



Effects of stress on sex change in *Crepidula cf. marginalis* (Gastropoda: Calyptraeidae)

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ARTICLE INFO

Article history:

Received 20 November 2011

Received in revised form 19 February 2012

Accepted 20 February 2012

Available online xxxx

Keywords:

Desiccation stress

Food stress

Panama

Plasticity

Protandry

ABSTRACT

It is well known that interactions with conspecifics can affect the size and timing of sex change but the potential effects of other environmental influences on sex change are rarely examined. Sex allocation theory predicts that increased mortality rates should decrease the optimal size at sex change. Therefore we examined how stress (early starvation and exposure to desiccation) affects the size at sex change in *Crepidula cf. marginalis* a protandrous marine snail. Our results showed significant effects of early starvation on sex change: animals responded to early starvation by losing male characteristics earlier and at smaller sizes, remaining longer in the transitional phase, and becoming female at larger sizes than animals that were provided adequate food throughout. Desiccation on the other hand, decreased the size at which the female reproductive morphology was attained but no other effects were detected. The results of the desiccation treatment are consistent with theoretical predictions that higher mortality rates should decrease size at sex change. The starvation treatment seems to indicate that animals reduce the allocation to reproduction under extreme stress by entering a non-reproductive transitional stage earlier. Instead of regaining the male phase when conditions improved, they grew and rapidly completed the transition to the female phase.

Published by Elsevier B.V.

1. Introduction

Stress impacts any number of aspects of an organism's life history. Studies with marine invertebrates have demonstrated effects of stress on virtually all life stages, as well as "carry-over" effects of stress experienced by one life stage on the performance of subsequent stages (Pechenik, 2006). For example, mussels raised under a range of stressful conditions (food stress and temperature stress) produced fewer, smaller eggs, which contained less lipid than eggs from unstressed females (Bayne et al., 1978); the rate of reproduction of asexual lineages of *Brachionus plicatilis* rotifers relative to sexual lineages increases with temperature stress (Snell, 1986); increased heavy metal concentration in food reduces egg production and egg viability in cladocerans and copepods (Hook and Fisher, 2001); and UV exposure increases embryonic mortality and retards development in intertidal gastropods (Przeslawski et al., 2005).

Stress has also been shown to affect the timing of sex change in fishes (Frisch et al., 2007; Perry and Grober, 2003). In most sex changing organisms size at sex change is influenced by interactions with conspecifics (e.g., molluscs: Chen et al., 1998; Collin et al., 2005; Hoagland, 1978; Warner et al., 1996; Wright, 1989. Polychaetes: Franke, 1986; Premoli and Sella, 1995), and fishes are no exception (e.g., Cole and Robertson, 1988; Cole and Shapiro, 1995; Robertson,

1972; Sakai et al., 2001; Warner and Swearer, 1991). Stress caused by aggression and monopolization of resources by dominant fish is thought to increase cortisol levels (Frisch et al., 2007). This may inhibit androgen synthesis and result in sex change; however the details of the mechanism by which this occurs is still unknown (Frisch et al., 2007; Perry and Grober, 2003). Stress can also alter sex allocation in simultaneous hermaphroditic invertebrates by either increasing relative allocation to growth over sexual reproduction, or increasing allocation to male function over female function (Aira et al., 2007; Hughes et al., 2003). However, it is unknown how stress influences sex change in invertebrates.

Stress, as a potential indicator of mortality rate, might alter the size or timing of sex change, because high mortality rates are predicted to reduce the size at sex change (Charnov, 1982). High mortality reduces the size at sex change by reducing the chance that any individual will attain a large size, thereby increasing sex ratio bias toward the first sex and increasing the benefits of earlier sex change. In most models of sex change age and size are linked so that a prediction of "later" sex change implies that individuals are both older and larger at sex change. When growth rates differ, however, age and size are uncoupled and the form taken by the "delay" in sex change depends on the relationship between reproductive success, size and age. For example at higher growth rates, a larger size at sex change could occur at a younger age but this is not necessarily the case. Increased mortality has been demonstrated in field populations to result in smaller, earlier sex change in fishes and shrimps (reviewed in Kazancıoğlu and Alonzo, 2010; Munday et al., 2006).

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The size distribution of the population with which animals interact is likely often a proximate cue for this response, since socially-mediated sex change has been demonstrated in most sex changers that have been studied (Charnov, 1982; Munday et al., 2006). However, socially-mediated sex change does not rule out a direct and independent influence of physical and other environmental factors on sex change. In marine invertebrates few studies have examined the direct effects of physical conditions on sex change independent of their effects on conspecific interactions. Here we do so by examining how stress, *per se*, affects sex change. We test if, as predicted by sex allocation theory, stress accelerates the size and timing of sex change in a species of *Crepidula* slipper snails.

Species of *Crepidula* and to some extent other species of marine slipper limpets in the family Calyptraeidae were some of the first sex-changing animals to be studied (Gould, 1917, 1919; Orton, 1909). These animals are sedentary, protandrous suspension-feeders, with well-documented socially-mediated sex change (Coe, 1938, 1953; Collin, 2006; Collin et al., 2005; Gould, 1917, 1919; Hoagland, 1978; Warner et al., 1996). We used a common species of calyptraeid, *Crepidula* cf. *marginalis*, to determine if food stress and desiccation stress alter the size, timing and duration of sex change.

2. Materials and methods

Juvenile *C. cf. marginalis* were collected by hand from within an approximately 2-hectare area at Playa Chumical (8.874°N, 79.644°W) near the town of Veracruz, Panama, during the spring tides in 2009. *C. cf. marginalis* are abundant attached directly to the underside or slightly buried edges of rocks. They usually occur in small clusters of one or a few females associated with a number of small males on the same rock. Size at sex change in the field occurs at 11.0 mm (c.i. = 10.3–12.2 mm) and on average lab raised animals change sex between 10.17 and 11.89 mm depending on the food ration provided (Mérot and Collin, 2012).

The shell length of all animals was measured with calipers to the nearest 0.1 mm and their sex was determined at the beginning of the experiment and every subsequent week. Males were identified by the presence of a penis behind the right tentacle, and females by a genital papilla (fgp) and capsule gland on the right side of the mantle cavity. Transitional individuals either have the organs of neither sex or of both sexes and males often show a reduction in penis size a week or two before it disappears. Small specimens without sexual characteristics were considered to be juveniles, which was verified by subsequent observations during the laboratory experiments. Small (3–5 mm) animals were allocated haphazardly among the different treatments within 2 or 3 days of being collected from the field. Animals were kept individually in 350 ml cups, at approximately 20 °C (18–21 °C) in 350 ml cups of fresh filtered seawater, which was replaced three times a week. Because we are interested in the influence of the environment only and not the confounding effects of interactions with conspecifics we raised each animals alone. They were fed the microalga *Isochrysis galbana* (strain T-iso).

To examine the influence of stress on sex change, we subjected the snails to one of 3 different treatments: early starvation ($n = 29$), desiccation ($n = 29$), and a control treatment ($n = 36$) with no desiccation and a high food ration. In the control treatment animals were given approximately 38×10^6 cells per day. The early starvation treatment consisted of 2.4×10^6 cells per day. This concentration was not sufficient to support growth beyond 8 mm and after 12 weeks the animals in this treatment had clearly stopped growing and appeared thin. They were then given the same ration as the control treatment until the end of the experiment. In the desiccation treatment animals were left without water for 3 hours during each water change but were otherwise treated in the same way as the control. In the field these animals often experience 3 hours of emersion during large amplitude tides, but remain immersed during the smaller amplitude tides (pers. obs.). All animals

were sacrificed 3 weeks after sex change was complete to verify they were females.

The effect of each treatment on (1) size at which the penis was lost and the size at which the fgp was first reported, and (2) the number of weeks until the penis was lost and the fgp first reported was examined using one-way ANOVA on log-transformed variables using the program R (R Development Core Team, 2010).

3. Results

In all treatments the juveniles grew quickly during the first 2–3 weeks, but growth diverged rapidly in the early starvation treatment by week 4 (RM MANOVA univariate tests $p < 0.05$; Fig. 1). None of the *C. cf. marginalis* in the starvation treatment grew between weeks 6 and 12; they lost the penis, did not develop any female characteristics, and appeared to be near starvation. Subsequent to the application of the higher food treatment in week 12 these animals grew as fast as those of similar size in the control treatment. Linear mixed model comparison of the week 5–12 of the control treatment and week 13–20 of the early starvation treatment showed no significant effect of treatment on growth ($F_{1, 292} = 2.79$; $p = 0.10$). Desiccation did not have a significant effect on growth rates over the period studied (Fig. 1).

Food stress had a significant effect on size and timing of sex change but desiccation stress had little effect on sex change. The size at which animals lose the penis is significantly smaller in the starvation treatment than in the control or desiccation treatments (ANOVA: $F_{2,91} = 17.8$; $p < 0.0001$; Fig. 2). The size at which animals first become female is smallest in the desiccation treatment, intermediate in the control treatment and largest in the early starvation treatment (all became female after they were fed the increased food ration) (ANOVA: $F_{2,90} = 35.1$; $p < 0.0001$ Fig. 2). Although it is a statistically significant difference the first female mean size is only 0.7 mm smaller in the desiccation treatment than in the control. The treatments also differed in timing of sex change. Animals in the control and desiccation treatments lost the male phase later than the early starvation treatment (ANOVA $F_{2,91} = 5.6$; $p = 0.005$; Fig. 2). In contrast animals became female earlier with control or desiccation than they did in the early starvation treatment ($F_{2,90} = 51.9$; $p < 0.001$; Fig. 2).

As part of normal sex change the penis shrinks prior to its complete disappearance. In our experiments, possibly due to the absence of females some males developed penes that were smaller than are

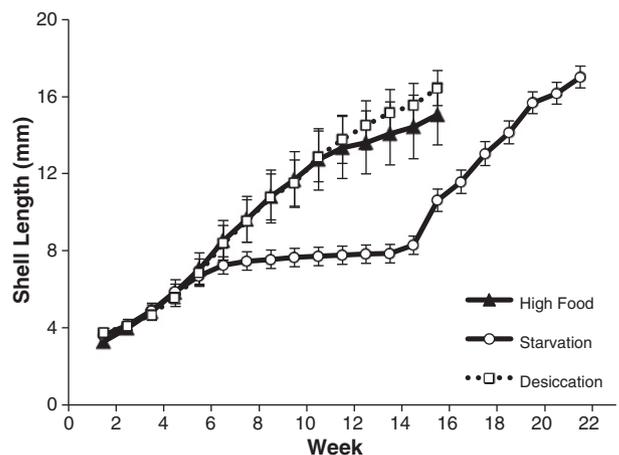


Fig. 1. Growth of *C. cf. marginalis* exposed to high food and two stress treatments. Bars represent a 95% confidence interval. The increase in food concentration in the early starvation treatment was implemented on week 12. The data for the control and desiccation treatment end before week 22 because animals were sacrificed 3 weeks after sex change was complete.

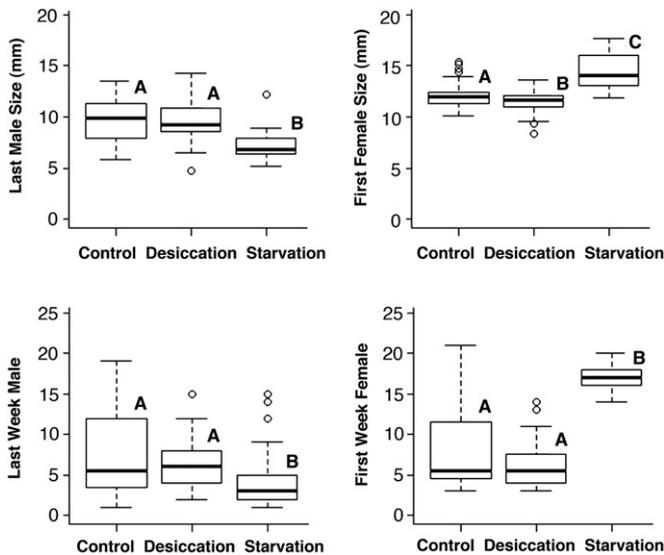


Fig. 2. Box plots for last size male, the first size female, the weeks until the last male observation and until the first female observation. The box is drawn between the quartiles, with a thick line at the median value. The whiskers indicate the range of the data, with outliers shown as circles. Groups marked with the same letter are not significantly different with Tukey's HSD post-hoc test.

often in our different treatments and in field collected animals. The frequency of males that did not develop a large robust penis did not differ between the treatments ($\chi^2 = 2.63$; $df = 2$; $p = 0.27$). The results do not qualitatively differ when our ANOVA analyses are repeated using the first week with the reduced penis instead of the last week a penis was reported.

4. Discussion

In populations with high mortality rates, sex change is predicted to occur at an earlier age or smaller size than in those with lower mortality (Charnov, 1982). To become female earlier is advantageous if life expectancy is short and the life-time reproductive output threatened by high mortality risk. Changes in mortality rates in the field due to fisheries have resulted in observed downward shifts in the size at sex change in both fish and shrimp (e.g., Charnov, 1981; Charnov, and Hannah, 2002; Hamilton et al., 2007; Kazancıoğlu and Alonzo, 2010). It seems likely that these downward shifts in size at sex change are the result of animals adjusting to altered social environments but other scenarios are possible. Animals could perceive the risk of mortality from external cues like the direct detection of predators or predation events, dead conspecifics in the immediate environment, or stressful physical conditions (e.g., our desiccation treatment). They may also assess their own internal levels of nutritional stress (our early starvation treatment), parasite load, or other aspects of body condition. Our experiment was designed to test for the direct effects of stress on sex change and not effects mediated via changes in population demographics.

Our results support the conclusions of previous studies with calyptraeid gastropods, that the duration of sex change and the age at sex change are highly plastic, but that size at sex change varies only moderately. Our desiccation treatment did show changes in the size at sex change consistent with the predictions. The size at which sex change was completed was slightly smaller in animals experiencing desiccation than it was in controls. The difference in size is so small (0.7 mm) that the biological impact of this change is unclear, and it is uncertain if it could be detected in animals from the field. Emersion is a potential cause of mortality in *C. cf. marginalis* and the conditions they were exposed to in the lab could be more severe than the conditions experienced in the field. This species occurs in

the rocky intertidal where they are exposed during most large amplitude low tides and can sometimes be exposed for as much as 5 hours. Since *C. cf. marginalis* are usually found attached to the under-sides or edges of rocks that are embedded in the soft substrate, *in situ* the surrounding sandy mud may buffer the animals from complete aerial exposure like our desiccation treatment. Laboratory emersion for 24 hours results in death *C. cf. marginalis* (pers. obs.), demonstrating that desiccation is associated with mortality risk.

Early starvation followed by increased food did not result in earlier sex change in the manner predicted if stress is an indicator of increase mortality. However this treatment did have significant effects on sex expression: very small *C. cf. marginalis* lost the penis and the female condition was not achieved by any animals until they were exposed to high food rations. The early loss of the penis probably indicates a survival response associated with reduced energy expenditure on sexual reproduction, rather than early sex change. Starved animals recovered fully, although none re-grew the penis despite being well within the normal male size range when food levels increased. Rather, they remained sexless until they reached the expected size at sex change, which generally took 3–4 weeks. When they did become female it was at a significantly larger size than the animals in the other treatments, showing delayed completion of sex change relative to body size. This is contrary to the prediction that “bad” conditions result in earlier sex change at smaller sizes and could produce dwarf females. It is consistent with the idea that animals forego reproduction in order to conserve energy under extreme nutritional stress. This long non-reproductive phase shows that physiological conditions can trigger a more complicated response than simply altering the timing of sex change, and that sex change could profitably be thought of a sequence of events and can be decoupled instead of a single event that happens at a specific moment.

In our experiments animals were raised alone to avoid the effects of interactions with conspecifics and potential completion for food. This means that they had no potential for reproduction. The response to food limitation may have been different in the presence of another animal where trade-offs between the immediate chance for reproduction and potential future reproduction could affect the size and timing of sex change. Under high-food conditions males kept with females usually grow very slowly and retain well-developed penes (Collin et al., 2005). This could reflect competition for food between large females and small males, or allocation of resources to reproduction instead of growth in the presence of a large female. Under food-limiting conditions, the presence of a reproductive female and possible immediate pay-offs to male reproductive allocation, more resources might have been allocated to male reproduction than in our solitary snails. These alternatives would be difficult to distinguish in *Crepidula*.

Unfavorable conditions terminating the male phase without the subsequent development of female structures have been reported in older studies on temperate *Crepidula* species (Coe, 1938, 1948). Food stress in simultaneous hermaphroditic invertebrates commonly results in the reduction of resource allocation to sexual function in favor of maintaining body condition (e.g., Locher and Baur, 2002; Schärer et al., 2005) and similar effects have been observed with other kinds of physical stress (e.g., Aira et al., 2007). In simultaneous hermaphrodites allocation to both male and female function increases when resources are abundant. Therefore, tradeoffs between male and female allocation are only evident when resources are limiting but not so limiting that reproduction is abandoned. This pattern of increased allocation to both sexes with increased resources is evident in *C. cf. marginalis*, which reduces the duration of the transitional phase during sex change when resources are abundant (Mérot and Collin, 2012). A study using a food concentration 4 times that of our starvation treatment (medium food in Mérot and Collin, 2012) also found that the male stage was lost at a smaller size than under abundant food conditions. At these medium food conditions the snail spent much longer time as males because growth was slower. This

could have ultimately resulted in higher life-time allocation to male function in terms of sperm production or realized paternity. To quantify the details of allocation to male function it would be necessary to employ destructive sampling to directly measure gonad size, sperm production or paternity.

Acknowledgements

We thank Maricela Salazar, Maria Fernanda Vinasco, Maria Lobato and especially Matt Starr for their help in the field and with animal maintenance. Matt Starr and Kaza Ansley commented on the manuscript and we thank them for their comments. This project was conducted as part of a masters internship (for C.M.) and the work was supported by the Smithsonian Tropical Research Institute.

Statement of Authorship

RC conceived and designed the study. CM and RC conducted the laboratory procedures and CM performed the data analysis. RC drafted the manuscript, but both authors contributed to writing and approved the final manuscript.

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