CONTRIBUTIONS TO ZOOLOGY 92 (2023) 349–361

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High degree of independence in the feeding apparatus of planarian flatworms

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RECEIVED: 23 MAY 2021; REVISED AND ACCEPTED: 25 APRIL 2023; PUBLISHED ONLINE 6 JUNE 2023; PUBLISHED IN ISSUE 1 AUGUST 2023
EDITOR: A. MINELLI

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Published with license by Koninklijke Brill NV | DOI: 10.1163/18759866-BJA10045
 | ISSN: 1383-4517 (print) 1875-9866 (online)

Abstract

In this contribution, feeding behaviour assays with the three species *Paucumara falcata*, *Dugesia* sp. and *Girardia* sp. were used to investigate the function of the pharynx during feeding and whether absence of feeding behaviour until full regeneration is a widespread phenomenon among planarians from different taxonomic groups. Our results showed that feeding behaviour of decapitated flatworms was inhibited. Intact worms responded only to pork liver pieces, but isolated pharynges were highly responsive to both pork liver pieces and pork liver extracts. After transverse cutting, the oral part of the isolated pharynx was responsive, while the aboral part showed no response to food items, suggesting that the oral portion of pharynx plays a crucial role during feeding.

Keywords

feeding behaviour – isolated pharynx – Platyhelminthes – Tricladida

Introduction

Freshwater planarians (Platyhelminthes, Tricladida) are predators that feed on a variety of invertebrate prey species, such as annelids, molluscs, crustaceans, and insect larvae (Jennings, 1962; Vila-Farré & Rink, 2018, and references therein). The feeding process includes chemotaxis, pharyngeal extension and, subsequently, ingestion of the food by the pharynx (Shimoyama et al., 2016). Feeding and movement of these worms have been used as marker behaviour in physiological and ecotoxicological studies on planarians, since such behaviour can be quantified more easily and objectively than, for example, reproduction or growth (Hellou, 2011; Rodrigues et al., 2016).

The pharynx of freshwater and marine planarians, as well as that of many terrestrial planarians, is a cylindrical muscular tube that during feeding is protruded from an opening in the ventral body wall that is generally called the mouth. The muscular pharynx penetrates the body of the prey and ingests fluids and small pieces of tissues by peristaltic muscular action (Sluys, 1989). Surprisingly, pharynges isolated from the body are able to perform this function without having any connection with the central nervous system, the pharyngeal nerve plexus apparently being a self-sufficient system (Miyamoto et al., 2020). This independent feeding action of isolated pharynges was observed already by the great naturalist Von Baer (1827), although his observations were generally not acknowledged by later workers (apart from Von Graff, 1912–17; Koehler, 1932; Reisinger, 1976) who observed the same phenomenon (Wulzen, 1917; Kepner & Rich, 1918; Viaud, 1954; Miyamoto et al., 2020). Only a few years after Von Baer's publication, Charles Darwin in 1832 observed in land planarians that "… after the rest of the animal was completely dead … this organ [the pharynx] still retained its vitality" (Darwin 1983, p. 25; see also Darwin, 1844). Another early observer was Leidy (1847), who noted that when one of the pharynges of the polypharyngeal species *Phagocata gracilis* (Haldeman, 1840) became isolated from the body, the organ gave the impression of being a live young worm. Such isolated pharynges may be obtained after physical amputation (under natural conditions through autoamputation, or during

laboratory experiments through surgical ablation) or chemical amputation (Viaud, 1949; Shiroor et al., 2018). This apparent individuality of the pharynx corresponds with the fact that it is the only organ in the planarian body incapable of regenerating an entire new animal, as it lacks neoblasts (Baguñà, 1976; Saló & Baguñà, 1989; Adler et al., 2014, and references therein).

Behavioural assays on their feeding and movements have been used to analyse the function of the nervous system involved in chemotaxis, thermotaxis, and thigmotaxis of planarians (Inoue et al., 2015). Previous studies showed that the feeding behaviour of decapitated planarians, such as *Dugesia japonica* Ichikawa & Kawakatsu, 1964 (Inoue et al., 2015; Shimoyama et al., 2016) and *Girardia tigrina* (Girard, 1850) (Bardeen, 1901; Sheiman et al., 2002) was inhibited, whereas the feeding of tail-amputated planarians was not affected (Shimoyama et al., 2016). The brain was considered to be a necessary requirement in the control and regulation of pharynx extension, while the pharynx may also send signals to the brain for its positioning and food localization (Inoue et al., 2015; Shimoyama et al., 2016; Miyamoto et al., 2020).

Regeneration studies revealed that after amputation of the distal part of the pharynx or removal of the entire pharynx, the worms exhibited no feeding behaviour until full regeneration (Ito et al., 2001; Sheiman et al., 2002). However, these conclusions were drawn from only three freshwater species of the family Dugesiidae, namely *D. japonica* (Ito et al., 2001), *G. tigrina* (Sheiman et al., 2002) and *Schmidtea mediterranea* (Benazzi, Baguñà, Ballester & del Papa, 1975) (Adler et al., 2014; Shiroor et al., 2018), via different experimental settings. Therefore, it is still unclear whether this is a widespread phenomenon among planarians from different taxonomic groups and different habitats, such as freshwater, brackish, and marine environments.

Therefore, our study aimed to examine whether the feeding behaviour of both the amputated worms and the isolated pharynx is conserved among planarian species dwelling in different habitats and belonging to different taxonomic groups. From that perspective, we examined the marine triclad *Paucumara falcata* Wang & Li, 2019 (Suborder Maricola Hallez, 1892) and the freshwater planarians *Dugesia* sp. and *Girardia* sp. (Suborder Continenticola Carranza, Littlewood, Clough, Ruiz-Trillo, Baguñà & Riutort, 1998) by studying the effect of amputation of their heads or tails on their feeding behaviour. Furthermore, as it has been suggested that a two-fold chemotropic response takes place during feeding, one being the movement of the worm towards the food item and the other formed by extension and movement of the pharynx (Wulzen, 1917), we have analysed also the feeding reaction of isolated pharynges.

Materials and methods

Collection and culturing

Specimens of *Dugesia* sp. were provided by Professor Wu from the Protein Science Laboratory of the Ministry of Education, Tsinghua University. Specimens of *Girardia* sp. were collected from a ditch at the outlet of the Changlingpi reservoir, Shenzhen, Guangdong Province, China (22°37'0″ N, 114°0'46″ E) (World Geodetic System 1984) and have been cultured in our laboratory for years. *Paucumara falcata* specimens were collected from a beach at Shenzhen, Guangdong Province, China (22°28'11" N, 114°32'12" E) (WGS84). All specimens were cultured at 26–27°C in 500 mL glass bowls and were fed with slices of pork liver for 3 h every two days.

Paucumara falcata was cultured in sea water with a salinity of 19‰, while *Dugesia* sp. and *Girardia* sp. were cultured in freshwater.

Feeding behaviour assay

Heads or tails of the worms were amputated using a surgical blade (fig. 1A, B). Isolated pharynges were obtained by cutting the anterior and posterior ends of the pharyngeal pocket with a surgical blade (fig. 1C). Subsequently, the pharynx was transferred to a glass slide, with a small amount of water, in order to prevent desiccation. In order to examine the feeding response of different portions of the isolated pharynges, they were divided into an oral part and a basal or aboral part by cutting through the middle of the organ (fig. 1D). Liver extracts were prepared by removing the connective tissue of the liver and then homogenizing it in water (0.1 g/mL) with a sonicator (Scientz-IID, Zhejiang, China). The homogenized samples were centrifuged at 6,000 g, and 4°C for 5 min. The supernatants were filtered using membranes with a pore size of 0.22 μm, while the filtrates were collected as extracts of the liver.

The following four experiments were performed: (1) to investigate which portion of the body is important for the feeding function, intact worms (fig. 2A, B, C) and amputated worms were kept in Petri dishes (each dish containing 10 individuals) and fed with pork

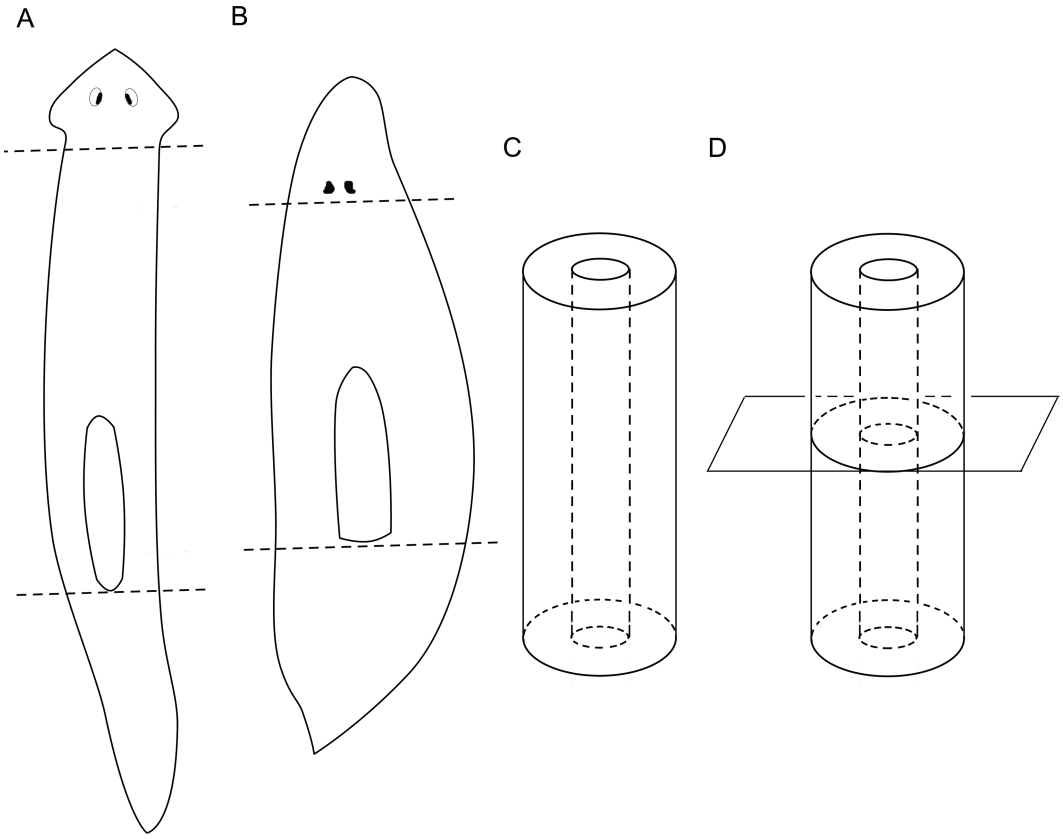

FIGURE 1 Schematic diagram of specimen preparation. A–B: amputation of *Dugesia* sp., *Girardia* sp. (A) and *Paucumara falcata* (B) (dashed line indicates level of transection); C: isolated pharynx; D: transection of isolated pharynx (upper part originally connected to the intestine and thus forming the aboral end, while the opposite portion concerns the oral end).

liver (0.01 g) for two hours per day. The liver was weighed before and after feeding and the food uptake was scored as percentage of liver weight consumption per day. In this experiment, normal, intact worms were used as the control group; (2) to determine whether the intact worms and isolated pharynges (fig. 2D, E, F) respond differently to solid food and water-soluble substances from the corresponding food, intact worms and isolated pharynges were fed with pieces of pork liver or incubated in pork liver extracts. Percentages of individual worms and pharynges that showed feeding response were scored during a period of two hours immediately after the food source had been dispensed. Feeding response of intact animals was defined in three steps: (a) movement toward the food, (b) extension of the pharynx, (c) ingestion of food by the pharynx. Response of isolated pharynges was defined as extension of the pharynx and ingestion of food; (3) to determine the efficacy of liver extracts, in terms of feeding behavioural response induction, isolated pharynges were incubated with a series of diluted pork liver extracts (0.1%, 1%, 10% series dilution of liver extracts). Isolated pharynges in freshwater (*Dugesia* sp. and *Girardia* sp.) or in sea water with a salinity of 19‰ (*P. falcata*) were used as the control groups. Percentages of pharynges with an ingestion response to the extracts were documented within two hours directly after the food source had been dispensed; (4) to determine which portion of the isolated pharynx is crucial for the feeding function, intact pharynx, aboral part, and oral part of the pharynx were fed with pieces of pork liver. In this experiment, intact pharynges were used as the control groups. Percentages of pharynges, or parts thereof, with ingestion responses to food were documented within two hours. Each of these four experiments was replicated six times.

Statistical analysis

Statistical analysis was carried out using the nonparametric Mann-Whitney U test for experiment #1, and experiment #4 (no feeding response in the aboral part of the pharynx, therefore, only the feeding responses of isolated intact pharynges and oral portions were statistically tested). Kruskal-Wallis test was applied for experiment #3. For experiment #2, no statistical test was required, since the response of the animals was 100% versus almost no response in the controls. A *P*-value < 0.05 was considered statistically significant in all tests. For data presentation, box-and-whiskers plots were constructed in R 4.2.2 (R Core Team, 2022) with ggplot2 (Wickham, 2016).

Results

Recovery of feeding after amputation of head or tail

In all three of the examined species, during the first and second days of the experiments, feeding of decapitated worms was significantly inhibited in comparison with the control groups. Food uptake behaviour restarted from day-3 post-amputation of the head, although in *Girardia* sp. and *P. falcata* food uptake in the decapitated group was still distinctly lower than that of the control group, i.e., intact worms (fig. 3). Although in the following days there were also significant differences between the decapitated animals and the control groups, the percentage of food uptake in the decapitated group recovered to a level similar (no statistically significant differences) to the control group at day-6, day-7, and day-8 post-amputation in *P. falcata*, *Girardia* sp. and *Dugesia* sp., respectively (fig. 3).

In the tail-removing experiments, only *Dugesia* sp. displayed feeding inhibition

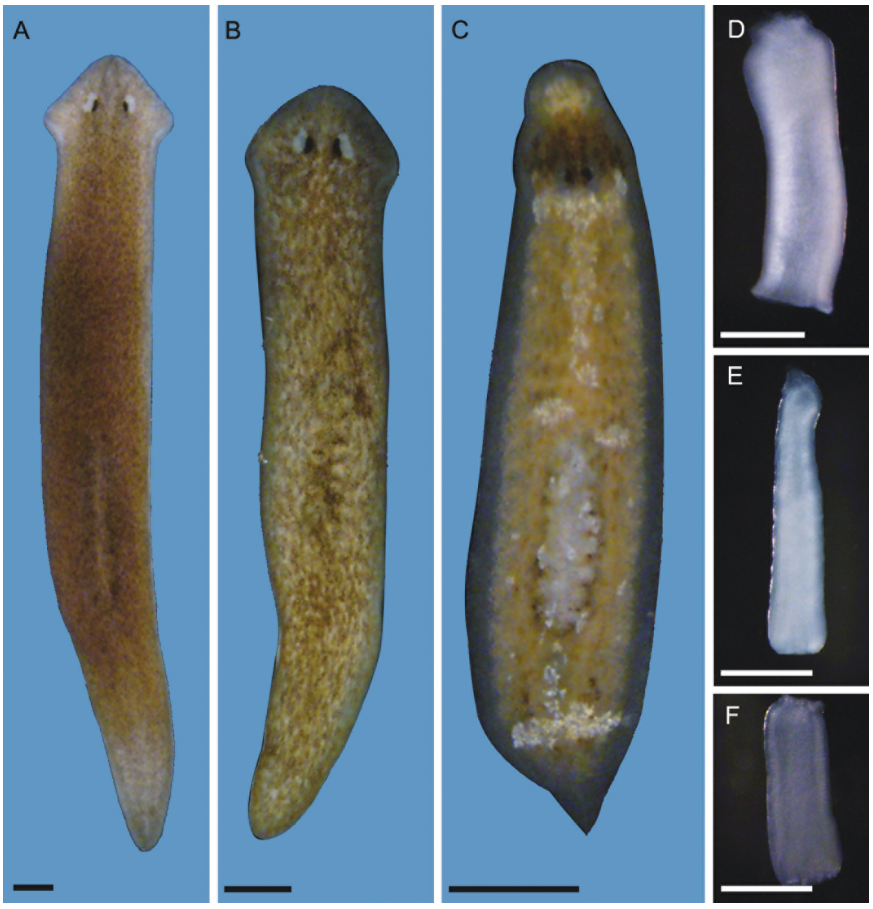

FIGURE 2 Three species and their isolated pharynges. A: *Dugesia* sp.; B: *Girardia* sp.; C: *Paucumara falcata*; D–F: isolated pharynges of *Dugesia* sp., *Girardia* sp. and *Paucumara falcata,* respectively (intestinal, aboral side at the top and oral end at the bottom; scale bars: 500 μm).

during the first day after amputation (scored at 2-hours post-amputation), but the feeding response was resumed at day-2 post-amputation. As for *Girardia* sp. and *P. falcata*, these animals began feeding within two hours after amputation (data not shown).

Comparison of feeding responses of intact flatworms and pharynges

Intact worms and their isolated pharynges were fed with two types of food, viz., pieces of pork liver and liver extracts. In all three species examined, the response of intact worms was 100% when pieces of pork liver were provided (fig. 4). However, when pork liver extracts were given, no response could be observed in all three species. In contrast, the isolated pharynges of all three species were highly responsive to both forms of food, with 82–93% average response to liver extracts. The isolated pharynges exhibited uptake of food at the oral opening, followed by peristaltic movements, which is similar to the activity of the pharynx in intact worms when it extrudes from the ventral side of body during feeding (see supplementary video S1 and S2: videos showing an intact worm and an isolated pharynx feeding on pork liver tissue). When

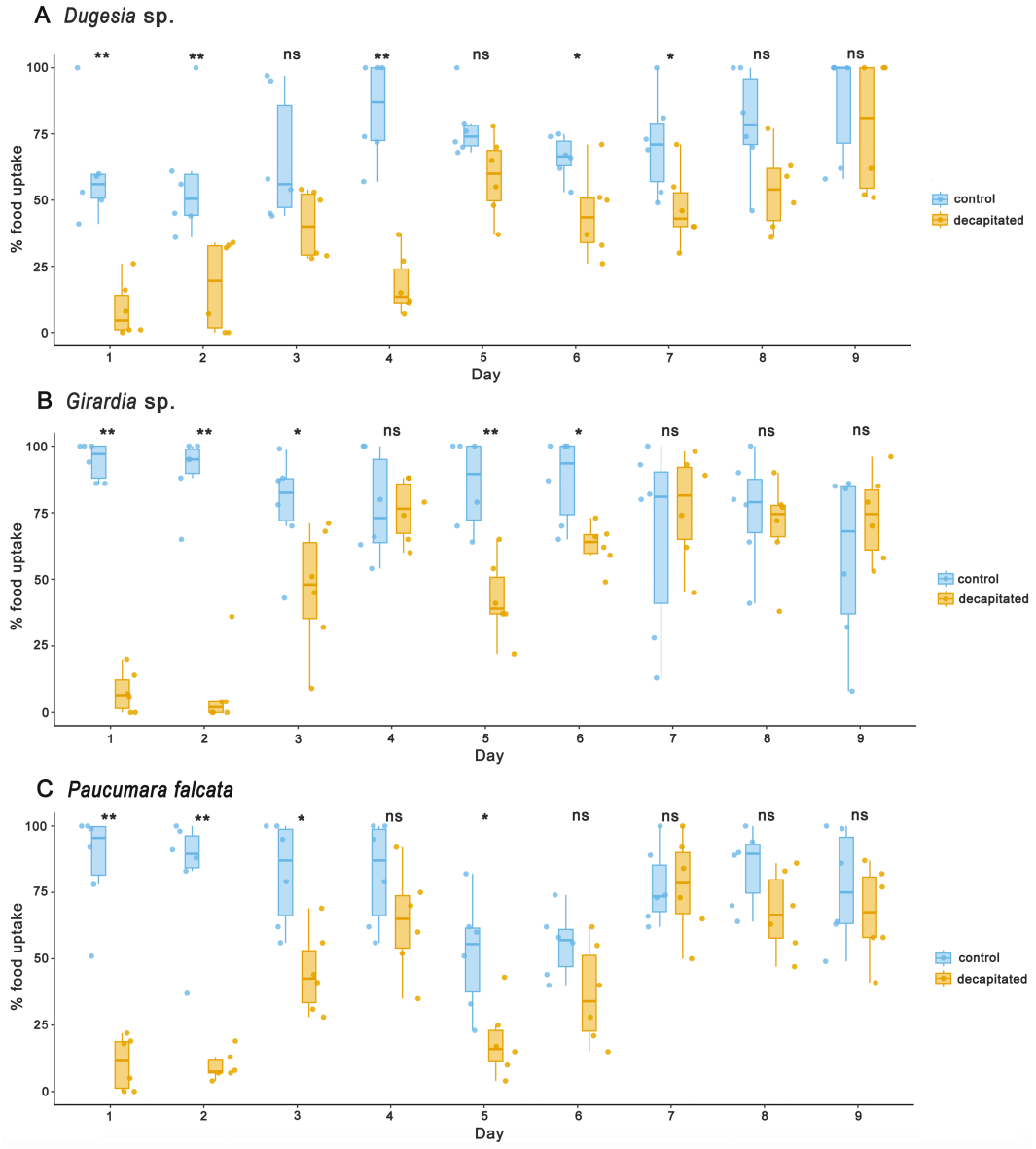

FIGURE 3 Recovery of feeding in three species after head amputation. Data represent percentage of food uptake from one to nine days post- amputation. A: *Dugesia* sp.; B: *Girardia* sp.; C: *Paucumara falcata* (nonparametric Mann-Whitney U test, $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant).

fed with different dilutions of the pork liver extracts, the feeding responses of isolated pharynges were concentration dependent (fig. 5). Specifically, the isolated pharynges of *Dugesia* sp. and *Girardia* sp. incubated with freshwater, or *P. falcata* incubated with brackish water without pork liver extracts, showed no feeding response at all, while the feeding response of isolated pharynges was greatest at 10% diluted liver extracts, reaching a response of 98 ± 4% in *Dugesia* sp. and *Girardia* sp., and 93 ± 5% in *P. falcata*. The inducing effect was weakened at lower concentrations. Even at the lowest concentration examined (0.1% dilution) the

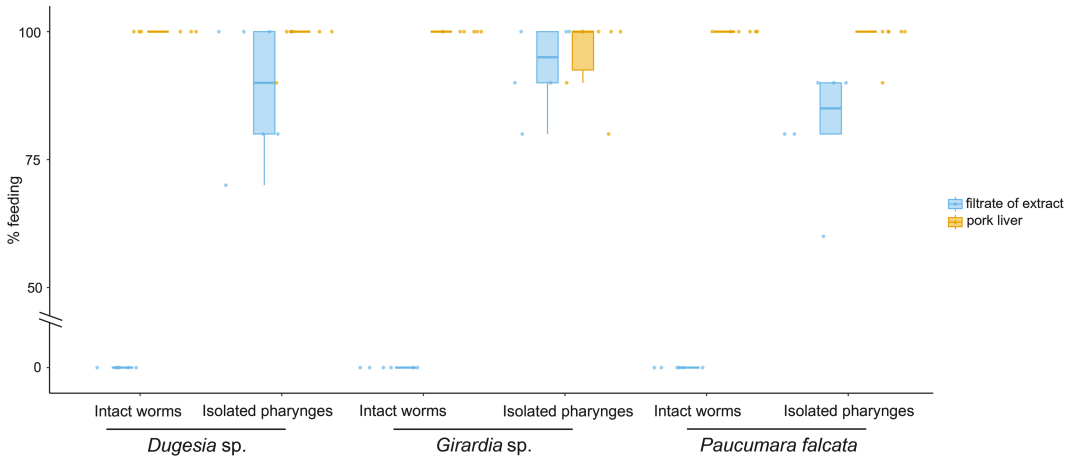

FIGURE 4 Percentage of intact worms and isolated pharynges showing feeding response to pieces of pork liver and liver extracts.

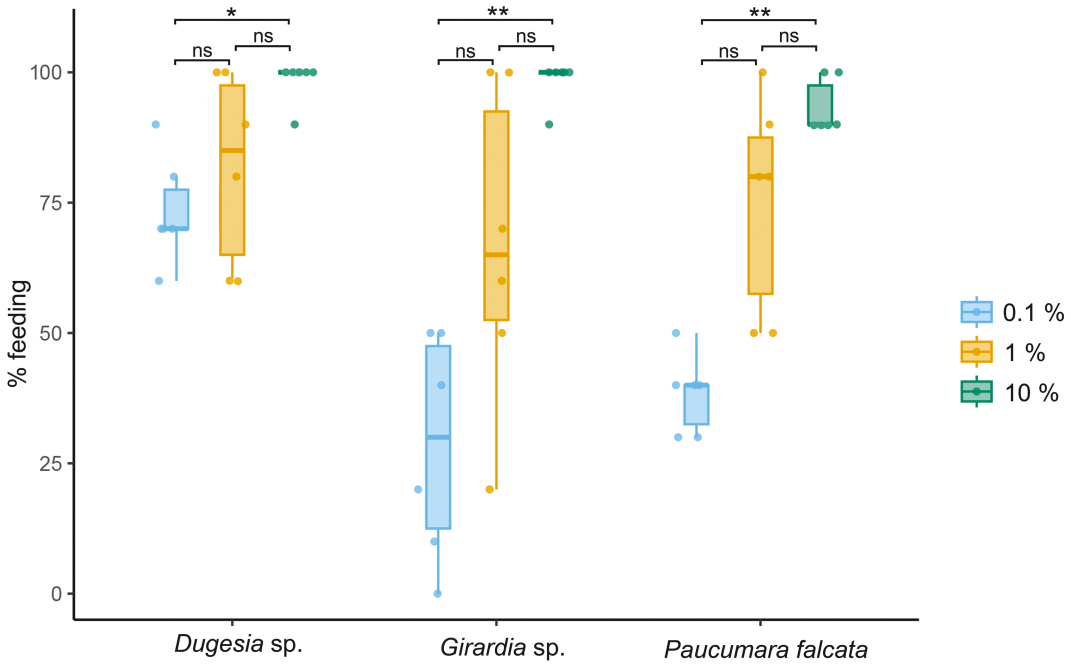

FIGURE 5 Inducement effects of serial dilutions of pork liver extracts on feeding response of isolated pharynges (Kruskal-Wallis test, $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant).

feeding response of isolated pharynges was still detectable in all three examined species, reaching 73 ± 10% in *Dugesia* sp., 28 ± 21% in *Girardia* sp., and 38 ± 8% in *P. falcata*.

Feeding response of oral and aboral portions of isolated pharynges

Based on the comparison of feeding responses of the oral and aboral portions of the isolated

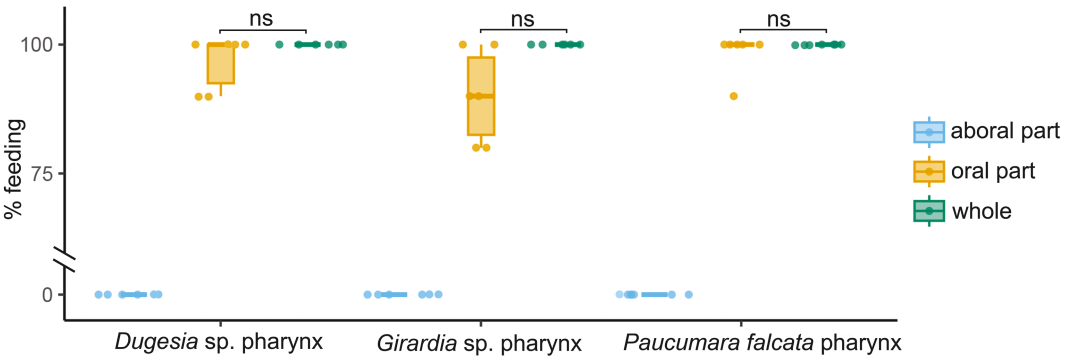

FIGURE 6 Percentage of feeding response of aboral, oral portions of pharynx and whole pharynx upon feeding (nonparametric Mann-Whitney U test for whole pharynges and oral portions of pharynges; ns, not significant).

pharynx, the oral portion exhibited obvious feeding reactions to pork liver tissue, with the percentage of response reaching 97 ± 5% in *Dugesia* sp., 90 ± 9% in *Girardia* sp., and 98 ± 4% in *P. falcata*. In addition, there were no statistically significant differences between intact pharynges and oral portions of pharynges (fig. 6). Specifically, in both intact pharynges and oral portions of pharynges, the oral opening expanded and, subsequently, engulfed the food item, whereafter it closed again. Subsequently, the engulfed pork liver tissue was transported towards the posterior, aboral part of the pharynx through peristaltic action of the pharyngeal musculature, and eventually expelled at the posterior end (fig. 7A1–C2). In contrast, in all three of the species examined, the aboral part of the pharynx did not show any response to pork liver tissue and finally shrank to form a sphere (fig. 7A3–C3).

Discussion

The results of amputation experiments showed that feeding in decapitated worms was completely inhibited, with no food uptake during the first two days after amputation, while it gradually recovered during regeneration (fig. 3). These results conform to those obtained for decapitated planarians by Shimoyama et al. (2016) and lend support to the notion that the brain plays an important role in the regulation of feeding behaviour (Inoue et al. 2015). Meanwhile, we found that in decapitated worms of all three experimental species, the food uptake began to increase at day-3, while statistically significant differences between decapitated and untreated worms were only observable by day-6 to day-8, depending on the species being examined. Therefore, it may be the case that the central nervous system is not fully recovered or functioning in these species until approximately day-7 post-amputation. However, this presumption requires further investigation by histochemical analyses of the neural system, as results solely based on bioassays cannot fully resolve this issue.

Planarian feeding behaviour has been interpreted as consisting of a twofold chemotropic response, with one being formed by movement of the worm towards the food item and the other by the activity of the pharynx (Wulzen, 1917; Sheiman et al., 2002). Movement of worms towards food was considered to be a response to chemical or physical stimulation from the food source (Sheiman et al., 2002). As shown by our results, intact

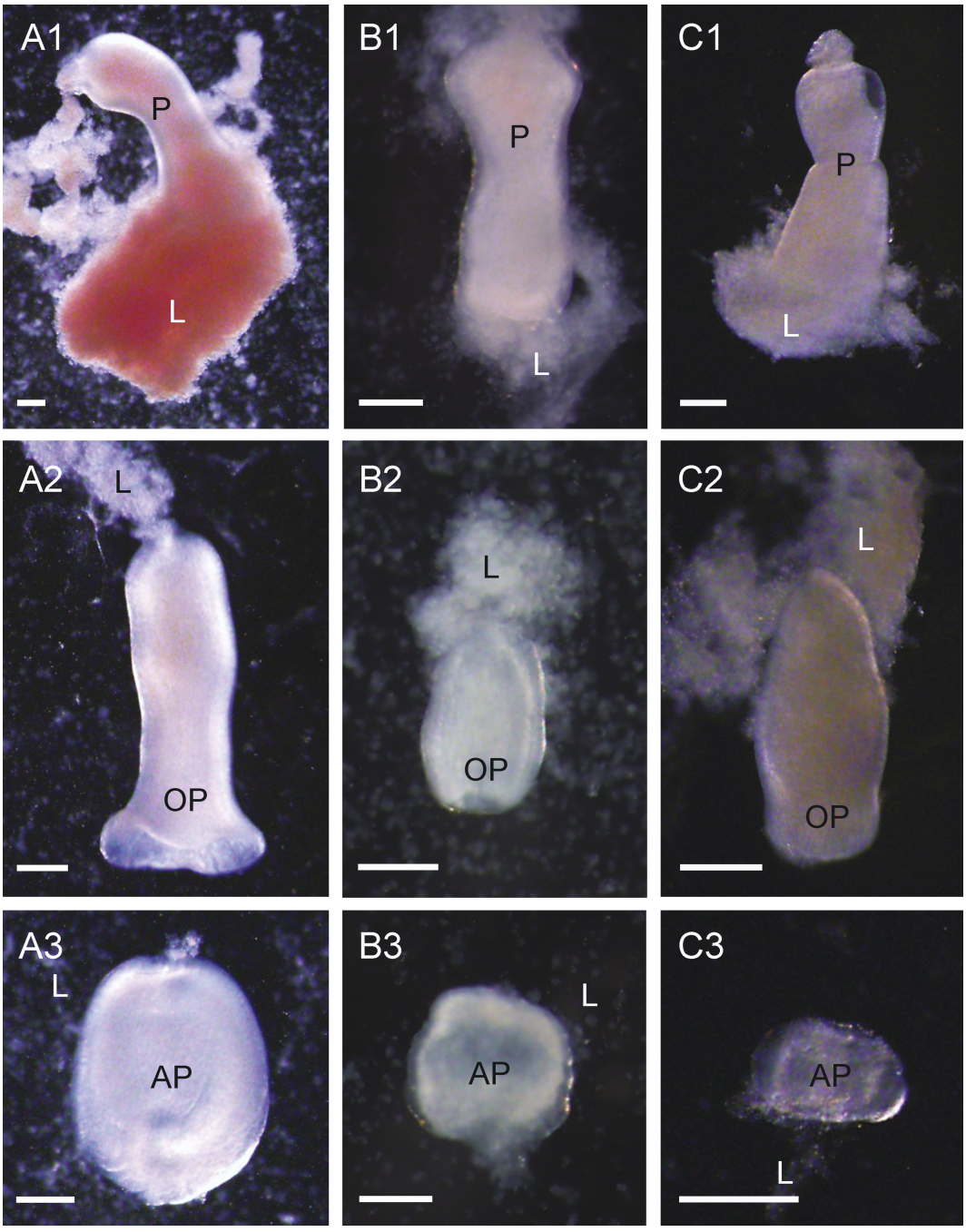

FIGURE 7 Feeding response of isolated complete pharynx, oral portion, and aboral portion of pharynges of (A) *Dugesia* sp., (B) *Girardia* sp., (C) *Paucumara falcata*. Panels no. 1 show intact pharynx, with liver tissue being transported through peristaltic movements and being expelled at the aboral end (top); panels no. 2 show oral portion of the pharynx, with liver tissue being expelled at the aboral end (top); panels no. 3 show unresponsive aboral portion of pharynx being contracted to a sphere. Abbreviations: L, liver tissue; P, pharynx; AP, aboral part of pharynx; OP, oral part of pharynx. In all figures: aboral part at the top and oral portion at the bottom; scale bars: 200 μm.

worms showed 100% response to liver pieces, but not to the filtered liver extract, which did not contain solid content. This suggests that physical stimulation predominates in eliciting a feeding response in the worm when it approaches the food source. In contrast, isolated pharynges were highly responsive to both the pieces of liver and the extracts, thus suggesting that the feeding reaction of the pharynx is stimulated through chemosensory perception of the soluble substances emanating from the solid food source.

Our results on the feeding behaviour of isolated pharynges basically corroborate the vivid, precise, albeit more anecdotal, accounts of Von Baer (1827) and Wulzen (1917). The intact isolated pharynges in all three of our experimental species exhibited peristaltic movements facilitating ingestion. Furthermore, the aboral portion of the isolated pharynx in all three species had no response to food items. In contrast, the oral part was highly responsive during feeding, while no significant differences were found between the feeding of the oral part and the intact isolated pharynx. These results are in good agreement with those from *D. japonica* (Ito et al., 2001; Miyamoto et al., 2020) and lend further support to the important role of the oral portion of the pharynx in feeding behaviour.

According to Kepner and Rich (1918), in an intact pharynx attached to the body of *Phagocata albissima* (Vejdovsky, 1883), the oral end and a sphincter muscle at the base of the pharynx are necessary for normal feeding movements. When in the isolated pharynx one or both of these two portions – oral end and sphincter – were removed, the remaining part generally showed no feeding response, although it may move about; occasionally, however, an isolated pharynx without its sphincter muscle, nevertheless, ingested food. The oral portion of the isolated pharynges either showed opening or closing movements or remained inactive, depending on the particular experiment. Later studies on *D. japonica* found also that when the oral portion of the pharynx was removed, there was no feeding response in either the fixed pharynx (Ito et al., 2001) or the isolated pharynx (Miyamoto et al., 2020).

Although in the present study we did not specifically study the pharyngeal nervous system, it is interesting to note that the pharynx contains automatic neural machinery and has sensory neurons and motor neurons, which constitute a simple reflex pathway and controls movements of pharyngeal muscle layers (Hanström, 1926). Interestingly, a study indicated that the chemosensory neurons that constitute nerve rings, have been detected also at the oral end of the pharynx (Okamoto et al., 2005). Therefore, the oral end of the pharynx, especially the pharyngeal nerve cord within this area, most likely plays an indispensable role in regulating its feeding action and reaction.

Interestingly, the high degree of neural autonomy of the tubular pharynx as reported here, and in other studies, for freshwater and maricolan planarians has been established also in other turbellarian flatworms, such as prorhynchids, polyclads, typhloplanids, and proseriates (Reisinger, 1976). It is not only the pharynx that exhibits a high degree of neural independence, but this holds true also for the intestine and thus for the entire stomatogastric system (Reisinger, 1976). In planarians, the presence of a gastrodermal nerve plexus was first demonstrated by Baguñà (1974) and presumably this plexus autonomically facilitates defecation via the pharynx, which is usually accompanied by convulsions and muscular contractions of the entire body (Hyman, 1951; Ball & Reynoldson, 1981) and by peristaltic action of the gut (Reisinger, 1976). According to Ehlers (1985), the stomatogastric plexus represents a primitive, plesiomorphic condition that evolved already in the common ancestor of all flatworms.

Acknowledgements

We thank Jia-Jia Chen, Wei-Xuan Li, Ying Yang and Xiao-Yu Song for assistance during sample culturing, and Dr. Si Chen for valuable comments on the present work. This study was supported by the Guangdong Undergraduate Training Program for Innovation and Entrepreneurship (Grant No. 201910590068), as well as grants from the Scientific and Technical Innovation Council of Shenzhen Government (grant nos. JCYJ20210324093412035 and KCXFZ20201221173404012) and Guangdong Natural Science Foundation (grant no. 2020A1515011117) to Yu Zhang. The authors like to thank two anonymous reviewers for their useful comments.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.22692226

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