

## STUDY ON THE PRESENCE OF CHEMICAL DEFENSE AGAINST PREDATORS IN THE EARLY STAGES OF THE OPISTHOBANCH *APLYSIA DEPILANS*

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### ملخص

دراسة تواجد الدفاع الكيميائي ضد المفترسات عند ذيلية الخياشيم *أبليزيا ديبيلنس* في مراحل النمو المبكرة : لقد تم الإثبات الفعلي لتواجد دفاعات كيميائية فعالة تستخدمها ذيلية الخياشيم *أبليزيا ديبيلنس* لحماية نفسها من الهجمات المفترسة. ومع ذلك ، لا توجد سوى بيانات قليلة عن هذه الدفاعات في المراحل الأولى من تطور هذه الرخويات ، حيث يكون البيض والفراخ والأحداث أكثر عرضة للافتراس ، بما أنها عديمة الحركة و بدون غلاف أو حماية جسدية أخرى. تم الإبلاغ عن عدد قليل من الكائنات المفترسة التي تتغذى على البيض أو أحداث ذيلية الخياشيم *أبليزيا ديبيلنس*، الأمر الذي يقودنا إلى التشكيك في أصل عدم الاستساغة لذا تم اختبار الدفاعات الخاصة عند ثلاث مراحل من النمو (البيض وحجمين من أحداث ذيلية الخياشيم *أبليزيا ديبيلنس*) ضد اثنين من المفترسات المتجاورة وهي أقحوان البحر أنيمونيا سيلكاتا وسرطان البحر كارسينيس أستيري تم استخدام علف اصطناعي مصنوع من مستخرجات *أبليزيا ديبيلنس* لاختبار الأصل الكيميائي للدفاعات.

وتبين ان البيض وأحداث *أبليزيا ديبيلنس* صدت كل من أنيمونيا سيلكاتا و كارسينيس أستيري حيث يبدو أن هذا الدفاع له أصل كيميائي.

كما لم تكن مجموعات البيض الثلاثة المختبرة مستساغة لكل من المفترسين ( $p < 0.05$ ). هذا الدفاع هو من أصل كيميائي لأن الحيوانات المفترسة استهلكت البيض بعد الاستخراج ورفضت الأعلاف الصناعية مع المستخرجات المدمجة. كما تم رفض الأحداث من قبل أقحوان البحر مع تقبل الفراخ و تم رفض الأحداث من قبل السرطانات. تم رفض التغذية الاصطناعية المصنوعة من مقتطفات للأحداث من قبل كل من الحيوانات المفترسة التي تم اختبارها

**الكلمات المفتاحية:** الافتراس ، الدفاعات الكيميائية ، الأحداث ، البيوض ، ذيلية الخياشيم *أبليزيا ديبيلنس* أنيمونيا سيلكاتا ، كارسينيس أستيري ، تونس ، البحر المتوسط.

### RESUME

**Etude de la présence de défense chimique contre les prédateurs chez les premiers stades de l'Opisthobranch *Aplysia depilans* :** L'existence de défenses chimiques efficaces utilisées par les opisthobranches pour se protéger des attaques de prédateurs est déjà établie. Ceci dit, peu de données existent sur ces défenses chez les premiers stades de développement de ces animaux. Les œufs, les recrues et les juvéniles peuvent être très vulnérables à la prédation, étant sessiles et ne disposant pas de coquille ou d'autre protection physique. Peu d'espèces de prédateurs ont été reportés se nourrir des œufs ou juvéniles de *Aplysia*, ce qui nous pousse à nous poser des questions quant à l'origine de cette non palatabilité. Les défenses de trois stades de développement des œufs et de deux tailles des juvéniles de *Aplysia depilans* ont été testées contre deux prédateurs sympatriques : *Anemonia sulcata* et *Carcinus aestuarii*. Des extractions et des aliments artificiels faits avec les extraits ont été utilisés pour tester l'origine chimique des défenses.

Les œufs et les juvéniles de *Aplysia depilans* ont repoussé *Anemonia sulcata* et *Carcinus aestuarii*. Cette défense semble être d'origine chimique.

Les trois groupes d'œufs testés n'ont pas été palatables aux deux prédateurs ( $p < 0,05$ ). La défense est d'origine chimique puisque les prédateurs ont consommé les œufs après extraction et rejeté les aliments artificiels avec les extraits incorporés. Les juvéniles ont été rejetés par *Anemonia sulcata* mais pas les recrues. Les deux tailles de juvéniles ont été rejetés par les crabes. Les aliments artificiels fabriqués avec les extraits des juvéniles ont été rejetés par les deux prédateurs testés.

**Mots clés:** défenses chimiques, juvéniles, œufs, prédation, *Aplysia depilans*, *Anemonia*, *Carcinus*, Tunisie, Méditerranée.

### ABSTRACT

The existence of efficient chemical defenses used by Opisthobranchs to get protection from predator's attacks is already established. However, little is known about the defenses in the early life stages of these animals.

Predation on eggs, recruits and juveniles can be very important since these stages are almost sessile, and have no physical protections like shells. Few predator species were reported feeding on *Aplysia* egg masses or juveniles, this may let someone ask about the origin of the deterrence of these early life stages. The deterrence of three developmental stages of the egg masses and two sizes of the juveniles of *Aplysia depilans* were tested against the sympatric predators. *Anemonia sulcata* and *Carcinus aestuarii*. Extractions and artificial diets made with the extracts were used to test the chemical origin of the defenses.

The egg masses and the juveniles of *Aplysia depilans* were deterrent to *Anemonia sulcata* and *Carcinus aetuarii* and the defense seems to be chemically mediated.

The three groups of eggs were deterrent to both of the predators tested ( $p < 0.05$ ). The deterrence was chemically mediated as the predators eat the extracted eggs and the two predators tested rejected the artificial diets where the extracts were incorporated. Juveniles but not recruits were deterrent to *Anemonia sulcata*. And both sizes of the juveniles were rejected by the crabs, the artificial diets made with the extracts from the juveniles repelled eating from both of the predators tested.

**Key Words:** predation, chemical defenses, juveniles, egg masses, *Aplysia depilans*, *Anemonia*, *Carcinus*, Tunisia, Mediterranean.

## INTRODUCTION

Predation is a universal biotic interaction that influences species abundance and distribution, affects population dynamics, changes community organization and functioning, and acts as an evolutionary force (O'Steen *et al.*, 2002; Finke and Denno, 2005; Losos *et al.*, 2006). These effects are a consequence of the high variability in predation at every level of biological and ecological organization (Michelli, 1997; Evans, 2004; Kolb *et al.*, 2007). Although no organism is free from predation, there are multiple variables that condition food selection (McLoughlin *et al.*, 2010) and predators show strong preferences over certain species (Hayward and Kerley, 2008; Abrams, 2010) or individuals of the same species (Genovart *et al.*, 2010). Predators concentrate on substandard individuals that are weak, sick, or show restricted physical conditions (Genovart *et al.*, 2010). For many species, age and size are surrogates of such substandard individuals, and there are numerous examples showing how the susceptibility to be preyed upon is inversely related to age and size (Fuiman and Magurran, 1994; Gosselin, 1997; Sogard, 1997; Clemente *et al.*, 2009; Porter and Bailey, 2007; Horning and Mellish, 2012).

Predator preferences also are strongly influenced by the prey capacity to defend. As a major source of mortality, species have developed a number of behavioral, structural, and chemical mechanisms to minimize predation (Abrams, 2001). Presence of one or several defensive mechanisms significantly decreases the vulnerability of species to predators (Schupp and Paul, 1994; Becerro *et al.*, 1997; Hülsmann and Wagner, 2007; Kishidia *et al.*, 2009; Hammill *et al.*, 2010; Kuprewicz and García-Robledo, 2010). Chemical deterrence is particularly spread in the marine benthos (Paul, 1992; Hay, 1996; McClintock and Baker, 2001; Paul *et al.*, 2011), which suffers a strong top-down control as compared to plankton or terrestrial ecosystems (Shurin *et al.*,

2002). Because the efficient and widespread use of chemical defenses to deter predators, it is suggested that chemical defenses evolved in response to predation, driving the evolution of multiple groups (Berenbaum, 1983; Feeny, 1991; Martin, 1995). Marine opisthobranch mollusks in particular show an evolutionary trend to shift from structural to chemical defenses (Faulkner and Ghiselin, 1983; Cimino and Ghiselin, 1998, 1999; Wägele and Klussmann-Kolb, 2005), which is unusual in other mollusks (Gosliner, 1994). From an evolutionary standpoint, chemical defenses must be functional prior to shell loss (Faulkner and Ghiselin, 1983; Wägele and Klussmann-Kolb, 2005), so they might have helped protect early developmental stages where structural defenses may be too weak to be effective. Today, a number of studies show that opisthobranch egg masses can be chemically rich and have several activities (Matsungana *et al.*, 1986; Avila *et al.*, 1991; Ebel *et al.*, 1999; Benkendorff *et al.*, 2000; 2001; Becerro *et al.*, 2006). Whether these chemicals and activities function in the field as chemical defenses against predators is less well known (Pennings, 1994; Becerro *et al.*, 2003a, 2003b). Our study targets this need and will test whether egg masses and juveniles of two Mediterranean opisthobranch mollusks are defended against generalist predators and whether the defense is chemically mediated.

Sea hares are a small group of herbivorous opisthobranchs with internal shells and a great variety of defensive mechanisms (Johnson and Willows, 1999). Some sea hares distribute defensive chemicals in the skin or outer tissues, which effectively deters predators (Carefoot, 1987; Pennings, 1990; 1994; Pennings *et al.*, 1999). This chemical strategy may be species specific as the external tissues of other sea hare species fail to deter predators (Pennings, 1994). Defensive compounds from the diet preferentially concentrate in the digestive gland and, despite they can deter predators at naturally occurring concentrations, it is unclear whether they play such

role in nature (Pennings and Paul, 1993; de Nys *et al.*, 1996). Sea hares also release ink and opaline when disturbed, which function as a defense against a variety of predators (Kicklighter *et al.*, 2005; Nusnbaum and Derby, 2010). Yet, use and effectiveness of these mechanisms may vary with ontogeny (e.g., the capacity to release ink and opaline is absent in eggs and must be acquired at some point during their development). Most information on sea hare chemical ecology is restricted to adult organisms.

Here we experimentally test whether five early developmental stages of *Aplysia depilans* are protected against the sympatric predators *Anemonia sulcata* and *Carcinus aestuarii* and whether protection is provided by chemical defenses. Sea hares deposit millions of eggs in benthic egg masses that are noodle like strands made of proteins and polysaccharides, making them prone to predation and fouling. Egg masses are often visually conspicuous remain in the substrate for over a week until embryos develop and planktonic larvae hatch (Switzer-Dunlap and Hadfield, 1977). To remain fully functional and survive until larvae are released, egg masses may rely on chemical defenses. The capacity of sea hare egg masses to deter predators and inhibit microbes is not universal and varies between species. The resulting veliger larvae must metamorphose and settle back onto benthic communities, where juvenile sea hares continue with their development and spend the rest of their life until they die after reproduction. Sea hare juveniles cannot rely on their tiny internal shell as a defense and may be able to use the chemical defensive mechanisms present in the adults. The number of predators known to naturally feed on sea hares is limited but includes anemone, crabs, fish, flatworms, nemertines, pycnogonids, opisthobranchs, and seastars (Winkler and Tilton, 1962; Pennings, 1990; Johnson and Willows, 1999; Rogers *et al.*, 2000). In our study area, the anemone *Anemonia sulcata* and the crab *Carcinus aestuarii* were very abundant predators that shared habitat with *Aplysia depilans*. Since both anemone and crabs are known to feed on a variety of diets including sea hares, we used them to test whether five early developmental stages (three egg and two juvenile stages) of *Aplysia depilans* were protected against sympatric generalist predators and, if so, whether protection is chemically mediated.

## MATERIAL AND METHODS

We collected all egg masses, juveniles, and predators in the locality of la Marsa, Golf of Tunisia. Egg masses were collected between June and July. We classified them in three distinct categories based on color, elasticity, and presence of moving embryos checked under a scope. Undeveloped eggs (und)

referred to those whitish, freshly laid, compact egg masses with minimum elasticity and without moving embryos under the scope. This developmental stage was no older than 5 days. Developed eggs (dev) were darker in color and embryos were clearly moving even at bare eye. This developmental stage corresponded to 4-7 days. Pre-hatched eggs (phe) had a brownish, noticeable darker color than dev eggs, and were also much softer and flexible, being ready for the forthcoming hatching. Egg masses at this stage had 7-10 days old.

We grouped juveniles of *Aplysia depilans* (hereafter *Aplysia*) in two distinct sizes that also corresponded with functional differences. We called recruits (rec) to individuals smaller than 5 mm in length and juveniles (juv) to those larger than 6 mm and smaller than 2 cm. We failed to observe *Aplysia* individuals smaller than 3 mm, so we were unaware of the actual size when recruits settled back to the benthic community. In our study, recruits and juveniles referred to the smallest and largest size classes we investigated, respectively. These groupings were in fact defined *ad hoc* and based on the ability to release secretions. We observed after our first collection that specimens below 5 mm lacked the capacity to release secretions while those larger than 6 mm did release a white secretion when disturbed. Whether the secretion was ink, opaline, or a combination of both is unknown but we decided to use that size range to define our recruit and juvenile categories because the capacity to release secretions is a critical defensive mechanism in sea hares. Recruits and juveniles were exclusively found on the green alga *Ulva lactuca*.

All live animals, including the anemone *Anemonia sulcata* (hereafter *Anemonia*) and the crab *Carcinus aestuarii* (hereafter *Carcinus*) were collected and placed underwater in several plastic bags (without mixing species), transferred to large aerated coolers (without mixing species), and taken to the laboratory within 2/3 hours. We also collected fresh *Ulva lactuca* (hereafter *Ulva*) so that sea hares could continue with their regular feeding activities while in the laboratory. Once in the laboratory, each species was transferred into a large aerated aquarium (20 liters of capacity) with filtered seawater and offered *Ulva* (*Aplysia*) or mussels (predators) on a daily basis until they were used in our experiments. Frozen mussels (*Mytilus edulis*) were used to feed predators and as control food in our experiments (see below). We ran three sets of experiments to test whether early developmental stages were deterrent and whether protection was chemically mediated. First, we offered our predators live eggs, recruits, and juveniles to test whether the five early developmental stages were defended against predation. Then, we used chemically extracted organisms to test whether early developmental stages were protected regardless their secondary chemistry (so structural defenses or low

nutritional quality could contribute to their defense). Finally, we incorporated extracts of the early developmental stages into an artificial diet to test whether secondary chemistry alone can deter feeding by our generalist predators.

### **Deterrence of live animals**

To run the experiment, we transferred anemones and crabs to individual containers. Each container was 1 liter and held a single predator. Typically, predators exhibited normal behavior in 5 to 10 minutes. We referred to normal behavior in anemones as the opening of their oral disk and extension of tentacles, and not being very active as when stressed for crabs. We discarded any anemone or crab that failed to demonstrate normal behavior after 30 min.

Because the factors leading to food consumption or rejection may vary between individual predators, we used the same individual predator for both treatment and control trials. We therefore repeated testing of the same individual, reducing heterogeneity in the predator response. This approach is particularly indicated when exposure to one treatment may affect the response of the next treatment (Sokal and Rohlf, 1995). We then tested for a significant change in predator behavior due to our treatment with the McNemar test for significance of changes (Sokal and Rohlf, 1995). We first offered predators our treatment, which was placed on the anemone tentacles or in front of the crab, between the claws. We then observed whether the treatment was eaten or rejected. We scored eaten when the food item was completely eaten in 60 min or less, even if it was initially rejected or mouthed several times; otherwise we scored that replicate as rejected. We then offered a small piece of mussel of the same size than the treated food and scored whether it was eaten or rejected (i.e., uneaten after 60 min). Both feeding trials (treatment and control) represented a single replicate and experiments consisted of about 20 replicates. All predators and preys were used only once. Data were tabulated as the number of predators that ate the treatment and rejected the control, rejected the treatment and ate the control, and ate or rejected both the treatment and the control.

### **Chemically mediated defenses**

The second set of experiments tested whether chemically extracted of early developmental stages were deterrent to anemone and crab predators. Experiments were identical to those described earlier, except that instead of live animals, all developmental stages had been extracted with a 1:1 mix of methanol/dichloromethane.

Freshly collected egg masses, recruits, and juveniles were frozen at -20°C upon arrival to the laboratory. Once frozen, each developmental stage was freeze-dried and exhaustively extracted with three times 30-

min long, sequential additions of 20 ml of the solvent mix for gram of tissue (dry mass). Egg masses were cut to their final size before extraction. Extracts for recruits and juveniles were obtained from whole animals. Control mussels were subject to the same extraction procedure to prevent confounding factors between treated and control foods. Solvent from the three extractions was pooled together and evaporated down under vacuum in a rotavap to obtain a residue that contained the secondary metabolites from each developmental stage. We weighed the residue to calculate the concentrations at which they occur in their original tissue on a dry mass basis. These concentrations were used in our third experimental set. Extractions were then stored in a freezer at -20°C until they were used. Extracted material was dried at room temperature to evaporate the solvent and then offered to predators as described earlier.

To test whether the secondary metabolites extracted from the early developmental stages inhibited feeding of the anemone and crab predators we added the extracts at naturally occurring concentrations (dry mass) in an artificial diet. The artificial diet consisted of 5g of freeze-dried, milled mussels mix with X g of a mixture of agar/carrageenan (15:85) in 80 ml of water. We added 2 ml of 1:1 DCM/MeOH with varying mass of extract to match the naturally occurring concentration of each early developmental stage. Control food was identical except that the 2 ml of solvent contained no extract. The mix was poured into rectangular molds that were cut to obtain the final control and treatment food cubes used in our experiment. Experiments were run as described earlier, except that rather than live or extracted animals we used artificial food cubes.

### **Statistical analyses**

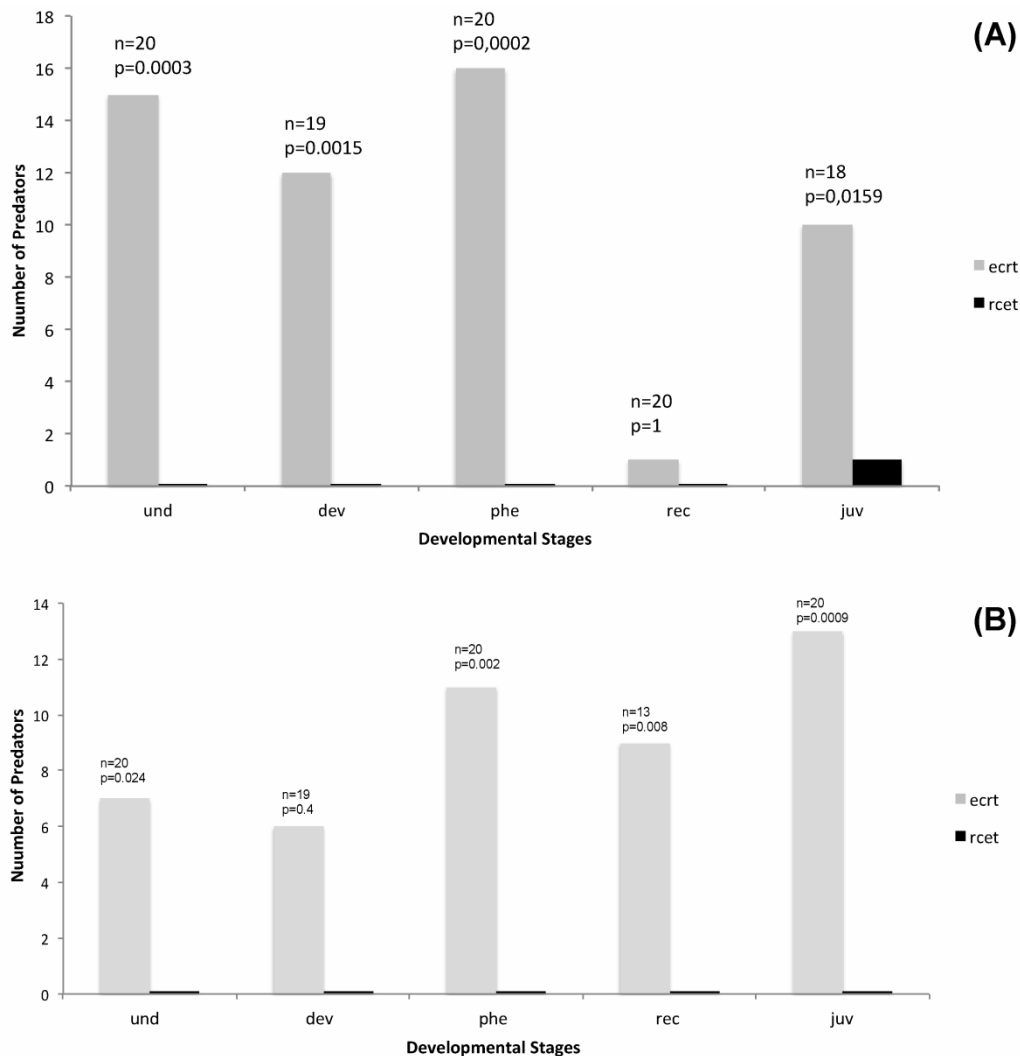
We run one experiment for each developmental stage and predator for a total of 30 experiments. McNemar test allowed testing for the effect of our treatment in each experiment. We then used meta-analysis to test whether the five developmental stages differed in their ability to deter predators and were equally effective against both predators. Meta-analysis provides a formal framework to combine results from independent studies or experiments and to test hypotheses beyond those tested by the individual studies or experiments. We assumed that each species or developmental stage has its own effect size, so we ran a mixed-effect model to account the variance between the experimental units. Effect sizes differed if their 95% confidence intervals (CI) exclude zero. Differences between species or developmental stages occur when their CIs do not overlap. Meta-analysis was run in R project.

## RESULTS

### Deterrence of live animals

The three developmental stages of egg masses (undeveloped, developed, and prehatched) and

juveniles significantly deterred feeding in *Anemonia sulcata* and *Carcinus aestuarii* (Figure 1) as compared to mussel control (McNemar tests). *Anemonia sulcata* failed to feed on the experiment with recruits (only one control item eaten).

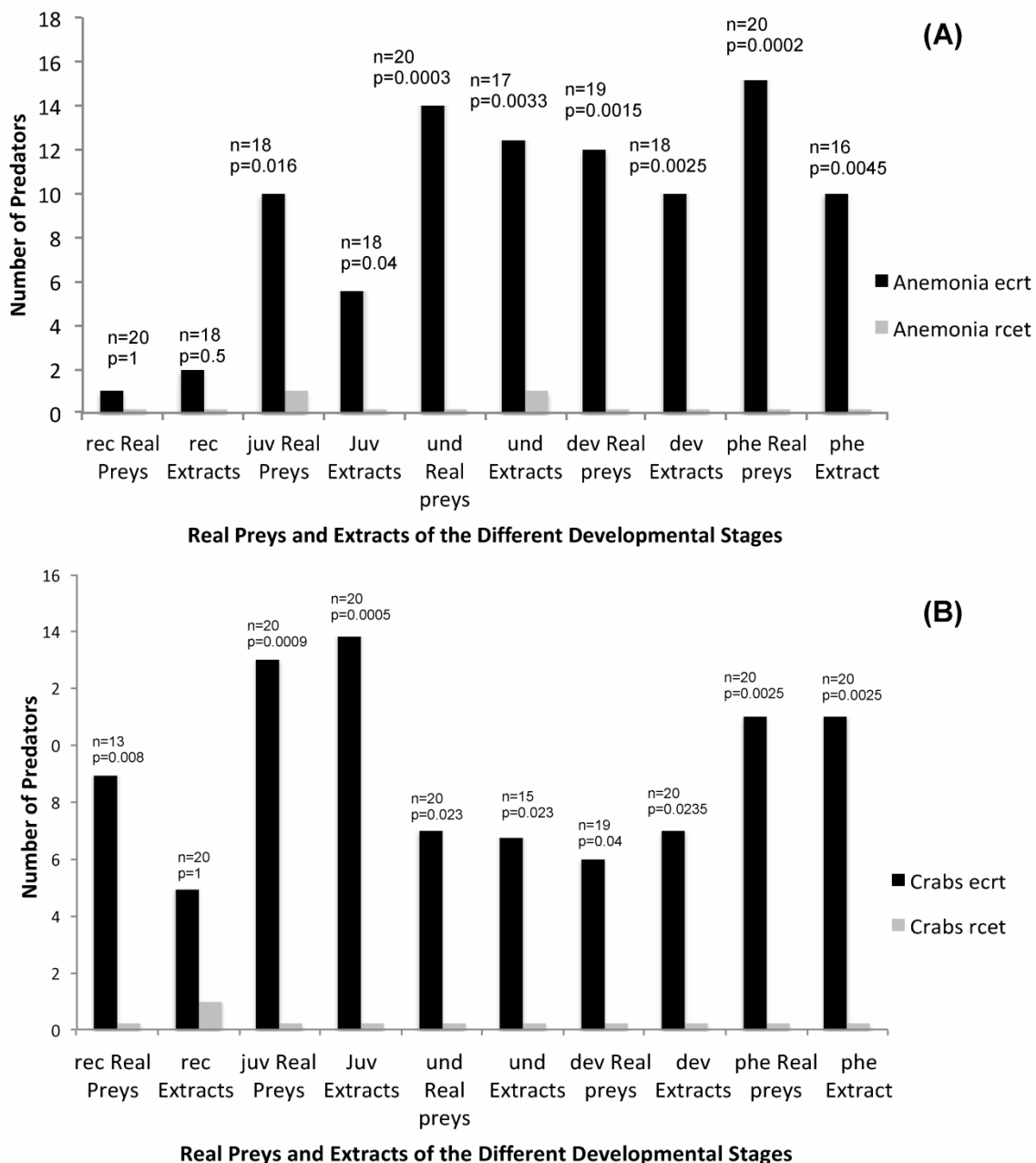


**Figure 1:** Test of the deterrence of egg masses and juveniles of *Aplysia depilans* to *Anemonia sulcata* (A) and to *Carcinus aestuarii* (B). Und, undeveloped eggs; dev, developing eggs; phe, pre-hatching eggs; rec, recruits; juv, juveniles; ecrt, predators that have eaten the controls and rejected the treatments; rcet, predators that have rejected the controls and eaten the treatments.

### Chemically mediated defenses

The three developmental stages of egg masses (undeveloped, developed, and prehatched) as well as the juveniles deterred feeding by the two predators tested as real preys and as extracts incorporated in artificial diets at naturally occurring

concentrations. As for the recruits, the real preys and the extracts were eaten by the anemones (McNemar tests,  $p = 1$  and  $p = 0.048$  respectively, Figure 2), but the crabs rejected the real preys (McNemar tests,  $p = 0.008$ ) and ate the extracts (McNemar tests,  $p = 0.22$ ).



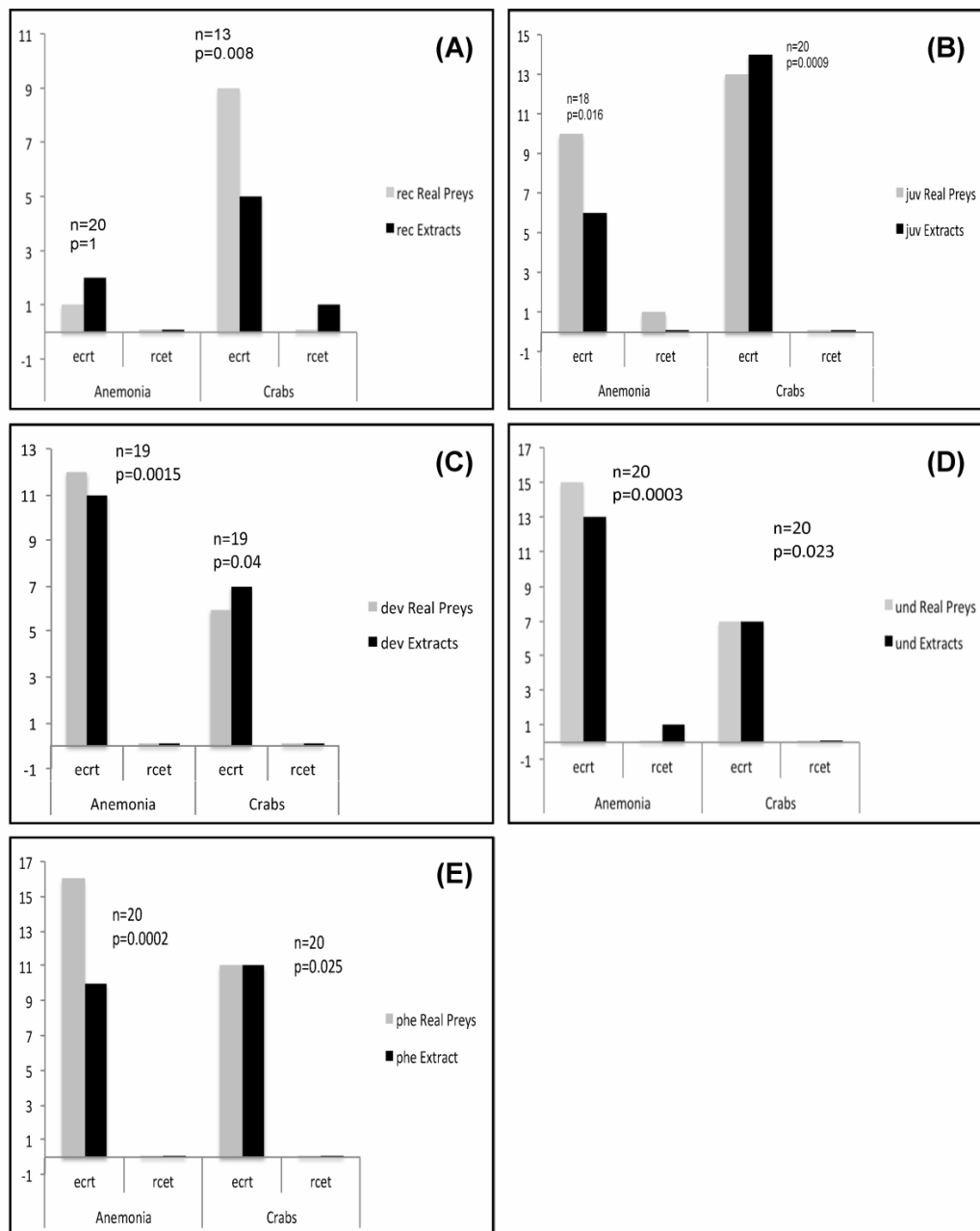
**Figure 2:** Test of the differences in the deterrence of real preys *versus* extracts ( $\text{CHCl}_2/\text{MeOH}$ ) of *Aplysia depilans* against (A) anemones and (B) crabs. Und, undeveloped eggs; dev, developing eggs; phe, pre-hatching eggs; rec, recruits; juv, juveniles; ecrt, predators that have eaten the controls and rejected the treatments; rcet, predators that have rejected the controls and eaten the treatments.

#### Predation by *Anemonia sulcata* *versus* *Carcinus aestuarii*

The three developmental stages of the egg masses and the juveniles were significantly deterrent for both of

the predators tested: the anemones as well as the crabs (McNemar tests, Figure 3).

However, the recruits were deterrent for the crabs (McNemar test,  $p = 0.008$ ) but were eaten by the anemones (McNemar test,  $p = 1$ ).



**Figure 3:** Test of the difference in the deterrence of *Aplysia depilans* eggs and juveniles (A, B, C, D and E) between anemones and crabs. Und, undeveloped eggs; dev, developing eggs; phe, pre-hatching eggs; rec, recruits; juv, juveniles; ecrt, predators that have eaten the controls and rejected the treatments; rcet, predators that have rejected the controls and eaten the treatments.

### Summary of experimental data

The different experiments are resumed in Table I.

**Table I:** Summary of experimental data showing the effect of different stages development of *Aplysia depilans* on feeding by anemones and by crabs. Und, undeveloped eggs; dev, developing eggs; phe, pre-hatching eggs; rec, recruits; juv, juveniles

		Effect on feeding by anemones	Effect on feeding by crabs
Different stages pf development of <i>Aplysia depilans</i>	UND	Rejected	Rejected
	DEV	Rejected	Rejected
	PHE	Rejected	Rejected
	Rec	Eaten	Rejected
	Juv	Rejected	Rejected
Real preys (RP) Versus extracts (E)	UND RP	Rejected	Rejected
	UND E	Rejected	Rejected
	DEV RP	Rejected	Rejected
	DEV E	Rejected	Rejected
	PHE RP	Rejected	Rejected
	PHE E	Rejected	Rejected
	Rec RP	Eaten	Rejected
	Rec E	Eaten	Eaten
	Juv RP	Rejected	Rejected
	Juv E	Rejected	Rejected

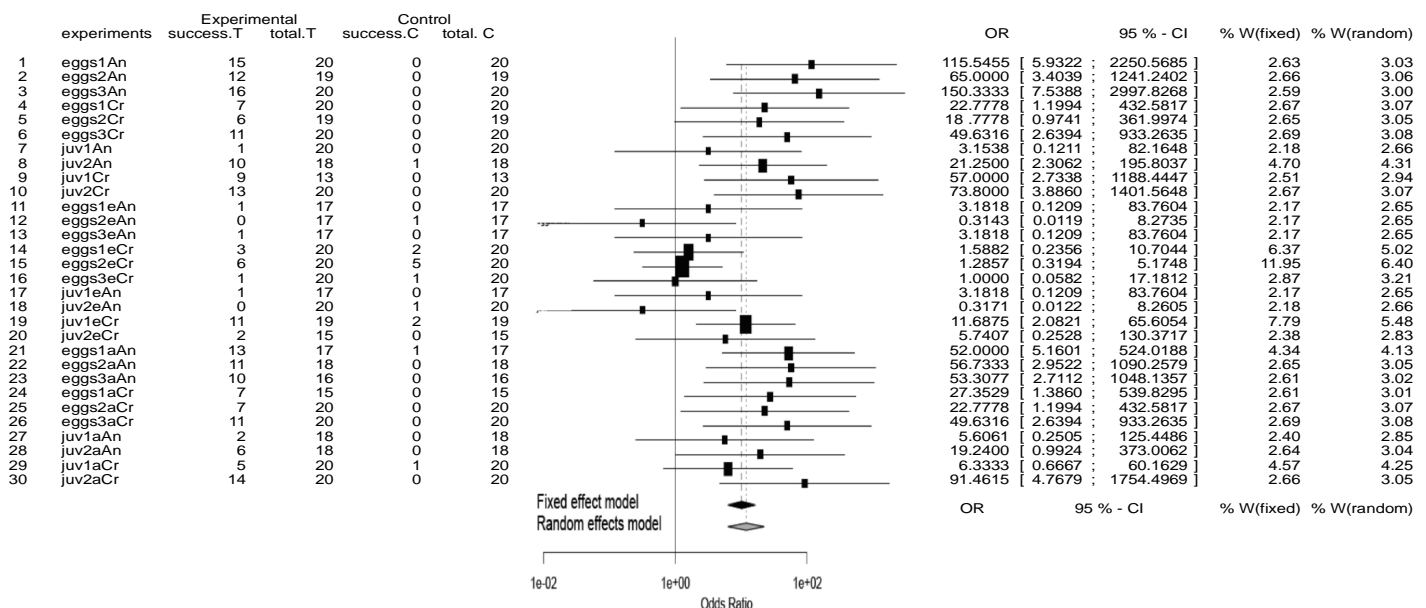
### Meta-analysis

We calculated odd ratios using meta-analysis (Table II) in order to have an over view on our results and to be able to compare between the different experiments on the one hand, and between predators and stages of developments on the other hand.

The odd ratios obtained were very high in the case of live eggs, recruits and juveniles for both predators tested except for the recruits and *Anemonia sulcata*,

indicating a strong deterrent effect of the three developmental stages, and the juveniles against the predators tested. The same observations are available when we look at the odd ratios of the artificial diets made with the extracts from the eggs and the juveniles. However, for the extracted eggs and juveniles, the odd ratios were low and the deterrence was not statistically significant.

**Table II:** Forest plot of the meta-analysis on binary data of the deterrence of early developmental stages of *Aplysia depilans* on *Anemonia sulcata* and *Carcinus aestuarii*





## DISCUSSION

The early life stages of *Aplysia depilans* are deterrent to crabs and anemones; secondary metabolites seem to be involved in the defense from the predator's attacks just like in adult *Aplysia*.

The different developmental stages of the eggs of *Aplysia depilans* are chemically defended against *Anemonia sulcata* and *Carcinus aestuarii*. The recruits were consumed by the *Anemonia* and rejected by the crabs and the juveniles were deterrent to both of the predators tested in this study. The deterrence in the juveniles was chemically mediated as shown by the extractions and the artificial diets made with the extracts.

Benthic egg masses are generally thought to play a protective role in the embryonic development of marine invertebrates; they must be adequately protected to ensure the long-term survival of the species. Few studies tested the defenses in the egg masses of sea hares.

In our study, the eggs were strongly rejected by both of the predators tested and the deterrence was chemically mediated as shown by the extraction and the artificial diets made with the extracts. Similarly, other studies confirm these results, in fact, Pennings (1994) showed that pieces of egg masses of *Aplysia juliana* were universally rejected by crabs and reef fish and that the extracts of the eggs deterred feeding by some reef fish.

Sea hares do not appear to secrete diet-derived compounds into their egg masses, yet these egg masses are unpalatable to generalist predators. For example, egg masses from the sea hare *Aplysia juliana* are chemically unpalatable to reef fishes, but diet-derived metabolites do not appear to be responsible of this unpalatability (Pennings, 1994).

The chemical origin of the defenses in the egg masses of sea hares is already shown. Having antipredatory compounds in the egg masses is considered as adaptative and prevents ovovores from ingesting the eggs (Derby, 2007), but the origin of the metabolites responsible of the defense is still unknown. In fact, the hypothesis that the secondary metabolites responsible of the deterrence of the egg masses may be obtained from the algal diet of the animal wasn't confirmed, but some studies showed that the egg masses contained the pigments of the algae, and that these pigments are transferred from the animal to the eggs within few days and can even predict if the algal diet of the animal is composed of green or red algae (Johnson and Willows, 1999; Switzer-Dunlap and Hadfield, 1977; Chapman and Fox, 1969; Carefoot *et al.*, 2000). However, the egg masses didn't contain the secondary metabolites of the algae, so, unless the pigments are responsible of the chemical defense, the deterrence is not diet related. The eggs of *Aplysia juliana* fed with a diet of green algae were as

deterrent as the eggs of the sea hares fed red or brown algae (Johnson and Willows, 1999). The sea hares *S. longicauda*, *D. auricularia* and *Aplysia californica* were fed natural diets or artificial diets containing secondary metabolites, but no secondary metabolites were found in the egg masses (Pennings and Paul, 1993). All these studies may indicate that the chemical defense in the egg masses of sea hares may be due to autogenic metabolites, and neither to the secondary metabolites nor to the pigments from the animal's diet. Further studies are needed to confirm these hypotheses.

Sea hares' egg masses, in addition to the defense against predators, were reported to have an important antibacterial activity against marine and terrestrial bacteria (Kamiya *et al.*, 1984). Benkendorff *et al.* (2001) showed that the antibacterial activity is more important in freshly laid eggs when compared with hatching ones.

All these mechanisms of defense against predators and pathogenic bacteria allow the embryos embedded in the egg masses to survive until the hatching which usually occurs 6 to 10 days after the egg masses are laid out. Free-swimming veliger larvae are released (Switzer-Dunlap and Hadfield, 1977). For these veligers, as the chemical protection of the eggs is no longer effective, it seems like they must rely only on the physical defense of their larval shell to get protection against predators. After the swimming period, the veligers begin to settle out of the water column onto preferred seaweeds that induce metamorphosis. During this settling period, they undergo their greatest amount of predation (McGinitie, 1934 in Johnson and Willows, 1999).

The chemical defenses used by the sea hares are multilayered and vary during the ontogeny. They act in at least three different ways: chemical defense, sensory disruption and phagomimicry (Derby, 2007). Chemical defense consists on inhibiting feeding by the predators by unpalatability or toxicity, sensory disruption: acting on the predators sensory system preventing normal function and leading to confusion and stopping the attack by the predator (Kicklighter *et al.*, 2005), and phagomimicry: secretion stimulating the predators sensory pathway involved in feeding causing the predator to attend to the secretions as if they were food allowing the sea hare to escape.

In our study, the recruits showed no deterrence against *Anemonia sulcata* and were eaten immediately. They had no active protection against predators and their passive defenses were not strong and efficient which made them vulnerable and easily attacked. This can also be explained by the fact that the secretions are in very small quantities to be effective and detected by the predators.

Our experiments showed that the recruits are not defended against *Anemonia* and were immediately eaten, but with crabs, the situation was different, as

they were not attacked. This may be due to strong passive defenses with effect on crabs, like the unpalatability of these juveniles to *Cracinus aestuarii* or to their very small size.

The juveniles released a cloud of a white secretion when attacked by the *Anemonia*. In fact, numerous observations suggest that cnidarian tentacles (probably the nematocysts) elicit defensive behavior in *Aplysia*, including ink and opaline secretion (Benkendorff *et al.*, 2001).

The chemical secretion can play a role on the protection of the *Aplysia* juveniles against the two predators tested in this study. Few studies dealt with the white ink (released by few sea hares as *Aplysia depilans* which releases both white and purple ink in addition to opaline, or *Aplysia juliana* which releases only white ink and opaline). The white secretion can play a role in the defense from predator's attacks as shown with crab (Pennings, 1994; Benkendorff *et al.*, 2001) if we consider that Pennings (1994) was describing the white ink in his paper and not the opaline, as suggested by Johnson and willows (1999) and this can explain the deterrence occurring with the *Anemonia* only with the juveniles that secreted this white secretion. The size of the juveniles can also explain their deterrence towards the predators tested, the passive defenses like the odor may be more effective and able to deter the predators, because bigger *Aplysia* specimens may produce more mucous, ink, opaline and odor than the small ones. In fact, Carefoot *et al.* (1999) showed that there is an isometric relationship of ink volume to body size. This suggests that small individuals even if they secrete chemical defenses, may not be as protected as the big ones unless the chemical defenses in the small individuals are more concentrated in active compounds. This is observed in some snake species that produce more toxic venom in their young stage compared to adults and in some spiders in which the potency of the venom increases in small animals.

The extracted recruits were eaten by the anemones but rejected by the crabs, these results may indicate that the deterrence in the non manipulated recruits and juveniles against the crabs is not only due to chemical defense, the size, the color or the texture may also be responsible of this unpalatability. The extracts from juveniles incorporated in an artificial diet were rejected by the crabs showing that a chemical defense exists and is responsible of the deterrence against crabs, at least for the juveniles.

At the early stages of post metamorphosis development, the defenses in sea hares can be passive, for which direct action on the nervous system is not required, consisting of the odor, the palatability, the size... The passive defenses are considered as the first lines of defense. The defenses can also be active, and must be activated, usually by

predator's attack, like releasing ink or opaline secretions.

Sea hares use different levels of defense to protect themselves from different predators and different stages of a predatory encounter. These defenses have different degrees of cost and effectiveness as well: the cost and benefit of passive chemical defenses, such as those found in the skin and mucous, differ from those of active chemical defenses, such as ink released only after a sustained predatory encounter (Nolen and Johnson, 2001). In fact, depending on the predator, sea hares may use different defensive mechanisms, for example, against the sea anemone *Anthopleura sola*, sea hares used only ink (and not opaline), this secretion caused retract of the *Anemonia* tentacles (Derby, 2007), in fact against sea anemones, ink is an unpalatable deterrent that causes tentacular withdrawal (Nolen *et al.*, 1995; Kicklighter and Derby, 2006). The ink is considered to be the best defense of sea hares against anemones, it is way more efficient than the passive defenses like the distasteful secondary plant toxins incorporated into the animal's skin (Nolen and Johnson, 2001). Sea hares with no ink supplies were easily captured by the anemones (Nolen and Johnson, 2001). This may explain the vulnerability of the recruits, which didn't secrete ink or opaline when attacked by the anemone, and were consequently immediately eaten, unlike the juveniles, which were able to escape, probably thanks to the white secretion they released. In fact, a sea hare is struck significantly less frequently if it released ink thus demonstrating a reduction in predatory attacks as a result of inking these results are supportive of similar studies of interactions between sea hares and predatory sea anemones (Nolen *et al.*, 1995) or spiny lobsters (Kicklighter *et al.*, 2005).

Ink seems to be used also as a deterrent for predatory crabs. In fact, *Aplysia* would concentrate some metabolites in its ink and uses them as deterrents against blue crabs (Kamio *et al.*, 2010). Kamio *et al.* (2010) showed that ink is highly effective and that opaline is moderately effective in suppressing feeding of crabs. In fact, a single deterrent compound can be not only deterrent against multiple consumer species (Cronin *et al.*, 1997; Hay *et al.*, 1998; Schnitzler *et al.*, 1998) but also have multiple roles including antipredatory, allelopathic, antifouling and antibacterial effects (Kubaneck *et al.*, 2002).

Rogers *et al.* (2002) showed that juvenile *A. parvula* experience high predation by fishes when compared to the adults (85% juvenile *A. parvula* eaten over 2 h compared to adults: 20% eaten). This suggests that sea hares may reach a size-escape from fish predation, the size being related to the efficiency of the chemical defenses.

The two examples of predators tested in this study showed that the defenses in the sea hares vary not only with the ontogeny, with the stage of

development (eggs, juveniles, adults), but also with the predator it's facing, some defense strategies being more efficient against the one or the other predator. The multiple lines of chemical defense can affect different predators, and some compounds may work on olfactory pathways and others through gustatory pathways. The different chemicals may affect the behavior of the predator through different sensory pathways and in different ways. Using a set of compounds with a variety of effects against a diversity of enemies will be the most effective defense in a marine ecological community with many species (Kamio *et al.*, 2010).

At the early stages of development, the active defenses are not very efficient, and the animal relies more on the passive defenses like the unpalatability, the odor and the size. These passive defenses can deter some predators like the crabs, but have no significant effects on other predators as it was shown with *Anemonia sulcata*. For this reason, sea hares' juveniles face huge predation; sea hares with only a passive chemical defense (distastefulness) were eaten 3.5 times more often than those with only an active chemical defense, i.e. ink (Nolen and Johnson, 2001).

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