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Resource Availability Drives Mating Role Selection in a Simultaneous Hermaphrodite, *Aplysia californica*

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Abstract

In simultaneous hermaphrodites, a clear conflict exists between sperm donor and sperm recipient roles; however, how such conflict is mediated remains up for debate. This study recorded and observed mating role selection as a function of resource availability in the simultaneous hermaphrodite, *Aplysia californica*. When food was plentiful animals participated in both sperm donor and recipient roles at relatively even frequency. However, when half of the mating pairs were placed on restricted diets, food limited animals participated in the sperm donor role at a significantly ($p < 0.05$) greater frequency than their *ad libitum* partners; however, the frequency of total successful mating events remained the same. The mass and frequency of eggs laid were also significantly ($p < 0.05$) correlated with parental food intake. These results demonstrate how mating strategies can change relatively quickly as a result of shifting environmental conditions and calls for a diverse framework to address these issues in simultaneous hermaphroditic mating systems.

Keywords

sexual selection; simultaneous hermaphroditism; mating role; *Aplysia*; resource allocation; experimental behavior; gender conflict

Introduction

Darwin (1859; 1871) observed that in a great many organisms, males are the sex more eager to mate while females are more choosy. Since this discovery, male and female roles have been observed to be in conflict across many different clades over concerns related to mating, offspring, and parental care among other factors (Chapman *et al.*, 2003; Arnqvist and Rowe, 2013). This conflict poses a unique problem for hermaphrodites, particularly simultaneous hermaphrodites who may act either as a sperm donor (“male”) or sperm recipient (“female”) for any given sexual encounter over the course of their mature lives. Not only do they need to select and obtain a partner, but also determine who will participate in which role, particularly if one of the roles offers a distinct advantage over the other. When individuals prefer the same role, gender conflict occurs (Wethington and Robert T Dillon, 1996).

Conventional thinking may lead to the belief that the role with the highest potential fitness, typically donor, will be unconditionally preferred in simultaneous hermaphrodites (Charnov, 1979; Greeff and Michiels, 1999; Michiels *et al.*, 2003; Anthes *et al.*, 2006b). This idea is demonstrated perhaps most strikingly in the marine flatworm, *Pseudoceros bifurcus*, which engage in behavior popularized as “penis fencing” in which pairs attempt to donate their sperm to one another *via* hypodermic insemination, while actively avoiding being inseminated themselves (Michiels and Newman, 1998). There is also evidence of opportunistic male strategies in sea slug species more related to *Aplysia californica*, the model used in this study (Michiels *et al.*, 2003; Anthes *et al.*, 2006a). However, there is also evidence to suggest that the recipient role is preferred, particularly in internal fertilizers like *A. californica* (Dall and Wedell, 2005; Leonard, 2005; Anthes *et al.*, 2006b). This preference may be the result of sperm storage and digestion mechanisms found in many simultaneous hermaphrodites (Angeloni *et al.*, 2003). Recipients are afforded a greater certainty that their gamete investment will result in viable offspring, whereas donors' invested gametes could go unfertilized, be digested etc.

No matter what role is selectively advantageous, there is still the question of how gender conflict is handled. One possible resolution is a reciprocal mating strategy. If both partners participate in both roles they assume the same costs and benefits, alleviating potential conflict (Sella, 1988). However, this strategy is vulnerable to “cheaters” who could assume the less-costly role initially and desert their partner. One way cheating behavior may be mitigated is through successively mating, in which partners alternate roles multiple times, releasing only a small portion of their gametes in each exchange. Such cooperative “egg-trading” behavior has been observed in several serranid fish species as well as in polychaete worms (external fertilizers) (Fischer, 1980; Axelrod and Hamilton, 1981; Sella and Ramella, 1999; Sella and Lorenzi, 2000; Erisman and Allen, 2006; Crowley and Hart, 2007; Hart *et al.*, 2016). In instances where the donor role has greater direct investment, one would expect to see reciprocal “sperm trading” instead, such as appears to be the case in several internally fertilizing gastropods (Leonard and Lukowiak, 1985; Anthes *et al.*, 2005; Jordaens *et al.*, 2005; Koene and Ter Maat, 2005; Facon *et al.*, 2008)

The simultaneous hermaphrodite *Aplysia californica* (J.G. Cooper, 1863) is an opisthobranch mollusk commonly employed as a model organism in neurophysiology (Frazier *et al.*, 1967). *Aplysia californica* are terminally reproductive and also mate unilaterally, *i.e.* one donor and one recipient for any given encounter (Carefoot, 1987). *Aplysia californica* are not thought to habitually engage in reciprocal mating behavior in the lab or the field (Pennings, 1991; Ludwig and Walsh, 2008).

For this study, two sets of controlled mating trials were designed. In the first, Mating I, all experimental animals were placed on unrestricted, *ad libitum* diets to establish mating role selection when both partners had equal access to resources. In the second, Mating II, trials were designed so that *ad libitum* animals were paired with animals on restricted diets to observe if mating role selection changed when one partner occupied a disadvantaged, resource-deprived position. Theory predicts that fitness gains in the donor role are proportionally greater when investments are minimal, and begin to plateau as more resources are devoted. Recipient roles by contrast are expected to have a more linear relationship

between fitness gains and investment (Vizoso and Schärer, 2007). Experimentally it has been shown that resource limitation impairs female function (but not male) in a simultaneously hermaphroditic snail (Janicke and Chapuis, 2016). It is therefore expected that resource deprived animals will participate in the donor role at a greater frequency than their *ad libitum* counterparts. Their *ad libitum* partners may respond in kind by selecting recipient more frequently; however, they may also insist on a reciprocating strategy, à la gamete trading hypothesis (Leonard and Lukowiak, 1985; Anthes *et al.*, 2006b) or refuse to mate altogether.

Materials & Methods

Rearing Conditions

A. californica used in this study were bred and reared in captivity at the NIH-funded National Resource for Aplysia at the University of Miami. Animals were reared in 16-liter polycarbonate cages submerged in communal fiberglass troughs that received a continuous flow of chilled (15°C) seawater to within 2.5 cm of the top of the cage. Each cage received seawater directly from a faucet, with perforations along the cage bottom to optimize flow. Animals were reared at 1/cage to prevent unobserved copulations. However, animals were likely aware of one another through chemical signals that may have been present in the communal water supply. Each cage was fitted with a weighted panel of perforated styrene “egg crate” to prevent escapes. Lighting was kept on a 14:10-h light:dark cycle. Water temperature ranged from 14.5°C to 15°C, approximating the animal's natural range along the California coast (Wayne and Block, 1992). Seawater O₂ concentration, pH, and salinity were all held at standard hatchery levels (Idrisi *et al.*, 2006). Cages were checked daily for the presence of clutches, which were removed and weighed using an electronic balance.

Original Experimental Animals

Twelve *Aplysia californica* specimens were selected from two unrelated sibling groups and weighed to the nearest 0.1 g using an electronic balance. Difference in weight between animals did not exceed 4 g. All animals were <50 g and not yet sexually mature (Gerdes and Fieber, 2006). The two sibling groups were age 265 and 241 days at the start of the experiment, designated as day 1. Due to a lack of growth, one animal was replaced during the second week, and two during the third.

Feeding

Animals were fed a diet of the red macroalgae *Agardhiella subulata*, which has been shown to be optimal for inducing growth in *A. californica* (Gerdes and Fieber, 2006). All animals began the experiment with *ad libitum*, unrestricted diets. Algae were weighed using an electronic balance before being placed in each cage. After 48 hrs any remaining algae was removed from cages and reweighed. Prior to weighing, algae was left to air dry for 45–60 min in an effort to minimize the effect excess water may have on mass. By subtracting the amount of algae removed from the amount of algae added, it was possible to calculate approximately how much algae was consumed over a 24 hour period for each animal. After three months of *ad libitum* feeding, half the animals were moved to a restricted diet. Restricted diets were tailored to each individual and calculated from food intake data during

the *ad libitum* period in relation to the animal's body weight. Restricted diets were calculated from the equation $Cm \times x(Fi/Bm)$ where $x=0.5$ for the first two weeks of restricted feeding and $x=0.25$ for the remainder of the experiment, Fi = mean food intake for a particular animal over a 30 day period of *ad libitum* feeding, and Bm = mean body mass for the same animal over the same 30 day period. Restricted diets were updated weekly based on the current mass of each animal, represented by Cm . *Ad libitum* diets were loosely based on each animal's previous feeding history; no *ad libitum* animals were observed to be without food at any point in the experiment.

Mating I

Mating I trials were conducted to observe mating role selection when all animals are on unrestricted, *ad libitum*, diets. Trials began on day 52 of the experiment when the sibling groups were 293 and 317 days old. Partners paired together were unrelated (Fig. 1). The effect of body size on role selection is well documented in simultaneous hermaphrodites (see Table 2 in Chaîne and Angeloni, 2005; (Dillen *et al.*, 2008; Dillen *et al.*, 2010; Nakadera *et al.*, 2015); however, the relationship has not been strongly supported in *A. californica* (Zaferes *et al.*, 1988; Pennings, 1991; Angeloni *et al.*, 2003). Nevertheless, potential mates were selected so that the percent difference in mean body mass between them did not exceed 30%; otherwise, pairs were established by random selection. Potential mates were placed together in mating cages and allowed one hour to engage in mating behavior. Mating behavior was shown to begin after ~20 minutes in previous experiments (Ludwig and Walsh, 2008). To prevent sperm depletion and to incentivize further mating, copulations were interrupted 15-30 min after mating initiated. *Aplysia californica* are not thought to be sperm limited from copulation periods lasting less than one hour (Yusa, 1996; Ludwig and Walsh, 2004; Ludwig and Walsh, 2008). Penis intromission was used as an indicator of successful mating. Sperm donor “attempts” were also recorded. An attempt was categorized by typical sperm donor behavior, in which the head and anterior portion of the foot lean forward between the recipient's parapodia (Leonard and Lukowiak, 1986); however, penis intromission was not observed after the 15-30 minute mating period. We assumed that animals who exhibited “attempts” were selecting the sperm donor role, but their partners were unwilling or unable to act as recipients. Animals were paired randomly under the described criteria for every trial. Trials were conducted once each day, 6 days per week.

Recess

On day 90, a 30-day recess period was imposed during which no mating trials were conducted. The objective of the recess was to allow the animals time to fully digest any stored allosperm as well as to acclimate to the new restricted diet for designated animals. *A. californica* has been shown to be capable of storing sperm for 22 days $\pm$ 3.6 days (mean $\pm$ SD) (Ludwig and Walsh, 2004). By allowing animals time to completely process any potential stored allosperm they can be said to be theoretically “reset” by preventing any overlap of cost/benefit associated with either mating role between mating periods (Ludwig and Walsh, 2004).

Mating II

Mating II trials were conducted to see how mating role selection is influenced when *ad libitum* animals are paired with those on restricted diets. After the 30 day mating recess, trials resumed on day 120 of the experiment, when the sibling groups were 360 and 384 days old. Pairs were selected so that animals were matched with an unrelated potential mate on a different diet: *ad libitum* animals were only paired with restricted animals, and *vice versa* (Fig. 1). Mating II trials also included additional supplemental animals to adjust for a lack of suitable potential mates. Otherwise Mating II trials were conducted as in Mating I.

Supplemental Animals

An increase in standard deviation of mean mass as the animals aged occurred during the experiment. To mitigate concern that there would be insufficient potential mates in the 30% difference range to constitute mating pairs, 10 animals were added to the experiment on the day 65 in addition to the original 12. These animals belonged to the same two sibling groups as the original groups but did not participate in Mating I trials. These animals were divided evenly into restricted diet and *ad libitum* groups (Fig. 1).

Analysis

Relative daily intake values used to compare between paired donors and recipients were calculated from a polynomial curve of all available data for each individual, $[y(x)/z(x)]$ where y is the intake on day x and z is body mass. Paired t-tests compared intake and mass between the donors and recipients of each copulation, in order to test the null hypothesis that donors and recipients were not significantly different.

Results

Growth

At the onset of the experiment animals averaged $43.2 \text{ g} \pm 1.2 \text{ g}$ (mean $\pm$ SD). For the first 40 days both growth and food intake proceeded at a relatively stable rate. Food intake began to plateau at approximately day 40 (age 281 and 305 days). All animals maintained steady growth through initial mating trials up until feeding regimens were changed on day 92, when sibling groups were 356 and 332 days old (see Fig. 2). At this point mean difference in growth between experimental animals was not significant (unpaired t-test; $t=0.62$, $df=10$, $p=0.55$). Mean mass of restricted animals achieved a maximum at $948.4 \text{ g} \pm 221.2 \text{ g}$, 102% of pre-diet mass 40 days after restricted diets were implemented. In contrast animals that remained on *ad libitum* feeding schedules achieved an average maximum mass of $1742.0 \text{ g} \pm 165.9 \text{ g}$, 188.1% of pre-diet values 49 days after restricted diets were implemented for selected animals. Two animals died, presumably from old age at 382 and 396 days, before the conclusion of the experiment on days 141 and 155, both from the *ad libitum* experimental group.

Mating I

26 successful copulations occurred over the 39 days of Mating I trials. Mating behavior was observed on the first day of trials and throughout Mating I. All participating animals except

one acted as both a sperm donor and sperm recipient over the course of Mating I. Donor attempts were observed 15 times for an average of 0.38 attempts per day (Table 1). Animals did not consistently alternate between sperm donor and recipient over multiple days. However, one instance of immediate reciprocal mating was observed wherein two animals acted as both donor and recipient simultaneously. Donors had less relative food intake than their recipients in 38% of copulations. Over this period relative food intake did not have a significant impact on mating role selection (Fig. 3, paired t-test; $t=0.6$, $df=25$, $p=0.55$).

Mating II

Fourteen successful copulations occurred over the 30 days of Mating II trials. Donor attempts were observed 18 times for an average of 0.60 attempts per day (Table 1). As with Mating I, mating behavior was observed on both the first and last day of trials. Nine of the original experimental animals successfully copulated at least once in Mating II trials. The lowest intake for an animal acting as a recipient was 3.14% of body mass per day. There were three instances of acting donors and four instances of attempted donors with intakes below the 3.14% mark. In Mating II trials donors had less relative food intake than their respective recipients in 85% of copulations (Fig. 3). Relative food intake had a significant (paired t-test; $t=2.4$ $df=13$, $p=0.03$) impact on mating role selection; animals who were consuming relatively less food per day were more likely to act as a sperm donor than a recipient.

A chi-squared test comparing mating role selection between family groups was not significant, *i.e.* families did not specialize in a particular role (chi-squared test; $\chi^2=1.6$, $df=1$, $p=0.21$). Body size was also not significantly correlated with mating role selection, at least within the established 30% margin in Mating I (paired t-test; $t=0.79$ $df=25$, $p=0.44$) or Mating II (paired t-test; $t=1.7$ $df=13$, $p=0.12$). Over the course of the experiment, some individuals were paired together multiple times. There were 13 total replicated pairs in Mating I, and 3 in Mating II.

Eggs

Eleven of the 12 original experimental animals laid at least one clutch over the course of the experiment. Each animal laid an average of 4.0 ± 2.3 clutches. Each clutch had an average mass of $37.9 \text{ g} \pm 29.0 \text{ g}$. *Ad libitum* animals had greater egg yield than restricted animals, in terms of both frequency and mass (Fig. 4, Fig. 5). Animals that were placed on restrictive diets had an average clutch mass of $26.7 \text{ g} \pm 19.5 \text{ g}$ compared to those that fed *ad libitum* for the entire experiment, which had an average clutch mass of $46.2 \text{ g} \pm 32.1 \text{ g}$. Both groups resembled one another closely in terms of both mass and frequency until diets were implemented for restricted animals on day 90 (Fig. 4, Fig. 5).

Discussion

As has been noted previously (Capo *et al.*, 2002; Fieber *et al.*, 2005; Gerdes and Fieber, 2006) the growth of individual animals varied considerably under laboratory conditions. Animals on *ad libitum* feeding regimens had greater standard deviations in weekly growth in addition to achieving an overall greater mass than animals on restricted diets. Food intake

has been shown to have a significant impact on growth (Kriegstein, 1977; Gerdes and Fieber, 2006). The shallower growth curves of animals on restricted diets show that the restricted diets implemented were severe enough to impose a physiological cost to experimental animals. In addition, growth curves of restricted animals plateaued shortly after diets were implemented, much earlier than their *ad libitum* counterparts. This is noteworthy because it has been previously observed that *A. californica* requires significantly less food once sexual maturity is reached (Gerdes and Fieber, 2006). Thus for diet to have an effect on mating role selection, it is important to establish that restricted diets are sufficiently severe to impose a cost. Animals who are sufficiently resource deprived will, presumably, have a preference for the less costly role.

Mating behavior was observed on both the first and last day of Mating I and Mating II trials, establishing that experimental animals remained sexually active for the duration of the experiment. Lack of observed reciprocation in Mating I further supports the idea that reciprocal mating is not a mechanism for mediating gender conflict in *A. californica* (Pennings, 1991; Ludwig and Walsh, 2008). In spite of this, one instance of immediate reciprocal mating was observed in which both partners exhibited both roles simultaneously. This observation shows that reciprocal mating is possible in *A. californica*; however, it is not common in hatchery reared animals. Mating I trials established that when all animals are fed *ad libitum* relative intake is not a factor that influences mating role selection. Animals will select either role regardless of their intake relative to potential partners (Fig. 3).

However, once *ad libitum* animals were paired with those on restricted diets in Mating II trials, significant impacts of food intake on mating role selection were discovered. Animals who had restricted diets were significantly more likely to adopt the sperm donor role than their *ad libitum* counterparts (Fig. 3). Some suggest female roles are less costly in simultaneous hermaphrodites; as “recipients” those who play female are not obligated to expend gametes, and may even receive nutritional benefit from digested sperm (Charnov, 1979; Visser *et al.*, 1994; Charnov, 1996; Eberhard, 1996; Greeff and Michiels, 1999; Chaine and Angeloni, 2005; Dall and Wedell, 2005). Instead the results suggest that the sperm donor role is less costly in *A. californica*, as it is the role preferred by animals who must partition their limited resources (Vizoso and Schärer, 2007; Schärer, 2009; Janicke and Chapuis, 2016).

Observations of donor attempts may show that mating roles are determined by the behavior of the resource deprived partner. Restricted animals more than doubled their rate of attempted donor behavior between Mating I and Mating II (Table 1) (chi-squared test; $\chi^2=4.1$, $df=1$, $p=0.04$). This increased frequency in the donor role is consistent between attempts and observed copulations. By comparison *ad libitum* animals attempted the donor role at the same rate in both Mating I and II (chi-squared test; $\chi^2=6.0E-4$, $df=1$, $p=0.98$), but assumed the recipient role in actual copulations more frequently in Mating II. This suggests that *ad libitum* animals accept both roles, and exhibit the recipient role more only because it is selected more frequently by their potential mates on restricted diets. However, it is worth noting that the frequency of donor attempts was not found to be significant across experimental groups in Mating I (chi-squared test; $\chi^2=0.74$, $df=1$, $p=0.39$) or Mating II (chi-squared test; $\chi^2=1.26$, $df=1$, $p=0.26$).

Interestingly, the proportion of successful copulations did not change significantly between Mating I and II (two-proportion z-test, $z=1.69$, $p=0.09$). This suggests a lack of increased gender conflict in Mating II. Animals did not refuse to mate with a resource-deprived partner at a greater frequency than when they were paired with an animal of similar condition.

Taken together, these data seem to suggest that both partners benefit from having the animal with the greatest access to resources act as a sperm recipient. This strategy ensures maximum offspring, as well-fed animals can lay more eggs and, presumably, are less likely to engage in sperm digestion. In terms of which partner controls this dynamic, two scenarios are possible. It could be that resource-deprived animals refuse to accept a more costly recipient role, as is suggested by the observation of donor attempts. However, it may also be the *ad libitum* animals refusing to donate sperm to a low-quality recipient who will not be able to produce as many eggs, and who may even eat the donated sperm. The lack of increased gender conflict suggests either that the passive partner accepts both roles, or that both partners are able to maximize fitness benefits by adopting complementary roles.

If nothing else this study clearly demonstrates that individual mating decisions are determined on a case by case basis rather than by obligate reciprocation or gamete-trading. This study also provides evidence that some simultaneous hermaphrodites are capable of assessing partner condition based on factors that do not seem readily apparent (food intake). Such capabilities have been suggested for other hermaphrodites (Michiels and Bakovski, 2000; Anthes *et al.*, 2006a; Velando *et al.*, 2008; Domínguez and Velando, 2013); however, the mechanisms themselves remain poorly understood (Anthes, 2010). *Aplysia californica* is an example of a simultaneous hermaphrodite that favors role flexibility in mating, and is able to change strategies over the course of a mating season as a result of shifting environmental variables and partner condition. These findings are supported by (Ludwig and Walsh, 2008) who came to a similar conclusion concerning multiple mating.

These findings support the Gender Ratio Hypothesis, which posits that simultaneous hermaphrodites make mating decisions based on potential fitness gains for each distinct mating event. Gender Ratio predicts that while the male role is generally preferred, the female role will typically be accepted as well (Anthes *et al.*, 2006b). This theory allows for a variety of different strategies dependent on the condition of both partners, rather than obligate dependence on any specific strategy. In Mating I trials it could be assumed that when mating occurred, the fitness gain in both roles were net positive and as a result there was not a strong incentive for animals to select one role over the other. However, in Mating II, restricted diet animals had decreased fitness in the recipient role as a result of their reduced egg output. It then becomes more profitable for both partners to allow the restricted animal to act as donor.

It should be noted that this study only observed interactions between two unrelated cohorts of animals. Any unaccounted factors that may exist between these particular groups due to genetic background or other sources were not taken into account. No attempt was made to thoroughly measure fertilization or sperm investment. It may be beneficial for future studies

to take these factors into account in order to obtain a more complete analysis of costs/benefits of mating role selection.

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Figure 1. Pools of potential pairs for both mating periods. In Mating I pairs were mated across families. In Mating II pairs were mated across families and diet regimen. Gray represents supplemental animals that were added to the experiment (See Methods).

Figure 2.

Mean (± 1 SD) growth and intake of both *ad libitum* and restricted diet experimental groups over the length of the experiment. Diets were imposed on day 90.

Figure 4.

Mass of egg clutches over the length of the experiment for *ad libitum* and restricted animals fitted to polynomial curves (Fieber *et al.*, 2005). Vertical line designates when restricted diets were implemented. *Ad libitum* animals laid larger egg clutches on average than those on restricted diets (unpaired t-test; $t=3.1$, $df=81$, $p<0.01$)

Figure 5.

total # of clutches laid by animals of both diets over the course of the experiment. Vertical line designates when restricted diets were implemented. *Ad libitum* animals laid eggs at a higher frequency than restricted animals (unpaired t-test; $t=2.3$ $df=81$, $p=0.02$)

Table 1

Average number of donor attempts observed per day for both diets.

	<i>Ad libitum</i>	Restricted	Total
Mating I	0.23	0.15	0.38
Mating II	0.23	0.37	0.60