Embryonic learning of chemical cues via the parents' host in anemonefish (*Amphiprion ocellaris*)

[1]Kazuko Miyagawa-Kohshima, [2]Coral group of the Okinawa Churaumi Aquarium*, [2]Hirokazu Miyahara, [2]Senzo Uchida

1: Wildlife Research Center of Kyoto University, 2-24 Tanaka-Sekiden-cho, Sakyo-ku, Kyoto 606-8203, Japan Phone: +81-75-723-1525, Fax: + 81-75-771-4394, E-mail: kohshima46@ybb.ne.jp
2:Okinawa Churaumi Aquarium, Ishikawa 424, Motobu-cho, Okinawa 905-0206, Japan, E-mail: h-miyahara@okichura.jp

*: Shuhei Odoriba, Daigo Okabe, Yuichiro Baba, Hideyuki Touma, Atsusi Takemoto, Naomi Yamanishi, Shohei Matsuzaki, Shunsuke Nagata, Yusaku Kanaya, Mariko Wakai, Hidekazu Koyanagi, Hajime Igei, Miyuki Nakazato (chief)

ABSTRACT

The species-specific host-recognition system of anemonefish was examined experimentally, with a particular focus on the function of imprinting using naive *Amphiprion ocellaris* juveniles. Anemonefish parents lay their eggs very close to their host anemone so the eggs are almost always touched by the host's body or tentacles. Here, we demonstrate the embryonic and immediate post-hatching learning of chemical cues via the parents' host in *A. ocellaris* through a host-exchange experiment with egg batches during hatching. The memory obtained from such imprinting operates at the time when juveniles first search for their hosts. Unexpectedly, innate recognition was found to exist not only in the symbiotic host species but also weakly in two non-partner species. Innate recognition alone is not sufficient. Imprinting via the parents' host complements innate recognition, leading to rigid species-specific host recognition. Imprinting by the parents' single host provides a sufficient cue for reaching the two host species. Furthermore, when combined with imprinting, innate recognition of non-partners serves to supplement the recognition of those species, leading to substitute partnerships that are only observed in some localities. Potential functions of imprinting in the host-recognition system are discussed. The "spare recognition hypothesis" and the necessity of clear distinctions between symbiotic and substitute species are also proposed here.

Keywords: Symbiosis; Anemonefish; Sea anemone; Imprinting; Embryonic learning; Host recognition

1. **Introduction**

After spending ca. 1 week in the pelagic stage, anemonefish larvae become juveniles (characteristic white bands appear), after which they enter the benthic stage and begin to look for hosts. Each anemonefish inhabits species-specific symbiotic anemone(s). Previous studies, both in laboratory aquaria (Miyagawa, 1989; Miyagawa and Hidaka, 1980) and in the sea (Elliott et al., 1995), have demonstrated that naive juvenile anemonefish reach their hosts by recognising chemicals emitted from symbiotic anemone(s). Visual cues do not play a large role in host recognition during their first encounter (Arvedlund et al., 1999; Arvedlund and Nielsen, 1996; Elliott et al., 1995; Miyagawa, 1989; Miyagawa and Hidaka, 1980).

The potential functions of host imprinting in this chemical recognition have been documented, focusing on an additional function that may supplement the recognition of substitute species in cases of host shortage, which leads to unusual partnerships in some localities (Miyagawa, 1989). The *Amphiprion perideraion–Heteractis crispa* partnership in the Ryukyu Islands, Japan (Hirose, 1985; Uchida et al., 1975) is considered a typical example of such substitute partnerships.

Arvedlund and Nielsen (1996) first demonstrated that imprinting by the parents' host is necessary for juveniles to recognise their symbiotic host in *A. ocellaris*. However, they conducted experiments with only *Heteractis magnifica*, one of two symbiotic partner anemones that *A. ocellaris* usually inhabits. *Amphiprion ocellaris* juveniles that hatched close to their other symbiotic host, *Stichodactyla gigantea*, could recognise both symbiotic anemones (Miyagawa, 1989). Therefore, the determination of whether juveniles that hatch adjacent to *H. magnifica* can also recognise both symbiotic species is needed to fully demonstrate that imprinting by a single parents' host provides a sufficient clue to reach both symbiotic species.

Several important questions remain. This chemical recognition is thought to be established on the basis of innate recognition. *Amphiprion melanopus* was thought to possess an innate preference for its symbiotic anemone *Entacmaea quadricolor* (Arvedlund et al., 1999), but the mechanism of this innate recognition has not yet been clearly documented.

The timing of the critical (sensitive) period of this imprinting also remains unknown. Newly hatched anemonefish larvae soon rise up to the water surface toward the afterglow of sunset, thereby avoiding the lethal touch of the parents' host's tentacles (Miyagawa, 1989). To survive, anemonefish have to look for hosts immediately after entering the benthic stage and therefore need to be imprinted before becoming juveniles. For these reasons, we predicted that imprinting occurs during both the pre-hatching and immediate post-hatching stages, and we conducted host-exchange experiments to test this hypothesis.

Lastly, the need for imprinting in such a rigid species-specific host-recognition system has not yet been explained. Such imprinting is thought to provide flexibility for adapting to changing environments. Therefore, we attempted to verify if imprinting results in unusual partnerships in some localities. The present study was conducted to resolve unanswered questions using hatching egg batches and naive juvenile anemonefish. Our main objectives were to verify the existence of basic innate (genetic) recognition and to determine how imprinting (learned) and innate recognition (hard-wired) work together in the host-recognition system; define the duration of the critical period; and establish the adaptive function of this imprinting.

2. Materials and methods

2.1. Sea anemones

Five species of anemonefish symbiotic anemones, *Stichodactyla gigantea* (Forsskal, 1775), *Heteractis magnifica* (Quoy and Gaimard, 1833), *Stichodactyla mertensii* (Brandt, 1835), *Heteractis crispa* (Ehrenberg, 1834) and *Entacmaea quadricolor* (Rueppell and Leuckart, 1828) were collected from the sea off the Motobu Peninsula (026.64N, 128.13E), close to the Okinawa Churaumi Aquarium, and kept in separate tanks to avoid any chemical contamination. Tanks were supplied with running natural seawater and kept under sunlight so that zooxanthellae in the anemone bodies could survive and maintain the health of the anemones; when zooxanthellae began to decline, large anemones were returned to the sea along with a few associated anemonefish. Several non-partner species of *A. ocellaris* that are partners of other anemonefish were also tested: *S. mertensii*, which is the partner of *Amphiprion sandaracinos* and *A. clarkii*; *H. crispa*, which is that of *A. clarkii* and *A. perideraion*; and *E. quadricolor*, which is the partner of *A. frenatus* in the Ryukyu Islands.

Experimental anemones are expressed using abbreviations for convenience: *S. gigantean* = *Sg*, *H. magnifica* = *Hm*, *S. mertensii* = *Sm*, *H. crispa* = *Hc* and *E. quadricolor* = *Eq*. Underlined names are symbiotic partner species of *A. ocellaris*.

2.2. Naive fish

Amphiprion ocellaris and *A. perideraion* juveniles used in this study were laboratory-bred at the Okinawa Churaumi Aquarium from July to October in 2007–2011. Parent anemonefish with host anemones were collected from the sea off the Motobu Peninsula.

Parent fish laid eggs closely adjacent to the host anemone on the wall or bottom of their rearing tank (100 L and 30 L). Breeding was done with a different anemonefish pair for each anemone species, but two pairs were used with *Hm* to confirm the results. A pair without a host (to obtain non-imprinted fish) laid eggs inside the wall of a PVC duct (10 cm diameter × 15 cm length); the next year, this pair was used to breed juveniles that were hatched adjacent to *Sm*.

The parental male cared for the eggs until hatching. After ca. 1 week, the eggs hatched after sunset and hatched larvae were collected by flashlight and gently transferred to a 100-L tank using a siphon or a plastic container. Thereafter, the larvae were kept isolated from any possible sensory contact with sea anemones until the experiments. Larvae were fed the marine rotifer *Brachionus plicatilis*; increasing amounts of *Artemia salina* nauplii were added as development proceeded. As juveniles grew, their diet was switched to frozen Copepoda and the dry fish food.

2.3. Trough experiment

From July to September, a PVC trough (200 cm long × 12 cm wide × 9 cm high) was used for the experiments (Fig. 1). The trough was marked every 40 cm to create five sections (I–V) for monitoring the behavioural responses of test fish. When the water temperature fell below 26.5°C at the end of October or the beginning of November, juveniles became fairly inactive and few reached section V in the 200-cm trough even though they showed some attraction. Therefore, to confirm their responses, a 150-cm trough was used with markings every 30 cm, which clearly confirmed their attraction or non-attraction.

The same experimental trough was used for experiments with different anemone chemicals. To avoid chemical contamination of the trough, the inside was completely covered with a thin (0.02 mm) polyethylene sheet, and overflow water was drained from a plastic tube attached to the sheet at the end of section I (Fig. 1) because anemone chemicals easily stick to PVC. After each experiment, the polyethylene sheet and drainage tube were removed and a new sheet and tube were used in every experiment.

Test fish were removed from the rearing tank in a glass beaker that was then placed at the end of section I and left for 10–15 min before the test to allow the fish to acclimate to the experimental conditions. Then, test juveniles were gently released into the trough at the closed end of section I. At first, fresh seawater was supplied as a control and then experimental seawater containing test anemone chemicals was continuously supplied by a vinyl tube at the far end of the trough (section V) at a flow rate of 75–85 mL min^{-1}.

Since overflowing seawater drained from the plastic tube at the end of section I, and judging from the behaviour of the tested fish, some portion of the symbiotic anemone chemicals seemed to

reach section I relatively fast (in a few minutes), being delivered near the water surface via the overflowing water. Seawater containing test anemone chemicals was poured into the trough continuously at approximately the same flow rate for every experiment in order to make an incline of concentration of chemicals from section V to I: concentration was thought to be the highest in section V. When seawater containing symbiotic anemone chemicals started to be poured into the trough, tested juveniles soon appeared to recognise something in the water, especially seeming to detect something just beneath the water surface, and started to swim around actively. They were observed to proceed toward and reach section V, seemingly following chemicals in the water.

During the control period with fresh seawater and for ca. 1–2 min after the introduction of seawater that contained chemicals from a test anemone, the behaviours and movements of the fish were observed for 30 min (60 min with non-imprinted and *Sg* juveniles), and the locations of test juveniles were recorded every 30 s. Each test was repeated three to five times with a new set (3–5 individuals) of juveniles.

The number of juveniles that reached or stayed in section V was used to judge whether juveniles were attracted to the test anemone chemicals. The average number of juveniles that reached or stayed in section V per observation period ("reach V value" hereafter) for each control and test condition was calculated, and values were compared among groups using Paired t-tests (see Results and Supplementary Data Fig. I–V).

For trough experiments with non-imprinted and *Sg* juveniles, each test anemone was placed in a container (10-L, 25-L, and 35-L containers were used according to anemone size), and seawater in the container that contained chemicals from the anemone was poured into the trough. The anemones varied greatly in size: *Sg*, ca. 25–40 cm diameter; *Hm*, 60–80 cm; *Sm*, 50–70 cm; *Hc*, 25–30 cm; *Eq*, 6–10 cm diameter (8–10 individuals of *Eq* were used together in each experiment). To keep the concentration of anemone chemicals roughly equal among the experiments, each test anemone was weighed and the amount of seawater placed in their respective containers was determined to be inversely proportional to the ratio of their weight: e.g. *Hm*, 6.2 kg with 20 L seawater and *Sg*, 3.4 kg with 11 L seawater.

In all experiments, other than ones with non-imprinted and *Sg* juveniles, seawater from the typical rearing tank for each anemone was used as the seawater containing test-anemone chemicals to prevent non-imprinted juveniles from responding to the dense concentration of symbiotic host chemicals. The volume of seawater in the rearing tanks of large anemones (*Hm* and *Sm*) was ca. 70 L and the volume with medium size anemones (*Sg* and *Hc*) was ca. 30 L. Seawater near the test anemone (within ca. 10–15 cm) was siphoned from the rearing tank and poured into the trough.

During all experiments, the same amount of fresh seawater that was poured into the trough was supplied to the container and the rearing tank. To obtain adequate results over a short

observation period, a folded gauze with attached anemone mucus was wound around the inlet tube tip (finished dimensions ca. 2 × 5 cm) in all trough experiments except those with non-imprinted and
Sg juveniles. During preparation, clean gauze (30 × 45 cm) was kept on the oral disc or attached to the column of each test anemone for more than 3 h before the experiment. Newly collected anemones were used for trough experiments as much as possible, while anemones without reduced zooxanthellae were used when necessary.

The imprinting rates of *Hm-* and *Sm-A. ocellaris* juveniles were not high, and therefore, the imprinting status of these juveniles was checked at the start of the trough experiments. Imprinted juveniles of each condition were kept separately from non-imprinted juveniles, and the trough experiments were then conducted using chemicals from other test anemones.

2.4. Host-changing manipulation

A long period of time is usually needed for an *A. ocellaris* pair to start breeding adjacent to a non-partner species host. Night observations have shown that anemonefish are unable to see in the dark. Therefore, after dark, "host-changing manipulations" were performed on the evening (i.e. ca. 1 day) before hatching. The *Hm* host anemone of a pair was replaced with *Hc* (Supplementary Data Fig. VI). The pair accepted the new host *Hc* and the parental male continued to take care of the eggs until hatching, as usual.

2.5. Host-exchange experiment

To determine the timing of the critical period, an egg batch needed to be transferred adjacent to a *Sg* anemone. The parents of non-imprinted juveniles were accustomed to laying their eggs inside the wall of a PVC duct, and therefore, this pair was made to associate with an *Sm* anemone. They laid eggs inside the wall of the same PVC duct that was cut in half adjacent to *Sm*.

At hatching, the parental male stirred the eggs by wagging and rubbing its body above the eggs. This behaviour appeared to promote hatching. Eggs did not start to hatch without this male behaviour; however, we noticed that once hatching started and toward the end of hatching, some eggs hatched without such male care. Therefore, although it was very difficult to determine the transfer timing in the dark (during our last attempt, we used a night vision device), after more than 60–70% of eggs had hatched in the parents' tank with *Sm*, the remaining eggs on the half-cut PVC duct were initially transferred into a small container filled with fresh seawater. The spawning duct was then quickly transferred to the rearing *Sg* tank and placed ca. 5 cm from the *Sg*'s oral disc to

prevent newly hatched larvae from being killed by the tentacles (Supplementary Data Fig. VII). We were careful to avoid transferring water and newly hatched larvae from the *Sm* tank and the small container into the *Sg* tank. To minimise the time during which the eggs were out of water, every transfer was done as quickly as possible just above the water surface of each tank.

Larvae that hatched in both the *Sg* and *Sm* (parents') tanks were scooped up and reared in separate tanks without any anemone until the experiment. All juveniles were then used in trough experiments with *Sg* and *Sm*, respectively, to confirm which anemone the juveniles had imprinted to. Initially about half of the juveniles were examined with *Sg*, after which non-attracted *Sg* juveniles were tested with *Sm*, while the remaining half were tested first with *Sm* and then with *Sg*.

2.6. Direct encounter experiment

After the trough experiments, some non-imprinted, *Sg*, and *Hm* juveniles were kept in separate tanks isolated from any anemone and then made to encounter an intact symbiotic *Sg* anemone in the aquarium. The fish were 192–246 days old (total length ca. 2.5–4.5 cm). The experimental aquarium (150 cm long × 45 cm wide × 50 cm high) was completely covered with a thin (0.05 mm) polyethylene sheet, and overflow water was drained from a PVC duct (35 cm high) located close to the end of section I. After the experiment, the sheet was removed and the PVC duct was washed with soap and rinsed. A new sheet and washed duct were used in every experiment to avoid contamination. The aquarium was marked every 30 cm to create five sections (I–V) for monitoring the behavioural responses of test fish.

Test fish were removed from the rearing tank using a transparent plastic bowl that was then floated on the surface of section I for 10–15 min before each test to acclimate the fish to the experimental conditions. Then, test juveniles were gently released into the aquarium near the end of section I. At first, fish locations were observed without an anemone for 30 min as a control, after which an opaque plastic plate was inserted between sections III and IV (without an anemone, juveniles tended to stay almost completely in section I). First, a plastic container was used to remove ca. 10 L of seawater from the aquarium in section V, and then an intact *Sg* was gently placed in section V. After the *Sg* was introduced, the partition was slowly removed and the experiment was started. Fresh seawater was continuously supplied at the end of section V at a flow rate 75–85 mL min^{-1}. The behaviours and locations of juveniles were recorded every 30 s for 30 min during every control and experimental period. Each test was repeated five times with a new set of four juveniles. The average number of juveniles that reached and stayed in section V was calculated for each control or experimental period, and statistical processing was identical to the trough experiments.

3. Results

3.1. Results of trough experiments with A. ocellaris

Trough experiments (Fig. 1) were conducted with naive *A. ocellaris* juveniles hatched from eggs under the following condition: without a host anemone, next to a symbiotic partner anemone (*Sg* or *Hm*) or next to a non-partner anemone (*Sm* or *Hc*). None of the tested juveniles were attracted to fresh seawater as the control prior to pouring seawater containing test anemone chemicals.

3.1.1. Juveniles hatched without a nearby host anemone: Non-imprinted juveniles

At first, non-imprinted juveniles were examined to verify innate recognition. Non-imprinted juveniles were able to innately recognise both symbiotic host anemones (*Sg* and *Hm*) to some extent; they were attracted to chemicals of *Sg* (paired t-test: t=7.4632, df=4, p-value=0.0017) and *Hm* (paired t-test: t=8.4973, df=4, p=0.0011) (Table 1; Fig. 2-A, 2-B; Supplementary Data Fig. I). However, their behaviours differed distinctly from those of juveniles that hatched normally next to their parents' host (Figs. 2-A', 2-B'). The former juveniles were not normally attracted to their symbiotic anemones, although they showed significant attraction compared to the control. Four characteristic behaviour patterns were observed. The first pattern (to *Sg,* 48% of tested fish, *n* =25; to *Hm,* 52.0%, *n* =25) was to move fairly straight to section V and stay near the inlet tube tip where seawater containing anemone chemicals was pouring in, but without showing any intimate approach to the tube tip itself. The second (to *Sg,* 32%; to *Hm,* 32.0%) was to move back and forth repeatedly between section I and IV or V, similar to behaviours observed in a previous study by Arvedlund and Nielsen (1996). The third (to *Sg,* 8.0%; to *Hm,* 0%) was to proceed slowly and stay near the boundary of section IV–V. The fourth (to *Sg,* 12%; to *Hm,* 16.0%) was to swim around and stay within section I alone, where they had been introduced.

The direction in which non-imprinted juveniles were attracted was not clear, and the fish that were attracted took a relatively long time to reach section V. Some juveniles reached section V and stayed there, but others did not swim straight toward section V or did not stay there for a long period. Judging from these behaviours, non-imprinted juveniles appeared to be at a substantial disadvantage in reaching their host compared to normally imprinted juveniles. However, most

importantly, the existence of innate recognition of symbiotic partner anemone species was definitively demonstrated.

Moreover, unexpectedly, some non-imprinted juveniles were also attracted to non-partner anemones *Sm* (paired t-test: t=3.1873, df=4, p=0.0333) and *Hc* (paired t-test: t=2.9125, df=4, p=0.0436) (Table 1; Fig. 2-C, 2-D; Supplementary Data Fig. I), although the attraction intensity was much weaker than to *Sg* and *Hm*. These individuals responded to the chemicals of *Sm* and swam less actively than with *Sg* or *Hm*, and several fish (20% of all tested fish, n=25) stayed near the inlet tube in section V, whereas others soon returned to section I. Non-imprinted juveniles were even more weakly attracted to *Hc*. Some individuals (32%, n=25) reached section V, but a few tested fish stayed for a brief period and were entirely indifferent to the inlet tube. These results show that non-imprinted juveniles can innately recognise *Hc*, although weakly. Non-imprinted juveniles were never attracted to the non-partner anemone *Eq* (paired t-test: t=1, df=4, p=0.3739)(Table 1; Fig. 2-E; Supplementary Data Fig. I).

3.1.2. Juveniles imprinted by Sg (S. gigantea): Sg juveniles, Juveniles imprinted by Hm (H. magnifica): Hm juveniles

Both *Sg* and *Hm* juveniles recognised both *Sg* (*Sg* juveniles to *Sg*, paired t-test: t=10.2638, df=4, p=0.0005; *Hm* juveniles to *Sg*, paired t-test: t=4.2758, df=4, p=0.0129) and *Hm* (*Sg* juveniles to *Hm*, paired t-test: t=3.5982, df=3, p=0.0135; *Hm* juveniles to *Hm*, paired t-test: t=11.9984, df=3, p=0.0012)(Table 1, 2; Figs. 2-A', 2-B', 3-A; Supplementary Data Fig. II, III). In short, imprinting on either symbiotic species was enough for individuals to recognise both symbiotic species; i.e. offspring can identify chemical cues to reach both symbiotic species, regardless of which species their parents inhabited. Tested juveniles quickly reached section V (Figs. 2-A', 2-B'), staying and gathering near the inlet tube tip for long periods. Marked differences were observed between *Sg* and *Hm* juveniles compared with non-imprinted juveniles in attraction intensity, affinity to chemicals and time taken to reach section V. Thus, imprinting clearly caused a quick and straight approach to, and strong affinity toward, the symbiotic anemones' chemicals.

Sg juveniles often approached and kissed the inlet tube tip and the wall behind the tube, and sometimes tried to eagerly dash into the tube tip. *Hm* juveniles also often kissed the mucus gauze that was wound around the inlet tube tip (see Methods 2.3.). *Sg* juveniles vibrated their bodies in the water pouring from the tube and *Hm* juveniles vibrated their bodies on the mucus gauze, similar to how juveniles usually rub their bodies on host tentacles. This intimate host-touching behaviour elicited by anemone chemicals was only observed in imprinted juveniles.

Note that *Sg* and *Hm* juveniles were never attracted to *Sm* (*Sg* juveniles to *Sm*, paired t-test: t=2.2953, df=4, p=0.0834; *Hm* juveniles to *Sm*, paired t-test: t= –1.6262, df=3, p=0.2024) (Table 1, 2, Figs. 2-C', 3-B; Supplementary Data Fig. II, III) and *Hc* (*Sg* juveniles to *Hc*, paired t-test: t=0.0346, df=3, p=0.9745; *Hm* juveniles to *Hc*, paired t-test: t=3.1770, df=3, p=0.0502) (Table 1, 2; Fig. 2-D', Fig. 3-C; Supplementary Data Fig. II, III). *Sg* juveniles were never attracted to *Eq* (paired t-test: t=2.7994, df=3, p=0.0679)(Table 1; Figs. 2-E'; Supplementary Data Fig. II). These results indicate that imprinting on host anemones suppresses the weak innate recognition of non-partner species (*Sm* and *Hc*). This clearly shows that the imprinting of symbiotic species complements rigid species-specific host recognition.

In trough experiments with *Hm*, 9-day-old *Sg* juveniles showed strange movements like small insects, wriggling and twirling their bodies on the trough bottom and suddenly moving straight to section V very quickly. They appeared to move in a taxis-like way rather than swimming normally.

3.1.3. Direct encounter experiment

Non-imprinted, *Sg* and *Hm* young fish were made to encounter an exposed symbiotic *Sg* in the aquarium. The results were significantly different between non-imprinted and imprinted fish (Fig. 4). All *Sg* (paired t-test: t=4.6243, df=3, p=0.0190) and *Hm* young (paired t-test: t=9.5139, df=3, p=0.0025) reached the *Sg* within 7–8 min (Table 3; Fig. 4-B, 4-C; Supplementary Data Fig. IV), and they soon began to kiss and touch it, rubbing against the tentacles while wagging their bodies. They moved around the oral disc, continually touching the tentacles, and entered among them.

However, numerous non-imprinted young were not attracted to (paired t-test: t=1.3061, df=5, p=0.2484) and did not reach the *Sg* during the 30 min observation period (Table 3; Fig. 4-A; Supplementary Data Fig. IV). Only 20.8% of non-imprinted individuals (n=24) reached the *Sg*, but it took them twice the time to reach it compared with *Sg* and *Hm* young. Moreover, it took them much longer to begin to touch and mount the tentacles, and intimate touching and kissing were seldom observed. Near the end of the observation period, a few fish began to touch the tentacles, but their affinity to them appeared to be very low and they did not slip among the tentacles. These results clearly show that non-imprinting is disadvantageous with regard to arriving at a host quickly, as well as hiding among its tentacles to escape from agonistic behaviours by adults and predations, even when individuals are grown.

3.1.4. Juveniles imprinted by Sm (S. mertensii): Sm juveniles

Amphiprion ocellaris juveniles were also expected to be imprinted by non-partners (*Sm* and *Hc*) because non-imprinted juveniles were innately able to weakly recognise these species. A pair of adult fish was made to associate with and breed beside a non-partner (*Sm*). The results demonstrated that *A. ocellaris* can be imprinted by a non-partner (*Sm*) when its eggs hatch adjacent to it. *Sm* juveniles were clearly attracted to *Sm* (paired t-test: t=15.5116, df=4, p=0.0001)(Table 2, Fig. 5-B) very similarly in the case of *Sg* and *Hm* juveniles to their symbiotic species.

Furthermore, strangely, *Sm* juveniles were not attracted to symbiotic *Sg* (paired t-test: t=0.6762, df=4, p=0.5360) and *Hm* (paired t-test: t=1.633, df=4, p=0.1778) (Fig. 5-A; Supplementary Data Fig. V). Some fish rapidly swam back and forth between sections I and V but never stayed in section V, whereas others did not move out of sections I and II, which was somewhat similar to the responses of non-imprinted juveniles. These results suggest that *Sm* juveniles would search exclusively for *Sm* and would be unlikely to ever reach their original symbiotic species (*Sg* and *Hm*) at their first encounter, which could result in a substitute partnership.

3.1.5. Juveniles imprinted by Hc (H. crispa): Hc juveniles

The "host-change manipulation" (section 2.4.) demonstrated that *A. ocellaris* was also able to be imprinted by *Hc*. However, the imprinting rate was remarkably low: only 5 of 38 individuals were imprinted during two attempts. Apparently, imprinting by *Hc* is rather difficult, although other causes may be at play. In addition, the attraction pattern of *Hc* juveniles to *Hc* was quite different from the patterns with *Sg* , *Hm* and *Sm* juveniles. The *Hc* juveniles moved very slowly to section V (Fig. 6-A) and acted as if they sensed something different in the chemicals of *Hc*. Even when they approached the mucus gauze, they turned their heads just before kissing it and rarely actually kissed it. Their behaviour was consistent with the fact that no ecological reports of *A. ocellaris–Hc* partnerships have actually been documented.

With chemicals of either *Sg* or *Hm* (Fig. 6-A'), *Hc* juveniles rapidly swam back and forth between sections I and V but never stayed in section V, which was similar to the behaviour of *Sm* juveniles with *Sg* and *Hm*. These results demonstrate that imprinting even occurs to non-partner species that are weakly innately recognised. Furthermore, this imprinting of non-partner species simultaneously suppresses the innate recognition of symbiotic species, in contrast to the case in which individuals are imprinted by symbiotic species. This indicates that imprinting functions to supplement the recognition of species other than symbiotic species, and that this mechanism likely creates substitute partnerships in some localities.

3.2. Changes in recognition with growth

Some changes in host recognition with growth were observed. Moreover, some grown *Sm* juveniles (older than 50 days) began to show a clear attraction to *Hc* (paired t-test: t= 2.847, df=4, p=0.0465) (Table 2; Fig. 5-C'; Supplementary Data Fig. V), although grown *Hm* juveniles never showed any attraction to *Hc* (paired t-test: t= –1.7493, df=2, p=0.2223) (Table 2; Fig. 3-C'; Supplementary Data Fig. III). *Sm* juveniles also began to show a weak attraction to *Sg* (paired t-test: t=3.2358, df=3, p=0. 048) and *Hm* (paired t-test: t=2.9346, df=5, p=0. 0325)(Fig. 5-A'; Supplementary Data Fig. V) and gradually tended to spend more time in section V with growth. One-year-old *Sm* juveniles that were reared without hosts still recognised *Sm* (paired t-test: t=4.6354, df=2, p=0. 0435) (Fig. 5-B"). However, they were more strongly attracted to *Hm* (paired t-test: t=30.4320, df=2, p=0. 0011) (Fig. 5-A"; Supplementary Data Fig. V) than *Sm*; i.e. the suppression from imprinting on *Sm* had disappeared and the recognition of *Hm* had sufficiently recovered within a year.

Moreover, some grown (older than 70–80 days) *Hm* juveniles started to be attracted to *Sm* (paired t-test: t=2.7503, df=5, p=0. 0403)(Table 2; Fig. 3-B'; Supplementary Data Fig. III), which also suggests the recovery of the innate recognition of *Sm*, although the response differed among the three broods examined. These results suggest that the suppression of other species recognition by imprinting via *Hm* starts to disappear in later juvenile stages (ca. 2–3 months). One-year-old *Hm* juveniles that were reared without hosts were attracted to *Hm* (paired t-test: t=7.9725, df=2, p=0. 0154)(Table 2; Fig. 3-A"; Supplementary Data Fig. III), and they also recognised *Sm* (paired t-test: t=3.5835, df=3, p=0. 0372)(Table 2; Fig. 3-B"; Supplementary Data Fig. III)

3.3. Critical period

A "host-exchange experiment" was conducted to determine when host imprinting occurs. The imprinting rates of *Sm* juveniles were not usually high, probably because eggs were laid on the inside curved wall of a half-cut PVC duct so that the host's oral disc and tentacles did not always touch the eggs. However, such a low imprinting rate was thought to be rather convenient for verifying if post-hatching imprinting occurs because non-imprinted embryos afford the opportunity for post-hatching imprinting even if pre-hatching imprinting can occur.

Sm juveniles were not attracted to *Sg*. Therefore, host exchange of an egg batch from *Sm* to *Sg* was conducted. After more than two-thirds of the eggs had hatched, the spawning PVC duct that was adjacent to *Sm* was placed closely adjacent to *Sg* in the *Sg* tank (see section 2.5). If *Sg* juveniles were found in the group that hatched in the *Sg* tank, the occurrence of post-hatching imprinting would be verified, and if *Sm* juveniles were found in the same group, the occurrence of pre-hatching

imprinting would also be verified. Indeed, both *Sg* and *Sm* juveniles were found in the group that hatched in the *Sg* tank (Table 4), clearly indicating that both pre-hatching and post-hatching imprinting had occurred. Thus, both embryonic and post-hatching imprinting were verified. The fish that hatched in the *Sm* tank consisted entirely of *Sm* and non-imprinted juveniles, and they were never attracted to *Sg*.

3.4. Imprinting in A. perideraion

Breeding of *A. perideraion* associated with *Hc* was attempted to confirm the function of imprinting to supplement substitute partnerships. However, because of difficulties in breeding *A. perideraion*, only eight juveniles survived from one attempt among four trials. Two of the eight individuals were attracted to the chemicals of *Hc* (Fig. 6-B); i.e. they were *Hc* juveniles, but they were not attracted to their symbiotic anemone *Hm* (Fig. 6-B'). If *Hc* represents another partner, *Hc* juveniles should also have been attracted to *Hm*, and *A. perideraion* should inhabit *Hc* in every region where these two species sympatrically occur. These results indicate that *Hc* is not a symbiotic partner but a substitute species for *A. perideraion*, although the sample size was very small.

4. Discussion

4.1. Crucial spawning positioning

Four anemonefish species were observed to display the same spawning site preferences in the field: eggs were laid adjacent to the host anemone's column or pedal disc. This spawning site preference is thought to be influenced by both host imprinting and predator protection at night (Arvedlund et al., 2000).

In this study, the highest imprinting rate (91.0%) was observed in *Sg* juveniles whose spawning position most closely resembled natural conditions in the sea. Unnatural spawning sites that were some distance from the host were likely responsible for the lower imprinting rates in *Hm* juveniles (30.5–67.6%, over four breedings) and *Sm* juveniles (37.8–62.0%, over six breedings). In the *Hm* case, the spawning site was ca. 10 cm from the host so that egg batches were rarely touched by the host's tentacles. A natural spawning positioning immediately adjacent to the host must be necessary to ensure pre- and immediate post-hatching imprinting. This crucial positioning is

456 probably the reason why the eggs are completely protected from host anemone stings (Elliott and
457 Mariscal, 1996; Miyagawa, 1989; Davenport and Norris, 1958).
458
459 *4.2. Pre- and post-hatching imprinting*
460
461 The development of the olfactory system in *A. melanopus* embryos was examined, and the
462 ontogenetic timing of the imprinting mechanism was thought to occur toward the end of embryonic
463 development (Arvedlund et al., 2001). The present study confirms this observation.
464 The water supply to the parents' tank and eggs with the host was stopped 30–60 min before
465 hatching; therefore, seawater in the tank was filled with host chemicals. However, even though all
466 newly hatched larvae stayed in host chemicals for 20–60 min before being transferred to rearing
467 tanks, every hatched group contained some non-imprinted individuals [non-imprinted rates were
468 9.0–69.5% over all breedings (12) in this study with various host species]. These results suggest
469 that post-hatching imprinting occurs over a limited period immediately after hatching. This strategy
470 is likely highly adaptive because newly hatched larvae soon rise up to the water surface and enter
471 their pelagic life. Therefore, pre-hatching imprinting must be very important for anemonefish.
472 However, the timing of the onset of pre-hatching imprinting is still unknown.
473 One of the chemicals of *Hc*, which is recognised by *A. perideraion*, has been identified as
474 "Amphikuemin" (Konno et al., 1990; Murata et al., 1986). The present study verified that
475 "Amphikuemin" is one of the chemicals that is supplemented by imprinting via *Hc.* Young *A.*
476 *perideraion* with plugged nostrils could recognise "Amphikuemin" (Miyagawa-Kohshima, pers.
477 obs.), whereas salmon with occluded nostrils were unable to return to their home river (Wisby and
478 Hasler, 1954). Potential candidates might be sensory-like organs scattered on the head surface
479 (observed by scanning electron microscopy) or taste organs. Embryos may receive their parents'
480 host chemicals through chemoreceptors, e. g. solitary chemosensory cells (Kotrschal, 1991), other
481 than their nostrils, during pre-hatching imprinting.
482
483 *4.3. Unique symbiotic life and strict social structure at each host, and a function of recovery of*
484 *innate recognition*
485
486 Anemonefish form groups with a size-based hierarchy (Allen, 1975): one breeding pair and
487 fewer than four subordinate fish are able to inhabit each host (Hattori, 2012; Buston, 2003).
488 Afterward, innate recognition recovers with growth, as shown in grown *Sm* and *Hm* juveniles, and it
489 is thought to also recover in juveniles that have associated with their host in the sea. The beginning
490 of recovery of innate recognition is thought to correspond to the time when juveniles are just

beginning to be evicted from their first host because the body size of evicted juveniles observed late in the breeding season (roughly July–September) in the sea (Miyagawa-Kohshima, pers. obs. at Kuroshima) seemed to closely resemble that of laboratory-bred juveniles (total length: 1.5–2.8 cm) at ca. 2–3 months. This recovery of innate recognition with growth must expand the range of choices for potential subsequent hosts and plays an important role with respect to the promotion of substitute partnerships, thereby enhancing juvenile survival.

4.4. Actual ecological documentation of substitute partnerships

At Madang, Papua New Guinea (Elliott and Mariscal, 1996, 2001), where the highest species diversity (nine) of anemonefish occurs, the actual occurrence of substitute partnerships is well represented. In this region, *A. percula* (closely related to *A. ocellaris*) inhabits *Sg*, *Hm* and even *Sm*, while *A. perideraion* inhabits *Hm*, *Hc* and even *Sg*. Five anemonefish species inhabit *Hm* and seven species inhabit *Hc* in this region. Therefore, symbiotic and substitute anemone species overlap among many anemonefish species.

Amphiprion sandaracinos and *A. leucokranos* were observed to cohabit one host with other anemonefish species, while others did not. *Amphiprion percula* and *A. perideraion*, which inhabit a common host *Hm*, usually have different distribution patterns among zones at Madang, and in rare cases, these two species occupy the same host simultaneously and are very aggressive toward each other (Elliott and Mariscal, 2001). Therefore, heterospecific evictions likely occasionally occur when juveniles of different anemonefish species recruit to the same host in this region.

Amphiprion ocellaris and *A. perideraion* occur in the Ryukyu Islands and Moluccas, Indonesia (Dunn, 1981). In Madang, *A. percula* and *A. perideraion* live sympatrically. In these areas, *A. perideraion* inhabits both *Hm* and *Hc* (an exception was reported on Lizard Island; Fautin, 1986). In these regions, *A. perideraion* must be obligated to inhabit *Hc* because of interspecific competition over *Hm* with *A. ocellaris* or *A. percula*, as well as heteroevictions after the establishment of its first association. Indeed, *A. perideraion* only inhabits *Hm* even though *Hc* also occurs in areas where neither *A. ocellaris* nor *A. percula* are found sympatrically, e.g. at Fiji (Allen, 1978; Dunn, 1981) and Eniwetok (Allen, 1972). Observations at Fiji and Eniwetok suggest that conspecific evictions do not promote substitute partnerships, while those on the Ryukyu Islands, Moluccas and Madang show that heterospecific evictions do promote substitution.

A particular note regarding the observations at Madang (Elliott and Mariscal, 2001) is that even with intense interspecific competition over symbiotic and substitute species among many anemonefish species, *A. perideraion* and *A. percula* do not blindly inhabit any species and clearly search for subsequent hosts using their innate recognition after experiencing heteroeviction: *A.*

526 *perideraion* can recognise *Hc* innately (Miyagawa, 1989), and *A. percula* is predicted to recognise
527 anemone *Sm* innately because it inhabits exactly the same symbiotic and a substitute species of *A.*
528 *ocellaris.*
529 Interspecific competition is not responsible for species-specific anemonefish–sea anemone
530 partnerships (Elliott and Mariscal, 2001). However, interspecific competition over common
531 symbiotic species is thought to be the primary contributor to the occurrence of substitute
532 partnerships in *A. perideraion* and *A. percula.*
533
534 *4.5. Hypothesis regarding spare recognition—potential substitute species of each anemonefish*
535
536 *Amphiprion melanopus* is not imprinted by *Heteractis malu*, which is not a symbiotic species
537 of *A. melanopus* (Arvedlund et al., 1999). This suggests that *A. melanopus* cannot recognise *H.*
538 *malu* innately as a potential host; i.e. *A. melanopus* does not have an innate template (Konishi,
539 1965) for *H. malu*. In this study, *A. ocellaris* did not recognise *Eq* innately; i.e. *A. ocellaris* does not
540 have an innate template for *Eq* and cannot be imprinted by it.
541 Anemone species which have been observed to be inhabited by any anemonefish have all been
542 considered "symbiotic" species so far, even though some anemonefish-anemone partnerships have
543 only been rarely observed in some localities. However, the present study revealed that two types of
544 partnerships exist in this symbiosis, symbiotic and substitute. It demonstrated that *Sm* and *Hc* are
545 potential substitute species for *A. ocellaris*; meanwhile, *Hc* has been observed as a substitute
546 species for *A. perideraion* at Madang and in the Ryukyu Islands. This additional function in the
547 chemical recognition system is unlikely to be limited to these two anemonefish species.
548 Here, we hypothesise that every anemonefish has innate templates for symbiotic species and
549 also spare templates for a few non-partner species, as do *A. ocellaris* and *A. perideraion*. In order to
550 know what species are programmed for spare recognition in each anemonefish, we re-summarised
551 anemonefish—sea anemone distribution data (Moyer & Yogo 2001; Elliott & Mariscal 2001,1996;
552 Fautin & Allen 1992; Dunn 1981), focusing on symbiotic species and predicted substitute species in
553 each anemonefish species complex (Table 5).
554 Partnerships that are observed in every region where two species occur sympatrically are
555 considered symbiotic partnerships. If in any region two species occur sympatrically but do not form
556 partnerships, these two species would not be considered symbiotic. Meanwhile, unusual
557 partnerships that have only been observed in some localities are judged to be substitute
558 partnerships. As distinguished in Table 5, anemone species are inhabited as either symbiotic or
559 substitute (later proposed as sub-symbiotic) by each anemonefish in each species complex. Table 5
560 indicates that each anemonefish likely has a few spare templates in its innate recognition, which

supports the "spare recognition hypothesis". It is also shown, symbiotic species seem to be common among anemonefish species in each species complex, while substitute species seem to show little variation among anemonefish species in each species complex. *Hc* is shown to be the most utilised substitute species by various anemonefish species.

4.6. Why are substitute partnerships only observed in some localities?

Although all anemonefish species likely have a few spare templates for substitute species in their innate recognition, very few substitute partnerships are actually observed. Whether a substitute partnership actually arises seems to depend on the ecological situation of each anemonefish in each habitat. The most relevant scenario is likely that anemonefish species that cannot cohabitate in a single host face interspecific competition over a common symbiotic host. The characteristic behaviours of anemonefish species, especially the size of their active range (i.e. how far they dare move to search for a subsequent host after being evicted from the first host), and the populations of common symbiotic and substitute species must largely be involved in the occurrence of substitute partnerships.

Even though the present study demonstrated that *A. ocellaris* has weak innate recognition for two non-partner anemones (*Sm* and *Hc*), the *A. ocellaris–Sm* partnership has only been supported by photographs (Allen, 1972) taken in the Philippines (Dunn, 1981). This partnership is rarely observed, probably because *A. ocellaris* is strongly dependent on its host, which it never swims far from (Miyagawa-Kohshima, pers. obs. at Kurosima), while in *A. perideraion*, migration between groups, although rare, has been observed (Hattori, 1995).

The ancestral species of each species complex has been suggested to have completed their differentiation for host preference at the centre of the distribution area, the Indo-Australian Archipelago (Allen 1980), and then to have dispersed and differentiated further, judging from almost identical host preferences among allopatric species in each species complex (Miyagawa, 1989). The additional function of the chemical recognition system to produce substitute partnerships might also have been established in the ancestral species of each complex in the same area. At the centre of the distribution area, high species diversity, intense interspecific competition and substitute partnerships must have already occurred among the ancestral species, as observed at Madang by Elliott and Mariscal (1996, 2001). Therefore, symbiotic and substitute species are fairly common within each complex beyond regional differentiation (Table 5).

However, farther from the centre of the distribution area, species diversity is much lower, which reduces the occurrence of host species overlap among sympatric anemonefish. Substitute partnerships only occur in localities where anemonefish face host shortages, especially those caused

by interspecific competition among sympatric species over common host species.

If precise quantitative ecological investigations similar to what Elliott and Mariscal (2001) undertook at Madang were conducted in regions where substitute partnerships do and do not occur, clear answers regarding the ecological conditions that promote substitute partnerships could be obtained. In such ecological investigations, information about the individual fish that are associated with each anemone needs to be collected, such as body size and developmental stage, e.g. newly recruited juveniles during the breeding season, young fish or a breeding adult pair. Such studies will provide more detailed information about substitute partnerships.

4.7. Necessity of making a clear distinction between symbiotic and substitute species

The different types of partnership, symbiotic and substitute, should not be thought of together as "symbiotic" because they arise through different mechanisms: one type is truly symbiotic and the other is spare. If these two types of partnerships are left mingled as "symbiotic", some confusion will arise in future studies.

Here, we propose that substitute partnerships should be distinguished from symbiotic relationships by calling them "sub-symbiotic" because a clear distinction between them will be especially necessary for resolving existing confusion and advancing our understanding of unsolved problems in this recognition system.

If this clear distinction is made, outstanding problems can be documented as follows. How do anemonefish innately recognise their symbiotic and sub-symbiotic species? How does imprinting by symbiotic species complement rigid species-specific recognition while suppressing sub-symbiotic species recognition? Why is imprinting by either host species sufficient for recognising both symbiotic species? How can imprinting by certain sub-symbiotic species supplement that species recognition while conversely suppressing the recognition of symbiotic species? How are sub-symbiotic species programmed into the innate recognition in each anemonefish?

Furthermore, unexpectedly, such a distinction also provides a clearer understanding of the protection mechanism. Early studies using *A. clarkii–Sg* (Mariscal, 1965, 1970a) and *A. bicinctus–Sg* (Schlichter, 1968, 1976) combinations indicated that anemonefish do not have protection against symbiotic anemone stings. However, later, 12 of 27 anemonefish species were discovered to have innate protection against their symbiotic anemones, with no counter examples (Elliott and Mariscal,1996; Miyagawa, 1989; Miyagawa and Hidaka, 1980) Therefore, one can reasonably assume that every anemonefish has innate protection against its symbiotic anemones. However, the reasons for such incompatible results in early studies remain unexplained. As a possible explanation, Table 5 indicates that the combinations examined in early studies are not symbiotic,

631 but sub-symbiotic; they are included among the four imperfectly protected combinations ([f)] marked
632 species) among sub-symbiotic species in the *clarkii* complex of the genus *Amphiprion*. These
633 species are thought to establish associations with each anemone through an "acclimation process",
634 exactly as indicated by Mariscal and Schlichter, although what happens to the fish body surface
635 during the "acclimation process" remains unclear. These can be thought of as special combinations,
636 even among sub-symbiotic species, because many innately protected combinations exist among
637 sub-symbiotic combinations. The well-known combinations of *A. clarkii* and *A. bicinctus* with *Sg*
638 seem to be especially unique, and further precise investigations are desirable, which may reveal
639 some clues about the ancient beginning of this relationship.
640
641 *4.8. The necessity of imprinting*
642
643 Why does this rigid species-specific host recognition in anemonefish need imprinting?
644 Imprinting is thought to provide two functions to ensure juvenile survival in the habitats where each
645 anemonefish lives. The first function is that imprinting complements innate recognition, leading to
646 rigid species-specific partnerships in each anemonefish species. Reaching their hosts as soon as
647 possible after entering the benthic stage is the top priority for anemonefish juveniles to survive.
648 Making a taxis-like prompt approach, as observed in very early stage *Sg* juveniles, following rigid
649 species-specific recognition of symbiotic host anemone(s) must be the most efficient method when
650 fish are small and still have poor swimming ability. Meanwhile, non-imprinted young fish had a
651 double handicap with respect to the prompt approach to their host and immediate hiding among its
652 tentacles. The results of the direct encounter experiment clearly demonstrate the necessity of
653 imprinting and show how disadvantageous it is to survival if juveniles are not imprinted.
654 Unlike imprinting in birds, which is involved in the recognition of their own species (Bolhuis
655 1991; Immelman, 1972; Lorenz, 1935), ecological imprinting such as that in anemonefish is
656 involved in the recognition of objects, e.g. hosts, habitat areas or food (Immelman, 1975). Rigid
657 recognition in anemonefish would not necessarily be advantageous throughout their entire life. If
658 juveniles are evicted from their first host, innate recognition is more advantageous for juveniles
659 when searching for subsequent hosts among species, including sub-symbiotic species. Olfactory
660 memory via imprinting is optimised when it is most needed; in anemonefish, this occurs at a very
661 early stage when first searching for a host, while in salmon, it occurs near the end of their life when
662 returning to their home rivers (Hasler and Scholz, 1983).
663 The second function of imprinting is to provide for sub-symbiotic partnerships to allow
664 adaptation to environmental changes, especially in cases of host shortage due to intense
665 interspecific competition. The configuration of anemonefish species that live sympatrically and the

population of each anemonefish-symbiotic anemone differ among regions. However, imprinting via the parents' host helps the next generation obtain clues to reach the most appropriate host species in the local habitat, reflecting the ecological situation of their parents. Juveniles that hatch adjacent to a sub-symbiotic species can avoid interspecific competition over common symbiotic host species because they only search for that sub-symbiotic species at their first encounter, as observed in *Sm* and *Hc-A. ocellaris* and *Hc-A. perideraion* juveniles in this study. This mechanism likely allows some anemonefish to survive among sympatric species whose host species overlap, as observed in the Ryukyu Islands and at Madang.

Anemonefish are buttressed by multiple innate protection mechanisms against symbiotic anemones (Miyagawa, 1989). The present study further demonstrates that this symbiosis is also buttressed by a chemical recognition system that consists of innate recognition and imprinting, which supports juvenile survival by helping them adapt to the ecological situation in each habitat.

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782

Figure captions

Fig. 1. Arrangement of the trough experiment.

PVC troughs (200 cm long × 12 cm wide × 9 cm high) were used for the experiments. The water was 5 cm deep in every experiment. The trough was marked every 40 cm, dividing it into five sections (I–V), to monitor the behavioural responses of test fish.

Fig. 2. Example of the average positions of five non-imprinted and *Sg*-*A. ocellaris* juveniles during a typical trough experiment over 60 min.

Seawater containing chemicals from each test anemone was poured into the trough at the end of section V.

solid line: average fish positions in experiment; dotted line: average fish positions in control; faint dotted line: range of fish occurrence in experiment

Fig. 3. Example of the average positions of three stages of *Hm* juveniles of *A. ocellaris* in response to various anemone chemicals during 30 min of observation during a typical trough experiment.

Seawater containing chemicals from each test anemone was poured into the trough at the end of section V.

solid line: average fish positions in experiment; dotted line: average fish positions in control; faint dotted line: range of fish occurrence in experiment

Fig. 4. Typical example of the average positions of five fish of non-imprinted, *Sg* and *Hm* young of *A. ocellaris* in response to an exposed symbiotic anemone *Sg* during 30 min of observation in a direct encounter experiment.

An exposed symbiotic anemone *Sg* was placed in section V of the aquarium.

solid line: average fish positions in experiment; dotted line: average fish positions in control; faint dotted line: range of fish occurrence in experiment

Fig. 5. Example of the average positions of three stages of *Sm* juveniles of *A. ocellaris* in response to various anemone chemicals during 30 min of observation in a typical trough experiment.

Seawater containing chemicals from each test anemone was poured into the trough at the end of section V.

solid line: average fish positions in experiment; dotted line: average fish positions in control; faint dotted line: range of fish occurrence in experiment

Fig. 6. Example of the average position of *Hc-A. ocellaris* and *Hc-A. perideraion* juveniles in response to chemicals from anemone *Hc* by which they were imprinted and symbiotic *Hm* during the 30-min observation

Seawater containing chemicals from each test anemone was poured into the trough at the end of section V.

Experiments were done in early stage: *Hc-A. ocellaris* juveniles (12–14day-old); *Hc-A. perideraion* juveniles (19–20 day-old)

solid line: average fish positions in experiment; dotted line: average fish positions in control; faint dotted line: range of fish occurrence in experiment

Glossary

Imprinting

Imprinting is the term used in psychology and ethology to describe any kind of phase-sensitive learning at a particular life stage (critical period) that is rapid and apparently independent of the consequences of behaviour. The well-known form of imprinting is filial imprinting. The influence of early stage experience is very important with respect to certain aspects of adult behaviour, especially with regard to the determination of sexual preferences, as well as to several other aspects of social and other behaviours (e.g. recognition of food, habitats, hosts).

Author contributions

All experiments were performed by K.M-K.. Laboratory breeding of *A. ocellaris* under various conditions was conducted by S.O., D.O. and K.M-K., and that of *A. perideraion* was performed by K.M-K.. A.T., S.N., and S.M. was partly involved in taking care of larvae and juveniles. H.T. constructed the plumbing for the experimental space seawater supply. S.O., D.O., S.N. and S.M. prepared the rearing aquaria and plumbing. S.O., D.O., Y.B., H.T., A.T., N.Y., M.N., S.M., S.N., Y.K., M.W., H.K. and H.I. engaged in rearing symbionts and were partly involved in collecting animals for experiments. S.O., S.M., Y.B., Y.K., D.O., M.W. A.T. and H.K. cultured marine rotifers and *Artemia*. M.N. and S.M. directed and supervised the Coral Group staff. S.U. and H.M. organised and supervised the study. The manuscript was written by K.M-K..

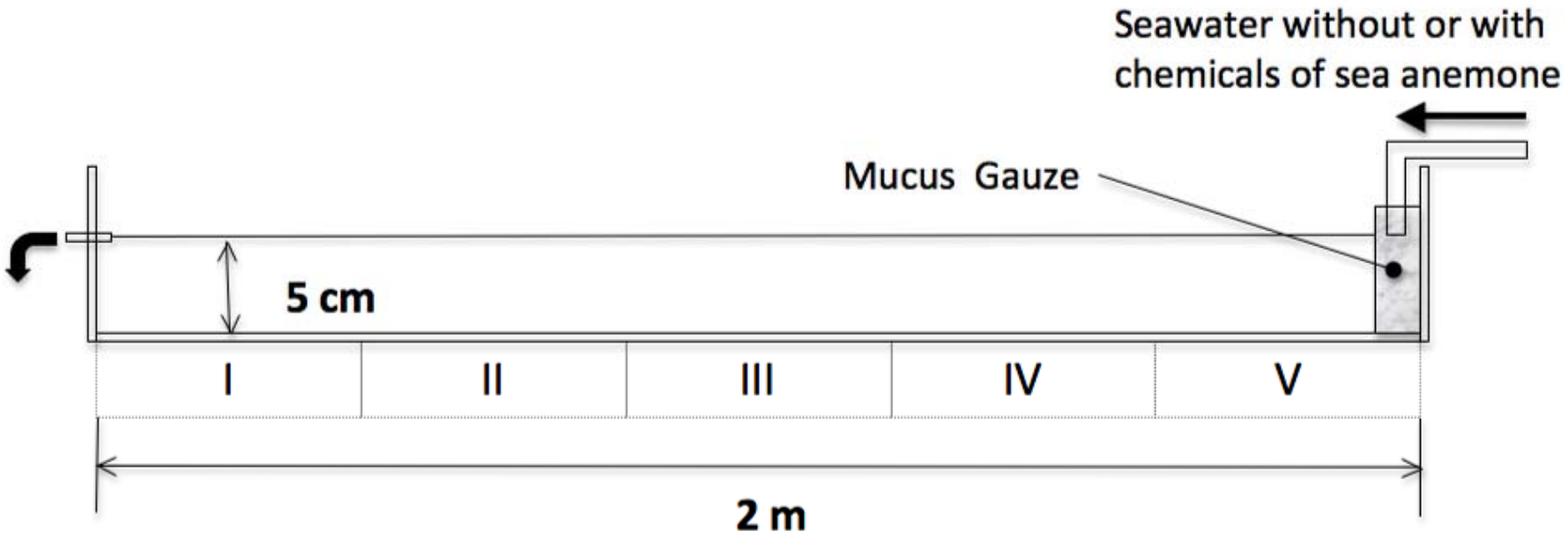

Seawater without or with
chemicals of sea anemone
Mucus Gauze
5 cm
I
II
III
IV
V
2 m

Fig. 1

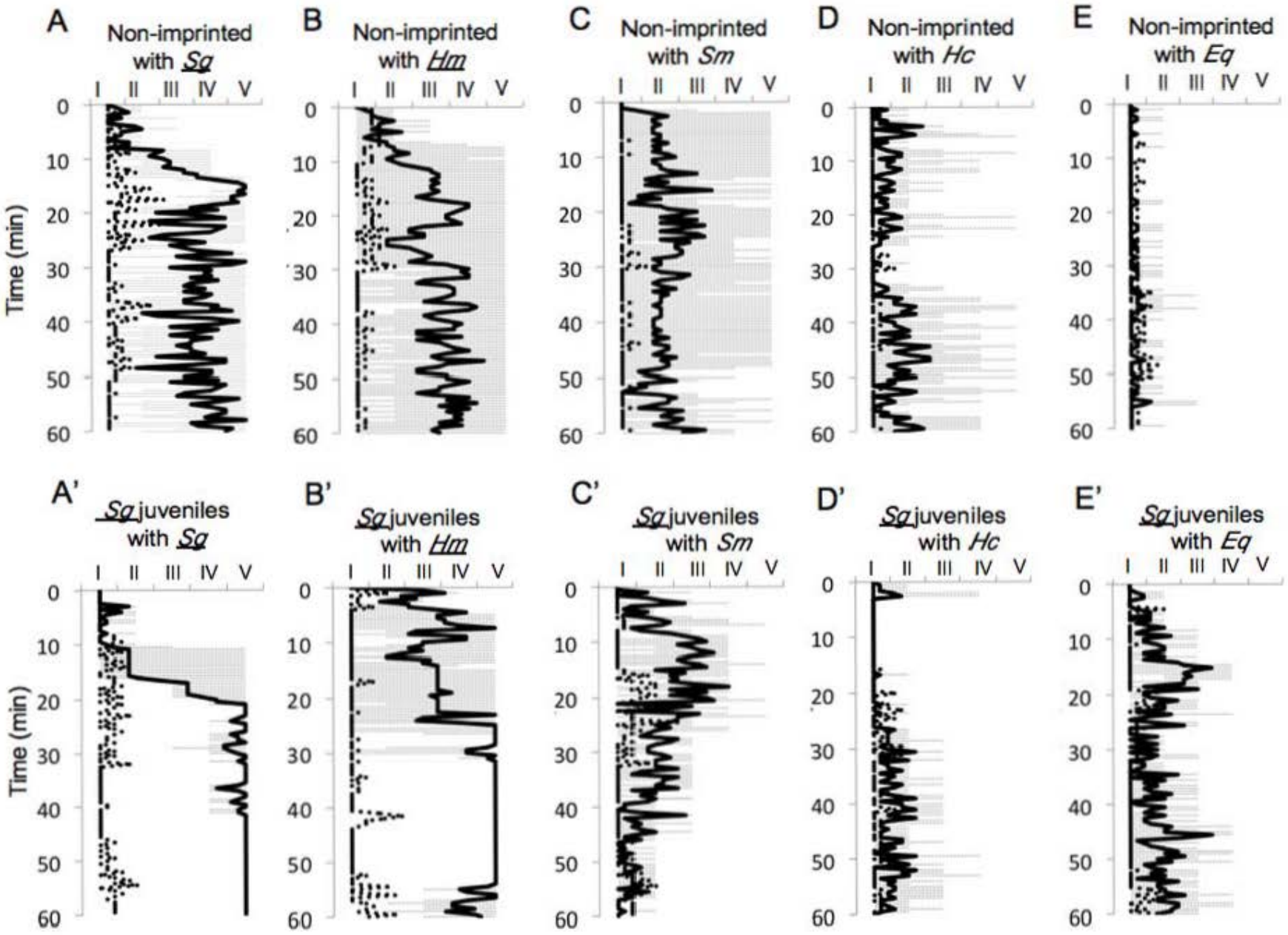

A
Non-imprinted with Sg
B
Non-imprinted with Hm
C
Non-imprinted with Sm
D
Non-imprinted with Hc
E
Non-imprinted with Eq
A'
Sg juveniles with Sg
B'
Sg juveniles with Hm
C'
Sg juveniles with Sm
D'
Sg juveniles with Hc
E'
Sg juveniles with Eq
Time (min)
I II III IV V
0 10 20 30 40 50 60

Fig. 2

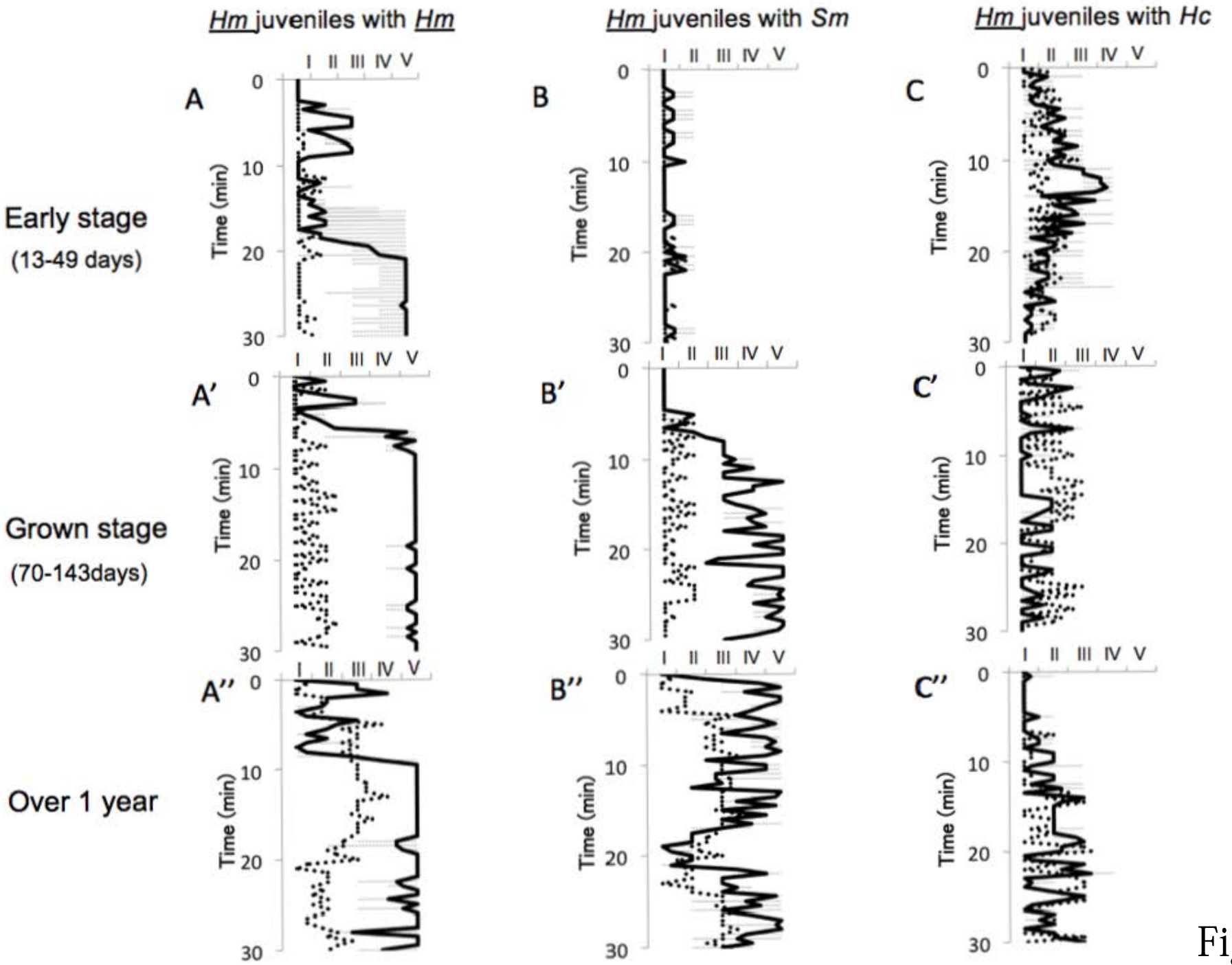

Hm juveniles with Hm
Hm juveniles with Sm
Hm juveniles with Hc
Early stage
(13-49 days)
Grown stage
(70-143days)
Over 1 year
A
A'
A''
B
B'
B''
C
C'
C''
I II III IV V
Time (min)
0
10
20
30

Fig. 3

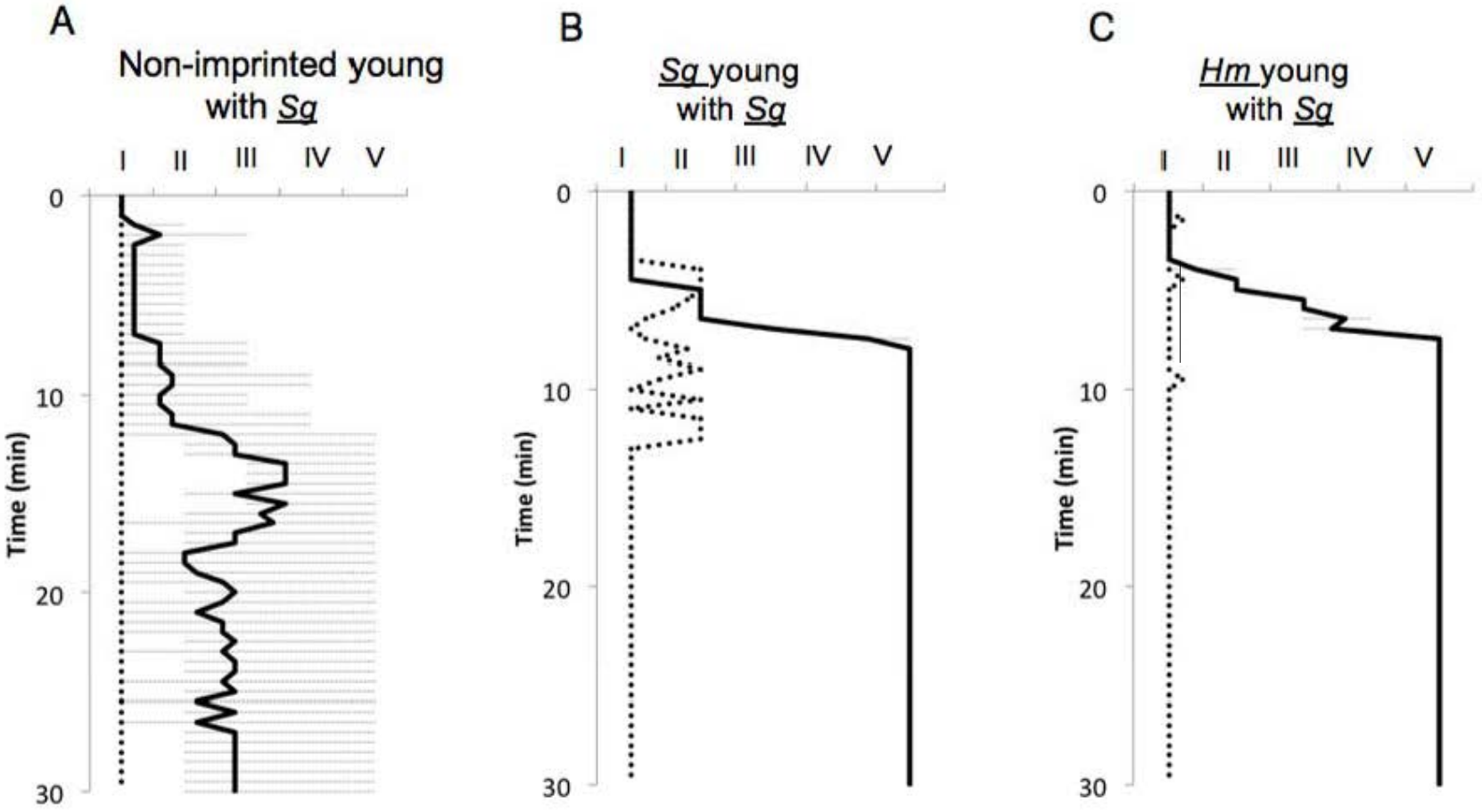

A
Non-imprinted young
with Sg
I
II
III
IV
V
0
10
20
30
Time (min)
B
Sg young
with Sg
I
II
III
IV
V
0
10
20
30
Time (min)
C
Hm young
with Sg
I
II
III
IV
V
0
10
20
30
Time (min)

Fig. 4

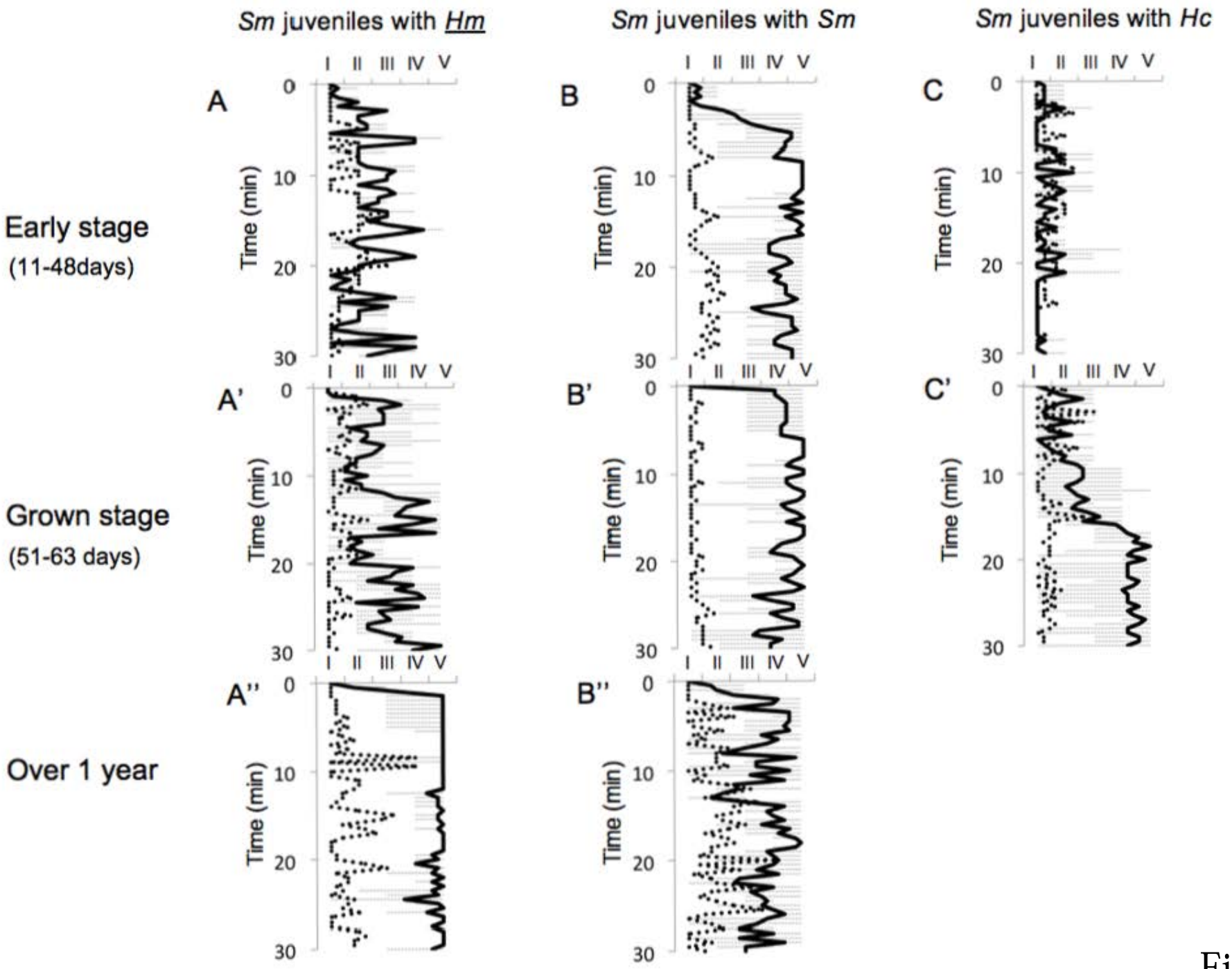

Sm juveniles with Hm
Sm juveniles with Sm
Sm juveniles with Hc
Early stage
(11-48days)
Grown stage
(51-63 days)
Over 1 year
A
A'
A''
B
B'
B''
C
C'
I II III IV V
Time (min)
0
10
20
30

Fig. 5

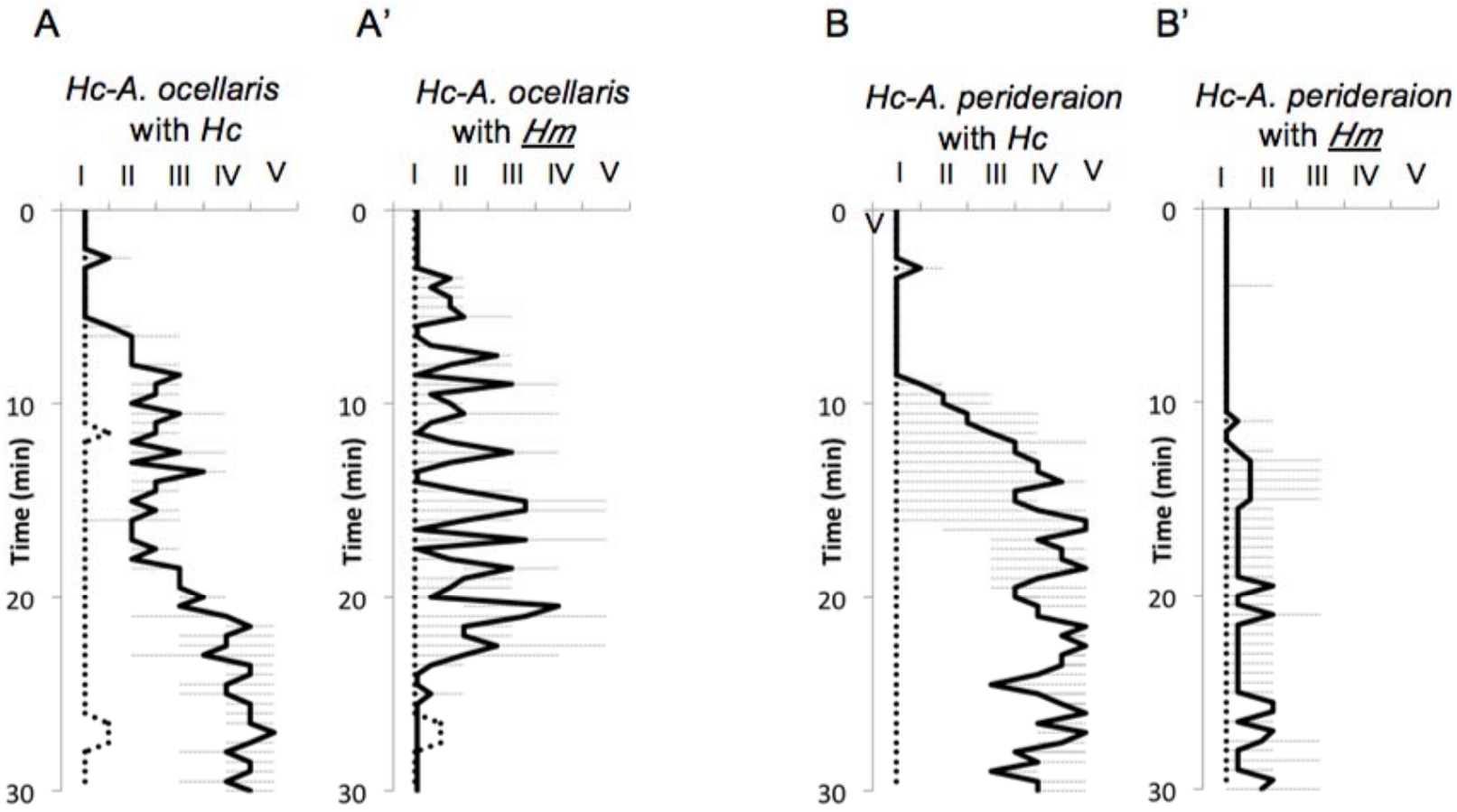

A
A'
B
B'
Hc-A. ocellaris
with Hc
Hc-A. ocellaris
with Hm
Hc-A. perideraion
with Hc
Hc-A. perideraion
with Hm
I II III IV V
0
10
20
30
Time (min)

Fig. 6

Table 1 Response of non-imprinted and *Sg* juveniles of *A. ocellaris* to symbiotic-partner anemones and non-partner anemones

	Non-imprinted Juveniles (N=5)			*Sg* juveniles (N=5)		
Sea Anemone	(Repl.)	Control	Anemone chemicals	(Repl.)	Control	Anemone chemicals
Sg	(5)	0.04±0.04	1.47±0.41	(5)	0.01±0.03	2.88±0.62
Hm	(5)	0.01±0.02	1.81±0.46	(4)	0	2.83±1.08
Sm	(5)	0.03±0.03	0.45±0.30	(5)	0	0.08±0.04
Hc	(5)	0.02±0.02	0.23±0.15	(4)	0	0.01±0.04
Eq	(5)	0	0.03±0.04	(4)	0	0.04±0.03

S. gigantean=Sg, *H. magnifica=Hm*, *S. mertensii=Sm*, *H. crispa=Hc* and *E. quadricolor=Eq* (Underlined names are symbiotic-partner species of *A. ocellaris*)

Average “reach V value” ± SD
“reach V value”: the average number of juveniles that reached or stayed in section V per observation period.

Non-imprinted juveniles: 11–49 day-old; *Sg* juveniles: 9–52 day-old

N= number of tested juveniles in each experiment.

Table 2 Response of early, grown stage juveniles, and over 1 year-old young of *Amphiprion ocellaris* imprinted by anemone *Hm* and *Sm* to symbiotic-partner anemones and non-partner anemones

A.ocellaris juveniles	Sea anemone	Stage of juveniles: Early stage Control	Early stage Anemone chemicals	Grown stage Control	Grown stage Anemone chemicals	Over 1 year Control	Over 1 year Anemone chemicals
		(N=4)		(N=3)		(N=4)	
Hm juveniles	*Sg*	(5) 0	1.39±0.72	(4) 0.06±0.08	1.69±0.61		
	Hm	(4) 0.03±0.05	0.67±0.13	(4) 0	1.23±0.75	(3) 0.12±0.21	2.56±0.49
	Sm	(4) 0.01±0.01	0	(6) 0.01±0.02	0.72±0.51	(4) 0.01±0.01	0.66±0.36
	Hc	(4) 0	0.04±0.03	(3) 0.06±0.06	0.03±0.05	(3) 0.02	0.02±0.04
		(N=5)		(N=3)		(N=5)	
Sm juveniles	*Sg*	(5) 0.03±0.07	0.13±0.22	(4) 0.08±0.10	0.66±0.43		
	Hm	(5) 0	0.01±0.02	(6) 0.01±0.01	0.28±0.22	(3) 0.15±0.15	4.37±0.13
	Sm	(5) 0.19±0.21	3.04±0.48	(5) 0	1.80±0.30	(3) 0.08±0.08	1.06±0.42
	Hc	(4) 0.03±0.06	0.01±0.03	(5) 0.02±0.04	0.57±0.63		

Average “reach V value” ± SD
“reach V value”: the average number of juveniles that reached or stayed in section V per observation period.
Early stage: *Hm* juveniles (13–49 day-old); *Sm* juveniles (11–48 day-old)
Grown stage: *Hm* juveniles (70–143 day-old); *Sm* juveniles (51–63 day-old)
N=number of tested juveniles in each experiment.

Table 3 Response of non-imprinted and *Sg* and *Hm* young fish of *Amphiprion ocellaris* to an exposed symbiotic anemone *Sg* in direct encounter experiment

A. ocellaris juveniles	Replication	Control	Exposed *Sg*
Non-imprinted young (N=4)	6	0	0.56±0.84
Sg young (N=4)	4	0	2.52±1.09
Hm young (N=4)	4	0	2.80±0.58

Average “reach V value” ± SD
“reach V value”: the average number of juveniles that reached or stayed in section V per observation period.
N= number of tested juveniles in each experiment.

Table 4 Results of the host-exchange experiment: imprinted rates by each anemone in Sm and *Sg* tanks

Hatched condition		Rates of imprinted juveniles: 1st experiment 2011/08/01 (%)	2nd experiment 2011/10/05 (%)
Before transferring (hatched in *Sm* tank)		(N=45)	(N=18)
	Sm juveniles	26.7	11.1
	Sg juveniles	0	0
	Non-imprinted	73.3	88.9
After transferring (hatched in *Sg* tank)		(N=84)	(N=64)
	Sm juveniles	45.2	14.1
	Sg juveniles	19.1	23.4
	Non-imprinted	35.7	62.5

N= number of tested juveniles in each tank.

Table 5 Partnerships between anemonefish and symbiotic species or predicted sub-symbiotic (substitute) species

Anemonefish Genus & Species complex	Anemonefish species (No.of symbiotic anemones)	Sea anemone Symbiotic species (No. of symbionts): symbionts species	Sea anemone Predicted sub-symbiotic species (No. of symbionts): symbionts species
genus *Premnas*	*Premnas biaculeatus* (2)	*Eq* (1): *P. bia*	*Hc* (1): *P. bia**
genus *Amphiprion* Percula complex	*Amphiprion ocellaris* (3) *Amphiprion percula* (4)	*Sg* (2): *A.oce*, *A.per* *Hm* (2): *A.oce*, *A.per*	*Sm* (2): *A. per*, *A. oce* **a)** *Hc* (1): *A. per* a)
Polymnus complex	*Amphiprion polymnus* (2) *Amphiprion sebae* (1) *Amphiprion latezonatus* (2)	*Sh* (2): *A.pol*, *A.seb*	*Hc* (1): *A. pol*, *A. late* **b)** *Eq* (1): *A. late*
Akallopisos complex	*Amphiprion akallopisos* (2) *Amphiprion nigripes* (1) *Amphiprion perideraion* (4) *Amphiprion sandaracinos* (2) *Amphiprion leucokranos* (3)	*Hm* (4): *A.aka, A.nig, A.peri*, *A.leu* *Sm* (3): *A.san*, *A.aka, A. leu*	*Hc* (3): *A. peri*, *A. leu*, *A. san* *Sg* (1): *A. peri* **c)** *Md* (1): *A. peri*
Ephippium complex	*Amphiprion ephippium* (2) *Amphiprion frenatus* (1) *Amphiprion mccullochi* (1) *Amphiprion melanopus* (3) *Amphiprion rubrocinctus* (2)	*Eq* (5): *A. eph*, *A. fre*, *A. mcc*, *A. mel*, *A. rub*	*Hc* (2): *A. eph*,*A. mel* *Hm* (1): *A. mel* *Sg* (1): *A. rub*
Clarkii complex	*Amphiprion clarkii* (10) *Amphiprion akindynos* (6) *Amphiprion allardi* (3) *Amphiprion bicinctus* (6) *Amphiprion chagosensis* (1) *Amphiprion chrysogaster* (5) *Amphiprion chrysopterus* (6) *Amphiprion fuscocaudatus* (1) *Amphiprion latifasciatus* (1) *Amphiprion omanensis* (3) *Amphiprion tricinctus* (4)	*Sm* (9): *A.cla*, *A. aki*, *A.all, A.bic* **e)**, *A.chrg, A.chrp*, *A.fus, A.latif, A.tri* *Sh* (5): *A. cla*, *A. aki, A. chrg* , *A.chrp*, *A. oma* **e)** *Hc* (6): *A.cla*, *A.aki*, *A.bic, A.chrp*, *A. oma, A. tri* *Eq* (9): *A. cla*, *A. aki, A. all, A. bic, A.* chag **e)** *A. chrp* , *A.* fus, *A. oma, A. tri*	*Hm* (5): *A. cla*, *A. aki, A. bic,A. chrg, A.chrp* *Sg* (3): *A.cla* **d), f)**, *A.aki* **c),f)**, *A.bic* **d),f)** *Ha* (7): *A. cla*, *A. aki*, *A. all, A.bic, A. chrg, A. chrp*, *A. tri* *Hl* (1): *A. cla* *Md* (1): *A. cla* *Ca* (1): *A. cla* **f)**

Sg=Stichodactyla gigantea, *Hm=Heteractis magnifica*, *Sm=S. mertensii*, *Hc=H. crispa, Eq=Entacmaea quadricolor*, *Sh=S. haddoni, Md=Macrodactyla doreensis, Ha=H. aurora, Hl=H. malu, Ca=Cryptodendrum adhaesivum*

Table based on field observation data mainly from Dunn (1981) and Fautin & Allen (1992), and supplemented by Elliott & Mariscal (2001,1996), Moyer & Yogo (2001).

Genera and species complexes of *Amphiprion* (Allen, 1972) are separated by horizontal lines.

☐ marked anemonefish species that has been demonstrated to have an innate protection against the anemone (Elliott & Mariscal, 1996; Miyagawa, 1989; Miyagawa & Hidaka, 1980).

* marked species has been demonstrated to have an imperfect protection against the anemone (Elliott & Mariscal, 1996; Miyagawa, 1989; Miyagawa & Hidaka, 1980; Schlichter, 1968; Gohar, 1948).

a) Data from photo by Allen in 1972 (Dunn. 1981).

b) This species is unclear whether a symbiotic or a sub-symbiotic species of *A. latezonatus.* Further ecological information (e. g. whether *A. latezonatus* inhabits only this anemone simply due to absence of *S. haddoni* in its habitat) is needed.

c) This partnership was reported by Elliot & Mariscal (1996).

d) *Sg* is thought to be a substitute species for *A. clarkii* because this partnership has only been observed in some localities (Elliott and Mariscal, 1996 ; Mariscal, 1969, 1970b; Saville-Kent, 1897). *Sg* is also thought to be a substitute species for *A. bicinctus* in the Red Sea according to the observation by Gohar (1948) and Schlichter (1968), who also showed the imperfect protection of *A. bicinctus* against *S. gigantea*.

e) This partnership was reported by Moyer & Yogo (2001).

f) Marked species has been demonstrated to have an imperfect protection against the anemone (Elliott & Mariscal, 1996; Miyagawa, 1989; Miyagawa & Hidaka, 1980; Schlichter, 1968; Gohar, 1948).

Fig. I The reach V values of the trough experiments with non-imprinted *A. ocellaris* juveniles to each test anemone's chemicals

"reach V value": the average number of juveniles that reached or stayed in section V per observation period.

Paired t-tests were used to detect whether tested juveniles were attracted to each test anemone's chemicals or not.
n = number of experiments

Fig. II The reach V values of the trough experiments with *Sg*-*A. ocellaris* juveniles to each test anemone's chemicals

The attraction to each test anemone's chemicals was judged to be significant using a paired t-test.
n = number of experiments

Fig. III The reach V values of the trough experiments with three stages (early, grown, and over 1 year) of *Hm*-*A. ocellaris* juveniles to each test anemone's chemicals

The attraction to each test anemone's chemicals was judged to be significant using a paired t-test.
n = number of experiments

Fig. IV Reach V values of non-imprinted, *Sg*, and *Hm* young fish of *A. ocellaris* to an exposed *Sg* in direct encounter experiments

The attraction to each test anemone's chemicals was judged to be significant using a paired t-test.
n = number of experiments

Fig. V The reach V values of the trough experiments with three stages (early, grown, and over 1 year) of *Sm-A. ocellaris* juveniles to each test anemone's chemicals.

The attraction to each test anemone's chemicals was judged to be significant using a paired t-test.
n = number of experiments

Fig. VI Diagram of host-changing manipulation

After dark, the host anemone *Hm* of a pair was replaced with *Hc* on the evening (i.e. ca. 1 day) before hatching.
The eggs hatched adjacent to *Hc* next evening, and some *Hc* juveniles were obtained.

Fig. VII Diagram of host-exchange experiment

1. After more than 60–70% of eggs had hatched in the parents' tank with *Sm*, the remaining eggs on the half-cut PVC duct were initially transferred into a small container filled with fresh seawater.
2. The spawning duct was then quickly transferred to the rearing *Sg* tank and placed ca. 5 cm from the *Sg*'s oral disc to prevent newly hatched larvae from being killed by the tentacles. Transferring water and newly hatched larvae from the *Sm* tank and the small container into the *Sg* tank was carefully avoided. Every transfer was done as quickly as possible just above the water surface of each tank.

Fig. I The reach V values of the trough experiments with non-imprinted *A. ocellaris* juveniles and each test anemone's chemicals

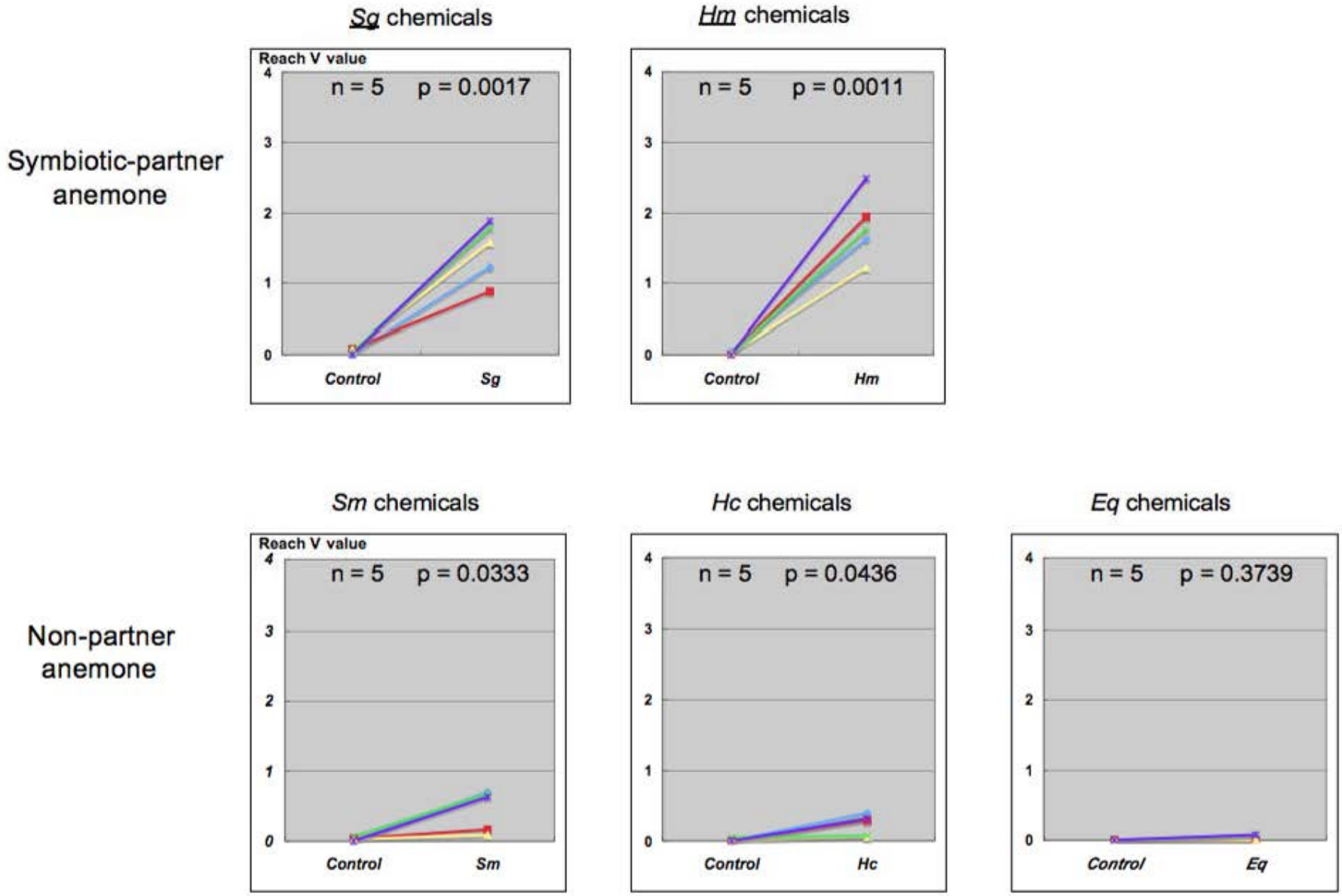


Fig. II The reach V values of the trough experiments with *Sg*-*A. ocellaris* juveniles and each test anemone's chemicals

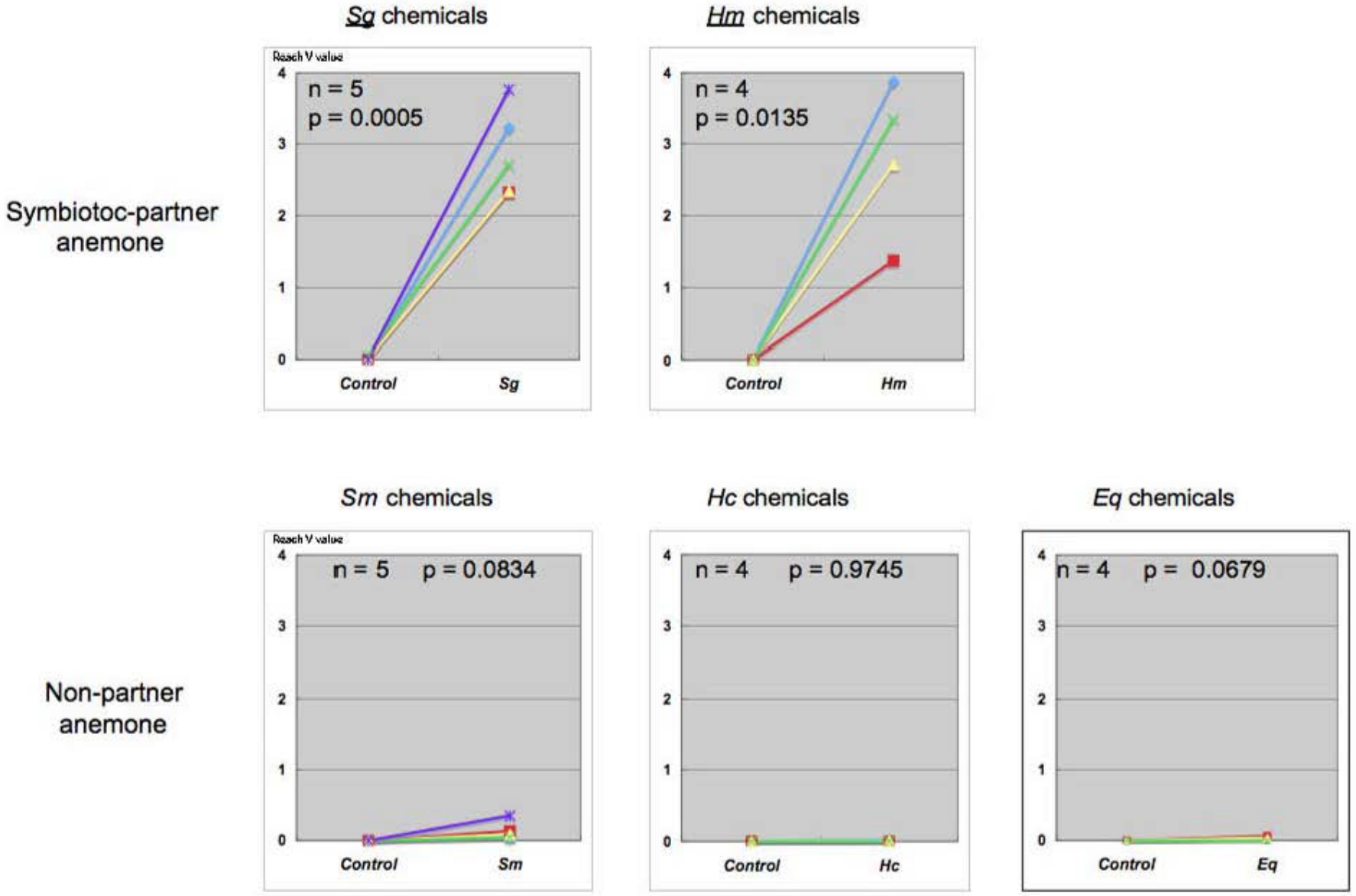


Fig. III The reach V values of trough experiments with early stage, grown stage, and over 1 year-old juveniles of *A. ocellaris* imprinted by *Hm* to each test anemone's chemicals

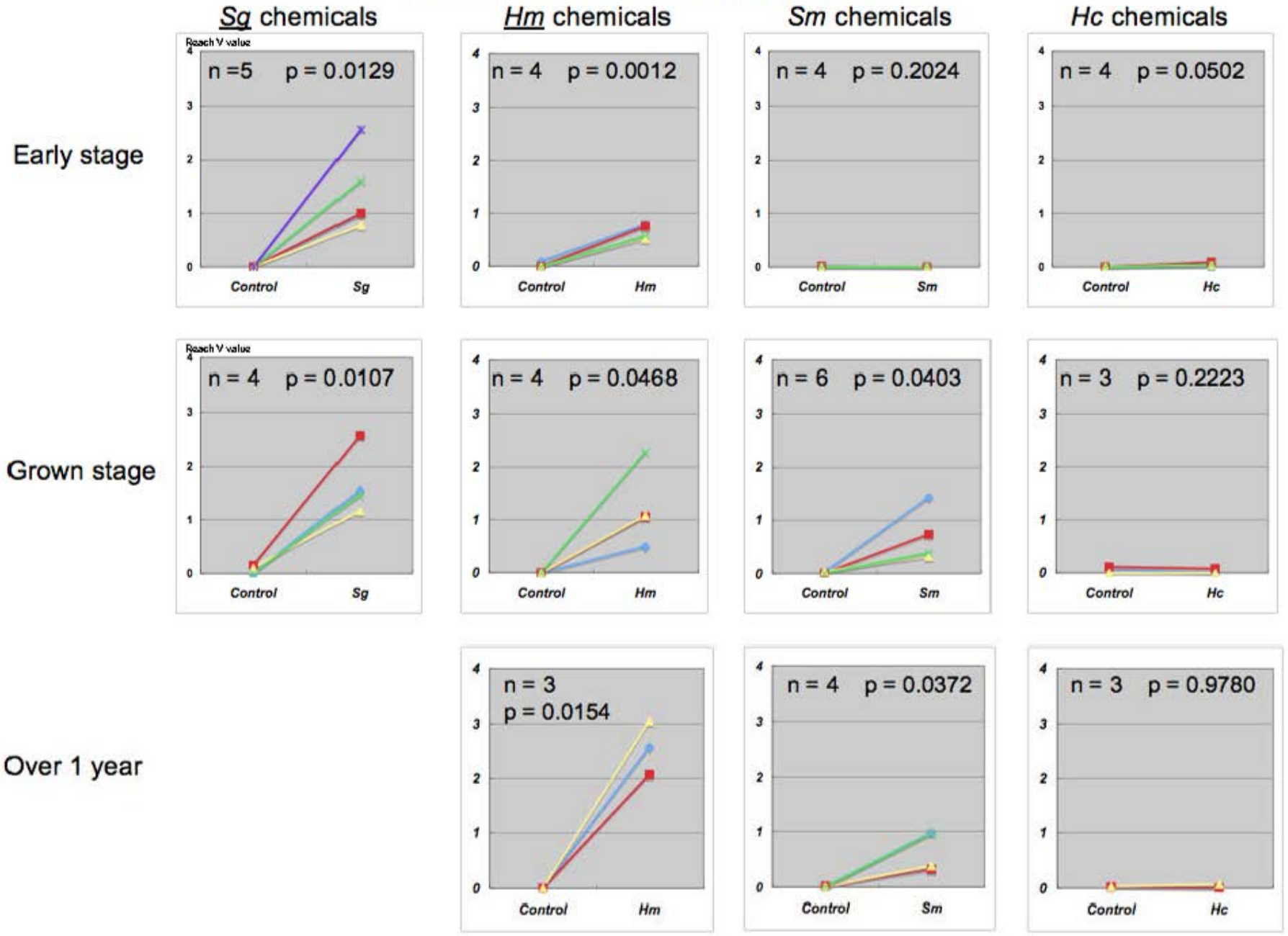
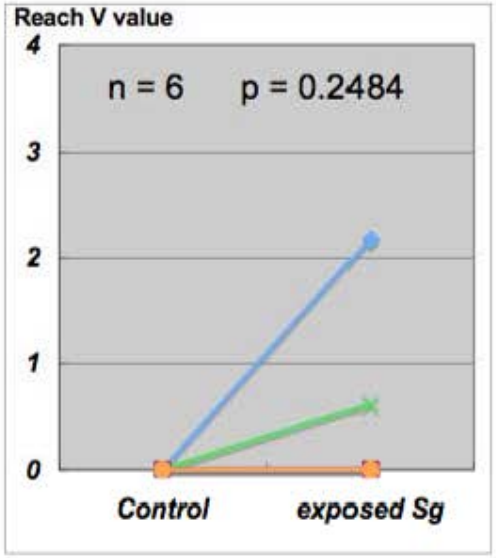
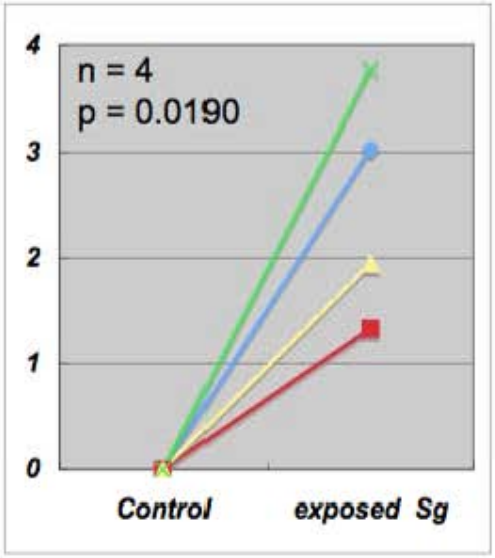


Fig. IV Reach V values of non-imprinted, *Sg* , and *Hm* young fish of *Amphiprion ocellaris* to an exposed *Sg* in direct encounter experiments

Non-imprinted young with an exposed *Sg*



Sg young with an exposed *Sg*



Hm young with an exposed *Sg*

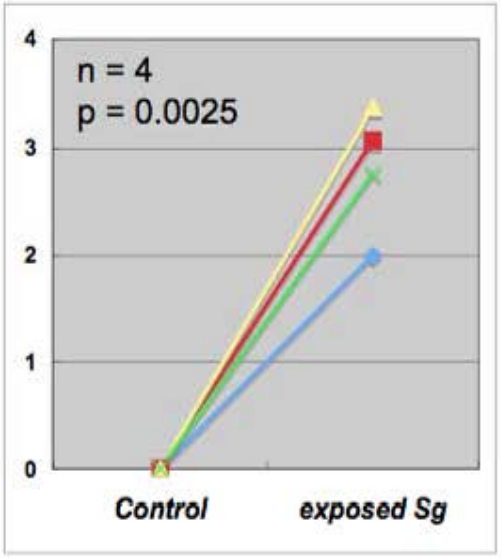


Fig. V The reach V values of trough experiments with early stage, grown stage, and over 1-year-old juveniles of *A. ocellaris* imprinted by *Sm* to each test anemone's chemicals

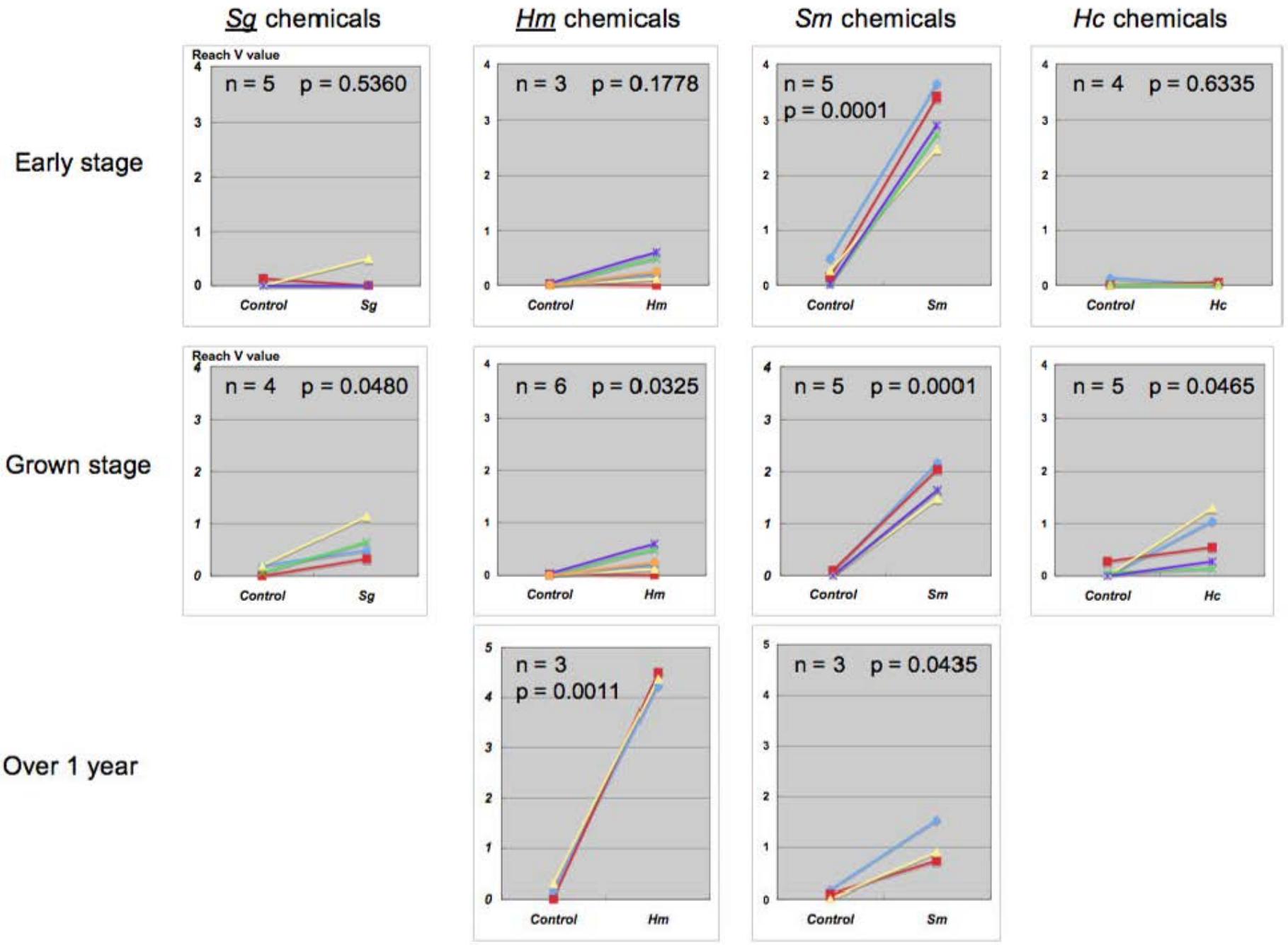


Fig. VI Diagram of host-changing manipulation

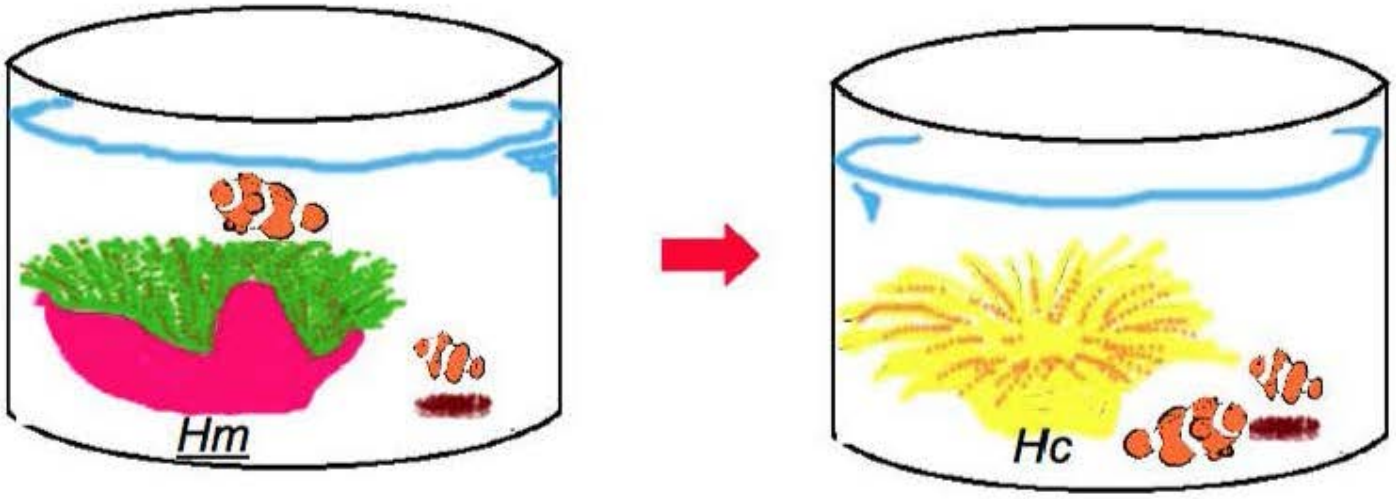


Fig.VII Diagram of host-exchange experiment