HOW HERMAPHRODITES AND MALES COEXIST: INSIGHTS FROM MATING SYSTEM AND LIFE HISTORY OF THE ANDRODIOECIOUS BARNACLE CHELONIBIA TESTUDINARIA

by

CHRISTINE EWERS

(Under the Direction of John P. Wares)

ABSTRACT

Androdioecy was first described by Darwin in his seminal work on barnacle diversity; he was the first to identify dwarfed males and large hermaphrodites in the same reproductive population. Despite Darwin’s evidence for androdioecy, it was declared absent from nature in the 1980s, only to later be rediscovered in phylogenetically diverse taxa. Today we realize that androdioecious systems of many plants and animals share astonishing similarities, particularly with regard to their evolutionary history and mating system. Barnacles, however, persist as an oddball with a seemingly different evolutionary trajectory. The present dissertation aimed to clarify the evolutionary dynamics of the androdioecious barnacle Chelonibia testudinaria (Linnaeus, 1758). Combining field assays, laboratory trials and genetic parentage assignment studies, I characterized its mating system and life history. I compare those findings to theoretical expectations and other, non-androdioecious systems, and show that males do not have a relative mating advantage, as expected by mating system theory, but that life history is sufficient to maintain androdioecy. Specifically, high mortality rates and early maturation of males maintains androdioecy in this system.
INDEX WORDS:  dwarf males, hermaphrodites, microsatellite markers, parentage assignment, growth rate, mortality, sex-specific life history
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TESTUDINARIA

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DEDICATION

I am dedicating this dissertation to my mentors: My father, who never got tired of explaining nature to me; my Diplom thesis advisor Dirk Brandis, who showed me the wondrous world of crustaceans; my doctoral advisor John P. Wares, who believed (in) and supported me during all my adventures; and my husband Daniel, who helped me discover my own potential, the river, and adventures in the first place.
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I am still astounded by the large number of people who were willing to help me collect barnacles. They are too numerous to name (see the acknowledgements of each chapter), but Pearse Webster (South Carolina DNR), Mike Arendt (South Carolina DNR), and Matt Ogburn (SERC) stand out for offering their support repeatedly. John Zardus, Dan Rittschof and Keith Crandall were tremendous mentors outside of my committee. It literally took a village to write this dissertation, and I am so grateful to know all its inhabitants!
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Androdioecy

Androdioecy is a sexual system characterized by reproductive populations of males and hermaphrodites. Other sexual systems, also referred to as breeding systems or reproductive systems, are dioecy (separate sexes), hermaphroditism (combined sexes), and gynodioecy (hermaphrodites and females). Androdioecy is the rarest of these four sexual systems (Weeks 2012), and has elicited disbelief and wonder since its discovery (see Darwin quote 1). It occurs in disparate plant and animal systems (Weeks 2012). Where it occurs in animals, it often evolved in several closely related species. Examples are rhabtodid nematodes, including the genetic model organism Caenorhabditis elegans, freshwater shrimp of the genus Eulimnadia and Thoracican barnacles, particularly the genus Scalpellum (Darwin 1851).

(1) “It is so novel a fact, that there should exist in the animal kingdom hermaphrodites, aided in their sexual functions by independent and, as I have called them, Complementing males, that a brief consideration of the evidence [...] will not be useless.” Charles R. Darwin, “Living Cirripedia” Volume 1, 1851
The rarity of androdioecy is matched by its evolutionary improbability. In order for androdioecy to be an evolutionary stable state (ESS), males and hermaphrodites must have equal fitness, despite inherently unequal reproductive capacity, and the general overabundance of male gametes (Bateman 1948). This male disadvantage makes it unlikely for males to compensate for their lack of female reproductive function through higher male reproductive success. Two different fields of theoretical biology aim to explain the evolution of androdioecy: mating system theory (including sex allocation theory), and life history theory. Briefly, mating system theory asks: Which characters should a mating system have to facilitate the maintenance of males and hermaphrodites (Charlesworth and Charlesworth 1978, Charnov et al. 1987, Lloyd 1975, Otto et al. 1993, Wolf and Takebayashi 2004)? Traits under consideration include self-fertilization and inbreeding depression, mating group size and differential mating success of the sexes. I discuss the connection between androdioecy and mating systems in more detail in chapter 2. Life history theory, on the other hand, asks how general life history or sex-specific life history traits facilitate androdioecy. I discuss general life history in chapter 1, and sex-specific life history in chapter 4.

The conditions under which androdioecy should evolve depend greatly on the ancestral state of the system and the evolutionary trajectory. If populations evolved from dioecious ancestors, females were replaced with self-fertilizing hermaphrodites. Hermaphrodites have an advantage over females because they can self-fertilize their eggs, which is particularly advantageous in sparse populations (Pannell 1997). Matings between hermaphrodites are less successful or even impossible, thus allowing males to be maintained as a means of outcrossing. Expectations are very different for systems with hermaphroditic ancestors that were invaded by males. It is hard to imagine a scenario under which males can invade successfully. In theory, small mating groups lead to female-biased hermaphrodites with limited male function. Males
could then invade such a population because hermaphrodites are effectively sperm- or pollen-limited. Sex-specific life history, such as higher survival rates of males, would further increase the relative fitness of males. Most empirical work has focused on populations with dioecious ancestors, and little empirical work has been carried out in androdioecious systems that originated from hermaphrodites. In the present dissertation, I provide empirical insight into an androdioecious population with hermaphroditic ancestors using field assays, laboratory trials and genetic parentage assignment studies.

**Barnacles and the study of sexual systems**

Barnacles allow us to ask broad evolutionary questions regarding sexual systems, mating systems, sexual selection, male dwarfism, phenotypic plasticity and speciation (Darwin 1851). This potential was recognized early by Darwin (1851), who dedicated eight years of his life to the categorization of all living and fossil barnacles (Darwin quote 2).

(2) “I am at work on the second vol. of the Cirripedia, of which creatures I am wonderfully tired: I hate a barnacle as no man ever did before, not even a Sailor in a slow-sailing ship. My first vol. is out: the only part worth looking at is on the sexes of Ibla & Scalpellum; I hope by next summer to have done with my tedious work.” Charles R. Darwin in a letter to W. D. Fox, October 24, 1852

(https://www.darwinproject.ac.uk/letter/entry-1489)

The present dissertation focuses on the evolution of sexual systems, the most interesting aspect of barnacles according to Darwin (Darwin quote 2). Recent observational studies have identified a continuum of sexual systems from purely hermaphroditism over androdioecy to
dioecy (Yusa et al. 2013), which is well-suited to clarify their evolutionary pathway. Barnacles are one of the few taxa that evolved androdioecy from hermaphroditism (Yusa et al. 2012). Androdioecy and dioecy evolved several times independently in the group (Kelly and Sanford 2010, Yusa et al. 2012). Males evolved the same characters every single time: they are dwarfed and attached to larger hermaphrodites (or females in dioecious systems), suggesting their importance. Darwin (1851) also pointed out that hermaphrodites in androdioecious systems have poorly-developed male organs (see Darwin quote 3), indicating that hermaphrodites may be limited in their male function, and males are able to fertilize the majority of eggs.

(3) “Regarding the final cause, both of the simpler case of the separation of the sexes, notwithstanding that the two individuals, after the metamorphosis of the male, become indissolubly united together, and of the much more singular fact of the existence of the Complemental males, I can throw no light; I will only repeat the observation made more than once, that in some of the hermaphrodites, the vesiculae seminalis were small, and that in others the probosciformed penis was unusually short and thin.” Charles R. Darwin, “Living Cirripedia” first vol. 1851

Studying barnacles can be extremely insightful, but has its limitations. Experimental studies are complicated by the complex life cycle of barnacles. Adult barnacles copulate via long penises. Embryos are brooded until free-swimming larvae are released. These larvae spend some time in the water column, dispersing widely, until they settle onto their final substrate. Barnacle larvae of many species have been reared, but getting them to mate with each other, to settle and grow into the adult form remains challenging. A few species settle successfully in experimental
settings. Such settlement experiments are important because they can shed light on the sex determination mechanism in barnacles (Gomez 1975, Svane 1986, Hoeg et al. submitted). Settlement experiments to date are limited to two out of 30 androdioecious species, the stalked barnacle Scalpellum scalpellum and the acorn barnacle Conopea galeatus. These two systems alone have given us great insight into the sex determination of barnacles. In Scalpellum scalpellum, 50% of larvae can metamorphose into males or hermaphrodites, and 50% can only metamorphose into hermaphrodites, suggesting the influence of both genetic and environmental elements (Svane 1975, Hoeg et al. submitted). Sex determination in Conopea galeatus, on the other hand, appears to be under complete genetic control: 25% of larvae become males, the remainder hermaphrodites (Gomez 1975).

Observational studies are often hindered by the fact that many barnacle species occur in inaccessible habitat, such as the deep sea and the polar regions. Adult barnacles are sessile, and are commonly attached to inanimate substrate, plants and animals. Curiously, many androdioecious species occur in the deep sea, or are attached to animals, making it difficult to study them (Buhl-Mortensen and Hoeg 2006, Kelly and Sanford 2010). I chose to study the androdioecious acorn barnacle Chelonibia testudinaria.

The study organism: Chelonibia testudinaria

Chelonibia testudinaria is a marine cosmopolitan epibiont. Adults use diverse marine animals as substratum, including sea turtles, manatees, swimming crabs, and horseshoe crabs. Host-specific morphotypes were previously described as distinct species (Darwin 1854, Hayashi 2013). However, recent molecular analyses indicate that C. testudinaria is a host generalist, and C. patula (Ranzani, 1818) and C. manati (Gruvel, 1903) are now considered synonyms of C.
testudinaria (Cheang et al. 2013, Zardus et al. 2014). Instead of host-specific divergence, genetic lineages are delineated by geographic affinities. The three major lineages are restricted to the Indo-West Pacific (IWP), Tropical Eastern Pacific (TEP) and Atlantic Ocean, respectively (Rawson et al. 2003). These lineages likely represent separate species based on their levels of genetic differentiation (Zardus et al. 2014).

C. testudinaria is androdioecious in the IWP and Atlantic lineages (Cheang et al. 2013, Zardus and Hadfield 2004, Zardus et al. 2014). Its status in the TEP lineage is unknown. The sister species to C. testudinaria sensu lato, Chelonibia caretta, is purely hermaphroditic, and attaches only to sea turtles. This means androdioecy either evolved two to three times independently, or has been maintained for four to five million years, based on the number of base pair substitutions in mitochondrial and nuclear DNA sequence data (Chapter 3). The latter is the more parsimonious explanation. Such longterm-maintenance is suggestive of its evolutionary stability. In this dissertation, I present further evidence that androdioecy is an evolutionary stable state.

I chose C. testudinaria as study organism because in comparison to other androdioecious barnacles, it is easy to collect (Kelly and Sanford 2010). It fouls a number of species that are either of economic or conservation concern, specifically blue crabs, horseshoe crabs and loggerhead sea turtles. I was able to collaborate with a number of organizations that worked on those hosts, and allowed me to collect barnacles from them. The main disadvantage of this species is that the sex determination mechanism is not yet known; where necessary, we assume its sex determination mechanism is genetic (Chapter 5). We do this, on the one hand, because sex determination is genetic in other androdioecious barnacles. On the other hand, most models aimed at explaining androdioecy assume genetic sex determination.
Why study rare phenomena and non-model systems?

Talking to people about the research I do goes something like this: “You study BARNACLES?!?! Are they animals? Andro-what? Males and hermaphrodites? That is odd... But tell me more. I will be thinking about this all afternoon...” What this kind of small talk reveals to me is that I study two things that most people never think about. Androdioecy is rare, and barnacles are, if anything, known as a nuisance. But I think that research on the unusual, the oddballs of nature explores the limits of evolutionary reality, or, to paraphrase Star Trek: “Barnacles / Androdioecy: the final frontier. We aim to explore strange new worlds, to seek out new life [...], to boldly go where no man has gone before.” It is the same reason Charles Darwin sailed around the world in the Beagle; an inherently human sense of curiosity, with the notion that we never know what will come from it. And who knows, maybe, one day, the potential of barnacles has been fully recognized, and children will ask each other, what Darwin's son George asked his acquaintance (Darwin quote 4).

(4) “Where does [your father] do his barnacles?” Charles Darwin’s son, George, talking to an acquaintance

While this scenario seems unlikely (even to me), barnacles make an interesting comparison to plant systems, which they rival in terms of diversity of sexual systems, mating systems, sexual selection, and phenotypic plasticity. Like plants, adult barnacles are sessile but disperse offspring widely. Like most plants, most barnacles are hermaphroditic.

Research on androdioecy will never be common; it is limited by the number of species that exhibit this sexual system, and to the accessibility of those species. Nonetheless, it is a phenomenon that allows us to integrate several biological disciplines, which are often too
complex to be integrated (Stearns 1992). In the present dissertation, I combine, to our best ability, life history evolution, sex allocation theory, mating system studies, and ecology to shift “the final frontier” a little further, to gain a little more insight into the evolution and maintenance of androdioecy.

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CHAPTER 2

GROWTH, MORTALITY, AND MATING GROUP SIZE OF AN ANDRODIOECIOUS BARNACLE: IMPLICATIONS FOR THE EVOLUTION OF DWARF MALES

Abstract

Androdioecy is a mating system characterized by the coexistence of hermaphrodites and males. It has evolved several times independently in ancestrally hermaphroditic barnacles. Life history and sex allocation theory suggest that dwarf males can occur in hermaphroditic populations with very small mating groups, low growth rates and high mortality rates. We tested these predictions in the androdioecious barnacle *Chelonibia testudinaria* (Linnaeus, 1758), an epibiont on loggerhead sea turtles, blue crabs, and horseshoe crabs. The potential number of mates is indeed very small on invertebrate hosts, but larger on sea turtles. Growth rates are host-specific, but comparable to those of other purely hermaphroditic barnacle species. The maximum age of hermaphrodites was less than three years, which is lower than in most purely hermaphroditic species. Our data suggest that small mating groups on some hosts and high mortality on all hosts contribute to the evolutionary origin and persistence of dwarf males.
Most adult barnacles are sessile crustaceans that mate with their neighbors via pseudo-copulation (Charnov 1987, Murata et al. 2001, see Barazandeh et al. 2013 for an exception). Thus, barnacle density and penis reach limit the number of mating partners. According to sex allocation theory, limited mating groups lead to hermaphroditism as an evolutionarily stable system (Charnov 1982, 1987). In concordance with theory, most barnacle species are hermaphroditic.

Androdioecy, a sexual system characterized by the coexistence of hermaphrodites and males, is generally rare (Charlesworth and Charlesworth 1978, Charlesworth 1984, Lloyd 1975, Pannell 1997, 2000, 2002, Weeks 2012). But in acorn and pedunculate barnacles, androdioecy has evolved independently several times from hermaphroditic ancestors (Kelly and Sanford 2010, Yusa et al. 2012). In all androdioecious barnacles, males are dwarfed and attach directly to larger hermaphrodites (Darwin 1851, Klepal 1987, Yusa et al. 2013).

The conditions under which males and hermaphrodites coexist are poorly understood. If males and hermaphrodites have the same life history, males can only continuously coexist with hermaphrodites by fertilizing at least twice as many eggs as hermaphrodites (Lloyd 1975, Charlesworth and Charlesworth 1978). Dwarf males, however, have a different life history than hermaphrodites. Vollrath (1998) suggests that dwarfing in males is associated with early maturation. Early maturation is advantageous in environments with low growth rates and high mortality rates. In such environments, dwarf males and hermaphrodites may have comparable fitness: While dwarf males have a higher probability of reaching maturity and reproducing, hermaphrodites that reach maturity generate offspring through both male and female function (Crisp 1983, Pannell 1997, Yamaguchi et al. 2012, 2013). The ecology of androdioecious
barnacle species lends indirect support for this hypothesis: many androdioecious species are epibionts, and assumed to be short-lived, others are deep-sea species, which are assumed to grow slowly (Kelly and Sanford 2010).

Sex allocation theory proposes that very small mating group size favors androdioecy (Charnov 1987, Urano et al. 2009, Yamaguchi et al. 2008). Hermaphrodites in very small mating groups should invest little resources into the male function as a result of reduced sperm competition between mates. Invading dwarf males should then be able to fertilize a large number of offspring. This theory is supported by a comparative study on pedunculate barnacles in which a positive association between the presence of dwarf males and the proportion of solitary hermaphrodites, a proxy for mating group size, was detected (Yusa et al. 2012). The proportion of solitary hermaphrodites varied from 16 to 100% for androdioecious species (Appendix of Yusa et al. 2012).

To date, empirical data on the life history of androdioecious barnacles are rare (Buhl-Mortensen and Hoeg 2006, Yusa et al. 2012), and growth and mortality rates have not been reported. Our goal was to estimate mating group size, growth and mortality rates of hermaphrodites and males of the androdioecious barnacle *Chelonibia testudinaria* (Linnaeus 1758) (Zardus and Hadfield 2004). *C. testudinaria* is a cosmopolitan epibiont of many marine animals, such as crabs, sea turtles and manatees (Cheang et al. 2013, Zardus et al. 2014).

We expect our results to match predictions of sex allocation and life history theory for androdioecious species. Sex allocation theory suggests that in androdioecious species, mating group size should be very small, and empirical work indicates that a significant proportion of hermaphrodites in androdioecious populations are without neighboring individuals (Urano et al. 2009, Yamaguchi et al. 2008, Yusa et al. 2010, 2012). Following predictions of life history
theory for the evolution of androdioecy, growth rates of hermaphrodites of *C. testudinaria*
should be lower than growth rates of other, purely hermaphroditic, barnacle species, and
mortality rates should be higher.

**Material and methods**

Field collections

Field data on size and number of hermaphrodites and males were used to estimate mating group
size, and growth rates from size frequency distributions, as well as assess age structure by
applying growth rate estimates to size measurements. Barnacles of the species *Chelonibia
testudinaria* (Linnaeus, 1758) were collected from loggerhead sea turtles (*Caretta caretta*
(Linnaeus, 1758)), blue crabs (*Callinectes sapidus* Rathbun, 1896) and horseshoe crabs (*Limulus
polyphemus* (Linnaeus, 1758)) along the US Atlantic coast from Delaware to Florida and in the
Gulf of Mexico along the coast of Mississippi between 2011 and 2013. All barnacles attached to
blue crab and horseshoe crab hosts were removed, counted and measured. In addition, host
carapace length and width without spines or tail was measured. For loggerhead sea turtle hosts,
all barnacles of a haphazardly chosen 10x10 cm square area of the carapace were removed,
counted and measured.

Barnacle size was measured as maximal rostro-carinal diameter (Figure 2.1) with calipers
to the nearest 100 μm. Barnacles with highly irregular shell shape were excluded from
measurement. All statistical analyses were carried out in the R environment (R Development
Core Team 2014).
Mating group size

Mating group size is defined as the number of hermaphrodites a hermaphrodite actually mates with (Charnov 1987). Therefore, mating group size can only be investigated by direct observations of copulation activity, or inferred by parentage assignment studies. Since these approaches are beyond the scope of the present study, we report the potential mating group size, defined by us as the number of hermaphrodites in mutual mating distance, i.e. within penis reach. This measure provides an upper limit for the number of mates a hermaphrodite can have. Similar estimates based on barnacle density have previously been used to approximate mating group size in barnacles (Kelly and Sanford 2010, Raimondi and Martin 1991). To calculate potential mating group size, we considered the area a large hermaphrodite can reach with its penis. The unextended penis is up to 25 mm long in large hermaphrodites of \textit{C. testudinaria} (CES, pers. obs.). Crisp (1983) noted that the penis of \textit{C. testudinaria} can probably double in length when extended. This means large hermaphrodites should be able to mate with individuals 50 mm away in any direction. This includes approximately an area of $\pi \times 50^2$ mm$^2$ or 7853 mm$^2$, and we report the number of hermaphrodites in this area as measure of potential mating group size. In addition, we counted the number of males per hermaphrodite, which may allow insights into mating system structure (Buhl-Mortensen and Hoeg 2006). To compare our results with previous studies (Yusa et al. 2012), we calculated the proportion of hosts which were fouled by a single hermaphrodite. Yusa et al. (2012) does not distinguish between hermaphrodites with males or without males, and neither do we.

For blue crabs and horseshoe crabs carrying \textit{C. testudinaria}, all individuals per host were counted. We calculated host carapace size by approximating the carapaces as arithmetic shapes, for which area calculations are simple: the blue crab carapace is rhomboid-shaped, while the
horseshoe crab carapace approximates an oval. Using the number of hermaphrodites per host, and the size of the host, we were able to calculate the potential mating group size. For hermaphrodites from loggerhead sea turtles, all hermaphrodites of a haphazardly chosen 10x10 cm square area of the carapace were counted, and the number of hermaphrodites in mutual mating distance calculated. We acknowledge that this approach is not ideal: barnacles are patchily distributed on the turtle shell (Hayashi and Tsuji 2008, Pfaller et al. 2008), and extrapolating from a single quadrant is adding uncertainty to our estimates. However, most loggerhead turtles in this study were heavily fouled by *C. testudinaria*, and we wanted to minimize the stress for the turtle. We assessed host effects with the non-parametric Kruskal-Wallis rank sum test for both the number of hermaphrodites in a mating group and the number of males per hermaphrodite.

Growth rates: Laboratory observational trials

Laboratory trials were conducted at the Duke Marine Laboratory in Beaufort, NC, USA, from April to July 2013, and continued at the University of Georgia, Athens, GA, USA, until August 2013. We conducted two sets of laboratory trials: observational trials with *ad libitum* food, and trials in running, single pass, sand filtered sea water.

For the trials with *ad libitum* food, we removed 39 hermaphroditic *C. testudinaria* from two loggerhead sea turtles. Two of the hermaphrodites from one of the turtles had each two males attached. The males died within the first two weeks of the study, and we did not estimate their growth. The loggerhead sea turtles had stranded on the North Carolina coast, and were examined by veterinarians after their rescue. At this point in time, we were able to remove barnacles from the turtle carapace with a small knife. Inadvertently, some of the turtle shell
remained on the underside of the barnacle, and likely facilitated survival of the barnacles. We also purchased six freshly shed blue crab carapaces with a total of 38 hermaphroditic barnacles, but no males. The majority of these carapaces were from females that overwintered and were experiencing their terminal molt in the spring. All barnacles were kept in sand filtered sea water with air bubblers and without water flow and fed \textit{ad libitum} daily with freshly hatched \textit{Artemia spp.} larvae. Water was exchanged twice a week.

For the laboratory trials in running sea water, seven live blue crabs with a total of 21 hermaphroditic \textit{C. testudinaria} were purchased from a local fisherman, and an additional live horseshoe crab with 11 hermaphroditic \textit{C. testudinaria} was collected at night during a full moon in the Radio Island Channel, Beaufort, NC. Blue crabs were placed in buckets in running sea water. The sea water contained most of the phyto- and zooplankton as food sources for barnacles. Horseshoe crabs were kept in a large tank in sand-filtered sea water at ambient temperature. All blue crabs and horseshoe crabs were fed \textit{ad libitum} every two days with fresh fish. No additional food was provided to the barnacles.

In both sets of trials, maximum rostro-carinal diameter of each barnacle was measured with calipers to the closest 100 \( \mu \text{m} \) approximately every two weeks. Linear growth rates were calculated as the difference between initial size and final size, divided by the number of days of laboratory rearing. We assessed host effects with non-parametric Kruskal-Wallis rank sum tests.

\textbf{Growth rates: Mesocosm observational trials}

\textit{Mesocosm} observational trials were conducted at the Duke Marine Laboratory from April to July, 2013. Five live blue crabs with a total of 11 hermaphroditic barnacles were purchased. Blue crabs were placed in minnow traps, of which the bottom part had been buried
into the intertidal zone as in Dickinson et al. (2006). Blue crabs survived air exposure for several hours, and none of the crabs died or molted during our trials. The crabs were fed every two days with fresh fish. No additional food was provided to the barnacles. Maximum rostro-carinal length of each barnacle was measured with calipers to the nearest 100 μm approximately every two weeks. Linear growth rates were calculated as the difference between initial size and final size, divided by the number of days from the start of the observational trial.

Growth rates: Size frequency distribution in natural populations

To calculate growth rates based on size frequency distribution of natural populations, we made two assumptions: recruitment is seasonal and growth is indeterminate. Seasonally limited recruitment and indeterminate growth are common in marine invertebrates (McDougall 1943, Todd and Doyle 1981). Under these assumptions, distinct size classes are present, reflecting different age classes. Preliminary data showed that there is considerable variance in size of barnacles from different host individuals, even among barnacles collected at the same date and location. This means that population-wide size comparisons, as carried out for other barnacles (Hines 1979, Moore 1935, Southward and Southward 1967), would not be successful. We therefore considered the host individual as the appropriate unit for size frequency distributions. This approach should also help control for differences in growth rates caused by different host behavior and ecology.

The size frequency distribution of each age class should approximate a normal distribution, and the overall size distribution should be multimodal in the presence of several age classes. Further preliminary analyses showed that only a few large individuals were present on any host, which makes it difficult to establish multimodality. Nonetheless, the few larger
individuals should skew the overall size frequency distribution and lead to significant deviations from the normal distribution.

To determine whether more than one size class was present on a host individual, we tested whether the size frequency distribution of barnacles of the same sex was significantly different from a normal distribution, using the chi-square test. We also carried out skewness tests that assess whether the data distribution is asymmetrical. In the presence of two or more size classes, the size distribution should have a longer tail, reflecting the presence of a larger size class with few individuals. In addition, we carried out dip tests for unimodality (Hartigan and Hartigan 1985). If the null hypothesis of unimodality is rejected, more than one size class is present.

The size distribution data of barnacles from any host that deviated significantly from the normal distribution, were skewed or did not conform to unimodality, had the highest potential of containing two or more size classes. These size distributions were plotted in a histogram and examined visually for the presence of distinct size classes. We considered two or more distinct size classes to be present when a host was fouled by several small and several large barnacles, but not by medium sized barnacles. The minimal gap that indicated the absence of medium-sized individuals was chosen to be 2 mm, which exceeds the lowest annual growth known for barnacles (Chthamalus dalli; Southward and Southward 1967). Thus we assumed that barnacles had to be of two different year classes if they were separated by more than two millimeters in size.

The visual inspection also clarified the number of size classes present on a host. We took the median of each distinct size class per host individual, and subtracted the median of the smaller size classes from the median of the larger size classes. We divided this difference by
364.25 days (1 sidereal year) to calculate linear daily growth rates. We assessed host effects with non-parametric Kruskal-Wallis rank sum tests.

Growth rates: Capture-recapture analysis

The South Carolina Department of Natural Resources manages a random-sampling, regional (33.1°N to 29.9°N) trawl survey to monitor, in conjunction with numerous collaborators, temporal changes in sea turtle catch rates, spatial distribution patterns, demographic structure, health, and foraging ecology (Arendt et al. 2012a, b, Deem et al. 2009, O'Connell et al. 2010). During the survey, pictures are taken of each turtle caught. Occasionally, turtles are recaptured in the same or subsequent year. We used the picture archive to compare turtle pictures from these capture-recapture events. We identified individual hermaphroditic barnacles on five turtles that were present in both a capture and a recapture picture. Of these turtles, three were recaptured within the same season and two turtles were recaptured in the subsequent year. We only used barnacles that were unambiguously identified in both pictures, those barnacles either being solitary on the turtle's carapace or attached to the head of the turtle. Males were too small to be identified from the pictures. We measured maximal rostro-carinal diameter with the software pixelstick v2.7 (Miller 2014). A board identifying the turtle ID present in all pictures was used for size calibration. Linear growth rates of barnacles were calculated as the difference between initial size and final size, divided by the number of days between capture events.
Growth rate comparisons

Growth rates of hermaphrodites may differ depending on host species (loggerhead sea turtle, blue crab, or horseshoe crab), method (laboratory trial with ad libitum food, laboratory trial under running sea water, mesocosm trial, size frequency analysis, or capture-recapture analysis) and barnacle sex (hermaphrodite or male). Not all methods were applied to barnacles from all host species: the mesocosm and running sea water trials were applied to barnacles from blue crabs only, while capture-recapture data were limited to barnacles from loggerhead sea turtles.

Growth rates estimated from barnacles on different hosts and growth rates estimated with different methods were inspected for normality before analyses. We carried out a two-way ANOVA with host species, method, and interaction term as explanatory variables. To assess the effect of different methods on growth rate estimates for barnacles on the same host species, we carried out Kruskal-Wallis rank sum tests. Wilcoxon rank sum tests were used post hoc to determine which host pairs were significantly different.

Age structure and mortality rate

The age of C. testudinaria populations was estimated for all barnacles collected in the field assays between April and August from 2011 to 2013, a time when data for all host species were available. We calculated age in days by dividing the size of field collected individuals by a host-specific median daily growth rate. The median growth rate was the median of all individual growth rate estimates obtained during the first part of this study. If a method yielded growth rate estimates that differed significantly from estimates taken under the most natural conditions, we excluded these divergent estimates. The methods can be ordered as follows from representing the
most natural conditions to the least natural conditions: size frequency distribution and capture-recapture analysis, mesocosm trials, laboratory trials under running sea water, and laboratory trials with \textit{ad libitum} food. Kruskal-Wallis rank sum tests compared median age of barnacles from different hosts. Wilcoxon rank sum tests were used post hoc to determine which host pairs were significantly different.

Too few growth rate estimates were obtained for males of \textit{C. testudinaria} to be accurate. Instead, we applied our estimates of the hermaphroditic growth rates to male size data as described for the hermaphroditic age estimation.

We used Hoenig's equation (Hoenig 1983) to calculate instantaneous annual mortality rates. He showed that the mortality rate (M) and the maximum age (t\text{max}) of several marine taxa are significantly correlated in the following way: \( M = \exp(1.44 - 0.982 * \ln(t_{\text{max}})) \). Thus, a low maximum age is the result of a high mortality rate. We used the highest observed age for hermaphrodites or males as \( t_{\text{max}} \).

Literature search

We searched the literature for information on growth rates, mortality rates, and maximal age of acorn barnacle species. Pedunculate species were not included in the analyses because it is not clear which size measurement is homologous to the maximal rostro-carinal diameter of acorn barnacles. We used combinations of the search terms “growth”, “mortality”, “age”, “barnacle”, and “cirripedia” in an online Google Scholar search (http://scholar.google.com, last access June 30, 2014), and also searched the reference lists in publications we found through the online search. As was noted above, barnacle growth rates can decrease with age, size, or the onset of
maturation (Crisp and Bourget 1985); therefore, we report growth rate estimates from the linear, pre-maturation phase of barnacle growth.

If only maximum age of a population was reported, we calculated instantaneous annual mortality rates from maximal age using the equation of Hoenig (1983).

Results

We collected and measured 2228 hermaphrodite and 998 male *C. testudinaria* from 40 loggerhead sea turtles, 109 horseshoe crabs and 159 blue crabs in field assays (Table 2.1). The maximal rostro-carinal diameter of hermaphrodites ranged from 0.5 to 60 mm, and the diameter of males ranged from 1 to 22 mm.

Mating group size

The median number of hermaphrodites in the potential mating group size area (2500π mm²) was significantly different between hermaphrodites from different host species (Kruskal-Wallis, p-value < 0.0001). It was 8.64 for loggerhead sea turtles, 0.28 for horseshoe crabs, and 4.55 for blue crabs (Figure 2.2A-C). The proportion of solitary hermaphrodites was 17.6% on blue crabs, 32.7% on horseshoe crabs and 0% on loggerhead sea turtles.

Males were found on 8.5% of hermaphrodites. The number of males per hermaphrodite did not differ significantly between host species (Kruskal-Wallis, p-value = 0.1647). Of hermaphrodites with males, 43.5% had one male attached, and the median was two males. The maximum number of males per hermaphrodite was 25 (Figure 2.2D).

Growth rate estimates
We obtained 148 growth rate estimates for hermaphrodites of *C. testudinaria*, 48 being based on size frequency distributions (field observations) and 100 on repeated measurements of individual barnacles (see Figure 2.3 for host- and method-specific sample sizes). We estimated five growth rates for males from size frequency distributions. Analysis of the hermaphroditic growth data by a two-way ANOVA with the growth estimation method and the host species as effects showed that host species was not significant (p-value = 0.5421), but method and interaction term were (method: p-value < 0.0001 and method-host interaction term: p-value = 0.0052). Because of the significance of the interaction term, we evaluated methods and hosts separately.

In the laboratory observational trial with *ad libitum* food, hermaphrodites from loggerheads and blue crabs did not grow at significantly different rates (Kruskal-Wallis, p-value = 0.21). Barnacles from loggerhead sea turtles had a growth rate of 60 ± 74 μm/day (SD), and those from blue crabs 81 ± 65 μm/day (SD) (Figure 2.3).

Growth rates for hermaphrodites from blue crabs and horseshoe crabs kept in running seawater were also similar (Kruskal-Wallis, p-value = 0.076). Hermaphrodites from blue crabs had a growth rate of 48 ± 29 μm/day (SD), and those from horseshoe crabs 26 ± 35 μm/day (SD) (Figure 2.3).

Of all collected host individuals, 16% had significant evidence for the presence of hermaphrodites from two or more size classes. Growth rate estimates based on these size-frequency distributions differed between barnacles from different hosts (Kruskal-Wallis, p-value < 0.0001). In particular, blue crab barnacles had a lower estimated growth rate of 30 ± 11 μm/day (SD) than those of loggerhead sea turtles, 60 ± 31 μm/day (SD), and horseshoe crabs, 61 ± 25 μm/day (SD) (Figure 2.3).
Of the 308 host individuals, 125 were fouled by males in addition to hermaphrodites. Of hosts with males, only five hosts had males with significant signs of more than one size class (4% of host individuals with males). Two of these hosts were loggerhead sea turtles, and we calculated male growth rates of 27 and 71 μm/day on them. Two hosts were blue crabs, with growth rates of 25 μm/day for both males on them. One host was a horseshoe crab, and the growth rate of that male barnacle was 36 μm/day. These data are too sparse for statistical comparison between host species, or with hermaphrodites of the same species, or with other species. Nonetheless, these growth rate estimates for males are within the range of those for hermaphrodites of *C. testudinaria*.

Growth rates were not method-specific for hermaphrodites from horseshoe crabs or loggerhead sea turtles (Kruskal-Wallis, p-value = 0.1077 for horseshoe crabs, and p-value = 0.1354 for loggerhead sea turtles), but were method-specific for barnacles on blue crabs (Kruskal-Wallis, p-value < 0.0001) (Figure 2.3). Growth rates calculated from size frequency distributions or mesocosm trials were lower than rates estimated from laboratory trials.

Repeating the ANOVA, this time excluding the growth rate estimates for blue crab barnacles obtained under laboratory conditions (as these appear to be divergent), hermaphrodites grew at host-specific rates (p-value < 0.0001). Hermaphrodites from loggerhead sea turtles grew significantly faster than hermaphrodites from blue crabs and horseshoe crabs. Table 2.2 summarizes the growth rate estimates for hermaphrodites from each host species used to calculate age structure.
Age structure and mortality rate

The median growth rate for barnacles from horseshoe crabs and loggerhead sea turtles was calculated from all growth rate estimates for the respective host. For barnacles from blue crabs, we used the median growth rate of estimates from size frequency distributions and mesocosm observational trials, as these most closely resemble natural conditions.

The median age of hermaphrodites was host-dependent (Kruskal-Wallis, p-value < 0.0001). Hermaphrodites from horseshoe crabs were significantly younger than hermaphrodites from loggerhead sea turtles and blue crabs (Figure 2.4A). The maximum age was 1000 days (2.75 years), but the median age was less than one year for hermaphrodites from all hosts (Table 2.2).

Male age was also host-dependent (Kruskal-Wallis, p-value < 0.0001) (Figure 2.4B). Males from blue crabs were oldest (median age 74 days), while the median age of males from horseshoe crabs was 36 days, and the median age of males from loggerhead sea turtles was 37 days (Table 2.2). The oldest male was an estimated 342 days old.

From the maximal age, we calculated an instantaneous mortality rate of 1.43 per year for hermaphrodites and 4.22 per year for males.

Literature search

We found 13 publications that reported growth rates and/or a maximum age for 21 hermaphroditic barnacle species, and one publication reporting growth rates from recently settled hermaphrodites of our study organism, the androdioecious C. testudinaria (Table 2.3). Mortality rates for barnacles were rarely reported (but see Hines 1979, Shkedy et al. 1995). Of the 15 reported maximum age estimates for hermaphroditic species, only Austromegabalanus
and Cantellius palidus had shorter maximal lifespans than hermaphroditic C. testudinaria, and no species had a shorter life span than male C. testudinaria. We calculated mortality rates from maximum age estimates (Hoenig 1983), and consequently the pattern of mortality rates was the same as the pattern of maximal age.

Growth rate estimates were considered not different from growth rates of C. testudinaria when the respective ranges overlapped. This was the case for ten of 14 species. Two barnacle species had higher reported growth rates than those for C. testudinaria in this study. Only Chthamalus dalli and Chthamalus fissus had lower growth rates than C. testudinaria. Recently settled specimens of C. testudinaria also had higher growth rates than those reported here (Sloan et al. 2014).

Discussion

Androdioecy, a sexual system characterized by the coexistence of hermaphrodites and males, has evolved repeatedly in barnacles. From sex allocation and life history theory, low numbers of mates, low growth rates, and high mortality rates should favor the evolution and maintenance of dwarf males in these systems (Charnov 1987, Urano et al. 2009, Yamaguchi et al. 2013). We found that mating groups of the epizoic androdioecious barnacle Chelonibia testudinaria are small on two host species, blue crabs and horseshoe crabs, but larger on loggerhead sea turtles. While mating groups are larger on loggerheads than on the other two hosts, we found that the proportion of males on these hermaphrodites does not differ between hosts. Growth rates of both hermaphrodites and males of C. testudinaria prove to be comparable to growth rates of purely hermaphroditic barnacle species, but the maximum age attained by this
species is lower than in most hermaphroditic barnacles: males do not live longer than one year, while hermaphrodites survive up to three years.

Implications for the evolution of androdioecy

Our findings suggest that small mating group size and high mortality played a role in the evolution and maintenance of androdioecy; in the present species these parameters differ from those of purely hermaphroditic barnacle species. This kind of comparison is a good starting point for understanding the conditions under which males can evolve and be maintained in hermaphroditic populations. Further studies should investigate growth, mortality, and mating group size in other androdioecious species. Androdioecious barnacles are commonly either epibiotic or deep-sea species (Kelly and Sanford 2010). We investigated an epibiotic species, and found mortality rates to differ from purely hermaphroditic species. Could deep-sea species have evolved androdioecy in response to low growth rates instead of high mortality? Comparisons between species of different habitats should be particularly elucidating.

It is evident that each host species provides a distinct environment for *C. testudinaria* with respect to mating group size and growth. Given the apparent absence of local adaptation (Cheang et al. 2013, Zardus et al. 2014), *C. testudinaria* should adopt a life history strategy that balances the requirements of each host and maximizes overall fitness. This balancing act may have led to the current situation, in which males and hermaphrodites coexist.

Differences in mating group size

Two different patterns of mating group size are evident among the three host species. On long-lived loggerheads, densities of hermaphrodites are very high, and we, like Caine (1986), did
not find any turtle with solitary hermaphrodites. On the shorter-lived invertebrate hosts, up to 33% of fouled hosts carried only one hermaphrodite, comparable to previous estimates for *C. testudinaria* on blue crabs (Key et al. 1997). Invertebrate hosts thus match empirical data of other androdioecious barnacle species (Yusa et al. 2012), and fulfill sex allocation's prediction of very low mating group size. *C. testudinaria* on loggerhead sea turtles show a pattern more typical of a hermaphroditic barnacle species.

Our potential mating group estimates provide an upper limit for mating group size for *C. testudinaria*. The actual number of mates for a given individual is not known, and can only be unveiled by direct mating observations or parentage assignment studies. This distinction is important because the theoretical model is based on the actual number of mates (Charnov 1987). Direct observations have shown that some barnacle species mate with multiple partners, while others restrict mating to a single individual (Anderson 1994, Charnov 1982, Murata et al. 2001). Parentage assignment studies confirm this pattern. Independent of density, the intertidal barnacle *Tetraclita rubescens* has rarely more than two mates (Kelly et al. 2012). In the stalked barnacle *Pollicipes elegans*, density of barnacles and mating group size are positively correlated, and multiple matings are common (Plough et al. 2014). We may be able to indirectly infer mating group size using sex allocation theory. Sex allocation theory predicts that male sex allocation of hermaphrodites should scale positively with the number of mates (Charnov 1982). If male sex allocation does not respond to barnacle density, we can infer that having more neighbors does not lead to more matings, and actual mating groups are small. *Tetraclita rubescens*, the only species with both estimates of actual and potential mating group size, follows this expectation (Kelly and Sanford 2010, Kelly et al. 2012). Assuming this framework to be valid, the acorn barnacles *Balanus glandula* and *Semibalanus balanoides* appear to have low numbers of mates.
(Hoch and Levinton 2012). In another species, *Catomerus polymerus*, mating group size scales with barnacle density, suggesting a large number of mates. The determinants of mating group size in barnacles are unknown, and direct observations or parentage assignment studies of *C. testudinaria* are needed to estimate actual mating group size.

A median of two males, but up to 25 males, settled on individual hermaphrodites. It is not clear how and whether this many males reproduce with the hermaphrodite they are attached to. The number of males per hermaphrodite reported for other androdioecious barnacles range from 0 to 6 (Buhl-Mortensen and Hoeg 2006, Yusa et al. 2010), similar to our findings. Does this range represent the evolutionarily stable number of males per hermaphrodite? This question has not received theoretical treatment. The answer will surely depend on the mode of sex determination in barnacles. Crisp (1983) suggests plastic sex determination for *C. testudinaria*, but sex determination in two other androdioecious barnacle species is, at least partially, genetic (Gomez 1975, Svane 1986). Further theoretical and empirical studies on the number of males per hermaphrodite and sex determination in barnacles should advance our understanding on the evolution and maintenance of androdioecy.

Age structure and mortality of *C. testudinaria*

Longevity of epibionts depends on host behavior and host survival. Our age estimates suggest that hermaphrodites of *C. testudinaria* can reach an age of almost three years, but that most barnacles live less than a year. Does this finding match estimates of host age and molting behavior?

On juvenile blue crabs and juvenile horseshoe crabs, frequent molting prevents the establishment of epibiotic communities altogether. Female blue crabs, on the other hand,
undergo a terminal molt to maturity after which they do not molt anymore, and larger male blue crabs molt less frequently. Adult blue crabs live on average six to twelve months, but sometimes reach three years (Abbe 1974, Darnell et al. 2009, Havens and McConaugha 1990, Ju et al. 1999, Millikin and Williams 1984). Horseshoe crabs take 11 years to mature, have a terminal molt, and live for several years afterwards (Ropes 1961, Shuster 1950, but see Carmichael et al. 2003). Loggerhead sea turtles live several decades (Parham and Zug 1997), and slough their carapace scutes throughout their lifetime, but the exact timing of sloughing is unknown.

Maximum life span of barnacles on loggerhead sea turtles is determined by the frequency of scute sloughing, active removal of epibionts or the negative effects of other epibiotas (Frick et al. 2000, Schofield et al. 2007). An independent estimate of epibionta longevity is provided by the time that satellite tracking devices stay attached to loggerhead sea turtles. Recent studies found that tracking devices attached to juvenile loggerhead sea turtles record between a month and almost two years, and on average around 200 days (Arendt et al. 2012c, McClellan and Read 2007).

In conclusion, host ecology indicates that epibionta may survive between six months and three years on their hosts. These estimates coincide well with our age estimates for *C. testudinaria* hermaphrodites.

Males are generally younger than hermaphrodites when assuming equal growth rates for both sexes. This is expected because males attach to hermaphrodites that have already settled on a host. Our assumption that males and hermaphrodites grow at the same rate was supported by the growth rates we obtained for males. We cannot exclude the possibility that males stop growing after an initial growth period (e.g., upon reaching maturity). If so, we have underestimated the age of males. Crisp (1983) suggests that males of *C. testudinaria* grow slower
than hermaphrodites due to space and food limitations present on the hermaphrodites’ shells. Only studies directly estimating growth rates throughout the lifetime of males will clarify this issue.

Differences in growth rates of *C. testudinaria*

This study cross-validates several independent methods of growth rate estimation, giving valuable insight into the growth pattern of *C. testudinaria*. In general, different methods for estimating growth rates on the same host gave comparable results. Among the three host species, growth rates for attached hermaphroditic barnacles calculated by different methods were only significantly different for barnacles from blue crabs, for which estimates based on size frequency distributions were significantly lower than rates observed in laboratory trials. This either suggests the laboratory conditions provide better growing conditions than natural conditions for *C. testudinaria* attached to blue crabs. It is also possible that estimates from size-frequency distributions are lower because these estimates include the colder winter season, when ectotherms have reduced growth rates. This is less likely because we did not find lower growth rates in barnacles from the other hosts (Figure 2.3).

*C. testudinaria* grew at host-specific rates under natural conditions. But when kept under the same conditions (laboratory observational trial), those isolated from different hosts grew at the same rate. This highlights the plasticity of growth rates as well as the influence of the host. In particular, host movement and host behavior could influence growth rates. It has been shown that many barnacles grow faster when they are exposed to swifter water currents, as such currents deliver food particles faster (Crisp 1960, Crisp and Southward 1961). In epibiotic species, the host largely determines current velocity. Loggerhead sea turtles move up to 20-40 km/day
(Abecassis et al. 2013, Foley et al. 2013, Hart et al. 2010, Renaud & Carpenter 1994), mature blue crabs on average five km/day (Carr et al. 2004, Wrona 2004), and horseshoe crabs two to six km/day (Watson and Chabot 2010). This suggests that barnacles on loggerhead turtles should grow fastest, in concordance with our findings.

In most species of barnacles, growth rates decelerate with size, age, maturity, or decreasing temperatures (Anderson 1994, Crisp and Bourget 1985). If this were the case for *C. testudinaria*, using individuals of different size to estimate growth rates would bias our growth rate estimates. However, growth rates that included the cold season (size-frequency distribution) did not differ from growth rates obtained in the warm season only (mesocosm trials). This suggests that growth rates of hermaphroditic *C. testudinaria* in the size range we investigated do not change seasonally. This seems surprising, but could explain the relatively large size of *C. testudinaria* hermaphrodites (CES, pers. obs.) in comparison to those of many other barnacle species. At least some large acorn barnacles, e.g. *Balanus nubilis, Austromegabalanus psittacus* and *C. testudinaria*, are sublittoral, thus able to feed constantly and may be able to grow at a relatively constant rate, independent of reproductive status or size.

Growth rates were much higher in another study of *C. testudinaria* (Sloan et al. 2014). There are several possible reasons for this discordance: Sloan et al. (2014) only reported on the first 6-36 days after settlement. In addition, the barnacles were attached to inanimate substrata and some of the sites were offshore, where strong currents could have increased growth rates. This curious discordance should be investigated further by following the growth of *C. testudinaria* throughout its life span. If *C. testudinaria* grows faster in the first days to months after settlement, we overestimated the age of *C. testudinaria* by applying our lower growth rates.
to size data. This does not change our conclusions for the evolution of dwarf males; it rather strengthens them by suggesting a shorter life span than the one we estimated.

Conclusion

The identification of causes for the evolution of life history patterns often relies on observational correlations, rather than manipulative experiments. These correlations only suggest the importance of certain factors, but cannot prove them. This is also the case in the present study. We cannot say with certainty what has led to the evolution of androdioecy in *C. testudinaria*. The data at hand suggest that of the investigated factors, small mating group size on some hosts and high mortality on all hosts are more likely to have favored the evolution of androdioecy than low growth rates. Such claims can become more powerful if they hold in several, independently evolved cases. Barnacles provide an ideal scenario for such comparative studies because androdioecy evolved several times independently in the group (Kelly and Sanford 2010, Yusa et al. 2012). Future studies should assess the relative importance of growth, mortality and mating group size in other androdioecious barnacle species, particularly paying attention to differences between epibiotic and deep-sea species. It may be that very different habitats select for the same reproductive strategy: the coexistence of males and hermaphrodites.

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Author contributions

CES wrote the manuscript and conducted field collections, measurements, and statistical analysis. MDA provided the capture-recapture pictures, and supported field collections and manuscript preparation. JPW helped design the observational trial, provided laboratory space, and supported field collections and manuscript preparation. DR was integral to the development of the experimental design, provided laboratory space, and supported field collections.

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Yamaguchi, S., Y. Yusa, K. Sawada and S. Takahashi. 2013. Sexual systems and dwarf males in
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Yamaguchi, S., Y. Yusa, S. Yamato, S. Urano and S. Takahashi. 2008. Mating group size and
evolutionarily stable pattern of sexuality in barnacles. Journal of Theoretical Biology
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Yusa, Y., M. Takemura, K. Miyazaki, T. Watanabe and S. Yamato. 2010. Dwarf males of
Octolasmis warwickii (Cirripedia: Thoracica): The first example of coexistence of
males and hermaphrodites in the suborder Lepadomorpha. The Biological Bulletin
218: 259-265.

sexual systems in barnacles. Integrative and Comparative Biology 53: 701-712.


Tables

Table 2.1. Summary of specimen collections, including sampling locations and years, number of *Chelonibia testudinaria* specimens and hosts collected, and organizations supporting field collections. The data only include barnacles collected from April to August of each year. These collections were used to assess mating group size, estimate growth rates based on size frequency distributions and age structure. The following organizations supported barnacle collections:

NCDNR: North Carolina Department of Natural Resources, NC, USA; MRD: MRD In-Water Sea Turtle Research, South Carolina Department of Natural Resources, SC, USA; CRP: Caretta Research Project, GA, USA; MSU: Mississippi Southern University, MS, USA; UGAMI: University of Georgia Marine Institute, GA, USA; UDEL: University of Delaware, DE, USA; LML: Loggerhead MarineLife Center, FL, USA; SERC: Smithsonian Southeast Ecological Research Center, VA, USA; DUKE: Duke Nicholas School of Environment, NC, USA; SEAMAP: SEAMAP, South Carolina Department of Natural Resources, SC, USA; BMC: Larry DeLancey, South Carolina Department of Natural Resources, SC, USA.

<table>
<thead>
<tr>
<th>Host species</th>
<th># host individuals</th>
<th># hermaphrodites</th>
<th># males</th>
<th>Years</th>
<th>US states</th>
<th>Organizations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loggerhead sea turtles</td>
<td>40</td>
<td>538</td>
<td>265</td>
<td>2012, 2013</td>
<td>GA, SC,  NC</td>
<td>NCDNR, MRD, CRP</td>
</tr>
<tr>
<td>Total</td>
<td>308</td>
<td>2228</td>
<td>998</td>
<td>2011-2013</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Linear growth rate estimates of maximal rostro-carinal basal diameter, size and age structure for *Chelonibia testudinaria* hermaphrodites and males from different hosts. Error is reported as standard deviation. The growth rate estimate for hermaphrodites from blue crabs is based on results from size frequency distributions and mesocosm observational trials.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Hermaphrodites</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median growth rate [μm/day]</td>
<td>Median age [days]</td>
</tr>
<tr>
<td>Blue crab</td>
<td>32 ± 24</td>
<td>170 ± 164</td>
</tr>
<tr>
<td>Horseshoe crab</td>
<td>55 ± 32</td>
<td>217 ± 190</td>
</tr>
<tr>
<td>Loggerhead sea turtle</td>
<td>74 ± 49</td>
<td>216 ± 185</td>
</tr>
</tbody>
</table>
Table 2.3. Range of growth rate estimates based on maximal basal rostro-carinal diameter measurements, maximum age and inferred instantaneous mortality rate of balanomorph barnacles reported in the literature and in this study. The instantaneous annual mortality rate (M) was calculated from maximum age ($t_{\text{max}}$) using the following formula: $M = \exp(1.44 - 0.982 \times \ln(t_{\text{max}}))$ (Hoenig 1983). Letters behind the growth rate and mortality rate estimates indicate rates that were higher (H) or lower (L) than estimates for adult hermaphrodites of *C. testudinaria*.

Sloan et al. (2014) reports growth rates for the first 6-36 days after settlement in mm²/day, which we converted to μm/day. Abbreviations: herm. = hermaphrodites.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Austromegabalanus nigrescens</em></td>
<td>-</td>
<td>2</td>
<td>2.14 H</td>
<td>Anderson 1994</td>
</tr>
<tr>
<td><em>Amphibalanus amphitrite</em></td>
<td>245 H</td>
<td>6</td>
<td>0.73 L</td>
<td>Crisp and Bourget 1985, Calcagno et al. 1998</td>
</tr>
<tr>
<td><em>Balanus balanus</em></td>
<td>36-130</td>
<td>-</td>
<td>-</td>
<td>Crisp and Bourget 1985</td>
</tr>
<tr>
<td><em>Balanus crenatus</em></td>
<td>220-284 H</td>
<td>&gt; 8</td>
<td>&lt; 0.55 L</td>
<td>Crisp and Bourget 1985, Varfolomeeva et al. 2008</td>
</tr>
<tr>
<td><em>Balanus eburneus</em></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>Grave 1933</td>
</tr>
<tr>
<td><em>Balanus hameri</em></td>
<td>33</td>
<td>5</td>
<td>0.87 L</td>
<td>Crisp and Bourget 1985, Moore 1935</td>
</tr>
<tr>
<td><em>Balanus glandula</em></td>
<td>47, 51, 208</td>
<td>8-9</td>
<td>0.49 L</td>
<td>Crisp and Bourget 1985, Vallarino and Elias 1997, Grave 1933, Hines 1979</td>
</tr>
<tr>
<td><em>Balanus perforatus</em></td>
<td>14-122</td>
<td>-</td>
<td>-</td>
<td>Crisp and Bourget 1985</td>
</tr>
<tr>
<td><em>Cantellius pallidus</em></td>
<td>-</td>
<td>2</td>
<td>2.14 H</td>
<td>Brickner et al. 2010</td>
</tr>
<tr>
<td><em>Chelonibia testudinaria</em> (herm.)</td>
<td>1400-1800 H</td>
<td>-</td>
<td>-</td>
<td>Sloan et al. 2014</td>
</tr>
<tr>
<td><em>C. testudinaria</em> (herm.)</td>
<td>32-74</td>
<td>&lt; 3</td>
<td>&gt; 1.43</td>
<td>This study</td>
</tr>
<tr>
<td><em>C. testudinaria</em> (males)</td>
<td>25-71</td>
<td>&lt; 1</td>
<td>&gt; 4.22</td>
<td>This study</td>
</tr>
<tr>
<td>Species</td>
<td>Length (L)</td>
<td>Width (W)</td>
<td>Height (H)</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>-----------</td>
<td>------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Chthamalus fissus</td>
<td>22</td>
<td>3</td>
<td>1.43</td>
<td>Hines 1979</td>
</tr>
<tr>
<td>Chthamalus stellatus</td>
<td>10-55</td>
<td>-</td>
<td>-</td>
<td>Crisp and Bourget 1985</td>
</tr>
<tr>
<td>Chthamalus dalli</td>
<td>2.8-7.5</td>
<td>&gt; 5</td>
<td>0.87</td>
<td>Southward and Southward 1967</td>
</tr>
<tr>
<td>Elminius modestus</td>
<td>25-138</td>
<td>-</td>
<td>-</td>
<td>Crisp and Bourget 1985, Crisp and Patel 1961</td>
</tr>
<tr>
<td>Megabalanus stultus</td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>Lewis 1992 (estimated from figure 5)</td>
</tr>
<tr>
<td>Semibalanus balanoides</td>
<td>23-160</td>
<td>&gt; 5</td>
<td>&lt; 0.87</td>
<td>Crisp and Bourget 1985, Moore 1934</td>
</tr>
<tr>
<td>Tesseropora rosea</td>
<td>-</td>
<td>10</td>
<td>0.44</td>
<td>Anderson 1994</td>
</tr>
<tr>
<td>Tectraclita rubescens</td>
<td>-</td>
<td>&gt; 5</td>
<td>&lt; 0.87</td>
<td>Villalobos 1979</td>
</tr>
<tr>
<td>Tectraclita squamosa</td>
<td>33</td>
<td>15</td>
<td>0.3</td>
<td>Hines 1979</td>
</tr>
<tr>
<td>Trevathana sarae</td>
<td>-</td>
<td>6</td>
<td>0.73</td>
<td>Brickner et al. 2010</td>
</tr>
</tbody>
</table>
Figure 2.1. Illustration of hermaphroditic *Chelonibia testudinaria* from a loggerhead sea turtle, indicating how size was measured (maximal rostro-carinal diameter). The individual has a small male attached to its shell. Scientific illustration by Melissa Merrill, University of Georgia, USA (http://dx.doi.org/10.6084/m9.figshare.1126308).
Figure 2.2. Distribution of potential mating group size of *Chelonibia testudinaria* hermaphrodites per host individual, as well as number of males per hermaphrodite. Data are shown as violin plots, a combination of a box plot and a kernel density plot (histogram). The potential mating group size is the number of hermaphrodites in an area that could be reached by the penis of a large hermaphrodite ($\pi*2*50^2$ mm$^2$). (A) Potential mating group size for hermaphrodites on blue crabs, (B) potential mating group size for hermaphrodites on horseshoe crabs, (C) potential mating group size for hermaphrodites on loggerhead sea turtles and (D) number of males per hermaphrodite for all hosts. For A-C, hosts without *C. testudinaria* are not shown. For D, hermaphrodites without males are not shown.
Figure 2.3. Linear growth rate estimates [$\mu$m/day] of hermaphroditic *Chelonibia testudinaria* estimated with different methods and host species. Method abbreviations are as follows:

Size.freq: size frequency distributions of field collected barnacles; lab.food: laboratory trial with *ad libitum* food; lab.water: laboratory trial under running seawater, without additional food; mesocosm: mesocosm trial; cap.recap: capture-recapture analysis. The numbers above each box plot indicates the sampling size.
**Figure 2.4.** Violin plots of inferred age distribution of hermaphrodites and males of *Chelonibia testudinaria*. A violin plot is a combination of a box plot and a kernel density plot (histogram). Specimens were collected between April and August 2011-2013. (A) hermaphroditic age distribution for each host, (B) male age distribution for each host. Asterisk indicates that blue crab age is calculated by applying growth rate estimates of size frequency distributions and mesocosm trials only.
CHAPTER 3
LONGTERM-MAINTENANCE OF HOST-SPECIFIC PHENOTYPIC PLASTICITY IN AN
EPIBIOTIC BARNACLE

Abstract

Phenotypic plasticity is the ability of a genotype to produce different phenotypes depending on the environment. Its importance in ecology and evolution is increasingly recognized. Recent work discovered host-specific phenotypic plasticity in the Pacific lineage of the epizoic barnacle Chelonibia testudinaria (Linnaeus 1758). We investigated genetic and morphological host-specific structure in the Atlantic lineage of C. testudinaria, which likely diverged from the Pacific lineage four to five million years ago. We find patterns of host-specific shell morphology identical to those of the Pacific lineage in the absence of genetic host-specific structure. The most parsimonious explanation for this astonishing similarity between the two lineages is that C. testudinaria maintained phenotypic plasticity over millions of years, suggesting its long-term stability.

Key words

Chelonibia testudinaria, Atlantic Ocean, Indo-West Pacific Ocean, Thoracica, morphology,
Caretta caretta, Limulus polyphemus, Callinectes sapidus
Introduction

The evolutionary role of phenotypic plasticity (PP), the ability of the same genotype to generate environment-dependent phenotypes, is much debated (West-Eberhard 1989). On the one hand, PP masks genotypes from selection based on their phenotype, thus slowing evolution down (Wright 1931, Stearns 1989). On the other hand, PP may allow populations to utilize new environments, which can lead to genetic assimilation, thus accelerating evolution (Waddington 1953, Stearns 1989, Schaum & Collins 2014). Under which conditions PP will be maintained is a difficult question, addressed often theoretically (Stearns 1989, Moran 1992, DeWitt et al. 1998, Pigliucci 2005). To better understand the maintenance of PP, it is desirable to identify empirical systems that maintain PP over evolutionary times.

The globally distributed epibiotic barnacle *Chelonibia testudinaria* (Linnaeus 1758) has the potential for long-term maintenance of PP. *Chelonibia testudinaria* uses diverse marine animals as substratum, such as sea turtles, manatees, swimming crabs, and horseshoe crabs. Host-specific morphotypes were previously described as distinct species (Darwin 1854, Hayashi 2013). However, recent molecular analyses indicate that *C. testudinaria* is a host generalist, and *C. patula* (Ranzani, 1818) and *C. manati* (Gruvel, 1903) are now considered synonyms of *C. testudinaria* (Cheang et al. 2013, Zardus et al. 2014). Instead of host-specific divergence, genetic lineages are delineated by geographic affinities. The three major lineages are restricted to the Indo-West Pacific (IWP), Tropical Eastern Pacific and Atlantic Ocean, respectively (Rawson et al. 2003). These lineages likely represent separate species based on their levels of genetic differentiation (Zardus et al. 2014).

In the West-Pacific lineage, morphotypes from crustaceans (mainly brachyuran crabs) and sea turtles differ at several ecologically important morphological features: feeding
appendage length, which is correlated to feeding efficiency; shell thickness, which likely affects how firmly barnacles are attached to their substratum; shell height, which determines the amount of drag, or how much force acts on dislodging the barnacle; and orifice length, the size of the “dorsal” opening of barnacles (Cheang et al. 2013). The presence of other lineages of \textit{C. testudinaria} in different ocean basins, but with very similar hosts, provides the rare opportunity to compare patterns of PP.

In the present study, we investigated the extent of PP in the Atlantic lineage of \textit{C. testudinaria}. First, we characterized a set of microsatellite loci for Atlantic populations. We used these loci as well as partial mitochondrial cytochrome oxidase subunit one (COI) DNA sequences to assess fine-scale pattern of host-specific differentiation between populations from the blue crab \textit{Callinectes sapidus} (Rathbun, 1896), the horseshoe crab \textit{Limulus polyphemus} (Linnaeus, 1758) and the loggerhead sea turtle \textit{Caretta caretta} (Linnaeus, 1758) collected along the Eastern US coast. We compared several morphological traits between the Atlantic and West-Pacific lineages of \textit{C. testudinaria}. Our results indicate negligible levels of host-specific genetic differentiation, but significant levels of morphological host-specificity. In particular, host-specific shell morphology of Atlantic populations is indistinguishable from West-Pacific populations. The most parsimonious explanation of this pattern is the long-term maintenance of PP.
Material and Methods

Microsatellite marker development

We extracted genomic DNA from a feeding appendage of a single large hermaphroditic *C. testudinaria* collected from a horseshoe crab with Gentra Puregene Tissue Kit (Qiagen), and measured DNA concentration with a Qubit 2.0 Fluorometer (Life Technologies). Genomic DNA was fragmented into approximately 700bp lengths (insert size) and shotgun-sequenced on an Illumina MiSeq sequencer (PE250). We quality-checked paired-end reads with FastQC (Andrews 2015). The software FASTQMCF was used to trim adapters, cut low quality ends and remove low quality reads and their mate-pair read (Aronesty 2011). We calculated the haploid genome size by first mapping reads to 52 nuclear single-copy gene fragments available from the acorn barnacle *Semibalanus balanoides* (Regier et al. 2010), calculating the median coverage, and then dividing the total number of amplified base pairs by the median coverage. We used 10 nuclear gene fragments amplified from other barnacle species, which were available in genbank.

We executed the perl script PALFINDER to identify short sequence repeat regions (Castoe et al. 2012). The script also calls PRIMER3, version 2.0.0, to identify primer pairs that span the repeat region (Rozen & Skaletsky 2000). The minimum number of n-mer repeat units was chosen as in Castoe et al. (2012). PRIMER3 parameters were the default values. The search resulted in a large number of potentially amplifiable loci (PALs), repeat regions for which primers were identified. We filtered the results by removing all PALs which occurred less than two times and more than the estimated genome coverage in the genomic reads. If the number of primer occurrences is low, the primer sequence may contain sequencing error. If the number of occurrences is higher than the expected genome coverage, the primer region may occur more
than once in the genome, leading to amplification of multiple loci (genomic regions), while we aimed to retain loci that occur only once in the genome.

Of the filtered PALs, we chose 48 PALs for trial amplification, which differed in kmer length, repeat motif and fragment size. Trials used the method of Schuelke (2000) to tag primers fluorescently. We amplified DNA of 16 _C. testudinaria_ individuals from all three host species (specimen sampling and DNA extraction specified below). Loci that amplified and scored consistently in all individuals were fluorescently labeled with 6-FAM, NED or HEX (Applied Biosystems, Custom Oligo Synthesis Center), and used in the following analyses.

Specimen collections

Up to seven individuals of _C. testudinaria_ per host individual were collected along the Eastern US coast (Delaware to Georgia) and in the Gulf of Mexico (Mississippi) from 2011 to 2015. Individuals were removed from the horseshoe crab _Limulus polyphemus_ (Linnaeus, 1758), the blue crab _Callinectes sapidus_ (Rathbun, 1896) and the loggerhead sea turtle _Caretta caretta_ (Linnaeus, 1758). These hosts were chosen for their abundance and easy access. We collected in collaboration with organizations working on the host species, or with appropriate collection permits. Barnacles were either preserved immediately in 95% EtOH, or frozen prior to preservation. We recorded latitude and longitude for all sampling sites.

Microsatellite marker amplification

Genomic DNA was extracted from feeding appendages of barnacles with the Chelex method (Walsh et al. 1991). We amplified 12 microsatellite repeat regions overall, multiplexing 3 loci per reaction for a total of four PCR reactions per individual. PCR amplifications were
performed in 20µl volumes containing final concentrations of 1x PCR buffer (Bioline), 5% bovine serum albumin 10 mg/mL (Sigma), 200 mM each dNTP, 2 nM MgCl, 0.5 mM each primer, 0.5 units of Promega GoTaq DNA Polymerase, and 1 µl template DNA. PCR conditions were as follows: 4 min initial denaturation, followed by 40 cycles with 45 sec denaturing at 94°C, 60 sec annealing at 55°C, 60 sec extension at 72°C and a final extension time of 10 min. The PCR were carried out in a MJ Research PCR Engine. HiDi and ROX500 size standard were added to each sample, and fragment length analysis was carried out at the Georgia Genomics Facility on an ABI 3730xl. Peaks were called and binned with the microsatellite plugin of Geneious version 8.1 (Kearse et al. 2012).

Microsatellite marker characterization

We inspected peak calls of all loci for fragment size consistency, using the R package MSATALLELE (Alberto 2009). MSATALLELE allows plotting of all peak calls of a locus in histogram form, thus facilitating visual binning of alleles. If bins could not be clearly assigned, the locus was not considered a true short sequence repeat, and excluded from the subsequent analysis.

We tested whether loci were in Hardy-Weinberg Equilibrium (HWE) by using 1999 Monte Carlo permutations, as implemented by the function HW.TEST in the R package PEGAS (Paradis 2010). We recorded the number of alleles, range of fragment sizes, and allelic richness of each locus. Genotyping error rates were calculated by repeating genotyping for approximately 20% of individuals. We amplified the markers for 24 individuals from Queensland, Australia, to assess if the markers could be used in cross-lineage analysis.
Mitochondrial cytochrome oxidase subunit one DNA sequences

We amplified partial cytochrome oxidase subunit one (COI) gene sequences for a subset of individuals following the protocol of Rawson et al. (2003). We combined the new sequence data with 10 GenBank sequences of Atlantic *C. testudinaria* (Zardus et al. 2014).

Genetic host-specific structure

We tested whether population differentiation exists between barnacles attached to different host species. We were not able to obtain samples for all hosts in all regions, and sampling sites were spread throughout the geographic range covered, due to the nature of collecting (e.g. collecting along transects rather than fixed sites). Thus we defined geographic regions as clusters of sampling sites. The lack of full-factorial sampling of regions and hosts makes it difficult to disentangle the effects of geography and host. We employed several strategies to deal with this difficulty, including subsetting the data and multivariate approaches. Analyses were carried out on the mitochondrial and nuclear microsatellite data sets separately.

We assessed levels of host-induced population structure in two ways: (1) we used the complete data sets for broad-scale analysis, and (2) restricted data samples taken between northern Florida and South Carolina, a geographic region where individuals from all host species were available (the “GA” region). To test for host-specificity in the GA data set, we compared the variance found within host-specific populations with the variance between populations on different hosts using analysis of molecular variance (AMOVA) (Excoffier et al. 1992). Host-induced population structure is indicated when variance between populations is significantly larger than variance within populations. We used AMOVA as implemented in the function AMOVA in the R package PEGAS (Paradis 2010). Significance was assessed with 1000 Monte-
Carlo permutations. In the complete data set, we accounted for geography by using both geographic regions and host species as explanatory variables. This multivariate AMOVA approach is implemented in the function `adonis` of the R package `vegan`. The dependent variable is a genetic distance matrix, and the explanatory variables were geographic regions and host species. As explanatory variables are added sequentially, the order of adding new variables can have an impact on the significance levels. We added either host or location first, and compared the results.

We calculated genetic distance matrices between all pairs of individual genotypes for implementation in the AMOVA. For the microsatellite data, we calculated these individual distance matrices based on Nei's distance (Nei 1972, Nei 1978), the genetic distance of Cavalli-Sforza & Edwards (1967), which is least sensitive to the presence of null alleles (Chapuis and Estoup 2007), and genetic distance based on relative dissimilarity (Prevosti et al. 1975) with the functions `nei.dist`, `edwards.dist` and `provesti.dist` of the package `POPR` (Kamvar et al. 2014) in the R environment, respectively. ANOVA's were carried out with each of these distance matrices. For the COI data, we computed genetic distances based on the transversional model of sequence evolution (function `dist.dna`, R package `ape`, model="K80"). Kimura's (1980) model was estimated to be the optimal DNA sequence model based on the Bayesian information criterion by `jmodeltest` version 2.1.5 (Darriba et al. 2012).

We further investigated host-induced population structure of the microsatellite data with a discriminant analysis of principal components (DAPC), implemented by the function `dapc` in the R package `ADEGENET` (Jombart 2008, Jombart et al. 2010). The DAPC algorithm summarizes the genotypic data, and identifies alleles that best differentiate pre-assigned groupings, in this case the host species. We conducted a DAPC with the complete data set and with data restricted
to the GA population. The number of PC’s to be retained was calculated based on the alpha score (Jombart et al. 2010), implemented in the function `OPTIM.A.SCORE` of the R package `ADEGENET`.

We calculated the mean assignment probability for each individual. If this probability is high, groups are well-discriminated by the genetic data. In addition, we calculated the contribution that each allele had to the pre-assigned groupings. Alleles with large contributions are useful in differentiating the data, and may indicate parts of the genome with host-limited gene flow.

Identifying the extent to which frequencies of alleles with significant contributions change among groups further highlights their relative significance.

Morphological host-specific structure

Populations of *C. testudinaria* consist of hermaphrodites, which attach directly to their host, and dwarfed males (complemental males *sensu* Darwin 1854), which attach to hermaphrodites (Crisp 1983, Zardus and Hadfield 2004, Cheang et al. 2013, Zardus et al. 2014). Males may experience currents and food availability differently by being attached to other, larger individuals, which could alter their morphology. We thus restricted our morphological analyses to hermaphrodites of *C. testudinaria*. We measured maximal basal shell diameter, maximal operculum length, shell height and shell thickness to the closest 100um with digital vernier calipers (Figure 3.1). We measured the length of the feeding appendage IV (hereafter called cirral length) with a ruler to the closest mm (Figure 3.1). The different accuracies were due to the different tools used to measure shell characteristics vs soft feeding appendages. For comparison to the IWP lineage (Cheang et al. 2013), we normalized operculum length, shell height, shell thickness, and cirral length by the basal shell diameter for data collected from loggerhead sea turtles and blue crabs. These two hosts are most similar to hosts in the Pacific study, which...
include three different sea turtle species and several species of brachyuran crab (Cheang et al. 2013). We compared the Atlantic and Pacific lineage using multivariate ANOVA's with host (brachyuran crab vs sea turtle) and oceanic basin (West-Atlantic vs IWP) as explanatory variables.

To further investigate host-specific morphology within the Atlantic Ocean, we regressed morphological traits of barnacles from blue crabs, horseshoe crabs and loggerhead sea turtles against age. Age was calculated based on host-specific growth rate estimates of the basal shell diameter (Ewers-Saucedo et al. 2015). A significant correlation indicates that morphology changes with age. In addition, if the factor host is significant, but not the interaction term between host and age, regression lines for different hosts are parallel. If age and interaction term are significant, but host itself is not, age has the same overall effect on the morphological trait (e.g. positive), but the slope differs between hosts. If all three variables are significant, both slope and height of the regression line depend on the host species. In summary, if either host or interaction term are significant, the host species has a significant effect on barnacle morphology.

Results

Microsatellite marker development

The MiSeq run generated 15,324,079 paired-end reads (35-251 bp long) with 81.05% > Q30. After quality control, 13,498,280 paired-end reads (19-251 bp long) remained, for a total of 6.2 Gb. The median genome coverage was 8x (min = 3, max = 24) for 52 nuclear single-copy gene fragments, and the haploid genome size is therefore approximately 800 Mb. The PALFINDER
script detected 629,990 microsatellite repeat regions, of which 5.38% were potentially amplifiable loci with forward and reverse primer (PALs). After removing PALs with more than eight or less than two occurrences of either forward or reverse primer in the sequence read data, 17,265 PALs remained. We chose 48 loci for trial amplification that differed in kmer and repeat motif. Of those 48 loci, 12 loci amplified and scored consistently throughout the trials, and were permanently tagged with fluorescently labeled dye (Appendix A, Table A1).

Specimen collections

We collected 436 *C. testudinaria* specimens along the eastern US coast of Delaware, Virginia, South Carolina, Georgia, Florida and Mississippi. Of these individuals 215 were collected from horseshoe crabs, 79 from loggerhead sea turtles and 142 from blue crabs. We attempted to genotype all individuals with microsatellite markers, and measured morphological traits on 100 barnacles per host species, covering the observed size range from 1mm to 60mm basal diameter. Microsatellite genotypes, morphological measurements and accompanying collecting information for each individual (host, latitude, longitude, location, date) are available on DataDryad (http://datadryad.org/).

Microsatellite marker characterization

We genotyped 387 individuals successfully at more than ½ of all loci (Figure 3.2). Visual inspection of peak call histograms revealed that peak calls of Ctest2 did not have clearly defined bins, and we excluded this locus from the analyses. The number of alleles of the 11 scorable loci ranged from six to 30. All loci showed homozygote excess, and no locus was in Hardy-Weinberg equilibrium (HWE) (Table 3.1). Calculating HWE only for individuals that amplified at all loci
(n=248), or only for individuals from horseshoe crabs collected in the GA region did not change the results (results not shown). Genotyping error rates ranged from 0 to 7.32% (Table 3.1).

All loci amplified in IWP individuals, indicating their usefulness in cross-lineage studies. One locus (Ctest31) was monomorphic. The expected heterozygosity, a measure of genetic diversity, was lower in six loci amplified in Pacific individuals (Table 3.1).

Mitochondrial partial cytochrome oxidase subunit 1 DNA sequences

We generated 158 sequences from individuals from all three host species throughout the range (Figure 3.2). Sequences were trimmed, aligned and error-checked in Geneious version 8.1 (Kearse et al. 2012). Final sequence length of the alignment was 642bp. Sequences are available on NCBI genbank under accession numbers KT793179 - KT793336.

Genetic host-specific structure

AMOVA of the GA population did not indicate host-specific population structure for either the microsatellite data (P > 0.900) or the COI data (P = 0.452), and neither did the multivariate AMOVA of the microsatellite data set (P > 0.281) nor the COI data set (P > 0.374) when the complete geographic range was considered.

Using the complete data set or the data set restricted to GA, DAPC had a low probability of assigning individuals accurately to their host species (complete: p=0.625, GA: p=0.595). High assignment probabilities are 0.8 or higher (Jombart et al. 2010). The proportion of preserved variance was high (complete: p=0.884, GA: p=0.805). Few alleles contributed to host-specificity, and their allele frequencies differed maximally 0.1653 between any of the host populations when considering only the GA population (Figure 3.3). Similar results were obtained when the
complete geographic range was considered (Appendix A, Figure A1). All results indicate little to no population genetic structure between host populations.

Morphological host-specific structure

We measured all shell and arthropodal characters on 307 Atlantic barnacles, 101 barnacles from the blue crab *Callinectes sapidus*, 101 barnacles from the horseshoe crab *Limulus polyphemus*, and 105 barnacles from the loggerhead sea turtle *Caretta caretta*. Barnacles ranged in basal diameter from 2mm to 60mm. We were able to use the morphological data of Cheang et al. (2013) for IWP populations of *C. testudinaria*, which consists of morphological measurements of 75 barnacles from the IWP, 59 from crabs (mostly the brachyuran crab *Scylla serrata*) and 17 from sea turtles (mostly the green turtle *Chelonia mydas* and the loggerhead sea turtle *Caretta caretta*), but see appendix S1 of Cheang et al. 2013 for more details on host species and sampling sites of the IWP population. The IWP barnacles ranged in basal shell diameter from 2.7mm to 65.6mm.

We first compared normalized morphological traits of Atlantic and IWP populations. For this comparison, we excluded barnacles from horseshoe crabs, as this host category was only sampled in the Atlantic population. All morphological traits differed significantly between barnacles from brachyuran crabs and sea turtles (*F*\(_{1,278}\) = 398 for cirral length, *F*\(_{1,278}\) = 479 for opercular length, *F*\(_{1,278}\) = 174 for shell height, *F*\(_{1,278}\) = 349 for shell thickness; *P* < 2e-16 for all traits). Within each host category, normalized shell characteristics did not differ between ocean basins (*F*\(_{1,278}\) = 1.03, *P* = 0.31 for opercular length, *F*\(_{1,278}\) = 2.85, *P* = 0.093 for shell height, *F*\(_{1,278}\) = 3.3, *P* = 0.07 for shell thickness) (Figure 3.4). Cirri, on the other hand, were significantly shorter
in barnacles from West-Pacific sea turtles than in barnacles from Atlantic loggerhead sea turtles ($F_{1,278} = 15, P = 0.0001$) when accounting for basal shell diameter.

When investigating host-specific morphologies of populations within the Atlantic, we considered barnacles from blue crabs, horseshoe crabs, loggerhead sea turtles. All morphological traits increased with age ($P < 0.001$ for all traits). Cirral length shows the fewest host-specific effects; only the interaction term of age with the blue crab – loggerhead contrast is significant (Table 3.2A). For shell height and opercular length, the host term itself is not important, which means the magnitude of the regression lines do not differ between host populations. Instead, most interaction terms are significant, which means the slopes differ in a host-specific manner (Table 3.2B, 3.2C). Shell thickness shows the most host-specific pattern; both host itself and interaction term are significant (Table 3.2D). Overall, barnacles from blue crabs have shorter cirri, are flatter, have smaller opercular openings and thinner shells than barnacles from horseshoe crabs and loggerheads of the same age (Figure 3.5). Differences between barnacles from loggerheads and horseshoe crabs are less pronounced. Where differences exist, barnacles from horseshoe crabs have a morphology intermediate between those from blue crabs and sea turtles.

Discussion

Our study revealed astonishingly similar patterns of phenotypic plasticity between two lineages of *C. testudinaria* that diverged several million years ago. The most parsimonious explanation for their high degree of similarity is common ancestry, and the long-term maintenance of phenotypic plasticity (PP). The long-term maintenance of this PP suggests that it...
is not transitionary but rather a stable state, maintained either by developmental constraints or as an adaptation.

Microsatellite loci characterization

Most loci display an excess of homozygotes. Homozygote excess can be caused by selection, the presence of null alleles, inbreeding, population substructure or large variance in reproductive success. Inbreeding is an unlikely cause because most barnacles are obligate outcrossers and *C. testudinaria* has a widely-dispersing planktonic larval phase. Selection cannot be excluded as an explanation, but selection on almost all markers appears unlikely. Population substructure may be present, but if so, is neither host-induced nor geographical. Large variance in reproductive success can cause homozygote excess (Hedgecock 1994), and has been invoked to explain homozygote excess in sea urchins (Addison & Hart 2004). If variance in reproductive success is present, the effective population size of *C. testudinaria* should be low (Hedgecock 1994). We estimated a theta of ten for the Atlantic *C. testudinaria* population using Watterson's estimator on the COI data, which suggests a very large effective population size of millions of individuals (data not shown). Thus the data at hand do not support the variance in reproductive success hypothesis. The most likely cause for homozygote excess is null alleles. Null alleles are ubiquitous in microsatellite markers, and are caused by mutations in the primer sequence. They become increasingly prevalent with increasing effective population size (Chapuis and Estoup 2007). Chapuis and Estoup (2007) show that simulated null allele frequencies were larger than 0.2 for all loci when the population mutation rate (theta) was one, the largest value simulated. We estimated null allele frequencies between zero and 0.28 for our microsatellite loci, well within the range of simulated data with large effective population size. Thus the observed
homozygote excess can be explained by the presence of null alleles. Null alleles lead to an upward-bias of population differentiation (Chapuis and Estoup 2007). This means our estimates of population differentiation are overestimates, and our conclusion that there is no population differentiation between host species remains unaltered by the presence of null alleles.

Genetic population structure

The number of markers in this study is relatively small, which leaves the possibility that the morphological divergence is caused by few genetic loci that are not in linkage with the microsatellite loci. This appears unlikely because many traits are host-specific, suggesting the involvement of many loci, thus most of the genome should show signs of divergence. Nonetheless, more extensive sampling of the genome should be carried out to exclude the possibility of locus-specific selection.

We used the infinite-sites model to estimate population divergence for both COI and microsatellite loci. We chose it over the microsatellite-specific stepwise mutation model because it is more powerful in identifying low levels of population differentiation, and gives lower mean square errors (Gaggiotti et al. 1999, Balloux & Goudet 2002). Zardus et al. (2014) showed a lack of population differentiation using mitochondrial and nuclear sequence data, suggesting that, if any, levels of population differentiation would be low. We show that even with rapidly-evolving microsatellite loci, no population differentiation could be detected.

Morphological population structure

The barnacle shell is the distinctive character of all barnacles and plays a crucial role in their ecology: it attaches barnacles to their substrate, and protects them from predation and

Each morphological trait has a unique response to the hosts. Cirral length differs little between barnacles from different hosts, and is most variable at any age. This suggests that other, unobserved factors influence cirral length. Shell height and opercular length differ little between individuals from horseshoe crabs and loggerhead sea turtles, but is distinct from individuals attached to blue crabs. In general, shell morphology (opercular length, shell height, and shell thickness) responds more to host environment than cirral morphology. It stands out that barnacles from blue crabs are more diverged than barnacles from horseshoe crabs and loggerhead sea turtles, even though both barnacles from blue crabs and horseshoe crabs were formerly assigned to the same species, *C. patula*.

Cheang et al. (2013) suggested that shell morphology develops either in response to substratum, hydrostatic pressure or flow velocity. Our results suggest that substratum is unlikely to cause the observed pattern because both horseshoe crabs and blue crabs have a smooth chitinious shell, but barnacle morphology differs significantly. Hydrostatic pressure and flow velocity, on the other hand, differ “in the right direction” between all three hosts: Sea turtles dive deep, and horseshoe crabs presumably spend significant time in deep water. Blue crabs are generally shallow-water inhabitants, even though females migrate into deeper water to spawn. Similarly, sea turtles move faster than horseshoe crabs, and blue crabs move slowest (Renaud &
Carpenter 1994, Carr et al. 2004, Wrona 2004, Hart et al. 2010, Watson & Chabot 2010, Abecassis et al. 2013, Foley et al. 2013). Thus we hypothesize that being in deep water or experiencing high flow velocities increases shell thickness, decreases shell height as well as opercular width. Two avenues of research may be used to test this hypothesis: on the one hand, we may collect morphological information on barnacles from other host species with known ecology, taking advantage of the wide host spectrum of *C. testudinaria*. We can then ask if fast moving species or species that occur in deep water carry *C. testudinaria* individuals with thick depressed shells. The drawbacks of this approach are that we know little about the host species and their behavior, and secondly that hosts differ in other aspects of their ecology that could impact barnacle shell development. Alternatively, we could use an experimental approach, and grow barnacles under high and low water flow, as well as high and low pressure conditions.

**Between-lineage pattern of PP**

While cirral length differed between Atlantic and Pacific individuals from the same hosts, host-specific shell morphology was indistinguishable between individuals from different lineages. This means that same pattern of host-specific morphology either evolved independently, or was present in the ancestor, and has been maintained ever since. Of these two explanations, maintenance is more parsimonious and the view we favor. Comparing fossils of *C. testudinaria* to present-day individuals could further clarify if the same morphology was expressed in the past as well.

Phylogenetic reconstructions provide an estimate of the time since divergence of the two lineages. COI sequences of the West-Pacific and Atlantic lineage differ 12 to 14% (estimated from Figure 4 of Cheang et al. 2013 and Figure 1 of the supplementary material of Zardus et al.
If COI diverges about 3% per million years in barnacles (Wares 2001), the two lineages diverged four to five million years ago. Taken together with the absence of host-specific genetic structure, it appears that *C. testudinaria* has retained phenotypic plasticity (PP) over millions of years.

The fact that host-induced PP has been maintained over millions of years is curious, given that host specialization is commonplace in barnacles (Mokady & Brickner 2001, Tsang et al. 2009, Tsang et al. 2014). A proximate explanation for this phenomenon is that *C. testudinaria* may be unable to distinguish between host species (Getty 1996). If *C. testudinaria* cannot identify the host species it settles on, its environment would seem unpredictable. In this scenario, morphological host-specificity arises as a response to environmental exposure rather than to a host cue itself. Ultimately, none of the host species alone may be able to support populations of *C. testudinaria* that are large enough to not go extinct.

Our study throws no light on the adaptive potential of host-specific phenotypic plasticity in *C. testudinaria*. We do not know if expressing host-specific morphology confers fitness advantages. Cheang et al. (2013) suggest that most of these differences are adaptive. It is certainly true that plasticity of barnacle shell morphology can be adaptive (Lively 1986a, b). It may be possible to test for its adaptive potential by comparing the performance of each morphotype in different environments. While it is not tractable to transplant individuals between hosts in the field, we may be able simulate different environments, raise individuals in certain environments, and then transplant them between environments. However, this experimental approach hinges on knowing the factors that induce certain shell morphologies.
Conclusion

Our study points out a likely case of long-term maintenance of phenotypic plasticity. It is curious that host-specificity has not arisen in this epizoic barnacle, and suggests the potential for the evolutionary stability of phenotypic plasticity.

Acknowledgements

This study would not have been possible without the many people who supported barnacle collections in the field, such as Pearse Webster (SCDNR), Jeff Schwenter (SCDNR), Mike Arendt (SCDNR), Matt Ogburn (SERC), Darcie Graham (USM), Emily Maung (UDel), Matthew Godfrey (NOAA), Mike Seebo (VIMS), and Riley Wares. We would like to acknowledge our funding sources, the National Science Foundation (NSF-OCE No. 1029526) and the University of Georgia Department of Genetics Hightower Award.

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Gruvel, A. 1903. Cirripédés opercules nouveaux ou peu connus de la collection du muséum.  


**Table 3.1.** Microsatellite loci characteristics. Abbreviations: obs. = observed, exp. = expected, IWP = Indo-West Pacific.

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<tr>
<th>Locus</th>
<th>Kmer range</th>
<th># alleles</th>
<th>Allelic richness by host</th>
<th>Heterozygosity</th>
<th>Genotyping error rate</th>
<th>Proportion null alleles</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Blue crab</td>
<td>Horseshoe crab</td>
<td>Loggerhead</td>
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<td></td>
<td></td>
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Table 3.2. The effect of age and host species on morphological traits. A: cirral length; B: opercular length; C: shell height; D: shell width. Significance, the probability that the observed t value is larger or smaller than the expected t value (Pr(>|t|)), was assessed with linear least-square regression.

(A) Cirral length

|                  | Estimate | Std. Error | t value | Pr(>|t|)    |
|------------------|----------|------------|---------|------------|
| age              | 0.02     | 0          | 14.71   | 2.91E-037  |
| blue crab vs horseshoe crab | 0.23     | 0.81       | 0.28    | 0.78       |
| blue crab vs loggerhead | 0.46     | 0.79       | 0.58    | 0.56       |
| loggerhead vs horseshoe crab | -0.23    | 0.77       | -0.3    | 0.76       |
| age : blue crab vs horseshoe crab | 0        | 0          | 1.54    | 0.13       |
| age : blue crab vs loggerhead | 0        | 0          | 2.46    | 0.01       |
| age : loggerhead vs horseshoe crab | 0        | 0          | -0.85   | 0.4        |

(B) Opercular length

|                  | Estimate | Std. Error | t value | Pr(>|t|)    |
|------------------|----------|------------|---------|------------|
| age              | 0.01     | 0          | 17.66   | 2.23E-048  |
| blue crab vs horseshoe crab | -0.57    | 0.44       | -1.31   | 0.19       |
| blue crab vs loggerhead | -0.64    | 0.43       | -1.5    | 0.13       |
| loggerhead vs horseshoe crab | 0.07     | 0.42       | 0.17    | 0.86       |
| age : blue crab vs horseshoe crab | 0.01     | 0          | 7.2     | 4.83E-012  |
| age : blue crab vs loggerhead | 0.01     | 0          | 9.32    | 2.52E-018  |
| age : loggerhead vs horseshoe crab | 0       | 0          | -1.84   | 0.07       |
### (C) Height

|                          | Estimate | Std. Error | t value | Pr(>|t|)  |
|--------------------------|----------|------------|---------|-----------|
| age                      | 0.02     | 0          | 26.49   | 1.17E-080 |
| blue crab vs horseshoe crab | 0.03    | 0.54       | 0.05    | 0.96      |
| blue crab vs loggerhead  | 0.87     | 0.53       | 1.66    | 0.1       |
| loggerhead vs horseshoe crab | -0.84  | 0.51       | -1.65   | 0.1       |
| age : blue crab vs horseshoe crab | 0.01  | 0          | 7.52    | 6.28E-013 |
| age : blue crab vs loggerhead | 0.01  | 0          | 4.86    | 1.87E-006 |
| age : loggerhead vs horseshoe crab | 0     | 0          | 2.78    | 0.01      |

### (D) Shell width

|                          | Estimate | Std. Error | t value | Pr(>|t|)  |
|--------------------------|----------|------------|---------|-----------|
| age                      | 0        | 0          | 8.25    | 4.86E-015 |
| blue crab vs horseshoe crab | -0.42  | 0.29       | -1.44   | 0.15      |
| blue crab vs loggerhead  | 0.72     | 0.28       | 2.52    | 0.01      |
| loggerhead vs horseshoe crab | -1.13  | 0.28       | -4.1    | 5.25E-005 |
| age : blue crab vs horseshoe crab | 0.01  | 0          | 10.44   | 5.46E-022 |
| age : blue crab vs loggerhead | 0.01  | 0          | 19.83   | 1.52E-056 |
| age : loggerhead vs horseshoe crab | -0.01 | 0          | -8.76   | 1.44E-016 |
Figure 3.1. Shell and arthropodal characters measured for morphological analysis in both Atlantic and Indo-West Pacific populations of *Chelonibia testudinaria*.
Figure 3.2. Collection map. The numbers next to sampling sites are the sample sizes, microsatellite markers on the left, partial COI sequences on the right. Colors denote host species. Black: blue crab; red: horseshoe crab; green: loggerhead sea turtle. GA: geographic region where all three hosts were sampled, which includes northern Florida, Georgia and South Carolina.
Figure 3.3. DAPC results of genotypic data of individuals from GA, the geographic region where all host species were sampled. (a) The first two principal components of the DAPC are plotted, using host species as prior grouping by which the data are partitioned. Colors identify the host species, and each dot represents an individual. (b) Bar graph of membership probabilities of each individual to the pre-assigned groupings. Colors denote groups, while each
bar represents an individual. (c) Bar graph of the relative contributions of alleles to the DAPC principal components. Alleles with contributions larger than 0.05 are considered significant. Each vertical bar represents an allele. (d) Line plot of allele frequencies with significant contributions for each pre-assigned grouping. Abbreviations: LD = principle component.
Figure 3.4. Comparisons among morphological traits of Atlantic and Indo-West Pacific (IWP) populations between two host categories, brachyuran crabs and sea turtles: (a) normalized cirral length, (b) normalized opercular length, (c) normalized shell height, (d) normalized shell thickness. In the Atlantic, we only sampled from blue crabs (*Callinectes sapidus*), while the IWP sample contains several species of crabs purchased at fish markets. For the turtle barnacles, we only collected barnacles from loggerhead sea turtles (*Caretta caretta*) for the Atlantic sample, but the Pacific sample contains barnacles from loggerhead, hawksbill and green sea turtles. All morphological parameters were normalized by basal shell diameter to reduce the effect of size. The asterisk denotes the only significant difference between morphological characters in the two ocean basins. All differences between host categories were significant.
**Figure 3.5.** Host-specific changes of morphological traits with age: (a) cirral length, (b) opercular length, (c) shell height, (d) shell thickness. Colors and plotting symbols denote the host species: blue crabs (black circle), horseshoe crab (red triangle), loggerhead sea turtle (green cross).
CHAPTER 4

THE MATING SYSTEM OF THE BARNACLE *CHELONIBIA TESTUDINARIA* (LINNAEUS, 1758) - UNUSUAL FOR AN ANDROdioECIOUS SPECIES

\[1\]

Abstract

Androdioecy was first described by Darwin in his seminal work on barnacle diversity; he identified dwarfed males and large hermaphrodites in the same reproductive population. Despite Darwin's evidence for androdioecy, it was declared absent from nature in the 1980s, only to later be rediscovered in phylogenetically diverse taxa. Today we realize that androdioecious systems of many plants and animals share astonishing similarities, particularly with regard to their evolutionary history and mating system. Barnacles, however, persist as an oddball with a seemingly different evolutionary trajectory. The present study assessed the mating system of the androdioecious barnacle *Chelonibia testudinaria*. In contrast to other androdioecious species, *C. testudinaria* does not self-fertilize, and mates rarely with more than two other individuals during a reproductive event. Males do not have a mating advantage over hermaphrodites, which is the norm among other androdioecious systems, and expected by theory. Taken together, the mating system of *C. testudinaria* is unusual in comparison to other androdioecious plants and animals, and its lack of a male mating advantage is unexpected.
The evolutionary stability of androdioecy, a sexual system characterized by the coexistence of males and hermaphrodites, depends on equal fitness of males and hermaphrodites. The sexes, however, are inherently unequal in reproductive capacity. The mating system is a natural starting point for understanding the evolution and maintenance of androdioecy. Equalizing reproductive success through a male mating advantage has been a common approach to explain the maintenance of androdioecy (Charlesworth and Charlesworth 1978, Charnov et al. 1987, Lloyd 1975, Otto et al. 1993, Wolf and Takebayashi 2004), and resonates with empirical data from phylogenetically diverse plant and animal species (Table 4.1).

Early theoretical work postulated that males can invade mating systems of cross-fertilizing hermaphrodites if they are able to fertilize twice as many eggs or ovules than the hermaphrodites (Charlesworth and Charlesworth 1978, Charnov et al. 1987, Lloyd 1975). This two-fold fertilization success is more readily reached in small mating groups, because it reduces competition between males and hermaphrodites (Charnov 1987). Moreover, sex allocation theory suggests that hermaphrodites should produce little sperm in small mating groups (Charnov 1982), thus making it more likely for males to fertilize more offspring than hermaphrodites. Any self-fertilization of hermaphrodites makes the stable coexistence of males and hermaphrodites less likely, unless costs of self-fertilization are high (Charlesworth and Charlesworth 1978, Pannell 2002b).

In contrast to these theoretical expectations, empirical work revealed rampant levels of self-fertilization in recognized androdioecious populations. It was subsequently shown that these populations evolved from dioecious ancestors (Table 4.1), and females gained the male function.
to assure fertilization of their own eggs, but not to mate with other individuals (Pannell 2002b, Weeks 2012). Effectively, females were replaced with self-fertilizing hermaphrodites. Males are maintained in these systems because they have some kind of mating advantage (Table 4.1). In these systems, large mating groups are advantageous for males, because they increase the probability of males to find mates (Pannell 2000). For example, males of the androdioecious plants *Mercurialis annua* and *Phillyrea angustifolia* have higher reproductive success in dense populations (Eppley and Pannell 2007, Vassiliadis et al. 2002), and males are often absent in sparse populations of *Schizopepon bryoniaefolius* and *Mercurialis annua* (Ishida and Hiura 2002, Dorken and Pannell 2008). These empirical results differ markedly from early theoretical expectations based on ancestrally hermaphroditic populations. Instead, most androdioecious species are ancestrally dioecious, invaded by self-fertilizing hermaphrodites (Table 4.1). Subsequent theoretical work has considered this evolutionary trajectory in more detail (Otto et al. 1993, Pannell 1997, Wolf and Takebayashi 2004).

Theoretical predictions for androdioecy with hermaphroditic ancestors remain largely untested. This seems surprising given that androdioecy was first described in a predominantly hermaphroditic taxon: free-living barnacles (Darwin 1851, Yusa et al. 2012). Darwin (1851) identified several androdioecious barnacle species in his seminal work on barnacle diversity. Since then, more than 30 androdioecious barnacle species have been described, and androdioecy evolved at least seven times independently within this taxon from hermaphroditic ancestors (Kelly and Sanford 2010, Yusa et al. 2012). Thus androdioecious barnacles are well-suited to test theoretical predictions for androdioecious species with hermaphroditic ancestors.

In the present study, we characterized the mating system of the androdioecious barnacle *Chelonibia testudinaria* (Linnaeus, 1758). *C. testudinaria*, now synonymous with *C. patula*
(Ranzani, 1818) and *C. manati* Gruvel, 1903 (Cheang et al. 2013, Zardus et al. 2014), occurs circumtropically on turtles, manatees, crabs and horseshoe crabs (Hayashi 2013) (Figure 4.1). Males in the system are dwarfed and attached to larger hermaphrodites (Zardus and Hadfield 2004). It is still unclear if sex determination is genetic (Crisp 1983). We used field assays and genetic parentage assignment to quantify self-fertilization, determine mating group size and factors that increase mating success. We use fertilization success as a proxy for mating success, assuming a direct link between mating success and fertilization success, and the absence of post-copulatory selection. A relevant determinant of mating success is proximity to the receptive hermaphrodite in other barnacle species (Kelly et al. 2013, Yuen and Hoch 2010), and we tested this effect in *C. testudinaria*. Given that adult barnacles are sessile, and copulate by stretching their penises towards the receptive hermaphrodite (Charnov 1987, Murata et al. 2001, but see Barazandeh et al. 2013), we further investigated the effect of penis length on mating success. We postulate that individuals with longer penises are more likely to reach another individual, and successfully mate with it. Moreover, males should be more successful than hermaphrodites in fertilizing broods, either through their inherent proximity to the hermaphrodite onto which they settle, or because hermaphrodites are sperm-limited, as a result of small mating groups.

We found that, as expected for an androdioecious population with hermaphroditic ancestors, self-fertilization was absent, and mating groups were small. Proximity to the receptive hermaphrodite and longer penises determined mating success of males and hermaphrodites. Interestingly, the sex (male or hermaphrodite) did not play a role. We propose that this is because a) males have generally shorter penises than hermaphrodites, and b) individuals had equal probability of mating success as long as they were close enough – which often included proximal hermaphrodites. Thus males were disadvantaged by their relatively short penises, and did not
gain an advantage when hermaphrodites were also close to the receptive hermaphrodite. While aspects of the mating system of *C. testudinaria* align well with theoretical predictions, the lack of a male mating advantage androdioecy refutes the hypothesis that the mating system alone is responsible for the maintenance of androdioecy in *C. testudinaria*.

**Material and methods**

We characterized the mating system of *C. testudinaria* with a combination of field assays and parentage assignment. We were able to collect complete mating group assemblages, consisting of all individuals that can mate with each other. This was possible because adult barnacles are sessile, and copulate with long penises. Thus all potential mates are within a certain distance of one another. We distinguish between potential mating groups which contain all individuals in potential mating distance to a focal hermaphrodite and actual mating groups which contain only individuals that mated with a focal hermaphrodite. Within actual mating groups, some individuals may sire only very few embryos, and are evolutionarily less relevant because they do not contribute many alleles to the gene pool of the next generation. Generally, we consider all mature hermaphrodites within 300mm to be in potential mating distance to a focal hermaphrodite, and all mature males attached to the focal hermaphrodite. We do not consider males attached to other hermaphrodites to be part of the potential mating group (Charnov 1987).
Field collections

We collected *C. testudinaria* specimens from three host species, loggerhead sea turtles (*Caretta caretta* (Linnaeus, 1758)), blue crabs (*Callinectes sapidus* Rathbun, 1896), and horseshoe crabs (*Limulus polyphemus* (Linnaeus, 1758)), along the US Atlantic coast and in the Gulf of Mexico between 2011 and 2015. All barnacles attached to blue crab and horseshoe crab hosts were removed and stored in 95% ethanol. Given the size of blue crabs and horseshoe crabs, all mature hermaphrodites attached to the same host individual are part of the potential mating group (Ewers-Saucedo et al. 2015). For loggerhead sea turtle hosts, we collected at most one isolated cluster of barnacles per host individual; each cluster was considered a potential mating group. We only collected clusters of barnacles that were separated from other any other barnacles by at least 300mm. We chose this distance because it exceeds the maximal mating distance for this species (Crisp 1983; Ewers-Saucedo et al. 2015), thus ensuring collecting all members of a potential mating group. We counted the number of hermaphrodites and males, and checked for the presence of embryos. We measured basal shell diameter and flaccid penis length with a ruler to the closest 1mm for all collected individuals. The penis extends during mating (Crisp 1983), and is therefore only a measure of relative reach. We assume that penises extend by the same factor independent of their size, so that a short flaccid penis is relatively shorter when extended than a long flaccid penis. We took scaled in-situ pictures of all potential mating groups, and marked the location of each individual barnacle for spatial analysis (Figure 4.1). From this initial collection, we chose potential mating groups for parentage assignment that contained a brooding hermaphrodite with embryos (the focal hermaphrodite), and up to 10 potential mates (the potential fathers of the brood). The upper limit of potential mating group size was chosen to limit genotyping effort, and to be able to distinguish the genotypes of all
potential mates; the smaller the number of potential mates, the smaller the probability that two mates have the same genotype (Kelly et al. 2013). We did not consider individuals without a penis as potential mates. Criteria for choosing the focal hermaphrodite were: presence of embryos in the mantle cavity, absence or presence of males, varying distance to potential mates, varying penis length of potential mates.

Genetic parentage assignment

DNA extractions followed the protocol of Casquet et al. (2012), but with 25ul of Chelex-Proteinase K solution for embryos, and 100ul solution for males and adult tissue. For small males, the complete body was used in DNA extractions. For hermaphrodites, we used part of a feeding leg. We genotyped all individuals initially at three polymorphic microsatellite DNA loci developed by Ewers-Saucedo et al. (submitted): Ctest9, Ctest11, and Ctest18. If three loci were not able to resolve paternity unambiguously, we genotyped mates and embryos at three additional loci (Ewers-Saucedo et al. submitted): Ctest31, Ctest36, and Ctest47. PCR conditions are those of Ewers-Saucedo et al. (submitted). We scored and binned microsatellites with the microsatellite plugin in Geneious version 8.1 (Kearse et al. 2012).

We assigned parents with the exclusion method, implemented in the R package SOLOMON (Christie 2013). The exclusion method reports whether the genotype of embryo and potential mate match, while taking the maternal genotype into account. We considered an individual to be the father of an embryo if it matches the embryos' genotype better than any other potential mate. We did not allow for any mismatches between embryo and mate. In some cases, missing data prevented us to match all loci. We were able to detect unsampled mates by the
presence of unmatched alleles in more than one embryo. In these cases, we inferred the genotype of the unknown mate by subtracting the maternal genotype from the embryo genotype.

Mating group size

We assessed the number of mates for a focal hermaphrodite in several potential mating group with different numbers of potential mates, some of which contained males. We define “the number of mates” as all individuals that sired any number of embryos. We propose that mates are only evolutionarily relevant when they sire a substantial number of embryos, as only those make a significant contribution to the gene pool of the next generation. It is not trivial to determine which mates are evolutionarily relevant, and which are rare enough to be irrelevant. We attempted to genotype a large number of embryos per focal hermaphrodite to distinguish between rare and evolutionarily relevant mates.

An objective framework to determine the number of evolutionarily relevant mates is provided by diversity indices and Hill numbers used in community ecology and information science. A diversity index reflects the number and distribution of “types” in a system. In community ecology, for example, types are species. In our case, each potential mate is a type, and the number of embryos it sired is the type abundance. Different diversity indices are more or less sensitive towards rare types by weighing types by their abundance. The sensitivity of an index to rare types is specified by its “order”; the higher the order, the less sensitive (Jost 2006). The diversity index can be converted into the effective number of types, called the Hill number (Hill 1973). The Hill number denotes the number of common types that we cannot distinguish in abundance. We chose the Hill number corresponding to the second order diversity index (also called the Simpson index) to provide an estimate of the number of non-rare, thus evolutionarily
relevant, mates (Simpson 1949, Jost 2006). We calculated the bias-corrected Hill number of the second order diversity index for each potential mating group with the functions “bcDiversity” in the R package **ENTROPART** (Marcon and Herault 2014). The mean of the Hill numbers of all mating groups provides our estimate of average actual mating group size.

We further tested if the number of mates increased as the potential mating group size increases. We fitted a linear model between the potential mating group size and the actual number of mates, both as the number of all mates and the number of effective mates.

Determinants of mating success

To test whether sex, distance to the focal hermaphrodite or penis length influence mating success, we increased our sample size with regard potential mating groups. We aimed to determine the evolutionarily relevant mates of potential mating groups where potential mates differed with regard to distance to the focal hermaphrodite, penis length and sex.

To minimize genotyping costs, we first assessed the number of embryos that needed to be genotyped to sample all evolutionarily relevant mates via rarefaction. Using the thoroughly sampled mating groups described above, we calculated the probabilities that all evolutionarily relevant mates were identified using a smaller number of embryos (Heck et al 1975, Hurlbert 1971) with the function “drarefy” in the R package **VEGAN** (Oksanen et al. 2015). A sufficient number of embryos was sampled when the probability of identifying all evolutionarily relevant mates was larger than 0.95. With this sampling strategy, we may considered an individual an evolutionarily relevant mate that only fertilized a small proportion of embryos overall, but it is unlikely that we did not identify an effective mate.
We analyzed the complete data set, as well as two subsets of the data; one subset contained only potential mating groups with males, the other subset contained potential mating groups without males. For each data set, we carried out conditional logistic regressions, which estimate regression model by maximizing the conditional likelihood, implemented in the function “clogit” of the R package \texttt{SURVIVAL} (Therneau 2015). Conditional logistic regressions are more appropriate than regular logistic regressions because the mating success of any individual depends on the phenotypes of the other individuals in the potential mating group (Kelly et al. 2013). The dependent binary variable was mating success, and explanatory variables were distance to the focal hermaphrodite in mm and penis length in mm in all data sets. Sex (male or hermaphrodite) was an additional explanatory variable in the complete data set, and the data set of potential mating groups with males. We compared models with all different combinations of the explanatory variables in an AIC model selection framework. The best model was the model with a Log_{10} evidence ratio (LER) of zero (Burnham and Anderson 2002, Snipes and Taylor 2014). If a model had a similarly good fit, it had a low LER.

After initial data visualization (Appendix B, Figure B1), it appeared that proximal potential mates had equal probability of mating success, but distant mates were disadvantaged. To test this observation, we divided the data evenly into individuals that were close to the focal hermaphrodite, and individuals that were further away. We carried out a conditional logistic regression for each of the subsets, with distance or penis length as explanatory variables. If distance is more important for more distant potential mates, it should only be significant in the subset of more distant individuals, while penis length should be significant in both subsets.

We compared distance to focal hermaphrodite and penis length between males and hermaphrodites with an analysis of variance (ANOVA). Mating group was a block factor to
account for the fact that penis length and distance should be considered relative to the other members of the mating group.

Occurrence of self-fertilization

We identified isolated hermaphrodites in our initial collection. We considered a hermaphrodite isolated if no other mature hermaphrodite or mature male was in potential mating distance. We assigned parentage to embryos of all brooding solitary hermaphrodites to determine if all embryos were sired by another individual, or self-fertilized. If the embryos were self-fertilized, we would only detect maternal alleles. Outcrossed embryos would have non-maternal alleles in addition to maternal alleles.

Results

Mating group size

We successfully determined parentage of 43 to 133 embryos for each focal hermaphrodite in 11 potential mating groups. The number of males on these hermaphrodites ranged from 0 to 5, and the number of hermaphrodites in potential mating distance ranged from 1 to 9 (Datadryad SI). The number of individuals that fertilized any embryos ranged from one to six, with an average of 2.67 per brood. However, siring success was not evenly distributed among mates (Figure 4.2). The number of evolutionarily relevant mates ranged from 1.02 to 2.42, with an average of 1.71 mates per focal hermaphrodite. The average mating group size was therefore three (1.71 + 1 focal hermaphrodite rounded to the next full number). Neither the
Determinants of mating success

The rarefaction analysis indicated that all evolutionarily relevant mates can be detected by genotyping 11 or more embryos with a probability of 0.95 or higher. Even genotyping as few as three embryos per focal hermaphrodite allows us to detect all evolutionarily relevant mates in six of the 11 potential mating groups. To assure detection of all relevant mates, we attempted to genotype more than 11 embryos per brood, and genotyped successfully 18 embryos on average. In 13 cases, we genotyped less than 11 embryos successfully (Datadryad SI). Exclusion of these potential mating groups from the analysis did not change the results, so they remained in the analysis.

We determined the evolutionarily relevant mates of 46 focal hermaphrodites, each focal hermaphrodite representing a different potential mating group. We already determined the evolutionarily relevant mates for four of these potential mating groups for which we had distance information available in the previous section. The number of hermaphrodites in the potential mating groups ranged from 1 to 13, and the number of males ranged from 0 to 5 (DataDryad SI). Of those potential mating groups, 19 contained both males and hermaphrodites as potential mates, and 27 contained hermaphrodites only. The largest distance of a potential mate to the focal hermaphrodite was 247mm, and the closest potential mate was 1.4mm, a male attached to the operculum of the focal hermaphrodite. Penis length of the potential mates ranged from 1 to 24mm.
The AIC model selection approach of the complete data set as well as the data set containing only potential mating groups with males suggested that the best model contains penis length, distance and their interaction term, but all models containing distance as explanatory variable were similarly good, as indicated by low LER (Table 4.2A; Appendix B, Table B1). All explanatory variables were highly significant in the best model with an overall high predictive power (p = 2.03e-12. Penis length or sex alone did not explain mating success well (Table 4.2A). In all models, proximity increased mating success, as did having a longer penis. In the full model, sex was significant. In stark contrast to our expectations, being male lowered the probability of mating success. Successful mates were proportionally closer to the focal hermaphrodite than all potential mates (Figure 4.3A), while the advantage of a longer penis was less pronounced (Figure 4.3B). When considering only potential mating groups without males, mating success was best explained by distance, but models with both distance and penis length had similar AIC values (Table 4.2B). All results taken together indicate that proximity is more important than penis length in predicting mating success, and males do not have an inherent mating advantage.

When repeating the conditional logistic regression including only individuals closer than 62 mm (the median distance for all individuals), distance was not significant (p = 0.0534), but penis length was (p = 0.0115). When including only more distant individuals, distance was significant (p = 0.000381), but penis length was not (p = 0.0947).

Males were significantly closer to the focal hermaphrodite than hermaphrodites of the same potential mating group (p = 4.363e-14). Males also had shorter penises than hermaphrodites of the same potential mating group (p = 1.562e-15) (Figure 4.4).
Occurrence of self-fertilization

We found 98 isolated hermaphrodites. Of these, two hermaphrodites were brooding. Parentage assignment of 11 embryos of each of these broods showed that each embryo had non-maternal alleles. In addition, all embryos genotyped in this study had paternal alleles. Thus we did not observe self-fertilization in *C. testudinaria*.

Discussion

We characterized the mating system of the androdioecious barnacle *Chelonibia testudinaria*. Unlike most androdioecious species that have been studied more thoroughly, *C. testudinaria* does not self-fertilize, and actual mating groups consist on average of only three individuals. Proximity to mates is the most important determinant of mating success. Despite males being attached to hermaphrodites, and consequently being very close to them, they do not have a mating advantage over hermaphrodites. Thus an important assumption for the evolution of androdioecy, high male mating success, does not apply to *C. testudinaria*. Instead, males may increase their relative fitness via life history traits, most likely through maturing earlier than hermaphrodites (Crisp 1983, Ewers-Saucedo et al. 2015).

Mating group size

Previous studies have linked androdioecy in free-living barnacles with small potential mating groups (Ewers-Saucedo et al. 2015, Kelly and Sanford 2010, Yusa et al. 2013). We show that the number of actual mates is even smaller. As pointed out by Charnov (1982), the actual number of mates is the evolutionary important entity considered by sex allocation theory. In
discordance with this sex allocation theory, hermaphrodites do not have reduced mating success, suggesting that they still produce enough sperm to be competitive mates. Curiously, *Tetraclita rubescens*, a free-living barnacle with a purely hermaphroditic sexual system, has equally small mating groups (Kelly et al. 2012). Taken together, these findings suggest that small mating groups do not lead to sperm-limited hermaphrodites in barnacles.

We discovered a consistent pattern of mating success: the majority of embryos were sired by one or two mates, while other potential mates sired few embryos, if any. A possible explanation of this pattern is that the first mate(s) to reach a receptive hermaphrodite fertilize the majority of her embryos, while mates that reach the hermaphrodite later can only sire a few remaining embryos. This explanation is supported by the fact that proximity to the receptive hermaphrodite increases mating success. Alternatively, a female has many mates, but can choose which mates' sperm fertilizes her brood. To date no study has linked mating success to fertilization success in barnacles, and few studies have looked at either one.

Several possible reasons can explain the presence of unknown mates, both in the analysis of mating group size (Figure 4.2), as well as in seemingly isolated hermaphrodites that were found brooding. On the one hand, mates could have died and fallen off the host before we were able to collect them. *C. testudinaria* hermaphrodites and males leave a short-lived faint mark on the host or hermaphrodite, so we would not be able to detect such events unless they happened very recently. Alternatively, hermaphrodites from different host individuals may get close enough to mate with each other. This is possible because hosts interact closely with each other during their mating activity, e.g. horseshoe crab males hold on to females, laying on top of them for hours.
Determinants of mating success

Mating success was significantly increased by proximity to the mate. This result is in concordance with other studies on barnacle mating systems (Kelly et al. 2013, Yuen and Hoch 2010), and may prove to be a general feature of barnacle mating systems. In addition, we found penis length to be important for mating success even though to a lesser degree. In the acorn barnacle *Tetraclita rubescens*, a measure related to penis length (male gonad weight), did not have a significant effect on mating success (Kelly et al. 2013).

Our results indicate that being too distant to the focal hermaphrodite reduces mating success. However, all individuals within 50 to 60mm have an equal chance of mating with the focal hermaphrodite. Within this radius, penis length becomes important for mating success, with individuals with longer penises being more likely to mate with the focal hermaphrodite. These observations together explain why males do not have an obvious mating advantage despite being close to the focal hermaphrodite: they have no advantage from being the closest potential mate if hermaphrodites are also close by because they have shorter penises than hermaphrodites. This means that males are most likely to be successful mates if hermaphrodites are not in proximity.

The observation that hermaphrodites have high mating success despite small mating group size contradicts Charnov's (1982) theory of sex allocation. Charnov (1982) predicts that hermaphrodites invest little into the male function when mating groups are small, making them sperm-limited, and less successful at fertilizing eggs or ovules. Empirical data consistent with sex allocation theory is abundant in both plants and animals (Baeza 2007, Dorken and Pannell 2008, Sanchez Vilas and Pannell 2012, Schärer 2009, Schärer and Ladurner 2003).
Self-fertilization

Self-fertilization is rarely observed in free-living barnacles (Barnes and Crisp 1956, Kelly et al. 2013). Therefore its absence in *C. testudinaria* is not surprising from a taxonomic perspective. It is also in concordance with theoretical expectations for androdioecious systems with hermaphroditic ancestors (Charlesworth and Charlesworth 1978, Pannell 2002b). It is, however, unusual when compared to other recognized androdioecious species, all of which are characterized by high levels of self-fertilization (Table 4.1).

The mates of the two isolated individuals that were brooding had either died and fallen off the host before we sampled, or were hermaphrodites attached to another host individual, brought within mating distance by close interactions of the host individuals.

Conclusion

The mating system of the androdioecious barnacle *C. testudinaria* differs markedly from the mating system of other recognized androdioecious species. This is not surprising given the different evolutionary trajectory of androdioecious barnacles. While small mating groups and the absence of self-fertilization conform with theoretical predictions for the maintenance of androdioecy from ancestrally hermaphroditic populations such as *C. testudinaria*, the lack of a male mating advantage is unexpected. The mating system is therefore not sufficient to explain the maintenance of androdioecy in *C. testudinaria*. Instead, we propose that sex-specific life history equalizes male and hermaphroditic overall fitness. Males differ from hermaphrodites in two aspects: they are attached to hermaphrodites, and are dwarfed relative to hermaphrodites. Proximity was proposed to lead to a male mating advantage (Charnov 1987, Urano et al. 2009, Yamaguchi et al. 2012), a notion not supported in *C. testudinaria*. The dwarfism of males has
been hypothesized to indicate early maturation and higher relative survival to maturity (Crisp 1983, Yamaguchi et al. 2012). Early maturation would increase the relative fitness of males, especially if mortality rates were high. Indeed, the mortality rate of *C. testudinaria* is higher than in other barnacle species with purely hermaphroditic sexual systems (Ewers-Saucedo et al. 2015). We propose that sex-specific life history is responsible for the maintenance of androdioecy in *C. testudinaria*.

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Therneau TM (2015) A package for survival analysis in S.


**Author contributions**

CES and JPW designed the study. CES, NBH and JPW conducted field collections. CES wrote the manuscript, organized field collections, and carried out data analyses. NBH and CES executed morphological measurements and genetic parentage assignments. JPW and NBH supported manuscript preparation.

**Data accessibility**

Parentage assignment calls, distance and penis length measurements: Dryad
Table 4.1. Mating systems of androdioecious plant and animal species for which extensive empirical data exist. For more complete lists including putative androdioecious species, see Pannell (2002a) and Weeks (2012). We follow Pannell (2002) in his definition of functional androdioecy, which does not include hermaphrodites with size-dependent sex allocation, e.g. the largest or smallest individuals are males, but reproduce as hermaphrodites in one point in their life. The fish *Serranus psittacinus* and *Serranus baldwini*, the corals *Goniastrea australensis* and *Stylophora pistillata* and shrimp of the genus *Lysmata* fall into this category (Baeza et al. 2009, Hastings and Petersen 1986, Kojis and Quinn 1981).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Mating behavior in absence of males</th>
<th>Male mating advantage</th>
<th>Ancestral system</th>
<th>References</th>
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<tr>
<td>Durango root (<em>Datisca glomerata</em>)</td>
<td>NA</td>
<td>Inbreeding depression present, high male fitness, males produce more pollen</td>
<td>dioecy</td>
<td>Rieseberg et al. 1993, Philbrick and Rieseberg 1994, Zhang et al. 2006</td>
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<tr>
<td>Annual vine (<em>Schizopepon bryoniaefolius</em>)</td>
<td>Substantial self-fertilization</td>
<td>Males produce more pollen</td>
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<td>Akimoto et al. 1999</td>
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<td>Annual mercury (<em>Mercurialis annua</em>)</td>
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<td>Males produce more pollen, disperse pollen further</td>
<td>dioecy</td>
<td>Eppley and Pannell 2007, Korbecka et al. 2011</td>
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<tr>
<td>Japanese Ash (<em>Fraxinus lanuginosa</em>)</td>
<td>Substantial self-fertilization</td>
<td>Inbreeding depression present</td>
<td>dioecy</td>
<td>Ishida and Hiura 1998, 2002</td>
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<td>Narrow-leaved mock privet (<em>Phillyrea angustifolis</em>)</td>
<td>Density-dependent self-fertilization</td>
<td>Inbreeding depression present, two self-incompatible hermaphroditic morphs</td>
<td>dioecy</td>
<td>Vassiliadis et al. 2002, Saumitou-Laprade et al. 2010</td>
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<tr>
<td>Nematode (<em>Caenorhabditis elegans</em>)</td>
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<td>Hermaphrodites are sperm-limited, male sperm is more competitive</td>
<td>dioecy</td>
<td>Ward and Carrell 1979, Wood 1988</td>
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<tr>
<td>Organism</td>
<td>Self-fertilization</td>
<td>Inbreeding depression</td>
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<td>Mangrove killifish (Kryptolebias marmoratus)</td>
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<td>Hermaphrodites are sperm-limited</td>
<td>Mackiewicz et al. 2006a, 2006b</td>
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Table 4.2. AIC model selection results. The best model has a LER of 0, and large LER indicate models with poor predictive power. (A) Analysis of potential mating groups with both hermaphrodites and males as potential mates. (B) Analysis of potential mating groups with hermaphrodites, but not males, as potential mates. Abbreviations: AIC = Akaike information criterion, AIC_c = AIC corrected for small sample size, w = Akaike weights, LER = Log_{10} evidence ratio.

(A)

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<th>LER</th>
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(B)

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<td>65.54</td>
<td>0.51</td>
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<tr>
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<tr>
<td>penis length</td>
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<td>103.86</td>
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<td>19.16</td>
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Figure 4.1. Labeling scheme, shown on a horseshoe crab with *C. testudinaria*. We labeled all hermaphrodites in a potential mating group in pictures, and the barnacle itself, and chose a brooding hermaphrodite as the focal hermaphrodite. All other hermaphrodites were considered potential mating partners.
**Figure 4.2.** The distribution of mating success within each brood. Each plot shows a mating group. Each bar is a potential mate, and the height of the bar corresponds to the proportion of embryos sired by each potential mate. Colors denote the gender of the mate: blue for males, grey for hermaphrodites, and white for unknown mates that were inferred from the genotyping data. The filled circles (and lines) represent the probability of identifying a mate from ten genotyped embryos, as calculated from rarefaction curves.
Figure 4.3. Determinants of mating success. (A) Distribution of the distance to the focal hermaphrodite for all potential mates (light grey bars) and for successful mates (shaded bars). (B) Distribution of penis length of all potential mates (light grey bars) and successful mates (shaded bars).
Figure 4.4. Box plots of penis length and distance to focal hermaphrodite for males and hermaphrodites. Data only includes mating groups with both males and hermaphrodites as potential mates.
CHAPTER 5
SEX-SPECIFIC LIFE HISTORY CAN MAINTAIN MALES AT HIGH FREQUENCIES IN AN ANDRO DioECIOUS BARNACLE

Abstract

Androdioecy, a sexual system characterized by the coexistence of hermaphrodites and males, is rare in nature. Theory suggests that a male mating advantage is most likely to maintain androdioecy, but sex-specific life history can also increase the relative fitness of males. We tested if survival to maturity is sex-specific in the androdioecious and epizoic barnacle *C. testudinaria*, and if those differences suffice to maintain androdioecy. We find that males matured when about a month old, while hermaphrodites matured when four to five years old. Relative larval mortality was between 1.05 and 10.6 times higher for males than for hermaphrodites. Fitting models of androdioecy showed that androdioecy is only evolutionary stable in *C. testudinaria* on loggerhead sea turtles, not on horseshoe crabs or blue crabs. However, given the high abundance of *C. testudinaria* from loggerhead sea turtles, androdioecy can be maintained on all hosts. The predicted male frequency of 0.3 fits empirical estimates of 0.4 reasonably well.
Introduction

One of the rarest sexual systems is androdioecy, the coexistence of males and hermaphrodites in reproductive populations (Weeks 2012). Its rarity is matched by the difficulty of males to match the reproductive output of hermaphrodites. Equal reproductive output, however, is necessary for the evolutionary stability of androdioecy - if the sexes do not differ in any other aspect (Lloyd 1975, Charlesworth and Charlesworth 1978, Charnov et al. 1987). In congruence with this theoretical expectation, males are more successful mates in androdioecious plants and animals (Rieseberg et al. 1993, Akimoto et al. 1999, Eppley and Pannell 2007, Ward and Carrell 1979, Sassaman 1995, Mackiewicz et al. 2006a, 2006b).

In addition to high male mating success, most theories consider an additional explanation for the maintenance of males and hermaphrodites: sex-specific differences in life history. Higher survival to maturity, in particular, should increase relative male fitness and, in consequence, the frequency of males (Lloyd 1975, Crisp 1983, Charnov 1987, Urano et al 2009, Yamaguchi et al. 2012). While the mating advantage hypothesis has been widely tested (e.g. Akimoto et al. 1999, Ishida and Hiura 2002, Pannell and Ojeda 2000, Weeks et al. 2014, Ellison et al. 2013), empirical evidence for the life-history hypothesis remains limited to sex-specific longevity (e.g. Weeks et al. 2014, Zucker et al. 2001). This is somewhat surprising given that males are commonly smaller than hermaphrodites (Weeks 2012), and small size is a possible sign of early maturation (Crisp 1983, Vollrath 1998). Maturing younger, however, increases survival to maturity if we assume equal mortality rates. Sexual size dimorphism is particularly pronounced in androdioecious barnacles (Darwin 1851, McLaughlin and Henry 1972, Klepal 1987), which are therefore good candidates to investigate sex-specific life history, especially sex-specific survival to maturity.
We investigated sex-specific life history in the androdioecious epizoic barnacle *Chelonibia testudinaria* (Linnaeus, 1785) by assaying populations of *C. testudinaria* along the East coast of the USA. We first tested the hypothesis that the age at maturation differs between hermaphrodites and males, and secondly that these differences provide an evolutionary explanation for the maintenance of androdioecy.

**Material and methods**

**Study organism**

Hermaphroditic *C. testudinaria* live attached to phylogenetically diverse marine animals (Hayashi 2013). These hosts provide a highly unstable and variable substrate, leading to high mortality rates and host-specific growth rates in *C. testudinaria* (Chapter 2). Males are dwarfed and attached to hermaphrodites (Crisp 1983, Zardus and Hadfield 2004), which are features of all male barnacles (Darwin 1851, Klepal 1987, Kelly and Sanford 2010). Reproduction and dispersal in *C. testudinaria* follows the typical barnacle schedule: Adult barnacles pseudo-copulate using a long penis; fertilized eggs are brooded for some time before being released into the water column as free-swimming larvae; larvae undergo six feeding naupliar stages and a non-feeding cypris stage, which attaches to suitable substrate; the attached cypris metamorphoses into the juvenile barnacle form.
Field collections and measurements

We collected *C. testudinaria* from the loggerhead sea turtles *Caretta caretta* (Linnaeus, 1758), the blue crab *Callinectes sapidus* Rathbun, 1896, and the horseshoe crab *Limulus polyphemus* (Linnaeus, 1758) along the US Atlantic coast from Delaware to Florida and in the Gulf of Mexico on the Mississippi coast from 2011 to 2015 as described in Ewers-Saucedo et al. (Chapter 2). We noted the prevalence of *C. testudinaria*, that is, how many host individuals were fouled by it. We measured basal rostro-carinal diameter of each barnacle with calipers to the nearest 100 μm. This size measurement was used to approximate age based on linear host-specific growth rates (Chapter 2). We determined the maturation status of a subset of individuals (males and hermaphrodites) by the presence of the penis. We also measured penis length to the closest 0.5mm with a ruler for a subset of individuals. Barnacles with highly irregular shell shape were excluded from the analyses.

Survival to maturity

The penis is a minimal requirement for male maturity. It is necessary for copulation, but it is developed before testis and vesicular seminalis are fully formed (Crisp 1983). Ewers-Saucedo et al. (Chapter 4) showed that individuals with a penis as short as 2-3mm can be successful mates, suggesting that penis presence is a good indicator of maturation. We tested for differences in age at maturation between hermaphrodites and males from different host species with logistic regressions.

The age at which penis length is zero provided an additional measure of age at maturation. To obtain this estimate, we carried out linear regressions for each sex and host with penis length as explanatory variable, and age as dependent variable. The intercept with the y-axis
is the desired estimate. While this approach is biologically incorrect (penis length does not explain age, the correct relationship is the opposite), it provides us with error estimates for the age of maturation.

Survival to maturation \((v)\) is the inverse of the product of mortality rate \((Z)\) and age at maturation \((m)\). Mortality rates differ between the sexes (Chapter 2). An inevitable cause of sex-specific mortality is that males are attached to older hermaphrodites, and die when the hermaphrodite dies. This mortality is likely to occur after individuals reach maturity. Two other mortalities need to be considered: larval mortality and juvenile mortality of barnacles that have settled, but not yet reached maturity. We expect differences in larval mortality between the sexes because of their unequal settlement opportunity assuming sexes are genetically determined. Male larvae have to settle on a hermaphrodite, while hermaphroditic larvae can presumably settle on any host (Charnov 1987). Male larvae have only a fraction of the settlement opportunities of hermaphrodites. We assessed this difference by comparing the prevalence of hermaphrodites on each host. The more host individuals are fouled by hermaphrodites, the higher the probability of male larvae to find a settlement location. The probability of hermaphrodite larvae to find a settlement location only depends on the abundance of hosts. Thus relative larval mortality is the proportion of hosts with barnacles, the barnacle prevalence. Hosts or hermaphrodites are never completely covered by \(C.\ testudinaria\) hermaphrodites and males, respectively, thus settlement is possible whenever the minimal requirements for each sex are met. We used prevalence estimates obtained during the field assays as well as values from the literature. It is not clear if juvenile mortality rates differ early in life. Given a lack of data on early-life mortality rates, we assume that mortality rates did not differ at this stage.
Empirical male frequencies

We calculated male frequencies as the number of mature males divided by the number of mature individuals (males plus hermaphrodites). We used the fitted logistic regression of penis presence (see age at maturation) to predict the maturation status of all collected individuals based on their age, sex and host. We determined an individual to be mature if the predicted probability of having a penis was larger than 0.5. We tested how well this model predicted penis presence by comparing predicted penis presence with actual assessments.

The scale at which male frequencies should be calculated depends on the scale at which male frequencies are determined. While the sex determination mechanism is unknown for *C. testudinaria*, host-specific genetic structure is absent (Ewers-Saucedo et al., submitted). Thus a population-wide male frequency might be most appropriate, even though the possibility of male frequency adjustment at a smaller scale cannot be excluded (Svane 1986). We calculated the population-wide male frequency, and male frequencies for each host individual. In the latter, we excluded host individuals without large hermaphrodites, as those are necessary for males to settle on. We used the male frequencies calculated per host individual to test if male frequencies differed between host species, month or year collected by means of ANOVA after ensuring normal distribution of the data.

Evolutionary significance of survival to maturity

We evaluated if differences in survival to maturity are sufficient to maintain androdioecy in *C. testudinaria*. We considered two measures: a minimal requirement similar to that proposed by Yamaguchi et al. (2012) and an evolutionary stable state (ESS) model (Lloyd 1975). The minimal requirement for the evolution of androdioecy is that the relative survival to maturity of
male over hermaphrodites exceeds two (Yamaguchi et al. 2012). A more quantitative approach was taken by Lloyd (1975). He constructed an ESS model, which predicts frequency of males based on outcrossing rate (t), strength of inbreeding depression (i), relative mating advantage of hermaphrodites over males (l) and relative survival to maturity of hermaphrodites over males (v):

\[
q = \frac{t - 2lv(t - i - ti)}{2iv(1 - r)(1 - l) + r(1 + v - 2iv)}.
\]

Ewers-Saucedo et al. (Chapter 4) showed that self-fertilization is absent (t=1, i=0) and that males do not have a mating advantage (l=1), thus reducing the model to:

\[
q = \frac{1 - 2v}{1 + v - 2v}
\]

Note that the minimal requirement is based on the male:hermaphrodite ratio, while the ESS model considers hermaphrodite:male ratios.

Results

Field collections and measurements

We collected 2800 hermaphrodites and 1293 males. We were able to collect *C. testudinaria* from all host species between April and August, and our analyses are based on those data. The smallest individuals were 1mm in basal diameter. Maximal size was 60mm for hermaphrodites and 25mm for males; estimated age ranged from 9 to 813 days for hermaphrodites and 14 to 364 days for males. Size and age distributions of *C. testudinaria* are described in more detail in Ewers-Saucedo et al. (Chapter 2). We checked for the presence of a penis in 1431 hermaphrodites and 390 males of varying age. Of those, 444 individuals were
collected from blue crabs, 773 from horseshoe crabs and 604 from loggerhead sea turtles. We measured penis length of 1073 hermaphrodites and 195 males.

Survival to maturity

The logistic regression showed that males matured at a significantly earlier age than hermaphrodites in all host-specific comparisons (p < 2e-16). Hermaphrodites on loggerhead sea turtles matured earlier than hermaphrodites on the other hosts (p < 1.35e-06), and males on loggerhead sea turtles matured earlier than males on other hosts (p < 0.00011) (Figure 5.1A). Using penis length to obtain estimates of age at maturation showed that hermaphrodites from horseshoe crabs matured significantly later than hermaphrodites from the other two hosts (p = 0.000321) (Figure 5.1B). Males from blue crabs were oldest when developing a penis. Males from horseshoe crabs were significantly younger (p = 0.0168), as were males from loggerhead sea turtles (p = 9.97e-09). Both logistic regression and linear regression estimated an average age of maturation for hermaphrodites between 120 and 190 days, and an average age at maturation for males between 24 and 45 days (Table 5.1A). Subsequent analyses are based on the age of maturation estimates from penis length regression because standard errors are available for those estimates.

We estimated that *C. testudinaria* was prevalent on 9.4% of blue crabs, 25.1% of horseshoe crabs and 95.3% on loggerhead sea turtles (Datadryad SI). We used these prevalences as proxies for relative mortality rates of males.
Empirical male frequencies

The male frequency for the complete sample of mature individuals was 0.399 (binomial CI = 0.37-0.43), and host-specific frequencies were comparable (Table 5.1B). Male frequencies varied substantially between host individuals (Figure 5.1C). Neither host (p=0.3700) nor year (p=0.1742) were able to explain this variation. Month was significant (p=0.0137) but a subsequent Tukey HSD test did not identify any significant pairwise differences (p > 0.1769 for all comparisons).

Evolutionary significance of survival to maturity

Based on the age at maturation and relative larval mortality, the relative survival to maturity (male:hermaphrodite ratio) ranged from 0.2519427 to 4.6608626 (Table 5.1A). Only males on loggerhead sea turtles had a at least a two times higher probability of reaching maturity than hermaphrodites, the minimal requirement for the evolution of androdioecy. Parameterizing the model of Lloyd (1975) with host-specific values for relative survival to maturity led to expected male frequencies between 0 and 0.727 (Table 5.1B).

Discussion

Androdioecy, the coexistence of males and hermaphrodites, is difficult to explain in evolutionary terms. It requires that males “make up” for their lack in female sexual function. Empirical studies to date have mostly explored how mating system and sex allocation facilitate androdioecy. Conversely, the effects of sex-specific life history are less frequently considered. In the present study, we show that higher survival to maturity of males alone provides an evolutionary
explanation for the maintenance of androdioecy in an epizoic barnacle – on one of the three sampled host species. This highlights the importance of large-scale sampling from diverse habitats to account for natural variability.

Male frequencies

Male frequencies were highly variable among host individuals, suggesting that settlement is a stochastic process. This is not surprising; barnacle larvae have to find a “moving target”, their host, and males have to find a host with larger *C. testudinaria*. We suggest that a population average captures the male frequency best because of a lack of host-specific or regional genetic population structure. However, larvae may be able to adjust their sex at settlement, and sensing which host species they will settle on may change their sex choice. Better understanding the scale at which male frequencies should be estimated, would clearly require understanding the sex determination mechanism. By calculating one population-wide male frequency, we assume that larvae are one homogenous group, that gets completely mixed during their free-swimming phase, and that *C. testudinaria* cannot adjust their sex at settlement.

Our empirical estimates of sex-specific life history suggest that only *C. testudinaria* on loggerhead sea turtles are able to sustain androdioecy. In the absence of host-specificity (Ewers-Saucedo et al., submitted), and under the assumption of complete genetic sex determination, the observed male frequencies are a fitness compromise across all hosts. Using rough estimates of the overall abundance of all hosts, and the prevalence and fouling intensity of *C. testudinaria*, we can calculate how many barnacles are attached to each host (Datadryad SI). Multiplying the relative abundance of *C. testudinaria* on each host with the predicted male frequency provides us with a new, averaged, expected male frequency. While estimates of host abundance should be
considered with caution, it appears that 48% of *C. testudinaria* in the Southeastern US are attached to blue crabs, 42% to loggerhead sea turtles, and only 10% to horseshoe crabs. The adjusted overall male frequency becomes 0.3, which is closer to the empirical male frequency of 0.4. *C. testudinaria* also settles on some other host species, such as manatees and terrapin turtles (Hayashi 2013). We do not know if those hosts are suitable to sustain androdioecy. However, given the low overall numbers of *C. testudinaria* on those hosts (Datadryad SI), they do not change our inference.

**Mortality rates**

In this study, we made the assumption that juvenile mortality rates of males and hermaphrodites are equal until they reach maturity. While males have an overall higher mortality rate than hermaphrodites (Chapter 2), mortality may increase only after they reach maturity, effective when the hermaphrodites they are attached to die. However, it is possible that males and hermaphrodites have different mortality rates because they experience different environments. Males are raised higher above the host surface, possibly facilitating dislodgment. On the other hand, the shell of the hermaphrodite provides a number of crevices that are often used by males (Crisp 1983, Zardus and Hadfield 2003). These crevices may prevent dislodgment. Further studies on juvenile mortality need to be conducted to clarify this issue. Such studies are challenging because they require the capture and re-capture of host individuals throughout a season. In addition, unequal settlement opportunity of the two sexes leads to frequency-dependent larval mortality. We expected higher male larval mortality because male larvae have to find a hermaphrodite to settle on, while hermaphroditic larvae can presumably settle on any host (Charnov 1987). We accounted for these differences by considering the
proportion of hosts fouled by *C. testudinaria*. The higher this proportion, the easier it should be for male larvae to find a settlement location.

Age at maturation

The age at maturation was similar among hermaphrodites and males from different host species, and most barnacles of the same sex matured within 30 days of each other (see confidence intervals). Males matured when about one month old, while hermaphrodites matured when four to five months old. Without differences in mortality, the early maturation of males would sustain androdioecy on all host species.

Implications for the evolution and maintenance of androdioecy

Androdioecy has been an enigma since its discovery by Darwin (1851). Explaining the evolution and maintenance of androdioecy has proven difficult, both theoretically (Charlesworth 1984) and empirically (e.g. Weeks et al. 2014). Our study highlights the importance of sex-specific life history, and how it can help (early maturation) and hamper (high male mortality) in the maintenance of androdioecy. Moreover, assessing life history across a broad range of habitats was necessary to explain androdioecy in *C. testudinaria*. The discrepancy between the empirical and expected male frequency is likely caused by inaccurate estimates of host abundance, or by sex-specific juvenile mortality. The difficulty of assessing these factors is a result of the difficulty of tracking marine animals in general, and the hosts of *C. testudinaria* in particular. However, given that all host species are of conservation concern, better abundance estimates and ways of tracking these animals may allow us to improve our estimates. In particular, we would predict that either juvenile mortality is lower for males than for hermaphrodites, or that
loggerhead sea turtles are more abundant than previously thought (Mike Arendt, In-Water Sea turtle Trawl project leader, pers. comm.).

Acknowledgements

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References


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Table 5.1. (A) Estimates of age at maturation of hermaphrodites ($m_h$) and age at maturation of hermaphrodites ($m_m$), relative larval mortality of males ($Z$), and relative survival to maturity of males ($v$). Hermaphrodite larval mortality and hermaphrodite survival to maturity is one. Age at maturation was estimated with logistic regression by assessing when the odds of having a penis was one (“log.”), and by assessing when the penis length was zero, using a linear regression of penis length against age (“linear”). Both estimates are provided here, but the subsequent analyses are conducted with the linear estimates because we were able to calculate the error around them.

(B) Estimates of the male frequency $q$, as predicted by fitting the model of Lloyd (1975) (“Lloyd”), and host-specific empirical estimates (“empirical”). Confidence intervals are in parentheses.

(A)

<table>
<thead>
<tr>
<th>Host</th>
<th>$m_h$ (log.)</th>
<th>$m_h$ (linear)</th>
<th>$m_m$ (log.)</th>
<th>$m_m$ (linear)</th>
<th>$Z$</th>
<th>$v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue crab</td>
<td>184.07</td>
<td>119.806 (102.874-136.73)</td>
<td>44.7 (26.259-63.1)</td>
<td>0.09</td>
<td>0.25</td>
<td></td>
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<tr>
<td>Horseshoe crab</td>
<td>189.58</td>
<td>156.257 (136.463-176.05)</td>
<td>36.156 (11.683-60.62)</td>
<td>0.25</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>Loggerhead</td>
<td>151.93</td>
<td>122.121 (100.519-143.72)</td>
<td>24.97 (-7.901-57.8)</td>
<td>0.95</td>
<td>4.66</td>
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</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Host</th>
<th>$q$ (Lloyd)</th>
<th>$q$ (empirical)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue crab</td>
<td>0</td>
<td>0.422 (0.387–0.457)</td>
</tr>
<tr>
<td>Horseshoe crab</td>
<td>0</td>
<td>0.33 (0.305–0.357)</td>
</tr>
<tr>
<td>Loggerhead</td>
<td>0.727 (0.465–0.988)</td>
<td>0.456 (0.428–0.48)</td>
</tr>
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Figure 5.1. Empirical estimates of age at maturation, and male frequencies. (A) Fitted probabilities of hermaphrodites (“H”) and males (“M”) to possess a penis given their age, host and sex. The 0.5 probability was taken as the average age of maturation. Colors denote the host: black = blue crab, red = horseshoe crab, green = loggerhead sea turtle. (B) Age plotted against penis length. The intercept with the y-axis denotes the average age at maturation. Colors and symbols as in A. Solid lines refer to hermaphrodites, dashed lines to males. (C) Empirical male frequencies per host individual, plotted by host species. Host individuals without large hermaphrodites were omitted. Note the large variance in male frequencies.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation aimed to understand androdioecy in an odd creature, the barnacle. Barnacles are unusual in several aspects (e.g. Ewers and Wares 2012), but have been highly successful (Newman and Abbott 1980). I began my work by putting together a checklist of sorts, prompted by theoretical expectations, and results of previous empirical work. The checklist is realized by the four chapters of my dissertation. Of my findings, the absence of a male mating advantage was most surprising (Chapter 4), followed shortly thereafter by the recognition that a males' life history is all that is needed to maintain androdioecy (Chapter 5). Combined with the possibility that androdioecy has been maintained for million of years in this species (Chapter 3), it suggests that androdioecy in *C. testudinaria* is not a transitional stage towards dioecy, but rather an evolutionary stable state.

**One for all, and all for one?**

My findings provide an explanation for the evolution and maintenance of androdioecy, but not for its transition to dioecy, as commonly proposed (Charnov 1987, Charlesworth 2006): Androdioecy is thought to be an intermediate stage between hermaphroditism and dioecy because, the smaller the mating group, the more female-biased hermaphroditic sex allocation. This sex allocation adjustment first allows the invasion of males. If mating groups get even smaller, hermaphrodites abandon the male function altogether, and the sexes become completely separate. We see these expectations fulfilled in a number of androdioecious barnacle species,
which have close dioecious relatives (Yusa et al. 2013). In *C. testudinaria*, on the other hand, we
do not see reduced reproductive success in hermaphrodites, even though mating groups are small
(Ch. 2). Therefore we infer that mechanisms leading to androdioecy and dioecy in *C.
testudinaria* differ from barnacle species with poorly-developed penises and close dioecious
relatives.

Interestingly, there are no dioecious barnacles within the acorn barnacles, only within the
other major clade of barnacles, the gooseneck barnacles. It may be that sex allocation adjustment
is limited to certain branches of the barnacle phylogeny, or to certain habitats – two factors that
are not independent in barnacles (Anderson 1994). Moreover, it emphasizes the idea that
different mechanisms are responsible for the invasion of males into hermaphroditic systems and
the conversion of hermaphrodites into females. It would be interesting to compare my results to
androdioecious barnacle species with close dioecious relatives, i.e. where androdioecy can
transition into dioecy.

**Caution! Missing information on sex determination**

My results are indicative of the adaptive nature of androdioecy: males have a high
relative fitness, and function to disseminate alleles faster. However, the last requirement of an
adaptative trait, its inheritance, has not been confirmed here. In the case of *C. testudinaria*, we do
not know the sex determination mechanism. Moreover, the models I used throughout my
dissertation assume genetic sex determination (Charnov 1987). So we must ask: Could the mode
of sex determination change the results? What if the system is completely based on
environmental sex determination, as suggested by Crisp (1983)? The answer is: I am not sure but
would certainly require adjustment of theoretical expectations. What I propose is that settlement
onto conspecifics is a genetic trait. I base this hypothesis on the anecdotal observation that some species “allow” settlement onto each other, some do not. For example, I have never seen small individuals on the congener of *C. testudinaria* sensu lato, *C. caretta*, but this observation needs to be confirmed with larger-scale sampling. If this settlement behavior has a genetic basis, it can be selected on. And settlement onto conspecifics may be the first step towards dwarfism, and abandoning the female function. This argument requires that barnacle larvae can recognize conspecific during the settlement process. Given the general gregarious settlement behavior of barnacles, and experimental evidence (Knight-Jones 1953, Crisp 1961, Dreanno et al. 2007, Matsumara and Quian 2014), conspecific recognition exists. We cannot exclude the possibility that androdioecious species lost the ability to recognize conspecifics, and their settlement behavior is the result of unspecific settlement. This would mean conspecific settlement is not adaptive. If fitness costs are high for those settled on others (as we might expect), the trait should never spread through a population. The next step will certainly be to investigate sex determination with settlement experiments, transplant experiments, and gene expression studies, which have provided valuable insight into the evolution of major life history traits of marine invertebrates (Kissinger and Raff 1998, Hoegh et al. submitted).

**Should the question be: Why is androdioecy rare?**

Given the high relative fitness of males over hermaphrodites, and the fact that this fitness increase is likely caused by early maturation of males and their short generation time, one could ask the question: Why are dwarf males not more common? We can certainly imagine that some individuals mature earlier, so there should not be a mechanistic hindrance. The answer will most commonly be that being small is dangerous and disadvantageous. Small individuals are more
susceptible to predation, poor competitors, and are unable to produce large amounts of pollen or sperm. Therefore the success of dwarf males should be restricted to habitats with low predator pressure, little resource competition and low levels of mate competition; this is precisely what we find in *Chelonibia testudinaria*. Its epibiotic life-style limits the occurrence of many predators, such as snails, and also makes it uninhabitable for most other barnacle species (Caine 1986, Key et al. 1997, Pfaller et al. 2008). In the second chapter, we discuss that mating groups are small, which also suggests that producing large amounts of sperm is not necessary. This theory is further supported by the fact that many androdioecious barnacle species are epibiotic (Kelly and Sanford 2010).

“Oligogamy” and self-fertilization in barnacles

Mating systems in barnacles are not well-studied. However, from various observational and experimental evidence, we can infer that mating groups are not always small, and sometimes scale with the potential mating group size (see Ch. 2 for definition). There has been a longstanding debate on the advantages of polyandry. What is the advantage for females to mate multiple times, if all eggs could be fertilized by mating only once? This is particularly of interest if the cost of mating is high. Answers suggest increased fitness through higher genetic variation (Pinzone and Dyer 2013, Gowaty 2013). In barnacles, the cost of mating is that of higher predation risk; during mating, their feeding legs are exposed, and the penis must reach all the way across to the receptive female. Even though predation would be sublethal in most cases, it incurs high fitness costs (Meyer and Byers 2005). Given the high cost for both mating partners, it is not surprising that monogamy, or oligogamy (mating with few), is sustained in barnacles.
We commonly think that mating systems in barnacles lack self-fertilization, which might pre-dispose them for the invasion of males (Lloyd 1975). However, some evidence suggest that as many as 50% of barnacle species – though not *C. testudinaria* (Chapter 2) – can self-fertilize. Under such mixed mating strategies, we would expect very different evolutionary strategies to evolve. The role of inbreeding depression can be pronounced (Chang and Rausher 1999), and should be explored further in these marine arthropod systems.

**Darwin, Charnov and barnacles: Back to the future**

The study of androdioecy in barnacles has a long and interrupted history. The ground work was laid by Darwin (1851, 1854), followed by a phase in which androdioecy was deemed impossible, to its instrumental role in defining sex allocation theory by Charnov (1982), and a renewed interest in the past ten years. We are just now beginning to understand ultimate and proximate causes of androdioecy. Ultimately, it appears that the life history, mating system and ecology have to come together just right to allow males and hermaphrodites to co-exist. Proximately, genetic sex determination mechanisms are prevailing (e.g. in plants and other androdioecious barnacles), even though environmental sex determination plays a role as well (Pannell 1997, Sassaman and Weeks 1993, Wolf et al. 2001, Gomez 1975, Svane 1986). It will be interesting to expand the sample size of androdioecious barnacles studied, and study the apparent continuum of sexual systems found in barnacles. At which point are sexes genetically determined? Are certain taxa of barnacles pre-adapted because they “allow” settlement on each other? How do different kinds of mating system facilitate androdioecy? Despite more than 160 years of research on androdioecy in barnacles, insights are hard to gain, and questions have multiplied - a common “side effect” of the scientific quest into the unknown. Future work may
not only lead us into the abstract unknown within the evolutionary space of possibility, but into the physical unknown: most androdioecious barnacles are constrained to the deep sea and the arctic regions.

“Man cannot discover new oceans unless he has the courage to lose sight of the shore.”
Andre Gide

References


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Knight-Jones, E. W. 1953. Laboratory experiments on gregariousness during setting on *Balanus balanoides* and other barnacles. The Journal of Experimental Biology 30:584-598.


APPENDIX A

Table A1. Microsatellite loci primer sequences and multiplex scheme. All loci were amplified at 55°C annealing temperature.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Dye</th>
<th>Multiplex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctest2</td>
<td>ACACACATCAGTGGACTCG</td>
<td>CAGTAAGCAGCTCTGTTCG</td>
<td>NED</td>
<td>BB</td>
</tr>
<tr>
<td>Ctest7</td>
<td>GTTATCCGTCATTCCATCC</td>
<td>GACGTAACCACCTTGTCG</td>
<td>6-FAM</td>
<td>AA</td>
</tr>
<tr>
<td>Ctest9</td>
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<td>TTGTACTGTCTTGTAAACGC</td>
<td>6-FAM</td>
<td>BB</td>
</tr>
<tr>
<td>Ctest10</td>
<td>ATACGCACAAAATCTACACC</td>
<td>TGTCCCTCTTACAGAGATCGG</td>
<td>HEX</td>
<td>BB</td>
</tr>
<tr>
<td>Ctest11</td>
<td>GTGTCACCTTTATGTCTTG</td>
<td>AGTTGAAAATACGCACGC</td>
<td>HEX</td>
<td>CC</td>
</tr>
<tr>
<td>Ctest12</td>
<td>ACCTGGGTGAGCATTCTGG</td>
<td>CATCTTTATGAGTCGAGG</td>
<td>HEX</td>
<td>AA</td>
</tr>
<tr>
<td>Ctest16</td>
<td>TCAGGTACAGCATTATGC</td>
<td>CAAGGACCATCAATTACC</td>
<td>6-FAM</td>
<td>CC</td>
</tr>
<tr>
<td>Ctest18</td>
<td>TCTATGACATCTCTCTGG</td>
<td>GAAATCATAAAAAAGGCGATGC</td>
<td>NED</td>
<td>AA</td>
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<tr>
<td>Ctest31</td>
<td>GTACGCCGAAGTTAAAGC</td>
<td>AGGTCTCTGAACAGTTATGCC</td>
<td>6-FAM</td>
<td>DD</td>
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<tr>
<td>Ctest32</td>
<td>AGAAATCCATAATCGTCTGG</td>
<td>ATACGACGGTACAGCACC</td>
<td>NED</td>
<td>CC</td>
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<tr>
<td>Ctest36</td>
<td>AGATATGGTGGAACGAGC</td>
<td>CACACATACCTCAACGAGC</td>
<td>HEX</td>
<td>DD</td>
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<td>Ctest47</td>
<td>GTGACACGATGACATAACG</td>
<td>ACAATTCCAGCTCTGTTCAGC</td>
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<td>DD</td>
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</table>
Figure A1. DAPC results of genotypic data of individuals from the whole range sampled. (a) The first two principal components of the DAPC are plotted, using host species as prior grouping by which the data are partitioned. Colors identify the host species, and each dot represents an individual. (b) Bar graph of membership probabilities of each individual to the pre-assigned groupings. Colors denote groups, while each bar represents an individual. (c) Bar graph of the
relative contributions of alleles to the DAPC principal components. Alleles with contributions larger than 0.05 are considered significant. Each vertical bar represents an allele. (d) Line plot of allele frequencies with significant contributions for each pre-assigned grouping.
APPENDIX B

Table B1. AIC model selection results for the complete data set (potential mating groups with and without males combined). The best model has a LER of 0, and large LER indicate models with poor predictive power. Abbreviations: AIC = Akaike information criterion, AICc = AIC corrected for small sample size, w = Akaike weights, LER = Log\textsubscript{10} evidence ratio.

<table>
<thead>
<tr>
<th>model</th>
<th>AIC</th>
<th>AICc</th>
<th>w</th>
<th>LER</th>
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</thead>
<tbody>
<tr>
<td>distance * penis length</td>
<td>142.34</td>
<td>142.43</td>
<td>0.7</td>
<td>0</td>
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<tr>
<td>distance + penis length</td>
<td>145.51</td>
<td>145.56</td>
<td>0.15</td>
<td>1.57</td>
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<tr>
<td>sex + distance + penis length</td>
<td>146.67</td>
<td>146.76</td>
<td>0.08</td>
<td>2.17</td>
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<tr>
<td>sex + distance</td>
<td>147.4</td>
<td>147.44</td>
<td>0.06</td>
<td>2.51</td>
</tr>
<tr>
<td>distance</td>
<td>150.02</td>
<td>150.04</td>
<td>0.02</td>
<td>3.81</td>
</tr>
<tr>
<td>penis length / distance</td>
<td>181.31</td>
<td>181.33</td>
<td>0</td>
<td>19.45</td>
</tr>
<tr>
<td>penis length / distance + sex</td>
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<td>181.51</td>
<td>0</td>
<td>19.54</td>
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<tr>
<td>penis length</td>
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<td>193.72</td>
<td>0</td>
<td>25.65</td>
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<tr>
<td>sex + penis.length</td>
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<td>194.63</td>
<td>0</td>
<td>26.1</td>
</tr>
<tr>
<td>sex</td>
<td>195.81</td>
<td>195.82</td>
<td>0</td>
<td>26.7</td>
</tr>
</tbody>
</table>
**Figure B1.** Effect of distance to the focal hermaphrodite on mating success. Each symbol denotes a potential mate. Blue circles denote males, black crosses hermaphrodites. Lines are drawn between potential mates of the same potential mating group.