

**Reproductive altruism, social diversity and host association in  
sponge-dwelling snapping shrimps, *Synalpheus***

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By

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## **APPROVAL SHEET**

This dissertation is submitted in partial fulfillment of  
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## **DEDICATION**

To my wife, Belinda, my son Clayton, my parents James and Florence, and God.

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## **AUTHOR'S NOTE**

The chapters that comprise this dissertation were written in manuscript format for scientific publication. At the time of writing, the citations for individual chapters are as follows: - Be sure to eliminate the stuff below for Chaps 3-5 if they are not yet published by press time.

### **CHAPTER 1.**

Chak S.T.C., Rubenstein D.R., Duffy J.E. 2015 Social Control of Reproduction and Breeding Monopolization in the Eusocial Snapping Shrimp *Synalpheus elizabethae*. *The American Naturalist* 186, 660-668.

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Chak S.T.C., Duffy J.E., Rubenstein D.R. 2015 Reproductive skew drives patterns of sexual dimorphism in sponge-dwelling snapping shrimps. *Proceedings of the Royal Society B: Biological Sciences* 282, 20150342.

### **CHAPTER 3.**

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### **CHAPTER 4.**

Chak S.T.C., Duffy E. Group advantage in communal and eusocial snapping shrimps.

### **CHAPTER 5.**

Chak S.T.C., Song B., McDowell J.R., Hultgren K., Duffy E. Host association of snapping shrimps in sponge holobionts.

## ABSTRACT

The diversity of animal social strategies has interested evolutionary biologists since the time of Darwin. Eusociality—the apex of animal sociality—traditionally characterized by cooperative offspring care, overlapping generations and reproductive division of labor, was until recently known only in insects and a few vertebrate species. The independent evolution of eusociality in shrimps in the genus *Synalpheus* offers a unique opportunity to test the generality of social evolution theories that are based mainly on insects and social vertebrates. The genus *Synalpheus* is particularly ideal for comparative analysis because their social organizations are highly diverse, yet they share very similar ecology of being sponge dwellers. Further, their close associations with sponges, in which many are considered microbial fermenters, allow one to test the ecological drivers of species diversity in *Synalpheus*.

In this dissertation, I first explored the nature and consequences of reproductive altruism in eusocial species. Chapter 1 showed that workers in eusocial *Synalpheus* retain reproductive capability, but reproduction of female workers is suppressed by the queen. Chapter 2 showed further that such reproductive inequity among females within a colony leads to potentially strong competition among females for reproductive opportunities, and is associated with reduced sexual dimorphism in eusocial *Synalpheus* species.

Second, I examined the evolutionary trajectories between and ecological advantages associated with different social organizations in *Synalpheus*. Chapter 3 shows that the two demographically distinct social organizations found in *Synalpheus*—communality and eusociality—have evolved via separate evolutionary trajectories and represent alternative social strategies. Chapter 4 further shows that these social strategies are associated with different aspects of ecological advantages conferred on *Synalpheus* living together.

Finally, the intimate association with host sponges constrains the lifestyle of *Synalpheus* and may be one factor that has predisposed their evolution of eusociality. In Chapter 5, I examined the association pattern of *Synalpheus* with their host sponges and found that the symbiotic microorganisms in sponges, rather than the phylogenetic histories of the host sponges, are a better predictor and potential driver of the host association pattern.

This dissertation has sought to test, and ended up challenging, several paradigms in ecology and evolution. My results suggest that 1) polymorphic reproductive soldiers may represent a natural transition towards eusociality, 2) reproductive monopolization can modulate the pattern of sexual dimorphism in social species, 3) communality and eusociality evolved from distinct trajectories and have different ecological advantages, and 4) symbiotic microorganisms may mediate biological interactions between their hosts and other organisms.



**REPRODUCTIVE ALTRUISM, SOCIAL DIVERSITY, AND  
HOST ASSOCIATION IN SPONGE-DWELLING  
SNAPPING SHRIMPS, *SYNALPHEUS***

## **INTRODUCTION**

Advanced sociality is one of the major transitions in the history of life (Smith & Szathmary 1997), allowing insects to populate the terrestrial ecosystems and humans to conquer earth (Wilson 1975, Wilson 2012). Although sociality is widespread in animals, the nature of animal societies differs widely among and within species (Wilson 1971, Brown 1978, Smuts et al. 1987, Choe & Crespi 1997, Solomon & French 1997, Duffy & Thiel 2007). Eusociality—characterized by overlapping generations, reproductive division of labor, and cooperative care of young (Michener 1969, Wilson 1971)—is the apex of animal sociality. Eusociality has independently evolved in multiple species of insects (Wilson 2008), crustaceans (Duffy 2007), and mammals (Jarvis et al. 1994). Studies of eusocial insects and other highly social vertebrates have enlightened our understandings of the intricate relationship between ecology and evolution.

Although most research on animal sociality has focused on terrestrial animals, eusociality has been known in the sponge-dwelling snapping shrimp (the genus *Synalpheus*) for more than 20 years (Duffy 1996a, 1998). There have been significant advances in understanding the colony dynamics (Duffy 1996a, Duffy & Macdonald 1999, Duffy et al. 2002, Tóth & Duffy 2005, 2008), evolutionary history (Duffy et al. 2000, Morrison et al. 2004, Hultgren & Duffy 2011, Hultgren et al. 2014, Hultgren & Brandt 2015), host associations (Duffy 1992, Hultgren & Duffy 2010, Hultgren & Duffy 2012, Hultgren 2014), population genetics (Duffy 1993, Rubenstein et al. 2008), sexual biology (Tóth & Bauer 2007, 2008), and systematics of *Synalpheus* (Duffy 1996b, c, Ríos &

Duffy 1999, 2007, Anker & De Grave 2008, Anker & Toth 2008, Macdonald et al. 2009, Hultgren et al. 2010, Hultgren et al. 2011, Anker et al. 2012, Hultgren et al. 2014, Hultgren & Brandt 2015). Several reviews have nicely summarized these works (Duffy et al. 2002, Duffy 2007, Hultgren et al. 2016). However, we still lack a complete understanding of **1) the nature and consequences of reproductive altruism in eusocial *Synalpheus*, 2) the evolution and drivers of different social organizations in this genus, and 3) the drivers of host associations between *Synalpheus* and sponges.**

*The nature and consequences of reproductive altruism in eusocial *Synalpheus**

Worker sterility and caste formation are two distinguishing characteristics of eusocial species that have fascinated scientists since Darwin. In Chapter 1, I test whether sterility accompanies eusociality and morphological differentiation in *Synalpheus*. I show using histology and experiments that workers in *Synalpheus elizabethae* are reproductively totipotent, and that female gonadal development and mating are mediated by the presence of a queen. Thus, eusocial shrimp workers retain reproductive totipotency despite signs of morphological specialization. The failure of most female workers to mature is instead facultative and mediated by the presence of the queen, ensuring her reproductive monopoly.

Sexual dimorphism is typically a result of sexual selection on male traits, but in social species where reproduction is monopolized by a few individuals in a group, selection on secondary sexual characteristics may be strong in both sexes. In Chapter 2, I examine the relationship between sexual dimorphism and sociality in eight communal

and eusocial *Synalpheus* species. In communal species where reproduction was shared more equitably, most members of both sexes were physiologically capable of breeding. However, in eusocial species where reproduction was monopolized by a dominant breeder (the queen), a large proportion of females were reproductively suppressed by the queen, suggesting strong reproductive conflict among females within the colony. As reproductive skew and female-female competition over reproduction increased among eusocial *Synalpheus* species, sexual dimorphism in fighting claw size (major chelae) decreased. Therefore, in social species where reproduction is monopolized by one or a few individuals in a group, selection on secondary sexual characteristics may be strong in both sexes, as shown in many cooperatively-breeding vertebrates and eusocial insects.

#### *The evolution and drivers of different social organizations in Synalpheus*

Social animals appear to form a continuum based on reproductive skew, but the assumption that reproductive skew is a continuous trait has not been tested explicitly. In Chapter 3, I test the evolutionary trajectory into eusociality in *Synalpheus* to evaluate whether the social organizations taken by *Synalpheus* represent a continuum of social evolution. I show that eusocial and communal species (with cohabiting females that share a nest but provision their own offspring) represent distinct evolutionary trajectories and endpoints, i.e., pair-forming species of *Synalpheus* did not transition into eusocial species via communal intermediates. This means that the appearance of a continuum among social organizations of *Synalpheus* based on reproductive skew (the degree of reproductive division of labor) is an artifact driven by convergent evolution.

Eusociality and communality appear to be widespread social strategies to overcome intense ecological pressure. In Chapter 4, I assess whether communal and eusocial *Synalpheus* species experience similar ecological advantages by forming large groups. I found that larger group sizes in eusocial *Synalpheus* are associated with wider host ranges and occupy higher proportions of their host sponges. In contrast, larger group sizes in communal species are associated with wider geographic ranges only. The unique host-related advantages in eusocial, but not communal species suggest that the evolution of large groups may be fundamentally different when kinship is involved. Therefore, the ecological success of eusocial *Synalpheus* may not be due to group advantage alone.

#### *Drivers of host association between *Synalpheus* and sponges*

The highly intimate association with host sponges constrains the lifestyle of *Synalpheus* and may predispose them to eusociality. Interestingly, sponges can be viewed as holobionts that comprise sponges and their communities of symbiotic microorganisms. Although these symbiotic microbes are known to expand the functional repertoire of their hosts, it is unclear if they can affect biological interactions between their hosts and other organisms. In Chapter 5, I test whether host association patterns in *Synalpheus* can be explained by the evolutionary history of sponges or the similarity of sponge bacterial communities. I found that when a shrimp pair is more closely related, their host sponges tend to have more similar bacterial communities, but surprisingly, these sponges tend to be phylogenetically more distantly related. The inverse relationship between shrimp and sponge phylogenetic similarity may be due to intense competition between shrimp species when eusocial species are involved. Most importantly, this study

suggests that microorganisms can mediate biological interactions, perhaps by affecting larval settlement or diet.

This dissertation has clarified the reproductive biology in eusocial *Synalpheus* and the evolution of different social organizations in this genus. It has also illuminated the association between *Synalpheus*, its sponge host, and the sponge microbiome.

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## **CHAPTER 1.**

# **SOCIAL CONTROL OF REPRODUCTION AND BREEDING**

## **MONOPOLIZATION IN THE EUSOCIAL SNAPPING SHRIMP**

***SYNALPHEUS ELIZABETHAE***

## **Abstract**

Understanding why individuals within altruistic societies forego reproduction to raise others' offspring has fascinated scientists since Darwin. Although worker polymorphism is thought to have evolved only in sterile workers, worker subcastes appear to be common among social invertebrates and vertebrates. We asked whether sterility accompanies eusociality and morphological differentiation in snapping shrimps (*Synalpheus*) – the only known marine eusocial group. We show that workers in *S. elizabethae* are reproductively totipotent, and that female—but not male—gonadal development and mating are mediated by the presence of a queen, apparently without physical aggression. In queenless experimental colonies, a single immature female worker typically became ovigerous, and no female workers matured in colonies with a resident queen. Thus, eusocial shrimp workers retain reproductive totipotency despite signs of morphological specialization. The failure of most female workers to mature is instead facultative and mediated by the presence of the queen, ensuring her reproductive monopoly.

## **Introduction**

Societies characterized by cooperation and reproductive altruism occur across the animal tree of life in a range of vertebrate and invertebrate taxonomic groups (Brown 1987; Buskirk 1981; Duffy 1996; Solomon and French 1997; Strassmann et al. 2000; Wilson 1971). Eusocial societies characterized by cooperative offspring care, overlapping generations, and reproductive division of labor (castes) (Michener 1969; Wilson 1971) represent the pinnacle of social evolution, but share many characteristics with other forms of altruistic groups. Although social animals have been suggested to form a continuum based on reproductive skew (Keller and Perrin 1995; Keller and Reeve 1994; Sherman et al. 1995), the monopolization of breeding positions appears to be maintained differently in different taxa (Crespi and Yanega 1995). Worker sterility—a defining characteristic of caste differentiation in eusocial species (Boomsma 2007; Boomsma 2009; Crespi and Yanega 1995)—occurs in a variety of obligatorily eusocial insects (e.g., several ant genera, corbiculate bees, vespine wasps, and higher termites) (Fletcher and Ross 1985; Ratnieks et al. 2006; Thorne et al. 2003; Wilson 1971) where workers are irreversibly committed to their non-reproductive roles (Boomsma 2013). In contrast, helpers in all cooperatively breeding vertebrates (birds and mammals), as well as workers in most facultatively eusocial insects (e.g., halictid bees, gall-forming thrips, and lower termites) (Chapman et al. 2002; Faulkes 1990; Hart and Ratnieks 2005; Hartke and Baer 2011) are totipotent and retain the ability to reproduce.

Most facultatively eusocial species outside of the Hymenoptera are ‘fortress defenders’ (Queller and Strassmann 1998) that nest within rich, concentrated food

sources (e.g., clonal gall-forming aphids, ambrosia beetles, wood-dwelling termites, thrips, and two species of mole-rats). However, very few—if any—of these fortress defenders other than the higher termites have evolved obligatory eusociality with an irreversible worker caste (Abe 1987; Noirot and Pasteels 1987). Why obligatory eusociality has evolved in higher termites but not other fortress defenders remains unclear, but may be related to food resources. Unlike most other fortress defenders, termites are central place foragers that obtain food from outside of the nest (Boomsma 2013; Heinze and Korb 2008; Higashi et al. 1991). Additionally, long-lived host fortresses have been hypothesized to play a key role in the evolution of obligate eusociality (e.g., the ambrosia beetle *Austroplatypus incomptus*, Boomsma 2013; Kent and Simpson 1992), though this idea has not been tested.

The evolution of an irreversible worker caste is thought to be an important precursor to the evolution of morphological polymorphism of workers in eusocial species (i.e., the presence of specialized worker subcastes) because it allows colony-level selection to enhance worker efficiency and reproductive fitness of the colony (Oster and Wilson 1979). In support of this hypothesis, sterile workers in ants and higher termites show the most extreme forms of worker morphological and ecological polymorphism (Wilson 1971). Yet, some fortress defenders express morphological differentiation among non-reproductives that are reproductively totipotent, including soldier neotenics in lower termites (Thorne et al. 2003) and the dispersive morph in naked mole-rats (O'Riain et al. 1996). Therefore, it has been hypothesized that the presence of polymorphic reproductive soldiers may represent a natural transition towards obligate eusociality (Boomsma 2013; Thorne et al. 2003). However, it is not yet clear whether worker sterility is a necessary

condition for the evolution of irreversible worker castes (i.e., obligatory eusociality), especially when observations have been limited to only a few lineages of insects and a single vertebrate group.

Exploring the generality of the relationship between worker sterility and worker polymorphism requires studying a range of animal lineages with a diversity of social systems. An ideal study species would be a non-insect, eusocial fortress defender, like the snapping shrimp *Synalpheus elizabethae*. *Synalpheus* shrimps live obligatorily within canals of live marine demosponges, which are stable, predator-free and typically long-lived fortresses (McMurray et al. 2008). Like most other fortress defenders, *Synalpheus* shrimps do not forage outside of the host sponge. Instead, they feed on host sponge tissues (Đuriš et al. 2011) that may contain a significant amount of sponge-associated bacteria (Hentschel et al. 2006; Webster and Taylor 2012). Within the Caribbean gambarelloides clade of sponge-dwelling *Synalpheus*, eusociality appears to have evolved independently at least four times (Duffy and Macdonald 2010; Duffy et al. 2000; Morrison et al. 2004). Eusocial *Synalpheus* species live in groups that can contain up to several hundred individuals and one or a few breeding queens (Duffy 2007). In *S. regalis*, workers are related on average by 0.5 and likely to be the offspring of a single queen (Duffy 1996). However, whether these eusocial *Synalpheus* have sterile workers and are obligatorily eusocial has not been tested.

Some eusocial *Synalpheus* species exhibit morphological differentiation between queens and worker (Duffy and Macdonald 1999), as well as among workers (as subcastes) (Duffy 1998; Duffy et al. 2002; Tóth and Duffy 2008), suggesting that this group may be very similar to other obligatory eusocial species. For example, in *S.*

*filidigitus*, the queen's major chela (snapping claw) is replaced with a smaller minor chela (Duffy and Macdonald 1999). Moreover, queens in most species have a brood pouch (i.e., extended pleura on the abdomen) that is not observed in female workers (Chak et al. 2015). This degree of morphological differentiation between queens and workers is suggestive of a true worker caste, similar to that observed in the eusocial ambrosia beetle (Kent and Simpson 1992). Additionally, large colonies of eusocial *Synalpheus* in several species exhibit a morphologically specialized group of large individuals that possess a bigger weapon (fighting claw) than other workers, and who are more active in colony defense but show no external signs of reproductive maturity (Duffy 1998; Duffy et al. 2002; Tóth and Duffy 2008). These shrimp workers resemble the morphologically specialized caste in many obligatory eusocial insects like ants and higher termites or facultatively eusocial species like lower termites and naked mole-rats that have reproductively totipotent workers.

Here we explore if the fortress defender *Synalpheus elizabethae* shows parallels with other social insects and vertebrates in how eusociality, worker sterility, and morphological differentiation have co-evolved. Our primary goal is to determine whether a eusocial species of *Synalpheus* has either reproductively totipotent workers that might represent an early stage of social evolution or sterile workers that might facilitate evolution of worker polymorphism as in the Hymenoptera (Boomsma 2013; Crespi and Yanega 1995). We first test whether workers in *Synalpheus elizabethae* have lost or maintained the ability to reproduce by examining gonadal development. We then report on experiments designed to determine if workers are capable of mating in the absence of the queen, and whether mediation of worker development involves aggression on the part

of queens or workers. Ultimately, our results will be important for understanding the nature of reproductive altruism and conflict in a taxonomic group that is similar to, but distinct from other eusocial fortress defenders.

## **Material and Methods**

### *Gonadal development of workers in wild colonies*

To assess whether workers are sterile or totipotent, we obtained samples of the eusocial species *Synalpheus elizabethae* Ríos and Duffy, 2007 from five colonies in the Bocas del Toro region of Panama. *S. elizabethae* are most abundant in the demosponge *Lissodendoryx colombiensis* (Zea and Van Soest 1986) in this region of the Caribbean (McGrew and Hultgren 2011). We collected whole sponges with SCUBA from depths of 2 to 8 m from subtidal sandy patches near the west coast of Isla Colon, Bocas del Toro (N 9.39712, W 82.31760) between August and October 2011. Sponges were transported while submerged in seawater to the Smithsonian Tropical Research Institute Bocas del Toro Research Station, where all shrimps inhabiting the sponges were removed and identified under light microscopy. Ovigerous individuals (hereafter queens) possessed visible embryos under the abdomen. Non-ovigerous individuals (hereafter workers) lacked visible embryos and were preserved in Davidson's fixative (3:3:2:1 of distilled water, 95% ethanol, 37% formaldehyde, and glacial acetic acid) for subsequent histological analysis to assess gonadal development. From the three largest colonies, we confirmed that *S. elizabethae* has a morphologically specialized group of large workers

that possesses stronger weapons like its close relatives (Tóth and Duffy 2008) (see Appendix B, available online).

We chose 20 workers from each colony for histological examination. All workers were above the maturation size - the size of the smallest individual in the population that had mature gonads (see appendix B). Carapace length (CL) and major chela length were measured from photographs as described by Tóth and Duffy (2008) using ImageJ v1.48 (Schneider et al. 2012). Specimens were decalcified overnight (in 0.1 g/ml sodium citrate in 22.5% formic acid), dehydrated, and then infiltrated and embedded in paraffin using standard protocols (Humason 1979). Sagittal sections (3-5  $\mu\text{m}$ ) were cut with a rotary microtome and mounted onto glass slides before staining with hematoxylin and eosin. Depending upon an individual's CL, we examined 6-12 sets of 3-5 continuous sections, each separated by 20-30  $\mu\text{m}$  until at least half of the specimen was sectioned.

We scored each specimen for the presence of sperm, testes, developing oocytes, young ova, or mature ova (Bell and Lightner 1988). Mature ova had lipid-filled cytoplasm and were distinctively larger cells than young ova. Sperm were highly basophilic with the distinct umbrella shape characteristic of decapod crustaceans, and were located in the testis, vas deferens, or in an enlarged sac near the gonad-opening (gonopore) at the base of the fifth walking leg. Individuals were ultimately categorized as: (i) mature males with sperm and testis; (ii) immature males with testis lacking sperm; (iii) mature females with mature ova; or (iv) immature females with developing oocytes or young ova. We quantified the amount of sperm in mature males as the proportional

area occupied by sperm cells in the enlarged sac located in the section that had the highest number of sperm for each specimen.

#### *Experimental analysis of worker reproductive capacity and physical aggression*

To determine the reproductive capacity of workers and whether breeding monopolization involves aggression on the part of queens or workers in *S. elizabethae*, we examined experimentally how the presence of the queen and the number of workers in the colony influenced worker gonadal development. We created vacant sponges as semi-natural habitats for the shrimps (see appendix B) in the lab at the Smithsonian Tropical Research Institute Bocas del Toro Research Station between June and August 2013. A pilot experiment showed that 22 days was the optimal experimental duration to observe worker development and reproduction (see appendix B). We report combined data from the pilot and main experiments, since results did not differ between the two.

We initiated the experiment by collecting *S. elizabethae* from 16 sponges and dividing the shrimp from each sponge into three groups: (i) one queen and six workers (1Q/6W); (ii) queenless with six workers (0Q/6W); and (ii) one worker only (0Q/1W). Each of these shrimp composition treatments had 16 replicates. After 22 days, we preserved all shrimps in Davidson's fixative for histological examination of gonadal development. To determine if the workers that became ovigerous had fertilized eggs, we compared the histology of eggs from ovigerous workers and from queens that remained ovigerous after the experiment. Finally, we recorded whether workers had lost their major chela as a measure of within-colony aggression.

### *Statistical analysis*

For the examination of gonadal development in workers from wild colonies, we determined whether mature and immature females differed in CL using a generalized linear mixed model with maturity as a fixed factor and colony as a random block (slopes and intercepts were allowed to vary among colonies). P-values were obtained from likelihood ratio tests. We also examined the relationship between CL and sperm amount with colony as a random block. We then determined whether the percentage of mature workers in each colony differed between males and females using a Student's t-test. All analyses were performed with R v3.0.1 (Team 2015).

Our analyses of the experimental manipulation were based on 24 replicate groups (i.e., groups of different shrimp composition treatment, each in a cup of vacant sponge) with an experimental duration of 22 days from both the pilot and main experiments. Although we initially had 48 groups (16 replicates of each of 3 treatments), we excluded 24 groups that either had vitellogenic workers at the beginning of the experiment, that contained a queen that died during the experiment, or that contained workers that were determined to have been all males or all females based on subsequent histological analyses (Table A2). Vitellogenic workers had a visible mass of small, developing ova internally within the cephalothorax between the stomach and the heart—we removed these replicates so that all workers at the beginning of the experiment had only immature gonads, thus no worker had any head start in development. To analyze the results of the experiment, we first determined how many females in each group became reproductively

mature (i.e., mature gonads as shown by histology). At the end of the experiment, most groups had only a single mature or ovigerous female worker (typically either ovigerous or with mature gonads; fig. A1 in appendix A, available online); hence, we categorized each group by the most developed female into ovigerous, mature, or immature. We then used two-tailed Fisher's exact tests to test whether worker development (of the most developed female in each sample) was affected by the absence of a queen (0Q/6W vs. 1Q/6W) or by social interactions (i.e., presence or absence of potential mate or potential competition with other same-sex individuals; 0Q/6W vs. 0Q/1W). Specifically, we tested whether the observed proportions of the three nominal groups (ovigerous, mature, and immature; focusing on the most developed female in each sample, Figure 1B and Table A1) were independent between treatments, using groups (as opposed to individual shrimps) as replicates. P-values were adjusted according to the Holm–Bonferroni method for multiple comparisons (Holm 1979). In the 0Q/6W treatment, we tested for a difference in CL between immature, mature, and ovigerous female workers using a one-way analysis of variance (ANOVA).

To investigate the effect of treatment on levels of social conflict among workers, we used data for experimental durations of 7, 11, and 22 days from the pilot and main experiments. We tested whether worker mortality (i.e., the proportion of dead workers) differed between the 1Q/6W and 0Q/6W treatments using Poisson regression. We then tested whether worker injury (i.e., the proportion of workers that had lost a major chela) differed between treatments using a generalized linear mixed model with binomial response.

## Results

### *Gonadal development of workers in wild colonies*

The five field-collected *S. elizabethae* colonies ranged in size from 84 to 344 individuals, containing 1 to 7 queens each (Table A1). Based on histology, none of the 112 adult workers we examined were sterile. Instead, all female ( $n = 52$ ) and male ( $n = 60$ ) workers examined histologically showed signs of gonadal development in which sperm, testis, or various stages of ova were present. However, the level of gonadal maturity differed by sex; only 17 of 52 female workers were mature (mean  $\pm$  SD =  $38.4 \pm 20.9\%$ ), whereas 58 of 60 male workers were mature (mean  $\pm$  SD =  $97.5 \pm 5.6\%$ ). Thus, significantly fewer female than male workers had mature gonads ( $t_5 = 6.06, P = 0.0023$ ) (Table A1, fig. 1A). Gonadal maturity was not related to size in either sex; immature and mature females were similar in CL ( $\chi^2_1 = 0.13, P = 0.72$ ) (fig. A2), and the amount of sperm did not correlate with CL in males ( $\chi^2_1 = 1.48, P = 0.22$ ).

### *Experimental analysis of worker reproductive capacity*

We analyzed each experimental group according to the degree of gonadal development of the single most developed female worker in the group. Of the 12 queenless groups of workers (0Q/6W) included in the final analysis, three groups developed a single mature, ovigerous worker (fig 2A, B) and six developed a mature but non-ovigerous worker (fig. 2C, D) (fig. 1B; Table A2). In contrast, in the six groups with multiple workers and a single queen (1Q/6W), no workers reached maturity. Thus, the presence of a queen

significantly affected worker gonadal development, and when queens were removed, workers started transitioning into new queens in significantly more groups than in those where queens were left intact (1Q/6W vs. 0Q/6W,  $P = 0.029$ , fig. 1B). Finally, female workers usually failed to mature when held alone (0Q/6W vs. 0Q/1W,  $P = 0.17$ , fig. 1B); only 1 of 6 cups in this treatment developed a mature worker. This pattern was consistent even in the few cases in which workers had initial gonadal development (i.e., with visible gonads at the start of the experiment), as ovigerous workers were still found only in queenless groups (0Q/6W, 1 of 4) (Table A3). Carapace length (CL, an index of body size) did not differ among mature, immature, and ovigerous female workers in the treatment where they co-occurred (0Q/6W) (one-way ANOVA,  $F_{2,26} = 0.41$ ,  $P = 0.67$ ).

Histological examination showed that workers that became ovigerous had fertilized eggs. Eggs from queens and eggs from ovigerous workers showed no structural difference: all eggs had differentiated cells characteristic of fertilized eggs (fig. A3). Although females in some species of alpheid shrimp can ovulate without mating, unfertilized eggs are much smaller than fertilized eggs and have a chalky appearance, making unfertilized eggs easily recognizable (Felder 1982; Knowlton 1973). In our study, all ovigerous female workers examined had large, non-chalky eggs similar in appearance to eggs of other queens in captivity and the wild, suggesting that they were fertilized.

#### *Experimental analysis of physical aggression*

Across experimental durations from 7 to 22 days, worker injuries (i.e., the loss of the major chela) were more frequent in queenless groups (0Q/6W) than in groups containing

a queen (1Q/6W) ( $\chi^2_1 = 9.87, P = 0.0017$ ). However, worker mortality did not differ among treatments ( $\chi^2_1 = 1.44, P = 0.23$ ) (fig. 3).

## Discussion

The combination of our field studies and lab experiments demonstrates that (i) workers in the eusocial snapping shrimp *Synalpheus elizabethae* are not sterile, with individuals of both sexes showing signs of gonadal development, and (ii) immature workers can mature and reproduce when the queen is absent. Our experimental results support observations of gonadal development in workers of four other eusocial species of *Synalpheus* (Chak et al. 2015). Therefore, worker sterility does not appear to have evolved in eusocial snapping shrimps, which is consistent with what has been found in other facultatively eusocial fortress defenders that show some degree of morphological specialization despite having reproductively totipotent workers (e.g., gall-forming thrips, lower termites, and naked mole-rats) (Boomsma 2013; Chapman et al. 2002; O'Riain et al. 1996; Thorne et al. 2003). Thus, the similarity among *Synalpheus* and these quite distinct lineages of facultatively eusocial animals supports the generality of a model for evolution of social organization that bears strong similarities among disparate animal taxa. Moreover, the recent evolution of eusociality in *Synalpheus* (Morrison et al. 2004) suggests that worker polymorphism may indeed evolve before workers achieve permanent sterility.

Although most male workers in *S. elizabethae* were reproductively mature (98%), most female workers (>60%) were reproductively immature. These reproductively immature females were similar in size to the mature females and were not sterile, since

our experimental manipulation revealed that they could develop into mature, ovigerous individuals with fertilized eggs in as little as three weeks after queen removal. These sex-specific patterns of gonadal development in *S. elizabethae* suggest that female—but not male—workers show reduced gonadal development. Our experimental manipulations further demonstrated that this reduced reproductive development is mediated by the presence of the queen. That is, in the presence of a mature queen, all female workers remained immature, but, when the queen was experimentally removed, a single female worker in most colonies became ovigerous, or at least developed mature gonads. Moreover, in nearly all cases, only one of the six workers became mature after queen removal, indicating that once a worker becomes a replacement queen, she can affect others' reproductive development.

Our results also suggest that workers are able and willing to mate with their nestmates in an artificial setting, highlighting potential hidden reproductive conflict in eusocial colonies of *S. elizabethae*. Despite the presence of reproductively immature female workers, we found that many female workers in this species had mature gonads in wild colonies (38.4%). However, none of the female workers in these wild colonies had “breeding dress”, a typical morphological modification in breeding female caridean shrimps in which a brood-pouch forms to hold spawned eggs under their abdomens (Bauer 2004). Therefore, despite being reproductively mature, all female workers remained unmated; only queens produced fertilized eggs in the wild. Therefore, eusocial *Synalpheus* are similar to many facultative eusocial species in which workers are ‘hopeful reproductives’ that only reproduce when the opportunity arises (Thorne et al. 2002).

Interestingly, although half of the natural colonies of *S. elizabethae* at our site in Panama had a single queen, some had as many as 20 (mean  $\pm$  SD =  $3.58 \pm 4.09$ ). In fact, there was a strong positive correlation between colony size and the number of queens among 72 colonies of *S. elizabethae* collected from Bocas del Toro from 2007 to 2013 (fig. A4). This may suggest that the degree to which queens can mediate worker development and monopolize breeding varies among colonies (Hultgren et al. 2016), and is probably limited by the number of individuals that the queen can influence (Keller and Nonacs 1993; Michener 1990; Strohm and Bordon-Hauser 2003), whether via behavior or chemical signals. In other words, as colony size increases, breeding monopolization becomes more difficult and other females are able to reproduce, as has been hypothesized previously (Rubenstein and Shen 2009). On the other hand, theory also predicts that reproductive sharing (i.e., having multiple queens per sponge) may help to reduce reproductive conflict, either between queens and workers or among workers, when larger colonies provide greater reproductive benefits (Rubenstein 2012; Rubenstein and Shen 2009). However, ecological constraints on queen mortality, queen longevity, dispersal, and independent colony founding may also explain the presence of multi-queen colonies (Bourke and Heinze 1994; Keller 1995).

Finally, our observation that worker injuries were lower when the queen was present than when the queen was removed indicates that the mechanism of worker breeding monopolization by queens in *S. elizabethae* and probably other eusocial *Synalpheus* species is not mediated by the queen's physical aggression towards workers, as is also true of various social invertebrates and vertebrates (Bell et al. 2014; Cant et al. 2014; Clarke and Faulkes 2001; Cronin and Field 2007; Liebig et al. 2005; Young and Bennett

2013). Chemical mechanisms like pheromones (Holman et al. 2010; Keller and Nonacs 1993; Le Conte and Hefetz 2008; Matsuura et al. 2010) from the queen seem likely to be responsible for suppressing female worker maturation given their widespread presence in crustaceans (Breithaupt and Thiel 2010) and arthropods in general (Blomquist and Bagnères 2010). Indeed, direct queen aggression and policing (Hoffmann and Korb 2011; Ratnieks et al. 2006) is unlikely in *Synalpheus* because queens in eusocial species tend to have smaller major chelae than workers (Tóth and Duffy 2008), and because colonies of most eusocial shrimps are likely too large for a queen to behaviorally prevent other pairs from mating. Additionally, the observation that worker injuries (but not mortality) increased in queenless groups of workers where a single worker eventually became ovigerous or mature suggests that there is indeed overt aggression when a queen dies and an opportunity arises for others to breed. In other words, workers appear to fight for succession to become the dominant breeder when a queen dies; this is similar to naked mole-rats (Clarke and Faulkes 1997) but not lower termites (Hoffmann and Korb 2011).

In summary, we have shown that workers of both sexes in the eusocial snapping shrimp *Synalpheus elizabethae* have retained reproductive capacity, as is true in many fortress defender eusocial species that exhibit food-shelter coincidence (Crespi 1994), but that the presence of a healthy queen can mediate gonadal development only in females. Breeding monopolization by the queen evidently occurs without physical aggression, but in queenless experimental colonies, workers fought for vacated breeding positions and ultimately only one filled the reproductive vacancy. Therefore, in facultatively eusocial shrimp, some degrees of ecological and morphological polymorphism can evolve in the absence of sterility (as is also true of non-social animals, West-Eberhard 2003).

Moreover, the sex-specific pattern of reduced worker development in eusocial shrimps appears to be unique among invertebrates. With a better understanding of the mechanisms that govern reproductive skew and colony dynamics in eusocial shrimps, we are moving towards a more unified appreciation of animal sociality across diverse lineages—vertebrates and invertebrates—and ecosystems, from the terrestrial to the marine.

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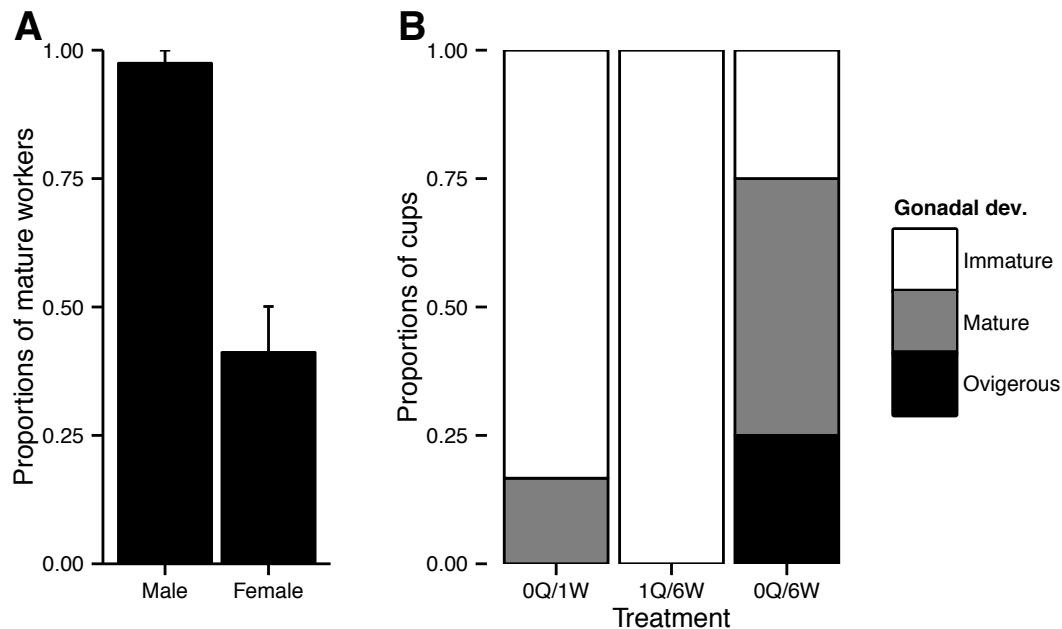
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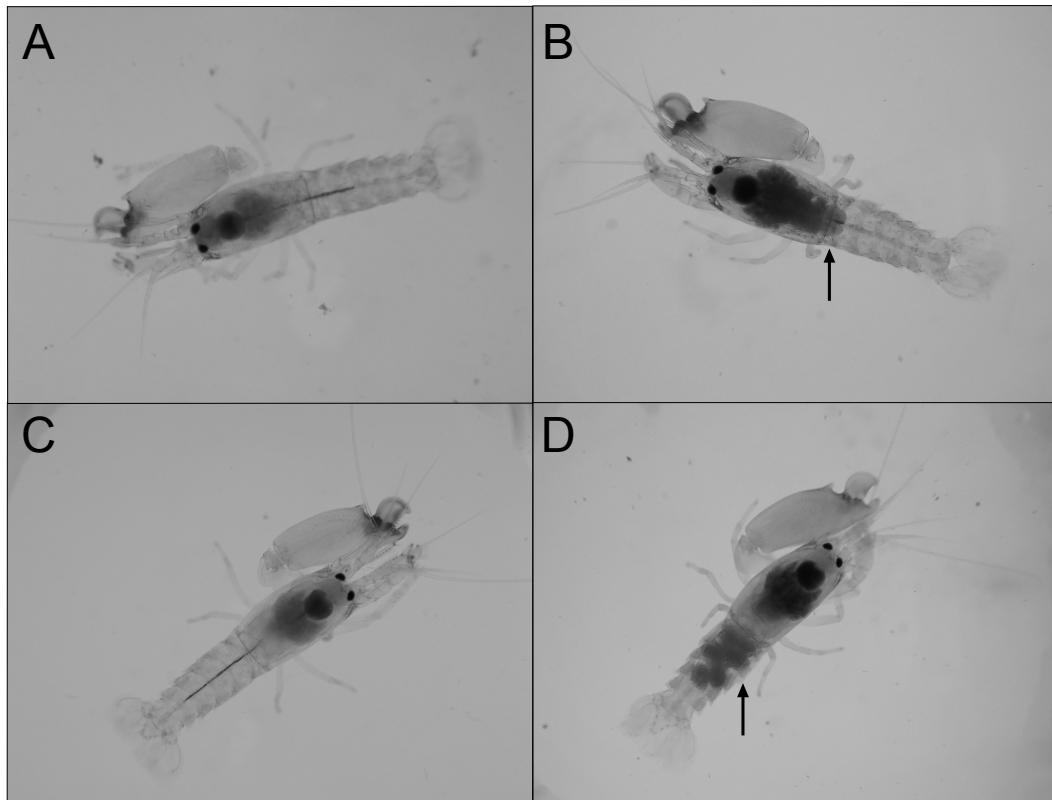
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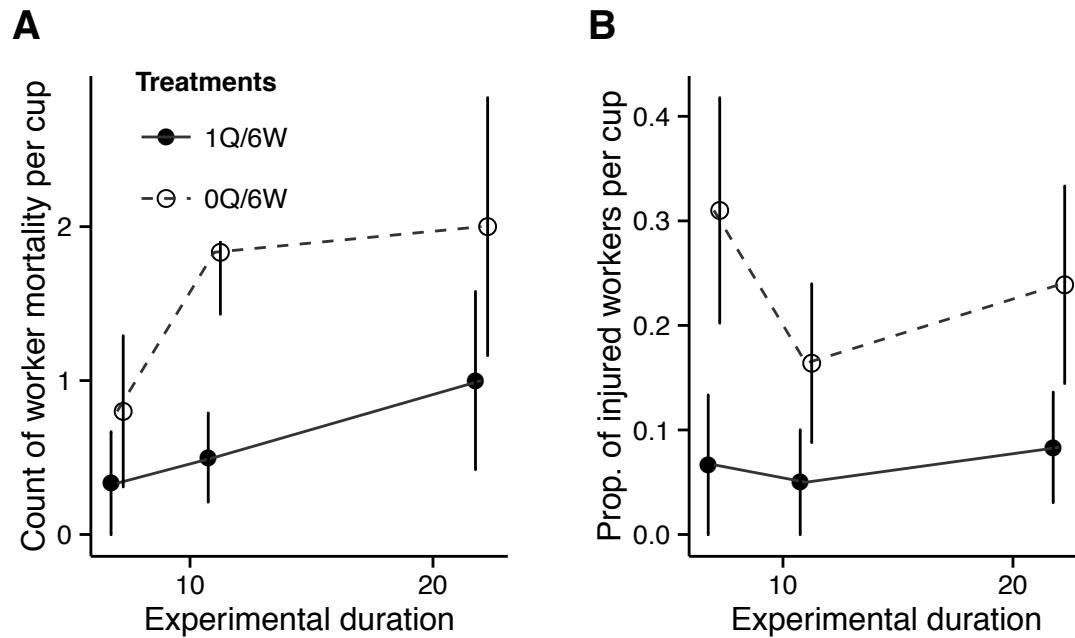
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**Figure 1.** Gonad development of workers (*A*) in wild colonies and (*B*) in experimental colonies of *Synalpheus elizabethae*. *A*, the mean ( $\pm$  SE proportion) of mature workers (mature + ovigerous) per colony was significantly higher for males than females in wild colonies ( $t_5 = 6.06, P = 0.0023$ ). *B*, degree of gonadal development of the most developed female worker in experimental groups after 22 days. The presence of a queen significantly suppressed worker gonadal development (1Q/6W vs. 0Q/6W,  $P = 0.029$ ), and female workers usually failed to mature when held alone (0Q/6W vs. 0Q/1W,  $P = 0.17$ ). Workers became ovigerous only in the absence of a resident queen and a potential mate (0Q/6W).



**Figure 2.** Reproductive development of two *Synalpheus elizabethae* workers from (A) immature into (B) mature and from (C) immature into (D) ovigerous in queenless experimental colonies. Arrows indicate developing gonad in B and eggs in D. In our experiment, the reproductive developments of all workers were further examined histologically.



**Figure 3.** (A) Mortality and (B) injuries (i.e., loss of major chela) among workers in treatments of different experimental duration with and without a queen (mean  $\pm$  SE). The presence of queens did not significantly influence worker mortality, but worker injury was more frequent in the absence of a queen.

## Appendix A: Supplemental Tables and Figures

**Table A1**

SEX AND MATURATION STATUS OF ADULT WORKERS FROM FIVE *S. ELIZABETHAE* COLONIES  
 FROM THE DEMOSPONGE *LISSODENDORYX COLOMBIENSIS* COLLECTED FROM BOCAS DEL  
 TORO, PANAMA.

COLONY NUMBER	COLONY SIZE	NO. OF QUEENS	TOTAL WORKERS SAMPLED	MATURE MALES	IMMATURE MALES	MATURE FEMALES	IMMATURE FEMALES	
						Yolk-filled ovaries	Small ovaries	Oocytes
223	350	7	35	14	2	3	15	1
224	84	2	20	8	0	4	8	0
234	74	1	20	13	0	3	4	0
236	142	5	19	12	0	2	5	0
237	204	3	18	11	0	5	2	0
Total			112	58	2	17	34	1

**Table A2**

GONADAL DEVELOPMENT OF THE WORKER THAT HAD THE MOST ADVANCED DEVELOPMENT  
IN EACH GROUP UNDER THREE EXPERIMENTAL TREATMENTS

GONADAL DEVELOPMENT	TREATMENT		
	0Q/1W	1Q/6W	0Q/6W
Immature	5	6	3
Mature	1	0	6
Ovigerous	0	0	3
Total	6	6	12

NOTE. – 1Q/6W: one queen and six workers; 0Q/6W: queenless with six workers (0Q/6W); and 0Q/1W: one worker only.

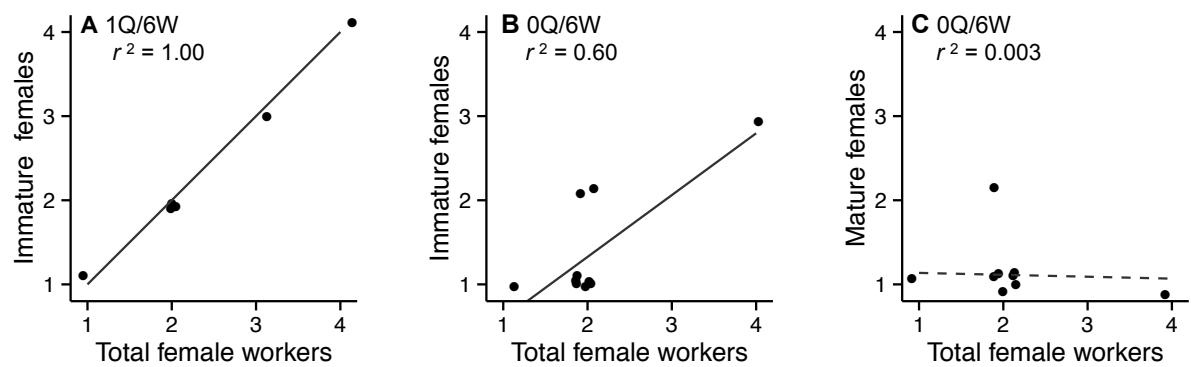
Numbers in the table represent counts of groups. We have excluded 10, 10 and 4 groups from treatments 0Q/1W, 1Q/6W and 0Q/6W respectively, because either workers had initial gonadal development, the queen died, the group had only male workers, or the group had only female workers.

**Table A3**

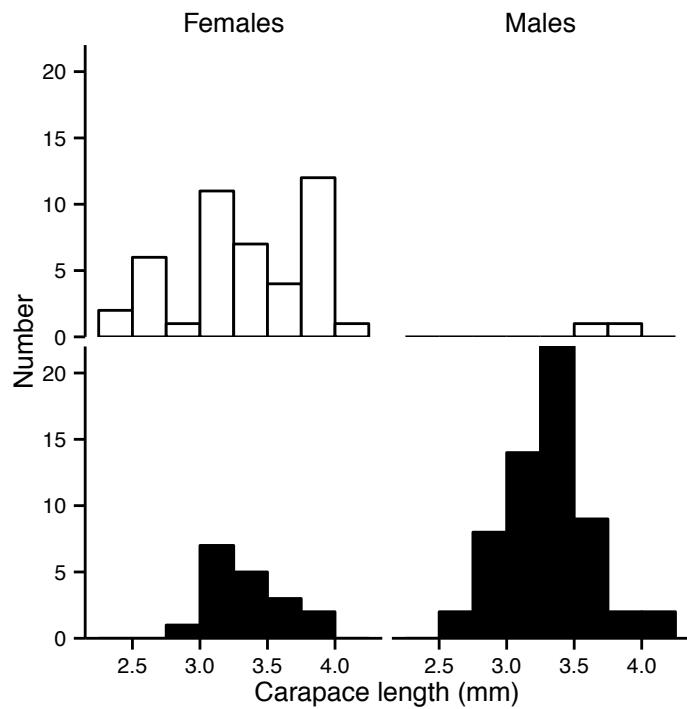
GONADAL DEVELOPMENT OF THE WORKER THAT HAD THE MOST ADVANCED DEVELOPMENT  
IN EACH GROUP UNDER THREE EXPERIMENTAL TREATMENTS WHERE WORKERS HAD INITIAL

## GONADAL DEVELOPMENT

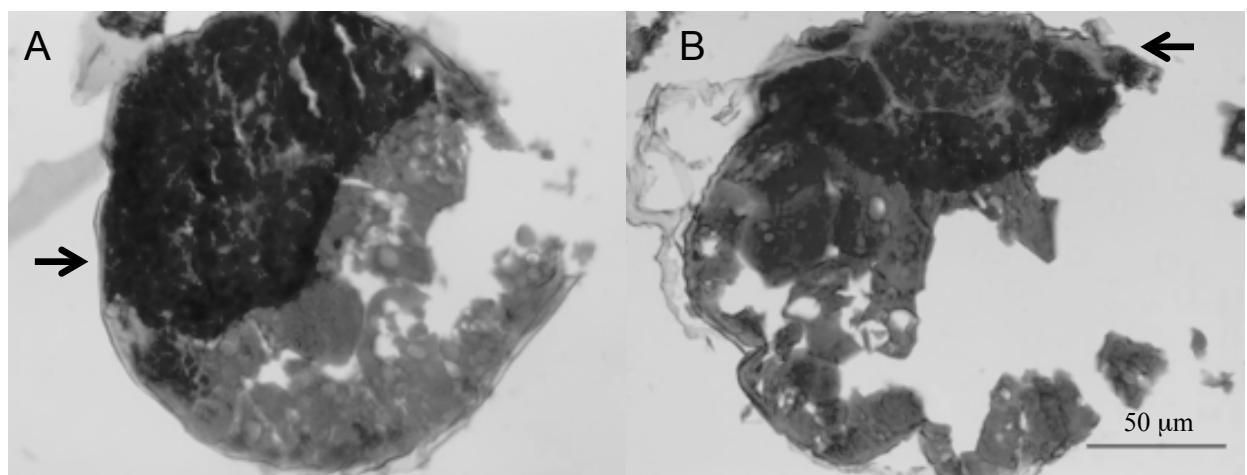
GONADAL DEVELOPMENT	TREATMENT		
	0Q/1W	1Q/6W	0Q/6W
Immature	NA	1	2
Mature	NA	1	1
Ovigerous	NA	0	1
Total	NA	2	4



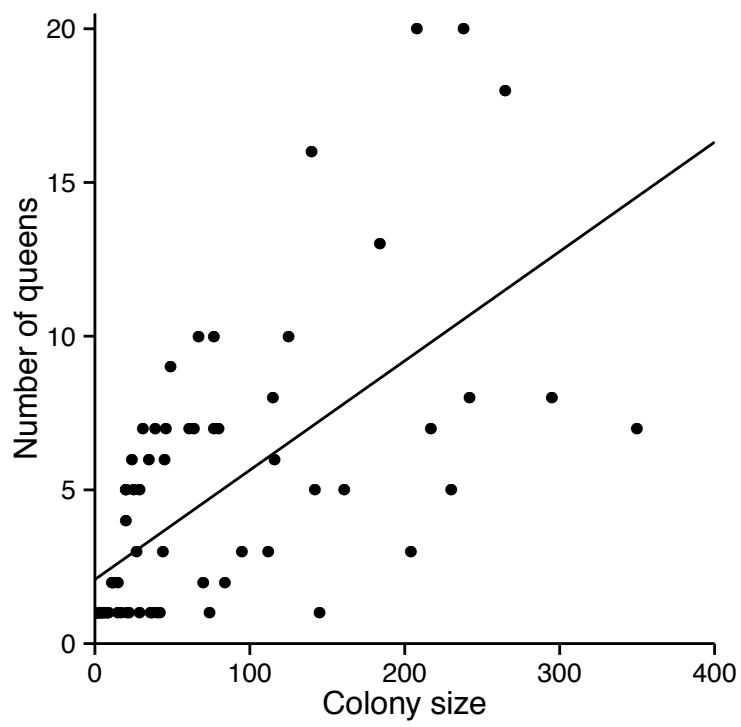
**Figure A1.** Number of immature females per experimental group versus the total number of females in treatments (A) with a queen and (B) without a queen, and (C) number of mature (i.e., ovigerous or with mature gonads) females per experimental group versus the total number of females in treatments without a queen. In (C), most groups without a queen (0Q/6W) developed a single mature female regardless of the total number of female workers present. Solid and dashed lines indicate significant and non-significant linear fits from GLM, respectively. Points are jittered to aid viewing.



**Figure A2.** Gonadal maturity of female and male workers as a function of body size (carapace length) from all sampled *S. elizabethae* colonies. White and black bars represent immature and mature workers, respectively.



**Figure A3.** Fertilized eggs from (A) a queen after 22 days and (B) an ovigerous worker after 22 days. Arrows indicate a group of multiplied embryonic cells with deeply stained nuclei.



**Figure A4.** The number of queens in a colony increases with colony size in the eusocial shrimp *Synalpheus elizabethae*. Data include 72 colonies collected from Bocas del Toro, Panama between 2007 and 2013 (linear regression:  $F_{1, 70} = 49.12, p < 0.0001$ , adj.  $r^2 = 0.40$ ).

## **Appendix B: Supplemental Materials and Methods**

### *Testing for morphological castes*

To confirm that *Synalpheus elizabethae* has a morphologically specialized group of large workers that possess stronger weapons like its sister species, *S. chacei*, *S. filidigitus*, and *S. regalis* (Tóth and Duffy 2008), we measured carapace length (CL) and fixed finger length of the major chela of workers from the three largest colonies (colony sizes = 142, 204, and 305 individuals). We plotted the allometry between finger length and CL (fig B1). We found that the largest colonies (colony 223) showed a diphasic allometry with an obvious break at 3 mm. Therefore, we performed linear regressions separately for individuals with CL larger and smaller than 3 mm for all colonies (Table B1), finding that the allometric slopes differed between large and small workers in the two largest colonies (colony 223 and 237). Therefore, similar to other eusocial *Synalpheus*, the allometry between chela size and CL in *S. elizabethae* exhibits a diphasic relationship, especially in large colonies. Thus, a subset of *S. elizabethae* workers resembles the morphologically specialized caste in many obligatory eusocial insects like ants and higher termites, or facultatively eusocial species like lower termites and naked mole-rats that have reproductively totipotent workers.

### *Estimating size of maturity*

To determine the size of maturation in *S. elizabethae*, we examined (i) the relationship between carapace length (CL) and major chela length (MCL), and (ii) the size distribution of colony 223 (i.e., the largest colony in which workers of the whole size

range were sampled) and the pooled sample. Size at first maturity in decapod crustaceans can be estimated by the breakpoint in the allometry regression (Hartnoll 1978), or by a logistic regression that determines the size class at which a randomly chosen individual has a 50% chance of being mature (Somerton 1980). In contrast to many other decapod crustaceans in which most individuals larger than the size-of-maturation are mature (Somerton 1980), we found in *S. elizabethae* that immature females were present in all size ranges and that the proportions of mature females were never above 50% in any size class (see fig A2 in appendix A). Moreover, there were no abrupt change in the allometry slope between CL and MCL (fig B2). Thus, we estimated the mature size of *S. elizabethae* as the size of the smallest mature female (2.75 mm) across colonies. We believe that this is an appropriate estimate because these putative adults were large enough to have mature gonad (i.e., mature ova).

#### *Creating vacant sponges as semi-natural habitats for shrimp*

Field-collected *L. colombiensis* were divided into 2-4 cm pieces to remove all macrofauna, and then ~50 mL of sponge tissue was allowed to regenerate for 5-8 days in plastic cups covered with mesh (cup volume = 100 mL, diameter = 6.2 cm, height = 7 cm, mesh size = 800  $\mu\text{m}$ ) and supplied with a constant flow of seawater released 30 cm above the cup. We immersed cups in 3 cm of water and placed them 15 cm apart to prevent water exchange. Before adding shrimps, we measured the underwater mass of each re-aggregated sponge and adjusted sponge mass by cutting away tissues so that each cup had 8 g of sponge tissue per shrimp; thus initial sponge mass depended upon the

number of shrimp in each cup, but had a similar ratio of sponge to shrimp. Sponge fragments typically annealed into a single piece after three days with minimal mortality. Treatments were blocked by individual sponge source (i.e., genotype) such that each sponge was divided into 12 cups, with each receiving one treatment (three shrimp composition treatments by four temporal treatments).

*Pilot experiment to determine optimal experimental duration*

*S. elizabethae* used in the pilot experiment were collected from six sponges from the same location sampled in 2011. We divided the shrimps into 12 groups corresponding to the 12 crossed treatments: three shrimp compositional treatments crossed with four temporal treatments, each with six replicates. The shrimp composition treatments included: (i) one queen and six workers (1Q/6W); (ii) queenless with six workers (0Q/6W); and (iii) one worker only (0Q/1W). We used the four temporal treatments (7, 11, 22, and 33 days) to determine the optimal experimental duration in which workers could become reproductively mature. We weighed all sponges and assessed worker gonadal development in each treatment by photographing all shrimps on a mini-light box before and after the experiment. Workers were classified visually as ovigerous (i.e., eggs were ovulated and carried under the abdomen), vitellogenic (i.e., a visible mass of small, developing ova internally within the cephalothorax between the stomach and the heart), or immature (i.e., no visible gonads, and hence, could be female or male). Histological examination of visually immature workers confirmed that these workers had only immature gonads.

Based on visual observations of workers in queenless groups (0Q/6W) in the main experiment, vitellogenic and ovigerous workers first appeared after 11 and 22 days, respectively (fig B3). Sponge masses in the experimental cups (each holding one group of shrimp) declined most in the first 7 days, and then continued to decline steadily (fig B4). By day 22, sponges averaged 55% of their original mass (SD = 15%). We noted that the loss of sponge tissue (i.e., white, brittle senescent tissue) was from the exterior and not from the interior sponge canals where shrimp reside. Moreover, worker mortality increased with time; on average 1.5 and 1.9 workers died after 22 and 33 days, respectively. Overall, sponge condition and shrimp survival were not ideal for the experimental duration of 33 days, but were acceptable for 22 days. Most importantly, we started to observe ovigerous workers after 22 days. Although there was a reduction in sponge mass and worker mortality after 22 days, this duration appears to be ideal for the determination of worker development.

### **Supplemental References**

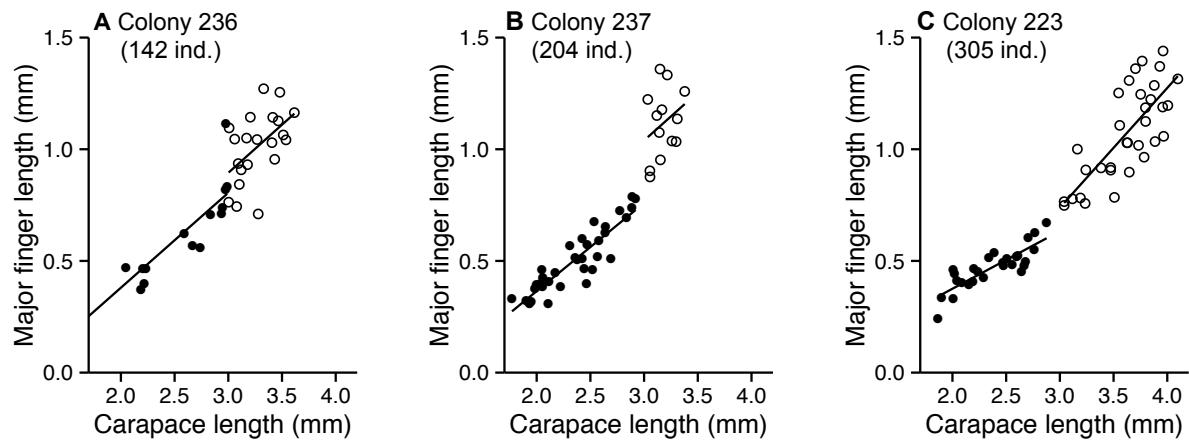
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**Table B1**

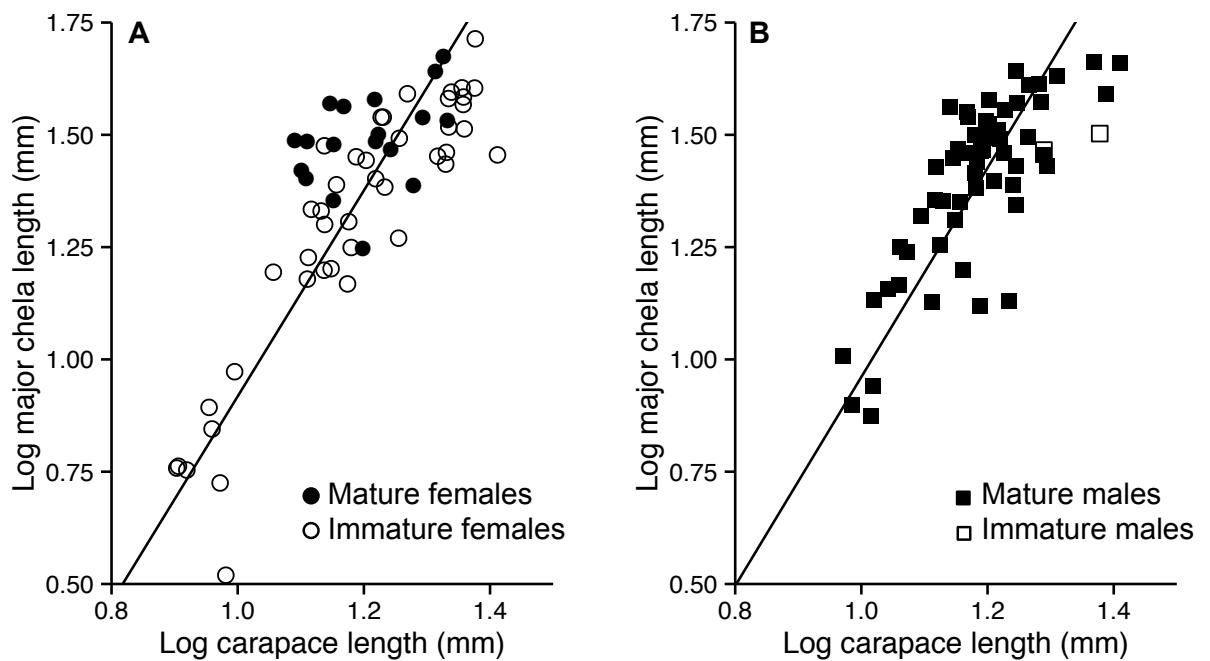
RESULTS OF REGRESSIONS BETWEEN FIXED FINGER LENGTH OF THE MAJOR CHELA AND  
CARAPACE LENGTH IN THREE COLONIES OF *S. ELIZABETHAE*.

COLONY	SIZE	DF	F	P	ADJ. $r^2$	SLOPE
223	Small	1, 25	61.89	< 0.0001	0.70	0.26
	Large	1, 31	44.36	< 0.0001	0.58	0.54
236	Small	1, 13	36.17	< 0.0001	0.72	0.43
	Large	1, 19	7.17	0.015	0.72	0.43
237	Small	1, 33	140.5	< 0.0001	0.81	0.39
	Large	1, 11	1.08	0.32	0.0062	0.43

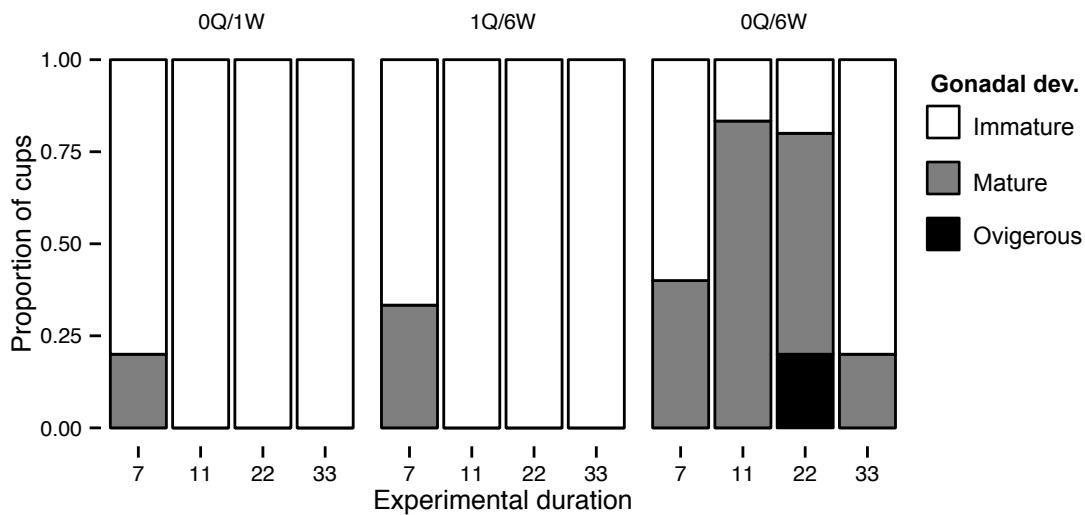
NOTE. – Each colony was divided into large ( $\geq 3$ mm CL) and small ( $< 3$ mm CL) size classes.



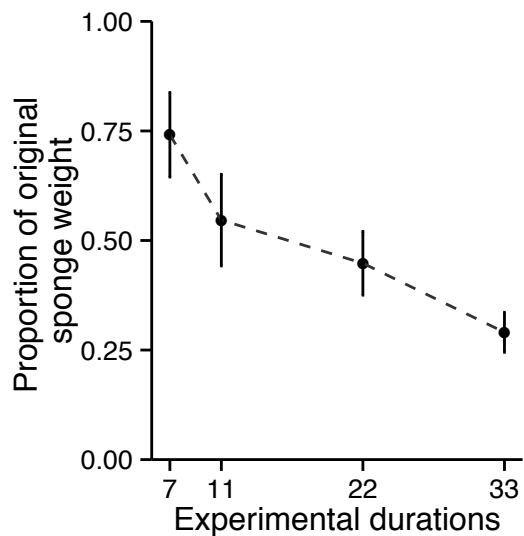
**Figure B1.** Allometry between fixed finger length of the major chela and carapace length in three colonies of *S. elizabethae*. Note that in large colonies (*B* and *C*), larger workers (open circles,  $\geq 3$  mm CL) had much larger finger lengths relative to carapace lengths compared to smaller workers (closed circles). Solid lines are fitted lines of linear regression.



**Figure B2.** Allometry between major chela length and carapace length (mm) in (A) females and (B) males in colony 223. The relationship between carapace and major chela length did not show an abrupt change in slope, which often indicates size of first maturity in Decapod crustaceans (Hartnoll 1978). Solid lines represent major axis regression (male:  $r^2 = 0.60$ ,  $\beta = 2.33$ , parametric  $p = 0.001$  (999 permutations); female:  $r^2 = 0.70$ ,  $\beta = 2.29$ , parametric  $p = 0.001$ ).



**Figure B3.** Worker gonadal development in the initial experiment with three treatments of shrimp group composition (0Q/1W, 0Q/6W, 1Q/6W) and four treatments of experimental duration (7, 11, 22, 33 days). Worker gonadal development in each cup was scored based upon the worker with the most advanced stage of gonadal development. Worker gonadal development was assessed visually, except on day 22 when all workers were preserved and examined histologically.



**Figure B4.** Decline in sponge underwater mass (mean  $\pm$  SE) relative to the starting mass in each temporal treatment.

**CHAPTER 2**

**REPRODUCTIVE SKEW DRIVES PATTERNS OF SEXUAL  
DIMORPHISM IN SPONGE-DWELLING SNAPPING SHRIMPS**

## **Abstract**

Sexual dimorphism is typically a result of strong sexual selection on male traits used in male-male competition and subsequent female choice. However, in social species where reproduction is monopolized by one or a few individuals in a group, selection on secondary sexual characteristics may be strong in both sexes. Indeed, sexual dimorphism is reduced in many cooperatively breeding vertebrates and eusocial insects with totipotent workers, presumably because of increased selection on female traits. Here we examined the relationship between sexual dimorphism and sociality in eight species of *Synalpheus* snapping shrimps that vary in social structure and degree of reproductive skew. In species where reproduction was shared more equitably, most members of both sexes were physiologically capable of breeding. However, in species where reproduction was monopolized by a single individual, a large proportion of females—but not males—were reproductively inactive, suggesting stronger reproductive suppression and conflict among females. Moreover, as skew increased across species, proportional size of the major chela—the primary antagonistic weapon in snapping shrimps—increased among females and sexual dimorphism in major chela size declined. Thus, as reproductive skew increases among *Synalpheus*, female-female competition over reproduction appears to increase, resulting in decreased sexual dimorphism in weapon size.

## **Introduction**

Sexual selection often results in sexual dimorphism in which morphological traits related to competition or mate attraction are more pronounced in males [1-3]. This occurs because males can typically increase their reproductive success more by mating multiply than can females [4, 5]. The ratio of receptive males to receptive females at any time, the operational sex ratio (OSR) [6], quantifies this intensity of male competition for mates [7]. In polygynous species, the OSR is often male-skewed and positively correlated to the degree of sexual dimorphism in traits related to mating competition, assuming all adults in the population are reproductively active [8]. Interestingly, this assumption is violated in many social animals living in societies with high reproductive skew where most individuals do not reproduce (i.e., helpers or workers).

In cooperatively breeding vertebrates, most helpers are totipotent but not reproductively active. In such systems, both males and females may experience strong intrasexual competition for mates or breeding opportunities, and thus have equally high (or even higher) variance in reproductive success than males [8-11]. Ultimately, selection on the same competitive traits used in intrasexual competition for access to mates, resources, or social rank can be as strong in females as in males of these social species [9, 11, 12]. For example, cooperatively breeding birds are generally not sexually dimorphic [13], and sexual dimorphism in plumage and body size is reduced in cooperative African starlings compared to their non-cooperative relatives [11]. Similarly, eusocial insects also tend to express low degrees of sexual dimorphism [14], and in facultatively eusocial species where totipotent (non-sterile) workers can replace the queen [e.g., 15, 16, 17], there may be strong competition among female workers to obtain breeding opportunities.

Aggressive interactions between queens and totipotent workers in many Hymenoptera (reviewed in [18]) suggest that eusocial insects exhibit high intrasexual competition among females. For example, in lower termites, neotenic workers of both sexes can replace the royal pair, but only after killing other neotenics [19, 20]. Moreover, in the hover wasp *Liostenogaster flavolineata* in which workers are totipotent, the degree of physical aggression among workers increases with rank [21]. Although sexual dimorphism appears to be reduced in a variety of social species, explicit tests of this hypothesis have been limited to comparisons of social and non-social species (e.g., [11]). A stronger demonstration of how sociality influences the evolution of sexual dimorphism would be to compare related species that vary continuously in their social structure, or their levels of reproductive skew (i.e., number of breeding positions per colony member).

The snapping shrimp genus *Synalpheus* is an ideal group within which to investigate the relationship between reproductive skew and sexual dimorphism because closely related species vary in social structure and show extreme elaboration of weapons used for, among other purposes, obtaining mates. Not only has eusociality evolved independently at least four times [22-24] in the group of approximately 45 West Atlantic (*Gambarelloides*) species [25], but reproductive skew varies continuously among species with different social structures: (i) *eusocial species* live in colonies with a single or a few ‘queens’ and a few to hundreds of non-breeding workers; (ii) *communal species* live in groups with multiple breeding pairs, typically with equal ratios of adult males and females; and (iii) *pair-living species* live in sponges with a single breeding pair per sponge [23, 26]. All species within the *Gambarelloides* groups live obligatorily in sponge canals, and therefore appear to face similar ecological constraints on social living.

In addition to their complex social behavior, snapping shrimps are also known for their extreme armament elaboration—one of the first pair of walking legs is enlarged to form a snapping claw (major chela) [27], which serves mainly as a weapon and signal in conspecific interactions [28-31]. The larger major chela in males [27, 28] is generally considered a sexually selected trait [32] because it mediates male-male competition for access to receptive females in caridean shrimps [33]. Therefore, we expected that larger major chela in females would also be selected for when competition among females is higher.

To study the relationship between reproductive skew and sexual dimorphism, we examined sex ratio variation, armament dimorphism, and reproductive skew in eight species of communal and eusocial *Synalpheus*. The five eusocial species represent most of the known eusocial species in *Synalpheus*; a few other rare species were only recently described from very small samples, and others have apparently gone locally extinct [34]. The three communal species that we studied represent the primary communal species that can reach large colony size similar to eusocial species. Since eusocial *Synalpheus* species have totipotent workers that show reduced reproductive development when the queens are present but compete for dominant breeding positions when queens are removed [35], we hypothesized that as reproductive skew increases (leading to fewer breeding positions per colony member), higher intrasexual competition among females for access to breeding opportunities would result in stronger selection on weapons in females and ultimately reduced sexual dimorphism. To test this hypothesis, we used both histology and scanning electron microscopy [36, 37] to determine the sex and degree of gonadal development of workers. First, we calculated the proportions of mature males and

females within colonies to estimate the degree of potential reproductive conflict in each species. Second, we used these data to estimate both the adult sex ratios (ASRs) and operational sex ratios (OSRs) as suggested by [38], since these metrics provide different but complementary information: ASR is more influenced by demographic processes, whereas OSR is more affected by individual mating prospects. Third, we measured sexual dimorphism based on female and male allometries between chela length and body size. Lastly, we calculated the eusociality index [39] for each species as a measure of reproductive skew (*sensu* [23]). We compared these metrics among species of *Synalpheus* to test three key predictions, namely that as reproductive skew increases, (i) reproductive conflict among females would increase, (ii) females would be expected to develop larger weapons, and (iii) sexual dimorphism would decrease. Ultimately, this study sheds new light on how patterns of reproductive skew influence the evolution of weapons and sexual dimorphism in social species that cannot be explained by operational sex ratio.

## Material and methods

### *Histology*

We sampled five eusocial *Synalpheus* species (*S. brooksi*, *S. chacei*, *S. duffyi*, *S. elizabethae* and *S. regalis*) and three communal species (*S. dardeaui*, *S. pectiniger* and *S. yano*) from Belize, Florida, Jamaica and Panama between 2003 and 2014. We collected whole sponges using SCUBA, and then removed and identified all shrimps inhabiting each sponge under light microscopy. Individuals of the same species from a single sponge were considered a colony. Ovigerous (i.e., egg-bearing) individuals and those

with a brood pouch were considered to be reproductive females (i.e., queens). Individuals with a brood pouch have extended pleura on the abdomen, which indicated a recent release of eggs or larvae, and were found only in communal species; 27 of these females were confirmed to have mature ova by histology. Subsets of non-ovigerous individuals were sexed using histology or scanning electronic microscopy (SEM).

We chose 20 non-ovigerous individuals from 4-6 colonies from each species for histological examination ( $n = 670$  total), visually excluding individuals of the smallest size class (i.e., juveniles). Specimens were preserved in Davidson's fixative (3:3:2:1 of distilled water, 95% ethanol, 37% formaldehyde and glacier acetic acid), decalcified overnight (in 0.1 g/ml sodium citrate in 22.5% formic acid), dehydrated, infiltrated, and embedded in paraffin using standard protocols [40]. Sagittal sections (3-5  $\mu\text{m}$ ) were cut with a rotary microtome and mounted onto glass slides before staining with hematoxylin and eosin. Depending upon an individual's carapace length, we examined 6-12 sets of 3-5 continuous sections, each separated by 20-30  $\mu\text{m}$  until at least half of the specimen was sectioned.

Individuals were sexed and classified as immature or mature based on gonadal development. Males were scored for the presence of sperm or testis. Sperm were highly basophilic with the distinct umbrella shape characteristic of decapod crustaceans, and were located in the testis, vas deferens, or in an enlarged sac near the gonad opening (gonopore) at the base of the fifth walking leg. Females were scored for the presence of developing oocytes, young ova, or mature ova according to Bell and Lightner [41]. Mature ova had lipid-filled cytoplasm and were distinctively larger cells than young ova.

Thus, individuals were categorized as (i) mature males with sperm and testis, (ii) immature males with only testis, (iii) mature females with mature ova, or (iv) immature females with developing oocytes or young ova. Additionally, a few specimens were considered to be hermaphrodites (i.e., intersex) when both a vas deferens and oviduct were found at the bases of the third and fifth walking legs (coxae of pereiopods), respectively [36]. Hereafter, we refer to these individuals as hermaphroditic, rather than intersex (*sensu* [37]), because of histological evidence of sequential hermaphroditism (see Supplemental Materials).

#### *Scanning electronic microscopy*

For sex determination using SEM, we sampled at least 10 non-ovigerous individuals (mean = 17.71; range = 11 to 24) in the adult size classes per colony for eusocial species and at least four individuals (mean = 4.82; range = 4 to 7) per colony for communal species (Table 1); the lower sample size in communal species was due to their smaller colony sizes. Ethanol preserved specimens were dehydrated with hexamethyldisilazane [42] and examined in the Microscopy and Imaging Facility at the American Museum of Natural History. Specimens were scored according to the presence of male gonopores on the bases of the fifth walking legs and/or female gonopores on the bases of the third walking legs [36]. Specimens were considered to be hermaphrodites when both male and females gonopores were present.

### *Reproductive maturity and sex ratios*

We measured carapace length (CL) and major chela length (MCL) from photographs (*sensu* [36]) using ImageJ v1.48 [44]. We estimated the size at maturity for each sex and species separately as the size of the smallest individuals that had mature gonads (see Supplementary Materials). Only individuals larger than the size at maturity were considered adults and used in subsequent analyses on proportions of mature males and females, sex ratios, and allometries. Although our delineation of maturation size as the size of the smallest individuals is somewhat arbitrary, a more stringent criterion produced results that were qualitatively similar (see Supplementary Materials).

Since our histological samples represented a subsample of non-ovigerous individuals in each colony (20 individuals out of a maximum colony sizes of 350 and 88 for eusocial and communal species, respectively), we estimated the number of mature and immature males or non-ovigerous females of non-ovigerous individuals, excluding reproductive females (i.e., those with eggs or extended pleura), based on proportions calculated from the subsample. The total number of mature females included both the observed number of reproductive females and the estimated number of mature non-ovigerous females. Thus, we calculated the (i) proportion of mature females as the number of mature females to total females, (ii) proportion of mature males as the number of mature males to total males, (iii) ASR for each colony as the number of males divided by the sum of females and males (mature and immature), and (iv) OSR as the number of mature males divided by the sum of mature female and mature males. For the proportions of mature females and males, four colonies were excluded from the analysis: two colonies of *S. regalis* that had no females and two colonies of *S. duffyi* in which all

colony members were smaller than the maturation size. To estimate potential reproductive conflict, we compared the proportions of mature females and males in communal and eusocial species using generalized linear mixed models with binomial responses with species and colony included as random factors. P-values were obtained from likelihood ratio tests. We further tested for the effect of body size on the difference in the proportion of immature females between communal and eusocial species (see Supplementary Materials).

Sex ratios from SEM were calculated from non-hermaphrodites, and ASRs of the entire colony were estimated as they were from histological data. Sex ratios from SEM could not be assessed for *S. duffyi* and *S. pectiniger* because all non-ovigerous individuals were hermaphrodites. Since SEM cannot assess the functional sex of a hermaphrodite (see Supplementary Materials), we performed subsequent analyses based on ASRs from histology. Excluding *S. duffyi* and *S. pectiniger*, for which these calculations were impossible, ASRs calculated from SEM and histology did not differ for any species (all  $t < 0.64$ , all  $p > 0.064$ ), except for *S. regalis* ( $t_{8.91} = 3.37$ ,  $p = 0.0084$ ; Table 1, Figure S1). We tested ASRs and OSRs against 0.5 (i.e., a 50:50 sex ratio) in each colony using G-tests of goodness-of-fit with sequential Bonferroni correction, and for each species using repeated G-tests [45]. When sex ratios varied significantly among colonies for a given species, we examined sex ratios for each colony instead of by species.

### *Social structure*

Social structure was estimated using a variation of the eusociality index [38], calculated as  $E = 1 - (2*Q)/N$  where N is colony size and Q is the number of reproductive females

(*sensu* [22, 23]). The eusociality index (E) incorporates both colony size and reproductive skew of a colony, making the simplifying assumption that all breeding individuals contribute equally to offspring production. We determined whether the proportion of mature females, ASR, and OSR were each correlated with E using linear regression; we also made similar comparisons based on categories of sociality (communal versus eusocial) (see Supplementary Materials).

### *Sexual dimorphism*

To quantify sexual dimorphism for each species, we first examined the allometry for each sex between the logarithm-transformed CL and MCL using a major-axis regression [46]. We used major-axis regression instead of ordinary least squares regression because we were interested in the underlying relationship between CL and MCL instead of predicting MCL from CL, and vice versa [47]. To determine if females develop larger weapons with increasing skew, we compared the difference in the allometric slopes of each species by sex and sociality (communal versus eusocial) using ANOVA and performed linear regressions between E and the allometric slopes of females and males.

We quantified sexual dimorphism for each species as the ratio of the male to female allometric slopes of MCL on CL. An allometry ratio = 1 means that females and males have the same allometric slope, whereas as an allometry ratio > 1 means that males have a larger allometric slope than females, and an allometry ratio < 1 means that females have a larger allometric slope than males. In other words, larger allometric ratios mean that males have a steeper increase in major chela size with carapace length than females.

We used a ratio of slopes instead of the difference because the magnitude of the

difference will be affected by the size of the species (range of mean species CL = 2.4 – 6.4 mm). Additionally, we compared the slopes instead of the intercepts because of a significant interaction between CL and sex in predicting MCL (generalized linear mixed models with species as random factor,  $\chi^2_1 = 63.6$ ,  $p < 0.0001$ ); hence difference in the intercept cannot accurately quantify sexual dimorphism.

We fit allometry ratio as a function of OSR, CL, and either sociality (communal versus eusocial, ANCOVA) or E (multiple regression). We included mean species CL as a covariate in our models to control for body size, since sexual size dimorphism often varies with body size [48]. The proportion of mature females was not used as a predictor because it has the same numerator as OSR. Importantly, to control for shared evolutionary histories, we calculated phylogenetic contrasts [49] for all variables using the R package *ape* [50] and repeated the regression analysis. A phylogenetic tree of the eight species was extracted from a Bayesian consensus tree consisting of 1958 bp from three genes (16S, COI and EF2) [51]. We also performed a linear regression of allometry ratio and OSR. All analyses were performed with R v3.0.1 [52].

## Results

### *Reproductive maturity and sex ratios*

Proportions of mature females and males, ASRs, OSRs and E for each species are shown in Table 1. Significantly more males were reproductively mature than females in both communal and eusocial species (communal:  $\chi^2_1 = 17.44$ ,  $p < 0.0001$ ; eusocial  $\chi^2_1 = 210.25$ ,  $p < 0.0001$ ; Figure S2a). The proportion of reproductively mature males in eusocial and communal species did not differ ( $\chi^2_1 = 0.33$ ,  $p = 0.57$ ; Figure S2a), but

eusocial species had a lower proportion of mature females than did communal species ( $\chi^2_1 = 4.52, p = 0.033$ ; Figure S2a). This is consistent with the finding for *S. elizabethae* [35], that female—but not male—workers in eusocial species were reproductively suppressed.

In most species, nearly all males were mature and the proportion of mature males did not vary with E ( $F_{1,6} = 0.47, p = 0.52$ , adj.  $r^2 = -0.083$ , Figure 1a). In contrast, the proportion of mature females was strongly negatively correlated with E ( $F_{1,6} = 11.18, p = 0.016$ , adj.  $r^2 = 0.59$ , Figure 1b), such that species exhibiting high reproductive skew had lower proportions of mature females. ASRs of most species from both histology and SEM averaged near 50:50 or slightly male-skewed, but many species showed high variability among colonies (Table 1, Figure S3). Although ASR was not significantly related to E ( $F_{1,6} = 1.243, p = 0.31$ , adj.  $r^2 = 0.034$ , Figure 1c), OSR increased strongly with E ( $F_{1,6} = 15.93, p = 0.0072$ , adj.  $r^2 = 0.68$ , Figure 1d), becoming more male-biased as skew increased (see also Table 1, Figure S1).

### *Sexual dimorphism*

We quantified sexual dimorphism for each species by the allometry of CL and MCL (Figure S4). Overall, allometric slopes did not differ between the sexes ( $F_{1,1} = 0.25, p = 0.62$ ), but eusocial species had steeper allometry, i.e., larger major chela for a given body size (CL), than communal species ( $F_{1,1} = 9.47, p = 0.0088$ ). The allometric slopes of male chelae were mostly positive or isometric, whereas the allometric slopes of female chelae were negative for communal species but positive for eusocial species (Figures S4 and S5). Moreover, allometric slopes increased with E in females ( $F_{6,1} = 10.95, p = 0.016$ ,

adj.  $r^2 = 0.58$ , Figure 1e) but not in males ( $F_{6,1} = 1.15, p = 0.32$ , adj.  $r^2 = 0.022$ , Figure 1f). Thus, in eusocial species with high reproductive skew, large females had proportionally larger chelae, whereas in communal species with lower skew, large females had proportionally smaller chelae.

Allometry ratio (i.e., the degree of sexual dimorphism in chela allometry) was significantly higher in communal species than eusocial species (Table 2, Figure 2a) and decreased with E (Figure 2b). Moreover, multiple regression showed that allometry ratio was significantly related to E and mean CL, but not to OSR, both using raw data (Table 2, Figure 3) and phylogenetically independent contrasts (Table 2, Figure S6). Critically, the allometry ratio decreased as E increased (i.e., greater skew), as CL increased (i.e., body size), but not as OSR increased, both using raw data (Table 2, Figure 3) and phylogenetically independent contrasts (Table 2, Figure S6). Finally, raw values of allometry ratio were not significantly correlated with OSR ( $F_{1,6} = 4.92, p = 0.068$ , adj.  $r^2 = 0.36$ , Figure 3d).

## Discussion

Selection on traits used for access to mates, resources, or social rank may be similarly strong in both sexes in social vertebrates and insects with totipotent workers because intrasexual competition is similarly strong in females as it is in males [9, 11, 12]. Here we show that patterns of sexual dimorphism in secondary sexual characteristics do not just differ between social and non-social species (*sensu* [11]), but instead vary continuously among closely related social species that differ in their degree of reproductive skew. In agreement with our predictions, as reproductive skew increased (indicated by increasing

E) across sponge-dwelling snapping shrimps, (i) potential reproductive conflict among females increased because fewer females were able to reach reproductive maturity, (ii) females—but not males—had larger competitive weapons (i.e., the snapping chela), and importantly (iii) sexual dimorphism of the snapping chela decreased. Moreover, sexual dimorphism in *Synalpheus* was well predicted by the degree of reproductive skew, but not by operational sex ratio.

The difference between adult sex ratio (ASR) and operational sex ratio (OSR) reflects mating prospects of individuals driven by underlying physiology or proximate mating opportunity [38]. In *Synalpheus*, sociality and the presence of reproductive suppression in species with high reproductive skew (suggested here and experimentally demonstrated in [35]) dramatically affected the transition from ASR to OSR. Both SEM and histological analysis indicated that the ASRs of eusocial and communal species were similar, indicating that *Synalpheus* species with very different social structures have similar demographic structures [38]. OSR deviated only slightly from ASR (and a 50:50 sex ratio) in low-skew species; however, OSR deviated considerably from ASR, being heavily male-biased, in high-skew species. The higher OSRs in high-skew species were due to sex-specific reproductive suppression in females, hence the prevalence of mature female workers—but not male workers—decreased. Although the positive relationship between OSR and E may seem circular, it is not: high values of E are the result of high queen to workers ratios (i.e., reproductive skew) regardless of the sexes and maturation status of the workers. Thus, the calculation of E is independent of OSR or the proportion of mature females or males in a colony.

Classical sexual selection theory predicts that when only males compete for

mates, sexual dimorphism should increase with OSR because as OSR increases (i.e., more reproductively mature males than females), males would evolve larger secondary sexual traits to compete more effectively for females [6, 7]. In contrast to this prediction, we found that sexual dimorphism in fighting chelae (as depicted by allometry ratios) actually decreased with OSR. However, after controlling for the effect of E and mean species body size, OSR no longer predicted sexual dimorphism. Although OSR did not drive the overall pattern of sexual dimorphism in *Synalpheus*, low-skew species did exhibit sexual dimorphism: males of communal *Synalpheus* species had proportionally larger major chelae than females. This is expected because in communal species males have easy access to female neighbors, and since males of caridean shrimps can mate multiple times within a molt-cycle [53, 54], they may have a higher variance in reproductive success (and be under stronger sexual selection) than females.

Why do *Synalpheus* species depart from the prediction that male-biased OSR should select for stronger sexual dimorphism? Similar to cooperatively breeding vertebrates and facultatively eusocial insects with totipotent workers, reduced sexual dimorphism may be driven by increased selection on females to compete with other females for reproductive opportunities and/or access to mates [10, 11, 55]. We have shown elsewhere that in the eusocial *S. elizabethae*, queens can suppress reproduction in female—but not male—workers [35]. The prevalence of immature female workers in high-skew *Synalpheus* species found in the present study is consistent with the hypothesis that the sex-specific reproductive suppression demonstrated in *S. elizabethae* is also operating in these other species. The immature workers in these species are reproductively primed for becoming replacement queens [35], hence, the potential for

reproductive conflict among females is high. Since most males are reproductively capable but the queen typically mates with a single one [56], competition among males and the variance of male reproductive success may remain high in eusocial *Synalpheus* species. Although it is unclear how often a worker could inherit a colony in eusocial shrimps, worker inheritance occurs in eusocial species like termites [18, 55, 57] and naked mole-rats [58]. Although species with multiple breeding females per colony (e.g., *S. brooksi* and *S. elizabethae*) are predicted to exhibit stronger intraspecific reproductive competition among females than species with a single breeder per colony [59], reproductive skew (i.e., E) incorporates the degree of breeding by multiple reproductives into the ratio of the number of queens to colony size. Therefore, we expect female-female competition to increase linearly with reproductive skew. In support of this idea, we have shown that (i) female allometric slopes were higher as reproductive skew increased and (ii) female *Synalpheus* had larger chelae in species with higher skew. This is strikingly similar to African starlings, in which females in cooperatively breeding species were more ornamented than non-cooperative species [11]. Finally, we have shown that sexual dimorphism decreased (i.e., became more monomorphic) with increasing skew.

The pattern we observed in sexual dimorphism of the major chela also reflects other aspect of social behavior in *Synalpheus*. The trend of relatively larger chelae in eusocial species (although only significant in females) suggests that eusocial species may be better competitors against rivals of the same size in conventional (non-sexual) competition, irrespective of their ability to cooperatively defend [30]; this is consistent with community-level data showing that eusocial species were more abundant than non-social species on Belizean coral reefs [23]. Larger weapons in eusocial species may be

adaptive for colony defense as in many social insects where selection acts on traits used to defend valuable resources that colonies control [60]. Therefore, chela size may be driven by both natural and sexual/social selection. Moreover, females in communal species had smaller major chelae at a given size (negative allometry) than males, while the pattern is reversed in eusocial species. This could reflect differential resource allocation, such that females in communal species allocate more resources to reproduction [61], whereas females in eusocial species allocate more resources to weaponry.

Although social vertebrates and invertebrates differ greatly in their ecology, life history, and genetic systems, social systems with strong reproductive skew appear to have a similar effect of reducing the degree of sexual dimorphism in weapons used in intrasexual competition in both kinds of animals. We have shown that species of snapping shrimps with high reproductive skew are sexually monomorphic in the snapping chela, despite having highly male-skewed operational sex ratios. This is likely a result of selection in eusocial species for larger antagonistic weaponry in females used in intrasexual competition for breeding opportunities, as supported by the rarity of reproductively mature females in highly skewed species [62]. Thus, this study not only supports the recent refocus on social competition among females in altruistic societies [10, 55, 62, 63], but it demonstrates consistent differences in patterns of sexual dimorphism among social species with different forms of altruistic societies, not just between social and non-social species.

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**Table 1.** Proportions of mature females and males, sex ratios (ASR and OSR), proportion of hermaphrodites (herm.) and eusociality index (E) in *Synalpheus spp.* from histology and scanning electron microscopy. Values inside brackets are standard errors.

social system	species	histology							scanning electron microscopy				
		sample size		prop. of mature female s	prop. of mature males	ASR	OSR	prop. of herm.	sample size		ASR	prop. of herm.	
		colo ny	in d.						E	colo ny			
eusocial	<i>S. brooksi</i>	5	12	0.38 (0.11)	0.96 (0.02)	0.63 (0.04)*	0.81 (0.05) #	0.00 (0.00)	0.79 (0.06)	3	58	0.55 (0.03)	0.04 (0.02)
	<i>S. chacei</i>	5	91	0.68 (0.09)	0.92 (0.03)	0.70 (0.05)*	0.75 (0.06)*	0.00 (0.00)	0.90 (0.02)	3	52	0.59 (0.05)	0.10 (0.06)
	<i>S. duffyi</i>	5	49	0.38 (0.31)	0.62 (0.19)	0.50 (0.22) #	0.85 (0.08)*	0.00 (0.00)	0.89 (0.06)	2	37	NA	1.00 (NA)
	<i>S. elizabethae</i>	5	11	0.41 (0.09)	0.97 (0.03)	0.54 (0.05)^	0.75 (0.04)^	0.00 (0.00)	0.96 (0.01)	3	58	0.59 (0.06)*	0.19 (0.06)
	<i>S. regalis</i>	6	92	0.26 (0.25)	1.00 (0.00)	0.86 (0.06)^	0.99 (0.01)^	0.00 (0.00)	0.97 (0.02)	5	96	0.60 (0.05) #	0.00 (0.00)
communal	<i>S. dardeaui</i>	4	31	0.63 (0.05)	0.96 (0.04)	0.48 (0.02)	0.59 (0.04)	0.00 (0.00)	0.34 (0.07)	5	24	0.64 (0.06) #	0.00 (0.00)
	<i>S. pectiniger</i>	5	10	0.83 (0.10)	0.97 (0.03)	0.65 (0.07) #	0.7 (0.05)*	0.15 (0.12)	0.51 (0.08)	1	4	NA	1.00 (NA)
	<i>S. yano</i>	5	65	0.95 (0.03)	0.97 (0.03)	0.57 (0.04)*	0.58 (0.03)*	0.00 (0.00)	0.23 (0.04)	3	25	0.61 (0.07)*	0.00 (0.00)

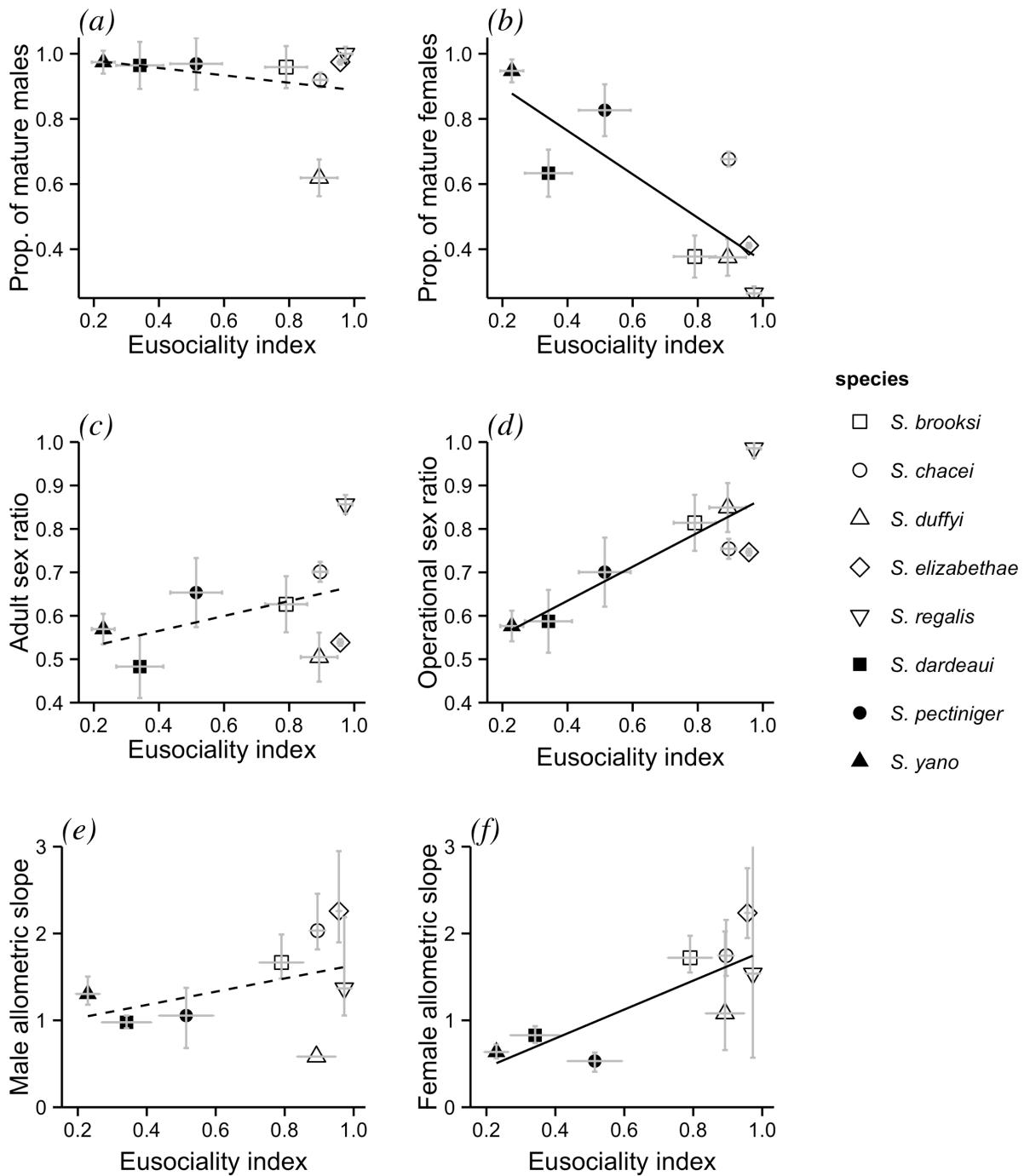
\* indicates sex ratio significantly deviated from 50:50

# indicates sex ratio varied significantly among colonies

^ indicates sex ratio varied significantly among colonies, but all colonies were significantly deviated from 50:50

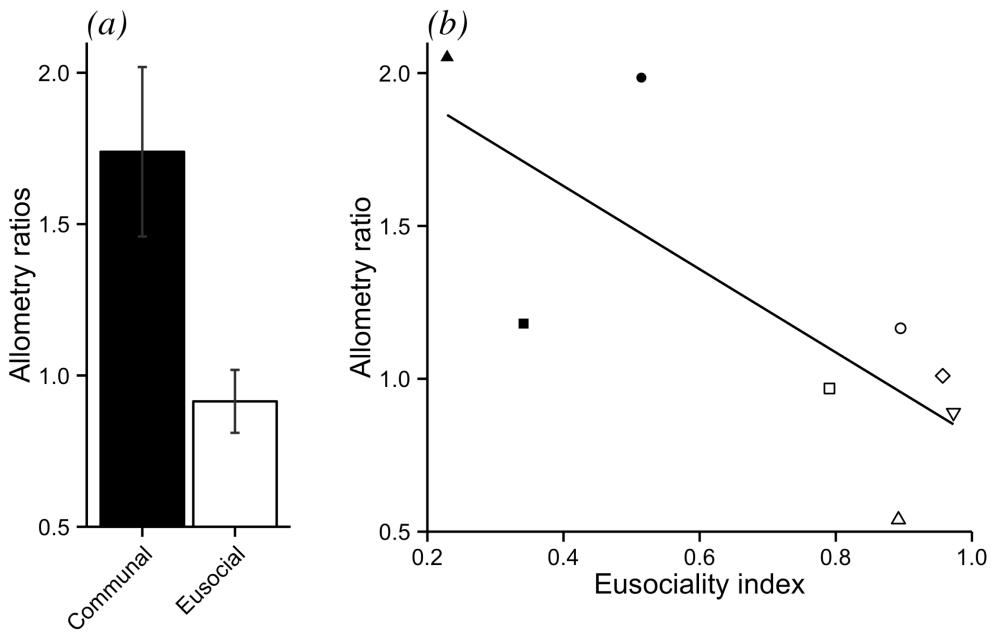
**Table 2.** Results of multiple regressions estimating the effects of (a) sociality or (b) eusociality index, carapace length and operational sex ratio on allometry ratio using raw data and (c) phylogenetic contrasts in *Synalpheus*.

data type	response variable	F	d.f.	p	adj. $r^2$	$\beta$	$t_I$	p
(a) raw data	overall model	13.22	3, 4	0.015	0.84			
	sociality	17.45	1, 4	0.014		-1.20	-4.18	0.014
	carapace length	11.18	1, 4	0.029		-0.36	-3.34	0.029
	operational sex ratio	2.10	1, 4	0.22		-1.51	-1.45	0.22
(b) raw data	overall model	7.56	3, 4	0.04	0.74			
	eusociality index	9.11	1, 4	0.034		-2.40	-3.02	0.039
	carapace length	7.30	1, 4	0.054		-0.40	-2.70	0.054
	operational sex ratio	0.44	1, 4	0.54		-0.94	-0.67	0.54
(c) contrasts	overall model	23.03	3, 3	0.013	0.92			
	eusociality index	40.30	1, 3	0.0079		-3.10	-6.35	0.0079
	carapace length	42.34	1, 3	0.0074		-0.44	-6.50	0.0074
	operational sex ratio	1.09	1, 3	0.37		-0.76	-1.05	0.37

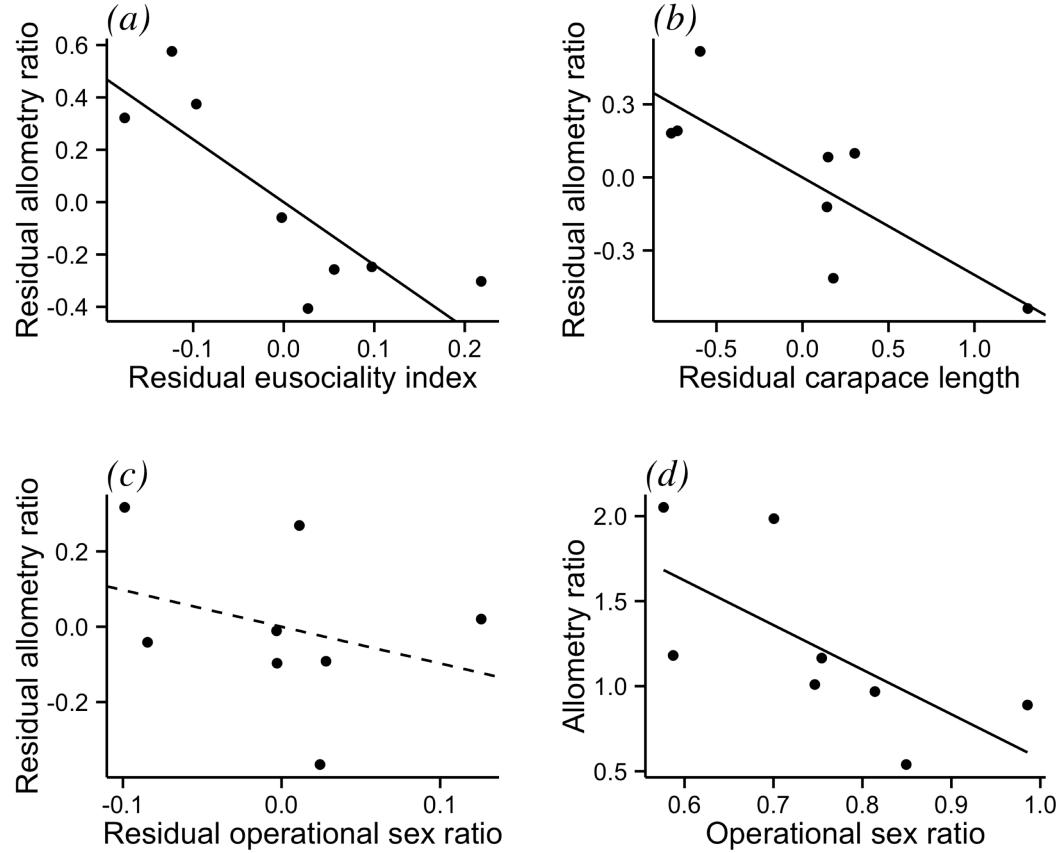


**Figure 1.** Relationships between eusociality index (E) and (a) proportion of mature males, (b) proportion of mature females, (c) adult sex ratio, (d) operational sex ratio, (e) male allometric slope, and (f) female allometric slope in *Synalpheus*. Grey error bars

indicate standard errors. Closed and open symbols represent communal and eusocial species, respectively.



**Figure 2.** Relationships between allometry ratio and (a) sociality and (b) eusociality index. White and black bars represent communal and eusocial species, respectively. See Figure 1 for symbols used in (b).



**Figure 3.** Relationships between allometry ratio, eusociality index (E), operational sex ratio (OSR) and carapace length (CL). Axes show (a) partial residual controlling for OSR and CL, (b) partial residual controlling for E and OSR, (c) partial residual controlling for E and CL, and (d) regression of raw allometry ratio and OSR. Solid and dashed lines indicate significant (at  $p < 0.05$ ) and non-significant regressions, respectively. In (a, b), the degree of sexual dimorphism in chela allometry (i.e., allometry ratio) decreased as reproductive skew (i.e., E) and CL increased. In (c, d) sexual dimorphism decreased with OSR but not after controlling for E and CL.

## **Supplemental material and methods**

### *Determination of size at maturity*

Since females appeared to mature at larger sizes than males, especially for eusocial species, we determined a size at maturity for each sex and each species separately as the size of the smallest individuals that had mature gonads. However, the eusocial species *S. regalis* had no mature females except the much larger ovigerous queens. For this species, we estimated the female maturation size as the male maturation size times 1.179 (S.E. = 0.04), which was the averaged fraction of maturation sizes between females and males in six species (excluding *S. pectiniger* because it had a higher male maturation size than female, as well as a high percentage of hermaphrodites).

We recognize that our delineation of maturation size as the size of the smallest individuals is somewhat arbitrary. For decapod crustaceans, maturation size is often defined as the size class where 50% of the individuals are mature ( $CL_{50}$ ) [1]. However, low sample sizes and the presence of reproductive suppression in eusocial species precluded accurate estimations of  $CL_{50}$  in our data. Our current delineation of maturation size could be lower than  $CL_{50}$ . To show that a larger maturation size would not qualitatively affect our conclusions, we defined a second maturation size as the 25<sup>th</sup> percentile size of the mature individuals in each sex. Despite using this more stringent criterion, the overall pattern of maturity remained the same: most males were mature in both eusocial and communal species, whereas a smaller proportion of females were mature in eusocial versus communal species (Figure S2b).

### *Discrepancy between results from SEM and histology*

In *S. brooksi*, *S. chacei*, and *S. elizabethae*, SEM identified a low proportion of hermaphrodites, but histology did not identify any intersex individuals (Table 1). In *S. duffyi* and *S. pectiniger*, SEM identified that all non-ovigerous individuals were hermaphrodites, but histology only identified a small number of hermaphrodites in each of these species (Table 1). These hermaphrodites, identified by histology, possessed both vas deferens and oviducts, but none of them had both male and female functional gonads; hence we did not observe a single instance of specimens having both functional testis and ova (i.e., intersex) in *S. duffyi* and *S. pectiniger*. This suggests that these species exhibit sequential hermaphroditism.

We selected specimens of *S. regalis* ( $n = 4$ ) and *S. duffyi* ( $n = 4$ ) to be examined both histologically and by SEM. In *S. regalis*, a species with no hermaphrodites, classifications of sex were identical using both methods. However, in *S. duffyi*, a species with 100% hermaphrodites, histological specimens with either male or female gonads were identified as hermaphrodites under SEM. Therefore, histology cannot adequately exclude the presence of hermaphroditism, but SEM cannot assess the functional sex of a hermaphrodite. Since calculating sex ratios requires knowing the sex of the specimen, we used histological data to calculate and analyze ASRs.

### *Reproductive maturity and sociality*

To test for an effect of size on maturity, we first looked for a size difference between immature and mature workers (separately for each sex) using generalized linear mixed models (GLMMs) with species and colony included as a random factors. *S. duffyi* was

excluded because there were no immature males. We then performed a logistic regression of sociality versus the odds of being mature, with CL as a covariate and species and colony included as random factors. Since eusocial species were generally smaller than communal species, we used Z-scores to standardize CL separately for each species.

We found that immature adult females had smaller CL than mature adults in both communal and eusocial species (communal:  $\chi^2_1 = 13.95, p < 0.0001$ ; eusocial:  $\chi^2_1 = 36.92, p < 0.0001$ ). The different levels of female maturity in eusocial and communal species remained significant ( $\chi^2_1 = 4.89, p = 0.027$ , Figure S2a) even after controlling for the effect of the smaller size of immature workers ( $\chi^2_1 = 96.40, p < 0.0001$ ). A unit increase in CL (Z-score) resulted in 9.52 higher odds of being mature in communal species, but only 0.37 higher odds in eusocial species (difference between odd ratios:  $Z = 2.60, p = 0.0093$ ). Therefore, eusocial species still had a much higher proportion of immature females than communal species, regardless of size. In contrast, immature adult males had smaller CL than mature adults in communal species ( $\chi^2_1 = 15.34, p < 0.0001$ ), but not in eusocial species ( $\chi^2_1 = 0.026, p = 0.87$ ).

#### *Sex ratio and sociality*

Using generalized linear mixed models with species and colony (nested within species) as random factors, we determined separately for each sex whether carapace length differed by degree of maturation (immature versus mature) and sociality (communal versus eusocial). Using weighted generalized linear mixed models with logit links, binomial error distribution and species as random factor, we determined whether (i) the

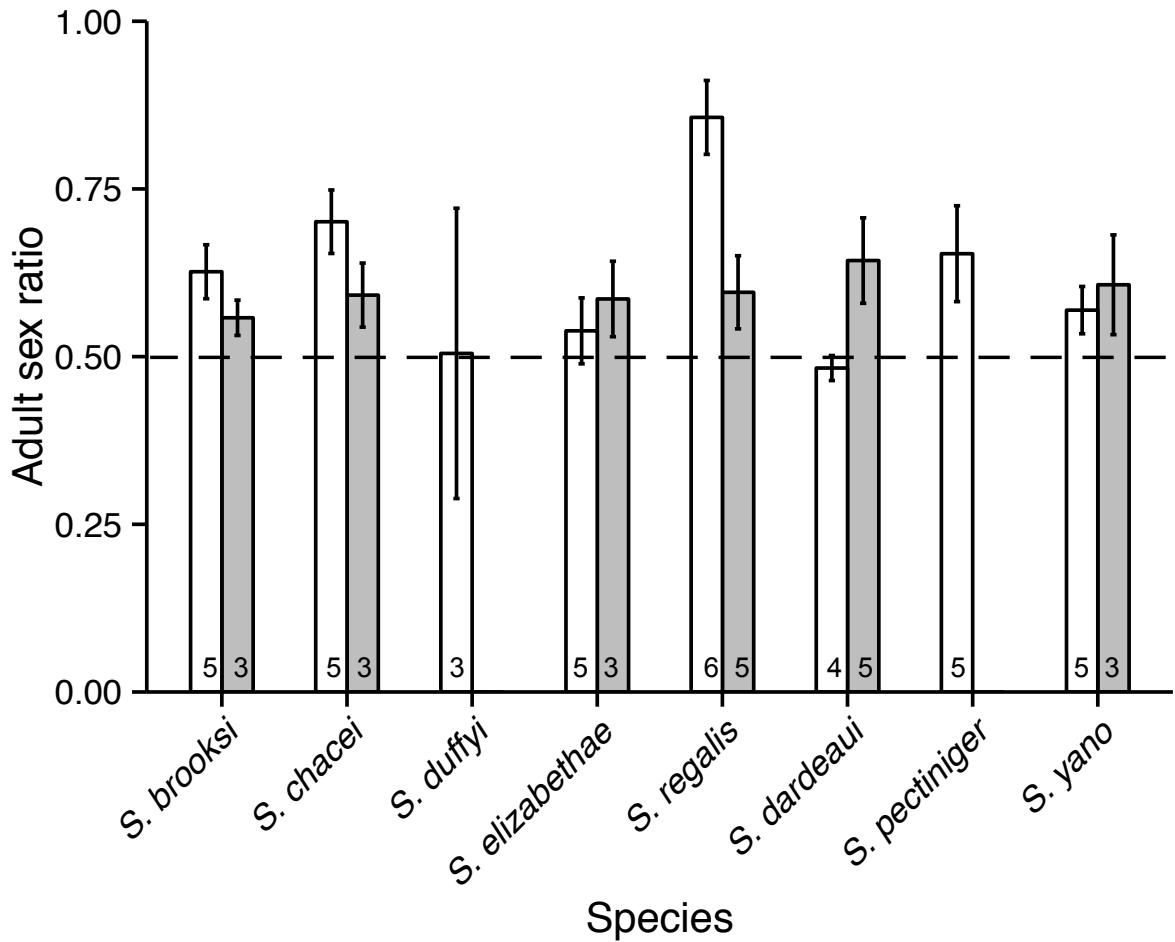
proportions of mature individual differed by sex and sociality and (ii) sex ratios differed by sociality and type (ASR versus OSR). For the SEM data, we determined whether adult sex ratios from SEM differed between communal and eusocial species using generalized linear mixed models with species included as a random factor.

We found that the ASRs of most eusocial species were slightly male-skewed, but OSRs were strongly male-skewed in all eusocial species (Table 1). In contrast, ASRs of communal species were generally even, and OSRs were only slightly male-skewed (Figure S7). Overall, OSRs were more highly male-skewed than ASRs, but the difference between OSR and ASR was more pronounced in eusocial species than in communal species (sociality \* type of sex ratio,  $\chi^2_1 = 6.18, p = 0.013$ ) (Figure S7). Moreover, OSRs were more highly male-skewed than ASR in eusocial species ( $\chi^2_1 = 29.18, p < 0.0001$ ), but not in communal species ( $\chi^2_1 = 0.93, p = 0.33$ ) (Figure S7). Although the ASRs did not differ between communal and eusocial species ( $\chi^2_1 = 0.29, p = 0.59$ ), OSRs were marginally higher in eusocial than communal species ( $\chi^2_1 = 3.20, p = 0.074$ ). Finally, ASRs calculated from SEM did not differ between communal and eusocial species ( $\chi^2_1 = 0.034, p = 0.85$ ).

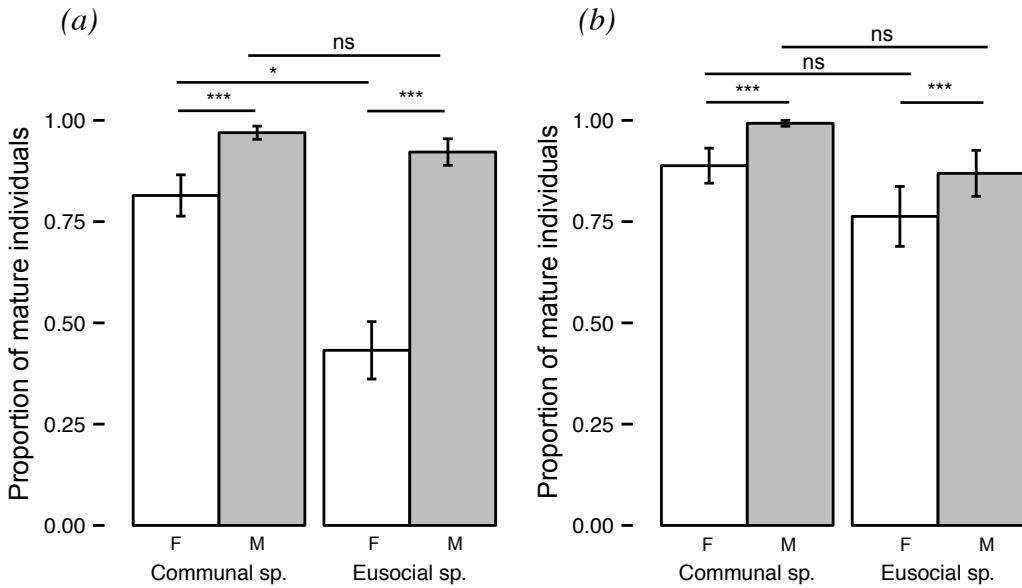
### **Supplemental references**

1. Somerton D.A. 1980 A Computer Technique for Estimating the Size of Sexual Maturity in Crabs. Can J Fish Aquat Sci 37, 1488-1494. (doi:10.1139/f80-192)

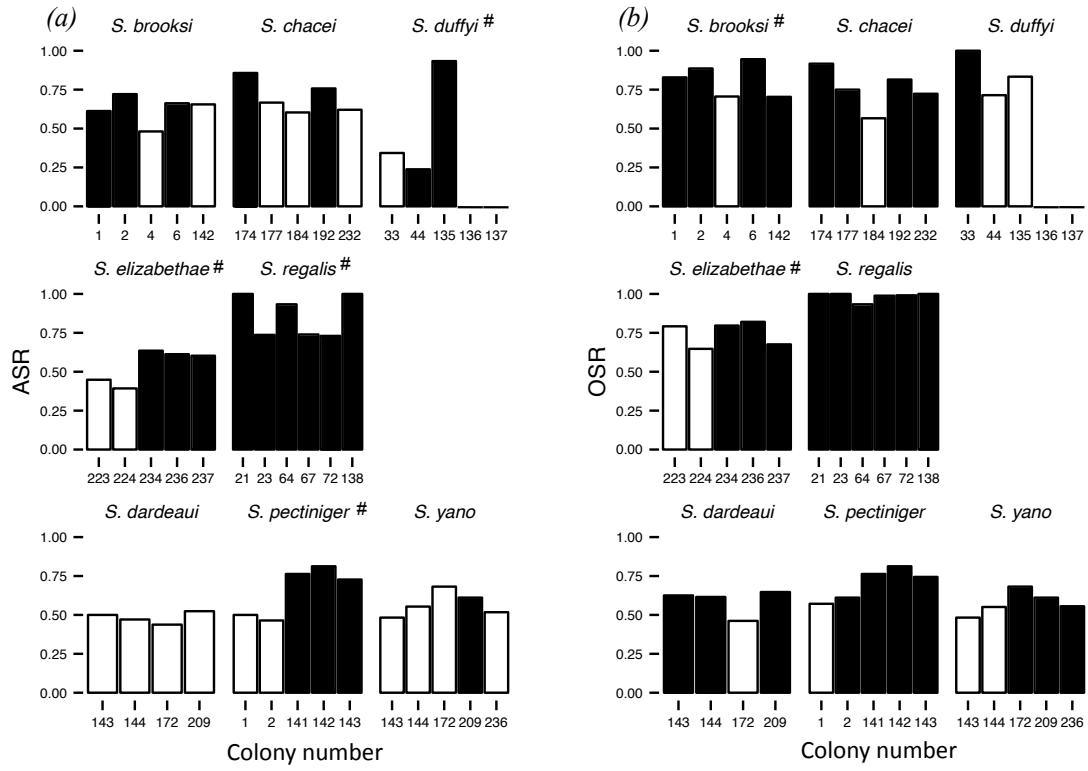
## Supplemental figures



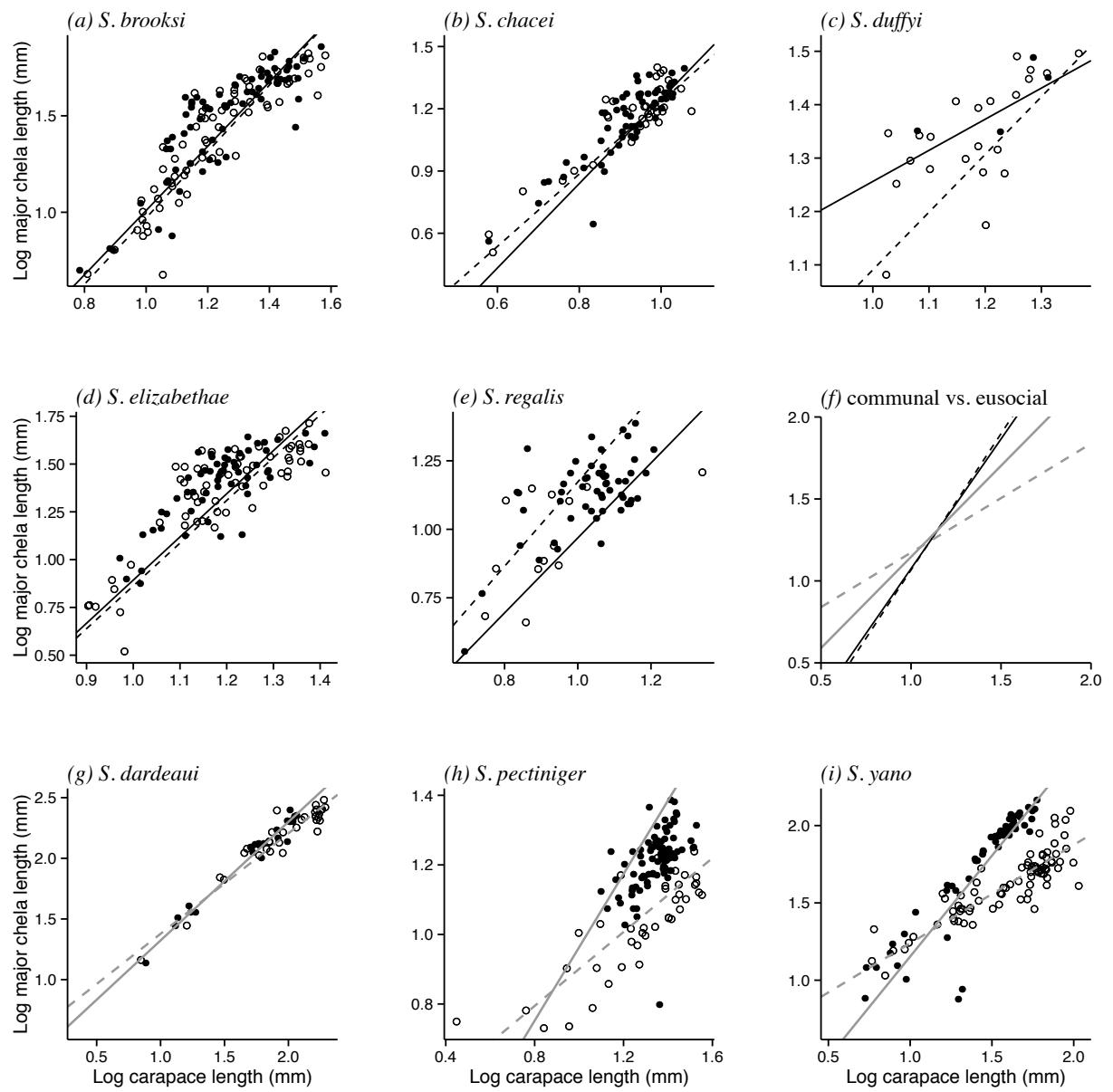
**Figure S1.** Comparison of mean  $\pm$  SE adult sex ratios (ASRs) from scanning electronic microscopy (grey bars) and histology (white bars). ASRs calculated from the two methods differed only in *S. regalis*. Numbers below each bar indicate numbers of colonies. ASR could not be calculated for *S. duffyi* and *S. pectiniger* from scanning electronic microscopy because both species had workers that were externally hermaphroditic.



**Figure S2.** Mean  $\pm$  SE proportion of mature females and by sociality based on maturing size defined as (a) the size of the smallest individual with mature gonad and (b) the 25<sup>th</sup> percentile of the sizes of individuals with mature gonad. White and grey bars indicate proportions of mature females and proportion of mature, respectively. Horizontal lines indicate significant ( $p < 0.05$  \*,  $p < 0.0001$  \*\*\*) or non-significant (ns) differences between pairs of proportions based on GLMM with species included as a random factor.

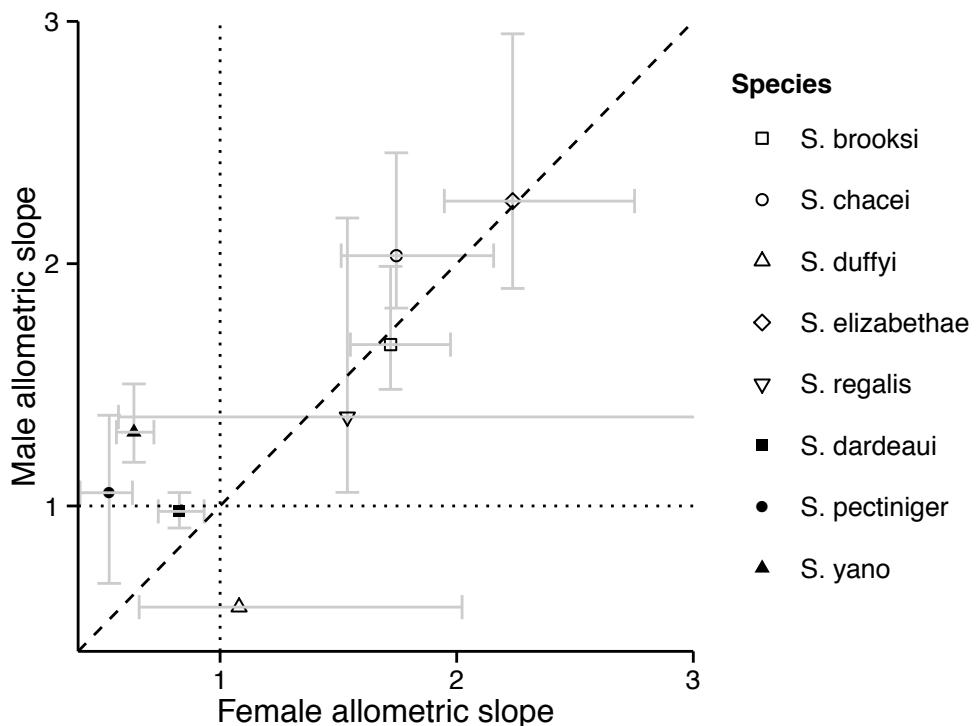


**Figure S3.** (a) Adult sex ratios (ASR) and (b) operational sex ratios (OSR) of each species by colony. Two colonies of *S. duffyi* were excluded because samples sizes of adult were too small. Black bars indicate significant differences from 50:50 for each colony. <sup>#</sup> after species names denotes that sex ratio varied significantly among colonies. In some species, despite sex ratios being heterogeneous among colonies, all colonies deviated from even sex ratios.

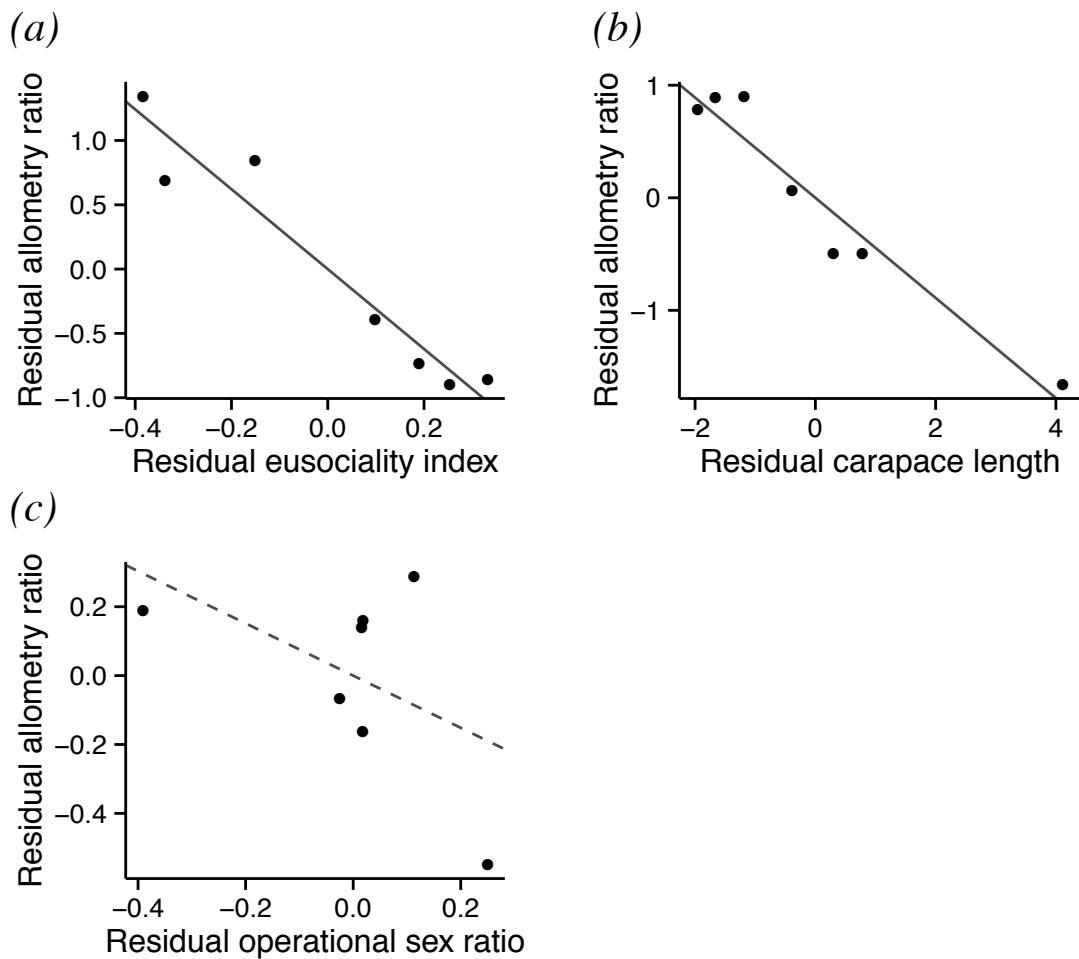


**Figure S4.** Size allometry (major-axis regression) between major chela and carapace length in eusocial (a, b, c, d and e) and communal (g, h and i) *Synalpheus* species. (f) provides a comparison between eusocial (black lines) and communal (grey lines) species. Open and closed circles indicate females and males, respectively. Dashed and solid lines are regression lines for females and males, respectively. For (f), regression lines are based

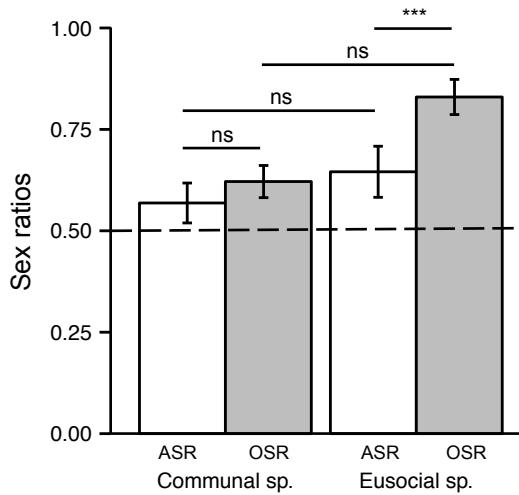
on the averaged major axis regression slopes and intercepts of eusocial and communal species.



**Figure S5.** Allometric slopes of females and males in communal (solid symbols) and eusocial species (open symbols). Error bars are 95% confidence intervals estimated by permutation (not estimated for male *S. duffyi* because of low sample size; upper confidence interval for female *S. regalis* was 6.229). The diagonal dashed line indicated a 1:1 relationship between allometric slopes of females and males; species lying on this line are sexually monomorphic, whereas species above this line are sexually dimorphic towards males, and vice versa. The horizontal and vertical dotted lines indicate where allometric slopes for each sex are isometric; species lying on this line have major chelae and carapaces of equal lengths, whereas species above this line have larger major chelae than carapaces.



**Figure S6.** Relationships between phylogenetic independent contrast values of allometry ratio, eusociality index (E), operational sex ratio (OSR) and carapace length (CL). The axes show (a) partial residual controlling for OSR and CL, (b) partial residual controlling for E and OSR, and (c) partial residual controlling for E and CL. Solid and dashed lines indicate significant (at  $p < 0.05$ ) and non-significant regressions, respectively.



**Figure S7.** Mean  $\pm$  SE adult (ASR) and operational (OSR) sex ratios in communal and eusocial species. ASRs (white bars) did not differ between eusocial and communal species, but OSRs (grey bars) were higher in eusocial species. Horizontal lines indicate significant ( $p < 0.0001$  \*\*\* ) or non-significant (ns) differences between pairs of ratios based on GLMM with species included as a random factor.

**CHAPTER 3**

**EVOLUTIONARY TRANSITIONS TOWARDS EUSOCIALITY IN**

**SNAPPING SHRIMPS**

## **Abstract**

The evolution of extreme reproductive division of labor (reproductive skew) and complex sociality is a remarkable phenomenon that has evolved independently in a variety of animal lineages. Among social animals, eusocial species appear to occupy the extreme end of a continuum based on reproductive skew. Using reproductive skew as a common denominator to understand the evolution of sociality assumes that solitary or pair-forming groups transitioned into eusociality via intermediates of low or medium skew. This assumption has yet to be tested broadly in different animal lineages except for within several Hymenopteran clades. Here we tested the evolutionary trajectory into eusociality using a clade of sponge-dwelling snapping shrimp, *Synalpheus spp.* that has high interspecific variation in social organization. We found that the social organizations of *Synalpheus* species clustered naturally into pair-forming, communal, and eusocial species based on demographic characteristics. Comparison among different models of social evolution showed that eusocial and communal species represent distinct evolutionary trajectories and endpoints. Thus, pair-forming species of *Synalpheus* did not transition into eusocial species via communal intermediates. Therefore, our data support a subsocial origin of eusociality in *Synalpheus*. Further, our findings caution that comparative analyses that assume a continuous scale of reproductive skew could be misleading.

## **Introduction**

Sociality is a the major transition in the history of life (Smith & Szathmary 1997), allowing insects to populate the terrestrial ecosystems and humans to conquer Earth (Wilson 1975). While sociality is widespread in animals, the nature of animal societies differs widely among and within species (Wilson 1971, Brown 1978, Smuts et al. 1987, Choe & Crespi 1997, Solomon & French 1997, Duffy & Thiel 2007). This variation potentially provides materials to understand the evolution of sociality—Darwin's "one special difficulty"—but it has been difficult to develop a unified theoretical framework to compare disparate lineages in the tree of life (Crespi & Yanega 1995, Sherman et al. 1995, Costa & Fitzgerald 1996b, Costa & Fitzgerald 1996a, Reeve et al. 1996, Wcislo 1997, Costa & Fitzgerald 2005, Lacey & Sherman 2005). While a social group can simply consist of a mating pair, many species form larger social groups in which reproduction can be evenly distributed or highly skewed among group members (Sherman et al. 1995). Eusocial species exhibit very high reproductive skew, or uneven reproduction among members, because a single queen usually monopolizes reproduction (especially in ants, bees and termites). In contrast, many animals exhibit medium to low skew where reproduction is distributed more evenly within the group (Brown 1987, Solomon & French 1997, Wcislo & Tierney 2009). This apparently continuous range of animal social organization across invertebrate and vertebrate taxa has raised the intriguing possibility that the continuum could be used to understand the evolutionary causes and consequences of sociality in terms of reproductive skew (Sherman et al. 1995, Lacey & Sherman 2005). An important assumption of this approach for comparative analyses across taxa is that reproductive skew is indeed a continuous trait, meaning that

highly skewed eusocial societies evolved via intermediate steps of medium to low skew. However, after decades of study, we still lack a complete understanding of the evolutionary trajectory from simple social organizations (i.e., solitary or pair-forming species) towards eusocial ones, and whether animal social organizations represent discrete or continuous variation (Rubenstein et al. in press).

Several models have been proposed to explain the evolutionary trajectory towards eusociality. For social insects, it is generally accepted that eusocial species evolved from a so-called “subsocial intermediate” in which offspring remain in the parental nest after they mature (Michener 1969, Danforth 2002, Boomsma 2009). Similarly, the extended-family model also suggests that vertebrates with high reproductive skew evolved through the retention of offspring (Emlen 1995, Emlen et al. 1995). Alternatively, Michener (1969) proposed in the parasocial hypothesis that aggregation of breeders of the same generation may develop into societies with strong reproductive skew. Michener (1974) defined this intermediate stage as communal breeding, in which females share a nest but provision their own offspring, forming groups of low reproductive skew. Although there has been little empirical support of the parasocial hypothesis, the idea was recently revived in an alternative model of the evolution of eusociality (Nowak et al. 2010) that has generated widespread controversy (e.g., Abbot et al. 2011, Boomsma et al. 2011, Bourke 2011, Ferriere & Michod 2011, Gardner et al. 2011, Herre & Wcislo 2011, Marshall 2011, Rousset & Lion 2011, Strassmann et al. 2011). Moreover, communal breeding societies with relatively low skew are more common in social insects and vertebrates than once realized (Rubenstein & Abbot in press). Importantly, these different

hypotheses for the evolution of extreme reproductive altruism disagree on whether or not eusocial societies passed through a low-skew intermediate during their evolution.

Clarifying the evolutionary trajectory towards eusociality could resolve a long-standing debate—whether eusocial species should be treated as the end of a continuum based on reproductive skew or instead as a discrete social organization type (Crespi & Yanega 1995, Sherman et al. 1995, Costa & Fitzgerald 1996b, Reeve et al. 1996, Costa & Fitzgerald 2005, Rubenstein et al. in revision). The theory of reproductive skew is potentially unifying in that skew can be used to quantify the reproductive partitioning in animal societies and provides a general framework to study the evolution of sociality within disparate social taxa (Keller & Reeve 1994, Rubenstein & Abbot in press). Sherman et al. (1995) further proposed to unify social animals on a “eusocial continuum” based on reproductive skew, where eusocial insects occupy the extreme end of the spectrum. Alternatively, others have argued that eusociality should be considered as its own, qualitatively distinct domain because eusocial societies express distinct adaptations such as morphological castes (Crespi & Yanega 1995) and reciprocal communication (Costa & Fitzgerald 1996a). Further, treating social organization as a continuum or using reproductive skew for comparative analyses across taxa makes an important but subtle assumption that reproductive skew is a continuous trait. This assumption equates with the parasocial hypothesis that eusocial species with high reproductive skew evolved via low-skew intermediates, but this hypothesis has remained controversial.

Studies of insects have laid the foundation for the evolution of sociality and have led to further studies in other social lineages. However, testing the evolutionary trajectory towards eusociality is difficult in some insect lineages. For example, in ants and termites,

the early stages of transition are ancient and absent in extant lineages (Thorne 1997, Barden & Grimaldi 2016, Engel et al. 2016); i.e., in corbiculate bees (Cardinal & Danforth 2011, Romiguier et al. 2016) and allodapine bees (Tierney et al. 2008), eusociality is the ancestral state of these socially diverse clades. Empirically, the subsocial model was supported by comparative analysis in the halictid bees (Danforth 2002), but not in the vespid wasps (Hines et al. 2007), although the biology of the latter group is not well studied. However, the subsocial model is consistent with the fact that all advanced eusocial insects are monogamous throughout their lifetimes (Boomsma 2007, Boomsma 2009), thus precludes the formation of communal intermediates. Hence, it is generally accepted that eusociality in insects likely evolved via the subsocial model, yet empirical evidence is equivocal. Therefore, we still lack a complete understanding of the evolutionary trajectory towards eusociality, especially in the role of communal breeders in the transition (Kocher & Paxton 2014).

Here we focused on snapping shrimps in the genus *Synalpheus* to understand the evolutionary trajectory towards eusociality, because the genus is socially diverse and eusociality has evolved recently and repeatedly at least four times (Duffy et al. 2000, Morrison et al. 2004, Duffy & Macdonald 2010). Additionally, *Synalpheus*, particularly the ~45 West Atlantic species in the Gambarelloides group (Hultgren & Duffy 2011, Hultgren et al. 2016), can provide a powerful comparative test of routes of social evolution because all species live obligatorily within canals of sponges; thus they face similar ecological pressures and constraints (Macdonald et al. 2006, Hultgren & Duffy 2010, Hultgren & Duffy 2012). Encompassing almost the full range of animal sociality, *Synalpheus* species vary in social organizations from pair-forming to communal to

eusocial (Hultgren et al. 2016). Eusocial *Synalpheus* species typically have a single queen or at most a few queens and a few to several hundred of non-breeding workers (Duffy 1996a), but workers are totipotent and retain the ability to reproduce (Chak et al. 2015b). Communal species live in groups with multiple breeding pairs, with roughly equal ratios of adult males and females (Chak et al. 2015a, Hultgren et al. 2016). Pair-forming species live in sponges with usually a single breeding pair per sponge (Duffy 2007, Duffy & Macdonald 2010, Hultgren et al. 2016); this social organizations is ancestral for the family and shared by most other alpheid snapping shrimps (Knowlton 1980, Rahman et al. 2003, Mathews 2007). Therefore, *Synalpheus* is an ideal group to test the evolutionary trajectory towards eusociality, especially for comparison with insects and vertebrates.

The goals of this study are to: 1) define the social organizations of *Synalpheus* species objectively using demographic data, 2) examine the evolutionary transitions among types of social organizations to determine how eusociality arose, and 3) determine if these social organizations represent continuous or discrete variations in social organization. Briefly, we used an extensive collection of *Synalpheus* amassed over 30 years and tested different models of evolutionary transitions between demographically-defined groups. Our results provide an empirical test of whether the evolutionary trajectory towards eusociality is direct or involves low-skew intermediates.

## Results

### *Demographic clustering*

The social organizations of many *Synalpheus* species have been noted in taxonomic descriptions (e.g., Ríos & Duffy 2007, Macdonald et al. 2009), but there has been no

attempt to systematically classify *Synalpheus* species into discrete social categories using quantitative data. To provide such a classification and analysis, we used demographic characteristics of 31 *Synalpheus* species within the gambarelloides group collected from the tropical West Atlantic (data from 1233 unique colonies; Table 1, and Table 2). We summarized the colony size (CS; i.e., the total number of female and male individuals in a sponge) and the number of ovigerous females (NOF) for each colony from each sponge. We also calculated the skewness of colony size and number of ovigerous females based on values on a log-2 scale (which better describes geometric population size growth). As an alternative measure to summarize colony size and number of ovigerous females, we calculated the eusociality index (E), a modified version of Keller and Perrin's (1995) eusociality index, as  $E = 1 - ((2 * NOF) / CS)$  (sensu Duffy et al. 2000, Duffy & Macdonald 2010). The eusociality index incorporates both colony size and the number of ovigerous females relative to colony size (i.e., reproductive skew (Sherman et al. 1995)), making the simplifying assumption that all breeding individuals contribute equally to offspring production.

We found that *Synalpheus* shrimp species naturally cluster into three distinct social categories (Figure 1A), using the Partitioning around Medoids (PAM) algorithm (Kaufman & Rousseeuw 2009) with different combinations of demographic variables (Supplementary Table 3). Twenty-two species were unambiguously clustered into one of the three groups (Figure 1C, 1D), while nine “intermediate” species cluster into different groups depending upon the input variables used (Supplementary Table 4, see Supplementary Materials). Demographic organizations of the three groups clearly correspond to social organizations of pair-forming, communal, and eusocial species

(Figure 1B). Essentially, species with small colony sizes ( $CS < 8$ ) are pair-forming, whereas species with larger colony sizes and many ovigerous females ( $CS \geq 8$  and  $NOF \geq 3$ ) are communal, and species with large colony sizes but few ovigerous females ( $CS \geq 8$  and  $NOF < 3$ ) are eusocial.

Pair-forming, communal, and eusocial species each exhibit different demographic characteristics. All demographic variables, except for the number of ovigerous females, were significantly different among the three social organization types (phylogenetically-informed Bayesian regression models, all models were significant at  $\Delta DIC > 8$ ; all post-hoc comparisons had  $MCMC p < 0.02$  and non-overlapping 95% credibility intervals; Supplementary Table 5). The number of ovigerous females did not differ between pair-forming and eusocial species ( $MCMC p = 0.30$ ) because a single female often monopolizes reproduction in eusocial species, despite their large colony size (Figure 2). The difference between the three social organization types is also apparent in bivariate relationships between demographic variables (Figure 1C and 1D).

### *Social transition*

We examined the evolutionary trajectories among types of demographically-defined social organizations to determine how eusociality arose in *Synalpheus*. Using 22 unambiguously clustered species, we constructed three models of continuous trait evolution (Figure 3) and tested which model is best supported given the evolutionary history of *Synalpheus*. We coded social organization continuously because species showed continuity in their demographic characteristics (Figure 1C and 1D). The best-supported social transition model (model 2 in Figure 3) agrees with the subsocial

hypothesis, in which eusocial species did not evolve from communal species, but instead arose directly from pair-forming species. Importantly, this result was robust to specification of the underlying evolutionary processes (Supplementary Table 6). The transition of pair-formers directly into eusocial species is consistently supported even when intermediate species were included (30 species, Supplementary Figure 1 and Supplementary Table 7) or when species with low sample sizes (< 6 colonies) were included (39 species, Supplementary Table 8) (see Supplementary Materials). Moreover, our model supports that the two species (*S. brooksi* and *S. elizabethae*; indicated by “×” in Figure 1C and 1D) that were clustered with either communal or eusocial species depending on the demographic variables used transitioned from eusocial species rather than from communal species, thus represents a secondary loss of eusociality (see Supplementary Materials). Mapping the social organizations on the *Synalpheus* phylogeny also reveals that eusocial clades or species examined were found within a more inclusive clade of pair-forming species (Figure 4).

## Discussion

Clarifying the evolutionary trajectory towards eusociality across different social lineages can elucidate whether animal social organizations represent discrete or continuous variation (Rubenstein et al. in press). It is especially important to clarify the role of communality in such a trajectory because communally breeding societies appear to be more common in social insects and vertebrates than once realized (Rubenstein & Abbot in press). In the socially diverse snapping shrimp genus *Synalpheus*, we found strong support for the evolution of eusociality via the classical subsocial route, i.e., through

accumulation of offspring of a single mated pair, which emphasizes the importance of close kin relations in the evolution of advanced social organizations (Hughes et al. 2008, Boomsma 2009, Lukas & Clutton-Brock 2012). We showed that *Synalpheus* species naturally clustered into groups of pair-forming, communal, and eusocial species, each with unique demographic characteristics. We further showed that eusocial *Synalpheus* species evolved directly from pair-forming species, whereas communal *Synalpheus* species also evolved directly from pair-forming species via a separate trajectory. Therefore, communal species are not intermediates between pair-forming and eusocial species. This supports the subsocial hypothesis for the evolution of eusociality in *Synalpheus* shrimps, because eusocial groups evolved from parent-offspring associations (nuclear families) (Michener 1969). This conclusion is further supported by the fact that all eusocial *Synalpheus* species have non-dispersing larvae that enable the formation of a closely related family group, whereas most other *Synalpheus* species have dispersing larvae (Duffy & Macdonald 2010).

There has been a long-standing debate on whether animal social organizations across taxa form a continuum (Keller & Perrin 1995, Sherman et al. 1995, Reeve et al. 1996) or discrete categories arising from basal organizations (Crespi & Yanega 1995, Costa & Fitzgerald 1996a, Boomsma 2009). Our results are consistent with elements of both hypotheses. First, the social organizations among species of *Synalpheus* appear to be continuous in that some species have intermediate demographic characteristics that cannot be unambiguously assigned to a single demographically-defined cluster (species indicated by + and × in Figure 1C and 1D). Second, *Synalpheus* species also fall uniformly along a continuous scale of reproductive skew (eusociality index, Figure 1D).

These intermediate demographic characteristics suggest that *Synalpheus* species appear to form a continuum, however, the evolution of social organizations along this continuum can take either of two directions. Specifically, communal species evolved from pair-forming species along one continuum, and eusocial species evolved from pair-forming species along another non-overlapping continuum (Model 2 in Figure 3); such a separation is also visible in the plot of ovigerous females versus colony size (Figure 1C). This divergence in trajectories can be considered compatible with the hypothesis that eusociality represents a distinct state from communal groups with lower reproductive skew. Therefore, the presence of reproductive skew in communal and eusocial species may have different evolutionary causes. Thus, caution should be taken when analyzing social species along a continuum of reproductive skew, as it may obscure patterns that operate separately along alternative social trajectories in species that form from the retention of offspring and those that do not. The presence of species that exhibit demographic characters of both communal and eusocial species (*S. brooksi* and *S. elizabethae*; “×” in Figure 1C and 1D) initially suggested a transition between the two social organizations. However, model comparisons consistently supported a model that had no transition between communal species and these intermediate species (see Supplementary Materials). Thus, these intermediate species likely had eusocial ancestors and later evolved demographic characteristics that are in between communal and eusocial species. In short, our results suggest that *Synalpheus* social organizations evolved continuously in two distinct trajectories, with communality and eusociality being two discrete end-points.

Finally, our findings support several recent developments in social evolution.

First, our results support the view that eusociality and communalism represent alternative solutions to environmental pressure (e.g., nest limitation) (Wcislo & Tierney 2009). In fact, the prevalence of communal species (~15% of all species, versus ~20% eusocial) and its multiple independent evolutions in *Synalpheus* (Figure 4) suggest that communalism may be an equally stable strategy as eusociality. A recent synthesis also found that communal breeding societies with relatively low skew are more common in social insects and vertebrates than once realized (Rubenstein & Abbot in press). Wcislo and Tierney (2009) proposed several benefits of communalism in defense and energetic saving, but this remains to be tested empirically. Second, our findings support the theory that lifetime monogamy by females favors the evolution of eusociality (Boomsma 2007, Boomsma 2009). In communal *Synalpheus*, lifetime absence of re-mating promiscuity is highly unlikely. This is because males have access to multiple female neighbors, and males of caridean shrimps can mate multiple times within a molt-cycle (Rahman et al. 2003, Bauer 2004). Further, fighting claw size is sexually dimorphic in communal species (Chak et al. 2015a), and thus males likely compete for sexually receptive females within their communal nest or sponge. Therefore, the fact that only pair-forming *Synalpheus* evolved eusociality reinforces the key role of close genetic relatedness in the evolution of eusociality (Duffy & Macdonald 2010), as seen in many other social invertebrates and vertebrates (Hughes et al. 2008, Boomsma 2009, Cornwallis et al. 2010).

Whether animal social organizations represent discrete or continuous variation is intrinsically linked to our understanding of the evolutionary trajectory towards eusociality. We found that social evolution in the snapping shrimp genus *Synalpheus*

involved two distinct trajectories in which communality and eusociality occupy different endpoints. Further, despite living in different ecosystems, evolution of eusociality in shrimps and insects both had a subsocial origin that is based on family groups; thus affirming the role of close genetic relatedness in the evolution of advanced societies.

## Methods

### *Collections*

We collected sponges and their associated macrofauna from shallow habitats in eight countries in the tropical West Atlantic from 1988 to 2014 (see Table S1 for details). In general, we collected either macroscopic sponges attached to hard substrates or cryptic sponges attached to or infilling between dead coral rubble using SCUBA (5-20 m) and snorkeling (< 5 m). We collected whole sponges and kept them submerged in seawater during transportation to field stations; in most cases, sponges were retained in flowing seawater until they could be processed. We subsequently dissected sponges and carefully removed all macrofauna from the internal canals of the sponge. We sorted *Synalpheus* shrimps by species and counted the number of ovigerous females and non-ovigerous individuals. Non-ovigerous individuals can be female or male (Tóth & Bauer 2007, Chak et al. 2015a), but they were not sexed in the field. All shrimp of the same species from the same sponge were considered a colony. Shrimps were preserved in 95% EtOH.

*Synalpheus* identification was based on taxonomy established by Coutiere (1909), Chace (1972) and Dardeau (1984), and supplemented by recent taxonomic descriptions and keys (Duffy 1996c, Ríos & Duffy 1999, MacDonald & Duffy 2006, Ríos & Duffy 2007, Anker & Toth 2008, Macdonald et al. 2009, Hultgren et al. 2010, Hultgren et al. 2011).

Uncertain specimen identifications were confirmed using COI and 16S sequences and established phylogenies (Duffy 1996b, Morrison et al. 2004, Hultgren & Duffy 2011, Hultgren et al. 2014).

### *Synalpheus* phylogeny

To construct the tree, we added morphological and molecular characters for several new species to previously published datasets for *Synalpheus* (Hultgren and Duffy, 2011; Hultgren et al., 2014; Morrison et al., 2004). Molecular data consisted of three loci: the mitochondrial 16S rRNA locus (16S), the 5' barcoding end of the mitochondrial cytochrome oxidase I gene (HCO), and a region of the 18S nuclear large ribosomal subunit (18S). Collection locations, voucher locations, and taxonomic information are summarized in Supplementary Table 9. DNA extraction, primers, amplification, and sequencing methods have been described previously (Hultgren et al., 2014). Forward and reverse sequences were aligned on SEQUENCHER v.4.8 (Gene Codes), and aligned with all existing *Synalpheus* sequences for that locus using the program MUSCLE (Edgar, 2004); since multiple studies have shown monophyly of individual species of *Synalpheus*, we trimmed the dataset to include one exemplary individual per species (Hultgren and Duffy, 2011). We used MacClade v.4.08 (Hultgren et al., 2014; D. Maddison and W. Maddison, 2005) to translate coding loci (HCO) to check for stop codons, which may indicate the presence of pseudogenes (Williams and Knowlton, 2001); none were detected. The structural loci 16S and 18S were difficult to align, and preliminary data from this study and simulation studies suggest better resolution after exclusion of ambiguously aligned positions. We used the program GBlocks v0.9 (allowed gap

positions = half) to exclude ambiguous parts of the alignment for 16S and 18S (Castresana, 2000; Talavera and Castresana, 2007), resulting in useable regions of 446 bp for 16S and 663 bp for 18S; the HCO region used was 669 bp. Finally, we used MrModelTest v2.3 (Nylander, 2004) to code the general model of evolution for each locus (HCO and 16S: GTR+I+G, rates = invgamma, 18S: K80+I+G, rates = invgamma). In addition to the sequence data, we used a set of 33 morphological characters, compiled (with slight modifications) from two previous published datasets (Hultgren and Duffy, 2011; Morrison et al., 2004). Morphological characters for the new species were scored by KMH.

We ran a partitioned Bayesian analyses in MrBayes v3.2.5 (Ronquist et al., 2012). Although we were missing data for 1-3 species for each set of data (HCO, 16S, 18S, morphological data; **Table S2**), we opted to utilize all taxa with data for at least 2 out of the 4 loci and treated all gap data as missing data. This was based on a preliminary analysis of our dataset and simulations suggesting inclusion of such taxa improved the accuracy of the final tree (Wiens, 2006; 2005). We ran Markov Chain Monte Carlo (MCMC) searches with four chains and two runs for  $2 \times 10^7$  generations, sampling the chain every 1000 generations. For all trees, we discarded the first 25% (standard deviation of split frequencies after this burn-in sample  $\leq 0.01$ ), and estimated support for nodes using Bayesian posterior probabilities (bpp).

We converted the tree into a clocklike phylogeny by estimating evolutionary rates using penalized-likelihood and verified the rates by cross-validation (Sanderson 2002). We fixed the age of the root at one and scaled the tree by the absolute rate using chronopl

in the *R* package *APE* (Paradis et al. 2004). We used this ultrametric tree for testing trait evolution using Bayesian phylogenetic mixed-models (Hadfield 2014).

### *Demographic clustering*

First, we used demographic characteristics to identify natural clustering of *Synalpheus* species. We tallied the colony size (CS; i.e., the total number of female and male individuals in a sponge) and the number of ovigerous females (NOF) for each colony from each sponge. We also calculated the skewness of CS and NOF (skCS and skNOF) based on values on a log 2 scale (which better describes geometric population size growth). As an alternative measure to summarize CS and NOF, we calculated the eusociality index (E), a modified version of Keller and Perrin's (1995) eusociality index, as  $E = 1 - ((2 * NOF) / CS)$  (sensu Duffy et al. 2000, Duffy & Macdonald 2010). The eusociality index incorporates both colony size and the number of ovigerous females relative to colony size (i.e., reproductive skew (Sherman et al. 1995), making the simplifying assumption that all breeding individuals contribute equally to offspring production. We focused the analysis within the monophyletic *Gambarelloides* species group (Banner & Banner 1975, Hultgren et al. 2014) and on species for which we had samples from more than 5 colonies (i.e., at least 6 different sponges). To obtain accurate demographic variables, we excluded all partially sampled sponges and colonies with a single individual or with no ovigerous females.

To identify natural clusters of *Synalpheus* species based on demographic properties, we employed the Partitioning around Medoids (PAM) algorithm (Kaufman & Rousseeuw 2009) using the *R* package *cluster* (Maechler et al. 2015). This algorithm

clusters objects about k medoids and minimizes the sum of the distances (Silhouette distance,  $s_i$ ) from each object to the closest medoid (Reynolds et al. 2006). To avoid subjective selection of input variables, we partitioned *Synalpheus* species based on seven combinations of five normalized variables, including CS, NOF, skCS skNOF, and E (Table S4). Each combination had at least CS and NOF or E as the main variables. For each combination of input variables, we ran the algorithm separately with 2 to 6 clusters ( $k$ ) and determined the best  $k$  value as the one that has the highest average Silhouette distance ( $s_i$ ) among the seven analyses with different input variables. The Silhouette distance measures how well an object fits into its own cluster rather than the nearest neighboring cluster (Reynolds et al. 2006), thus the highest Silhouette distance means the most discrete clustering. After selecting the best number of clusters, we identified the species that always clustered into the same group among all seven analyses (hereafter ‘unambiguous species’), and species that did not have a consistent group assignment that were clustered into different groups in different analyses (hereafter ‘intermediate species’).

To explore how demographic metrics contributing to clustering of the unambiguous species, we built a classification tree (Breiman et al. 1984) using CS, NOF, skCS and skNOF with the *R* package *rpart* (Therneau et al. 2015). The *rpart* algorithm performs recursive partitioning to create decision rules for predicting a categorical outcome. According to the criteria of the classification tree, we assigned *post hoc* groupings to 18 species with less than 6 colonies; these species were only used for supplementary analysis of social transitions.

The PAM analyses suggested that *Synalpheus* species naturally clustered into three groups, which conformed to pair-forming, communal, and eusocial categories based on the demographic characteristics (see Results for more detail). We explored differences in demographic and social traits between 21 species that are unambiguously grouped into these three groups, excluding the ‘intermediate’ species. First, we used Bayesian phylogenetic mixed-models (Hadfield & Nakagawa 2010) in the R package *MCMCglmm* (Hadfield 2010) to test whether the variables NOF, CS, skCS, and E differed among groups. We checked for normality visually and with the Shapiro-Wilk normality test, and log transformed skCS and square root transformed NOF. We used the usual inverse-Gamma (0.001, 0.001) distribution as the prior distribution for the residual variance and the variance components (i.e., random effect based on the phylogeny) (Hadfield 2014). Model significance was assessed using Deviance Information Criteria (DIC; Spiegelhalter et al. 2002) against a null model. *Post hoc* comparison between social organizations were assessed by testing whether the 95% credibility intervals (CI) overlapped zero, or by MCMC p-values (Pennell et al. 2014).

### *Social transitions*

We investigated the transitions among species with different social organizations (i.e., pair-forming, communal, eusocial, and the two intermediate forms), by mapping the distribution of social organizations onto the *Synalpheus* phylogeny and reconstructing

transitions among social states. Our primary goal was testing whether eusociality evolved directly from pair-forming species, or via communal species as a transition. We coded the social organizations as continuous traits in various configurations from 1 to 5 following seven different social transition models (Figure 1). We treated social organizations as continuous instead of discrete traits because species showed continuity in their demographic characteristics (Figure 2C and 2D). The coding configurations allowed directional transitions between social organizations coded as adjacent numbers (e.g., 2 <-> 3 <-> 4), but prevented the direct transition between the two social organizations coded as non-adjacent numbers (e.g., 1 and 5). To conform with the PAM results, we always coded Intermediate 1 adjacent to either pair-forming or communality, and Intermediate 2 adjacent to either communality or eusociality (Figure 3). We tested the fit of these competing social transition models using *fitDiscrete* in the R package *geiger* v2.0.3 (Hadfield 2010). In addition, because the evolution process underlying trait evolution was unknown, we used six different evolutionary processes: Brownian motion (BM), BM+ $\lambda$ , BM+ $\kappa$ , Ornstein-Uhlenbeck (OU), ACDC, and white noise. The BM process assumes a Brownian motion of continuous trait evolution (Felsenstein 1973). The additional parameters  $\lambda$  and  $\kappa$  estimate the amount of phylogenetic signal (Pagel 1999) and a degree of branch length transformation conforming to punctuational view of evolution (Pagel 1994), respectively. The OU process assumes stabilizing selection and the ACDC process assumes adaptive radiation in which character evolution rate can accelerate or decelerate (Blomberg et al. 2003). The white noise process assumes that character evolved in random, independent of the phylogeny. To maximize the chance of finding the optimal solution in the likelihood space, we ran each model with 1000 random starting points.

Finally, we compared the resulting 30 models with Akaike Information Criterion adjusted for small sample sizes (AICc) (Akaike 1998). Although the *Synalpheus* phylogeny has a polytomy, analyses with all possible resolved trees yielded identical results. We excluded *S. microneptunus* in this analysis because it was assigned to all three social organizations. Excluding *S. microneptunus* did not affect the analysis because *S. microneptunus* is nested within a eusocial clade (*S. duffyi* and *S. cayoneptunus*; Figure 4) and likely had a eusocial ancestor. We performed two supplementary analyses to (i) use only unambiguously clustered species and excluded intermediate species, and (ii) include species with *post hoc* assignments of social organizations. Results from these analyses were reported in Table S7 and S8.

## References

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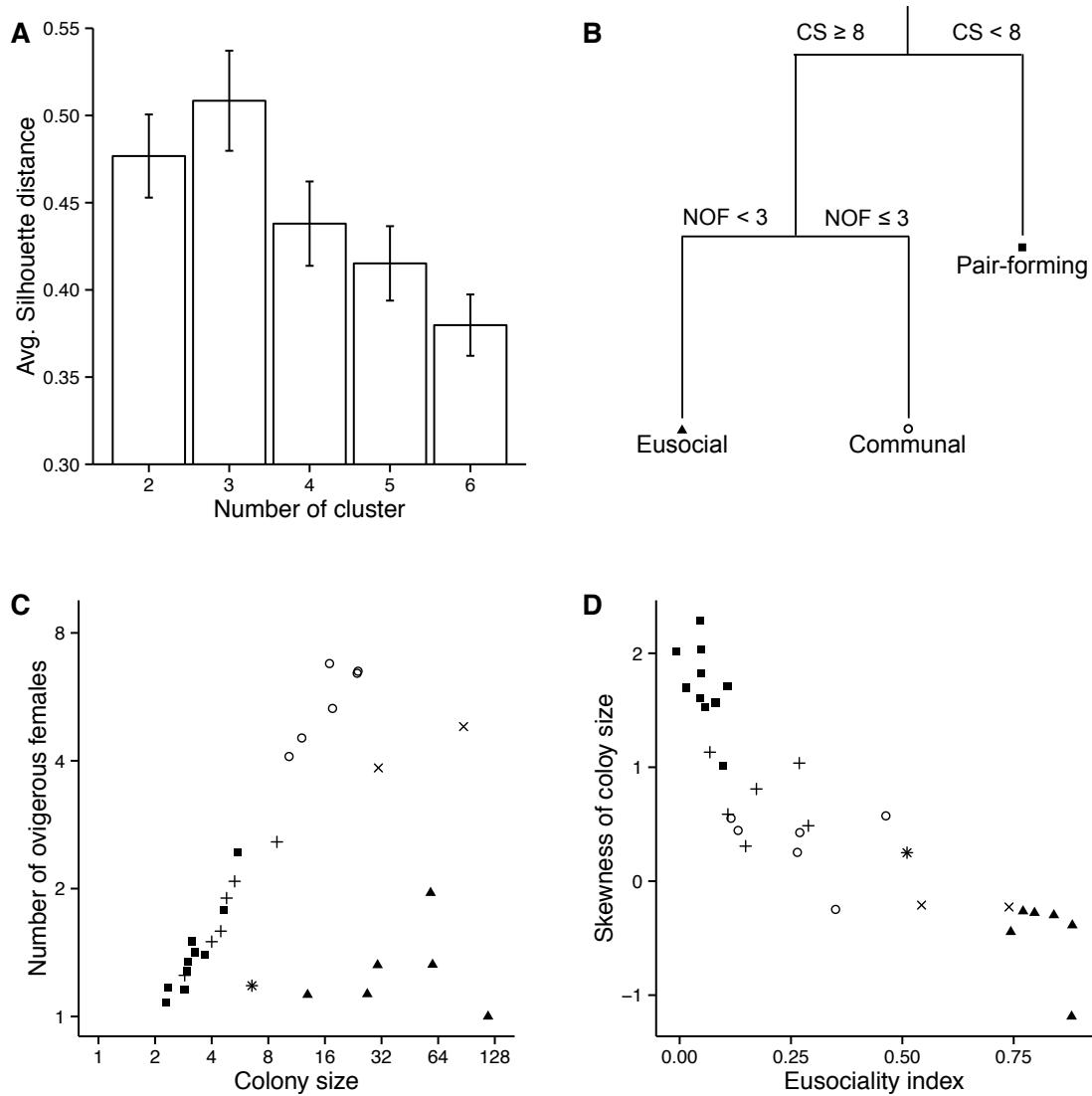
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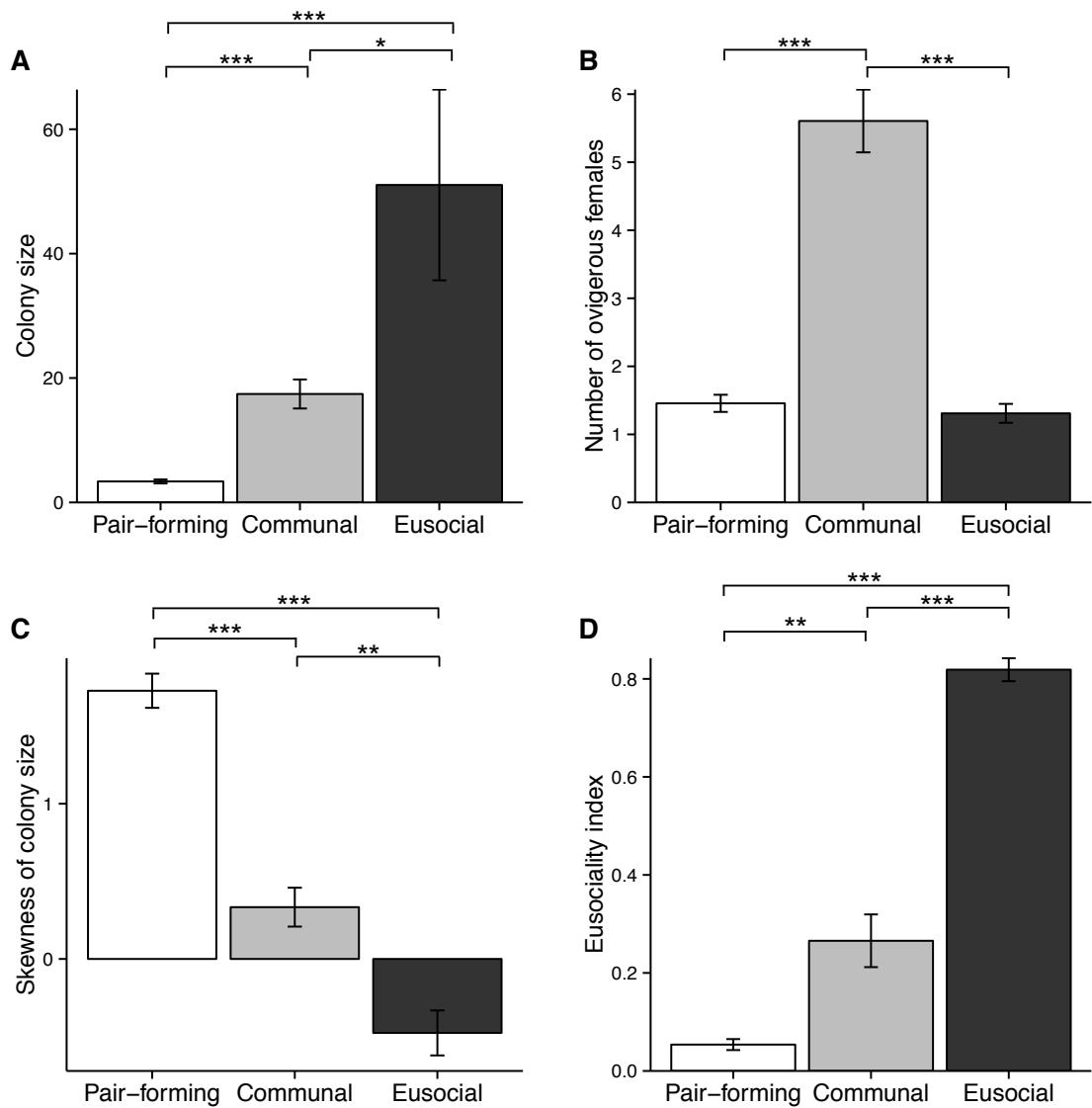
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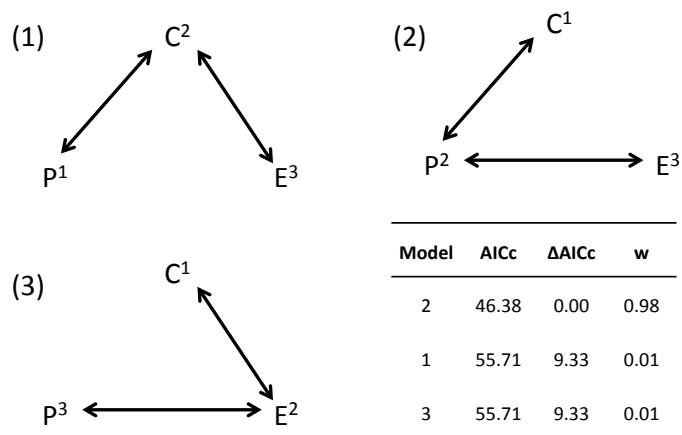
**Figure 1. Demographic classification of social states in *Synalpheus* shrimp.**

(A) Optimum number of social categories (clusters) as determined from average Silhouette distances ( $s_i$ ) from PAM analyses with different  $k$  (number of clusters) and different input variables. The high  $s_i$  for  $k=3$  indicates that *Synalpheus* species are best separated into three clusters. (B) Classification tree used to predict the three clusters, namely pair-forming, communal, and eusocial. (C, D) Bivariate plots of two sets of variables that resulted in the highest average  $s_i$ .

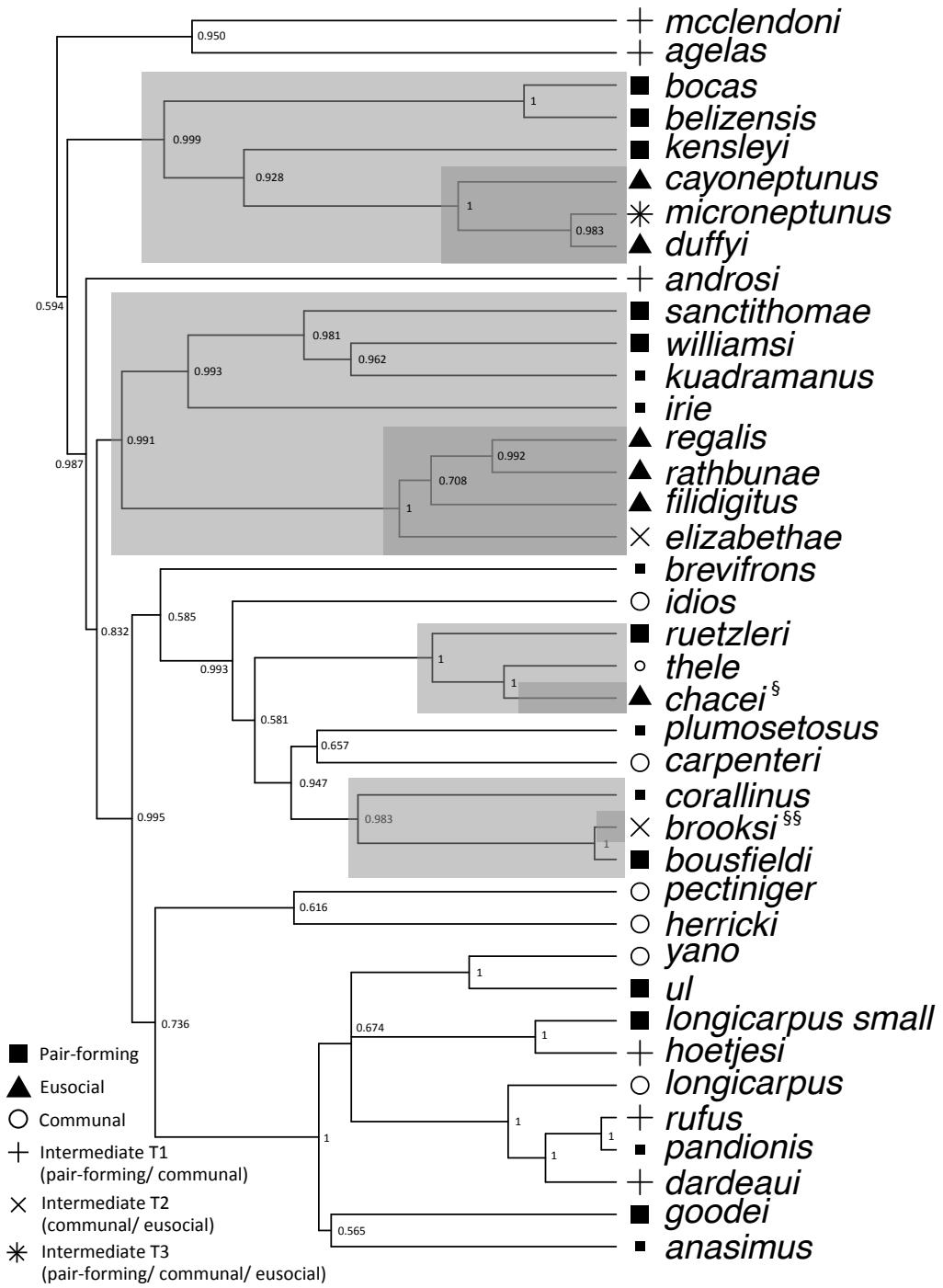
Solid squares, open circles and solid triangles represent pair-forming, communal and eusocial species, respectively, that were unequivocally identified in all PAM analyses regardless of input variables. +, × and \* represents intermediate species that were clustered with either pair-forming or communal species, either communal or eusocial species, and all three groups, respectively. In C, the number of ovigerous females and colony size are shown on geometric scales.



**Figure 2. Demographic properties of pair-forming, communal, and eusocial species.** Comparisons between social organization were performed using phylogenetic generalized least square regressions. Error bars indicate standard errors. Brackets indicate significant differences between groups (MCMCp \*\*\* <0.0001, \*\* <0.01, \* 0.02).



**Figure 3. Comparison of three models of continuous social traits evolution among pair-forming (P), communal (C), and eusocial (E) species.** The best model (model 2) suggests that eusociality evolved from a pair-forming state without a transition via communality. The analysis was based on 22 species that were unambiguously clustered into three social organization types. The inserted table shows the model comparison results based on the BM model, which was best supported among six different evolutionary processes. w represents Akaike weight. Superscripts show how we coded social organizations as continuous traits.



**Figure 4. Phylogeny of social evolution in *Synalpheus*.** Bayesian consensus tree, constructed with 16S, 18S, and COI sequence data and ultrametrized into a clocklike phylogeny. Symbols at the tips represent social organizations assigned from PAM analyses. Small shapes represent species with less than six colonies

so that social organizations were inferred. Numbers at nodes represent Bayesian posterior probability values. Highlighted clades show that eusocial species only evolved within more inclusive clades of pair-forming species. In §, although the eusocial species *S. chacei* has a communal sister species (*S. thele*), these two species together had a pair-forming sister species. The most parsimonious explanation is that they both evolved from pair-forming ancestors, which agrees with the best-supported social trait evolution model (Figure 3). In §§, although *S. brooksi* has mixed demographic characters of communal and eusocial species, it evolved within a clade of pair-forming species, and thus agrees with the model that intermediate species T2 represent eusociality being secondarily lost or reduced (Supplementary Figure 2).

## Supplementary Information

### *Demographic characteristics of intermediate species*

PAM analysis with CS and NOF and with E and skCS yielded the highest  $s_i$ . Therefore, we plotted these two bivariate relationships with the respective groupings in Figure 1C and 1D. Examination of these bivariate relationships helped explain why the seven ‘intermediate’ species clustered with different groups depending on the input variables used. *S. agelas*, *S. androsi*, *S. dardeaui*, and *S. hoetjesi* clustered with either pair-forming or communal species. This is because they were more similar to pair-forming species in terms of NOF and CS (Figure 1C), but more similar to communal species in terms of skCS and E (Figure 1D). *S. brooksi* and *S. elizabethae* clustered with either communal or eusocial species. This is because they were more similar to communal species in terms of NOF (Figure 1C) and yet more similar to eusocial species in terms of CS, skCS, and E (Figure 1C and 1D). *S. microneptunus* clustered with either with all three groups because it was in between pair-forming and eusocial species in terms of NOF and CS (Figure 1C), and in between communal and eusocial species in terms of skCS and E (Figure 1D).

### *Social transitions: supplementary analyses and results*

First, we used only 22 unambiguously clustered species, thus excluded nine intermediate species. We coded the social organizations as continuous traits in various configurations from 1 to 3 following three different models of transitions (Figure S2). Depending on the coding configurations, each model excluded direct transitions between social organizations that were coded as 1 and 3. Model fitting and model comparison were described in the main text. The model with no direct transition between communality and eusociality (model 2) was best-supported regardless of the evolutionary model used (Table S7). Brownian motion (BM) was the best-supported evolutionary model.

Second, in a separate analysis we included nine species with colony sizes less than six and assigned *post hoc* social organizations according to their demographic characteristics and the classification tree. We coded the social organizations as 1 to 7 as described in the main text (Figure 3) and carried out the same model fitting and model

comparison procedures. As in the main analysis, the model with no direct transition between communalism and eusociality (model 3) was best-supported regardless of the evolutionary model used (Table S8); BM+ $\lambda$  and white-noise were the best supported evolutionary models ( $\Delta\text{AICc} < 0.97$ ).

**Supplementary Table 1.** *Synalpheus* colony characteristics and social organizations. Social organizations were assigned according to the PAM analyses. Species marked with \* had colony sizes less than six; their social organizations were assigned according to classification tree (Figure 1B).

	<i>Synalpheus</i> sp.	Social organizations	No. of colonies	Mean colony size	Skewness of log2(colony size)	Mean no. of ovigerous females	Skewness of log2(ovigerous females)	Mean eusociality index
1	<i>agelas</i>	Intermediate 1	37	5.297	0.308	2.081	0.694	0.148
2	<i>anasimus</i>	Pair-forming *	1	2.000	-	1.000	-	0.000
3	<i>androsi</i>	Intermediate 1	10	4.800	1.132	1.900	0.713	0.068
4	<i>belizensis</i>	Pair-forming	25	2.880	1.566	1.160	1.855	0.081
5	<i>bocas</i>	Pair-forming	25	3.680	1.607	1.400	1.211	0.046
6	<i>bousfieldi</i>	Pair-forming	62	5.532	1.820	2.435	1.876	0.048
7	<i>brevifrons</i>	Pair-forming *	5	2.600	0.704	1.200	1.500	0.067
8	<i>brooksi</i>	Intermediate 2	112	30.777	-0.210	3.848	0.214	0.543
9	<i>carpenteri</i>	Communal	38	12.053	0.552	4.526	0.504	0.115
10	<i>cayoneptunus</i>	Eusocial	16	12.938	-0.445	1.125	3.615	0.743
11	<i>chacei</i>	Eusocial	56	30.482	-0.264	1.321	1.398	0.771
12	<i>corallinus</i>	Pair-forming *	1	2.000	-	1.000	-	0.000
13	<i>dardeaui</i>	Intermediate 1	111	8.892	0.487	2.577	0.856	0.289
14	<i>duffyi</i>	Eusocial	31	26.871	-0.278	1.129	2.213	0.797
15	<i>elizabethae</i>	Intermediate 2	70	87.329	-0.227	4.814	0.448	0.740
16	<i>filidigitus</i>	Eusocial	37	59.757	-0.298	1.324	2.617	0.840
17	<i>goodei</i>	Pair-forming	25	2.960	1.526	1.280	1.425	0.057
18	<i>herricki</i>	Communal	32	10.313	0.253	4.094	0.565	0.264
19	<i>hoetjesi</i>	Intermediate 1	17	4.471	0.809	1.588	1.278	0.172
20	<i>idios</i>	Communal	32	17.563	-0.248	5.313	0.309	0.350
21	<i>irie</i>	Pair-forming *	2	2.000	-	1.000	-	0.000
22	<i>kensleyi</i>	Pair-forming	14	3.143	2.034	1.500	2.280	0.048
23	<i>kuadramanus</i>	Pair-forming *	1	2.000	-	1.000	-	0.000
24	<i>longicarpus</i>	Communal	8	24.000	0.573	6.500	-0.372	0.463
25	<i>longicarpus small</i>	Pair-forming	32	3.000	1.697	1.344	1.733	0.015
26	<i>mccleldoni</i>	Intermediate 1	8	2.875	0.587	1.250	1.155	0.108
27	<i>microneptunus</i>	Intermediate 3	11	6.545	0.250	1.182	1.650	0.511
28	<i>orapilosa</i>	Pair-forming *	1	2.000	-	1.000	-	0.000
29	<i>pandionis</i>	Pair-forming *	5	3.000	0.502	1.600	0.638	-0.060
30	<i>pectiniger</i>	Communal	56	23.750	0.426	6.429	0.622	0.270
31	<i>plumosetosus</i>	Pair-forming *	1	4.000	-	2.000	-	0.000
32	<i>rathbunae</i>	Eusocial	22	58.318	-0.387	1.955	0.761	0.882
33	<i>regalis</i>	Eusocial	92	117.880	-1.187	1.000	-	0.880

34	<i>ruetzleri</i>	Pair-forming	29	3.276	1.014	1.414	1.823	0.098
35	<i>rufus</i>	Intermediate 1	6	4.000	1.036	1.500	1.789	0.269
36	<i>sanctithomae</i>	Pair-forming	24	2.333	2.013	1.167	2.545	-0.008
37	<i>thele</i>	Communal *	5	15.600	-0.082	8.000	-0.075	-0.015
38	<i>ul</i>	Pair-forming	41	4.610	1.710	1.780	2.422	0.108
39	<i>williamsi</i>	Pair-forming	25	2.280	2.291	1.080	4.695	0.045
40	<i>yano</i>	Communal	129	16.930	0.445	6.775	0.593	0.131

**Supplementary Table 2. Sampling locations for *Synalpheus* from eight Caribbean countries.**

Country	Region	No. of sponge	Year collected
Bahamas	Various	154	1989, 2001
Barbados	West coast	290	2008
Belize	Carrie Bow Cay	5534	1990, 1993, 1994, 1995, 1996, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2009, 2012, 2014
Cuba	South coast	112	2011
Curacao	South coast	457	2008
United States	Florida Keys	2248	1990, 1995, 2005, 2006, 2012, 2013, 2014, 2015
Jamaica	Discovery Bay	1054	2008, 2012
Panamá	Isla Colón	4654	2003, 2007, 2008, 2009, 2011, 2013
Panamá	Pacific	39	1991, 1993
Panamá	San Blas	1276	1998, 1990, 1991, 1992, 1993

**Supplementary Table 3. Average Silhouette distances ( $s_i$ ) from seven (Partitioning around Medoids) PAM analyses (A - G) using different demographic variables and different pre-assigned numbers of clusters ( $k$ ).**

PAM analyses	Demographic variables	$k = 2$	$k = 3$	$k = 4$	$k = 5$	$k = 6$
A	CS, NOF	0.525	0.623	0.512	0.470	0.459
B	CS, NOF, skCS	0.480	0.545	0.474	0.413	0.394
C	CS, NOF, skNOF	0.450	0.488	0.352	0.430	0.381
D	CS, NOF, skCS, skNOF	0.393	0.469	0.368	0.379	0.310
E	E, skCS	0.588	0.578	0.508	0.479	0.400
F	E, skNOF	0.461	0.415	0.437	0.313	0.364
G	E, skCS, skNOF	0.440	0.441	0.413	0.422	0.350
Average		0.477	0.508	0.438	0.415	0.380

**Supplementary Table 4.** Cluster assignments ( $k = 3$ ) of *Synalpheus* species from seven PAM analyses (A - G) using different demographic variables.

<i>Synalpheus</i> sp.	A	B	C	D	E	F	G	Social organization
<i>agelas</i>	2	2	2	1	1	1	1	Intermediate 1
<i>androsi</i>	2	2	2	2	2	1	1	Intermediate 1
<i>belizensis</i>	2	2	2	2	2	2	2	Pair-forming
<i>bocas</i>	2	2	2	2	2	2	2	Pair-forming
<i>bousfieldi</i>	2	2	2	2	2	2	2	Pair-forming
<i>brooksi</i>	1	1	1	1	3	1	1	Intermediate 2
<i>carpenteri</i>	1	1	1	1	1	1	1	Communal
<i>cayoneptunus</i>	3	3	3	3	3	3	3	Eusocial
<i>chacei</i>	3	3	3	3	3	3	3	Eusocial
<i>dardeaui</i>	2	1	1	1	1	1	1	Intermediate 1
<i>duffyi</i>	3	3	3	3	3	3	3	Eusocial
<i>elizabethae</i>	1	1	1	1	3	3	1	Intermediate 2
<i>filidigitus</i>	3	3	3	3	3	3	3	Eusocial
<i>goodei</i>	2	2	2	2	2	2	2	Pair-forming
<i>herricki</i>	1	1	1	1	1	1	1	Communal
<i>hoetjesi</i>	2	2	2	2	1	2	1	Intermediate 1
<i>idios</i>	1	1	1	1	1	1	1	Communal
<i>kensleyi</i>	2	2	2	2	2	2	2	Pair-forming
<i>longicarpus</i>	1	1	1	1	1	1	1	Communal
<i>longicarpus small</i>	2	2	2	2	2	2	2	Pair-forming
<i>mcclendoni</i>	2	2	2	2	1	2	1	Intermediate 1
<i>microneptunus</i>	2	3	2	3	1	1	3	Intermediate 3
<i>pectiniger</i>	1	1	1	1	1	1	1	Communal
<i>rathbunae</i>	3	3	3	3	3	3	3	Eusocial
<i>regalis</i>	3	3	3	3	3	3	3	Eusocial
<i>ruetzleri</i>	2	2	2	2	2	2	2	Pair-forming
<i>rufus</i>	2	2	2	2	1	2	2	Intermediate 1
<i>sanctithomae</i>	2	2	2	2	2	2	2	Pair-forming
<i>ul</i>	2	2	2	2	2	2	2	Pair-forming
<i>williamsi</i>	2	2	2	2	2	2	2	Pair-forming
<i>yano</i>	1	1	1	1	1	1	1	Communal

**Supplementary Table 5.** Effects of social organizations on demographic variables in *Synalpheus*. Deviance Information Criterion (DIC), a Bayesian analog to AIC was used to test for model significance against null models. *Post hoc* tests were assessed by testing whether the 95% credibility intervals (CI) overlapped zero, or by MCMC p-values (MCMCp).

Response variable	ΔDIC	Post-hoc comparisons	Lower CI	Upper CI	MCMCp
Colony size	20.1	Eusocial - Communal	2.113	0.276	0.02
		Eusocial - Pair-forming	2.631	4.248	<5e-05
		Communal - Pair-forming	1.545	2.944	<5e-05
Skewness of colony size	8.06	Eusocial - Communal	-0.275	-1.259	0.004
		Eusocial - Pair-forming	-1.035	-0.597	<5e-05
		Communal - Pair-forming	-0.628	-0.226	<5e-05
No. of ovigerous females	13.4	Eusocial - Communal	-1.609	-2.559	<5e-05
		Eusocial - Pair-forming	-0.459	0.144	0.30
		Communal - Pair-forming	0.581	1.155	<5e-05
Eusociality index	27.3	Eusocial - Communal	0.694	0.404	<5e-05
		Eusocial - Pair-forming	0.623	0.876	<5e-05
		Communal - Pair-forming	0.085	0.312	0.002

Supplementary Table 6. Comparison of models of continuous trait evolution using 22 species of *Synalpheus* that were unambiguously assigned to one of three clusters (pair-forming, communal, and eusocial). See Figure S2 for details of models 1-3. The two best models are in bold. AIC: Akaike Information Criterion adjusted for small sample sizes; w: Akaike weight.

Models	AICc	$\Delta\text{AICc}$	AIC weight
<b>BM 2</b>	<b>46.380</b>	<b>0.000</b>	<b>0.469</b>
BM+ $\kappa$ 2	48.945	2.565	0.130
EB 2	49.076	2.696	0.122
OU 2	49.082	2.702	0.122
BM+ $\lambda$ 2	49.082	2.702	0.122
White noise 2	53.730	7.350	0.012
BM 1	55.708	9.328	0.004
BM 3	55.708	9.328	0.004
BM+ $\kappa$ 1	57.344	10.964	0.002
BM+ $\kappa$ 3	57.344	10.964	0.002
OU 1	57.572	11.192	0.002
OU 3	57.572	11.192	0.002
EB 3	57.572	11.192	0.002
EB 1	57.572	11.192	0.002
BM+ $\lambda$ 1	58.324	11.944	0.001
BM+ $\lambda$ 3	58.324	11.944	0.001
White noise 1	59.035	12.655	0.001
White noise 3	59.035	12.655	0.001

**Supplementary Table 7.** Comparison of models of continuous trait evolution using 30 *Synalpheus* species with more than 5 colonies (pair-forming, communal, eusocial, intermediate 1, and intermediate 2). See Figure 3 for details of models 1-5. The two best models are in bold. AIC: Akaike Information Criterion adjusted for small sample sizes; w: Akaike weight.

Models	AICc	$\Delta$ AICc	AIC weight
<b>BM+<math>\lambda</math> 3</b>	<b>96.46</b>	<b>0.00</b>	<b>0.55</b>
BM+ $\kappa$ 3	99.47	3.00	0.12
White noise 3	99.58	3.12	0.12
White noise 2	100.52	4.06	0.07
OU 3	101.92	5.45	0.04
EB 3	101.92	5.45	0.04
BM+ $\lambda$ 2	102.83	6.37	0.02
OU 2	103.00	6.54	0.02
EB 2	103.00	6.54	0.02
BM+ $\kappa$ 2	106.38	9.91	3.85E-03
White noise 4	109.29	12.83	8.98E-04
BM 3	109.56	13.09	7.86E-04
OU 4	111.77	15.31	2.60E-04
EB 4	111.77	15.31	2.60E-04
BM+ $\lambda$ 4	111.77	15.31	2.60E-04
BM+ $\lambda$ 1	112.38	15.91	1.92E-04
White noise 1	113.78	17.31	9.53E-05
BM+ $\kappa$ 4	114.62	18.16	6.25E-05
OU 1	116.25	19.79	2.76E-05
EB 1	116.25	19.79	2.76E-05
White noise 5	116.61	20.15	2.31E-05
BM+ $\kappa$ 1	117.18	20.72	1.74E-05
BM+ $\lambda$ 5	117.85	21.38	1.25E-05
OU 5	119.00	22.53	7.00E-06
EB 1	119.00	22.53	7.00E-06
BM+ $\kappa$ 5	120.71	24.24	2.98E-06
BM 4	124.86	28.40	3.73E-07
BM 2	127.85	31.38	8.39E-08
BM 5	128.28	31.82	6.75E-08
BM 1	130.07	33.60	2.76E-08

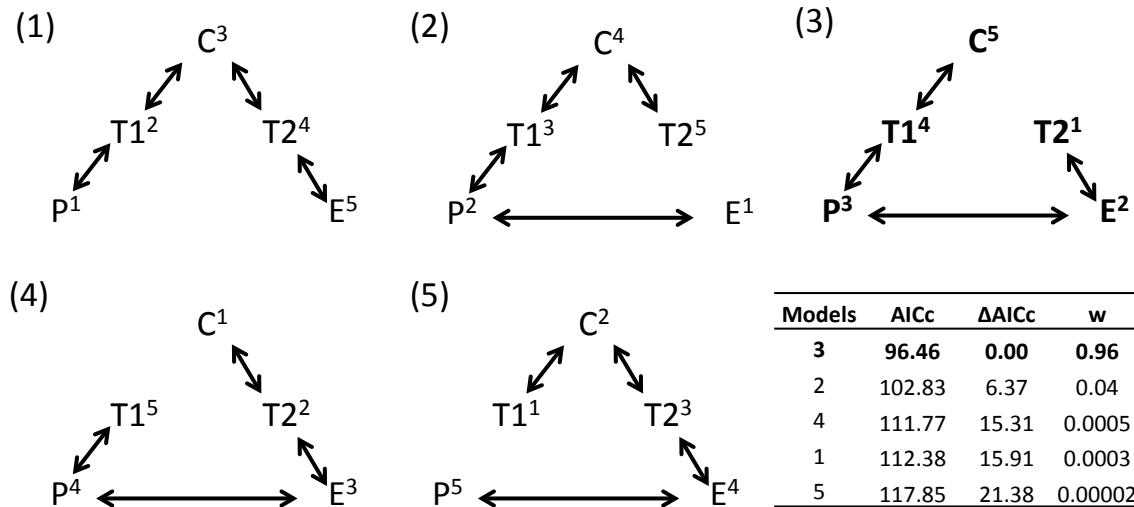
Supplementary Table 8. Comparison of models of continuous trait evolution using 39 *Synalpheus* species including ones with less than 6 colonies (pair-forming, communal, eusocial, intermediate 1, and intermediate 2). See Figure 3 for details of models 1-5. The two best models are in bold. AIC: Akaike information criterion adjusted for small sample sizes; w: Akaike weight.

Models	AICc	$\Delta$ AICc	w
<b>BM+<math>\lambda</math> 3</b>	<b>117.94</b>	<b>0.00</b>	<b>0.39</b>
<b>White noise 3</b>	<b>118.92</b>	<b>0.97</b>	<b>0.24</b>
White noise 2	120.64	2.70	0.10
OU 3	121.26	3.31	0.07
EB 3	121.26	3.31	0.07
BM+ $\lambda$ 2	123.00	5.06	0.03
OU 2	123.00	5.06	0.03
EB 2	123.00	5.06	0.03
BM+ $\kappa$ 3	123.87	5.92	0.02
White noise 4	133.48	15.53	1.66E-04
BM+ $\kappa$ 2	135.34	17.39	6.57E-05
OU 4	135.68	17.74	5.52E-05
EB 4	135.68	17.74	5.52E-05
BM+ $\lambda$ 4	135.84	17.90	5.10E-05
BM+ $\lambda$ 1	137.74	19.80	1.97E-05
BM 3	140.26	22.31	5.61E-06
BM+ $\kappa$ 1	140.92	22.97	4.03E-06
White noise 1	141.51	23.56	3.00E-06
BM+ $\kappa$ 4	143.50	25.55	1.11E-06
EB 1	143.87	25.92	9.22E-07
OU 1	143.87	25.92	9.22E-07
White noise 5	146.35	28.41	2.66E-07
OU 5	148.72	30.77	8.16E-08
BM+ $\lambda$ 5	148.72	30.77	8.16E-08
EB 5	148.72	30.77	8.16E-08
BM 4	153.66	35.71	6.90E-09
BM 2	158.22	40.28	7.04E-10
BM+ $\kappa$ 5	158.40	40.45	6.46E-10
BM 1	158.85	40.91	5.14E-10
BM 5	190.50	72.56	6.90E-17

**Supplementary Table 9.** GenBank sequence information for the *Synalpheus* species used in phylogeny. "n. sp." indicates undescribed species. In Collection #, FLMNH UF = Florida Natural History museum specimens; VIMS = Virginia Institute of Marine Science; OUMNH = Oxford University Museum of Natural History; MNHN = Muséum National d'Histoire Naturelle, Paris; AA indicates held by Arthur Anker. M: Morphology included. (Table prepared by K. Hultgren)

Species	Collection Country, Date	Collection#	16S	HCO	18S	M
<i>Alpheus percyi</i> (outgroup)	Hawaii, 2006	FLMNH UF-12372	KJ4776 94	KJ4776 97	KJ4943 95	y
<i>Synalpheus bocas</i>	Jamaica, 2008	VIMS 08JAM7402	HQ435 426	KJ5950 41	KJ5952 49	y
<i>Synalpheus duffyi</i>	Jamaica, 2008	VIMS 08JAM7401-2	HQ435 444	KJ5950 78	KU682 633	y
<i>Synalpheus corallinus</i>	Jamaica, 2008	VIMS 08JAM7001	HQ435 441	KU980 212	KU682 630	y
<i>Synalpheus irie</i>	Jamaica, 2008	VIMS 08JAM2802	HQ435 456	KJ5951 06	KJ5952 94	y
<i>Synalpheus belizensis</i>	Curaçao, 2008	VIMS 08CU3701	HQ435 423	KJ4776 96	KU682 627	y
<i>Synalpheus elizabethae</i>	Panama (WA), 2008	VIMS 08P12504	HQ435 446	KU980 213	KU682 634	y
<i>Synalpheus herricki</i>	Curaçao, 2008	VIMS 08CU3202	HQ435 449	KJ5950 95	KU682 635	y
<i>Synalpheus hoetjesi</i>	Curaçao, 2008	VIMS 08CU2901	HQ435 452	KJ6250 37	KJ5952 93	y
<i>Synalpheus plumosetus</i>	Jamaica, 2008	VIMS 08JAM2704	HQ435 471	KU980 220	KU682	y
<i>Synalpheus microneptunus</i>	Breach Reef, Barbados	VIMS 08BR6001	HQ435 463	KJ5951 10	KU682 640	y
<i>Synalpheus ul</i> <i>Synalpheus "near</i> <i>carpenteri" n. sp.</i>	Barbados, 2008	VIMS 08BR8703	HQ435 482	KJ6250 44	KU682 648	y
	Panama (WA), 2008	VIMS 08P1501	HQ435 482	KU980 216	KU682 641	y
<i>Synalpheus chacei</i>	Belize, 2009	VIMS 09CBC501	HQ435 440	KJ5950 59	KJ5952 65	y
<i>Synalpheus carpenteri</i>	Belize, 2009	VIMS 09CBC1504	HQ435 439	KJ5950 52	KJ5952 628	y
<i>Synalpheus agelas</i>	Belize, 2009	VIMS 09CBC1508	HQ435 419	KU980 32	KJ5953 45	y
<i>Synalpheus williamsi</i>	Belize, 2009	VIMS 09CBC5102	HQ435 484	KJ4776 224	KJ5952 38	y
<i>Synalpheus goodei</i>	Belize, 2009	VIMS 09CBC5404	HQ435 448	KJ4777 98	KJ5952 79	y
<i>Synalpheus obtusifrons</i>	Belize, 2009	VIMS	HQ435 466	KJ4777 03	KJ4943 96	y
<i>Synalpheus ruetzleri</i>	Belize, 2009	VIMS 09CBC6201	HQ435 475	KJ5951 36	KJ5953 13	y
<i>Synalpheus androsi</i>	Belize, 2009	VIMS 09CBC6502	HQ435 421	KJ6250 33	KJ5952 46	y
<i>Synalpheus regalis</i>	Belize, 2009	VIMS 09CBC7002	HQ435 474	KJ6250 42	KU682 645	y
<i>Synalpheus filidigitus</i> <i>Synalpheus "near</i> <i>sanctithomae" n. sp.</i>	Belize, 2009	VIMS 09CBC7603	HQ435 447	KJ5950 79	KJ5952 75	y
	Belize, 2009	VIMS 09CBC7804	HQ435 464	KJ6250 43	KJ5952 43	y
<i>Synalpheus pandionis</i>	Belize, 2009	VIMS 09CBC8403	HQ435 468	KJ5951 26	KJ5953 05	y
<i>Synalpheus idios</i>	Belize, 2009	VIMS 09CBC8803	HQ435	KJ6250	KU682	y

				455	38	636	
<i>Synalpheus ankeri</i>	Panama (WA), 2009	VIMS 09P6903-2	HQ435 473	KJ6250 41	KJ5952 42	y	
<i>Synalpheus brooksi</i>	Panama (WA), 2009	VIMS 09P4911-2	HQ435 438	KJ6250 35	KJ5952 52	y	
<i>Synalpheus brevifrons</i>	Belize, 2009	VIMS 09CBC2704	HQ435 435	KJ6250 34	KJ5952 51	y	
<i>Synalpheus dominicensis</i>	Belize, 2009	VIMS 09CBC6303	KJ4776 95	KJ4777 02	KU682 632	y	
<i>Synalpheus bousfieldi</i>	Belize, 2009	VIMS 09CBC3605	HQ435 431	KJ5950 42	KJ5952 50	y	
<i>Synalpheus yano</i>	Belize, 2009	VIMS 09CBC3802	HQ435 485	KJ5951 61	KJ5953 39	y	
<i>Synalpheus "longicarpus small" n. sp.</i>	Panama (WA), 2009	VIMS 09P9101-2	HQ435 459	KJ5950 25	KJ5952 40	y	
<i>Synalpheus pectiniger</i>	Jamaica, 2008	VIMS 08JAM8801	HQ435 470	KJ5951 29	KJ5953 07	y	
<i>Synalpheus kensleyi</i>	Panama (WA), 2007	VIMS 07P1204	HQ435 458	KJ6250 39	KJ5952 95	y	
<i>Synalpheus occidentalis</i>	Panama, 2007	OUMNH* (AA-07-087)	AY344 748	KJ5951 25			y
<i>Synalpheus parfaitti</i>	São Tomé, 2006	MNHN-IU-2010-4150	KJ5952 23	KJ5951 27	KJ5953 06		
<i>Synalpheus "near brevifrons" n. sp.</i>	Madagascar, 2008	AA-14255-2	KJ5951 72	KJ5950 22	KJ4943 94		
<i>Synalpheus anasimus</i>	Florida, USA, 2001	VIMS 94A-3	AY344 735				y
<i>Synalpheus gambareloides</i>	Croatia, 2001	VIMS gambCR01	AY344 751				y
<i>Synalpheus rathbunae</i>	Panama (WA), 2001, 2008, 2009	VIMS 92P09, 09P9704, 08P3501	AY344 767	KU980 221	KU682 644	y	
<i>Synalpheus "cf. brooksi" n. sp.</i>	Jamaica, 2012	VIMS 12JAM3201	KU682 626	KU980 219	KU682 643	y	
<i>Synalpheus "near herricki" n. sp.</i>	Jamaica, 2012	VIMS 12JAM8002	KU682 625	KU980 217	KU682 642	y	
<i>Synalpheus "near pectiniger" n. sp.</i>	Panama (WA), 2008	VIMS 08P12704	KU980 218	KU980 214	KU682 637	y	
<i>Synalpheus kuadramanus</i>	Jamaica, 2012	VIMS 12JAM9501	KU980 214	KU980 215	KU682 639	y	
<i>Synalpheus mcclendoni</i>	Jamaica & Barbados, 2008	VIMS 08JAM6128; 08BR1413	HQ435 462	KU980 215	KJ5953 639	y	
<i>Synalpheus thele</i>	Jamaica, 2008	VIMS 08JAM8914, 8924	HQ435 481	KU980 223	KU682 35	y	
<i>Synalpheus rufus</i>	Jamaica, 2008	VIMS 08JAM5902, 5903	HQ435 477	KU980 222	KU682 646	y	
<i>Synalpheus dardeaui</i>	Belize, 2009	VIMS 09CBC3105, Hurt- 801	HQ435 442	KJ6250 36	KU682 631	y	
<i>Synalpheus bannerorum</i>	Panama (EP), 2007	AA--07-176a-b	KJ5951 78	KJ6250 45	KJ5952 48	y	
<i>Synalpheus fritzmuelleri</i>	Jamaica, 2008	VIMS 08JAM4706, VIMS fritPA06	AF2307 98	KJ5950 81	KJ5952 77	y	
<i>Synalpheus sanctithomae</i>	Belize, 1994; Barbados, 2008	VIMS 94CBC3401, VIMS 08BR1201	AY344 768	KJ5951 39	KU682 647	y	
<i>Synalpheus longicarpus</i>	Panama (WA), 2001, Florida, USA, 2004	VIMS 93P1812, AA04-003b1	AY344 758	KJ6250 40	KU682 638	y	
<i>Synalpheus cayoneptinus</i>	Florida, 2013	VIMS 13FK2901, 2903	KM204 180	KM204 166	KU682 629	y	



**Supplementary Figure 1. Comparison of five models of continuous social trait evolution among pair-forming (P), communal (C), and eusocial (E) species.** The best model (model 3) suggests that eusociality evolved from a pair-forming state without a transition via communality. The analysis was based on 22 species that were unambiguously clustered into three social organization types and eight intermediate species that were clustered with either pair-forming and communal species (T1), or between communal and eusocial species (T2). The inserted table shows the model comparison results based on the BM+  $\lambda$  model, which was best supported among six different evolutionary processes. w represents Akaike weight. Superscripts show how we coded social organizations as continuous traits.

## **CHAPTER 4**

### **GROUP ADVANTAGE IN COMMUNAL AND EUSOCIAL SNAPPING**

**SHRIMPS**

## **Abstract**

Eusociality and communalism (cohabiting females that share a nest but provision their own offspring) appear to be widespread social strategies to overcome intense ecological pressure. While communal species may benefit from having large group sizes, it has been proposed that the same group benefit is enough to drive the evolution of eusociality, in which close relatedness between group members is not the cause, but a consequence of eusociality. Here we tested whether communal and eusocial species exert similar ecological advantages in the socially diverse snapping shrimp genus *Synalpheus*. We found larger group sizes in eusocial *Synalpheus* are associated with wider host ranges and occupy higher proportions of their host sponges. In contrast, larger group sizes in communal species are associated with wider geographic ranges only. The unique host-related advantages in eusocial, but not communal species suggest that the evolution of large groups may be fundamentally different when kinship is involved. Therefore, the ecological success of eusocial *Synalpheus* may not be due to group advantage alone.

## **Introduction**

Eusociality has been considered the pinnacle of social evolution that allowed eusocial insects like ants, bees and termites to dominate terrestrial habitats (Wilson 2013). The ecological advantage may be a consequence of the altruistic cooperative behavior and caste organization within eusocial colonies that promote colony competitiveness when

species are under ecological strains (Emlen 1982, Rubenstein & Lovette 2007, Sun et al. 2014). It has also been proposed that the group advantage in colony defense is the primitive driving force that predisposes the evolution of eusociality (Wilson & Holldobler 2005, Nowak et al. 2010). However, there have been few empirical tests of this ecological benefit of social life (but see Tibbetts & Reeve 2003, Smith et al. 2007, Duffy & Macdonald 2010) because it requires comparisons of closely related solitary/pair-forming and group-living species in the same or similar habitats (Wcislo & Tierney 2009).

On the other hand, communal—cohabiting females that share a nest but build, provision (and oviposit) in their own cells (Michener 1974)—appears to be an alternative strategy to cope with ecological pressure such as intense competition for nests (Wcislo & Tierney 2009). Recent examinations of social animals revealed that communal species are more common than once thought (Rubenstein & Abbot in press), and represent distinct evolutionary trajectory from eusociality (Chapter 3). However, little is known about the ecological context where communality evolved (Wcislo & Tierney 2009). Wcislo and Tierney (2009) proposed that communal Hymenoptera might benefit from improved passive nest defense and energetic saving from shared nest construction and maintenance. Importantly, the group advantage argument of Wilson and Hölldobler (2005) applies equally to communal species in that the group advantage in colony defense can also promote the formation groups of communal species. The key difference between communal and eusocial colonies, apart from maximum group sizes, is the presence of kin structure in eusocial colonies. Therefore, comparing the group benefit attained by communal and eusocial species can reveal the importance of kin structure and

may explain why workers (in facultatively eusocial species) would remain in their nest as “hopeful reproductives” instead of pursuing independent reproduction.

Here we focused on the socially diverse snapping shrimp genus *Synalpheus* to test the ecological advantages associated with communality and eusociality. Communality and eusociality have evolved independently multiple times from pair-forming species in the monophyletic West Atlantic Gambarelloides clade (Chapter 3). Eusocial *Synalpheus* species form colonies up to several hundred individuals, but have a single or a few reproductive female “queens”, whereas communal species form groups up to 20 individuals, with roughly equal sex ratios (Chapter 3, Chak et al. 2015). Preliminary molecular analyses suggest that mating pairs in communal colonies are unrelated (D.R. Rubenstein personal communication). This agrees with their biology that communal species have planktonic larvae that disperse from their natal sponge once hatched (Duffy & Macdonald 2010). Therefore, larvae that settled on the same sponge to form a mating pair are typically not closely related (LeFèvre & Bourget 1992) (although it is not impossible; e.g., Grosberg & Quinn 1986). Further, all species in the Gambarelloides clade are obligate sponge dwellers in which adults feed, mate and reproduce within sponge canals. They apparently venture outside of their host sponges only during dispersal as larvae, or as adults in eusocial species that have non-dispersing larvae (Tóth & Bauer 2007, Duffy & Macdonald 2010). The similar ecology and high social diversity of *Synalpheus* make this group ideal for testing the ecological benefit associated with group living.

Duffy and Macdonald (2010) showed that among *Synalpheus* species in Belize, abundance, sponge occupancy and host ranges increased with the degree of reproductive

skew (i.e., breeding inequality among workers and queen in a colony). However, a recent analysis showed that although the degree of reproductive skew appears to be increasing continuously from pair-forming, communal to eusocial *Synalpheus* species, communal and eusocial species evolved along separate evolutionary trajectories (Chapter 3). Therefore, intermediate skew in communal species and high skew in eusocial species are formed along different evolutionary trajectories. Hence, comparing pair-forming, communal and eusocial species on the same scale of reproductive skew may not reveal true patterns in the evolution of sociality.

Here, we test the ecological benefit of group living by comparing pair-forming with communal and eusocial species separately. We used group size (the total number of individual of the same species in a sponge) as the main predictor for various variables of ecological benefit because we were primarily interested in the ecological advantage associated with group formation. Specifically, we hypothesized that group size is positively related to 1) host range 2) geographic range, 3) relative abundance within sponge, and 4) occupancy among individual sponges. Therefore, our goal is to compare the group advantages of communal and eusocial *Synalpheus* and indirectly test the effect of kinship on promoting group advantage.

## Materials and methods

### *Geographic and effective host ranges*

We quantified host associations and geographic ranges of *Synalpheus* shrimps using data from 28 years of surveys from eight countries throughout the tropical West Atlantic (see Chapter 3). For geographic ranges, we tallied the number of regions where each

*Synalpheus* species were found (i.e., countries, including Bahamas, Barbados, Belize, Cuba, Curacao, USA-Florida, Jamaica and Panamá). For host ranges, we considered that a *Synalpheus* species is associated with a sponge species when we have observed this association in > 3 samples. We used presence-absence of associations instead of frequencies because sponges were not sampled in a way that can be used to make inferences about their natural abundances. Because some *Synalpheus* species were found to use sponge hosts that are closely related (see supplementary material), the effective number of phylogenetically distinct hosts may better represent their host ranges. We calculated this using Hill numbers (Hill 1973, Chao et al. 2010) that quantify the effective number of maximally distinct lineages. We calculated phylogenetic diversity in the order  $q = 1$ , corresponding to the exponential of Shannon entropy (Jost 2007, Chao et al. 2010):

$$\text{effective host range} = \exp(H_p/T)$$

where  $H_p$  is phylogenetic entropy (Allen et al. 2009), and T is the mean base change. We used a sponge phylogeny from Chapter 5, which is a Bayesian consensus tree based on COI and 28S sequences. We converted this tree into an ultrametric one by fixing the age of the root at 1 and scaling the tree by the absolute rate estimated using penalized-likelihood and verified using cross-validation (Sanderson 2002). Analyses were done in R (R Core Team 2015) using packages *ape* (Paradis et al. 2004) and *entropart* (Marcon & Hérault 2014).

#### *Relative abundance per sponge and sponge occupancy*

Relative abundance per sponge quantifies the extent to which a species dominates a sponge when the sponge has other congeneric occupants. For each sponge occupied by

multiple shrimp species, we calculated the group size of each shrimp species divided by the total number of shrimps in the sponge. For each *Synalpheus* species, we averaged the relative abundance per individual sponge for each sponge species that it occupied, then averaged the relative abundances among sponge species. These measurements of relative abundance are not confounded by sampling efforts of the sponge species, because sampling more sponge will only lead to measurements that are more accurate.

Sponge occupancy quantifies the relative frequency of occupation of sponges by a *Synalpheus* species. For each sponge species, we calculated the proportion of sponges in which the focal shrimp species was found. For each shrimp species, we averaged the proportions among its host sponge species. This proportion-based calculation avoided potential bias due to unequal sample sizes of different sponge species, hence it has controlled for sampling effort.

#### *Data partitioning*

Because communal and eusocial species evolved from pair-forming species in different evolutionary trajectories (see Chapter 3), we partitioned the data into two sets of species to reflect these two distinct trajectories and tested the relationship between group size and ecological advantages within each group separately. Group PC represents pair-forming and communal species, but excludes pair-forming species from clades that led only to eusocial species (figure 1, e.g., the *paraneptunus* clade). Group PE represents pair-forming and eusocial species, but excludes pair-forming species from clades that led only to communal species (figure 1, e.g., the *longicarpus* and *rathbunae* clades). See Chapter 3 for details of social organization of each species.

### *Phylogenetic regressions*

We ran four regression models to test the ecological benefit associated with group size, separately for PC and PE groups. We tested whether colony size was correlated with 1) effective host range (the number of sponge species used as hosts, controlling for host phylogeny), controlling for geographic range as a measure of sampling effort, 2) geographic range, controlling for raw host range as a measure of sampling effort (the raw number of sponge species used as hosts), 3) relative abundance per sponge, controlling for shrimp body size, and 4) sponge occupancy (mean proportion of individual sponges occupied). We controlled for the effect of either geographic range and effective host range in models 1 and 2 because species with wider geographic range may have a wider host range. We controlled for the effect of body size in model 3 because species with smaller body size would be expected to be more abundant in a sponge with a given availability of space or food, all else being equal. The variance inflation factor, a measure of the degree of multi-collinearity between independent variables, was smaller than 2 for all models with two predictors, meaning that these variables were not correlated strongly enough to affect the results of the regression models (O'Brien 2007). We estimated the slope ( $\beta$ ) between colony size and the response in these models using Monte Carlo Markov chain generalized linear mixed models (MCMCglmm) with the R package *MCMCglmm* (Hadfield 2010). We used the usual inverse-Gamma (0.01, 0.01) distribution as the prior distribution (Villemereuil & Nakagawa 2014) for the residual variance and the variance components (i.e., the random effect based on the phylogeny), but results were not sensitive to relaxing the prior to inverse-Gamma (0.001, 0.001). We

ran 500,000 iterations with the first 10% as burn-in and sampled every 200 iterations to obtain at least 2000 effective samples of the MCMC chain. A phylogenetic signal ( $\lambda$ ) was co-estimated in the model. We calculated the 95% credibility intervals of  $\beta$  to test if it was different from zero. We also reported  $pMCMC$ , which is the smaller probability of either  $\beta < 0$  or  $\beta > 0$ . Variables were transformed to conform to normality (Table 1).

For the above analyses, we used the most recent *Synalpheus* phylogeny (from Chapter 3). Briefly, we ran Bayesian analyses on three loci (16S, 18S, and COI) and 33 morphological characters. The final Bayesian consensus tree was based on DNA only, but with three constraints in resolving polytomies that were resolved in the full data (DNA + morphology) tree. We converted the tree to an ultrametric one, as described for the sponge phylogeny.

## Results

We analyzed how group size predicts effective host ranges, geographic ranges, relative abundances per sponge, and sponge occupancies for 34 *Synalpheus* species (table 1). Among pair-forming and eusocial species (group PE), larger group size was associated with larger effective host range and greater sponge occupancy (Table 2, Figure 2).

Among pair-forming and communal species (group PC), larger group size was associated with wider geographic range (Table 2, Figure 2). Although each sponge can support multiple pairs of communal species, they do not have higher abundance than pair-forming species because they often share the same sponges with eusocial species, in which the later are often more abundant.

## **Discussion**

Recent studies show that communality is a widespread social system that is demographically different (Rubenstein et al. 2016) and evolutionarily distinct (Wcislo & Tierney 2009, Chak et al. Submitted - Chapter 3) from eusociality. Both social systems can result in large group sizes, but the relative benefits of larger group sizes in communality vs. eusociality are rarely tested empirically (Wcislo & Tierney 2009). Here we show that in *Synalpheus* snapping shrimps, large group sizes in eusocial species are associated with wider host ranges and higher occupancies than pair-forming species. In contrast, larger group sizes in communal species are associated with wider geographic ranges than pair-forming species, but not with other host-related variables. Therefore, larger group sizes in both communal and eusocial species appear to have some, perhaps adaptive, ecological advantages, but these advantages are manifested differently.

Our findings that eusocial *Synalpheus* generally have wider host ranges and occupy higher proportions of their host sponges corroborate a previous, more geographically restricted study (Duffy & Macdonald 2010) to highlight that ecological superiority is closely linked to the evolution of eusociality in shrimps. In this respect, the evolution of eusociality in the ocean bears similarity to that of eusocial insects that attained ecological dominance in the terrestrial ecosystems (Wilson 2013), although the dominance of eusocial shrimps is restricted to congeners and specialized niche within reefs. This ecological superiority could be a consequence of the altruistic cooperation and caste organization within eusocial colonies that increase the effectiveness of colony defense and to secure food sources. However, it has been hypothesized that larger groups first evolved simply due to the adaptive advantage of enhanced nest defense, and that kin

structure within colonies came secondarily as an indirect consequence of group formation, i.e., by the addition of offspring that remain in the nest (Wilson & Hölldobler 2005, Nowak et al. 2010). Although it may be difficult to test the isolated effect of kinship or group benefit in eusocial species, communal species, in which group formation does not involve kinship, can be used to directly test the effect of group benefit. Here, we found that communal *Synalpheus* species do not experience the same ecological advantages in terms of greater host range and host occupancy as eusocial species. This suggests that kin structure within colonies yields additional benefits for *Synalpheus* species, beyond those of group formation (communality) alone. This host-related ecological dominance attained by eusocial species means that workers might have more incentive to remain in their nest as “hopeful reproductives” instead of pursuing independent reproduction. On the other hand, communal *Synalpheus* species appear to have wider geographic ranges. This suggests that larger communal groups may be advantageous in other aspects, for example, in enhancing colony-level fecundity as suggested by Wcislo and Tierney (2009) and to enhance dispersal, but this remains to be tested.

In conclusion, we found that in *Synalpheus* snapping shrimps, group benefits in communal and eusocial species are manifested differently. The unique host-related advantages in eusocial, but not communal species suggest kinship is important for eusocial species to achieve their ecological dominance in *Synalpheus*.

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Table 1. Host ranges (effective and raw), geographic ranges, group size, relative abundances per sponge, and sponge occupancies (mean proportion of individual sponges occupied) for 34 *Synalpheus* species.

<i>Synalpheus</i>	Raw host range	Effective host range	Geographic range	Mean group size	Median female carapace length (mm)	Relative abundances per sponge	Sponge occupancies	Social organization	Group PC	Group PE
<i>agelas</i>	2	1.066	7	5.297	4.432	0.534	0.235	Intermediate 1	Y	
<i>androsi</i>	1	1.000	3	4.800	3.867	0.507	0.123	Intermediate 1	Y	
<i>belizensis</i>	2	2.000	4	2.880	4.902	0.670	0.086	Pair-forming		Y
<i>bocas</i>	3	2.359	2	3.680	4.413	0.218	0.063	Pair-forming		Y
<i>bousfieldi</i>	5	5.000	5	5.532	3.999	0.720	0.103	Pair-forming	Y	Y
<i>brevifrons</i>	1	1.000	1	2.600	3.146	-	-	Pair-forming	Y	Y
<i>brooksi</i>	8	6.849	5	30.777	4.030	0.715	0.146	Intermediate 2		Y
<i>carpenteri</i>	2	1.066	5	12.053	3.088	0.658	0.297	Communal	Y	
<i>cayoneptunus</i>	2	2.000	1	12.938	3.430	-	0.065	Eusocial		Y
<i>chacei</i>	8	6.849	2	30.482	2.491	0.891	0.336	Eusocial		Y
<i>dardeaui</i>	3	3.000	2	8.892	8.744	0.250	0.184	Intermediate 1	Y	
<i>duffyi</i>	2	2.000	2	26.871	3.495	0.948	0.167	Eusocial		Y
<i>elizabethae</i>	3	3.000	4	87.329	3.708	0.769	0.155	Intermediate 2		Y
<i>filidigitus</i>	3	2.359	2	59.757	2.380	0.992	0.236	Eusocial		Y
<i>goodei</i>	1	1.000	2	2.960	4.755	0.816	0.353	Pair-forming	Y	
<i>herricki</i>	2	2.000	4	10.313	4.817	0.677	0.359	Communal	Y	
<i>hoetjesi</i>	2	2.000	3	4.471	5.505	0.399	0.027	Intermediate 1	Y	
<i>idios</i>	5	3.967	5	17.563	4.752	0.674	0.454	Communal	Y	
<i>kensleyi</i>	2	2.000	3	3.143	5.283	0.917	0.059	Pair-forming		Y
<i>longicarpus</i>	1	1.000	3	24.000	5.960	0.505	0.158	Communal	Y	
<i>longicarpus small</i>	2	2.000	2	3.000	5.262	0.579	0.188	Pair-forming	Y	
<i>mcclendoni</i>	2	2.000	2	2.875	4.000	0.771	0.021	Intermediate 1	Y	
<i>microneptunus</i>	1	1.000	1	6.545	3.665	0.953	0.083	Intermediate 3		
<i>pandionis</i>	1	1.000	3	3.000	5.579	0.653	0.012	Pair-forming	Y	Y
<i>pectiniger</i>	1	1.000	4	23.750	4.200	0.370	0.295	Communal	Y	
<i>rathbunae</i>	1	1.000	1	58.318	2.934	0.899	0.327	Eusocial		Y
<i>regalis</i>	3	3.000	3	117.880	3.623	0.985	0.312	Eusocial		Y
<i>ruetzleri</i>	1	1.000	2	3.276	3.821	0.808	0.095	Pair-forming	Y	Y
<i>rufus</i>	1	1.000	2	4.000	4.818	0.115	0.025	Intermediate 1	Y	

<i>sanctithomae</i>	3	3.000	5	2.333	3.123	0.727	0.080	Pair-forming	Y
<i>thele</i>	4	4.000	4	15.600	2.930	0.489	0.023	Communal	Y
<i>ul</i>	6	6.000	6	4.610	4.581	0.683	0.050	Pair-forming	Y
<i>williamsi</i>	1	1.000	5	2.280	4.026	0.943	0.229	Pair-forming	Y
<i>yano</i>	4	3.340	5	16.930	5.675	0.589	0.109	Communal	Y

Table 2. Results of MCMCglmm regressions to test the ecological advantage associated with increasing group size in eusocial and communal *Synalpheus* snapping shrimps.  $\beta$ : mean posterior slope estimate, CI: Bayesian credibility interval,  $pMCMC$ : the lower of the probabilities of  $\beta$  being larger or smaller than zero,  $\lambda$ : phylogenetic signal.

Groups	Models	Responses	Predictors	$\beta$	95% CI	$pMCMC$	$\lambda$	
Eusocial	1	log(effective host range)	log(group size)	<b>0.255</b>	<b>0.018 - 0.492</b>	<b>0.039</b>	0.863	
			log(geographic range)	0.508	-0.043 - 1.026	0.060		
	2	log(geographic range)	log(group size)	-0.14745	-0.387 - 0.114	0.222	0.969	
			log(raw host range)	0.42262	-0.050 - 0.918	0.090		
	3	(relative abundance) <sup>2</sup>	log(group size)	0.041	-0.090 - 0.163	0.479	0.834	
			body size	-0.062	-0.265 - 0.127	0.492		
	4	sqrt(sponge occupancy)	log(group size)	<b>0.063</b>	<b>0.003 - 0.114</b>	<b>0.030</b>	0.720	
	Communal	1	log(effective host range)	log(group size)	0.011	-0.452 - 0.463	0.963	0.132
				log(geographic range)	0.509	-0.230 - 1.248	0.184	
		2	log(geographic range)	log(group size)	0.24304	-0.012 - 0.485	0.058	0.087
				log(raw host range)	0.33795	0.020 - 0.635	0.032	
		3	(relative abundance) <sup>2</sup>	log(group size)	-0.094	-0.226 - 0.021	0.119	0.298
				body size	-0.037	-0.120 - 0.044	0.373	
		4	sqrt(sponge occupancy)	log(group size)	0.085	-0.034 - 0.199	0.151	0.443

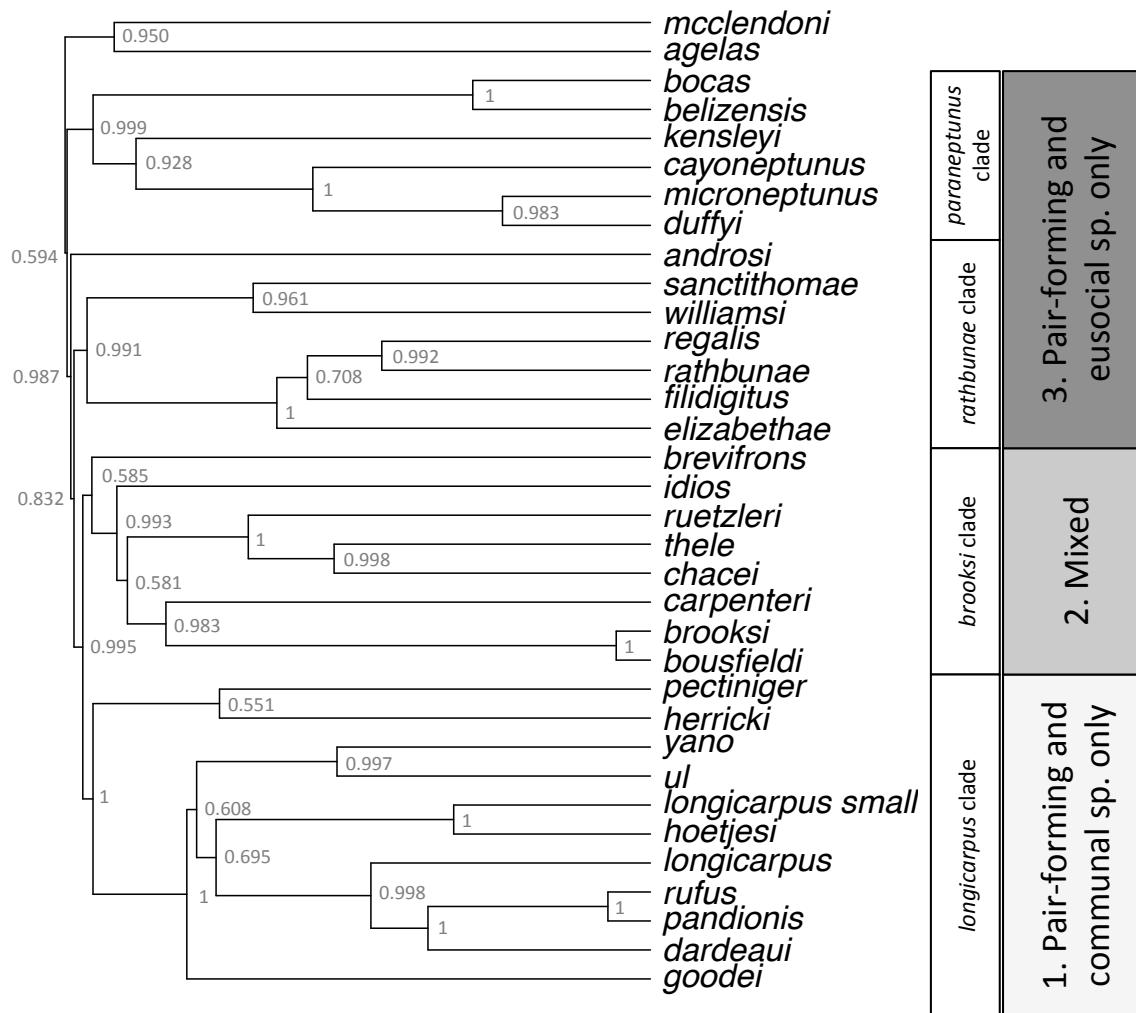


Figure 1. Molecular phylogeny of *Synalpheus*. Boxes on the right indicate the four major clades, and the social organizations in species within these clades. The “mixed” clade include species that are pair-forming, communal and eusocial. In our analysis, group PC (pair-forming and communal) includes species in clade 1 and the pair-forming and communal species in clade 2. Group PE includes species in clade 3 and pair-forming and eusocial species in clade 2. Node supports are Bayesian posterior probabilities. Species that were sampled less than 3 times were excluded.

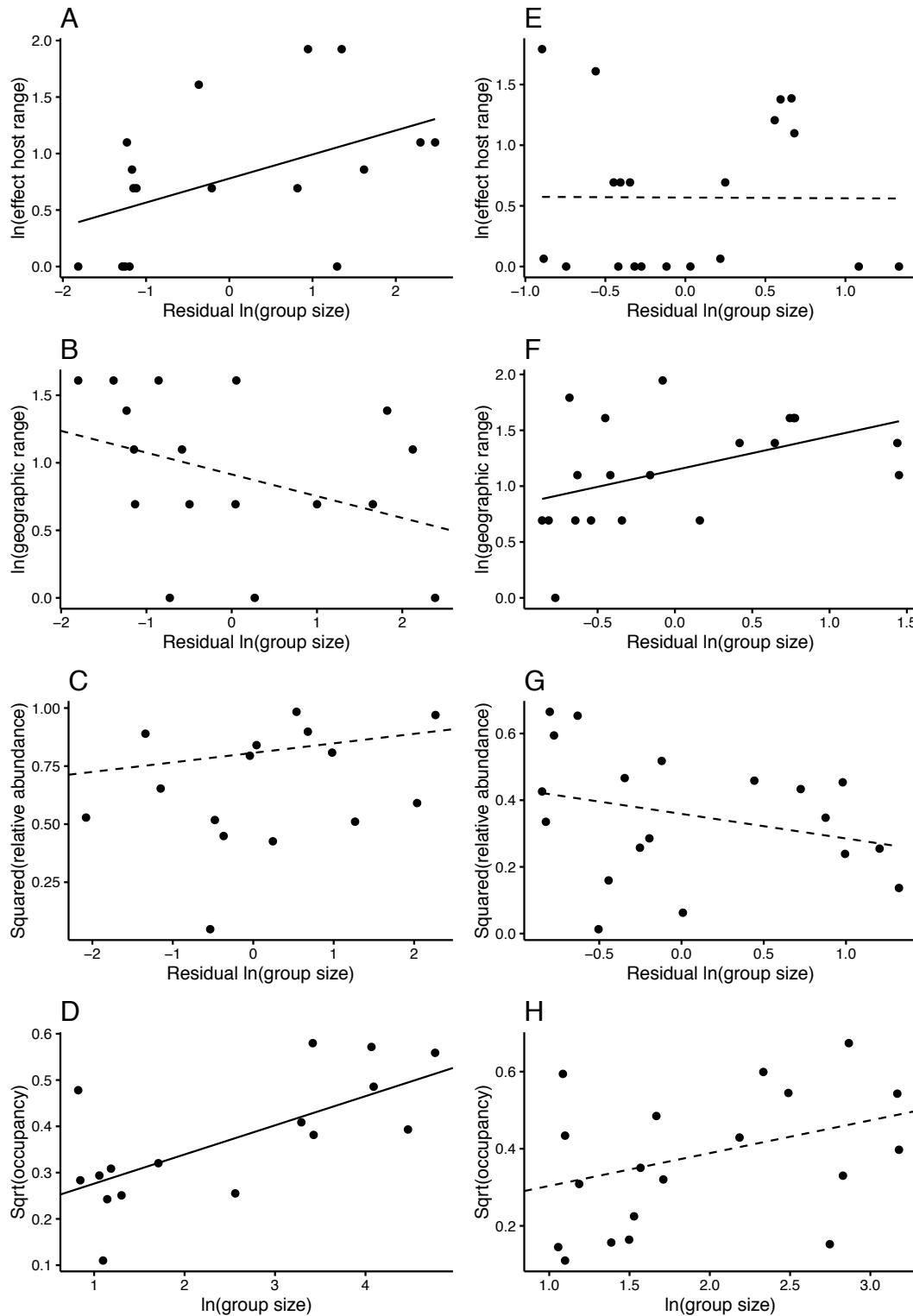


Figure 2. Relationships between group size and (A, E) effective host range, (B, F) geographic range, (C, G) relative abundance per sponge and (D, H) sponge occupancies among pair-forming and communal species (group PC, left column) and among pair-forming and eusocial species (group PE, right column). Solid and dashed lines represent significant and non-significant regressions. Axes show partial residuals controlling for (A, E)  $\ln(\text{geographic range})$ , (B, F)  $\ln(\text{effective host range})$  and (C, G) median female carapace length.

## **Supplementary Materials**

### *Net relatedness of host sponges*

We quantified whether each *Synalpheus* species uses sponge hosts that are more phylogenetically closely related than expected using the net relatedness index (NRI) (Webb et al. 2002). NRI represents the standardized effect size of phylogenetic relatedness, which we used to compare sponge hosts of different shrimp species by controlling for potential differences in the number of hosts.

$$\text{NRI} = -1 * (\text{MPD}_{\text{obs}} - \text{MPD}_{\text{random}}) / \text{sdMPD}_{\text{random}}$$

where  $\text{MPD}_{\text{obs}}$  is the mean pairwise phylogenetic distance of the hosts of a shrimp species, and  $\text{MPD}_{\text{random}}$  is the pairwise phylogenetic distance of the randomly assembled hosts. Positive values of NRI indicate that the sponge hosts are more phylogenetically related than randomized communities of sponge hosts. We did this calculation with *picante* in R (Kembel et al. 2010), using 999 random communities of sponge hosts generated using the independent swap method (Gotelli & Entsminger 2003).

We found that apart from 11 shrimp species that use a single sponge hosts, 11 out of 23 species have positive NRI, meaning that these species have hosts that are more closely related than random (Figure S1).

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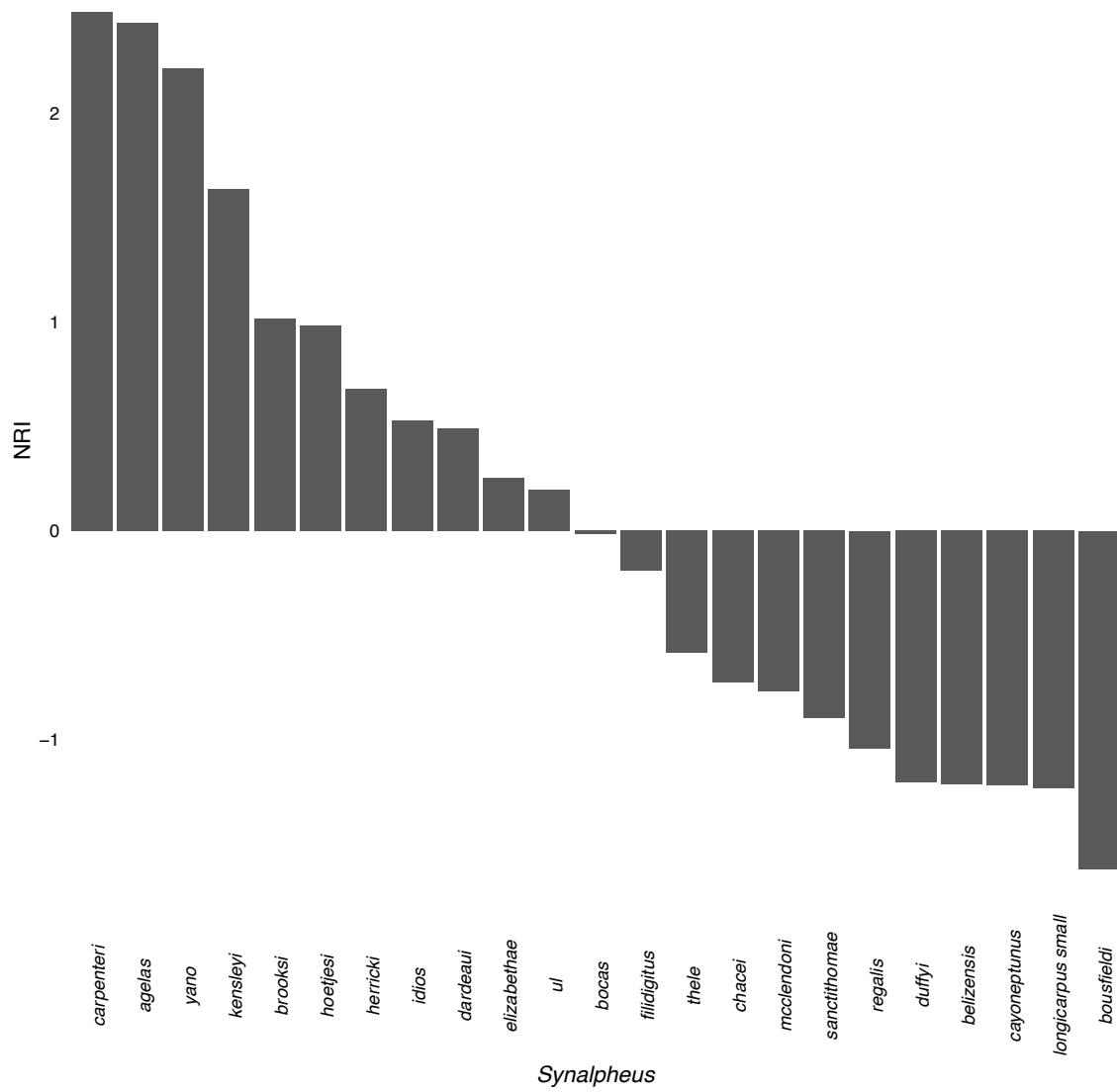


Figure S1. Net relatedness index of sponge hosts used by *Synalpheus* species.

## **CHAPTER 5**

### **HOST ASSOCIATION OF SNAPPING SHRIMPS IN SPONGE**

#### **HOLOBIONTS**

## **Abstract**

Many ecologically important organisms such as coral and sponges can be viewed as holobionts that comprise the primary hosts and their symbiotic microorganisms.

Although symbiotic microbes often expand the functional repertoire of their hosts (e.g., photosynthesis, cellulose digestion), it is often unclear how far the microbial symbiosis reaches to affect biological interactions between their hosts and other organisms. Here, we focused on a group of sponge-dwelling snapping shrimps to test whether host association patterns in shrimps can be explained by the evolutionary history of the host sponges or the similarity of sponge bacterial communities. We found that when a shrimp pair is more closely related, their host sponges tend to have more similar bacterial communities, but surprisingly, these sponges tend to be phylogenetically more distantly related. The inverse relationship between shrimp and sponge phylogenetic similarity may be due to intense competition between shrimp species, or that sponge traits that affect host specificity of shrimps have no phylogenetic signal. Most importantly, our findings suggest that microorganisms may mediate biological interactions, perhaps by affecting larval settlement or diet. This study illustrates that a holistic analysis of holobionts can bring a more comprehensive understanding of ecological patterns.

## **Introduction**

Holobionts are individuals of different species that are physically associated for significant portions of their life history (Margulis 1993, Gordon et al. 2013). Many ecologically important organisms such as coral and sponges are increasingly being viewed as holobionts that comprise the hosts and their symbiotic microorganisms (Hentschel et al. 2006, Blackall et al. 2015). These symbiotic microbes can expand the functional repertoire of their hosts, for example in performing nitrogen metabolism in sponges (Fiore et al. 2010, Webster & Taylor 2012), photosynthesis in corals (Baker 2003) and lignocellulose digestion in termites (Brune 2014). However, it is unclear whether holobiont associations affect larger-scale ecological and evolutionary processes, although coral reef ecology and evolution as driven by scleractinian coral holobionts (*Symbiodinium*) provides a notable exception (put your favorite ref here – perhaps something from Ruth Gate's GeoSymbio group).

Clarifying the pattern of biological associations, such as host specificity and the interdependency between organisms, is especially important for highly diverse ecosystems such as coral reefs, that are considered an evolutionary source of marine biodiversity (Kiessling et al. 2010). The biodiversity of coral reefs is not only comprised of the corals that provide their frameworks, but also by the high diversity of small organisms living within and on corals (Knowlton et al. 2010) and sponges (Wulff 2006). We use de Bary's (1879) original definition of symbiosis as species living together (Lewin 1982) to describe this tight association between hosts and the associated organisms, but the term is also applicable to host and microbes. The diversity of symbiotic organisms that live within their hosts may be driven by speciation after

disruptive selection for use of different host resources, i.e., speciation after host-shift, and has been found in fish (Munday et al. 2004), mollusks (Faucci et al. 2007, Krug 2011) and crustaceans (Morrison et al. 2004, Tsang et al. 2009, Hurt et al. 2013). In general, speciation after host-shift results in a host specificity pattern in which more closely related symbionts would use more closely related hosts (Vienne et al. 2013).

A tight association between the phylogenetic histories of hosts and symbionts assumes that the host phylogeny is an adequate proxy for host traits that mediate host specificity, meaning that these traits are not formed by convergent evolution. However, host traits that mediate host specificity could also be related to microorganisms in holobionts. Recent studies are beginning to reveal that symbiotic microorganisms play an important role in mediating biological interactions of the hosts with, for example, bacteria and viruses in humans (Cogen et al. 2010a, Cogen et al. 2010b, Pfeiffer & Virgin 2016), bacteria and pathogens in corals (reviewed in Thompson et al. 2014), and parasitic microfungi in fungus-growing ants (Oh et al. 2009), through chemical interactions (e.g., by producing selective antibiotics). Many corals and sponges are often hosts to a wide variety of invertebrates (Wulff 2006, Gibson et al. 2011) that rely on chemical cues to settle on or find hosts (Hay 2009). However, whether and how the microorganisms in these holobionts have contributed to the characteristically high diversity of these groups has not been explored.

Here, we test the contributions of host and microbes in explaining patterns of host specificity between sponges and snapping shrimps in the genus *Synalpheus*. *Synalpheus* shrimps in the monophyletic *Gambarelloides* clade (Hultgren et al. 2014) live obligately inside the canals of different sponges in the tropical West Atlantic (Duffy 1992,

Macdonald et al. 2006, Hultgren & Duffy 2010). Sponges offer a protective and stable habitat for the majority of the shrimp's life cycle. Therefore, shrimp population subdivision has been shown to be related to different sponge use (Duffy 1996b). Also, sponge traits (canal size and volume) can partly predict local communities of the inhabiting *Synalpheus* species (Hultgren & Duffy 2010). Hence, we expect that host specificity of *Synalpheus* to be mediated, at least in part, by host-shifts between different sponges. On the other hand, the diversification of *Synalpheus* could also be driven by difference in the microbial communities in their host sponges. Sponges harbor highly diverse symbiotic microbial communities in their mesohyl matrix (Hentschel et al. 2006, Taylor et al. 2007, Webster & Taylor 2012), which could comprise as much as 40% of sponge tissue volume (Vacelet 1975). The bacterial communities of sponges are highly species-specific and generally stable over environmental, geographical and temporal gradients (Erwin et al. 2012, Björk et al. 2013, Medina et al. 2013, Olson & Gao 2013, Cárdenas et al. 2014, Erwin et al. 2015). Moreover, closely related sponges may or may not have more similar microbial communities (Schmitt et al. 2011, Schöttner et al. 2013, Easson & Thacker 2014). We hypothesize a strong relationship between *Synalpheus* and microbes because *Synalpheus* shrimps feed directly on sponge tissues (Ruetzler 1976, Ďuriš et al. 2011) and the microbial communities may produce chemical cues (Pawlik 1992, Hadfield & Paul 2001) that are used to guide larval settlement in marine invertebrates (Pawlik 1992, Hadfield & Paul 2001). Therefore, the aim of this study is to test whether the patterns of host specificity in *Synalpheus* can be explained by the evolutionary history of sponges and the similarity of sponge bacterial communities.

## **Materials and methods**

### *Shrimp-sponge associations*

We quantified the communities of *Synalpheus* shrimps in sponges using 28 years of survey data on populations of *Synalpheus* spp. from the tropical West Atlantic. Sampling methods and localities are reported in Chapter 3 (Chak et al. submitted). We considered that a *Synalpheus* species is associated with a sponge species (i.e., a presence of shrimp-sponge link) when we have observed this association in > 3 samples. We did not use the frequency of observing each shrimp-sponge association to indicate host preference because sponges were not sampled randomly and do not represent the natural abundance of sponges. We used those *Synalpheus* species that associate with *only* the 13 sponges we examined in this study (see next section). In other words, we excluded any *Synalpheus* species that used any sponge species not sampled in our study. *Synalpheus* identification was based on taxonomy established by Coutiere (1909), Chace (1972) and Dardeau (1984), and supplemented by recent taxonomic descriptions and keys (Duffy 1996c, Ríos & Duffy 1999, MacDonald & Duffy 2006, Ríos & Duffy 2007, Anker & Toth 2008, Macdonald et al. 2009, Hultgren et al. 2010, Hultgren et al. 2011).

### *Similarity of bacterial communities between sponges*

We collected samples of sponge species that host *Synalpheus* shrimps from shallow habitats in the tropical West Atlantic (Belize, Barbados, Curacao, Florida Keys, Jamaica, Panamá, Table S1). These sponges are either macroscopic sponges attached to hard substrates or cryptic sponges attached to or infilling between dead coral rubble. Whole

sponges were collected using SCUBA (5-20 m) and snorkeling (< 5 m), kept submerged in seawater during transportation to field stations, and retained in flowing seawater until they could be processed. We subsequently dissected sponges and carefully removed all macrofauna from the internal canals of the sponge. From each sponge, we removed 5-10 ml of tissue from the internal lining of the canals using sterile scissors or forceps and preserved tissue in 95% ethanol for DNA extraction to characterize the bacterial communities or to reconstruct the sponge phylogenetic trees (see next section).

We extracted DNA of symbiotic bacteria from ~5 mm<sup>3</sup> of sponge tissue using the FastDNA SPIN Kit for Soil with lysing matrix A (MP Biomedicals, Solon, OH) following the manufacturers' protocols. Universal primers (27F and 338R) of 16S rRNA genes were used to amplify hypervariable V1 and V2 regions (Chakravorty et al. 2007). The 338R primer had Ion Torrent adapter A fused with eight barcode nucleotides and the 27F primer included the Adapter trP1 (Hamady et al. 2008) (see Supplementary Materials). The polymerase chain reaction (PCR) contained 1 × Qiagen PCR buffer (with 15 mM MgCl<sub>2</sub>), 0.6 mM dNTPs, 0.2 mM of each primers, 0.1 mg/ml BSA, 2.5 U Taq polymerase, and 20 ng DNA template in a 25 µl reaction vessel. PCR conditions involved an initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 20 s, followed by 5 min at 72 °C. We ran the PCR product in a gel and excised the ~380 bp band for purification using the Qiagen QIAquick PCR Purification Kit. Quality and concentration of the PCR products were further checked on the Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, CA). Equimolar PCR products were pooled and sequenced on an Ion Torrent PGM sequencer (Life Technologies, Grand

Island, NY) using the Ion 318 Chip Kit V.2 following the Ion Torrent 400 bp sequencing kit protocol.

We used the online RDP Pipeline Initial Process (Cole et al. 2014) to bin sequences according to the barcodes, trim primer sequences, and remove all sequences shorter than 150 bp. Acacia v.1.53.b0 (Bragg et al. 2012) was used to de-noise the trimmed sequences and remove all sequences with quality scores < 25 (as in Song et al. 2014). Chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011) in the FunGene Pipeline (Fish et al. 2013). Subsequent analyses were conducted with the Mothur pipeline (Schloss et al. 2009) using the SILVA rRNA database (v.119) (Pruesse et al. 2007) for alignment and classification. Detailed codes are provided in the Supplementary Materials.

A total of 576,345 sequences were obtained from 35 sponge samples representing 13 sponge species. Based on 97% sequence similarity 87,697 OTUs (operational taxonomic units) were identified in bacterial symbionts. For each sponge sample, we calculated relative abundance of each OTU by dividing the number of sequences assigned to each OTU by the total number of sequences in each sample. Preliminary analysis with weighted-UniFrac distance (Lozupone & Knight 2005) showed that bacterial communities among samples of the same sponge species clustered closely together only for some sponge species, and that there is no obvious trend of clustering based on sampling location and years (Figure S1). Therefore, to incorporate the variability of bacterial communities within a sponge species, we pooled samples of the same sponge species by averaging the relative abundance of OTUs in each sample, resulting in 13 samples. Averaging the relative abundance instead of the raw

number of sequences avoids uneven averaging due to variability of sequencing depth among samples.

Using the 13 pooled samples, each representing a sponge species, we calculated the weighted UniFrac distance between each sponge species. The weighted UniFrac incorporates both the relative abundance of OTUs among samples and the phylogenetic distance between OTUs (Lozupone & Knight 2005). Therefore, a shorter UniFrac distance means that a pair of sponge species has a more similar phylogenetic composition of bacterial communities. The phylogenetic distances between OTUs were based on a maximum likelihood tree built using FastTree (Price et al. 2010). A recent simulation study supported the use of relative abundance and weighted UniFrac for clustering analyses based on 16S pyrosequencing (McMurdie & Holmes 2014). These calculations were carried out using the R package Phyloseq (McMurdie & Holmes 2013). We built a neighbor-joining tree based on the pairwise UniFrac distances between sponges and used it for subsequent analyses.

#### *Phylogenetic relationships between sponges*

We extracted sponge DNA from tissue samples using either the Qiagen DNeasy Blood & Tissue Kit or the MP Biomedicals FastDNA SPIN Kit for Soil with lysing matrix A following the manufacturers' protocols. The quality of each extraction was checked using agarose gel electrophoresis and DNA concentration was quantified using a NanoDrop 2000 (Thermo Scientific). Some samples were further purified using the Zymo Research Genomic DNA Clean & Concentrator after unsuccessful amplifications. We amplified

the cytochrome c oxidase subunit I (COI) gene and genes coding for the 28S subunit of the ribosomal RNA (28S, covering the C1, D1, and C2 domains) using primers from Morrow et al. (2012) and Borchiellini et al. (2004) (see Supplementary Materials). The polymerase chain reaction (PCR) for both COI and 28S contained 1 × Qiagen PCR buffer (with 15 mM MgCl<sub>2</sub>), 0.6 mM dNTPs, 0.6 mM of each primers, 0.1 mg/ml BSA, 0.03 U Taq polymerase, and 2.5 ng/μl DNA template in a 10 μl reaction vessel. Annealing temperatures (T<sub>A</sub>) were 49 °C for COI and 51 °C for 28S. PCR conditions involved an initial denaturing at 94 °C for 4 min, annealing at T<sub>A</sub> for 2 min, and extension at 72 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 51 °C for 1 min, and 72 °C for 1 min, and end with an extra extension at 72 °C for 4 min. The size-specific PCR products were excised from an agarose gel after electrophoretic separation and the excised fragment was cleaned using the Qiagen QIAquick PCR Purification Kit. We sequenced the PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM® 310xl Genetic Analyzer. Forward and reverse contigs were assembled and trimmed using Sequencher v.5.4.1 (Gene Codes, Ann Arbor, MI).

We constructed the phylogenetic history of 13 sponge species that host *Synalpheus*, using 25 samples. We aligned COI sequences with MUSCLE (Edgar 2004) following (Morrow et al. 2012) and 28S sequences with MAFFT with the Q-INS-i algorithm that uses information on the secondary structure of the 28S molecule (Katoh & Standley 2013) following Thacker et al. (2013). We used the online server of GBlocks v0.91b (allowing gap positions within the final blocks) to exclude ambiguous parts of the alignment (Castresana 2000, Talavera & Castresana 2007). Then we ran a partitioned Bayesian analysis using MrBayes (Ronquist et al. 2012) from the Cyberinfrastructure for

Phylogenetic Research (CIPRES) Science Gateway (Miller et al. 2011). We used the most general model (GTR + G + I) because the Bayesian method is shown to be more sensitive to under-specification than to over-specification of the evolutionary model (Huelsenbeck & Rannala 2004). We ran Markov Chain Monte Carlo (MCMC) searches with four chains and two runs for  $1 \times 10^7$  generations, sampling the chain every 1000 generations, and discarded the first 25% as burn-in (standard deviation of split frequencies reached 0.009 after burn-in). We estimated node supports using Bayesian posterior probabilities (bpp). We used the sponge *Plakortis angulospiculatus* as the outgroup, which is in the Class Homoscleromorpha that is basal to the Demospongiae (Erpenbeck et al. 2007, Kober & Nichols 2007). Since samples of the same sponge species are monophyletic in the Bayesian tree (see Results), we subset the tree into a smaller tree with 13 species that were analyzed for bacterial communities; the sponge phylogeny and the bacterial analysis used the same sponge sample for nine sponge species.

#### *Phylogenetic similarity between *Synalpheus* species*

We quantified the phylogenetic similarity between *Synalpheus* species using the most recent *Synalpheus* phylogeny from Chapter 3. Briefly, we ran Bayesian analyses on three loci (16S, 18S, and COI) and 33 morphological characters. The final Bayesian consensus tree was based on DNA only, but with three constraints in resolving polytomies that were resolved in the full data (DNA + morphology) tree. We used this tree to depict phylogenetic similarity between pairs of *Synalpheus* species in subsequent analyses.

### *Data Analysis*

First, we tested whether the sponge similarity based on bacterial communities reflects the phylogenetic relationship among sponges. Such a relationship was reported for a set of Caribbean sponges (Easson & Thacker 2014), and would mean that similarity between sponges and that between bacteria within sponges is highly correlated and potentially redundant. For the sponge phylogeny, we computed pairwise distances between sponge species using the cophenetic function in APE (Paradis et al. 2004). Then we tested for a correlation between the pairwise distances between sponges and the UniFrac distances between sponges based on the bacterial communities using a Mantel test (Mantel 1967).

To test whether more closely related shrimps use more closely related sponges or sponges with more similar bacterial communities, we generated a list of all possible pairs of the 29 *Synalpheus* species (406 pairs). For each shrimp pair, we calculated their phylogenetic similarity (shrimpPS) as  $1 - \text{PSV}$ , where PSV is the phylogenetic species variability (PSV) of the two shrimp species. PSV quantifies the inverse of how species of a community are phylogenetically related, independent of species richness (Helmus et al. 2007). PSV equals to one when all species are equally related (i.e., from a star phylogeny), and approaches zero when groups of species become more closely related. Thus, the shrimp species pair is more closely related when shrimpPS (i.e.,  $1 - \text{PSV}$ ) approaches one. For each of the 406 shrimp pairs, we tallied the sponge species (both unique and shared) used by the pair, and calculated the phylogenetic similarity between the sponges (spongePS) in the same way as  $1 - \text{PSV}$ . In this case, the shrimp pair could have 2 or more sponge hosts, but the number of species involved does not affect the

values of PSV. Again, higher spongePS means that sponges used by the shrimp pair are more phylogenetically similar. Finally, for each shrimp pair, we calculated the bacterial community similarity of their host sponges (bacterialCS) using a neighbor-joining tree between sponges based on UniFrac distances. In this case, higher bacterialCS means that the sponge hosts of a shrimp pair have more similar bacterial communities.

We used generalized linear models and a permutation approach to test the relationships between shrimpPS, spongePS, and bacterialCS. If more closely related shrimps use more closely related sponges, we expected positive relationships between shrimpPS and sponges. Similarly, if more closely related shrimps use sponges with more similar microbial communities, we expected positive relationships between shrimpPS and bacterialCS. We first tested a full regression model to predict shrimpPS as a function of spongePS, bacterialCS and the interaction between spongePS and bacterialCS. We used gamma regression with log link (McCullagh and Nelder 1989) to model the positively skewed predictor variable (shrimpPS). We excluded the interaction effect in our final model based on Bayes factor ( $e^{\Delta BIC/2}$ ), which depicts the likelihood of the final model versus the full model, or vice versa (Jarosz & Wiley 2014). Further, because eusocial species appear to be more competitive and could affect shrimp communities in sponges, we tested an additional model that included the interaction between sociality and each spongePS and bacterialCS. We coded a shrimp pair as eusocial when at least one shrimp species was eusocial; other shrimp pairs were coded as non-eusocial. We square root transformed all variables because they were positively skewed (Tabachnick & Fidell 2007). We used a randomization test to assess the significance of the model parameters because data on shrimp pairs may not be statistically independent. We generated 10,000

random *Synalpheus* trees from our original tree by randomizing tip labels using phyloshuffle in the R package phylotools (Zhang et al. 2015). From each random tree, we calculated shrimpPS<sub>random</sub> and ran the regressions again. We only randomized the predictor variable shrimpPS because it is the generation of shrimp-pair data that resulted in non-independency. Using the random distribution of each coefficient (e.g.,  $\beta$  for spongePS), we calculated  $p_{\text{random}}$  as the proportion of times (out of 10,000 iterations) the random coefficient were more extreme than our observed coefficient. Therefore,  $p_{\text{random}}$  tests the null hypothesis that the observed coefficient is significantly different from that generated using shrimpPS<sub>random</sub>.

Note that it may appear that one could test for whether closely related shrimp use sponges that are closely related (or with more similar bacterial communities) using distance-based cospeciation analyses that test for patterns of cophylogeny between hosts and associated taxa (Vienne et al. 2013). However, *Synalpheus* communities within most sponges are phylogenetically closely related (Hultgren & Duffy 2012). This poses a problem for using cophylogenetic analysis because patterns of non-random host associations (i.e. signal of cophylogeny) can be driven by sponges being used by phylogenetically similar shrimps. We presented cophylogenetic analyses in the Supplementary Materials, which shows that significant cophylogenetic patterns were mostly driven by the sponge species being used by phylogenetically similar shrimps.

## Results

Our sample included 76 associations between 13 sponge species and 29 *Synalpheus* species (Table 1). Our samples covered 80% of *Synalpheus* species in the West Atlantic

Gambarelloides group, 60% of their host sponges and 84% of all observed associations between *Synalpheus* and sponges. Although most *Synalpheus* species used a few hosts, some species were host generalists and used up to seven different sponge species (Table 1). On the other hand, most sponge species were used by a few *Synalpheus* species, but some had up to 11 *Synalpheus* species (Table 1).

We quantified the similarity among host sponges used by *Synalpheus* shrimps based on their symbiotic bacterial communities (Figure 1, Figure 2B, S2 and S3) and their phylogenetic history (Figure 2A; tree with all samples is shown in Figure S4). The phylogenetic relationship between *Synalpheus* species is shown in Figure 3. In the set of sponges we sampled, the sponge phylogeny was not correlated to the sponge similarity based on bacterial communities ( $r = -0.136, p = 0.917$ ). This was also apparent in that the topology of the sponge phylogeny compared to the tree that depicts the bacterial similarity between sponges (Figure 3). Therefore, it is logical to separately test the contributions of sponges and their symbiotic bacteria to host associations in shrimps.

We tested whether more closely related shrimps (shrimpPS) use more closely related sponges (spongePS), whether more closely related shrimps (shrimpPS) use sponges with more similar microbial communities (bacterialCS). We found a non-significant, but negative relationship between shrimpPS and spongePS (Model 2, Table 2, Figure 4). This means that there is a weak signal that more closely related shrimps tended to use more distantly related sponges. On the other hand, there was a positive relationship between shrimpPS and microbialCS (Model 2, Table 2, Figure 4). This means that more closely related shrimps tend to use sponges with more similar microbial communities. Further, we tested whether having a eusocial shrimp in a shrimp pair (sociality) would

affect these relationships, and found a significant interaction between sociality and shrimpPS, but not microbialCS (Model 3, Table 2). Specifically, the trend that closely related shrimps tend to use sponges with more similar microbial communities is more apparent when eusocial species were involved (Figure 4B).

## Discussion

The ecological functions of corals and sponges, two major components of tropical coral reefs, are strongly linked to their symbiotic microorganisms. They are also hosts to a diverse suite of animals that contribute to the extraordinary biodiversity of reefs, many of which are obligate associates. We do not know whether the symbiotic microorganisms of these hosts contribute to the host specificity of other obligately associated animals. Here, we showed that in sponge-dwelling snapping shrimps of the genus *Synalpheus*, when a shrimp pair is more closely related, 1) their host sponges tended to have more similar bacterial communities and 2) these sponges tended to be phylogenetically more distantly related. Therefore, our results suggest—surprisingly—that in sponge holobionts, the phylogenetic history of sponge and their symbiotic bacterial communities have opposite effects on the host specificity of sponge-dwelling snapping shrimps.

The sponge holobiont includes the sponge host and the highly diverse symbiotic microbial communities in their mesohyl matrix (Hentschel et al. 2006, Taylor et al. 2007, Webster & Taylor 2012). Here we found evidence that these symbiotic microorganisms can mediate the pattern of host (sponge) association in snapping shrimps. Specifically, the observed pattern that more phylogenetically similar shrimps use sponges with more similar microbial communities can be explained in several ways. First, many small

marine herbivores (mesograzers) specialize on chemically-defended sessile invertebrates (e.g., macroalgae and sponges) that are not preferred by fishes (Hay 1996). These mesograzers, many of which are crustaceans, are often undeterred or even attracted by the same host metabolites that deter fish feeding. In sponges, many of these secondary metabolites are likely synthesized by microbes (Bewley et al. 1996, Unson et al. 1994, Schmidt et al. 2000, Piel 2004, Balskus 2014, Hardoim & Costa 2014). Thus, the microbial communities may produce unique blends of chemical cues (Pawlik 1992, Hadfield & Paul 2001) that differentiate these sponges and may be used to guide larval settlement (Pawlik 1992, Hadfield & Paul 2001), hence helping to structure the pattern of host use in snapping shrimps. Becerra (1997) studied the plant genus *Bursera* and the associated beetle genus *Blepharida* and found a greater influence of host plant chemical similarity than host phylogeny in explaining the host association patterns. Therefore, although there remains uncertainty about how microbial metabolites specifically mediate larval settlement in *Synalpheus*, chemicals from microbes could have important and common, but underappreciated influence on biological interactions (Hay 2009). On a finer scale, shifting to a different host may be related to a change in diet. Snapping shrimps appear to feed on sponge tissues (Ruetzler 1976, Ďuriš et al. 2011) that are loaded with bacteria (Vacelet 1975) and they may be able to selectively feed or assimilate specific microorganisms from their diet (Abreu et al. 2007). Therefore, diet specialization on a specific community of bacteria may be a hidden drive of host-mediated differentiation in shrimps (Duffy 1996b) and may lead to that closely related shrimp pairs using sponges that have similar microbes. Finally, both bacterial communities in sponges and host specificity of shrimps could be responding to other sponge traits, such as canal

size. While size matching between sponge canal size and shrimp body size is important in host specificity of *Synalpheus* (Hultgren & Duffy 2010), little is known about how sponge architecture affects the communities of symbiotic bacteria, nor is there a consensus on how the sponge microbial communities are determined (Webster 2014). Therefore, although we found that more related shrimps use sponges that have more similar bacterial communities, the mechanisms of this relationship remains to be tested.

It is surprising to find that more closely related shrimps use sponges that are more distantly related. Although host use of *Synalpheus* is dependent on sponge morphological traits such as canal size (Hultgren & Duffy 2010), these traits may not have a phylogenetic signal. In fact, the gross morphological characters of sponges such as shapes may be highly variable even within the same species (e.g., Becerro et al. 1994). The many convergent traits in the relatively simple anatomy of sponge species have been the major challenge of sponge systematics (Erpenbeck & Worheide 2007, Redmond et al. 2013). Therefore, the sponge phylogeny may not capture the similarity among host characters that *Synalpheus* species respond to during host shift; the similarity among sponge microbial communities appears be a more accurate predictor. On the other hand, a pattern of divergent hosts use is expected if competition between shrimp species is intense (i.e., competitive exclusion) (Webb 2000). This competitive effect may be especially prominent in shrimp pairs that contain eusocial species, because they can cooperatively defend their hosts (Duffy 1996a, Duffy et al. 2002, Tóth & Duffy 2005) and tend to out compete phylogenetically related congeners that use the same host (Hultgren & Duffy 2012). In support of competitive exclusion, among *Synalpheus* shrimp pairs that contained eusocial species, the negative relationships between shrimp

phylogenetic similarity (shrimpPS) and sponge phylogenetic similarity (spongePS)

became more prominent than when shrimp pairs did not contain eusocial species.

Therefore, we speculate that the competitiveness of eusocial species may involve in shaping the host association pattern in *Synalpheus*.

In conclusion, we found that in sponge holobionts, the phylogenetic history of sponge and their symbiotic bacterial communities have opposite effects on the host specificity of sponge-dwelling snapping. More closely related shrimps tend to use sponges that have more similar microbial community, and sponges that are more distantly related. Our results suggest possible roles in microbially-derived chemicals, as well as competition exclusion between shrimp species, in driving the host use pattern between snapping shrimps and sponges. Most importantly, we have shown that beyond expanding the functional repertoire of their hosts, microorganisms can also mediate biological interactions of their host. Hence, a holistic analysis of holobionts can reveal hidden ecological patterns and clarify complex interaction between organisms.

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Table 1. Associations between *Synalpheus* species and sponges in tropical West Atlantic.

Column and row totals are the total number of host species used per shrimp and the total number of shrimps per host, respectively. Numbers after species name are sample sizes.

<i>Synalpheus</i>	<i>Agelas clathrodes</i> (154)	<i>Agelas dispar</i> (49)	<i>Aiolochroia crassa</i> (16)	<i>Calyx podatypa</i> (61)	<i>filmy encrusting sponge</i> (8)	<i>Hyattella intestinalis</i> (194)	<i>Hymeniacidon caerulea</i> (103)	<i>Lissodendoryx columbiensis</i> (207)	<i>Neopetrosia cf. proxima</i> (116)	<i>Neopetrosia cf. subtriangularis</i> (131)	<i>Neopetrosia rosariensis</i> (113)	<i>Oceanapia</i> sp. (32)	<i>Spirastrella</i> sp. (81)	Total
<i>agelas</i> (251)	1	1	0	0	0	0	0	0	0	0	0	0	0	2
<i>androsi</i> (109)	0	0	0	0	0	1	0	0	0	0	0	0	0	1
<i>ankeri</i> (17)	0	0	0	1	0	0	0	0	0	1	0	0	0	1
<i>belizensis</i> (71)	0	0	0	0	0	1	0	0	0	1	1	0	0	4
<i>bocas</i> (93)	0	0	0	0	0	0	0	0	1	1	1	0	0	3
<i>bousfieldi</i> (400)	1	0	1	0	0	1	1	0	1	1	0	0	0	6
<i>brevifrons</i> (23)	0	0	0	0	1	0	0	0	0	0	0	0	0	1
<i>carpenter</i> (549)	1	1	0	0	0	0	0	0	0	0	0	0	0	2
<i>carpenteri small</i> (16)	1	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>cayoneptunus</i> (205)	0	0	0	0	0	0	0	0	1	1	0	0	0	2
<i>duffyi</i> (951)	0	0	0	0	0	0	0	0	1	1	0	0	0	2
<i>elizabethae</i> (8154)	0	0	0	0	0	1	1	1	0	0	0	0	0	3
<i>filidigitus</i> (4439)	0	0	0	0	0	1	0	0	1	1	0	1	0	4
<i>goodie</i> (72)	0	0	0	1	0	0	0	0	1	0	0	0	0	2
<i>herricki</i> (351)	0	0	1	0	0	1	0	0	0	0	0	0	0	2
<i>hoetjesi</i> (83)	0	0	0	0	0	1	0	1	0	0	0	0	0	2
<i>kensleyi</i> (22)	0	0	0	1	0	0	0	0	0	0	0	0	0	1
<i>longicarpus small</i> (81)	0	0	0	1	0	0	0	1	0	1	0	0	0	3
<i>mclendonii</i> (32)	1	1	0	0	0	1	0	0	0	0	0	0	0	3
<i>microneptunus</i> (77)	0	0	0	0	0	0	0	0	1	1	0	0	0	2
<i>pandionis</i> (21)	0	0	0	0	0	1	0	1	0	0	0	0	1	3
<i>rathbunae</i> (1824)	0	0	0	0	0	0	0	0	0	0	1	0	0	1
<i>regalis</i> (13357)	0	0	0	0	0	1	0	0	1	1	0	0	0	3
<i>ruetzleri</i> (30)	0	0	0	0	0	0	1	0	0	0	0	0	0	1
<i>sanctithomae</i> (100)	1	0	0	0	0	1	1	0	1	0	0	0	0	4
<i>thele</i> (126)	1	0	0	0	0	1	1	0	0	0	1	0	0	4
<i>ul</i> (191)	1	0	0	1	0	0	1	1	1	1	0	0	1	7
<i>williamsi</i> (53)	0	0	0	0	0	0	1	0	0	0	0	0	0	1
<i>yano</i> (2540)	0	0	0	1	0	1	1	1	0	0	1	0	0	5
Total	8	3	2	6	1	13	8	6	11	10	4	2	2	

Table 2. Gamma regression (log-link) results in models predicting phylogenetic similarity of shrimp pairs (shrimpPS).  $\beta$ : estimated slope coefficient, Bayes factor: probability of one model against the indicated model, BIC: Bayesian information criteria, microbialCS: bacterial community similarity of host sponges of a shrimp pair,  $p_{\text{random}}$ : p-value to test the null hypothesis that  $\beta$  is significantly different from that generated from randomized shrimpPS, spongePS: phylogenetic similarity between the sponges used by a shrimp pair.  $\beta$ 's for the interactions with sociality are relative to non-eusocial species.

Predictor	Model 1		Model 2		Model 3	
	$\beta$	$p_{\text{random}}$	$\beta$	$p_{\text{random}}$	$\beta$	$p_{\text{random}}$
spongePS	-0.952	0.070	-0.616	0.097	-1.660	0.009
microbialCS	0.884	0.251	2.32	0.041	3.647	0.033
spongePS x microbialCS	4.404	0.131				
spongePS x sociality					1.261	0.021
microbialCS x sociality					-1.117	0.252
$\chi^2$	13.08		12.05		20.83	
d.f.	3		2		4	
p	0.004		0.002		0.0003	
BIC	-776.42		-761.863		-754.640	
Bayes factor	0.066 (against model 2)		15.219 (against model 1)		0.027 (against model 2)	

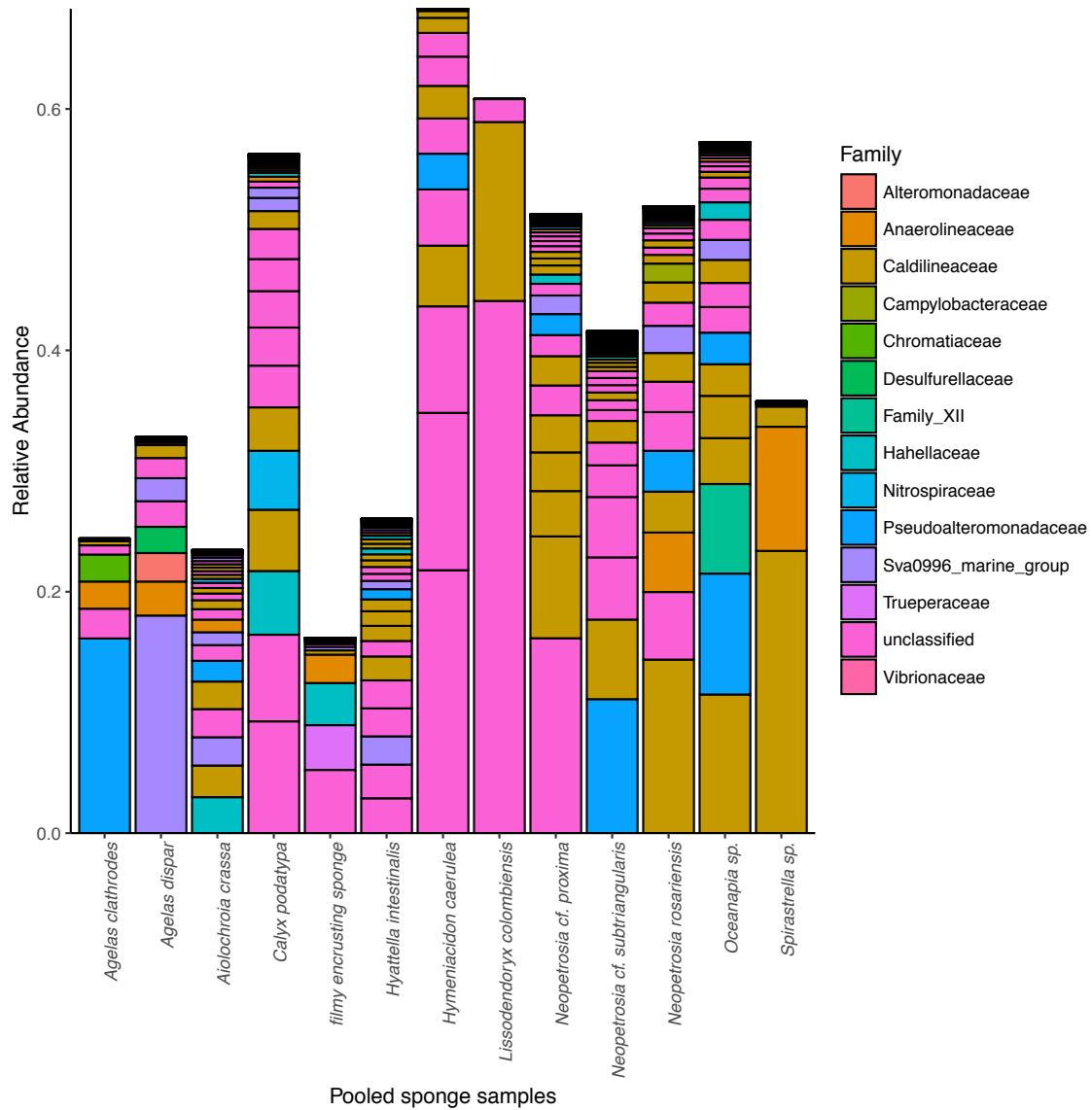


Figure 1. Relative abundance and bacterial families of the top 100 OTUs among 13 pooled sponge samples.

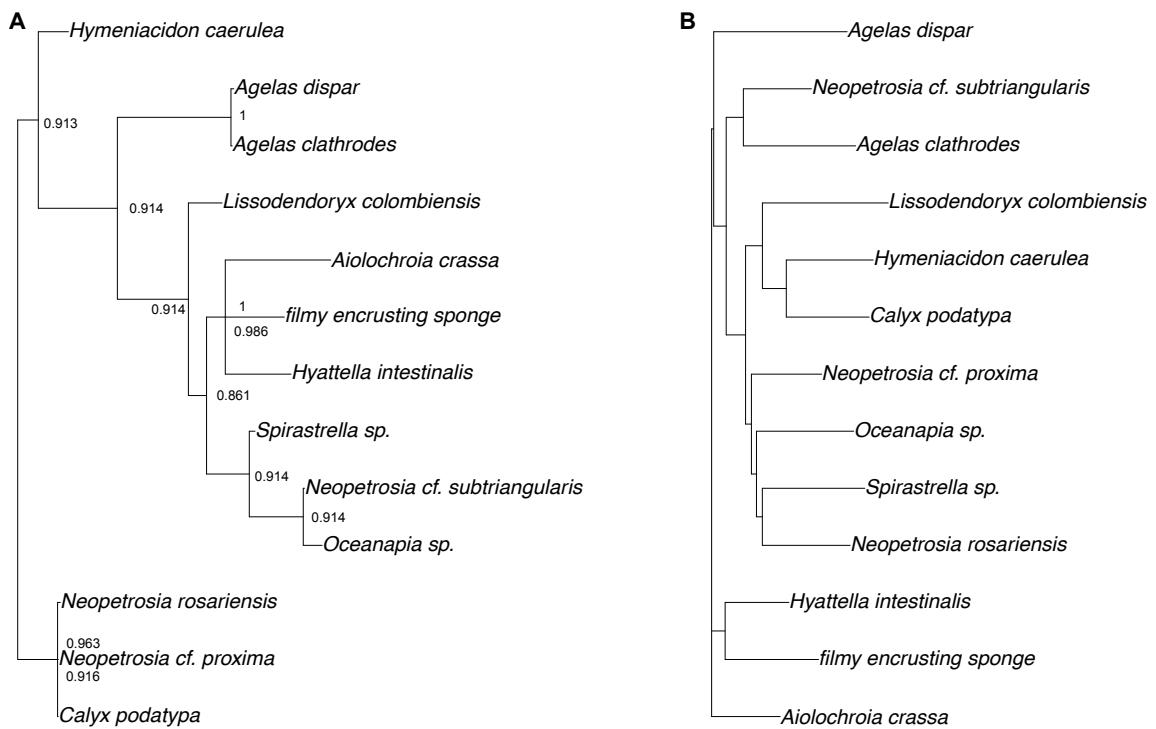


Figure 2 (A) Phylogenetic relationships between sponges used by *Synalpheus* and (B) neighbor-joining tree of UniFrac distances between sponges based on their symbiotic bacterial communities. Node supports are based on Bayesian posterior probabilities.

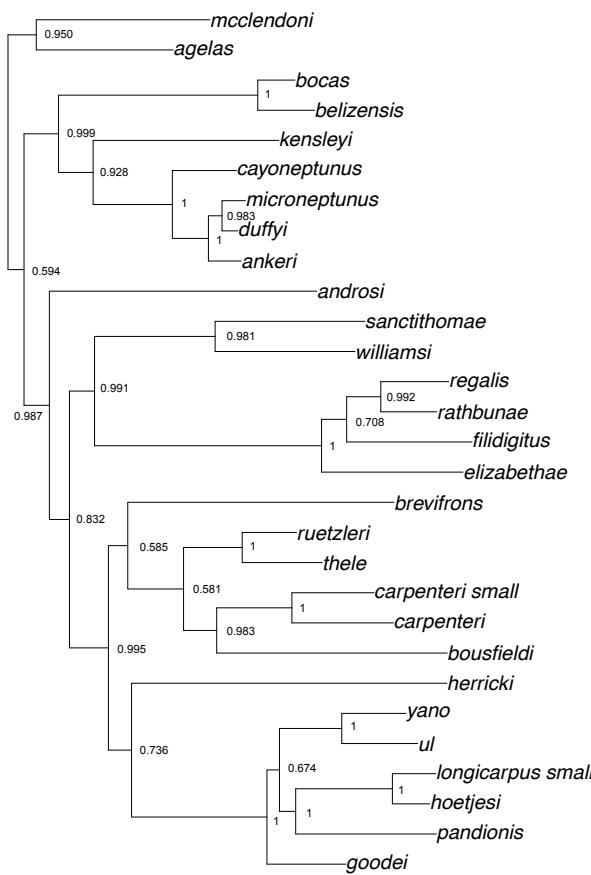


Figure 3. Phylogenetic relationships between *Synalpheus* species used in this study. Node supports are based on Bayesian posterior probabilities.

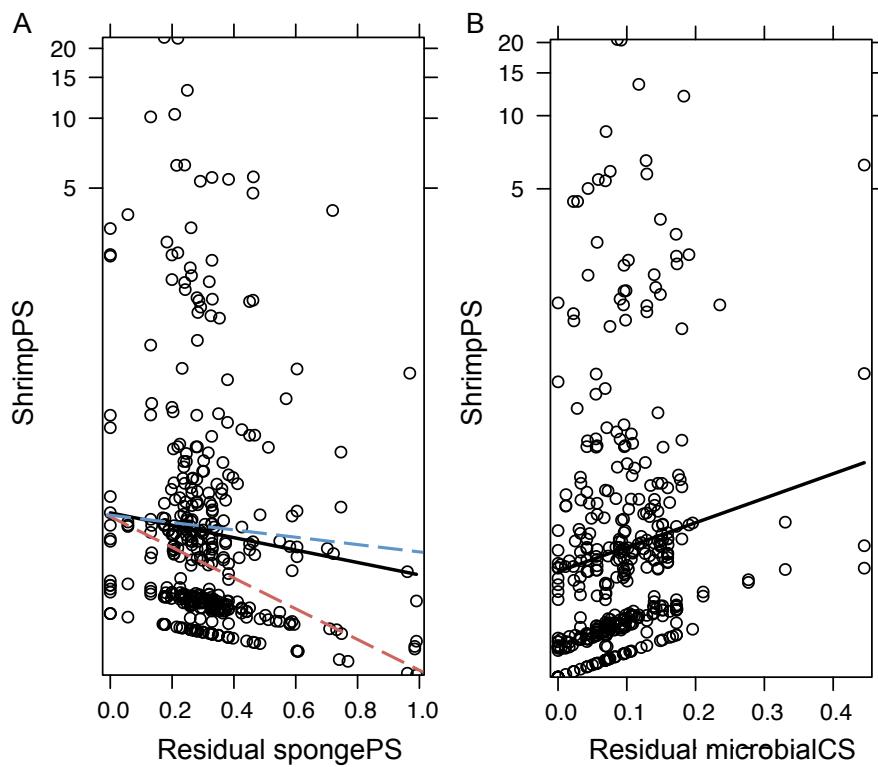


Figure 4. Relationships between phylogenetic similarity between shrimp pairs (shrimpPS) and (A) bacterial community similarity between sponges (microbialCS) and (B) phylogenetic similarity between sponges (spongePS). Axes show (A) partial residual controlling for spongePS and (B) partial residual controlling for microbialCS. Solid lines represent regressions of the main effects. In (B), dashed lines represent the interaction between spongePS and sociality (red: eusocial, blue: non-eusocial).

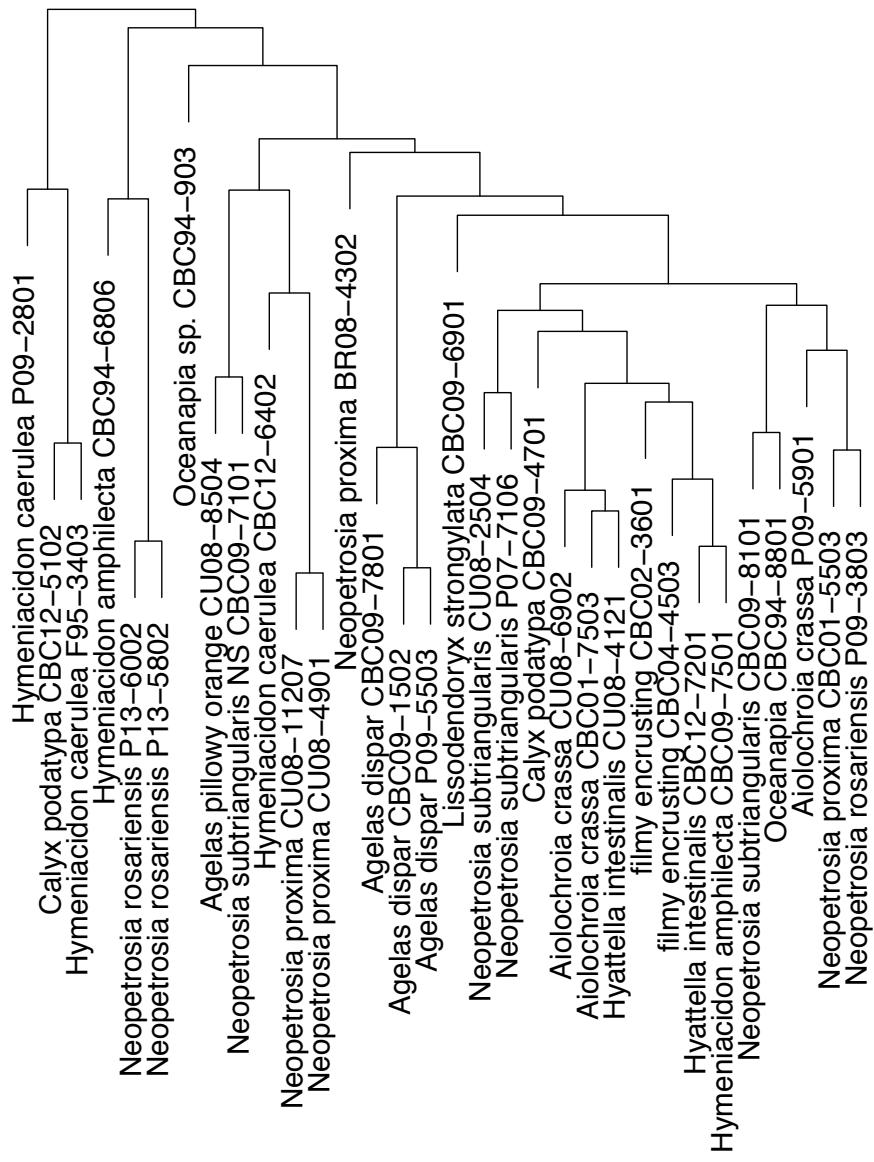


Figure S1. UPGMA cluster of weighted UniFrac distances between bacterial communities from 23 sponge samples.

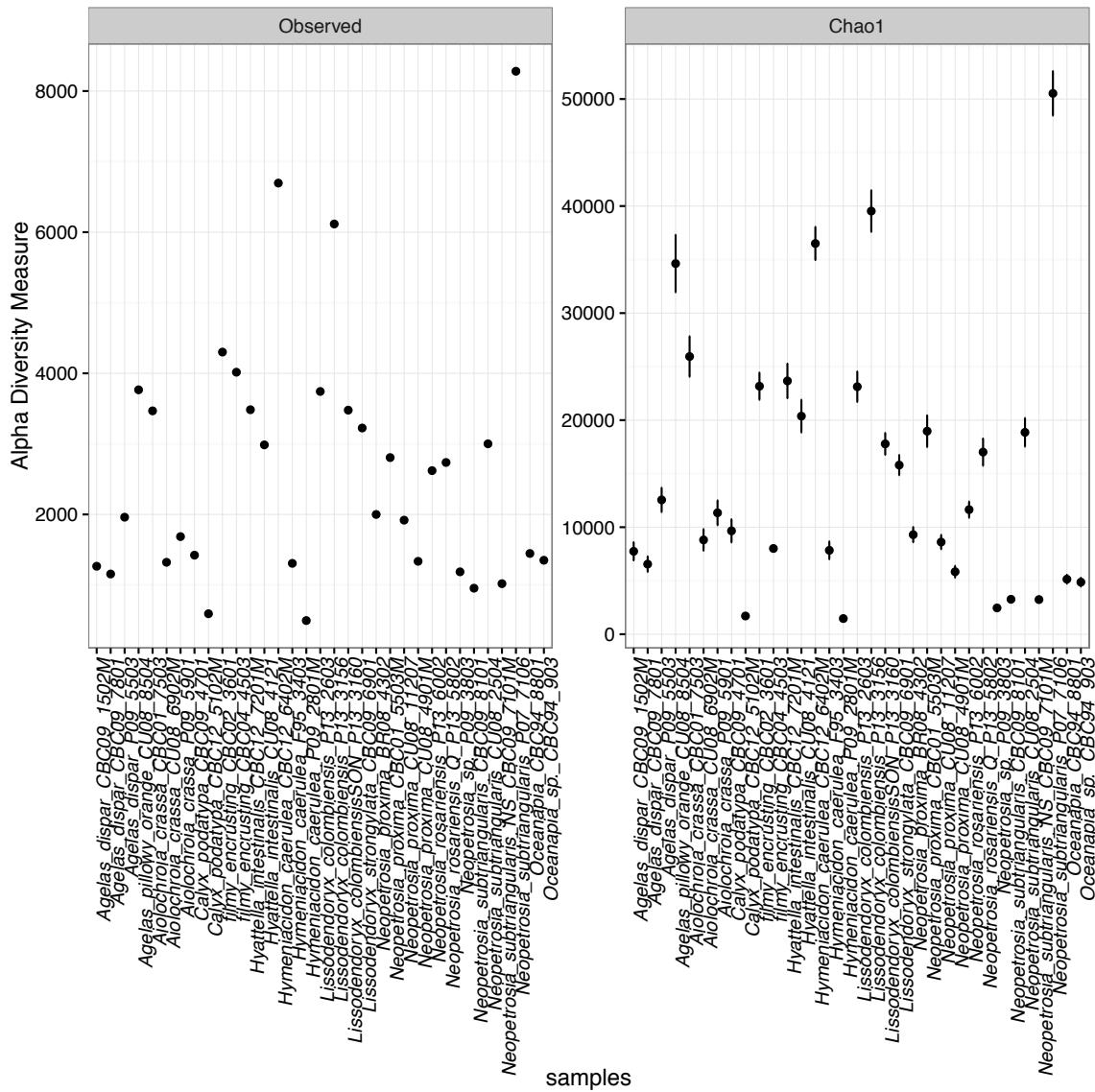


Figure S2. Observed and bias-corrected Chao1 richness estimate for the microbial communities in 33 sponge samples. The Chao1 richness is a non-parametric estimate that adds a correction factor to the observed number of OTUs to account for the probability of not observing an OTU based on mark-release-recapture stats (Chao1 = richness +  $n_1^2 / 2n_2$ , where  $n_1$  is the number of singletons and  $n_2$  is the number of doubletons).

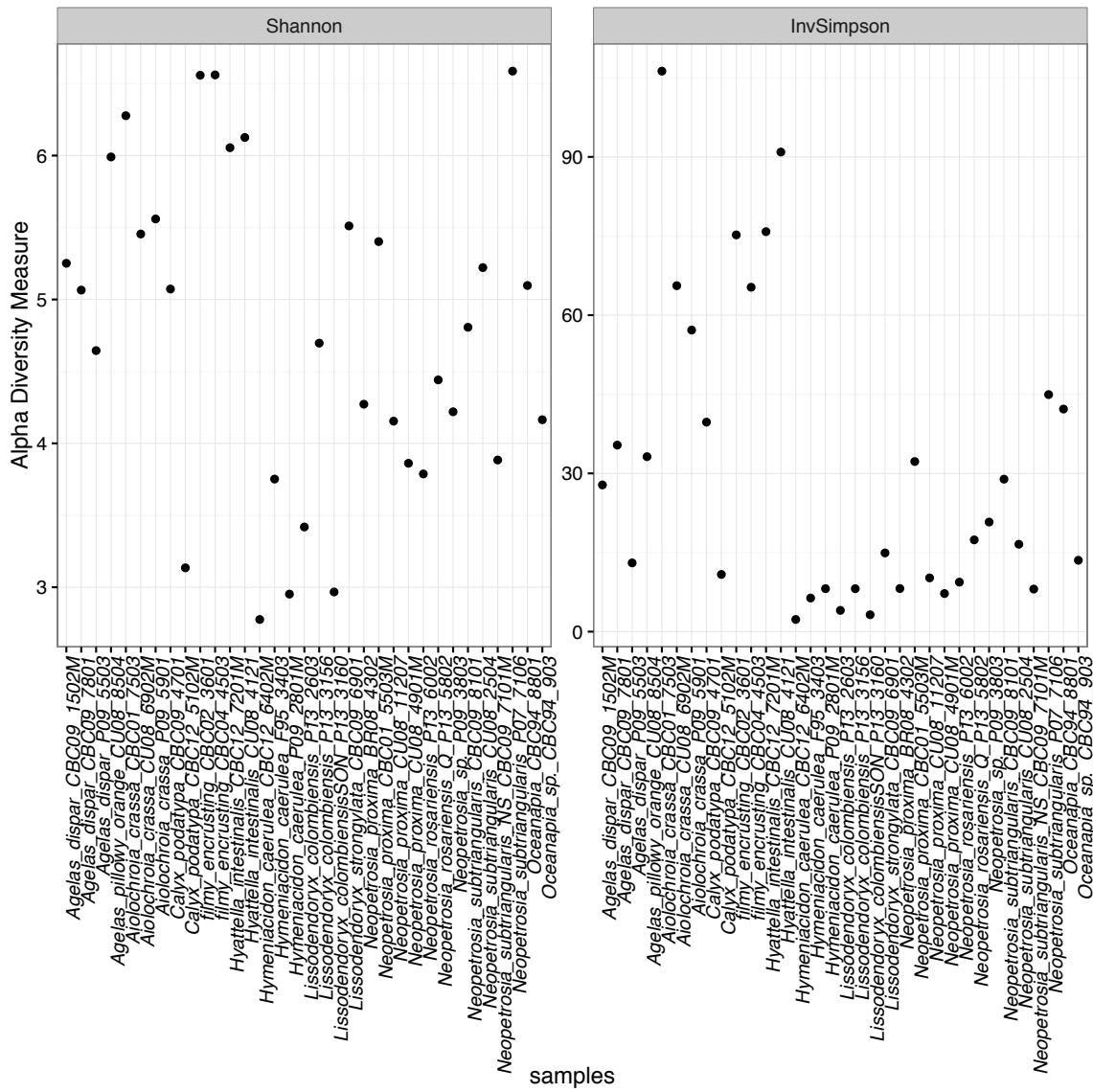


Figure S3. Shannon and inverse Simpson diversity indices for the microbial communities in 33 sponge samples. Shannon index accounts for both richness and evenness, whereas the inverse Simpson index gives roughly the number of very abundant OTUs.

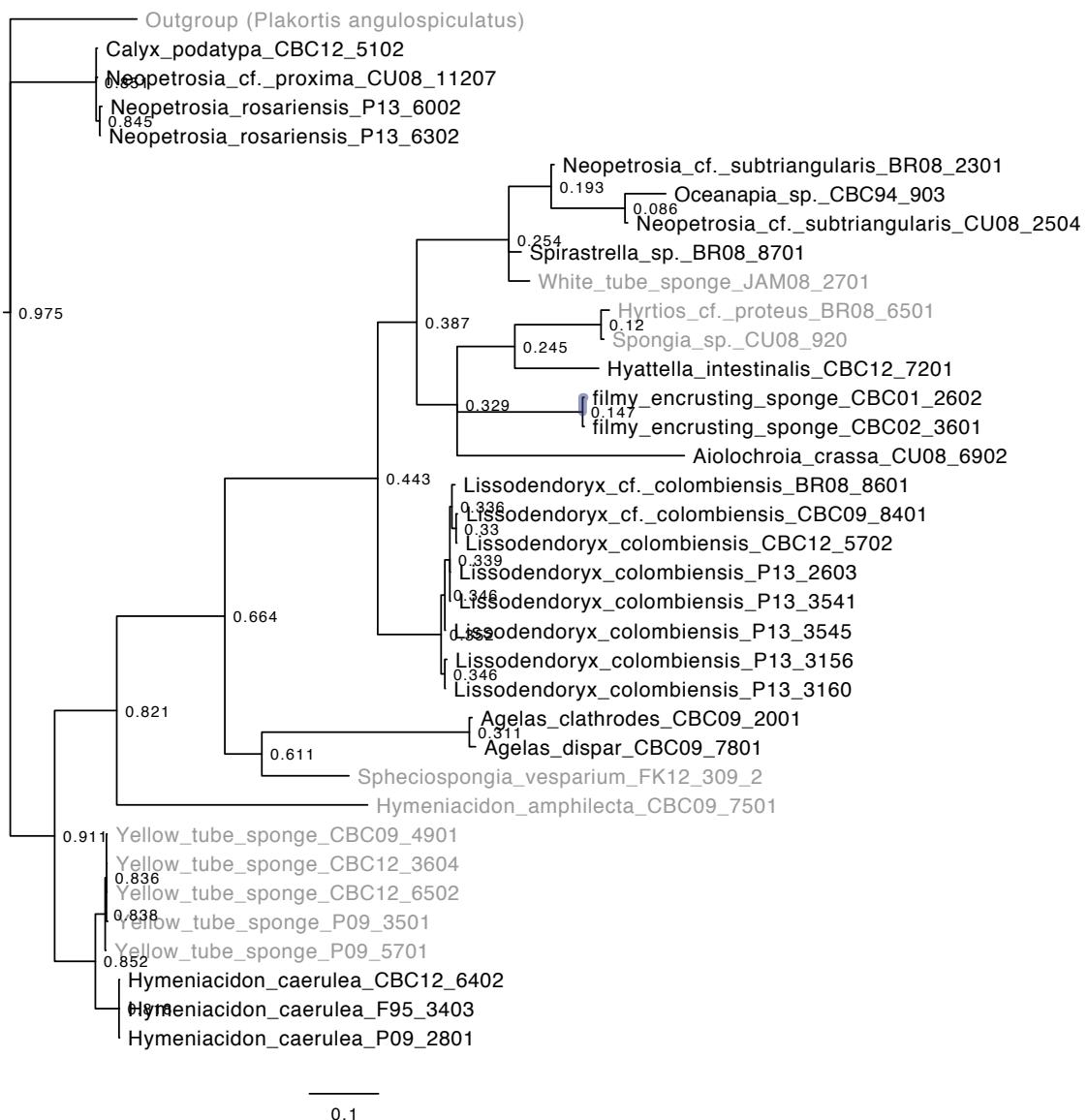


Figure S4. Sponge phylogeny with all samples. Node supports are Bayesian posterior probabilities. Sample names in grey are samples not used in this study.

## **Supplementary Materials**

### *PCR primers*

Sponge COI:

dgLCO1490: 5'-GGTCAACAAATCATAAAGAYATYGG-3'

dgHCO2198: 5'-TAAACTCAGGGTGACCAAARAAYCA-3'

Sponge 28S:

C'1: 5'-ACCCGCTGAATTAAAGCAT-3'

Ep3: 5'-ATKCGYTTCCCTCCYACCGG-3'

Bacteria 16S:

### *Cophylogenetic analyses*

#### *Shrimp-sponge cophylogeny*

We used the distance-based method (Vienne et al. 2013) ParaFit (Legendre et al. 2002) to test for the cophylogeny between *Synalpheus* and sponge. We did not use event-based methods which are mostly developed to analyze cospeciation between host and parasites (Vienne et al. 2013) rather than speciation after host-shift which we were testing. For both methods, we used the shrimp-sponge association matrix, *Synalpheus* phylogenetic distances, and sponge phylogenetic distances, calculated using the command ‘cophenetic’ in APE, for the analyses. Phylogenetic distances were converted to Euclidean distances by taking the square root of the patristic distance (de Vienne et al. 2011) instead of using the typical Cailliez (1983) adjustment which would lead to distortion. In ParaFit, we calculated the ParaFitGlobal statistic to tests the null hypothesis that the evolution of

shrimps and sponges are independent, meaning that they are randomly associated. The value of ParaFitGlobal are functions of the shrimp and sponge phylogenetic distances and of the set of association links in principle coordinate space, thus this value is not directly interpretable. We performed 10,000 permutations to each row of shrimp on the association matrix to test if the observed ParaFitGlobal is different from random.

Further, we identified associations between shrimp and sponge that contributed to the overall cophylogeny. We calculated the test statistic ParaFitLink1( $k$ ), which quantifies the reduction of overall cophylogeny when an association  $k$  is removed. ParaFit calculates a permutation p-value to assess if an association is random given the coevolutionary structure, and we considered associations with  $p < 0.05$  as significant associations. While the choice of a cut-off p-value is not straightforward because of multiple testing (Balbuena et al. 2013), we used 0.05 as a cut off only to select associations that are relatively important.

### *Shrimp-bacteria congruency*

We used the same method, ParaFit, to test for a congruent pattern between *Synalpheus* phylogeny and the similarity between sponges based on bacterial phylogenetic communities. Here, we used the shrimp-sponge association matrix, *Synalpheus* phylogenetic distances, and UniFrac distances between sponge species calculated using the bacterial community data. Therefore, the only difference between this analysis and the above shrimp-sponge analysis is how the similarity between sponges was depicted.

### *Cophylogenetic results*

We tested for patterns of cophylogeny or congruency between the shrimp phylogeny and either the sponge phylogeny or the similarity of microbial communities. We found a significant pattern of cophylogeny between *Synalpheus* and their host sponges (ParaFitGlobal = 5.627,  $p = 0.011$ ). There is also a significant congruent pattern between the phylogenetic similarity of *Synalpheus* and bacterial phylogenetic communities of their host sponges (ParaFitGlobal = 0.220,  $p = 0.008$ ). Given significant patterns of cophylogeny/congruency, we tested for associations that made important contributions to the overall cophylogeny. ParaFit identified 23 (out of 76) significant associations that contributed to the overall cophylogenetic pattern in the shrimp-sponge analysis and 18 associations that contributed to the overall congruent pattern in the shrimp-bacteria analysis. Nine of these associations are shared in both shrimp-sponge and shrimp-bacteria analyses. Examination of the tanglegrams in Figure S5 and S6 shows that many of these significant associations involved phylogenetically similar shrimps using the same sponge. This suggests that the cophylogenetic pattern is driven by similar shrimps sharing the same sponge, instead of by more similar shrimps using more similar hosts. Therefore, cophylogenetic analysis cannot adequately test our hypothesis that more similar shrimps use sponges host that are more phylogenetic similar, and that have more similar bacterial communities.

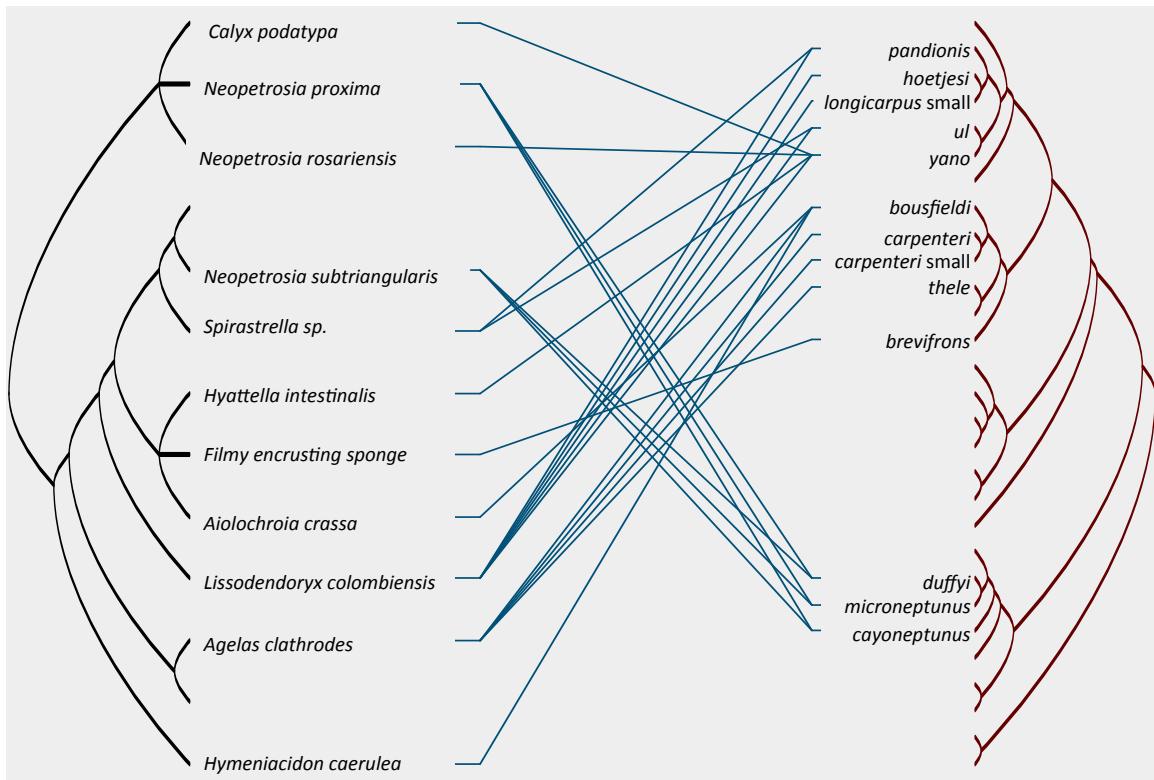


Figure S5. Significant associations in the cophylogenetic patterns between sponge phylogeny (left) and shrimp phylogeny (right).

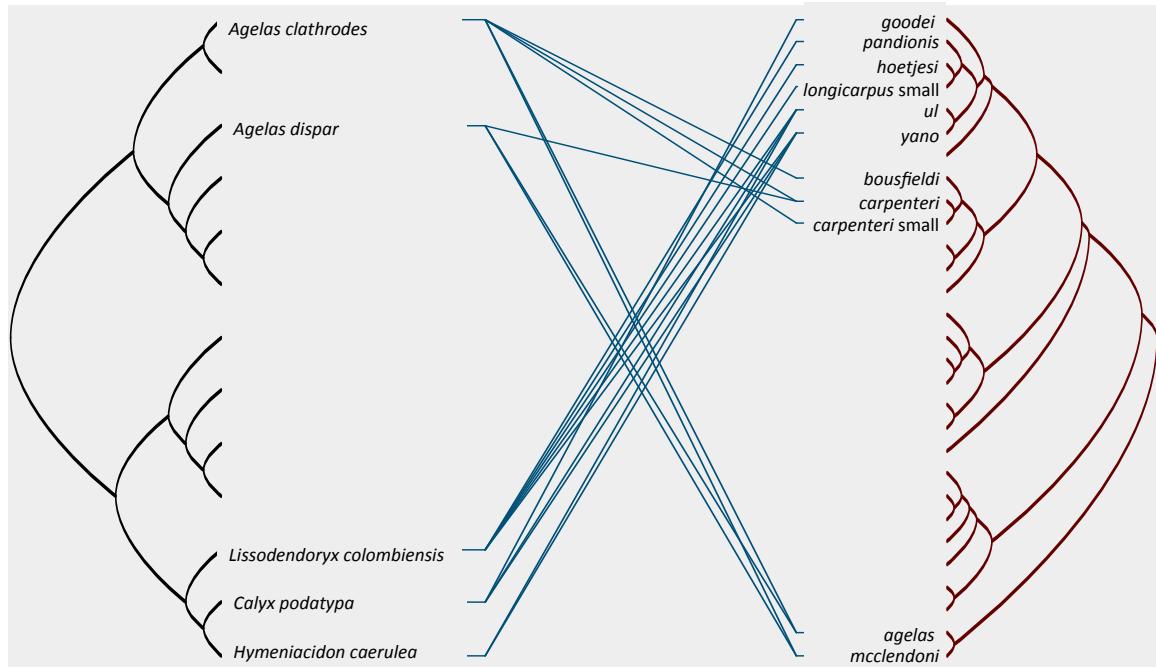


Figure S6. Significant associations in the congruent patterns between bacterial similarity between sponges (left) and shrimp phylogeny (right).

## *Mothur analysis pipeline*

1. Prepare chimera checked files from Acacia
  - Put all XXXX\_all\_tags.fasta file in one folder
2. Merge files and generate groups file
  - mothur > make.group(fasta=sample1.fasta-sample2.fasta-sample3.fasta, groups=A-B-C)
  - mothur > merge.files(input=sample1.fasta-sample2.fasta-sample3.fasta, output=merged.fasta)
  - mothur > count.groups(group=mergegroups)
3. Get unique sequence and generate .names file
  - mother > unique.seqs(fasta=merged.fasta)
    - Output:  
merged.names (728721 seq)  
merged.unique.fasta (254280 seq)
  - Use summary.seqs(fasta=merged.unique.fasta) and count.seqs(name=merged.names, group=mergegroups) to check the number of seq you get.
  - Renames files:  
merged.unique.fasta → trim.fasta,  
merged.names → trim.names, and  
mergegroups → trim.groups
4. Align to SILVA database
  - mothur > align.seqs(fasta=trim.fasta, reference=silva.seed\_v119.align, flip=T, processors=32)
    - Output files:  
trim.align  
trim.align.report,  
trim.flip.accnos
  - mothur > filter.seqs(fasta=trim.align)
  - mothur > summary.seqs(fasta=trim.filter.fasta, name=trim.names)
    - Determine the optimal end point for the use in the next step
  - mothur > screen.seqs(fasta=trim.filter.fasta, name=trim.names, group=trim.groups, minlength=200, maxhomop=8, optimize=start, criteria=90, processors=16)
    - Output files:  
trim.filter.good.fasta  
trim.filter.bad.accnos  
trim.good.names  
trim.good.groups
  - mothur > unique.seqs(fasta=trim.filter.good.fasta, name=trim.good.names)

- Output files:
  - trim.filter.good.names
  - trim.filter.good.unique.fasta

## 5. Precluster & chimera check

- mothur > pre.cluster(fasta=trim.filter.good.unique.fasta, name=trim.filter.good.names, group=trim.good.groups, processors=16)
  - Output files
    - trim.filter.good.unique.precluster.unique.names
    - trim.filter.good.unique.precluster.unique.fasta
    - trim.filter.good.unique.XXXX.precluster.map
- mothur > chimera.uchime(fasta=trim.filter.good.unique.precluster.fasta, name=trim.filter.good.unique.precluster.names, group=trim.good.groups, processors=16)
  - Output files:
    - trim.filter.good.unique.precluster.uchime.chimeras
    - trim.filter.good.unique.precluster.uchime.accnos
- mothur >
 

```
remove.seqs(accnos=trim.filter.good.unique.precluster.uchime.accnos,
fasta=trim.filter.good.unique.precluster.fasta,
name=trim.filter.good.unique.precluster.names, group=trim.good.groups)
```

## 6. OTU based analysis

- mothur > dist.seqs(fasta=final.fasta, cutoff=0.15, processors=16)
- mothur > cluster.split(column=final.dist, name=final.names, large=T, cutoff=0.1, method=*furthest*, processors=16)
  - Output files:
    - final.fn.sabund
    - final.fn.rabund
    - final.fn.list
- mothur > make.shared(list=final.fn.list, group=final.groups, label=unique-0.03-0.05-0.07-0.09)
  - Output file: final.an.shared
- mothur > count.groups(shared=final.fn.shared, group=final.groups)
  - Output files: final.count.summary
- mothur > classify.otu(list=final.fn.list, name=final.names, group=final.groups, taxonomy=final.nr\_v119.wang.taxonomy)
- mothur > clearcut(fasta=final.fn.0.03.rep.filter.fasta, DNA=T)
  - Output file: final.an.0.03.rep.filter.tre

remove.rare

Check if I've removed those within R...

## **CONCLUSIONS**

## **Summary**

In this dissertation, I explored 1) the nature and consequences of reproductive altruism in eusocial *Synalpheus*, 2) the evolution and drivers of different social organizations in this genus, and 3) the drivers of host association between *Synalpheus* and sponges. I showed that eusociality in *Synalpheus* is facultative, meaning that workers retain reproductive capability that is suppressed by the queen (Chapter 1). Such reproductive inequity among females leads to potentially strong competition among females for reproductive opportunities within a colony, and is associated with reduced sexual dimorphism in eusocial *Synalpheus* species (Chapter 2). Further, the two demographically distinct social organizations—communality and eusociality—have evolved via separate evolutionary trajectories and represent alternative social strategies (Chapter 3). These social strategies may have evolved due to different aspects of ecological advantages (Chapter 4). Finally, diversification of *Synalpheus* appears to be more strongly related to the symbiotic microorganisms in sponges, compared with the phylogenetic histories of their sponge hosts (Chapter 5).

## **Broader Context**

Central to studying the sociality in shrimps is to expand our general understanding of animal sociality. In this dissertation, I have made several advances in the understanding of ecology and evolution.

The evolution of an irreversible worker caste is thought to be an important precursor to the evolution of morphological polymorphism of workers in eusocial species (i.e., the presence of specialized worker subcastes) (Oster & Wilson 1979), because sterile workers in ants and higher termites show the most extreme forms of worker morphological and ecological polymorphism (Wilson 1971). In Chapter 1, I found that eusocial shrimp workers retain reproductive totipotency despite signs of morphological specialization. This is analogous with the soldier neoteny in lower termites (Thorne et al. 2003) and the dispersive morph in naked mole-rats (O'Riain et al. 1996). Therefore, the presence of polymorphic reproductive soldiers may represent a natural transition towards obligate eusociality (Thorne et al. 2003, Boomsma 2013).

Sexual dimorphism is typically a result of strong sexual selection on male traits used in male-male competition and subsequent female choice (Thornhill & Alcock 1983, Andersson 1994). However, in social species where reproduction is monopolized by one or a few individuals in a group, selection on secondary sexual characteristics may be strong in both sexes (Hauber & Lacey 2005, Clutton-Brock et al. 2006, Rubenstein & Shen 2009, Young & Bennett 2013). In Chapter 2, I found that eusocial *Synalpheus* species have reduced sexual dimorphism in major chela allometry. This corroborates studies of birds (Rubenstein & Lovette 2009) and mammals (Clutton-Brock et al. 2006) to demonstrate consistent differences in patterns of sexual dimorphism among social species with different forms of altruistic societies.

Social organizations across invertebrate and vertebrate taxa appear to form a continuum based on reproductive skew. This pattern raised the intriguing possibility that

this continuum could be used to understand the evolutionary causes and consequences of sociality (Sherman et al. 1995, Lacey & Sherman 2005). Here, an important assumption is that reproductive skew is indeed a continuous trait, meaning that low skew societies evolved into societies of intermediate and eventually highly skewed eusocial societies. In Chapter 3, I found that high-skew eusocial *Synalpheus* species evolved directly from pair-forming species, whereas intermediate-skew communal species evolved directly from pair-forming species via a separate trajectory. Therefore, assuming a continuous scale of reproductive skew in comparative analyses across *Synalpheus* could be misleading when different levels of skew were a result of convergent evolution.

Recent theoretical assessment proposed that ecological advantages of large groups are sufficient to initiate the evolution of eusociality and that close relatedness between group members is not the cause, but a consequence of eusociality (Wilson & Hölldobler 2005, Nowak et al. 2010). In Chapter 4, I compared the ecological advantages of eusocial *Synalpheus* species with communal species that form large but unrelated groups. I found unique host-related advantages in eusocial, but not communal species. This suggests that the evolution of large groups with kin structure is fundamentally different from that of large groups without kin structure. Therefore, the ecological success of eusocial species may not be achievable without the effect of kinship.

Many ecologically important organisms such as corals and sponges are increasingly being viewed as holobionts that are comprised of the hosts and their symbiotic microorganisms (Margulis 1993, Hentschel et al. 2006, Gordon et al. 2013, Blackall et al. 2015). These microbes can expand the functional repertoire of their hosts (Muscatine 1980, Fiore et al. 2010, Webster & Taylor 2012, Brune 2014), but it is

unclear whether these they can also affect biological interactions between their hosts and other organisms. In Chapter 5, I found that the evolutionary history of *Synalpheus* is well reflected by the similarity between symbiotic bacteria communities in sponges, but not by the sponge phylogeny. Therefore, beyond expanding the functional repertoire of their hosts, microorganisms can also mediate biological interactions of their host at a different trophic level.

## Outlook

A common theme in this dissertation is the use of comparative phylogenetic methods. However, many results are correlational or observational in terms of phylogenetic history. Relating to eusocial colony dynamics, we still lack mechanistic understandings of 1) how queens suppresses reproduction in workers, 2) how new eusocial colonies are established, 3) how and whether workers inherit a colony when the queen dies, and 4) how multiple queens affect relatedness and competition within colonies. Relating to the evolution of social diversity, we still lack a clear understanding of 1) how communal colonies are established, 2) how pair-forming, communal and eusocial species interact to form communities within sponges (but see Hultgren & Duffy 2012), and 3) how and why abbreviated development (the hatching of crawling larvae instead of larvae that disperse by swimming) evolved in eusocial species. Finally, relating to host association, we still need a more complete understanding of how competition, diet, dispersal and evolutionary history of *Synalpheus* species shape their association with sponges and sponge microbes.

Recent advances in studying the evolution of animal sociality are moving towards genomic approaches to understand the genomic mechanisms of altruism and the

consequences of sociality. Similar ventures in snapping shrimps are lagging, but are important because *Synalpheus* is the only marine animal and only crustacean genus known to have evolved eusociality. Initial assessment of genome size among *Synalpheus* species shows strong variability between and within species (Jeffery et al. in press), whereas eusocial species tend to have larger genome sizes (Hultgren et al. in prep). Further investigation should explore how sociality is related to genome size through testing mechanisms of genome changes (Gregory 2011). For instance, the increase in genome size from solitary to eusocial species could involve adaptive changes such as gene duplication and local expansion of introns; whereas changes in genome size between species of the same social organization may involve neutral, non-adaptive changes like genome-wide expansion of introns and transposable elements. These hypotheses can be tested using transcriptomic and high-coverage genome sequencing.

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## **VITA**

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