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Can a sponge feeder be a herbivore? *Tylodina perversa* (Gastropoda) feeding on *Aplysina aerophoba* (Demospongiae)

MIKEL A. BECERRO[1]*, XAVIER TURON[2], MARIA J. URIZ[1] and JOSE TEMPLADO[3]

[1]*Center for Advanced Studies (CEAB, CSIC), E-17300 Blanes, GI, Spain*
[2]*Department of Animal Biology (Invertebrates), University of Barcelona, 645 Diagonal Avenue, E-08028 Barcelona, Spain*
[3]*National Museum of Natural History (CSIC), Jose Gutierrez Abascal 2, E-28006 Madrid, Spain*

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Feeding biology in mollusks has important biological, ecological and evolutionary implications because many of the characteristics we observe in mollusks arise from their co-evolution with diet organisms. We investigated the relationship between the opisthobranch *Tylodina perversa* and the sponges *Aplysina aerophoba* and *Aplysina cavernicola* in order to ascertain the trophic interactions between them. The opisthobranch preferred specimens of *Aplysina aerophoba* inhabiting shallow overdeep waters, ectosome of *Aplysina aerophoba* over choanosome, and showed no preference for *Aplysina cavernicola*. The sponge *Aplysina cavernicola* lacks the cyanobacteria abundant in the ectosome of *Aplysina aerophoba*. Our study shows that the opisthobranch *Tylodina perversa* actively selects for sponges or sponge zones with high concentration of cyanobacteria, i.e. only a fraction of the ingested material is of animal origin. Addition of cyanobacteria to symbiont-free sponge material induced a shift in mollusk preference. Our results cast doubt over the widely recognized qualification of *Tylodina perversa* as a carnivorous sponge feeder and show evidence that cyanobacteria determine the opisthobranch food selection. Whether this is an isolated example of how symbionts may determine trophic interactions between hosts and predators or it is widespread in benthic organisms remains an open question that requires further investigation. *Biological Journal of the Linnean Society*, 2003, **78**, 429–438.

ADDITIONAL KEYWORDS: Aplysinid sponges – predator–prey interactions – sponge/microsymbiont/opisthobranch co-evolution – trophic relationships.

INTRODUCTION

Mollusks are extremely diverse organisms that display numerous ecological strategies. These strategies are based upon key characteristics such as their shell, chemical defenses, feeding biology, or reproduction and life cycles, among others. Feeding biology is a research area with important biological, ecological and evolutionary implications because many characteristics that we observe in mollusks arise from their co-evolution with diet organisms. For example, opisthobranch mollusks are mainly specialized in feeding on organisms that are somehow protected from predation and are low preference prey for other generalist predators (Faulkner, 1992; Avila, 1995; Cimino & Ghiselin, 1999; Cimino *et al.*, 2001). Many prey organisms are chemically defended and the capacity of the mollusk to sequester secondary metabolites from their prey is a well-known trait often associated with shell reduction or loss, and has contributed to drive the evolution of this group (Faulkner & Ghiselin, 1983; Cimino & Ghiselin, 1998). Feeding biology has certainly been a major factor in gastropod success (Kohn, 1983) and is an important topic when considering the overall evolution of gastropods, particularly opisthobranchs (Willan, 1984).

Opisthobranchs are excellent organisms for the study of evolutionary phenomena (Cimino & Ghiselin, 1998, 1999). With over 4000 known species belonging

*Corresponding author. E-mail: mikel@ceab.csic.es

to several orders, some key characteristics in Opisthobranchs, such as shell reduction or loss, have occurred independently among lineages (Gosliner & Ghiselin, 1984). Feeding habits of the genus *Tylodina* may be particularly valuable from an evolutionary perspective because *Tylodina* is among the most primitive genera of the entire Opisthobranchia (Willan, 1987). Members of this genus exclusively feed on sponges of the family Aplysinellidae, order Verongida (Willan, 1984, 1998).

North-western Mediterranean rocky bottoms may locally hold large populations of the sponges *Aplysina aerophoba* Schmidt 1862 and *Aplysina cavernicola* (Vacelet 1958) (e.g. Uriz *et al.*, 1992) and the tylodinid *Tylodina perversa* (Gmelin, 1790) (Cervera *et al.*, 1988; Doneddu & Manunza, 1990). The two sponge species share similar skeleton and morphology (Vacelet, 1959), secondary chemistry (Ciminiello *et al.*, 1997) and may overlap in their depth distribution. These species are so similar that there is controversy as to whether they are two distinct species or just two ecotypes of the same species (Voultsiadou-Koukoura, 1987). Despite these similarities, *A. aerophoba* and *A. cavernicola* have contrasting ecological requirements (Wilkinson & Vacelet, 1979) and also differ in some secondary metabolites that are species specific (Ciminiello *et al.*, 1997). Although the two sponge species may overlap in their depth distribution, *A. aerophoba* has more photophilous preferences than *A. cavernicola,* which is usually found at greater depths or in caves and under overhangs in shallow waters. Also, *A. cavernicola* lacks the photosynthetic cyanobacteria symbionts present in *A. aerophoba* (Vacelet, 1970, 1975). *Tylodina perversa* lives in close association with the sponge *Aplysina aerophoba* (Ros, 1976; Doneddu & Manunza, 1995) on which it feeds without producing the death of the sponge. *Tylodina perversa* is highly cryptic on *Aplysina aerophoba* from which it incorporates many chemicals (Teeyapant *et al.*, 1993) including the pigment uranidine responsible for the rapid blackening of the sponge and nudibranch when they are exposed to air (Cimino *et al.*, 1984; Cimino & Sodano, 1994). The opisthobranch also has aerothionin – a compound exclusive to *A. cavernicola* – and it is unclear whether *T. perversa* produces aerothionin within its tissues by biotransformation or is also able to feed on *A. cavernicola* and incorporate this compound from the diet (Ebel, Marin & Proksch, 1999).

Our study aims to investigate patterns of consumption of *Tylodina perversa* in relation to (1) the two *Aplysina* species present in the Mediterranean, (2) the ecological distribution of the prey species, (3) the diverse parts of the sponge, and (4) the presence of cyanobacterial symbionts and cell types producing the secondary metabolites of the sponge. Our data provide insights on the feeding biology and ecology of *Tylodina perversa* and open up new ecological and evolutionary perspectives not only for sponge feeders, but also for other predator groups feeding on prey with important symbiotic populations.

MATERIAL AND METHODS

Study site and quantification techniques

Els Caials is a small bay in the natural park of Cape Creus, next to the Spanish–French Mediterranean border. The bay includes a shallow flat area up to a depth of 6 m and a deep coralligenous reef area at a depth of 30 m. The shallow area contains numerous sand zones, boulders, and rocks dominated by seaweeds and the seagrass *Posidonia oceanica*, sea urchins and sponges including abundant *Aplysina aerophoba*. The deep coralligenous reef area contains a number of big platforms with small overhangs and crevices dominated by the alga *Lythophyllum incrustans* and many ascidians, bryozoans and sponges, including *Aplysina cavernicola*. A natural channel with vertical walls and boulders at its bottom connects these two areas. We observed *A. aerophoba* in the channel down to a depth of 15 m while *A. cavernicola* was not observed in the same locality until a depth of 30 m.

We used nine 5 m-long random transects at depths between 0–5, 5–10 and 10–15 m (three transects at each depth) to quantify the number of *Aplysina aerophoba*. Preliminary visual inspections showed that *Aplysina cavernicola* is restricted to the 30-m coralligenous reef area so we did not quantify its abundance along a depth gradient. To measure the opisthobranch distribution in the field, we haphazardly selected 20 specimens of *A. aerophoba* at depths between 0–5, 5–10 and 10–15 m, and *A. cavernicola* at depths of 28–32 m. We recorded the size of these sponges by measuring their maximum diameter and counted the number of *T. perversa* per sponge. We used analysis of variance (ANOVA) to test for differences in the abundance of *A. aerophoba* along depth, and analysis of covariance (ANCOVA) to test for differences in the number of *T. perversa* on *Aplysina* spp., the covariate being sponge size.

Feeding choice experiments

We ran a series of choice experiments to determine the preferences of *Tylodina perversa* and to test possible causes of such preferences. We ran all the choice experiments in the laboratory with a continuous flow system delivering seawater directly from the sea. All sponge and opisthobranch specimens were collected in Els Caials, placed in a cooler with seawater and an air-

stone, and transferred to the aquarium with running seawater within 4 h after collection. We ran all the experiments after an adaptation period of 20 h (to prevent changes in the natural opisthobranch behaviour due to collecting and transferring stress) and before the following 48 h (to prevent any sponge alteration that may affect the opisthobranch selection).

We used one opisthobranch per tank and ran paired assays. We offered every opisthobranch two pieces of sponge placed at opposite corners of the tank. Treatments were randomly placed at each trial in the corners so that light or flow conditions are factored out of the experiment. Sponge pieces and opisthobranchs were used only once to avoid significant results due to individual preferences. We offered opisthobranchs a choice between (1) *A. aerophoba* and *A. cavernicola*, (2) *A. aerophoba* from shallow (0–5 m) and deep (10–15 m) habitats, and (3) ectosome and choanosome of *A. aerophoba*. All sponge pieces were cut off from the apical part of the oscular 'chimneys'. The ectosome was peeled off the choanosome so that both ectosome and choanosome came from the same chimney of the sponge. We scored a selection when the opisthobranchs selected and started feeding on one of the sponge pieces, which occurred usually within 5 min of the start of the experiment. When the opisthobranchs simply passed over a sponge piece we extended the observation period by 5 min to see if a selection was finally made; if no selection was made the trial was disregarded.

To ascertain the role of photosynthetic symbionts in the opisthobranch choice, we also selected 10 specimens of *A. aerophoba* at a depth of 3 m and set up a transplant experiment. We transplanted parts of every specimen to depths of 3 m (transplant control) and 30 m (depth treatment). The remaining part of every sponge was left untouched at its original place (absolute control). We took a small piece of the sponge from every treatment at the beginning and at the end of the experiment ($t = 0$ and $t = 6$ weeks, respectively) and quantified levels of chlorophyll *a* as an estimation of the amount of photosynthetic cyanobacteria in the sponge. Chlorophyll quantification is the most useful chemical method for estimating the total quantity of photosynthetic organisms (Parsons, Maita & Lalli, 1984). The cyanobacterium *Aphanocapsa feldmani* is the only known source of chlorophyll *a* in the sponge (Vacelet, 1970, 1975; authors' histological observations) and therefore levels of chlorophyll *a* are directly related to the amount of cyanobacteria in the sponge and their physiological condition. We extracted overnight 100 mg of sponge dry mass with 5 mL of acetone (90%). Quantification of chlorophyll *a* in the solutions was done by a UV-visible recording spectrophotometer Shimadzu 2100 measuring extinctions at wavelengths of 630, 645, 665 and 750 nm and following the spectrophotometric equations by Jeffrey & Humphrey (1975). We used repeated analysis of variance (ANOVAR) to test for differences in chlorophyll concentration in the treatments along time.

After 6 weeks, the sponge pieces were taken to the laboratory to run choice experiments with *T. perversa* as previously explained. We offered opisthobranchs a choice between parts of the same sponge from (1) the absolute control and transplant control, and (2) from the transplant control and depth treatment. With this approach, we factored interspecimen variation in *A. aerophoba* out of the experiment and prevented this factor from affecting the selection of the opisthobranch.

As we failed to isolate enough cyanobacteria from *Aplysina aerophoba*, we isolated cell fractions with and without cyanobacteria from the sponge *Petrosia ficiformis*. These fractions were coated onto pieces of choanosome from *Aplysina aerophoba* and offered to *Tylodina perversa* in a choice experiment to specifically test the role of cyanobacteria in the selection process of the opisthobranch. To obtain the cyanobacteria-rich and cyanobacteria-poor fractions, we cut off small pieces of the ectosome of *P. ficiformis*, ground them in a mortar, and filtered them through a 25-μm mesh to avoid cell aggregations and debris. The filtered cell suspension was placed in a centrifuge at 500 r.p.m. for 2 min. Cell components precipitated on the bottom while the cyanobacteria remained in suspension. The cyanobacterial suspension was pipetted out, transferred to another cubette, and centrifuged at 3000 r.p.m. for 10 min. We repeated this procedure several times to obtain enough amounts of cell components and cyanobacteria to coat these two fractions onto small pieces of choanosome from *A. aerophoba*. We stored these fractions frozen at –40 °C until we defrosted and resuspended them prior to running the experiments. Both fractions followed the same procedures and were coated onto the pieces of choanosome just before we started each replicate in any of the feeding choice experiments. We offered *T. perversa* a choice between a piece of choanosome of *A. aerophoba* coated with cyanobacteria and a piece coated with cell components. We used a small syringe to carefully deposit the fractions onto the piece of choanosome. In this way a layer formed over their surfaces which remained stable long enough to perform the assay. The pieces of choanosome were used only once and were coated anew at each replicate.

Feeding deterrence experiments

We collected five specimens of *Aplysina aerophoba*, *Tylodina perversa*, and five egg masses of *T. perversa* (the opisthobranch lays yellow, ribbon-like egg masses on *Aplysina aerophoba*) to determine whether or not

they are chemically protected from fish predators. All the replicates were individually manipulated. Samples were frozen at –40°C, freeze-dried, weighed and exhaustively extracted with a 1 : 1 mixture of dichloromethane (DCM) and methanol (MeOH). The extract was filtered, evaporated down, and the remnant weighed to calculate the percentage yield of crude extract in the sponges, opisthobranchs and egg masses. Then, the five replicates within each sample type were pooled together to obtain the crude extract used in the feeding deterrence experiment.

We incorporated the extracts from *A. aerophoba*, *T. perversa* and egg masses of *T. perversa* into small, artificial fish food pellets (Sera Granumarin). Extracts were dissolved in 1 mL of the DCM : MeOH mixture and added to 0.3 g of pellets (>150 pellets). The amount of extract added varied to match natural concentrations (dry mass basis) in the sponge, opisthobranch and egg mass. One millilitre of the mixture solvent was added to controls. After evaporation of the solvent we offered control and treatment pellets to the damselfish *Chromis chromis*, one of the most abundant fish in the area. Feeding deterrent experiments were run at Sta. Anna point, Blanes, at a depth of 5 m. We introduced each treatment in a plastic syringe so that only one pellet is given at a time to the fish. A diver released sufficient amount of untreated pellets to gather a large number of fish around the diver. We started the experiment when fish surrounding the divers repeatedly poked at the syringe containing the untreated pellets. We then offered *C. chromis* the following sequence of pellets: control, *A. aerophoba*, control, *T. perversa*, control and egg mass of *T. perversa*. Syringes aimed at different directions each time to prevent the same or a few fish dominating the consumption of pellets. This sequence was repeated until no pellets remained. We set up two categories to evaluate the fish behaviour as a response to the pellets. We scored a pellet as 'eaten' when the pellet was eaten by the fish at first trial or in less than five attempts and 'rejected' when pellets were ignored or continuously rejected for five or more attempts. Data were analysed using log–linear models (probability values are Bonferroni adjusted).

RESULTS

Patterns of distribution

At our study site, *Aplysina aerophoba* and *Aplysina cavernicola* have contrasting depth distributions. The abundance of *A. aerophoba* decreased with depth (Fig. 1, ANOVA, $P = 0.021$) while we exclusively found *A. cavernicola* at a depth of 30 m (coralligenous community). The number of *Tylodina perversa* on specimens of *A. aerophoba* significantly decreased with depth (Fig. 2, ANCOVA, $P = 0.045$), while the percentage of sponges with *T. perversa* also showed a strong pattern of decrease with depth, which was close to being statistically significant (Fig. 2, log–linear model, $P = 0.051$). We found no *T. perversa* on *A. cavernicola*.

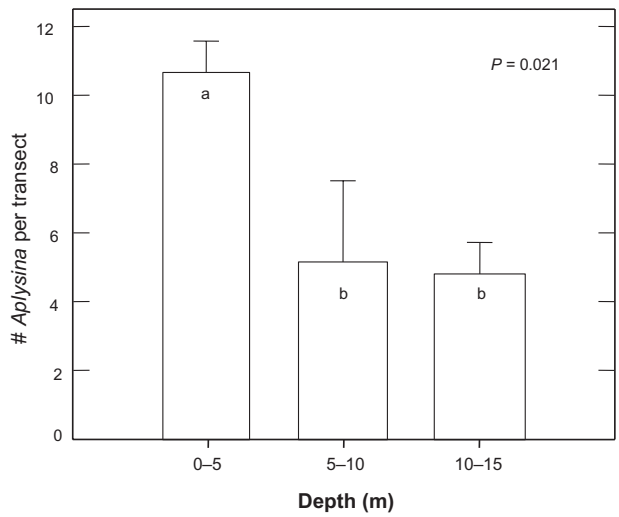

Figure 1. Density of the sponge *Aplysina aerophoba* with depth in the locality of Els Caials. Bars represent average number of specimens (mean ± 1 SE) found in three 5-m long transects at each of the depth ranges sampled. Mean differences between depths tested with one-way ANOVA. Bars sharing letters do not differ statistically at $P = 0.050$ (Tukey HSD). See 'Material and methods: study site and quantification techniques' for more information.

Feeding choice experiments

In laboratory feeding choice experiments, *Tylodina perversa* significantly preferred *Aplysina aerophoba* over *Aplysina cavernicola* ($P = 0.004$, Table 1). When given a choice between shallow (0–5 m) and deep (10–15 m) specimens of *A. aerophoba*, *T. perversa* significantly preferred specimens from 0 to 5 m over those from 10 to 15 m ($P = 0.002$, Table 1). When given a choice between the ectosome and choanosome of *A. aerophoba*, the opisthobranch significantly preferred the ectosome over the choanosome ($P = 0.011$, Table 1).

In the field, chlorophyll *a* levels decreased significantly after a 6-week period in the 30-m transplants, while they remained at before-experiment levels in the 3-m transplants and in the sponge parts that were not transplanted (one-way ANOVAR, 'time × treatment' interaction term: $F = 3.742$, $P = 0.038$; we show the *P*-values for the time effect within each treatment in Fig. 3). When given a choice between these treatments, *T. perversa* significantly preferred sponge parts transplanted at 3 m (transplant control) over parts

Table 1. Laboratory feeding choice experiments conducted with *Tylodina perversa*. 'Selection' shows the actual selection of the opisthobranchs out of the total number of replicates (N) per experiment. P values calculated with a binomial distribution with $p = q = 0.5$ for all the experiments

Feeding choice	N	Selection	P
Aplysina aerophoba vs. *Aplysina cavernicola*	8	8 : 0	0.004
Aplysina aerophoba			
Shallow (0–5 m) vs. deep (10–15 m)	16	14 : 2	0.002
Ectosome vs. choanosome	10	9 : 1	0.011
Control (3 m) vs. transplant control (3 m)	29	16 : 13	0.428
Transplant control (3 m) vs. depth treatment (30 m)	28	24 : 4	0.001
Choanosome with cyanobacteria vs. choanosome with cells	16	13 : 3	0.011

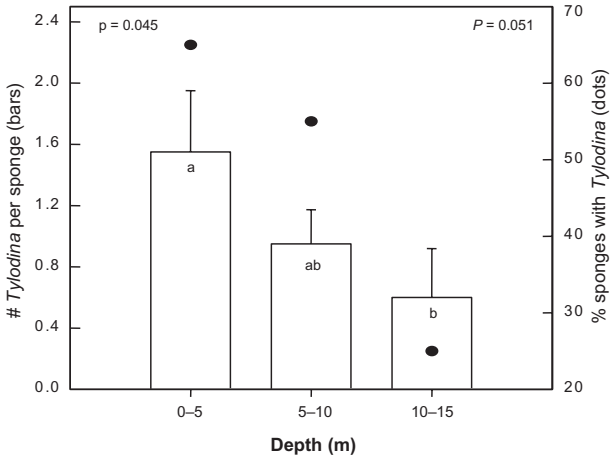

Figure 2. Density of the opisthobranch *Tylodina perversa* in the locality of Els Caials as indicated by the average number of specimens (mean ± 1 SE) per sponge (bars, left axis) and percentage of sponges with opisthobranch (dots, right axis). Mean differences in the number of opisthobranchs per sponge between depths tested by one-way ANCOVA with sponge size as covariate. Bars sharing letters do not differ statistically at $P = 0.050$ (Tukey HSD). Differences in the number of sponges with and without opisthobranchs between depths tested with a loglinear model. See 'Material and methods: study site and quantification techniques' for more information.

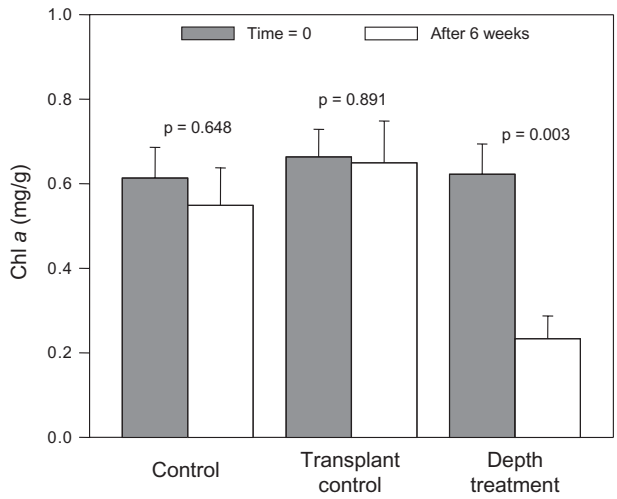

Figure 3. Concentration of chlorophyll *a* (mg mL^{-1}) in *Aplysina aerophoba*. Bars represent average chlorophyll concentration (mean ± 1 SE) in sponges transplanted at 30 m (depth transplant), 3 m (transplant control), or not transplanted (control) at the start of the experiment (filled bars) and after 6 weeks (empty bars). Mean differences in chlorophyll concentration between time and treatments tested with one-way ANOVAR. Probabilities given are for the time effect within each of the three treatments (paired *t*-test).

transplanted at 30 m (depth treatment) ($P < 0.001$, Table 1). The opisthobranch showed no preference between sponge parts transplanted to 3 m and the sponge parts that were not transplanted (absolute control) ($P = 0.428$, Table 1).

When given a choice between choanosome of *A. aerophoba* coated with cell fractions from the sponge *Petrosia ficiformis* with and without cyanobacteria, *T. perversa* significantly preferred choanosome coated with sponge material with cyanobacteria over choanosome coated with sponge material without cyanobacteria ($P = 0.011$, Table 1).

Feeding deterrence experiments

In field experiments and at naturally occurring concentrations, crude extracts from *A. aerophoba*, *T. perversa*, and egg masses of *T. perversa* significantly deterred feeding by the damselfish *Chromis chromis* (Fig. 4, log–linear model, $P < 0.001$). However, extracts from *T. perversa* and its egg masses were more deterrent than extracts from *A. aerophoba* ($P = 0.009$ and $P = 0.022$, respectively). There were significant differences in the percentage yield of crude extract between these species (Fig. 5, one-way ANOVA, $F = 14.745$, $P = 0.001$), with a lower percent-

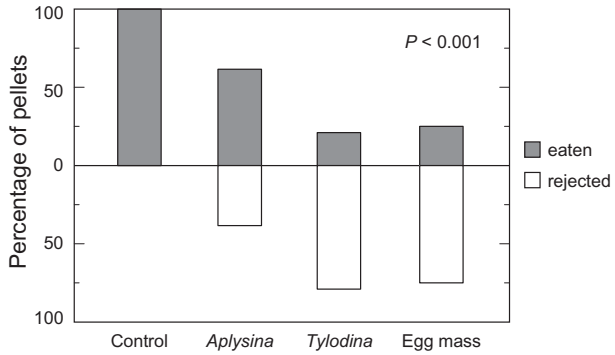

Figure 4. Feeding deterrence of the crude extract of *Aplysina aerophoba*, *Tylodina perversa*, egg masses of *Tylodina perversa*, and control food towards natural fish consumers in the field. Bars represent the percentage number of control pellets eaten (filled bars) and rejected (empty bars) in the field by the generalist fish predator *Chromis chromis*. Differences between the number of pellets eaten and rejected between treatments tested with a loglinear model.

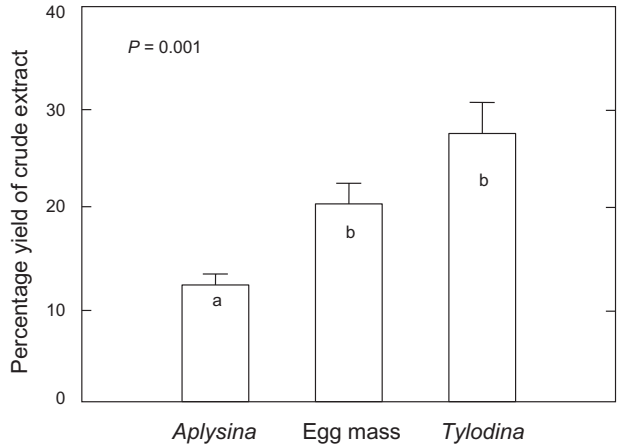

Figure 5. Percentage yield of crude extract per dry mass (mean ± 1 SE) in tissues from *Aplysina aerophoba*, *Tylodina perversa* (without shell) and egg masses of *Tylodyna perversa*. Mean differences in percentage yield of crude extract tested with one-way ANOVA. Bars sharing letters do not differ statistically at $P = 0.050$ (Tukey HSD).

age yield in *A. aerophoba* compared to *T. perversa* (Tukey HSD, $P < 0.001$) and its egg masses (Tukey HSD, $P = 0.033$), while we found no significant differences between the opisthobranch and its egg masses (Tukey HSD, $P = 0.065$).

DISCUSSION

In the Mediterranean, *Tylodina perversa* feeds on the sponge *Aplysina aerophoba*, although chemical studies show that *Tylodina perversa* shares secondary chemistry with *Aplysina aerophoba* and *Aplysina cavernicola* (Ebel *et al*., 1999). Our study shows that *T. perversa* has a strong preference for *A. aerophoba* over *A. cavernicola*. Moreover, this study provides evidence that suggests cyanobacteria – and not true sponge components – are responsible for the opisthobranch feeding choices, which may explain the ecological distribution of *T. perversa*.

The ecological distribution of *Tylodina perversa* matches that of *Aplysina aerophoba*, both decreasing their abundance with depth. Despite the chemical, structural and morphological similarities between *A. aerophoba* and *A. cavernicola*, the opisthobranch *T. perversa* is exclusively found in the zone where *A. aerophoba* is abundant although it can be occasionally found on substrates other than *A. aerophoba*. *Tylodina*'s pattern of distribution correlates well with chlorophyll concentration in *Aplysina* species at a species, population, and within-sponge level. We found no *Tylodina* on *A. cavernicola*, which lacks cyanobacteria (i.e. chlorophyll). We also found significantly higher number of sponges with *Tylodina* in specimens of *A. aerophoba* inhabiting shallow waters as compared to specimens from deeper waters (i.e. specimens with higher and lower concentration of cyanobacteria, respectively) and a strong trend to decrease the density of *Tylodina* per sponge with depth. We experimentally tested these observations and found evidence to support the correlation between chlorophyll levels and feeding choice of *T. perversa*. No *Tylodina* selected *A. cavernicola* over *A. aerophoba*, and when given a choice between specimens of *A. aerophoba* from shallow and deep waters, *Tylodina* significantly selected for shallow water specimens.

This positive relationship between chlorophyll levels and feeding choice of *Tylodina* also occurs at a within-specimen level. When given choices of sponge parts from the same specimen, *Tylodina* significantly selects those parts untouched or transplanted at the same depth (high concentration of cyanobacteria) over parts transplanted at a depth of 30 m (i.e., significant reduction of chlorophyll concentration at the end of the experiment). Also, *Tylodina* actively selects the ectosome over the choanosome of the same sponge specimen (high vs. low concentration of chlorophyll levels, respectively). This matches observation of feeding habits in the field, where *Tylodina* feeds on the ectosome of the sponge, leaving behind shallow scars noticeable for the distinct coloration between the green/bluish, cyanobacteria-containing ectosome and the yellow, cyanobacteria-free choanosome. It is worth noting that scars are limited to no more than the first 2 mm of the sponge thickness, the cyanobacteria-rich ectosome.

The biology of specialist predators has received con-

siderable attention, especially because many of the prey species have defense mechanisms (e.g., chemicals and nematocysts) that the predator can sequester and use for its own defense (Thompson, 1976; Pawlik *et al*., 1988; Becerro *et al*., 2001). Noteworthy examples include many dorid opisthobranchs and sponges (Faulkner & Ghiselin, 1983; Karuso, 1987; Avila, 1995; Cimino & Ghiselin, 1999). Chemical concentrations can vary between predator and prey, usually with the former having a higher concentration of compounds than the latter (Pawlik *et al*., 1988; Becerro *et al*., 2001). *Tylodina perversa* shares a number of metabolites with *Aplysina aerophoba* and *Aplysina cavernicola* (Teeyapant *et al*., 1993), including aerophobin-2 and aerothionin, which the opisthobranch concentrates in the mantle, mucus and egg masses (Ebel *et al*., 1999). The concentration and distribution of secondary metabolites in soft tissues, mucus and egg masses is considered indirect evidence for a defensive role of these compounds, a hypothesis that remains to be experimentally tested. Our data show significantly higher percentage yield of crude extract in *T. perversa* and its egg masses than in *A. aerophoba*. Data from our feeding experiment show that, at natural occurring concentrations, crude extracts from *T. perversa* and its egg masses are significantly more deterrent against generalist fish than the crude extract from *A. aerophoba*. It is highly likely that aerophobin-2 and other closely related brominated alkaloids are responsible for the deterrent activity of the sponge and the mollusk and its egg masses. However, because we isolated no compound, the possibility that other metabolites may account for the inhibitory activity of the extracts cannot be ruled out. Also, it is worth noting than aerothionin is not present in *A. aerophoba* but only in *A. cavernicola*, so the presence of this compound in the opisthobranch must be due to either a previous encounter with *A. cavernicola* or to biotransformation of sequestered compounds by *Tylodina* (Ebel *et al*., 1999). Both observational and experimental data in our study suggest biotransformation as the most likely explanation for the presence of aerothionin in *Tylodina*.

Tylodina perversa feeds on *A. aerophoba*, but given the high concentration of cyanobacteria in the ectosome of the sponge, it seems reasonable to question whether *Tylodina* feeds on true sponge or symbiotic components, or whether it needs both sponge cells and cyanobacteria for its diet. Yamamuro (1999) pointed out that most herbivorous heterotrophs grazing on seagrass beds significantly depend on epiphytic cyanobacteria rather than seagrass leaves. The hypothesis that symbiotic cyanobacteria are important food sources for the opisthobranch could explain why there are more *Tylodina* on shallow sponges and, also, why there are higher densities of *Tylodina* per sponge in shallow waters. According to this hypothesis, higher cyanobacterial densities would imply higher food sources per sponge surface area and therefore more resources to sustain a larger *Tylodina* population. Even so, *Tylodina* sequesters defensive brominated alkaloids from *Aplysina* (Teeyapant *et al*., 1993) and concentrates and distributes these compounds in the mantle, mucus and egg masses (Ebel *et al*., 1999). Because brominated metabolites are not found in the cyanobacteria but in the spherulous cells of *A. aerophoba* (Turon, Becerro & Uriz, 2000), which are mainly distributed in the periphery of the sponge (Uriz *et al*., 1996), it seems clear that *T. perversa* ingests true sponge components. At this point, the relative contribution of sponge and symbiotic components in the diet of *Tylodina* is unknown but it seems reasonable to think that, given the amount of cyanobacteria ingested by *Tylodina*, they may play a significant role in the opisthobranch diet. An alternative hypothesis could be that *Tylodina* uses cyanobacterial products to identify the sponge, but obtains no extra nutritional benefit from ingesting cyanobacterial material. However this hypothesis fails to explain why, once the food source is found, *Tylodina* exclusively feeds on the cyanobacteria-rich ectosome and not on the cyanobacteria-free choanosome (proportionally more nutritious under this assumption). Differences in toughness (or in other structural or chemical traits) between the sponge ectosome and choanosome or radular limitations in the opisthobranch might also explain this behaviour, so this alternative hypothesis cannot be completely ruled out.

Beyond the biological and ecological consequences of our data, our study poses questions from an evolutionary perspective. Diet is a determinant factor to consider in the evolution of opisthobranchs (Thompson, 1976; Salvini-Plawen, 1988; Mikkelsen, 1996) and there is a recurrent discussion about the primitive feeding mode – herbivore or carnivore – of the ancestral opisthobranch gastropod and the evolutionary changes in trophic strategy in opisthobranch evolution. Some authors consider herbivorous diet as plesiomorphic in opisthobranchs, with carnivory evolving independently among lineages (Mikkelsen, 1996), while other authors consider carnivorism the plesiomorphic mode of feeding from which herbivory arose in various gastropod clades (Haszprunar, 1985; Rudman & Willan, 1998). Although still speculative at this point, the affinity of *T. perversa* for cyanobacteria allows its carnivorism to be questioned. From an evolutionary perspective, the feeding habits of *T. perversa* could be interpreted either as a transition step from herbivorism to carnivorism (or the other way around) or it may be an evolutionary endpoint in itself.

Incorporating recent advances in phylogenetic research, Cimino & Ghiselin (1999) outlined an evolu-

tionary scenario that begins with an opisthobranch–pulmonate common ancestor that was herbivorous and had some diet-derived chemical defense. According to these authors, the ancestors of Nudibranchia and Notaspidea switched to feeding upon sponges and gained protection from metabolites contained in them (Cimino & Ghiselin, 1999). Our data might be consistent with this hypothesis, with *Tylodina* as a transitional step close to the ancestor of opistobranchs. A possible evolutionary scenario may consist on a microherbivorous cephalaspidean-like ancestral opisthobranch from which the extant herbivorous Cephalaspidea, plus Sacoglossa, Anaspidea, Thecosomata and *Tylodina*, evolved. Lineages switching to carnivorism could have evolved independently from the basal herbivorous lineage.

Alternatively, it could also be that *Tylodina* is a recent unique convert to herbivorism rather a link to a vegetarian ancestor and predation might be the ancestral condition from which herbivory evolved. Among the primitive Vetigastropoda clade, that originated during the Late Cambrian, true herbivorous members arose after the Palaeozoic era (Vermeij & Lindberg, 2000). They also include some groups of microphagous, sponge-eating or tunicate-eating snails originating from bellorophontoid ancestors during the late Palaeozoic or Triassic. Thus, feeding on sessile invertebrates may be the plesiomorphic mode of feeding from which herbivory arose in various gastropod clades (Vermeij & Lindberg, 2000). The evolution from carnivorism to herbivorism has been demonstrated at least in some clades of herbivorous collumbelids within the otherwise carnivorous buccinoid gastropods (deMaintenon, 1999). We can find an example of opisthobranchs foraging on specific symbionts in nudibranchs of the genus *Phyllodesmium* Ehrenberg, 1831. Primitive species just feed on soft corals (with or without zooxanthellae), while highly evolved species depend on certain soft corals with zooxanthellae, which have led to the evolution of nudibranch–zooxanthellae symbiosis (Rudman, 1991). Our data show that *Tylodina* is able to feed on sponges with low concentration of cyanobacteria, but prefers those sponges with high cyanobacteria content. If cyanobacteria do provide a nutritious source for the opisthobranch, *Tylodina* might have switched during evolution from a cyanobacteria-free prey to a more nutritious cyanobacteria-rich prey, without losing benefits from feeding on the sponge (e.g. sequestration of secondary metabolites, Teeyapant *et al*., 1993). At this point, we have no evidence to support or refute the competing hypotheses: that *Tylodina* represents a transition step from carnivorism to herbivorism, that the opposite is true, or that it is an evolutionary endpoint in itself with no further implications. We need a more thorough knowledge of phylogenetic relationships and feeding ecology of related species and groups before reaching a conclusion.

Many studies on sponge-feeding opisthobranchs overlook potentially relevant points about the diet itself and the origin of the chemical substances being translocated through the food chain. Many sponges are in fact complex communities where diverse symbionts – autotrophic (cyanobacteria) or heterotrophic (bacteria) – interact with the true sponge cell populations. A point of contention has been the role of these symbionts in the production of the chemical substances that act as defence mechanisms of both the sponge and its associated symbionts. In contrast, the role of these same symbionts on the feeding ecology of the predator has been largely ignored. When a predator is said to feed on a sponge and sequester its defensive substances, it is highly relevant to ascertain whether the predator actually feeds on sponge matter or on symbionts, and to relate these results with the compartment producing the chemicals of interest. In addition, from both a biological and evolutionary point of view, ascertaining the true diet of a mollusk can vary its consideration as a carnivore predator and help explain the uncertain transition(s) to and from herbivorism and carnivorism in several lines of evolution. To our knowledge, *Tylodina perversa* is the first example in which the role of photosynthetic symbionts in the feeding of a sponge grazer is demonstrated. Some dorid nudibranchs also feed on sponges hosting symbiotic cyanobacteria. The Mediterranean dorid *Peltodoris atromaculata* Begh, 1880 feeds almost exclusively upon the demosponge *Petrosia ficiformis* (Poiret, 1789), which hosts a dense population of the same cyanobacteria species found in *Aplysina aerophoba* (Cattaneo-Vietti, Schiaparelli & Chiantore, 2001). A large number of opisthobranch mollusks are known to exhibit different degrees of host–symbiont integration with chloroplasts or zooxanthellae from their prey species. These associations have been described in a number of species of sacoglossa (Ros & Marín, 1991) and in the suborders Dendronotoidea, Aeolidoidea and Arminoidea (Kempf, 1991). However, the feeding relationship between the sponge symbionts and the feeding habits of the mollusks remain open to investigation and could provide further information on the role of symbionts in the biology, ecology and evolution of both hosts and associated predators.

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