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ARTICLE

Defensive spray by a semiaquatic osmylid larva (Insecta: Neuroptera) for both aquatic and terrestrial predators

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Abstract
Chemical secretions are an effective means by which insects can deter potential enemies. Several terrestrial insects spray these liquids directionally toward enemies, but little is known about spraying behavior in aquatic and semiaquatic insects. The larvae of *Osmylus hyalinatus* (Neuroptera: Osmylidae) are semiaquatic, inhabiting the edges of small streams and ponds where they encounter multiple enemies on land and in water. The larvae of this osmylid sprayed a hyaline liquid from the anal opening if disturbed in either air and water, although the spray appeared slightly viscous in water. The liquid was stored in the posterior half of the hindgut and sprayed directionally toward an artificial stimulus. Spraying allowed the larvae to escape biting by ants, and to repel them in 90% of encounters. Spraying caused the regurgitation of 71% and 60% of all larvae swallowed by terrestrial frogs and aquatic newts, respectively. Aquatic fishfly larvae released 30% of captured larvae due to spraying. Most of the larvae that repelled ants or were regurgitated by amphibians survived, but those released by fishfly larvae were killed by heavy biting with the mandibles. This is the first report of effective liquid spraying by insects in water, and also within the order Neuroptera.

Keywords Amphibian predators · Ant avoidance · Chemical defense · Osmylidae · Spraying behavior

Introduction

The sequence of predation comprises encounter, detection, approach, capture, and consumption (Endler 1991). Most prey species develop morphological, physical, behavioral, and chemical defenses that are effective for escaping predation at specific stages of this sequence (Nelsen et al. 2014; Walker et al. 2018; Sugiura 2020a). Fluid-spraying is of a diverse range of such defensive traits (Eisner et al. 2005), and is used as a defensive tactic by many terrestrial animal groups including mammals (Stankowich 2012; Fisher and Stankowich 2018), birds (Swennen 1974), reptiles (Rosenberg et al. 1984; Middendorf and Sherbrooke 1992; Melville et al. 2004; Sherbrooke and Middendorf 2004; Berthé et al. 2013), amphibians (Brodie and Smatresk 1990), velvet worms (Baer et al. 2017), scorpions (Eisner et al. 2005; Nisani and Hayes 2015), whip scorpions (Eisner et al. 2005), spiders (Yap and Li 2009), and insects (Eisner et al. 2005). In water, however, defensive spraying is scarce and only reported in marine mollusks such as octopuses, squids and sea hares (Vincent 2005; Kamio et al. 2010; Love-Chezem et al. 2013), deep-sea pocket sharks (Claes et al. 2020), deep-sea shrimps (Inouye et al. 2000), and ostracods (Rivers and Morin 2012). In general, defensive sprays produced by terrestrial animals include venomous and chemically stimulating substrates (Eisner et al. 2005; Nelsen et al. 2014), whereas spraying by aquatic animals is used to disappear themselves from the predators, using sprayed material as smokes, decoys, or smell disruptors (Vincent 2005; Love-Chezem et al. 2013). The differences in the media of terrestrial (air) and aquatic (water) environments greatly affect the effectiveness of liquid spraying by prey. Water is denser and more viscous than air, such that sprayed material does not spread rapidly, instead floating for long periods (Denny 1993). Sprayed material is expected to attach directly to the predator's surface in air, but not to attach in water as it floats and is gradually diluted. Thus, the evolutionary background of defensive spraying differs physically between terrestrial and aquatic animals.

The larvae of some groups of Osmylidae (Insecta: Neuroptera) are water-dependent and live in wet or moist zones

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along streams and ponds, where they feed (through sucking) on soft-bodied insects. Water-dependent species are often observed to crawl underwater, and are considered semiaquatic (Martins et al. 2018), whereas other groups of osmylid larvae are distinctly terrestrial and can be found under bark in drier habitats or on vegetation far from water bodies (New 1986; Winterton et al. 2017; Martins et al. 2018). Semiaquatic species may be attacked by both terrestrial and aquatic predators. In central Japan, the potential predators of osmylid larvae, such as *Osmylus hyalinatus*, are insectivorous birds, frogs, fishing spiders, and ants on land and carnivorous fish, amphibians such as larval salamanders and newts, and predatory aquatic insects such larvae as dragonfly (Odonata), dobsonfly (Megaloptera: Corydalidae: Corydalinae), and fishfly (Megaloptera: Corydalidae: Chauliodinae) in water (Fig. 1). However, the defensive capabilities of prey against multiple types of predators has been rarely studied (Sugiura 2020a), and little attention has been given to prey species that must defend against both terrestrial and aquatic predators.

In this study, first we describe the spraying behavior of semiaquatic osmylid larvae in air and water, and next we compare the effects of their sprays on prey-swallowing terrestrial frogs, prey-biting terrestrial ants, prey-swallowing aquatic newts, and prey-biting aquatic fishfly larvae. Finally, the evolutionary divergence of chemical defense is discussed within the order Neuroptera and between water and land habitats.

Materials and methods

Spraying behavior

The last (3rd) instar larvae of *O. hyalinatus* were collected during late February to early March in 2020 from substrates such as moss-covered stones and wet fallen tree twigs at the edges of small hill streams in Hanno City, Saitama Prefecture, central Japan. Species identification of these larvae followed Matsuno and Yoshitomi (2016) and Matsuno (2017). The larvae were kept individually in small plastic cups (50 mm in diameter, 35 mm deep) containing several wet fallen leaves and living mosses at 15 ± 1 °C (14-h:10-h light:dark light cycle). Seven larvae were examined for the direction of spraying by stimulating them with forceps from the right ($N = 4$) or left side ($N = 3$) on a dark-colored board (Fig. 2a, b). Three larvae were kept at ca. 4 °C for one day and then put into a freezer (− 20 °C) for 20 min, because precooling was required to prevent self-spraying in the freezer. After melting, the larvae were dissected in insect Ringer (0.9 g NaCl, 0.02 g $CaCl_2$, 0.02 g KC1, and 0.02 g $NaHCO_3$ in 100 mL distilled water) to observe their internal organs under a stereoscopic microscope (×10). The other three larvae were treated to exhaust the stored sprayed material by repeated (ten or more times) stimulations with forceps before freezing. Comparisons between these intact and treated larvae allowed us to determine which part of the larva held the spray material. The body length from the anterior margin of the head to the abdominal tip, excluding the hook apparatus, was measured in five randomly selected live larvae.

The last-instar larvae were also collected from late October to early December in 2020 at Hanno City and Hidaka City, Saitama Prefecture, and on 6 February 2021 at Moroyama Town, Saitama Prefecture. Ten larvae stimulated in water using forceps from the right ($N = 5$) and left side ($N = 5$) were video recorded from the outside of an aquarium (Fig. 2c). The larval body length was measured for five live individuals.

The last-instar larvae of two other *Osmylus* species, *O. pryeri* ($N = 2$) and *O. decoratus* ($N = 1$), were also collected at small hill streams at Yokoze Town on 6 April 2020, Higashichichibu on 4 March 2021, and Hanno City on 10 April 2020, Saitama Prefecture. These three larvae were stimulated from the right side with forceps on the dark-colored board to examine their spraying behavior.

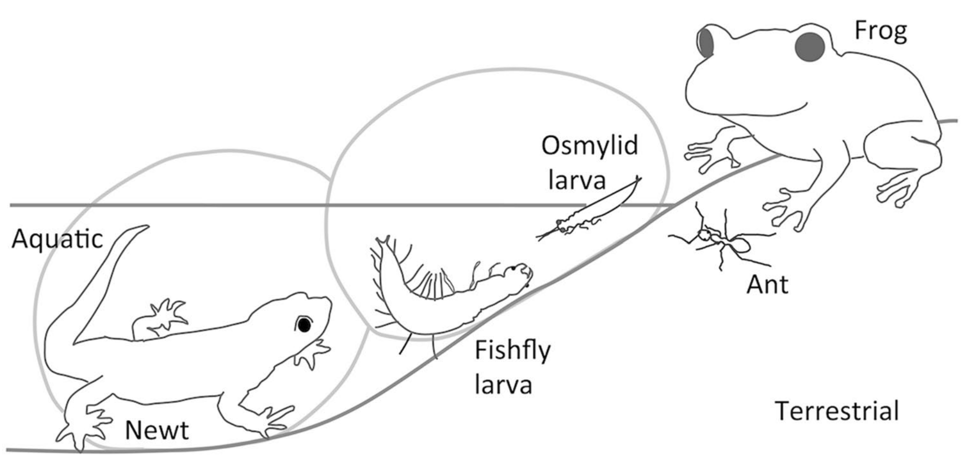

Fig. 1 Schematic drawings of potential predators of semi-aquatic osmylid larvae, on land (e.g. frogs and ants) and in water (e.g. newts and fishfly larvae), at the edge of a small, stony stream

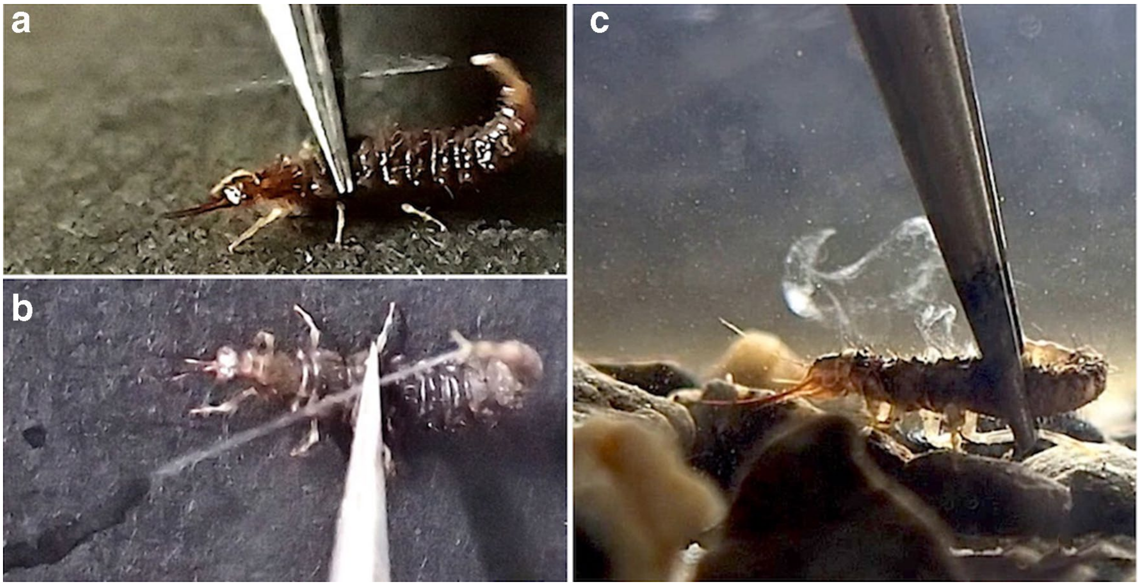

Fig. 2 Liquid spraying from an anal opening by the last-instar osmylid larvae, *Osmylus hyalinatus*, stimulated with forceps. **a** Lateral view in air. **b** Dorsal view in air. **c** Lateral view in water

Effects on aquatic predators

Ten last-instar larvae of the fishfly *Parachauliodes japonicus* (Megaloptera: Corydalidae: Chauliodinae) were collected on 3 and 5 March 2020 from small hill streams at Hanno City. After measuring the head width at the widest point using a slide caliper, these larvae were kept individually in glass vessels (65 mm diameter, 90 mm high) at 15 ± 1 °C (14-h:10-h light:dark cycle). The rearing vessels included well-aerated tap water, not exceeding 5 mm in depth, and stones as refuges. Water was replaced and one living last-instar larva (ca. 13 mm in body length) of the chironomid *Propsilocerus akamusi* was provided daily. Feeding experiments were conducted at 20 ± 1 °C on 8 March 2020. One chironomid larva was dropped from forceps to touch the larval head region, because this predator recognizes prey from tactile cues (Hayashi 1985). If the chironomid larva was eaten, then one intact osmylid larva was dropped gently to touch the fishfly larva as it clung to a small piece of leaf, to prevent artificial spraying. We observed the larval feeding behavior and measured the durations of manipulation (from biting prey with mandibles to beginning to swallow it) and eating (from the start to end of prey swallowing using the maxillae and fore- and mid-legs) with a stopwatch. If the larva released the prey, then the time between biting and releasing was measured as the manipulation duration. On 14 March 2020, the same feeding experiments were performed using the treated osmylid larvae to exhaust the stored sprayed material by repeated (ten or more) stimulations with forceps. These treated larvae were then washed in sufficient water and immediately given to the predator.

Sexually mature newts *Cynops pyrrhogaster* (six males, seven females) were collected on 11 and 17 March 2020 from a small pond at Hachioji City, Tokyo, central Japan. In the laboratory, we measured the snout to vent length (SVL) of each newt and kept them individually in plastic containers (220 mm × 130 mm, 135 mm deep) at 20 ± 1 °C (12-h:12-h light:dark cycle). Each container included well-aerated water, not exceeding 30 mm in depth, and stones as refuges. Water was replaced every 2 or 3 days. On each experimental day from 14 to 26 in March, a newt that had been starved for 2–9 days was given one chironomid larva to confirm its willingness to eat at 20 ± 1 °C. If the newt ate the larva, then one treated osmylid larva was put in front of the newt with forceps. We recorded the feeding behavior and occurrence of successful swallowing or regurgitation after swallowing. Next, one intact osmylid larva was gently placed in front of the newt while it clung to a small piece of leaf or moss to prevent artificial spraying, and the feeding behaviors were recorded. This method of prey presentation differed from that for the fishfly larvae because food recognition by learning has been documented in amphibians (Suboski 1992). Two male newts that never ate chironomid larvae, and one male that ate a chironomid larva but was unresponsive to the provided osmylid larvae, were omitted from the analysis. Lower feeding rates in males may be related to breeding activity in spring in this newt species (Akiyama et al. 2011; Ihara 2013). The regurgitated larvae were kept individually in small plastic cups with wet fallen leaves at 20 ± 1 °C (14-h:10-h light:dark cycle) and occasionally given chironomid larvae to examine their survivorships. All newts were released to the capture pond soon after the experiments.

Effects on terrestrial predators

Ten froglets of *Glandirana rugosa* were obtained by rearing from an egg mass collected on 3 June 2019 from the Misawa River, Minano Town, Saitama Prefecture. Hatched tadpoles were kept in plastic containers (130 mm × 170 mm, 55 mm deep) at 20 ± 1 °C (14-h:10-h light:dark cycle) and provided commercially available turtle food (Tetra ReptoMin Stick, Spectrum Brands Japan, Yokohama) without food shortage. Water was changed every other day. The larval period of this frog is long and metamorphosis usually occurs after overwintering in the field (Matsui and Maeda 2018). After metamorphosis in spring to summer in 2020, froglets were put in plastic containers that were the same size as those used for larval rearing, but without water, and kept at the room temperatures (14–32 °C). The larvae (ca. 8 mm in body length) of broad-horned flour beetles *Gnatocerus cornutus*, cultured following Okada et al. (2019), were provided every day as food. On the day of the feeding experiments (13, 14, or 17 November 2020) at 20 ± 1 °C, individual froglets that had been starved for one day were provided with one beetle larva. If the beetle larva was eaten, the treated osmylid larva was provided using forceps. Next an intact larva was placed gently in front of the froglet while it clung to a small piece of leaf or moss. The behavior of froglets was monitored using a video camera. Three froglets that did not eat any beetle larvae were omitted from the analysis. The body size of froglets was measured as SVL after the experiments. The regurgitated osmylid larvae were kept in small plastic cups containing wet fallen leaves and living mosses, at 20 ± 1 °C under a natural photoperiod, and occasionally given chironomid larvae.

Ten foragers of the ant *Formica japonica* were randomly selected from two colonies that were collected at Hachioji, central Tokyo, and cultured at 25 ± 1 °C (14-h:10-h light:dark cycle). On 10 and 11 December 2020, one intact osmylid larva was gently introduced into a Petri dish (55 mm in diameter, 15 mm deep) containing wet filter paper (55 mm diameter, Whatman 2) on the bottom. One ant forager that had been walking outside the colony chamber was then introduced gently. On 8 and 11 February 2021, the same feeding experiments were conducted using the treated osmylid larvae. In both experiments at 25 ± 1 °C, the behaviors of the paired larva and ant were video-recorded for 5 min, except for one pair that was recorded until the end of first ant attack. After recording, ant's head width at the widest part and the body length from the front of the head to abdominal tip were measured using an ocular micrometer of the stereoscopic microscope (×10). After the experiment, the osmylid larvae were kept as described for the froglet experiment.

Some of the digital videos of these prey-predator behaviors were posted on the Movie Archives of Animal Behavior (MOMO) website.

Statistical analyses

The body sizes of prey and predators were presented as mean ± standard deviation (SD) with ranges. Paired *t* tests were used to detect the effects of prey type on the feeding time of the fishfly larvae. Frequency data for fed and unfed predators were compared using χ^2 tests.

Results

Spraying behavior

Disturbed larvae of *O. hyalinatus* lifted and vented the abdomen like a scorpion and vigorously splayed hyaline liquids from an anal opening in air (Fig. 2a, b). Although chemically unidentified, the sprayed liquid had a pungent smell. When stimulated with forceps from the right side, the larvae sprayed to the right front ($N = 3$), and when stimulated from the left, they sprayed to the left front ($N = 3$). In water, the disturbed larvae also sprayed liquid to the stimulated sides ($N = 5$ for each direction) (Fig. 2c). The liquid did not form a vigorous stream in water, but floated and finally sank to the bottom. The last-instar larvae of *O. pryeri* and *O. decoratus* also sprayed hyaline liquids directionally toward stimuli held in the air using forceps.

Repeated stimulation reduced the amount of sprayed liquid, and later a small droplet to form. Differences in internal organs between intact and treated larvae indicated that the spraying material was stored in the posterior part of the hindgut, which inflates in the intact larvae ($N = 3$) but deflates in the treated larvae ($N = 3$) (Fig. 3). When this part of the hindgut was broken, the hyaline liquid leaked out, causing the same smell observed in the air spraying experiment.

Defensive effects of spraying

The last-instar osmylid larvae used in the spring experiments were 10.9 mm in mean body length ($N = 5$, SD = 0.6, Range 9.2–14.3). The last-instar larvae of fishflies were 6.81 mm in mean head width ($N = 10$, SD = 0.56, Range 5.95–7.55). Three (30%) of 10 larvae released the captured, intact osmylid larva during manipulating it with the mandibles (for a digital video, see http://www.momo-p.com/showdetail-e.php?movieid=momo210215oh01b&embed=on), but all (100%) fed on the treated larvae (Fig. 4a). The difference in frequency between these two prey types was statistically marginal ($\chi^2 = 3.53$, $df = 1$, $P = 0.06$). The mean manipulating time was 299.5 ± 134.2 s for the intact larvae and 85.7 ± 47.5 s for the treated larvae (paired *t* test; $t = 2.30$, $df = 9$, $P < 0.05$), but the eating time did not differ between these two prey types (138.7 ± 22.4 s and 128.0 ± 18.2 s, respectively: paired *t* test; $t = 0.08$, $df = 6$, $P = 0.94$). The

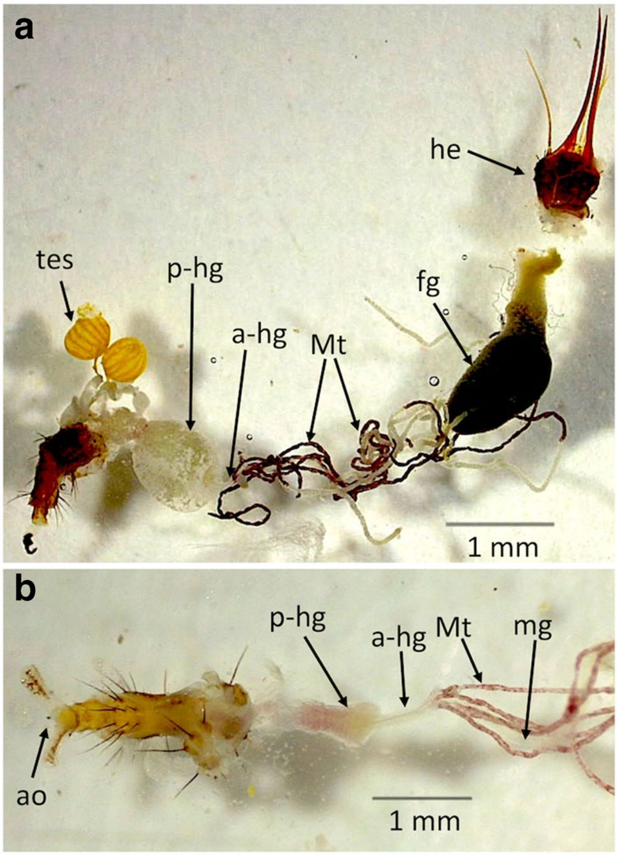

Fig. 3 Internal organs of the last-instar osmylid larvae, *O. hyalinatus*. **a** Intact larvae. **b** Treated larvae, in which spraying material was exhausted by repeated stimulations with forceps. The digestive system ends at the foregut. The anterior part of the very fine midgut closes and Malpighian tubules open near the end of the midgut, which connects to the hindgut. *a-hg* anterior part of hindgut, *ao* anal opening, *fg* foregut, *he* head, *mg* midgut (fine and hyaline, unclear in this photograph), *Mt* Malpighian tubules, *p-hg* posterior part of hindgut, *tes* developing testis

fishfly larvae usually ceased prey manipulation after spraying liquids in water (observed based on its smell), but after liquid sedimentation they resumed manipulation. Three larvae that had been bitten once by fishflies and released did not survive.

The newts were 59.8 mm in SVL ($N=10$, SD = 6.0, Range 51.9–67.1) and only one (10%) of 10 individuals regurgitated the treated osmylid larvae after swallowing (Fig. 4b). In contrast, six (60%) of 10 newts regurgitated swallowed intact larvae (for a digital video, see http://www.momo-p.com/showdetail-e.php?movieid=momo210215lh01b&embed=on). These frequencies of regurgitation differed statistically between the two prey types ($\chi^2=5.49$, $df=1$, $P<0.05$). All larvae were alive at regurgitation, one (14%) of which was unfortunately lost and one (14%) died after 7 days. However, five (71%) survived for 14.8 days ($N=5$, SD = 6.3, Range 5–20) and prepupated in cocoons between the wet fallen leaves.

The last-instar osmylid larvae used in winter experiments were 8.4 mm in mean body length ($N=5$, SD = 1.5, Range 6.6–10.7). The mean SVL of the froglets was 20.3 mm ($N=7$, SD = 1.7, Range 18.2–22.9). When given the treated osmylid larva, one (14%) of seven froglets regurgitated it, but when given the intact one, five (71%) regurgitated it (Fig. 4c) (for a digital video, see http://www.momo-p.com/showdetail-e.php?movieid=momo210215oh02b&embed=on). The frequency of regurgitation differed statistically between the prey types ($\chi^2=4.67$, $df=1$, $P<0.05$). The regurgitated froglets extruded the tongue and scratched it with their forelimbs to peel off the attached osmylid larva. Six larvae were alive at regurgitation, but five (83%) of them died after 7.0 days ($N=5$, SD = 2.9, Range 5–12). Only one (17%) prepupated in cocoons after 65 days.

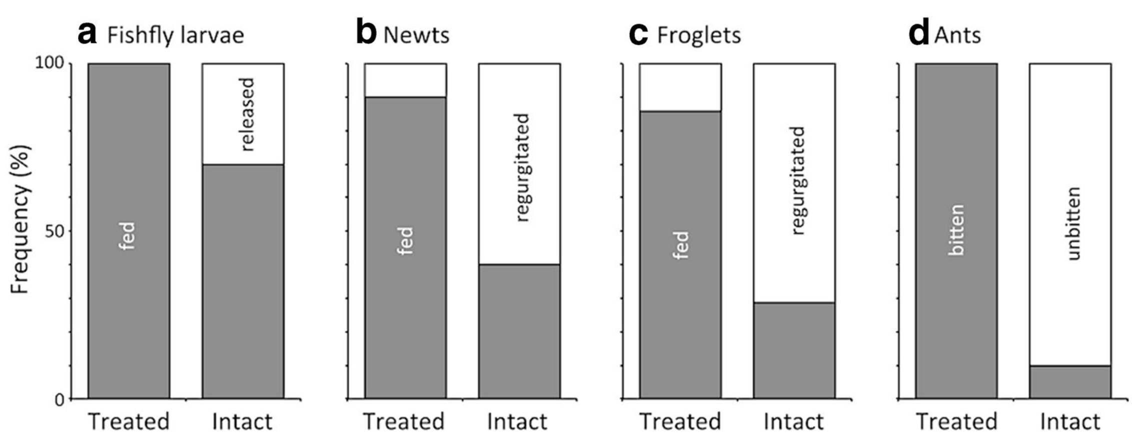

Fig. 4 Feeding success in **a** fishfly larvae *Parachauliodes japonicus* ($N=10$), **b** newts *Cynops pyrrhogaster* ($N=10$), **c** froglets *Glandirana rugosa* ($N=7$), and **d** ant foragers *Formica japonica* ($N=10$ in each). In each experiment, individual predators were provided with treated or intact last-instar osmylid larvae *O. hyalinatus* singly. In treated larvae, spraying material was exhausted by repeated (ten or more times) stimulations with forceps and washed well immediately prior to each feeding trial

Ants were the smallest predators in the present study; 1.21 mm ($N=20$, SD = 0.065, Range 1.10–1.30) in head width and 5.83 mm ($N=20$, SD = 0.58, Range 4.45–7.70) in body length. Ten treated osmylid larvae were all bitten by ants (Fig. 4d). Ant attacks were intermittent. The first attack continued for 161 s ($N=10$, SD = 145, Range 17–507), during which the osmylid larvae lifted the abdominal tip 12 times ($N=10$, SD = 8, Range 5–30) (for a digital video, see http://www.momo-p.com/showdetail-e.php?movieid=momo210215oh04b&embed=on). The reason that ants temporarily stopped attacking the larvae remains unclear, but some ants appear to have been repelled after repeated lifting of abdominal tip, which suggests the secretion of a small amount of chemicals from the abdominal tip despite artificially induced exhaustion immediately prior to the experiment. In contrast, only one (10%) of the ten intact larvae was bitten (Fig. 4d). The bitten larva lifted the abdomen to spray six times, but the ant continued to attack for 118 s until it was repelled. Each of the other nine intact larvae sprayed an average of 1.2 times ($N=9$, SD = 0.4, Range 1–2) during ant attacks lasting an average 0.46 s ($N=9$, SD = 0.36, Range 0.1–1.3). Thus, 90% of intact larvae successfully repelled ants without being bitten. The frequency of ant biting differed between the treated and intact larvae ($\chi^2=16.36$, $df=1$, $P<0.001$). Ants were deemed to have been repelled by larvae when they exhibited abnormal leg movements after spraying (for a digital video, see http://www.momo-p.com/showdetail-e.php?movieid=momo210215oh03b&embed=on). However, the ants seemed to recover from these abnormal leg movements within minutes of being transferred to a new Petri dish. Finally, 7 (70%) of 10 treated larvae and 9 (90%) of 10 intact larvae prepupated in cocoons after 30.5 days ($N=16$, SD = 20.8, Range 15–97). Other four larvae died after 1–24 days without preputation.

Discussion

Spraying in air and water

Larvae of the osmylid *O. hyalinatus* sprayed hyaline liquid from the anal opening by bending the abdomen dorsally when tactile stimulation was provided. In air, the liquid was sprayed as a stream directed to the stimuli, finally forming several droplets on the substrate (Fig. 2b). In water, spraying was less powerful and the sprayed material appeared to be slightly viscous, first floating and finally sinking. The liquid had a pungent smell when sprayed in both air and water, but its chemical composition remains unknown. Differences in the physical characteristics of air and water influenced the dispersal of the spray, which reached farther in air with low density but floated in dense water. Exocrine glands and chemical defenses in freshwater invertebrates have been documented in Platyhelminthes (flatworms), Nemertini worms, and water mites (Hydrachnidia), but are not widespread as in those living in terrestrial or marine environments (e.g. Dettner 2010). Aquatic insects with chemical glands, such as adephagan beetles and water bugs, do not spray. They merely secrete fluid onto their body surface (Dettner 2019). Larvae of the stonefly *Pteronarcys dorsata* expel a milky, cloudy fluid (hemolymph) into water through pores located on the trochanteral segments of the metathoracic legs when captured by crayfish (Moore and Williams 1990). The crayfish then release the larvae and spent cleaning their mouth parts and antennae.

The osmylid larvae are the first insect group found to vigorously spray a defensive fluid in water. Examined larvae of two other osmylid species, *O. pryeri* and *O. decoratus*, also spray, suggesting this behavior is common among the family Osmylidae. However, there is no information of defensive spraying in other semiaquatic and terrestrial genera of this family (Aldrich and Zhang 2016; Walker et al. 2018). Therefore, we cannot discuss the evolutionary origin of this unique spraying behavior, despite the availability of global molecular phylogeny data for Osmylidae (Winterton et al. 2017).

The material sprayed from osmylid larvae is stored in the posterior part of the hindgut and exhausted by repeated spraying. Among terrestrial neuropterans, adults of Chrysopidae and Osmylidae species have prothoracic scent glands that secrete chemicals when disturbed (Blum et al. 1973; Güsten and Dettner 1991; Güsten 1996). The chrysopid larvae also secrete a droplet at the anal opening as a defense against ants to which their abdominal tip is oriented (LaMunyon and Adams 1987). The droplet triggers ant grooming if it becomes attached to the ant's head or antennae (LaMunyon and Adams 1987). This viscous substance, stored in the hindgut, is probably derived from Malpighian tubules that open to the posterior end of the midgut (LaMunyon and Adams 1987). Larval neuropterans have a dead-end digestive system at the foregut, and the midgut and hindgut are independent of food digestion (Walker et al. 2018). Therefore, spray material stored in the posterior hindgut is not derived from food, but is secreted from Malpighian tubules or other unidentified glands that open into the mid- and hindgut. The chrysopid larvae cease producing this substance when nearing pupation, because the larvae form cocoons before pupation using silk produced in Malpighian tubules and secreted from the anal opening (LaMunyon and Adams 1987; Sutherland et al. 2010). Larvae of the osmylid *Spilosmylus flavicornis* form coarse cocoons from silk (Kawashima 1957). The osmylid examined in this study also pupated in the coarse cocoon. Therefore, it would be interesting to examine the chemicals of these defensive and cocoon materials in the chrysopid and osmylid larvae.

Effects on multiple predators

Defensive secretions can be classified into three categories based on their reaction mechanisms (Pasteels et al. 1983): sticky, slimy, or entangling secretions acting mechanically rather than chemically; non-specific irritants acting as common repellents for animals; and true poisons with specific physiological processes. A wide range of potential predators threaten insect species, and these three defense types are not equally effective against all predators. The oymylid larvae are semiaquatic, inhabiting the edges of small streams and ponds where they encounter multiple types of enemies living both on land and in water. Chemical defense strategies include the use of multiple target-specific chemicals and single chemicals effective against most targets (Rojas et al. 2017). The target-specific chemical may be more efficient for repelling enemies, but are costly to prepare for use on multiple targets. The material sprayed by the osmylid larvae seems act as an irritant, repelling both terrestrial and aquatic predators. This suggests lower defense costs against multiple enemies.

Defensive effects also differ among predators with different feeding behaviors, such as prey swallowing, biting with mandibles, and stinging with mouthparts (Shinohara and Takami 2020). We examined four types of predators in this study; aquatic fishfly larvae that bite with strong mandibles, aquatic newts that swallow prey with water, terrestrial frogs that swallow prey using a sticky tongue, and small ants that bite with mandibles.

Larvae of the fishfly *P. japonicus* are large predatory aquatic insects found under stones at the edges of small streams (Hayashi 1989). These larvae were collected in the same streams as osmylid larvae in this study. Their prey are captured with large mandibles and manipulated with the maxillae and fore- and occasionally mid-legs before swallowing (Hayashi 1985). In this study, intact osmylid larvae sprayed fishfly larvae upon captured, and 30% of them were released after longer manipulation, perhaps due to the repellent effect of the sprayed material. However, the osmylid larvae were unable to survive the strong bite of this predator.

The newt *C. pyrrhogaster* is common in small ponds and streams, particularly in its reproductive seasons from late autumn to early summer in central Japan (Akiyama et al. 2011). Most aquatic predators, such as fish, larval salamanders, and newts, swallow prey items whole without biting, and their prey escape without being injured by morphological and chemical defenses. For example, the long abdominal spines of dragonfly larvae reduce predation risk by fish, which spit out the larvae after swallowing (Mikolajewski and Rolff 2004; Mikolajewski and Johanssen 2004; Johansson et al. 2017). In our experiments, 60% of intact osmylid larvae swallowed by newts were regurgitated alive, and 71% went on to prepupate. Thus, the sprayed material was highly effective in allowing the osmylid larvae to escape predation by repelling aquatic swallowing predators.

The frog *R. rugosa* prefers streamsides as its main habitat, where the osmylid larvae also live. Frogs capture prey using a sticky tongue and swallow them whole. Frogs are often cited as model predators, and tend to reject chemically defended and spiny insects (Sugiura 2020a). In our experiments, 71% of intact osmylid larvae swallowed by frogs were regurgitated following spraying. The regurgitated larvae died shortly after regurgitation, with only 17% surviving to prepupation. This was much lower than the survival rate (71%) after escaping from newt predation. Aquatic predators swallow and regurgitate their prey with water, contributing to their safe escape, whereas terrestrial frogs swallow the prey using a sticky tongue and regurgitate it by scratching with their forelimbs. Although frog forelimbs lack claws, some physical pressure may threaten prey survival after regurgitation.

The ant *F. japonica* is common in central Japan. Ants are typically used as model predators because the workers hunt other arthropods to feed (Sugiura 2020a). Most osmylid larvae escaped from ant biting by spraying. Ants are small predators that bite using small mandibles, resulting in higher survivorship among the attacked osmylid larvae.

In general, natural selection seems to favor tough bodies that resist handling by predators. For example, the small, hard beetle *Regimbartia attenuate* can escape from the vents via the digestive tract even if swallowed by frogs (Sugiura 2020b). In adult butterflies, body toughness appears to be correlated with unpalatability, because unpalatable species must tolerate manipulation by predators until they can escape (DeVries 2003). The osmylid larvae have apparently soft bodies and seems to exhibit lower tolerance to predator's manipulation. However, they can escape mandibular attacks by ants, suggesting that their bodies are somewhat resilient. Insectivorous birds that feed at streamsides, such as wagtails, eat semiaquatic insects (Davies 1976). Larval manipulation using forceps likely simulates capture by a small bird beak. However, no larvae died after treatment with forceps in this study, suggesting that the osmylid larvae that escape bird predation may survive the attempt.

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Author contributions TI found the spraying behavior and all authors contributed to completion of this study; particularly PY and FH reviewed the spraying behavior of terrestrial and aquatic animals, TI and FH performed experiments and analyses, and FH wrote the manuscript which was finally checked by all authors.

Data availability Authors make all the data available to readers upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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