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Note

Phyllosomas of smooth fan lobsters (*Ibacus novemdentatus*) encase jellyfish cnidae in peritrophic membranes in their feces

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Abstract: Jellyfish possess venomous cnidae on their tentacles to capture and consume marine zooplankton. Nevertheless, the planktonic larvae of the smooth fan lobster (*Ibacus novemdentatus*), known as phyllosoma, prey on jellyfish and successfully ingest both tentacle tissue as well as constituent cnidae, despite the presence of the venom-filled explosively penetrant cnidae or nematocysts. In the present study, we hypothesized that phyllosomas have mechanical and/or physiological resistance to internal envenomation by ingested nematocysts. To test this hypothesis, we examined the feces of phyllosomas (n=5) that were fed with Japanese sea nettle (*Chrysaora pacifica*) and found both undischarged as well as discharged cnidae surrounded by peritrophic membrane. We surmise that this membrane may mechanically insulate the lining of the midgut from stinging nematocysts to avoid injection of jellyfish venom into the phyllosomas' body by nematocyst tubule penetration. We then tested physiological sensitivity of the phyllosomas (n=10) to crude extract of tentacle cnidae injected into their bodies. For this experiment, we used a crude venom extract prepared from nematocysts isolated from tentacles of a rhizostome jellyfish (*Nemopilema nomurai*) after exposure to high salt which disrupted tentacle integrity, and phosphate-buffered saline as a control. Nine out of 10 animals died after the injection of crude venom extract, while none of the animals died in the control group. These results indicate that the defense of phyllosoma larvae against the toxin of jellyfish is a combination of mechanical inactivation of the ingested nematocysts and chemical digestion of the toxin in the midgut rather than physiological resistance against the toxin.

Key words: feeding ecology, nematocyst, plankton, Scyphozoa, toxin, venom

Jellyfish consume planktonic crustaceans such as copepods (Ishii & Tanaka 2001) and shrimp (Carrette et al. 2002) utilizing various types of tentacle cnidae including penetrant, venom-filled nematocysts (Tardent 1995, Nagai 2012, Ponce et al. 2013). During nematocyst discharge, venom-filled tubules explosively penetrate the cuticle of the prey (Tardent & Holstein 1982, Purcell 1984) and inject their venom through the tubules (Lotan et al. 1996, Yanagihara et al. 2002). Despite the potency of venom in jellyfish, phyllosomas of spiny (Palinuridae) and slipper (Scyllaridae) lobsters often associate with gelatinous zooplankton, including jellyfish (Booth et al. 2005, Ates et al. 2007, Ohtsuka et al. 2009, O'Rorke et al. 2014). Indeed the phyllosomas of *Ibacus novemdentatus* Gibbes, 1850 (Decapoda: Achelata: Scyllaridae) have been reported to ride and prey on cnidarian jellyfish in the wild (Shojima 1963). In the laboratory, planktonic-phase phyllosomas were fed exclusively moon jellyfish (*Aurelia aurita* s.l.) (Wakabayashi et al. 2012b). The phyllosomas can consume a variety of jellyfish (Wakabayashi et al. 2012a), including species harmful to humans because their long tubules can penetrate our skin (e.g., *Carybdea brevipedalia* Kishinouye, 1981) (Kitatani et al. 2015). Phyllosomas attach to the exumbrella of jellyfish and consume all of the tentacles, then eat the rest of the body (Wakabayashi et al. 2012a). By consuming tentacles, phyllosomas introduce nematocysts into their digestive tracts and consequently risk internal envenomation by the ingested nematocysts. Potential protective mechanisms to circumvent this could include 1) specialized digestive tract lumen surfaces acting as a physical barrier to nematocyst discharge, or 2) phyllosomas' immunity to the venom injected in their body. Hindgut and foregut in crustaceans are cuticle-lined and thus mechanically protected (Mikami & Takashima 1993, Greenaway 2001). In contrast, the midgut is not cuticle-lined (Mikami et al. 1994) and thus relatively vulnerable. There are three possible mechanisms whereby phyllosomas can feed on intact nematocyst-loaded tentacles of jellyfish. The first is to ingest the nematocysts without inducing discharge, as aeolid nudibranchs do. Nudibranchs prey on cnidarians and sequester the nematocysts (Edmunds 2009) into defensive organs—the kleptocnidae (Garese et al. 2012). In this case, nudibranchs

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ingest intact nematocysts without making them discharge and send them through the digestive system to form kleptocnidae on their body surface. The second mechanism is encasing nematocysts in a peritrophic membrane (Lehane 1997) to mechanically protect the midgut. The peritrophic membrane is present in adult Scyllaridae (Johnston & Alexander 1999). A third potential mechanism is to have physiological resistance to injected venom, which could be obtained through mutations of target proteins making them resistant to the venomous molecules (Barchan et al. 1992, Voss & Jansa 2012). Defensive molecules in hemolymph (Fredrick & Ravichandran 2012) might play a role to neutralize the venom. To explore these hypotheses of possible mechanisms of phyllosomas' tolerance to jellyfish venom, we examined if: (1) nematocysts of Japanese sea nettle *Chrysaora pacifica* (Goette, 1886) in the feces of phyllosomas are discharged; (2) if nematocysts in the feces are surrounded by peritrophic membrane; and (3) if phyllosomas are tolerant to crude extract from venom of the rhizostome jellyfish *Nemopilema nomurai* Kishinouye, 1922. A coherent study, using one species of jellyfish would have been ideal, however three different species of jellyfish were used due to physical and operational constraints combined with a limited number of cultured phyllosomas in this study; *A. aurita* s.l. was used as food because it is abundant enough for culturing phyllosoma, *C. pacifica* was used for observation of feces, *N. nomurai* was used for preparation of crude venom, because it provided the amounts of tentacles necessary for the experiments.

An ovigerous female of *Ibacus novemdentatus* (carapace length 65 mm) was purchased from commercial fisherman in Hirado, Nagasaki Prefecture, Japan, and kept in a recirculating tank system. Phyllosomas derived from the ovigerous female were collected from the tank and reared individually using mesh cases as described elsewhere (Wakabayashi et al. 2016). We defined stage I as the phyllosoma immediately after hatching, stage II as the phyllosoma after first molt, and stage III as the phyllosoma after the second molt, and so on. Each stage in the phyllosoma phase (Sekiguchi et al. 2007) is usually associated with a single instar in *I. novemdentatus* (Wakabayashi & Tanaka 2012).

To observe nematocysts in feces, fresh tentacles removed from *C. pacifica* were used because live individuals are available from Tokyo Bay as described elsewhere (Wakabayashi et al. 2012b). *C. pacifica* was selected from the species that are available in Tokyo Bay for this purpose because the morphology of their nematocysts can be readily observed using light microscopy. Five of the phyllosomas at stages either V or VI were starved until their digestive tracts were void. The phyllosomas were fed with the tentacles and observed for a few hours until they passed feces. The feces were collected by pipet from the bottom of the mesh cases and viewed under a microscope (BX-50W, Olympus).

In an effort to assess a potential host resistance, the physiological response of phyllosomas to injected venom was examined. Crude venom extract was prepared from tentacle nematocysts of *N. nomurai*. Excised *N. nomurai* tentacles (10 ml) were added to 20 ml of 3 M NaCl and kept at 4°C for three days, at which point tentacle integrity was lost to yield a cell and cnidae slurry. This solution was filtered through a nylon stocking prior to centrifugation of the filtrate at 1,100×*g* for 30 min. The pellet from the centrifugation containing nematocysts was used to extract venom. A weight of 0.5 g (wet) of the nematocyst pellet was mixed with 1.0 g of glass beads of diameter 0.5 mm and 1 ml of 10 mM phosphate buffer pH 7.0 in a plastic tube equipped with a mini-bead beater (BioSpec Products, Bartlesville). To extract the venom from intact nematocysts, the tube was agitated using the mini-bead beater at 4,800 oscillations/min for 30 s and cooled down on ice for 30 s (Carrette & Seymour 2004). This procedure was repeated ten times. Extracted venomous liquid was collected from the tube by pipet, brought up to 1.3 ml with 10 mM phosphate buffer pH 7.0, and used as crude venom extract. The crude venom extract was injected using a syringe (SuperFlex 5182-3496, Agilent) into the abdominal muscle of the phyllosomas (n=10) from the ventral side at a dose of 3 μl crude venom extract per g body weight. Individuals that were injected with the phosphate buffer were used as a 'sham' control. The movements of appendages, including pereiopods and third maxillipeds, were observed by eye for 1.5 h, then every 20 min up to 5 h after the injection. Animals that survived were observed again 24 h after the injection. This experiment could not be blind because the injected crude venom extract was not transparent and appeared different from the phosphate buffer. Numbers of animals that died within 24 h were compared between a test and a control using a two-tailed sign test at a significance level of 5% in the statistical program R (R Foundation for Statistical Computing) (http://www.r-project.org) version 3.0.2. *N. nomurai* individuals were collected from the Sea of Japan with the research vessel *Kaiyo-maru No. 7* (lat. 38°00′ to 37°30′N and long. 137°30′ to 138°30′E) using an LC-net (larvae catcher, Nichimo Ltd.), with 10×10 m of mouth opening and 30×30 mm of mesh opening, including cod-end, that was horizontally towed at a depth of 10 m for 5 min at a constant speed of 1 m/s. We used the largest phyllosomas at stage VI (weight 0.40–0.79 g) as smaller animals may not be tolerant of physical damage caused by the syringe needle during the injection.

Our observations of feces from five phyllosomas were consistent with the observation of discharged nematocysts in feces from wild-caught scyllarid phyllosoma by Sims Jr. and Brown Jr. (1968). The nematocysts in the feces (Fig. 1A, B) were discharged and covered by a sheath-like material; we interpret this to be a peritrophic membrane. Peritrophic membranes are non-cellular secreted layers that separate ingested material from the gut epithelium. In insects and crustaceans, peritrophic membranes are produced in the midgut trunk (Martin et al. 2006). Nematocyst tubules were not observed on the outer surface of the peritrophic membrane (Fig. 1A), indicating that the tubules do not penetrate the membrane and that the membranes function as a mechanical barrier to protect the midgut from nematocysts. The proportion of nematocysts that were discharged in the midgut could not be

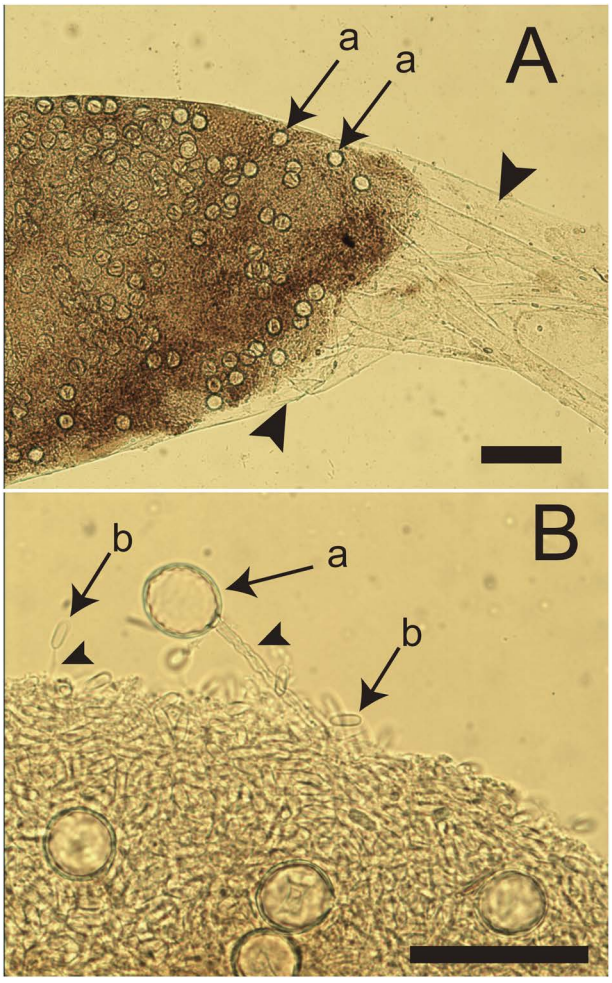

Fig. 1. Light microscopic images of feces of phyllosoma. A: Feces. Scale bar is 100 μm. Within the feces, nematocysts (a), are encased in a peritrophic membrane (arrowheads). B: Contents of feces. Scale bar is 50 μm. The peritrophic membrane of the feces was torn using forceps, and the contents of the feces were observed. Nematocyst tubules, which are discharged from the nematocyst capsule (indicated by arrows a, b), are indicated by arrowheads. There are at least two types of nematocysts, having either large capsule (a) or small capsule (b), and some part of both of them is discharged. A zoomed-out image mosaic of this fecal matter is available as a supplemental figure (https://www.jstage.jst.go.jp/browse/pbr).

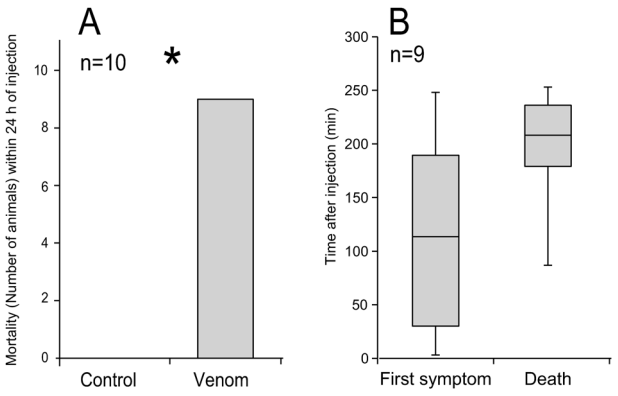

Fig. 2. Responses of the phyllosomas to venom injection. A: Number of phyllosomas of *Ibacus novemdentatus* that died within 24 h of injection with crude venom extract from *Nemopilema nomurai* and phosphate buffer as control. * significant difference ($P<0.01$ by two-tailed Sign test). B: Boxplot of latency to show first symptom of intoxication and to death after injection of crude venom extract from *N. nomurai.* Values are medians, interquartile ranges, minima, and maxima.

determined because various stimuli that occur during experimental manipulation before their ingestion and after their egestion can induce discharge; however, even if a small portion of discharge occurs in the midgut, discharged proteinous venoms (Nagai 2012) may not be highly toxic in the midgut as they may be enzymatically digested, and toxicity inactivated before being absorbed into the digestive gland.

In our venom-injection experiment, the ratio of the wet weight of nematocysts used for the extraction of crude venom, to the phyllosoma's wet weight was 0.12%. Since generally, the volume of hemolymph in crustaceans is 30.6% of the body weight (weight /weight), the ratio of nematocyst wet weight to hemolymph wet weight was 0.38%. For example, a 500 mg phyllosoma was injected with crude venom extract from 580 μg of nematocysts. This dose was selected as it was found sufficient to kill a freshwater shrimp in 20 min in a preliminary experiment. Before the injection, the phyllosomas were swimming by continuous fanning of pereiopodal exopods and grooming body surfaces by continuous motion of the third maxillipeds. Injection of crude venom extract extracted from the nematocysts of *N. nomurai* proved lethal to the phyllosomas. Within 24 h of injection of the crude venom extract, 90% of the phyllosomas (n=10) died, as determined by a complete lack of movement (Fig. 2A). In contrast, all phyllosomas (n=10) survived injection of the phosphate-buffered saline. The difference between these two groups of animals was significant ($P<0.05$, $Z=-2.89$ by Sign test). The latency to show the first symptoms, being intermittent stoppage of fanning of pereiopodal exopods, ranged from 3 to 248 min. The median value of the latency was 83.5 min. The first and the last dead individuals were observed 87 min and 253 min after the injection of crude venom extract, respectively. The median value of time to die was 208 min (Fig. 2B). Grooming was more persistent than fanning, and in all cases grooming started to decline following termination of the fanning movement. Additionally, one phyllosoma that showed a reduction in fanning recovered without showing any changes in grooming, and survived to complete all planktonic stages. Therefore, persistent grooming by the third maxillipeds might be responsible for maintaining their basic biological activities such as respiration (Kamio et al. 2015). Individuals injected with phosphate buffer showed no changes in their behavior during the observation period.

Thus, no systemic protective response, including neutralizing resistance, was observed in the phyllosomas of *I. novemdentatus* upon exposure to injected jellyfish crude venom extract. In contrast, evidence was observed to support digestive tract mechanisms to evade internal envenomation by ingested

nematocysts during digestion. In addition to the digestive adaptation of the peritrophic membrane, phyllosomas exhibit other adaptive characteristics to facilitate stable associations with planktonic jellyfish. To stay on and consume jellyfish, the phyllosomas grip and manipulate jellyfish by pointed dactyli at the ends of their pereiopods (Wakabayashi et al. 2012a). Similar morphological adaptations occur in hyperiid amphipods: they have hook-like structures on the dactyli to catch gelatinous animals (Laval 1980). Continuous grooming behavior using the elongated third maxillipeds is a strategy to avoid fouling by mucus secreted from jellyfish (Kamio et al. 2015). In addition to behavioral aspects, the mechanism of prey processing may eliminate envenomation by ingested nematocysts. The phyllosomas have a filter-press structure to consume the body fluid of prey, and which permits only fluids and the finest particles (e.g. 1 μm in diameter in spiny lobster *Sagmariasus verreauxi* (H. Milne Edwards, 1851) to enter the digestive gland (Johnston & Alexander 1999, Simon et al. 2012). Therefore, the filter-press might mechanically prevent nematocysts [large ones have 10 μm diameter (Östman 2000)] from entering the digestive gland. Through a combination of behavioral and morphological adaptations phyllosomas avoid envenomation by jellyfish nematocysts.

The present study used two species of jellyfish that co-occur with the phyllosomas of *Ibacus novemdentatus*, and thus our results should reflect ecologically-relevant predator-prey interactions. However, since each jellyfish species was used to test only one of the hypothetical mechanisms, future studies should use each tested jellyfish species in all assays. In addition, the mechanisms of action of the jellyfish venoms require investigation as phyllosomas may also have biochemical adaptations to cope with envenomation.

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Competing interests

No competing interests declared.

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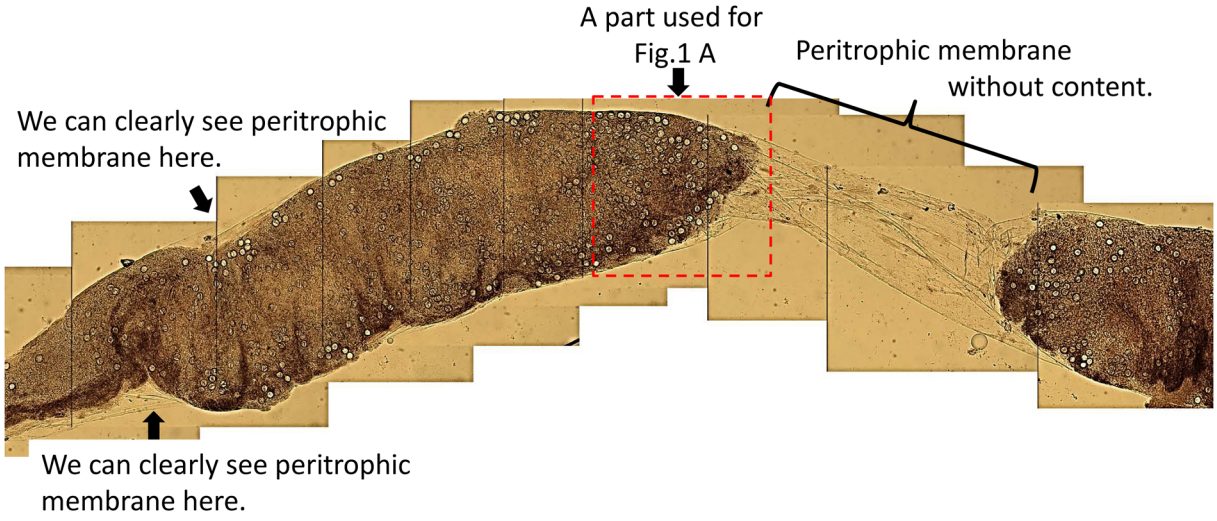

Supplemental Figure.