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The bug-killer fly *Gymnosoma rotundatum* (L.) (Diptera: Tachinidae) forms the respiratory funnel independently of the host's immune response

Research Paper

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Abstract

In internal parasitism, the respiration strategy within the host's body is as essential as evading attack from the host's immune system. Tachinid flies are parasitoids of terrestrial arthropods, mostly insects, during their larval stage. To obtain oxygen while living in the host body, they build a cylindrical structure known as the respiratory funnel at the aperture opened by the tachinid larva on the host integument or trachea. These funnels can be divided morphologically into sheath and cone types. Previous research on sheath-type funnels revealed that they are derived from the encapsulating substance produced by the host's immune system. In contrast, the cone-type funnels cover part of the body of the larval tachinid and may be constructed independently from the host immune system. To determine the mechanisms of cone-type funnel formation, histological observations were carried out on *Gymnosoma rotundatum* (L.) (Diptera: Tachinidae), which possesses this type of funnel. The respiratory funnel of *G. rotundatum* was found to be derived from the tube-shaped faeces wrapped with the peritrophic membrane and excreted by the fly larva, not from host tissue or haemocytes. Additionally, secretory glands putatively involved in the funnel formation were discovered around the larval anal plate of *G. rotundatum*. A comparison of funnel types within Tachinidae revealed that Phasiinae and Dexiinae have cone-type funnels, which may be created by the same mechanism as in *G. rotundatum*. These new findings suggest that funnel formation that does not use the host immune system is relevant to tachinid phylogeny.

Introduction

As respiration is one of the essential biogenic processes for organisms, various respiratory organs and strategies have evolved. Especially, endoparasitoids have developed a range of respiratory strategies for obtaining oxygen since they reside within a host body, which is sealed off from the outside and under hypoxic conditions. Respiratory strategies include not only the ability to take in oxygen but also the evasion of host immunity that interferes with breathing. Insects have an immune system that can eliminate invading foreign bodies like parasites (Lackie, 1988), and one of the cellular immune systems is encapsulation with haemocytes (Lavine and Strand, 2002). In the hymenopteran endoparasitoids, the females inject venom and polyDNA viruses to prevent encapsulation. This allows the larvae to absorb dissolved oxygen from the host body via their epidermis (Gullan and Cranston, 2014). In the dipteran endoparasitoids, most of the larvae acquire outside air by attaching their posterior spiracles to the host's tracheal system or the integumental hole (Clausen, 1940; Askew, 1971; Eggleton and Belshaw, 1993). It is widely known that most tachinid larvae use a respiratory funnel to breathe (Salt, 1968).

The role of the funnel in the respiration of tachinid flies has been recognised for a long time (Pantel, 1910; Valigurová *et al.*, 2014). Also, the wall of the funnel is believed to function as a shelter to avoid suffocation by the host's immune response because it mostly covers the larval body (Herting, 1960; Eggleton and Gaston, 1992; Belshaw, 1994; Valigurová *et al.*, 2014; Yamashita *et al.*, 2019). Previous research on funnel formation demonstrated that the funnels are composed of the host epidermis (or tracheal tissue, depending on the connecting point) and the parasitoid-caused scabs on the host tissues (Pantel, 1910; Clausen, 1940). In addition, studies have demonstrated the presence of host haematocytes within the respiratory funnels of *Exorista larvarum* and *Drino inconspicuoides*, parasitoids of lepidopteran larvae (Salt, 1968; Valigurová *et al.*, 2014; Yamashita *et al.*, 2019; fig. S1e, f). In the case of *E. larvarum*, the funnel walls consist of a multilayered structure derived from the host's haemocytes (Valigurová *et al.*, 2014). Therefore, it is thought that the formation mechanism for these respiratory funnels

involves the encapsulation reaction of the host, which is the ability of immune cells to produce a membrane to contain foreign substances in the body (Mellini, 1990; Valigurová *et al*., 2014). In all situations previously studied, the respiratory funnels are generated by host-derived material and the immunological response of the host, and it is unclear to what extent the tachinid larvae actively construct the respiratory funnels.

There are known wide variations in the shape and size of the respiratory funnels (Clausen, 1940). One type, referred to here as the sheath-type, covers the entire body of the larva (fig. S1a, b). The second type, which we refer to as the cone-type, covers just a part of the larval body (fig. S1c). While cone-type respiratory funnels are semitransparent and made of thin material (Clausen, 1940), sheath-type respiratory funnels are thickened and partly darkened by melanisation (Clausen, 1940; Mellini, 1990; Valigurová *et al*., 2014; Yamashita *et al*., 2019). These differences suggest that the developmental process for each funnel type is distinct. However, the research mentioned above regarding funnel formation and components has focused on the sheath-type funnel.

In this study, to reveal the construction process of the cone-type respiratory funnel, we established a rearing method for the tachinid fly *Gymnosoma rotundatum*. The larvae and respiratory funnels were histologically examined using confocal laser scanning microscopy and transmission electron microscopy (TEM). Additionally, we compared the respiratory funnel of tachinid flies to evaluate how widely used the cone-type is in the family Tachinidae.

Materials and methods

Establishment and maintenance of host and tachinid colonies

The tachinid fly *G. rotundatum* is a solitary endoparasitoid parasitizing adult stink bugs, primarily species in the family Pentatomidae (Tschorsnig, 2017). Eggs laid on the host's body, and upon hatching the first instar larvae penetrate into the host and feed on its internal tissues until reaching the late third instar. They subsequently exit the host's body and pupate (Higaki, 2003; Higaki and Adachi, 2011). To maintain a stable population of *G. rotundatum* within our laboratory, we initially focused on rearing its primary host, the stink bug *Plautia stali* Scott, 1874 (Hemiptera: Pentatomidae). Nymphs and adults of *P. stali* were collected from Fukuoka City, Japan (N33°35′57.94, E130°13′23.95) in 2017 and kept in plastic cages (20 cm × 30 cm, 5 cm high) containing raw peanuts, soybeans and water (0.5% ascorbic acid and 0.25% L-cysteine added). Adult bugs that were 3 days after their final moult were used to establish the fly colony.

Adults of *G. rotundatum* were collected from Fukuoka City, Japan (N33°35′57.94, E130°13′23.95) in 2017 to establish the fly colony. The females were kept individually in plastic cages (13 cm dia., 9.5 cm h.) and fed 20% aqueous honey. To parasitise the bugs, five female flies and 20–30 female adult bugs were put in cylindrical mesh bags (38 cm dia., 50 cm h.) and the flies were allowed to lay eggs on the bugs for 2–5 h. A moist tissue was connected to the top of the mesh bag as a water source. Parasitised bugs were kept until the mature third instar larvae emerged (generally within 19 days post-oviposition). The puparia of the flies were kept on damp cotton in Petri dishes until emergence. For mating, a female was placed with a male in the plastic cage same as previously mentioned. Most pairs initiated mating almost immediately. After 24 h, the males were removed and the females were transferred to a mesh bag containing bugs for oviposition. All rearing procedures were conducted at 25°C with an L16:D8 photoperiod.

Funnel observation

Parasitised stink bugs were periodically dissected in phosphate-buffered saline (PBS) to observe the process of respiratory funnel formation. This dissection process was carried out over 18 days following parasitisation, involving a total of 99 stink bugs. On average, approximately five stink bugs were dissected each day at 24 h intervals. Notably, the fly larvae exhibited significant variability in developmental rates, with first instar larvae appearing between 1 and 8 days after the oviposition, second instar larvae between 3 and 11 days, and third instar larvae between 7 and 18 days. Ultimately, this study examined 20 first instars, 44 second instars and 35 third instars with a Leica S9D stereomicroscope (Leica, Germany).

Fluorescence observation of funnel structure

Fluorescence observation with confocal microscopy was used to observe the structural characteristics of the larval respiratory funnel of *G. rotundatum*. Specifically, three larvae, each at the early and late stages of the second instar, were used for this analysis. The larvae were fixed in a 10% formaldehyde/PBS solution and kept at 4°C in PBS. Samples were stained for 24 h with 1.5% (wt/vol) Congo-red in PBS. The samples were then transferred to distilled water, left for 20 min, then rinsed many times with PBS until the solute component of Congo-red was no longer visible. The specimens were afterward placed on slides and immersed in glycerine. Observations were conducted utilizing a confocal laser scanning microscope FV3000 (Olympus, Tokyo, Japan) with an excitation wavelength of 561 nm and an ×10 or ×20 Apochromat objective.

X-ray micro-CT scanning

A host bug parasitised by *G. rotundatum* was observed without dissection using an X-ray micro-CT scanning system (SKYSCAN1172, Bruker, Germany) to visualise the fly larva inside the host. A stink bug, which had been parasitised for 14 days, was selected for this analysis. The stink bug was dehydrated and fixed in 50, 70, 90, 95 (wt/vol) and 100% ethanol for 4–6 h each and then stained for 24 h with 3% (wt/vol) iodine (KI/I2 at a ratio of 2:1) in 100% ethanol. After staining, the specimen was fixed in hexamethyldisilazane (HMDS) for 24 h, replaced with fresh HMDS and air-dried. Subsequently, 3600 X-ray projections were digitised for the specimen at 0.1° intervals across 180° using 80 kV, 50 W and a 2000 ms exposure period. The projections were taken at an angle of 180° with an exposure duration of 2000 ms. 3.8 m x–y–z voxels were produced using a modified Feldkamp filtered back-projection algorithm. The micro-CT data were processed using 3D approaches using CTAnalyzer (version 1.13, Bruker), and the colouring was finished using Adobe Photoshop (CS6).

Paraffin sectioning

Paraffin-embedded sections of second instar larvae were prepared to investigate the internal morphology of the larvae and their respiratory funnels. Parasitised *P. stali* were dissected to obtain

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Table 1. Comparison of the respiratory funnels and ecological features in Tachinidae

Subfamily	Species	Funnel type	Funnel colour	Funnel connecting point	Order of host	Host species[a]	Collection site and year[a]	References
Phasiinae	*Gymnosoma rotundatum*	Corn	White with purple margins	Thoracic trachea	Hemiptera	*Plautia stali* (Pentatomidae)	Reared in the laboratory, 2017	fig. 1
	Gymnosoma inornatum	Corn	Semi-transparent white	Thoracic trachea	Hemiptera	*Eysarcoris ventralis* (Pentatomidae)	Itoshima City, Fukuoka Pref., 2019	fig. 5
	Gymnosoma philippinense	Corn	Semi-transparent khaki	Thoracic trachea	Hemiptera	*Nezara viridula* (Pentatomidae)	Makurazaki City, Miyazaki Pref., 2017	fig. 5
	Catharosia sp.	Corn	–	Thoracic trachea	Hemiptera	*Ligyrocoris diffusus* (Rhyparochromidae)	–	Thorpe and Harrington (1979)
	Clairvilliops breviforceps	Corn	Yellowish green	Thoracic trachea	Hemiptera	*Leptocorisa chinensis* (Alydidae)	Fukuoka City, Fukuoka Pref., 2019	fig. 5
	Clairvilliops sp.	Corn	Semi-transparent white	Thoracic trachea	Hemiptera	*Cletus punctiger* (Coreidae)	Itoshima City, Fukuoka Pref., 2019	fig. 5
	Ectophasia rotundiventris	Corn	Brown with purple margins	Thoracic trachea	Hemiptera	*Plautia stali* (Pentatomidae)	Soeda Town, Fukuoka Pref., 2020	fig. 5
Dexiinae	*Euthera* sp.	Corn	Semi-transparent white	Thoracic or abdominal trachea	Hemiptera	*Cantao ocellatus* (Scutelleridae)	Amami Oshima, Kagoshima Pref., 2020	fig. 5
	Prosena siberita	Corn	–	Trachea	Coleoptera	*Popillia japonica* (Scarabaeidae)	–	Clausen *et al.* (1927)
Exoristinae	*Drino inconspicuoides*	Sheath	Yellowish white with dark brown bases	Epidermis	Lepidoptera	*Mythimna separata* (Noctuidae)	–	Yamashita *et al.* (2019)
	Exorista japonica	Sheath	Yellowish white with dark brown bases	Epidermis	Lepidoptera	*Mythimna separata* (Noctuidae)	Reared in the laboratory, 2019	fig. S1
	Exorista larvarum	Sheath	Yellowish white with dark brown bases	Epidermis	Lepidoptera	*Galleria mellonella* (Pyralidae)	–	Valigurová et al. (2014)
	Pales pavida	Sheath	–	Epidermis	Lepidoptera	*Euproctis chrysorrhoea* (Erebidae)	–	Herting (1960)
	Panzeria rudis	Sheath	Yellow, light-dark brown	Epidermis	Lepidoptera	*Panolis flammea* (Noctuidae)	–	Prell (1915)
	Parasetigena silvestris (*as P. segregata*)	Sheath	Yellow, light-dark brown	Epidermis	Lepidoptera	*Lymantria dispar* (Erebidae)	–	Prell (1915)
Tachininae	*Ormia brevicornis*	Sheath	–	Abdominal epidermis	Orthoptera	–	–	Nutting (1953)
	Tachina nupta	Sheath	Yellowish white with dark brown bases	Epidermis	Lepidoptera	*Mythimna separata* (Noctuidae)	Reared in the laboratory, 2019	fig. S1

[a]Information on the specimens utilised in the study.

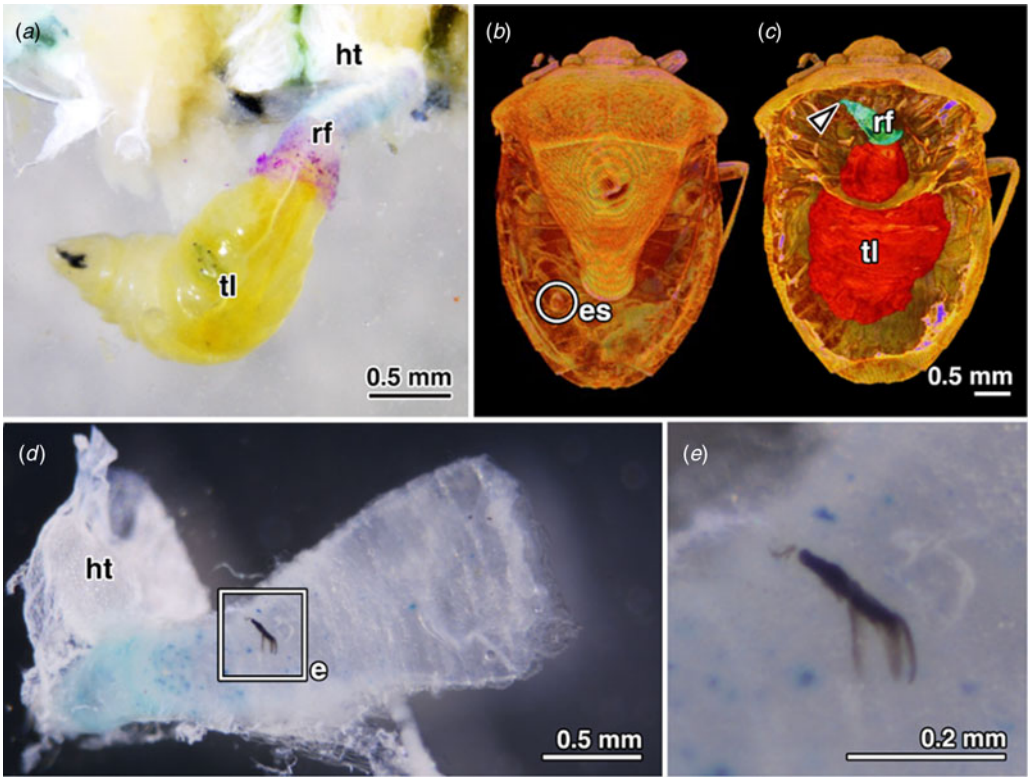

Figure 1. Larva and respiratory funnel of *G. rotundatum*. (a) Second instar larva with the respiratory funnel. The respiratory funnel covers only the caudal part of the larval body. (b, c) X-ray tomography of the third instar of *G. rotundatum* (red) with respiratory funnel (green) within the adult *Plautia stali*. The egg was laid on the host abdominal tergite, and the respiratory funnel was formed at the thorax. (d) The respiratory funnel of the second instar. It contains the exvium of the first instar larva with the cephaloskeleton (e). The white arrowhead indicates the connecting position of the respiratory funnel and host trachea. Abbreviations: es, eggshell; ht, host trachea; rf, respiratory funnel; tl, tachinid larva.

the fly larvae and respiratory funnels in the PBS buffer 2–14 days after oviposition. Subsequently, six fly larvae from the first to third instar were selected for this analysis. Specimens were fixed in FAA fixative (formalin-acetic acid-alcohol fixative, formalin: acetic acid: ethanol = 6:1:16) and dehydrated in 70, 90, 95 (wt/vol) and 100% ethanol for 1–6 h each. Specimens were then infiltrated with pure xylene and embedded in paraplast (Sigma-Aldrich, St. Louis, MO, USA). Sections (5 µm thick) were made using a microtome. These sections were placed on microscope slides and immersed in pure xylene for deparaffinisation, then hydrated in 100, 95, 70% (wt/vol) ethanol and finally in tap water for 5 min each. Tissues were stained with haematoxylin and eosin. After staining, sections were washed in tap water and then dehydrated in 70, 95, 100% (wt/vol) ethanol, and pure xylene for 5 min each. Tissues were embedded in Canada balsam (50% pure xylene added) and observed under a light microscope (BX51; Olympus).

Transmission electron microscopy (TEM)

TEM imaging to investigate the internal ultrastructure of epidermal tissue of the *G. rotundatum* larvae was contracted to Hanaichi UltraStructure Research Institute, Co., Ltd. (Aichi, Japan). This analysis examined two second instar larvae, with their funnels removed prior to fixation. The larvae were fixed in phosphate-buffered 2% glutaraldehyde and then post-fixed in 2% osmium tetra-oxide in an ice bath for 3 h. Specimens were then dehydrated in graded ethanol and encapsulated in epoxy resin. Ultramicrotome-obtained ultrathin sections were stained with uranyl acetate for 10 min and lead staining solution for 5 min prior to TEM observation (H-7600; HITACHI, Tokyo, Japan).

Taxonomic comparison of respiratory funnels among tachinid flies

Four subfamilies are recognised in the family Tachinidae: Phasiinae, Dexiinae, Exoristinae and Tachininae (Herting, 1984; O'Hara and Wood, 2004; Cerretti *et al*., 2014). In addition to *G. rotundatum* (a member of the Phasiinae), the following eight species representing the four subfamilies were available for breeding and study in our laboratory: *Clairvilliops breviforceps* (van Emden), *Clairvilliops* sp., *Ectophasia rotundiventris* (Loew), *Gymnosoma inornatum* Zimin and *G. philippinense* (Townsend) (all Phasiinae); *Euthera* sp. (Dexiinae); *Exorista japonica* (Townsend) (Exoristinae); and *Tachina nupta* (Rondani) (Tachininae). These were examined for morphological comparison of their respiratory funnels. Specimens were obtained both by rearing wild-caught hosts and from lab-bred parasitoid colonies. Each host and collection information are shown in table 1. Before tachinid larval egression, host insects were kept in plastic cages or mesh bags. All rearing procedures were undertaken at 25°C with L16:D8 photoperiod. After the egression of the tachinid larvae, the host bodies were dissected in PBS buffer to examine the structure of the respiratory funnels remaining within them using a Leica S9D stereomicroscope (Leica, Germany) equipped with a single-lens reflex camera D5200 (Nikon, Japan).

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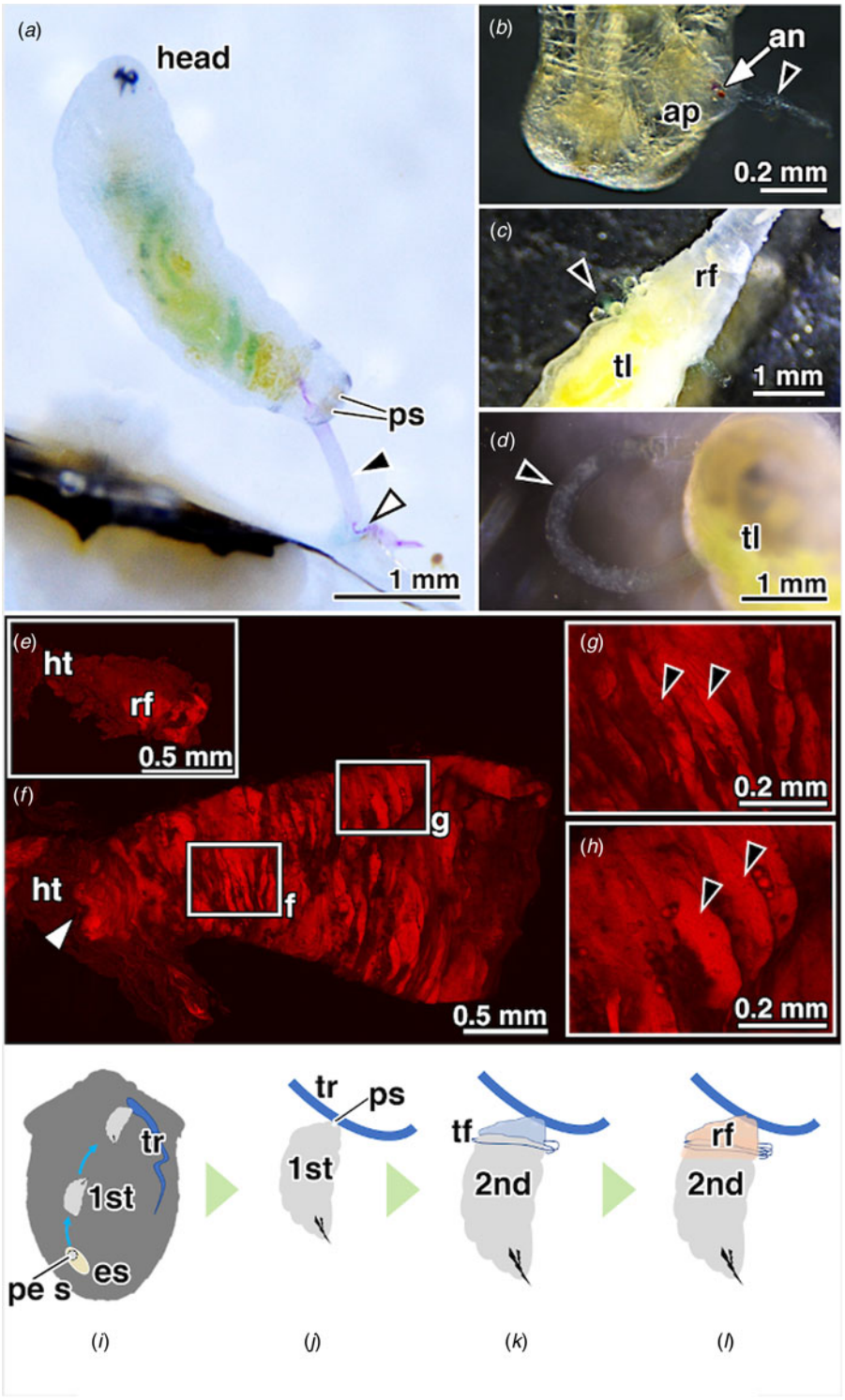

Figure 2. Structure and formation process of the respiratory funnel of *G. rotundatum*. (a–d) Second instar larvae with the tubular faeces. (e–h) Confocal laser scanning microscope images of the respiratory funnels of second instar larvae. (i–l) The respiratory funnel formation scenario. The second instar larva excretes tubular faeces, and its end is connected with the host tissue (a). Tubular faeces is continuously excreted from the anus (b) and wrap around the caudal part of the larva (c). Faeces before becoming part of the respiratory funnel (d). During the initial forming stage, the respiratory funnel is made mainly by torn host tracheal tissue (e), and the subsequently formed part consists of tubular faeces (f). The tubes in the wall of the respiratory funnel become wider as it approaches the rim (g, h). The respiratory funnel formation process is presumed as follows: upon hatching, the larva penetrates the host body from behind the egg and migrates to the prothorax of the host (i). The first instar larva connects its posterior spiracles to the host trachea (j). After ecdysis, the second instar larva excretes the tubular faeces, wraps it around the body (k) and secretes mucus from the glands surrounding the anal lobe to hardening excrement (l). White arrowheads indicate the connecting point of the respiratory funnel/tubular faeces and host tracheae. Black arrowheads indicate tubular faeces. Ordinal numbers indicate the larval instars. Abbreviations: an, anus; ap, anal plate; es, eggshell; ht, host trachea; pe s, penetration site; ps, posterior spiracle; rf, respiratory funnel; tf, tubular faeces; tl, tachinid larva.

Results

Formation and structure of the respiratory funnel of G. rotundatum *larva*

Funnel formation and structure was elucidated in *P. stali* through dissection of parasitised individuals at different times during the larval development of *G. rotundatum*. Eggs were laid on the abdominal tergite of the host. The first instar larvae hatched by piercing a hole in the ventral side of the eggshell along with the host exoskeleton, and then immediately invaded the host body. Once inside the host, these first instars stayed in the hemocoel and moved towards the thoracic trachea. None were observed to form a respiratory funnel. Funnel formation began in the early second instar, and the cone-type respiratory funnel composed of semitransparent, partially purple material was formed by the middle of the second instar (fig. 1a). The respiratory funnel kept growing as the larvae matured, but did not cover their entire bodies; only the caudal portion was covered.

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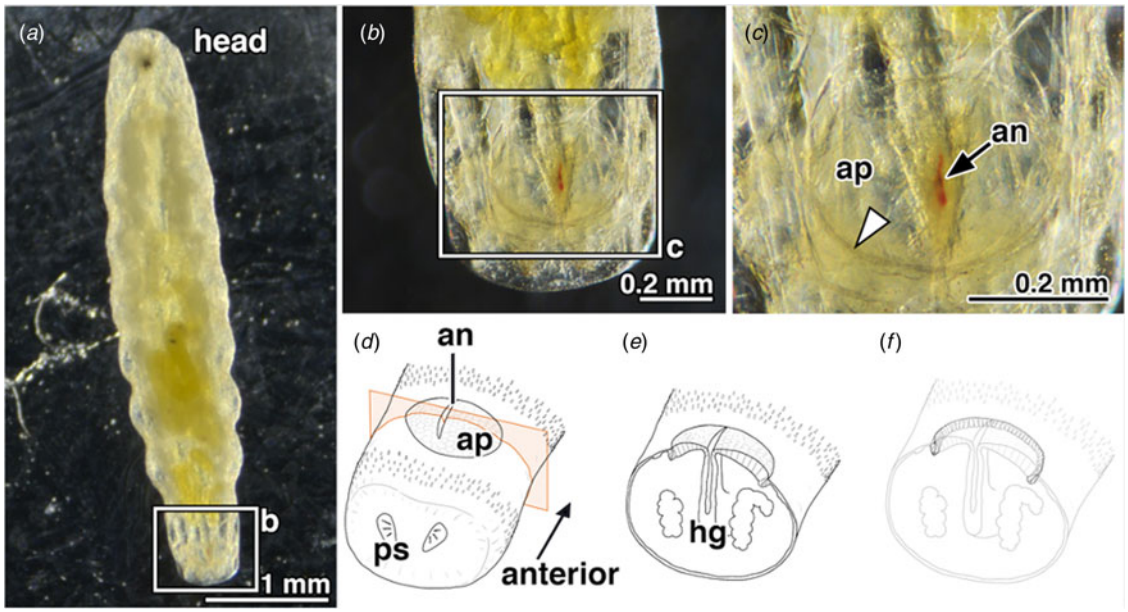

Figure 3. Structure of the anal plate in the second instar larva of *G. rotundatum*. (a–c) Whole body and its caudal part in ventral view. (d–f) Diagram of the anal plate. The anal plate of *G. rotundatum* is larger than that of the other species examined (b; see also fig. S2). The anal plate surrounds the anus, bordered by a slit-shaped opening (c, d). The anal plate comprises thick epidermal cells (e) and is surrounded by a secretory gland-like structure (f). White arrowhead indicates the slit-shaped opening. Abbreviations: an, anus; ap, anal plate; hg, hindgut; ps, posterior spiracle.

Regardless of the penetration point of the first instars, the funnels were attached to the host's trachea in the metathorax without exception (fig. 1b, c).

Funnel formation started during the early second instar, with the larva attaching its body to host tissue with tubular faeces wrapped in the peritrophic membrane (fig. 2a) that originated in the midgut of the tachinid larva. The tubular faeces were then rolled around the larva, becoming fused with, and part of the respiratory funnel (fig. 2b–d). Notably, we observed the presence of host tracheal tissue and larval exuvium within the initially formed basal part of the respiratory funnel (figs 1d, e, 2e). In essence, the funnel was constructed by wrapping faeces around the host's tissues and larval exuvium of the first moult. Therefore, the funnel wall was created by stacking a number of thin tube-like structures and did not contain cellular structure (figs 2f, S1d), in contrast to the sheath-type respiratory funnels which are made by host immune cells and contain a large number of cells (fig. S1e, f, Valigurová *et al.*, 2014). This tube-shaped material forming the respiratory funnel wall became wider as it approached the respiratory funnel rim (fig. 2f–h), indicating the continuous formation of the funnel as new excreta built up along the funnel's edges as the larva grew.

Histological structures of larval anal plates

The respiratory funnels were observed to be continuously formed from faeces that were shaped into a tube of very robust construction. The mechanism producing such robust, tubular faeces was determined by examination of the anal plate of the second instar *G. rotundatum*. This was found to be much more developed than that of larvae with sheath-type funnels (figs 3, S2). Considering the potential significance of this plate in the production of tubular faeces, we analysed the internal structure of the anal plate through paraffin sectioning and TEM. The anal plate of *G. rotundatum* consisted of hexagonal epidermal cells thicker at the edges (fig. 4a, b). A narrow outward opening slit was observed at the lobe edge (figs 3c, 4c–h). The slit exhibited a folded structure as it penetrated farther into the epidermal cell layer (fig. 4g), and the cells around the slit had a larger nucleus (fig. 4c). Around the slit, the cells contained rough-surfaced endoplasmic reticulum with numerous ribosomes and several secretory vesicles (fig. 4g). Therefore, these cells and slits appear to be structures for synthesizing and secreting a substance, suggesting that these larvae produce secretions to stiffen faeces and construct funnels. The tubular faeces are highly resilient and did not break apart readily when stretched.

Comparison of the respiratory funnel in Tachinidae

The respiratory funnels of the seven observed species of Phasiinae and Dexiinae (three *Gymnosoma*, two *Clairvilliops*, one *Ectophasia* and one *Euthera* species) were of the cone type and covered only the caudal end of the larvae, and all were connected to the host tracheae. The cone-type funnel was consistently formed in the host thorax in almost all species examined, except in *Euthera* sp. where the location of the funnel connection varied between individuals and could be found in either the thorax or abdomen (fig. S3a–c). All examined cone-type respiratory funnels were semitransparent, but colouration varied among species as follows: almost colourless in *G. inornatum* and *Euthera* sp.; entirely pale brown in *Clairvilliops* sp. and *G. philippinense*; brown and partially green in *C. breviforceps*; almost colourless with purple margin in *G. rotundatum*; and almost brown with purple margin in *E. rotundiventris* (fig. 5). In contrast to these, in *Exorista japonica* and *Tachina nupta* the respiratory funnels were of the sheath type consisting of a thick milky-white, partially blackish-brown wall covering the entire body of the larvae (figs S1b, c). These funnels were attached to the epidermis of the host.

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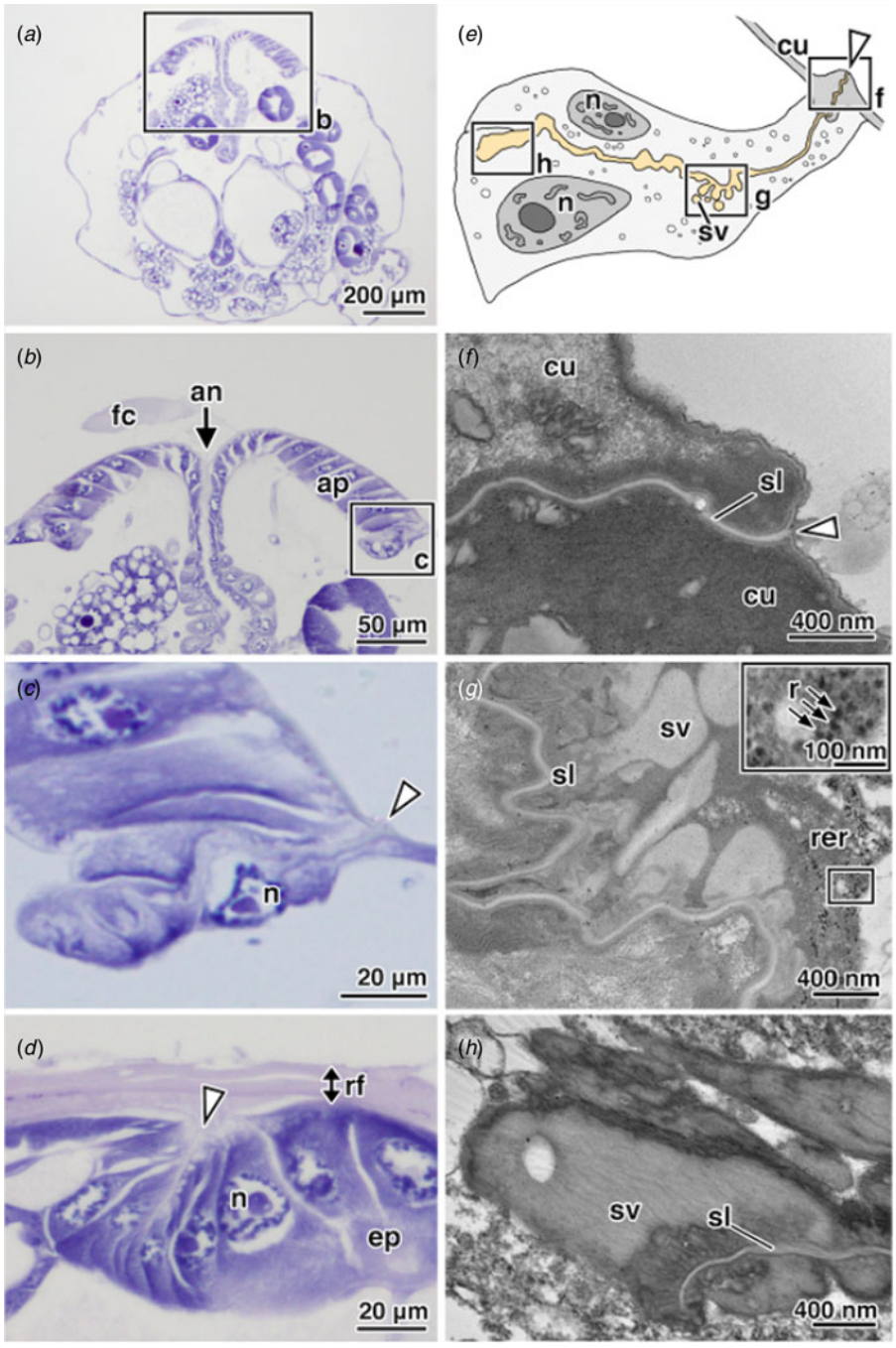

Figure 4. Anal plate and gland-like structures in the second instar larva of *G. rotundatum*. Inner structures of the anal plate in cross-section (a–c) and longitudinal section (d). Diagram and ultrastructures in a gland-like organ adjacent to the anal plate (e–h). Thickened epidermal cells of the anal plate line both sides of the anus (a, b), with slit-shaped openings (f) and gland-like organs located at the outermost edge of the plate (c, d). The cells compose gland-like organs containing secretory vesicles adjacent to the slit and rough-surfaced endoplasmic reticulums accompanied by numerous ribosomes (g, h). White arrowheads indicate the opening sites. Abbreviations: an, anus; ap, anal plate; cu, cuticle; ep, epidermal cell; fc, faeces; n, nucleus; r, ribosome; rer, rough-surfaced endoplasmic reticulum; rf, respiratory funnel; sl, slit; sv, secretory vesicle.

Discussion

The respiratory funnels of Tachinidae have previously been interpreted as sheath-type structures formed during the immune response of the host to a foreign invader (Salt, 1968; Valigurová *et al.*, 2014; Yamashita *et al.*, 2019). However, our study focusing on the cone-type funnel has revealed that it is actively constructed by tachinid larvae through a mechanism independent of the host's immune response. Our histological investigation of the cone-type respiratory funnel of *G. rotundatum* suggests that the funnel is composed of tubular faeces mixed with the peritrophic membrane and formed independently of the host immune system (fig. 2i–l). This is notably distinct from the sheath-type funnel produced by the host during an encapsulation process induced by the host immune system (Valigurová *et al.*, 2014; Yamashita *et al.*, 2019). As noted in previous studies (Pantel, 1910; Clausen, 1940), even in the case of a cone-type, the respiratory funnel in its early stages seems to be composed of injured tracheal tissue from the host (fig. 2e). Its wall also contains larval exuvium, which may contribute to the structural reinforcement (fig. 1d–e). Furthermore, since encapsulation and melanisation by the host immunological response do not occur during the funnel formation, *G. rotundatum* must independently conduct structural stabilisation of the funnel. We found that the secretory gland around the anal plate may create a secretion that holds tubular faeces together to preserve the solid structure of the funnel (figs 3d–f, 4). The anal plate is a well-known organ in dipteran larvae (Teskey, 1981)

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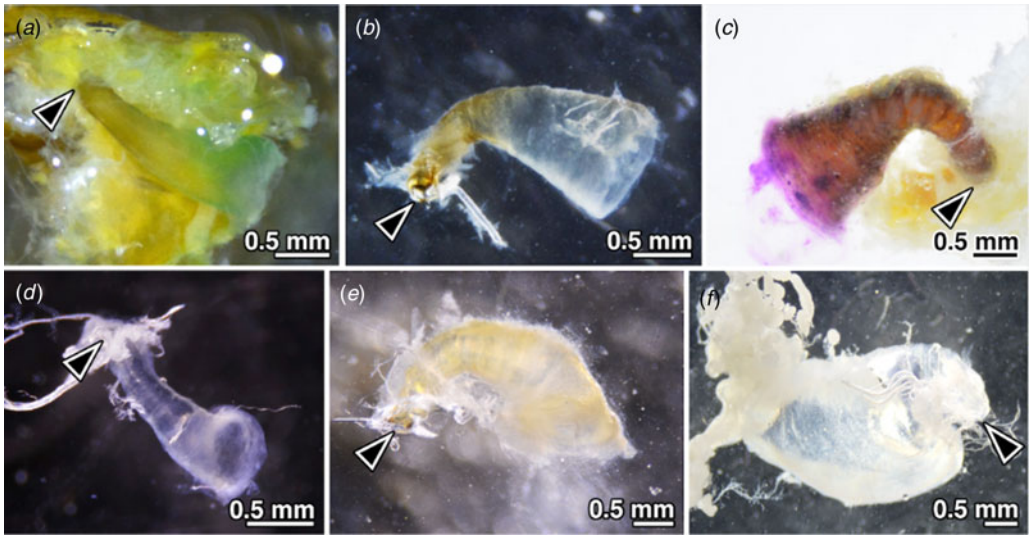

Figure 5. Morphological comparison of the respiratory funnels among Phasiinae (a–e) and Dexiinae (f). (a) *Clairvilliops breviforceps*; (b) *Clairvilliops* sp.; (c) *Ectophasia rotundiventris*; (d) *Gymnosoma inornatum*; (e) *G. philippinense* (f) *Euthera* sp. Arrowheads indicate connecting sites of host tracheae and respiratory funnels.

and is thought to be responsible for the release of saline to control its concentration in the body when living in water or comparable situations (e.g. decaying fruit or animal corpses) (Schwantes and Seibold, 1991; Durham and Grodowitz, 2012). The secretory gland found in *G. rotundatum* is the only example in the Diptera, suggesting that this organ is an evolutionary novelty for funnel formation. Alternatively, the initial saltwater drainage mechanism by the anal plate may have been co-opted for acquiring a secretory organ for funnel development.

All examined cone-type funnels were semitransparent and layered structures with clearly constant shapes (fig. 5), in contrast to the sheath-type with melanised opaque and irregular shapes. The colourations of cone-type funnels varied among species (fig. 5), which may indicate that the faeces from which the funnels were derived contained pigments from the host tissue ingested by the tachinid larvae. For instance, the respiratory funnel of *G. rotundatum* was primarily white but sometimes exhibited green or reddish-purple hues (fig. 1a, d). This is compatible with the absence of colour inside the host's body cavity, the reddish-purple dorsal epidermis and the green ventral epidermis. The respiratory funnel of *C. breviforceps* was brownish or green (fig. 5a), the same colour as the tissues of its host *Leptocorisa chinensis*. While the sheath-type funnel has been shown to be formed by the immune response of the host rather than by the parasitoid (Valigurová *et al.*, 2014; Yamashita *et al.*, 2019), there had not been clear evidence that the cone-type funnel was formed by the tachinid larva itself. The morphological stability, colour and position of formation, as well as the materials of the cone-type funnel revealed in this study, strongly support that this funnel is formed by the activity of the tachinid larva, suggesting that it is fundamentally different from the sheath-type funnel.

Tachinid larvae with these two different types of funnels may exhibit variation in the developmental stage at which the respiratory funnel plays the role of a breathing tube or 'snorkel'. The sheath-type funnel is often abandoned in the late stages of larval development, as the larva begins to respire without the funnel (Valigurová *et al.*, 2014). However, the cone-type funnel is used until just before larval emergence, at least in *G. rotundatum* and *Euthera* sp. examined here (fig. 1c). Therefore, there appears to be a significant difference in behaviour and respiration between the larvae with these two types of funnels, at least in the late stages of larval development.

In addition to its role as a 'snorkel' for respiration, the respiratory funnel has been hypothesised to serve as a 'shelter' to help tachinid larvae avoid attack from the host's immune system. By encasing the larval body with materials supplied by the host, the sheath-type funnel avoids subsequent immune reactions (Eggleton and Gaston, 1992; Belshaw, 1994; Valigurová *et al.*, 2014; Yamashita *et al.*, 2019). However, neither host immune cells nor encapsulating membranes were identified in the cone-type funnels of *G. rotundatum* (fig. S1f), nor did the funnel cover the whole body (fig. 1a). It is improbable that such a funnel provides a sufficient barrier to the host immune response. Features of the cone-type funnel detailed in this study suggest that *G. rotundatum* may have undiscovered mechanisms to suppress the immune response other than the respiratory funnel. In *D. inconspicuoides*, it has been suggested that melanisation of the encapsulation membrane is suppressed by substances derived from the larvae (Schwier *et al.*, 2021). However, in the species observed in this study, it appears that the formation of the encapsulation membrane itself is being suppressed, or evaded. It is crucial to elucidate host specificity through parasitisation experiments, and to investigate how host immunity is circumvented through an immunological approach.

The phylogenetic relationships among the four subfamilies of Tachinidae have been proposed by molecular analyses, with Exoristinae and Tachininae (for the most part), and Phasiinae and Dexiinae, being sister groups, respectively (Stireman *et al.*, 2019). However, there is little clear evidence other than genetic information to support these results, as many morphological and ecological characteristics have been acquired and lost multiple times (Stireman *et al.*, 2019). In this study, the cone-type funnel was found in Phasiinae and the single Dexiinae, whereas the sheath-type was in Exoristinae and Tachininae, suggesting that the distribution of funnel types within this family might reflect the phylogenetic relationships among the subfamilies. On the other hand, it is suggested that there is no correspondence between funnel type and ecological characteristics such as host

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(table 1). *Prosena siberita*, a dexiine species known to parasitise coleopteran larvae, has been reported to have a cone-type funnel covering only the caudal end of the abdomen (Clausen *et al.*, 1927), in addition to phasiine and dexiine species that parasitise adult stink bugs. In Diptera, the endoparasitoid habits are found in many families such as Bombylidae, Calliphoridae, Nemestrinidae, Phoridae, Rhinophoridae, Sarcophagidae and Tachinidae (Clausen, 1940; Feener and Brown, 1997). Among these, the larvae of Rhinophoridae and Nemestrinidae have been reported to possess tubular structures that are functionally and morphologically similar to the respiratory funnels of Tachinidae (Thompson, 1920, 1934; Prescott, 1961; Wood, 1987). Further studies should investigate the larval respiratory methods in different endoparasitoid groups.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485324000221

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