

Case Study

The cyanide gland of the greenhouse millipede, *Oxidus gracilis* (Polydesmida: Paradoxosomatidae)

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Abstract

Although the greenhouse millipede, *Oxidus gracilis*, is distributed worldwide, there is little work using modern tools to explore its morphology. We used confocal laser scanning microscopy (CLSM) to image the cyanide glands of *Oxidus gracilis*. Glands from adult millipedes were dissected out before imaging, and we were able to image glands of juveniles through the cuticle due to the strong autofluorescence of the gland extract. We can report that CLSM is a promising technique to non-invasively investigate the development and mechanisms of polydesmid cyanide glands.

Keywords

Oxidus gracilis, cyanide gland, Diplopoda, Polydesmida, CLSM

Background

The following work is the result of the "Know Your Insect" graduate course in the Department of Entomology at the Pennsylvania State University, taught during the fall semester of 2016. In short, the course provides the opportunity for a small group of

students to learn more about the insects they are studying. Each student focuses on an anatomical feature of interest and leads the class in a lecture, discussion, and live dissection. The students also have the opportunity to image their anatomical feature of interest using confocal laser scanning microscopy (CLSM), relying on the autofluorescence of arthropod tissue (Haug et al. 2011).

Through work on decomposer communities in Pennsylvanian agricultural fields, KP came across the greenhouse millipede, *Oxidus gracilis*. *O. gracilis* was the only millipede species she collected in corn and soybean fields in the summer of 2016, and they dominated her samples (over 35% of macroinvertebrates collected in pitfall traps). Because *O. gracilis* is an invasive species, KP was curious to learn more about their invasion, the morphological and ecological mechanisms that allow them to reach such high densities, and implications of their presence for decomposition and nutrient cycling.

Overall KP's case was unique for this course, as her "insect" is not an insect at all but rather a diplopod; while working with a diplopod provided some challenges, we encountered some exciting surprises.

New information

As with many other arthropod groups, interest in studying millipede morphology has varied over time. Overall, diplopods have received little attention compared to other arthropod groups (Sierwald and Bond 2007). Despite its global distribution, it is somewhat surprising that *O. gracilis* is no exception. Research on the cyanide glands of *O. gracilis* has focused on the exudate chemistry of adults (Duffey et al. 1977, Taira et al. 2003). We could not find any morphological studies on the cyanide glands of *O. gracilis*, and while other Polydesmid cyanide glands have been dissected out and studied (Eisner et al. 1963, Marek and Moore 2015, Duffey 1981), this appears to be the first use of confocal laser scanning microscopy (CLSM) to image Polydesmid cyanide glands.

We are also excited to report that, at least up to the 5th larval stadium, the cuticle is transparent enough to image the highly auto-fluorescing storage chamber without dissection. We believe that non-invasive imaging of the glands has multiple potential applications; it could be used to do live imaging of when glands are activated, to observe the growth and development of the glands across stadia, and to determine the amount of defensive secretions released under different stressors.

During dissection of the adult specimens, we noticed heavy infestation nematodes. It would be interesting to know if these nematodes act as a significant top-down control on *O. gracilis* populations, although high millipede densities in our sample site would lead us to hypothesize they have little control.

Overview of the cyanide glands in millipedes

Cyanide glands (=repugnatorial glands, Minelli (2015)) are found in the order Polydesmida and are just one of three defensive gland types found in diplopods. Paul Marek and Tom McCoy (Marek and McCoy 2016) have produced some light microscopy images of a beautifully dissected cyanide gland from *Pachydesmus crassicutis*, which provides a good representation of polydesmid gland morphology.

Cyanide glands are usually present in just over half the body segments - 5, 7, 9, 10, 12, 13, 15-19 - and are located in the paired, flange-like pleural keels (Shear 2015). The glands consist of a large reservoir containing the precursor compound (e.g. mandelonitrile) connected to a smaller reaction chamber via a muscularized valve. It is in this smaller reaction chamber where the precursor compound is mixed with enzymes which break down the mandelonitrile into hydrogen cyanide and benzaldehyde (Duffey 1981). The reaction products are released through ozopores in the cuticle surface. In addition to hydrogen cyanide and benzaldehyde, *O. gracilis* produces various phenolic compounds which may serve defensive functions against specific predators. For a more thorough review of the biochemistry, physiology, and ecology of millipede chemical defenses, see Shear (2015).

Methodology

Field collection and rearing of *Oxidus gracilis*

We collected adult *Oxidus gracilis* millipedes from a corn field at the Russell E. Larson Agricultural Research Center at Rock Springs, in Centre County Pennsylvania, USA, during summer and fall of 2016.

After summer storms, moist soil and high humidity provided for easy surface collection of adult *Oxidus gracilis*. We collected approximately 100 adults on 28 July 2016; once in the lab, we kept the millipedes in a clear plastic container with a 1-2 cm layer of field soil and a handful of moistened straw. We maintained the colony of 100 adults for four weeks; after three weeks (18 August 2016) we observed clusters of eggs and second instar larvae. However, it was difficult to maintain the colony under proper humidity - too dry and the colony desiccated, too wet and fungus overgrew the colony. The majority of the colony was lost by mid-September; interestingly, this coincided when adult field populations began to decline. In their hypothesized native range of Japan, *Oxidus gracilis* has been observed to have a similar life history - millipedes mature in summer, lay eggs in fall, and overwinter as late larval stages (Murakami 1962, Murakami 1966). It is probable that their annual life cycle means field-collected adults will die in the fall, regardless of rearing conditions. In early October, although populations were much sparser, we collected additional adults and juveniles for microscopy and to re-start the lab colony.

Specimen preparation and dissection

Adult specimens collected in September were fixed in 80% ethanol. Upon dehydration in ethanol, the cyanide gland became impossible for us to remove – likely due to the gland tissue collapsing. Because of this observation, adult specimens collected in October were kept alive until dissection. At the time of dissection, we placed the millipedes in a petri dish containing 0.1 M dibasic phosphate buffer (pH=7.4) and quickly severed the head from the body using dissecting scissors. Timing of this process seems to be critical; if the millipede is disturbed too much before the dissection, the glands' storage chambers may be emptied which makes it hard to see the clear gland tissue against the fat body.

After decapitation, the body rings were carefully separated from each other and from the digestive system. Only the segments containing cyanide glands (rings 5, 7, 9, 10, 12, 13, and 15-19) were dissected further under light microscopy; before each ring dissection we checked for the gland ozopore on each keel to ensure we were dissecting rings with cyanide glands (Suppl. material 1).

For each ring dissection, the dorsal tergite and ventral sternite were bisected so each keel could be dissected independently. Care was taken to remove fat body and cuticle to reveal the storage and reaction chambers of each gland.

We use two, fifth stadium juvenile millipedes for additional imaging. Noticing how transparent the cuticle was, and knowing how fluorescent the storage chambers were from the adult dissections, we did not attempt to fully dissect out the glands. From one juvenile we isolated body rings with ozopores and we left the other juvenile largely intact, only severing the head to reduce movement.

Confocal Laser Scanning Microscopy

Specimens were examined with an Olympus FV10i Confocal Laser Scanning Microscope using two excitation wavelengths: 473 nm, and 559 nm. We detected autofluorescence using two channels with emission ranges of 490–590 nm (green pseudocolor), and 570–670 nm (red pseudocolor), respectively. We generated volume rendered micrographs and media files with ImageJ (Schneider et al. 2012) using maximum intensity projection.

Observations on the cyanide glands using dissections and Confocal Laser Scanning Microscopy

Adults

Some glands were easily identified under a light microscope by a characteristic brownish-yellow droplet suspended within the storage chamber. Droplets like this have been observed across polydesmids and are presumed to be the precursor mandelonitrile (Duffey 1981); interestingly, it is still unknown how exactly these droplets are stabilized. The quick

release required for successful defense limits the likelihood that the precursor is stored as either a glycoside or emulsion (Duffey 1981); but without stabilization, the mandelonitrile could react and internally generate cyanide and cause damage to gland tissue. Possibly, the hydrophobicity of mandelonitrile is enough to stabilize the droplet in an aqueous environment, or other compounds (benzoic acid, fatty acids, and fatty acid esters) may play a role in stabilization.

Most storage chambers were difficult to see or not found at all, likely due to destruction of the delicate storage chambers during dissection, or the millipedes evacuating their storage chambers upon disturbance.

We successfully dissected out a storage chamber from one cyanide gland which we imaged on the CLSM using autofluorescence (Figs 1, 2). As expected, the millipede exoskeleton brightly fluoresced and we were able to observe the thin wall of the storage chamber. We were initially surprised to see how brightly the gland extract fluoresced because it is thought to be a mixture of only of small molecules, while arthropod autofluorescence is usually attributed to large, complex molecules (Haug et al. 2011). It is possible that the strong autofluorescence of the gland extract is based on the aromaticity and cyanogenic structure of mandelonitrile and its derivatives (Duffey 1981).

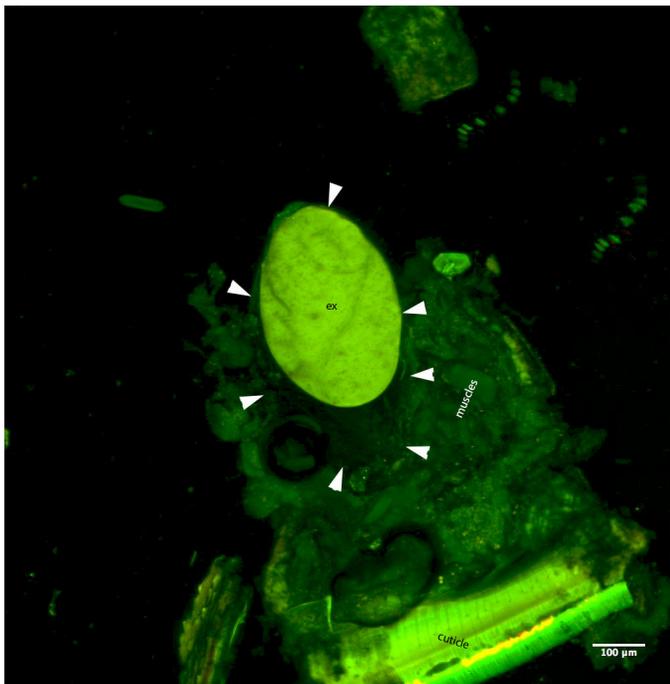


Figure 1.

CLSM volume rendered micrograph showing the cyanide gland of *Oxidus gracilis* (arrows pointing the wall of the cyanide gland, ex=strongly autofluorescing gland extract).

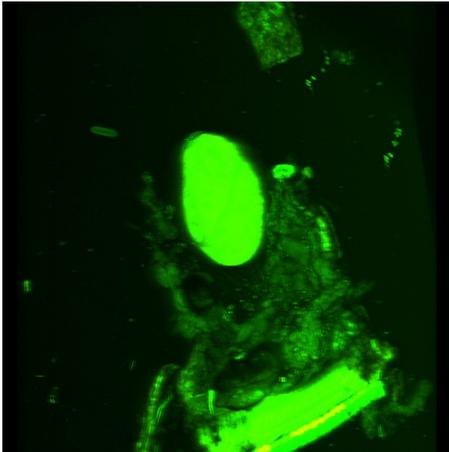


Figure 2.

CLSM volume rendered media file showing the cyanide gland of *Oxidus gracilis* (gland extract is the overexposed droplet).

Juveniles

We were unable to observe the cyanide glands from the dissected juvenile millipede; it is possible the gland structures are even more delicate in juveniles, and therefore more prone to tearing during dissection. However, because the cuticle is transparent in the 5th larval stadium, we successfully imaged the strongly fluorescing storage chambers through the cuticle of the intact juvenile millipede (Figs 3, 4). In one case, we were also able to observe the reaction chamber and valve (Fig. 4).

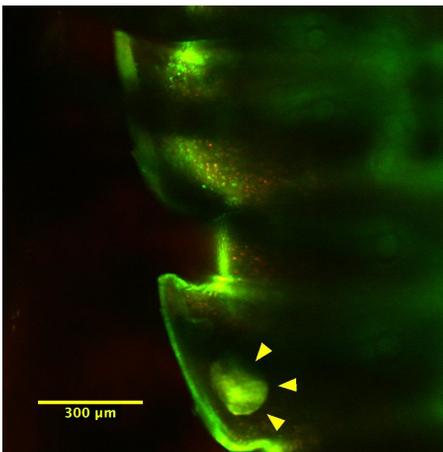


Figure 3.

Top view of the juvenile millipede; the bright field in the lower flange is a gland storage chamber.

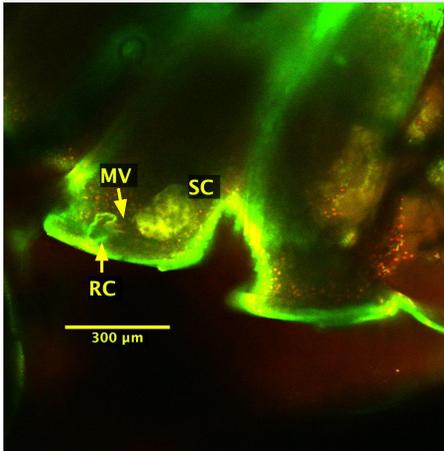


Figure 4.

Top/rotated view of the juvenile millipede. SC = storage chamber, RC = reaction chamber, MV = muscularized valve connecting the two chambers.

Nematode infestation in adults

Upon dissection, we observed some of the adult specimens contained numerous nematodes (Suppl. material 1). *O. gracilis* can succumb to nematode infection, but have been shown to survive low level infections of an unidentified rhabditid nematode (Poinar and Thomas 1985).

Relevance to ongoing research

One of us (KP) is studying the effects of insecticides on macrodecomposer activity in corn and soybean fields in Pennsylvania. Before this field season, her advisor mentioned the prevalence of millipedes in some fields, and her fieldwork this summer confirmed this – over 35% of specimens in pitfall traps (in a maize and soy field at our University's research farm) were millipedes (KP, *pers. obs.*). Of these, virtually all of them were *Oxidus gracilis*. With such a dominant millipede species, we were interested, although not entirely surprised, to discover it was an invasive species.

Invasive decomposers are prevalent – invasive earthworms are often dominate agricultural and forest decomposer communities throughout the United States (Baker et al. 2006). Researchers have documented the ecological changes associated with earthworm invasion fronts in forest systems – loss of the O horizon (organic litter layer), reduced soil carbon, and altered nutrient distribution in the soil horizon, all of which can dramatically disrupt nutrient cycling (Resner et al. 2015, Jennings and Watmough 2016). As invasive earthworms may be able to out compete native millipede species (Snyder et al. 2013), we hypothesize that an invading millipede species, especially one that reaches extraordinary

population densities, may similarly influence invertebrate communities and soil structure and function much like invading earthworms.

Decomposer food web

Of course, agricultural systems aren't pristine – they've been invaded by both destructive species (slugs and other crop pests) and beneficial species (carabid beetles, earthworms). We are interested in how *O. gracilis* fits into this novel food web - how do they interact with pests, predators, and other decomposers? Some farmers worry that the millipedes may be damaging their crops, although *O. gracilis* refused to eat live plants in laboratory experiments (Causey 1943).

We chose to image the cyanide glands of *Oxidus gracilis* because the defenses of this invasive species potentially tie into their interactions with generalist predators found in central Pennsylvania. Around 90% of plant biomass is recycled through decomposers, meaning the bulk of plant-based energy travels to predators not through herbivores, but through decomposers (Allison 2006, Polis and Strong 1996). More and more, ecologists recognize the importance of how 'brown food webs' link to the more frequently studied 'green food webs' (Wolkovich et al. 2014). In an agricultural context, it is important to understand how generalist predators interact with decomposers; if generalist predators can depend on decomposers as alternative prey, then the decomposer community can play an indirect role in controlling pest populations (Symondson et al. 2000).

If decomposers are acceptable, but less desirable prey, they can sustain predator populations when pest pressures are low, but not interfere with control when pest populations increase. Unfortunately, it is difficult to predict how valuable decomposers are for pest management because interactions between generalist predators and decomposers are poorly documented. The generalist predator community in Pennsylvania field crops is dominated by carabid beetles and spiders. Some carabid beetles reject earthworms and isopods (Best and Beegle 1977), while other species readily accept earthworms or juvenile millipedes as alternative prey (Brunke et al. 2009, Symondson et al. 2000). Certain spiders have been shown to heavily rely on decomposers, namely collembolans (Agusti et al. 2003). For millipedes especially, predation has rarely been studied, and most references to predators are based on casual observation and anecdotes (Sierwald and Bond 2007). More studies on millipede-predator interactions, and the defensive mechanisms involved, can aid our understanding of the role of decomposers in pest management.

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Supplementary material

Suppl. material 1: Cyanide Gland Pore

Authors: KA Pearsons

Data type: image

Filename: Screen Shot 2016-11-27 at 16.33.47.png - [Download file](#) (560.75 kb)