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作品名稱　Sequentially bidirectional gastrovascular flows in highly branched digestive tracts of panocerid flatworm

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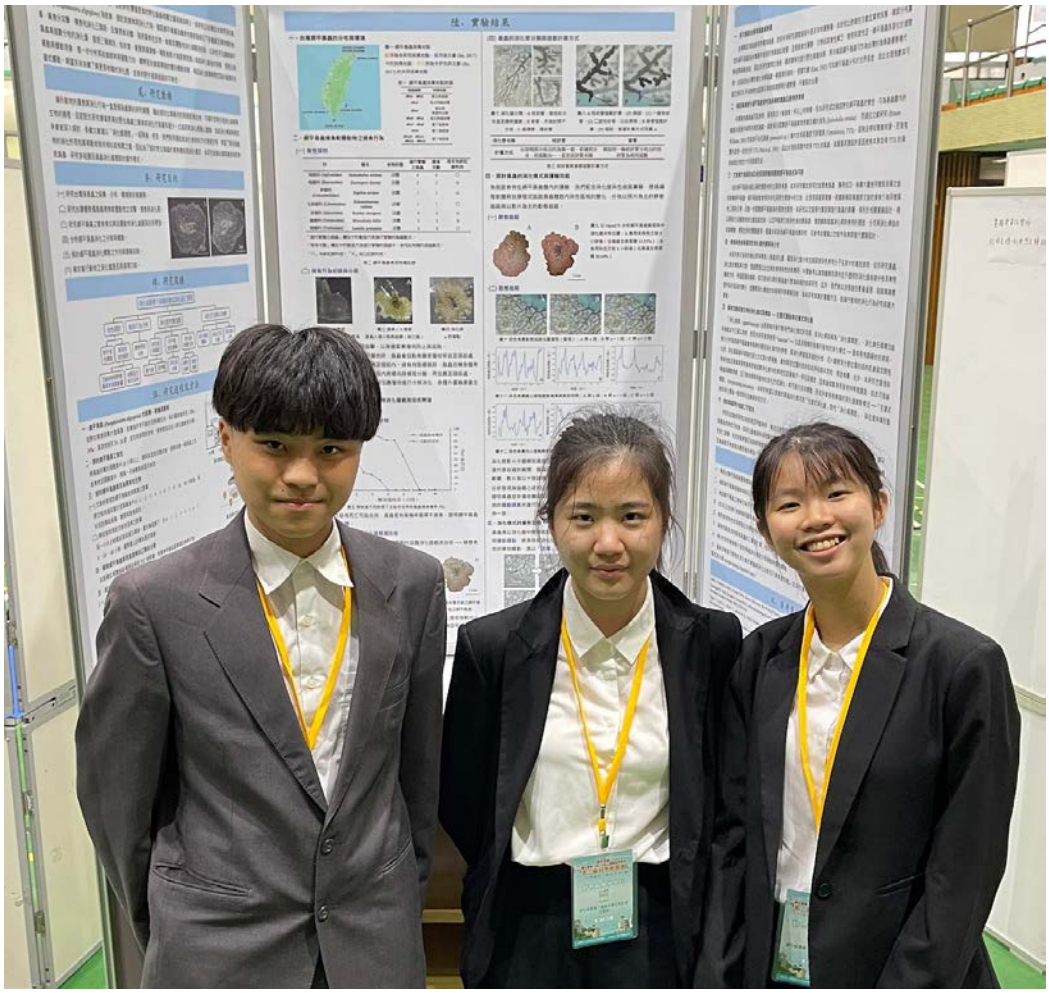
關鍵詞　peristalsis, segment movement, post-stain tracking

作者簡介



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基於對生物科學的熱愛，身為竹科實中數資班的我們組成了科展團隊，從高一下開始研究海洋扁蟲的消化行為。我們憑藉創意與持續嘗試的勇氣，在歷經挫折後，成功開發出獨創的扁蟲消化道染色與分析方法，並針對扁蟲的消化期進行深入探討。我們很幸運有機會踏上專題研究的道路，並在其中獲得源源不斷的感動與美好，也感謝一路以來協助我們的維邦老師和支持我們的家長，讓我們能在科學的旅程中義無反顧的追求自我。

Abstract

Examination of the predation behavior of polyclad flatworms is extremely rare. This study collects *Paraplanocera oligoglena*, the most common species in Taiwan. Tank-based feeding experiments reveal that *Paraplanocera oligoglena* can prey on several species of gastropods, such as sea snails and sea hares. Predation behavior encompasses attack, invasion and ingestion periods. This research pioneers the use of stained clam and static image analysis to observe the highly branched digestive system of flatworms. The sequentially bidirectional flow of gastrovascular cavity is first found in polyclad flatworms by the post-stain active tracking technique. Measuring peristalsis movement in inward and outward directions and segmented movement, the contraction frequencies are roughly the same in subsequent order of given branches. Confirmation is provided that the circular membrane-like muscles within the digestive tract are the main driving force for transporting and mixing food. The food dyeing technology used in this experiment also provides the possibility of future research on food chains in the wild.

Background

Koopowitz (1970) detailed the feeding behavior of the polyclad, *Planocera gilchristi*. The planocerid flatworms were collected from intertidal zones and kept in a container with periwinkle (*Oxystele*) as their prey. Complex feeding behaviors including prey recognition, stalking, capture, extraction and swallowing were observed (Koopowitz 1970). Newman and Cannon (2003) documented the predation behavior of an undescribed *Planocera* sp. that consumed an entire cowrie from Micronesian waters (Fig. 1). Ritson-Williams et al (2006) found an undescribed *Planocera* sp. containing the deadly neurotoxin tetrodotoxin (TTX) from a tropical Pacific island, suggesting that planocerid flatworms can utilize TTXs to capture several species of gastropod snails (Ritson-Williams et al 2006). Drawing on insights from these feeding experiments, it is apparent that Planoceridae flatworms are active predators of certain gastropod sea snails.

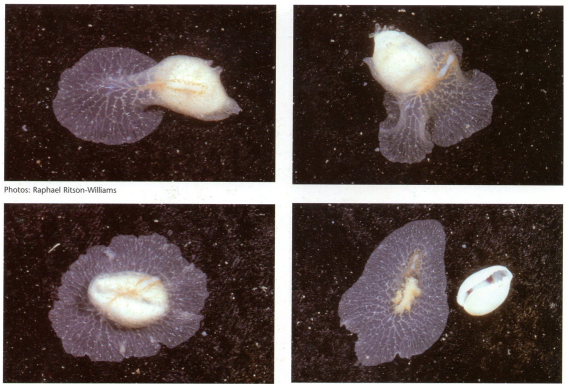


Fig. 1. A planocerid polyclad feasts on an entire cowrie in Micronesia waters. (Derived from Newman and Cannon 2003)

After predation on periwinkle, the flatworm's pharyngeal folds move down and engulf the entire shell, which is then drawn into the mouth. Approximately 24 hours after feeding, the undigested parts of the snail are regurgitated through the mouth (Koopowitz 1970). Once in the pharynx, the food moves to the main gastrovascular cavity, the gut with numerous branches distributing nutrients throughout the body (Jennings 1957; Newman and Cannon 2003). Jennings (1957) described the dietary habits of *Leptoplana tremellaris*, an acotylean flatworm that feeds on small animals such as polychaetes, isopods, and amphipods. The food enters the flatworm's branched guts and is partially broken down by enzymatic activities in the lumen before entering the cells. Tsuyuki et al (2020) provided insights into the feeding habits of *Pericelis flavomarginata*, a cotylean flatworm that preys on red color scale worms. Within 1–2 hours of feeding, they observed a gradual reddening of the flatworm's intestinal branches. Comparing the freshly fed specimen with a starving one, the reddish intestinal contents made the gut branches of the polyclad worm more visible (Tsuyuki et al 2020).

Newman and Cannon (2003) proposed "the multibranched system may be added by small muscular valves (sphincters) along the branches that can regulate flow along them and thus provide waves of contraction (peristaltic movement) to fill or clear the gut." However, no reports on the ingestion movement within the digestive system have been presented by the original founders, Newman and Cannon (2003), or any other subsequent researchers since 2003.

The gastrovascular cavity, integral to both digestion and nutrient distribution, constitutes the basic body plan of cnidarians, including jellyfish, corals and hydras (Campbell and Reece 2005). Shimizu et al (2004) provided evidence of digestive movement in hydras (Fig. 2), elucidating how the diffuse nerve net controls digestion and circulation through peristalsis and segmentation movements (Shimizu et al 2004; Shimizu and Okabe 2007). Although the gastric cavity of hydra is a blind sac, the segmentation movement in the cavity might result from the bouncing at the cavity's end,facilitating back-and-forth transfers in the cavity (Shimizu et al 2004).

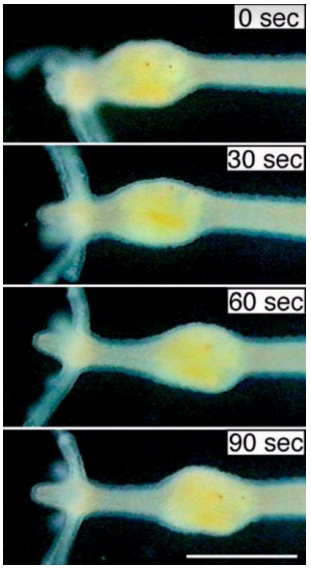


Fig. 2. Esophageal reflex-like movement of a hydra which had ingested five *Artemia* when the tentacles were removed. (Derived from Shimizu et al. 2004)

Harmata et al (2013) delved into the gastrovascular flow in cnidarian phylum, exploring hydroids and octocorals. They described sequentially bidirectional flow or simultaneously bidirectional flow with baffles separating the flow described (Harmata et al. 2013). In the absence of a true circulatory system, the gastrovascular cavity distributes the partially digested material through the entire body (Fig. 3). Myoepithelial contractions from the center of the colony drive the sequentially bidirectional flow in hydroid species (Harmata et al. 2013). Avian et al (2022) employed resin endocasts and 3D X-ray computed microtomography reveal the branched gastrovascular system of *Rhizostoma pulmo* (Rhizostomatidae) in which inwards and outwards flows are simultaneously observed in the peculiar double hemi-canal structure. These innovative techniques also suggest various openings of the gastrovascular system may function as a through-gut apparatus in cnidarians (Avian et al 2022).

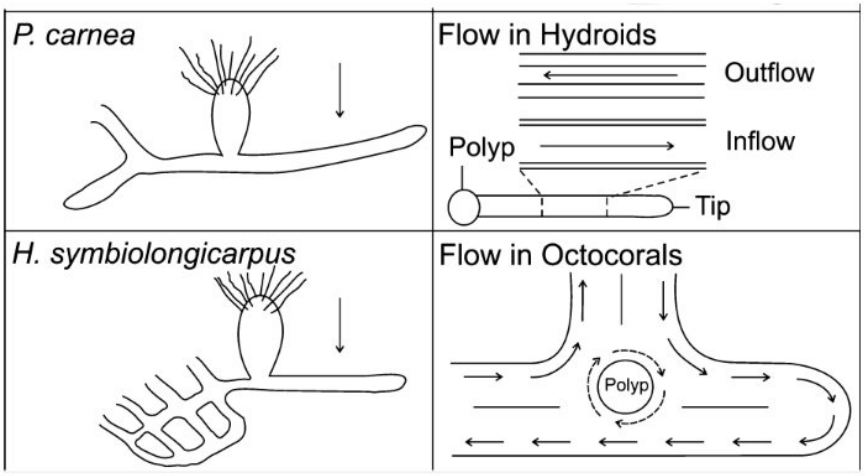


Fig. 3. Schemata illustrating the general morphologies of *Podocoryna carnea* and *Hydractinia symbiolongicarpus*. Arrows indicate locations imaged for the gastrovascular flow. The *P. carnea* hydroids species exhibited sequentially bidirectional flow, while the gastrovascular system of *A. amboinensis* octocorals species exhibited simultaneously bidirectional flow. (Derived from Harmata et al. 2013)

Polyclad flatworms are free living flatworms that have branched intestines radiated into a peripheral network (Faubel 1983). Similar to these cnidarians, these flatworms feature an incomplete gut known as a gastrovascular cavity (Brusca et al. 2016). However, the intricacies of the gastrovascular flow in polyclad flatworms remain relatively unexplored, with Newman and Cannon's description (2003) providing the most detailed insights. This prompts questions about the distinctions in peristaltic movement between polyclad flatworms and cnidarians, as well as the functional role of small muscular valves along their highly branched digestive tracts.

To address these inquiries, we collected *Paraplanocera oligoglena* (Schmarda, 1859) (Polycladida: Planoceridae) from the intertidal pools in Taiwan. *P. oligoglena*, a globally distributed polyclad flatworm (Hyman 1953; Newman and Cannon 2005; Soutullo et al 2021), was initially documented inTaiwan by Kato (1943) in Suao and has been featured in various Chinese field guide publications (Chen, 2001). Despite being one of the most prevalent polyclad flatworms along Taiwanese coasts (Jie 2017), its intertidal food webs remain unexplored.

Simulating the feeding experiments of Jennings (1957), Koopowitz (1970), Ritson-Williams et al (2006) and Dittmann et al (2019), we assume that the videotaping of *Paraplanocera oligoglena* might help us learn more details about planocerid predation behavior. Employing a stained food technique, adapted from the of Wells and Sebens' method (2017) as the bait for the flatworms, we aim to closely observe digestive tracts movements. This approach seeks to unravel the dynamics of the gastrovascular flow in the highly branched digestive system of these flatworms.

Materials and Methods

Paraplanocera oligoglena, the polyclad flatworms under study, were gathered from tidal pools around Taiwan and transported to school (Fig. 4). They were placed in a glass tank with a circulating filtration system. The seawater's salinity was maintained at 32‰ - 34‰ and the temperature was regulated at 24°C - 26°C. The artificial seawater quality was maintained by regularly removing food residue and adding nitrifying bacteria.

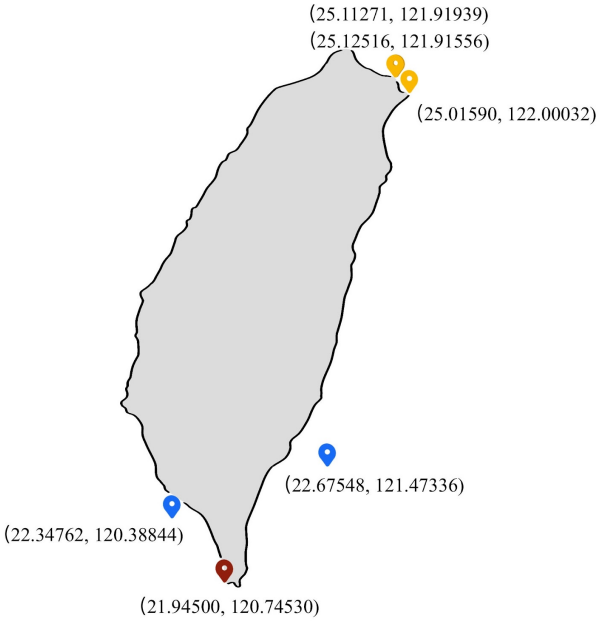


Fig. 4. The geographic information of the collected *Paraplanocera oligoglena* (Schmarda, 1859) of this study around Taiwan.

Long-term videotaping in tanks

All the gastropods used in feeding tests were collected from the same collection sites of *Paraplanocera oligoglena*, including sea snails with or without operculum, sea slugs and sea hare (Table 1). The transparent glass tank was equipped with fluorescent lamps and a JVC GZ-RX500BTW for extended video recording. The flatworms were isolated for 24 hours before initiating recording. After the feeding sessions, continuous filming documented the predation behavior. Long-term recordings were established in tanks, and subsequent analysis focused on discerning patterns in flatworm predation behavior.

Bait preparation

According to Teng et al's research (2022), alternative baits were successfully employed to study oyster leech behavior.In our tank experiments, we extended this approach by using clams as alternative baits. While not a natural prey for *Paraplanocera oligoglena* in the wild, using clams as food substitutes facilitated the sustained observation of wild flatworms in the laboratory.

Market-sourced Asian hard clams (*Meretrix lusoria*) were dissected, and their meat served as food for the flatworms. Prepared meats were held in isolated boxes for varying time spans, ranging from 1 to 8 hours, before being offered to the flatworms. The feeding rate was calculated as the percentage of the clams consumed by the flatworms.

The clam's gills were removed to make slide specimens. These slides were then positioned under a microscope, and the cilia on the gill surface were observed over varying time intervals, ranging from 1 hour to 48 hours. The aim was to ascertain the vitality of the clams by determining whether the cilia on the gills were exhibiting movement. A correlation analysis was performed, plotting the relationship between the time elapsed since dissection (freshness) and the survival rate of the clams.

Stained clam meat was dyed with 0.1% methylene blue overnight, a method modified from those of Wells and Sebens (2017). The stained clam meat served as bait for flatworms, and the blue color in their digestive tracts served as an indicator of consumption. Photographs of the ventral sides of the two flatworms were taken hourly to observe the transportation of food within their highly branched digestive system.

Post-stain processing and tracking

Post-stain image processing involves two primary methods: static processing and active tracking, focusing on picture analysis and video analysis respectively. Both techniques necessitate image transformation (Harris et al 2020) and utilize OpenCV (Open Source Computer Vision Library) (Bradski 2000) and Matplotlib (Hunter 2007) for image analysis and subsequent data graphing. In active tracking, Pandas (McKinney 2010) is employed to provide calculated percentage points for each time point.

For post-stain static processing, flatworms in the ingestion period, post-feeding on stained bait, are positioned in a petri dish beneath an even light source, with white paper lining the surface for uniformity. After capturing photos of the flatworms, background removal is followed by converting the original image to an 8-bit grayscale format and applying a Gaussian blur filter for noise reduction. Adaptive thresholding creates a binary image with white lines on a black background, identifying blue-colored areas in contrast. The binary image is then converted to a color image (BGR format) to match the original's dimensions. Simultaneously, a blank red image is generated with identical dimensions. Combining the processed image with the red image overlays the red lines onto the original, marking the flatworm's blue-dyed regions. Percentage calculation involves dividing the marked areas' pixel count by the flatworm's total.

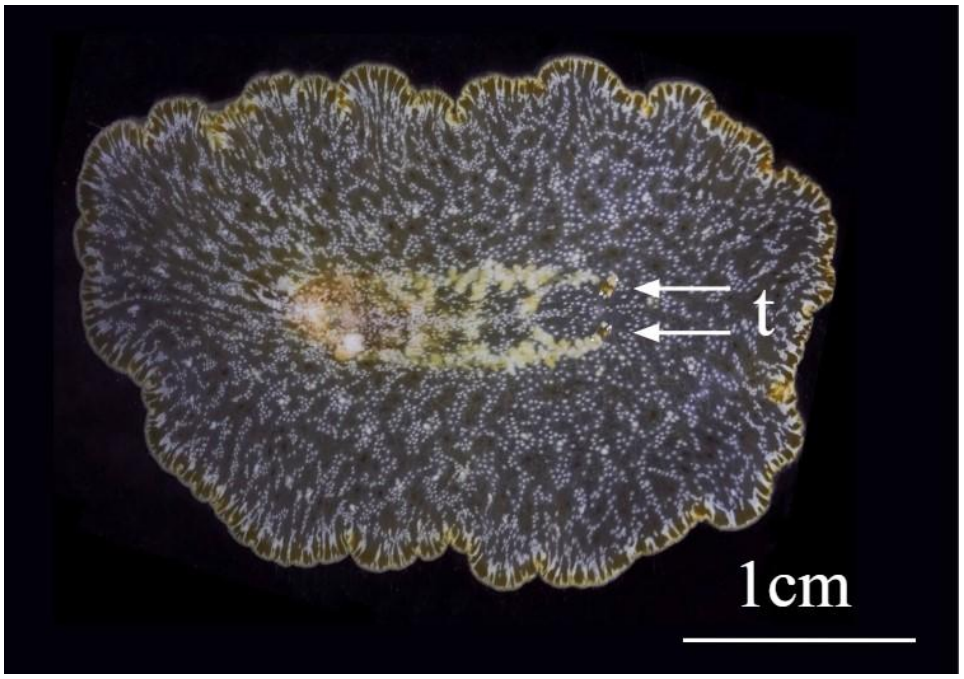
In post-stain active tracking, flatworms in the ingestion period were moved to the side of the transparent glass tank,with a white plastic divider at the back for an even background. Uniform white light surrounds the attachment side,and a digital camera records the ingestion period of flatworms. The recordings, sped up tenfold, undergo processing in a manner similar to static processing. However, instead of analyzing the entire flatworm, designated areas with a small stretch of the digestive tract are selected and continually updated. Tracking enables tracing of the selected area despite the flatworm's movement. Data points, complete with time stamps and percentages, are then exported and plotted using Pandas and Matplotlib, respectively.

Results

This study involved the collection of *Paraplanocera oligoglena* (Schmarda, 1859) by snorkeling in various places such as Magang (25°01'03.0"N 122°00'00.8"E) , Longdong Bay (25°06'48.1"N 121°55'13.1"E) and Houbihu (21°56'19.3"N 120°44'44.1"E) around Taiwan (Fig. 4). The flatworms are found in crevices or under rocks and then collected by hand brushing from September, 2022 to April, 2023. A total of 17 individuals were utilized in the feeding studies. Synthesizing findings from Kato (1943), Jie (2017) and the present study, it is evident that *P. oligoglena* has the highest capture rate among all recorded polyclad flatworms in Taiwan's collecting history. This shows evidence that *P. oligoglena* is apparently the most dominant species in Taiwan.

P*araplanocera oligoglena* has an oval to circular body shape, characterized by a translucent light brown to dark brown background color (Fig. 5). There are several white and brown irregular spots and dots, forming a dark brown reticulated pattern (Jie 2017). Given the transparent and slender composition, the pharynx and main intestine are easily visible from the ventral side. The main intestine radiates and branches from the pharynx toward the distal margin,constituting the transparent branched digestive system is so thin and narrow that it is barely detectable to the naked eye.

(A)



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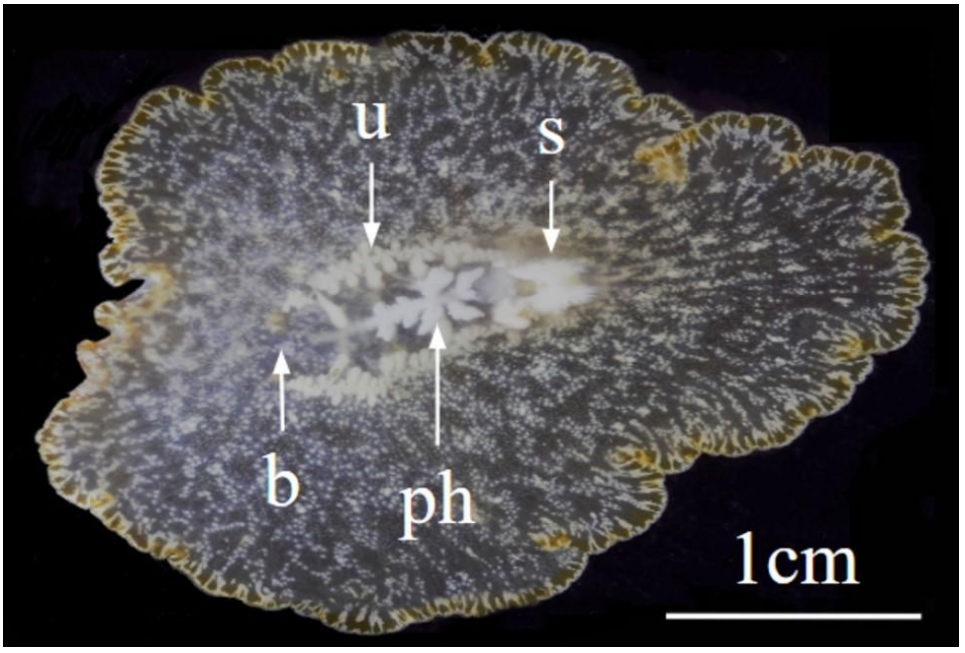


Fig. 5. *Paraplanocera oligoglena* (Schmarda, 1859) specimens collected on August 5th, 2022, at Longdong Bay. (A) Dorsal view. Anterior end on the right. (B) Ventral view. Anterior end on the left. b, brain; ph, pharynx; sexual organ; t, tentacle; u, uterus.

Snails and sea hares are eaten

In the feeding experiment, gastropod snails, including nudibranchs and sea hares sourced from the same collection sites as *Paraplanocera oligoglena* are utilized. Videotaping is utilized to observe whether the food bait is consumed or not. The consumed species include those with and without operculum, and the outcomes are cataloged in Table 1. This includes representatives from at least eight different gastropod families,with species such as *Euplica scripta* (Columbellidae) (Fig. 10) and the *Stylocheilus striatus* (Aplysiidae) observed to be consumed, both in their natural habitat and in tanks (personal observations).

Table 1. *Paraplanocera oligoglena* (Schmarda, 1859)predation observed in feeding experiments.

Family	**Species**	**Shell**	**Operculum**
Aplysiidae	*Stylocheilus striatus*	×	×
Aglajidae	*Melanochlamys* sp.	×	×
Buccinidae	*Enzinopsis lineata*	○	×
Columbellidae	*Euplica scripta*	○	×
Littorinidae	*Echinolittorina radiata*	○	×
Muricidae	*Reishia clavigera*	○	○
Trochoidea	*Monodonta labio*	○	○
Turbinidae	*Lunella granulata*	○	○

"○" represent possessing shell or operculum; "×" represnt lacking.

Predation periods under long-time videotaping

Attack period on sea slugs

In the attack phase, when *Paraplanocera oligoglena* senses its prey, active prey such as the active *Stylocheilus striatus* (sea hare), it rapidly stalks and extends its body toward the prey.Upon contact, the flatworm swiftly wraps itself around the prey until it is completely covered and unable to escape (Fig. 6). Notably, when targeting *S. striatus,* the sea hare often produces a lot of purple ink during the encounter. Throughout this process, *P. oligoglena* refrains from protruding its pharynx or detaching the autonomic pharynx described by Teng et al (2022). In the case of other slow-moving gastropods, the flatworm's attack involves moving toward the shell and initiating the process of enveloping it within its body. The attack on sluggish slow moving sea snails is less conspicuous in comparison to shell-less sea hares.

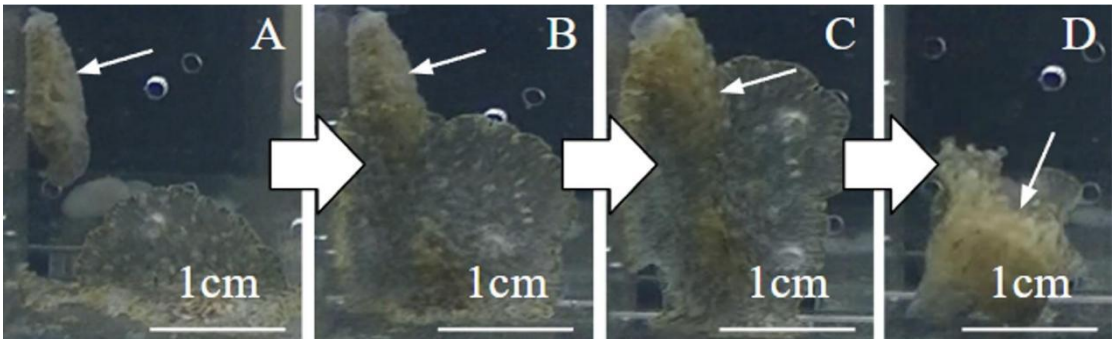


Fig. 6. Serial pictures from A to D show the attack behavior of *Paraplanocera oligoglena* on sea hare, *Stylocheilus striatus* (indicated by white arrow).

Nevertheless, the attempt to attack *Hypselodoris maculosa* (nudibranch) proved unsuccessful. They fought for nearly 20 minutes within the tank. The flatworm persisted in its efforts until the sea slug released a white milky chemical. At that point, the flatworm conceded and retreated, as observed firsthand (Fig. 7).

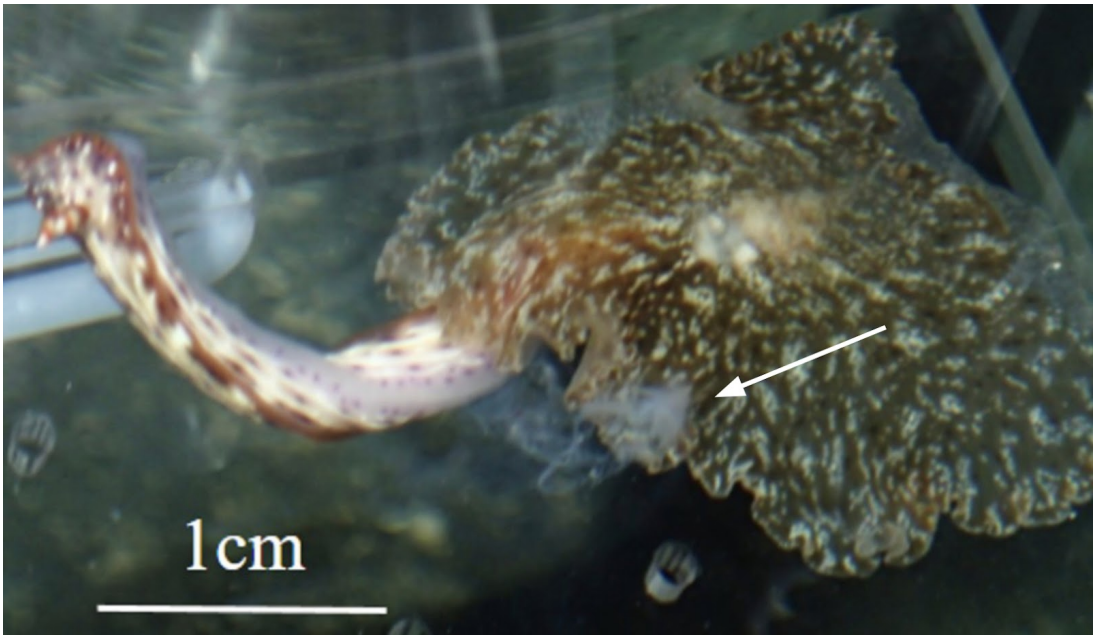


Fig. 7. *Paraplanocera oligoglena* attempts to attack *Hypselodoris maculosa* (nudibranch) and then fails. White arrow indicates the sea slug releases a white milky chemical when being attacked.

Invasion period

After attacking and initiating predation on sea hares, *Paraplanocera oligoglena* extends its margin to fully envelop the prey and then actively maneuvers the entire prey to its mouth opening. Subsequently, the flatworm's pharynx protrudes from the mouth opening toward the prey.

For shells without operculum, such as *Enzinopsis lineata* (Buccinidae), *Euplica scripta* (Columbellidae), and *Echinolittorina radiata* (Littorinidae), the flatworms intricately twist and turn the entire shell before protruding their pharynx inside the shell (Fig. 8). Approximately 6 to 12 hours later, the flatworm separates the entire snail body from its shell. It then withdraws from the empty shell,wrapping its meal as it departs.

Fig. 8. The invasion of *Paraplanocera oligoglena* on *Enzinopsis lineata* (Buccinidae) indicated by the white arrow. The flatworm twists and turns the entire shell,eventually protruding its pharynx inside the shell. This period takes roughly 6 to 12 hours to complete on shells without operculum.

For sea snails with operculum, such as *Reishia clavigera* (Muricidae), *Monodonta labio* (Trochoidea), and *Lunella granulata* (Turbinidae), *Paraplanocera oligoglena* envelopes the entire shell as previously described. Subsequently, the flatworms extend their pharynx into the shell and attempt to remove the protective covers before efficiently extracting the snail meat. This process requires a longer duration, taking approximately 8 to 24 hours to complete.

Ingestion period

During the ingestion period, the food wrapped inside the pharynx cavity undergoes decomposition and digestion (Fig. 9). The rhythmic contraction of the highly branched digestive system reveals the movement of ingestion.

Multiple attachment points are arranged in a row at the outer body margin (Fig. 9). The flatworm changes its position several times during ingestion, and after each settling, the adhesive points become visible again. This period spans 6 to 12 hours.

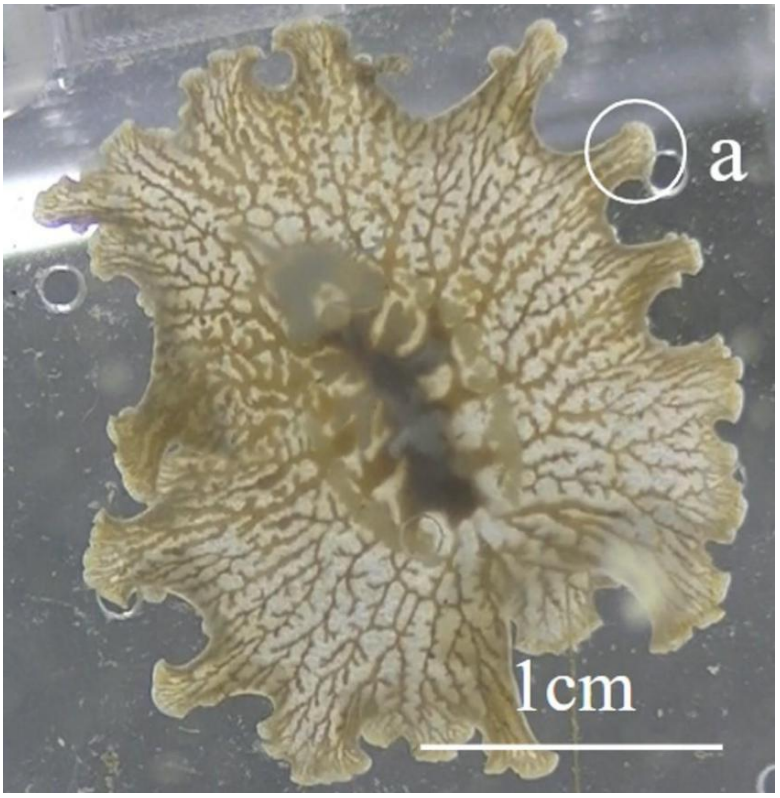


Fig. 9. The ingestion process of *Paraplanocera oligoglena.* A white circle highlights one of the attachment points surrounded at the outer margin. The digested food rhythmically moves along the highly branched digestive system. This phase spans 6 to 12 hours.

Upon completion of the digestive phase, the flatworm expels the residual waste products from its pharynx, and the flatworms disengage freely.

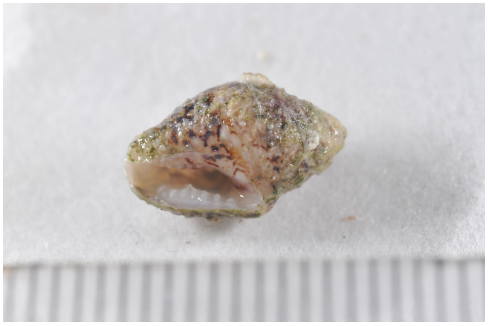
(A)  (B) 

Fig. 10. *Paraplanocera oligoglena* found feeding on *Euplica scripta* (Columbellidae) at Magang, 28th February 2014 and regurgitated (A) empty shell and (B) food pellet with radula right after feeding..

Flatworm's Preference for Fresh Meat

In the feeding experiment, clam meat was extracted as bait through dissection every hour before being presented to flatworms for predation rate calculation. Notably, flatworms exhibited the highest predation rate on clam meat harvested within the first hour (Fig. 11). Subsequently,the predation rate gradually declined, and flatworms ceased consuming food from the six-hour-old baits. This is assumed to be related to the freshness of the baits. At the same time, a compound microscope was utilized to observe the activity of flagella on the surface of the clam's gills to ascertain the clam's viability. It was also observed that the survival rate of the clam significantly decreased eight hours after dissection (Fig. 11). Therefore, it is inferred that flatworms are not detritivore.

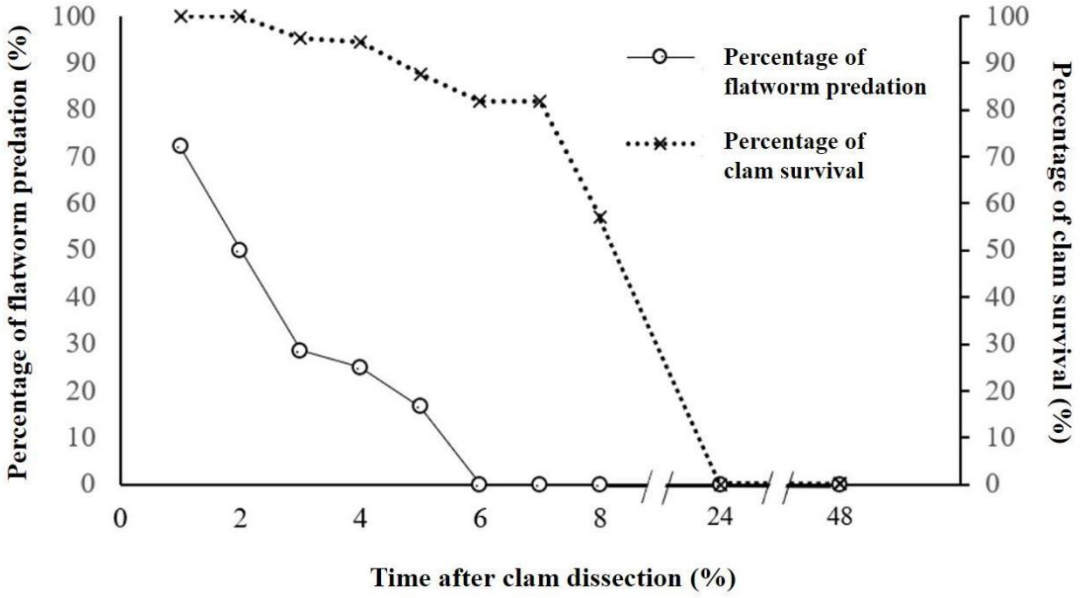


Fig. 11. The curve depicting*Paraplanocera oligoglena* predation percentage on clam bait and the survival percentage of clams per hour after being extracted from the shell.

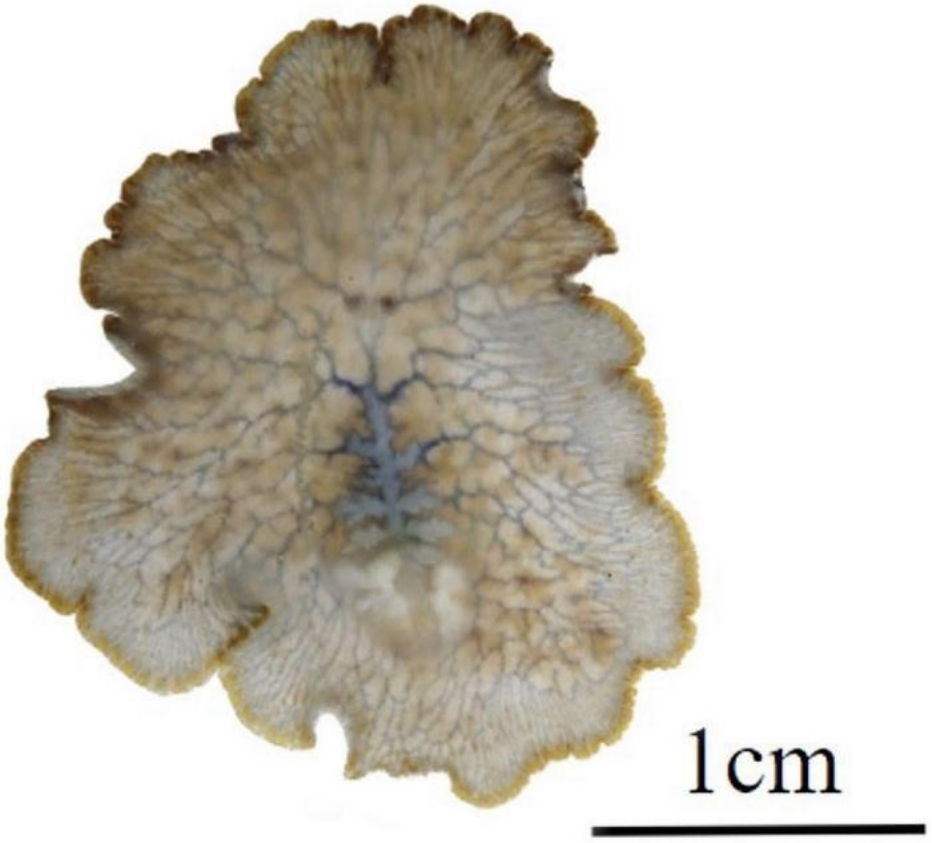
Digestive system shown by stained food

The digestive system of *Paraplanocera oligoglena* is both transparent and highly branched, making it well-suited for tracking the passage of digested food (Fig. 5). To observe the digestive movement of flatworms, we modified the staining technique from Wells et al (2017) and developed it into the in vivo digestive tract observation technology,utilizing 0.1% methylene blue-stained clam as bait for the feeding experiment.

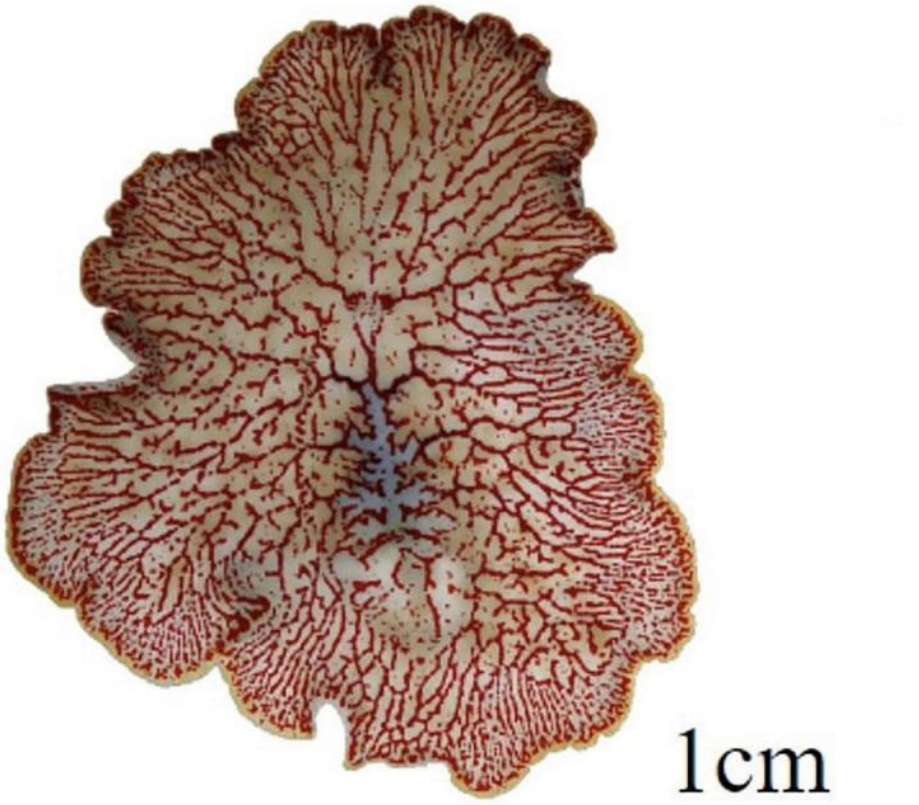
The results reveal that the digestive system of *Paraplanocera oligoglena* comprises blind sacs, simple loops, and predominantly highly branched branches. Blind sacs are distributed along digestive tracts, resembling tubes with closed ends that do not continue to branch. Circulative simple loops connect adjacent branches to form a ring tract, and are sporadically distributed along the digestive tract. The branched tract is a defining characteristic of the flatworm's digestive system, extending from the pharynx and branching into fork-like bifurcations to the body's margin edge. The diameter of the digestive tract gradually diminishes as the branches bifurcate, evenly distributed around most areas of the body (Fig. 12A). Apparently, there are no openings at the outermost ends of the branched tract, differing from the through-gut apparatus described by Avian et al (2022).

The widest tract, originating from the central portion or pharyngeal region, branches into a narrower secondary tract that further radiates into an even narrower tertiary tract. It has been calculated that the highest order of digestive tracts in *Paraplanocera oligoglena* can extend up to the narrowest tract, the sixth order branches. Some branches may not be clearly seen, suggesting that the real order number of its highly branched tract may surpass the sixth order.

Using a post-stain image process, OpenCV and Matplotlib, the image was converted to 8-bit and filtered with the HSV range of the color stained by methylene blue. Areas identified as blue were then colored red using a red marker. The entire body with marked areas is presented here (Fig. 12B), illustrating the extreme complexity of the gastrovascular cavity of *Paraplanocera oligoglena*.



(A)



(B)

Fig. 12. Post-stain image of*Paraplanocera oligoglena* (A) with methylene blue stained bait. (B) Image processing using static tracking technique. The red color indicates regions involving stained food. The processed graph illustrates that the digestive system of *P. oligoglena* consists of blind sacs, simple loops and mostly highly branched branches.

Gastrovascular flow shown by post-stain active tracking

In accordance with Shimizu et al (2004), the digestive movement of hydra includes esophageal-like movement, segmented movement and defecation-like movement. Similarly, we have observed three distinctive digestive movements in *Paraplanocera oligoglena*. As the ingestion period begins, the flatworm spontaneously contracts its pharynx, gradually pushing the food into the digestive tract.

The digestive movement within the flatworm's branched tract, extending from the pharynx to the most remote margin, is evident in the post-stain image. Surprisingly, the 0.1% methylene blue stained bait has revealed sequentially bidirectional gastrovascular flows for the first time. Videotaping the digestive tract with dyed bait has shown that food transport occurs bidirectionally through the entire body via peristalsis. The segmented movement is also clearly indicated by secondary staining. Unlike the segmented movement in hydra, the flatworm exhibits regular and centrifugal sphincter contractions inwards to the outermost branches or the highest order end (Fig. 13A to D). It then undergoes centripetal movement from the outermost or narrowest branches outward to the pharynx or the first-order digestive tract (Fig. 13E to H), resulting in a rhythmic movement pattern. Although blind sacs are distributed along the tract, there are no flows originating from any blind sac between the two ends of the branched tract.

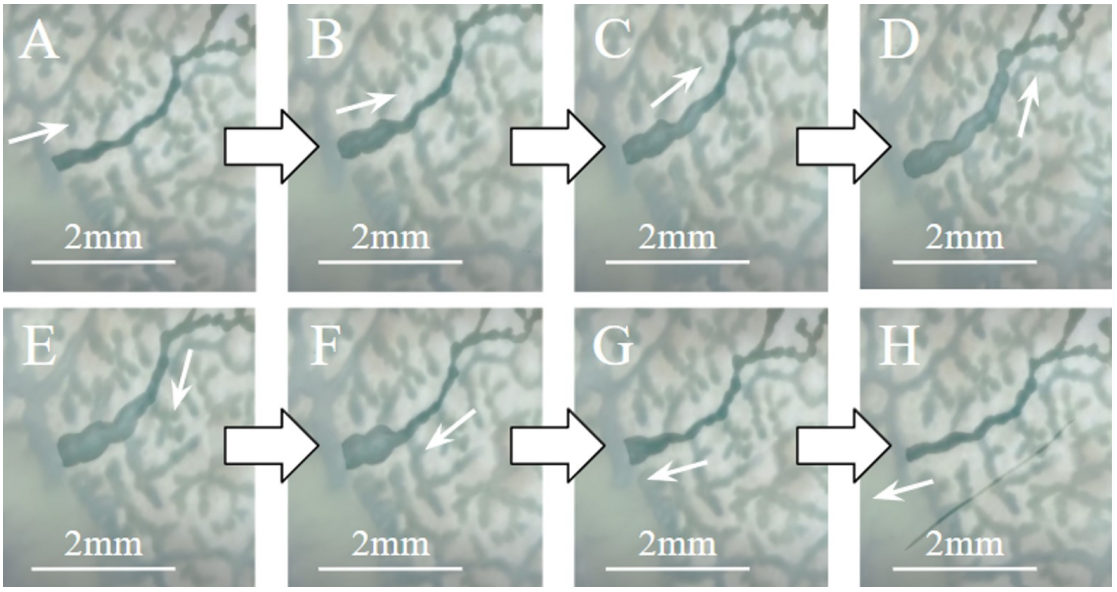


Fig. 13. Serial pictures from A to D show the inward gastrovascular flow and E to H show the outward flow in the same track of *Paraplanocera oligoglena* (flow direction indicated by white arrow).

In post-stain active tracking, we designate the order of ‘n’ as the given order of the observing branch,followed by the subsequent digestive tract of the next first order as n+1, the next secondary order as n+2, and the higher tertiary order as n+3. Using OpenCV and its tracker package, we track n, n+1, n+2, n+3 order branches bidirectionally. We then cooperate with Matplotlib to plot the staining area ratio within the selected range against time. In the video, the contraction begins as the digestive tract of the flatworm narrows. Consequently, it can be deduced that the wave trough of the curve represents the duration of contraction, and the period between two peaks and wave troughs is measured as a peristalsis cycle (Fig. 14).

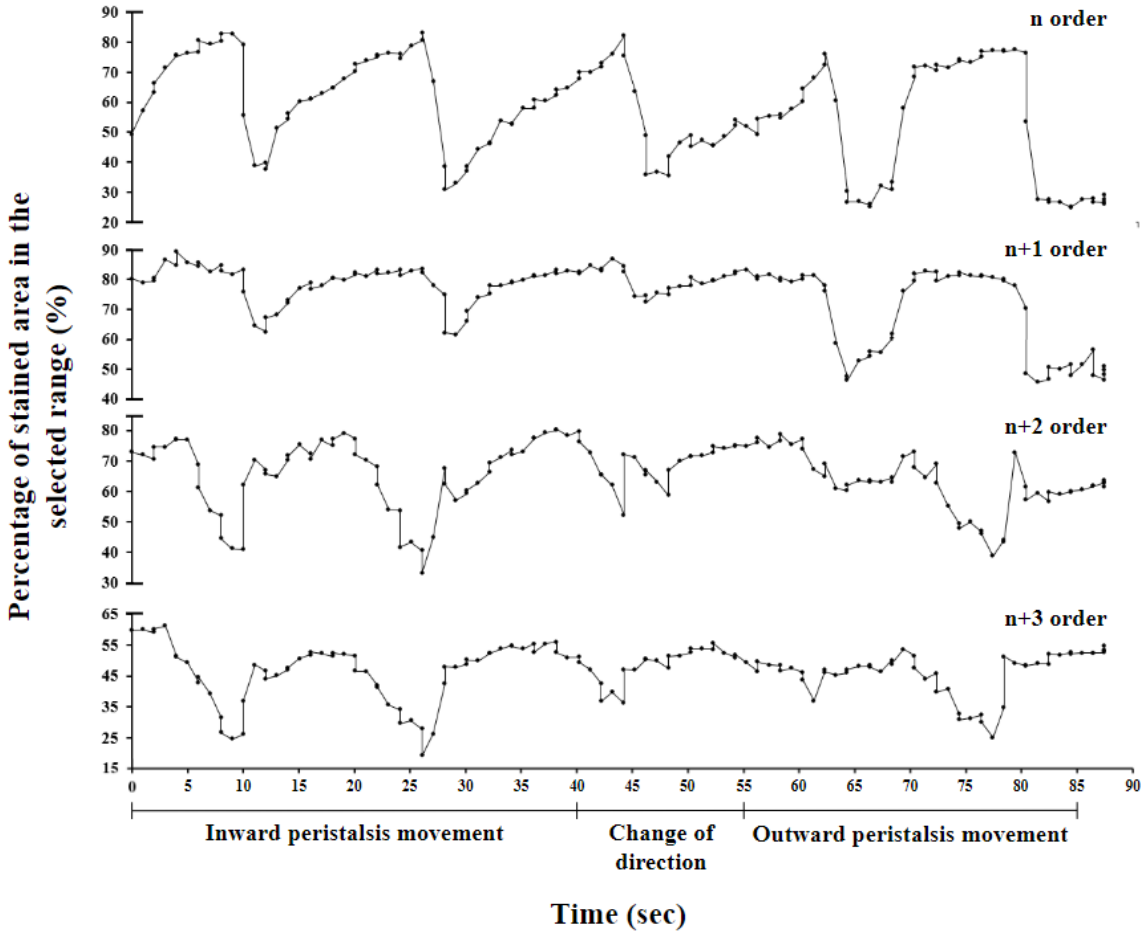


Fig. 14. Post-stain active tracking of *Paraplanocera oligoglena* in given order branches n, n+1, n+2, n+3. Inward peristalsis movement occurs from the second 0 to 40. The bidirectional gastrovascular flow changes direction during the second 40 to 55. Outward peristalsis movement is observed from the second 55 to 85. The wave trough of the curve indicates the duration of contraction, and the period between two peaks and wave troughs represents a peristalsis cycle.

It is noteworthy that the contraction frequency and cycle of the digestive tract maintain a constant speed in the same direction, regardless of the branches of higher or lower order tract (Fig. 15).

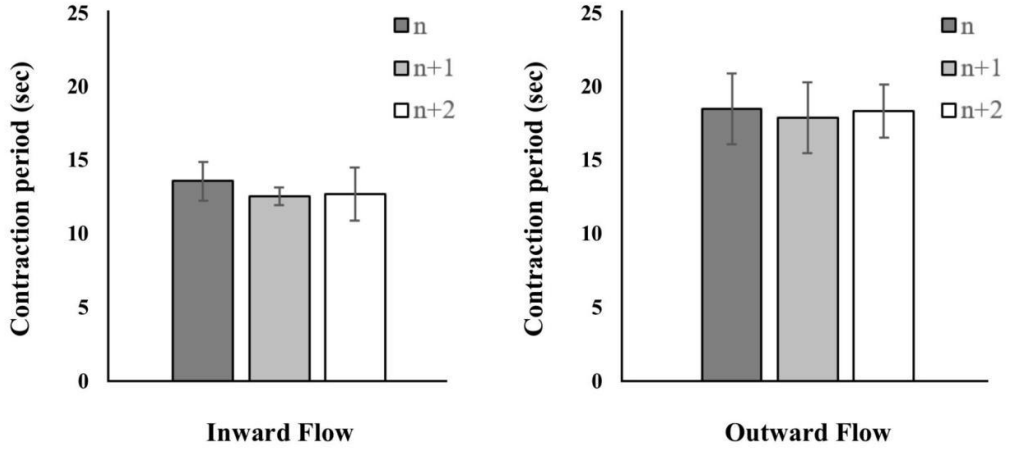


Fig. 15. Contraction cycles of *Paraplanocera oligoglena* in given order branches 'n,' 'n+1,' 'n+2.' The two-directional contraction cycles continually progress.

This result suggests that *Paraplanocera oligoglena* does not rely solely on the pharynx contraction for transporting food. The evident segmented movements indicate that flatworms engage the contraction of the tract itself in the process. Fixed segment positions contract regularly and sequentially, resulting in rhythmic movement patterns in both directions. Regardless of the direction, the movement remains consistent and sequentially bidirectional, albeit at an uneven rate.

The digestive movement of *Paraplanocera oligoglena* dramatically decreases after about one day and comes to a complete stop within two days (Fig. 16). These findings provide evidence that peristaltic and segmented movements serve as functions of the flatworm's gastrovascular cavity, and the sequentially bidirectional flow is a digestion behavior observed in polyclad flatworms.

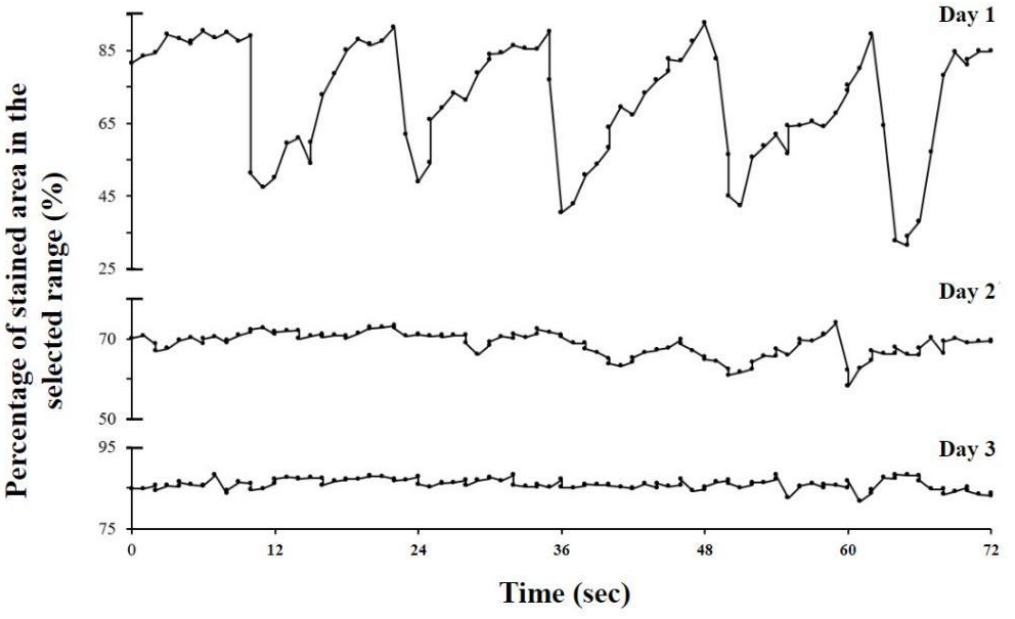
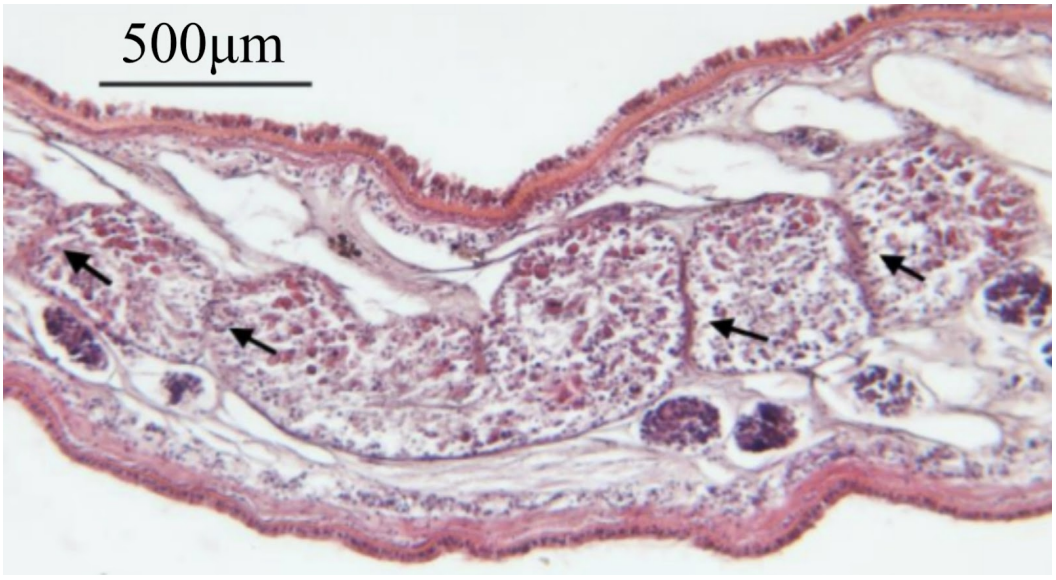


Fig. 16. The graph illustrates contraction cycles in the digestive tract of *Paraplanocera oligoglena* on the first, second, and third days.

Upon concluding the ingestive period, the flatworm regurgitates the remaining food residues back to the pharynx and expels them. This behavior resembles the defecation reflex observed in hydra (Shimizu et al 2004), hence termed a defecation-like movement.

Circular membrane-like muscle inside digestive tract

To comprehend the anatomical structure within the digestive tract of *Paraplanocera oligoglena*, we conducted microscopic observations on serial sections of the digestive tract. Our findings reveal that circular membrane-like muscle structures are evenly distributed along the tract (Fig. 17A). Furthermore, we calculated the average distance between these circular membranous muscles in digestive tracts of low, medium and high orders, discovering that the average distance increases in lower-order tracts and decreases in higher-order tracts. This aligns with Newman and Cannon's (2003) description of small muscular valves along the branches of Planocera sp., supporting our results (Fig. 17B). We infer that these circular membranous muscles serve as contracting and pushing forces within the digestive tract.



(A)

(B)

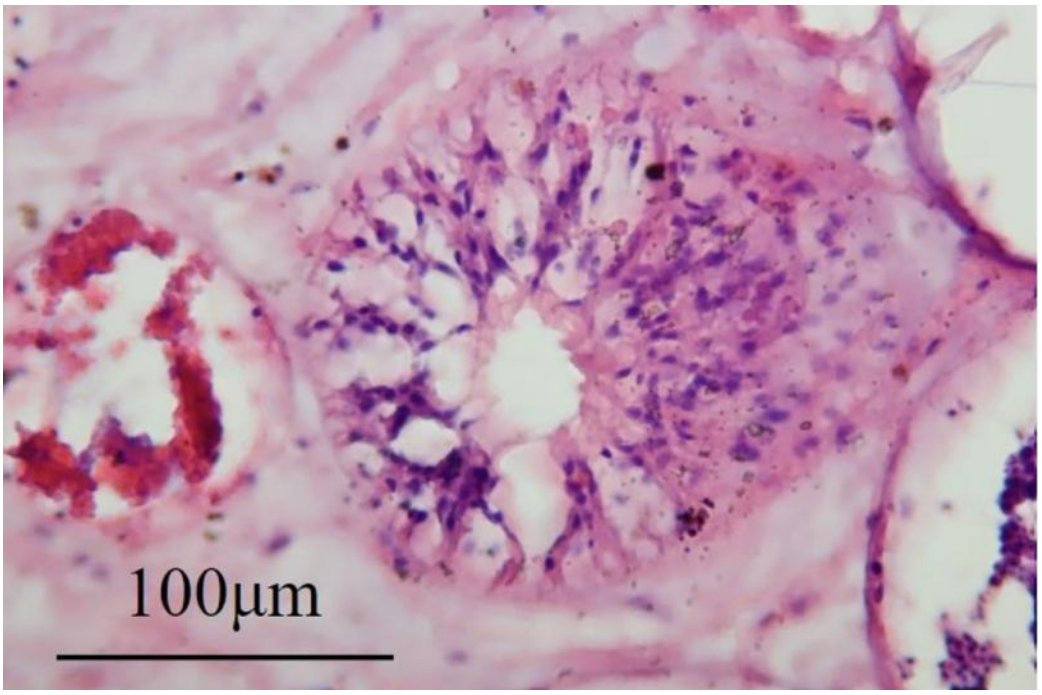


Fig. 17. Inside the digestive tract of *Paraplanocera oligoglena* (A) Longitudinal section highlighting circular membrane-like muscles(black arrows). (B) Cross section of circular membrane-like muscles.

Discussion

There are over fifty polyclad flatworm species in the waters near Taiwan (Jie 2017). Most of those species are seldom mentioned, being among the rarest. However, the focus of this study, *Paraplanocera oligoglena,* stands out as it is relatively easy to collect from intertidal zones across Taiwan's waters. Laboratory feeding experiments revealed a diverse diet, encompassing various gastropods such as sea snails and sea hares (refer to Table 1). This broad range of food sources may contribute to the widespread distribution of flatworms in Taiwan.

Notably, sea hares (Aplysia) are known to contain components of TTX (Paul and Pennings 1991), a defensive mechanism against natural predators. This prompts speculation about the predation relationship between *Paraplanocera oligoglena* and TTX-bearing sea hares like *Stylocheilus striatus*. It remains unclear whether the predation behavior of planocerid flatworms involves the utilization of chemicals to capture gastropod snails.

Although this study also revealed that *Paraplanocera oligoglena* consumes Asian hard clams (*Meretrix lusoria*), a bivalve from the order Bivalvia, confirmation through field observations is lacking. Therefore, it is inappropriate to infer *P. oligoglena* is a natural predator. The widespread availability of clams in local markets makes them a convenient substitute for bait preparation, facilitating the extended maintenance of flatworms in tanks. This extended duration is crucial for observing behaviors such as predation rates on fresh baits and bidirectional digestion movements with stained food.

Wells and Sebens (2017) developed a novel method to mark sea anemones using neutral red, methylene blue, and fluorescein through injection in laboratory and field studies. We modified the procedure and used these dyes to prepare stained clams for *Paraplanocera oligoglena*. However, after consuming dyed bait with luciferin, observing the results in the flatworms' digestive system proved challenging, even under blue light in a dark room (personal observation). Notably, neutral red was excluded from bait preparation due to its potential interference with their transparent brown body color pattern. In this secondary staining experiment, 0.1% methylene blue emerged as the most suitable dye for observing the digestive tract. The food dyeing technology employed in this experiment opens avenues for future research on food chains in the wild.

In both post-stain static processing and active tracking, methylene blue dye not only indicates the widest primary order but also the narrowest order tracts of the highly branched digestive system. Merely using OpenCV to analyze the digestive tract resulting in difficulty identifying the exact pathway due to incomplete and discontinuous branches. However, when employing OpenCV to analyze the image or video in post-stain bait test, the digestive tract had been secondarily dyed blue by methylene blue, and most are complete and continuous branches of the digestive tract that wildly distribute and reach every portion of its body. Additionally, during the digestion period, polyclad flatworms often remain almost stationary for more than six hours (Gammoudi et al 2017; Tang et al 2022). Therefore, this novel experimental method contributes to observing and tracking the movements of the digestive tract in real time. It is likely that many polyclad flatworms have a long, stationary period during the ingestion period, suggesting that future studies of image static processing and video active tracking methods can be applied to more flatworm species.

The gastrovascular bidirectional flow was reported by Harmata et al in 2013. Now it has been proved in polyclad flatworms. The sequentially bidirectional flow found in *Paraplanocera oligoglena* uses circular membrane-like muscles in the digestive tract to perform two directional peristalsis. Partially digested food is transported back and forth at a constant speed in the digestive system, so it also has the segmentation function of mixing and transporting food. This might not practically be similar to hydrozoans (Harmata et al 2013), because the digestive tract of flatworms is more highly branched. The bidirectional flow needs more complex nerves to coordinate with tracts in every different order level. These more complex nerves regulate the low order branches and the high order branches to keep the same direction and at the same speed. And then, the flatworm's nerve system sequentially coordinates the digestive branched system moving in reverse.

Newman and Cannon (2003) initially described the multibranched system of *Planocera* sp., suggesting the presence of small muscular valves along the branches. The muscular valves are proved by this study. We found that circular membrane-like muscle structures (Fig. 17) are distributed along the digestive branches. These structures can regulate flow within the tract, providing waves of contraction to fill or clear the gut (Newman and Cannon 2003). Following secondary staining with stained bait, the sequentially bidirectional flow is identified in *Paraplanocera oligoglena* (Fig. 13, 14, 15). The description parallels descriptions found in cnidarians (Harmata et al 2013) but is unprecedented in flatworms.

The digestive movement cessation two days after feeding provides evidence that the peristaltic and segmented movements primarily serve digestive functions, rather than circulatory function in polyclad flatworms (Fig. 16). This result might explain why polyclad flatworms tend to be larger and more mobile than other flatworms (Newman and Cannon 2003), but still require an extremely thin body plan for efficient diffusion and interchange of materials with their environment.

Wells and Sebens (2017) proposed a staining technique capable of marking individuals in distinguishable patterns for short-term studies, such as examining predator-prey interactions and movement. This study demonstrates the effectiveness of this marking technology in helping observers identify prey-predator relationships. Given that methylene blue remains detectable in dye-receiving flatworms, and the experimented flatworms did not show immediate reluctance in subsequent feeding tests. In the future, it's worth considering the application of marking technology through food chains in tidal pool animals as well as tracking flatworm behavior in their natural habitat.

Conclusions

1. *Paraplanocera oligoglena* is confirmed as the most common species in Taiwan.

2. Feeding experiments conducted in tanks reveal that *Paraplanocera oligoglena* can prey on various gastropod species, including sea snails and sea hares.

3. Predation behavior involves attack, invasion and ingestion periods.

4. This is the first time stained clam and static image analysis has been used to observe the highly branched digestive system of flatworms.

5. The post-stain active tracking technique has revealed, for the first time, a sequentially bidirectional flow within the gastrovascular cavity of polyclad flatworms.

6. The assessment of peristalsis movement in both directions, along with segmented movement, indicates that the contraction frequencies are roughly equal.

7. It is confirmed that the circular membrane-like muscles within the digestive tract are the main driving force for transportation and mixing of food.

References

Avian M, Mancini L, Voltolini M, Bonnet D, Dreossi D, Macaluso V, Pillepich N, Prieto L, Ramsak A, Terlizzi1 A, Motta, G. 2022. A novel endocast technique providing a 3D quantitative analysis of the gastrovascular system in *Rhizostoma pulmo*: An unexpected through-gut in cnidaria. Plos one **17**: e0272023. doi: 10.1371/journal.pone.0272023

Bradski G. 2000. The openCV library. Dr. Dobb's Journal: Software Tools for the Professional Programmer. **25**: 120-123.

Brusca RC, Moore W, Shuster SM. 2016. *Invertebrates*. Sinauer Associates Incorporated. Third edition. UK.

Campbell NA, Reece JB. 2005. Biology. Pearson Education. International edition. US.

Chen YS (2001) Seashore life (1), 700 Intertidal species in Taiwan. Taipei, Taiwan. Recreation Press (in Chinese).

Dittmann IL, Dibiasi W, Norena C, Egger B. 2019. Description of the snail-eating flatworm in marine aquaria, *Pericelis tectivorum* sp. nov.(Polycladida, Platyhelminthes). Zootaxa **4565**: 383-397.

Faubel A. 1983. The Polycladida, Turbellaria. Proposal and establishment of a new system. Part I. The Acotylea. Mitt hamb zool Mus Inst 81:189–259.

Harmata KL, Parrin AP, Morrison PR, McConnell KK, Bross LS, Blackstone NW. 2013. Quantitative measures of gastrovascular flow in octocorals and hydroids: toward a comparative biology of transport systems in cnidarians. Invertebr Biol **132**: 291-304.

Harris CR, Millman KJ, van der Walt SJ, Gommers R, Virtanen P, Cournapeau D, Wieser E, Taylor J, Berg S, Smith NJ, Kern R, Picus M, Hoyer S, van Kerkwijk MH, Brett M, Haldane A, Fernandez del RJ, Wiebe M, Peterson P, Gerard-Marchant P, Sheppard K, Reddy T, Weckesser W, Abbasi H, Gohlke C, Oliphant TE. 2020. Array programming with NumPy. Nature **585**:357–362. doi: 10.1038/s41586-020-2649-2

Hunter JD. 2007. Matplotlib: A 2D graphics environment. Comput Sci Eng **9**: 90-95.

Hyman LH. 1953. The polyclad flatworms of the Pacific coast of North America. Bull Am Mus Nat Hist **100**: 265–392.

Gammoudi M, Ben Ahmed R, Bouriga N, Ben-Attia M, Harrath AH. 2017. Predation by the polyclad flatworm *Imogine mediterranea* on the cultivated mussel *Mytilus galloprovincialis* in Bizerta Lagoon (northern Tunisia). Aquac Res **48:** 1608–1617. doi:10.1111/are.12995

Jennings JB. 1957. Studies on feeding, digestion, and food storage in free-living flatworms (Platyhelminthes: Turbellaria). Biol Bull **112**: 63-80.

Jie WB. 2017. Taxonomy and Phylogeny of Polyclad Flatworms (Platyhelminthes) of Taiwan. Ph.D. Dissertation, National Sun Yat-sen University, Taiwan.

Kato K. 1943. Polyclads from Formosa. Bull Biogeogr Soc Jap **13**: 69-77.

Koopowitz H. 1970. Feeding behaviour and the role of the brain in the polyclad flatworm, Planocera gilchristi. Anim Behav **18**: 31-35.

McKinney W. 2010. Data structures for statistical computing in python. In Proceedings of the 9th Python in Science Conference. **445**: 51-56.

Newman LJ, Cannon LRG. 2003. Marine Flatworms: the world of polyclad flatworms. CSIRO Publishing, Collinwood, Australia.

Paul VJ, Pennings SC. 1991. Diet-derived chemical defenses in the sea hare Stylocheilus longicauda (Quoy et Gaimard 1824). J Exp Mar Biol Ecol **151**: 227-243.

Schmarda LK. 1859. Neue wirbellose Thiere beobachtet und gesammelt auf einer Reise urn die Erde 1853 bis 1857. Bd 1: Turbellarien, Rotatorien und Anneliden. 1 Halite: 66 pp. W. Engelmann, Leipzig.

Shimizu H, Koizumi O, Fujisawa T. 2004. Three digestive movements in Hydra regulated by the diffuse nerve net in the body column. J Comp Physiol **190**: 623-630.

Shimizu H, Okabe M. 2007. Evolutionary origin of autonomic regulation of physiological activities in vertebrate phyla. J Comp Physiol **193**: 1013-1019.

Soutullo P, Cuadrado D, Norena C. 2021. First study of the Polycladida (Rhabditophora, Platyhelminthes) from the Pacific Coast of Costa Rica. Zootaxa **4964**: 363-381.

Tsuyuki A, Oya Y, Jimi N, Kajihara H. 2020. Description of *Pericelis flavomarginata* sp. nov. (Polycladida: Cotylea) and its predatory behavior on a scaleworm. Zootaxa **4894**: zootaxa.4894.3.6 doi: 10.11646/zootaxa.4894.3.6

Wells CD, Sebens KP. 2017. Individual marking of soft-bodied subtidal invertebrates in situ–A novel staining technique applied to the giant plumose anemone *Metridium farcimen* (Tilesius, 1809). Plos one **12**: e0188263.

【評語】050018

這項研究對台灣常見的多齒板蟲 Paraplanocera oligoglena 的捕食行為進行了觀察，並利用染色技術和靜態影像分析來研究其高度分支的消化系統。稀有的捕食行為研究：多齒板蟲的捕食行為研究非常罕見，這項研究為這一領域帶來了新的認識。

1. 新技術的應用：運用染色蚌類和靜態影像分析技術來觀察多齒板蟲的消化系統，這在研究方法上是創新的。

2. 消化系統的詳細觀察：首次發現多齒板蟲胃血管腔中的循序雙向流動，提供了消化系統功能的新見解。

3. 深入理解多齒板蟲的生理機制：這項研究有助於深入理解多齒板蟲的消化過程和生理機制。

4. 對生態系統的潛在影響：研究多齒板蟲的捕食行為有助於了解海洋生態系統中的食物鏈和能量流動。

5. 促進未來研究的可能性：所使用的食物染色技術為未來在野外進行食物鏈研究提供了新的方法。

研究建議：

1. 特定物種的限制：研究僅對特定的多齒板蟲物種進行，其結果可能不適用於其他物種或其他生態環境。建議應多參考文獻，補充背景資料。
2. 未來需要探討消化道的腸道微生物、化學物質及營養吸收，讓試驗更完整。