Article

No Tail No Fail: Life Cycles of the Zoogonidae (Digenea)

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Abstract: The Zoogonidae is the only digenean family where known cercariae lack the tail but actively search for the second intermediate host. However, the data on the zoogonid life cycles are scarce. In the present study, we elucidated and verified life cycles of the Zoogonidae from the White Sea. Using rDNA data, we showed that *Pseudozoogonoides subaequiporus* utilizes gastropods from the family Buccinidae as the first intermediate host and protobranch bivalves as the second one. This life cycle can be facultatively truncated: some cercariae of *P. subaequiporus* encyst within the daughter sporocysts. Molecular data also confirmed previous hypotheses on *Zoogonoides viviapus* life cycle with buccinid gastropods acting as the first intermediate hosts, and annelids and bivalves as the second intermediate hosts. We demonstrated the presence of short tail primordium in the developing cercariae of both species. Based on the reviewed and our own data, we hypothesize that the emergence of tailless cercariae in the evolution of the Zoogonidae is linked to the switch to non-arthropod second intermediate hosts, and that it possibly happened only in the subfamily Zoogoninae. Basally branching zoogonids have retained the ancestral second intermediate host and might have also retained the tail.

Keywords: cercariae; metacercariae; Zoogonidae; *Pseudozoogonoides subaequiporus*; *Zoogonoides viviapus*; Buccinidae; life cycles; evolution; White Sea

1. Introduction

Life cycles of the Digenea are complex, typically with two or three hosts, alteration of reproductive modes and two free-dwelling larval stages, miracidium and cercariae. Locomotion of miracidia is provided by ciliated epithelial cells. Cercariae lack cilia and move using their tail—a specialized muscular hind part of the body. The tailed cercaria is most likely a plesiomorphic condition for the Digenea [1–3].

Cercarial tails are diverse in structure and function, depending on the specific transmission pattern. The tail gets modified to facilitate swimming (in the Heterophyidae and Lepocreadiidae [4–6]) and floating (in the Diplostomidae and Cyathocotylidae [7,8]), to attract the next host (in the Bivesiculidae and Azygiidae [9]) and even to penetrate the second intermediate host (in the Hemiuroidea [10,11]). Alternatively, in a number of the digenean taxa there is a clear evolutionary trend towards tail reduction. In the Opecoelidae and Lepidapedidae, tails are very short and used for attachment, not locomotion [12,13]. Tails are also reduced in the cercariae of the digeneans with secondary dixenous life cycles. Those cercariae do not leave the intermediate host and transform into infective metacercariae therein [14–16].

A special case is the family Zoogonidae, parasites of marine and freshwater fish [17]. Known zoogonid cercariae lack the tail, but they leave the first intermediate host and actively crawl over the substrate in a leech-like manner to find and infect the second intermediate host which is typically a slow-moving benthic invertebrate [18,19]. However, our

general knowledge on the zoogonid life cycles stays rather poor since they are completely reconstructed only for five species [19–23]. The question is: how do the Zoogonidae, having such a bizarre cercariae strategy, thrive as a cosmopolitan parasite group, abundant in various communities, from the intertidal to the abyssal zone [24,25]?

The main goal of this study was to elucidate and verify the life cycles of the Zoogonidae from the White Sea: *Pseudozoogonoides subaequiporus* (Odhner, 1911) and *Zoogonoides viviparus* (Olsson, 1868). The second objective was to study the development of the zoogonid cercariae to check upon the tail primordium. Finally, we aimed to review published data on the Zoogonidae life cycles and speculate on the possible premises of the tailless cercariae emergence.

2. Materials and Methods

2.1. Sampling

Sampling was conducted at the Keret Archipelago, White Sea, in 2018–2021. We examined four fish species to find maritae of the Zoogonidae: Atlantic wolffish *Anarhichas lupus* Linnaeus, 1758, common dab *Limanda limanda* (Linnaeus, 1758), Arctic flounder *Liopsetta glacialis* (Pallas, 1776) and European flounder *Platichthys flesus* (Linnaeus, 1758). To detect sporocysts, cercariae and metacercariae of zoogonids we dissected a variety of subtidal invertebrates listed in Table 1.

Table 1. List of the dissected White Sea benthic invertebrates and their infection with zoogonid life cycle stages.

Host			Number of Dissected Individuals	Zoogonid Infection
Gastropoda	Buccinidae	*Buccinum undatum* Linnaeus, 1758	2174	+
		B. scalariforme Møller, 119	19	+
		Neptunea despecta (Linnaeus, 1758)	103	+
	Naticidae	*Cryptonatica affinis* (Gmelin, 1791)	270	-
		Euspira pallida (Broderip & GB Sowerby I, 1829)	24	-
		Amauropsis islandica (Gmelin, 1791)	3	-
	Capulidae	*Ariadnaria borealis* (Broderip & G. B. Sowerby I, 1829)	28	-
	Margaritidae	*Margarites helicinus* (Phipps, 1774)	17	-
	Muricidae	*Boreotrophon clavatus* (G. O. Sars, 1878)	8	-
Annelida	Nereididae	*Nereis pelagica* Linnaeus, 1758	11	-
	Pectinariidae	*Cistenides hyperborea* Malmgren, 1866	9	-
	Nephtyidae	*Nephtys* sp.	8	+
	Polynoidae	*Lepidonotus squamatus* (Linnaeus, 1758)	5	-
	Orbiniidae	*Scoloplos armiger* (Müller, 1776)	4	-
	Trichobranchidae	*Terebellides stroemii* Sars, 1835	1	-
	Lumbrineridae	*Scoletoma fragilis* (O.F. Müller, 1776)	1	-

Table 1. *Cont.*

Host			Number of Dissected Individuals	Zoogonid Infection
Bivalvia	Mytilidae	*Musculus discors* (Linnaeus, 1767)	31	-
	Cardiidae	*Serripes groenlandicus* (Mohr, 1786)	66	+
		Ciliatocardium ciliatum (Fabricius, 1780)	39	+
	Nuculanidae	*Nuculana pernula* (O. F. Müller, 1779)	394	+
	Yoldiidae	*Yoldia hyperborea* (Gould, 1841)	78	+
		Portlandia arctica (Gray, 1824)	6	-
	Nuculidae	*Ennucula tenuis* (Montagu, 1808)	5	-
Decapoda	Paguridae	*Pagurus pubescens* Krøyer, 1838	12	-
	Pandalidae	*Pandalus montagui* Leach, 1814	8	-
	Thoridae	*Eualus gaimardii* (H. Milne Edwards, 1837)	4	-
	Crangonidae	*Crangon crangon* (Linnaeus, 1758)	2	-
		Sabinea septemcarinata (Sabine, 1824)	1	-
Ophiuroidea	Ophiuridae	*Ophiura robusta* (Ayres, 1852)	67	-
	Ophiopholidae	*Ophiopholis aculeata* (Linnaeus, 1767)	40	-

2.2. Morphological Analysis

For the whole mounts, zoogonid maritae and metacercariae extracted from the cyst were heat-killed and then fixed in 96% ethanol. We used a pair of insect pins to rupture the cyst wall and extract metacercariae. Sporocysts and cercariae were fixed in Shaudin's solution, or mercury (II) chloride with acetic acid, or Zenker's solution for whole mounts and histological study. To identify mucoid substances, we used metachromatic staining with toluidine blue. Acetic carmine staining was used to visualize general structure. Histological sections were stained with Azur II-eosin. All procedures were the same as previously described [26]. Whole mounts were observed using a compound microscope Leica DM 2500 (Leica Microsystems, Wetzlar, Germany), and photos were taken with a Nikon DS Fi3 camera (Nikon, Tokyo, Japan). Measurements were conducted in Fiji software [27]. All measurements are in micrometers, the range of values is followed by the mean in parentheses. Visualization of flame cells arrangement, digestive and muscular systems in cercariae was performed by means of confocal laser scanning microscopy (CLSM). Specimens were fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) for 6–24 h, then washed in PBS and stored in PBS with 0.1% sodium azide (NaN_3) at 4 °C. Specimens were stained with TRITC-labeled phalloidin (Sigma Aldrich, Darmstadt, Germany, P1951) in PBS (500 ng/mL), incubated for 60 min and washed in PBS for 20 min. Finally, specimens were mounted in Fluoroshield™ medium with DAPI (Sigma Aldrich, Darmstadt, Germany, F6057) and examined under the CLSM Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany).

2.3. Molecular Analysis

For molecular analysis, we utilized ethanol preserved samples. Eighteen isolates of putative and identified zoogonids were used (Table 2). To extract DNA, samples were taken from 96% ethanol and dried completely, incubated in 200 µL of 5% Chelex® 100 resin (Bio-Rad, Hercules, California, USA) solution with 0.2 mg/mL of proteinase K at 56 °C for 4 h, then kept for 8 min at 90 °C and centrifuged at $16,000 \times g$ for 10 min. The supernatant containing DNA was then transferred to a new tube and stored at −20 °C. Several primers were used to amplify fragments of the ribosomal operon (Table 3). Amplifications were performed in 20 µL reaction mixture containing 4 µL of ScreenMix-HS (Evrogen, Moscow,

Russia), 0.5 μL of forward and reverse primers (10 pmol/μL), 2 μL of the DNA and 13 μL of PCR grade water (Evrogen, Moscow, Russia). PCR products were stained with ethidium bromide and visualized through electrophoresis on a 1% agarose gel. Sequencing was carried out with PCR primers on an ABI Prism 3500xl genetic analyzer (Applied Biosystems, Waltham, Massachusetts, USA). To analyze and edit chromatograms and to build alignments, we used Geneious Prime 2022.0.1 (https://www.geneious.com (accessed on 10 November 2022)). We used three datasets: the first one to link the life cycle stages (28S and 5.8S+ITS2 rDNA), the second and the third ones to resolve the phylogenetic position of *Pseudozoogonoides subaequiporus* (28S rDNA). All the information on GenBank sequences used in the second and the third datasets is listed in Supplementary Table S1. The maximum likelihood (ML) analysis was conducted for all datasets at the CIPRES Science Gateway (https://www.phylo.org (accessed on 10 November 2022)) using RAxML [28] with 1000 bootstrap iterations. The stability of clades was assessed using non-parametric bootstrapping with 1000 pseudoreplicates. The Bayesian analysis was conducted for the second and the third datasets using MrBayes v.3.2.6 [29] with 1,000,000 generations. The model of nucleotide substitution was estimated as GTR+G (28S) and K2+G (5.8S+ITS2) for the first dataset (Figure 1), as GTR+G+I for the second dataset (Supplementary Figure S1) and as GTR+G for the third dataset (Figure 2), using JModelTest [30] in MEGA 11 [31] under the maximum likelihood model.
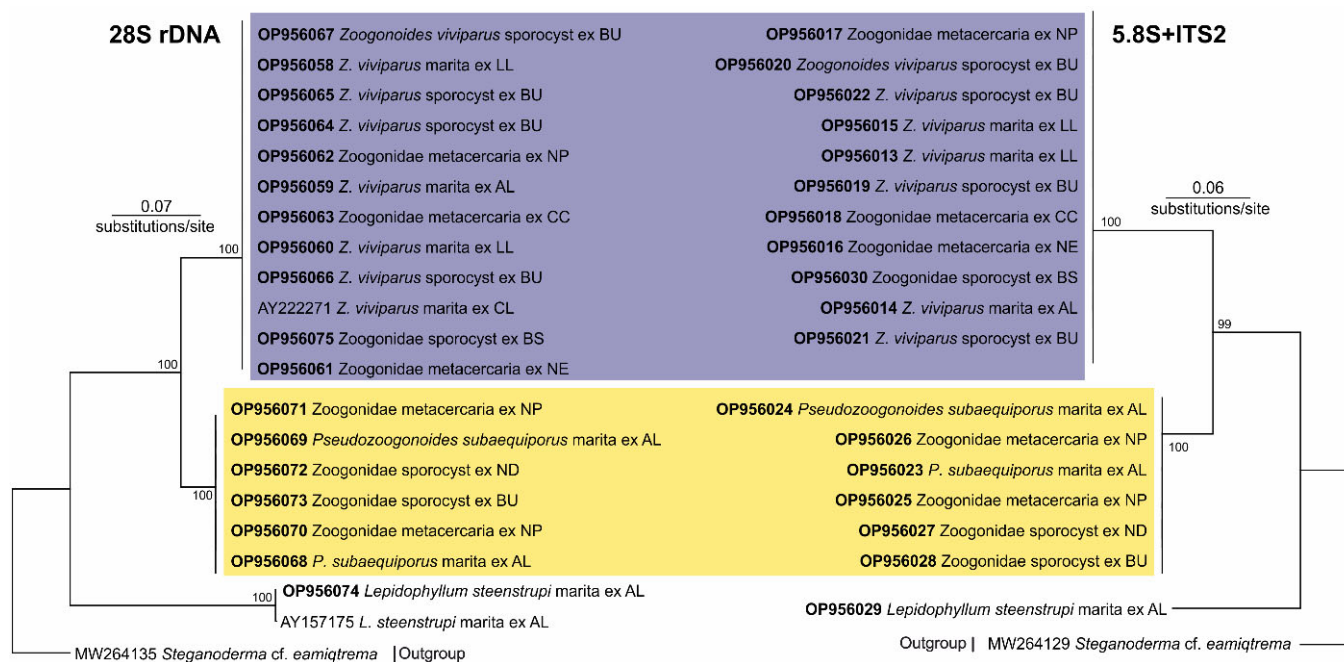

Figure 1. Maximum likelihood phylogenetic trees based on the 28S and 5.8S+ITS2 rDNA sequence data. Bootstrap values are printed in nodes. GenBank accession numbers of newly generated sequences are indicated in bold. Blue and yellow squares mark life cycle stages of *Zoogonoides viviparus* and *Pseudozoogonoides subaequiporus*, respectively. Scale bar shows the substitution rate. Abbreviations: AL—*Anarhichas lupus*; BU—*Buccinum undatum*; BS—*Buccinum scalariforme*; CC—*Ciliatocardium ciliatum*; CL—*Callionymus lyra*; LL—*Limanda limanda*; ND—*Neptunea despecta*; NE—*Nephtys* sp.; NP—*Nuculana pernula*.
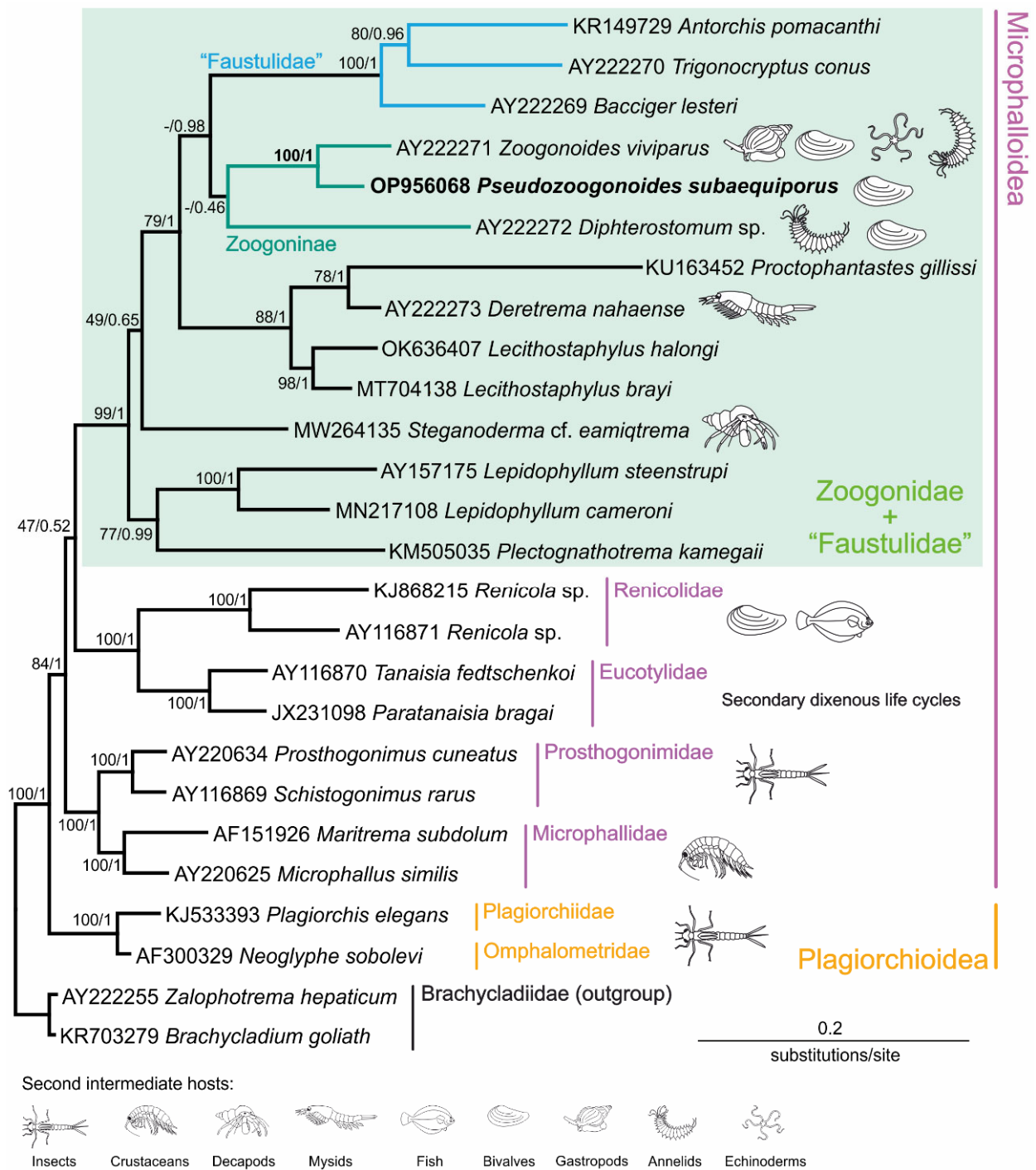
Figure 2. Phylogenetic position of *Pseudozoogonoides subaequiporus* (indicated in bold) based on 28S rDNA sequence data analysis inferred with Bayesian inference (BI) and maximum likelihood (ML). Nodal support: ML/BI. Scale bar shows the substitution rate.

Table 2. Isolates of putative and identified Zoogonidae from the White Sea, their origin and GenBank accession numbers.

ID	Species	Stage	Host Species	GenBank Accession Numbers	
				28S rDNA	5.8S rDNA+ITS2
G7.3	*Zoogonoides viviparus*	Marita	*Limanda limanda*	OP956058	OP956013
G7.2	*Z. viviparus*	Marita	*Anarhichas lupus*	OP956059	OP956014
A5.36	*Z. viviparus*	Marita	*L. limanda*	OP956060	OP956015
G7.8	Putative *Z. viviparus*	Metacercaria	*Nephthys* sp.	OP956061	OP956016
G7.5	Putative *Z. viviparus*	Metacercaria	*Nuculana pernula*	OP956062	OP956017
G7.4	Putative *Z. viviparus*	Metacercaria	*Ciliatocardium ciliatum*	OP956063	OP956018
G7.9	Putative *Z. viviparus*	Sporocyst with cercariae	*Buccinum undatum*	OP956064	OP956019
G7.10	Putative *Z. viviparus*	Sporocyst with cercariae	*B. undatum*	OP956065	OP956020
A5.37	Putative *Z. viviparus*	Sporocyst with cercariae	*B. undatum*	OP956066	OP956021
A5.38	Putative *Z. viviparus*	Sporocyst with cercariae	*B. undatum*	OP956067	OP956022
G7.6	*Pseudozoogonoides subaequiporus*	Marita	*A. lupus*	OP956068	OP956023
G7.7	*P. subaequiporus*	Marita	*A. lupus*	OP956069	OP956024
D9.6	Putative *P. subaequiporus*	Metacercaria	*N. pernula*	OP956070	OP956025
D9.7	Putative *P. subaequiporus*	Metacercaria	*N. pernula*	OP956071	OP956026
D9.4	Putative *P. subaequiporus*	Sporocyst with cercariae and metacercariae	*Neptunea despecta*	OP956072	OP956027
D11.3	Putative *P. subaequiporus*	Sporocyst with cercariae and metacercariae	*B. undatum*	OP956073	OP956028
G7.1	*Lepidophyllum steenstrupi*	Marita	*A. lupus*	OP956074	OP956029
AG4.12	Zoogonidae gen. sp.	Sporocyst with germ balls	*B. scalariforme*	OP956075	OP956030

Table 3. PCR primers and thermocycling conditions; in all reactions, initial denaturation was at 95 °C for 5 min and final extension was at 72 °C for 10 min.

Product	Primer	Sequence (5′-3′), Forward (F) and Reverse (R)	Thermocycling Profile	Reference
18S rDNA	18S1A	F, GGCGATCGAAAAGATTAAGCCATGCA	94 °C—1 m 52 °C—1 m 72 °C—1 m ×35	[32] primers and conditions
	32	R, CGAAGTCCTATTCCATTATTC		
	652	F, GCAGCCGCGGTAATTCCAGCTC		
	28	R, AGCGACGGGCGGTGTGT		
28S rDNA	digl2	F, AAGCATATCACTAAGCGG	95 °C—30 s 54 °C—30 s 72 °C—2 m ×40	[33] primers and conditions
	1500R	R, GCTATCCTGAGGGAAACTTCG		[34] primers and conditions
5.8S+ITS2	3S	F, GTACCGGTGGATCACGTGGCTAGTG	94 °C—30 s 55 °C—30 s 72 °C—1 m ×30	[35] primers
	ITS2.2	R, CCTGGTTAGTTTCTTTTCCTCCGC		

2.4. Visualization

We used CorelDraw® 2017 software to create the schemes of parasite life cycle stages as well as intermediate and definitive hosts.

3. Results

3.1. General Observations

In the studied White Sea fish, we found zoogonid maritae of three species. *Zoogonoides viviparus* was obtained from the three examined flatfish species and from Atlantic wolffish. *Pseudozoogonoides subaequiporus* and *Lepidophyllum steenstrupi* Odhner, 1902 were found only in Atlantic wolffish. Sporocyst and cercariae similar to the ones described by Lebour [36] and Køie [19] as *Z. viviparus* were obtained from the common whelk *Buccinum undatum* Linnaeus, 1758. Zoogonid sporocysts with morphologically different cercariae and also encysted metacercariae were found in *B. undatum* and *Neptunea despecta* (Linnaeus, 1758). These larvae resembled maritae of *P. subaequiporus*. In one more whelk species, *B. scalariforme* Møller, 119, we found zoogonid sporocysts at the earlier stage of development, containing only the germ balls. Other dissected gastropods were free from zoogonid sporocysts (Table 1). Metacercariae similar to *Z. viviparus* maritae were found in several bivalve species and in the annelid *Nephtys* sp. We also recovered another type of zoogonid metacercariae, morphologically similar to maritae of *P. subaequiporus*, from protobranch bivalve *Nuculana pernula* (O. F. Müller, 1779). All the other examined benthic invertebrates were not infected with zoogonid metacercariae (Table 1).

3.2. Molecular Data and Life Cycles Elucidation

To link life cycle stages from intermediate hosts and maritae from definitive hosts we obtained 1244–1263 b.p. sequences of the 28S rDNA fragment (domains D1–D3) for all 18 isolates (Table 2). We also included 28S rDNA sequences of *Z. viviparus* (AY222271) and *L. steenstrupi* (AY157175) maritae into the analysis. *Steganoderma* cf. *eamiqtrema* Blend and Racz, 2020 (MW264135) was added as an outgroup. After trimming to match the shortest sequence, the alignment was 1269 b.p. long, including gaps. Three distinct groups were evident in the maximum likelihood (ML) tree (Figure 1). The first one comprised *Z. viviparus* from GenBank (AY222271), newly obtained putative and identified isolates of *Z. viviparus* and the zoogonid sporocyst from *B. scalariforme* (Figure 1). The second group incorporated putative and identified isolates of *P. subaequiporus* (Figure 1). Both groups had no intraspecific variability in 28S fragments and differed from each other in 86 positions. The third group comprised sequences of *L. steenstrupi*, the one from GenBank (AY157175) and the one newly obtained from the White Sea isolate (Figure 1). These sequences differed by a single nucleotide substitution (C/A) in the position 293.

For all the 18 isolates, we also sequenced a 431–545 b.p. long fragment containing partial 5.8S, ITS2 and the beginning of the 28S rDNA (Table 2). After trimming to match the shortest sequence, the alignment was 456 b.p. long, including gaps. Sequence of *S.* cf. *eamiqtrema* (MW264129) was added as an outgroup. In the ML tree, our sequences are grouped the same way as in the 28S rDNA analysis (Figure 1). The clades including *Z. viviparus* and *P. subaequiporus* isolates had no intraspecific variability and differed from each other in 97 positions. Sequence of *L. steenstrupi* differed from these groups in 133 and 91 positions, respectively (Figure 1).

We also generated a 1378 b.p. long sequence of 18S rDNA fragment for one isolate of *P. subaequiporus* marita and deposited it in the GenBank under accession number OP956057.

3.3. Phylogenetic Position of Pseudozoogonoides subaequiporus

As we obtained the first molecular data for *P. subaequiporus*, they were used to resolve its phylogenetic position. Analysis was run with two datasets: the first one comprised 51 sequences and the second one just 26 sequences. The resulting phylogenetic trees showed the same relationship between families and within the Zoogonidae. The tree based on

the larger dataset is shown in Supplementary Figure S1. Below we describe results of the analysis inferred from the smaller dataset.

The alignment comprised 26 sequences of 28S rDNA fragments and yielded 1231 characters, including gaps. Phylogenetic trees inferred with ML and Bayesian approaches revealed almost the same topology. The Bayesian tree is presented in Figure 2; ML bootstrap support values are additionally mapped onto it next to the posterior probability (PP) values for all the ML-supported nodes.

Similarly to previous studies [37–40], the Zoogonidae was resolved within the Microphalloidea as a well-supported clade, though containing some members of the family Faustulidae (Figure 2). The latter is known to be polyphyletic as several faustulid species fall into the superfamily Gymnophalloidea [41–43], thus we place this family name into the quotation marks. The clade Renicolidae + Eucotylidae was inferred as a sister group to the Zoogonidae with a low BI and ML support. *Pseudozoogonoides subaequiporus* grouped together with *Z. viviparus* in a well-supported clade within the Zoogoninae (Figure 2). Monophyly of the latter was poorly supported. The former Lepidophyllinae was resolved as highly paraphyletic, and the Zoogoninae was inferred as the closest relative of the "faustulids", similarly to previous studies [37–43].

3.4. Infection Data and Morphological Descriptions

3.4.1. Maritae of *Zoogonoides viviparus*

Locality: White Sea, Keret Archipelago.

Hosts and infection rates: *Anarhichas lupus* (prevalence 25%, N = 28; mean intensity = 5.7); *Limanda limanda* (91%, N = 116; MI = 21.1); *Liopsetta glacialis* (37%, N = 27; MI = 7.6); *Platichthys flesus* (52%, N = 79; MI = 19.3).

Other reported hosts: 20 fish species (summarized in [44]).

Sites: Intestine, occasionally urinary bladder

Vouchers: Paragenophores (four slides) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: Measurements in Table 4.

Remarks: Ovigerous specimens located predominantly in rectum.

3.4.2. Metacercariae of *Zoogonoides viviparus*

Locality: White Sea, Keret Archipelago.

Hosts and infection rates: Bivalvia: *Nuculana pernula* (2.1%, N = 394; MI = 1.3); *Yoldia hyperborea* (Gould, 1841) (1.3%, N = 78; MI = 1); *Serripes groenlandicus* (Mohr, 1786) (10.6%, N = 66; MI = 1.1); *Ciliatocardium ciliatum* (Fabricius, 1780) (5.1%, N = 39, MI = 1.5). Annelida: *Nephtys* sp. (25%, N = 8; MI = 2.5).

Other reported hosts: Various benthic invertebrates (summarized in [44]).

Sites: Bivalvia: Mantle, siphons, gills, oral lobes, visceral mass, adductor muscles, digestive gland. Annelida: Somatic musculature, gut wall.

Vouchers: Syngenophores (seven slides) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: [Measurements based on 7 fixed specimens from bivalves. See also Table 4].

Body of metacercaria extracted from cyst 385 (341–446) × 115 (101–137), oval, with narrowed anterior and posterior ends. Tegument spinose. Stylet present (five of six metacercariae). Oral sucker subterminal, oval, 68 (59–81) × 73 (60–99). Ventral sucker oval, 99 (82–114) × 94 (82–132). Sucker ratio 1:1.47 (1.33–1.68). Prepharynx very short, pharynx oval, 23 (18–36) × 30 (24–34), esophagus long. Ceca reaching posterior third of ventral sucker; lumen present. Excretory vesicle in hindbody, small, oval. Two testes primordia behind ventral sucker, 39 (23–57) × 31 (18–47) and 37 (25–57) × 31 (21–44). Cirrus-sac primordium 95 (81–115) × 23 (19–27). Ovary primordium 31 (21–41) × 28 (18–39), between testes, dorsally. Other primordia of female reproductive system (oviduct, Laurer's canal, vitellarium, uterus and metraterm) present.

Remarks: Of 29 recovered metacercariae 13 were dead: four of five from *Nephtys* sp.; three of eight from *S. groenlandicus*; two of three from *C. ciliatum*; four of 12 from *N. pernula*. Morphology of studied metacercariae agrees with the previous descriptions [19,45].

Table 4. Dimensions of the White Sea Zoogonidae life cycle stages.

	Zoogonoides viviparus (Maritae) *n* = 10	*Z. viviparus* (Metacercariae) *n* = 6	*Z. viviparus* (Cercariae) *n* = 11	*Pseudozoogonoides subaequiporus* (Maritae) *n* = 12	*P. subaequiporus* (Cercariae) *n* = 11
Length	766 (665–831)	385 (341–446)	388 (337–451)	747 (622–929)	301 (248–385)
Width	246 (206–281)	115 (101–137)	139 (125–158)	295 (237–341)	118 (98–136)
Forebody, length	273 (227–294)	172 (151–196)	179 (153–242)	255 (219–304)	128 (96–180)
Length: forebody ratio	1:0.36 (0.31–0.39)	1:0.45 (0.40–0.50)	1:0.46 (0.43–0.54)	1:0.34 (0.29–0.39)	1:0.42 (0.35–0.48)
Oral sucker	114 (88–137) × 127 (102–144)	68 (59–81) × 73 (60–99), *n* = 7	73 (63–79) × 73 (65–83)	129 (107–152) × 130 (113–146)	58 (50–66) × 57 (50–66)
Ventral sucker	187 (168–222) × 182 (164–202)	99 (82–114) × 94 (82–132), *n* = 7	103 (93–119) × 87 (71–98)	138 (127–156) × 137 (121–182)	60 (54–65) × 60 (55–66)
Sucker ratio	1:1.66 (1.43–1.99)	1:1.47 (1.33–1.68) *n* = 7	1:1.40 (1.19–1.63)	1:1.08 (0.97–1.27)	1:1.05 (0.92–1.24)
Stylet	-	-	16 (12–20) × 5 (4–6) *n* = 9	-	13 (10–16) × 4 (3–5) *n* = 8
Pharynx	37 (30–55) × 47 (43–50)	23 (18–36) × 30 (24–34), *n* = 7	23 (17–36) × 29 (26–32)	40 (28–50) × 45 (36–53)	19 (15–21) × 23 (21–25)
Esophagus	84 (71–95)	52 (36–77)	61 (46–76)	78 (44–97)	43 (26–69)
Cirrus-sac	261 (210–296) × 52 (44–65)	95 (81–115) × 23 (19–27), *n* = 4	87 (62–102) × 19 (17–23)	278 (203–325) × 74 (64–89)	67 (56–85) × 14 (12–15)
Ovary	96 (77–121) × 72 (60–89)	31 (21–41) × 28 (18–39), *n* = 5	28 (24–30) × 23 (20–27)	102 (81–150) × 93 (75–144)	18 (17–20) × 15 (14–17)
Testis, anterior	87 (65–123) × 72 (53–94)	39 (23–57) × 31 (18–47), *n* = 5	48 (42–57) × 36 (33–40)	112 (78–144) × 83 (64–103)	25 (16–30) × 18 (12–23)
Testis, posterior	83 (67–113) × 75 (63–101)	37 (25–57) × 31 (21–44), *n* = 5	45 (40–50) × 33 (27–37)	99 (78–138) × 80 (67–112)	25 (18–30) × 19 (14–23)
Eggs	64 (56–76) × 28 (23–31)	-	-	70 (61–79) × 30 (27–33), *n* = 10	-

3.4.3. Sporocysts, Developing and Infective Cercariae of *Zoogonoides viviparus* (Figure 3A–C and Figure 4)

Locality: White Sea, Keret Archipelago.

Hosts and infection rates: *Buccinum undatum* (0.9%, N = 2174); *B. scalariforme* (5.2%, N = 19).

Sites: Digestive and reproductive glands. In heavily infected whelks, daughter sporocysts are also located in the kidney and body wall near esophagus and stomach. Infective cercariae commonly accumulate in the kidney.

Vouchers: Isogenophores (two slides) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description:

Daughter sporocysts (Figure 4A)

[Measurements based on 10 fixed specimens from *B. undatum*].

Sporocysts oval, 904 (476–1178) × 343 (252–443). Brood cavity occupying almost all inner space, containing germ balls, developing and infective cercariae. Birth pore terminal

at anterior body end. Germinal mass subterminal at posterior body end, embedded in parenchyma.

Cercariae (Figure 3A,B and Figure 4B,C)

[Measurements based on 11 fixed specimens. See also Table 4].

Tailless distome xiphidiocercariae. Body 388 (337–451) × 139 (125–158), oval, with narrowed anterior and posterior ends. Tegument spinose. Oral sucker subterminal, oval, 73 (63–79) × 73 (65–83). Ventral sucker oval, 103 (93–119) × 87 (71–98), closer to posterior end. Sucker ratio 1:1.40 (1.19–1.63). Prepharynx very short, pharynx oval, 23 (17–36) × 29 (26–32), esophagus long, 61 (46–76). Ceca reaching posterior third of ventral sucker; lumen present. Cerebral ganglion dorsal to prepharynx. Stylet in pocket within oral sucker, dorsally to mouth opening, 16 (12–20) × 5 (4–6), single-pointed, two-layered; external refractive layer with two small shoulders; posterior end of internal layer naked. One group of 17 large unicellular penetration glands in forebody. Five glands without ducts. Twelve ducts of penetration glands arranged according to formula (2 + 3 + 1) + (1 + 3 + 2). Each duct with individual pore near anterior edge of oral sucker. Excretory vesicle small, oval. Excretory system of "Mesostoma" type. Main collecting ducts dividing into anterior and posterior parts at posterior third of ventral sucker. Excretory formula 2[(2 + 2) + (2 + 2)] = 16. Two testes primordia 48 (42–57) × 36 (33–40) and 45 (40–50) × 33 (27–37), in hindbody, symmetrical, anterior testis slightly larger. Cirrus-sac primordium anterior to ventral sucker. Vasa efferentia fusing at cirrus-sac primordium. Ovary primordium between testes, dorsally, 28 (24–30) × 23 (20–27). Vitellarium primordium smaller, ventral to ovary. Forming Laurer's canal posterior to ovary. Uterus primordium running anteriorly, its dilated distal part close to cirrus-sac primordium.

Developing cercariae (Figures 3C and 4D)

In underdeveloped cercariae, a very short knob-like tail primordium was evident. It disappears during further development; therefore, infective cercariae are tailless. In underdeveloped cercariae, mucoid substances were found in the mucoid gland cells which lie ventrally, three pairs in the forebody and two in the hindbody. During the final stages of cercarial development, the mucoid substances are transferred into the tegument which becomes swollen.

Remarks: Morphology of studied cercariae agrees with the previous descriptions of this species [19,36,46]. Remarkable features revealed in our study are the number of the penetration glands and their ducts, stylet structure, structure of the excretory and genital systems, number of the mucoid glands and presence of the tail primordium in developing cercariae.

3.4.4. Maritae of *Pseudozoogonoides subaequiporus*

Locality: White Sea, Keret Archipelago.

Host and infection rates: *Anarhichas lupus* (93%, N = 28; MI = 38.1).

Other reported hosts: Six fish species [44,47].

Site: Intestine.

Vouchers: Paragenophores (three slides) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description: Measurements in Table 4.

Remarks: Ovigerous specimens located predominantly in rectum.

3.4.5. Metacercariae of *Pseudozoogonoides subaequiporus* (Figure 5)

Locality: White Sea, Keret Archipelago.

Hosts and infection rates: *Neptunea despecta* (1 of 103); *Buccinum undatum* (1 of 2174); *Nuculana pernula* (1%, N = 394; MI = 1).

Sites:

Bivalvia: Mantle, siphons.

Vouchers: Hologenophore (single slide) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.
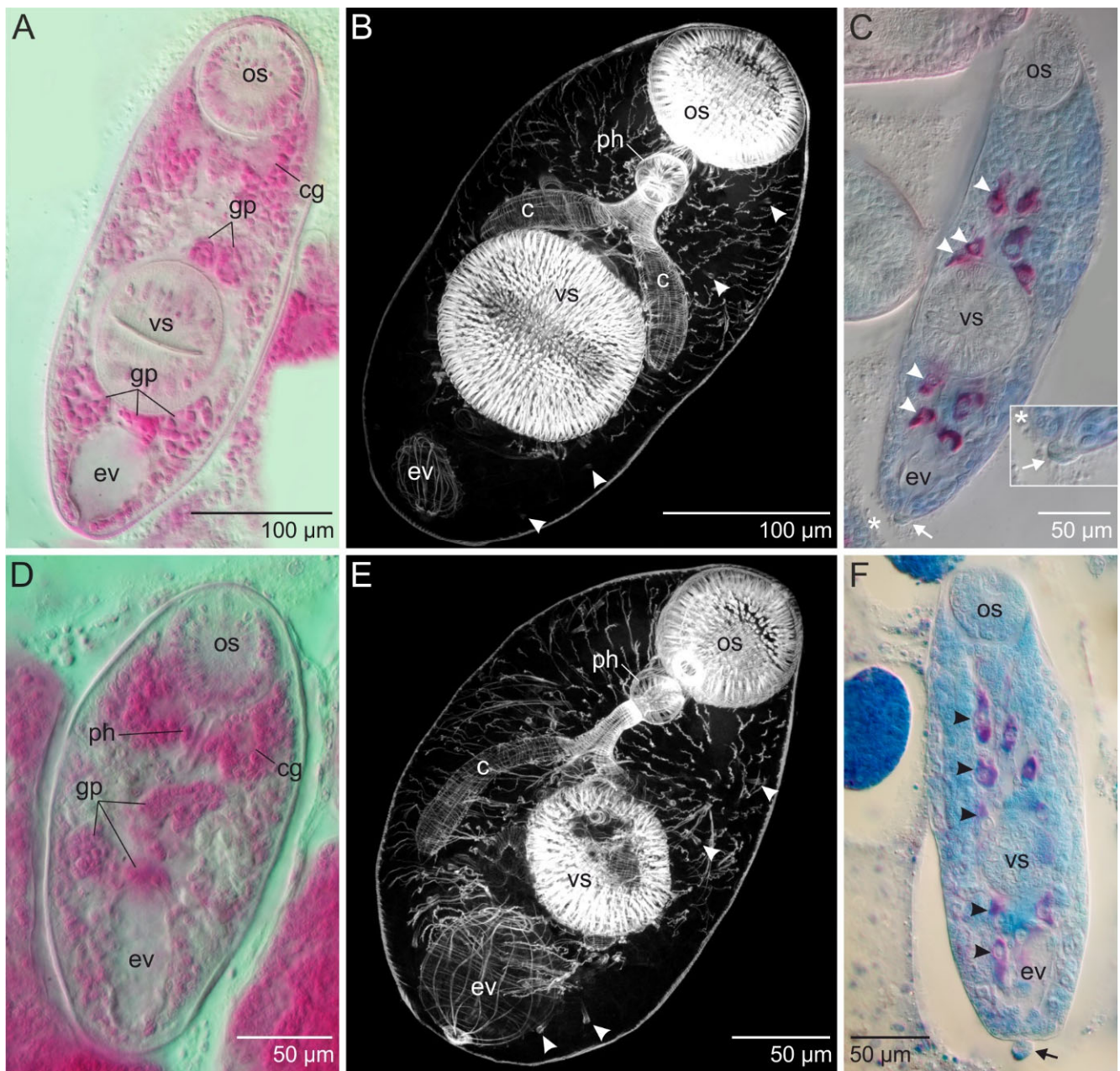
Figure 3. *Zoogonoides viviparus* (**A–C**) and *Pseudozoogonoides subaequiporus* (**D–F**) cercariae. (**A,D**) Infective cercariae, general structure, acetic carmine staining and differential interference contrast (DIC). (**B,E**) Infective cercariae, flame cells, digestive and excretory systems, confocal laser scanning microscopy and TRITC-phalloidin staining. (**C,F**) Developing cercariae, mucoid gland cells and the tail primordium, toluidine blue staining and DIC. Abbreviations: c—cecum; cg—cerebral ganglion; gp—genital system primordium; os—oral sucker; ph—pharynx; t—testis primordium; vs—ventral sucker. Arrowheads indicate flame cells (**B,E**) and mucoid gland cells (**C,F**). Arrows indicate the tail primordium. Asterisk marks magnified fragment in square of (**C**).

Description:

Metacercariae enclosed in oval cyst, 153 (123–187) × 137 (116–157) (measurements based on 22 fixed specimens from *N. despecta* and *B. undatum*). Following description based on single hologenophore from *N. pernula*. Body of metacercaria extracted from cyst 275 × 119, oval, with slightly narrowed posterior end. Tegument spinose. Oral sucker subterminal, oval, 53 × 59. Ventral sucker roundish, 49 × 50. Sucker ratio 1:0.92. Prepharynx very short, pharynx oval, esophagus long. Ceca ending behind ventral sucker; lumen

present. Excretory vesicle in hindbody, small, oval. Two testes primordia behind ventral sucker, 40×30 and 40×31. Cirrus-sac primordium 63×23. Ovary primordium 23×23, between testes, dorsally. Other primordia of female reproductive system (oviduct, ootype, Laurer's canal, vitellarium, uterus and metraterm) present.

Remarks: Two of four metacercariae recovered from *N. pernula* were dead. Zhukov [47] claimed he found metacercariae of *P. subaequiporus* in mesenteries of small flounders from the Sea of Japan, but since he provided neither dimensions nor figure, we are unable to compare his finding with our results.
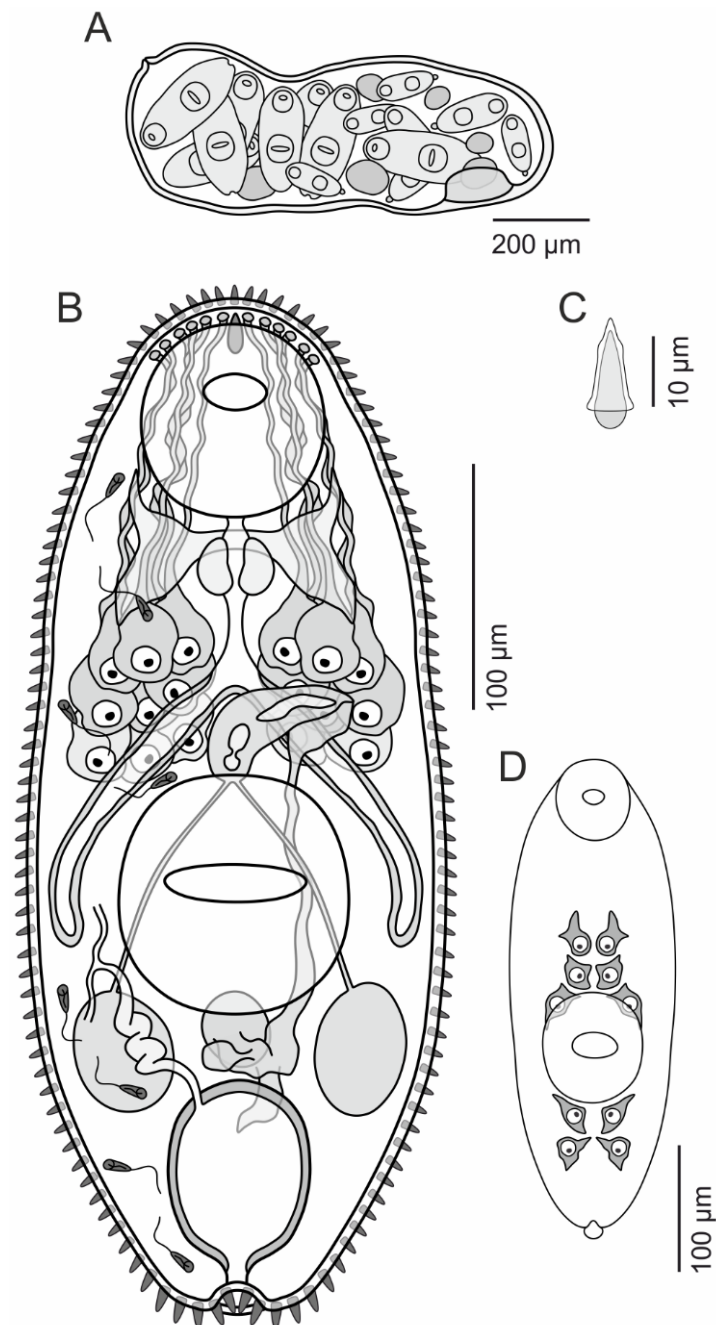
Figure 4. Drawings of life cycle stages of *Zoogonoides viviparus*: daughter sporocyst (**A**), infective cercaria body structure (**B**), stylet, ventral view (**C**), mucoid gland cells and the tail primordium in underdeveloped cercaria (**D**).

3.4.6. Sporocysts, Developing and Infective Cercariae of *Pseudozoogonoides subaequiporus* (Figure 3D–F and Figure 6)

Locality: White Sea, Keret Archipelago.

Hosts and infection rates: *Neptunea despecta* (1 of 103); *Buccinum undatum* (1 of 2174).

Sites: Digestive and reproductive glands.

Vouchers: Isogenophores (eight slides) deposited in the collection of the Department of Invertebrate Zoology of Saint Petersburg University.

Description:

Daughter sporocysts (Figure 6A)

[Measurements based on 20 fixed specimens, 10 from *N. despecta* and 10 from *B. undatum*].

Sporocysts elongated, 955 (528–1879) × 269 (171–350). Broad brood cavity containing germ balls, developing and infective cercariae and encysted metacercariae. Birth pore terminal at anterior body end. Germinal mass subterminal at posterior body end, embedded in parenchyma.

Cercariae (Figure 3D,E and Figure 6B,C)

[Measurements based on 11 fixed specimens. See also Table 4].

Tailless distome xiphidiocercariae. Body 301 (248–385) × 118 (98–136), oval, with slightly narrowed posterior end. Tegument spinose. Oral sucker subterminal, roundish, 58 (50–66) × 57 (50–66). Ventral sucker roundish, 60 (54–65) × 60 (55–66), closer to posterior end. Sucker ratio 1:1.05 (0.92–1.24). Prepharynx very short, pharynx oval, 19 (15–21) × 23 (21–25), esophagus long, 43 (26–69). Ceca ending behind ventral sucker; lumen present. Cerebral ganglion dorsal to prepharynx. Stylet in pocket within oral sucker, dorsally to mouth opening, 13 (10–16) × 4 (3–5), single-pointed, two-layered; external refractive layer with two small shoulders; posterior end of internal layer naked. One group of 12 large unicellular penetration glands in forebody. Two glands without ducts. Ten ducts of penetration glands arranged according to formula (3 + 2) + (2 + 3). Each duct with individual pore near anterior edge of oral sucker. Excretory vesicle elongated, oval. Excretory formula 2[(2 + 2) + (2 + 2)] = 16. Two testes primordia 25 (16–30) × 18 (12–23) and 25 (18–30) × 19 (14–23), in hindbody, symmetrical. Cirrus-sac primordium anterior to ventral sucker. Vasa efferentia fuse at cirrus-sac primordium. Ovary primordium between testes, dorsally, 18 (17–20) × 15 (14–17). Vitellarium primordium small, bipartite, ventral to ovary. Ootype primordium between testes, ventrally. Forming Laurer's canal posterior to ovary. Uterus primordium running anteriorly, its dilated distal part close to cirrus-sac primordium.

Developing cercariae (Figures 3F and 6D)

In underdeveloped cercariae, a very short knob-like tail primordium was evident. It disappears during further development; therefore, infective cercariae are tailless. In underdeveloped cercariae, mucoid substances were found in mucoid gland cells which lie ventrally, three pairs in the forebody and two in the hindbody. During the final stages of cercarial development, the mucoid substances are transferred into the tegument which becomes swollen.

Remarks: Encysted metacercariae were more abundant in the infected specimen of *B. undatum* (collected at the end of June) than in the infected specimen of *N. despecta* (collected at the end of July).
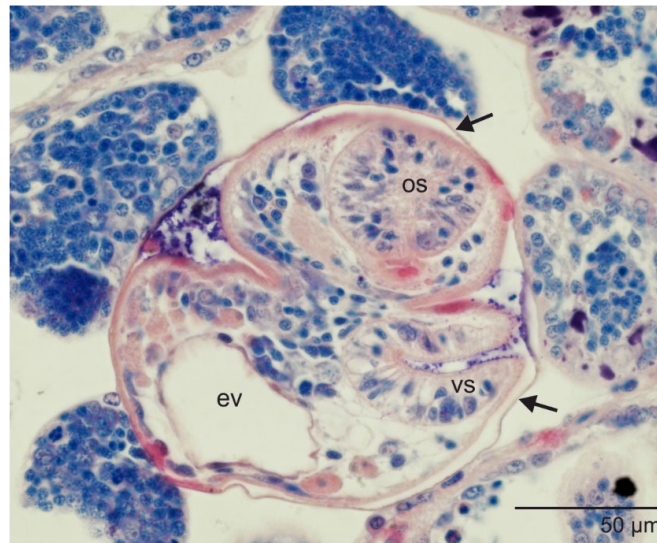
Figure 5. Histological section through infected whelk *Neptunea despecta* showing encysted metacercariae of *Pseudozoogonoides subaequiporus* within daughter sporocyst, Azur II-eosin staining. Abbreviations: ev—excretory vesicle; os—oral sucker; vs—ventral sucker. Arrows indicate cyst wall.
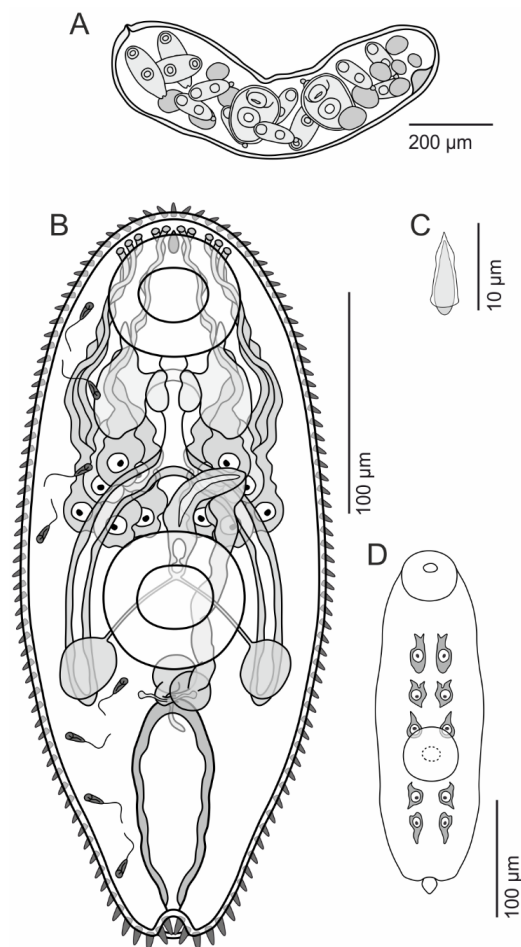

Figure 6. Drawings of life cycle stages of *Pseudozoogonoides subaequiporus*: sporocyst (**A**), infective cercaria body structure (**B**), stylet, ventral view (**C**), mucoid gland cells and the tail primordium in underdeveloped cercaria (**D**).

4. Discussion

The first hypothesis on the life cycle of *Zoogonoides viviparus* was proposed by Lebour [36]. She found sporocysts and tailless distome xiphidiocercariae in a common whelk, *Buccinum undatum*, and suggested these were conspecific with maritae of *Z. viviparus*. Later, based on both natural and experimental evidence, Køie [19] showed that the first intermediate host of *Z. viviparus* was indeed *B. undatum*, and the second ones were mollusks, annelids and brittle stars. By molecular data, we confirmed that buccinid gastropods *B. undatum* and *B. scalariforme* are the first intermediate hosts, and bivalves and annelids are the second intermediate hosts of this species (Figure 7A).

Køie [19] proposed brittle stars, and particularly *Ophiura albida* Forbes, 1839, to be the most important second intermediate hosts of *Z. viviparus* in Danish waters, considering high infection and survival rates of metacercariae. Studies of Scott in Canada indicated that brittle stars could serve as the second intermediate host of *Pseudozoogonoides subaequiporus* as well, judging from higher infection rates with maritae of this species in bigger flatfishes, which consume more echinoderms [48,49]. However, *O. albida* is absent in the White Sea [50], and we did not find any zoogonid metacercariae in the abundant local brittle stars, *O. robusta* (Ayres, 1852) and *Ophiopholis aculeata* (Linnaeus, 1767). We found metacercariae of *Z. viviparus* only in the annelids *Nephtys* sp. and several bivalve species from the families Cardiidae, Nuculanidae and Yoldiidae. Such infection routes match feeding preferences of the White Sea definitive hosts of *Z. viviparus*: local flatfishes feed predominantly on annelids and bivalves, and Atlantic wolffish consume mainly hard-shelled invertebrates ([51,52]; our unpublished observations), including bivalves.

We elucidated the three-host life cycle of *P. subaequiporus* using molecular data and demonstrated that it utilizes buccinid gastropods *Neptunea despecta* and *B. undatum* as the first intermediate hosts, and bivalves as the second intermediate hosts (Figure 7B). We found metacercariae of *P. subaequiporus* in a single bivalve species, *N. pernula*. Other benthic invertebrates also should be considered as potential second intermediate hosts of this species. However, the main transmission route of *P. subaequiporus* to the Atlantic wolffish in the White Sea is likely through direct consumption of the infected first intermediate hosts, buccinid gastropods, containing encysted metacercariae (Figure 7B). Shulman-Albova [53], therefore, correctly predicted that *P. subaequiporus* has a facultatively dixenous life cycle based on high infection rates of the Atlantic wolffish.

According to the results presented here and published earlier, the range of the first intermediate hosts of the Zoogonidae comprises gastropods from the Trochoidea (Vetigastropoda), Buccinoidea (Neogastropoda) and Naticidae (Littorinimorpha) (reviewed by Barnett et al. [54]). However, we disagree with the idea that naticid gastropods harbor zoogonid sporocysts and cercariae proposed by Palombi [20]. He did not find zoogonid infection in the Naticidae himself, but hypothesized *Cercariae crispata* Pelseneer, 1906 from *Euspira pulchella* (Risso, 1826), belongs to the species *Diphterostomum brusinae* (Stossich, 1888). However, from Pelseneer [55] drawings it is evident that the structure of digestive system, shape of the excretory bladder, and sucker position do not match zoogonid cercariae or metacercariae. Instead, these larvae strongly resemble metacercariae of the Microphallidae. The host, naticid gastropod, and the absence of cyst makes us suspect that *Cercariae crispata* should be identified as *Microphallus pseudopygmaeus* Galaktionov, 1980. This species has a secondary dixenous life cycle and utilizes a great variety of gastropods as the first intermediate hosts [56].
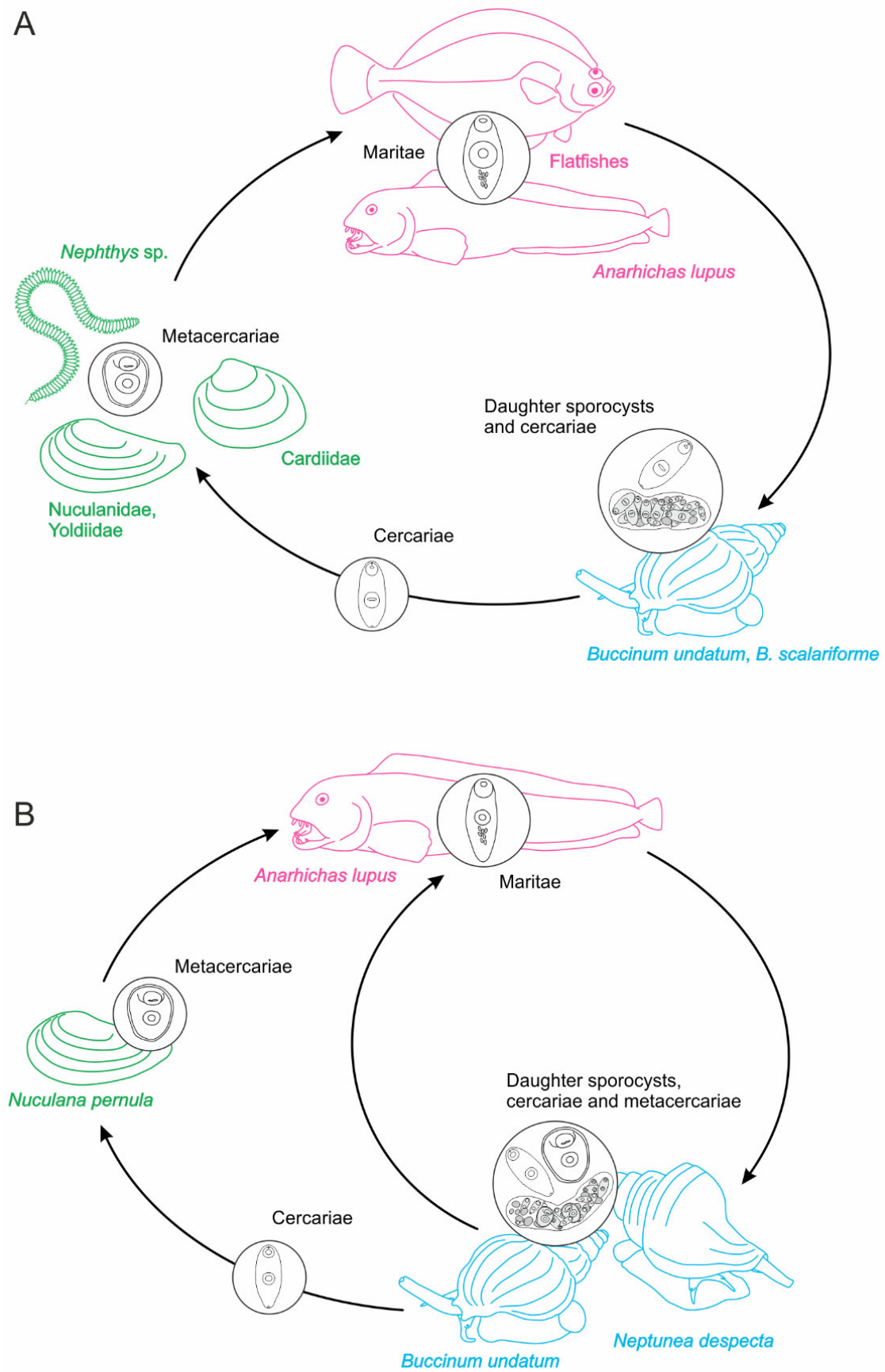
Figure 7. Life cycle schemes of *Zoogonoides viviparus* (**A**) and *Pseudozoogonoides subaequiporus* (**B**) based on the data retrieved at the White Sea.

Sharing the same first (common whelk) and second (bivalve *N. pernula*) intermediate hosts by *Z. viviparus* and *P. subaequiporus* may lead to misidentification of their cercarial and metacercarial stages. The most reliable discriminative feature is the sucker ratio: ventral sucker in *Z. viviparus* is relatively larger in all the ontogenetic stages. The body size can also help to tell *Z. viviparus* and *P. subaequiporus* apart: cercariae and metacercariae of *Z. viviparus* are larger (Table 4).

Studied material allowed us to specify two important morphological traits of the zoogonid cercariae. The first one is the presence of five pairs of mucoid glands at the ventral body side of developing cercariae (Figures 5D and 6D). Such a pattern is unique among Xiphidiata since cercariae of the other Microphalloidea and Plagiorchioidea have just four pairs of the mucoid glands [57–59]. Previously, only Køie [46] noted the presence of mucoid glands in developing cercariae of *Z. viviparus*, though she did not identify their number. The second significant trait is a short knob-like tail primordium in the developing cercariae (Figures 5D and 6D). This primordium disappears during further development and the infective cercariae become tailless. Previously, only Shimura and Ito [60] noted the tail primordium in immature larvae of *Cercaria brachycaeca* Shimura & Ito, 1980, putative Zoogonidae. The presence of tail primordium proves that tailless zoogonid cercariae evolved from an ancestor with a tail.

What are the benefits of tail loss? Apparently, it is increased longevity of cercariae as tail-based locomotion requires a lot of energy. The lifespan of actively swimming cercariae is quite short and commonly does not exceed 24 h [61]. However, in short-tailed cercariae the longevity is higher [62–66]. Tailless zoogonid cercariae have a lifespan between 24 and 120 h [19,46,60,67–70], and low temperature prolongs it up to 8–12 days [67].

Together with the longevity benefit, tail loss obviously makes cercariae less motile, and that must affect the range of second intermediate hosts. Judging from the known life cycles, an arthropod second intermediate host is a common plesiomorphic feature for the Microphalloidea and Plagiorhioidea in general, and the Zoogonidae in particular (Figure 2). The only zoogonid group with documented tailless cercariae is the subfamily Zoogoninae, and their range of second intermediate hosts is wide and includes a variety of benthic invertebrates, such as polychaetes, gastropods, bivalves, sea lilies, brittle stars and sea urchins, but never arthropods ([19,21–24,45,67,70–75]; Figure 2). Conversely, in the basally branching Zoogonidae (*Limnoderetrema minutum* (Manter, 1954) and *Steganoderma* spp.), the second intermediate host range is restricted to arthropods: shrimps, crabs, hermit crabs and mysids ([38,76–79]; Figure 2).

Although from some studies one may conclude that all zoogonid cercariae are tailless [80], this inference seems to be premature since we have no data on the cercariae of the basally branching zoogonids. These species have the ancestral life cycle pattern, and their cercariae could have retained the tail. It may turn out arthropods are too active to be chased by tailless cercariae.

Additionally, metacercariae of the Zoogoninae and the basally branching zoogonids differ in their growth capacity. Metacercariae of *Steganoderma* spp. are large, up to five millimeters, and both *Steganoderma* spp. and *L. minutum* sometimes start egg production within the second intermediate host [38,76–79]. All the metacercariae of the Zoogoninae are much smaller (their length does not exceed 600 μm), and they never reach sexual maturity in the second intermediate host [19,22–24,45,67,71–75]. Thus, growth capacity seems to be the cost of the host switch to diverse non-arthropod invertebrates.

Based on the reviewed and retrieved data, we hypothesize that two major events in the evolution of the Zoogonidae, tail loss and switch to non-arthropod second intermediate host, might have co-occurred in the Zoogoninae lineage. This led to lower host specificity and growth capacity of the metacercariae in this group.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15010121/s1, Table S1: List of species and 28S rDNA sequences used in this study (the second and the third datasets); Figure S1: Analysis of the second dataset. Phylogenetic position of *Pseudozoogonoides subaequiporus* (indicated bold) based on 28S rDNA

sequence data analysis inferred with Bayesian inference (BI) and maximum likelihood (ML). Nodal support: ML/BI. Scale bar shows the substitution rate. Model of nucleotide substitution: GTR+G+I. Refs. [81–101] are in the supplementary materials.

Author Contributions: D.K. conceived and designed the study. G.K., A.U., V.K., O.S., A.G. (Arseniy Gubler) and D.K. conducted sampling. G.K., D.K., A.G. (Anna Gonchar) and A.G. (Arseniy Gubler) performed molecular analyses. G.K., O.S. and D.K. performed morphological analyses. G.K., D.K. and A.G. (Anna Gonchar) wrote the article. D.K. received funding. All authors have read and agreed to the published version of the manuscript.

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