A new accumulation assay of *Schistosoma mansoni* miracidia using square capillary glass tubes

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Abstract

Current control measures for schistosomiasis have only been partially successful in endemic areas due to socioeconomic constraints. One possibility for controlling the disease is to aim at the miracidial stage of the trematode to avoid infecting intermediate snail hosts by introducing more attractive substances for miracidia in the environment. Here, we introduce an accumulation assay of *Schistosoma mansoni* miracidia using a square glass tube for analysis of the positive responses of miracidia toward several substances, including snail-conditioned water of *Biomphalaria glabrata*, *Bulinus globosus* and insusceptible snails collected in the Nagasaki area in Japan. The substances are not proteins because miracidia accumulated in boiled snail-conditioned water and the secretion or emission level of substances depended on the feeding conditions of *Biomphalaria glabrata*. The present study also showed that substances emitted from *Biomphalaria glabrata* with a molecular weight around 10 kDa accumulated *Schistosoma mansoni* miracidia. Further, we showed that *Schistosoma mansoni* miracidia did not accumulate in response to mono- or disaccharides tested in the study.

Keywords: *Schistosoma mansoni*, Accumulation, *Biomphalaria glabrata*, Miracidium, Capillary glass tube

1. Introduction

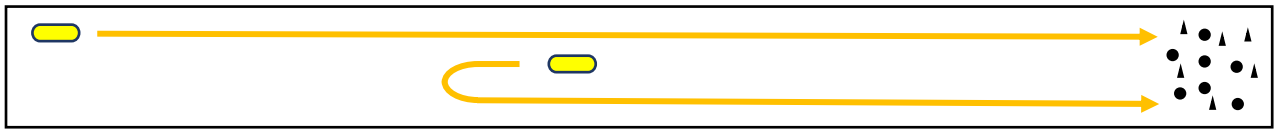
Schistosomiasis, also known as Bilharziasis or snail fever, is a tropical parasitic disease caused by blood-dwelling fluke worms of the genus *Schistosoma* (Gryseels et al., 2006). This disease affects 200 million people worldwide, and 600 million are at risk of infection (Chitsulo et al., 2000). Some 66 million people were treated with praziquantel in 2015, but an estimated 219 million people continue to require treatment for schistosomiasis (WHO, 2016). However, the emergence of drug-resistant parasites is a threat to the main strategy for control of schistosomiasis using praziquantel (Doenhoff et al., 2002, WHO, 2011, Wang et al., 2012). Thus, development of new complementary tools is needed for control of schistosomiasis.

Three major parasite species cause schistosomiasis in humans: *S. mansoni* transmitted by *Biomphalaria* snails and *S. japonicum* transmitted by *Oncomelania* snails cause intestinal and hepatic schistosomiasis; and *S. haematobium* transmitted by *Bulinus* snails causes urinary schistosomiasis (Gryseels et al., 2006). Three other human-infecting species, *S. intercalatum*, *S. mekongi* and *S. guineensis*, are of local importance. Interestingly, miracidia of these parasites have specific intermediate snail hosts and cannot develop to cercariae in the wrong hosts. Thus, control of schistosomiasis may be possible using decoy snails and capture of miracidia in non-intermediate hosts. However, this may cause disruption of ecosystems in the area in which the decoy snails are introduced. Using attractive substances from host snails is an alternative way to capture miracidia and prevent them from invading intermediate snails.

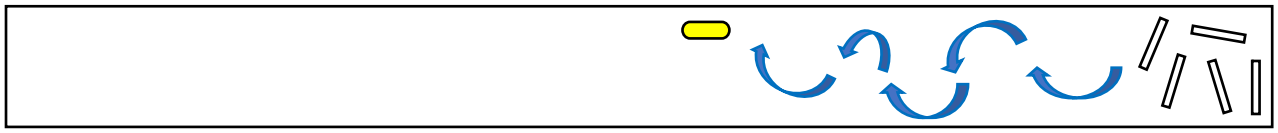
The search for host snail-derived or synthetic substances to which *S. mansoni* miracidia

will respond has lasted for more than 70 years. Short-chain fatty acids, amino acids, sialic acids, ions, glucose, 80 kDa glycoprotein, >300 kDa glycoconjugates and other compounds have been identified as candidates, but with different findings in terms of the molecular size (MacInnis, 1965; MacInnis et al., 1974; Stibbs et al., 1976; Plorin and Gilbertson, 1985; Dissous et al., 1986; Haberl and Haas, 1992; Haberl et al., 1995; reviewed in Haas et al., 1995). These differences may be due to use of different systems and techniques for observation of chemotaxis and chemokinesis (increase of random movement) of miracidia. Also, the terms "(chemo)taxis" and "(chemo)kinesis" have been used for the same response of miracidia to substances, and these terms are further subjectively classified as chemo-ortho-kinesis, chemo-klino-kinesis, chemo-klino-taxis and chemo-tropo-taxis (MacInnis, 1965), which has caused confusion (reviewed in Saladin, 1979). To improve the clarity, in this study we use miracidial "accumulation" as an objective state with a combination of positive taxis and kinesis in developing a new analytical method using a square capillary glass tube (Fig. 1).

(A) (Chemo)taxis



(B) (Chemo)kinesis



(C) Accumulation

Fig. 1. Schematics of accumulation of miracidia. (Chemo)taxis is a response of straight path swimming of miracidia (A). (Chemo)kinesis is a response of non-directed turning of miracidia (B). Accumulation is a combined response with (chemo)taxis and (chemo)kinesis (C). Yellow ovals indicate miracidia. Open boxes, closed circles and closed triangles indicate substances inducing a response of miracidia in snail-conditioned water. **(colored figure)**

2. Materials and Methods

2.1. Parasite and snail strains

A Puerto Rican strain of *S. mansoni* (NIH-Sm-PR-1) was routinely maintained by passage through 6-week-old female ICR mice (Japan SLC, Inc. Hamamatsu, Japan) and *Biomphalaria glabrata* snails (Newton's NIH Puerto Rican/Brazilian M-line) (Matsumura et al., 1991) in the animal facility in the Biomedical Research Center of Nagasaki University. *Bulinus globosus* snails (originating from Mwachinga Community, Kwale District, Kenya), the intermediate host of *Schistosoma haematobium*, have been kept in our laboratory since the 1980's and were also used in the study. *B. glabrata* and *B. globosus* snails were fed on lettuce for 7 days prior to experiments. The experimental protocol was approved by the Ethical Review Committee for Animal Experimentation of Nagasaki University (Approval number: 2005011626). All animal experiments were performed in accordance with the guidelines for animal experimentation in the Biomedical Research Center at Nagasaki University.

2.2. *Isolation of* S. mansoni *eggs*

Isolation of *S. mansoni* eggs was performed using a method modified from that of Katsumata (1988). Briefly, at 8 weeks post-infection with 200 *S. mansoni* cercariae, the infected mice were anesthetized with isoflurane inhalation, the hepatic portal vein was cut, and the livers was perfused with 30 ml of sodium citrate buffer (0.75% trisodium citrate and

0.85% NaCl in H_2O, pH 7.4) via the cardiac left ventricle, followed by perfusion with 20 ml of phosphate-buffered saline (PBS). The removed liver was dipped into PBS and left to stand overnight at room temperature. It was then minced with scissors and a blender, and incubated with 0.1% actinase E (Kaken Pharmaceutical Co., Ltd.) in PBS in an Erlenmeyer flask for 3 hr at 37℃ with shaking. After centrifugation at 2,000 rpm for 5 min at room temperature, the supernatant was discarded and the pellet was further digested by incubation with 0.01% actinase E and 0.05% collagenase (Wako) in PBS for 2 hr at 37℃. The digest was centrifuged and washed 3 times in PBS at 2,000 rpm for 5 min at room temperature. The egg pellet was transferred into a 15-ml tube and washed twice in PBS with brief spinning. The harvested eggs were kept in PBS at room temperature overnight and then used in experiments.

2.3. Preparation of miracidia of S. mansoni

Fresh eggs of *S. mansoni* were mixed with dechlorinated tap water (DTW) and transferred to a 15-ml tube with the lower half covered with aluminum foil. The tube was placed under sunlight or an artificial light at 26ºC. Miracidia hatched from the eggs concentrated at the top of the water by phototaxis. The miracidia were collected and used for further studies.

2.4. Preparation of snail-conditioned water from B. glabrata *and* B. globosus

Snail-conditioned water (SCW) of *B. glabrata* or *B. globosus* was prepared by placing two

2-mm snails in 1 ml of DTW in a well of a 24-well multiple well plate overnight. After spinning down solid materials in the culture at 2,000 rpm for 5 min, 800 µl of the supernatant was collected and used undiluted as SCW. Further, 500 µl of the SCW was fractionated using Amicon ultra filter tubes (Merck Millipore Ltd.) with molecular weight (MW) cutoffs of 100,000, 50,000, 30,000, and 10,000. The filtrate and residue were adjusted to 500 µl with DTW and used in experiments. In use of a mixture of the filtrate and residue, a half volume of the filtrate and residue after fractionation was mixed and another half volume of the filtrate and residue was adjusted to 250 µl with DTW. One microliter of a sample was used for each accumulation assay. In some experiments, boiled (100ºC, 10 min) SCW and SCW from starved *B. glabrata* were used.

2.5. Non-susceptible snails collected in Nagasaki, Japan

Stenothyra japonica, *Assiminea hiradoensis*, *Angustassiminea castanea*, *Gyraulus spirillus* and *Physella acuta*, all of which are non-susceptible to *S. mansoni* infection, were collected in the Nagasaki area and used for accumulation and penetration assays. *B. glabrata* was used as a positive control in these assays. The snails were kept under the same conditions as for *B. glabrata*, with one snail per well of a 24-well plate in 1 ml DTW overnight prior to collection of SCW of the snails. The sizes of the snails used for accumulation and penetration assays (Table 1) were measured using electronic calipers (Mitutoyo Co.). For the accumulation assay

in this setting, 0.5 µl of SCW were used for each experiment.

Table 1. Sizes of snails used in the accumulation and penetration assays

Snail species (n=5)	Length (Mean ± SD[a], mm)	Width (Mean ± SD, mm)
Stenothyra japonica	4.1 ± 0.2	2.5 ± 0.1
Assiminea hiradoensis	6.3 ± 0.3	4.0 ± 0.3
Angustassiminea castanea	2.9 ± 0.2	1.7 ± 0.1
Gyraulus spirillus	3.8 ± 0.4	3.2 ± 0.4
Physella acuta	4.2 ± 1.0	2.2 ± 0.2
*Biomphalaria glabrata**	6.6 ± 0.4	5.4 ± 0.4
*Biomphalaria glabrata***	7.6 ± 1.4	6.6 ± 1.0

*Snails used only in accumulation assay.

**Snails used only in penetration assay.

[a] Standard deviation.

2.6. Preparation of mono- and disaccharide solutions

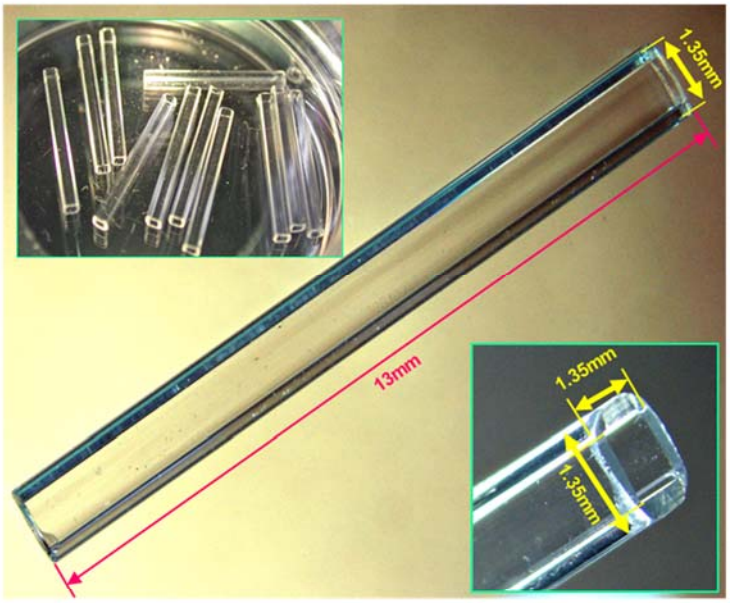
Solutions (all 10 mM) of galactose, mannose, glucose, *N*-acetylgalactosamine (Fujifilm Wako Pure Chemical Corporation), lactose, maltose, and trehalose (Tokyo Chemical Industry Co. Ltd.) were prepared in DTW and used in accumulation assays.

2.7. Experimental procedures for accumulation analyses

A both-end-open square glass capillary tube (13×1.35×1.35 mm, Hilgenberg GmbH, Germany; Fig. 2A) was used for analysis of accumulation of miracidia. The capillary tube was divided into three equal areas: zones A, B and C. Zone C was defined as the zone where

the samples were loaded, Zone B as the middle area, and Zone A as the other end of the tube (Fig. 2B). Each tube was filled with 9.5-10.0 µl of DTW containing 3-10 miracidia per tube freely swimming for 30 sec before addition of 0.5 or 1 µl of SCW or other samples to one end of the tube (Fig. 2B, Zone C). The miracidia were used within 3 hr after hatching when they were still fresh. Movement of miracidia was recorded from 30 sec before to 1 min after sample loading under a J-scope video recording system (Satotech)-equipped SMZ745 stereoscopic microscope (Nikon). The number of miracidia in each zone was counted every 5 sec and the proportion (%) of miracidia was calculated as: proportion in Zone A, B or C (%) = 100 × (number of miracidia in each zone) / (total number of miracidia used in the experiment).

(A)



(B)

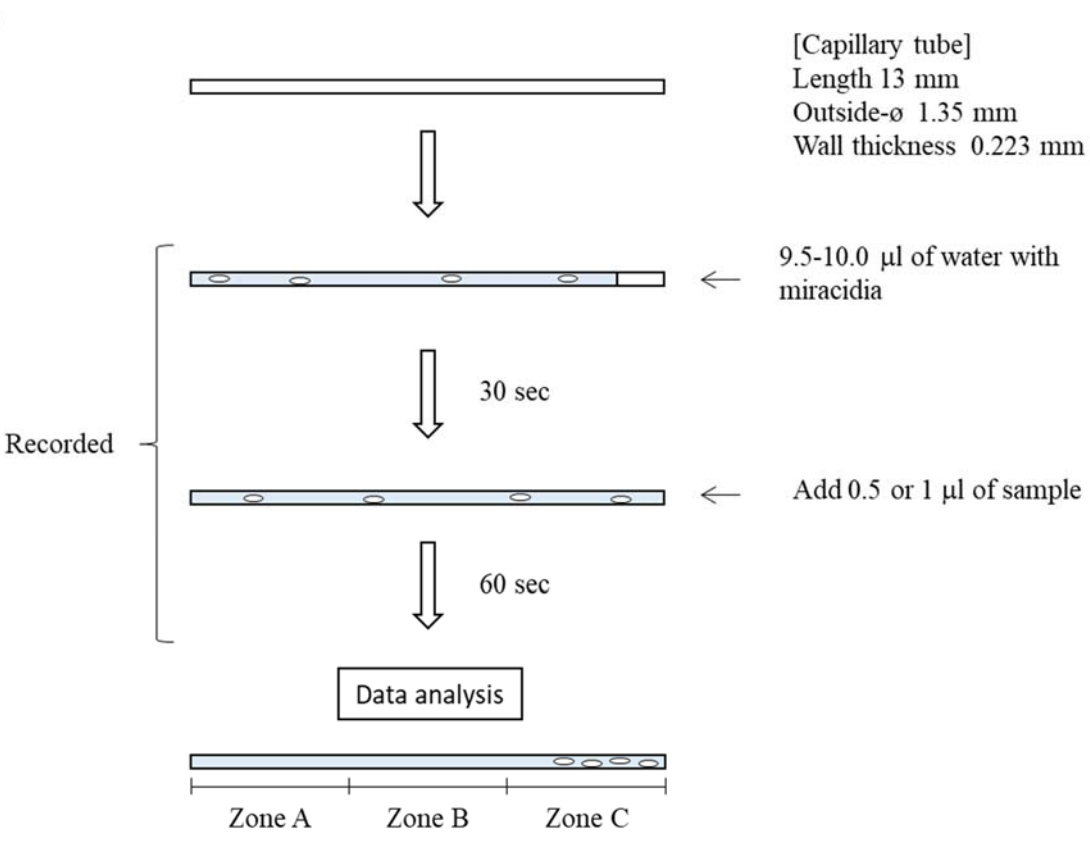


Fig. 2. Images of capillary glass tubes (A) and scheme of the study (B). White ovals indicate miracidia. **(colored figure)**

2.8. Experimental procedures for the penetration assay

Non-susceptible snails and *B. glabrata* described in section 2.5. and Table 1 were used for the assay. Ten *S. mansoni* miracidia were added to a well of a 24-well plate (Sumitomo Bakelite Co., Ltd.) and one snail was added together with 1 ml DTW. The number of miracidia that had not attached to or penetrated the snail were counted first at 10 min and then at 15-min intervals up to 130 min (i.e. at 10, 25, 40, 55, 70, 85, 100, 115 and 130 min) using a stereoscopic microscope. Five independent studies were conducted.

2.9. Statistical analysis

Comparisons of the number of miracidia remaining swimming in SCW of insusceptible snails with that of *B. glabrata* were conducted using a Mann-Whitney test in Epi-Info software (ver. 7, Centers for Disease Control and Prevention, Atlanta, GA, USA).

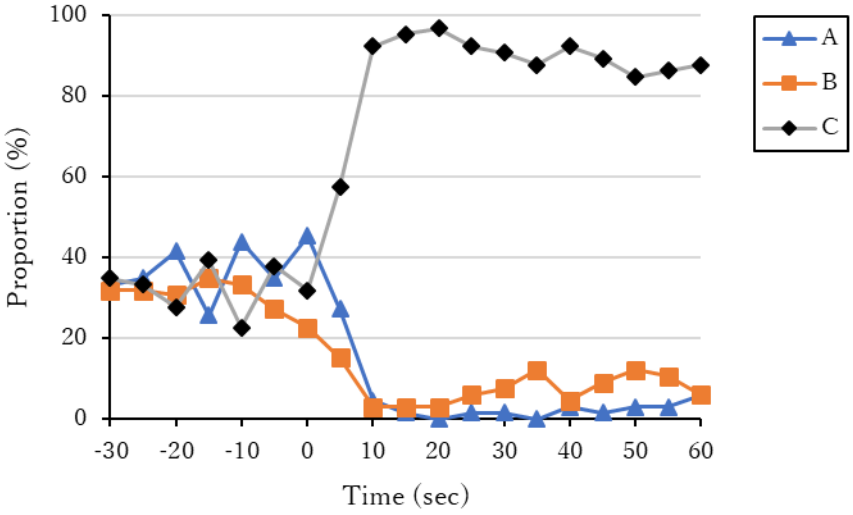
3. Results

3.1. Accumulation of S. mansoni *miracidia by SCW of* B. glabrata *and* B. globosus

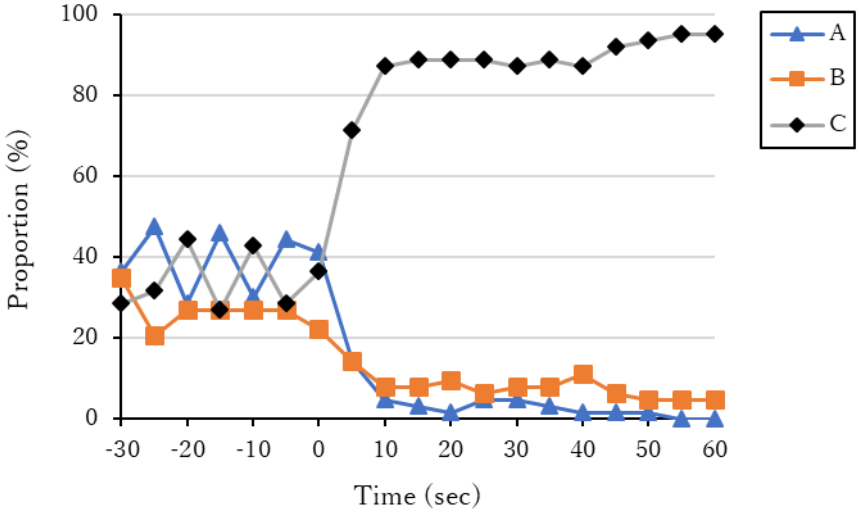
Studies using a cast-iron center chamber with four side arms, coverslip chambers and Plexiglas choice-chambers have been performed to identify attractants for *S. mansoni* miracidia (Etges and Decker, 1963; Roberts et al., 1979; Haberl et al., 1995). However, data

obtained in these studies have been somewhat unclear, with the effects of waves, current and vibration affecting miracidial responses not considered when the samples were applied. To minimize these stimuli, we used square capillary glass tubes in the current study (Fig. 2A). A schematic diagram of the study is shown in Fig. 2B. Directly after loading susceptible *B. glabrata* SCW into a tube in which *S. mansoni* miracidia were freely swimming (time 0), the miracidia swam straight to Zone C and accumulated in this zone with non-directed turning (Fig. 3A, Movie 1). The same reaction of the miracidia occurred with insusceptible *B. globosus* SCW (Fig. 3B, Movie 2), but not with DTW (Fig. 3C, Movie 3). These results indicate that the substances causing accumulation of miracidia were derived from both snail species.

(A) Biom (n=66)



(B) Bulinus (n=63)



(C) Water (n=71)

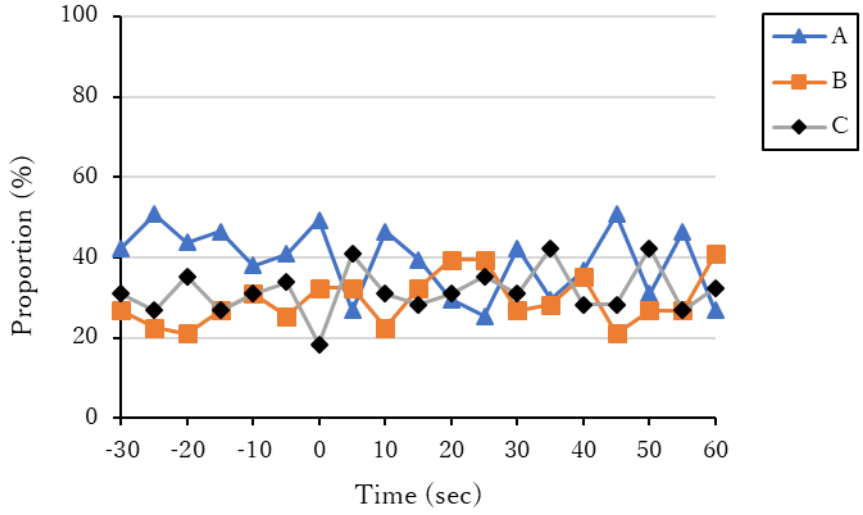
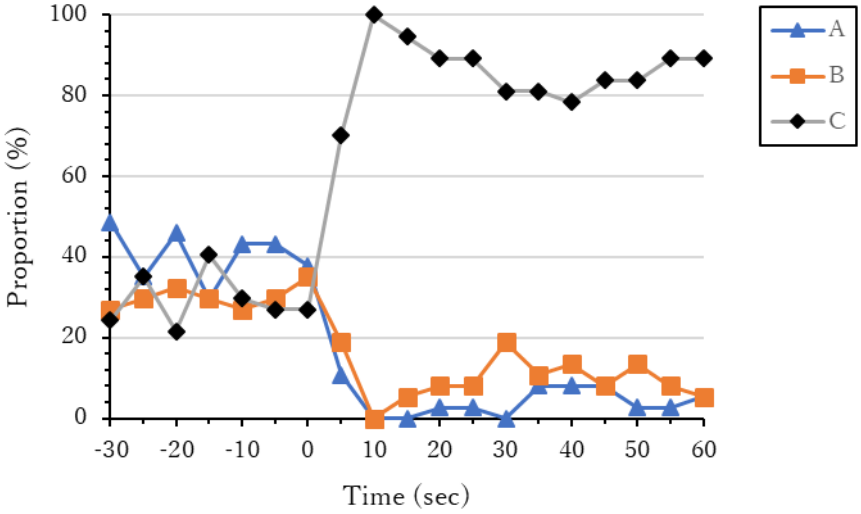


Fig. 3. Accumulation of *S. mansoni* miracidia in response to *B. glabrata* SCW (A), *B. globosus* SCW (B), and DTW (C). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown for each time point. The samples were loaded at time 0. The numbers of miracidia exposed to the stimulants in (A) to (C) were 66, 63 and 71, respectively.

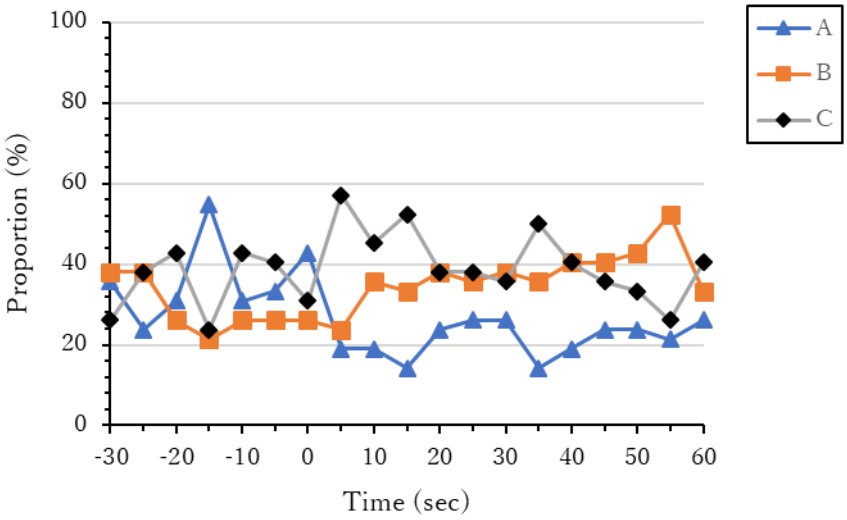
3.2. Effect of feeding conditions of B. glabrata *on accumulation of* S. mansoni *miracidia in SCW*

To characterize the substances in SCW of *B. glabrata* accumulating the miracidia, we first examined if secretion or emission of the substances depended on the feeding conditions of the snails. Fed or starved *B. glabrata* snails were prepared and SCW was collected as described above. As shown in Figure 4, *S. mansoni* miracidia were attracted to SCW from fed snails, but not to SCW from starved snails. In moving toward SCW from fed snails, miracidia accumulated in Zone C of the tubes (Fig. 4A), but did not respond or accumulate in this zone with SCW from starved snails (Fig. 4B). These results suggest that the feeding condition of the snail affects the amount of substances in SCW that attract miracidia.

(A) Fed (n=37)



(B) Starved (n=42)



(C) Water (n=37)

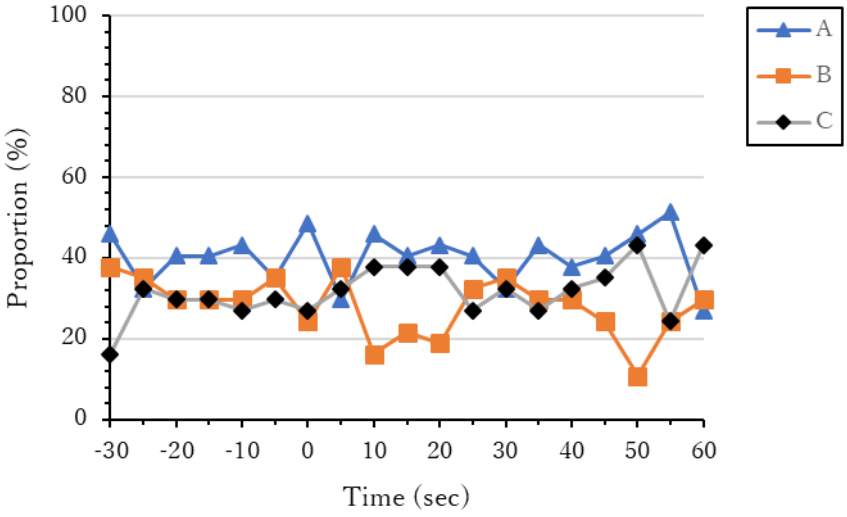
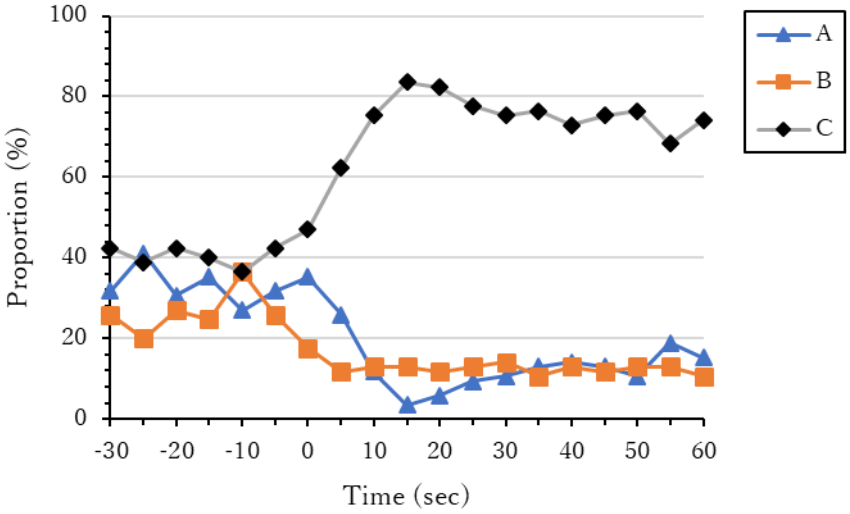


Fig. 4. Accumulation of *S. mansoni* miracidia in response to fed *B. glabrata* SCW (A), starved *B. glabrata* SCW (B) and DTW (C). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. Samples were loaded at time 0. There were 37, 42 and 37 miracidia exposed to stimulants in (A), (B) and (C), respectively.

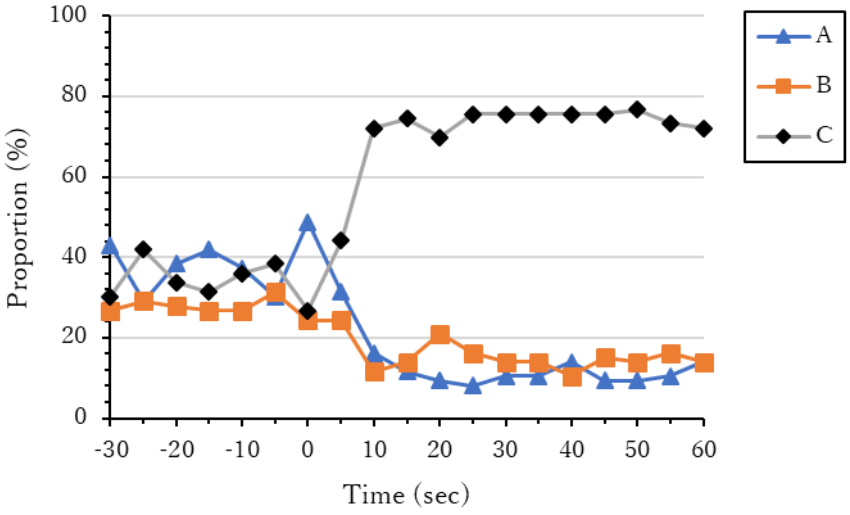
3.3. Examination of the potential of proteins to be attractive substances in SCW

To examine if proteins in *B. glabrata* SCW attract *S. mansoni* miracidia, boiled SCW was used in the accumulation assay (Fig. 5). Miracidia accumulated in boiled (100ºC, 10 min) SCW (Fig. 5B) to the same extent as in control SCW (Fig. 5A). This result strongly suggests that the substances in *B. glabrata* SCW that accumulate *S. mansoni* miracidia are not proteins.

(A) 26ºC (n=85)



(B) 100ºC (n=86)



(C) Water (n=57)

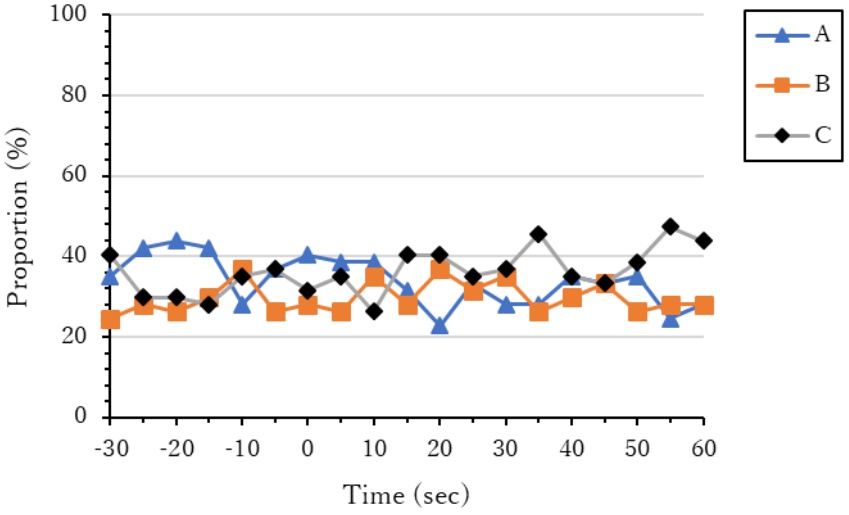
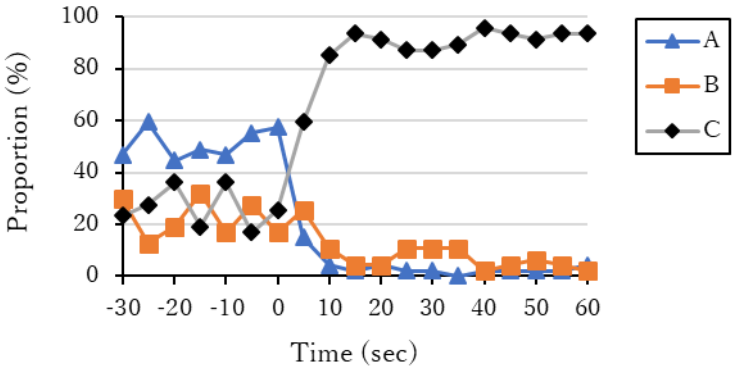


Fig. 5. Accumulation of *S. mansoni* miracidia in response to control *B. glabrata* SCW (A), boiled *B. glabrata* SCW (B) and DTW (C). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. Samples were loaded at time 0. There were 85, 86 and 57 miracidia exposed to the stimulants in (A), (B) and (C), respectively.

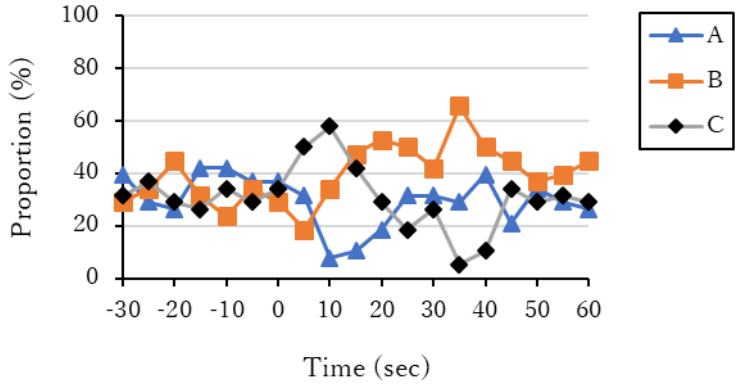
3.4. Molecular size of substances accumulating miracidia in SCW of B. glabrata

A 80 kDa glycoprotein in *B. glabrata* extracts and a glycoconjugate in SCW of the snail with MW >300 kDa have been shown to stimulate development of *S. mansoni* miracidia (Dissous et al., 1986; Haberl and Haas, 1992). However, the size of substances attracting miracidia remains uncertain. Therefore, we tried to confirm the molecular size of the substance(s) in *B. glabrata* SCW that stimulated accumulation of miracidia in our system. The miracidia accumulated in Zone C in response to SCW and substances with MW <100 kDa (Fig. 6A and 6C, Movies 4 and 6). Substances with MW >100 kDa stimulated non-directed turning of miracidia, but did not accumulate miracidia in Zone C (Fig. 6B, Movie 5). The miracidia did not show any responses when DTW was loaded in the tube (Fig. 6D, Movie 7).

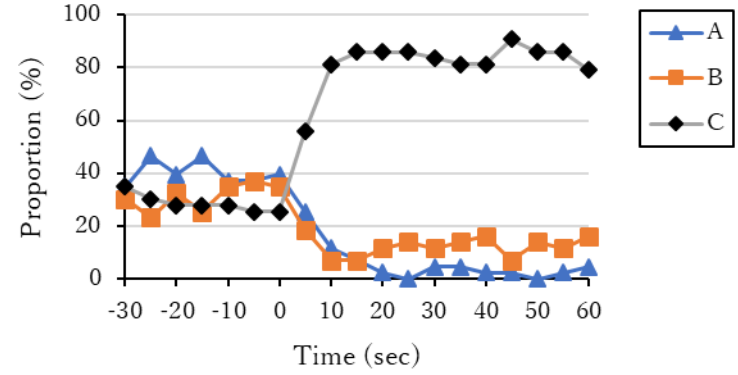
(A) SCW (n=47)



(B) Over 100 kDa (n=38)



(C) Under 100 kDa (n=43)



(D) Water (n=39)

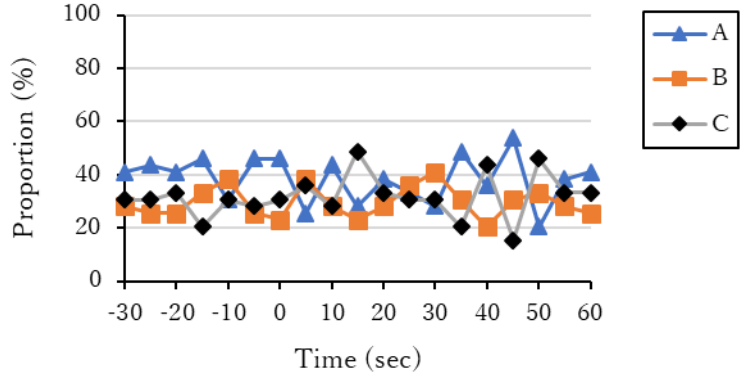


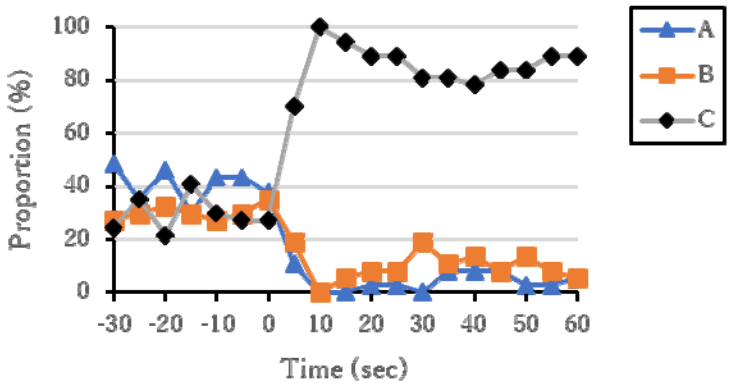
Fig. 6. Accumulation of *S. mansoni* miracidia in response to *B. glabrata* SCW (A), *B. glabrata* SCW fraction >100 kDa (B), *B. glabrata* SCW fraction <100 kDa (C) and DTW (D). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. The samples were loaded at time 0. The numbers of miracidia exposed to the stimulants in (A) to (D) were 47, 38, 43 and 39, respectively.

Macromolecules of MW >30 kDa in SCW of intermediate host snails have been shown to attract *S. japonicum*, *S. haematobium* and *S. mansoni* miracidia (Haas et al., 1991; Haberl et al., 1995). Therefore, we fractionated *B. glabrata* SCW using molecular filtration tubes with MW cutoffs of 50 kDa (Fig. S1) and 30 kDa (Fig. S2), and conducted assays. *S. mansoni* miracidia accumulated in Zone C in response to the <50 kDa fraction (Fig. S1C), as well as to SCW (Fig. S1A). In contrast, the miracidia showed non-directed turning, but did not accumulate in Zone C, in response to the >50 kDa fraction (Fig. S1B). Interestingly, *S. mansoni* miracidia accumulated in response to the <30 kDa fraction (Fig. S2C) and to a mixture of the filtrate and residue (Fig. S2D), as well as to SCW (Fig. S2A). Conversely, the miracidia showed non-directed turning, but did not accumulate in Zone C, in response to the >30 kDa fraction (Fig. S2B).

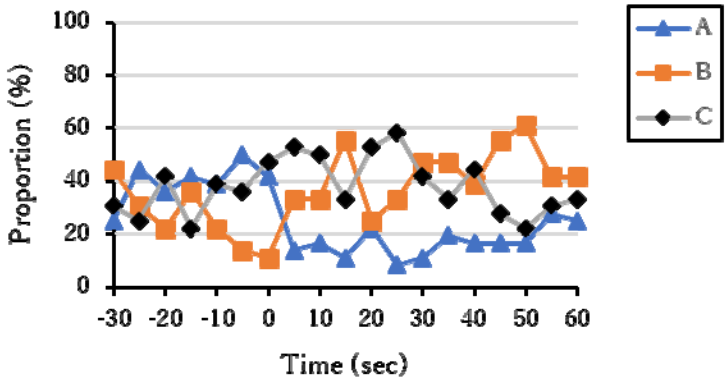
In contrast with previous studies (Haas et al., 1991; Haberl et al., 1995), *S. mansoni* miracidia were accumulated by substances with MW <30 kDa in Zone C (Fig. S2). To further

verify the molecular size of the substances, we fractionated SCW using molecular filtration tubes with a molecular weight cutoff of 10 kDa and conducted assays. *S. mansoni* miracidia showed non-directed turning, but did not accumulate, in response to the >10 kDa fraction (Fig. 7B, Movie 9) and accumulated with the <10 kDa fraction (Fig. 7C, Movie 10). Remarkably, the miracidia accumulated less in Zone C in response to the <10 kDa fraction compared with SCW, but once the fraction was mixed with the >10 kDa fraction (Fig. 7D, Movie 11), the miracidia accumulated in the mixture in Zone C to the same extent as with SCW (Fig. 7A, Movie 8).

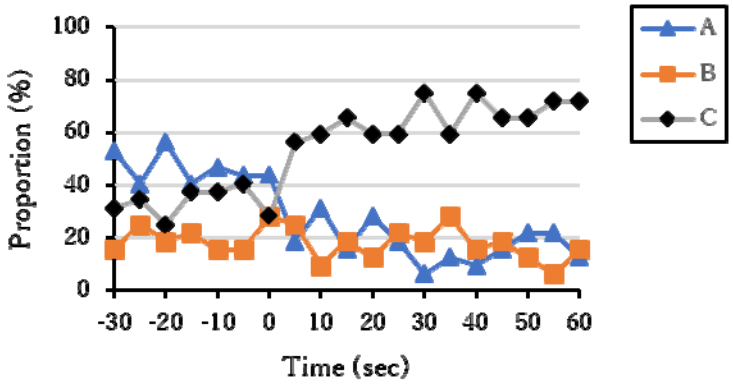
(A) SCW (n= 37)



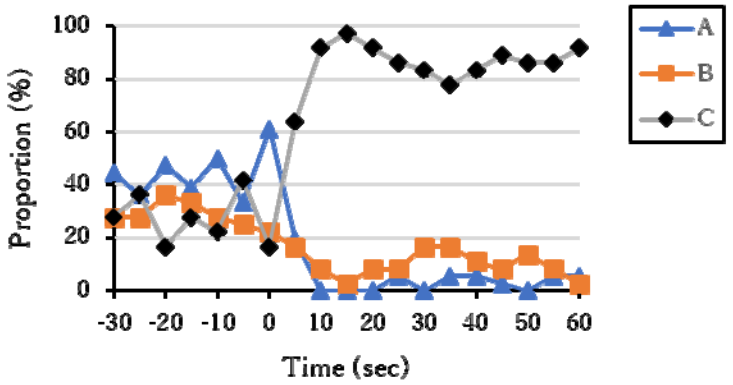
(B) Over 10 kDa (n= 36)



(C) Under 10 kDa (n= 32)



(D) Mix (n= 36)



(E) Water (n= 37)

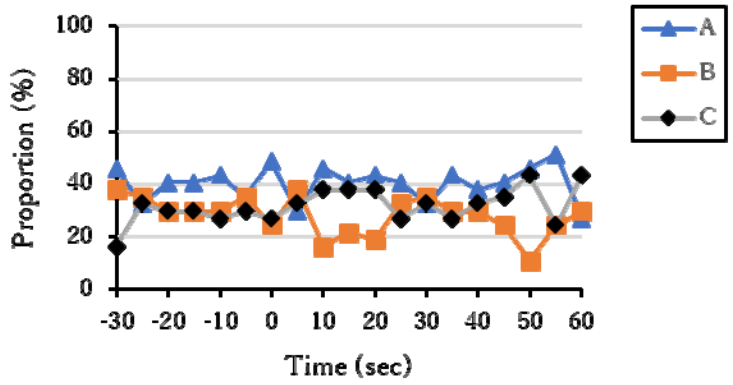


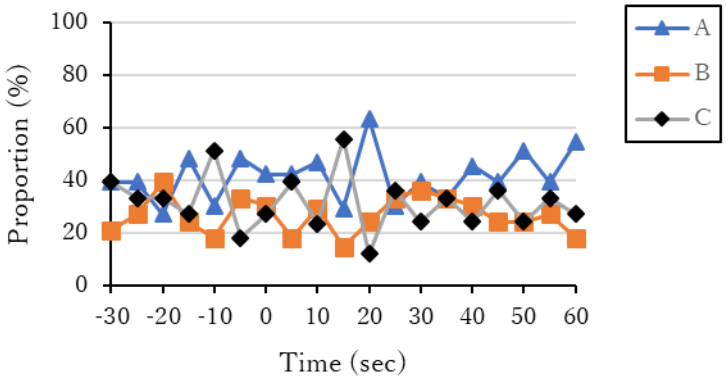
Fig. 7. Accumulation of *S. mansoni* miracidia in response to *B. glabrata* SCW (A), *B. glabrata* SCW fraction >10 kDa (B), *B. glabrata* SCW fraction <10 kDa (C), mixture of the fractions (D) and DTW (E). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. The samples were loaded at time 0. The numbers of

miracidia exposed to the stimulants in (A) to (E) were 37, 36, 32, 36 or 37, respectively.

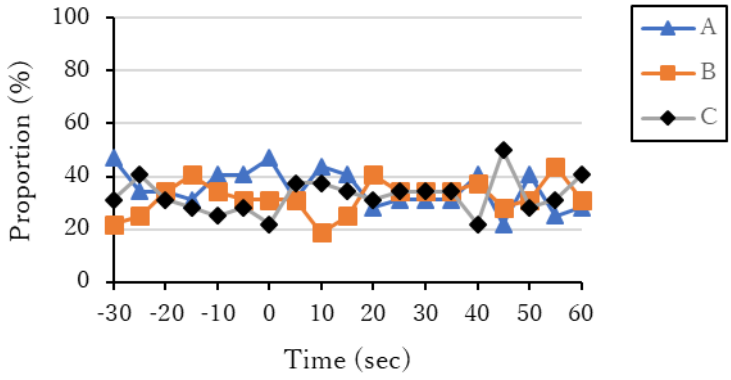
3.5. Verification of mono- and di-saccharides as attractants for S. mansoni *miracidia*

In previous literatures, small molecules such as fatty acids, amino acids, ions and sugars have been suggested as attractants for *S. mansoni* miracidia (MacInnis, 1965; MacInnis et al., 1974; Stibbs et al., 1976; Sponholtz and Short, 1976; Plorin and Gilberston, 1985). Glycoconjugates derived from mucous of the snails might attract miracidia, and glucose and a glycoconjugate have been reported to stimulate and attract miracidia (Plorin and Gilberston, 1985; Haberl and Haas, 1992; Haberl et al., 1995). Furthermore, substances of MW <10 kDa can accumulate miracidia in Zone C (Fig. 7) and these substances are not proteins (Fig. 5). To verify whether sugars could be these substances, monosaccharides (glucose, galactose, mannose, *N*-acetylgalactosamine) and disaccharides (lactose, trehalose, maltose) were studied as possible attractants for miracidia, but none accumulated miracidia in Zone C in our system (Fig. 8). This indicates that other substances with MW <10 kDa in SCW or a combination of substances including the tested sugars induce accumulation of miracidia.

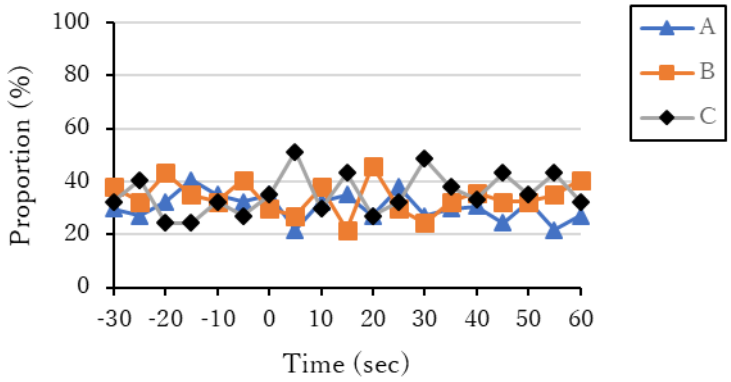
(A) Glucose (n= 33)



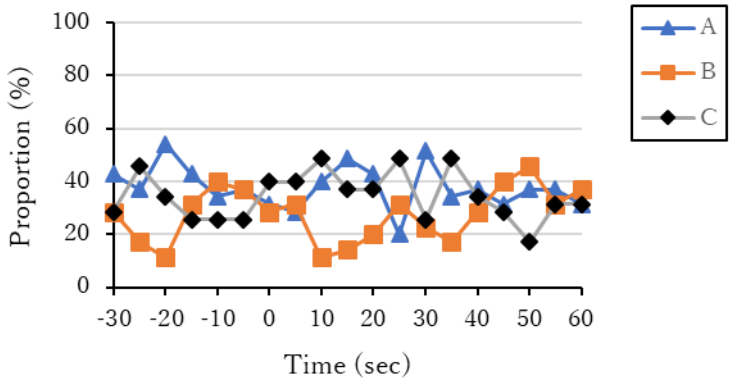
(B) Galactose (n= 32)



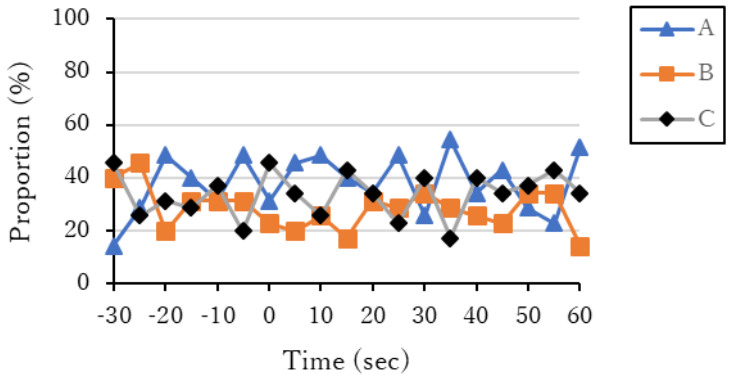
(C) Mannose (n= 37)



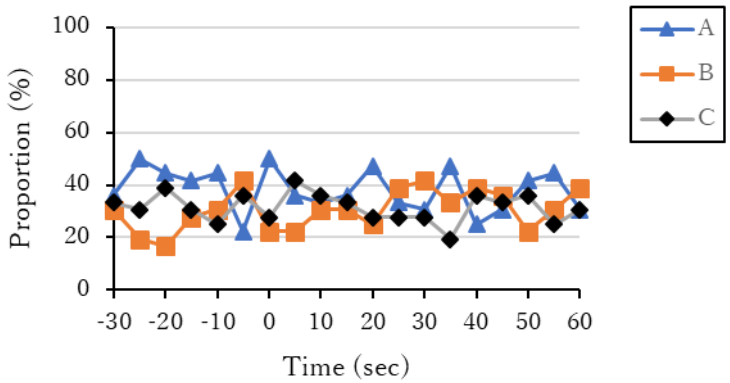
(D) GalNAc (n= 35)



(E) Lactose (n= 35)



(F) Trehalose (n= 36)



(G) Maltose (n= 35)

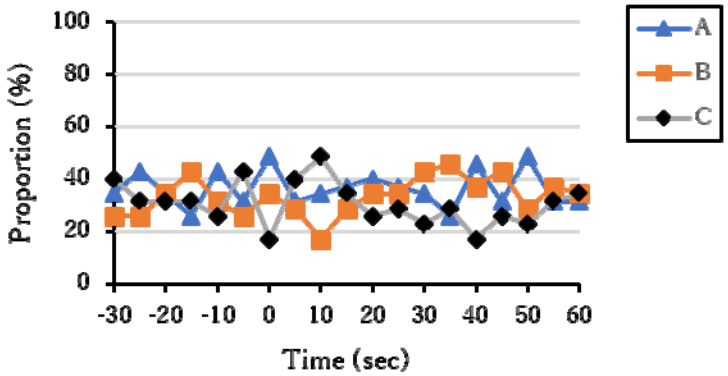


Fig. 8. Accumulation of *S. mansoni* miracidia in response to mono- or disaccharides. Glucose (A), galactose (B), mannose (C), *N*-acetylgalactosamine (GalNAc) (D), lactose (E), trehalose (F) and maltose (G) (all 10 mM) were tested. Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. The samples were loaded at

time 0. The numbers of miracidia exposed to the sugars in (A) to (G) were 33, 32, 37, 35, 35, 36 and 35, respectively.

3.6. Decoy effects of non-susceptible snails collected in Nagasaki, Japan

3.6.1. Accumulation of S. mansoni *miracidia by SCW of non-susceptible snails*

Chernin (1970) has shown that SCW of insusceptible snails can attract *S. mansoni* miracidia. Using our established accumulation assay, we examined if SCW of snails inhabiting the Nagasaki area could attract miracidia. SCW samples of *Stenothyra japonica*, *Assiminea hiradoensis*, *Angustassiminea castanea*, *Gyraulus spirillus* and *Physella acuta* were used in the assay (Fig. 9). SCW of *B. glabrata* was used as a positive control. *S. mansoni* miracidia were attracted weakly to *S. japonica* SCW (Fig. 9A), but did not accumulate in Zone C with SCW of *A. hiradoensis*, *A. castanea* or *G. spirillus* (Fig. 9B-D). The proportion of miracidia accumulated by *P. acuta* SCW was comparable to that by *B. glabrata* SCW (Fig. 9E, F).

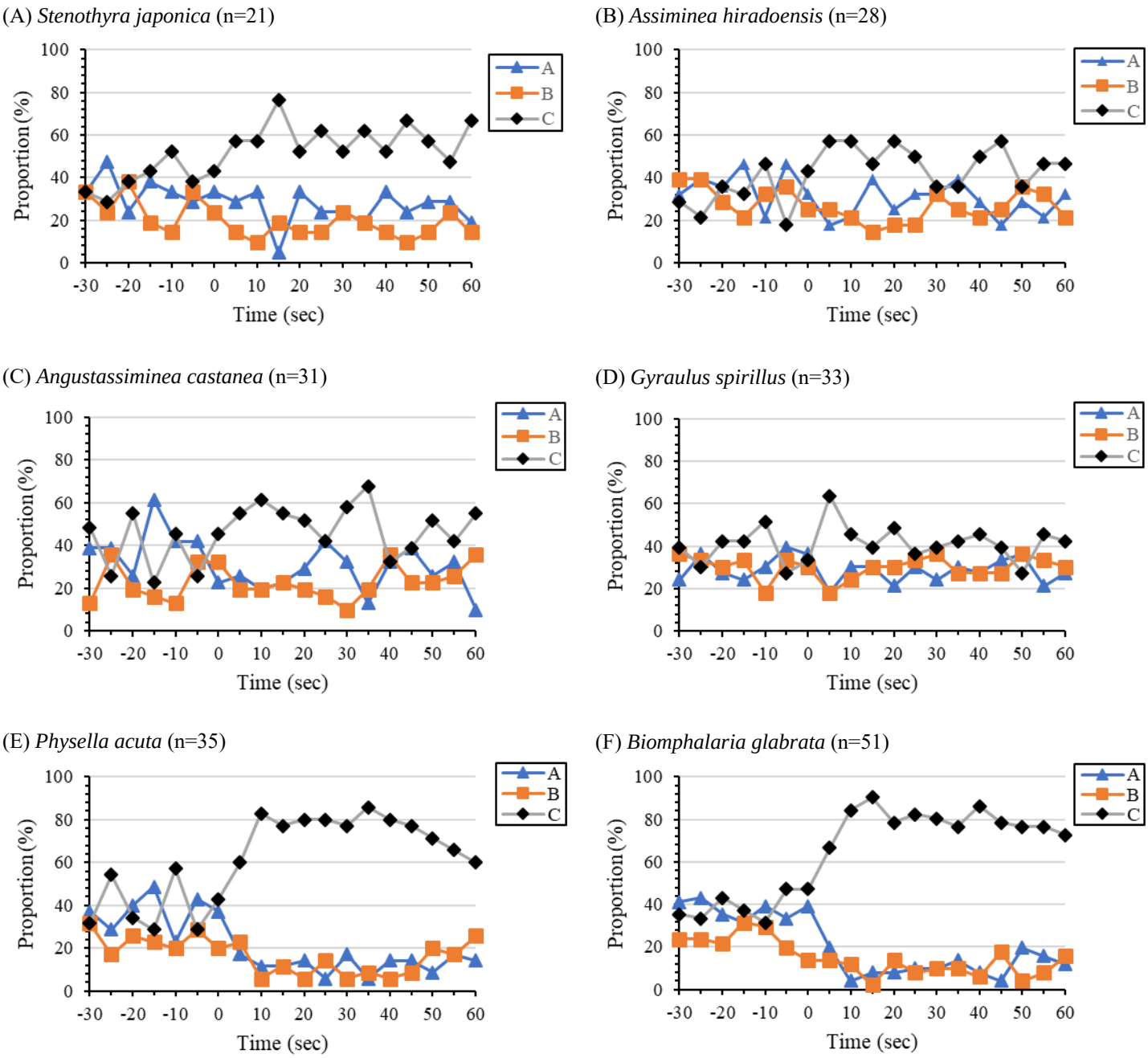
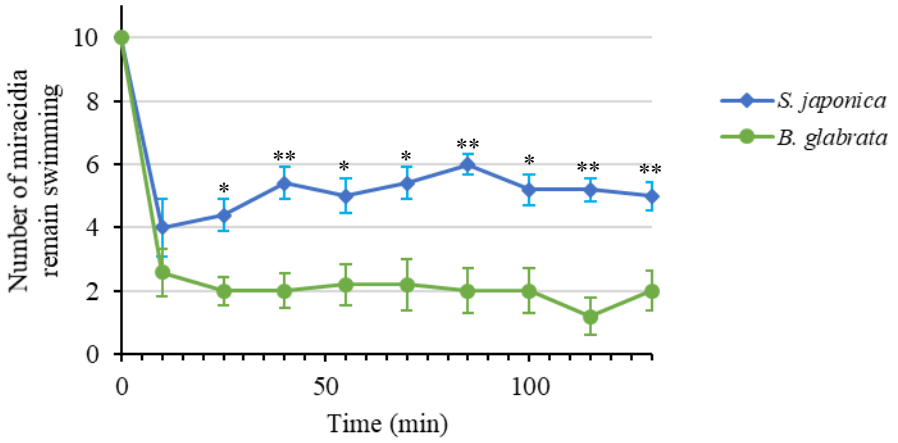


Fig. 9. Accumulation of *S. mansoni* miracidia in response to *S. japonica* SCW (A), *A. hiradoensis* SCW (B), *A. castanea* SCW (C), *G. spirillus* SCW (D), *P. acuta* SCW (E) and *B. glabrata* SCW (F). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown for each time point. Samples were loaded at time 0. There were 21, 28, 31, 33, 35 and 51 miracidia exposed to the stimulants in (A) to (F), respectively.

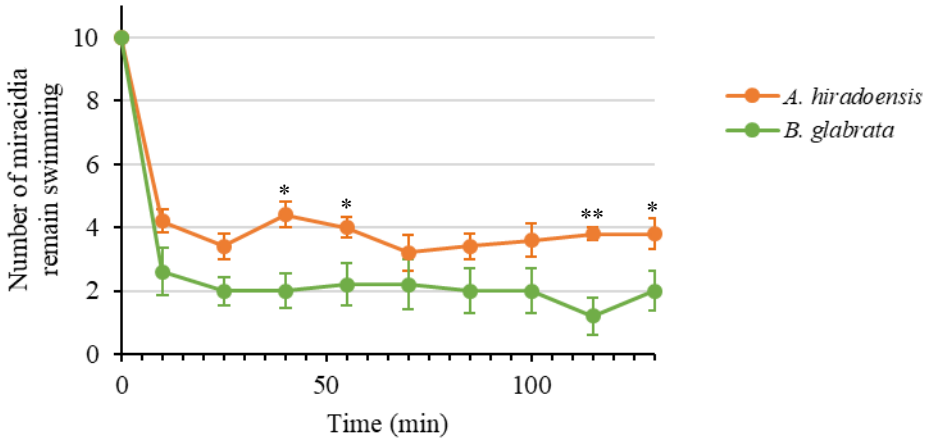
3.6.2. Attachment and penetration of S. mansoni *miracidia in non-susceptible snails*

To assess whether accumulation of *S. mansoni* miracidia toward insusceptible snail SCW is associated with attachment to and penetration of snails by miracidia, a penetration assay was conducted. In the assay, ten miracidia were incubated with a snail for the indicated time in Figure 10 and miracidia remaining swimming in the well were considered to be unattached and unpenetrated. An average of 5 *S. mansoni* miracidia attached to or penetrated *S. japonica* over the observation time period, compared to an average of 8 miracidia that attached to or penetrated *B. glabrata* (Fig. 10A). The miracidia showed similar trends of attachment and penetration toward *A. hiradoensis* and *A. castanea* (Fig. 10B, C). An average of 6 miracidia attached to or penetrated the snails after 70 min of incubation. The number of miracidia remaining swimming in the wells was slightly higher than in the wells with *B. glabrata* throughout the observation time period. *S. mansoni* miracidia showed similar trends of attachment and penetration toward *G. spirillus* and *P. acuta* (Fig. 10D, E). At 10 min and 25 min of incubation, significantly higher numbers of miracidia remained swimming in both groups compared with that with *B. glabrata*. However, in both groups, an average of 8 miracidia attached to or penetrated the snails after 40 min of incubation, which was comparable to that observed with *B. glabrata*.

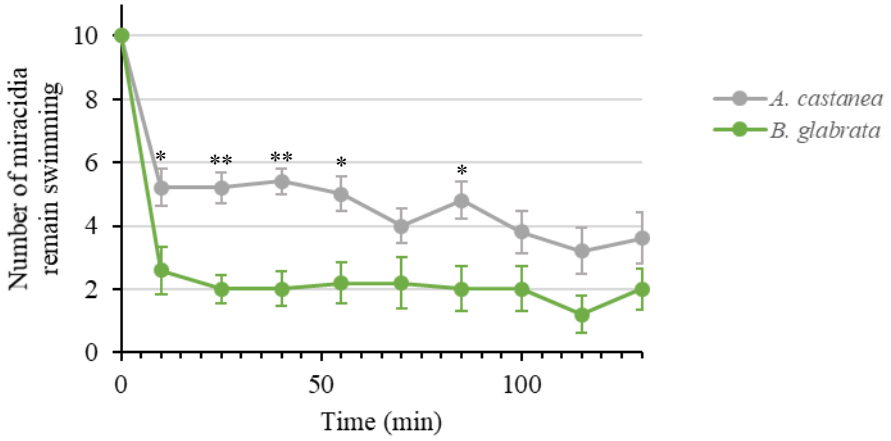
(A)


Number of miracidia remain swimming
10
8
6
4
2
0
0
50
100
Time (min)
*
**
*
*
**
*
**
**
S. japonica
B. glabrata

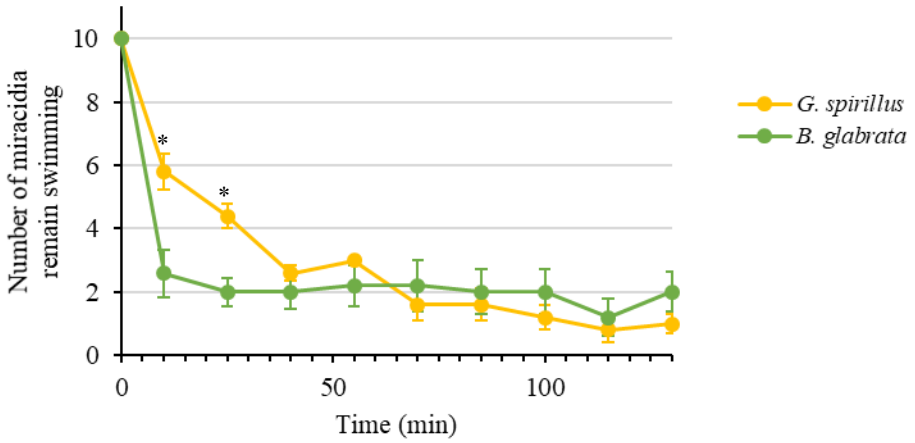
(B)


Number of miracidia remain swimming
10
8
6
4
2
0
0
50
100
Time (min)
*
*
**
*
A. hiradoensis
B. glabrata

(C)


Number of miracidia remain swimming
10
8
6
4
2
0
0
50
100
Time (min)
*
**
**
*
*
A. castanea
B. glabrata

(D)



(E)

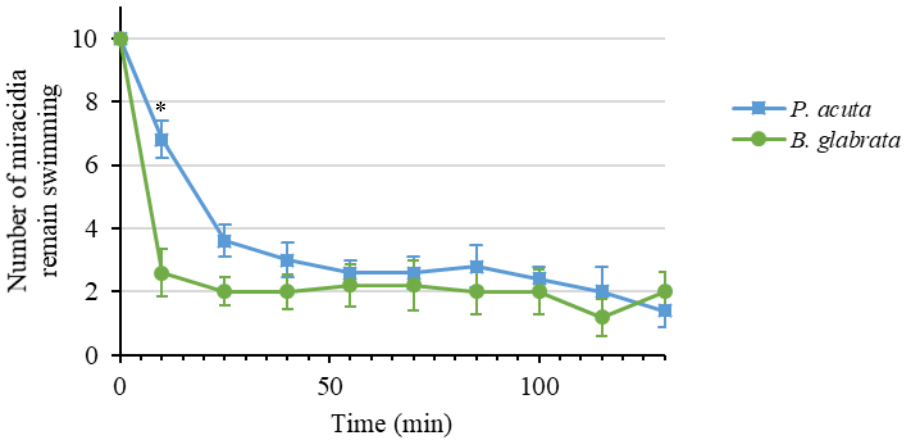


Fig. 10. Numbers of *S. mansoni* miracidia remaining swimming after exposure to *S. japonica* (A), *A. hiradoensis* (B), *A. castanea* (C), *G. spirillus* (D) and *P. acuta* (E). The number of miracidia remaining swimming after being exposed to *B. glabrata* is shown as a reference. Results from 5 independent studies are shown as mean ± standard error. *p < 0.05, **p < 0.01 for the number of miracidia remaining swimming with a non-susceptible snail vs. that with *B. glabrata* (Mann-Whitney test).

4. Discussion

Here we introduced a system using a square capillary glass tube to focus on accumulation of miracidia and identify the molecular size of substances inducing accumulation. We first showed that SCW of insusceptible *B. globosus* was able to attract *S. mansoni* miracidia similarly to susceptible *B. glabrata* (Fig. 3). This in agreement with the results reported by Chernin (1970) showing accumulation of miracidia in SCW from insusceptible snail species. Interestingly, no cercaria developed from miracidia attached to *B. globosus* even after 30-day culture of infected snails in our hands (data not shown). Snail-conditioned water of insusceptible snails from the Nagasaki area also attracted *S. mansoni* miracidia (Fig. 9). The miracidia accumulated in response to *P. acuta* SCW and attached to or penetrated the snails to the same extent as with *B. glabrata* snails (Figs. 9E, 10E). *S. japonica* SCW accumulated miracidia at a lower level compared to *B. glabrata* SCW (Fig. 9A), but a significantly lower number of miracidia attached to or penetrated *S. japonica* snails compared with *B. glabrata* (Fig. 10A). Conversely, *G. spirillus* SCW did not accumulate *S. mansoni* miracidia (Fig. 9D), although miracidia could attach to or penetrate the snails to the same extent to that with *B. glabrata* (Fig. 10D). These results suggest that the substances involved in accumulation of miracidia in response to SCW differ from those involved in the attachment and penetration of miracidia in snails.

The mechanisms through which miracidia can attach and penetrate, but cannot develop to

the next stage of larva, in non-susceptible snails were thoroughly studied by Chernin and Perlstein (1969) and Combes and Moné (1987). These results have encouraged use of decoy snails in schistosomiasis endemic areas for more than 40 years to control the disease (Laracuente et al., 1979; Johnson et al., 2009). However, although eliminating the fluke-mediated disease by introducing decoy snails is attractive, there are complex environmental factors that need to be considered carefully before the method is put into practice. For example, Johnson et al. (2009) showed that though co-existence of insusceptible snail species reduced the infection prevalence of and cercarial release from host snails, the introduced snails also increased egg production by host snails. This suggests that introduction of decoy snails in an endemic area creates improved conditions for the fluke.

Another way to control schistosomiasis is to use attractants for miracidia of *S. mansoni* to guide the larva away from the snail habitat. Most studies have tried to identify a single molecule that attracts miracidia and have shown chemokinetic responses that mainly involved the orientation of *S. mansoni* miracidia to snail hosts (Chernin, 1970; Samuelson, 1984). Haas et al. (1991) further found that the chemotactic host finding ability of *S. mansoni* miracidia was less effective than that of *S. japonicum* miracidia. However, straight path approaches of *S. mansoni* miracidia were apparent in response to SCW of *B. glabrata* (Fig. 3A) and some of other SCW fractions. Careful observations indicated that straight path swimming of miracidia toward Zone C occurred first, followed by accumulation upon reaching the loaded SCW

(Supplemental Movie S1).

We hypothesize that at least two substances are involved in promotion of accumulation of miracidia. This is because miracidia showed non-directed turning, but did not accumulate, in response to the residue of SCW fractionated with MW cutoffs of 100 kDa, 50 kDa and 30 kDa, but positively responded and accumulated with the filtrate of the samples (Figs. 6, S1, S2). Further experiments revealed that one of the substances had a MW of about 10 kDa because miracidia accumulated less with the SCW filtrate fractionated with a MW cutoff of 10 kDa compared to SCW, but once the filtrate was mixed with the residue, the miracidia accumulated in response to the mixture to a similar degree compared to SCW (Figs. 7C, D). This may indicate that there are several substances required for inducing miracidial accumulation. This hypothesis explains why a mono- or di-saccharide alone in DTW cannot induce accumulation of miracidia, although Plorin and Gilberston (1985) showed a positive response of miracidia to 10 mM glucose. To confirm our hypothesis, there is a need to identify substances in SCW and check whether a combination of these substances can elicit miracidial accumulation.

A *B. glabrata* protein pheromone *Bg*Temptin with a molecular weight of ~12 kDa that attracts snails was recently identified (Pila et al., 2017). This protein could be a candidate substance that also attracts *S. mansoni* miracidia. However, this was not the case because *S. mansoni* miracidia accumulated in response to *B. glabrata* SCW incubated at 100ºC for 10

min, at which *Bg*Temptin lost its attractive potency for the snails (Pila et al., 2017). This result also suggests that the attractive substances are not volatile.

Secretion or emission of the substances attracting *S. mansoni* miracidia depended on the feeding conditions of *B. glabrata* (Fig. 4). This is reasonable because well-fed and healthy snails are better suited than starved and unhealthy snails for *S. mansoni mi*racidia to develop to cercariae.

Our method using a capillary tube has the potential to identify substances that accumulate miracidia using small samples. The tubes are washable, reusable and can be viewed under a microscope. However, as with other *in vitro* methods, the environment of miracidia in tubes is unlike the conditions in nature. Nevertheless, our method is simple, does not require special training, and may also be useful for other purposes. Using this system, we would like to examine if *S. mansoni* miracidia accumulate in response to SCW of resistant *B. glabrata* strains, such as BS-90.

Declaration of interest

The authors state there are no potential conflicts of interest.

Acknowledgments

We would like to thank Dr. Hiroshi Fukuda (Okayama Univ.) for identifying the species of snails collected in the Nagasaki area. We thank Dr. Masaaki Shimada for his critical reading of the manuscript. We would also like to thank Mr. Stephen Kiluva and Ms. Kyoko Masuda for support and help with the studies.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data statement

All relevant data are within the paper and its supporting materials here.

Abbreviations

DTW, dechlorinated tap water

kDa, kilodalton

MW, molecular weight

PBS, phosphate-buffered saline

SCW, snail-conditioned water

Author statement

Mitsumasa Miura: conceptualization, methodology, validation, investigation, data curation.

Yoshinori Mitsui: conceptualization, formal analysis, data curation, writing - original draft, writing - review and editing.

Yoshiki Aoki: conceptualization, formal analysis, data curation, writing - original draft, writing - review and editing.

Kentaro Kato: conceptualization, methodology, resources, validation, formal analysis, investigation, data curation, writing - original draft, writing - review and editing, visualization, supervision, project administration.

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Movie captions

Movie 1. vs Biom SCW (Fig. 3A)

Movie 2. vs Bulinus SCW (Fig. 3B)

Movie 3. vs water (Fig. 3C)

Movie 4. vs SCW (Fig. 6A)

Movie 5. vs >100 kDa (Fig. 6B)

Movie 6. vs <100 kDa (Fig. 6C)

Movie 7. vs water (Fig. 6D)

Movie 8. vs SCW (Fig. 7A)

Movie 9. vs >10 kDa (Fig. 7B)

Movie 10. vs <10 kDa (Fig. 7C)

Movie 11. vs mixture (Fig. 7D)

Movie 12. vs water (Fig. 7E)

Legends for Supplemental figures

Fig. S1. Accumulation of *S. mansoni* miracidia in response to *B. glabrata* SCW (A), *B. glabrata* SCW fraction >50 kDa (B), *B. glabrata* SCW fraction <50 kDa (C) and DTW (D). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. The samples were loaded at time 0. The numbers of miracidia exposed to the

stimulants in (A) to (D) were 56, 46, 61 and 47, respectively.

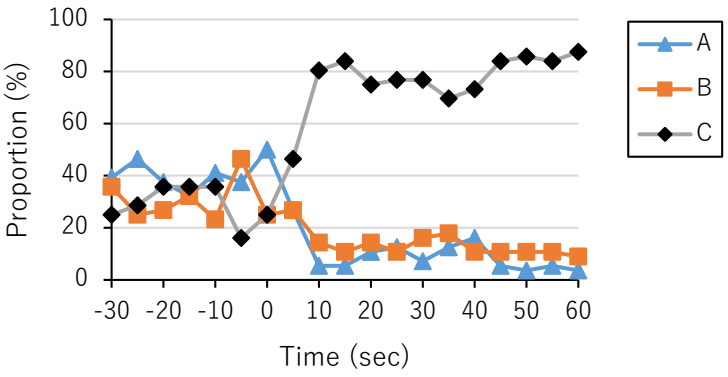
Fig. S2. Accumulation of *S. mansoni* miracidia in response to *B. glabrata* SCW (A), *B. glabrata* SCW fraction >30 kDa (B), *B. glabrata* SCW fraction <30 kDa (C), mixture of the fractions (D) and DTW (E). Proportions in Zone A (triangles), Zone B (boxes) and Zone C (diamonds) are shown at each time point. The samples were loaded at time 0. The numbers of miracidia exposed to the stimulants in (A) to (E) were 37, 44, 26, 33 and 35, respectively.

Legend for Supplemental Movie S1

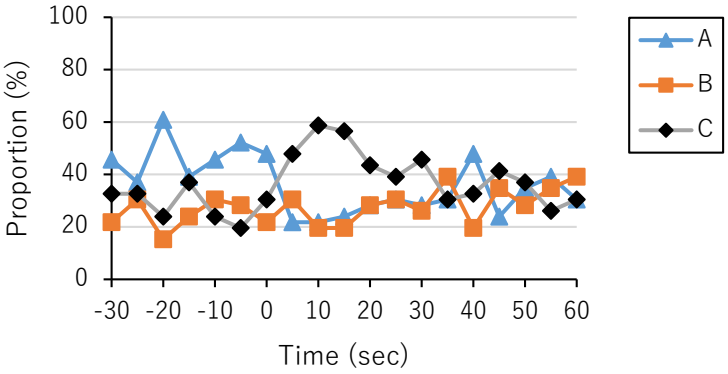
0.04% trypan blue solution was added to *B. glabrata* SCW and 1 µl of the sample was loaded. The loaded SCW diffused and the edge reached the border between Zone B and Zone C of the tube, as observed by the blue color of the solution.

Miura M et al. Supplemental Figure S1

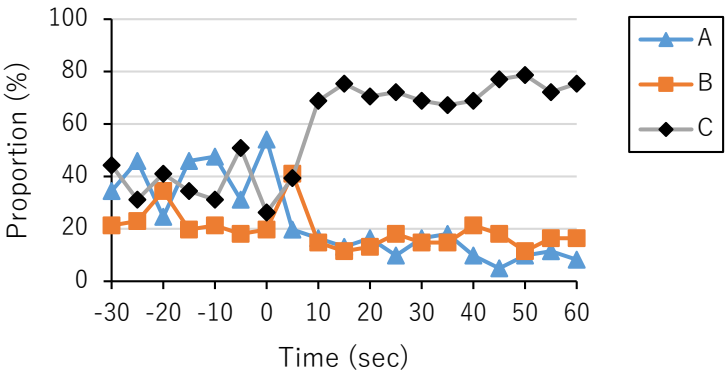
(A) SCW (n=56)



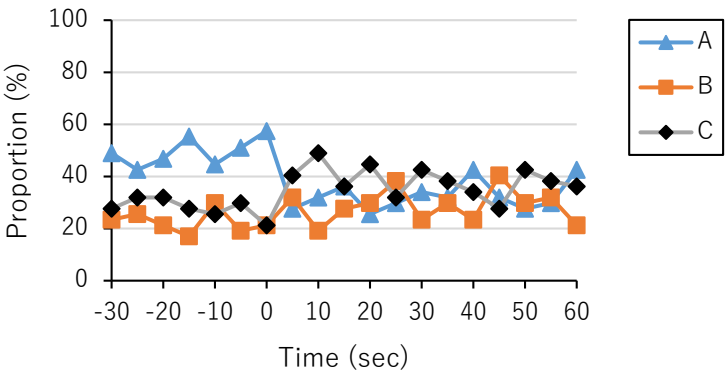
(B) Over 50 kDa (n=46)



(C) Under 50 kDa (n=61)

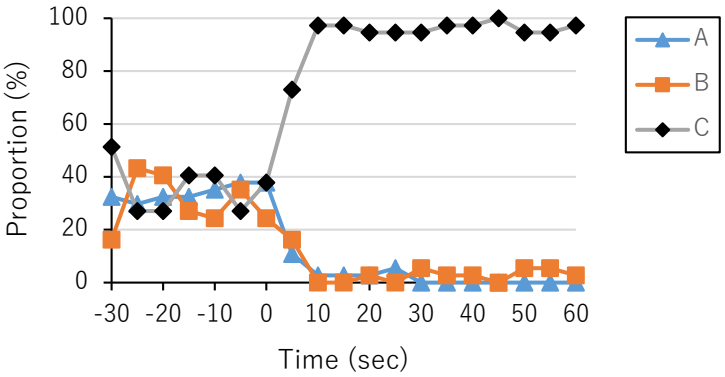


(D) Water (n=47)

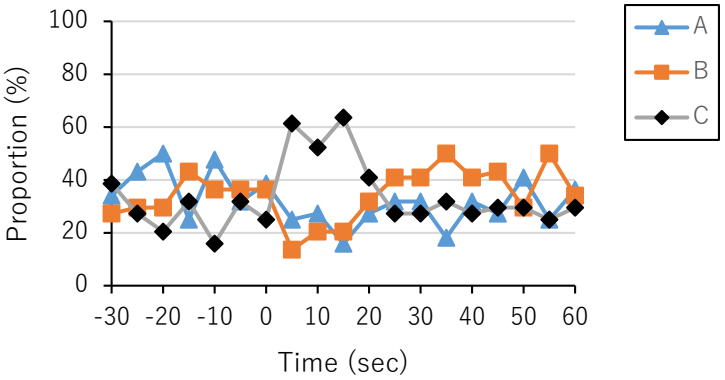


Miura M et al. Supplemental Figure S2

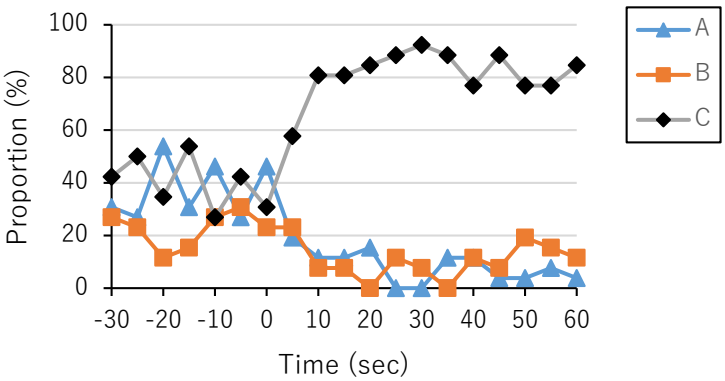
(A) SCW (n= 37)



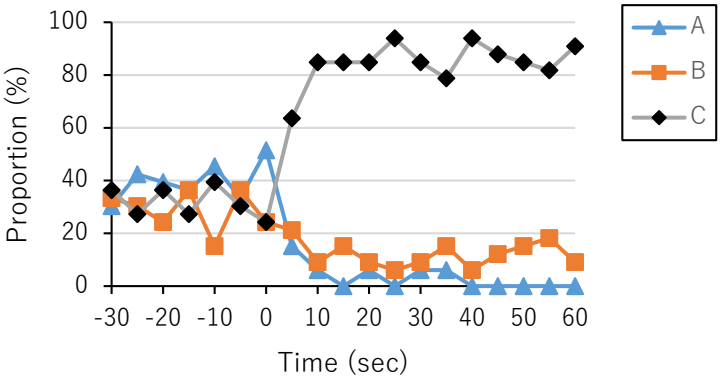
(B) Over 30 kDa (n= 44)



(C) Under 30 kDa (n= 26)



(D) Mix (n= 33)



Miura M et al. Supplemental Figure S2 (continued)

(E) Water (n= 35)