Research Article

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The *Proteocephalus* species-aggregate (Cestoda) in sticklebacks (Gasterosteidae) of the Nearctic Region, including description of a new species from brook stickleback, *Culaea inconstans*

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Abstract: A survey of the species of the *Proteocephalus*-aggregate from sticklebacks (Actinopterygii: Gasterosteidae) is provided. The occurrence of three species in North America is confirmed: (i) *Proteocephalus filicollis* (Rudolphi, 1802), which has been reported from the three-spined stickleback, *Gasterosteus aculeatus* Linnaeus, in the northeastern part of North America (Newfoundland); (ii) *Proteocephalus pugetensis* Hoff et Hoff, 1929 occurs also in *G. aculeatus*, but in northwestern North America (British Columbia and Washington); and (iii) *Proteocephalus culaeae* sp. n., which is described from the brook stickleback, *Culaea inconstans* (Kirtland), in Manitoba (Canada). Another species, *Proteocephalus ambiguus* (Dujardin, 1845), a specific parasite of the nine-spined stickleback, *Pungitius pungitius* (Linnaeus), and type species of the genus, has also been found in North America (Alberta, Canada), but its vouchers are in poor condition and cannot be reliably assigned to this species. Both species reported from three-spined stickleback differ from each other by the shape of the scolex (rounded in *P. filicollis* versus continuously tapered towards the anterior extremity in *P. pugetensis*) and the apical sucker (widely oval to subspherical in frontal view in *P. filicollis* versus flattened in *P. pugetensis*). *Proteocephalus culaeae* sp. n. is characterised by a short body composed of a few, continuously widened proglottids, a short scolex narrower than the strobila and devoid of an apical sucker, a short, pyriform cirrus sac, no vaginal sphincter, and few testes. A key to species of the *Proteocephalus*-aggregate from sticklebacks is provided.

Keywords: tapeworms, species diversity, systematics, identification key, freshwater fish, Gasterosteiformes, North America

Tapeworms (Cestoda) are common and widespread parasites of freshwater fishes in North America (Hoffman 1999, Scholz and Kuchta 2017), but little attention has been paid to this group of endoparasitic helminths over the past several decades (Scholz and Choudhury 2014). Therefore, taxonomic studies based on a critical examination of type and voucher specimens from museum collections supplemented by a morphological evaluation of newly collected, properly fixed material are being carried out on fish tapeworms of North American freshwater fishes.

Scholz et al. (2019) presented a survey of tapeworms of the *Proteocephalus*-aggregate (see de Chambrier et al. 2004), which parasitise centrarchid and percid fishes in North America, i.e., bass (*Micropterus* spp.), perch (*Perca flavescens* Mitchill) and pikeperch (*Sander* spp.). Here we provide a robust baseline for future biodiversity, ecologically, and evolutionary studies on fish tapeworms. Therefore, the species of the *Proteocephalus*-aggregate that occur in sticklebacks (family Gasterosteidae of the order Gasterosteiformes) in the Nearctic region are reviewed.

Sticklebacks are small, elongated fishes. They are characterised by the absence of scales and their skin is protected by a variable number of hard, thin, bony plates on the sides of the body. Sticklebacks are carnivorous, feeding on small animals such as insects, crustaceans and fish larvae. They occur in fresh, brackish and marine waters in temperate regions of the Northern Hemisphere. Currently, 18 species in five genera are recognised (Froese and Pauly 2020).

Sticklebacks are fishes of negligible economic importance and thus their parasites, overall, have attracted only moderate interest. However, one species, the three-spined stickleback, *Gasterosteus aculeatus* Linnaeus, has been an iconic model for evolutionary studies and host-parasites interactions for several decades (Bell and Foster 1994, McKinnon and Rundle 2002) as well the subject of numerous investigations into its interactions with a larval cestode specific for it, the diphyllbothriidean *Schistocephalus solidus* (Müller, 1776) (e.g., Heins et al. 2010a, b). Plerocercoids of this cestode may alter behaviour and reprodu-

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ctive capacity of infected fish (Barber et al. 2000, Barber and Svensson 2003). In recent years, parasite communities in eastern North American populations of threespine stickleback have been analysed for patterns and processes in their parasite communities (Poulin et al. 2011).

Three species of *Proteocephalus* Weinland, 1858 have been described from sticklebacks: *Proteocephalus ambiguus* (Dujardin, 1845) from nine-spined stickleback, *Pungitius pungitius* (Linnaeus); *Proteocephalus filicollis* (Rudolphi, 1802); and *Proteocephalus pugetensis* Hoff et Hoff, 1929, both from *G. aculeatus* (see de Chambrier et al. 2017). In addition, Hoffman (1999) reported in the Host-Parasite list unidentified tapeworms (*Proteocephalus* sp.) from *P. pungitius*, *Apeltes quadracus* (Mitchill), *G. aculeatus*, and blackspotted stickleback, *Gasterosteus wheatlandi* Putnam.

In the present paper, a survey of the species of the *Proteocephalus*-aggregate from sticklebacks (Actinopterygii: Gasterosteidae) is provided and a new species is described from the brook stickleback, *Culaea inconstans* (Kirtland).

MATERIALS AND METHODS

The present study is based on the examination of available voucher specimens of the *Proteocephalus*-aggregate from sticklebacks (Gasterosteidae) in North America (for which no type specimens are available) and specimens of *Proteocephalus* spp. collected from sticklebacks in British Columbia and Manitoba, Canada. Tapeworms collected by AC were rinsed in 0.6% or 0.9% NaCl solution after removing them from the host intestine and fixed in hot 4% formaldehyde solution or killed in hot water with a quick subsequent transfer to AFA. Tapeworms were stained with acetocarmine or Ehrlich's hematoxylin, dehydrated in an ascending series of ethanol, cleared in methyl salicylate or xylene and mounted in Canada balsam on slides.

The following museum abbreviations were used in the paper: CMNPA – Canadian Museum of Nature Parasite Collection, Ottawa, Canada; HWML – Harold W. Manter Laboratory, Lincoln, Nebraska, USA; IPCAS – Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic; Nfld – The Rooms Provincial Museum, Newfoundland and Labrador, St. John's Newfoundland, Canada; UAPC – University of Alberta Parasite Collection, Edmonton, Canada; USNM – Smithsonian National Museum of Natural History, Washington, D.C., USA. Fish names follow Froese and Pauly (2020).

RESULTS

Morphological evaluation of newly collected and museum specimens of proteocephalid tapeworms found in sticklebacks (Gasterosteiformes: Gasterosteidae) in the Nearctic region confirmed the presence of three species including one new to science. The occurrence of another species specific to the nine-spined stickleback has to be confirmed. A survey of these four taxa that may occur in North America is provided in alphabetical order.

Proteocephalus ambiguus (Dujardin, 1845) Weinland, 1858 (occurrence of this species in North America has to be verified) Fig. 1A

Synonyms: *Taenia ambigua* Dujardin, 1845; *Proteocephalus filicollis* (Rudolphi, 1802) *auct. in part*

Material studied: One slide with fragments of a contracted, deformed specimen from Lake Superior, collected on 22 May, 1985 (USNM 1375661); 2 specimens identified as *Proteocephalus* sp. from South Baymouth, Manitoulin District, Ontario, Canada, collected by A.O. Dechtiar in 1969 (CMNPA 1987-2692, 2693), 1 specimen from unknown locality, D.J. Marcogliese (CMNPA 1993-0007), and 3 specimens misidentified as *P. filicollis* (UAPC 9968.01s, 9968.02s and 9968.03s) and provisionally considered here as *P. ambiguus*. For material from Europe, see Scholz and Hanzelová (1998).

Type and only host: nine-spined stickleback, *Pungitius pungitius* (Linnaeus) (Gasterosteiformes: Gasterosteidae).

Type specimens: Not known to exist.

Type locality: Rennes, France.

Distribution: Europe (British Isles, Estonia, Finland, France, Germany, Netherlands, Norway, Poland, Russia, Sweden), North America (Alberta – provisional identification).

Morphological descriptions: Willemse (1968), Rødland (1983), Scholz and Hanzelová (1998), Scholz et al. (1998).

Representative DNA sequences and phylogenetic relationships: Currently available sequence data originate from a single specimen of *P. ambiguus* from *P. pungitius* collected by L. Rolbiecki in Poland: (i) ITS-2 + 5.8S (DQ427096) region of the rRNA gene array and V4 region of *ssrDNA* (DQ427100) by Scholz et al. (2007). A phylogenetic analyses by Scholz et al. (2007) revealed that *P. ambiguus* forms a sister lineage to the Palaearctic species *P. thymalli* (Annenkova-Chlopina, 1923) from graylings (*Thymallus* spp.), and distant in position from the clade comprising *P. filicollis* (see fig. 1 in Scholz et al. 2007).

Remarks: *Proteocephalus ambiguus* is the type species of the genus and a specific parasite of the nine-spined stickleback (Scholz and Hanzelová 1998). The validity of this rare species was questioned repeatedly and *P. ambiguus* was considered a synonym of *P. filicollis* by numerous authors (e.g., La Rue 1914, Yamaguti 1959, Freze 1965, Schmidt 1986, Dubinina 1987, Rego 1994). However, Willemse (1968) and Rødland (1983) provided evidence that *P. ambiguus* and *P. filicollis* are distinct species. Scholz et al. (1998) and Scholz and Hanzelová (1998) considered *P. ambiguus* to be a valid species. Validity of *P. ambiguus* was later supported by molecular data (Scholz et al. 2007), but only sequences of ITS-2 + 5.8S region and V4 region of the *ssrRNA* gene are available and no ethanol-fixed material is currently available for a more detailed molecular study, especially sequencing the *lsrRNA* and COI genes.

There appears to be no published record of *P. ambiguus* from North America (see Hoffman 1999, Gibson et al. 2005), but there are a few voucher specimens from *P. pungitius* identified as *P. ambiguus* (USNM 1375661). Unfortunately, all specimens are in poor condition, being either decomposed and/or deformed (Fig. 1F), which impedes their reliable identification. The specimen USNM 1375661 differs from *P. ambiguus* in several diagnostic characteristics: (i) the strobila is robust and composed of very short and wide proglottids with parallel margins (data not shown) (proglottids of *P. ambiguus* are rectangular to oblong, with slightly convex lateral margins, i.e., barrel-shaped; see fig. 6A, B in Scholz and Hanzelová 1998); (ii) the scolex of the voucher is double bent, but the apical

Fig. 1. **A** – *Proteocephalus ambiguus* (Dujardin, 1845) from *Pungitius pungitius* Linnaeus, Karelia, Russia, scolex; **B** – *Proteocephalus filicollis* (Rudolphi, 1802) from *Gasterosteus aculeatus* Linnaeus, Scotland, UK, scolex (both redrawn from Scholz and Hanzelová 1998); **C** – juvenile *Proteocephalus macrocephalus* (Creplin, 1825) from *P. pungitius*, Nova Scotia, Canada, scolex (see Marcogliese and Scholz, 1999; IPCAS C-209/4); **D, E, H–J** – *Proteocephalus pugetensis* Hoff et Hoff, 1929 from *G. aculeatus*, British Columbia, Canada; **D, E** – scolex; **H** – mature proglottid; ventral view; **I** – terminal genitalia; dorsal view; **J** – gravid proglottid; ventral view; **F** – *Proteocephalus* sp. (unidentifiable) from *P. pungitius*, Lake Superior (USNM 1375661); scolex; **G** – *Proteocephalus* ‘*filicollis*’ from *Micropterus* sp., Down Lake, Michigan (USNM 1349988); frontal section of terminal genitalia; note a well-developed, ring-like vaginal sphincter typical of *P. longicollis* [Zeder, 1800]).

sucker seems to be relatively deep (width or diameter 30 µm, height 20 µm – Fig. 1F) compared to that in *P. ambiguus*, which is more flattened, only 9–12 µm high (see fig. 1C, D in Scholz et al. 1998); (iii) the number of the testes in the North American specimen is slightly higher (46–53; $n = 3$) than that of *P. ambiguus* in Europe (32–46 according to Scholz and Hanzelová 1998).

Three specimens from nine-spined stickleback from Medley River, Cold Lake, Alberta, Canada, accessioned as *P. filicollis* (UAPC 9968.01s-03s), resemble *P. ambiguus* from Europe in their gross morphology and available comparative data (Table 1) and may be conspecific with it. Although the apical sucker appeared to be shallow as in *P. ambiguus*, only a few proglottids were barrel shaped, a characteristic feature of *P. ambiguus*. All three specimens were from Cold Lake, Alberta, but they do not appear to be part of the major survey by Leong and Holmes (1981) because the accession number UAPC 9968 is not in their published study. Due to the poor condition of the specimens, we consider this identification as *P. ambiguus* provisional until fresh specimens can be collected from nine-spined sticklebacks in Alberta.

None of the other vouchers of *Proteocephalus* tapeworms from *P. pungitius* in Canada (CMNPA 1987-2692, 2693, 1993-0007) could be assigned to *P. ambiguus* with confidence either, especially because of their poor quality. Two mature stained and mounted specimens from *P. pungitius*,

CMNPA 1987-2692 and 2693, were 8.5 and 10.9 mm long, respectively, with proglottids becoming longer than wide posteriorly. These worms did not possess the barrel-shaped proglottids characteristic of *P. ambiguus*. Judging by the condition of the suckers, the scolex of CMNPA 1987-2692 appears to be poorly preserved and an apical disc could not be discerned. The apical sucker of *P. ambiguus* is small and shallow (Fig. 1A) and consequently would be prone to disintegration in poorly preserved material. In some other respects, namely its small size, few mature and gravid segments, shape of the scolex, placement of the suckers, and general disposition of the ovary and vitellarium, the worm resembles the new species from brook stickleback, *Culaea inconstans*, being described here.

The scolex of CMNPA 1987-2693 appears partially contracted such that the suckers appear cup-shaped and directed anteriorly. There appeared to be traces of a sunken apical sucker in this specimen but we cannot be certain. Other features such as the ovary and vitellarium do not distinguish it from the other species found in sticklebacks. The testes in both these specimens could not be accurately differentiated. In conclusion we cannot assign either of these specimens to any known species of *Proteocephalus* with confidence. CMNPA 1993-0007 was a small fluid preserved specimen; it did not appear to be in a condition suitable for preparing a stained whole mount.

Fig. 2. *Proteocephalus filicollis* (Rudolphi, 1802) from *Gasterosteus aculeatus* Linnaeus, Newfoundland, Canada (Nfld; Slide C-2, Threlfall Collection; specimens collected by Hanek and Threlfall 1970c). **A** – whole worm; **B** – scolex; **C** – gravid proglottid; dorsal view; **D** – pregravid proglottid, ventral view.

It is obvious that the occurrence of *P. ambiguus* in North America remains to be confirmed and molecular data for comparison with European populations should be obtained.

Proteocephalus filicollis (Rudolphi, 1802) Weinland, 1858
Figs. 1B, 2

Synonym: *Taenia filicollis* Rudolphi, 1802

Material studied: 1 specimen of *P. filicollis* from *Gasterosteus aculeatus*, Newfoundland, collected by Hanek and Threlfall (1970c) (Nfld; Slide C-2, Threlfall Collection) vouchers (longitudinal sections of gravid specimens and cross sections of scoleces) identified as “*P. filicollis*” from *Micropterus* sp., Down Lake, Michigan, USA (H.B. Ward Collection; USNM 1349988); for material from Europe – see Scholz and Hanzelová (1998). Three specimens from nine-spined stickleback from Medley River, Cold Lake, Alberta, Canada, accessioned as *P. filicollis* (UAPC 9968.01s-03s) have been discussed in the section on *P. ambiguus*.

Type host: *Gasterosteus aculeatus* Linnaeus (Gasterosteiformes: Gasterosteidae).

Additional reported hosts (all should be verified): *Culaea inconstans* (North America – see Remarks),

Pungitius pungitius (Europe; most probably misidentification of *P. ambiguus*) (Gasterosteiformes: Gasterosteidae); records from *Coregonus artedii* Lesueur, *C. nigripinnis* (Millner) and *C. prognathus* Smith [*nomen dubium*] by Benedict (1900), Watson and Dick (1979), and Leong and Holmes (1981) in Michigan, Manitoba and Alberta, respectively, and from *Micropterus* sp. in Michigan, are likely misidentifications.

Type specimens: Not known to exist.

Type locality: Greifswald, Germany.

Distribution: Circumboreal (Europe, Russia, North America – Canada, USA).

Morphological descriptions: Willemse (1968), Rødland (1983), Scholz et al. (1998), Scholz and Hanzelová (1998).

Life cycle: Diaptomid and cyclopoid copepods (*Eudiaptomus gracilis* [Sars], *Cyclops strenuus* Fischer, *Eucyclops serrulatus* [Fischer], and *Thermocyclops oithonoides* [Sars]) serve as natural or experimental intermediate hosts of this tapeworm, whose life cycle was studied only in Europe by Meggitt (1914), Kuczkowski (1925), Hopkins (1959), and Willemse (1968); no data are available from North America.

Representative DNA sequences and phylogenetic relationships (based on European

samples only): ITS2 – AY551162, and partial *ssrDNA* (V4 region) – AF335506 (Hypša et al. 2005); partial *lsrDNA* (D1–D3 region) – AJ388636, and partial large subunit (16S) mitochondrial rDNA (*rrnL*) – AJ389484 (Zehnder and Mariaux 1999). All molecular phylogenetic analyses invariably revealed *P. filicollis* as a sister taxon to *P. macrocephalus* (Creplin, 1825), a specific parasite of eels (*Anguilla* spp.) in the Palearctic and Nearctic Region, with *P. pearsei* as a sister lineage (Scholz et al. 2019).

Remarks: This species is a specific parasite of three-spined stickleback (Scholz and Hanzelová 1998). Hoffman (1967) reported unpublished records of *P. filicollis* from *Culaea inconstans* in North Dakota and Wisconsin (USNM 1373829), but morphology of these specimens was not described. These tapeworms may in fact belong to the new species from brook stickleback described in the present paper (see below).

Hanek and Threlfall (1970a–c) reported *P. filicollis* from *G. aculeatus* in Newfoundland, Canada. Examination of a single voucher specimen (Nfld-Slide C-2, Threlfall Collection) confirmed its species identification (Fig. 2). This specimen represents the only reliable record of the parasite in North America.

Examination of vouchers of *P. filicollis* from *Micropterus* sp. (USNM 1349988) revealed that they do not belong to this species. The scolex on slide labelled A.R.C. 57 seems to belong to *P. fluviatilis*, whereas a much smaller scolex with a large apical sucker on the slide labelled A.R.C. 55c belongs to a plerocercoid of *Proteocephalus ambloplitis* (Leidy, 1887). Adult specimens longitudinally sectioned on the slides labelled A.R.C. 55 and 55b possess a well-developed ring-like vaginal sphincter 26–28 µm in diameter (see Fig. 1G), thus markedly differing from *P. filicollis*, which does not possess any vaginal sphincter (Scholz and Hanzelová 1998). A similar ring-like vaginal sphincter is present only in *Proteocephalus longicollis* (Zeder, 1800) (syn. *P. exiguus* La Rue, 1911), which is a common parasite of salmoniform fishes in the Holarctic region (see Scholz and Hanzelová 1998, Hanzelová and Scholz 1999).

Benedict (1900) and several subsequent authors (see McDonald and Margolis 1995, Hoffman 1999 for references) reported tapeworms from whitefish (*Coregonus* spp.) as *P. filicollis*, but these in fact belonged to *P. longicollis* (see La Rue 1914, Scholz and Hanzelová 1998).

***Proteocephalus pugetensis* Hoff et Hoff, 1929**

Figs. 1D,E,H–J, 3

Material studied: three gravid and two mature specimens from *Gasterosteus aculeatus*, British Columbia, collected by one of us (AC) from Little Campbell River, British Columbia, on 27–29 April 1998.

Type and only known host: *Gasterosteus aculeatus* Linnaeus (Gasterosteiformes: Gasterosteidae).

Type specimens: Not known to exist.

Type locality: Tide pools near Smith's Cove, Seattle, Washington, USA.

Distribution: Canada (British Columbia), USA (Washington).

Life cycle: Guberlet (1929) found metacercariae identified as *P. pugetensis* in *Cyclops* sp.

Morphological description: Hoff and Hoff (1929).

Representative DNA sequences and phylogenetic relationships: No molecular data are available. In its morphology, *P. pugetensis* fits into the diagnosis of the *Proteocephalus*-aggregate and likely belongs to this monophyletic lineage (de Chambrier et al. 2015).

Remarks: *Proteocephalus pugetensis* was described from *G. cataphractus* (= syn. of *G. aculeatus*) by Hoff and Hoff (1929). The original description including illustrations was detailed, even though observations were largely based on histological sections (“Finer histological details were worked out from frontal sections 10–15 thick and from serial cross-sections.” – Hoff and Hoff 1929).

Type specimens of *P. pugetensis* are not known to exist and probably were never deposited (no information about types was provided in the original description). Nevertheless, comparison of tapeworms from British Columbia with those described by Hoff and Hoff (1929) revealed their conspecificity. The tapeworms from Washington and British Columbia are almost indistinguishable in their morphology and measurements, including (i) size of the body (25 mm according to Hoff and Hoff 1929 and 23–29 mm in the new material); (ii) shape of the body, which tapers continuously towards the anterior end (Fig. 2); (iii) small scolex (scolex width 100 µm in the original material and 105–118 µm at the level of the suckers in the new material), which is narrower than indistinct neck region (“The neck is always wider than the scolex”) and first proglottids (Fig. 1D); (iv) a very small, flattened vestigial (“rudimentary”) sucker, 23 µm in diameter in specimens from USA and 15–23 µm (6–13 µm in thickness) in specimens from Canada (Fig. 1D,E); (v) small, sublaterally situated suckers, 27–39 µm and 40–45 µm in diameter (Hoff and Hoff 1929 and the present study, respectively), with a very shallow cavity (Fig. 1D,E); (vi) a small, pyriform cirrus sac, the length of which represents less than 1/4 of the proglottid width (Fig. 1H–J); (vii) the absence of a vaginal sphincter (Fig. 1H, I); and (viii) a few lateral uterine diverticula (“Typically, there are six diverticula on the side where the cirrus pouch is located and seven on the opposite side” – Hoff and Hoff 1929; Fig. 1H).

The original description of *P. pugetensis* did not mention the presence of numerous and large, widely oval gland cells beneath the tegument of the scolex, which are very prominent in the specimens from British Columbia (Fig. 1E,G). Hoff and Hoff (1929) also reported 30–40 testes arranged in a single layer dorsal to the uterus, but the present study has revealed that some testes are in an incomplete second layer and that the number of the testes, which were counted from line drawings of last mature and first gravid proglottids, is in fact higher, 47–67 (mean 57; n = 8).

Even though the original description of *P. pugetensis* was of good quality and the species is considered valid (see below), its differentiation from *P. filicollis* was not based on accurate data on the latter species. Hoff and Hoff (1929) stated “*P. pugetensis* differs from *P. filicollis* in having (1) a fifth rudimentary sucker, (2) smaller functional suckers, (3) fewer testes and these in one layer.”

Fig. 3. *Proteocephalus pugetensis* Hoff et Hoff, 1929 from *Gasterosteus aculeatus* Linnaeus, British Columbia, Canada. Whole worm; dorsal view; testes omitted in all but one (fifth proglottid in the middle).

In fact, the fifth rudimentary (vestigial) sucker is also present in *P. filicollis*, the suckers slightly overlap in their size, being actually somewhat larger in *P. filicollis* (38–93 μm in diameter – Scholz et al. 1998), and the arrangement and number of the testes are in fact almost identical as in *P. filicollis*, i.e., in one layer with some testes in the second incomplete layer; the number of the testes in *P. filicollis* varies from 22 to 86 (Scholz and Hanzelová 1998).

Both species are actually very similar to each other, but they are considered as two separate species because of a different shape of the scolex, which is continuously tapered towards the anterior extremity in the former species (Figs. 1E, 2) versus rounded, and distinct from the neck in *P. filicollis* (Fig. 1B; see also fig. 1L, M and 4A–C in Scholz et al. 1998), as well as being narrower in *P. pugetensis* (100–118 μm versus 114–231 μm in *P. filicollis*). Finally, the apical sucker is flat in *P. pugetensis* versus widely oval to subspherical in frontal view in *P. filicollis* (compare Fig. 1D,E with Fig. 1B; see also fig. 1N–P in Scholz et al. 1998).

The existing data, albeit without accompanying genetic evidence, indicate that these two species occur allopatrically in three-spined stickleback in North America: *P. filicollis* occurs in the northeastern part of North America (and in Europe and northern Russia), whereas *P. pugetensis* is endemic to the northwestern part of North America (British Columbia and Washington).

Proteocephalus culaeae sp. n.

Fig. 4

ZooBank number for species:

[url:lsid:zoobank.org:act:FAF5CD33-17B5-4D7B-96D7-EF81BD59E4F5](https://zoobank.org/act:FAF5CD33-17B5-4D7B-96D7-EF81BD59E4F5)

Material studied: 5 adult specimens collected by one of us (AC) from brook stickleback *Culaea inconstans*, caught in the upper wetlands area of the River Brokenhead, Manitoba, Canada, on 19 July 1997; 1 adult specimen identified as *P. filicollis* from *C. inconstans*, Lake Sixteen, Price County, Wisconsin, USA, collected by Becky Lasee on 11 October 1981 (USNM 1373829).

Description (based on five mature specimens from *Culaea inconstans* in Manitoba, Canada; measurements in micrometres unless otherwise stated, with means and number of measurements in parentheses when appropriate): Proteocephalidae, Proteocephalinae, *Proteocephalus*-aggregate. Total body length 4.4–6.0 mm (5.4 mm; $n = 5$), maximum width 420–621 (538; $n = 5$). Strobila acraspedote, anapolytic, slightly, but continuously widening towards posterior end (Fig. 4A), consisting of about 13–21 proglottids: 9–14 immature (up to appearance of spermatozoa in vas deferens), 1–2 mature (up to appearance of eggs in uterus), 2–4 pregravid (up to appearance of hooks in oncospheres), and 1–2 gravid. Immature proglottids much wider than long to wider than long, 108–340 $\times$ 326–581 (length: width ratio 1 : 1.32–4.76; $n = 37$), mature proglottids wider than long, 239–397 $\times$ 419–580 (length: width ratio 1 : 1.06–1.93; $n = 7$), pregravid proglottids variable in shape, from wider than long to much longer than wide, 333–1,186 $\times$ 365–611 (length: width ratio 1 : 0.32–1.71; $n = 16$), gravid proglottids almost quadrate, 570–589 $\times$ 565–621 (length: width ratio 1 : 0.99; $n = 2$) (Fig. 4A,E,H).

Anterior end widely round to almost blunt (Fig. 4B–D), with scolex indistinctly separated from neck region (Fig. 4A–D), 167–230 wide ($n = 4$) at level of posterior margin of sucker, narrower than wide, indistinct neck region (Fig. 4B–D). Suckers subspherical, 61–88 $\times$ 54–76 ($n = 19$), directed sublaterally, with shallow cavity, relatively small

Fig. 4. *Proteocephalus culaeae* n. sp. from *Culaea inconstans* (Kirtland), Manitoba, Canada. **A** – whole worm; **B** – anterior end; **C** – anterior extremity; note absence of an apical sucker; **D** – scolex of *P. 'flicollis'* (= *P. culaeae*) from *C. inconstans*, Lake Sixteen, Wisconsin, USA (USNM 1373829); **E** – mature proglottid, ventral view; **F** – eggs; **G** – terminal genitalia, ventral view; **H** – gravid proglottid, ventral view. Holotype (**A**, **B**, **F**, **G**; USNM 1618957); paratype (**C**, **E**; IPCAS C-840).

compared to width of scolex (Fig. 4B–D). Apical sucker absent; numerous cells with granular content between suckers and in apical part of scolex (Fig. 4B,C). Whole surface covered with dense microtriches about 3 long (drawn in Fig. 4G).

Inner longitudinal musculature weakly developed (cross sections unavailable). Two pairs of narrow, almost straight osmoregulatory canals; ventral canals wider, 5–9 in diameter, with lateral canals in some proglottids (Fig. 4E), dorsal canals narrower, 2–3 in diameter (Fig. 4E,H).

Testes medullary, ovoid to subspherical, $33\text{--}67 \times 30\text{--}59$, almost always in 1 irregular layer, 21–35 in number (28; $n = 25$). Testes form 2 wide, preovarian fields median to vitelline follicles and osmoregulatory canals, separated posteriorly and confluent near anterior margin of proglottids (Fig. 4E). Testes present also in pregravid and gravid proglottids (Fig. 4H).

Vas deferens strongly coiled, with loops forming relatively small field lateral to uterine diverticula in pregravid and gravid proglottids (Fig. 4G,H). Cirrus sac widely pyriform, thin-walled (Fig. 4E,G,H), $106\text{--}143 \times 48\text{--}78$ wide ($n = 25$), cirrus sac length: width ratio 1 : 0.38–0.69 ($n = 25$), length of cirrus sac represents 21–34% (24%; $n = 25$) of proglottid width. Internal sperm duct voluminous, coiled, occupies proximal half of cirrus sac (Fig. 4G). Cirrus short, muscular, representing about 41–47% of length of cirrus sac. Common genital atrium narrow, deep (Fig. 4E,G,H), alternating irregularly, slightly pre-equatorial to equatorial, at 33–53% (43%; $n = 21$) of length of proglottid from its anterior margin (Fig. 4E,H).

Ovary medullary, bilobed, with narrow isthmus and ovarian lobes surpassing osmoregulatory canals laterally (Fig. 4E,H). Length of ovary, i.e., width of ovarian lobes, 66–376, i.e., 18–32% of proglottid length ($n = 21$); total width of ovary (horizontal) 230–460, i.e., 63–78% of proglottid width ($n = 21$). Mehlis' gland subspherical to spherical, $55\text{--}78 \times 60\text{--}84$ ($n = 8$). Relative ovarian size (see de Chambrier et al. 2012), 10–14% ($n = 2$).

Vaginal canal sinuous proximally; seminal receptacle elongate ovoid, thick-walled, situated anterior to ovarian isthmus (Fig. 4E); terminal (distal) part of vaginal canal (*pars copulatrix vaginae*) always anterior to cirrus sac (Fig. 4E,G,H), surrounded by chromophilic cells; vaginal sphincter absent (Fig. 4G).

Vitelline follicles medullary, forming 2 narrow lateral bands between anterior margin of proglottids and anterior margin of ovary, absent at level of cirrus-sac and vagina on ventral side (Fig. 4E,G,H). Length of bands represents 73–86% ($n = 11$) and 65–79% ($n = 11$) of length of proglottid on poral and aporal side, respectively.

Uterus medullary, with type 2 development (see de Chambrier et al. 2004). Uterine stem lined with chromophilic cells appearing in last immature proglottids; in mature proglottids, uterine lumen gradually extends from base to apex into each digitate diverticula lined with chromophilic cells as in pregravid and gravid proglottids. Uterus with 4–8 and 5–8 lateral diverticula on poral and aporal sides, respectively (Fig. 4H); width of uterus represents up to 3/5 of proglottid width.

Eggs spherical, hyaline outer envelope not observed in whole mounts; embryophore bilayered, with external layer 30–34 in diameter ($n = 22$) and internal envelope 25–30 in diameter ($n = 13$); oncosphere subspherical, $21\text{--}23 \times 20\text{--}21$ ($n = 23$), with 6 embryonic hooks 10–11 (median pair) and 9–10 (lateral pairs) long (Fig. 4F).

Taxonomic summary

Type host: Brook stickleback, *Culaea inconstans* (Kirtland)

(Gasterosteiformes: Gasterosteidae).

Type locality: Upper wetlands area of the River Brokenhead, Manitoba, Canada ($50^{\circ}22'56''\text{N}$; $96^{\circ}42'56''\text{W}$).

Additional localities: USA (Wisconsin).

Site of infection: Anterior intestine.

Infection rate: 1 of 24 adult brook stickleback collected on 19 July 1997 was infected with 6 tapeworms (prevalence 4%); total length of infected fish was 5.3 cm. In addition, 54 and 35 adult brook stickleback collected from the River Brokenhead on 28 June 1997 and 13 July 1997, respectively, were not infected with this tapeworm.

Type material: Holotype (USNM 1618957; one complete, stained specimen as a permanent whole mount on a slide); two paratypes (HWML 216326, 216327; two whole mounts with two specimens; one without scolex); one paratype (IPCAS C-840; one whole mount with one specimen without terminal proglottids); one paratype (MHNG-PLAT-137302; one whole mount with a complete specimen).

Etymology: Specific name refers to generic name of its definitive host.

Representative DNA sequences and phylogenetic relationships: No molecular data are available. The new species is placed in the *Proteocephalus*-aggregate based on its morphology (see below).

Differential diagnosis. The new species belongs to the *Proteocephalus*-aggregate as defined by de Chambrier et al. (2004) because it possesses all morphological characteristics typical of this Holarctic group of cestode parasites of freshwater fishes, such as a simple scolex, the testes tightly packed in the proglottids, lateral bands of vitelline follicles not exceeding the anterior or middle part of the ovary, uterine development type 2 according to de Chambrier et al. (2004), and uterine diverticula occupying most of the width of gravid proglottids.

Proteocephalus culaeae sp. n. differs from all species of the aggregate (and also the remaining species placed in the non-monophyletic genus *Proteocephalus* Weinland, 1858) including other species from sticklebacks, i.e., *P. ambiguus*, *P. filicollis* and *P. pugetensis*, by its size, which is very small (maximum length 6 mm), and a body that gently and gradually widens from a small, blunt scolex towards the posterior extremity, and composed of only about 20 proglottids, with less than 15 immature proglottids, 1 or 2 mature proglottids and a few (3–4) pregravid and gravid proglottids.

Two small, insufficiently described species of *Proteocephalus* from cavefishes (Percopsiformes: Amblyopsidae) in Kentucky, namely *Proteocephalus chologasteri* Whittaker et Hill, 1968 (total length 4.2–9.7 mm) from *Chologaster agassizi* Putman and *Proteocephalus poulstoni* Whittaker et Zober, 1978 (total length 5.5 mm) from *Amblyopsis spelaea* DeKay (see Whittaker and Hill 1968, Whittaker and Zober 1978), differ from the new species by the shape of the body, and in the possession of an apical sucker (42 μm and 25–47 μm in diameter, respectively), which is absent in the new species. The new species can also be distinguished from most congeneric taxa by a low number of the testes (21–35) and uterine diverticula (4–8 on each side), small suckers, the diameter of which repre-

Table 1. Selected morphological and biometrical characteristics of the species of the *Proteocephalus*-aggregate from sticklebacks (Gasterosteidae).

Species	<i>P. ambiguus</i>	<i>P. ambiguus</i> *	<i>P. filicollis</i> ¹	<i>P. filicollis</i>	<i>P. pugetensis</i> ²	<i>P. culaeae</i> sp. n.
Host	<i>Pungitius pungitius</i>	<i>Pungitius pungitius</i>	<i>Gasterosteus aculeatus</i>	<i>Gasterosteus aculeatus</i>	<i>Gasterosteus aculeatus</i>	<i>Culaea inconstans</i>
Geographical origin	Europe	North America	Europe	NE North America	NW North America	North America
Total length (mm)	6–16	7.4–12.6	up to 60	12	25 (23–29) ³	4.4–6.0 (mean 5.4)
Shape of proglottids ⁴	slightly wider than long, rectangular to oblong	mature quadrate to rectangular, gravid longer than wide	variable in shape (two forms)	wider than long	wider than long (last gravid proglottids quadrate)	wider than long; last proglottids quadrate to elongate
Number of proglottids	14–33	21–30	dozens	34	dozens	< 22 (9–14 immature, 1–2 mature, 2–4 pregravid, 1–2 gravid)
Scolex width ⁵ (µm)	157–220	130–140	114–231	131	100 (105–118) ³	167–230
Scolex shape	round, as wide as indistinct neck	round to truncate, as wide or slightly wider than neck	globular	somewhat globular	tapering	widely round to almost bluntly ended
Apical sucker (width; µm)	20–32	25–30	19–32	19	23 (15–23) ³	absent
Testis number	32–46	not determined	25–86	40–44	30–40 (47–67) ³	21–35
Cirrus shape	pyriform	pyriform	pyriform	pyriform	pyriform to elongate	pyriform
Vaginal sphincter	absent	absent	absent	absent	absent	absent

Explanations: *occurrence in North America has to be confirmed; ¹data on “narrow” and “wide” forms from Europe – Scholz and Hanzelová (1998); ²Hoff and Hoff (1929); ³present data in parentheses; ⁴mature, pregravid and gravid proglottids; ⁵width at the level of the posterior margin of the suckers.

sents only slightly more than 1/3 of the scolex width, and absence of an apical sucker (present in most species of the *Proteocephalus*-aggregate – see Scholz et al. 1998, 2007, 2019).

The specimen from *C. inconstans* collected in Wisconsin deposited as *P. filicollis* (USNM 1373829) is considered conspecific with the new species as it is indistinguishable from specimens from Manitoba. The total length of this specimen from Wisconsin is 5.2 mm and consists of 8 immature, 1 mature, 2 pregravid and 1 gravid proglottids.

Key to identification of species of the *Proteocephalus*-aggregate from sticklebacks in North America

(*occurrence in North America should be confirmed)

- 1 (2) Apical sucker absent; tiny worms (4.4–6.0 mm in total length); in brook stickleback (*Culaea inconstans*) ***P. culaeae* sp. n.**
- 2 (1) Apical sucker present; larger worms (total length > 6 mm, usually more than 10 mm); in other sticklebacks 3
- 3 (4) Small tapeworms (total length 6–16 mm), with mature and gravid proglottids oblong and with slightly convex lateral margins (barrel-shaped); in nine-spined stickleback (*Pungitius pungitius*) ****P. ambiguus***
- 4 (3) Larger tapeworms, > 20 mm in total length, with mature and gravid proglottids usually wider than long, with parallel lateral margins; in three-spined stickleback (*Gasterosteus aculeatus*) 5
- 5 (6) Scolex rounded, distinct from the neck (Fig. 1A); apical sucker subspherical in frontal view (Fig. 1B); in northeastern part of North America ***P. filicollis***
- 6 (5) Scolex continuously tapered towards anterior extremity (Fig. 1D); apical sucker flat in frontal view (Fig. 1C); in brackish coastal waters and freshwater drainages of the Pacific northwestern coast of North America ***P. pugetensis***

DISCUSSION

As many as four species of the *Proteocephalus*-aggregate may occur in sticklebacks (Gasterosteidae) in North America, including one species described herein as new from the brook stickleback. Two of these species, *P. pugetensis* and *P. culaeae* sp. n., are endemic to North America, whereas *P. ambiguus* and *P. filicollis* also occur in the Palearctic Region (Scholz and Hanzelová 1998, Scholz et al. 2007). However, the occurrence of *P. ambiguus* in North America has to be confirmed because all voucher specimens of these taxa studied by the present authors were misidentified or could not be identified to the species level. Interestingly, three-spined stickleback, *Gasterosteus aculeatus*, serves as the definitive host for two allopatrically occurring species of the *Proteocephalus*-aggregate: *P. filicollis* in the northeastern part of North America and *P. pugetensis* in its northwestern part.

Both species of *Proteocephalus* from three-spined stickleback are rather similar to each other in their morphology, and molecular data on *P. pugetensis* are pending to confirm that it is actually a valid species separate from *P. filicollis*. In contrast, the newly described species from the brook stickleback differs conspicuously in its morphology from all the remaining species of the *Proteocephalus*-aggregate. Unfortunately, the only material available was collected more than 20 years ago, and was not suitable for DNA analysis. All four species that may occur in North American sticklebacks seem to be strictly host-specific (oioxenous), i.e., they each occur in a single species of definitive host (Scholz et al. 2007).

Marcogliese and Scholz (1999) identified larvae (plerocercoids) of tapeworms found in nine-spined stickleback, *P. pungitius*, four-spined stickleback, *Apeltes quadracus*, and three-spined stickleback, *G. aculeatus*, from Sable Island, Nova Scotia, Canada, as *Proteocephalus macrocephalus* based on scolex morphology (Fig. 1C). However,

no molecular data are available to confirm this species identification.

As for most other helminths in North American freshwater fishes, there is limited information about the ecology and life cycles of any of the proteocephalid tapeworms parasitic in North American sticklebacks (Hoffman 1999). In contrast, all available data on any stickleback proteocephalid were obtained in Europe, where seasonal cycles in the occurrence and maturation of *P. filicollis* and its development were intensively studied (see Chubb 1982 and Scholz 1999 for review).

The brook stickleback, which is the type host of the new species of *Proteocephalus* described herein, is a small freshwater fish that is widely distributed in North America, especially in the northern part of the eastern United States and throughout much of central and southern Canada. This species inhabits clear, cool streams and lakes. In Manitoba, it is found in “quiet, weedy waters, in stream headwaters, ponds, prairie pothole lakes and human-made impoundments” (Stewart and Watkinson 2004). The type locality of *P. culaeae* is an upstream headwater reach of the Brokenhead River, a narrow meandering brook that flows gently through marshy upland bogs and forms quiet pools and weedy habitat ideal for brook sticklebacks.

The brook stickleback eats small invertebrates, algae and insect larvae (Scott and Crossman 1973, Becker 1983, Stewart and Watkinson 2004). The typical first – and often only – intermediate hosts of *Proteocephalus* spp. are copepods, and the quiet habitat of brook stickleback is conducive to the transmission of these tapeworms. Nevertheless, from all available accounts, *P. culaeae* appears to be rare in both Manitoba and Wisconsin, the two locations with bonafide records of this tapeworm.

The parasite fauna of the brook stickleback includes all principal groups of helminth parasites, with allocreadiid trematodes of the genus *Bunodera* Railliet, 1896 representing a dominant component as to species richness (Margolis and Arthur 1979, McDonald and Margolis 1995, Choudhury and León-Règagnon 2005; see also Gibson et al. 2005). Two species of tapeworms identified as *Proteocephalus pugetensis* in Lake Ontario, Canada (Dechtiar and Christie 1988), which may also belong to the new species (= *P. culaeae*), and plerocercoids of the diphyllbothriidean *Schistocephalus solidus* in Algonquin Park lakes (Dechtiar et al. 1989) and in Alberta, Canada (Shostak and Roberts 2000), were reported from this fish host. The latter tapeworm may also represent a new species of *Schisto-*

cephalus Creplin, 1829, considering a strict host specificity of species of the genus at the level of fish intermediate hosts (Dubinina 1980). Amin (1990) reported unidentified species of *Proteocephalus* from this fish in Wisconsin.

The discovery of a new species of tapeworm in the northern regions of North America, which has a long tradition of fish parasitology (Hoffman 1999), may appear surprising, but it reflects the rather lamentable situation in Canada and the USA, where the taxonomy and systematics of freshwater fish parasites have been in a general state of neglect for several decades (Scholz and Choudhury 2014). Future effort should focus on collecting new material suitable for molecular studies to clarify phylogenetic relationships and taxonomic status of the cestode parasites of sticklebacks and other fish hosts.

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