Niche Limits of Symbiotic Gut Microbiota Constrain the Salinity Tolerance of Brine Shrimp

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abstract: Symbiosis generally causes an expansion of the niche of each partner along the axis for which a service is mutually provided. However, for other axes, the niche can be restricted to the intersection of each partner's niche and can thus be constrained rather than expanded by mutualism. We explore this phenomenon using *Artemia* as a model system. This crustacean is able to survive at very high salinities but not at low salinities, although its hemolymph's salinity is close to freshwater. We hypothesized that this low-salinity paradox results from poor performance of its associated microbiota at low salinity. We showed that, in sterile conditions, *Artemia* had low survival at all salinities when algae were the only source of carbon. In contrast, survival was high at all salinities when fed with yeast. We also demonstrated that bacteria isolated from *Artemia*'s gut reached higher densities at high salinities than at low salinities, including when grown on algae. Taken together, our results show that *Artemia* can survive at low salinities, but their gut microbiota, which are required for algae digestion, have reduced fitness. Widespread facultative symbiosis may thus be an important determinant of niche limits along axes not specific to the mutualistic interaction.

Keywords: symbiosis, mutualism, *Artemia franciscana*, adaptation, salinity.

Introduction

The fundamental niche of a genotype results from its adaptation to its environment, meaning that natural selection has optimized a suite of traits so as to favor increased reproductive abilities in this environment (Antonovics 1976). Implicit, but central to this view, is the idea that this process occurred by genetic changes (beneficial mutations or combination thereof) in the line of descent to this genotype (Antonovics 1976; Barton and Partridge 2000; Holt 2009). However, this view of niche evolution becomes necessarily limited when dealing with organisms that have a mutualistic partner. The niche of an organism, then, is not only the product of its own genes but also an extended phenotype of the genes of its partner (Dawkins 1983), such that the (co)evolution of each partner will affect the other partner's niche (Saffo 1992; Case et al. 2005). This mutual functional and evolutionary dependence further entails that mutualistic partners not only expand each other's niches through provision of specific services but also share each other's niche limitations along niche axes unrelated to their interaction. On these axes, the resulting niche is the intersection of the niches of each partner, rather than their union, as is the case for the axis of the interaction (see fig. 1). Here, the words "union" and "intersection" are used as in set theory (for two sets A and B, intersection $A \cap B$ vs. union $A \cup B$), consistent with their use in the classical fitness set theory (Levins 1962). This phenomenon may often go unnoticed, as it involves dimensions of the niche that are not directly related to the mutualistic partnership, but it may be an important determinant of the niche of a broad variety of organisms. In particular, it is important to understand whether it is restricted to the somewhat specific case of obligate mutualisms or whether it also matters for the much broader class of nonobligate mutualisms, such as that between animals and their gut microbiota.

It is well known that symbiotic associations impact the ecological niche. Positive biotic interactions are often thought to mostly cause an extension of the niche (Bruno et al. 2003; Afkhami et al. 2014), afforded by the services traded by mutualistic partners, such as provision of nutrients (Paul et al. 2007; Ley et al. 2008; Akman Gündüz and Douglas 2009) and resistance to the abiotic and biotic (pathogens/predators) environment (Mueller et al. 2011; Koch and Schmid-Hempel 2012), among others. Research on mutualism even led to the idea of a holobiont, an emergent supraorganism encompassing a host and its community, as opposed to the focus on individual "focal species" (Margulis 1998; Feldhaar 2011; Hansen and Moran 2013).

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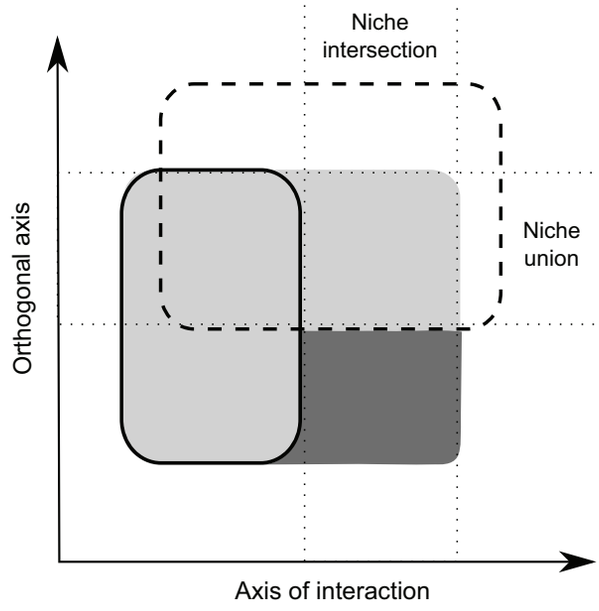

Figure 1: Effect of mutualism on niche limits. The niche of a focal species is represented through the presence (light shaded area) or absence (continuous line) of its mutualistic interactor, whose niche is delimited by a dashed line. The *X*-axis represents environments where the focal species can benefit from a service traded in the mutualism (the service is useless on the left, but becomes increasingly beneficial on the right). In our study system, the focal specie is the host (*Artemia*). The environments on the left are environments with a type of food that can be digested by *Artemia* in the absence of microbiota (yeast), whereas the environments on the right are those where the available food requires the presence of microbiota to be digested (algae). More generally, services traded can be as diverse as defense against predators or pathogens, provision of nutrients, or improved tolerance to some abiotic factors, among others (see "Introduction"). In general, this service differs among interactors (e.g., the reciprocal service from *Artemia* to microbiota is probably not related to algae digestion). In environments tolerated well by both partners, the mutualism causes a niche expansion along the axis of interaction (the light shaded area expands to the right), resulting in niche union. The *Y*-axis represents environmental variables that are not directly related to the service traded in the mutualism for the focal species ("orthogonal axis"). In our case, it represents the salinity gradient (low salinity at the bottom). Along these orthogonal axes, the mutualism can cause a constraint on niche extension (dark shaded area) in environments where the host needs the service traded by the mutualist. In these environments, the niche of the symbiont and the host cannot exceed the intersection of each of their niches when isolated.

Extension of the niche along the interaction axis is sometimes demonstrated by a reduction of the corresponding environmental range after removal of the symbiont (Boettcher et al. 2000; Zimmer et al. 2002; Pike and Kingcombe 2009; Rosengaus et al. 2011). However, in fact, there are also costs to mutualistic interactions, as revealed by the loss of symbionts when their benefit decreases (Thrall et al. 2008). These costs include producing one good to trade for another (e.g., nectar production for pollen transportation; Bronstein 2001) or susceptibility to cheaters (e.g., opportunists using goods without reciprocity; Case et al. 2005), among others. One such cost, on which we focus here, concerns adaptation on niche axes not directly involved in the interaction. Being dependent on a symbiont may entail a constraint on niche extension along orthogonal axes in conditions not tolerated by the symbiont. A general illustration of this phenomenon is given in figure 1, where the niche of a focal species is represented in presence (light shaded area) or absence (continuous line) of its mutualist interactor, whose niche is also delimited by a dashed line. Along the vertical axis (not directly involved in the interaction), the niche of an organism cannot exceed the intersection between its niche in isolation and that of its partner. This can cause a constraint on niche extension along this axis (dark shaded zone in fig. 1). Overall, the mutualism effect thus combines an extension along the niche axis of the traded services and a constraint on niche extension along other axes.

Although similar ideas have been discussed in the literature (Bronstein 2001; Case et al. 2005; Hansen and Moran 2013), the conceptualization of the ecological impact of mutualisms as a combination of niche union (along the axis that corresponds to the mutual service) and intersection (along other axes) has rarely been explicitly articulated. Furthermore, empirical studies addressing related questions rarely provide a comprehensive view of the process based on manipulative evidence. For instance, climate change or biological invasions cause range mismatches between mutualistic partners (Parker 2001; Stanton-Geddes and Anderson 2011; Jevanandam et al. 2013; Warren and Bradford 2013), leading several authors to recently call for more systematic integration of biotic interactions in the study of niche limits under climate change (Case et al. 2005; Travis et al. 2006; Sexton et al. 2009; Gilman et al. 2010). However, most studies of range mismatches rest on correlative evidence (Montllor et al. 2002; Ness et al. 2004; Ferrari et al. 2012; Hansen and Moran 2013), and geographic ranges can inform us on ecological niches only for species that are at equilibrium with respect to climate (Araujo and Pearson 2005). In other words, a crucial step that is generally lacking in such studies is the demonstration that the absence of the mutualist results from its maladaptation to the local environment (rather than historical or anthropic contingency; e.g., pollinator in Kjellberg and Valdeyron 1990). More direct evidence comes from studies conducted in the laboratory that show that the environment can destabilize a symbiotic interaction, causing a decrease in host fitness. Several studies investigate the effect of symbiont removal on host survival under different types of environmental stress, such as oil pollution (Newton and McKenzie 1995), antibiotics (Rosengaus et al. 2011; Willing et al. 2011), and high temperature (Rosenberg et al. 2007, 2009; Prado et al. 2010; Wernegreen 2012). These studies

clearly point out that environmental stresses have the potential to disrupt mutualistic interactions, with large phenotypic impact on the host. However, they do not partition the effects of such stresses on both the symbionts and the hosts. To our knowledge, Dunbar et al. (2007) is the only study that fully demonstrated constraints on the niche of a host (for dimensions other than the mutualistic service) caused by lack of adaptation of its mutualistic symbiont. The authors showed, by switching obligate symbionts (*Buchnera*) in aphid hosts, that a point mutation in the symbiont's genome reduced the thermal tolerance of their host. However, in general, even though niche intersection effects are likely to occur in many systems, they remain poorly documented. Studies involving facultative symbionts usually lack a partition of environmental effects on each of the partners, whereas in obligatory symbiosis, the niches of each partner are by definition difficult to study independently.

In this article, we investigate the influence of symbiotic microbes on niche limitations using the brine shrimp (*Artemia*) as a model system. *Artemia* are Branchiopoda that live in continental hypersaline environments, from salt marshes to lakes, where their abiotic niche is strongly structured by salt concentration (van Stappen 2002). This predominant axis of their niche offers a puzzling and unexplained situation, which can be labeled the low-salinity paradox. Brine shrimps are not found in nature at salinities below 40 g/L (Lenz and Browne 1991). This distribution is often explained by the presence of fish predators at low but not high salinities (Camargo 2002; Litvinenko et al. 2007). Although this is certainly an important factor in the wild, experiments also show that *Artemia* have strongly reduced juvenile survival at low salinities in the laboratory without predators, which probably contributes significantly to their niche limits (Abatzopoulos et al. 2003; Baxevanis and Abatzopoulos 2004; Castro-Mejía et al. 2011). The underlying mechanism could be that brine shrimps have a low physiological tolerance to low salinity, like most marine organisms. However, *Artemia* conserved their ancestral near-freshwater hemolymph thanks to very efficient sodium-potassium adenosine triphosphatase pumps that regulate inner medium in the face of salinities up to 140 g/L (Holliday et al. 1990; Weekers et al. 2002). Hence, we do not expect a physiological cost at low salinities for the brine shrimp. On the contrary, low salinity induces lower salt excretion and thus lower adenosine triphosphate consumption, which should cause less metabolic cost relative to high salinity. From the physiological standpoint, there is thus a paradox. Given the biology and evolutionary history of brine shrimps, what causes *Artemia* to have poor tolerance of low salinities?

Brine shrimps feed mostly on unicellular algae, such as *Dunaliella salina*, found in salt marshes and lakes (Lenz and Browne 1991). Most animals are unable to feed on such algae without specialized microbiota that provide them with essential nutrients (vitamins or amino acids), help them digest complex molecules (e.g., long carbohydrates such as cellulose), or eliminate toxins (Burroughs et al. 1950; Karley et al. 2002; Brune and Ohkuma 2011). Salinity in brine shrimp guts is the same as in the external environment (Geddes 1975; Holliday et al. 1990) and can thus directly affect growth and survival of microbiota. Here, we hypothesize that and investigate whether poor adaptation of brine shrimp gut microbiota causes the poor performance of brine shrimp at low salinity; in other words, we explore whether the fundamental and abiotic niche of *Artemia* results primarily from the process of adaptation of its gut microbiota rather than of its own genome.

Material and Methods

Artemia *Survival Experiment*

Artemia franciscana is a sexual species of brine shrimp from North America. Like all species of this genus, it can produce both active larvae (nauplii) and encysted diapause eggs (cysts), depending on environmental conditions. Cysts are produced as resistant forms and can hatch into nauplii larvae after a dormancy period ranging from a few days to several years. In our experiments, we used *A. franciscana* cysts obtained in 2007 from the Great Salt Lake in Utah (hereafter GSL07). Great Salt Lake *Artemia* were massively introduced in Aigues-Mortes salt ponds (Languedoc-Roussillon, France) in 1979 (Rode et al. 2013*b*) and are now coexisting in the salt ponds with parthenogenetic endemic populations. These cysts were provided by the *Artemia* Reference Center (reference ARC1710).

Axenic Culture and Microbiota Transmission. We first determined whether bacteria could be vertically transmitted through cysts. Because cysts are at the gastrula development stage, it is indeed possible that vertical transmission of gut microbiota occurs from the mother *Artemia* directly into the gastrula. To test this, we removed the chorion layer that acts like a shell and protects the cysts. This operation, called decapsulation, consists in soaking cysts in bleach, and it is often used to improve hatchability in brine shrimp cultures (Clegg 1986), but here our aim was to eliminate bacteria that would be on the surface of the chorion. We performed this experiment for 0–30 min, in increments of 5 min, until all cysts took on a light orange color. To evaluate whether some bacteria were still present after decapsulation, we performed polymerase chain reaction (PCR) using universal bacterial primers (63f: 5′-CAG GCC TAA CAC ATG CAA GTC-3′ [Marchesi et al. 1998]; B6r: 5′-TTG CGG GAC TTA ACC CAA CAT-3′ [Manceau and Horvais 1997]) on crushed decapsulated cysts (30 μL Phusion mix, 6 μL sterile water, 1 μL of each primer, and 2 μL of crushed decapsulated cysts;

94°C for 3 min; 35 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 1.5 min; and finally 72°C for 7 min; fig. 2).

Survival Experiment. To determine the influence of microbiota on the salinity tolerance of *Artemia*, we first measured juvenile survival in an experiment involving four treatments at both high and low salinities. Earlier studies have indicated that juvenile survival is a strong determinant of variation in overall fitness. For instance, using a full demographic projection model, Sukumaran and Grant (2013) demonstrated that the elasticity of population growth rate (Caswell 2001) is strongest with respect to juvenile survival in *A. franciscana*. More specifically, *A. franciscana*'s low performance at low salinity was shown to be due mostly to high juvenile mortality (Castro-Mejía et al. 2011). Technically, investigating niche limits would require determining environmental conditions where the population growth rate becomes negative. This is not possible under laboratory conditions that do not include the effects of density dependence and resource fluctuation of predator or of parasites, which all largely reduce demographic performance. However, if large differences in juvenile survival are found in the laboratory, they are likely to also have a large demographic impact in the field. Furthermore, the functional importance of microbiota to juveniles is expected to carry over to adults that have similar diet, thus also affecting survival at later ages.

We did a full factorial experiment with conditions being axeny (sterile or nonsterile), diet (algae or yeast), and salinity (low or high). When brine shrimps were grown with autoclaved algae as a food source under standard nonaxenic conditions, we expected to observe the low-salinity paradox (i.e., that brine shrimps have lower survival at low salinity under natural conditions). When brine shrimps were grown with the same food source but under axenic conditions, we expected very poor performance at either low or high salinity if gut microbiota are important for algae digestion. Finally, processed yeasts are easily digestible and, in particular, do not require gut microbiota to be assimilated (Coutteau et al. 1990). If gut microbiota cause the low-salinity paradox, we expected high survival at both salinities with yeast food source, regardless of sterility condition. However, because yeast is not a natural food source of *Artemia* and can also be used as a food source by bacteria, we did not have strong expectations regarding the relative performance of axenic versus nonaxenic conditions with yeast as a food source (yeast may either interact positively or negatively with microbiota in nonaxenic conditions).

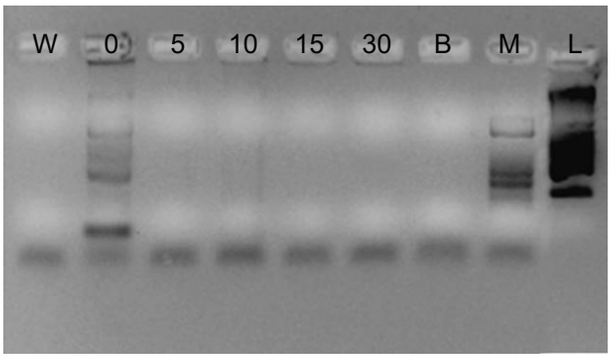

Figure 2: Test for vertical transmission in cysts. Numbers (0, 5, 10, 15 and 30) indicate the duration of the bleach treatment on cysts in minutes. Our negative controls showed no amplification, confirming the absence of contaminant DNA in our polymerase chain reaction mix. Our positive control displayed several fragments of different sizes, demonstrating the efficiency of our primers. B = negative control with brine; L = ladder; M = positive control of the nonsterile hatching media; W = negative control with sterile water.

The GSL07 cysts were hatched in sterile conditions (Makridis et al. 2000; Dhont and Sorgeloos 2002). Cysts were hydrated for 1 h in sterile water and decapsulated using bleach for 10 min (i.e., all chorion layers were removed). They were then rinsed in sterile water and placed in a 400-mL autoclaved solution of brine (5 g/L) containing a small amount of *Dunaliella salina* algae. Cysts were left to hatch in the sealed bottle for 3 days at ambient temperature under continuous light. Hatched nauplii were then placed in sterile and nonsterile brine solutions and fed with a sterilized food source of either *D. salina* or *Saccharomyces cerevisiae* (Superlevure, Gayelord Hauser). Brine solutions at 5 g/L and 80 g/L were obtained by diluting saturated brine (250 g/L) collected directly in the Aigues-Mortes salt ponds with osmosed water. In the sterile treatments, these brine solutions were autoclaved and checked for salinity before and after sterilization. Algae and yeast food solutions were autoclaved independently; 300 mL of those food solutions were added for 1 L of sterile or nonsterile brine solutions. This ensured that the sterile and nonsterile conditions did not differ in terms of possible denaturation of food source by the autoclave. For each treatment, six nauplii per tube were transferred in 25 sterile 50-mL Falcon tubes containing 40 mL of brine solutions. This transfer was performed under a laminar flow hood to preserve sterile conditions. The tubes were closed to maintain sterility, leaving enough air to allow respiration for the duration of the experiment. The full experiment was performed twice at two different dates to ensure reproducibility. Each time the experiment was performed, a total of 900 nauplii were evenly distributed among the nutrition treatments (nonsterile algae, sterile algae, and sterile yeast) and salinities (5 g/L or 80 g/L). Tubes were placed vertically and randomly using a Latin square design (Saville and Wood 1991) and were incubated for 4 days at 25°C and with a 12L : 12D photoperiod. At the end of this period, tubes were emptied in a net (120-μm mesh), and surviving nauplii were counted under a binocular.

Statistical Analysis of Nauplii Survival. Data from the two iterations of the experiment were analyzed jointly. We used generalized linear models (GLMs) with binomial error distributions to test our hypothesis about the influence of axeny, diet, and salinity on the survival of nauplii 8 days after hatching. Several possibly confounding factors might have an influence on our results and were included in our model selection. First, there may be an effect of experiment date or plate position (one parameter per stove level). We also controlled for an effect of light, with five levels depending on the distance of tubes to the light source. Model selection was performed in R (R2.14.2, available at http://www.r-project.org) using the MuMIn package (Barton 2013) and was based on quasi Akaike information criterion (QAIC; Akaike 1974; Peng et al. 2006). QAIC corrects for potential overdispersion in the data. Overdispersion estimate ($\hat{c}$) was calculated from the residual deviance between the best model (based on Akaike information criterion score) and a saturated model, where one survival probability is estimated per tube. Results from this model selection are presented in table 1 and figure 3. To determine the significant differences in survival among conditions in the best model, we performed pairwise post hoc comparisons based on two-tailed *Z*-tests (see appendix, available online), penalizing for multiple testing (Bonferroni correction). This analysis indicated which combinations of axeny, diet, and salinity were significantly different from each other.

Microbiota Growth Experiment

To determine whether *Artemia* gut microbiota were able to digest algae and grow at low salinities, we isolated microbiota from *A. franciscana* guts and measured their population growth capacity in different media. We used a full-factorial design with two treatments: diet (rich media with a combination of sources of carbon vs. poor media with autoclaved algae as the sole source of carbon) and a gradient of salinity (from freshwater to saturation). Because the composition of the rich media is more diverse and easily accessible, we expected that bacteria would grow better on rich media than on poor media. The poor media were also used as a test for algae digestion by gut microbiota. We expected a lower growth rate in the poor media than in the rich media, regardless of salinity, and a screening of bacteria specialized in algae digestion. However, if gut microbiota are constraining the niche of *Artemia*, we also expected that bacteria growth would be inhibited at low salinities in both media.

For the extraction of gut microbiota, adult *A. franciscana* from the Aigues-Mortes salt ponds in France (sampling in

Table 1: Generalized linear model selection for *Artemia* survival experiment

Model	*K*	logLik	AIC	QAIC	ΔQAIC	w_i	Parameters
Best QAIC model	18	−703.50	1,443.00	666.63	.00	.58	Axeny × salinity + axeny × food + plate + date
Double interactions	19	−703.36	1,444.73	668.50	1.87	.23	Axeny × salinity + axeny × food + food × salinity + plate + date
Full interaction + plate + date	20	−703.10	1,446.20	670.27	3.64	.09	Axeny × food × salinity + plate + date
Best AIC model	25	−693.99	1,437.97	672.10	5.48	.04	Axeny × food + axeny × salinity + date × plate
Double interactions + plate × date	26	−693.83	1,439.67	673.96	7.33	.01	Axeny × food + axeny × salinity + food × salinity + date × plate
Best QAIC model + light	22	−702.85	1,449.70	674.05	7.42	.01	Axeny × salinity + axeny × food + plate + date + light
Full interaction + plate × date	27	−693.52	1,441.03	675.68	9.05	.01	Axeny × food × salinity + date × plate
Full interaction	8	−790.25	1,596.50	724.39	57.76	.00	Axeny × food × salinity
Independent effects	4	−824.52	1,657.04	747.11	80.48	.00	Axeny + food + salinity
Axeny	2	−836.35	1,676.71	753.72	87.09	.00	Axeny
Food	2	−1,012.26	2,028.52	911.41	244.78	.00	Food
Salinity	2	−1,017.86	2,039.72	916.43	249.80	.00	Salinity
Saturated	400	−275.65	1,351.31	1,040.54	387.00	.00	Date × tube

Note: Saturated model and Akaike information criterion (AIC) best model helped in estimating the overdispersion parameter value. Parameters of models are salinity (high or low), food (algae or yeast), axeny (sterile or nonsterile), date (experiment date), light (five levels of light condition), plate (position of the plate in the experiment), and tube (tube number in the experiment). K = the number of parameters for each model; logLik = log likelihood; QAIC = quasi-likelihood AIC corrected for overdispersion, where the overdispersion estimate $\hat{c} = 2.23$; w_i = Akaike weights; ΔQAIC = QAIC differences.

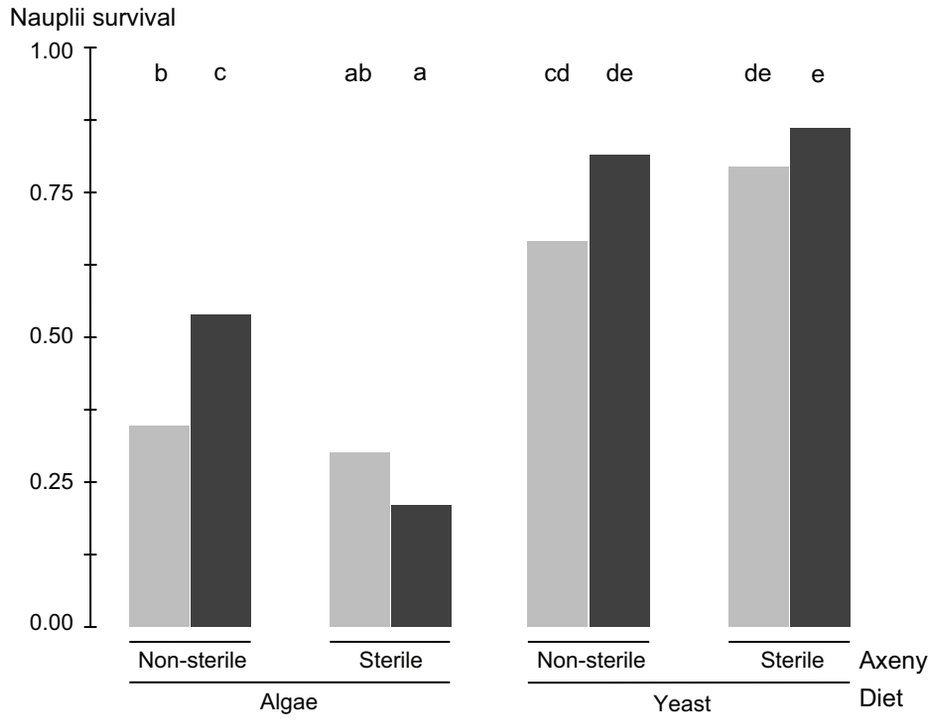

Figure 3: Survival of brine shrimp juveniles at low and high salinities under different food and axeny conditions. The bars represent nauplii survival estimates from the generalized linear model termed "best quasi Akaike information criterion model" in table 1. Light gray represents low salinity (5 g/L), and dark gray represents high salinity (80 g/L). Axeny (sterile or nonsterile) and diet (algae or yeast) correspond to the combination of treatments used to raise nauplii. Letters represent statistical comparison of categories corresponding to post hoc analysis (see table A1, available online). Nauplii survival estimates sharing the same letter are not significantly different.

the Fangouze pond: 43.504455, 4.224652; salinity, 170 g/L on February 13, 2013) were washed using ethanol to eliminate external bacteria and then rinsed with sterile water. They were then crushed, and the homogenate was filtered using a vacuum pump and a 1-μm filter to concentrate the microbiota inoculum. This bacterial community was grown in marine medium (25 g/L of Marine Broth 2216; Difco, Fisher Scientific) for one night at 30°C and conserved in a 15% glycerol solution at −80°C. The rich media contained 0.5% yeast extract, 0.5% casamino acid, 0.5% $MgSO_4$ $7H_2O$, 0.3% trisodium citrate, and 5% sodium chloride (Wang et al. 2009). The poor medium was composed with 1,000 cell/mL of algae. We used a low density of food in the poor media to prevent the senescence of algae (degradation of chlorophyll to nonfluorescent catabolites; Hörtensteiner 2004) from modifying optical density, thus interfering with the increased optical density caused by bacterial growth. We used a TECAN Infinite 200 microtiter plate reader to measure growth curves at different salinities (0, 20, 40, 60, 80, 100, 140, 180, 220, and 250 g/L). To quantify bacterial growth along the salinity gradient, we used carrying capacity (maximal optical density after 24 h; OD_{max}) for three replicates per treatment. Results from this experiment are presented in figure 4.

Reinoculated Artemia *Survival Experiment*

To identify bacteria involved in algae digestion in *Artemia*, we used a candidate-species approach. A recent study found that *Salinivibrio* was prevalent in guts of nauplii and adult brine shrimps sampled from Israeli salt ponds with minimal salinity of 71 g/L and 89 g/L, respectively (Tkavc et al. 2011). We thus expect that the inoculation of axenic nauplii with isolates of *Salinivibrio* from the gut of wild *Artemia* might help restore, at least partially, the wild-type phenotype (i.e., lead to higher survival in hypersaline environments, defined as 80 g/L in our experiment). To ascertain that *Salinivibrio* bacteria were not used as a food resource by *Artemia* nauplii, which are nonselective filterers, we exposed nauplii to gut bacteria for a short inoculation period (only 2 h) and then grew them in sterile medium.

Salinivibrio *Isolation and Inoculation Protocol.* We first isolated colonies of *Salinivibrio* by spreading some *A. franciscana* homogenate from Aigues-Mortes (Fangouze pond) on a solid marine medium (Marine Broth 2216) with 5% of agar. Single bacterial colonies were picked out for conservation and characterization. Sampled colonies were grown in

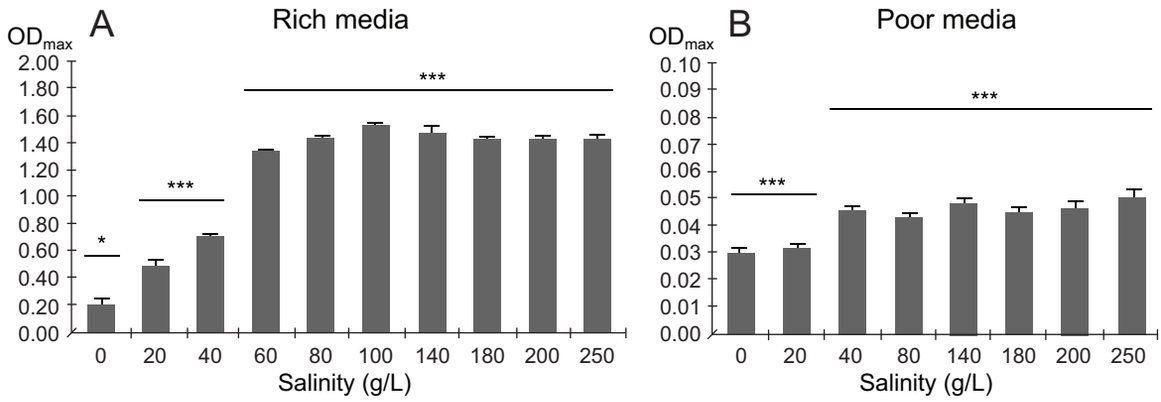

Figure 4: Salinity niche of bacteria community from *Artemia* gut. Graphs display maximal absorbance (OD_{max}) recorded at each salinity for communities grown in a rich medium with yeast extract as the main metabolic resource (*A*) and a poor medium containing only algae for metabolic use (*B*). Error bars represent standard deviation. Lines represent categories determined by ANOVA tests (rich medium with three salinity categories: $F = 167.9$, df $= 2$, $P = 1.22 \times 10^{-6}$; poor medium with two salinity categories: $F = 51.05$, df $= 1$, $P < 10^{-3}$).

liquid marine medium for one night at 30°C and conserved in a 15% glycerol solution at −80°C. The same colonies were used for genus identification, and PCR was performed using universal bacterial primers (63f: Marchesi et al. 1998; B6r: Manceau and Horvais 1997; see above). PCR products were sequenced (GenoScreen; Lille, France), and the sequences were blasted against the National Center for Biotechnology Information prokaryote database to identify the isolates corresponding to *Salinivibrio*.

Artemia cysts (GSL07) were hatched in sterile conditions (see above for details). After 3 days under continuous light and ambient temperature, hatched nauplii were transferred for 2 h into three types of inoculation solutions: (1) a nonsterile brine adjusted to 80 g/L by diluting saturated brine (250 g/L) collected directly in the Aigues-Mortes salt ponds with osmosed water, (2) a sterile brine (solution 1 autoclaved), and (3) a reinoculated brine obtained by adding overnight fresh culture of one *Salinivibrio* strain to solution 2. The latter was done independently for six *Salinivibrio* strains. For each inoculation treatment, 25 replicated groups of six nauplii were transferred, in sterile conditions, into Falcon tubes containing 40 mL of brine solutions and sterilized algae as food source (*D. salina*; see above for details). The experiment was performed twice at two different dates with three different strains of *Salinivibrio* used in both iterations of the experiment, plus three other strains of *Salinivibrio* used only in the second performance of the experiment. In each iteration of the experiment, the nauplii (750 in the first iteration and 720 in the second iteration) were evenly distributed among the treatments. Tubes were placed horizontally to eliminate light effect (see appendix), randomized using a Latin square design (Saville and Wood 1991), and incubated for 4 days at 25°C with a 12L : 12D photoperiod. At the end of this period, tubes were emptied in a net (120-μm mesh), and the number of surviving nauplii per tube was counted under a binocular.

Reinoculation Experiment Statistical Analysis. Data from the two experiments were analyzed jointly. We used GLM with binomial error distributions to test our hypothesis about the influence of *Salinivibrio* inoculation on the survival of nauplii 8 days after hatching. Because there may be an effect of experiment date or plate position (one parameter per stove level), we included these confounding factors in our model selection. Model selection was performed in R using the MuMIn package (Barton 2013) and was based on Akaike information criterion (AIC; Akaike 1974). We used AIC-based selection, because data were not overdispersed ($\hat{c} = 0.97$). Overdispersion was calculated from the residual deviance between the best model (based on AIC score) and a saturated model where one survival probability is estimated per tube. Results from the model selection are presented in table 2 and figure 5. To determine the significant differences in survival within and among conditions, we used a post hoc AIC-based analysis. This analysis helped us to determine which combinations of treatments were significantly different. Combinations tested are displayed in table 3.

Results

Data underlying the results are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.366nd (Nougué et al. 2015).

Artemia *Survival Experiment*

Axenic Culture and Microbiota Transmission. For our axenic treatments to be valid, we first needed to rule out the

Table 2: Generalized linear model selection for reinoculation *Artemia* survival experiment

Model	K	logLik	AIC	ΔAIC	w_i	Parameters
Best AIC model	9	−209.51	437.02	.00	.74	Condition + date
Best AIC model + plate	16	−204.08	440.16	3.14	.15	Condition + date + plate
Condition + plate	15	−206.01	442.02	5.00	.06	Condition + plate
Condition	8	−213.61	443.22	6.20	.03	Condition
Condition + date × plate	21	−201.85	445.7	8.68	.01	Condition + date × plate
Saturated	264	−85.93	699.87	262.85	.00	Tube × date

Note: Best Akaike information criterion (AIC) model and saturated models helped in estimating the overdispersion parameter value ($\hat{c} = 0.97$). Parameters of models are condition (sterile, nonsterile, and six levels of *Salinivibrio* strains), date (experiment date), plate (position of the plate in the experiment), and tube (tube number in the experiment). K = number of parameters for each model; logLik = log likelihood; w_i = Akaike weights; ΔAIC = AIC differences.

possibility that bacteria were vertically transmitted through cysts. We detected the presence of bacteria by amplification only for homogenate of cysts that were not bleach decapsulated (fig. 2). The size of the amplified fragments for the homogenate of nondecapsulated cyst was different from that for the positive control consisting in nonsterile brine. This could be explained by a differential composition of bacterial communities between the growing medium and the lumen of the brine shrimps' gut or by a difference in origin of the brine (Aigues-Mortes, France) and the cysts (Great Salt Lake, Utah). Nonamplification on cysts that were bleach decapsulated from 5 to 30 min (fig. 2) indicated that bacteria on or under the surface of the cyst shell were entirely removed. Cysts were still able to hatch after 30 min in bleach (result not shown), which indicates that bleach does not enter the embryo. Hence, bacteria are not present inside of the embryo but only on the cyst's chorion, and cysts are therefore sterile after decapsulation.

Influence of Microbiota on Artemia *Juvenile Survival.* We measured juvenile survival in an experiment involving four treatments at both high and low salinities. The survival analysis indicated a moderate overdispersion ($\hat{c} = 2.23$) that required a model selection based on QAIC to correct for the variability in our data set. The models that best describe the data on *Artemia* survival include large and significant axeny × salinity and axeny × diet interactions (table 1). These effects were consistent across the two replicated experiments (the term interaction with date is absent from all best models). The best model also included a plate effect and experimental date effect. We will return to these effects below, but overall these results demonstrate that the global interaction between the four treatments and salinity pattern is the same for all replicates. Parameter estimations and post hoc analyses indicated that our results were consistent with the hypothesis that microbiota are responsible for the reduced survival of *Artemia* at low salinity on

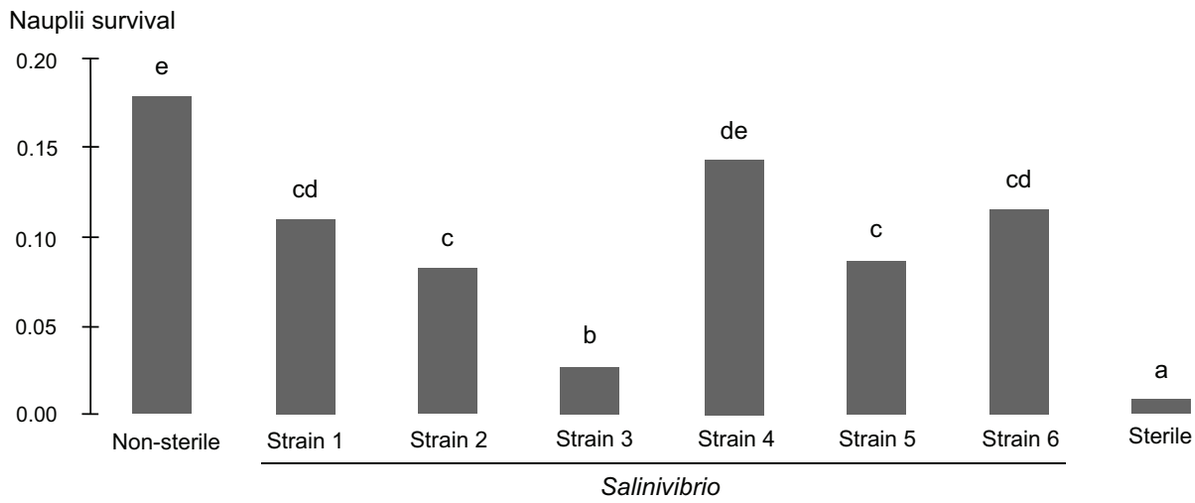

Figure 5: Survival of brine shrimp when inoculated with different *Salinivibrio* strains. Nauplii survival estimates are calculated from the effect sizes in the generalized linear model termed "best Akaike information criterion model" in table 2. Sterile and nonsterile conditions correspond to experimental controls. *Salinivibrio* strains (1–6) correspond to nauplii inoculated with various strains of *Salinivibrio* isolates. Letters represent categories corresponding to post hoc analysis (table 3). Survival rate estimations sharing the same letter are not significantly different.

Table 3: Post hoc Akaike information criterion (AIC)–based analysis for reinoculation *Artemia* survival experiment

		Salinivibrio strain											
Post hoc model	Nonsterile	S1	S2	S3	S4	S5	S6	Sterile	K	logLik	AIC	ΔAIC	w_i
Group F	d	c	c	b	d	c	c	a	5	−210.38	430.75	.00	.33
Group E	e	d	c	b	e	c	d	a	6	−209.75	431.49	.74	.23
Group G	d	c	c	b	c	c	c	a	5	−210.86	431.72	.97	.20
Group D	f	d	c	b	e	c	d	a	7	−209.55	433.11	2.36	.10
Group C	e	c	b	a	d	b	c	a	6	−211.05	434.10	3.35	.06
Group A	g	e	c	b	f	d	e	a	8	−209.54	435.07	4.32	.04
Group B	f	d	b	a	e	c	d	a	7	−211.04	436.07	5.32	.02
Best AIC model	g	f	c	b	g	d	e	a	9	−209.51	437.02	6.27	.01
Group H	c	c	c	b	c	c	c	a	4	−215.47	438.95	8.20	.01
Group I	c	b	b	b	b	b	b	a	4	−219.75	447.51	16.76	.00

Note: To determine which treatments were significantly different, we tested several models (groups A–I) where treatments with the same letter are set to have the same nauplii survival value. The best group is the model with the highest w_i. K = number of parameters for each model; logLik = log likelihood; w_i = Akaike weights; ΔAIC = AIC differences.

an algae diet. This pattern is presented in figure 3 (for observed data results, see appendix). In treatments mimicking natural conditions, with standard (nonaxenic) culture and an algae diet, *Artemia* have significantly lower survival at low salinity (35% survival) than at high salinity (54% survival). Under axenic conditions with an algae diet, *Artemia* displayed similarly low survival at either low or high salinity (<30% survival). With a yeast diet, *Artemia* displayed high survival (>80% survival) and comparable survival at both low and high salinities in both sterile and nonsterile treatments.

As mentioned above, the best model included two other effects in addition to the axeny × salinity and axeny × diet interactions. The plate and date effects are significant in our best QAIC model. We hypothesized that this results from the vertical position of the tubes and the uneven plate repartition in the stove, causing unequal light and temperature exposure (light effect might also explain overdispersion in our replicates). To test these hypotheses, a third experiment (see appendix) was performed that was aimed at entirely removing light effects. In this experiment, tubes were placed horizontally (instead of vertically) with equal exposure to light and therefore with the same influence of light and temperature. As expected, the best model had only axeny × salinity and axeny × diet interactions and was significantly better than the same model with a plate effect. Moreover, there was no light effect in this experiment, and overdispersion was strongly reduced compared with the two other experiments ($\hat{c} = 0.75$). Other results were entirely consistent with earlier findings.

Microbiota Growth Experiment

The microbiota associated with *Artemia* were able to grow on both the rich and the poor media. This indicates that at least some of the bacteria are able to digest algae. The OD_{max} of the cultures in the two growing media are depicted in figure 4, ranging from null to saturation salinity. As expected, the mean OD_{max} was globally higher on the rich medium (fig. 4*A*) than on the poor medium (fig. 4*B*). The carrying capacity was substantially reduced at low salinities in both the rich and the poor media. The carrying capacity in the rich media plateaued around $OD_{max} = 1.44$ for high salinities (between 80 g/L and 250 g/L; fig. 4*A*) but significantly decreased to 0.71 and 0.21 for salinities of 20–40 g/L and 0 g/L, respectively (ANOVA with three salinity categories: $F = 167.9$, df = 2, $P < 10^{-5}$). In the poor medium, carrying capacity remained around $OD_{max} = 0.047$ for salinities above 40 g/L (fig. 4*B*) but switched to lower OD_{max} values around 0.031 for salinities under 20 g/L (ANOVA with two salinity categories: $F = 51.05$, df = 1, $P < 10^{-3}$). Hence, regardless of the media, bacteria isolated from the gut of the *Artemia* grew less well at low salinities (<40 g/L), where brine shrimps tend to have reduced survival.

These results involved bacteria retrieved from brine shrimps living in high-salinity brine in the field (170 g/L). Tkavc and collaborators (2011) showed that salinity has an impact on the composition of the bacteria retrieved from the gut of *Artemia*. We thus also sampled gut bacteria from *Artemia* living at low and medium salinities (40 and 80 g/L, respectively). These samples were isolated in different salinity conditions (0, 40, and 80 g/L), and their carrying capacity (OD_{max}) was similarly measured along a salinity gradient. For all samples, we found that bacteria grew better at high salinities than at low salinities. The salinity of origin (i.e., the salinity of the environment from which the *Artemia* were obtained in the field) affected only the importance of the difference between OD_{max} at low and high salinity, whereas the salinity of isolation increased or decreased the height of the plateau in OD_{max} (see appendix).

Reinoculated Artemia *Survival Experiment*

To identify bacteria involved in algae digestion in *Artemia*, we used *Salinivibrio* as a candidate species. We isolated strains of this bacterial genus from guts of wild *Artemia*. We found that the growth of those strains presented the same pattern across salinities as that of the overall bacterial gut community (see appendix). We used those strains to perform the reinoculation experiments (fig. 5). We found that nauplii inoculated with *Salinivibrio* strains survived significantly better than nauplii in sterile conditions, which supported *Salinivibrio* as a good candidate to explain the low-salinity paradox in *Artemia*. This recovery was only partial compared with nonsterile conditions, suggesting that other interactions (involving different *Salinivibrio* or other bacteria) might be involved in algae digestion. The recovery was also variable among different *Salinivibrio* (from 3% to 14% nauplii survival depending on strains). These patterns were consistent across replicates within experiments and between the two replicated experiments (we did not detect an interaction between conditions and experiment date in the GLM; table 2). Note that these reinoculation experiments consistently showed higher mortality than our other experiments following nauplii survival. The likely reasons are that (1) in all treatments, brine shrimps were exposed to a very limited amount of microbiota (only 2 h of inoculation), which probably limited their digestion, and (2) the brine shrimps were manipulated and transferred more often in the reinoculation experiments.

Discussion

Microbiota and Adaptation to Salinity in Artemia

We used the brine shrimp and its gut microbiota as a model system to investigate how interspecific interaction with a symbiotic partner results in union of the partners' niches along the axis of the interaction but intersection of their niches along orthogonal axes. This is illustrated in figure 1. The horizontal axis in this graph corresponds to the service traded by the symbiont to its host. In our case, this service is the ability to digest algae, but it could be protection against pathogens or provision of water and nutrients, for instance. Clearly, a mutualism causes an expansion of the niche along this axis, and conversely, removing the symbiont experimentally will result in niche contraction, as discussed (and graphically illustrated) recently by Afkhami et al. (2014), for example. In contrast, the vertical axis in figure 1 is seldom discussed in the literature on mutualism. It represents other niche dimensions not related to the particular service traded by the symbiont (here, tolerance to salinity). Along these dimensions, the range of environments accessible to the host is necessarily reduced by intersection with the range of environments that its symbiont can tolerate. In other words, in conditions that require the service provided by the symbiont, there is necessarily a constraint on niche extension along axes orthogonal to this service (dark shaded zone in fig. 1); at best, the niche along these axes is unchanged if the symbiont's tolerance limits are broader than its host's.

We conducted a set of experiments that jointly provide support for such a combination of niche union along the axis of the service traded but niche intersection along other axes for the brine shrimp and its microbiota. In the absence of the symbiotic partner (sterile treatments), *Artemia* has high survival at high and low salinities when fed on yeast but low survival at both salinities when fed on algae. This corresponds to the area delimited by a continuous line in figure 1 (niche in the absence of interaction). With the symbiont (nonsterile condition in our experiments), *Artemia* can survive on algae, but juvenile survival is severely reduced at low salinity. Hence, compared with the niche without interaction, the niche with the interaction (shaded area in fig. 1) expands toward algae along the horizontal axis (light shaded area in fig. 1). Along the other axis, the niche with the interaction is constrained by the very poor performance of the gut microbiota with respect to survival at low salinities (dark shaded area in fig. 1). Our experiments on bacterial growth (fig. 4) show that this is caused by the low tolerance of the microbiota to low salinity (niche limited by dashed line in fig. 1), some of which are likely to digest algae (fig. 4*B*). Our reinoculation experiments with *Salinivibrio* strains isolated from adult wild *Artemia* identify these strains as good candidates for mediating these effects, because they partially restore survival when reinoculated in axenic nauplii. These bacterial strains also exhibit very limited growth below salinity of 40 g/L, consistent with the prediction that their niche constrains the niche of the host. Overall, in natural conditions, where algae are the prominent food source, the niche limit of *Artemia* at low salinity is thus probably largely set by their microbiota.

Although we do not evaluate fitness in the field, our results do provide a functional basis for understanding constraints on *Artemia*'s niche at low salinity. The niche of *Artemia* is known to exclude low salinity (<40 g/L), even in the absence of predators, but this niche limit remains poorly understood, notably because of the absence of clear physiological constraints for *Artemia* at low salinity. We report a substantial effect of salinity on the proportion of surviving juveniles over only 4 days of experiment (42%, 31%, and 52% proportional decrease in survival in experiments 1, 2, and 3, respectively; see fig. 3 and appendix). Extrapolating these results to a full demographic impact in the field (or even just in the laboratory) is beyond reach with our data, but juvenile survival is known to be a prominent determinant of *Artemia*'s demographic performance (Sukumaran and Grant 2013). In the nonsterile treatment, our results on juvenile survival are consistent with earlier studies on the low-salinity paradox

(McCarraher 1970; Castro-Mejía et al. 2011), so we are confident that our experiments reveal important factors that constrain *Artemia*'s niche at low salinity.

Niche limits are generally thought to result from ecological trade-offs, whereby mutations conferring adaptation to some conditions exhibit antagonistic pleiotropic effects under other conditions (Antonovics 1976; Barton and Partridge 2000). Here, the antagonism underlying the food-salinity trade-off is not set by *Artemia*'s genes but by interaction with the genes of its microbiota. This is particularly remarkable given that salinity is a prominent axis of *Artemia*'s niche (van Stappen 2002), overriding other abiotic parameters, such as ionic composition or temperature (Bowen et al. 1985; Vanhaecke et al. 1987; Browne et al. 1988), or biotic parameters, such as fish and bird predators or parasites (van Stappen 2002; Rode et al. 2013*a*). Thus, in this case study, the symbiont plays a prominent ecological role, because it constrains a major axis of *Artemia*'s niche.

Several features of *Artemia* and its microbiota that are also relevant to the more general issue of symbiont-mediated constraints on the niche remain to be fully elucidated. First, the candidate bacteria that we reported (*Salinivibrio*) may be only a part of the microbial community involved in the mutualism. Second, it remains to be shown whether there is strong specificity between *Artemia* species or genotypes and their gut symbionts. Third, because host-microbiota specificity generally depends on environmental factors (Spor et al. 2011), it would be important to investigate whether *Artemia* can switch their symbionts under different environmental conditions, as is done by corals with their zooxanthellae (Berkelmans and Van Oppen 2006; Reshef et al. 2006) or leafcutter ants with their mutualistic fungi (Mueller et al. 2011). In more complex symbiotic communities (e.g., plant ectomycorrhizal networks; Selosse et al. 2006), symbiont switching can even lead to the establishment of a symbiont "market" mediated by the niche limits of all possible partners (Noë and Hammerstein 1995; Cowden and Peterson 2009; Bever et al. 2010; Kiers et al. 2011). In *Artemia*, the overall gut bacterial community does change across salinities (Tkavc et al. 2011), but this may not directly concern the mutualistic community. If brine shrimp could switch their symbionts depending on salinity, the low-salinity paradox could be overcome in a form of symbiont-mediated phenotypic plasticity. Nevertheless, in our case, there is very limited evidence in favor of this symbiont-switching hypothesis. As we have shown, bacteria from the gut of *Artemia* collected at low or high salinity did not grow differently along the salinity gradient (and both grew very poorly at low salinities; see appendix). Furthermore, the low-salinity paradox, commonly reported in *Artemia* research (see in van Stappen 2002; Castro-Mejía et al. 2011), is at odds with this symbiont switching idea. However, it would be worthwhile to inspect this hypothesis in more detail and with a more functional approach to understand whether acclimation to a changing salinity can be partly attributed to a switching of facultative symbionts. This phenomenon may be important in the first stages of invasions involving large shifts in salinity in euryhaline organisms, for which genetic changes in the physiological determinants of the tolerance curve have already been demonstrated (Lee 1999; Lee et al. 2011, 2012; Kozak et al. 2013). Fourth, the possibility to acquire or change microbiota may be dependent on age or development. *Artemia*'s diet switches from stored maternal resources to an algal diet during their larval development. Whether the early established microbiota community changes later in life or remains constant is open to further investigation, which may shed a new light on the underlying mechanisms of larval versus adult survival under different environmental conditions.

Empirical and Conceptual Implications

Beyond their conceptual importance for evolutionary and ecological thinking, these findings have direct relevance to the study of traits within species. Interactions with the microbiota may contribute to generating large experimental error in the laboratory if they are not controlled for (which they rarely are). This is especially true in experiments that study variation (e.g., heritability and plasticity) of highly integrated traits (e.g., fitness components) in organisms that acquire their microbiota from their environment rather than from direct maternal transmission.

Regarding the underpinnings of environmental tolerance curves, a key tool in the context of species persistence in the face of climate change (Deutsch et al. 2008; Chevin et al. 2010; Lande 2014), our findings fit within the broad $G_1 \times G_2 \times E$ niche concept (Vale et al. 2008): the range of environments (E) suitable for a focal species (G_1) depends not only on its adaptation to the environment ($G_1 \times E$) but also on its interactions with symbionts ($G_1 \times G_2$), adaptation of these symbionts to the environment ($G_2 \times E$), or even interactions with symbionts that depend on the environment ($G_1 \times G_2 \times E$).

For mutualistic interactions, $G_1 \times G_2 \times E$ takes the form of niche expansion along the axis of the interaction, combined with niche intersection on other axes (fig. 1). This effect of biotic interaction on the niche adds to the more commonly acknowledged exclusion effect between antagonists, which is central to the concepts of realized versus fundamental niche. Combining intersection-union with exclusion provides an integrated understanding of how biotic interactions transform the abiotic niche in more complex ecosystems involving more actors. For instance, if a pathogen's niche envelope was added to figure 1, the interplay of these simple rules (union-intersection and exclusion) would define a clearer set of possible effects of any interaction on the niche of the focal species.

Conclusion

Almost every living macroorganism has symbiotic interactions with microorganisms, whose influence on the adaptation of the host is only starting to be elucidated (Feldhaar 2011; Hansen and Moran 2013). Our case study of a nonobligatory association between *Artemia* and its gut microbiota show that the niche limit of brine shrimp at low salinities might not be directly caused by its history of adaptation or by mutations in the *Artemia* genome from its freshwater ancestor. Instead, it probably results from its dependence, for algae digestion, on bacterial microbiota that do not tolerate low salinity. That symbionts and their hosts have to share niche limits along all axes, not just those directly involved in their interaction, is probably a major cost of symbiotic mutualism, which opens a rich array of interesting questions in evolutionary ecology.

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