More or Less? The Effect of Symbiont Density in Protective Mutualisms

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ABSTRACT: Symbionts can provide hosts with effective protection from natural enemies, but it can sometimes come at a cost. It is unclear to what extent the density of symbionts modulates the cost and benefits of conferred protection. Here, we use a meta-analysis of 103 effect sizes from a broad taxonomic range of protective symbioses to show that the degree of both protection and cost afforded to hosts is a positive function of symbiont density. We found that the effects of symbiont density on protection and cost are robust across ecological contexts. Density-function relationships did not vary with host type, symbiont localization, or transmission mode, nor did they vary with the method of density manipulation. Taken together, our results suggest that symbiont density can be a key variable determining the costs and benefits of a protective interaction.

Keywords: protective symbiosis, defense, symbiont density, host-symbiont interactions, density dependence.

Introduction

Symbionts can provide their hosts with a source of protection against natural enemies (Bronstein et al. 2006; King 2019). Such protective interactions occur across phylogenetically disparate taxa, from intracellular bacteria that inhibit viral replication in insect hosts (Martinez et al. 2014) to ants that protect plants by biting mammalian herbivores (Goheen and Palmer 2010). In many of these interactions, the simple presence of a given symbiont is not sufficient to ensure a protective effect. The density of the symbiont can also influence symbiotic phenotypes (Holland et al. 2002; Nyholm and McFall-Ngai 2004; Cunning and Baker 2014; Laughton et al. 2014) and may directly determine the effectiveness of symbiont-conferred protection. For example, in antiherbivore symbioses a higher density of symbionts can enhance the patrolling power on host plants (Janzen 1966; Rocha and Bergallo 1992). Similarly, for host-microbe symbioses, greater densities of protective microbes may generate higher toxin levels (King et al. 2016; Ballinger and Perlman 2017), increase a competitive advantage against an enemy (Paredes et al. 2016), or heighten immune regulation (Pan et al. 2012), thus providing more effective protection.

Utilizing symbionts for protection can be harmful to the host (Frederickson et al. 2012; King and Bonsall 2017), just as host-encoded responses against enemies are generally considered to be costly (Antonovics and Thrall 1994; Schmid-Hempel 2003; Graham et al. 2005). For example, mites protect potter wasps against lethal parasitoids, but in the absence of this enemy, developmental costs ensue as mites feed on host hemolymph (Okabe and Makino 2010). High symbiont densities may increase costs, generating greater exploitation of host resources or causing "collateral damage" from symbiont protection mechanisms (e.g., nonspecific toxin activity or tissue damage; Ballinger and Perlman 2017).

When investing in protective symbionts, hosts can face changes to costs and benefits as a direct result of symbiont density (for examples, see fig. 1). The role of density in determining the net benefit may also be critical for determining whether the symbiont or the host immune system provides the best protection strategy for the host (King and Bonsall 2017). Trade-offs between protection and cost as a function of symbiont density have been suggested in the interaction between *Drosophila* and the bacterial symbiont *Wolbachia*. Here, the extent of protection against viruses is directly related to within-host *Wolbachia* density (Osborne et al. 2012; Chrostek et al. 2013; Martinez et al. 2014). However, costs also scale, with high *Wolbachia* densities shortening host life span (Chrostek et al. 2013; Martinez et al. 2015). Trade-offs between host protection and host longevity might explain symbiont dynamics in natural populations of *Drosophila*. Low-density variants of *Wolbachia* have been selected to replace high-density

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Figure 1: A diversity of protective symbioses show evidence for a trade-off between protection and cost as a function of symbiont density. Increases in symbiont density are associated with increases in protection (blue) and cost (red) in interactions between mosquitoes and *Wolbachia* bacteria (Lu et al. 2012), aquatic gastropods and *Chaetogaster* worms (Hopkins et al. 2013), Vespidae wasps and *Ensliniella* mites (Okabe et al. 2010), and *Acacia* trees and *Crematogaster* ants (Palmer et al. 2013). Photo credits: *top row, left to right*—James Gathany, Public Health Image Library; Scott Bauer, USDA Agricultural Research Service, Bugwood.org; Christoph Kornmilch; Cpeeters, https://www.antwiki.org; *bottom row, left to right*—Zhiyong Xi, Michigan State University; Michal Maňas; Zoologische Staatssammlung München, Arthropoda varia section; Will Ericson [CASENT0904507], from https://www.antweb.org.

variants, despite their inferior antiviral protection (Riegler et al. 2005; Chrostek et al. 2013).

Might symbiont density be a key variable that mediates transitions along the parasitism-mutualism continuum? Many symbioses can shift along the continuum (Bronstein 1994; Drew et al. 2021), and changes to symbiont density may be a path by which mutualism breakdown can occur, as net benefits for hosts transition to net costs (Fonseca 1993; Sachs and Simms 2006; Hopkins et al. 2017). In some systems, maximum levels of protection are reached at low to medium symbiont densities, with only costs accruing at higher densities. Among acacia trees, levels of protection against browsing elephants saturate at medium densities of symbiotic ants, but the cost of reduced fruit production continues to be paid (Palmer and Brody 2013). Branchiobdellidan worms protect host crayfish from colonization by epibionts and parasites, but at high densities the worms begin consuming host gill tissue (Brown et al. 2012), potentially shifting the interaction toward a net detrimental effect for the host. Whether these relationships are general is crucial for the applied use of protective symbionts for disease management (Ford and King 2016), food security (Le Cocq et al. 2017; Mendiola et al. 2020), and ecosystem conservation. For example, when using defensive symbionts in biocontrol, it would be valuable to know the optimum titer for applying to crops threatened by pathogens or insect pests (Ownley et al. 2008; Saldajeno and Hyakumachi 2011). These relationships may also identify threshold densities that symbionts should be maintained below in crop systems, before costs to yield emerge.

Here, we use a meta-analytical approach to examine the generality of the relationship between symbiont density and the level of protection and cost for hosts. To this end, we searched published literature for studies of protective symbioses and quantified the effect of symbiont density via a standardized effect size measure (correlation coefficient, r), using multilevel models to account for the nonindependence of effect sizes due to the same study, host species, or symbiont genus. Overall, we demonstrate that the degree of both protection and cost is a positive function of symbiont density across taxonomically diverse symbioses. We recognize that assessing the effect of symbiont density can be complex, due to interactions with many ecological factors (e.g., temperature, enemy factors) and systems where symbiont density cannot be manipulated easily (e.g., intracellular symbionts). Where feasible, we evaluated the effect of ecological factors of host type, symbiont localization, transmission mode, and enemy type, in addition to the method of density manipulation and how protection or cost was measured. Across all ecological and methodological contexts tested herein, the degree of protection and cost remained a positive function of symbiont density. We suggest that further studies, testing the density dependence of protective phenotypes, are required to corroborate this.

Material and Methods

Literature Search

To identify relevant studies for inclusion in the meta-analysis, we searched the Web of Science database for

abstracts including the keywords "symbiont," "microbe," "endophyte," "density," "titre," "load," "defence," and "protect." Search terms were truncated with asterisks and arranged using Boolean logic operators to expand the search and avoid missing studies with alternative keyword spellings. This search retrieved 3,606 studies published from 1900 to February 2021, from which abstracts were filtered for relevance before full-text reading (for a PRISMA flow diagram, see fig. S1; figs. S1–S3 are available online). Studies were partitioned into two data sets, those exploring the protection conferred to hosts as a function of symbiont density and those exploring the cost (in the absence of an enemy). We did not seek to address the shape of the cost/benefit–density relationship or directly quantify the relationship between protection and cost, as very few studies used more than two different symbiont densities or measured both protection and cost within single experiments. Systems were included only if a protective effect has been demonstrated that is directly relevant for host fitness. For this reason, four studies reporting an effect of symbiont density on vector-borne microbes were not included, as harm from the "enemy" to the vector host has not been shown. Studies included in the final data set ($n = 34$) were subjected to forward and reverse searching to identify additional studies ($n = 24$), bringing the total number of studies to 58.

Extraction of Effect Sizes

To determine the strength of association between the density of protective symbionts and the benefits/costs to hosts, we calculated a standardized effect size (correlation coefficient, r) based on 68 effect sizes from 42 studies of protection and 35 effect sizes from 16 studies of cost. When density measurements were binned into ordinal variables and no correlation coefficient was reported, available values were transformed into r as follows. Where Z or χ^2 statistics were available, r was approximated by $z/\sqrt{N}$ and $\sqrt{\chi^2/N}$, respectively, else r was calculated from the mean and variance of low-density versus high-density groups (Koricheva et al. 2013) or approximated from odds ratios by the ϕ coefficient (Yule 1912; Bonett 2007; Field and Gillett 2010). For articles where the necessary values were not reported and data were not publicly available, we obtained data by contacting authors or extracting data points using web plot digitizer 4.2 (http://plotdigitizer.sourceforge.net), which has been validated as a reliable method of data point extraction (Drevon et al. 2016). All correlation coefficients underwent Fisher's Z transformation,

$$z = \frac{1}{2}\ln\left(\frac{1+r}{1-r}\right),$$

whereby the distribution of z is normalized and the variance approximates $1/\sqrt{N-3}$, which is independent of the strength of correlation (Fisher 1921). Determination of N was by the host sample size used for the original test statistic or, in the case of transformation, from means by combining the sample size of the groups. For presentation purposes, Fisher's z values were back transformed into correlation coefficients by

$$r = \frac{e^{2Zr} - 1}{e^{2Zr} + 1}.$$

Meta-analytical Model

A number of studies reported multiple effect sizes. To account for this dependency, multilevel models were fitted for both the protection data set and the cost data set under restricted maximum-likelihood estimation in R version 1.2.1335 (R Core Team 2013) using the package metafor (Viechtbauer 2015). Nesting effect ID within study ID in the multilevel model allows differentiation of variation in effect sizes due to sampling variation within studies and between studies (Raudenbush and Bryk 1985; Fernández-Castilla et al. 2020). To further account for nonindependence in the data set, symbiont genus (protection: $n = 27$; cost: $n = 15$) and host species (protection: $n = 36$; cost: $n = 16$) were included as crossed random effects. This approach allows multiple representations of the same host species and symbiont genus to be accounted for (Fernández-Castilla et al. 2019).

Univariate models were used to assess the contribution of additional biological and methodological factors to the overall effect estimate (for full definitions and inclusion criteria, see table S1, available online). These factors included broad categories of host type ("animal" vs. "plant") and the type of enemy a symbiont predominantly protects against ("animal enemy" vs. "microbial enemy"). We also explored whether the localization of symbionts, either on the surface of the host or within host bodies ("exo-" vs. "endo-"), contributed to the observed variation and whether the predominant mode of symbiont transmission mattered ("horizontal" vs. "vertical"). Regarding methodological context, we explored whether effects varied with the method of symbiont density manipulation. Here, we categorized by studies that physically manipulated symbiont densities ("physical," e.g., by the addition of known symbiont numbers) versus those that utilized inherent "natural" differences in density ("natural variation," e.g., due to location or variation in host population). We also examined whether the measure used to assess protection ("host survival/fitness" vs. "damage sites" vs. "enemy inhibition") or cost ("host fitness based" vs. "longevity" vs. "developmental") to the host affected effect size estimates. Likelihood ratio tests

using maximum likelihood estimation and Wald (χ^2) tests were used to assess the overall significance of each variable.

Assessing Publication Bias

To assess whether effect size data sets were directionally biased due to favored publication of significant results or specific outcomes, we visualized funnel plots (figs. S2, S3), performed regression tests, and estimated fail-safe numbers. We did not use trim-and-fill analyses (Duval and Tweedie 2004) to assess publication bias, as these have not been validated for use with multilevel meta-analytical models (Assink and Wibbelink 2016). Fail-safe numbers (n_{fs}) were calculated via the weighted Rosenberg method (Rosenberg 2005), to estimate the number of negative studies required to drive the observed mean effect to above a .05 significance level. For both protection ($n_{fs} = 3{,}705$) and cost ($n_{fs} = 2{,}027$), fail-safe N was above the threshold considered for a robust analysis, where n_{fs} is greater than $5N + 10$ (N = number of effect sizes in the data set; Rosenthal 1991). Altogether, these tests suggest that the protection and cost data sets are unlikely to be affected by publication bias.

Results

Association between Symbiont Density and Protection

Overall, across diverse host and symbiont taxa (table 1), symbiont density had a small but significant positive effect on the level of protection afforded to hosts (fig. 2*A*, full model, $r = 0.320$, SE = 0.065, $Z = 4.96$, 95% CI = 0.19–0.45, $P < .001$). In some systems, particularly microbial ones, it is common to look at protection across strains of a symbiont and correlate this with differences in strain density. To ensure such comparisons across strains were not biasing our overall effect, we ran the same model with effects ($n = 19$) from such studies removed and the result remained similar ($r = 0.278$, SE = 0.061, $Z = 4.43$, 95% CI = 0.15–0.39, $P < .001$). For the full model, the overall heterogeneity (I^2) in effect sizes was high (85.9%) based on the Higgins and Thompson classification, suggesting that a large amount of variance is attributable to true effects and only 14.1% is due to sampling variance. Such high heterogeneity is known to be widespread in ecology and evolution meta-analyses (Senior et al. 2016).

Although some of these analyses had limited statistical power, we found no evidence that the protection-density relationship differed between broad groupings of host type (fig. 3*A*, plant/animal, $^{\text{omnibus}}\text{QM}^{\text{df:1, 66}} = 0.12$, $P = .7270$) or the type of enemy symbionts protect against (fig. 3*B*, animal enemy/microbial enemy, $^{\text{omnibus}}\text{QM}^{\text{df:1, 66}} = 0.4982$, $P = .4803$). The effect of density did not differ significantly with the localization of symbionts (fig. 3*C*, endo-/exo-, $^{\text{omnibus}}\text{QM}^{\text{df:1, 66}} = 0.03$, $P = .8586$) or with transmission route (fig. 3*D*, horizontal/vertical, $^{\text{omnibus}}\text{QM}^{\text{df:1, 66}} = 0.23$, $P = .6336$). How protection was measured across systems (fig. 3*F*, host fitness based/damage sites/enemy inhibition, $^{\text{omnibus}}\text{QM}^{\text{df:2, 65}} = 7.48$, $P = .0238$) significantly contributed to the observed variation in the protection-density relationship; however, other methodological factors, such as the method of symbiont density manipulation (fig. 3*E*, natural variation/physical, $^{\text{omnibus}}\text{QM}^{\text{df:1, 66}} = 0.15$, $P = .7027$), did not have a detectable effect.

Within these categories a number of groups showed a strong effect of symbiont density on protection, indicated by effect estimates deviating significantly from zero. For symbionts localized within host bodies (endosymbionts), there was significant evidence for a positive effect of density on protection ($r = 0.33$, 95% CI = 0.17–0.49, $P < .0001$). Endosymbiont is a category largely composed of microbial symbionts, whereas exosymbionts have a high proportion of symbionts that are animal species. Estimates for symbionts associated with plant hosts ($r = 0.34$, 95% CI = 0.16–0.53, $P < .001$) were also associated with a significant effect, as were both horizontally ($r = 0.30$, 95% CI = 0.13–0.46, $P = .0003$) and vertically ($r = 0.36$, 95% CI = 0.15–0.57, $P = .0006$) transmitted symbionts. Symbionts that protect against microbial enemies ($r = 0.38$, 95% CI = 0.22–0.55, $P < .0001$) showed a notably significant effect of density on protection. Interestingly, studies that utilized natural variation in symbiont densities ($r = 0.34$, 95% CI = 0.17–0.52, $P < .001$) or used visible tissue damage as a measure of protection ($r = 0.59$, 95% CI = 0.27–0.86, $P < .0001$) were associated with stronger effects of density on protection.

Association between Symbiont Density and Cost

Symbiont density had a significant positive effect on the degree of cost (fig. 2*B*, full model, $r = 0.378$, SE = 0.12, 95% CI = 0.13–0.62, $Z = 3.04$, $P = .0023$). Heterogeneity was high ($I^2 = 91.9\%$). The analysis excluding effects ($n = 14$) generated from comparisons across strains reduced the effect, but it remained positive ($r = 0.133$, SE = 0.12, 95% CI = −0.11 to 0.37, $Z = 1.074$, $P = 0.283$). We found no evidence that the cost-density relationship differed between broad groupings of host type (fig. 4*A*, plant/animal, $^{\text{omnibus}}\text{QM}^{\text{df:1, 33}} = 1.57$, $P = .2095$) or by the type of enemy symbionts protect against (fig. 4*B*, animal enemy/microbial enemy, $^{\text{omnibus}}\text{QM}^{\text{df:1, 33}} = 0.1711$, $P = .6791$). The effect of density on cost did not differ significantly with the localization of symbionts (fig. 4*C*, exo-/endo-, $^{\text{omnibus}}\text{QM}^{\text{df:1, 33}} = 0.1158$, $P = .7336$) or transmission mode (fig. 4*D*, horizontal/vertical, $^{\text{omnibus}}\text{QM}^{\text{df:1, 33}} = 2.801$, $P = .0942$). When considering methodological

Table 1: Overview of host and symbiont taxa included in the meta-analysis

Host (common ID)	Host genus	Symbiont type	Symbiont genus	Protection	Analysis
Plant host:					
European spruce	*Picea*	Fungus	*Phialocephala*	Antibacterial	P and C
Cotton plant	*Gossypium*	Fungus	*Beauveria*	Antimicrobial	P and C
Acacia tree	*Acacia*	Ant	*Crematogaster*	Antiherbivore	P and C
Spanish elm	*Cordia*	Ant	*Azteca*	Antiherbivore	P
Neotropical tree	*Cecropia*	Ant	*Azteca*	Antiherbivore	P
Tropical tree	*Leonardoxa*	Ant	*Aphomomyrmex*	Antiherbivore	P
Tropical tree	*Macaranga*	Ant	*Crematogaster*	Antiherbivore	P
Cucumber	*Cucumis*	Fungus	*Colletotrichum*	Antiherbivore	P
		Fungus	*Fusarium*	Antimicrobial	P and C
		Fungus	*Glomus*	Antimicrobial	P and C
Soybean	*Glycine*	Fungus	*Piriformospora*	Antiworm	P
Arabidopsis	*Arabidopsis*	Bacterium	*Pseudomonas*	Antiherbivore	P and C
Wheat	*Triticum*	Bacterium	*Bacillus*	Antifungal	P
Tobacco	*Nicotiana*	Aphid	*Myzus*	Antibacterial	P
		Fungus	*Beauveria*	General	P and C
Nightshade	*Solanum*	Bacterium	*Pantoea*	Antibacterial	P
Animal host:					
Crayfish	*Cambarus*	Worm	*Cambarincola*	General	P and C
		Worm	*Xironodrilus*	General	P
Fruit fly	*Drosophila*	Bacterium	*Wolbachia*	Antiviral	P and C
Mosquito	*Culex*	Bacterium	*Wolbachia*	Antimicrobial	P
Mosquito	*Aedes*	Bacterium	*Wolbachia*	Antiviral	P
Owlet moth	*Helicoverpa*	Virus	*Baculovirus*	Antiviral	P
Potter wasp	*Allodynerus*	Mite	*Ensliniella*	Antiparasitoid	P and C
Oil-collecting bee	*Tetrapedia*	Mite	*Roubikia*	Antifungal	P
Black bean aphid	*Aphis*	Bacterium	*Hamiltonella*	Antiparasitoid	P and C
Pea aphid	*Acyrthosiphon*	Bacterium	*Spiroplasma*	Antiparasitoid	P and C
Pea aphid	*Acyrthosiphon*	Bacterium	*Regiella*	Antifungal	P
Carrion beetle	*Nicrophorus*	Mite	*Poecilochirus*	Antiblowfly	P and C
Tick	*Rhipicephalus*	Bacterium	*Rickettsia*	Antiapicomplexa	P
		Bacterium	*Coxiella*	Antiapicomplexa	P
Nematode	*Caenorhabditis*	Bacterium	*Enterococcus*	Antibacterial	P and C
Ram's horn snail	*Helisoma*	Worm	*Chaetogaster*	Antiworm	P
Bladder snail	*Physa*	Worm	*Chaetogaster*	Antiworm	C
Bryozoa	*Bugula*	Bacterium	*Endobugula*	General	C

Note: Protection = the type of protection the symbiont provides; symbionts that protect against multiple classes of microbes (e.g., viruses, bacteria, and fungi) are listed as antimicrobial, and those that defend against multiple threats (e.g., antimicrobial and antiherbivore) are listed as general. Analysis = host-symbiont combination is included in protection analysis (P) or cost analysis (C).

context, neither the type of cost measurement (fig. 4*E*, devo-/fitness/longevity/other, $^{\text{omnibus}}\text{QM}^{\text{df:3, 31}} = 1.44, P = .6962$) nor the method of density manipulation (fig. 4*F*, natural variation/physical, $^{\text{omnibus}}\text{QM}^{\text{df:1, 33}} = 0.68, P = .4098$) explained variation in study outcomes.

Within these categories, significant evidence for a positive effect of symbiont density on cost was observed for animal hosts ($r = 0.45$, 95% CI = 0.20–0.80, $P = .0011$), symbionts that defend against animal enemies ($r = 0.42$, 95% CI = 0.10–0.66, $P = .011$), and endosymbionts ($r = 0.41$, 95% CI = 0.11–0.71, $P = .0082$). Symbionts whose predominant transmission route is vertical were also associated with a strong effect of density on cost ($r = 0.64$, 95% CI = 0.26–1.0, $P = .0009$). Studies that used hosts with natural variations in symbiont density ($r = 0.47$, 95% CI = 0.14–0.81, $P = .0052$) and those that measured longevity as the cost factor ($r = 0.60$, 95% CI = 0.16–1.0, $P = .0074$) showed significant evidence for a positive effect of density on cost.

Discussion

Shifts in symbiont densities can generate marked changes to symbiont phenotypes (Wernegreen and Wheeler 2009)

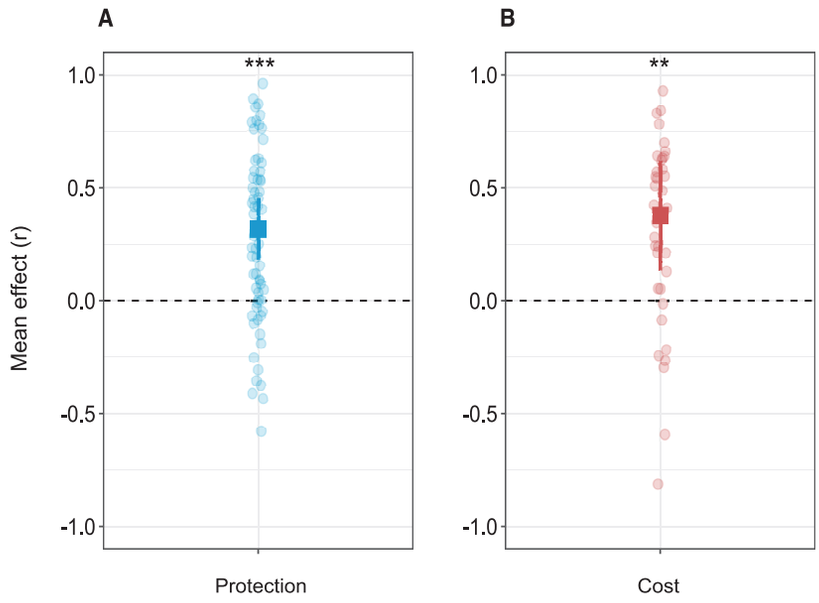


Figure 2: The extent of both protection (*A*; $r = 0.32$, $P < .001$) and cost (*B*; $r = 0.38$, $P = .0023$) conferred to hosts is a positive function of symbiont density in protective mutualisms. Mean effects from multilevel models are plotted with 95% confidence intervals; asterisks denote significance levels ($^{**}P < .01$, $^{***}P < .001$). The individual effects underlying each analysis are shown jittered.

and can cause previously beneficial interactions to become antagonistic (Sachs and Simms 2006; Brown et al. 2012). Despite these important outcomes, the general effect of symbiont density in protective mutualisms remains unquantified across systems. We hypothesized that the size of the persistent symbiont population may influence protective phenotypes and costs. Our analysis found broad support for a positive relationship between symbiont density and the magnitude of both protection and cost conferred to hosts. The effect of density may vary with context, just as theoretical models broadly suggest that cost-benefit relationships depend on the traits of hosts, symbionts, and the environment (Bronstein 1994; Herre et al. 1999). However, our meta-analysis detected consistent density-function effects across variation in host and enemy type, symbiont localization, and transmission mode. This result suggests that the effect of density may operate across the biological contexts tested herein. However, more studies are necessary to expand the resolution of the tested biological contexts. For example, studies with a greater breadth of enemy types (symbionts that protect against parasitoids were underrepresented in our analysis) and transmission strategies (bacteria dominated the vertically transmitted class of symbionts) would be valuable in corroborating our current findings.

Our results provisionally suggest that cost-protection trade-offs as a function of symbiont density may operate across the tree of life. Future studies could explicitly test for a trade-off by varying symbiont density (over a comprehensive range) and measuring both protection and cost within single experiments. Depending on the system, fitness changes due to the symbiosis could be measured over successive symbiont or host/symbiont generations. Microbial symbionts and hosts with rapid generation times (e.g., Aphids, *Caenorhabditis elegans*, *Arabidopsis*, *Nicotiana*) may prove particularly tractable systems here. If increases in density lead only to small advances in protection but equal gains in cost, then there may be selection for hosts to evolve mechanisms to regulate symbiont density.

The ability of hosts to regulate their symbionts (Fonseca 1993; Foster et al. 2017; Kokolo et al. 2020; Xiang et al. 2020)—or indeed of symbionts to regulate themselves (Kondo et al. 2005)—may be particularly important if host susceptibility (e.g., across life history) or ecological conditions of enemy attack (e.g., decrease in enemy prevalence) change. Hosts can benefit by controlling symbiont density at a point whereby symbiont-mediated protection is optimized relative to the costs. A number of symbioses appear to demonstrate such density regulation via physiological, behavioral, or structural mechanisms. In insects, modulation of endosymbiont titer commonly occurs across life history stages (Herren and Lemaitre 2011; Laughton et al. 2014). Cray fish remove ectosymbionts to densities shown to provide net benefits to the host (Brown et al. 2012; Thomas et al. 2013; Farrell et al. 2014) and at certain life stages (Farrell et al. 2014; Skelton et al. 2016; Thomas et al. 2016). Others have suggested

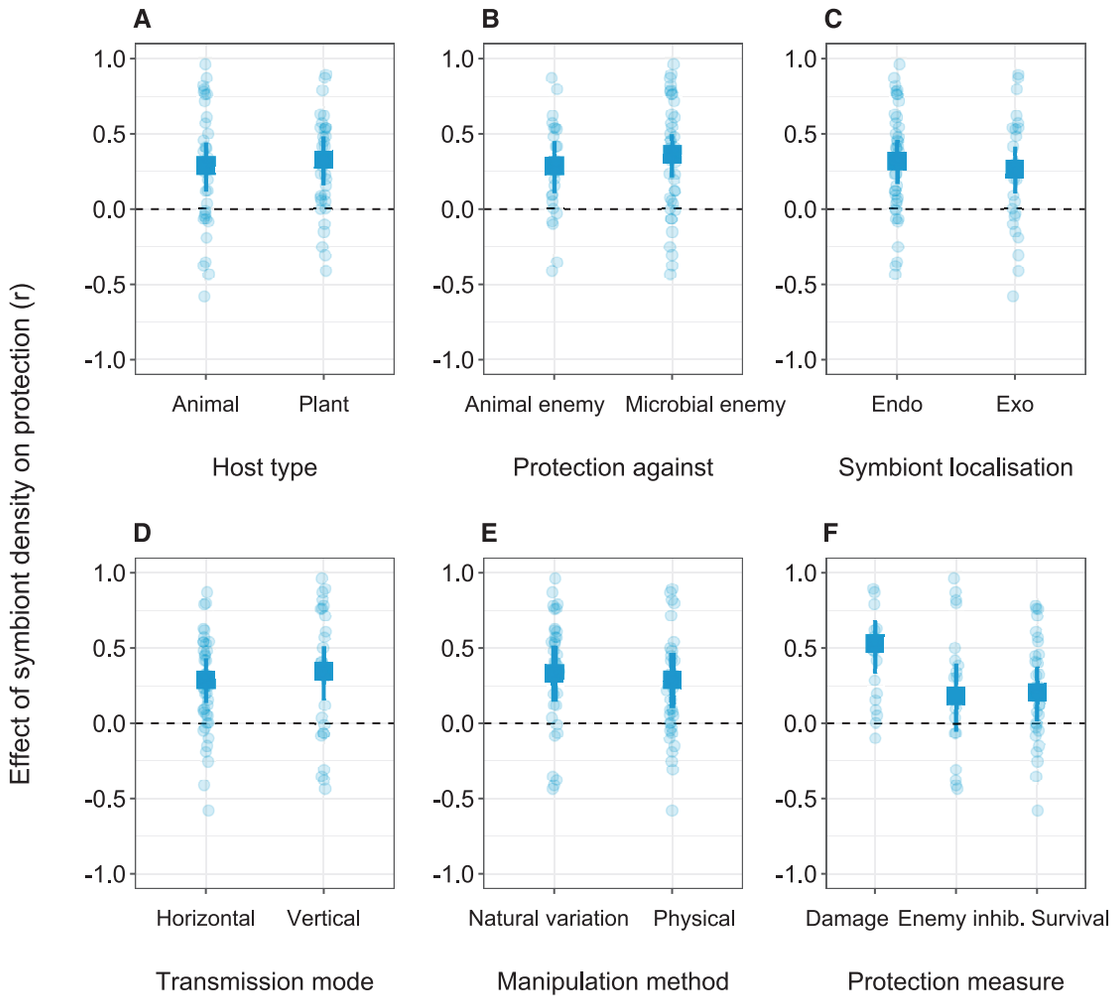


Figure 3: Variation in the effect of symbiont density on protection to hosts was not significantly impacted by host type (*A*), the type of enemy a symbiont defends against (*B*), symbiont localization (*C*), transmission (*D*), or density manipulation method (*E*; $P > .05$). *F*, How protection was measured did have a detectable effect on the density-protection relationship ($P < .001$). Mean effects and 95% confidence intervals from multilevel models are shown, with individual effect sizes jittered.

that in Hymenoptera, acarinarium structures (mite pockets) with smaller openings have been selected (Giordani 1985; Makino and Okabe 2003) to regulate the number of beneficial mites transmitting (Okabe and Makino 2010). In many cases it is unclear whether the host, the symbiont, or a coadaptive process drives density regulation. However, such diverse regulatory mechanisms in hosts may have evolved to optimize the net benefit received from protective symbionts.

While we show a significant positive effect of symbiont density on protection and cost, our results highlight the need to explore the shape of the relationship between these variables (Holland et al. 2002; Frederickson and Gordon 2009). The relationship is unlikely to be linear; larger and larger armies do not necessarily provide greater and greater benefit. Host protection may saturate at medium symbiont densities (Rocha and Bergallo 1992; Hopkins et al. 2017), after which symbionts transition to being net parasites as they consume host resources (Fonseca 1993). Such cases may produce a unimodal relationship between symbiont density and overall host performance. For acacia trees, the protection provided by ants against elephants saturates relatively quickly with increasing numbers of ants, but the cost of hosting continues to grow and is reflected in decreased fruit production (Palmer and Brody 2013). This fitness cost occurs presumably in part because acacias provide ants with metabolically costly nesting spaces and carbohydrate-rich food rewards (Palmer and Brody 2013). Hosts may be more willing to pay greater symbiont-associated costs or maintain densities above

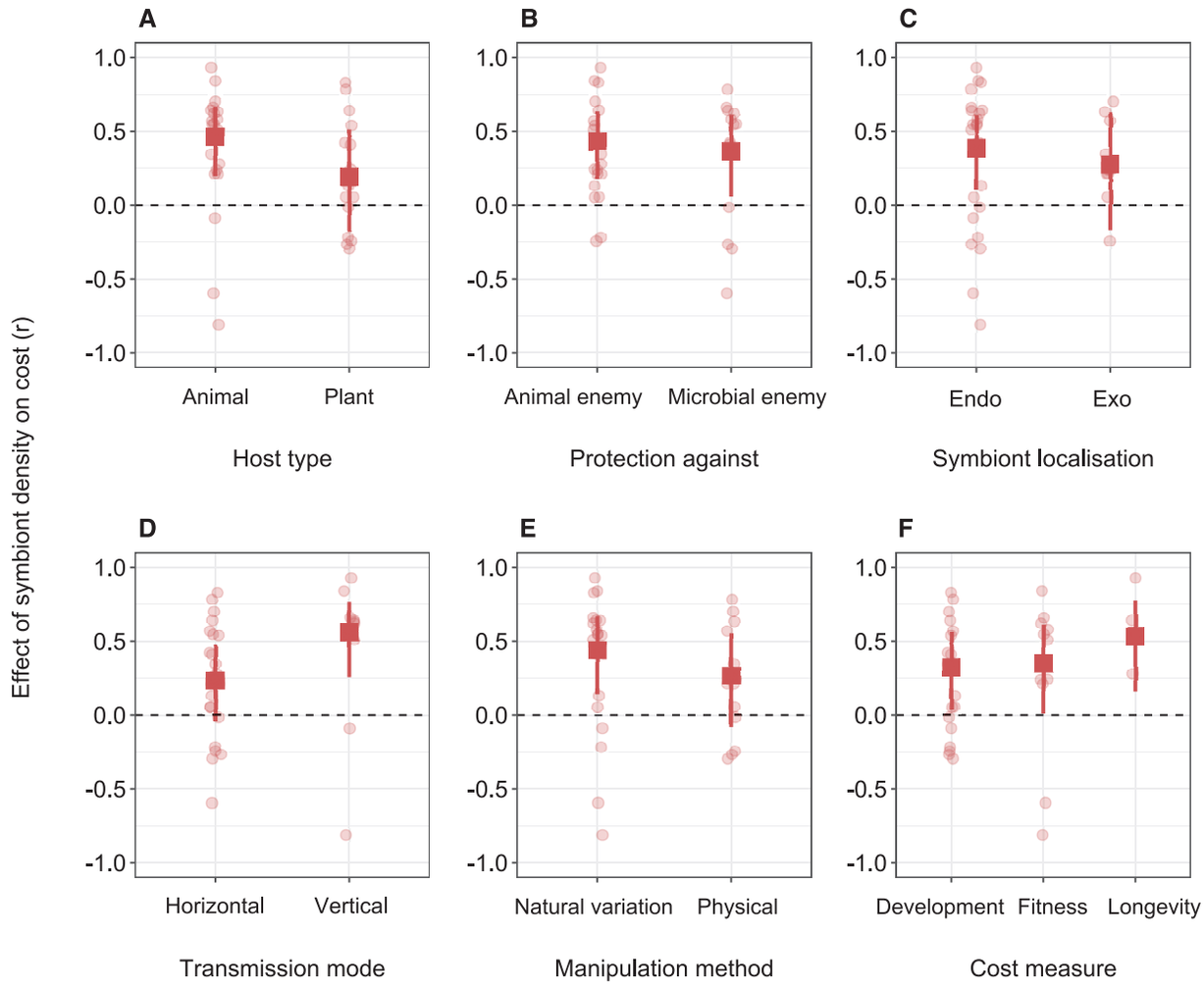


Figure 4: Variation in the effect of symbiont density on costs conferred to hosts was not significantly impacted by host type (*A*), the type of enemy a symbiont defends against (*B*), symbiont localization (*C*), transmission (*D*), density manipulation method (*E*), or how cost was measured (*F*; $P > .05$). Mean effects and 95% confidence intervals from multilevel models are shown, with individual effect sizes jittered.

optimum when the fitness consequences of an attack/infection are particularly high (e.g., interactions with large herbivores, parasitoids, or castrating parasites). For the host crayfish, *Cambarus chasmodactylus*, there are also clear nonlinear relationships between its symbiont's density and net host benefit. Crayfish with intermediate densities of protective branchiobdellian symbionts enjoy enhanced growth rates, yet at high densities host growth is reduced compared with symbiont-free individuals (Brown et al. 2012). Consequently, a nonlinear relationship between symbiont density and net benefit emerges (Fonseca 1993) and may tip the interaction toward parasitism. Changes to symbiont density may thus play a role in dynamic movements along the parasitism-mutualism continuum within an individual host's lifetime.

The effect of density on protection may also be an underestimate if symbionts provide multiple services to hosts (McLean et al. 2020). Multifunction symbioses could reduce trade-offs as a result of density, with more benefits provided by the same number of symbionts. Such interactions may be more widespread than currently realized. It is emerging that ants defending plants against herbivore attack often provide additional protection against pathogens (Offenberg and Damgaard 2019), yet assessments of protection level, such as grazing damage, are unlikely to fully incorporate this benefit.

The effect of density on both protection and cost was higher for symbionts that localize within ("endo-") host bodies compared with exosymbionts (although a statistically significant difference was not detectable). The endo- category

is largely composed of microbial symbionts that often protect against other microbial enemies. Density may be more crucial here, as symbionts might have to match large effective population sizes when defending against microbial threats. Conversely, thresholds for protection may be reached at lower densities of exosymbionts (e.g., branchiobdellia annelids and mites), which may physically defend against a single attacking individual at a time (e.g., a parasitoid wasp). Furthermore, endosymbionts are generally restricted to the host as a resource base, and greater symbiont densities may translate into heightened resource use paid for by the host. In contrast, exosymbionts may supplement nutritional needs from other non-host-based sources. This lack of dependence on hosts may be reflected in weaker associations between symbiont numbers and costs.

Additional factors likely affect the relationship between symbiont density and protection/cost. These factors may include complex genotype interactions across all players in the tripartite interaction (Vorburger and Gouskov 2011; Cayetano et al. 2015; Mathé-Hubert et al. 2019), the density of the attacking player (Thomas et al. 2013; Hopkins et al. 2017), other interacting species (Gaume et al. 1998), and variation between symbiont strains or populations (Martinez et al. 2014; Mathé-Hubert et al. 2019; Parker et al. 2021).

Interestingly, studies that utilized natural variation in symbiont densities were associated with greater effects for both protection and cost compared with those that physically manipulated symbiont densities in a more controlled manner. This result may reflect protection and costs being heightened by interactions with the wider ecological community. Symbiont densities in the natural variation group may also reflect host condition to a greater degree. Markedly high symbiont densities could reflect a compromised host that is unable to control its proliferating symbiont. Such compromised hosts may be avoided by attackers, but this avoidance may be interpreted as symbiont conferred protection. More generally, how protection is measured appears to influence the relationship between symbiont density and protection. Studies that measured tissue damage were associated with the strongest effects; however, this measure is used mainly for plant hosts. This result highlights potential challenges of comparing across systems, where some measures of protection will be more relevant (or feasible) than others.

For many protective symbioses, the mechanistic details of protection remain unknown. While not addressable in our analysis, we suspect the mechanism of protection will influence the evolutionary potential of symbiont density in generating variation in protection magnitude. Symbionts that protect hosts via the production of a defence compound, such as a toxin (Degnan and Moran 2008; Kroiss et al. 2010; Hamilton et al. 2016), can be selected for higher or more potent toxin production per symbiont (King et al. 2016). As toxins are frequently used by symbionts to inhibit their own competitors (Oliver and Perlman 2020), this outcome could also prove adaptive for the symbiont. Such cases would generate weak correlations between symbiont density and protection. Conversely, other mechanisms of protection may be density dependent to a greater degree. For symbionts that protect hosts via resource competition with enemies (Paredes et al. 2016) or patrolling power (Janzen 1966; Goheen and Palmer 2010), population density may be the major determinant of competitive ability or protection success. Under such mechanisms we may expect to see the effect of symbiont density on protection remain relatively stable over evolutionary time.

This study focused on pairwise interactions between hosts and symbionts. However, in nature protective effects can often be observed at the level of the symbiont community. Multiple taxa can interact to produce a protective or synergistic effect; this is observed for symbioses involving microbes (Oliver et al. 2014; Xie et al. 2014; Vannier et al. 2019; Mazorra-Alonsa et al. 2020) as well as macrobes (Palmer et al. 2010; McKeon et al. 2012). Thus, might density at the level of the whole symbiont community have a similar effect in determining the magnitude of protection and cost, and perhaps more so than symbiont diversity? In the protective bacterial symbiosis of hoopoe birds, egg shell bacterial community density, rather than diversity, contributes to greater hatching success (Martín-Vivaldi et al. 2014). Similarly, increases in skin microbiota density slow disease progression in amphibians (Bletz et al. 2018), and in the human gut microbiome disruptions to microbial loads appear to be more important drivers of disease susceptibility than taxonomic changes (Vandeputte et al. 2017). We show that the density of protective symbionts correlates positively with the magnitude of protection and cost conferred to hosts. A finding that may explain why host taxa frequently evolve mechanisms to regulate the numbers of beneficial symbionts. Future work should test for direct trade-offs in protective mutualisms as a function of symbiont density and also seek to establish the effect of density at the level of whole symbiont communities.

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Statement of Authorship

G.C.D. and K.C.K. designed the study and collected the data, G.C.D. carried out the analysis, and both authors contributed to writing the manuscript.

Data and Code Availability

The data sets and code used in this study are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.crjdfn35g; Drew and King 2021).

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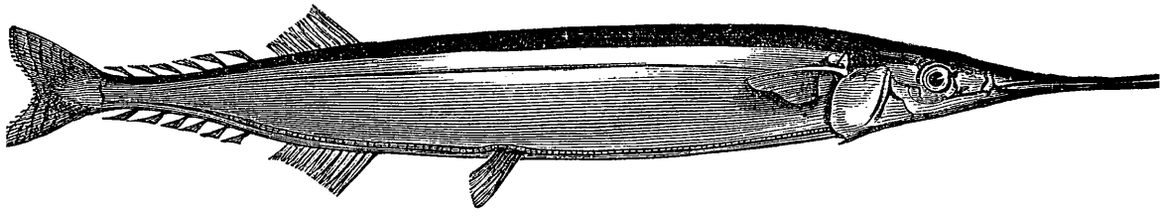
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"The Bill-fish (*Scomberesox Storerii*) [figured], which but fifteen years since I saw stranded on the shore by the thousands, driven in by its devouring pursuers, has gradually decreased, till at the present time it has nearly, if not quite, been driven away, and I think that during the past year there was not one specimen seen at Provincetown." From "The Habits and Migrations of Some of the Marine Fishes of Massachusetts" by James H. Blake (*The American Naturalist*, 1870, 4:513–521).