Field experiments on individual adaptation of the spider crab Inachus phalangium to its sea anemone host Anemonia viridis in the northern Adriatic Sea

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Abstract

We studied the adaptation of the spider crab *Inachus phalangium* (Fabricius, 1755) to one of its sea anemone host species, *Anemonia viridis* (Forsskål, 1775) in the coastal region of Rovinj, Croatia. Similar to other brachyuran species, *Inachus* spp. generally lives within the anemone to obtain protection from possible predators. Using removal and reintroduction experiments, this study investigates the protection mechanism and shows a loss of adaptation after a period of 10 days when the crabs are taken out of their host and kept solitary. Thirty-nine anemones from two different trial sites were marked individually and the inhabiting crabs were isolated to be released back into their individual hosts later. The reactions of the anemones were closely observed and characterized to determine the respective state of crab adaptation. As 35 out of 39 individuals provoked a defense/attack reaction of the anemone, it is concluded that the crabs possessed some sort of non-permanent protection mechanism that was lost during the test run (chi-square test, $p < 0.00014$). All tested crabs re-inhabited their host anemones within a maximum of 20 minutes after they had been reintroduced and stung by the anemones. Therefore, habituation to the host’s defense/attack mechanism is acquired individually and not genetically inherent to the species. The results are compared to adaptation and protection data on other decapod crustaceans and some anemonfishes.

Keywords: Field experiment, symbiosis, adaptation, sea anemone, spider crab.

Introduction

Symbiotic life patterns are widely distributed in nature. Marine decapods associate with a variety of other organisms in tropical, subtropical or temperate waters. Besides the classical example involving cleaner shrimp and fishes, associations to polychaetes, echiurids, molluscs, brachiopods, tunicates, sea anemones, echinoderms, sponges and corals probably form most of the marine cases (Ross, 1983). Cnidarians seem to play a prominent role as hosts for crustaceans (Balss, 1956-1957; Patton, 1967) providing shelter from predators (mostly fish and molluscs), food or rendez-vous-places for mating. Different abiotic and biotic factors, such as nutritional conditions, host abundance and intra- and interspecific relations, result in different types of symbiosis. Some species form long term relationships, while others tend to migrate from one host to the next on a short term basis (Thiel et al., 2003; Calado et al., 2007). Some live solitarily or in male-female associations, monopolizing and eagerly defending their hosts from every intruder. This constitutes a classical symbiosis, as harmful organisms are kept away from the host. For example, the anemone *Bartholomea annulata* is protected by its symbiotic snapping shrimp *Alpheus armatus* against the fireworm *Hermodice carunculata* (Smith, 1977). Other symbions form multiple intra- or even interspecific associations. Depending on food availability and other circumstances, a mutual symbiosis can develop into parasitic forms of symbiosis (Wirtz & Diesel, 1983; Wirtz, 1997).

Sea anemones use mechanically and/or chemically triggered nematocysts (Thorington & Hessinger, 1988) within their tentacles for paralyzing prey or for defense against enemies. The selectivity of mechanisms triggering discharge is unknown (Berking & Herrmann, 2005). A few decapod and fish species have managed to adapt to these nematocysts. They do not cause a defense or attack reaction and therefore are able to stay inside the anemones’ tentacle areas (Mebs, 2009). Previous studies of decapod-anemone relationships are based mainly on individual observations, presence/absence lists (Wirtz & Diesel, 1983; Wirtz, 1997; Patzner, 2004) or laboratory studies (Levine & Blanchard, 1980; Crawford, 1992; Giese et al., 1996; Calado et al., 2007). Melzer & Meyer (2010) undertook series of field experiments to elucidate the respective ranges of adaptation within various decapod crustaceans and some anemonfishes.
genera and among single individuals belonging to the same species. ‘Anemone-naïve’ individuals, which live outside of anemones, possess no defense system and are stung when coming into contact with anemone tentacles. In contrast, ‘habituated’ individuals of the same species, i.e. those that live inside anemones, provoke no stinging reaction when they are transferred from one anemone to another. Therefore, a specific process of adaptation has been assumed, along with the notion that adaptation is an individual condition which occurs in a few decapod species only but not present in individuals living outside of the anemones. So far, experiments have not shown that single individuals may occur in both, habituated and non-habituated conditions and, thus, may acquire or lose protection. However, a number of laboratory experiments have shown that acclimatization can be forced (Khan et al., 2003) or lost over time (Levine & Blanchard, 1980; Crawford, 1992).

The present study focused on the relationship between the spider crab *Inachus phalangium* (Fabricius, 1775) (family: Inachidae, superfamily: Majoidea, order: Decapoda) and the Snakelocks anemone, *Anemonia viridis* (Forsskål, 1775) family: Actiniidae, order: Actiniaria), in the northeastern Mediterranean Sea off Rovinj, Croatia. *Inachus phalangium* is found throughout sublitoral zones of the Mediterranean Sea and Eastern Atlantic at depths between 0.5 m and 20 m (Zariquiey Alvarez, 1968), mostly in close, preferably single association to sea anemones, with *A. viridis* as the prevalent host species (Weinbauer et al., 1982; Wirtz & Diesel, 1983; Đuriš et al., 2013). *Inachus* crabs seldom leave their hosts, except for feeding or molting, migrating to another anemone or when fleeing from larger conspecifics. In addition, male specimens are known to leave their hosts in search for females (Wirtz & Diesel, 1983).

Since the background of the decapod-anemone protection system is unknown, we designed follow-up experiments to test the hypothesis of individually obtained protection from nematocysts, using the readily accessible species pair *Inachus phalangium* and *Anemonia viridis*. To evaluate individual habituation, a series of removal and reintroduction experiments was undertaken during which individual decapods were removed and kept away from their host anemones for a period of time, then were returned to check whether they had lost their protection ability.

In contrast to earlier experiments, we also investigated habituation responses in situ, in order to minimize potential causes of artifactual results.

**Material and Methods**

The experiments were conducted over a period of four years in the months of August and September 2011–2014. The two main trial sites, Val Faborsa Bay (45.11775° N 13.616472° E) and Saline Bay (45.118194° N 13.620806° E), are situated around 5 km north of the city of Rovinj, Croatia. A peninsula divides these two cove-like sections of coastline formed by Croatia’s typical rocky geology. The shore area consists of a more or less linear rock-slope that is up to 4 meters deep depending on the tidal stage. It is dotted with two species of Actinaria, *Aiptasia mutabilis* (Gravenhorst, 1831) and *Anemonia viridis*; the latter is much more abundant and was used exclusively in the experiments. The anemones grow scattered all over the shore or form dense meadows, as in a 50 sqm area of Saline Bay. The bottom of the bay is covered with fine, at times muddy sediment, occasionally populated with *A. viridis* on loose substrate.

Specimens of *I. phalangium* were retrieved and returned by snorkeling. A total of 39 anemone-inhabiting *I. phalangium* were removed from depths of 0.2–2.5 m during the daytime (9 a.m. to 5 p.m.). When these individuals were reintroduced to their original hosts in situ after 10 days, the anemones were checked for any defense/attack reactions. Additional observations of reacclimatization behavior of such formerly adapted crabs were undertaken in September 2013 and 2014. Photographic documentation was performed using Canon IXUS 400 and 800, and Olympus μ Tough underwater cameras.

**General design of the experiments**

Habituated *I. phalangium* individuals were isolated from their hosts; upon their reintroduction after 10 days the anemones were observed for any (defensive or predatory) reaction (Fig. 1 A–D).

For permanent individual identification, anemones were marked under water using colored plastic binders fixed to the ground (Fig. 1 F). A total of 39 binders were placed, using three different colors to indicate the respective search area, further subdivided by an additional sequence of colored plastic straw units.

Black units symbolized three distances from the shoreline (small, intermediate and large). This proved to be very helpful, especially when tides were strong. In some sections of the study area black units could be neglected, as the anemones were located in a single line parallel to the shore.

A second color signified groups of anemones in sections of the bays, a third one in ascending numeric order identified the individual host. This way a characteristic code was generated for each anemone/crab pair (Fig. 1 E).

The location of each marked anemone was registered in a map, and further details were added to help relocate it. Weatherproof crayon was used to mark coastal structures.

A standardized progression was used for capturing decapods. To select for specimens showing habituation, each individual was monitored first, especially for any interactions with the tentacles. Generally, *Inachus* rests at the anemone's stem or when fleeing from larger conspecifics. In addition, *Inachus* rests at the anemone's stem or between the tentacles and seeks shelter within the host when approached by a snorkeler, touching the tentacles with all its body parts in the process. When the crab did not
do so, it was gently pushed into the tentacles with a plastic binder tip (special care was taken not to touch the anemone with the plastic binder). Only individuals not provoking any reaction from the anemone, hence considered as habituated, were caught and kept individually in a vial. Each vial was marked with the same color coding as the anemone, and placed in an outdoor flow-through basin at the Center for Marine Research (Ruđer-Bošković-Institute) in Rovinj.

**Specific design of the removal and reintroduction experiments**

Anemones are rather easy to spot on the rock slope between 0.2 and 2.5 m depth (Fig. 2 A). With few exceptions, each anemone housed at least one *Inachus*. Over a period of 4 years (2011–2014) 39 *I. phalan*gium individuals were collected from 39 anemones of the two trial sites. Each individual was carefully put in a 100 ml vial using a new binder to extract it from the anemone. The vial was closed with gauze netting, colorcoded and stored in a shaded storage container on the shore during the day. When sampling was completed all vials were put in a 200 liter outdoor tank with fresh seawater supply (Fig. 1 G). They were checked twice a day for vitality of the crabs, the necessity to change water (e.g., if a vial had fallen over) and other eventualities. The crabs were not fed or interfered with in other ways. Identification of the species was postponed until after the release test, as in-

![Fig. 1: A – D Schematic illustration of the removal and reintroduction experiment. A Habituated *I. phalan*gium sits in close contact to anemone without being stung. B Crab is taken out of anemone; the latter is marked with a binder (see F). C Crab is isolated for 10 days in the institute’s water tank, marked with corresponding binder (see G). D Crab is returned to its source anemone, and reaction is observed. E Binders with characteristic markings: example of a black binder with one black unit (close to shore), one blue unit (anemone group blue) and two red units (second individual of group blue); abbreviation BBL blb2r. F Example of individual underwater marking of an anemone. G Storage of vials in a 200 l flow-through tank with constantly renewed seawater.](http://epublishing.ekt.gr)
tense cleaning is needed to uncover features hidden by camouflage algae and sponges.

During this study period none of the *Inachus* showed particular abnormalities. Most of them slowly lost their camouflage decoration if they had any in the beginning.

Releasing the crabs took place in the same sequence and at approximately the same time of day as collecting.

Each crab vial was held 5-10 cm above the corresponding anemone. When the vial was opened and slightly turned downwards, the decapod started to climb out. The constant movement of the rather long tentacles of *Anemonia viridis* created a first crab-anemone contact on its legs that already showed the respective type of response. Then the complete reintroduction followed as the crab was released completely above the center of the anemone, into which it slowly sank.

Most observed interactions were distinct, allowing a clear assignment to either of two result classes, ‘no reaction’ or ‘reaction’; the latter was further divided into ‘strong’ and ‘weak’ reactions. Unclear interactions were rated as ‘no reaction’.

The two alternative reaction results can be characterized as follows:

1. ‘Reaction’. First contact of anemone tentacles with the *Inachus*’s legs resulted in the crab withdrawing back into the vial. This indicated that a strong reaction was to follow. Once the decapod was reintroduced completely, the anemone immediately switched to active movement. Tentacles in direct contact were glued to the crab’s carapax or body appendages, tentacles further away were bent towards the intruder and also stuck to it (Fig. 2 B–C). An extreme form twice encountered was that the crab was sucked in completely. In response, the crabs either tried to flee or stayed motionless. Moving decapods showed unambiguously that tentacles were glued to their bodies. When decapods did not move they were gently pushed through the tentacles, which revealed whether or not there was attachment. When the result was classified as a ‘reaction’, ‘strong’ reactions were distinguished from ‘weak’ ones by active movement of the tentacles versus passive adherence to a moving crab.

2. ‘No reaction’. First contact showed no reaction at all. *Inachus* tried to climb out of the vial unhindered. When placed into the anemone, no reaction was noticed either. Tentacles did not change behavior nor stick to the crab, which could move freely and was not harmed. Usually, *Inachus* climbed to the edge of the anemone but remained in close contact to the tentacles. When the crab did not move by itself, the “push-test” also gave no reaction result.

Results were noted on an underwater tablet, and each crab was returned to the laboratory for identification. The crabs were identified under stereo microscopes using Zariquey Alvarez (1968). Five of the studied *Inachus* were conserved in 4% formaldehyde in seawater, and deposited as reference specimens in the ‘Arthropoda varia’ collection at the Zoologische Staatssammlung München.

**Specific design of the “reacclimatization” experiments**

To further examine reacclimatization, all 34 remaining crabs were returned to their respective source anemones and subjected to a second and final release test. Each individual was placed on the ground next to the anemone

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**Fig. 2:**

A: Regular resting position of *Inachus phalangium* within *Anemonia viridis*; note tentacles not glued to the crab. B – C: different *I. phalangium* attacked by *A. viridis* after introduction; note tentacles glued to various parts of crab body.
but out of reach of the tentacles; as it entered the host, their behavior was observed and also documented with underwater cameras.

Results

Removal and reintroduction

Table 1 summarizes the reaction results obtained from the 39 crab/anemone pairs. Thirty-five of the tests were classified as showing a reaction, i.e. that the crab was attacked by the anemone. The results were negative or unclear in 4 experiments only.

The observations were tested against the null hypothesis of random reaction by the anemone using the chi-square test. No differentiation between strong and weak reactions was made here, as it would be irrelevant to the evaluation of decapod adaptation. The loss of previous adaptation is highly significant, with X² = 14.513 and p = 0.00014.

Reacclimatization

After the first release into the anemone, most _I. phalangium_ individuals showed very similar behavior. Upon getting stung, they climbed out of the anemone and rested out of the tentacles’ reach. Soon thereafter they started an acclimatization pattern that lasted up to 20 minutes. Beginning with the chelipeds or the first pair of walking legs (second pair of pereiopods), they started making contact to a few tentacles. After a while they were attacked less and less. Some clipped off small parts of the tentacles, bringing them to the mouth or sticking them onto the legs and carapax.

The same pattern was observed during the second release. When placed on the ground next to the anemone, most _I. phalangium_ individuals immediately started approaching the host. Getting stung at first contact, they either stopped moving or, predominantly, started climbing slowly into the tentacles, retreating only a little when covered by tentacles too intensely. The hind pairs of pereiopods always stayed connected to the ground to ensure a safe hold and to avoid getting pulled in by the anemone. Eventually they came to rest inside the anemone without any reaction from the tentacles.

Discussion

In the entire testing region _Inachus phalangium_ was found associated with anemones without exception, generally solitary, sitting inside or at the rim of the anemone and covered by its tentacles. The present study focuses on the question whether or not adaptation between the host anemone and symbiotic decapod is acquired individually and temporary (e.g., can be lost) or whether it is genetically inherent to the species _I. phalangium_.

The results show that any previous adaptation was lost after a period of 10 days in 35 out of the 39 individuals. All tested anemone/ _Inachus_ pairs were considered as initially adapted to each other, as the decapods had been sampled while sitting in direct contact to the tentacles without triggering any response.

During their isolation the crabs were not manipulated in any way. Therefore, the morphological structure of the carapax does not influence the state of adaptation, as it would remain unchanged after the isolation time.

On some individuals we observed a reduction in surface cover materials during isolation that is partly due to decapod activity, as _Inachus_ spp. are known to feed on such decoration (Wirtz & Diesel, 1983; Martinelli et al., 2006). Not all crabs were decorated initially, however, and not every crab’s decoration vanished. Therefore, the effect of the macro-decoration on habituation is considered as negligible. On the other hand, it is conceivable that any micro-decoration, e.g. with mucus or some other cover, might have been lost as well during the period for which the decapods were separated from their host anemones.

Anemone discharge is triggered mechanically and/or chemically (Thorington & Hessinger, 1988). Despite the mechanical stimulus provided by _I. phalangium_ individuals crawling through the tentacles, adapted crabs provoke no reaction. Our experiments therefore appear to have changed chemical stimuli that are either present or absent depending on the state of adaptation.

Weinbauer, Nussbaumer and Patzner (1982) assumed “the existence of a protective substance in or upon the integument of the crab”. In their experiments _Inachus phalangium_ was thoroughly washed in acetone affecting any chemical substances but none of the carapax structures. It was then released into _Anemonia sulcata_ (= _A._

Table 1: Summary of removal and reintroduction tests from 2011 to 2014.

<table>
<thead>
<tr>
<th>Date of catch</th>
<th>Origin</th>
<th>Total n</th>
<th>‘Weak reaction’</th>
<th>‘Strong reaction’</th>
<th>‘Reaction’</th>
<th>‘No reaction’</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.08.2011</td>
<td>Val Faborsa Bay</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>26.08.2012</td>
<td>Saline Bay</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>25.08.2013</td>
<td>Val Faborsa Bay</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>27.08.2013</td>
<td>Saline Bay</td>
<td>18</td>
<td>7</td>
<td>8</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>25.08.2014</td>
<td>Val Faborsa Bay</td>
<td>10</td>
<td>2</td>
<td>8</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>Totals</td>
<td>39</td>
<td>11</td>
<td>24</td>
<td>35</td>
<td>4</td>
</tr>
</tbody>
</table>
viridis). The protection was completely removed and all 11 individuals caused a very strong reaction of the anemone. It is possible, though, that some acetone residue might have remained on the carapax and triggered the reactions.

Regarding the present study a similar conclusion can be drawn. Most likely an external chemical layer protects the crabs that are lost after a period of time, possibly dissolved in sea water. Melzer and Meyer (2010) found I. phalangium, together with other decapod species, to be protected when initially located within their host anemone, and still protected when transferred to other anemones of the same species. We observed that habituated I. phalangium individuals as well as individuals after reacclimatization did not provoke any attack behavior by the anemones. In any case it remains unclear where such protective layers might come from and what they may be made of. Possible origins seem to be that I. phalangium either produce protective coating themselves or acquire it from the anemone via direct contact. The former is found in some species of sponges that appear to be chemically defended (Green, 1977; Pawlik et al., 1995; Wilcox et al., 2002). Similarly, the nudibranch Aeolidia papillosa produces nematocyst inhibiting mucus (Greenwood et al., 2004), but Davenport (1962) failed to prove the existence of such inhibition in actinians and their commensals. In the absence of a potential host Inachus might cease the production of such coating to avoid the corresponding energy costs. However, judging from the behavior of unadapted crabs after their release into an anemone, it seems more likely that Inachus gain protection by acquiring material from the anemones.

All individuals we investigated started an acclimatization pattern to gradually increase contact with the tentacles; thus, close contact seems to be essential. Some crabs were observed clipping off small parts with their chelipeds and bringing them to the mouth or sticking them onto the legs and carapax. No individual has been documented to become adapted without touching any tentacles.

Similar observations have been made in laboratory studies on the Great Spider Crab, Hyas araneus (Davenport, 1962), the cleaner shrimps Periclimenes rathbunae, P. anthophila and Thor amboinensis (Levine & Blanchard, 1976, 1980), the spider crab I. phalangium (Weinbauer et al., 1982) or on Periclimenes anthophila acclimatizing to Condylactis gigantea (Crawford, 1992). All of these showed a loss of adaptation after a period in isolation or after cleaning, and an acclimatization pattern similar to the one we observed in the field. Consequently, it can be concluded that this is a general pattern in these taxa.

In addition to these laboratory experiments, Melzer and Meyer (2010) proved an individual adaptation of a few decapod species in the field. Members of a given species were either adapted to their anemone hosts, as they were sitting among the tentacles without causing a reaction, or non-adapted when found outside of them. The latter individuals provoked an immediate reaction when transferred onto anemones. Thus, adaptation must be regarded as not innate to all members of a species. As the present study demonstrates, the adaptation of I. phalangium to its host Anemonia viridis is individually acquired, as preadapted crabs lose their protection in phases without contact to their host, and later regain it through reacclimatization.

It remains unclear whether the crab simply covers itself in anemone mucus or whether a passive or active chemical reaction with anemone components is effected on the crab’s carapax. Similarly open is the possibility that the loss of adaptation takes place on the host’s rather than on the decapod’s side. Transfer tests of non-habituated decapods found outside an anemone host (Melzer & Meyer, 2010) indicate an essential role of the decapod, as otherwise habituated anemones would react equally to outer and inner crabs.

In general, all of these decapods seem to show individual adaptation to their hosts, with inter- and intra-specifically different time spans for loss and regaining of the adaptation, which also varies in time and effort depending on host species.

Comparable circumstances have been studied widely in anemone fishes, which are known to cover themselves with anemone mucus to avoid being netted. However, the precise details of this immunity have also remained unclear in this case (Nedosyko et al., 2014), with contradictory results suggesting either acquired (Schlichter, 1975) or innate immunity (Lubbock, 1980, 1981; Foster, 1975 unpublished). As a conclusion, it appears that there is no general mechanism within anemone fish species. The adaptation can be innate, acquired from anemones or self-produced, inhibit nematocyst discharge or mask its stimuli. Discussions even include a function of anemone substances in addition to an inherited adaptation, whereby a pre-existing protection may be enhanced by the anemone.

Unlike fish, decapods need to moult for growing, thus repeatedly discard the old carapax. A special case occurs in Inachus, which stops moulting after a final moult of puberty (Hartnoll, 1963). Wirtz & Diesel (1983) found I. phalangium to leave its host for moulting. As protection seems to be lost, the crab is forced to take the risk of predation to avoid being attacked by the anemone. This also indicates that protection is not innate, as otherwise the crab would remain adapted. Contradictory observations were made with freshly moulted Periclimenes, which were either still protected from the anemone (Crawford, 1992) or provoked a strong reaction (Levine & Blanchard, 1980).

Rather little research has been undertaken on crustacean adaptations to their host anemones, and fish associations have remained somewhat mysterious as well. Although these marine symbioses are among the most familiar, very little is known and results reported on the causes and mechanisms are even contradictory at times. Our study places the spider crab Inachus phalangium among the multitude of crustacean and fish species sym-
biotic to sea anemones, and demonstrates an individual habituation phase, as the species is not generally immune. We assume a mechanism involving acquisition of anemone substances, as no decapod has ever been shown to adapt without close contact to the host’s tentacles.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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