

Doctoral Thesis

**Evolutionary ecology of complex sexual systems  
in marine animals**

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## Table of contents

Abstract	3-4
General Introduction	5-22
Study 1	23-36
Dwarf males in the epizoic barnacle <i>Octolasmis unguisiformis</i> and their implications for sexual system evolution	
Study 2	37-54
Sexual and mating systems in an androdioecious barnacle: testing the within-species effect of group size	
Study 3	55-83
Be a good loser: a theoretical model for subordinate decision making about bi-directional sex change among harem fish	
Study 4	84-100
Ecological background of sexual plasticity: population fluctuation and life history of a bi-directional sex changer	
General Discussion	101-115
Acknowledgements	116
References	117-140

# Abstract

Marine animals sometimes show complex sexual systems which can be described as combinations of different sexuality such as androdioecy and bi-directional sex change, offering unique opportunities to study sexual system evolution. The broad aim of my thesis is to test the hypothesis that different sexuality within a complex sexual system can be explained by spatiotemporal fluctuation of mating characteristics.

Barnacles have diverse sexual systems. I confirmed androdioecy in a barnacle *Octolasmis unguisiformis* with small mating groups, and demonstrated different life history between dwarf males and hermaphrodites. The theoretically predicted correlation between sex ratio and mating group size was tested and not significant. These results may indicate that different factors affect within- and cross-species patterns of barnacle sexuality.

Bi-directional sex change in fishes, or reversed sex change in basically protogynous species, is induced by the cohabitation of multiple males. I studied why and when subordinate males prefer sex change over dispersal, although most of them are polygynous. I constructed a theoretical model of decision making and predicted that low density and risk of dispersal favor sex change. My field survey on a bi-directionally

sex changing goby *Trimma caesiura* demonstrated a large population fluctuation, suggesting that temporal low density caused by fluctuation promotes sex change.

In summary my studies generally supported the effect of mating characteristics on sexual system evolution, although other factors such as dispersal cannot be ignored. I emphasize a potential of integrating different approaches to study sexual systems in marine animals with limited available information.

## **General Introduction**

Marine environments host diverse organisms. Marine animals are not only diverse taxonomically (Appeltans et al. 2012), but also represent ecological diversity different from terrestrial ones, in many aspects which evolutionary ecologists are interested in. Such diversity includes many life history parameters (Strathmann 1990), mode of fertilization (Henshaw et al. 2014; Monro & Marshall 2015), kin relationship (Kamel et al. 2010) and so on. In this thesis, I focus on the evolutionary ecology of sexual systems among marine animals, with special reference to complex sexual systems.

#### *Sexual system, sex allocation and mating system*

Sexual system is a distribution of male and female functions within a population or a species (Leonard 2013). Although virtually all tetrapods and terrestrial arthropods are gonochoristic (dioecious, i.e., each individual has only one sexual function throughout its lifetime), marine animals represent diverse sexual systems, including simultaneous or sequential hermaphroditism. Simultaneous hermaphrodites have both sexual functions at the same time. Sequential hermaphrodites have only one sex at a time, but change their sex during their lifetime, in protogynous (female to male) or protandrous (male to female) directions (Policansky 1982).

Evolution of diverse sexual systems are analyzed under the framework of sex

allocation theory (Charnov 1982; West 2009), which examines optimal allocation of reproductive resources into male and female functions. Examples of sex allocation include offspring sex ratio in dioecy, timing and direction of sex change and resource allocation by simultaneous hermaphrodites. It is widely recognized that mating systems, the patterns of the number of available mates (see Turner 1986), strongly affect the evolution of sexual systems and sex allocation (Ghiselin 1969; Charnov 1982), through local mate competition and sex-specific effect of size.

The most well-known effect of mating systems on sex allocation is local mate competition in dioecy. When brothers (sons of the same mother) compete each other over mating with limited number of females, it is wasteful for mothers to produce many sons. Thus female-biased sex ratio should be favored under local mate competition (Hamilton 1967; Charnov 1982). Similarly, in simultaneous hermaphrodites, limited mating group size causes competition among sibling sperm (local sperm competition) and thus favors female-biased sex allocation (Fig.1.1; Charnov 1982; Schärer 2009). This prediction is supported from many, but not all, studies using a wide range of animals (reviewed by Schärer 2009). Such local sperm competition makes the fitness return of male investment diminishing. This diminishing return to one sexual function should disfavor specialized male or female and favors simultaneous hermaphroditism (Charnov 1982). In addition,

limited mate availability may favor simultaneous hermaphroditism by mate assurance, since hermaphrodites can mate with all conspecific individuals while gonochorists can mate with individuals with the opposite sex only (Tomlinson 1966; Ghiselin 1969). Hermaphrodites may also reproduce via self-fertilization when they achieved no mates (Tomlinson 1966; Ghiselin 1969).

Another effect of mating systems on sex allocation explains the pattern of sex change (Fig. 1.2). If a few dominant individuals monopolize reproductive opportunity as males, small subordinates should mature as females and change sex to male after becoming large and dominant. In contrast, if such monopolization is difficult and mating occurs randomly with respect to size or dominance, small individuals should be males because they cannot produce many eggs. Dioecy is favored when males and females have similar relationship between size and reproductive success. This “size-advantage” hypothesis (Ghiselin 1969) is strongly supported by the study of various organisms, especially marine teleost fishes (Warner 1984; Kuwamura & Nakashima 1998; Munday et al. 2006a). Recent phylogenetic comparative studies also support this hypothesis (Molloy et al. 2007; Erisman et al. 2009, 2013; Kazancıoğlu & Alonzo 2010).

Under sexual reproduction, each offspring must have one genetic mother and one genetic father. Thus total reproductive success via male function must be identical to that



via female function (Fisher 1930). Due to this condition (called Fisher condition), optimal sex allocation for each individual depends on the allocation of other individuals. Especially it primarily depends on the allocation tactics of potential mating partners or competitors. This is clearly illustrated by sex changing fishes in which the timing of sex change is socially controlled (Munday et al. 2006a).

### *Complex sexual systems*

In this thesis, I consistently focus on more complex systems than simple systems like dioecy, simultaneous hermaphroditism, protogyny and protandry. Here, complex systems are defined as mixtures of more than one simple systems. For example, the mixture of dioecy and simultaneous hermaphroditism forms androdioecy (males and hermaphrodites), gynodioecy (females and hermaphrodites) and trioecy (males, females and hermaphrodites) (Weeks 2012; Leonard 2013). When individuals have both abilities of protogynous (female to male) and protandrous (male to female) sex change, the sexual system is bi-directional sex change, that is, they can change sex repetitively in both ways (Munday et al. 2010). Other examples of complex sexual systems include diandry (mixture of pure males and protogynous hermaphrodites) and dygyny (pure females and protandrous hermaphrodites). Complex sexual systems represent historical and

conceptual intermediates between different simple systems, and offer a unique opportunity to compare individuals with different sexuality within species. Below I discuss these benefits.

Complex sexual systems are sometimes supposed to mediate evolutionary transitions between simple systems in the evolutionary history (Charlesworth & Charlesworth 1978; Weeks et al. 2006; Weeks 2011). Especially, gynodioecy in plants (Charlesworth & Charlesworth 1978) and androdioecy in animals (Weeks et al. 2006) are hypothesized to play a key role as stepping stones in the evolution from hermaphroditism to dioecy or *vice versa*. This hypothesis is supported by phylogenetic studies. For example, hermaphroditic species of limnadiid clam shrimps were estimated to have evolved from dioecious ancestor through androdioecious states (Weeks et al. 2009). Therefore the understanding of the selective advantage of those complex systems will contribute to the understanding of how transitions of sexual system occur.

Even when they were not the historical intermediates, they can bridge the gap of biological understanding between different systems. For example, gonadal structures of bi-directional sex changers are sometimes similar to that of simultaneous hermaphrodites (Cole 1990; St. Mary 1998; Kobayashi et al. 2005; Munday et al. 2010). Ecological conditions favoring androdioecy in barnacles are theoretically supposed to be

intermediate between that favoring dioecy and that favoring hermaphroditism (Yamaguchi et al. 2008, 2013d). Therefore, complex systems will provide a clue to understand the relationship between different systems by filling the conceptual gap between the systems.

Another important advantage of studying complex sexual systems is that we can compare individuals with different sexuality within the same population. Previous studies taking this advantage include the comparison of fitness between males and hermaphrodites in an androdioecious clam shrimp (Weeks et al. 2014), that of life history parameters between males and hermaphrodites in an androdioecious barnacle (Ewers-Saucedo et al. 2015) and that of physiological costs between protandrous and protogynous sex change in a bi-directionally sex changing goby (Munday & Molony 2002). Using within-species/population comparison of sexuality, we can examine the ecological or social conditions favoring each sexual phenotype under a relatively constant background.

Different sexualities observed within a complex sexual system should associate with spatiotemporal variation of mating system, given the hypothesized link between mating and sexual systems. This prediction is my working hypothesis throughout this thesis, even though I studied different topics using different approaches. The first topic is androdioecy in thoracican barnacles and I conducted field survey and histological

examination. The second one is bi-directional sex change in teleost fishes, and I conducted field survey and theoretical analysis. Below I briefly review these two topics.

#### *Androdioecy and male dwarfism in thoracican barnacles*

Androdioecy, coexistence of pure males and hermaphrodites, is more common than gynodioecy and trioecy in animals (Weeks et al. 2006; Weeks 2012; Leonard 2013). Examples include a nematode *Caenorhabditis elegans*, a mangrove killifish *Kryptolebias marmoratus* and many thoracican barnacles.

Thoracican barnacles are a group of marine crustacean found in a wide range of marine habitats (Anderson 1993). While their larvae swim and disperse, they are almost immobile after settlement and metamorphosis. Since they primarily reproduce via copulation using penes (Murata et al. 2001; for exceptions see Barazandeh et al. 2013, 2014, 2015), they can only mate with neighboring individuals. This enables easy estimation of potential mating group size as a number of individuals within the reach of penes.

Ever since Darwin (1852), diverse and evolutionarily labile sexual systems in barnacles have attracted evolutionary ecologists (Charnov 1987; Kelly & Sanford 2010; Yamaguchi et al. 2012; Yusa et al. 2013). They have three sexual systems: simultaneous

hermaphroditism, dioecy and androdioecy. Most of them are hermaphroditic. However, some species include pure males in addition to hermaphrodites, forming androdioecious systems. There are also some species in which pure males coexist with pure females rather than hermaphrodites, forming dioecious systems. In addition, males are always small and attach to the body surface of their mating partners (hermaphrodites in androdioecy and females in dioecy), and thus called “dwarf males”. Importantly, dwarf males and pure females evolved several times independently from their hermaphroditic ancestor (Høeg 1995; Yusa et al. 2012; Pérez-Losada et al. 2012; Lin et al. 2015).

Mating systems, especially mating group size for potential hermaphrodites, are supposed to be a key factor in the evolution of sexual systems in barnacles (Charnov 1987; Yamaguchi et al. 2008, 2012, 2013d). As noticed above, simultaneous hermaphrodites should allocate less resource to male function when mating group size is small, due to local mate (sperm) competition (Fig.1.1). This weakens sperm competition and enables dwarf males to achieve enough reproductive success to evolve (Fig.1.3). Under extremely small groups, hermaphrodites cease allocation to male function and become pure females because they have few partners to give sperm. In summary, dwarf males and pure females should be adaptive under small mating group size. This prediction is supported by phylogenetic comparative studies. Dwarf males and females are likely to

evolve in lineages with smaller group size (Yusa et al. 2012) and ones living in deep seas where density is generally low and thus group size should be small (Lin et al. 2015).

In this thesis I focused on the adaptive significance of dwarf males in androdioecious systems, because the origin of dwarf males should be the key step in the evolution of sexual systems in barnacles (Yamaguchi et al. 2012). While there is a plenty of theoretical studies on this topic (Charnov 1987; Yamaguchi et al. 2007, 2008, 2012, 2013a, b, d), within-species relationship between mating and sexual system is not tested enough (but see Spremberg et al. 2012; Ewers-Saucedo et al. 2015).

*Octolasmis unguisiformis*, an endangered pedunculate barnacle symbiotic to intertidal crabs of genus *Macrophthalmus* (Kobayashi & Kato 2003; Kato 2012), is the study species of these chapters. Since this species lives on the body surface of small crabs, I can estimate the upper limit of mating group size as a number of matured hermaphrodites per host crab. Although small individuals attached to conspecific were described by Kobayashi & Kato (2003), it has not been examined whether they are dwarf males or not. Thus, I first confirmed the existence of dwarf males and hence androdioecy in this species by histological examinations and then tested the within-species correlation between mating and sexual systems.

### *Bi-directional sex change in teleost fishes*

Functional sex change is a reproductive strategy adopted by a wide range of organisms (Policansky 1982; Vega-Frutis et al. 2014), including numerous teleost fishes (Sadovy & Liu 2008; Kuwamura et al. in prep.). They include both protandrous and protogynous species and the direction of sex change corresponds to mating systems, as predicted by the size-advantage hypothesis (Warner 1984). However, recent studies revealed diverse and complex strategies about sex change (Kuwamura & Nakashima 1998; Munday et al. 2006a) which are beyond the classical dichotomy of protandry and protogyny. Bi-directional sex change is one of them. While no example on this phenomena in fishes had been known until recently (Reinboth 1980), increasing reports from various taxa of teleosts (e.g. Manabe et al. 2013; Kuwamura et al. 2016) suggests that it is rather prevalent.

All known teleost species with this ability are basically protogynous (Munday et al. 2010). That is, individuals first mature as females, and large and dominant ones change sex into males. When more than one males cohabit within a group, the subordinate males undergo reversed sex change to females. This pattern is confirmed for many species using both field and captive observations (Munday et al. 2010).

Why do subordinate males change sex? Because most of them are polygynous (Munday et al. 2010), dominant males achieve higher fitness than females. Thus

remaining as males should be optimal if possible. However, males of these species are mutually aggressive and thus cannot cohabit stably (Sunobe & Nakazono 1993). Subordinate males are forced to choose between dispersal to other habitat or reversed sex change. When expected fitness of the latter is larger than the former, reversed sex change should be adaptive as “the best of bad situations” (Kuwamura & Nakashima 1998). For example, if dispersal is extremely risky, reversed sex change may be better than dispersal (Munday et al. 2010). Kuwamura et al. (2002, 2011, 2014a) proposed a “low density hypothesis” that reversed sex change should be promoted by low density because males are more likely to become bachelors and more difficult to find mates. However, these hypotheses are not tested by mathematical modeling, preventing quantitative predictions. In addition, while the effect of population density is predicted, the extent of population fluctuation in bi-directional sex changers and its effect on sex change has not been examined. Furthermore, while the effect of sex-specific growth rate is supposed to be important for bi-directional sex change in coral-dwelling gobies (Kuwamura et al. 1994a; but see Munday 2002), it has not been studied in other group of hermaphroditic gobies. In this thesis, I aim to fill these gaps by mathematical modeling and field survey.

In the field survey, I studied a marine goby *Trimma caesiura*. Sex change, including bi-directional one, evolved in several lineages within family Gobiidae (Cole



2010). Gobies of genus *Trimma* are notable by their ability to change sex rapidly (Sunobe & Nakazono 1993; Manabe et al. 2008; Sakurai et al. 2009). This ability is enabled by their unique gonadal structure which contains both testicular and ovarian parts (Kobayashi et al. 2005). The ability of bi-directional sex change is also suggested for *T. caesiura* based on histology (Cole 1990; Munday et al. 2010). Although a few cases of naturally occurring reversed sex change in *T. okinawae* were reported by Manabe et al. (2007a), ecological or life-historical background of this ability is not examined. I studied population dynamics of *T. caesiura* to provide background information to discuss how spatiotemporal variation of abundance and hence mating opportunity shaped extreme sexual plasticity in *Trimma*. In addition, because sex-specific growth rate is supposed to be an important factor in other genera of gobies (Kuwamura et al. 1994a; Nakashima et al. 1996; but see Munday 2002) but is not examined in other sex changing gobies, I also measured growth rate by the mark-and-recapture method.

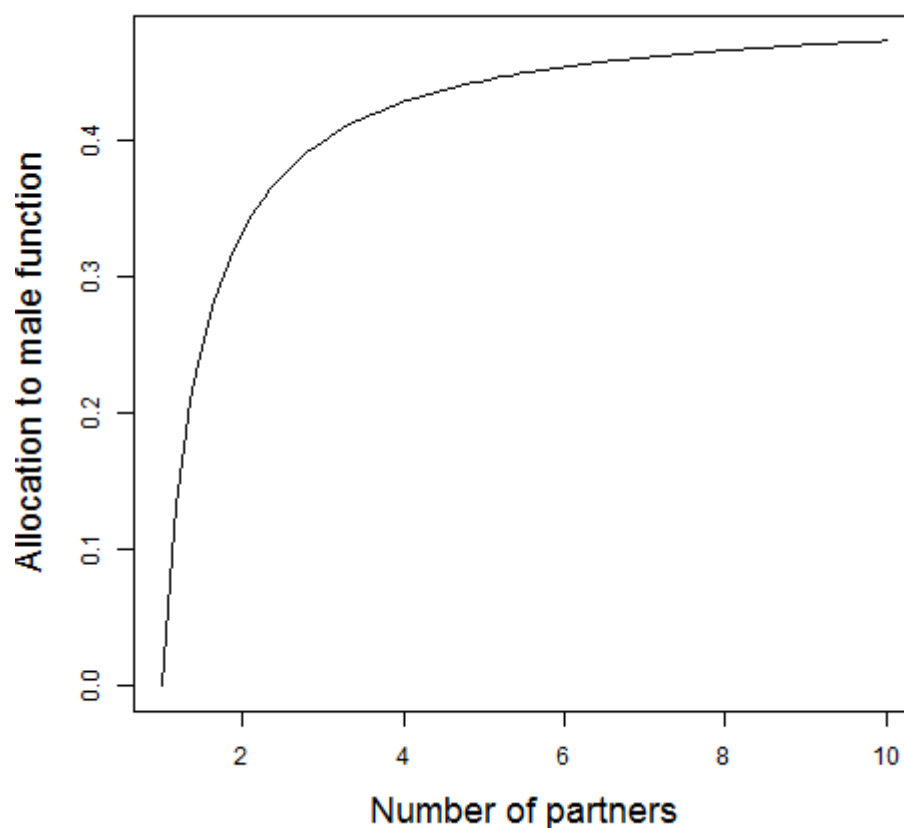
#### *Approaches in evolutionary ecology*

As noted above, I adopted different approaches in chapters of this thesis, that is, long-term field survey, theoretical modeling, histological examination and within-species comparison using natural variation. I chose these approaches to satisfy what is most

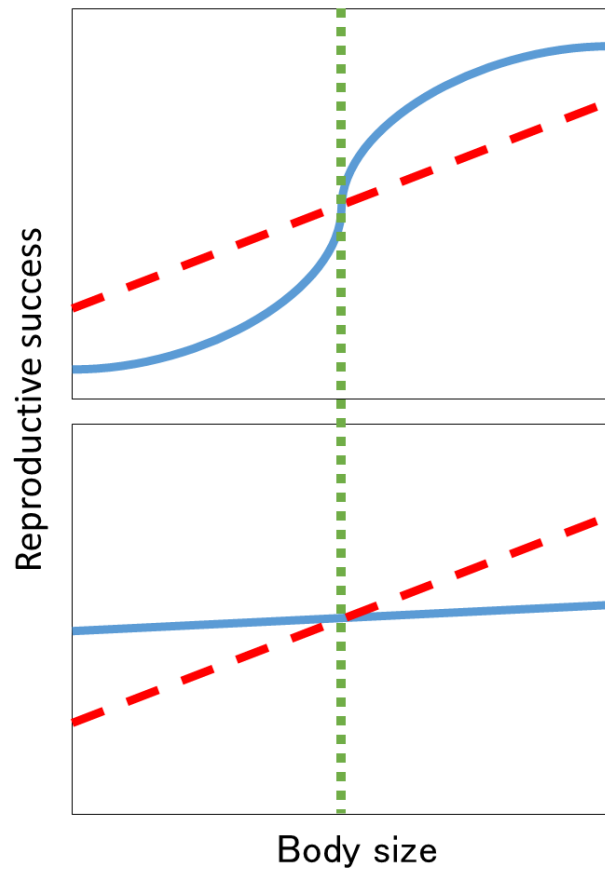
necessitated in each topic. Although there are a plenty of experimental and histological studies on bi-directional sex change in gobies (e.g. Sunobe & Nakazono 1993; Kuwamura et al. 1994a; Nakashima et al. 1995, 1996; Munday et al. 1998; Munday 2002; Munday & Molloy 2002; Kobayashi et al. 2005; Manabe et al. 2008, 2013; Sakurai et al. 2009; Cole 2010), theoretical and ecological aspects of this phenomena has rarely been studied. In contrast, while there is a clear theoretical hypothesis to test for androdioecious barnacles (Charnov 1987; Yamaguchi et al. 2012), the test using within-species variation is extremely limited (but see Spremberg et al. 2012). Thus I did it after confirming androdioecy in the study species by histology. This is why I adopted different approaches even though I consistently addressed the same problem, that is, the effect of varying mating systems on sexual systems.

This thesis is a product of collaboration between different disciplines behind different approaches: evolutionary/behavioral ecology, population ecology, histology, natural history and theoretical/mathematical biology. I hope this thesis provides a case illustrating how different domains of biology can be integrated for the common goal of understanding evolution of biodiversity.

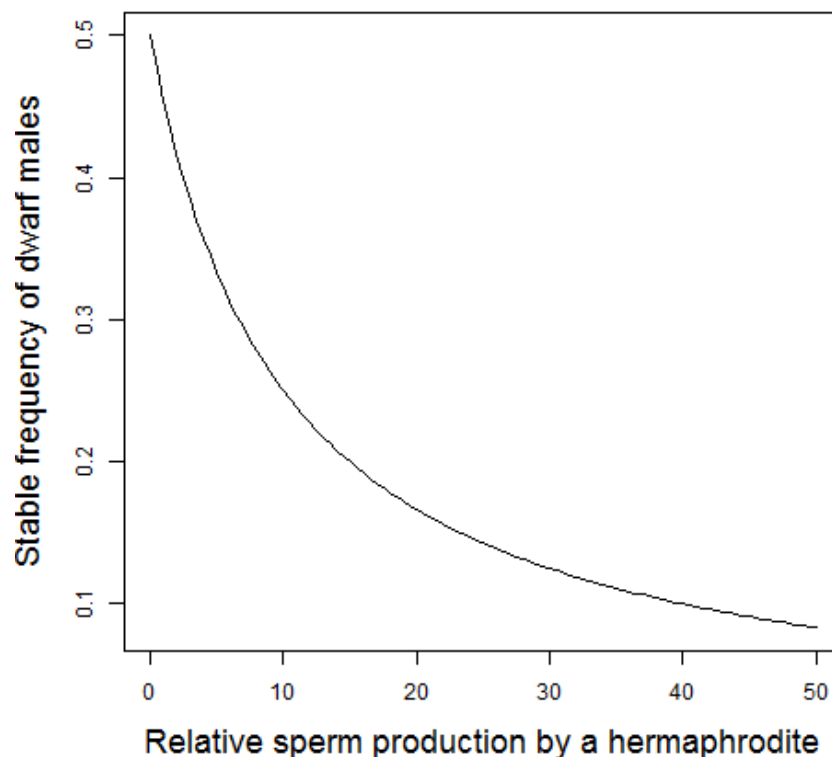
**Fig. 1.1:** Effect of local mate (sperm) competition on evolutionarily stable sex allocation (see Charnov 1982). Horizontal axis is the number of partners for a hermaphrodite (i.e. mating group size except for the focal individual), and vertical axis is the proportion of reproductive resource allocated to male function (male/total) at the evolutionarily stable strategy. Hermaphrodites should become female-biased if they have few partners to donate sperm, to avoid competition among sibling sperm. As the mating group size increases, the stable allocation approaches asymptotically to 0.5 (equal allocation).



**Fig. 1.2:** Schematic graph of size-advantage model (see Charnov 1982). Solid and dashed lines represent the relationship between reproductive success and body size among males and females, respectively. Dotted line represents optimal size at sex change. The top and bottom graphs correspond to polygyny and random mating. Female size-fecundity relationship has a positive slope, and is independent of mating systems because it represents physiological limitation of egg production. In polygynous species (top), small and subordinate males achieve little reproductive success, but larger and dominant ones achieve very high success. Thus individuals should first mature as females and then change sex to male after becoming large. In random mating (bottom) reproductive success of males is almost independent of their size because even small males can produce enough sperm. Thus individuals should change sex from male to female. The optimal size at sex change corresponds to the intersection of the curves of reproductive success for males and females.



**Fig. 1.3:** Predicted frequency of dwarf males in androdioecious barnacles (Charnov 1987; see also Yamaguchi et al. 2012). Horizontal axis is the amount of sperm production by a hermaphrodite divided by that by a dwarf male, and vertical axis is the evolutionarily stable frequency of dwarf males. Although a hermaphrodite can produce more sperm than a dwarf male at least potentially, its sperm production reduces by local mate competition under small mating group size (see Fig.1.1). Thus smaller values of relative sperm production correspond to smaller group size. Higher frequency of males is predicted under smaller group size and hence smaller amount of sperm production by a hermaphrodite. The maximum number of males per hermaphrodite (see Charnov 1987) is assumed to be 10.



## Study 1

**Dwarf males in the epizoic barnacle *Octolasmis unguisiformis*  
and their implications for sexual system evolution**

## Introduction

Specifying the evolutionary conditions promoting diverse sexual systems among organisms, such as hermaphroditism and dioecy (gonochorism), is one of the central goals of evolutionary ecology (Ghiselin 1969; Charnov 1982; Leonard 2010). Although sexuality is not evolutionarily labile in many animal taxa (Leonard 2013), some groups show very high diversity in their sexual systems, offering the opportunity to test hypotheses on the patterns and causes of evolution of sexual systems (e.g., Yusa et al. 2012; Erisman et al. 2013; Mathers et al. 2013). Thoracican barnacles, sessile marine crustaceans, are one such group (Darwin 1852; Charnov 1987; Høeg 1995; Kelly & Sanford 2010; Yamaguchi et al. 2012; Yusa et al. 2012, 2013).

Three systems of sexuality are known in thoracican barnacles: hermaphroditism (simultaneous hermaphrodites only), androdioecy (simultaneous hermaphrodites and males), and dioecy (females and males). Because androdioecy may be a transitional state between dioecy and hermaphroditism (Weeks et al. 2006; Weeks 2012), the adaptive significance of pure males in androdioecious populations is key to understanding the evolution of sexual systems. In both dioecious and androdioecious barnacle species, males are much smaller than conspecific females or hermaphrodites, and thus termed “dwarf males” (Vollrath 1998; Yusa et al. 2012). In this paper, I avoid the term “complemental



males” (which means androdioecious males, specifically), and use “dwarf males” in a broad sense which include both dioecious and androdioecious males, because there is no morphological distinction between them (Høeg 1995; Yusa et al. 2010, 2012; Spremberg et al. 2012). They live on the bodies of conspecific females/hermaphrodites throughout their lives. Males of pedunculate (gooseneck) barnacles are always attached to a specific, more or less confined area on non-male individuals (Yusa et al. 2012).

*Octolasmis unguisiformis* is a rare lepadomorph barnacle that lives on the carapace of the intertidal crab *Macrophthalmus milloti* and related species (Kobayashi & Kato 2003; Sawada & Yoshida unpubl. data). It is the only barnacle species that appears in the “Red Data Book” listing threatened fauna from tidal flats in Japan (Kato 2012). Kobayashi & Kato (2003) reported two classes of individuals: individuals attached to the crab carapace directly (hereafter “crab-attached”), and small individuals attached to crab-attached individuals of the same species (hereafter “conspecific-attached”). Although conspecific-attached individuals are morphologically similar to crab-attached ones, the sexual status of conspecific-attached individuals is unknown. Similar conspecific-attached individuals in a congener, *Octolasmis warwickii*, are males (Yusa et al. 2010). The same holds true for the lepadid barnacle *Alepas pacifica* (Yusa et al. 2015). In *Octolasmis angulata*, maleness is enhanced in artificially conspecific-attached

individuals, even though neither naturally conspecific-attached individuals nor males are known (Yusa et al. 2013). In contrast, conspecific-attachment, either natural or artificial, does not affect sexuality in the lepadomorph barnacle *Poecilasma kaempferi* (Yamaguchi et al. 2014b). Thus, I tested if conspecific-attached individuals of *O. unguisiformis* were dwarf males or not.

Interestingly, the site of attachment of conspecific-attached individuals of this species ranges from the capitulum to the peduncle (Fig. 2.1A). This enabled us to distinguish the effects of attachment to conspecifics itself, and to specific sites of attachment on conspecifics. I also investigated crab-attached individuals that were adjacent to large conspecifics to distinguish the effects of conspecific-attachment and proximity to conspecifics.

## Methods

I collected the host crabs *Macrophthalmus milloti* (three individuals) and *Macrophthalmus serenei* (one individual) carrying *Octolasmis unguisiformis* from the intertidal mud flat of Yabuchi-jima Island, Okinawa, southern Japan (26°19' N, 127°55' E). Collection was conducted by hand with a shovel or yabbie pump. In line with a previous report (Kobayashi & Kato 2003), only female crabs carried barnacles. I fixed

the specimens in 100% ethanol and then removed barnacles from the crabs. I measured the capitulum length of barnacles to the nearest 0.01 mm.

I examined six crab-attached individuals (individuals A–F) and seven conspecific-attached individuals (G–M). The capitulum length (mm) of each individual is shown in Table 2.1. Individuals G–H and I–M were attached to crab-attached individuals C and D, respectively. Some conspecific-attached individuals (I and J) were attached to the capitulum of conspecifics, but others (G, H, K, L, M) were attached to the peduncle. To distinguish the effect of direct attachment to conspecifics from the effect of neighboring individuals, two small crab-attached individuals (E and F) that were adjacent to a large one (D) (less than 1 mm in distance between the bases of peduncles) were included.

After decalcifying with aqueous Bouin's solution, I dehydrated and cleared samples in a graded ethanol-xylol series, and embedded them in paraffin wax. Serial sections (10  $\mu$ m thick) were cut in sagittal (A–D, F–K, M) or frontal (coronal: E, L) planes, and stained with Mayer's hematoxylin and eosin Y.

## **Results**

The results of the histological investigation are summarized in Table 2.1. All individuals that were sectioned sagittally had penes, but I could not determine the presence or absence

of a penis in the two individuals sectioned frontally (individuals E and L).

Conspecific-attached individuals had relatively well-developed penes (Fig. 2.1B). Of the seven conspecific-attached individuals, six also had spermatogonia and a seminal vesicle filled with sperm (Fig. 2.1B). Although the smallest conspecific-attached individual (J; 0.69 mm in capitulum length) did not have sperm, it had spermatogonia. However, none of these conspecific-attached individuals had eggs in their mantle cavities, or ovaries in their peduncles. Thus, they had male function but no female function.

In contrast, crab-attached individuals had much smaller penes for their sizes (Fig. 2.1C,D). Crab-attached individuals larger than 2.0 mm in capitulum length (C, D) had fertilized eggs in their mantle cavities and ovaries in their peduncles (Fig. 2.1C) as well as a relatively small amount of sperm and spermatogonia. Smaller crab-attached individuals (A, B, E, F) did not have any reproductive organs other than small penes (Fig. 2.1D), although they were of similar size to conspecific-attached individuals that had spermatogonia and seminal vesicles.

The relationship between capitulum length and development of male function (indicated by the presence or absence of sperm) for crab- or conspecific-attached individuals is summarized in Fig. 2.2. Although my data were not suitable for statistical tests owing to the problem of separation (Albert & Anderson 1984) and a small sample

size due to the rarity of members of this species, conspecific-attached individuals clearly developed their male function at a much smaller size than those that were crab-attached.

## **Discussion**

I found that conspecific-attached individuals of *Octolasmis unguisiformis* were dwarf males. They had mature sperm and penes at much smaller sizes than the maturation size of crab-attached individuals (about 2 mm in capitulum length, Kobayashi & Kato 2003). In addition, no conspecific-attached individuals were ovigerous, consistent with the idea that they were dwarf males. In contrast, large crab-attached individuals had penes and ovaries, and were ovigerous. In other words, morphologically they were hermaphrodites. Thus, this species has coexisting males and hermaphrodites, i.e., it is androdioecious (Weeks et al. 2006).

The existence of dwarf males in this species is consistent with theoretical predictions. Several studies have predicted that dwarf males among barnacles should evolve when mating group size is small, because sperm competition is weak in small groups (Charnov 1987; Yamaguchi et al. 2008, 2013d; see also the review of Yamaguchi et al. 2012). Because *O. unguisiformis* lives in small groups (only one or a few mature hermaphrodites per crab, Kobayashi & Kato 2003), my finding is compatible with this

prediction. In addition, Yamaguchi et al. (2013a, b) argue that ephemeral habitats and limited space favor dwarf males owing to growth limitation. The habitat of *O. unguisiformis* is extremely ephemeral, because it is lost by molting of host crabs, and may be spatially limited because the barnacles attach only on specific sites on the host (Kobayashi & Kato 2003). These temporal and spatial limitations should be exacerbated on small hosts (up to only ~20 mm in carapace breadth in *Macrophthalmus milloti*: Barnes 2010), which probably have short intermolt durations and lifespans. In contrast, *Poecilasma kaempferi*, a species without dwarf males, lives in dense aggregations on large hosts (William & Moyse 1988; Yamaguchi et al. 2014b), which probably live longer and molt less frequently. The relative importance of natural selection (ephemeral and limited habitat) and sexual selection (weak sperm competition in small mating groups) in the evolution of dwarf males in *O. unguisiformis* is unknown and needs further investigation. For pedunculate barnacles in general, Yusa et al. (2012) found that dwarf males are more likely to evolve when group size is small, supporting the importance of sexual selection, while the possibility of natural selection is not excluded (Yusa et al. 2013). Detailed examination of between- or within-species variation of the frequency of males and social or ecological conditions may help to address this question (e.g. Spremberg et al. 2012).

It is interesting that all conspecific-attached individuals were males irrespective of attachment site (capitulum or peduncle), while crab-attached ones were not males even if they were very close to large individuals of *O. unguisiformis*. This result is consistent with the general pattern that barnacle males attach to conspecifics, but not to specific sites on conspecifics.

A relationship between attachment site and sexuality can be explained by two possible proximate mechanisms: selective attachment by larvae with genetically predetermined sex, or environmental sex determination affected by the substratum type. Although sex determination among thoracican barnacles is largely unknown (Crisp 1983b; Yusa et al. 2013), Callan (1941) suggested that sex determination was environmental in *Scalpellum scalpellum*, and Gomez (1975) argued that it was genetic in *Conopea galeata*. Combination of these two mechanisms was also suggested in *S. scalpellum* (Svane 1986; see also Spremberg et al. 2012). In this study, I do not have data that bear on mechanisms of sex determination in *O. unguisiformis*, although the phenotypically plastic sexuality of its hermaphroditic congener *O. angulata* (Yusa et al. 2013) suggests the possibility of environmental effects in *O. unguisiformis*, as well.

My result shows that the exact attachment site on a conspecific does not affect differentiation into dwarf males or settlement of male larvae, though conspecific

attachment itself is important. In contrast, virtually all larvae of the pedunculate barnacle *S. scalpellum* which are attached to substrata other than a specialized area (the receptacle) on the conspecific capitulum develop into hermaphrodites, even if they are on the conspecific body surface (Svane 1986; Spremberg et al. 2012). Male position is also very confined and specialized in other scalpellid species (Buhl-Mortensen & Høeg 2006, 2013). Even though males in some other scalpellid species are not found in specialized areas (e.g. *Scillaelepas* spp.; Newman 1980), all known males of pedunculate barnacles are attached in more or less confined areas (Yusa et al. 2012). This makes a sharp contrast between *O. unguisiformis* and all other pedunculate barnacles, suggesting different mechanisms of interaction between larvae and adults, and in turn different relationship between attachment site and fitness of males.

In some barnacles, dwarf males may have the potential to grow up into hermaphrodites (Crisp 1983a; Yusa et al. 2010, 2015). Weeks (2012) suggested that androdioecy with dwarf males may represent sequential changes of sex expression in the lifetime of individuals. However, the potential for this is undoubtedly very limited for *O. unguisiformis*, if present at all. I as well as Kobayashi & Kato (2003) found no cases of large, ovigerous, conspecific-attached individuals. Further, the differences in sexual development between conspecific- and crab-attached individuals shows that they are not



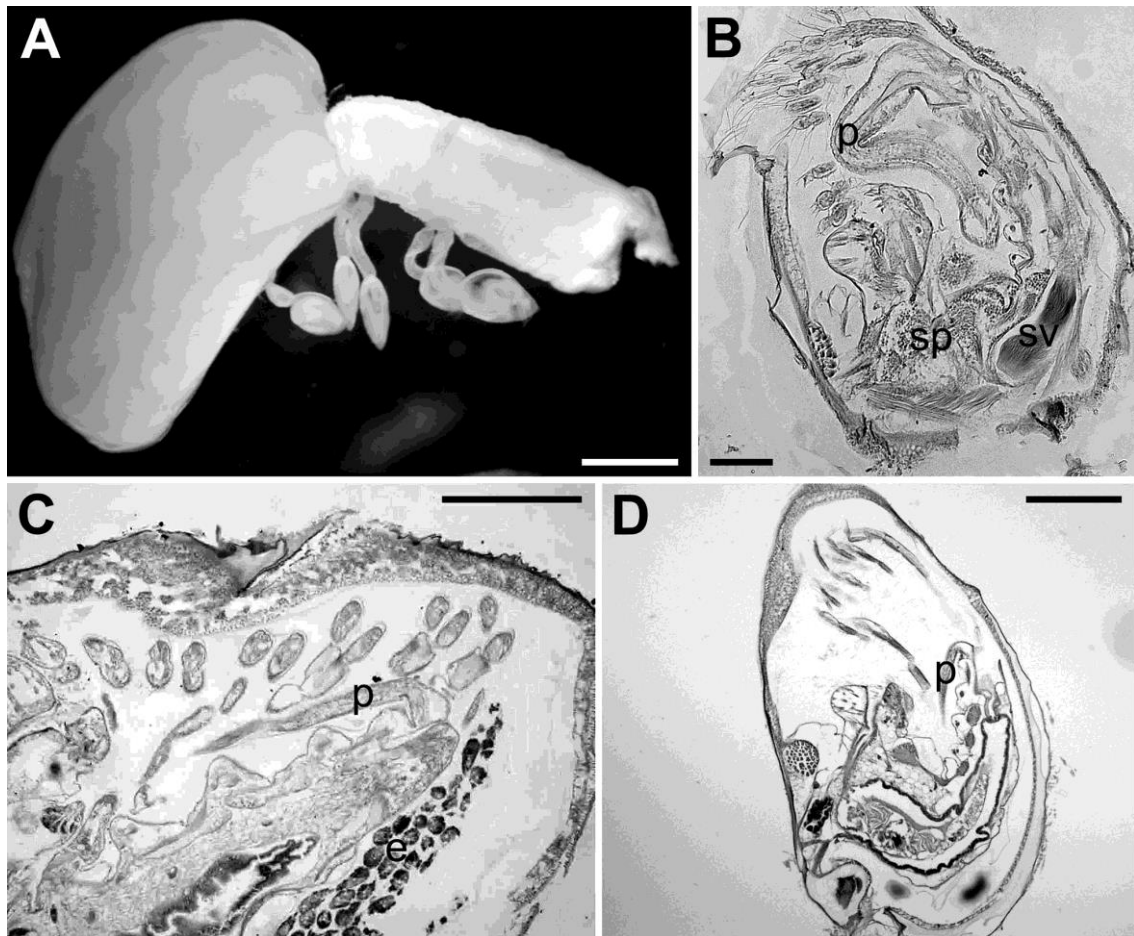
in different stages of one life-history trajectory, but instead are expressing two different strategies. Such differences exist even in a species in which males have the potential to be hermaphrodites (Yusa et al. 2010). Therefore, dwarf males are not simply small-sized hermaphrodites with a protandric tendency, but individuals with a fundamentally different strategy than normal hermaphrodites. In the evolution of androdioecy, it is likely that dwarf males arose from conspecific-attached hermaphrodites without modification of reproductive patterns (like *P. kaempferi*; Yamaguchi et al. 2014b) to have a different sexuality and life history (i.e. maleness and smallness), due to different selective pressures (ecological or sexual) (Yamaguchi et al. 2008).

My morphological observations on crab-attached barnacles indicate that they are hermaphrodites, which is consistent with a previous report (Kobayashi & Kato 2003). However, further investigations are required to determine they are functionally hermaphroditic, because their penes and the amount of sperm they carry are so small.

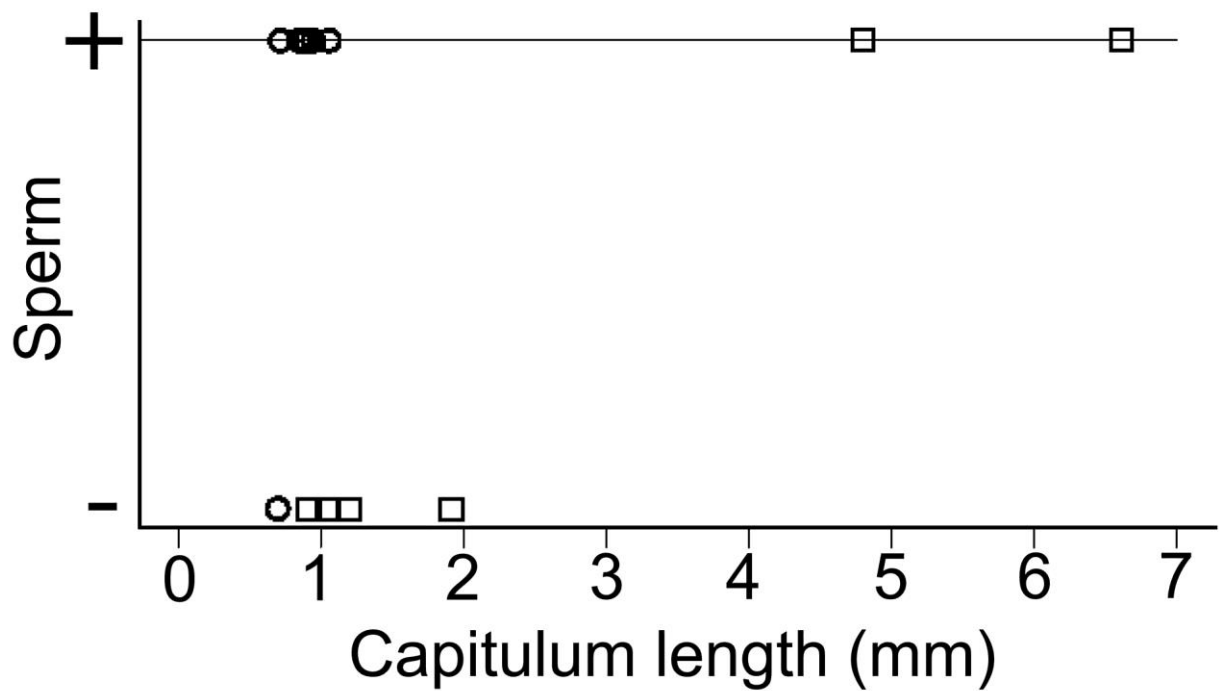
**Table 2.1.** Summary of measurements and results of histological examination of *Octolasmis unguisiformis*. For each reproductive organ, +, -, and ? indicate presence, absence, and undetermined, respectively. Crab hosts 1-3 were *Macrophthalmus milloti*, and crab host 4 was *M. serenei*. CL, capitulum length.

Barnacle ID	CL (mm)	Substratum	Penis	Sperm	Spermatogonia	Eggs	Ovary	Plane sectioned
A	1.20	crab 1	+	-	-	-	-	sagittal
B	1.91	crab 2	+	-	-	-	-	sagittal
C	6.62	crab 3	+	+	+	+	+	sagittal
D	4.80	crab 4	+	+	+	+	+	sagittal
E	1.04		?	-	-	-	-	frontal
F	0.91		+	-	-	-	-	sagittal
G	1.05	conspecific C,	+	+	+	-	-	sagittal
H	0.84	peduncle	+	+	+	-	-	sagittal
I	0.88	conspecific D,	+	+	+	-	-	sagittal
J	0.69	capitulum	+	-	+	-	-	sagittal
K	0.94	conspecific D,	+	+	+	-	-	sagittal
L	0.89	peduncle	?	+	+	-	-	frontal
M	0.71		+	+	+	-	-	sagittal

**Fig. 2.1.** **A.** A large crab-attached animal (individual D) and five small ones attached to it, two on the capitulum and three on the peduncle. Scale bar=1 mm. **B.** Sagittal section of the capitulum of a small conspecific-attached individual (individual H, 0.84 mm in capitulum length). Scale bar=0.1 mm. **C.** Sagittal section of the capitulum of a large crab-attached individual (individual D, 4.80 mm in capitulum length). Scale bar=0.5 mm. **D.** Sagittal section of the capitulum of a small crab-attached individual (individual B, 1.91 mm in capitulum length). Scale bar=0.5 mm. e, eggs; p, penis; sp, spermatogonia; sv, seminal vesicle filled with sperm.



**Fig. 2.2.** Relationship between capitulum length and presence or absence of sperm for conspecific-attached (circles) and crab-attached (squares) individuals. Conspecific-attached individuals mature as males at a much smaller size than do crab-attached individuals.



## Study 2

**Sexual and mating systems in an androdioecious barnacle:  
testing the within-species effect of group size**

## **Introduction**

The evolutionary conditions for diverse sexual systems, such as hermaphroditism and dioecy, remain largely unsolved (Ghiselin 1969; Charnov 1982; Leonard 2010, 2013).

Complex sexual systems including androdioecy (coexistence of males and hermaphrodites), gynodioecy (females and hermaphrodites) and trioecy (males, females and hermaphrodites) can provide a unique insight to understand evolutionary processes because the evolutionary transitions between pure hermaphroditism and dioecy may be mediated by such complex systems as stepping stones (Weeks et al. 2006; Weeks 2012).

For example, phylogenetic studies suggest that dioecious species among thoracican barnacles evolved from hermaphroditic ancestor through androdioecious systems (Yusa et al. 2012; Lin et al. 2015). That is, it is estimated that males originated in hermaphroditic populations first to form androdioecy and then pure females originated to form dioecy. In addition to the historical importance, theoretical studies suggest that androdioecy should evolve under intermediate conditions between hermaphroditism and dioecy (e.g. Yamaguchi et al. 2008, 2013d).

Thoracican barnacles have attracted evolutionary ecologists interested in sexual systems (Charnov 1987; Kelly & Sanford 2010; Yamaguchi et al. 2012; Yusa et al. 2013), ever since Darwin's (1852) finding of dwarf males. While most of them are simultaneous

hermaphrodites, some species exhibit androdioecy and dioecy with dwarf males. Those males always attach to the body surface of their sexual partners (hermaphrodites or females).

The key prediction derived from theoretical studies (Charnov 1987; Yamaguchi et al. 2008, 2012, 2013d) is the association between sexual and mating systems. The prediction is based on the effect of “local mate competition” on sex allocation. When number of sperm recipients is limited for a hermaphrodite, it is wasteful to produce much sperm because competition among sibling sperm becomes intensive. Thus small mating group size should favor female-biased sex allocation (Charnov 1982; see Fig. 1.1 in General Introduction). This prediction is supported by several, though not all, empirical studies (Schärer 2009), including ones on barnacles (Raimondi & Martine 1991; Hoch & Levinton 2012; but see Kelly & Sanford 2010). Female-biased sex allocation by hermaphrodites weakens sperm competition within a mating group and enables dwarf males to achieve sufficient fertilization success to become frequent (Charnov 1987; Yamaguchi et al. 2012; see Fig. 1.3 in General Introduction). If mating group size is so small that hermaphrodites do not have enough partners to donate sperm, they should abandon male function and become pure females. The predicted association between sexual system and mating group size is supported by cross-species comparative studies.

Yusa et al. (2012) found that androdioecy and dioecy tended to evolve in lineages with small mating group size. Lin et al. (2015) found deep-sea species are more likely to evolve into androdioecious and dioecious. Given the low density of deep-sea animals, it is also compatible to the prediction.

However, the predicted correlation between mating group size and the frequency of dwarf males within androdioecious species has rarely been tested, despite their importance to understand evolutionary transitions and patterns (Yamaguchi et al. 2012). Spremberg et al. (2012) reported that the presence of males on a hermaphrodite does not correlate with solitariness in *Scalpellum scalpellum*. Ewers-Saucedo et al. (2015) found that mating group size differs among different host species in *Chelonibia testudinaria*, but the frequency of males does not. Both of these studies do not support the prediction, although not directly tested it.

In this study, I focused on a crab-symbiotic barnacle *Octolasmis unguisiformis*. This species lives on the body surface of small intertidal crabs of the genus *Macrophthalmus* (Kobayashi & Kato 2003; Sawada et al. 2015: Study 1). This species is androdioecious and their sexuality corresponds to the attachment site (Sawada et al. 2015: Study 1). Namely, crab-attached barnacles are simultaneous hermaphrodites and conspecific-attached ones are dwarf males. I used this correspondence between sexuality



and substrata to estimate their sexuality. However, since the above result was obtained by a histological observation, their functional sexuality under natural conditions was not absolutely confirmed. Thus I tested the male function of dwarf males and hermaphrodites by exploring their contribution to the production of embryo by neighboring individuals.

Since barnacles primarily mate through copulation via penes (Murata et al. 2001; but see Barazandeh et al. 2013, 2014, 2015), upper limits of mating group size can be estimated as the number of matured hermaphrodites within the reach of penes (Ewers-Saucedo et al. 2015). Although this idea is based on an assumption that the reach of penes can be precisely estimated, it is not always the case as barnacle penes are extensible (Klepal et al. 1972) and phenotypically plastic (Neufeld & Palmer 2008; Neufeld 2011; Hoch 2008, 2009, 2010; Hoch & Reyes 2015). The estimation is easier for individuals on a small and isolated substratum which presumably carries one mating group (e.g. sea-urchin spines in Spremberg et al. 2012). I used number of matured hermaphrodites per host crab as an indicator of mating group size, because *O. unguisiformis* lives on small and solitary crabs.

## Methods

I collected crabs of genus *Macrophthalmus* carrying from the intertidal mud flats in

Katsuren Peninsula, the main island of Okinawa, Kasari Bay, Amami Island and Yabuchi-jima Island, Okinawa. Collection was conducted by hand with a shovel or yabbie pump. Some of the specimens from Yabuchi-jima were already used in Sawada et al. (2015: Study 1). I fixed the specimens in 100% ethanol and then removed barnacles from the crabs. I measured the capitulum length of barnacles to the nearest 0.01 mm. I also used specimens described in Sawada et al. (2015: Study 1) collected in Yabuchi-jima in the same way. In total I examined 37 matured hermaphrodites from 27 crabs.

Based on the results of the previous studies (Kobayashi & Kato 2003; Sawada et al. 2015), I categorized individuals into three reproductive statuses by size and attachment site. All conspecific-attached individuals were categorized as dwarf males. Among crab-attached individuals, individuals larger than 2 mm in capitulum length and smaller than this threshold were categorized as juveniles and matured hermaphrodites, respectively. The upper limit of mating group size on each host was estimated the number of matured hermaphrodites per host. I examined the presence or absence of embryos in the mantle cavities of matured hermaphrodites as an evidence for male function of at least one neighboring individuals, by removing shell plates partly under a binocular microscope. For matured hermaphrodites dissected for histological examinations in Sawada et al. (2015: Study 1), the presence or absence of embryos was determined by the observation

of sections.

I used separate generalized linear mixed models for the presence of embryos and the number of males for each matured hermaphrodite (i.e. crab-attached individual larger than 2mm in capitulum length), as responsive variables. For the presence of embryos, the presence/absence of hermaphroditic partner(s) (i.e. at least one other matured hermaphrodite on the same host) and the presence/absence of male partner(s) (i.e. at least one dwarf male on the focal hermaphrodite) were incorporated as explanatory variables. For the number of males, mating group size estimated by the above mentioned method was incorporated. In addition, capitulum length of the focal hermaphrodite was also incorporated to both models as an explanatory variable to control the possible effect of body size on reproductive status. In addition, an identity of host crabs was also incorporated as a random factor to control the non-independence among hermaphrodites on the same hosts. I used a binomial distribution for the presence or absence of embryos and the Poisson distribution for the number of males as error structures. All interactions were removed from the model if not significant by Wald test. Significance of each explanatory variable was also tested by Wald test. All statistical analyses were conducted using the statistical software *R* ver. 3.2.1 (*R* Core Team 2015).

## Results

My results supported that morphological dwarf males function as males. In the analysis of the presence or absence of embryos (Fig. 3.1), larger hermaphrodites were more likely to carry embryos, marginally significantly (Fig. 3.1(a)). In addition, the presence of male partner(s) significantly increased the probability of carrying embryos (Fig. 3.1(b)).

However, following results did not support morphological hermaphrodites and correlation between male frequency and mating group size. The presence of hermaphroditic partner(s) did not significantly correlate with the presence of embryos (Fig. 3.1(b)), although the relationship was positive. In the analysis of the number of males, larger hermaphrodites had more males, marginally significantly (Fig.3.2(a)). However, estimated mating group size did not correlate with the number of males (Fig.3.2(b)). All results were summarized in Table 3.1.

## Discussion

I found a positive and significant effect of dwarf males on the presence of embryos by their partners. This result demonstrates their functional maleness in *Octolasmis unguisiformis*, combined with the developed male reproductive organs observed histologically (Sawada et al. 2015: Study 1). However, my result did not support male

function of morphological hermaphrodites, since the presence of hermaphroditic partner(s) did not correlate with the presence of embryos significantly, although the trend was positive. A possible explanation is that this species is functionally dioecious, even though morphologically androdioecious. A similar situation is supposed for *Scillaelepas* spp. in which penes of morphological hermaphrodites seem to be too small to work (Newman 1980). The male organs in hermaphrodites of *O. unguisiformis* were also relatively undeveloped, although they had sperm (Sawada et al. 2015: Study 1). It is possible that mating group size in this species is so small that functional females are favored. However, the non-significance may be a result of insufficient statistical power. Since animals may lack embryos just because they were not in the ovigerous stage of their reproductive cycles, not because they were infertile, the efficient sample size which can distinguish the effect of hermaphroditic partners were small. Functional sexuality of these individuals should be determined by a behavioral observation and/or a genetic paternity analysis.

This study did not support the predicted negative correlation between mating group size and the number of males. First, if *O. unguisiformis* is functionally dioecious rather than androdioecious, lack of correlation is reasonable because functional females do not cause sperm competition. Second, it is possible that the within-species variation of

the frequency of males cannot be explained by mating group size even in functionally androdioecious species. This is consistent with the previous within-species studies mentioned in Introduction (Spremborg et al. 2012; Ewers-Saucedo et al. 2015). Although the number of species examined in this aspect is too small (only 3 spp., i.e. *Scalpellum scalpellum*, *Chelonibia testudinaria* and *O. unguisiformis*), this consistency suggests an interesting possibility that within- and cross-species variations are explained by different variables.

The theoretical studies predicting the effect of mating group size examined the patterns of evolutionarily stable frequency of dwarf males (Charnov 1987; Yamaguchi et al. 2008, 2012, 2013d), and those studies were supported by cross-species comparative studies (Yusa et al. 2012; Lin et al. 2015). In other words, these studies primarily consider the long-term evolutionary adaptation, rather than plastic response to short-term conditions (see Schärer 2009; Yamaguchi et al. 2012). It is not surprising that the prediction is supported by cross-species variations but not by within-species ones, because the prediction primarily focused on the former. Although it is likely that intense sperm competition in larger mating groups is unfavorable for dwarf males at within-species level as well, the prediction at within-species level should depend on proximate mechanisms of sex determination, larval settlement and the interaction between them.

Unfortunately, we know little about them for barnacles.

Mechanism of sex determination in dioecious and androdioecious thoracican barnacles is not specified (Crisp 1983b; Yusa et al. 2013). The correspondence between attachment site and sexuality may be explained by both environmental sex determination and sex-specific settlement behavior. Larvae may be sexually undifferentiated and determine their sex based on their attachment site, that is, they differentiate into males or hermaphrodites/females when they attach to conspecific body surface or other substrata, respectively. Callan (1941) suggested this mechanism for *Scalpellum scalpellum* based on karyotype analysis, rudimentary oogonia in males and a wrongly differentiated individual. However, Gomez (1975) found that larvae of *Conopea galeatus* differentiated into both males and hermaphrodites when metamorphosis was induced by a juvenile hormone mimic without any substrata, arguing against Callan (1941). This result suggested genetic sex determination, or at least, sex determination before settlement. If this is the case, the correspondence between attachment site and sexuality is a result of selective settlement that genetically male larvae choose conspecific bodies and genetically female/hermaphroditic larvae choose other substrata. A more complex hypothesis was proposed (Svane 1987; Høeg et al. 2015). In *S. scalpellum*, virtually all individuals on receptacles (specialized areas to carry males) of conspecific

hermaphrodites are dwarf males, and others are hermaphrodites (Callan 1941; Svane 1987; Buhl-Mortensen & Høeg 2006; Spremberg et al. 2012; Høeg et al. 2015). Svane (1987) reported that more larvae settled on receptacles and differentiated into males when more adult hermaphrodites were present. In addition, some of the larvae settled on conspecific receptacles (i.e. larvae which differentiate into males if not manipulated) differentiated into hermaphrodites when they were artificially removed from conspecific bodies just after settlement (Høeg et al. 2015). Although these results indicate environmental sex determination, the proportion of larvae settled on receptacles and differentiated into males never exceeded 50 percent (Svane 1987). Thus it is suggested that half of larvae are genetically determined hermaphrodites and never settle on conspecific receptacles, and the latter half are plastic in terms of attachment site and sex differentiation (Svane 1987; Spremberg et al. 2012; Høeg et al. 2015). It is also possible that different taxa within Thoracica have different mechanisms of sex determination, given the several independent evolutionary origins (Pérez-Losada et al. 2012; Yusa et al. 2012; Lin et al. 2015) and morphological diversity (Klepal 1987; Lin et al. 2015) of dwarf males in this taxon.

To predict within-species variation of sex ratio among mating groups with different sizes, larval settlement mechanism and ability of assessing environment also



matter. If larvae have genetically determined sex and arrive at habitats randomly, they sometimes need to abandon the opportunity of settlement and to search for other habitats to find suitable groups. Such habitat choice should be risky especially for deep-sea or symbiotic species and thus larvae should not be choosy about habitats with respect to mating group size. In this case, within-species variation of the frequency of males is determined stochastically and may not correlate with group size as shown by this study, while cross-species correlation can appear as a result of optimization of genetic sex ratio through natural selection.

In contrast, environmental sex determination can cause such a correlation not only in cross-species level, but also within-species level. If sex is determined by substrata and larvae can choose substrata (conspecific bodies or others), they can adjust their sex based on their conditions. Thus the predicted correlation can realize if larvae are more likely to attach conspecifics on smaller groups. However, larvae may utilize another cue of environmental conditions. For example, when the longevity of habitats is limited, larvae arrived later may not have enough time to grow up into hermaphrodites/females, and thus should mature early as dwarf males (Yamaguchi et al. 2013b). Such temporal limitation should be important for symbiotic species in which the longevity of habitats is limited by host death or molting. It is likely that *O. unguisiformis* suffers from such

limitation because they use small crabs as hosts. In this case, mating group size may not be a primary factor to determine the optimal sexual differentiation.

To draw a quantitative prediction under such a complex interaction of proximate mechanisms, sophisticated experiments on larval settlement are obviously required. Although the presence of planktonic larval phase in barnacles makes such experiments difficult, observations of larval settlement in captivity has been succeeded for several species of barnacles (e.g. Spremberg et al. 2012). The method to rear larvae of *O. unguisiformis* is currently under development (Y. Yusa pers. comm.). Although the substrata in this species (living crabs) are not suitable for controlled experiments, at least some aspects will be revealed once the methodology is established.

In addition to the need of sophisticated experiments, I also emphasize the importance of examining more species. Only three barnacle species, including *O. unguisiformis* in this study, has been examined with respect to within-species variation of the frequency of males, as noticed above (Spremberg et al. 2012; Ewers-Saucedo et al. 2015). This is too limited to draw a general conclusion since thoracican barnacles include more than 30 species known as androdioecious (Kelly & Sanford 2010) and much more species may be androdioecious but not described, suggested by the increasing number of recent findings (e.g. Yusa et al. 2010, 2012, 2015; Lin et al. 2015;

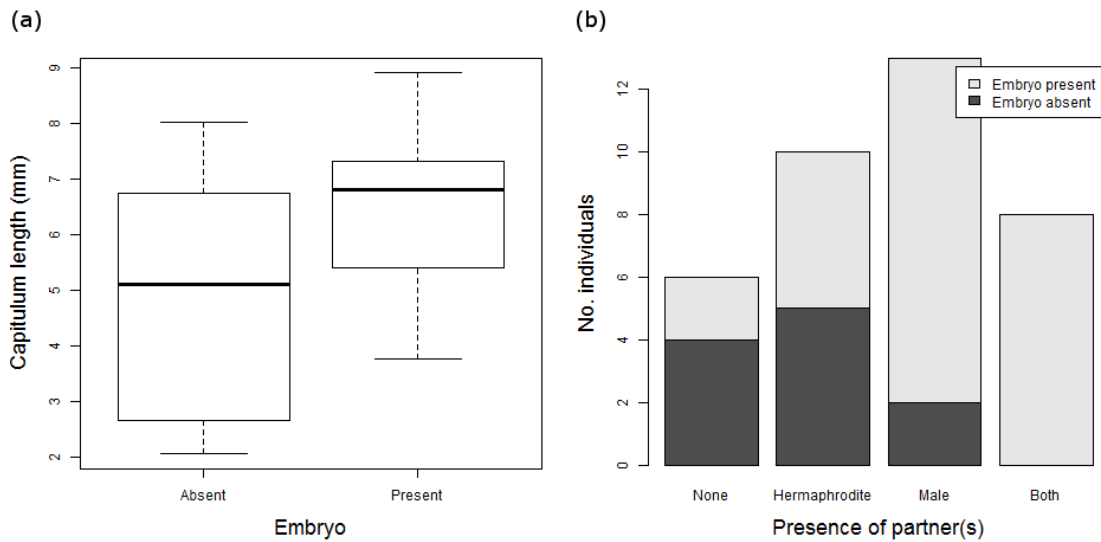
Sawada et al. 2015; Y. Yusa pers. comm.). Fortunately, the number of males on hermaphrodites can be counted by museum specimen without a serious damage to the specimen (e.g. Spremberg et al. 2012). Although mating group size is more difficult to estimate, estimation of the upper limit is possible especially for species using relatively small substrata, as I did in this study.

**Table 3.1:** Summary of statistical analysis

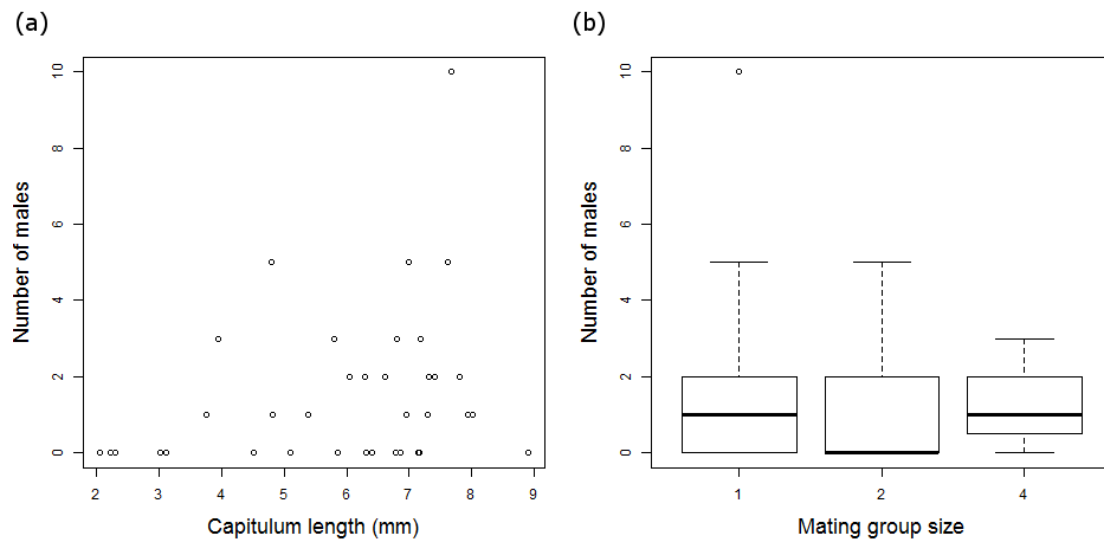
Response	Explanatory	Coefficient	SE	$z$	$p$
Presence of embryos	Capitulum length	0.5056	0.2878	1.757	0.0789
	Hermaphroditic partner(s)	1.6546	1.0898	1.518	0.1289
	Male partner(s)	2.5527	1.0635	2.400	0.0164
Number of males	Capitulum length	0.2740	0.1469	1.865	0.0621
	Mating group size	-0.0817	0.3403	-0.240	0.8103

**Fig.3.1:** The presence or absence of embryos. (a) A relationship with capitulum length.

Vertical axis is capitulum length in mm and horizontal axis is the presence or absence of embryos. (b) A relationship with the presence or absence of potential partners. Vertical axis is the number of matured hermaphrodites with different reproductive status (gray: presence of embryos, black: absence of them) and horizontal axis is the presence or absence of potential partner(s) with different sexualities (None: no potential partners present, Hermaphrodite: having at least one matured hermaphrodite but no male on the body, Male: at least one male but no matured hermaphrodite, Both: having both at least one matured hermaphrodite and at least one male).



**Fig.3.2:** The number of males. (a) Effect of capitulum length (mm). (b) Effect of estimated mating group size.



## Study 3

**Be a good loser:**

**a theoretical model for subordinate decision making about  
bi-directional sex change among harem fish**

## **Introduction**

Most animal societies are not egalitarian and thus show reproductive skew in favor of dominant individuals relative to subordinates (Nonacs & Hanger 2011). Then, why do subordinates remain in a group, rather than disperse to other habitat? Although this question was originally proposed and has been mainly studied for cooperatively breeding species (Emlen 1982; Brown 1987; Wong & Balshine 2011), the same question can be addressed in non-cooperative breeding societies with high reproductive skew (Gardner et al. 2003; Wong 2010; Wong & Buston 2013).

In contrast to gonochorists which usually compete over reproductive status with same-sex individuals, hermaphrodites can adjust their sexuality to their conditions including social status (Baeza & Baur 2004; Wong et al. 2012). Socially controlled sex change provides a good example of such adjustment. Functional sex change is a reproductive strategy adopted by a wide range of organisms (Policansky 1982; Vega-Frutis et al. 2014). Theoretically, individuals should first mature as a sex with weaker size-based reproductive skew, and then change into a sex with stronger skew after attaining large or dominant (Charnov 1982). This model is called as “size-advantage hypothesis” (Ghiselin 1969) and supported by numerous researches especially on marine fishes (Warner 1984; Charnov 1982; Kuwamura & Nakashima 1998; Munday et al.



2006a). The timing of sex change is often controlled by social status in teleost fishes (Robertson 1972; Ross 1990). For example, in a harem fish in which a dominant male monopolize all eggs produced by harem females, it is almost impossible for subordinates to reproduce as males. Hence they should first mature as females and then change their sex into males when they grow up and become dominant. In other words, harem mating systems usually favor protogynous sex change (Warner 1984), and such change is associated with dominant status (Ross 1990). In this case, subordinates can do their best by choosing a sex with weaker skew. However, they sometimes adopt alternative tactics to heighten their social status, including dispersal (Sakai et al. 2001; Manabe et al. 2007b), growth acceleration (Sakai 1997; Hamaguchi et al. 2002) and bachelor sex change in which females change sex without disappearance of harem males and become bachelor males (Moyer & Zaiser 1984; Aldenhoven 1986; Iwasa 1991; Sakai 1997; Takamoto et al. 2003; Yamaguchi et al. 2013c).

The most dynamic example of such interaction between sexuality and social status is bi-directional sex change among fishes (Kuwamura & Nakashima 1998; Munday et al. 2006a; Munday et al. 2010). This ability is known in more than 30 species (Kuwamura et al. 2016). Many of them are harem-polygynous (except for monogamous gobies and facultatively polygynandrous damselfish) and, as predicted by size-advantage

hypothesis, basically protogynous (Munday et al. 2010). When two males accidentally cohabit, the smaller and subordinate one changes sex back to females (Sunobe & Nakazono 1993; Kuwamura et al. 2002, 2011, 2014a, b; Sakai et al. 2003; Wittenrich & Munday 2005; Manabe et al. 2007a; Sakurai et al. 2009; Kadota et al. 2012; but see Manabe et al. 2008). Although such reversed sex change seems adaptive as the best of bad situations for subordinates (Kuwamura & Nakashima 1998), alternative tactics are also possible. Subordinate males can remain as males by dispersal to take over a harem with a smaller resident male than themselves or in which the male disappeared recently, or to wait for the recruitment of new females as bachelors (Kadota et al. 2012; Kuwamura et al. 2014a). Because harem males can achieve much higher fitness than females in polygynous species, such alternative tactics might be favored.

Two factors are hypothesized to facilitate reversed sex change. First, if the mortality risk associated with inter-group dispersal is high, dispersal may no longer be adaptive (Nakashima et al. 1996; Munday 2002; Munday et al. 2010). High risk of movement constrains dispersal, even if successful dispersal could be beneficial (Emlen 1982). Reversed sex change by subordinates can evolve as an alternative to risky dispersal. Bi-directional sex change is relatively common in cryptic, symbiotic, and/or demersal fishes (Munday et al. 2010), such as gobies of genera *Trimma* (Sunobe & Nakazono 1993;

Manabe et al. 2007a, 2008; Sakurai et al. 2009) and *Gobiodon* (Nakashima et al. 1996; Munday et al. 1998; Cole & Hoese 2001; Munday 2002). Since these fishes suffer from high risk of predation when they are away from their habitats or hosts, it supports the risk-of-movement hypothesis. However, several non-cryptic and pelagic fishes in which the risk of movement is apparently small also conduct bi-directional sex change (Munday et al. 2010). These examples can be explained by limited mate availability caused by low density (Kuwamura et al. 2011, 2014a, b), because reversed sex change is experimentally induced by female removal in such fishes including *Labroides dimidiatus* and *Centropyge ferrugata* (Kuwamura et al. 2011). This can induce reversed sex change not only by producing bachelor males (Kuwamura et al. 2002, 2011, 2014a, b) but also by reducing the benefit of harem males. Small harem size and total fecundity caused by low density weaken the reproductive skew among males on average, and thus dispersal to be a dominant becomes less advantageous.

Another unsolved problem about bi-directional sex change is conditional decision making by subordinate or bachelor males. The status of dominant males provide high reproductive success in polygynous species, as noticed above. Therefore, it is predicted that only males with less opportunity to become dominant choose to change sex while males with larger opportunity choose other options. In other words, decision

making about sex change should be conditional not only on environmental factors but also on the conditions of individuals. This prediction is supported by the observation that males often re-acquire females without sex change after mate loss (Kadota et al. 2012; Kuwamura et al. 2014a, 2015). However, the norm of such decision making has not been studied empirically or theoretically. It is also unknown how such decision making should interact with extrinsic environmental conditions to form an optimal conditional strategy. Given the importance of sex change as a tactic to cope with reproductive skew based on social dominance (see above), understanding such decision making should contribute the understanding of coevolution between sexual plasticity and social structures among hermaphroditic species. In this study, I examine the effect of environmental factors and the patterns of decision making by subordinates using a mathematical modeling, to understand the mode of social living based on sexual plasticity.

## **Model**

I assume a basically protogynous and polygynous fish with a harem mating system. Protogynous sex change is assumed to be socially controlled in a manner that follows “suppression model” (Ross 1990). In other words, only the largest and dominant individual in a group becomes a male and fertilize all the eggs produced by females. The

total fecundity by resident females of a harem  $R$  is assumed to be identical for all harems. When two males accidentally cohabit within a group, the smaller one becomes a subordinate. Subordinates must decide whether to change sex to female and remain in the group, or to disperse to another group, depending on the expected reproductive success associated with each tactic. If it becomes a female, its fecundity is  $as - c$ , where  $s$  is the body size,  $a$  is a coefficient representing size-fecundity relationship and  $c$  is the fecundity cost of sex change. If it disperses, it dies with a probability  $d$ , or invades another harem chosen randomly and causes male cohabitation again.

First, I suppose a male invaded to a harem with a resident male for whatever reason. The expected reproductive success of a male which just has invaded to a randomly-chosen harem is denoted by  $V(x)$ , where  $x$  is the body size of the invader. The body size of the resident male is denoted by  $y$ . Because invasion occurs at random, the probability distribution of  $y$  reflects the size distribution of harem males  $D(y)$ . Below I explain how the expected reproductive success of the invader male was calculated for all possible cases of invasion, which is summarized in Table 4.1. See also Fig. 4.1 for graphical representations.

If the invader is larger than the resident (i.e.  $x > y$ ), the invader takes over the harem. Then the resident becomes subordinate and chooses dispersal or sex change (Fig.

4.1(a)). Reproductive success of the invader is identical to the total fecundity of resident females (i.e.  $R$ ) when the former resident male disperses, and to total fecundity of resident females plus fecundity of the former resident male (i.e.  $R + ay - c$ ) when the former resident male changes sex. If the invader is smaller than the resident (i.e.  $x < y$ ), the resident keeps his dominant (harem-male) position. Then the invader becomes subordinate and chooses dispersal or sex change (Fig. 4.1(b)). When the invader disperses again and attempts to invade another harem, reproductive success of the invader is  $(1 - d)V(x)$ , because the next attempt of invasion gives the same expected success as the previous one if it does not die during dispersal. When the invader changes sex, the reproductive success is the fecundity as a female (i.e.  $ax - c$ ).

The expected reproductive success (abbreviated to RS) of an invader male with size  $x$ ,  $V(x)$ , is represented by a summation of two terms (correspond to the winning and the losing cases) as follows:

$$V(x) = \sum_{x>y} D(y)\Delta y * (\text{RS when resident } (y) \text{ is smaller than invader } (x)) + \sum_{x<y} D(y)\Delta y * (\text{RS when resident } (y) \text{ is larger than invader } (x)) \quad (1)$$

where  $D(y)\Delta y$  represents the frequency of resident males whose sizes are between  $y$  and  $y + \Delta y$ . The first term represents RS of the invader male when he is larger than the resident, and depends on the decision making (sex change or dispersal) by the resident.

The second term represents RS of the invader male when he is smaller than the resident, and depends on the decision making by himself. Given decisions by subordinates are assumed to be made in such a way as to maximize the expected RS of themselves, the tactic with larger expected RS is always chosen. Thus Eq. (1) can be represented as follows:

$$V(x) = \int_{s_{\min}}^x D(y) * \{(R + ay - c) * \varphi(y) + R * [1 - \varphi(y)]\} dy + \int_x^{s_{\max}} D(y) * \{(ax - c) * \varphi(x) + (1 - d)V(x) * [1 - \varphi(x)]\} dy \quad (2)$$

where

$$\varphi(s) = \begin{cases} 1 & \text{if sex change is advantageous for subordinates with size } s \\ 0 & \text{if dispersal is advantageous for subordinates with size } s \end{cases}.$$

Eq. (2) is simplified as follows:

$$V(x) = \int_{s_{\min}}^x D(y) * \{R + (ay - c) * \theta[ay - c, (1 - d)V(y)]\} dy + \int_x^{s_{\max}} D(y) * \max[(1 - d)V(x), ax - c] dy \quad (3)$$

where

$$\theta[X, Y] = \begin{cases} 1 & \text{if } X > Y \\ 0 & \text{if } X < Y \end{cases}$$

and

$$\max[X, Y] = \begin{cases} X & \text{if } X > Y \\ Y & \text{if } X < Y \end{cases}.$$

Note that the both sides of Eq. (3) include  $V(s)$  ( $s = x, y$ ). I determine the form of function  $V(s)$  which approximately meets the equation using an algorithm described

below. I assume a size distribution of resident males  $D(y)$  as a probability density, and the minimum size and the maximum size of males as  $S_{\min} = 1$ , and  $S_{\max} = 21$ , respectively. See Results for the specific distributions I assume. An initial form of function  $V(s)$  was given as  $V(s) = 0$  for all possible  $s$ . Then I calculate the right side of the Eq. (3) for all possible  $s$  and compare it with the left side, i.e. the initial values of  $V(s)$ . If  $\sum_{s=S_{\min}}^{S_{\max}} |(\text{left side}) - (\text{right side})| > \delta$ , where  $\delta$  is a value close enough to zero (0.005), I calculate a weighted average of both sides as  $V_{\text{next}}(s) = w * (\text{left side}) + (1 - w) * (\text{right side})$ , where  $w = 0.01$ . Then I replace the initial form of function  $V(s)$  by the updated form  $V_{\text{next}}(s)$ . This procedure is repeated until both sides become close enough to meet  $\sum_{s=S_{\min}}^{S_{\max}} |(\text{left side}) - (\text{right side})| < \delta$ .

I calculate the expected RS of dispersal and sex change for all sizes of males using the final  $V(s)$  determined above. The relative advantage of sex change is calculated by subtracting the RS for dispersal from the RS for sex change. The positive value of it indicates that sex change is favored, and the negative one indicates that dispersal is favored. A decision-making strategy using this criterion is evolutionarily stable.

I examine the dependence of the relative advantage on size distribution of harem males and on environmental parameters for all size-class of males.



## Results

*When male size-distribution follows power functions*

First, I adopt a simpler assumption for the size-distribution of males. The size-distributions of harem resident males  $D(y)$  are assumed to follow the standardized power function  $y^m$ , where  $m$  represents the power-law index ( $m = 0, 0.5, 1, 2, 3$ ), that is,

$$D(y) = \frac{y^m}{\int_{S_{\min}}^{S_{\max}} y^m dy} \quad (4)$$

Note that the distribution skewed toward larger half of the size range except for the case of  $m = 0$ . I plotted it in Fig. 4.2(a).

Fig. 4.2(b) shows the relative advantage of sex change by subordinates when the size distribution of resident males follows Eq. (4). Relative advantage of sex change is the reproductive success associated with sex change (i.e. fecundity as females),  $as - c$ , minus the expected reproductive success associated with dispersal to another harem,  $(1 - d)V(s)$ . Sex change is advantageous if it is positive, and dispersal is advantageous if it is negative. Fig. 4.2(b) indicates that the relative advantage of sex change is positive from the minimum male size  $S_{\min}$  to a certain threshold size  $S_T$ , when  $m \geq 1$  ( $m = 1, 2, 3$ ). In other words, sex change is advantageous for smaller subordinate males. Since

larger subordinate males are likely to find a resident male smaller than himself when dispersed, dispersal is advantageous than sex change for them. The positive relationship between relative advantage and size in the regions with negative relative advantage, seen in Fig. 4.2(b), is a result of size-fecundity skew for females (larger females produce more eggs).

The threshold size increased with an increasing  $m$  when  $m \geq 1$ , that is, even relatively larger males should change sex under larger values of  $m$ . This is because under large  $m$ , dispersed males are likely to encounter larger males than themselves and hence not able to take over a harem. This favors reversed sex change over dispersal.

In contrast, when  $m$  equals 0 or 0.5, the relative advantage of sex change is always negative, indicating that dispersal is always advantageous than sex change. This is because under small values of  $m$ , high frequency of small-sized resident males provides a chance of takeover to dispersed males.

#### *When male size-distribution follows beta functions*

In the previous section I calculated a threshold size of sex change when the probability density of resident male size is highest at the maximum size  $S_{\max}$ , except for the case of  $m = 0$  which corresponds to the uniform distribution. Although such assumptions gave

us a simplified view of how size-distributions affect, it is more realistic to assume that only a small proportion of a population attains the maximum size. The proportion of the resident males with the minimum size should also be low because protogynous sex change by small individuals is rare. To reflect these facts, I assume a distribution in which the probability density at the maximum and minimum size becomes zero. This is a modified beta distribution which is represented by the following equation:

$$D(y) = \frac{(y - S_{\min})^{\alpha-1} (S_{\max} - y)^{\beta-1}}{\int_{S_{\max}}^{S_{\min}} (y - S_{\min})^{\alpha-1} (S_{\max} - y)^{\beta-1} dy} \quad (5)$$

where  $\alpha$  and  $\beta$  are the indices of beta distributions. Note that  $D(S_{\max}) = D(S_{\min}) = 0$  in this distribution, unlike other distribution such as Gaussian distributions. The mean  $E(y)$  and the variance  $V(y)$  of size in this distribution are calculated as follows:

$$E(y) = \int_{S_{\min}}^{S_{\max}} y D(y) dy = \frac{\alpha S_{\max} + \beta S_{\min}}{\alpha + \beta}$$

$$V(y) = E(y^2) - [E(y)]^2 = \frac{\alpha \beta (S_{\max} - S_{\min})^2}{(\alpha + \beta)^2 (\alpha + \beta + 1)}$$

The previous section revealed that the skewness of the male size distribution is an important factor for the threshold size of sex change. Since it is reasonable to assume that the distribution is biased toward larger size in basically protogynous species, I chose the values of  $\alpha$  and  $\beta$  which result in such skewed distributions. I show the results when  $\beta$  varies under fixed  $\alpha$  ( $\alpha = 8$ ). I summarized the mean and variance for each value of

$\beta$  in Table 4.2. As  $\beta$  increases, the size distributions shift toward right (Fig. 4.3(a)).

As  $\beta$  increases, the threshold size of sex change decreases, that is, only smaller males change sex (Fig. 4.3(b)). When the size-distribution biased toward larger size, sex change is also advantageous for relatively larger males (see solid lines in Fig. 4.3(a) and 4.3(b)), because dispersed males are likely to encounter larger resident males.

#### *Effects of parameters on the threshold size of sex change*

I examine the effect of each parameter ( $a, c, d, R$ ) on the threshold size of sex change under the modified beta distribution in which  $(\alpha, \beta) = (8, 4)$  (the solid line in Fig. 4.3(a)), where  $a$  is the coefficient of size-fecundity relationship,  $c$  is the fecundity cost of sex change,  $d$  is the risk of dispersal and  $R$  is the total fecundity of resident females in a harem. The result is shown in Fig. 4.4. The quantitative pattern of Fig. 4.4 is robust to the change of parameters.

As the coefficient size-fecundity relationship  $a$  increases, the threshold size of sex change also increases (Fig. 4.4(a)), because  $a$  directly increases the reproductive success of sex-changed individuals. Under  $a = 1.2$ , the curve of reproductive success crosses the x-axis twice. The smaller cross point is the threshold size  $S_T$  of sex change and individuals smaller than the point change sex. The larger cross point indicates that

the larger males also change sex, because they can produce many eggs after changing sex under large values of  $a$ .

As the fecundity cost of sex change  $c$  increases, the threshold size  $S_T$  of sex change slightly decreases (Fig. 4.4(b)). This is because the reproductive success of sex-changed individuals decreases by the cost. When it is as large as 2, dispersal is advantageous for the minimum-sized individuals, producing another cross point than the threshold size. This is because the cost exceeds the potential fecundity of the minimum-sized individuals.

As the risk of dispersal  $d$  increases, the threshold size  $S_T$  of sex change increases (Fig. 4.4(c)), indicating that even the larger individuals changes sex. When dispersal incurs high mortality risk, subordinates should change sex and remain at their habitat.

When the total fecundity of resident females  $R$  is large ( $R = 30, 50$ ), there is a threshold size  $S_T$  of sex change and the threshold size correlated negatively with  $R$ . This is because relatively large males can take over other harems by dispersal and fertilize eggs produced by resident females. When  $R$  is small ( $R = 10$ ), all males irrespective of size chooses sex change, because they can achieve high fitness by egg production by themselves than the total fecundity of hare males (Fig. 4.4(d)).

Fig. 4.5 is a contour graph of the threshold size  $S_T$  of sex change under varying risk of dispersal  $d$  and the total fecundity of resident females  $R$ . The threshold size increases (i.e. even larger males change sex) as  $d$  increases or as  $R$  decreases. Under high  $d$ , all males change sex to females when they become subordinates.

## Discussion

In this study, I analyzed the adaptive behavior by subordinate individuals under a potentially plastic sexuality, i.e. bi-directional sex change. I specified possible environmental conditions favoring reversed sex change, and predicted size-dependent decision making about reversed sex change in harem and basically protogynous populations.

### *Ecological factors favoring bi-directional sex change*

My model found that two ecological factors, that is, the high mortality during movement (large  $d$ ) and/or low total fecundity of a harem (small  $R$ ), favor reversed sex change over dispersal. The former factor directly reduces the fitness of dispersing individuals. The latter factor reduces the benefit for a harem male, and hence the benefit of repetitive dispersal to look for a patch without a larger male. These findings are consistent with

previous hypotheses (risk-of-movement and low-density) and empirical studies. Therefore, my model gives mathematical validation to these hypotheses.

As noticed in Introduction, bi-directional sex change is relatively common among fishes with limited ability of dispersal (Munday et al. 2010), and explained by the risk of dispersal. And examples of experimental induction by mate-removal among non-cryptic fishes can be explained by limited mate availability caused by the removal, which correspond to low density under natural conditions (Kuwamura et al. 2011, 2014a, b, 2015). However, if population density is consistently low to prevent polygyny, protogynous sex change should no longer be favored, breaking the prerequisite of reversed sex change. Reversed sex change by basically protogynous species is predicted to occur under accidental low density in populations with regularly higher density (Kuwamura et al. 2011, 2014a, b). The extent of population fluctuation may play a key role in the evolution of bi-directional sex change (see also Study 4).

My model starts from the occurrence of two-male cohabitation for whatever reason. When protogynous sex change is perfectly controlled socially in a harem, such cohabitation occurs only through the inter-group dispersal by at least one male, which is unlikely among fishes with extremely high risk of dispersal. Here is a dilemma, that is, although high risk of movement favors reversed sex change, it reduces the chance of such

change to occur (Nakashima et al. 1996). Although this dilemma is invisible in experimental studies which induce reversed sex change by forcing male cohabitation, it should not be ignored to understand the natural condition of reversed sex change. The simplest solution to this dilemma is to assume an intermediate risk of dispersal. In a cryptic goby *Trimma okinawae*, which can change sex bi-directionally and rapidly, low ranked females sometimes disperse to other group to improve their social status (Manabe et al. 2007b), suggesting the risk is not so high to prevent any dispersal. However, given the ecological and behavioral diversity of bi-directional sex changers (Munday et al. 2010), it is unlikely that all of them share a common and appropriate extent of the risk. Instead of assuming a constant and intermediate risk, it is reasonable to assume that the risk of dispersal varies within a population, for example depending on the distance between habitats (Wong 2010). Bachelor males may first moves to neighboring patches with low risk, and when all of them are preoccupied by larger males, they may choose to change sex rather than disperse to a remote patch with high risk (Nakashima et al. 1996; Kuwamura et al. 2014a). Therefore, in addition to the fluctuation of density discussed above, a spatiotemporal variation of the risk of dispersal may also favor bi-directional sex change.



### *Size-based conditional strategy*

As well as the general condition favoring bi-directional sex change, I also revealed the factors affecting decision making by subordinate males. My model found that not necessarily all individuals adopt the same tactic, that is, conditional strategy based on body size should be adaptive under a certain condition. Sometimes the largest males, sometimes the smallest males are predicted to change sex when they become subordinate, based on the environmental parameters.

The former prediction that largest males change sex should realize when size-dependent reproductive skew among males is not so high relative to size-fecundity relationship of females. It is even possible that being large females is better than being harem males, under limited total fecundity per harem and/or steep size-fecundity relationship (Muñoz & Warner 2003, 2004; Yamaguchi et al. 2013c). If this condition is consistently met in the population, my assumption of basic protogyny is invalid. The facts that bi-directionally sex changing fishes are basically protogynous (Munday et al. 2010) and that in these fishes the largest generally becomes male (except for *Trimma kudo*, see Manabe et al. 2008) suggest the condition is unusual. Therefore, I do not focus on these cases.

The more important and realistic prediction is that smaller males are more likely

to change sex to females. This is because smaller ones are more difficult to find a harem with a male smaller than themselves. Indeed, this prediction is compatible with the traditional size-advantage model which predicts that smaller individuals should act as females under polygyny (Ghiselin 1969). However, the decision making regarding reversed sex change has not been studied, partly because most studies were done in captivity and thus do not allow individuals to disperse. Although there are a few field researches, number of observed sex change is small (Kuwamura et al. 2002, 2011, 2014a, b; Manabe et al. 2007a; Kadota et al 2012) and not enough attention is paid for individuals who do not change sex, preventing the quantitative analysis of decision making.

Under certain conditions, only the smallest males are predicted to change sex. In this case, reversed sex change should rarely occurs in the wild, because small males are generally rare in basically protogynous populations. This may explain why reversed sex change is rarely observed in the field even in species which have a potential to do it. As noticed above, most reports of reversed sex change in the field is based on one or a few cases (e.g. Kuwamura et al. 1994a, 2002, 2011, 2014a, b; Manabe et al. 2007a; Kadota et al. 2012), even for species which change sex easily in captivity, such as *Trimma* (Sunobe & Nakazono 1993). Although a cleaner wrasse *Labroides dimidiatus* has been extensively studied with respect to sex change in the field (Robertson 1972; Robertson & Choat 1974;

Kuwamura 1981, 1984; Nakashima et al. 2000; Sakai et al. 2001), reversed sex change without experimental manipulation has never been observed. Such rarity makes any field investigation of bi-directional sex change extremely difficult. Based on my model, it is predicted that I can observe more cases of sex change by focusing on small males.

#### *Possible effects of time and life history*

In this model, I do not incorporate the time course and focused on the immediate decision making based on current conditions. This simplification is justified because reversed sex change is a tactic to manage accidental and temporary change of local social conditions, rather than to long-term average conditions. While it is possible to interpret the parameters in my model to implicitly include long-term expectations and thus my main conclusion will hold at least qualitatively, specifying them explicitly will contribute to detailed understanding of evolutionary conditions and comparison with empirical data. Here I discuss possible effects of temporal factors to provide a basis for future expansions.

First, both sex change and dispersal take time during which animals cannot reproduce. Interestingly, duration for each direction of sex change varies among fish taxa (Sakai et al. 2003), suggesting different selective pressures. The fecundity cost of sex change ( $c$ ) in my model can be interpreted to include the opportunity loss of reproduction

during sex change, although it is possible that the time cost works in a different way, especially when the breeding season is restricted.

Second, as time goes by, each individual undergo various changes, including death, growth, recruitment, and so on. Those life history factors are suggested to affect the evolution of subordinate decision making in group living animals (Arnold & Owens 1998; Covas & Griesser 2007), and likely to affect sex change as well. For example, death of a dominant male offers opportunity to become a harem male for subordinate females, including ones underwent reversed sex change. Thus reversed sex change may function not only as a tactic to achieve fitness by producing eggs as females, but also a tactic to go back in the social queue and wait until the opportunity of “comeback”. In contrast, recruitment of newly-settled females should select against sex change, because bachelor males can achieve partners just by waiting, without takeover of other harems. Indeed, males often achieve partners in this way and thus do not change sex after the experimental mate removal (Kuwamura et al. 2011, 2014a). In addition, sexually dimorphic growth rate is supposed to play an important role for sex change among monogamous coral-dwelling gobies (Kuwamura et al. 1994a; Nakashima et al. 1996; but see Munday 2002), especially to explain why these gobies are basically protogynous even if they are monogamous (Kuwamura et al. 1994a). Again, it is possible to interpret that the assumed

values of fecundity include the expected changes in the future, although the effects may not be as simple as I assumed in this model.

### *Conclusion*

This study emphasizes an aspect of sex change as a social tactic. My model predicts that ecological factors including the high risk of dispersal and low population density should favor reversed sex change over dispersal, supporting previous hypotheses. These hypotheses are quite similar to the ones for cooperative breeding or group living, such as ecological constraint hypothesis and mate limitation hypothesis (Brown 1987; Pruett-Jones & Lewis 1990). This indicates the underlying common theme between these phenomena: decision making by subordinate individuals. In addition, I predict a size-dependent conditional strategy of sex change and dispersal. Not only sex allocation theories for hermaphroditic strategies (e.g. Charnov 1982), but also the theoretical framework for group living in general was helpful to understand my result, even though it primarily assumes gonochoristic animals. I hope this study stimulates studies on the unique form of society based on sexual plasticity (Wong et al. 2012) through the unified framework to understand both gonochoristic and hermaphroditic societies.

**Table 4.1:** Criterion of decision making and the reproductive success. I denote  $x$  and  $y$  as the size of the invader male and that of the resident male, respectively.  $V(s)$  represents the expected reproductive success when an male of size  $s$  invades into a randomly chosen harem.

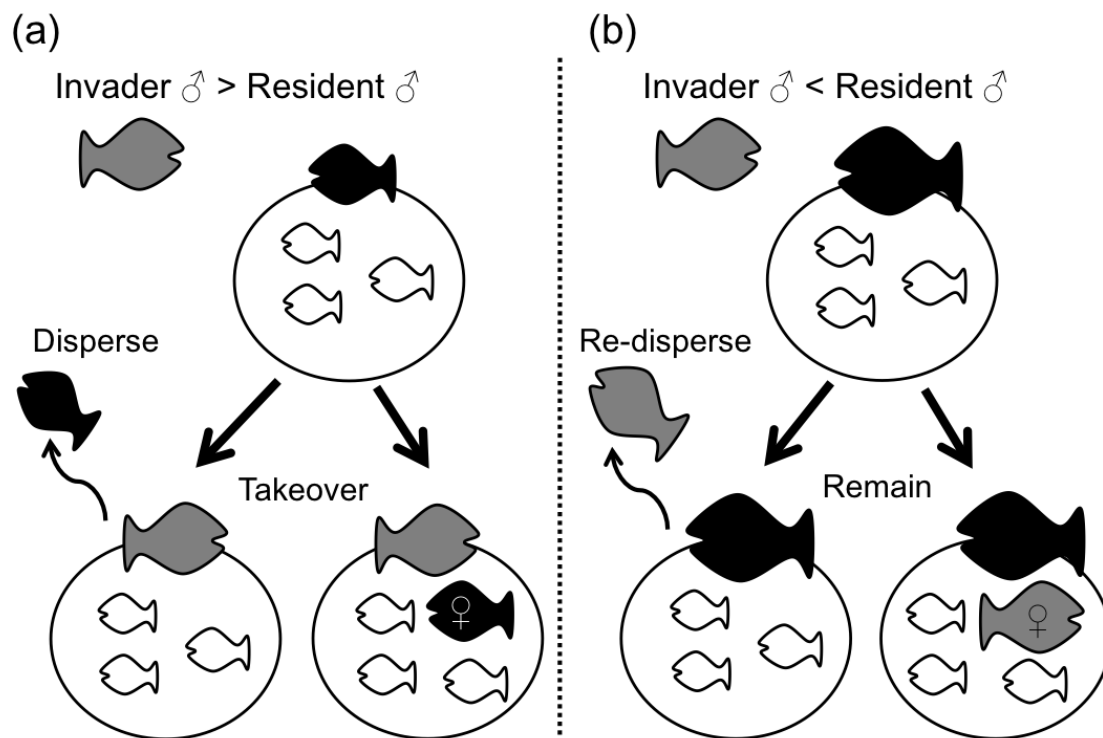
Magnitude relation of male size	Criterion of decision making	Decision of the invader	Decision of the resident	RS <sup>a</sup> of the invader
$x > y$	$(1 - d)V(y)$ $> y - c$	Takeover	Disperse	$R$
	$(1 - d)V(y)$ $< y - c$	Takeover	Sex change	$R + ay - c$
$x < y$	$(1 - d)V(x)$ $> ax - c$	Re-disperse	Remain	$(1 - d)V(x)$
	$(1 - d)V(x)$ $< ax - c$	Sex change	Remain	$ay - c$

<sup>a</sup> Reproductive success

**Table 4.2:** Mean, variance and standard deviance in the modified  $\beta$  distribution when  $\beta$  varies and  $\alpha = 8$ .

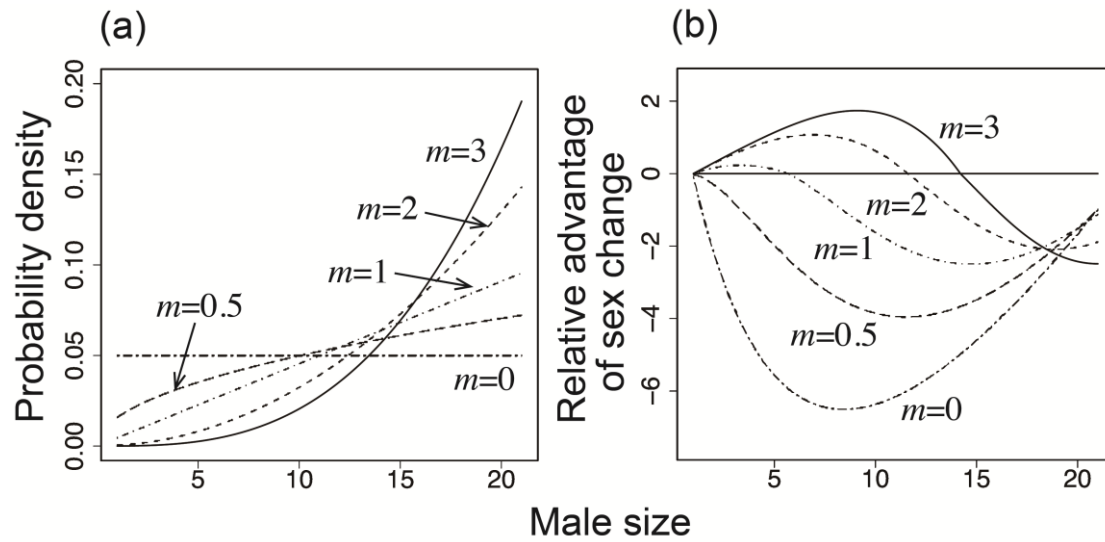
$\beta$	Mean	Variance	Standard deviance
4	14.33	6.84	2.61
5	12.43	6.43	2.55
8	11.00	5.88	2.43

**Fig.4.1:** Model assumption about decision making. (a) If the invader male is smaller than the resident male, the resident remains as a harem male. The invader decides whether to disperse again or change sex to female, according to the expected reproductive success. (b) If the invader is larger than the resident, the invader takes over the harem. The resident decide whether to disperse or change sex.



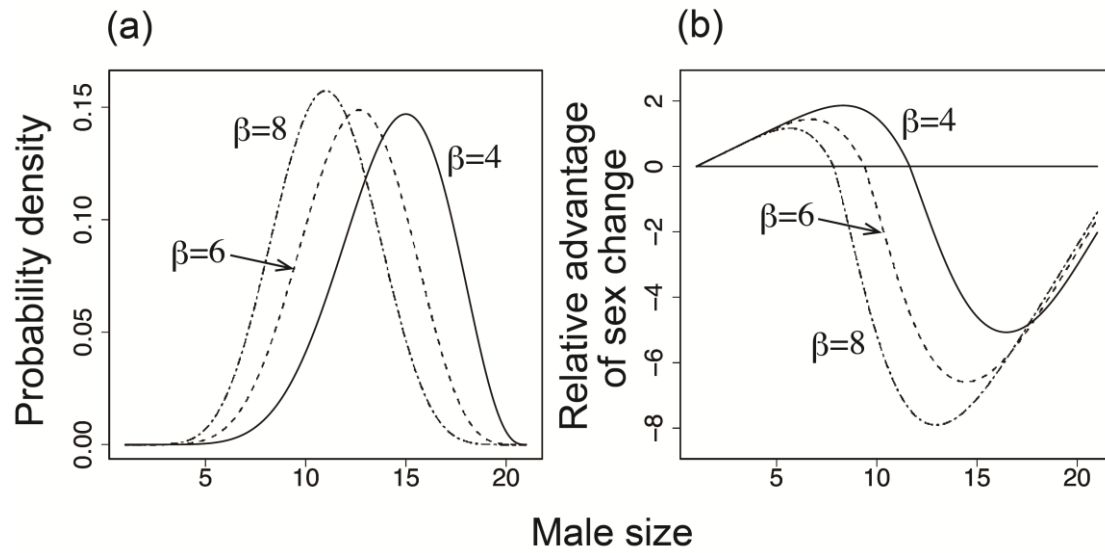
**Fig.4.2:** The advantage of sex change when male-size distribution obeys power functions.

(a) Male-size distributions represented by standardized power functions of size,  $y^m$ , where  $m$  is the power-law index ( $m = 0, 0.5, 1, 2, 3$ ). (b) The threshold size of sex change depending on the power-law index  $m$ . The horizontal axis is male size and the vertical axis is the relative advantage of sex change (i.e. fitness associated with sex change minus fitness associated with dispersal). The other parameters are  $a = 10$ ,  $d = 0.3$ ,  $c = 1.0$  and  $R = 30.0$ .

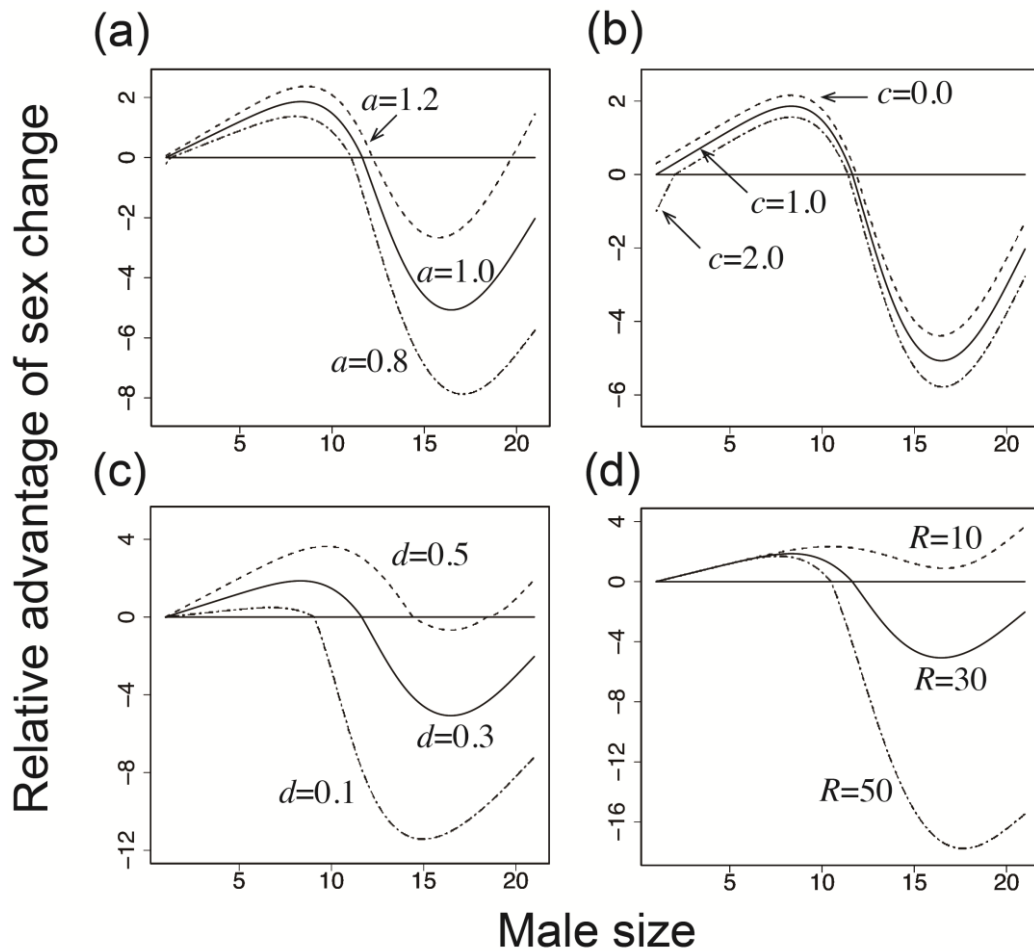




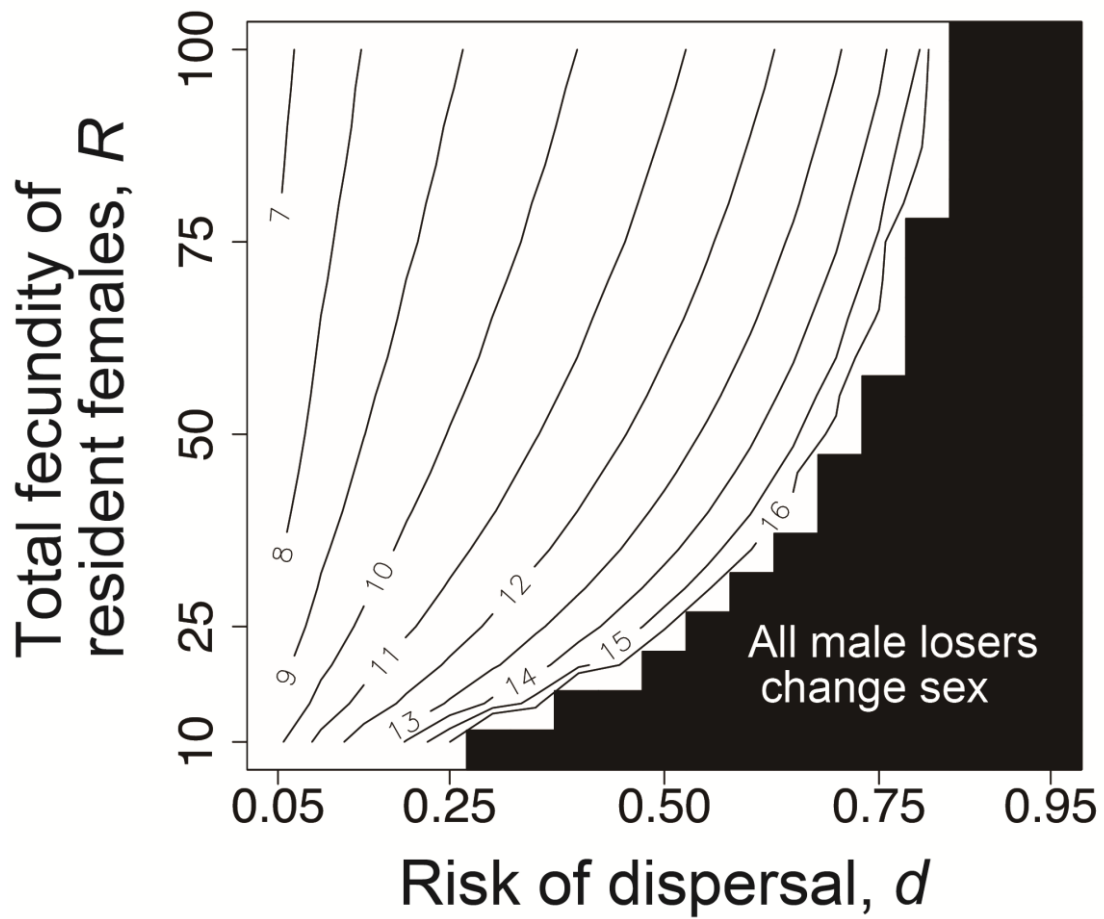
**Fig.4.3:** The advantage of sex change when male-size distribution obeys modified  $\beta$  distributions. (a) Male size distributions. (b) The threshold size of sex change depending on the index  $\beta$ . The other index  $\alpha$  was fixed to 8. The horizontal axis is male size and the vertical axis is the relative advantage of sex change. The other parameters are the same as Fig. 4.2(b).



**Fig.4.4:** Effect of four factors ( $a$ ,  $c$ ,  $d$ ,  $R$ ) on the relative advantage of sex change when male size distribution obeys the modified  $\beta$  distributions with  $(\alpha, \beta) = (8, 4)$ . (a) The result when the coefficient of size-fecundity varies. The other parameters are  $d = 0.3$ ,  $c = 1.0$  and  $R = 30.0$ . (b) The result when the fecundity cost of sex change varies. The other parameters are  $a = 10$ ,  $c = 1.0$  and  $R = 30.0$ . (c) The result when the risk of dispersal varies. The other parameters are  $a = 10$ ,  $c = 1.0$  and  $R = 30.0$ . (d) The result when the total fecundity of resident females varies. The other parameters are  $d = 0.3$  and  $c = 1.0$ .



**Fig.4.5:** The contour graph for the threshold size of sex change, depending on the risk of dispersal  $d$  and the total fecundity of resident females  $R$ . The black region shows that all males change sex to females when lost (became subordinates). The other parameters are fixed as  $a = 0.3$  and  $c = 1.0$ .



## Study 4

**Ecological background of sexual plasticity:  
population fluctuation and life history of  
a bi-directional sex changer**

## **Introduction**

Sex change, or sequential hermaphroditism, is a reproductive strategy adopted by a wide range of animals and plants (Policansky 1981; Vega-Frutis et al. 2014). The adaptive significance of sex change is generally explained by “size-advantage hypothesis” (Ghiselin 1969; Warner 1975; Charnov 1982; Munday et al. 2006a). According to this hypothesis, the occurrence and direction of sex change should depend on the mating system. For example, polygynous species in which a few largest and dominant males monopolize many females are predicted to be protogynous. This association between mating systems and sex change is supported mainly based on the studies for teleost fishes (Warner 1984; Erisman et al. 2009, 2013; Kazancıoğlu & Alonzo 2010). However, theoretical studies also suggested the potential importance of other non-mating factors such as growth, nutrition and mortality in the evolution of sex change (Charnov 1986; Iwasa 1991; Brooks & Iwasa 2010; Yamaguchi et al. 2013c).

Bi-directional sex change, in which individuals change sex in both ways repetitively, is also known for various organisms (Policansky 1982), and especially well-studied in teleost fishes (Munday et al. 2010). It is explained by inter-group dispersal hypothesis which is an expansion of size-advantage hypothesis (Nakashima et al. 1995; Munday et al. 2010). In basically protogynous species, inter-group dispersal of males

sometimes causes the coexistence of multiple males in a group. Then the smaller and subordinate male changes sex into female (reversed sex change), because it is no longer large enough to reproduce as a dominant male. This hypothesis was supported by both field and captive studies describing sex change by smaller males under male cohabitation (reviewed by Munday et al. 2010; but see Manabe et al. 2008).

Since these fishes are polygynous, dominant males can achieve higher fitness than females. Thus it might be adaptive for subordinate males to disperse and find a group without larger males (Study 3). The risk of dispersal was proposed to explain why subordinate males change sex rather than disperse (Munday 2002; Munday et al. 2010). In addition, occasional low density caused by spatiotemporal fluctuation of population density may lead to facultative monogamy, accidental mate loss and difficulty to find females, also favoring reversed sex change (Kuwamura et al. 2002; 2011). This low density hypothesis was proposed and tested by artificially creating low density by removing individuals (Kuwamura et al. 2011, 2014a, b, 2016). However, natural fluctuation of demography in bi-directional sex changers has rarely been studied (but see Kuwamura et al. 1994b). Examination of demography in these fishes is required.

Gobies (family Gobiidae), one of the most species-rich families of vertebrates (Nelson 2006), include several lineages with the ability of sex change (Cole 2010). Some

of them undergo bi-directional sex change (Munday et al. 2010). Some of hermaphroditic gobies exhibit unique features, making this taxa particularly important for the study of sex change. First, extremely rapid sex change (often completes within less than 1 week) in both directions is observed in two genera, *Trimma* and *Lythrypnus* (Sunobe & Nakazono 1993; Reavis & Grober 1999; Manabe et al. 2008; Sakurai et al. 2009). This sexual plasticity is enabled by their unique gonadal structure, that is, both ovary and testis are present at the same time but only one on them is active (Cole 1990; St. Mary 1998; Kobayashi et al. 2005). Understanding such extreme sexual plasticity should contribute not only to the study of sexuality but also that of phenotypic plasticity in general.

Second, the patterns of sex change in two genera of coral-dwelling gobies, *Paragobiodon* and *Gobiodon*, are supposed to be explained by a modified version of “growth-rate advantage” hypothesis (Kuwamura et al. 1994a; Nakashima et al. 1996). They are basically protogynous (with an ability of reversed sex change) even though they mate in monogamous pairs (Lassig 1977; Kuwamura et al. 1994a; Nakashima et al. 1996; Munday et al. 1998; Munday 2002), apparently contradicting the size-advantage hypothesis which predicts an association between polygyny and protogyny. This contradiction can be solved by sex-specific growth rate with faster rate in females than males. The faster growth rate in females, together with the sex-changing norm that smaller

and larger ones in a cosexual pair becomes females and males respectively, results in size-assortative pairs (Kuwamura et al. 1994a), and is advantageous for both sexes because reproductive success of a pair depends on the sizes of both egg-guarding males and egg-producing females to a similar extent (Kuwamura et al. 1993). This is a modified version of the “growth-rate advantage hypothesis” (Iwasa 1991) which predicts sex change from sex with faster growth to sex with slower growth. To my knowledge, it is the only application of growth-rate advantage hypothesis on fish sex change. Thus testing sex-specific growth in other hermaphroditic gobies is strongly required, but has not conducted. For example in genus *Trimma*, sex-specific growth rate was only tested for two presumably gonochoristic species (Winterbottom & Southcott 2008; Winterbottom et al. 2011), in which growth is sex-specific in one species (*T. nasa*) but not in the other (*T. benjamini*).

In this study, I conducted a long-term mark-and-recapture investigation and report growth pattern and population fluctuation in a marine goby *Trimma caesiura*, which is likely to be able to change sex bi-directionally (Cole 1990; Munday et al. 2010). Although little is known for its ecology, it was reported that this species lives beneath the overhanging reef structures within coral reefs and feeds mainly on copepods (Saeki et al. 2005). I aimed to elucidate the background ecological characteristics underlying extreme



sexual plasticity in this genus.

## **Materials and Methods**

I conducted a field investigation of *Trimma caesiura* at the coral reef of the Sesoko Island, Okinawa, southern Japan. I chose 3 areas (I, II and III) along the reef edge in which individuals of *T. caesiura* distribute continuously. The depth of these areas were about 1 to 5 meters. Area I, II and III were investigated from April 2008 to September 2010, from May 2008 to September 2010 and from June 2009 to February 2010, respectively. All individuals in the areas were collected as exhaustively as possible, by a hand-net using SCUBA. I measured the standard length of the collected individuals to the nearest 0.1 mm using calipers. Individuals were sexed by the inspection of the shape of genital papillae, which corresponds to functional sex (long and tapered posterior in males, and bulbous in females) in gobies including *Trimma* (Sunobe & Nakazono 1993; Manabe et al. 2008). Individuals with short and undeveloped papillae were categorized as immature. All individuals, except for 15 immature individuals which were too small to mark, were marked individually by the injection of fluorescent dye. Then I released them to their sites they collected in the next day of collection. I repeated this procedure with intervals of one to a few months. When sex of each individual changed, the individual was judged to have

changed sex.

The sexual size dimorphism and sex-specific growth rate were statistically tested using separate linear models. For the former, the standard length of all adults at the initial collection of each individual was analyzed by fitting a model with sex as an explanatory variable. For the latter, the amount of growth (difference of standard length between two measurements) for consecutive collections on adults were analyzed by fitting a model with sex and the standard length at the prior collection as explanatory variables. To control the variation of collection intervals, I used only the sets of measurement with intervals of 27-29 days, since this range included approximately one-fourth of consecutive measurements. No individuals had more than one sets of such measurements and experienced sex change during such intervals. The interaction between sex and standard length was removed from the models after confirming non-significance by likelihood ratio test. In both analyses, p-values were calculated by likelihood ratio test. All analyses were conducted by a statistical software *R* ver. 3.2.1 (*R* Core Team 2015).

## **Results**

I recorded 296 measurements for 171 individuals in total, consisting of 29 males, 82 females and 60 immature individuals when they collected for the first time. Standard

length of males, females and juveniles at the initial collection of each individual were shown in Fig. 5.1. Among adults, males were significantly larger than females ( $n=111$ ,  $b=2.958\pm0.519$ ,  $t=5.698$ ,  $p<0.001$ ). Among adults collected twice at the intervals of 27-29 days, relationship between growth, size and sex was shown in Fig. 5.2. The smaller individuals grow significantly more ( $n=31$ ,  $b=-0.083\pm0.039$ ,  $t=-2.124$ ,  $p=0.031$ ), but the growth did not differ between the sexes significantly ( $b=0.085\pm0.244$ ,  $t=0.350$ ,  $p=0.713$ ).

I observed six cases of sex change in total, based on the shapes of urogenital papillae (Table 5.1). Only one of the six cases was protandrous (male to female) change and the remaining five cases were protogynous (female to male). Throughout the periods of survey, population abundance fluctuated greatly in all of 3 survey areas (Fig. 5.3-5.5). Sex ratios of adults also fluctuated. For example, sex ratios (males/adults) in Area I (Fig. 5.3) varied from approximately 0.21 (in April 2008) to 0.67 (in Feb. 2009). Note that both sex ratio and abundance fluctuated not only intra-annually (possibly reflect seasonal variation) but also inter-annually.

## **Discussion**

### *Sexual and mating systems*

I found male-biased sexual size dimorphism and generally female-biased sex ratio in

*Trimma caesiura*. These patterns are similar to those of other hermaphroditic species, such as *T. okinawae* (Sunobe & Nakazono 1990, 1993; Manabe et al. 2007a), *T. kudo* (Manabe et al. 2008) and *T. yanagita* (Sakurai et al. 2009). In contrast, presumably gonochoristic species such as *T. nasa* (Winterbottom & Southcott 2008) and *T. benjamini* (Winterbottom et al. 2011) represent female-biased size dimorphism (in the former) or monomorphism (in the latter) and only slightly female-biased sex ratio (in both). In addition, I observed 5 protogynous and 1 protandrous sex change based on morphological inspection of urogenital papillae. Although detailed behavioral and histological examinations are needed to confirm functional sexuality, this result and the gonadal structure (Cole 1990) strongly indicate basically protogynous hermaphroditism with an ability of reversed sex change in this species. The sex-size distribution and sex ratios suggested polygynous mating systems, also in consistent with a hermaphroditic congener *T. okinawae* (Sunobe & Nakazono 1990) and with most other fishes with the ability of bi-directional sex change (Munday et al. 2010).

#### *Growth rate and sex change among gobies*

Growth rate did not differ between the sexes in *Trimma caesiura*. Therefore, sexual size dimorphism should be a result of protogynous sex change, not of sex-specific growth rate

as in *T. nasa* (Winterbottom & Southcott 2008). In hermaphroditic coral-dwelling gobies, *Paragobiodon* and *Gobiodon*, females grow faster than males (Kuwamura et al. 1994a; Nakashima et al. 1996). As mentioned in Introduction, the modified version of growth-rate advantage hypothesis based on this sex-specific growth was proposed to explain the pattern of sex change in these two genera (Kuwamura et al. 1994a; Nakashima et al. 1996). That is, the smaller individual in a pair becomes a female and grows rapidly to form a size-assortative pair.

This modified growth-rate advantage hypothesis was criticized. First, it cannot explain why sex change rarely occurs in heterosexual pairs with smaller males and larger females among those gobies (Kuwamura et al. 1994a; Munday 2002). This indicates that the primary function of sex change is mate-acquisition under high risk of mate-search (Munday 2002). Nevertheless, we have no other hypothesis to explain the sex-changing norm that smaller and larger ones in a cosexual pair becomes females and males respectively in these gobies. Even if the primary advantage of sex change itself is mate acquisition, growth-rate advantage might determine the pattern how sex change occurs. Second, Munday et al. (2006b) reported a cooperative growth regulation that the smaller individuals grow faster irrespective of size in *Gobiodon*. If sex-specific growth rate is a result of such growth regulation based on size hierarchy, rather than a physiological

constraint limiting growth in males, the assumption of growth-rate advantage hypothesis does not hold. However, males in size-matched pairs grow faster than females (Munday et al. 2006b), suggesting a role of physiological constraints to some extent. A sophisticated experiment to distinguish growth regulation and physiological constraints is required.

Another problem for growth-rate advantage hypothesis is its generality. As far as I know, it has been only applied to the two genera of coral-dwelling gobies despite the diversity of sex-changing gobies and other teleosts. Interestingly, several species of genus *Priolepis*, a close relative to *Trimma* (Thacker & Roje 2011), also follow the “smaller-female, larger-male” pattern of bi-directional sex change and form size-assortative pairs (Sunobe & Nakazono 1999; Manabe et al. 2013). It is likely that the modified growth-rate advantage hypothesis can also be applied to this genus (Manabe et al. 2013) like coral-dwelling gobies. However, two assumptions of the hypothesis, i.e. sex-specific growth rate and the dependence of reproductive success on sizes of both mates, are not confirmed for *Priolepis*. This study is not a direct test of the modified growth-rate advantage hypothesis because *Trimma caesiura* is probably polygynous. However, I demonstrated that the sex-specific growth is not universal among hermaphroditic gobies, calling for the need to examine the growth pattern in other species.

In addition to the effect on sex change, the evolution of sex-specific growth rate itself is an important biological problem. In some gonochoristic gobies, males grow faster than females in polygynous species but not in monogamous species, probably because of different selective pressures on male size and different costs of paternal egg guarding (Hernaman & Munday 2005). Hermaphroditic species can manage the sex-specific effect of size on fitness by size-dependent sex change (Wong et al. 2012), not by sex-specific growth. Therefore, growth rate may not only affect sex change, but also be affected by sex change strategies. Such possible interaction has not been fully understood.

#### *Population fluctuation and sexual plasticity*

Low density hypothesis (Kuwamura et al. 2011, 2014a, b, 2015) argues that occasional low density favors bi-directional sex change because it causes bachelor males and prevents mate acquisition by them. I found that both inter-annual and intra-annual fluctuations of population are highly drastic in *Trimma caesiura*. In terms of the hypothesis, this fluctuation might cause the selective pressure for the ability of bi-directional sex change.

The effect of low density is supported by the induction of reversed sex change by experimental mate-removal (Kuwamura et al. 2011, 2014a, b, 2015) and by the

theoretical modeling (Study 3). However, if density is consistently low so that males rarely dominate multiple females, protogynous sex change should not evolve. Therefore it is inconsistent with the fact that most fishes with the ability of bi-directional sex change are primarily protogynous (Munday et al. 2010). A similar dilemma also matters for the risk of dispersal hypothesis, another evolutionary hypothesis for this phenomenon. If dispersal is too risky so that any dispersal in adult stage is not adaptive, accidental cohabitation of males cannot occur and thus there should be no opportunity of reversed sex change (Nakashima et al. 1996).

Spatiotemporal fluctuation of environmental conditions may solve these dilemmas (see also Study 3). If population density fluctuates over time as demonstrated by this study, reversed sex change should be adaptive under temporal low density while basically protogynous sex change is also favored by temporal high density (see Wong et al. 2005). Spatial variation of density may also have a similar effect, that is, generally polygynous species sometimes include local populations with low density and facultative monogamy (Kuwamura et al. 2002). This situation probably occurred in the study population of *T. caesiura* because population fluctuation sometimes led to almost equal or even male-biased sex ratios (Fig. 5.3-5.5). In such situation, the risk of dispersal may also fluctuate. For example, it is likely that the risk of dispersal for coral-dwelling gobies



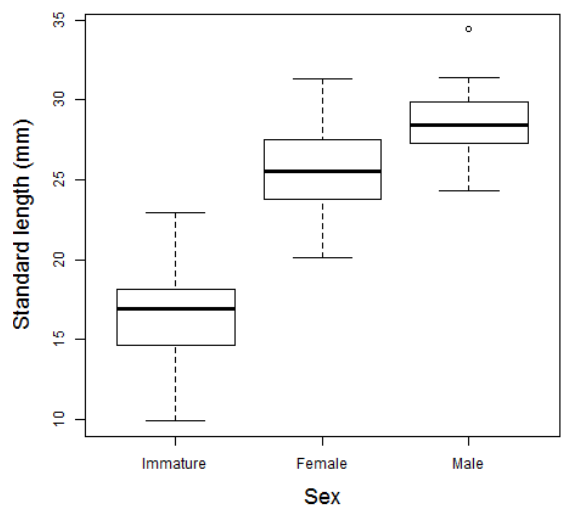
depends on the distance between corals (Wong 2010). Thus bachelor males can first disperse to nearby habitats to search females, and change sex into females only when all nearby habitats were occupied by larger males (Nakashima et al. 1995; Kadota et al. 2012). Investigation of such spatiotemporal fluctuations over a long time or a wide range will be required to understand the evolution of sexual plasticity.

It must be noted that I observed only 1 case of reversed sex change despite a long-term investigation of a largely fluctuating population. It suggests that males may adopt other tactics to cope with social changes caused by the fluctuation. Such tactics may include waiting for the recruitment of newly-settled females (Kadota et al. 2012). The decision-making about reversed sex change should be studied (Study 3).

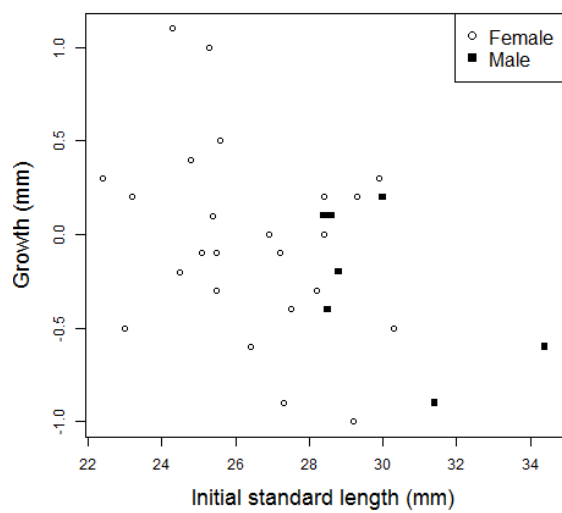
**Table 5.1:** Observed sex change (SC) based on the morphology of urogenital papillae.

Direction of SC	Last collection before SC		First collection after SC	
	Date	SL (mm)	Date	SL (mm)
Protogyny	May 3, 2008	29.5	Jun 26, 2008	29.3
	Feb 16, 2009	25.1	Aug 13, 2009	26.0
	Oct 29, 2009	20.8	Mar 5, 2010	26.3
	Mar 1, 2010	29.6	Sep 13, 2010	30.7
	Mar 4, 2010	25.3	Sep 14, 2010	26.2
Protandry	Jun 2, 2009	28.1	Aug 20, 2009	28.2

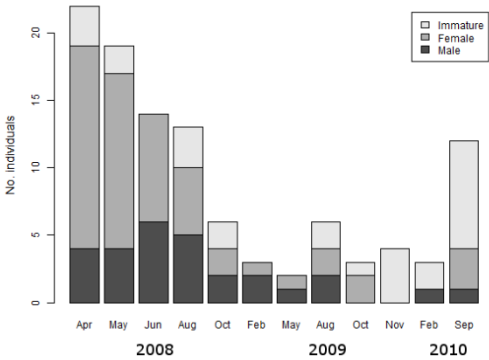
**Fig.5.1:** A boxplot showing the standard length of males, females and immature fish at the initial collection of each fish.



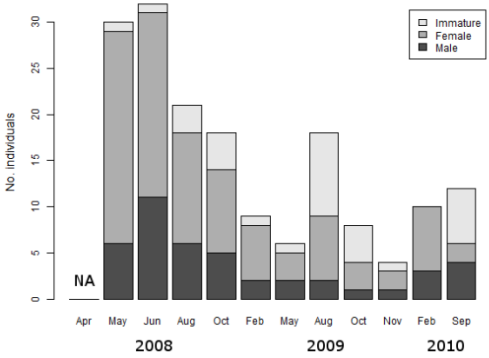
**Fig.5.2:** Relationship among growth (posterior standard length in mm minus prior one, for individuals collected at the intervals of 27-29 days), prior length and sex among adults.



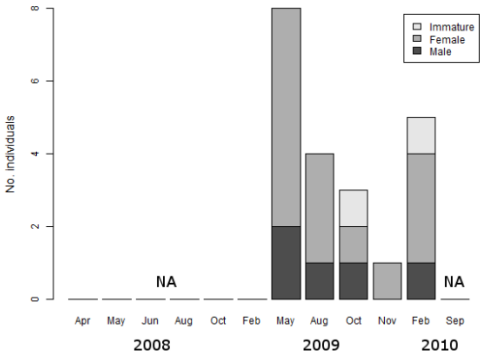
**Fig.5.3:** Population fluctuation of Area I



**Fig.5.4:** Population fluctuation of Area II



**Fig.5.5:** Population fluctuation of Area III



## **General Discussion**

In this thesis, I consistently focused on the relationship between mating and sexual systems, using androdioecious barnacles and sex changing fishes as model systems. As discussed in General Introduction, my working hypothesis is that the patterns of various sexual phenotypes in complex sexual systems correlates with variable local mating systems. This is partly supported by my studies.

Study 1 and 2 used a crab-symbiotic barnacle *Octolasmis unguisiformis* to test the hypothesis that barnacle sexual system correlates with mating group size (Charnov 1987; Yamaguchi et al. 2012). The results partially supported the link between sexual and mating systems although its link was not straightforward: while androdioecy in this species reported by Study 1 was compatible to the hypothesis given the small group size in this species, the frequency of dwarf males did not correlate with mating group size, as reported in Study 2.

Study 3 analyzed the evolutionary conditions for bi-directional sex change theoretically, and examined how the accidental change of mating system (male cohabitation) leads to reversed sex change, under varying risk of dispersal and mate availability. In Study 4 I found a large fluctuation of population abundance in a goby *Trimma caesiura*, and discussed possible effect of accidental low density on bi-directional sex change (see also Kuwamura et al. 2014a).

Although my result generally supports the link between sexual and mating systems, I also found that sexuality is not just a mating tactic. Study 1 confirmed that males and hermaphrodites have different life history trajectories indicated by different size at maturation. Study 2 failed to find a correlation between mating group and sexuality, leading to propose an effect of larval sex determination and settlement behavior. Study 3 predicted that reversed sex change may not be just a mating behavior but also a social behavior to balance costs and benefits of group living. In Study 4, I hypothesized the effect of population fluctuation on sexual plasticity, although its effect may work through altering mating conditions. Below I discuss these non-mating factors, life history, dispersal and social dominance. Note that all of these factors and mating systems are not mutually exclusive or independent, because there must be some kind of interactions among them. I dedicate distinct sections for them just to discuss in detail, not implying that they are independent and alternative hypotheses.

### *Life history*

Sex allocation theory studies allocation of reproductive resources into male and female functions. Organisms also allocate resources to survival or growth, as well as reproduction. Optimal resource allocation between reproductive and non-reproductive

functions is a subject of life history theory (Stearns 1992). Although sex allocation and life history theories have been developed separately (Zhang & Wang 1994), the interaction between them should not be ignored (Yamaguchi et al. 2013d; Yusa et al. 2013), especially when sex and size interact to determine individual reproductive success. If males and females adopt different life history strategies, sexual size dimorphism accrues (e.g. Vollrath & Parker 1992). Sexual size dimorphism can also result from size-dependence in sex allocation and sex determination. For example, in a fish *Menidia menidia*, sexual differentiation depends on the season in when they are born. This leads to sexual size dimorphism at their first reproductive season (Conover 1984). Simultaneous hermaphrodites also often modify their sex allocation depending on body size (Schärer 2009). The extreme case of this pattern is size-dependent sex change, in which individuals radically change their sexual function based on size (Cadet et al. 2004).

Both of my study species show sexual size dimorphism (Study 1 and 4). Sexual size dimorphism in *Trimma caesiura*, as well as other species of the same genus (Sunobe & Nakazono 1990, 1993; Manabe et al. 2007a, 2008; Sakurai et al. 2009), is explained by basically protogynous sex change. Theoretically, it is also possible that the size difference between male and hermaphrodites in androdioecious barnacles, can also be explained by ontogenic change from dwarf males to hermaphrodites (Weeks 2012).



However, it is unlikely in *O. unguisiformis* because no large conspecific-attached individuals were found, as discussed in Study 1. Even when males have a potential to grow up into hermaphrodites (Crisp 1983a; Yusa et al. 2010, 2015), the possibility is limited (Yusa et al. 2012). Most importantly, conspecific-attached individuals have different size at sexual maturation, suggesting different life history trajectories. Given the tradeoff between growth and reproduction assumed by life history theory, earlier maturation by dwarf males may suppress their growth in compared with immature hermaphrodites. The sex-specific growth is obvious in some scalpellid barnacles (Nilsson-Cantell 1931; Buhl-Mortensen & Høeg 2006, 2013) in which males have no or rudimentary foraging organs (cirri) and hence cannot grow at all. Although Ewers-Saucedo et al. (2015) reported similar growth rate between males and hermaphrodites in *Chelonibia testudinaria*, their sample size is too small to conclude. In addition to sex-specific growth, sex-specific mortality is also likely to underlie the sexual size dimorphism in barnacles. Dwarf males may have higher mortality because they die when their partners die (Yamaguchi et al. 2008). In epibionts of arthropods, including *O. unguisiformis*, existence of resident individuals indicates that long time has been passed since the last molting of hosts. Since dwarf males settle after their partners, they do not have so long time to grow up.

In addition to causing sexual size dimorphism at the proximate level, life history factors may affect the evolution of sexual systems at the ultimate level. For example, the patterns of sex change should be affected by sex-specific growth or mortality (Charnov 1986; Iwasa 1991; Kuwamura et al. 1994a; Brooks & Iwasa 2010; Matsumoto et al. 2011; Yamaguchi et al. 2013c). It is predicted that individuals should first mature as sex with faster growth or lower mortality. Kuwamura et al. (1994a) explained basically protogynous sex change in monogamous coral-dwelling gobies by this “growth-rate advantage” (but see Munday 2002 and Study 4). While there are several monogamous but basically protogynous gobiid species (Sunobe & Nakazono 1999; Manabe et al. 2013), sex-specific growth rate has not been confirmed except for coral-dwelling species. I found consistent growth rate between the sexes after controlling initial size in *Trimma caesiura* (Study 4), not surprisingly because they are probably polygynous, but casting a doubt on the commonality of sex-specific growth rate in hermaphroditic gobies. Testing sex-specific growth rate in monogamous gobies are required. In the theoretical model (Study 3), I did not incorporate growth rate for simplicity and to focus on short-term tactics. As discussed in the chapter, incorporating temporal factors including growth and mortality remains as a future task.

The evolutionary effect of life history is also supposed for barnacle sexual

systems. Yamaguchi et al. (2008, 2013a, b, d) predict that limited growth due to spatiotemporal limitation or poor food availability favors dwarf males, because they do not need to grow. This prediction was partly supported by the general tendency that deep sea or symbiotic species are likely to have dwarf males (Kelly & Sanford 2010; Yusa et al. 2012; Lin et al. 2015). However, it is difficult to test the effects of mating systems and various aspects of life history on sexual systems separately, because these factors usually confound (Yusa et al. 2013). In case of my study species *O. unguisiformis*, food abundance and spatial limitation are assumed to be relatively uniform because all individuals were collected in the subtropical intertidal flats from a few species of closely-related and morphologically resemble crabs. It is possible that temporal limitation caused by host molting cycles varies.

Although I focused on fishes and barnacles above, the effect of life history on sexual systems should be applicable to other organisms as well. For example, Loya & Sakai (2008) attributed the different timing of sex change in two species of fungoid corals to different life history parameters such as growth and mortality.

### *Dispersal*

Organisms disperse in different stages in their life history and thus interact with many

aspects of life history (Bonte et al. 2012). Among my study species, barnacles cannot disperse at the adult stage and thus disperse only in the larval period. Therefore, larval settlement behavior is central to determine their group composition. As discussed in Study 1 and 2, the interaction between larval settlement and sex determination has not been understood, although the correspondence between their attachment site and sexuality (Study 1) indicate the presence of at least some kind of such interaction. One possibility is environmental sex determination in which substrata induces sexual differentiation of larvae without predetermined sex, as argued by Callan (1940). Another possibility is that sex is genetically determined, or at least determined before larval settlement, and larvae choose substrata suitable for their genetic sex (Gomez 1975). Such interactions may become more complicated if barnacles have a mixture of genetic and environmental sex determination mechanisms, as argued by Svane (1986; see also Spremberg et al. 2012; Høeg et al. 2015). Such interactions not only cause substrata-sexuality correlation, but may affect the within-species variation of sexuality. It may be why the result of Study 2 – i.e., a non-significant correlation between mating group size and the frequency of males – did not support the theoretical prediction. In addition to choosing substrata and sex, larvae may choose the habitat patch to settle based on its environmental (food availability, spatiotemporal limitation) or mating (number or sex ratios of previously settled

individuals) factors. For example, larvae of barnacles *Octolasmis* spp. which live on the gill chamber of crabs, prefer to settle on newly-molted crabs (Jeffries et al. 1989). And barnacle cyprid larvae often settle gregariously (Crisp 1985) probably to acquire mates. Although these settlement behaviors should affect the pattern of sexuality, the effect has not been understood either empirically or theoretically.

Larval dispersal also affect the sexual systems of animals with mobile adult stage, although it is not the only way of habitat choice. In the female-removal field experiments to induce reversed sex change, males sometimes acquire mates not by changing sex but by the recruitment of newly settled juveniles which will mature as females in the near future (Kuwamura et al. 2011). The recruitment of potential mates offers another way to acquire mates for bachelor males: just waiting. Therefore, frequent recruitments may select against the evolution of bi-directional sex change. Another kind of possible interaction between sexuality and larval dispersal is illustrated by a complex sexual system not focused on my studies: diandry (coexistence of pure males and protogynous sex changers). Frequency of pure males in diandric wrasses is known to correlate with reef size (Warner & Robertson 1978), providing a classical case analyzed by sex allocation theory (Charnov 1982). It may be a result of sex-specific settlement behaviors by larvae (Rogers et al. 2013). However, it can be explained by environmental sex

determination based on the social condition after settlement (Munday et al. 2006c). Note that this problem is almost identical to that of barnacle settlement and sex determination explained above. The common difficulty to study these problems is to study planktonic larval behavior. Later I discuss this topic.

The ability of dispersal by adults varies among animals with mobile adults. Many goby species are small and benthic, and thus has a relatively limited ability of dispersal. As discussed in Study 3 and 4, such limitation may underlie the evolution of sexual plasticity. In the well-recognized scenario of bi-directional sex change, when multiple males cohabit accidentally in a polygynous and basically protogynous species, the subordinate male changes sex into female (Munday et al. 2010). However, if he disperses to other habitats where he can be a dominant male, it is beneficial since dominant males achieve higher fitness than females under polygyny. High risk of dispersal should select against dispersal and favor reversed sex change as the best of bad situations (Nakashima et al. 1995; Munday et al. 2010; Study 3). However, cohabitation of males will not occur under extremely and consistently high risk of dispersal (Nakashima et al. 1995). Thus the spatiotemporal variation of the risk of dispersal may play a key role, as discussed in Study 3.

### *Social dominance*

Many animal societies are not egalitarian, that is, dominant individuals achieve high fitness at the expense of subordinate ones (Krause & Ruxton 2006). In Study 3 I focused on an aspect of reversed sex change as a tactic to manage such reproductive skew. Here I expand this idea to sexuality in general.

Social control of sex change is widely recognized as a mechanism controlling the timing of sex change in animals, especially in fishes (Ross 1990; Munday et al. 2006a). For example, in relatively small groups, dominant individuals become harem males and suppress protogynous sex change by others (suppression model). From a standpoint of subordinates, being females is a tactic to avoid being evicted as rival males and to achieve reproductive success as possible. Based on this view that sexuality is a social tactic, it is not surprising that subordinates sometimes adopt various alternative tactics. Below I illustrate such tactics using examples of harem and basically protogynous fishes. Females in *Labroides dimidiatus* (Sakai et al. 2001) and *Trimma okinawae* (Manabe et al. 2007b) sometimes disperse to other harems to heighten their social rank and achieve dominant male status earlier. Females of *Centropyge ferrugata* decrease their frequency of spawning and allocate resources to growth acceleration under the presence of similar-sized females (Sakai 1997; Hamaguchi et al. 2002). In addition, females sometimes

undergo protogynous sex change without the disappearance of dominant males (early sex change *sensu* Moyer & Zaiser 1984). Sakai (1997) categorized early sex change into two types: bachelor sex change and harem-fission sex change. In case of bachelor sex change among angelfishes (Moyer & Zaiser 1984; Aldenhoven 1986) and a triggerfish (Takamoto et al. 2003; Yamaguchi et al. 2013c), sex-changed males become bachelor and have non-reproductive periods. Since bachelor males represent higher growth rate or lower mortality, such sex change provides long-term benefit (Aldenhoven 1986; Iwasa 1991; Yamaguchi et al. 2013c). In harem-fission sex change, sex-changed males acquire some of the females from the harems they belonged to and make new harems consisting of those females (Sakai 1997). These tactics should increase their reproductive success by becoming dominants earlier. Reversed sex change in bi-directional sex changers is one of such tactics, which occurs when a former dominant individual lose its social status (West 2009). I emphasize that it is just one of several tactics available for subordinates and thus decision-making among tactics is essential to understand bi-directional sex change (Kadota et al. 2012; Study 3).

It is worthy to note that the set of alternative tactics available for subordinate sex changers are similar to that for helpers in cooperative breeders (Brown 1987) or for reproductively suppressed individuals in groups (Gardner et al. 2003; Wong 2010). It is



not surprising because these problems form a part of general problem of subordinate decision making under reproductive skew. Placing sex change into the theoretical framework of social dominance will promote the general understanding of both hermaphroditic and dioecious societies (see Wong et al. 2012; Study 3).

Such dominance may not be important for barnacles because their adults show little, if any, social interaction except for mating. However, territorial behavior by cyprid larvae of a thoracican barnacle *Austrominius modestus* (Crisp 1961) suggest the possibility of social interaction, though not persisting long enough to form a social dominance. Larval competition among males over access to females is supposed to be an important selective pressure for sexual dimorphism and sex determination in rhizocephalan (parasitic) barnacles (Yamaguchi et al. 2014a).

#### *In defense of integrative approach for evolutionary ecology*

As discussed above, some of complex sexual systems are thought to evolve under low density, small group size and fluctuating populations. Unfortunately, species with such characteristics are not good subjects of research, because it is difficult and uncertain to achieve enough sample size. In addition, larval settlement behavior, which may play an important role to explain the variation of sexuality (see *Dispersal* section above), is

virtually impossible to observe under natural conditions and also difficult for captive experiments. This makes any controlled experiments or observations difficult.

An obvious solution to this problem is to develop a method to examine each species in detail. Development of protocols to rear and observe planktonic larvae in laboratory will contribute greatly (e.g. Lang 1976; Spremberg et al. 2012; Archambeault et al. 2015). The larval rearing methodology for the study species of Study 1 and 2, *Octolasmis unguisiformis*, is currently under development (Y. Yusa, pers. comm.). Various biological methods including otolith examination (e.g. Rogers et al. 2013) and molecular marker (e.g. Buston et al. 2009) also help to indirectly estimate larval behavior. However, despite the effort to develop and apply advanced technologies to diverse species, the number of species available for such detailed studies is still limited. Obviously, choosing easy-to-study species is a good strategy. However, it may bias our understanding on biodiversity, because such species are biased sample of whole biodiversity.

In this thesis, I compiled several methodology used in different disciplines of biology, that is, histology (Study 1), specimen-based comparison using natural variation (Study 2), theoretical modeling (Study 3) and long-term field investigations (Study 4). As discussed in General Introduction of this thesis, I chose these approaches to satisfy what is most necessitated in each topic. Due to the above mentioned difficulty, it is difficult to

draw clear-cut conclusions from single approach. However, the combination of approaches gave us biologically interesting insights. I demonstrated a potential of such integrative approach to study evolutionary ecology of diverse natural history among marine animals.

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