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COLLECTING AND PROCESSING BRANCHIURANS

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INTRODUCTION

Branchiurans are ectoparasites of primarily fishes, hence their common name of "fishlice." Branchiurans also use amphibians and invertebrates as hosts. Fishlice are most often attached to the body or fins of fishes but can be found inside the mouth or on the gills. The subclass Branchiura contains a single valid family, Argulidae Rafinesque, 1815, and four valid genera with approximately 157 valid species. *Argulus* O. F. Müller, 1785 contains about 129 valid species that occur in marine, estuarine, and freshwater habitats (Poly, 2008).

Branchiurans are dorsoventrally compressed with a circular to oval shield-like carapace, four pairs of swimming thoracic legs, and an unsegmented posterior abdomen (Fig. 1). Total length of adults ranges from a few millimeters to over 30 mm, with adult females often growing larger than males. Most species possess a pair of suction cups (modified first maxillae) in late juvenile and adult stages (Wilson, 1902).

COLLECTION

Live fishlice are semitransparent and can be overlooked easily on the host, even when in plain view (Fig. 1A). Their movement will often give away their location, as will their pair of eyes, pigment patterns, and the presence of yellow, opaque eggs in gravid females or white testes in males. Following fixation in formalin or preservation in ethanol, the body will become opaque and turn white, yellow, or green, depending on species and preservative, with contrasting pigment patterns, which can be green, brown, purple, or black (Fig. 1B). Fishlice can be removed most easily from their hosts (or other objects to which they could be attached) with a pair of fine-tipped forceps by sliding the tips (with a gap of one to several mm between the tips) under the specimens and lifting them off their hosts. Pinching a specimen with the forceps can damage it. A small metal spatula can also be effective for removing fishlice. Because they are able to move about freely on their hosts and even leave the host, fishlice can avoid detection or capture and can be easily lost. Techniques for collecting branchiurans are, for the most part, the same as those directed at catching the hosts on which they reside.

Active Sampling

Types of active sampling methods include seining, hook-and-line fishing, trawling, and electrofishing. In my experience, trawling is not likely to yield many, if any, fishlice, because of the amount of mechanical disturbance and ample opportunities for the fishlice to leave the host, in contrast to parasitic copepods, which can be permanently affixed to their hosts. Electrofishing, hook-and-line fishing, and seines are the best methods for catching fish while not losing fishlice. For studies aimed at determining prevalence and intensity of infestation, hook-and-line fishing is likely to yield the best results, followed by electrofishing if individual fishes are shocked and then captured and held separately. Seines also might provide relatively unbiased data, but the number of host specimens captured can not be regulated as effectively with a seine. Any contact of a fish host with other fishes, objects, the examiner's hands, or the ground increases the chance of fishlice being rubbed off or transferred elsewhere. Plankton nets have been effective for collecting free-swimming fishlice but usually not in great numbers. In one case, however, at least 178 specimens were collected in a plankton tow in Yunnan Province, China (Hsiao, 1950).

Passive Sampling

Trammel, hoop, and gill nets, minnow traps, and other gear deployed in a set location are examples of passive gear for sampling fish hosts. Other devices used to collect fishlice directly without capturing the host include light traps and insect emergence traps. Light traps have not been used often for collecting fishlice, but Engelmann (1973) collected a variety of aquatic insects and crustaceans, including fishlice, and *Argulus longicaudatus* Wilson, 1944 has been collected in several Illinois lakes with light traps (Poly, unpublished). Positive phototaxis has been observed for both larval *A. siamensis* Wilson, 1926 and larval and adult *A. japonicus* Thiele, 1900 (Tokioka, 1936; Sundara Bai, 1981; Yoshizawa and Nogami, 2008). Aagaard (1978) used

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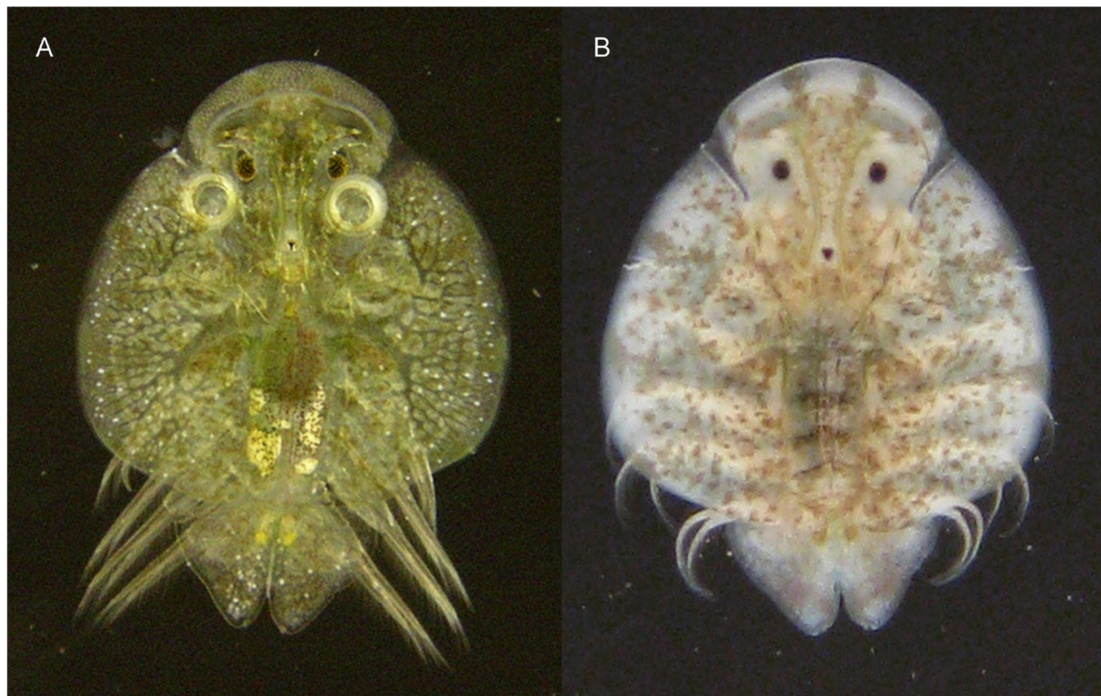

Fig. 1. Dorsal view of *Argulus americanus* (female, 4.6 mm TL), South Carolina, USA: A, live specimen; B, same specimen preserved in 70% ethanol.

emergence traps to collect aquatic insects in Lake Målsjøen (Norway) and discovered the effectiveness of these traps for collecting adult *A. coregoni* Thorell, 1864. Both light traps and emergence traps appear to be potentially useful not only to secure specimens for taxonomic or biological studies but also to estimate population size (Engelmann, 1973) or even to control infestations by reducing populations in aquaculture ponds or in other culture situations where abundant ectoparasitic crustaceans cause host mortalities (Pahl et al., 1999a, b).

Preservation

Common preservatives and fixatives (see Martin, 2016) that have been used for fishlice include ethanol or isopropyl alcohol, various dilutions of formaldehyde, or alcohol-formalin-acetic acid. Specimens placed in 10% formalin fix well, but the fringes of the suction cups of adult fishlice are usually curled, making it difficult to view important taxonomic characters. Specimens preserved in 10% formalin, then transferred to ethanol for storage, can exhibit distention of the exoskeleton (pers. obs.). Specimens of *A. yucatanus* Poly, 2005 preserved in 4% formalin did not show curled suction cups, and when transferred to 70% ethanol several years later did not exhibit any noticeable effects (Poly, 2005). Fixation in hot water for a few seconds and preservation in 70% ethanol have been used, and the method appeared to work well, especially for flaring the thoracic appendages, leaving them all projected laterally and well separated (Poly, 1997). The long-term condition of those specimens, however, has not been evaluated.

Fishlice preserved and stored in 70% ethanol have shown no adverse effects due to either initial preservation or long-term storage, and appendages do not become rigid as with other preservatives, making specimens easier to manipulate and less prone to damage during examination. Preservation and storage in 70% ethanol (absolute preferred over denatured) appears to be the most suitable for fishlice, and Levi (1966) advocated the use of alcohol for invertebrate preservation in general, citing the excellent condition of centuries-old specimens. Addition of glycerin to ethanol is not recommended for storing fishlice. Specimens destined for use in molecular studies should be best maintained frozen or preserved in 95-100% absolute ethanol.

From personal experience, the best preservation procedure involves removing the specimens from the host and keeping them in ambient water in vials, jars, or bottles for an hour or so, during which time the fishlice swim and wash away the residual fish mucus (see Sutherland and Wittrock, 1986). This first step saves much time in avoiding the removal of coagulated fish mucus from specimens. Fishlice should then be placed in a Petri dish or other flat-bottomed container with their ventral surface in contact with the bottom of the dish to ensure that the suction cups are affixed to the bottom and thus spread out. A small amount of 70% ethanol is applied with a pipette or dropper so as to have only a shallow layer in the dish no deeper than the specimens so that the specimens will tend to remain still rather than swim. The dish should be covered to prevent evaporation of the alcohol and dessication of the specimens. The fishlice are left in the dish for one to several hours, at which point additional alcohol can be added or the specimens can be transferred to a vial or jar for more permanent storage. This method ensures that the suction cups are spread out and that their supporting rods can be viewed easily, the legs are extended laterally, and the thorax and abdomen are straight. Special fixatives or preservatives can be used rather than ethanol if

specimens will be used for histological or other studies (see Maidl, 1911; Madsen, 1964). As an alternative, hot water could be applied using a dropper to individual specimens in a Petri dish to fix them before transfer to ethanol.

Another advantage of keeping fishlice alive briefly prior to preservation is to allow gravid females to deposit eggs. Eggs in the thorax, and in the carapace of some species, can obscure views of other features of interest, and the thorax (and sometimes carapace) often will be swollen due to the presence of numerous eggs. This swelling increases the thickness of the specimen, making viewing under high magnification more difficult. Gravid females will often lay eggs within a few hours after removal from their host. The eggs can be preserved, studied alive, or allowed to develop and hatch (Lutsch and Avenant-Oldewage, 1995; unpublished data).

As with most biological specimens, fishlice pigments are affected by exposure to fixatives, preservatives, and light. Collections of fishlice should be kept in cabinets or low-light areas to maintain natural pigments, which can be useful for identification. The use of lactic acid, glycerin, potassium hydroxide, and other clearing agents for permanent clearing, often with staining, is common (Masson and Delamare Deboutteville, 1962; Cressey, 1972; Sutherland and Wittrock, 1986; Gresty et al., 1993; Rushton-Mellor, 1994). This is not recommended for type specimens of fishlice because useful information about the pigment pattern will be lost and the overall condition of the specimen is diminished. If specimens have been photographed to document the pigment pattern, permanent clearing will not be such a problem. Hoyer's medium (and modified Hoyer's medium) has been used by zoologists and botanists for temporary clearing and temporary and permanent slide mounts (Mitchell and Cook, 1952; Masson and Delamare Deboutteville, 1962; King and Robinson, 1970; Upton, 1993), and it has been effective for temporary clearing of fishlice (Poly, 2005). An opaque specimen becomes completely transparent after several minutes in Hoyer's medium, yet the opaque condition returns with the pigment intact when placed back into ethanol. Specimens must not be left in Hoyer's medium for an extended time (>1 hour) because of shrinkage of tissue when returned to ethanol. Upton (1993) provided an excellent review of Hoyer's medium, its numerous modifications and names (e.g., Berlese's fluid), and correct and incorrect formulae, including the formula of Puri (1931) used by the author.

Various methods have been recommended for reconditioning dried crustacean specimens, including the use of ethylene glycol, trisodium phosphate, Decon 90, various liquids under vaccum, and boiling specimens in 85% alcohol (Van Cleave and Ross, 1947; Levi, 1966; Thompson et al., 1966; Marhue, 1983; Jeppesen, 1988). Dried type specimens of fishlice placed in Hoyer's medium became clear, rehydrated, and pliable, appearing normal. Cunningham (1972) noted that "[Hoyer's] will usually restore dried tissue to natural-like form..." Hoyer's medium should be equally useful for temporary reconstitution of dried specimens of other small crustaceans.

Fig. 2. Slide mount of *Argulus pugettensis* (female, approx. 7.1 mm TL), California, USA, showing crystallization of mounting medium (Turtox CMC-10).

MICROSCOPY

Parasitic copepods are often dissected to study appendages in detail, including dissection using the wooden-slide method of Humes and Gooding (1964). This method can be applied to fishlice. Unless mounted on slides or stored in microvials with the specimen, however, small appendages can be lost or separated from the original specimen more easily than if left *in situ*. From personal experience, fishlice appendages, including the second maxillae and all swimming legs, can be studied quite well without dissection. The ventral surfaces of these appendages can be seen well in ventral view in their normal state, but the dorsal surfaces of most of these appendages are covered by the carapace. The carapace is quite flexible and can be folded back, exposing the dorsal surfaces of all these appendages as well as the respiratory areas on the carapace. A cover slip on a temporary slide mount in ethanol can be used to pin down the folded carapace, holding it in place while these structures are examined. Detailed study of adults, especially type specimens, can be achieved best on specimens that have not been mounted permanently on glass slides. Examining specimens as temporary slide mounts in ethanol permits adequate study of the external anatomy for species descriptions and identifications without irreversible alteration of specimens. Temporary slide mounts in Hoyer's medium also can be made, but permanent mounts are not recommended, especially for type specimens (Coddington, 1983; Kabata, 1986; Upton, 1993). Permanent slide mounts often experience problems over time; for example, crystallization of mounting media such as Turtox CMC-10 and Permount® (Fig. 2). Clearing, staining, sectioning, and mounting on slides may be necessary and can be useful for studies that do not involve type specimens (Madsen, 1964; Gresty et al., 1993). Larval fishlice can be mounted between two cover slips to allow for dorsal and ventral views (Lutsch and Avenant-Oldewage, 1995).

Scanning Electron Microscopy (SEM)

Removal of the carapace for an unobstructed dorsal view of the thoracic appendages or any other desired dissections should be done after fixation/preservation and prior to cleaning for SEM. Preserved specimens can be cleaned by brief sonication in ethanol or by using manual agitation or a shaker table with mild detergent in water; cleaning methods were covered by Felgenhauer (1987).

Preparation of fishlice for SEM can be accomplished with a variety of effective methods. Felgenhauer (1987) recommended initial fixation of specimens in 3% glutaraldehyde, postfixation in osmium tetroxide, then dehydration in

a graded series of ethanol in order to reduce charging problems. The same method has been used to prepare specimens for transmission electron microscopy (see Rushton-Mellor and Whitfield, 1993; Gresty et al., 1993). Lutsch and Avenant-Oldewage (1995) preserved larvae of *A. japonicus* in ethanol, then rehydrated and freeze-dried them, followed by coating with gold. I have successfully used the abbreviated method of Rupp (1990) with slight modifications (Poly, 2003, 2005; see also Scotto, 1980), beginning with specimens preserved and stored in 70% ethanol or fixed in 4% formalin and transferred to 70% ethanol. Variations in the preparation procedures have been used with fine results (Shimura, 1983; Sutherland and Wittrock, 1986; Gresty et al., 1993; Rushton-Mellor and Whitfield, 1993).

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