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Microhabitat distribution and behaviour of Branchiobdellidan *Holtodrilus truncatus* found on the freshwater shrimp *Neocaridina* spp. from the Sugo River, Japan

Research Article

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Abstract: A study was performed on the microhabitat distribution and some aspects of behaviour of the ectosymbiotic branchiobdellidan *Holtodrilus truncatus* (Annelida, Clitellata) found on the freshwater shrimp that inhabit the Sugo River, Hyogo Prefecture, western Japan. Observations on shrimp that were collected from the Sugo River (2003 to 2011) confirmed that the host shrimp is *Neocaridina* spp. (Atyidae). The attachment location on the host shrimp was predominately between the 1st pleopod and the 5th pereopod (55.3%). The reproductive method of *H. truncatus* is hemaphroditism. The cocoon was found only inside the carapace of the host shrimp. The cocoon was transparent and contained a maximum of 14 juvenile worms (developing embryos). When hatching approached, *H. truncatus*'s worms became elongated and slender, and only one worm hatched out at a time. When *Holtodrilus truncatus* was removed from its host and was maintained in river water without any food, it survived for a maximum of 46 days. In a host exchange experiment, where we provided several other freshwater shrimp species, Palaemonidae fed on *H. truncatus*. Moreover, *Palaemon paucidens* and *Macrobrachium nipponense* from Lake Biwa also preyed upon *H. truncatus*. The possible symbiotic relationship between *H. truncatus* and *Neocaridina* spp. (family Atyidae) is further discussed.

Keywords: *Branchiobdellidan • Holtodrilus truncatus • Behaviour • Neocaridina spp. • Survival time • Host exchange experiment*

1. Introduction

In 2003, Niwa *et al*. [1-4] discovered that the ectosymbiont annelid, *Holtodrilus truncatus* (ZIHU3066, Hokkaido University, Japan), which had previously been reported present only in China (Henan and Guangdong Provinces), was attached to the freshwater shrimp *Neocaridina* spp. in the Sugo River (Figure 1), Hyogo Prefecture, western Japan. The Japanese endemic species *Neocaridina denticulata denticulata* is a freshwater shrimp that is distributed mainly in western Japan [5]. However, many live *Neocaridina* spp. were imported from China and South Korea to be used as live bait for sport fishing in Japan [6,7]. *H. truncatus* has not been previously reported in Japan [2]. *H. truncatus* may have been imported unintentionally into Japan together with bait shrimp, and later dispersed and settled after being discarded by sport fishers in the freshwater environment of the Sugo River [7,8]. Branchiobdellidans (Annelida) and temnocephalidans (Platyhelminthes) are both known to be ectosymbionts of decapod crustaceans. Their original geographical distributions are separate; the former is found in the northern hemisphere and the latter in the southern hemisphere [4,9]. However, the Sugo River in western Japan is an exceptional area, as both the branchiobdellid *H.truncatus* and the temnocephalid *Scutariella japonica* (Matjašič, 1990) can be found here together [1,3,4]. These species attach to the same host, *Neocaridina* spp., but their behaviour is not entirely clear. There are many unknown factors that affect the symbiotic relationship between *H. truncatus* on the host shrimp. We forcibly separated *H. truncatus*

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from its host with forceps, in order to determine how long it is able to survive without their host [10]. Additionally, because the ecological relationship of this ectosymbiont and its host is not well understood, we also examined the ability of *H. truncatus* to live with other host freshwater shrimp (*Neocaridina* spp., *Caridina japonica*, *Procambarus Clarkii*, Palaemonidae, *Palaemon paucidens*, *Macrobrachium nipponense*) [11].

2. Experimental Procedures

A total of 271 host shrimp was collected from the Sugo River, Hyogo Prefecture, from 2003 to 2011. The location of shrimp collecting were Station 1: N34°56'31.9", E134°38'19.0", Station 2: N34°55'28.5", E134°38'26.3", and Station 3: N34°51'05.3", E134°38'50.1" (Figure 1).

2.1 Method of collecting host shrimp

The shrimp were usually collected by the sweeping method for about 30 minutes to 2 hours using Dip-nets (mesh size 2 mm with a diameter 36 cm depth of 32 cm). Collected *Neocaridina* spp. were put into a cylindrical plastic tank of diameter 22 cm, 20 cm depth and external air supply, and were brought back to the Rokko Island High School. The collected shrimp were maintained at 20°C in an aquarium (60×30×36 cm 57.0-L) equipped with a ZENSUI MC-75E AQUARIUM COOLER. Breeding during an experiment with the plastic bucket ace type (15-L) of diameter 31×30 cm containing an external air supply. Each live shrimp was put into a 2.0-L plastic bottle of 30 cm (cut 6 cm from the top), and was bred. Imaging of these observations were done using a Victor Company of Japan colour video TM-150S type with the NIKON Type120 ECLIPSE E600 microscope, which were printed out by the digital colour printer by MITSUBISHI CP700DSA. The magnification was ×4 time and ×10.

2.2 Attachment ratio of *H. truncatus*

The experiments were ran from July 2nd to November 7th, 2011. When investigating the attachment ratio of *H. truncatus* to its host shrimp *Neocaridina* spp. collected from the Sugo River Stations 1 and 2 in 2011, we observed that the temnocephalid *S. japonica* was present on some occasions as well (152 individuals).

2.3 Survival of *H. truncatus*

The experiments were ran from October 3rd, 2007 to January 7th, 2008. Further collection at Station 3 of 14 *Neocaridina* spp. (2 male and 12 female host shrimp) produced a total of 15 *H. truncatus*. *H. truncatus* was maintained in the laboratory alive in laboratory dishes

Figure 1. Map showing sampling stations along the Sugo River, Hyogo Prefecture, Western Japan.

(90.0 mm in diameter) under continuous lighting at 24°C, and in vial containers (100 ml) that were kept at room temperature and had a loose lid to allow for aeration. They were cultured without live food; but in nature they eat protozoa, small crustaceans, and attached algae. The specimens were all cultured in river water obtained from their collection site, and their survival time was determined.

2.4 Host exchange experiment

The experiments were ran from May 1 to July 23, 2006. To test if *H. truncatus* would attach to alterative freshwater shrimp hosts, we collected *H. truncatus* from *Neocaridina* spp. shrimp from the Sugo River. These were placed together with 28 potential freshwater shrimp hosts of various species to investigate symbiotic attachment. Potential hosts used for this experiment included the following: eight *Neocaridina* spp. (1 from the Sugo River, 3 from Lake Biwa Shiriuchi Minamihama, and 4 from Lake Biwa Hayasaki), two *Caridina japonica* (from Kochi) and four *Procambarus clarkii* (3 from the Sugo River, 1 from Lake Biwa Hayasaki). The five *H. truncatus* individuals were separated from the host shrimp of *Neocaridina* spp. When it changed from the original host, Palaemonidae ten (5 from China Beijing,

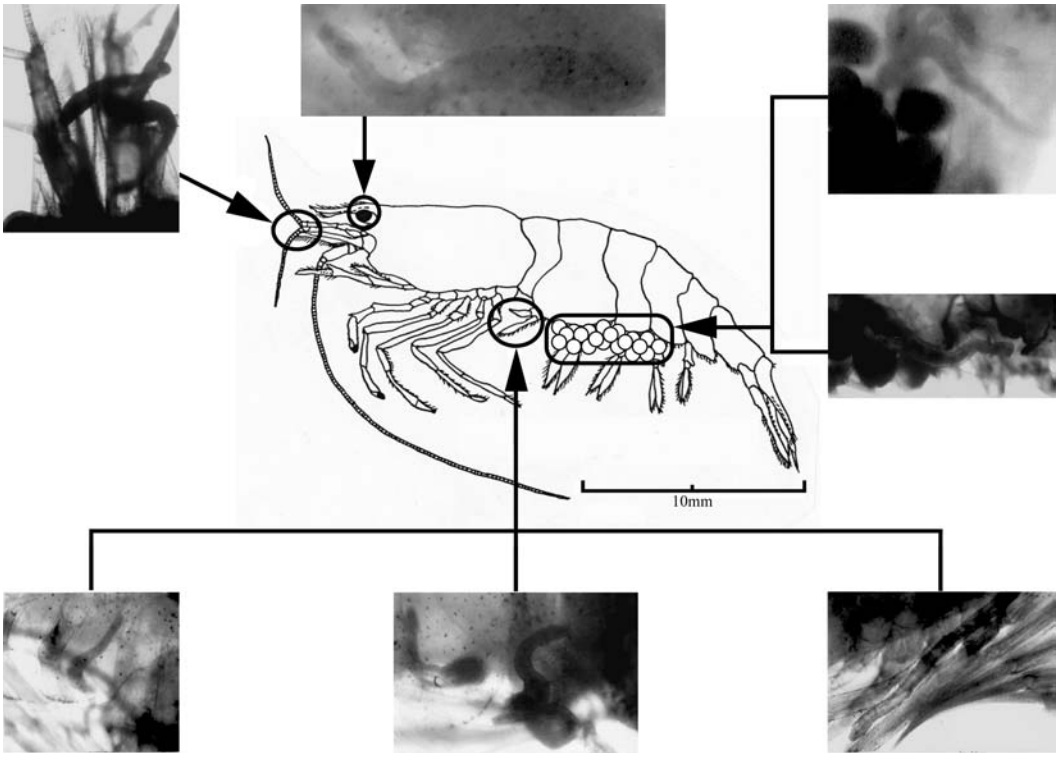

Figure 2. Illustration and photographs showing the distribution of *Holtodrilus truncatus* in the host shrimp *Neocaridina* spp. The arrows show microhabitats occupied by worms.

5 from Shanghai) three *Palaemon paucidens* (from Lake Biwa Shiriuchi Minamihama) one *Macrobrachium nipponense* (from Lake Biwa Hayasaki) were also used for potential hosts. One host was supplied at a time, and visual observation was carried out immediately and every 30-minutes after each introduction for 7-10 hours. It was observed that *H. truncatus* specimens were preyed upon during the observations, as recorded and checked by video photography. Video recordings were conducted for a maximum of 8 hours, and the camera was positioned under a transparent container to be able to observe the mouthparts of the shrimp, which are located facing the bottom. The timing was analyzed closely and the moment of predation was recorded.

3. Results

We found that *H. truncatus* was most abundant in *Neocaridina* spp. collected from Station 1, while none were found in shrimp from Station 2, which is located further downstream. We also detected the presence of another ectosymbiont, *S. japonica*, in Station 1 and 2 in 2011. *Holtodrilus truncatus* tended to be attached to its host shrimp, *Neocaridina* spp. at several anatomical locations (Figure 2). The most favored was between the 1st pleopod and the 5th pereopod (55.3%), followed by the carapace (17.0%), the base of the eye (15.6%), the antennule (8.5%), and around the egg mass (3.6%) (n=141 host shrimp). Regarding *H. truncatus*'s reproductive behaviour, it is known that this species is a hermaphrodite. The cocoon was globular, its diameter ranged from 0.58 to 0.76 mm (average of 0.64 mm SD±0.05, n=23) and cocoons were observed in the shrimp's gill chamber through the semitransparent carapace. The cocoon was transparent and contained a maximum of 14 juvenile worms (developing embryos). When hatching approached, *H. truncatus*'s worms became elongated and slender, and hatched one worm at a time. The cocoon was only found in the gill chamber of *H. truncatus*. Newly hatched worms from the cocoon were transparent and immediately entered the gill chamber of the host shrimp. Eggs of *S. japonica* could be observed inside the gill chamber, but we could not observe any interaction nor predation between these two organisms. We should also mention the peculiar breathing behaviour exhibited by *H. truncatus* that occurs inside the host shrimp's gill chamber, which it routinely enters and leaves. After *H. truncatus* entered the host shrimp's gill chamber, it shook its head violently and beat it into its host's scaphognathite. This strange action of head shaking was observed on four different

days (August 1st and 8th, 2006; September 1st, 2006; August 2nd, 2007).

We also observed an interesting aggregation behaviour of *H. truncatus* on live host shrimp when numerous freshwater shrimp were collected and maintained in captivity. Many captive host shrimp died, and as a result *H. truncatus* migrated from the unhealthy hosts to the healthy shrimp that remained alive. To give an example, we found that a single unovigerous female shrimp could host as many as 39 *H. truncatus* (Figure 3). To determine how long *H. truncatus* would survive when separated from the host shrimp, we conducted observations in the laboratory. Because the age of the specimens differed when we separated *H. truncatus* from the host shrimp, the survival period also varied. Ten individuals kept in 100 ml vials survived for 7 to 46 days, while the three that were maintained in dishes survived for 12 to 21 days. However, after removing *H. truncatus* from the host and maintaining them in river water obtained from the collection site without additional food, we found that *H. truncatus* could survive for a maximum of 46 days. Variation in survival time was attributed to the different environmental conditions encountered in the dishes and vials. *H. truncatus* kept in vials would sometimes migrate up the walls of the container toward the water surface, while those maintained in dishes were always on the bottom. We detected a decrease in motion and change in colouration of *H. truncatus*'s body, which became cloudy, on the day before they died. *H. truncatus* depends strongly upon its host shrimp, but we determined that they can survive without any external food source for up to 46 days [10].

We made video recordings of *H. truncatus* during the host shrimp exchange experiment. In the case of *Neocaridina* spp., *H. truncatus* began to attach to its host shrimp after 30 minutes, and all 5 individuals supplied attached within 3 hours after exposing themselves to their new host. *Neocaridina* spp. has a semitransparent carapace which permits the viewing of the gill chambers including the attached *H. truncatus*. When we supplied *Caridina japonica* as the host shrimp, we observed 3 to 4 *H. truncatus* fastened to it when initially exposed, and they attached firmly after 30 minutes. *H. truncatus* also attached itself to *Procambarus clarkii* from the beginning of the exposure, and were not preyed upon by the host even by the following day. We encountered difficulties with *Procambarus clarkii*, because their carapace is not transparent and it does not allow for the viewing of *H. truncatus* inside the carapace; but because *H. truncatus* disappeared from the laboratory dish this suggests that they had migrated inside the body of the host. Predation by Palaemonidae, *Palaemon paucidens* and *Macrobrachium nipponense* was monitored by video photography. Host shrimp Palaemonidae from China (Shanghai and Beijing) and maintained in dishes, were supplied with five *H. truncatus*, which were separated from their original host (*Neocaridina* spp.). Palaemonidae readily consumed *H. truncatus*. Moreover, *Palaemon paucidens* and *Macrobrachium nipponense* (from Lake Biwa, Japan) also preyed upon *H. truncatus* [11].

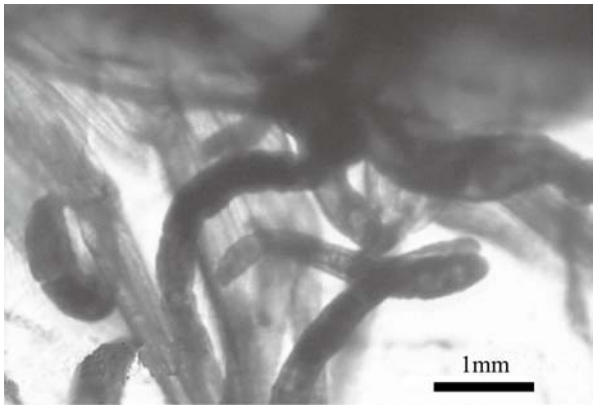

Figure 3. Photograph of unovigerous female shrimp, showing that it can host up to 39 *H. truncatus*.

4. Discussion

During our observations we clarified some new aspects of the behaviour of *H. truncatus*, and several aspects of its relationship with its host: These include how long it can survive alone, and whether it can attach to other freshwater shrimp hosts besides *Neocaridina* spp. Sugo River Station 2 is the location where we first discovered *H. truncatus* in 2003. During our observations we found that the preferred attachment location for *H. truncatus* on its host shrimp was between the 1st pleopod and the 5th pereopod, though it could also be found in other body parts. This region has an advantage because it offers ready access to the gill chamber carapace and eggs of the host. The maximum number of developing embryos found in a cocoon from *H. truncatus* was 14, some of which gradually became long and slender during development.

We could observe developing embryos hatching and the juveniles emerging by looking through the carapace of the host shrimp, but unfortunately this view was soon covered by the gill of the host. We only report the occurrence of the cocoon in this study, but a similar process has been observed in *H. truncatus* developing embryos hatching from the pleopods of host shrimp found on Miyako Island, Okinawa [12]. While *H. truncatus* was seen through the carapace of the host

shrimp, we observed a peculiar behavior that might be related to oxygen uptake. *H. truncatus* shook its head vigorously up and down in the direction of the host shrimp's scaphognathite, an action that likely increases the intake of oxygen. Although the function of this behaviour is not clear, perhaps *H. truncatus* is taking in oxygen from the respiration current generated in this location [13]. This study also found a useful method to collect *H. truncatus* in large quantities, which can be a time consuming activity. If numerous host shrimp are collected and maintained in buckets, their physical condition will deteriorate. *H. truncatus* will migrate to and concentrate on a few healthy host shrimp, which can then be used in further studies.

As a result of our experiment, we were able to determine that the maximum survival time of *H. truncatus* away from a host shrimp and without any supplemental feeding can be as long as 46 days. Another branchiobdellidan, which sometimes attaches to crayfish, has been documented alive in the laboratory and refrigerator for 8 weeks (2012, Gelder., personal communication) and in the laboratory for up to 8 months [14]. However, we are not sure yet about the relationship between the ectosymbiont and host shrimp. Palaemonidae and Atyidae have different feeding behaviours, and this may have affected depredation on *H. truncatus*. Moreover, one of the reasons why branchiobdellidans have not been discovered on *Procambarus clarkii* in Japan [15] is that, while other freshwater shrimp such as Atyidae have a transparent carapace, *Procambarus clarkii* has an opaque carapace and their internal organs are therefore invisible. It is still the same now that branchiobdellidans are not attached to the *Procambarus clarkii* to Japan [16]. The product from China and the *Procambarus clarkii* of Taiwan are also the same. However, in Europe, since there is an example, it could be suggested that the introduced *Procambarus clarkii* into Japan lacked branchiobdellidans or lost them in early time (2012, Ohtaka, personal communication). It is still not very clear whether *H. truncatus* is a symbiont or a parasite. Research conducted in North America has determined that crayfish hosting many branchiobdellidans in their gills have higher growth and less mortality rates than those that do not [17]. Branchiobdellidans feed on organic matter on the crayfish gills, and thus clean them, which may indicate a certain degree of mutualism [4,15,17]. In our case, *H. truncatus* attaches to its host shrimp *Neocaridina* spp. without any known adverse effects. *Neocaridina* spp. with host eggs infested with *H. truncatus* still successfully hatch and lead healthy lives. Further research must confirm whether this relationship is beneficial for the host shrimp, as in the case mentioned above with crayfish.

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