Sequestration and Possible Role of Dietary Alkaloids in the Sponge-Feeding Mollusk *Tylodina perversa*

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Abstract. Opisthobranchs of the genus *Tylodina* are found at exceedingly distant geographical regions in the marine environment but are always associated with sponges of the order Verongida (e.g., *Aplysina* species) which serve as prey for these gastropods. We investigated the chemical ecology of the Mediterranean species *T. perversa* that commonly feeds on *A. aerophoba*. The gastropod sequesters a set of sponge-derived brominated isoxazoline alkaloids which are accumulated in the mantle and egg masses and are furthermore exuded as part of the mucus when the animal is molested. Based on the documented feeding deterrent properties of the sponge alkaloids against fish, it is speculated that the sequestered sponge alkaloids serve also as a defense for *T. perversa*. Interestingly, specimens of *T. perversa* that were either collected while feeding on *A. aerophoba* or had been kept on these sponges under controlled conditions for several weeks almost always contained the brominated alkaloid aerothionin, which is not detected in *A. aerophoba* but occurs in the sibling species *A. cavernicola* instead. The latter sponge is also accepted as a food source by the gastropod, at least under experimental conditions. The possible origin of aerothionin in *T. perversa* is discussed.

12.1 Introduction

Opisthobranchs of the genus *Tylodina* (Notaspidea, Tylodinoidae, Tylodinidae) inhabit the marine environment at exceedingly distant geographical regions. While *T. corticalis* is found in the South Pacific from Southern Queensland, around the southern Australian coast to south-western Australia, *T. fungina* dwells in the Eastern Pacific from Southern California to the Galapagos Islands (Gabb 1865; Willan 1984, 1987, 1998). The Mediterranean Sea is inhabited by *T. perversa* (Gmelin 1791; Riedl 1983), which occasionally can also be found in the north-eastern Atlantic as far as the British Isles (Gainey and Turk 1997). Interestingly, all *Tylodina* species live in close association with sponges of the order Verongida (Willan 1984; Faulkner 1992; Teeyapant et al. 1993). The Mediterranean slug *T. perversa* is usually found on the yellow demosponge *Aplysina aerophoba* (Riedl 1983; Teeyapant et al. 1993; Doneddu and

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Progress in Molecular and Subcellular Biology
Subseries Marine Molecular Biotechnology
G. Cimino, M. Gavagnin (Eds.): Molluscs
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Manunza 1995). This sponge inhabits mostly sun-exposed hard substrates in shallow depths between less than 1 and up to 15 m (Riedl 1983; Pansini 1997). Often on a single *A. aerophoba* individual, several specimens of *T. perversa* can be found, together with their yellow egg ribbons.

The color of *T. perversa* as well as its mucus and egg ribbons is due to uranidine (Fig. 12.1, structure **5**), the same pigment that is found in the sponge *A. aerophoba* (Cimino et al. 1984; Teeyapant et al. 1993; Cimino and Sodano 1994). This, as well as feeding scars that are usually present concomitantly with the opisthobranchs on the sponge, makes it obvious that *T. perversa* preys on *A. aerophoba*. Often the slugs burrow a cavity into the sponge tissue that is large enough to accommodate the whole gastropod. In this case, their limpet-shaped shell protects them towards the opening of the cavity in the sponge, while outside the sponge the tiny shell is no efficient defense, as it only covers part of the soft body of the mollusc.

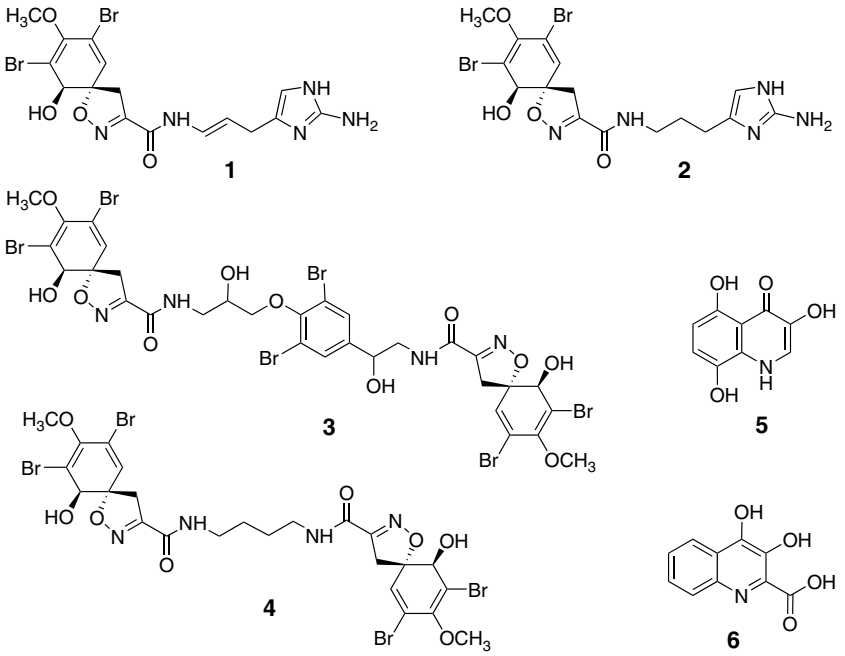

Fig. 12.1. Structures of alkaloids found in *Tylodina perversa* tissues in the course of our experiments: aplysinamisin-1 (**1**), aerophobin-2 (**2**), isofistularin-3 (**3**), aerothionin (**4**), uranidine (**5**), 3,4-dihydroxyquinoline-2-carboxylic acid (**6**)

Sponges of the order Verongida are interesting from a chemical point of view as they accumulate brominated isoxazoline alkaloids (e.g., Fig. 12.1, structures **1**–**4**) at high concentrations comprising more than 10% of their dry weight (Albrizio et al. 1994; Aiello et al. 1995; Ciminiello et al. 1994a,b, 1995, 1996a,b, 1997, 1999, 2000). These structurally unique

secondary metabolites are thought to be biogenetically derived from 3, 5-dibromotyrosine (Tymiak and Rinehart 1981). Whereas the spirocyclohexadienylisoxazoline moiety is identical for most of these alkaloids, the compounds differ by the nature of amine substituents linked to the carbonyl group adjacent to the isoxazoline ring (Fig. 12.1). Several isoxazoline alkaloids from sponges of the genus *Aplysina* (order Verongida) have recently been shown to act as strong feeding deterrents against marine fishes such as *Blennius sphinx* and are thought to play a crucial role in the chemical defense of the sponges against predatory fish (Thoms et al. 2004).

The Mediterranean Sea hosts two species of the order Verongida: *A. aerophoba* and *A. cavernicola*. While the former is abundant in shallow water at locations with high sun exposition, the habitat of the latter is restricted to shadier environments, such as underwater caves or deeper water of about 40 m (Wilkinson and Vacelet 1979; Pansini 1997; Thoms et al. 2003a). Despite these differences in the ecological requirements of the two Mediterranean *Aplysina* species, they share many similarities in their morphology (Vacelet 1959) as well as in their secondary metabolite pattern (Ciminiello et al. 1997; Thoms et al. 2004). There is even controversy as to whether they are really two distinct species or rather two ecotypes belonging to the same species (Voultsiadou-Koukoura 1987; Heim 2003). Nevertheless, some distinctive differences between the two sponges exist with regard both to their chemistry and to their microbiology. Only *A. aerophoba* possesses uranidine (**5**), the characteristic pigment that polymerizes quickly upon air exposure and thereby changes the color of the sponge from yellow to black (Cimino et al. 1984). *A. cavernicola* presents 3,4-dihydroxyquinoline-2-carboxylic acid (**6**) instead, which in comparison to uranidine (**5**) is far more stable (Fattorusso et al. 1971). While many isoxazoline alkaloids such as aerophobin-2 (**2**) and aplysinamisin-1 (**1**) can be found in both species, apparently only *A. cavernicola* accumulates aerothionin (**4**; Ciminiello et al. 1997). Both Mediterranean *Aplysina* species harbor a vast and diverse community of heterotrophic bacteria in their tissues (Friedrich et al. 1999, 2001; Hentschel et al. 2001; Thoms et al. 2003a). *A. aerophoba* is in addition associated with cyanobacteria of the species *Aphanocapsa feldmanii* that thrive in large numbers in its surface tissue (Vacelet 1971; Rützler 1985).

According to Becerro et al. (2003), the Mediterranean opisthobranch *T. perversa* is exclusively found in the zone where *Aplysina aerophoba* is abundant. To our knowledge, it has not yet been reported that *T. perversa* also feeds on the sibling species *A. cavernicola*. Thus, the slug is usually considered to be a specialist predator on *A. aerophoba* (Riedl 1983; Doneddu and Manunza 1995). Becerro et al. (2003) suggested that the cyanobacteria present in the sponge tissue are responsible for the gastropod's feeding choices rather than true sponge compounds, which

could explain a preference of *T. perversa* for *A. aerophoba* over the cyanobacteria-free *A. cavernicola* in nature.

Our study on *T. perversa* deals with the secondary metabolite uptake by the opisthobranchs when preying on *Aplysina* sponges. We report on the ecological aspects of this process as well as on the fate of the metabolites in the soft bodies of the slugs. Further aspects examined are the feeding preferences of *T. perversa* and the impact of different prey sponges on the alkaloid pattern in the tissues of the slug.

12.2 Sequestration of Alkaloids from the Prey Sponge by *Tylodina perversa*

As long ago as 1993, when we isolated compounds from *T. perversa* individuals that had been collected from *A. aerophoba* in the Canary Islands and analyzed the compound structures by ^{1}H NMR, ^{13}C NMR and mass spectrometry, we observed the presence of sponge alkaloids in the opisthobranchs (Teeyapant et al. 1993; Teeyapant 1994). Since the concentrations of these brominated compounds in *T. perversa* appeared too large to account for their presence only in the alimentary tract (30–45%, compared to the total alkaloid concentration in the respective prey sponges), we assumed their accumulation also in organs other than the digestive tissues.

To our surprise, in addition to the secondary metabolites present in *A. aerophoba*, we found the isoxazoline alkaloid aerothionin (Fig. 12.1, structure **4**) in crude extracts of *T. perversa* (Teeyapant et al. 1993). Aerothionin is considered a characteristic compound for *A. cavernicola* (Ciminiello et al. 1997), which had not been observed at the collection site of the slugs. This first observation was later confirmed in our subsequent studies on *T. perversa*: irrespective of the fact that the opisthobranchs were always collected from aerothionin-free *A. aerophoba* and irrespective of the absence of *A. cavernicola* at the respective collection sites, nearly all of the *T. perversa* specimens analyzed were found to contain considerable amounts of aerothionin (**4**; Teeyapant et al. 1993; Ebel et al. 1999; Thoms et al. 2003b).

A subsequent study analyzed in detail the uptake of *A. aerophoba* metabolites by *T. perversa* (Ebel et al. 1999). Opisthobranchs were collected from *A. aerophoba* individuals off the Mediterranean coast at L'Estartit, Spain, and kept along with this sponge in seawater tanks for more than one week. Thereafter, the opisthobranchs were dissected and studied for sequestered sponge-derived alkaloids. The amount of each individual alkaloid in the different tissue samples was determined by HPLC-UV, using calibration curves obtained for the respective isolated

compound. The sponge metabolites (the brominated alkaloids as well as the pigment uranidine, **5**) were present at high concentrations in the hepatopancreas as well as in the mantle tissue of the *T. perversa* individuals (Table 12.1). Mucus, egg ribbons and feces that had been produced during captivity likewise contained considerable concentrations of these natural products. Of all organs of *T. perversa* investigated in this study, the mantle tissue exhibited the highest total alkaloid concentration (43.3 μmol g^{-1} dry weight compared to a concentration of 62.0 μmol g^{-1} dry weight in *A. aerophoba*), followed by egg ribbons (29.6 μmol g^{-1} dry weight) and the hepatopancreas (24.0 μmol g^{-1} dry weight). Mucus and feces contained brominated alkaloids at a concentration of 21.5 and 16.1 μmol g^{-1} dry weight, respectively.

Table 12.1. Concentrations (μmol g^{-1} dry weight) of alkaloids in various tissues derived from the opisthobranch *Tylodina perversa* and in the sponge *Aplysina aerophoba* collected in L'Estartit (Spain)

	Tylodina perversa					*Aplysina aerophoba*
Compound	mantle	hepatopancreas	egg masses	mucus	feces	
Aplysinamisin-1 (**1**)	2.0 ± 0.6 (4.6 ± 1.4)	4.7 ± 1.9 (19.8 ± 7.8)	1.1 ± 1.1 (3.7 ± 3.9)	0.8 ± 0.3 (3.7 ± 1.3)	1.4 ± 0.1 (8.4 ± 0.5)	16.9 ± 3.5 (27.3 ± 5.6)
Aerophobin-2 (**2**)	34.5 ± 2.9 (79.7 ± 6.6)	9.3 ± 2.5 (38.7 ± 10.6)	26.8 ± 5.3 (90.7 ± 17.9)	16.6 ± 1.6 (77.5 ± 7.5)	6.6 ± 1.0 (41.1 ± 6.1)	32.1 ± 6.6 (51.8 ± 10.6)
Isofistularin-3 (**3**)	0.7 ± 0.7 (1.6 ± 1.5)	9.6 ± 1.4 (40.1 ± 6.0)	0.3 ± 0.2 (0.9 ± 0.7)	0.6 ± 0.3 (2.8 ± 1.6)	7.9 ± 0.6 (48.9 ± 3.5)	13.0 ± 2.1 (20.9 ± 3.3)
Aerothionin (**4**)	6.1 ± 2.0 (14.1 ± 4.6)	0.3 ± 0.3 (1.5 ± 1.1)	1.4 ± 1.1 (4.7 ± 3.7)	3.4 ± 1.4 (16.0 ± 6.7)	0.2 ± 0.3 (1.5 ± 2.2)	n.d. –
Total conc. (mg g^{-1})	24.6 ± 15.2	20.4 ± 6.6	19.2 ± 13.7	12.5 ± 13.6	14.2 ± 13.3	51.2 ± 17.7

Values in brackets represent relative proportions (%) of the respective alkaloids compared to the total brominated alkaloid content in the tissue. Total concentrations comprise additional brominated alkaloids detected in minor amounts and are expressed in mg g^{-1} dry weight to facilitate easier comparison with other chemical studies on opisthobranchs. n.d. Not detected

Comparison of the alkaloid patterns in the different opisthobranch organs analyzed (including mucus, egg ribbons, feces) revealed a distinctive selectivity in the sequestration of the respective isoxazoline alkaloids. Aerophobin-2 (**2**) was the major alkaloid in the mucus, the egg ribbons and in the mantle tissue of *T. perversa*, comprising between 77.5% and 90.7% of all quantified compounds (Table 12.1). In the host sponges, however, this compound made up for only 51.8% of all alkaloids present. In contrast, the isofistularin-3 (**3**) proportion was considerably lower in the opisthobranchs (0.9–2.8%) than in the sponge tissue (20.9%).

Apparently, isofistularin-3 (**3**) was selectively excreted by the opisthobranchs, as in the feces the relative concentration of this compound was 48.9% (total alkaloid content set at 100%) and thus considerably higher

than in the sponge tissue. Reasons for a selective excretion of isofistularin-3 (**3**) versus a selective accumulation of aerophobin-2 (**2**), however, are yet unclear. Both isoxazoline alkaloids revealed to be equally strong feeding deterrents against the Mediterranean fish species *Blennius sphinx* when tested at their natural concentrations as found in *A. aerophoba* (Thoms et al. 2004). Possibly, the accumulation of isoxazoline alkaloids in exposed tissues such as mantle and egg ribbons as well as in the mucus that is exuded when the opisthobranchs are molested, acts as a chemical defense. Experimental proof for this hypothesis is so far lacking, as the alkaloids have only been tested for feeding deterrent properties at concentrations as found in the sponges, which are approximately twice as high as the alkaloid concentrations in organs (e.g., mantle) of *T. perversa*.

As reported in our first study on *T. perversa* (Teeyapant et al. 1993), our second approach (Ebel et al. 1999) confirmed the presence of aerothionin (**4**) in *T. perversa*, even though this isoxazoline alkaloid is characteristic for *A. cavernicola* but is not detected in *A. aerophoba*. Separate analysis of the respective opisthobranch tissues revealed the highest concentrations of aerothionin (**4**) in the mantle (6.1 ± 2.0 µmol g^{-1} dry weight) and in the mucus (3.4 ± 1.4 µmol g^{-1} dry weight), whereas all other tissues analyzed contained this compound at much lower concentrations (Table 12.1). Again no aerothionin (**4**) was found in *A. aerophoba*, the sponge from which the opisthobranchs had been collected and on which they had preyed under controlled conditions for one week.

12.3 Choice Feeding Experiments with *Tylodina perversa*

To determine whether *A. cavernicola* could serve as a host sponge for *T. perversa* in nature, we performed choice feeding experiments and offered three different sponges including *A. aerophoba*, *A. cavernicola* and (as a control) *Axinella damicornis* to specimens of *T. perversa* held in seawater tanks (Thoms et al. 2003b). Other than the *Aplysina* species, *Axinella damicornis* accumulates alkaloids of the bromopyrrole type. All sponges used for the experiments had a similar yellow color. The opisthobranchs were placed at a starting point equally distanced at about 15 cm from each of the three sponges. We scored a selection when they had crawled completely onto one of the sponges. The sponges were randomly placed in the tanks at each trial to avoid a possible influence of light and flow conditions on our data.

In only 8.3% of a total of 48 individual choice feeding experiments did the opisthobranchs show a preference for *A. damicornis*, whereas in over 90% there was a clear preference for one of the *Aplysina* sponges. However, *T. perversa* apparently did not distinguish between the two *Aplysina*

species, as preference for *A. cavernicola* (43.8%) was almost equal to that for *A. aerophoba* (47.9%). This result contradicts the observations of Becerro et al. (2003), who found that *T. perversa* exclusively selects *A. aerophoba* when given the choice between this sponge and *A. cavernicola.*

12.4 Impact of Different *Aplysina* Sponges on the Alkaloid Patterns in *Tylodina perversa*

The acceptance of the *A. cavernicola* as a host sponge and food source for *T. perversa* enabled us to perform long-term feeding experiments with the opisthobranchs under controlled conditions and to investigate the impact of the respective prey sponge on the alkaloid profiles in the opisthobranchs. Specimens of *T. perversa* collected in the Mediterranean Sea close to Banyuls-sur-mer, France, were kept for more than two weeks in tanks together with individuals of *A. aerophoba*, followed by an additional feeding period of two weeks on *A. cavernicola* (Thoms et al. 2003b). In a second experiment, we kept *T. perversa* for a period of more than five weeks exclusively on *A. aerophoba.* At the end of each experiment, we dissected the slugs for subsequent HPLC analysis of the mantle and the hepatopancreas. In addition, egg ribbons that had been produced during the experiment were likewise investigated by HPLC. The alkaloids in the respective samples were quantified, based on calibration curves obtained with previously isolated compounds.

After two weeks feeding on *A. cavernicola*, the alkaloid profile of *T. perversa* had clearly shifted towards the profile of this species when compared to the alkaloid patterns of control opisthobranchs that were dissected and analyzed immediately after collection from *A. aerophoba.* The most pronounced similarity of the alkaloid pattern from specimens of *T. perversa* to the pattern of *A. cavernicola* was observed for the hepatopancreas, where high concentrations of aerothionin (Fig. 12.1, structure **4**; 17.1 ± 6.7 μmol g^{-1} dry weight) and the *A. cavernicola* pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**; 45.6 ± 14.8 μmol g^{-1} dry weight) were found (Fig. 12.2).

Moreover, for specimens of *T. perversa* that had been kept on *A. aerophoba* for more than five weeks, the closest similarity of the alkaloid pattern (compared to the host *A. aerophoba*) was also found for the hepatopancreas. Here, the overall concentration of isoxazoline alkaloids in the hepatopancreas amounted for 110.5 ± 36.5 μmol g^{-1} dry weight with aplysinamisin-1 (**1**) as the major constituent (57.1 ± 20.1 μmol g^{-1} dry weight). Thus, the total alkaloid content as well as the amount of aplysinamisin-1 in the hepatopancreas of *T. perversa* feeding on *A. aerophoba* from Banyuls-sur-mer (Fig. 12.2) was considerably higher than found in our previous

study involving individuals that had been collected at L'Estartit, Spain and kept on *A. aerophoba* for one week (Table 12.1; Ebel et al. 1999). This finding probably reflects intra-specific differences of alkaloid concentrations and patterns of *A. aerophoba* individuals collected at different geographical sites.

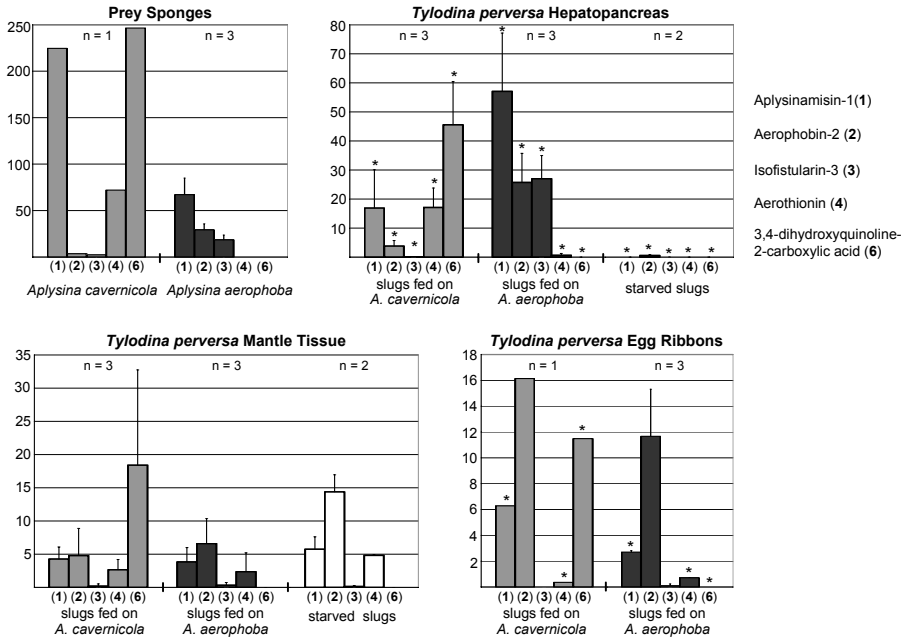

Fig. 12.2. Concentrations (μmol g^{-1} dry weight) of alkaloids in various tissues derived from the opisthobranch *Tylodina perversa* (after two weeks of feeding on *A. cavernicola*, after five weeks of feeding on *A. aerophoba*, after two weeks of starvation) and in their prey sponges. Vertical *bars* represent standard deviation. Numbers of compounds refer to Fig. 12.1. *Asterisks* indicate significant difference in different treatments of the experiment ($P < 0.05$)

The results obtained in the feeding experiments demonstrate a close relationship between the alkaloid profile of the hepatopancreas of *T. perversa* and the alkaloid profile of their most recent host sponge. This close similarity was further exemplified by an additional experiment where specimens of *T. perversa* were starved for two weeks following a five-week feeding period on *A. aerophoba* (Thoms 2004). After starving, the hepatopancreas of the slugs contained almost no alkaloids (total content of isoxazoline alkaloids 0.6 ± 0.2 μmol g^{-1} dry weight), thus demonstrating that dietary alkaloids have to be replenished continuously.

In analogy to the metabolite profiles of the hepatopancreas, the profiles of the mantle tissue of individuals that had fed on *A. cavernicola* for a period of two weeks prior to dissection differed from that of opisthobranchs reared on *A. aerophoba* with regard to the pigments of the respective prey sponges found therein. While the former contained

the *A. cavernicola* pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**) at concentrations of 18.4 ± 14.3 μmol g^{-1} dry weight, in the latter the *A. aerophoba* pigment uranidine (**5**) was found instead; but due to its instability upon contact with air, it could not be accurately quantified. However, with regard to their isoxazoline alkaloid profiles and contents, the mantle tissues of the two differently fed groups of opisthobranchs were almost identical. This also held true for the aerothionin (**4**) content, which in individuals that had preyed for more than two weeks on aerothionin-yielding *A. cavernicola* amounted to 2.7 ± 1.5 μmol g^{-1} dry weight, whereas in those that had fed on *A. aerophoba* the aerothionin (**4**) concentration amounted to 2.4 ± 2.8 μmol g^{-1} dry weight (Fig. 12.2). Interestingly, no depletion of isoxazoline alkaloids was observable in the mantle tissue of *T. perversa* individuals that were starved for more than two weeks following feeding on *A. aerophoba*. The total isoxazoline alkaloid content detected in starved opisthobranchs was even higher (25.1 ± 4.6 μmol g^{-1} dry weight) than in the mantle tissue of gastropods of the two other groups (13.1 ± 7.7 and 11.9 ± 6.5 μmol g^{-1} dry weight, respectively). The aerothionin (**4**) concentration in starved *T. perversa* individuals amounted to 4.8 ± 0.1 μmol g^{-1} dry weight, compared to 2.4 ± 2.8 μmol g^{-1} dry weight in slugs that had fed on *A. aerophoba* for more than five weeks and 2.7 ± 1.5 μmol g^{-1} dry weight in those that fed for over two weeks exclusively on *A. cavernicola* (Fig. 12.2).

Similar to their mantle tissues, the egg ribbons produced by opisthobranchs following feeding on *A. aerophoba* or *A. cavernicola* under controlled conditions showed no appreciable changes in isoxazoline alkaloid profiles that could reflect differences in alkaloid composition of their host sponges. However, the egg ribbons produced by the slugs after feeding on *A. cavernicola* for more than two weeks contained considerable amounts of the pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**), characteristic for this sponge. As the starved opisthobranchs had not produced any egg ribbons during captivity, it was not possible to analyze the metabolite pattern of eggs.

12.5 Conclusions

In the course of our studies on *T. perversa*, we were able to show that the opisthobranchs not only tolerate the defense metabolites of their prey sponges but even sequester them (Teeyapant et al. 1993; Ebel et al. 1999; Thoms et al. 2003b). Unequivocal evidence for this finding was provided by the observation that in all analyzed tissues of the slugs the pigment of the respective sponge on which *T. perversa* had been feeding previously [uranidine (**5**) in the case of *A. aerophoba*, 3,4-dihydroxyquinoline-2-

carboxylic acid (**6**) in the case of *A. cavernicola*] was found (Thoms et al. 2003b). Generally in gastropods, the hepatopancreas produces digestive enzymes and reabsorbs nutrients from the gut (Götting 1996). Chemical analysis of this organ from specimens of *T. perversa* kept in captivity revealed an alkaloid pattern essentially identical to that of the slug's most recent prey sponge (Thoms et al. 2003b). Moreover, an experiment involving starved *T. perversa* individuals demonstrated that the dietary alkaloids have to be replenished continuously in order to keep up their normal levels in the hepatopancreas, as after two weeks of starvation this organ was found to be almost alkaloid free (Thoms 2004).

Sequestration of sponge alkaloids turned out to be far more selective in the mantle tissue and in the egg ribbons of *T. perversa* than in the opisthobranch's hepatopancreas. When specimens of *T. perversa* were kept on *A. cavernicola* after an initial feeding period on *A. aerophoba*, the mantle tissue displayed an enrichment of aerophobin-2 (**2**; present in both *Aplysina* species) and a concomitant depletion of isofistularin-3 (**3**; only present in *A. aerophoba*) when compared to the alkaloid profile of *A. aerophoba* (Ebel et al. 1999; Thoms et al. 2003b). The enrichment of aerophobin-2 (**2**) could possibly be due to a conversion from aplysinamisin-1 (**1**) within the slug, as the structures of these compounds differ only in a double bond. Isofistularin-3 is possibly excreted selectively by the slugs as it is found enriched in the feces (Ebel et al. 1999). It seems likely that the selective sequestration of sponge alkaloids by *T. perversa* serves an ecological purpose as: (a) the alkaloids are preferentially accumulated in exposed tissues such as mantle and egg ribbons and are secreted as part of the mucus when the slugs are molested and (b) the alkaloids were shown to possess pronounced feeding deterrent properties when tested against the Mediterranean fish *Blennius sphinx* (Thoms et al. 2004). However, experimental proof for the feeding deterrent activity of *A. aerophoba* compounds at concentrations as found in the tissues of the slugs is still lacking. Nevertheless, Becerro et al. (2003) were able to show that a crude extract of *T. perversa*, as well as extract of its egg ribbons, had a stronger anti-feeding effect towards the Mediterranean fish *Chromis chromis* than a crude extract of *A. aerophoba*. As the slug tissues hold considerably lower concentrations of the isoxazoline alkaloids than the sponge, the involvement of other so far unknown compounds for their chemical defense is likely. This is also indicated by the study of Becerro et al. (2003), who found that *T. perversa* as well as its egg ribbons yielded a higher percentage of crude extract per dry weight than *A. aerophoba.*

Interesting but still unresolved is the origin of aerothionin (**4**) that is almost always found in *T. perversa* individuals collected from *A. aerophoba* in the wild. According to a number of recent reviews on the chemical ecology of opisthobranchs (e.g., Avila 1995; Cimino et al. 1999; Cimino and Ghiselin 1999, 2001; Gavagnin et al. 2000), three different ecological scenarios can explain the accumulation of secondary metabolites in their tissues:

1. De novo biosynthesis of the compounds by the slug itself (or by its associated microorganisms)
2. Biotransformation of diet-derived compounds
3. Accumulation of diet-derived compounds

Prominent examples for compounds that are products of the secondary metabolism of opisthobranchs formed by de novo biosynthesis are polypropionates found in tissue of the sacoglossans *Cyerce cristallina*, *Elysia viridis* and *Ercolania funerea* (Vardaro et al. 1991; Di Marzo et al. 1991, 1993; Gavagnin et al. 1994). However, in the case of *T. perversa*, de novo synthesis of aerothionin (**4**) seems rather unlikely, as the isoxazoline alkaloids accumulated in *Aplysina* sponges (e.g., Fig. 12.1, structures **1**–**4**) are structurally unique and almost exclusively found in sponge species of the order Verongida (Albrizio et al. 1994; Aiello et al. 1995; Ciminiello et al. 1994a,b, 1995, 1996a,b, 1997, 1999, 2000).

The opisthobranch *Ascobulla ulla* converts the algal-derived compound caulerpenyne into ascobullin-a and -b during detoxification of these potentially harmful metabolites (Gavagnin et al. 2000). The Mediterranean slug *Hypselodoris orsini* detoxifies scalaradial sequestered from the sponge *Cacospongia mollior* by transforming it into the less toxic deoxoscalarin (Cimino et al. 1993). These examples show that transformation processes of diet-derived metabolites occur in marine opisthobranchs and could possibly explain the occurrence of aerothionin (**4**) in *T. perversa* after feeding on *Aplysina aerophoba*. However, if aerothionin (**4**) is formed in *T. perversa* from other isoxazoline alkaloids, such as isofistularin-3 (**3**) or aerophobin-2 (**2**), this biotransformation reaction would not only involve cleavage of the putative precursors at the amide bond(s) but also re-assembly of two spirocyclohexadienylisoxazoline moieties and insertion of one putrescine unit, thereby giving rise to aerothionin (**4**). As there is no experimental evidence for this rather complex bioconversion in the slugs, this hypothesis remains speculative at the moment.

Long-term storage of dietary metabolites in gastropods as observed in this study for starved specimens of *T. perversa* is no isolated case for gastropods. A further example is the terrestrial slug *Chondrina clienta* that sequesters the anthraquinone parietin from its diet, the lichen *Xanrhoria parietina* (Hesbacher et al. 1995). Even after four weeks of feeding on a parietin-free diet, this anthraquinone could be detected in the tissue of slugs that had been collected while feeding on the lichens. This example could indicate that the sponge *A. cavernicola* is the actual source of aerothionin (**4**) found in *T. perversa*. The observation that, unlike *A. aerophoba*, no specimens of *A. cavernicola* were observed at the sampling sites of *T. perversa* is not necessarily a valid argument against this assumption but rather reflects differences in the ecology of both sponges. Whereas *A. aerophoba* grows exposed and can be found even at low water depths (around 1 m) *A. cavernicola* generally lives hidden in

caves or at greater depths and is thus harder to find. Further corroboration for the hypothesis that *A. cavernicola* is the actual source of aerothionin (**4**) found in *T. perversa* was provided by our choice feeding experiment which revealed that both *A. aerophoba* and *A. cavernicola* are similarly attractive to the gastropods (Thoms et al. 2003b). Thus, *A. cavernicola* could also be a host for *T. perversa* in nature, even though the gastropods have so far not been observed feeding on this particular sponge.

However, some inconsistencies remain if *A. cavernicola* is regarded as the source for aerothionin (**4**) found in *T. perversa*. First, in the long-term feeding experiment with *T. perversa* on *A. cavernicola*, we demonstrated that, in addition to the isoxazoline alkaloids, the pigment (**6**) is sequestered by the gastropods. However, the *A. cavernicola* pigment (**6**) was not found in any of the individuals that had been collected in their natural environment and that contained aerothionin (**4**). Second, while the amount of sequestered parietin in the above mentioned example *Chondrina clienta* decreased over time on a parietin-free diet (Hesbacher et al. 1995), in specimens of *T. perversa* that had preyed on *A. aerophoba* for more than five weeks under controlled conditions, the aerothionin (**4**) content in mantle tissue remained largely unchanged (Thoms et al. 2003b). This result is even more surprising, as in the course of the feeding experiment the slugs had produced large amounts of aerothionin-containing egg ribbons, which would have been expected to result in a measurable loss of aerothionin (**4**). The situation is even more complex, as recently some *Aplysina* specimens were collected from Croatia that yielded aerothionin (**4**) in addition to the *A. aerophoba* pigment uranidine (**5**; Heim 2003; Thoms 2004). The true taxonomic status (chemical race, new species?) of these chemically most unusual *Aplysina* specimens is unclear, as is the question whether similar sponges might also occur elsewhere in the Mediterranean Sea where they could be accessible for *T. perversa*. Thus, the origin of aerothionin in *T. perversa* is still an unsolved question and warrants further studies on the fascinating prey–predator relationship of *Tylodina* gastropods and *Aplysina* sponges.

Acknowledgments. We would like to thank PD Dr. Ute Hentschel (Universität Würzburg, Germany), Prof. Franz Brümmer (Universität Stuttgart, Germany) and Prof. Werner E.G. Müller (Universität Mainz) for their cooperation and stimulating discussions. This study was supported by a grant of the BMBF (project: Center of Excellence BIOTECmarin). C.T. gratefully acknowledges a scholarship (Feodor Lynen Fellowship) from the Alexander von Humboldt-Stiftung, Bonn.

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