CHEMICAL IMPRINTING OF *AMPHIPRION CHRYSOPTERUS* EMBRYOS AND *A. MELANOPUS* JUVENILES BY HOST ANEMONES

BY

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Title: Imprinting of *Amphiprion chrysopterus* embryos and *A. melanopus* juveniles by host anemones.

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Steven S. Amesbury, Chairperson, Thesis Committee

Anemonefishes (Amphiprioninae, Pomacentridae) habituate with 10 species of host actinians found throughout the Indo-Pacific region. Each species of anemonefish frequently associates with one or more host anemone species. *Amphiprion chrysopterus* and *A. melanopus* individuals were studied between January 1998 and June 1999 at the University of Guam Marine Laboratory in order to determine if preference for particular host actinians is due to chemical imprinting during development. In an experimental tank, juvenile *A. chrysopterus* (22 and 26 days old) that had been exposed as embryos to the host anemone, *Heteractis crispa,* swam significantly more often towards that species of host given a choice between *H. crispa* and another host anemone *Entacmaea quadricolor*. In contrast, *A. chrysopterus* exposed to *E. quadricolor*, as embryos, swam significantly more often toward that species over *H. crispa*. Naive *A. chrysopterus*, i.e., those with no contact with a host anemone at any time during development, were not significantly attracted to either host. This anemonefish species was therefore considered to have been imprinted by chemicals secreted by the anemone to which it was exposed during embryonic development. All *Amphiprion melanopus* individuals were raised naive. Groups Ia, IIa, and IIIa were exposed to host anemone treated seawater for 48 hours

when they were 18-19 days old. Groups Ib, IIb, and IIIb were exposed to host anemone treated seawater for 48 hours when they were 26-27 days old. Group Ia individuals exposed to *H. crispa* treated seawater swam toward *E. quadricolor* significantly more often than *H. crispa*. Group Ib also swam toward *E. quadricolor* more often but not significantly so. All individuals exposed to *E. quadricolor* treated seawater swam toward that anemone species significantly more often than *H. crispa*. Naive *A. melanopus* also chose *E. quadricolor* significantly more often than *H. crispa*. These results indicate that *A. melanopus* was not imprinted post-hatching and has an innate preference for *E. quadricolor*.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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Introduction

Larval behavior of reef fishes during colonization of a reef is important in determining the settlement patterns of tropical reef fishes (Sancho et al. 1997). The recruitment of coral reef fishes is dependent upon the selection of appropriate habitats on which to settle. Recruitment and settlement patterns influence reef fish populations and which portions of the reef they occupy. How selection takes place and the basis behind the preference for certain habitats is of fundamental importance to the understanding of reef fish recruitment.

It is labor intensive and technologically demanding to raise certain species of reef fish larvae to the point where they are competent to settle. Therefore, few studies have been performed on the behavioral responses of reef fish larvae and juveniles to cues that may influence where they settle. Anemonefishes have been consistently raised in aquaria for decades and therefore provide a unique opportunity to examine the way a specific habitat, the host anemone, is distinguished on the reef.

Twenty-eight species of anemonefishes (27 *Amphiprion* spp., one *Premnas* sp.) and 10 host anemone species are found throughout the tropical latitudes of the Indian and Pacific oceans. The Indo-Australian-Philippine area is presumed to be the biogeographical center of anemone and anemonefish diversity (Thresher 1984, Fautin and Allen 1992). The principal advantage of the symbiotic relationship between *Amphiprion* species and host anemones is protection, for both symbiont and host (Fautin 1991, Fautin and Allen 1992).

Anemonefishes spawn year round with seasonal peaks being possible, especially in more temperate waters, e.g. Japan (Ross 1976, Myers 1999). Spawning commonly takes place near the full moon, though in Guam it has been observed semimonthly (Ross 1976, Telluck, pers. comm. 1996, Myers 1999). Fautin and Allen (1992) suggested this type of lunar reproductive cycle might aid the male in nest guarding duties. In contrast, Ross (1976) observed a second clutch of *A. melanopus* eggs hatched almost two weeks after the full moon. This would indicate that males do not depend upon moonlight to aid in guarding the nest.

Capsule-shaped eggs are laid in a nest next to the host anemone's pedal disk (Ross 1976, Myers 1991, 1999, Fautin and Allen 1992, Fautin 1992, Telluck, pers. comm. 1996). The eggs are attached to the substrate with thin filaments and are innately protected from the host's tentacles (Allen 1972, Thresher 1984, Miyagawa 1989). In *Amphiprion chrysopterus*, cell cleavage begins after 3 hours, and the embryo begins to develop after 28 hours (Allen 1972). The entire incubation period lasts seven or eight days, depending upon water temperature (Allen 1972, Thresher 1984, Telluck, pers. comm. 1996). During this time the eggs are guarded, cleaned and fanned by the male (Thresher 1984, Fautin and Allen 1992). Before hatching, parents nibble and bite the tentacles and pedal disk causing the anemone to draw back from where the eggs were laid. This behavior allows the newly hatched larvae, which are not immune to the host's nematocysts, to avoid any contact with the anemone's tentacles (Miyagawa 1989). Hatching of larvae usually occurs one to two hours after sunset (Ross 1976, Myers 1991, Fautin and Allen 1992). In aquaria, the new larvae, about 3-4 mm in length, first drop to

the bottom of the tank, then swim toward the surface after a few minutes (Fautin and Allen 1992, Telluck, pers. comm. 1997).

After hatching, the dark-pigmented larvae are positively phototactic and therefore rise in the water column toward the moon or starlight. This behavior avoids tentacle contact and would expedite movement of the larvae away from the reef substrate, which harbors various predators. Rising in the water column also assists the passive dispersal of larvae. Ross (1976) found that the water heights on the hatching days of *A. melanopus* (+2 and +13 days from the full moon) were significantly greater than days 90° out of phase in the lunar cycle (± 7days). Ross (1976) therefore surmised that the lunar cyclic nature of spawning was due to tidal rhythm. *A. chrysopterus* eggs were observed hatching three days after the full moon, one day after the highest tide exchange of the month in Guam (personal observation). There is much variation in the length of the planktonic larval stage amongst pomacentrids (Allen 1991). *Amphiprion* larvae float in the water column between six and 22 days (Miyagawa 1989, Myers 1991, 1999, Fautin and Allen 1992, Elliott et al. 1995, Telluck, pers. comm. 1996). During this period the pigmentation fades and the larvae become translucent (Elliott et al. 1995).

The larval stage ends when the individual settles out of the water column and develops the typical coloration of benthic anemonefishes. The metamorphosis from translucent larva to juvenile coloration takes less than 24 hours (Elliott et al. 1995, Telluck, pers. comm. 1996). The juvenile must find a species-specific host anemone since it is highly vulnerable to predation without such protection. Once the fish has recruited to

a suitable host, Ross (1976) calculated a longevity period for *A. melanopus* on Guam of about four years.

Shick (1991) suggested that host anemones evolved certain mechanisms, e.g. chemical attractants, thereby luring symbionts and dramatically increasing their chances for survival. Some anemones attract more symbionts than others. *Entacmaea quadricolor* is the host for the largest number of anemonefishes, 13 species, including at least three that occur in no other anemone (Dunn 1981, Shick 1991, Fautin and Allen 1992). This anemone is the most abundant and geographically widespread host anemone in the Indo-Pacific (Mariscal 1970, Fautin 1985). Fautin (1986) proposed that the profusion of *E. quadricolor* throughout the Indo-Pacific has been the consequence of the symbiosis with anemonefishes. *Amphiprion* species show territorial behavior and aggressiveness towards other fishes, thereby protecting their host (Godwin and Fautin 1992). Host anemones without anemonefishes may be preyed upon by a wide assortment of fishes, including members of the Chaetodontidae, Tetraodontidae, Ostraciidae, Sparidae, Haemulidae, Cottidae, Notacanthidae, Pleuronectidae, Triakidae, and Chimaeridae (Fautin 1986, Shick 1991, personal observation). Fautin and Allen (1992) removed *Amphiprion* species from a number of *E. quadricolor* in a repopulation experiment. In each location (the Great Barrier Reef, Enewetak Atoll, and Papua New Guinea), the anemone host usually disappeared. Butterflyfish were observed with their mouths in the reef where there were once anemones. On Guam, *Chaetodon auriga* has been observed nipping at *E. quadricolor* tentacles when *A. melanopus* symbionts were distracted (personal observation). Other explanations for present distribution patterns of anemones also exist,

e.g. success has been due to the actinarian capability of reproducing asexually, or the ability to live on various hard substrata.

Amphiprion species use chemoreceptive mechanisms, e.g. olfaction, to inform themselves of the environmental chemistry. Environmental constraints, i.e. time, space, currents and turbulence, have led to the structure and physiology of *Amphiprion* species, and their chemoreceptor organs (Atema 1985). Swimming up and down, and side to side in the water column allows the fish to home in on the source of the odor or chemical mixture, i.e. the host anemone. Murata et al. (1986) described *A. peridérion* individuals demonstrating a "seesawing" behavior when aplysinopsin, a chemical isolated from *H. crispa*, was added to the experimental tank. Anemonefishes likely recruit to a host at night, thereby avoiding diurnal predators and aggression from resident anemonefishes (Elliott et al. 1995). It is therefore logical that chemoreception is the dominant sensory cue regulating *Amphiprion* larval behavior.

Though no clear pattern of species-specificity has been found, reasons for the preference by anemonefishes for certain anemone species have been suggested by Fautin (1986). One, stochastic processes are involved. Two, the fishes learn or have an innate host preference. It has been demonstrated that chemical processes are the primary way in which juveniles locate host anemones (Miyagawa and Hidaka 1980, Murata et al. 1986, Miyagawa 1989, Elliott et al. 1995). Three, certain *Amphiprion* species out-compete others for the preferable anemone species. Specialists, those fish that live in a limited number of host species, are usually better competitors and will secure the more preferred anemone species. It is believed that those *Amphiprion* species that occur with only one or

two species of anemones, e.g. *A. frenatus*, are better competitors than are those that occur on a greater variety of anemone hosts, e.g. *A. clarkii*. *Amphiprion clarkii*, geographically the most widespread and least discriminating symbiont, occurring in nine out of the 10 available hosts, is considered by Miyagawa (1989) to represent an early stage in the evolution of the symbiosis.

Habitat preference is another process with which anemonefishes possibly discriminate appropriate host anemones. Fishes may have a preference for current swept environments (seaward reefs), inner lagoon reefs, or sandy substrates. In Palau, *A. melanopus* is only found with *E. quadricolor* on shallow reefs (<60 ft.) and on wrecks within the lagoon where there is little current and high turbidity. *Amphiprion clarkii*, *A. chrysopterus*, and *A. perideraion* are usually found on the barrier reef, where strong currents and low turbidity occur (personal observation).

Each species of host anemone manufactures a chemically distinct mucus that contains biochemicals by which *Amphiprion* juveniles find them (Murata et al. 1986, Miyagawa 1989, Shick 1991, Fautin and Allen 1992, Elliott et al. 1995). *A. perideraion* and *A. ocellaris* showed searching behavior for the chemicals amphikuemin, from *H. crispa*, and tyramine and tryptamine, from *S. kenti* (*Stichodactyla haddoni*), respectively (Murata et al. 1986). These experiments suggest that patterns of species specificity exist between anemonefishes and host anemones, i.e. each anemone species uses a different chemical attractant for each species of symbiont.

Miyagawa (1989) established that 60-100 day old naive juveniles, i.e. ones that had been isolated from their host anemone after hatching, recognized their symbiotic anemones

by chemical rather than by visual means. However, the amount of time larvae spent in the tank with the anemone before isolation was not stated (Miyagawa 1989). Because isolation occurred only after hatching, there is also the possibility of pre-hatching influence (imprinting) by the host anemone.

Because eggs are laid close to the host anemone, the oral disc and tentacles may overhang or touch the eggs frequently. As the male anemonefish fans and mouths the eggs during incubation, anemone-specific chemicals could influence the embryos (Ross 1976, Miyagawa 1989). Most anemonefishes recognize several species of hosts, e.g. *A. ocellaris* juveniles that were hatched near *Stichodactyla gigantea* also recognized *H. magnifica*, implying that the recognition of host secretions is innate. It is not known whether the hosts share a common synomone or not (Miyagawa 1989). It is unlikely that fish recognize different hosts by the same chemical since secreted chemicals from different anemone species cause different behaviors (Murata et al. 1986, Miyagawa 1989). Pre- or post-hatching imprinting may augment the recognition system of anemonefishes (Miyagawa 1989). Anemonefishes preferences for some host anemones varies with geography, but why this occurs is unknown (Fautin 1986).

The objective of this study was to determine, through experimental analysis, whether *Amphiprion chrysopterus* embryos and *A. melanopus* juveniles are chemically imprinted by host anemones. If imprinting by a host anemone occurs, then juvenile fish of each species should be more inclined to search for that same species of host anemone during their settlement period. This behavior would be similar to imprinting in salmon, which occurs on distinctive odors of the natal tributary during the presmolt and smolt

stage. Hara (1986) suggested that the ability to 'home' is an acquired trait, rather than an inherited one. Salmon use mixtures of odors, as opposed to a single chemical, to identify their natal stream (Hara 1986). *Amphiprion* species appear to use one or more chemical substances, secreted by the host anemone to locate a preferable host (Miyagawa and Hidaka 1980, Murata et al. 1986, Miyagawa 1989).

Patterns of species-specificity may be partially explained if host anemones chemically imprint *Amphiprion* embryos. Imprinting is a behavioral adaptation, occurring at an early stage in the life of an individual, that establishes the perception of what its future habitat should include. In this case, a chemical, or chemicals, would affect the *Amphiprion* embryos so that juveniles, ready to settle, would be attracted, via olfactory cues, to a host anemone secreting the same chemical(s). Imprinting the next generation of *Amphiprion* species would assure that the longer-lived hosts acquire a long-term supply of symbionts. Imprinting may supplement anemonefishes' innate recognition system and could also explain why some partnerships vary with geography (Mariscal 1970, Dunn 1981, Miyagawa 1989). *A. perideraion* habituates with *H. magnifica, H. crispa, Macrodactyla doeensis,* and *S. gigantea* (Fautin and Allen 1992). Fautin (1986) observed that *H. magnifica* was the only host anemone that *A. perideraion* associated with at Lizard Island, Queensland, although *H. crispa* also occurs there. At the other end of its geographic range, the coastal region of Japan, both *H. magnifica* and *H. crispa* were used as hosts (Fautin 1986).

In Guam, *A. chrysopterus* rarely associates with *H. mertensii*, and is occasionally found with *H. crispa* and *H. magnifica* (Fautin and Allen 1992, personal observation). In

Palau, *A. chrysopterus* is found with *H. mertensii* occasionally, *H. crispa* and *H. magnifica* commonly, and *H. aurora* rarely (personal observation). Fautin and Allen (1992) found *A. chrysopterus* habituating with *E. quadricolor* as well, though this symbiosis was not personally observed in either Guam or Palau (Fautin 1991, personal observation). These associations may have to do with the local availability of each species of anemone which may, in part, be due to how well they recruit symbionts, or the habitat in which each is normally encountered. This phenomenon may also be explained by chemical imprinting. Sabol (1991) suggested that imprinting may also be a reason for some anemonefish species not requiring acclimation when being introduced to a particular anemone species. This study represents the first experimental analysis of chemical imprinting of *Amphiprion chrysopterus* and *A. melanopus.*

Experimental Design

This study took place at the University of Guam Marine Laboratory from January 1998 through June 1999. Two species of anemonefish, *Amphiprion chrysopterus* and *A. melanopus*, were used as test subjects. Within each species, all experimental individuals were the offspring of the same mating pair. Fish were reared in tanks at the Guam Department of Commerce fish hatchery, in Barrigada. Fish were transferred from the hatchery to holding tanks at the UOG Marine Laboratory once they had developed juvenile coloration.

Anemones were collected from fringing reefs in Pago Bay, Mangilao, and Jones Beach, Ipan. Each actinian species was kept in separate tanks to avoid chemical contamination. The tanks were fed with a flow-through seawater system and kept under natural light in order to keep the anemones healthy.

A. chrysopterus Host Exposure History

Experienced fish were obtained from eggs that had been laid on a ceramic tile, raised with a host anemone that was placed in the tank one day after the eggs were laid. The tile with the eggs was removed from the tank after six days, close to the time of hatching, and placed in a separate tank. Naive fish were produced from eggs laid, incubated and hatched in tanks with no host anemone. Two experienced broods (Group Ia and Ib) were raised with *Heteractis crispa* and two others (Group IIa and IIb) were raised with *Entacmaea quadricolor*. Naive fish were in Group IIIa and IIIb (control groups). Experiments were conducted two times, once with 22 day-old (Groups Ia, IIa, IIIa) and once with 26 day-old (Groups Ib, IIb, IIIb) *A. chrysopterus* juveniles. Each

group was from a different brood. The fishes were old enough to have developed the ability to recognize chemical cues from potential host anemones and were ready to settle.

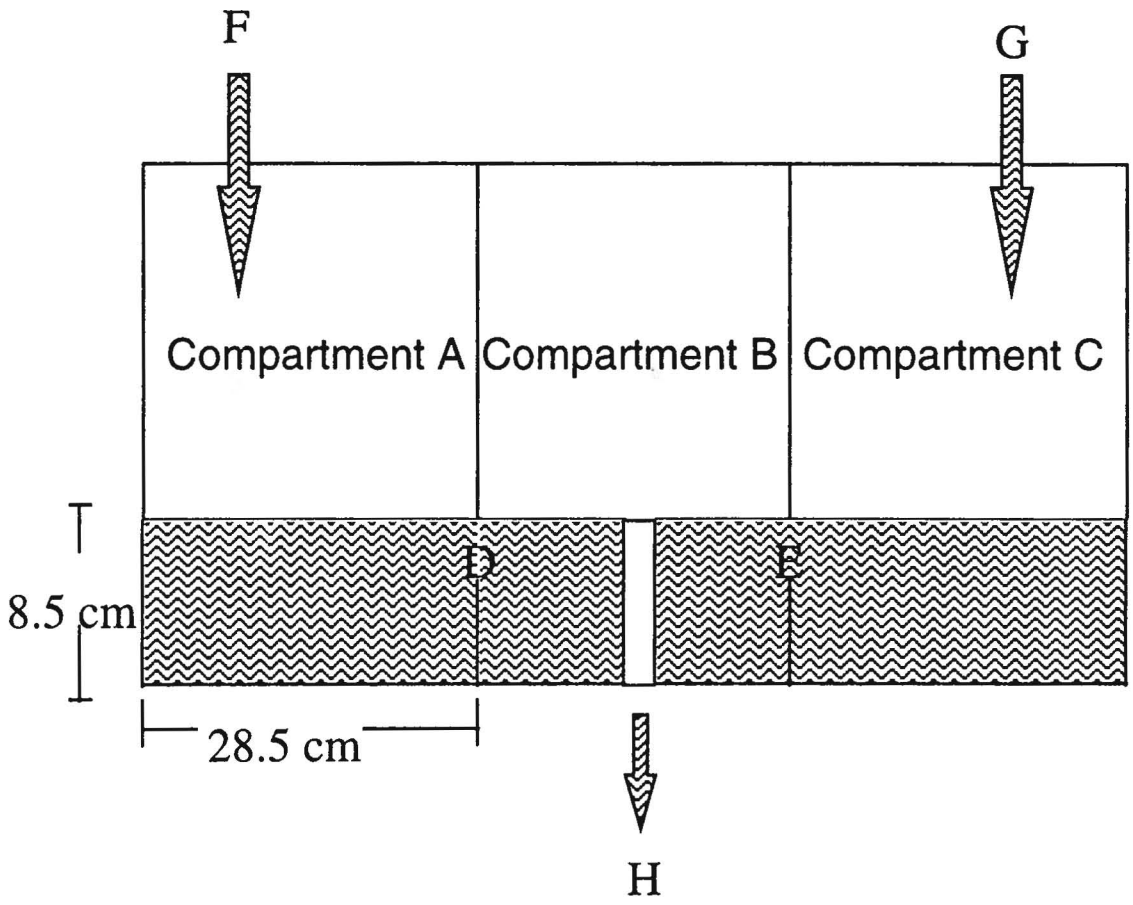
A. melanopus Host Exposure History

All of the *A. melanopus* were originally raised naive, then exposed to seawater run over either *H. crispa* or *E. quadricolor* when juveniles. Group Ia, IIa , and IIIa were from one brood and groups Ib, IIb, and IIIb were from a second brood. Group Ia *A. melanopus*, 18 days old, and group Ib, 26 days old, were continually exposed to seawater run over *H. crispa* for 48 hours. Group IIa *A. melanopus*, 18 days old, and group IIb, 26 days old, were exposed to seawater run over *E. quadricolor* for 48 hours. Groups IIIa and IIIb *A. melanopus* (control groups) remained completely naive, never having had any type of contact with a host. Experiments were conducted one time for each group of *A. melanopus*. All individuals within groups Ia, IIa, and IIIa were 20 days old when tested. Individuals from groups Ib, IIb, and IIIb were 28 days old when tested.

Experimental Tank

The experimental tank (fig. 1) was constructed of Plexiglass and consisted of three adjacent 28.5 x 19 x 8.5 cm compartments (A, B, and C). Seawater was fed (F and G) into the end compartments, at 900 ml/min, and drained (H) from the middle one. One fish, from Group I, II, or III, was removed from the holding tank, using a beaker, and placed in the middle compartment (B) of the experimental tank. Holes, five cm in diameter and 1.5 cm above the bottom of the tank, were drilled in the dividing compartment walls (D and E). This permitted the fish to move between adjacent compartments. Each fish was allowed to acclimate to the experimental surroundings for five minutes before water,

Figure 1. Experimental tank



siphoned from tanks containing the host anemones, was added. Fish that moved out of the middle compartment (B) during this acclimation period were discarded from the results.

Seawater flowed into the experimental tank at both end compartments (A and C) (figure 1). After the acclimation period for the fish, seawater from separate tanks containing *H. crispa* and *E. quadricolor* was siphoned into the end compartments at 100 ml/min. Seawater flowing into tanks containing host anemones was turned off two hours before experiments began in order to maximize the amount of anemone secretions in the siphoned seawater. Anemone-treated water being siphoned into the experimental tank was randomly assigned before each run to either compartment A or C. As seawater from the hosts tanks was added, the first move the fish made out of the middle compartment (B) and into one of the adjacent compartments was recorded as evidence of chemical attraction to the particular host actinian in that compartment. The location of the fish (A, B, or C) was recorded every 15 seconds for a total of 10 minutes. These data were used to determine the relative amount of time that each group of fish spent in each compartment. The general behavior of the fish was also noted.

All experiments were conducted under natural light conditions between 0800 and 1600 hours. After each experiment, the tank was completely drained and rinsed to avoid any chemical contamination in the next run. The groups were not tested simultaneously. Various current systems and the relative concentrations of the chemical cues needed to attract a fish were not addressed. A flow-through seawater system provided background

noise, or dispersed mixture components, to the fishes, but the odor source was deemed sufficient to stand out against the background water chemistry.

Results

Once seawater from the tanks containing the host actinians was siphoned into compartment A and C, the fishes, regardless of which symbiont species was being used, displayed consistent searching behaviors to chemical stimuli (Atema 1985). Searching behaviors included swimming up and down (seesawing), and back and forth. In general, the fishes became more energetic once anemone treated seawater was added to the tank.

A. chrysopterus Experiments

Group Ia and Ib *A. chrysopterus* individuals chose to swim first into the compartment receiving *H. crispa*-treated seawater significantly more often than into the compartment receiving *E. quadricolor* treated seawater (Table 1). Group IIa and IIb *A. chrysopterus* chose to swim first into the compartment receiving *E. quadricolor* treated seawater, significantly more often than the compartment receiving *H. crispa* treated seawater (Table 1). Naive individuals from group IIIa and IIIb were observed to randomly choose a compartment into which to swim first, indicating no preference (Table 1).

Group Ia and Ib *A. chrysopterus* spent significantly more time in the compartment receiving *H. crispa*-treated seawater, than in the compartment receiving *E. quadricolor* treated seawater (Grp Ia Paired T-test, p= .0369; Grp Ib Paired T-test, p= .0010) (fig. 2, 3). Group IIa and IIb *A. chrysopterus* spent significantly more time in the compartment receiving *E. quadricolor* treated seawater, than in the compartment receiving *H. crispa* treated seawater (Grp IIa Paired T-test, p= .0001; Grp IIb Paired T-test, p= .0062) (fig. 2, 3). Group IIIa *A. chrysopterus* did not spend significantly more time in either the

Table 1. *Amphiprion chrysopterus* and *A. melanopus* individuals were tested for anemone preference using initial movements into compartment containing host anemone treated seawater as evidence.

		No. of tested fish choosing			
*A. chrysopterus**	exposed to	*H. crispa*	*E. quadricolor*	G value	
Group Ia	*H. crispa*	14	5	4.4389	$p < .05$
Group Ib	*H. crispa*	16	2	12.3953	$p < .001$
Group IIa	*E. quadricolor*	2	14	10.1241	$p < .005$
Group IIb	*E. quadricolor*	6	15	3.9849	$p < .05$
Group IIIa	naive (control)	7	6	0.7699	$p < .5$ n.s.
Group IIIb	naive (control)	4	11	3.3960	$p < .1$ n.s.
*A. melanopus***					
Group Ia	*H. crispa*	3	11	4.8599	$p < .05$
Group Ib	*H. crispa*	3	9	3.1395	$p < .1$ n.s.
Group IIa	*E. quadricolor*	2	14	10.1241	$p < .05$
Group IIb	*E. quadricolor*	3	14	7.7230	$p < .01$
Group IIIa	naive (control)	5	14	4.4389	$p < .05$
Group IIIb	naive (control)	4	12	4.1859	$p < .05$

**A. chrysopterus* were exposed to anemone treated seawater as embryos.

***A. melanopus* (groups Ia, IIa, IIIa) were exposed to anemone treated seawater as 18-19 day-olds. Group Ib, IIb and IIIb *A. melanopus* were exposed to anemone treated seawater as 26-27 day-olds.

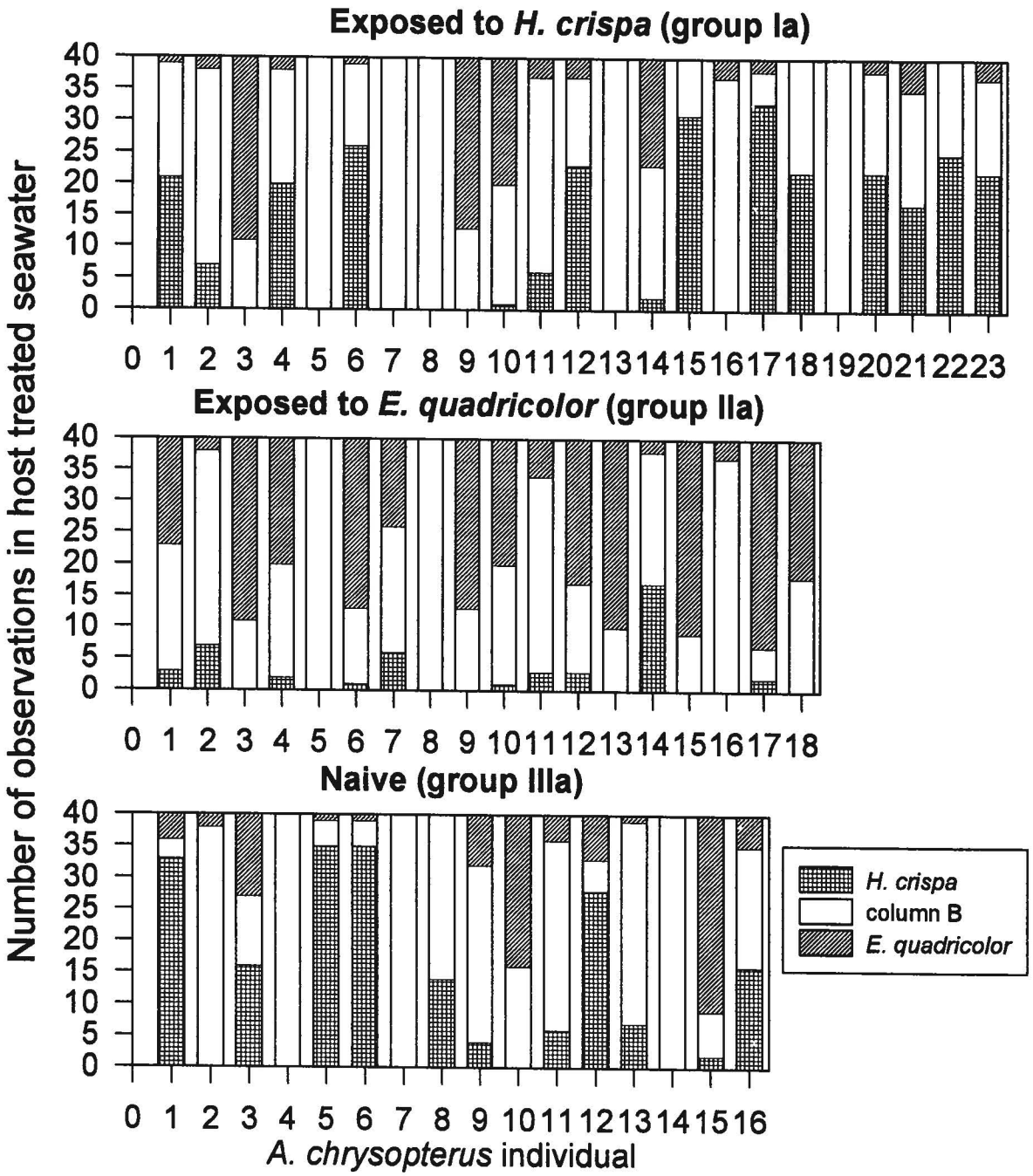


Figure 2. Relative time spent by *Amphiprion chrysopterus* individuals (groups Ia, IIa, IIIa) in experimental tank compartments over a 10 min. period. Group Ia paired T-test, p= .0413, group IIa paired T-test, p= .0001, group IIIa paired T-test, p= .1039. Observations were made every 15 seconds. One compartment had an inflow of *H. crispa*-treated water. The middle compartment received and drained water from both of the end compartments. One compartment had an inflow of *E. quadricolor*-treated water.

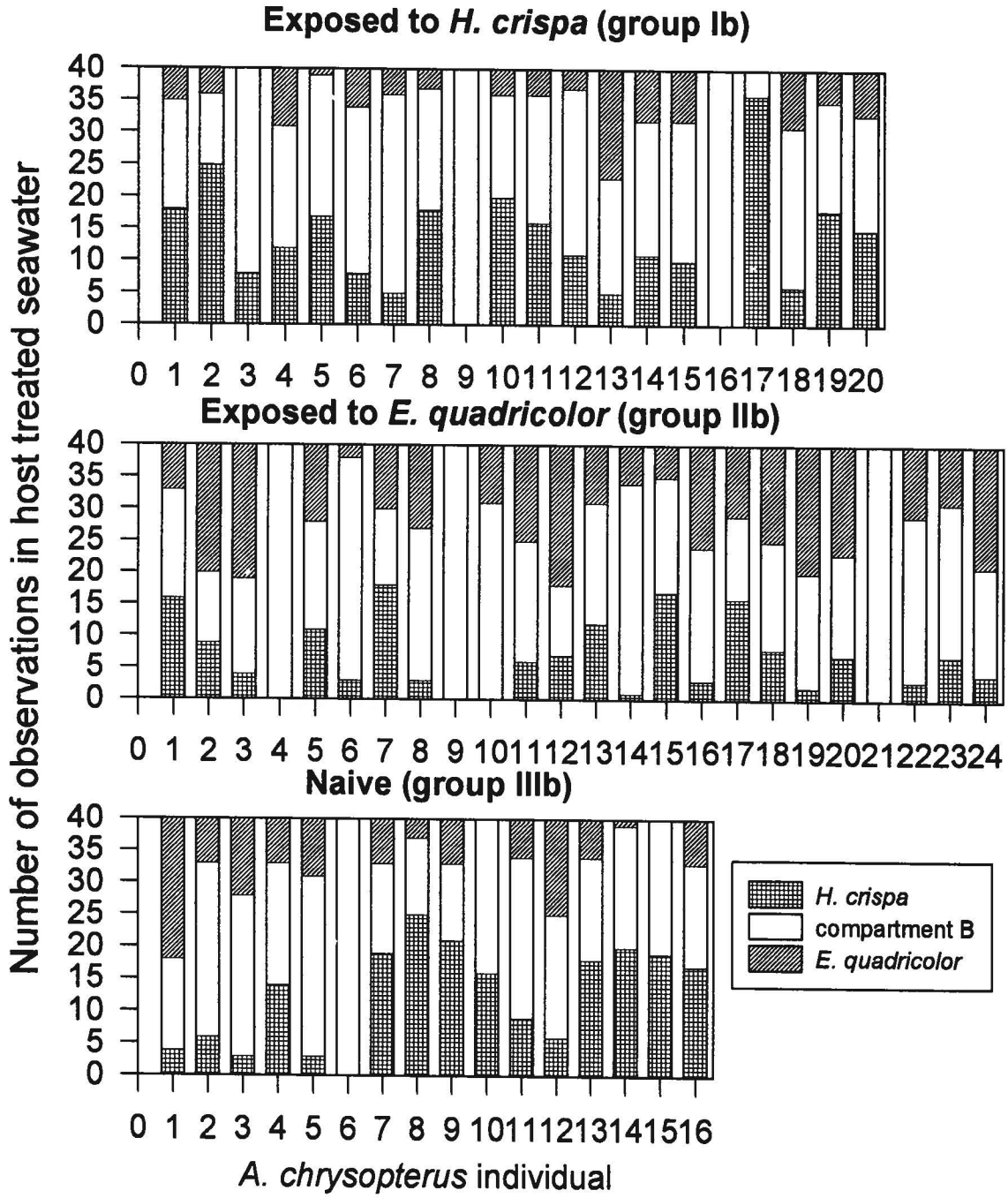


Figure 3. Relative time spent by *Amphiprion chrysopterus* individuals (groups Ib, IIb, IIIb) in experimental tank compartments over a 10 min. period. Group Ib paired T-test, p= .001, group IIb paired T-test, p= .0062, group IIIb paired T-test, p= .0371. Observations were made every 15 seconds. One tank compartment had an inflow of *H. crispa*-treated water. The middle compartment received and drained water from both of the end compartments. One compartment had an inflow of *E. quadricolor*-treated water.

compartment receiving *H. crispa*- or *E. quadricolor*-treated seawater (Paired T-test, p= .1039 n.s.) (fig. 2). Group IIIb *A. chrysopterus* spent a significant amount of time in the compartment receiving *H. crispa* treated seawater (Paired T-test, p= .0371) (fig. 3).

***A. melanopus* Experiments**

Group Ia *A. melanopus* individuals chose to swim significantly more often into the compartment receiving *E. quadricolor* treated seawater first, than into the compartment receiving *H. crispa*-treated seawater (Table 1). Group Ib did not swim into one compartment significantly more often than the other at $\propto = .05$, though the group was significant at $\propto = .1$ for the *E. quadricolor* compartment. Group IIa and IIb *A. melanopus* chose to swim significantly more often into the compartment receiving *E. quadricolor*-treated seawater first, than into the compartment receiving *H. crispa* treated seawater (Table 1). Group IIIa and IIIb *A. melanopus* also chose to swim significantly more often into the compartment receiving *E. quadricolor* treated seawater first, than into the compartment receiving *H. crispa* treated seawater (Table 1).

Group Ia *A. melanopus* did not spend a statistically significant amount of time in either the compartment receiving *E. quadricolor*- or *H. crispa*-treated seawater (Paired T-test, p= .0369) (fig. 4). Though group Ib's initial movement into host anemone treated seawater was not significant, a significant amount of time was spent by the group in the *E. quadricolor* compartment (Paired T-test, p= .0069) (fig. 5). Group IIa and IIb *A. melanopus* spent a significantly greater amount of time in the compartment receiving *E. quadricolor*-treated seawater than in the compartment receiving *H. crispa*-treated seawater (grp IIa Paired T-test, p= 5.12E-05; grp IIb Paired T-test, p= .0177) (fig. 4, 5).

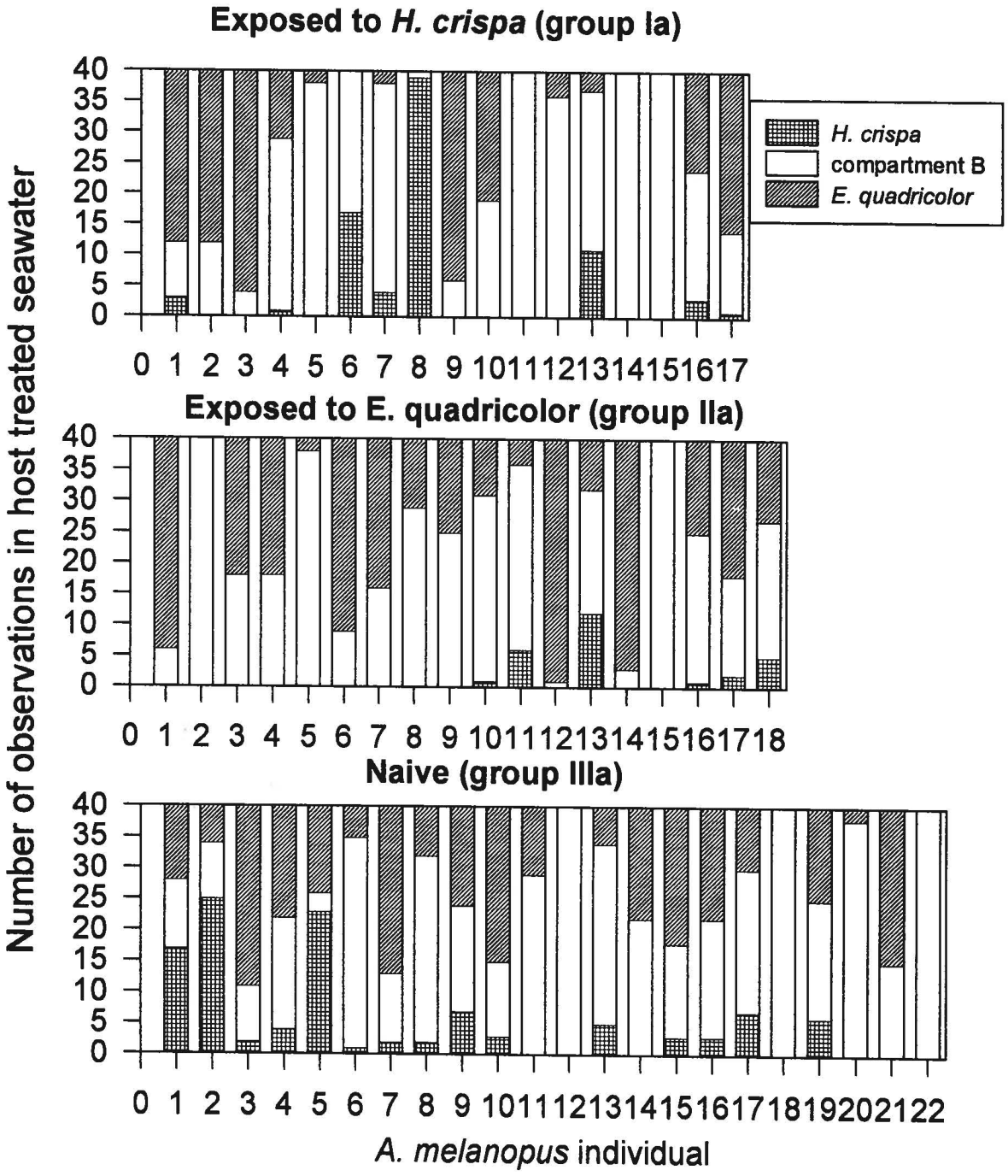


Figure 4. Relative time spent by *Amphiprion melanopus* individuals (groups Ia, IIa, IIIa) in experimental tank compartments over a 10 min. period. Group Ia paired T-test, p= .0483, group IIa paired T-test, p= 5.12E-05, group IIIa paired T-test, p= .0020. Observations were made every 15 seconds. One tank compartment had an inflow of *H. crispa*-treated water. The middle compartment received and drained water from both of the end compartments. One compartment had an inflow of *E. quadricolor*-treated water.

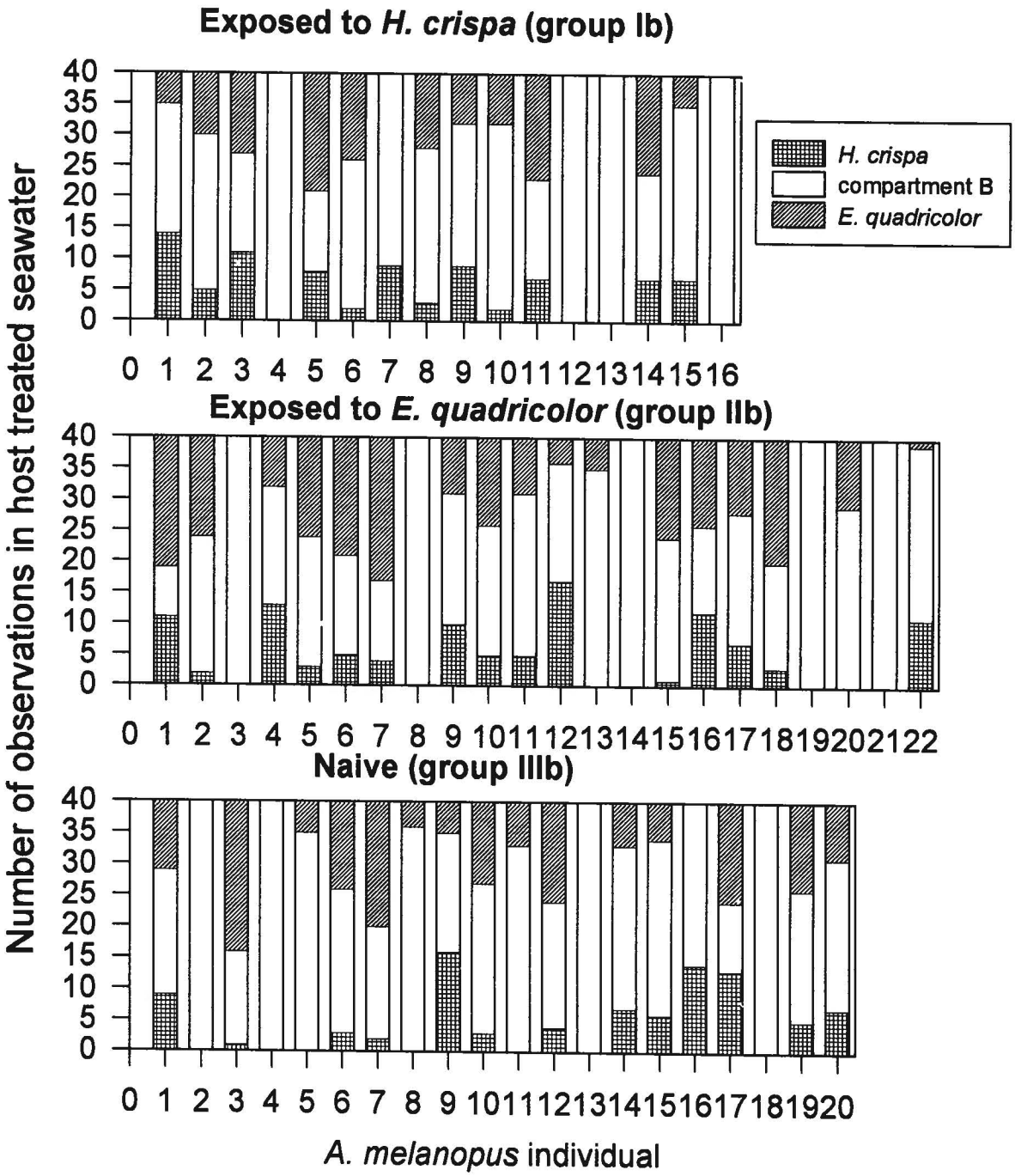


Figure 5. Relative time spent by *Amphiprion melanopus* individuals (groups Ib, IIb, IIIb) in experimental tank compartments over a 10 min. period. Group Ib paired T-test, p= .0069, group Iib paired T-test, p= .0177, group IIIb paired T-test, p= .0235. Observations were made every 15 seconds. One tank compartment had an inflow of *H. crispa*-treated water. The middle compartment received and drained water from both of the end compartments. One compartment had an inflow of *E. quadricolor*-treated water.

Group IIIa and IIIb *A. melanopus* spent a significantly greater amount of time in the compartment receiving *E. quadricolor* treated seawater, than the compartment receiving *H. crispa* treated seawater (grp IIIa Paired T-test, p= .0020; grp IIIb Paired T-test, p= .0235) (fig. 4, 5).

Discussion

Results of *A. chrysopterus* experiments indicate that this species is imprinted during embryonic development by exposure to natural host anemones. Fautin (1991) theorized that if species do imprint, then environmental conditions, not genetics, affect the developmental course of an individual in regard to host specificity. "Such behavior would promote assortative mating, and therefore could be an important mechanism of sympatric speciation" (Fautin 1991). If the identical synamone, a chemical secreted by a species that influences the behavior or development of another species, that the fish are attracted to, were being secreted by both host anemones, then the preference for a particular anemone species would not be significantly different. Therefore, it can be inferred that *A. chrysopterus* recognizes hosts by distinct synomones, or combinations of chemicals, secreted by each anemone species.

Results of *A. melanopus* experiments indicate that this species' settling behavior is not influenced by post-hatching exposure to a host anemone's secretions. At the age of 20 and 28 days old, *A. melanopus* has an innate preference, or inherited instinct, for *E. quadricolor*. Five out of six groups of *A. melanopus* were significantly attracted to *E. quadricolor* treated seawater in this study. Group Ib was only significant at $\propto = .1$, perhaps because of a small N. Therefore, the behavioral development of *A. melanopus* is fixed early in their life history, probably before they hatch. Specialist species, those that habituate with only one or two host species, e.g. *Premnas biaculeatus*, are more likely to use innate mechanisms to find and associate with their host (Fautin 1991). *A. melanopus*

is found exclusively in association with *E. quadricolor* in Guam and Palau (Myers 1991, personal observation).

Though it was not tested, there is the possibility that pre-hatching exposure to host anemones could imprint *A. melanopus*, thereby influencing post-hatching behavior, i.e. host selection. Group Ia and Ib, exposed to *H. crispa* seawater, spent a significant amount of time in the *E. quadricolor*-treated seawater compartment. This indicates that *E. quadricolor* has an effective chemical attractant that is preferred by *A. melanopus* over chemicals secreted by *H. crispa.* Field observations support these experimental findings. As was stated above, *A. melanopus* has consistently been found in association with *E. quadricolor* in Guam and Palau, though it has also been observed with two other host species elsewhere (Myers 1991, Fautin and Allen 1992, personal observation). *A. melanopus* needs to be studied more in depth to conclude where it lies within the spectrum from generalist to specialist

The current data agree with previous studies that have shown that juvenile anemonefishes, both generalists and specialists, use chemical cues to locate and select certain host actinians (Miyagawa and Hidaka 1980, Murata et al. 1986, Miyagawa 1989, Elliott et al. 1995). Generalists, e.g. *A. clarkii*, are more likely to rely on behavioral mechanisms, i.e. imprinting and acclimation (Fautin 1991). Specialists, e.g. *P. biaculeatus*, are more likely to use innate mechanisms to find and associate with their host (Fautin 1991).

Most *A. chrysopterus* and *A. melanopus* individuals were attracted to both anemone species, as indicated by the timed experiments. Though they may have spent

more time in one compartment than another, they actively searched in both. It can be deduced from this data that both *H. crispa* and *E. quadricolor* produce a chemical(s) that *Amphiprion* species can identify. This supports Miyagawa's (1989) statement that recognition of host secretions is innate.

The results of the present study suggest that *A. chrysopterus* falls between generalist and specialist extremes. It would therefore be expected that *A. chrysopterus* should be more widely spread geographically compared to *A. melanopus*, since it has a wide range of hosts to recruit into. In actuality, *A. chrysopterus* and *A. melanopus* are found within the same geographical area, throughout Indonesia and Micronesia (Myers 1991, Fautin and Allen 1992). *Premnas biaculeatus*, a symbiont that associates with *E. quadricolor* only, is another example of a geographically widespread specialist, found throughout the Indo-Malayan Archipelago (Fautin and Allen 1992). The geographical range of these anemonefish species is an indirect consequence of how successful, in terms of distribution and abundance, their host anemones are, as well as stochastic processes, learned and innate preferences, and competition (Fautin 1986, Murata et al. 1986, Miyagawa 1989, Shick 1991, Fautin and Allen 1992, Elliott et al. 1995). *E. quadricolor* is a common actinian within lagoons and on fringing reefs throughout the Indo-Pacific hosting at least 13 species of anemonefishes (Fautin 1991, Fautin and Allen 1992). This may allow fishes that associate with *E. quadricolor*, especially those that are highly competitive, to spread as far as the host's range. *A. melanopus* and *P. biaculeatus* are also aggressive competitors, able to out-compete less aggressive symbionts for *E. quadricolor*. As stated above, chemical imprinting may serve as a supplement to an

inherited recognition system, especially for anemonefish specialists (Miyagawa 1989). This may partially explain why partnerships vary throughout the Indo-Pacific (Dunn 1981, Miyagawa 1989).

The Amphiprioninae, falling within a spectrum of generalists and specialists, exhibit two behavioral strategies of recruitment. One, generalists, that can colonize at least several different host species, use less desired anemones, or habitats, escaping both inter and intra-specific competition in order to proliferate. Generalists are able to recruit to new geographic areas more easily being able to utilize a greater number of host species throughout the Indo-Pacific. Two, specialists rely upon their ability to outcompete other symbionts for desired hosts. This supports Fautin's (1986) competition theory explaining species-specificity.

Conclusion

In the field, all anemonefish embryos are exposed to chemicals secreted by the host actinian of the parent fish. It would be logical for anemonefishes to be imprinted by chemicals secreted by host anemones, to assist them later on in recognizing the host when recruiting to a reef. This study has demonstrated that imprinting of *A. chrysopterus* embryos by host anemones does affect their settlement behavior. Settlement behavior of Amphiprioninae determines recruitment patterns of anemonefish species on the reef.

A. melanopus were not imprinted post-hatching and have an innate recognition of a chemical(s) secreted by *E. quadricolor*. If imprinting occurs, it probably affects the embryos and may serve as a supplement to inherited recognition of certain chemicals secreted by host anemones.

As stated above, anemonefishes tend to fall into a spectrum between generalists and specialists. Generalists may be more prone to chemical imprinting than specialists which probably have innate preferences and host specificities. Isolating attractant chemicals from each of the 10 host anemones would give a clear picture of what synamones induce responses in *Amphiprion* species (Murata et al. 1986). An experiment covering a broader range of *Amphiprion* species and exposing their embryos to a variety of natural and unnatural host actinians would provide a greater understanding of the ways in which these fish recruit and have speciated over time and space.

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