

Erection of Ceratonova n. gen. (Myxosporea: Ceratomyxidae) to Encompass Freshwater Species C. gasterostea n. sp. from Threespine Stickleback (Gasterosteus aculeatus) and C. shasta n. comb. from Salmonid Fishes

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Citation	Atkinson, S. D., Foott, J. S., & Bartholomew, J. L. (2014). Erection of Ceratonova n. gen.(Myxosporea: Ceratomyxidae) to Encompass Freshwater Species C. gasterostea n. sp. from Threespine Stickleback (Gasterosteus aculeatus) and C. shasta n. comb. from Salmonid Fishes. Journal of Parasitology, 100(5), 640-645. doi:10.1645/13-434.1
DOI	10.1645/13-434.1
Publisher	American Society of Parasitologists
Version	Accepted Manuscript
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RH: ATKINSON ET AL. – *CERATONOVA GASTEROSTEA* N. GEN. N. SP.

ERECTION OF *CERATONOVA* N. GEN. (MYXOSPOREA: CERATOMYXIDAE) TO ENCOMPASS FRESHWATER SPECIES *C. GASTEROSTEA* N. SP. FROM THREESPINE STICKLEBACK (*GASTEROSTEUS ACULEATUS*) AND *C. SHASTA* N. COMB. FROM SALMONID FISHES

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ABSTRACT: *Ceratonova gasterostea* n. gen. n. sp. is described from the intestine of freshwater *Gasterosteus aculeatus* L. from the Klamath River, California. Myxospores are arcuate, 22.4 +/- 2.6 μm thick, 5.2 +/- 0.4 μm long, posterior angle 45 +/- 24°, with 2 sub-spherical polar capsules, diameter 2.3 +/- 0.2 μm , which lie adjacent to the suture. Its ribosomal small subunit sequence was most similar to an intestinal parasite of salmonid fishes, *Ceratomyxa shasta* (97%, 1,671/1,692 nt), and distinct from all other *Ceratomyxa* species (<85%), which are typically coelozoic parasites in the gall bladder or urinary system of marine fishes. We propose erection of genus *Ceratonova* to contain both intestinal, freshwater species and reassign the salmonid parasite as *Ceratonova shasta* n. comb.

Some 10% of the ~2,200 known myxosporean species (Cnidaria: Myxosporea) belong to Family Ceratomyxidae Doflein, 1899 with most taxa in the extraordinarily speciose genus *Ceratomyxa* Thélohan, 1892 (Eiras, 2006; Lom and Dykova, 2006; Gunter and Adlard, 2010). Most *Ceratomyxa* species infect the gall bladder or urinary system of marine teleosts and elasmobranchs. The long-standing taxonomic outlier is *Ceratomyxa shasta* Noble, 1950, which infects primarily the intestine of salmonid fishes and has a well-characterized, freshwater life

cycle that involves actinospore development in a freshwater polychaete *Manayunkia* sp. (Bartholomew et al., 1997). Five other nominal *Ceratomyxa* species have been described from freshwater fish hosts, and all sporulate in the gall bladder (Gunter et al., 2009; Azevedo et al., 2013).

Ribosomal DNA sequence data have shown repeatedly that *C. shasta* is phylogenetically distant from a well-supported super-clade of coelozoic marine ceratomyxids (Fiala, 2006; Gunter et al., 2009; Fiala and Bartošová, 2010; Gleeson and Adlard, 2012). While no DNA sequence data are available for the 5 gall bladder-infecting freshwater taxa, the phylogenetic distance of *C. shasta* to its relatives, and its sporulation in the intestine have prompted discussion of subdividing the genus (Gunter et al., 2009; Gleeson and Adlard, 2012). Herein we describe a novel, freshwater ceratomyxid parasite from threespine sticklebacks (*Gasterosteus aculeatus*). The parasite sporulates in the intestine, is genetically very similar to *C. shasta* and dissimilar to all gall bladder-infecting, marine ceratomyxids. We propose erection of a new myxosporean genus, *Ceratonova*, to contain these 2 phylogenetically distinct, intestinal, freshwater species. Accordingly, *C. shasta* is renamed *Ceratonova shasta*, and regarded as the type species, with the stickleback parasite, *Ceratonova gasterostea* as its only known congener.

MATERIALS AND METHODS

Threespine sticklebacks, *Gasterosteus aculeatus* L., were collected by Yurok Tribal Fisheries biologists using a beach seine in the Klamath River, California. In July and August 2009, 23 fish were collected from the mainstem river near the mouths of Blue Creek (river kilometer [rkm] 25.9), Tectah Creek (rkm 34.9) and Roach Creek (rkm 50.8). The fish were processed for routine histology and qPCR assay at the California-Nevada Fish Health Center. The intestinal tract of each fish was subdivided: half placed in Davidson's fixative, embedded,

cut at 5 μm and stained with Giemsa, or hematoxylin and eosin; the other half had total DNA extracted and assayed (True et al., 2010) using the *Ceratomyxa shasta* qPCR described in Hallett and Bartholomew (2006). qPCR-positive DNA samples were sent to Oregon State University (OSU) for DNA sequencing.

In July 2010, 12 sexually mature fish were collected from the Klamath River, near the mouth of Pecwan Ck (rkm 40.2). Two fish (samples 6447 and 6448) were placed in Davidson's fixative for histology and processed as in 2009. The remaining 10 fish (samples M10.12.KR1-10) were frozen, sent to OSU and necropsied. Gut, kidney, liver, gall bladder and gills were examined. Parasite spores ($N \geq 10$) from 3 fish (KR4, 6, 7) in wet mount were imaged under bright field or Nomarski interference contrast illumination. Measurements were made from these images and myxospores were described according to published guidelines (Lom and Arthur, 1989; Gunter et al., 2009) with the modification that spore thickness was measured along the axis of each valve cell, rather than normal to the sutural plane (curvature of the valve cells and small posterior angle meant measurement normal to the suture was not informative). Sample KR7 had many spores and was designated as type. Spores were air-dried onto slides, then stained with Diff-Quik (Dade Behring Inc., Newark, Delaware) and a coverslip mounted with Permount (Fisher Scientific, Waltham, Massachusetts). A slide with dry, unstained, uncovered spores was archived as a DNA sample, together with tissue preserved in 95% ethanol and 10% formalin. Intestinal tissue that contained spores was refrozen prior to total DNA extraction using a DNeasy Blood & Tissue Kit per manufacturer's instructions (Qiagen Inc., Valencia, California).

Samples from 2009 and 2010 were analysed by PCR using different primer pairs. In 2009, the small subunit rRNA gene (ssrRNA) was amplified thus: first round ERIB1 (ACCTGGTTGATCCTGCCAG; Barta et al., 1997) with ERIB10

(CTTCCGCAGGTTACCTACGG; Barta et al., 1997); second round with ERIB1 and ACT1r (AATTTACCTCTCGCTGCCA; Hallett and Diamant, 2001), or Myxgen4F (GTGCCTTGAATAAATCAGAG; Diamant et al., 2004) with ERIB10. In 2010, single round reactions were used on genomic DNA or ERIB1-ERIB10 template, using primers ERIB1 with Cs1591R (TCATGTTGAATACGTCTCTG; Atkinson and Bartholomew, 2010a). The internal transcribed spacer region 1 (ITS-1) was amplified in a single round using Cs1482f (CCTGCTCTGAGAAGAGTGG; Atkinson and Bartholomew, 2010a) with CsGenR1 (AGGGATCCACCGTTAAC; Atkinson and Bartholomew, 2010b). PCRs were performed using published reaction chemistries, cycling conditions and methods (Atkinson and Bartholomew, 2010a). Amplicons were sequenced at the OSU Center for Genome Research and Biocomputing, using an ABI BigDye Terminator Cycle Sequencing Kit v3.1 and ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, California). Sequence chromatograms were examined to identify polymorphic loci. Sequences were aligned, assembled and compared among samples in Bioedit (Hall, 1999). The *ssrRNA-ITS-1* consensus sequence of sample KR7 was deposited in the NCBI nucleotide database and used to conduct a BLASTn similarity search to determine affinity with other myxosporeans in the database.

DESCRIPTION

Ceratomyxa Thélohan, 1892

Diagnosis: Amended (bold) after Gunter & Adlard (2010): **Coelozoic parasites of marine fishes, exceptionally in freshwater. Myxospores** elongate, generally crescent-shaped or arcuate, occasionally sub-spherical or oval; shell valves significantly exceeding in length half of axial diameter of spore. Shell valves conical or sub-hemispherical, often pliable rather than rigid as in other genera. Sub-spherical polar capsules located and open close to suture line, in plane

perpendicular to it, at anterior pole of spore, but exceptionally open laterally from central suture line. Binucleate sporoplasm may fill spore cavity completely; in some species, 2 uninucleate sporoplasms have been reported. Trophozoites usually disporic, sometimes mono- to polysporic.

***Ceratonova* n. gen.**

Diagnosis: Parasites of freshwater and anadromous fishes, and freshwater polychaete worms. In fish, vegetative stages histozoic with typically disporic pseudoplasmodia and mature spores in intestine and occasionally other organs. Myxospores crescent-shaped in side-sutural view, ovoid in apical view. Shell valves pliable. Sub-spherical polar capsules located and open close to suture line at anterior pole of spore. Binucleate sporoplasm.

Taxonomic summary

Taxonomic affinities: *Ceratonova* is in Family Ceratomyxidae.

Type species: *Ceratonova shasta* n. comb. (syn. *Ceratomyxa shasta* Noble, 1950).

Other species: *Ceratonova gasterostea* n. sp.

Etymology: The generic name refers to both the horn-like shape of the valve cells and the presumed more recent evolution of this genus from marine ancestors.

***Ceratonova gasterostea* n. sp.**

Developmental stages and myxospores in vertebrate host, Figs. 1-2.

Diagnosis: With characters of the genus. Multicellular, histozoic vegetative stages in lamina propria, intestinal blood vessels and epithelium (Fig. 1A - C). Disporic (occasionally monosporic) pseudoplasmodia and mature myxospores in intestinal lumen (Fig. 1D, E).

Myxospores strongly arcuate in side-sutural view, posterior angle $45 \pm 24^\circ$, ovoid in apical and valvular views (Fig. 2). Valve cells, 2, approximately equal-sized, smooth surface, 22.4 ± 2.6 μm thick, 5.2 ± 0.4 μm long. Suture protrudes slightly from the spore surface. Polar capsules,

2, sub-spherical, diameter 2.3 +/- 0.2 μm , at spore apex adjacent to suture. Polar filament, coiled 4-5 times, extruded length $\sim 30\mu\text{m}$.

Taxonomic summary

Type vertebrate host: Gasterosteus aculeatus (L.)

Type invertebrate host: Unknown.

Site of infection: Vegetative development histozoic in lamina propria, intestinal blood vessels and epithelium, sporogony in intestinal lumen.

Type locality: Klamath River, California, near mouth of Pecwan Creek 41°20'34.8"N, 123°51'21.6"W

Prevalence: ~50% (7/15 fish examined by histology, 6/10 necropsied fish).

Specimens deposited: Two slides with Diff-Quik stained air-dried myxospores (accession numbers G465690 and G465691), infected intestinal tissues in 95% ethanol (G465692) and formalin fixed (G465693) from fish M10-12-KR7 in the Parasitology Collection at the Queensland Museum, Brisbane, Australia. DNA sequence data ssrRNA-ITS-1 gene sequence 2,010 nt in the NCBI database, accession number KF751186.

Etymology: The specific name “gasterostea” refers to the genus of the vertebrate host.

Remarks

Early sporogonic stages of *C. gasterostea* were apparent in histological sections of intestinal blood vessels and lamina propria, with both immature and maturing spore stages in the intestinal lumen. No host response was evident. Necropsied fish had no *C. gasterostea* spores in liver, gall bladder or kidneys. In kidneys, myxospores of *Myxobilatus gasterostei* were seen in 8/10 fish and *Myxidium cf. gasterostei* in 1/10 fish. Nematomorph larvae were observed encysted in the intestinal wall of 1/10 fish. *Ceratonova gasterostea* has thicker and more curved valves

than *C. shasta*, the only other intestinal, freshwater congener that has been described. The posterior angle of *C. gasterostea* varied considerably (45 +/- 24°; Fig. 2) and we regarded this metric to be of limited taxonomic use for this species. By qPCR assay, 14/22 fish tested positive, with Cq values 23-33. Only *C. gasterostea* could be amplified from these fish. Almost complete parasite ssrRNA gene sequences were obtained from 1 fish in 2009 and 4 in 2010, and were identical with each other. Additional 3' end ssrRNA sequences from 9 fish in 2009 were identical to all other sequences in the hypervariable region around position 1,700. In both *C. gasterostea* and *C. shasta*, 3 differences were present in the binding region of primer ERIB10, which explained the poor amplification we observed with that primer for both species. ITS-1 sequences were obtained from 8 fish in 2009 and 4 in 2010. Sequences were highly similar between fish, though indels and polymorphisms were present in several sequences. ITS-1 similarity with *C. shasta* GQ358729 was only ~78% over 280 nt. *Ceratomyxa shasta* was not amplified from any fish infected with *C. gasterostea*.

A BLASTn search of the NCBI database showed highest similarity (97%: 1,692/1,744 nt) with *C. shasta* GQ358729. No other highly similar species were found; next highest total scores were with marine taxa, e.g. *Ellipsomyxa gobii* GQ229235 (85%: 1,416/1,671 nt), *Sigmomyxa sphaerica* JN033225 (85%: 1,410/1,663 nt), *Enteromyxum scophthalmi* AF411335 (82%: 1,354/1,643 nt). The only higher score (91%) was with shorter sequence *Sinuolinea* sp. IF-2013 JX996022 (457/503 nt). The most similar marine, coelozoic *Ceratomyxa* species was *C. melanopteri* JF911817 (85%: 1,132/1,337 nt).

DISCUSSION

A long-standing taxonomic outlier to all other *Ceratomyxa* spp. is *C. shasta* Noble, 1950, as it has a well-characterised freshwater life cycle, is phylogenetically distant, and sporulates in

the intestine of the fish host rather than gall bladder (Bartholomew et al., 1997; Gleeson and Adlard, 2012). We discovered a novel freshwater ceratomyxid species that was genetically highly similar to *C. shasta* and sporulated in intestine of threespine sticklebacks. This led us to propose erection of a new myxosporean genus, *Ceratonova*, to contain these 2 species: *C. shasta* n. comb. as type and *C. gasterostea* n. sp. as the only described congener.

Morphologically, myxospores of *Ceratomyxa* spp. and *Ceratonova* spp. are superficially indistinguishable: bilaterally symmetric, crescent shaped valve cells with centrally located suture and apical polar capsules. Yet these morphological similarities distract from the biological differences between the genera. *Ceratonova* species have freshwater life cycles, intestinal spore development and *ssrRNA* gene sequences distant to all *Ceratomyxa* spp. Incorporation of multiple character sets has been recommended repeatedly to overcome ambiguities that arise from the historical, purely morphological discrimination of myxosporean species (e.g. Kent et al., 2001; Gunter et al., 2009; Fiala and Bartošová, 2010; Molnár, 2011). We adopted the holistic approach of incorporating host, tissue and *ssrRNA* sequence data to place *C. gasterostea* and *C. shasta* in a new genus.

Geographic distribution

It is unlikely that *C. gasterostea* is geographically widespread outside the Pacific Northwest of North America. Its vertebrate host, threespine stickleback, occurs commonly across the holarctic, as both fully freshwater and anadromous forms (Wootton, 1984). Stickleback parasite fauna has been characterized extensively and, though emphasis has been placed often on macroparasites (e.g., Zander, 2007; Poulin et al., 2011), myxozoans have been observed commonly, especially *Sphaerospora elegans* (Feist et al., 1991) and *Myxobilatus gasterostei* (Atkinson and Bartholomew, 2009). We consider it unlikely, therefore, that if *C. gasterostea*

occurs widely in sticklebacks that it has been hitherto unrecognized. In the Pacific Northwest, a few surveys have recorded stickleback myxozoan parasites (Lester, 1974; Hoffman, 1999; Atkinson and Bartholomew, 2009). We found only a single reference to “*Ceratomyxa* sp.” in sticklebacks (Lester, 1974). This report notes its prevalence in sticklebacks from salt water near Vancouver, Canada, a locality some 800 km north of where we found *C. gasterostea* n. sp. The parasite is recorded from intestines, though there is no species description, and no follow-up studies were conducted (R. J. G. Lester, pers. comm.). Though these sticklebacks were sampled from salt water, they were infected also with *S. elegans*, a freshwater myxozoan. They conceivably acquired both myxozoan parasites while rearing in freshwater. We consider it likely that *Ceratomyxa* sp. and *C. gasterostea* are related closely, if not the same organism. This would suggest that the parasite infects stickleback populations from California to Canada – a similar range to *C. shasta* in salmonids.

Two studies present survey data that support the hypothesis that more freshwater ceratomyxid species remain to be described in the Pacific Northwest. Arai and Mudry (1983) found “*Ceratomyxa* sp.” in the intestine of 1/43 largescale suckers, *Catostomus macrocheilus*, collected from the headwaters of McGregor River, British Columbia, sympatric with *C. shasta* in salmonids. No further information is available for this taxon. Previously, we discovered a *Ceratomyxa*-like partial ssrRNA-ITS-1 sequence “genotype X” in environmental water samples from the upper Klamath River basin (Atkinson and Bartholomew, 2010b). The sequence was never identified from a host, either vertebrate or invertebrate, hence no spore morphology or tissue data are available. “Genotype X” was more similar to *Ceratonova* species *C. shasta* (88%) and *C. gasterostea* (82%) over ~500 nt, than with any *Ceratomyxa* species. Additional sampling

of infected fish from these river basins is needed for rediscovery and description of these putative *Ceratonova* spp.

Life cycle

The life cycle of *C. gasterostea* is unknown, though most likely involves a specific, obligate invertebrate host. Phylogenetically, ceratomyxid species cluster in the myxozoan ‘marine clade’ (Fiala, 2006). The few marine clade life cycles that are known all involve a polychaete worm host (Bartholomew et al., 1997, 2006; Kjøie et al., 2007). *Ceratonova gasterostea* is related most closely to *C. shasta*, which has a well-characterized life cycle that involves the freshwater polychaete “*Manayunkia speciosa*” (Bartholomew et al., 1997). The polychaete hosts another marine clade myxozoan, *Parvicapsula minibicornis* (Bartholomew et al., 2006), and we consider it likely that it is the host also of *C. gasterostea*. This worm has been recorded from many rivers in the Pacific Northwest (Hazel, 1966; Holmquist, 1973; Stocking and Bartholomew, 2007). Recently, we have identified that this Pacific Northwest *Manayunkia* species is distinct genetically from *Manayunkia speciosa* Leidy, 1883 from the East coast and Great Lakes of North America (unpubl. data). The limited geographic range of this putative invertebrate host would explain restriction of *C. gasterostea* to the Pacific Northwest, despite the cosmopolitan nature of the vertebrate host, and presence of other *Manayunkia* species in the Northern Hemisphere (Bick, 1996; Schloesser, 2013).

Marine versus fresh water species of Ceratomyxidae

The majority of taxa in family Ceratomyxidae infect marine fishes, with the speciose genus *Ceratomyxa* containing predominantly gall bladder-infecting parasites. We have erected a fourth genus, *Ceratonova*, to incorporate the 2 freshwater species that sporulate in the intestine of the fish host. Five other nominal *Ceratomyxa* species are reported from freshwater fish in

geographically distant localities (Lom and Dyková, 2006; Azevedo et al., 2013). All of these species are coelozoic in the gall bladder or urinary system, with spore morphology consistent with *Ceratomyxa* and no DNA sequence data to impel reallocation to different genera at this time. Given the large ‘reservoir’ of marine species it is interesting that so few successful introductions into freshwater have occurred. If all Ceratomyxids require polychaete hosts, as is suggested by invertebrate host correlations with myxosporean phylogenies (Holzer et al., 2007), then freshwater invasion is probably constrained by lack of availability and diversity of potential freshwater polychaete hosts.

Implications for Klamath River research

The close geographic and genetic relatedness of *C. shasta* and *C. gasterostea* present some practical challenges. We aligned the DNA sequence of *C. gasterostea* with qPCR primers Cs-1034F, Cs-1104R and CsProbe-1058T used to assay *C. shasta* in water samples, fish tissues and polychaetes (Hallett and Bartholomew, 2006). The *C. gasterostea* sequence was identical to the oligonucleotides thus could be expected to produce a false positive for *C. shasta*. This explains the *C. shasta* qPCR assay results of the stickleback samples in 2009: although 14/22 fish were unambiguously positive, only *C. gasterostea* could be amplified and sequenced from these fish. Primer non-specificity is not likely to be a concern for Klamath River studies that focus on localities upstream from Rkm ~50, the highest point at which we detected *C. gasterostea*. Environmental samples from the lower Klamath River, however, could contain mixtures of both species. The *C. gasterostea* qPCR amplicon had a 2 nt insertion relative to *C. shasta*, which means it could be possible to distinguish the 2 species using the current qPCR with high-resolution melting curve analysis. Future assay development should take into account the existence of *C. gasterostea* when designing primers.

ACKNOWLEDGMENTS

We thank Yurok Tribal Fisheries biologists for fish collections, Kim True (USFWS) for performing the *C. shasta* qPCR assays, and Sascha Hallett (OSU) and an anonymous reviewer for critical input on the manuscript. Funding for this project was provided by the Bureau of Reclamation through Cooperative Agreement R09AC20022, Cooperative Ecosystems Study Unit 3FC810873.

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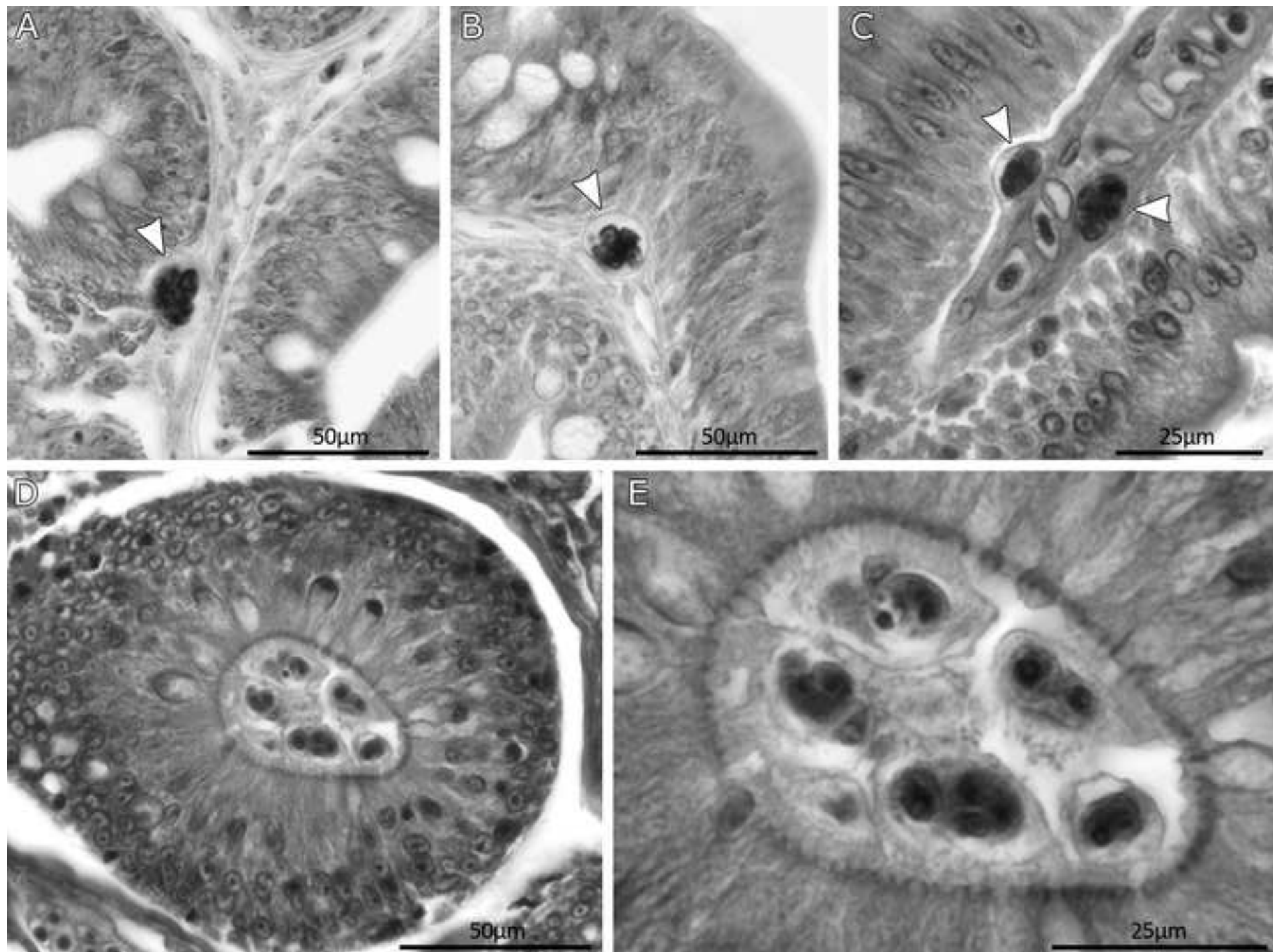
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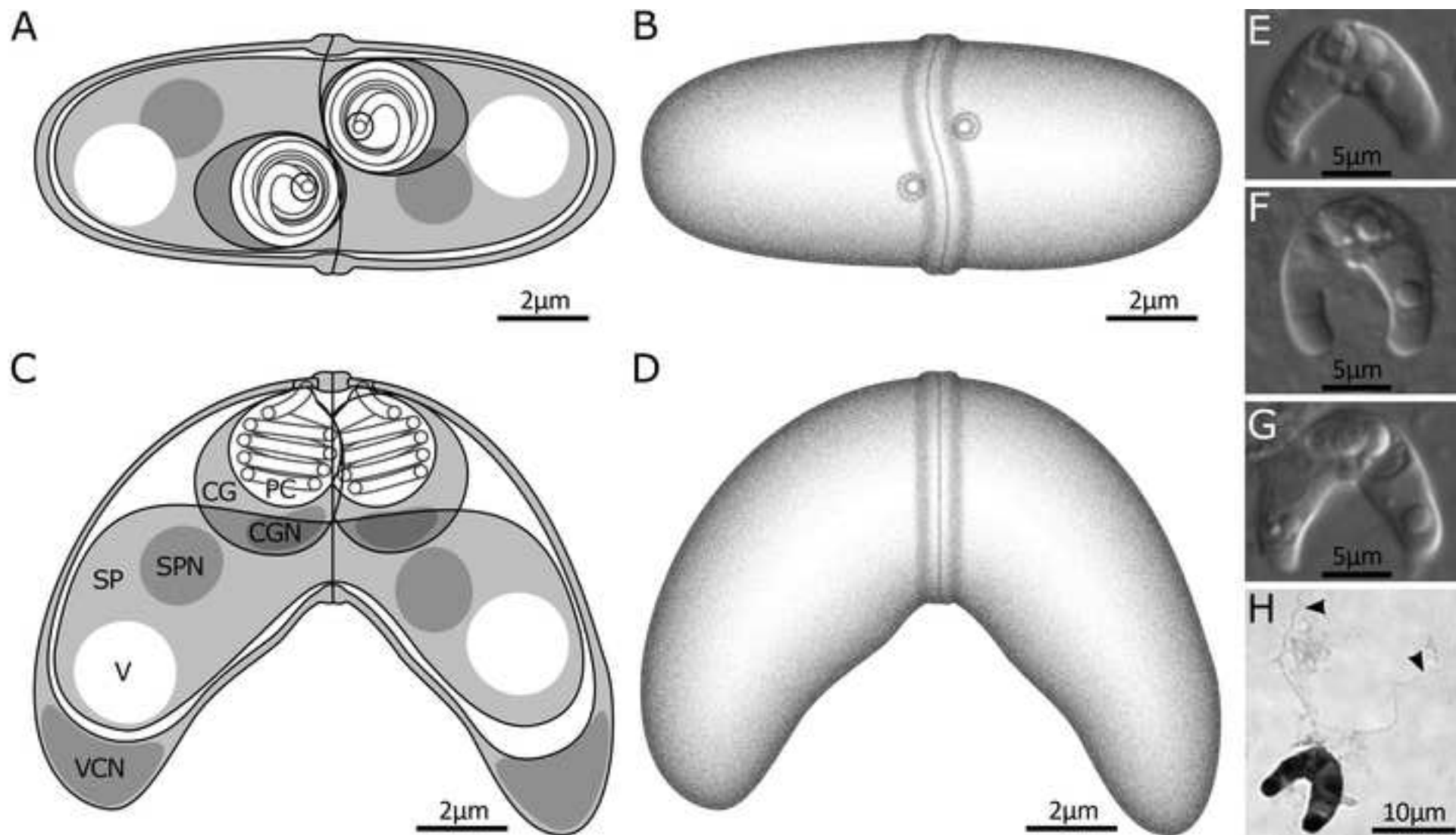
Figure 1. Histological sections of intestine from threespine stickleback collected in the lower Klamath River, California. (A-C) Multicellular parasite stages (arrowed) are apparent in lamina propria, close to capillaries. (D, E) Maturing myxospores in intestinal lumen.

Figure 2: *Ceratonova gasterostea* sp. n. Illustrated spore in apical (**A, B**) and side sutural views (**C, D**), showing internal structures (A, C) and external appearance (B, D). All cell components are paired, only 1 of each pair is labelled: VC = valve cell; VCN = valve cell nucleus; V = vacuole; SP = sporoplasm; SPN = sporoplasm nucleus; PC = polar capsule with coiled filament; CG = capsulogenic cell; CGN = capsulogenic cell nucleus; several sporoplasmosomes are often present within spores, but not shown in figure for clarity. Apical view illustrates the pores through which the polar filaments are fired. (**E-G**) Nomarski interference contrast images showing normal range of spore morphology, all images at same scale. (H) Diff-Quik stained spore with extruded polar filaments (arrowed).

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Figure
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