

Article

The Ascidian–Amphipod Association between *Phallusia mammillata* (Cuvier, 1815) and *Leucothoe richiardi* (Lessona, 1865) in the Mar Grande of Taranto (Mediterranean Sea, Italy)

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Abstract: Amphipods are widespread in the marine environment, and some have adopted a symbiotic lifestyle, such as numerous species of the genus *Leucothoe* (Leach, 1814). Unfortunately, few examples of such a relationship are known for the Mediterranean Sea. In the present study, we report for the first time the symbiotic relationship between *Leucothoe richiardi* (Lessona, 1865) and the ascidian *Phallusia mammillata* (Cuvier, 1815) from the Mar Grande of Taranto. Two samplings were carried out in November 2021 and May 2022, and the population structure of *L. richiardi* was also analyzed in relation to host weight. *L. richiardi* was found to live its entire cycle in *P. mammillata*. The amphipod population was dominated by females, and in most cases, juveniles of various sizes were found together with adults, indicating prolonged parental care. The host selection by the amphipods proved to be random and was determined by the abundance/availability of ascidians rather than their weight. The ascidians showed no signs of disturbance by the inhabiting amphipods, although they were present in large numbers, and we assumed that the relationship between *L. richiardi* and *P. mammillata* was commensal.

Keywords: symbiosis; Amphipoda; Tunicata; commensalism; Mediterranean Sea



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1. Introduction

The term “symbiosis” (from the Greek *syn*, “together”, and *biōsis*, “to live”) was coined to describe the “living together of unlike organisms” [1]. Since its introduction, the new term has generated much confusion, particularly about which interactions should be considered symbiotic [2]. The current broad definition refers to any biological association between two dissimilar organisms called symbionts (often the larger partner is also called the host), with some degree of physical and potentially long-lasting connection, regardless of the implications for the fitness of either organism [3]. The nature of host-symbiont associations can vary by environmental context and move along a parasite–mutualist continuum depending on the effects on the host [3–5]. In mutualism, both interacting organisms benefit from each other, whereas in parasitism, only the symbiont/parasite benefits at the expense of the host, which is harmed in some way. Interestingly, in commensalism, again, only the symbiont benefits from the association, while the host apparently neither benefits nor is harmed. In many cases, it is indeed difficult to determine the nature of the symbiotic association. Moreover, the terms mutualism and symbiosis have often been used interchangeably in the past, contributing to general confusion. However, analysis of population dynamics and specific traits of associated organisms can provide important clues to understanding their relationship [6].

Amphipods are among the most abundant and diverse invertebrates in the marine environment. They are brooding crustaceans adapted to a variety of lifestyles, and some have specialized to live in symbioses [7]. For example, the family Leucothoidae (Dana, 1852) includes many sibling species of the genus *Leucothoe* (Leach, 1814), which generally live as commensals in sessile organisms, such as sponges, bivalves, and ascidians [8]. Such large host organisms can exert strong selective pressure on the associated symbionts, leading to strictly local adaptations in host use and thus increasing the likelihood of sibling speciation [9,10]. It is, therefore, not surprising that numerous new *Leucothoe* species have recently been discovered worldwide, often in their ascidian host [11–17].

Ascidians are sessile benthic suspension-feeders that occur worldwide in both solitary and colonial forms and are often dominant components of fouling assemblages in enclosed environments [18,19]. Solitary ascidians are known to host a range of symbiotic crustaceans, including parasitic and/or commensal ascidicolid and notodelphyid copepods and leucothoid amphipods [18]. Due to their relatively large size and long lifespan, they ensure a stable internal microhabitat in which leucothoids can find shelter and have sufficient space and food to reproduce, provide extended care for juveniles, and possibly exhibit advanced social behavior; in some cases, juveniles may even ‘inherit’ the ascidians in which they were born when their parents die [20–22].

Tropical ascidians have been particularly studied in this regard, and *Phallusia nigra* (Savigny, 1816) alone is known to host at least six *Leucothoe* species, numerous copepods, and even a polychaete as symbiotic partners worldwide [23]. In contrast, *Phallusia mammillata* (Cuvier, 1815) is one of the largest and most abundant ascidians in the Mediterranean [19], commonly known to host several parasitic/commensal copepods [24,25]. Nevertheless, there seems to be only one documented case of symbiotic association with an amphipod (i.e., *Leucothoe denticulata* Costa, 1851) from the French Atlantic coast to date [26].

In this study, we investigated the symbiotic association between the amphipod *Leucothoe richiardi* (Lessona, 1865) and the ascidian *P. mammillata* from the Mar Grande of Taranto (southern Italy). We analyzed the occurrence and population structure of *L. richiardi* in *P. mammillata* specimens and evaluated their relative abundance to host size with the aim of gaining initial insights into the host use pattern and behavior of this ascidian-inhabiting amphipod.

2. Materials and Methods

2.1. Study Area

This study was conducted in the fish farm “Maricoltura Mar Grande”, located in a confined area of the Mar Grande of Taranto (40°25′56″ N; 17°14′19″ E) (Figure 1). The basin has an area of 35.5 km² and a maximum depth of 42 m. The temperature shows seasonal variations typical of coastal regions of the Ionian Sea, with an average annual value of about 18 °C, while the salinity is about 38 and almost uniform throughout the year. The fish farm covers an area of 0.06 km² and is located about 600 m from the coast. It consists of 15 cages operating at a depth of 7–12 m and producing about 100 tonnes/year of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) and sea bream *Sparus aurata*, Linnaeus, 1758.

As part of a life project, the fish farm was converted into an integrated multi-trophic aquaculture system (IMTA). Three long lines supported by buoys were installed around the fish cages to grow the bioremediating organisms envisaged for the project (i.e., seaweed, mollusks, sponges, and polychaetes). Some of the target species cultured for bioremediation were grown by natural recruitment on coconut fiber ropes (10 m in length) that were used as fouling collectors. A total of 196 bare ropes (fouling collectors) were attached to the long lines in October 2018, and a large biomass was easily obtained at the end of the first production cycle (June 2020), which lasted about one and a half years (for a more detailed description of the IMTA system, see [27]). Three additional production cycles were carried out (2019–2021, 2020–2022, and 2021–2023) with the number of ropes held constant in each cycle to ensure the same bioremediation capacity, and the growth of other potentially bioremediating species was also preliminarily assessed in these subsequent

cycles. Indeed, natural fiber ropes proved to be an excellent substrate for a variety of fouling species that can act as “biofilters”, including ascidians [28]. In particular, *P. mammillata* showed an average density of about ten individuals per rope during the first months of the 2021–2023 experimental production cycle [29]. During the monitoring of these production cycles, the presence of some brightly colored amphipods living in *P. mammillata* became evident. Furthermore, these amphipods were not observed in other ascidians or substrates in the area.



Figure 1. Map of the study area. The yellow stars indicate the location of the Mar Grande of Taranto in Italy. The red stars indicate the location of the fish farm in the Mar Grande of Taranto. The satellite images are taken from Google Earth.

2.2. Sampling and Laboratory Activities

Two sampling campaigns in November 2021 and May 2022 were conducted. In each sampling campaign, a total of 23 individuals of *P. mammillata* were collected by divers from several randomly selected collectors. Specimens of *P. mammillata* were immediately placed in individual plastic containers to reduce manipulation as much as possible and transported to the laboratory. A scalpel was used to cut the tunic of the ascidians lengthwise to examine the interior for any amphipods present. Any amphipods found were counted and isolated in individually labeled test tubes for each ascidian and preserved in 70% alcohol for later

analysis. The soft tissues of each ascidian were then removed from the tunics and dried at 70 °C for 24 h. The dry weight of *P. mammillata* individuals was used as a proxy for their biomass.

All amphipods were identified under a Nikon SMZ 800N stereomicroscope using the dichotomous keys available for the Mediterranean Sea [30]. Then, using a stereomicroscope (Nikon SMZ 25) equipped with a DS-Ri2 video camera and a video-interactive image analysis system (NIS-Elements BR 4.30.02 Nikon Instruments software), amphipods were measured along their dorsal surface from rostrum to telson, while they were divided into maturity classes according to head-to-telson length and counted: juvenile if smaller than 4 mm, otherwise adult [20]. Adult amphipods were then differentiated by sex based on the presence of oostegites and a smoother palm on gnatopod 2 in females as opposed to males (Figure 2 A,B). Finally, adult females were differentiated into ovigerous and non-ovigerous based on the presence of eggs in the abdomen and counted.

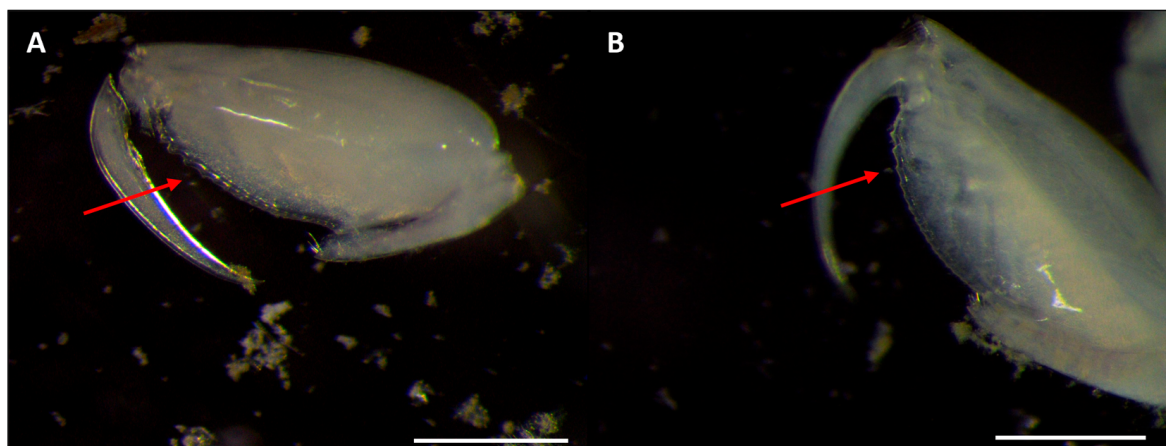


Figure 2. Detail of a male gnatopod 2 with clearly visible rough palm (A). Detail of a female gnatopod 2 with clearly visible smooth palm (B). Red arrows indicate the position of the palm in the gnatopod. Length of scale bars: 0.5 mm.

2.3. Data Processing

The following parameters were recorded in both sampling periods: the dry weight of each ascidian, the number, head-to-telson length, and maturity class (i.e., juvenile, adult male, non-ovigerous female, and ovigerous female) of amphipods found in each ascidian. The average number of amphipods found per ascidian (total number of amphipods/total number of ascidians) and the percentage of symbiotic association (number of ascidians with symbionts/total number of ascidians) were then calculated.

The head-to-telson length of amphipods was analyzed for each sampling period using the Kruskal–Wallis test and Dunn’s post hoc test to test for differences between maturity classes. Welch’s *t*-test was used to test for differences in the dry weight of ascidians and the number and length of amphipods in each maturity class between sampling periods. The dry weight of each ascidian was then correlated by linear regression with the total number of juveniles and adults (adult males, non-ovigerous females, and ovigerous females) it contained for each sampling period. Significance was set at a critical level of 95% ($p < 0.05$). All analyses were performed using Microsoft® Excel® for Microsoft 365 MSO (version 2306).

3. Results

Specimens of amphipods were found throughout the inhalant siphon to the branchial basket at both sampling times. Ascidian-dwelling amphipods were identified as *L. richiardi* based on the color of the living specimens and the presence of a small but clearly visible notch at the base of the posterior margin of epimeron 3 followed by a little process (Figure 3A,B).

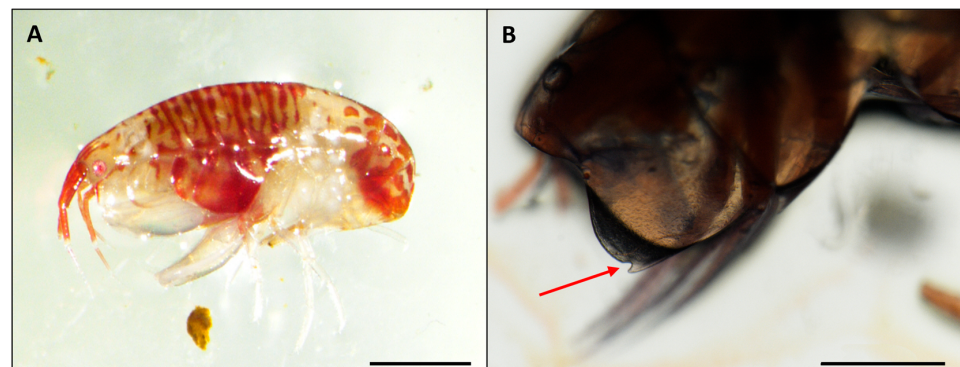


Figure 3. A living juvenile of *L. richiardi* found in *P. mammillata* (A). Detail of the notch and process (red arrow) at the base of epimeron 3 (B). Length of scale bars: 1 mm (A); 0.5 mm (B).

The total number of amphipods and mean head-to-telson length for each sex and maturity class from both sampling periods are shown in Table 1. There was no significant difference between the length of non-ovigerous females ($t_{(24)} = 2.06$, $p = 0.03$) and ovigerous females ($t_{(9)} = 2.26$, $p = 0.02$) between November and May (Table 1). In contrast, juvenile amphipods were found to be larger in November than in May ($t_{(36)} = 2.03$, $p < 0.01$), while males were larger in May than in November ($t_{(19)} = 2.09$, $p = 0.03$).

Table 1. Number of individuals (N°) and mean head-to-telson length (mm) with relative results of Welch's *t*-test for each sex and maturity class of the amphipod *L. richiardi*.

Maturity Class	November		May		Amphipod Number			Amphipod Length		
	N°	Length	N°	Length	df	<i>t</i>	<i>p</i>	df	<i>t</i>	<i>p</i>
Juvenile	24	3.14 ± 0.67	89	2.34 ± 0.67	18	2.10	0.19	36	2.03	<0.01
Adult male	18	7.66 ± 1.22	14	9.27 ± 2.32	38	2.02	0.89	19	2.09	0.03
Non-ovigerous female	63	6.82 ± 1.59	21	6.31 ± 2.67	38	2.02	0.15	24	2.06	0.51
Ovigerous female	4	10.58 ± 0.64	10	10.70 ± 1.10	28	2.05	0.03	9	2.26	0.82
Total	109	6.29 ± 2.26	134	4.31 ± 3.30	21	2.08	0.44	233	1.97	<0.01

Note: Significant *p*-values are given in italics.

In November, only 1 out of 23 ascidians had no crustaceans (occurrence = 95.65%). In the remaining 22 ascidians, a total of 109 specimens of *L. richiardi* were found. Most of them ($n = 66$) were females, including 4 with eggs, 19 were males, and 24 were juveniles; the sex ratio in the population was female dominated ($\approx 1 \text{ } \sigma : 3 \text{ } \varphi$). In May, 5 out of 23 specimens of *P. mammillata* were found without amphipods inside (occurrence = 78.26%). In the remaining 18, 134 specimens of *L. richiardi* were found. In total, there were 14 males and 31 females, 10 of whom had eggs, and the majority ($n = 89$) were juvenile; the sex ratio was female dominated ($\approx 1 \text{ } \sigma : 2 \text{ } \varphi$). In addition, a few individuals of the commensal copepod *Ascidicola rosea* (Thorell, 1859) were also found in some ascidians together with amphipods.

In November, the number of juveniles and males in ascidian hosts ranged from 0 to 6 and 0 to 3, respectively. Males were found in 50% of ascidians, while females were found in 90.91% of ascidians. Males cohabited with females in 40.9% of ascidians and with other males in 22.72%; the rest were inhabited by females only. Juveniles were found in six ascidians and always with other adults. The four ovigerous females were found in different ascidians.

In May, ascidian hosts contained between 0 and 48 juvenile specimens and between 0 and 3 males. Males were found in 61% of the ascidians and females in 77.7%. Female-male cohabitation was observed in 44.4% of the cases, while co-occurrence of males was observed in 11.1% of the cases. Juveniles were found in seven ascidians and only in one case without adults. Ovigerous females were found in 50% of the ascidians, and co-occurrence with another ovigerous female was observed in a single case.

The average dry weight of *P. mammillata* was significantly higher ($t_{(18)} = 2.10, p < 0.001$) in May (1.089 ± 0.652 g) than in November (0.415 ± 0.112 g), while the average number of amphipods found in ascidians showed no differences ($t_{(21)} = 2.08, p = 0.44$) between May (5.83 ± 11.42) and November (4.74 ± 4.99). The number of juveniles, males, and non-ovigerous females also showed no differences between November and May (Table 1), while the number of ovigerous females was significantly higher in May than in November ($t_{(28)} = 2.05, p = 0.03$). Linear regression showed a weak and non-significant relationship between ascidian dry weight and the number of adult/juvenile *L. richiardi* in both sampling periods, with a positive slope in November (Figure 4; adults: $R^2 = 0.1239, p = 0.108$; juveniles: $R^2 = 0.0721, p = 0.226$) and a negative one in May (Figure 5; adults: $R^2 = 0.0085, p = 0.716$; juveniles: $R^2 = 0.0249, p = 0.532$).

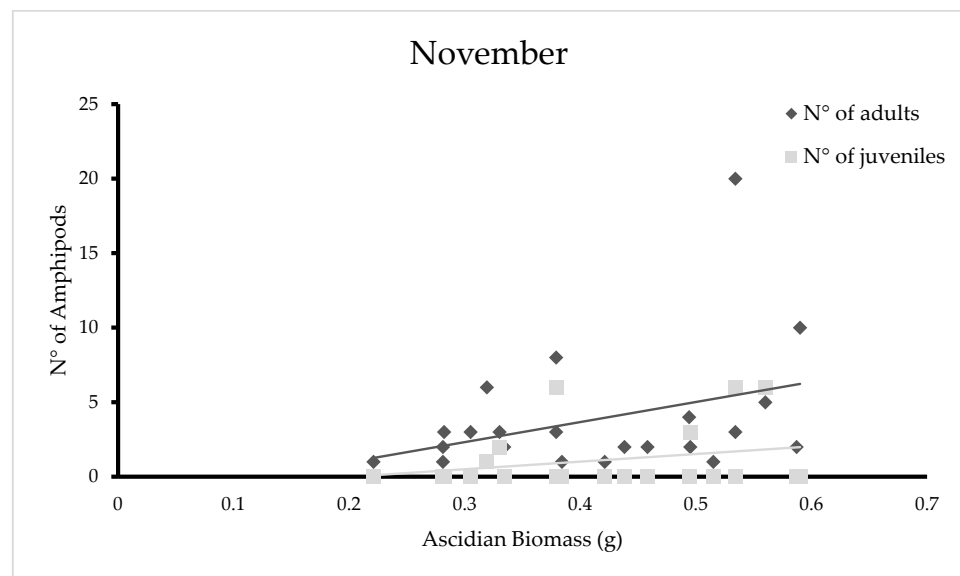


Figure 4. Linear relationship between dry weight (g) of the ascidian *P. mammillata* and the number of juveniles (light squares) and adults (dark diamonds) of *L. richiardi* in November.

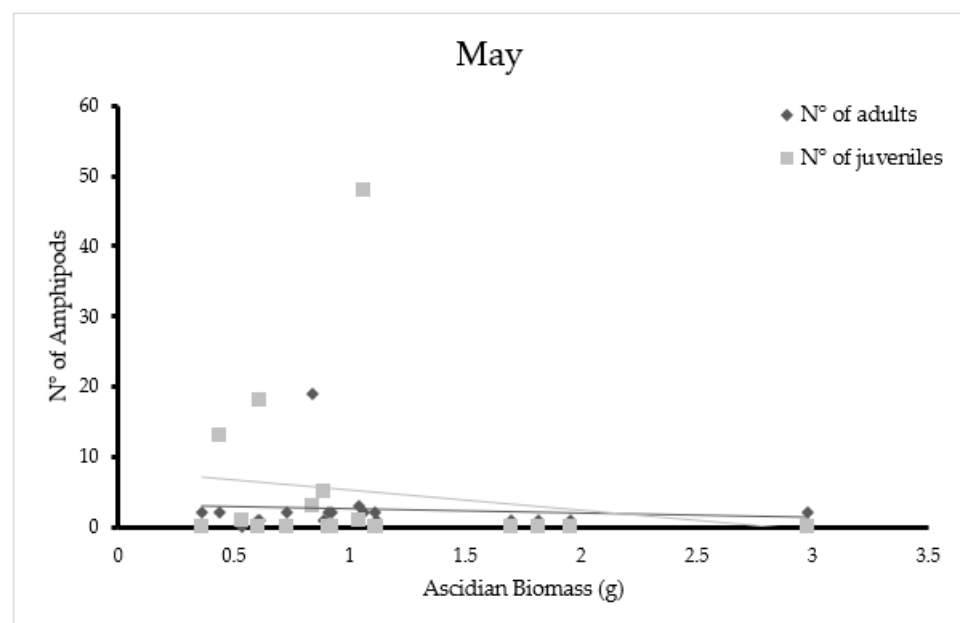


Figure 5. Linear relationship between dry weight (g) of the ascidian *P. mammillata* and the number of juveniles (light squares) and adults (dark diamonds) of *L. richiardi* in May.

4. Discussion and Conclusions

Several marine amphipods are known worldwide to dwell temporarily or throughout their lives in a variety of sessile host organisms, with leucothoids showing some affinity for ascidians [13,26]. The global list of leucothoids inhabiting ascidians is quite long, even when excluding newly described species (for a review, see [31]). These associations are reported worldwide, from the poles to the tropics, yet strikingly few examples are known from a long-explored basin such as the Mediterranean Sea [30,32]. A total of thirteen *Leucothoe* species are listed in the Mediterranean, but the respective ascidian hosts are known for only three of them [30,31]. However, one of these three cases should not be counted among those reported in the Mediterranean, as *Leucothoe furina* (Savigny, 1816) was found in the ascidian *P. nigra* from the Red Sea [33]. In a few cases, such as *L. richiardi*, the leucothoid is reported in detail to live in a variety of habitats, including coarse sand, among algal or seagrass rhizoids, and with sponges or ascidians, but no species name was given for any of the hosts [31].

Due to the high degree of morphological similarity among leucothoids, most newly described species have previously been misidentified as *Leucothoe spinicarpa* (Abilgaard, 1789), resulting in an unrealistic cosmopolitan distribution of what is now considered a species complex [34,35]. This has also led to *L. spinicarpa* being incorrectly associated as a commensal with multiple hosts worldwide. For example, the species was reported in 2009 by Cantor et al. [36] in association with the ascidian *P. nigra* from Ubatuba Bay on the southern Atlantic coast of Brazil. Six years later, Ramos et al. [37] questioned the validity of the previous record and reported *Leucothoe wuriti* (Thomas and Klebba, 2007) as a commensal of *P. nigra* from the same location. Finally, Senna et al. [12] described a new species, *Leucothoe angraensis* (Senna, Andrade, Ramos, and Skinner, 2021), also associated with *P. nigra* in Ilha Grande Bay, about 100 km north of Ubatuba Bay. Given the high morphological similarity, the occurrence in the same host, and the relatively short distance between the bays, it was assumed that the first two cases were also specimens of the new species and the *P. nigra*/*L. angraensis* association is currently considered the correct symbiotic match [23]. In some cases, *L. richiardi* may also have been misidentified in the past in the Mediterranean region, where it is reported to be a generalist commensal of several hosts, especially ascidians. However, there is no doubt that the amphipods found in the ascidian *P. mammillata* in the present study belong to the species *L. richiardi*, as the distinctive coloration of the living specimens, together with the small notch at the base of epimeron 3 makes this species unmistakable. Considering that, to our knowledge, *P. mammillata* seems to host leucothoids only on the French Atlantic coasts [26], the association reported in this study is new for the Mediterranean Sea.

Population analysis of *L. richiardi* revealed that all life stages were present in *P. mammillata* at both sampling times, suggesting that amphipods spend their entire life cycle in their ascidian host [20,21]. The wide variability in the length of non-ovigerous and ovigerous females and juveniles (Table 1) indicates that adults can reproduce more than once while living in *P. mammillata* [36,38] and that juveniles remain in their parents' host for a relatively long time after hatching. In addition, juveniles were frequently observed with other adults, in some cases ovigerous females, indicating that juveniles benefit from extended parental care during their stay, which increases survival and growth rates [22,39].

All maturity classes of *L. richiardi* (i.e., juveniles, males, non-ovigerous, and ovigerous females) were present in large numbers in both November and May and with the exception of ovigerous females, there were no significant differences between the sampling periods. The amphipod population housed in *P. mammillata* was female dominated in both November ($\approx 1 \sigma : 3 \varphi$) and May ($\approx 1 \sigma : 2 \varphi$), but with two samplings covering only half a year, this may result in a less accurate estimate of sex ratio. For example, in a two-year study, females of the sponge-dwelling gnathid isopod *Elaphognathia cornigera* (Nunomura, 1992) were reported to disappear during the colder season [40]. However, a female-dominated sex ratio is not unusual for amphipods and may be related to several factors such as food availability, longevity between sexes, local concentration of females due to migration, or

male predation [41]. For example, there is evidence of sex-specific predation in corophiid amphipods [42,43], whose males emerge from the sediment in search of a female to reproduce, exposing themselves to higher predation. When males of *L. richiardi* were present at both sampling times (in about 50–60% of ascidians), they were found primarily with other females, probably to use *P. mammillata* as a mating ground. Furthermore, although ovigerous females were present in both months, their numbers were significantly higher in May, at a time when there were also larger males and, thus, probably more fully ripe adults and/or better competitors for mating.

Phallusia mammillata is one of the largest ascidians growing on the fouling collectors in the Mar Grande of Taranto [28] and can provide sufficient space and food for *L. richiardi* to live and reproduce. Thus, the size of *P. mammillata* may have been a limiting factor in the number of amphipods hosted. However, linear regression showed no significant relationships between ascidian biomass and the number of amphipods at either sampling time, suggesting a commensal relationship [20,21,37]. Although the sampling was designed to cover both cold and warm seasons, since many benthic marine invertebrates, such as ascidians, exhibit a seasonal cycle in relation to activities such as growth and reproduction [18], only two sampling periods may have biased the lack of relationship between amphipod numbers and host biomass.

Density-dependent effects are known to be important in altering the nature of symbiotic relationships along a parasite–mutualist continuum, whereby mutualistic and/or commensal organisms would act as parasites when their density exceeds a certain threshold [44,45]. In the case of the association between the colonial ascidian *Diplosoma virens* (Hartmeyer, 1909) and notodelphyid copepods, for example, the parasitic copepods are reported to have an inhibitory effect on the sexual reproduction of host colonies, with colonies from heavily parasitized populations having a lower number of eggs/embryos per zooid than those from less parasitized populations [46]. However, the number of *L. richiardi* living in *P. mammillata* increased by a factor of about five on average, with no visible effect on host biomass.

The individuals of *P. mammillata* were much larger in May than in November, yet the number of amphipods showed no significant differences between sampling times. Interestingly, the highest number of amphipods was found in medium-sized ascidians with a dry weight of about 0.3–0.9 g, which were the most abundant at the sampling site (see Figures 4 and 5). Thus, host selection by leucothoids appears to be a random process determined by the abundance of ascidians (i.e., the probability of finding them) rather than their size. Indeed, spatial and temporal variations in host abundance may lead to variations in the density of *Leucothoe* spp. in their hosts [23] and may explain the results of Cantor et al. [36], who found a positive correlation between *P. nigra* biomass and the number of amphipods hosted. In addition, the results of Cantor et al. [36] were based on a single sampling, which increased the random component in the study of the population of *L. angraensis* (see [12]) and its relationship with *P. nigra*. Biotic interactions such as competition and predation, as previously suggested for sponge-associated gammarids [47], or parasitism may also affect the number of amphipods hosted. There is strong evidence that the presence of a parasite suppresses oviposition and the development of secondary sexual characteristics in host amphipod females [48] and that competition with another commensal species could limit the growth of either competitor, as in the case of *Dulichia rhabdoplastis* (McCloskey, 1970) [49] or *L. furina* [50].

In conclusion, the results of the present study indicate a commensal relationship between the amphipod *L. richiardi* and the ascidian *P. mammillata* from the Mar Grande of Taranto. While the ascidian had neither an advantage nor a disadvantage as a host, the associated amphipod seems to have found an ideal “home” to live and safely raise offspring, regardless of the size of the host. Apparently, it is not important how big the home is, but that one is found. Further studies to investigate the biological cycle of *P. mammillata* and the effects of the presence of *L. richiardi* on key host parameters such as growth rate and reproductive output are desirable to better understand the nature of their symbiotic

relationship. In addition, searching for *L. richiardi* in other potential hosts could help to clarify the specificity of such a relationship. In this context, it is important to emphasize that other large ascidians that could serve as potential hosts, such as the alien species *Styela plicata* (Lesueur, 1823), were also present in large numbers in the same area. However, although the phenomenon was not quantitatively examined, no specimen of *L. richiardi* was observed colonizing these ascidians. Lastly, genetic characterization of juvenile/adult specimens of *L. richiardi* in their hosts would provide valuable information about their parental connection and behavior.

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