited in GenBank (Table 2). Our observations indicate that bacteria from infected hemocoel come from the krill stomach (supporting

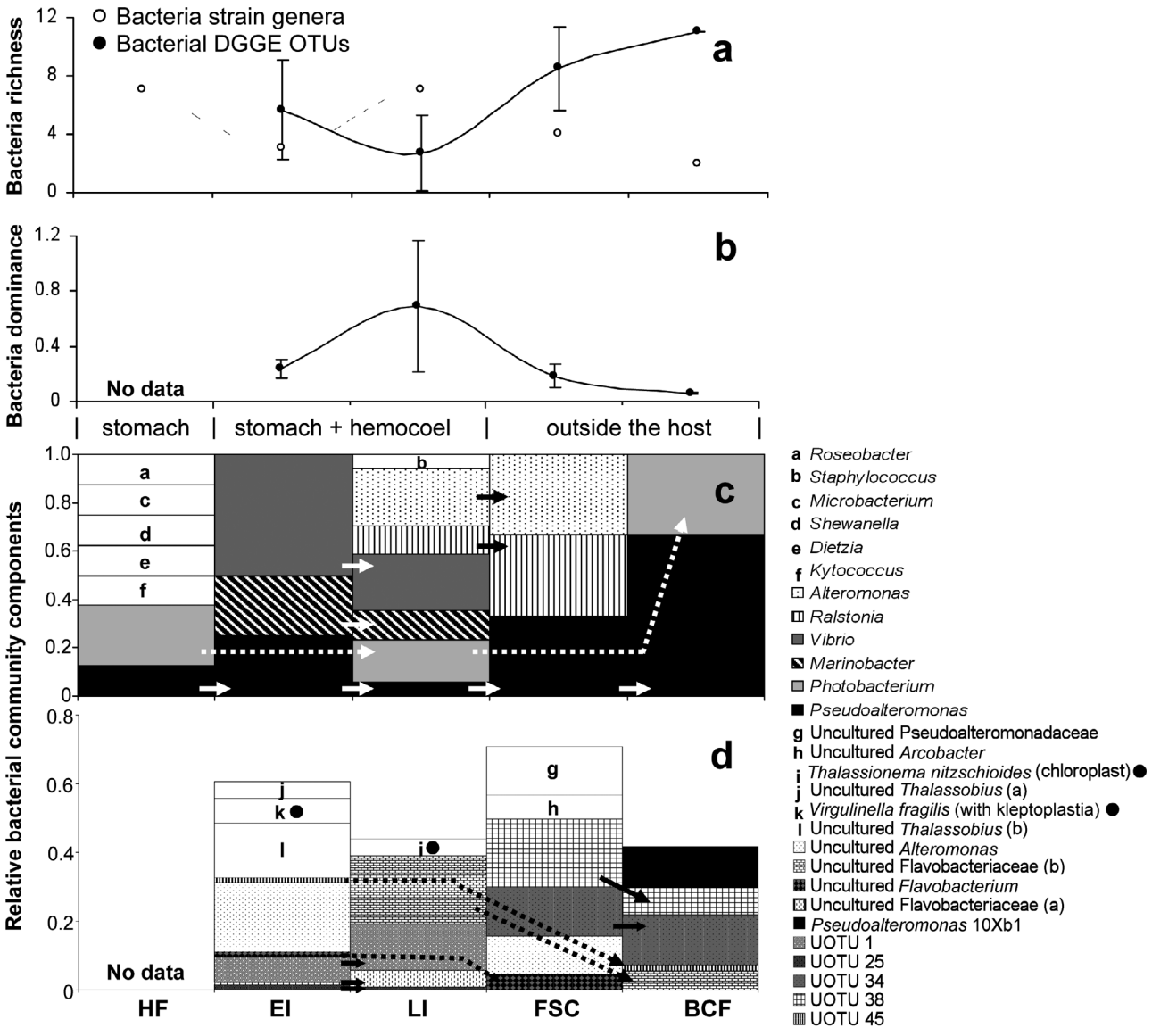
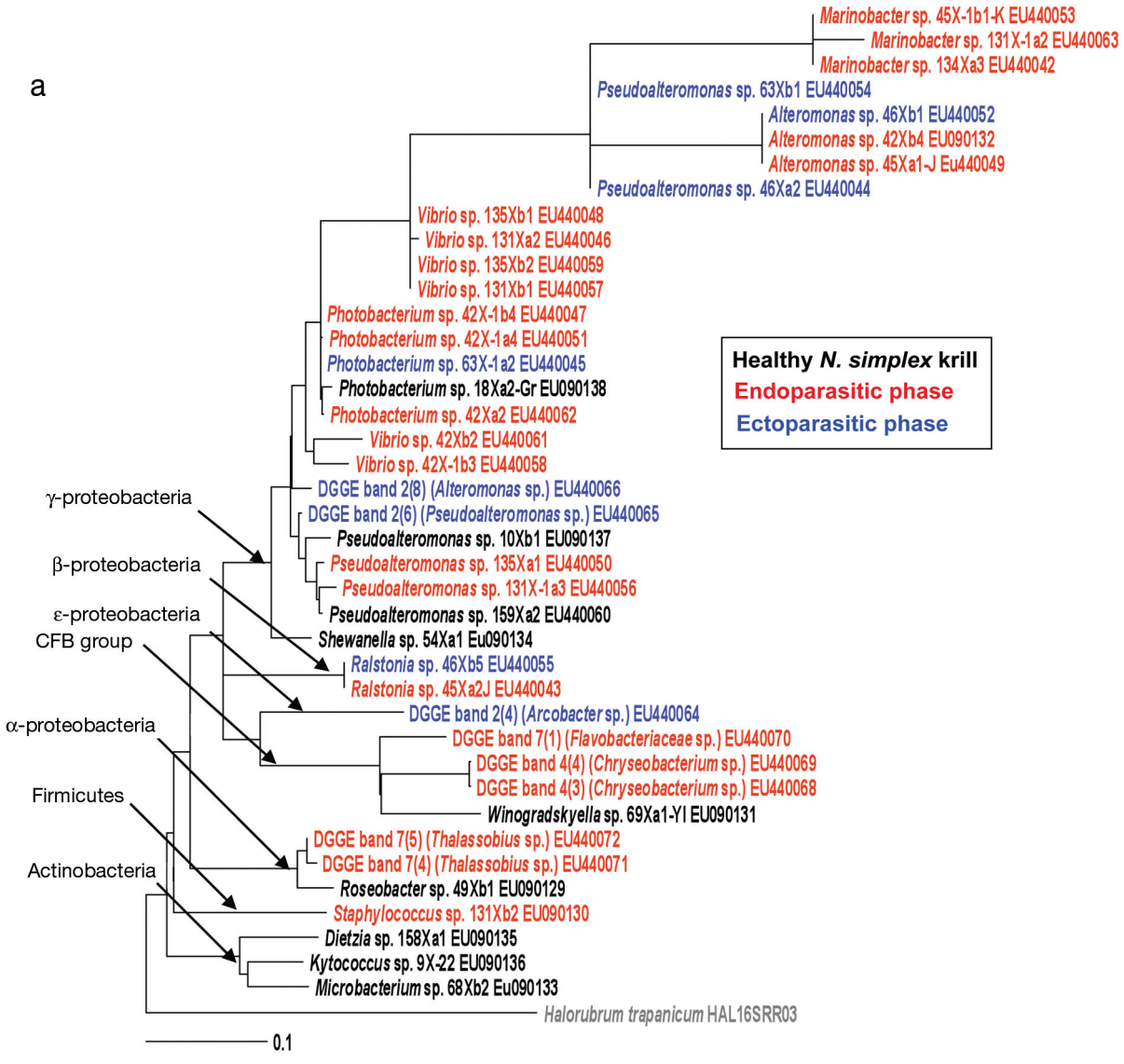
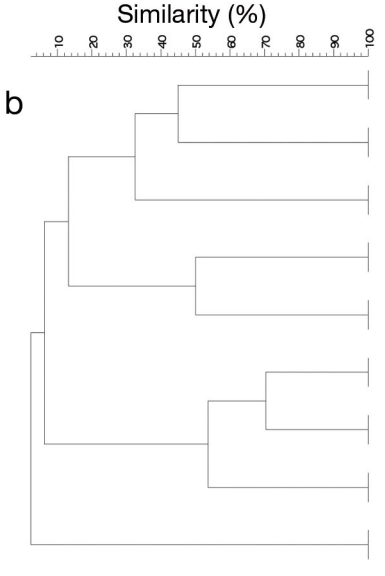


Fig. 2. Succession in the bacterial assemblage in *Nyctiphanes simplex* stomach and hemocoel, comparing healthy female krill (HF) with all parasitoid phases (EI: early infection; LI: late infection; FSC: free-swimming ciliate; BCF: bacteria–ciliate filament). (a) Richness of bacterial strain genera (dashed line) and of molecular operational taxonomic units (MOTUs) obtained by 16S rDNA PCR-DGGE (solid line), (b) Shannon dominance index of MOTUs, (c) relative frequency of bacterial strain genera, and (d) relative abundance of MOTUs. UOTU: unidentified operational taxonomic unit; black dots indicate MOTU sequences of 16S rDNA of eukaryotic chloroplasts (diatoms). Arrows indicate the presence of different bacteria observed in consecutive stages of infection

Fig. 3. Genetic sequences of bacteria in healthy and infected krill. (a) Phylogenetic tree based on 16S rDNA sequence positions 389 through 818 (*Escherichia coli* numbering) for the bacteria and DGGE bands isolated from bacteria from infected and healthy *Nyctiphanes simplex* tissue. The tree was rooted with the archeon *Halorubrum trapanicum* as the out-group (in gray). Code branch label: most related genera, strains, and GenBank accession number for cultured strains or DGGE lane, band number, most related genera, and GenBank accession number for uncultured operational taxonomic units (OTUs). Bacteria isolated from stomachs of healthy krill are shown in black; sequences obtained from the endoparasitic phase are shown in red; and sequences from free-swimming ciliates and ciliate-bacteria filament stages are shown in blue. (b) Cluster analysis of the DGGE separation patterns of bacteria 16S rDNA amplified from infected *N. simplex* tissue, free-swimming ciliates, and filaments. Each lane shows the infection stage of the krill, OTU-richness (*R*), Shannon diversity index (*H*), OTU dominance index (*D*), and Shannon's evenness (*E*). K1 and K2 indicate DNA was extracted from a specific krill. FSC: free-swimming ciliates





Infection stage	*R*	*H*	*D*	*E*
Early infection	4	0.953±0.063	0.688±0.046	0.312±0.046
Late infection	6	1.331±0.005	0.743±0.003	0.257±0.003
Late infection	6	1.644±0.023	0.918±0.013	0.082±0.013
Late infection	1	N/A	N/A	N/A
Early infection ^{K2}	3	0.891±0.042	0.811±0.038	0.189±0.038
Filaments ^{K1}	11	2.252±0.013	0.939±0.005	0.061±0.005
FSC ^{K1}	11	2.131±0.021	0.890±0.009	0.110±0.009
Early infection	10	1.824±0.067	0.792±0.029	0.208±0.029
Early infection	1	N/A	N/A	N/A

Table 2. GenBank accession numbers of the 16S rRNA sequences of isolated bacteria and DGGE bands. The identity percent was assigned with the most related sequence deposited in GenBank. Almost all sequences were obtained from the tissues of female *Nyctiphanes simplex*, showing the strain or the DGGE band lane. In the strain column, numbers outside the parentheses are the DGGE lane numbers and numbers inside the parentheses are the band numbers. Superscripts K1 and K2 (for 2 krill specimens) indicate that bacteria or DNA were extracted from the stomach or hemocoel of that specimen. The other bacteria and DNA were obtained from stomachs or hemocoels, but not from both organs of the same specimen. Nd: *Nematoscelis difficilis* (only healthy specimens); M: *Nyctiphanes simplex* male; all others were *N. simplex* healthy females

Strain sequences / bacterial community sequences (DGGE bands)	GenBank accession no.	Length (bp)	Most related sequence	Identity (%)
Healthy stomach				
Strain 158Xa1	EU090135	1421	*Dietzia* sp. PETBA17	100
Strain 9X-22	EU090136	1423	*Kytococcus sedentarius* DSM 20547	99
Strain 68Xb2	EU090133Nd	1424	*Microbacterium* sp. 3227BRRJ	99
Strain 18Xa2-Gr	EU090138	1457	*Photobacterium* sp. MOLA 61	99
Strain 10Xb1	EU090137	1446	*Pseudoalteromonas* sp. JL1003	99
Strain 159Xa2	EU440060^{M}	846	*Pseudoalteromonas* sp. NBRC 101683	99
Strain 49Xb1	EU090129^{M}	1371	*Roseovarius pacificus* strain 81-2	99
Strain 54Xa1	EU090134	1457	*Shewanella fidelia* KMM3589	99
Strain 69Xa1-Yl	EU090131Nd	1415	*Winogradskyella poriferorum* UST030701-295	100
Early infection (hemocoel)				
DGGE band 11(3)	EU440073	540	*Virgulinella fragilis* W613-28 16S chloroplast	100
DGGE band 7(1)	EU440070	557	Uncultured bacterium clone RESET_28E09	98
Strain 134Xa3	EU440042^{K1}	838	*Marinobacter* sp. strain FO-NAM10	100
DGGE band 7(4)	EU440071	540	Uncultured bacterium clone PROA52B_74	100
DGGE band 7(6)	EU440072	551	Uncultured bacterium clone N8_12_C_6D_46	100
Early infection (stomach)				
Strain 135Xa1	EU440050^{K1}	845	*Pseudoalteromonas tetraodonis* strain NBRC 103034	99
Strain 135Xb1	EU440048^{K1}	847	*Vibrio splendidus* strain GHrC13	100
Strain 135Xb2	EU440059^{K1}	838	*Vibrio splendidus* strain GHrC13	100
Late infection (hemocoel)				
Strain 45Xa1-J	EU440049^{K2}	1441	*Alteromonas macleodii* strain. Balearic Sea AD45	99
DGGE band 4(3)	EU440068	560	Uncultured marine bacterium clone J9-A10	97
DGGE band 4(4)	EU440069	571	Uncultured bacterium clone OMS-B13	97
Strain 45X-1b1-K	EU440053^{K2}	843	*Marinobacter* sp. FO-NAM10	99
Strain 131X-1a2	EU440063	845	*Marinobacter aquaeolei* OC-11	99
Strain 131X-1a3	EU440056	841	*Pseudoalteromonas marina* strain DHY3	100
Strain 45Xa2J	EU440043^{K2}	1434	*Cupriavidus* sp. DPN1	99
Strain 131Xb2	EU090130	1455	*Staphylococcus aureus* subsp. *aureus* ST228	100
DGGE band 4(2)	EU440067	541	*Thalassionema nitzschioides* strain p111 chloroplast[a]	94
Strain 131Xa2	EU440046	845	*Vibrio pomeroyi* strain CAIM 1314	99
Strain 131Xb1	EU440057	846	*Vibrio splendidus* strain GHrC13	100
Late infection (stomach)				
Strain 42Xb4	EU090132^{K2}	1441	*Alteromonas macleodii* strain Balearic Sea AD45	99
Strain 42X-1b4	EU440047^{K2}	847	*Photobacterium* sp. S3901	100
Strain 42X-1a4	EU440051^{K2}	837	*Photobacterium* sp. LC1-200	100
Strain 42Xa2	EU440062^{K2}	847	*Photobacterium* sp. S3901	99
Strain 42X-1b3	EU440058^{K2}	839	*Vibrio fischeri*	99
Strain 42Xb2	EU440061^{K2}	845	*Enterovibrio* sp. IMCC17013	99
Free-swimming ciliates				
DGGE band 2(8)	EU440066^{K2}	466	*Alteromonas* sp. H17	96
Strain 46Xb1	EU440052^{K2}	1448	*Alteromonas macleodii* strain. Balearic Sea AD45	99
DGGE band 2(4)	EU440064^{K2}	541	Uncultured bacterium clone KSTye-VF1-B-003	100
DGGE band 2(6)	EU440065^{K2}	439	*Pseudoalteromonas tetraodonis* strain BH26	97
Strain 46Xa2	EU440044^{K2}	837	*Pseudoalteromonas prydzensis* strain CAIM 381	100
Strain 46Xb5	EU440055^{K2}	1434	*Cupriavidus* sp. DPN1	99
Ciliate–bacteria filaments				
Strain 63X-1a2	EU440045^{K2}	845	*Photobacterium* sp. S3901	100
Strain 63Xb1	EU440054^{K2}	840	*Pseudoalteromonas prydzensis* strain CAIM 381	100

[a]95% similar to the uncultured bacterium clone Tc63Tet2mes (EU290450) isolated from the sponge *Tethya californiana*, but *T. nitzschioides* (94% similarity) is a common diatom in the Gulf of California that we interpret as the most likely sequence

our trophic *Pseudocollinia* infection hypothesis) because of (1) the presence of two *Alteromonas* strains (45Xa1-J and 42Xb4 with 99% similarity in the 16S rRNA sequences) in the stomach and hemocoel of a single infected krill (late infection); (2) 16S rRNA sequences with 99% similarity that are taxonomically assigned to *Photobacterium* (strains 18Xa2-Gr and 42X-1a4) from stomachs of healthy and infected krill (late infection) and filaments (obtained from several krill); (3) the same 16S rRNA sequences of *Vibrio* (strains 135Xb1 and 131Xb1) from the stomach at an early infection stage and the hemocoel at a late infection stage (Table 2); and (4) sequences from DGGE bands that correspond to the 16S rRNA from chloroplasts of the diatom *Thalassionema nitzschioides* (Grunow) Mereschkowsky, 1902 and the foraminifera *Virgulinella fragilis* Grindell & Collen, 1976, found in the hemocoel of infected krill in the early and late stages (Fig. 2d, Table 2), which indicate transfer of undigested contents from the stomach to the hemocoel at the early and late infection stages in krill (Fig. 2). Overall, the similarities of bacterial strains detected in stomachs of healthy krill and bacterial strains detected in the hemocoel of krill infected with *P. brintoni* ciliates provide indirect evidence that ingested ciliates inhabit the krill stomach and later colonize the krill hemocoel when *P. brintoni* enter the hemocoel by drilling through some part of the krill's digestive system (Fig. 2, Table 2).

DISCUSSION

We specifically tested the hypothesis of a *Pseudocollinia brintoni* trophic infection mechanism by comparing bacterial assemblages in the stomach of healthy krill and the hemocoel of parasitized krill. We obtained molecular evidence that at least part of the bacterial assemblages in the hemocoel was similar to those in the stomach of healthy krill, suggesting that when *Pseudocollinia* ciliates infect the krill, bacteria in the hemocoel use a trophic infection mechanism. A similar case occurs when the opportunistic bacteria *Psychrobacter* and *Pseudoalteromonas* infect the Antarctic krill *Euphausia superba*, causing black spots (melanized nodules) on the cuticle (Miwa et al. 2008). Although we never detected black spots in infected or healthy *N. simplex* specimens, we think bacteria can reach the hemocoel when *P. brintoni* ciliates infect the krill, most likely when the krill eat the free-swimming or filament–ciliate phase. Observed bacteria in krill stomachs and hemocoel may be pathobionts (resident microbes with pathogenic potential) that can be beneficial, but under certain circumstances, can cause disease and/or death (Parmentier & Michel 2013). If *Pseudocollinia* colonization is related to pathogen or bacterial colonization at molting, it is highly unlikely that *Pseudocollinia* infection would be biased to female infection because both sexes have similar intermolt periods and relatively similar growth rates (Gómez-Gutiérrez et al. 2012b). Although overall little is known about krill immune systems, Zhao et al. (2013) obtained and purified an antimicrobial polypeptide from *E. superba* that could destroy the cell cytoplasmic membrane and could inhibit cell division at the logarithmic phase against the pathogenic bacteria *Staphylococcus aureus.*

When infected krill die, *Pseudocollinia* ciliates leave the empty carcass and begin to encyst, forming a ciliate–bacteria filament (Gómez-Gutiérrez et al. 2006, 2012a). This is an atypical place for ciliate encystment, since apostome phoronts (cysts of other better known taxa like species of the family Foettingeriidae) are usually located on the surface of their crustacean hosts and more typically attach to the setae of their appendages (Bradbury 1994, Landers et al. 2006). Our shipboard observations suggest that 4 previously postulated apostome ciliate infection mechanisms for decapods and copepods, viz. (1) cuticle penetration (*Synophrya* spp. or *Terebrospira* sp.), (2) infection through mechanical wounds (Bradbury & Goyal 1976, Johnson & Bradbury 1976, Bradbury 1994), (3) vertical transmission from progenitors to offspring, thus far never observed in *Pseudocollinia* infection in larvae and juveniles (Gómez-Gutiérrez et al. 2006, 2012a), and (4) sexual infection of males with phoronts in the appendages to females though the gonopore, do not satisfactorily explain how *Pseudocollinia* infects krill (Gómez-Gutiérrez et al. 2006, 2012a, Landers et al. 2006). The phoront cysts that usually attach to krill appendages were previously believed to be *Pseudocollinia* phoronts; however, they are actually epibiont apostome ciliates of the genus *Gymnodinoides* that feed on exuvial fluids after the krill molt (exuviotrophic ciliates; Landers et al. 2006). Although krill cannibalism could be a possible infection vector, it is unlikely to be the transmission pathway that causes *Pseudocollinia* epizootic events, such as the occurrence observed in Oregon (Gómez-Gutiérrez et al. 2003).

Euphausia pacifica actively eat marine snow (>0.5 mm diameter), even when other sources of food are available (Dilling et al. 1998, Dilling & Brzezinski 2004). *Thysanoessa inermis* and *T. raschi* are also suspected to consume detrital sources (Falk-Petersen

1981). *N. simplex* may eat filaments that are densely ridden with *Pseudocollinia* phoronts, which is similar to marine snow or detritus material. This hypothesis would explain the initially high density of phoronts with a rapid excystation process to trophont cells during the early infection stage. However, previous shipboard experiments that exposed healthy, starved female krill and other potential zooplanktonic crustacean female hosts (holoplanktonic decapods and mysids) to *Pseudocollinia* filaments were unsuccessful (Gómez-Gutiérrez et al. 2012a). This suggests that the shipboard conditions cannot mimic the habitat where *Pseudocollinia* completes its life cycle. Although it is unknown whether this parasitoid ciliate infects an intermediate host, our observations indicate that krill are the definitive host for these apostome ciliates and the host where *Pseudocollinia* ciliates likely spend most of their life cycle (long relative duration of association).

In an attempt to find which life phases are most frequently infected with *Pseudocollinia* ciliates, and to assess the relative potential vulnerability during the krill life cycle, we explored the possibility of a trophic infection mechanism since species of the genus *Nyctiphanes* change their feeding habits as they grow (Kanaeva & Pavlov 1976, Ritz et al. 1990). Although krill can shrink from molt to molt when exposed to unfavorable thermal or feeding conditions (Marinovic & Mangel 1999, Shaw et al. 2010, Gómez-Gutiérrez et al. 2012b), the adult life span of *N. simplex,* estimated with a modified von Bertalanffy growth model from Lavaniegos (1992), is between 61 and 389 d (9–19.2 mm TL; our largest *N. simplex* ever recorded) (Fig. 4a). To date, only large adult females in the population were found to be parasitized with *P. brintoni* (Gómez-Gutiérrez et al. 2012a), suggesting that they are the most vulnerable segment of the population (Fig. 4a,b). Although most parasitoids attack juvenile hosts, some parasitoids, including mermithid nematodes, Nematomorpha, fecampiid flatworms, Microspora, some braconid Hymenoptera, and some Diptera, consume adult hosts (Lafferty & Kuris 2009). Hence, *N. simplex* females, with a sac-spawning reproductive strategy, are vulnerable to *P. brintoni* infection for more than three-fourths of their potential life span, from individuals at first maturity to the maximum size recorded (Fig. 4a,b). Within the *Pseudocollinia* life cycle, this is a long relative duration for a parasite–host association (Parmentier & Michel 2013). The preference of *Pseudocollinia* to infect females is also true for krill species with a broadcast spawning strategy. This sex bias prevalence has been previously discussed (Gómez-Gutiérrez et al. 2006, 2012a), and it is still unclear whether it is caused by distinct intersex behavior or, as proposed in Gómez-Gutiérrez et al. (2015, this issue), that differences in fatty acid content of the sexes favor *Pseudocollinia* development in females. Shipboard incubations carried out in different experiments with temperature ranging between 16 and 18°C indicate that the *P. brintoni* endoparasitic phase is considerably shorter (~36%) than the life phase outside the host (~64%; Fig. 4c). After the tomite cells kill the krill, they form ciliate–bacteria filaments, which under laboratory

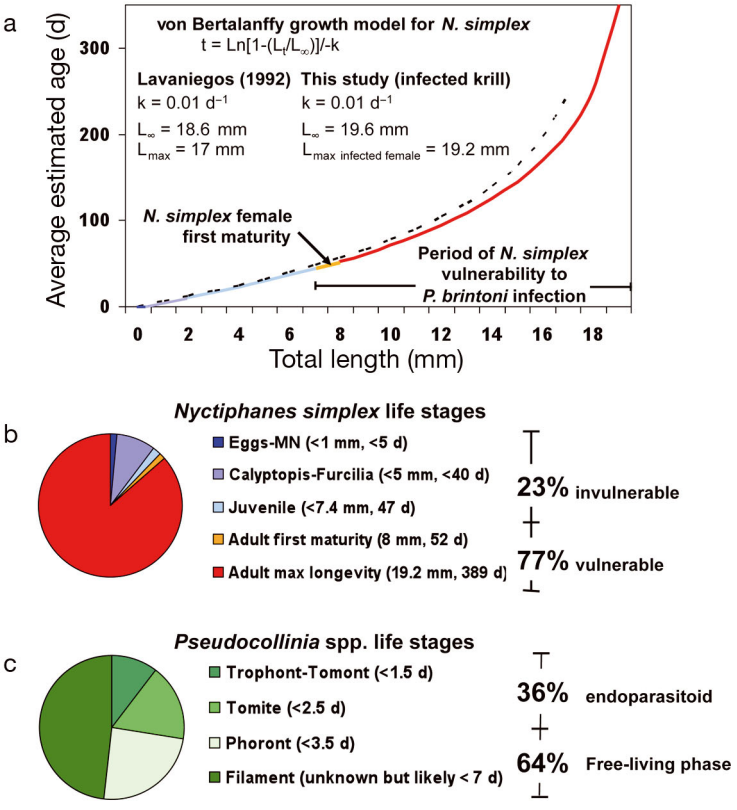


Fig. 4. Conceptual model of the life span of the *Pseudocollinia brintoni* infecting the subtropical sac-spawning krill *Nyctiphanes simplex*. All ciliate life stages and rates of development were observed, showing observations of the host life sequence stages of krill during shipboard incubation. (a) Von Bertalanffy growth models to estimate *N. simplex* average age, as a function of body length (neglecting potential body shrinkage), modified from Lavaniegos (1992). Relative duration of each developmental stage of (b) *N. simplex* and (c) *Pseudocollinia brintoni*. The duration of the encysted (phoront) stage of filaments in nature is still unknown

conditions lasted at least 7 d. The time phoront cells can survive before infecting a new host is still unknown.

The complex *P. brintoni*, bacteria, and *N. simplex* interaction that we propose is likely to represent the life cycle that occurs in the other 7 *Pseudocollinia*–krill interactions known for abundant krill species in the northeastern Pacific, northwestern Atlantic, and Antarctic Sea (Kulka & Corey 1984, Capriulo & Small 1986, Capriulo et al. 1991, Stankovic & Rakusa-Suszczewski 1996, Gómez-Gutiérrez et al. 2003, 2006, 2012a, Lynn et al. 2014). Four of these krill species (*E. pacifica*, *E. superba*, *T. inermis*, and *T. raschi*) are the target of a commercial fishery for animal and human consumption (Nicol & Endo 1999). They form massive swarms and are consumed by many predators, including blue whales, fin whales, and commercially harvested fish, such as salmon and hake, as well as a variety of seabirds.

Parasitoid ciliates of krill may be analogous to trophically transmitted castrators or predators since they decrease the victim's fitness to zero (Lafferty & Kuris 2002, 2009). Since krill cannot physiologically defend against *Pseudocollinia* infections, the relatively small detection rate of these parasitoids (<10%) among all zooplankton samplings of the same regional population suggests that *Pseudocollinia* ciliates have high transmission success within a krill swarm, but not between krill swarms (highly aggregated). Krill social behavior is apparently needed for completion of the parasite life cycle at a micro-scale (infection within the swarm); however, social aggregation seems to cause highly heterogeneous and patchy krill distribution patterns at a mesoscale level that result in an adaptive strategy to deter parasitoid infection at larger spatial scales (distances between krill swarms) (Kuris et al. 1980, Hamner 1984, Ritz 1994, Ritz et al. 2011). Because krill form aggregations with highly heterogeneous geographical distributions, aggregations may protect them against predators, parasites, and parasitoids at the mesoscale level (infection between aggregations), but facilitate horizontal infection of specimens within the infected swarm. Parasitoids can infect neighbors of krill in a dense swarm, but in theory, ciliate transmission stages have relatively less success at infecting individuals in new aggregations. Otherwise, parasitoids would be broadly distributed, like apostome exuviotrophic ciliates that infect >70% of krill populations (Landers et al. 2006), where evidence indicates that *Pseudocollinia*-infected krill are highly concentrated and heterogeneously distributed (Capriulo et al. 1991, Gómez-Gutiérrez et al. 2003, 2006, 2012a). This hypothesis agrees with the idea that swarming (individuals swimming in different directions) and schooling (individuals swimming coordinately in the same direction) behavior in invertebrates and fish is highly adaptive (Ritz 1994, Ritz et al. 2011), even if it might be dangerous when parasitoids infect individuals in a krill swarm or school. In waters of northwestern Mexico, abundance of the subtropical krill *N. simplex* is 6 to 22 times higher than the second-most abundant temperate krill *Nematoscelis difficilis*. *N. simplex* has a more diverse assemblage of parasites (5 ectoparasites, 1 mesoparasite, and 6 endoparasites) than *N. difficilis* (4 known ectoparasites; Gómez-Gutiérrez et al. 2010) and 1 endoparasite (Morales-Ávila et al. 2015). This diversity and host abundance suggests that *Pseudocollinia* ciliates (and other endoparasites) can successfully complete their life cycles by preferentially infecting the most abundant krill hosts, those that typically form massive swarms. We speculate that krill abundance and behavior may impose co-evolutionary interactions between krill and parasitoid ciliates. Given the low prevalence per sampling station, one could argue that krill are quite successful in defending against this parasite except under certain conditions — females with large biomass vulnerable to *Pseudocollinia* infection. What is clear is that when a krill has a beige carapace (early infection), it cannot stop the *Psedocollinia* population growth inside the host and dies in a short time frame.

Co-diversification of 4 *Pseudocollinia* species and 6 krill species in the northeastern Pacific was recently rejected (Lynn et al. 2014), suggesting that *Pseudocollinia* ciliates may infect any krill species worldwide, but probably with better chances to complete their life cycle by infecting the regionally most abundant krill species.

To date, *Pseudocollinia* ciliates have been rarely detected in krill species that typically have low population abundance, such as *Thysanoessa gregaria* (Gómez-Gutiérrez et al. 2006), perhaps because they do not achieve a threshold in biomass to support a parasitoid life cycle strategy or they are not present in sufficiently large biomasses to enable the ciliates to find new hosts. All of our investigations suggest that the *Pseudocollinia*–krill association is more widespread in the order Euphausiacea than previously recognized (Gómez-Gutiérrez et al. 2003, 2006, 2012a). Current evidence indicates that *Pseudocollinia* also infect krill in the northwestern Atlantic (Lynn et al. 2014), erroneously identified as sporozoans when discovered in this region (Kulka & Corey 1984) and very likely in the Antarctic Sea, infecting

the Antarctic krill *E. superba* (reported as 'unidentified endoparasitic ciliates...that may have a negative (lethal) consequence for the host'; Stankovic & Rakusa-Suszczewski 1996). Thus, currently it is known that parasitoid ciliates infect at least 8 krill species in 3 genera and infect species with broadcast- and sac-spawning reproductive strategies (Lynn et al. 2014). These overlooked parasitoids may exert population control at scales from the swarm to the species level and should be investigated in other krill species, particularly those that attain huge biomass standing-stocks and tend to form dense swarms that play a significant part in the trophic pathways of pelagic ecosystems or of commercial interest. Our observations of *Pseudocollinia* causing massive epizootic events leading to sinking carcasses that accumulate on the seafloor (Gómez-Gutiérrez et al. 2003) might explain the phenomenon of massive krill 'dead body rain' that serves as a main food source for abyssal benthos (mostly ophiuroids) observed in the South Atlantic (Sokolova 1994). The physiology, life cycle, and impact of parasitoid ciliate marine zooplankton in the pelagic realm deserve continued investigation.

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