Behavioral Ecology Vol. 14 No. 4: 554–560

Multiple mating, paternity, and body size in a simultaneous hermaphrodite, *Aplysia californica*

Lisa Angeloni,[a] Jack W. Bradbury,[b] and Ronald S. Burton[c]
[a]Section of Ecology, Behavior and Evolution, Division of Biology, University of California, San Diego, La Jolla, CA 92093-0116, USA, [b]Cornell Library of Natural Sounds, Cornell University, Ithaca, NY 14850, USA, and [c]Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202, USA

Sperm displacement and sperm competition prove difficult to measure, but are crucial elements in predicting sex allocation strategies of sperm-storing hermaphrodites. Body size is predicted to affect sex allocation so that within a population, large animals invest a greater proportion of resources in female function than do small animals. These mating strategies depend on sperm displacement abilities and lead to similar levels of paternity across body sizes despite differences in resource level. The present study investigated mating patterns, multiple paternity, and sperm competition in a field population of a simultaneously hermaphroditic sea slug, *Aplysia californica* (California sea hare). Animals mating in the female role were larger than the mean for the population, indirectly supporting theoretical predictions for increased investment in female function with body size. However, contrary to predictions, animals mating in the male role were not different in size from the population mean or the animals they inseminated. Individual tagging revealed that sea slugs are capable of moving across distances that allow for the sampling of many potential mates, and that they mate repeatedly in both sexual roles. Microsatellite paternity analysis demonstrated that multiple mating in the field leads to multiple paternity, and last-sperm donors achieve high levels of paternity. There was no effect of body size on paternity. Further paternity studies are needed to reveal the mechanisms of sperm precedence patterns in *A. californica*. *Key words:* multiple paternity, opisthobranch, sea hare, sex allocation, sperm competition. *[Behav Ecol 14:554–560 (2003)]*

Darwin (1874: 260, 613) suggested that traits affecting mating success in one sexual role would not evolve when both sexes are combined in the same individual. This historical misconception contributed to a lack of understanding of sexual selection in simultaneous hermaphrodites, which is only recently being explored. Although precopulatory sexual selection on traits related to mate acquisition may be weaker in hermaphrodites, limiting the potential for exaggerated elaboration (Greeff and Michiels, 1999), we now know that sexual selection can occur in hermaphrodites (Arnold, 1994; Charlesworth and Charlesworth, 1987; Charnov, 1979, 1996; Morgan, 1994), and that sperm competition and intersexual conflict are likely common (Charnov, 1979; Michiels, 1998). Unlike dioecious equivalents, a hermaphrodite can adjust the amount of resources allocated to mating in the male and female roles in response to current conditions and sexual selection pressures. By the same token, dividing resources between reproduction in both sexual roles constrains the amount of resources optimally committed to any sexually selected strategy below that seen in a corresponding dioecious species. There is thus a dynamic interaction between the processes of sex allocation and other sexually selected strategies in animal hermaphrodites (Angeloni et al., 2002; Baur, 1994; Charnov, 1996; DeWitt, 1996; Greeff and Michiels, 1999; Haase and Baur, 1995; Peters and Michiels, 1996).

The typical invertebrate hermaphrodite has a sperm storage organ, which can lead to sexual selection in the form of sperm competition (Michiels, 1998). This forces sperm donors (animals mating in the male role) to compete for maximum contribution to the sperm supply within sperm recipients (animals mating in the female role). Theoretical models of sex allocation in hermaphrodites have emphasized the importance of measuring sperm displacement and sperm competition for understanding hermaphroditic mating strategies (Angeloni et al., 2002; Charnov, 1996; Greeff et al., 2001; Pen and Weissing, 1999; Petersen, 1991). Fitness return to investment in sperm is a key factor determining sex allocation strategies, but has proven difficult to measure. The anatomy of hermaphroditic reproductive tracts, including organs for sperm storage and possibly sperm digestion, has been well described (see Adiyodi and Adiyodi, 1988; Tompa et al., 1984). However, for most hermaphrodites, little is known of the actual fate of sperm after it is transferred to a mate (Michiels, 1998; but see Beeman, 1970; Rogers and Chase, 2001, 2002), and most studies assume that copulation results in insemination and subsequent fertilization of eggs.

Body size may affect the resources an individual has for reproduction, as well as the size of its internal sperm storage organ, thereby affecting mating strategies (Charnov, 1996). Under a wide range of population conditions, a recent model predicts larger animals with more resources for reproduction should invest a lower proportion of resources in sperm (therefore a greater proportion in eggs) than do small animals (Angeloni et al., 2002). In addition, an animal that can adjust sperm versus egg production depending on the size of a current mate should invest more resources in sperm when mating with a large animal than when mating with a small animal (Angeloni et al., 2002). Body size affects the *proportion* of resources invested in sperm; large animals have greater *absolute* resource levels but invest a smaller proportion of those resources in sperm than do small animals, equalizing actual sperm production and transfer with respect to body size. This leads to predictions that hermaphrodites, like

Address correspondence to L. Angeloni, who is now at the Department of Biology, Colorado State University, Fort Collins, CO 80523-1818, USA.

Received 15 December 2001; revised 22 October 2002; accepted 4 November 2002.

animals with separate sexes, should achieve similar levels of sperm displacement regardless of the body size of the sperm donor or recipient (Angeloni et al., 2002; Birkhead and Parker, 1997; Parker and Simmons, 1994). Thus, under many conditions, sex allocation theory predicts that larger animals should emphasize female function, smaller animals should invest more in male function, and paternity patterns should be consistently similar across body sizes. These modeling efforts further emphasize the importance of measuring sperm displacement and sperm competition processes as a crucial part of understanding sex allocation and mating strategies.

We expected predictions for size-dependent sex allocation to translate into observable size-dependent mating strategies in a field population of a simultaneous hermaphrodite. In the present study, we investigated the effects of body size on mating patterns and sperm competition in the opisthobranch sea slug, *Aplysia californica* (California sea hare).

METHODS

Study organism

Opisthobranchs are nonselfing simultaneous hermaphrodites with highly complex and variable reproductive anatomies (Ghiselin, 1965; Thompson, 1976). Opisthobranchs have sperm storage organs, and one species has been shown to digest sperm (Beeman, 1970), a phenomenon that is probably widespread throughout the group (Ghiselin, 1965). Although some opisthobranchs mate reciprocally so that two individuals simultaneously inseminate each other, *A. californica* usually transfers sperm unilaterally, with one animal donating sperm while the other receives it (Pennings, 1991a). Occasionally, an individual will donate and receive sperm at the same time by mating within a chain of three or more animals, in which each transfers sperm to the one ahead of it.

A. californica is one of the most extensively studied opisthobranchs because it has been used as a model for neurobiological research (Kandel, 1979) and its distribution, natural history and mating behaviors have been described (see Audesirk, 1979; Carefoot, 1987; Kupferman and Carew, 1974; Leonard and Lukowiak, 1986; MacGinitie, 1934; Pennings, 1990, 1991a,b; Winkler and Dawson, 1963). Like many opisthobranchs, *A. californica* has an annual life cycle with indeterminate growth, showing an early onset of reproduction and continued growth until the end of life. This generates mating populations with large size differences between sexually mature animals (Pennings, 1991b), facilitating research on the effects of body size on mating patterns and sperm competition.

Mating patterns

We studied mating patterns of a population of *A. californica* within a 30 × 30-m rocky intertidal site at Bird Rock Tide Pools, San Diego County, California, USA, during low tides (ranging from 0.3 to −1.3 ft) on 10 sampling days (June–August 1998). This site included pools with high densities of *A. californica*. Each *A. californica* within the study site was weighed, and its mating status was classified into one of four categories: (1) not mating, (2) mating as sperm donor, (3) mating as sperm recipient, and (4) mating simultaneously as both sperm donor and sperm recipient. Mating status was easily established by inserting a finger between parapodia to determine if adjacent animals were linked by an extended penis.

We pooled data on masses of mating animals across all 10 sampling days to increase the sample size for detecting the effects of body size on mating patterns. Before pooling data across sample days, we standardized body mass to control for growth during the study. To do so, we subtracted from each individual's body mass, the mean mass of the population for the day it was sampled, and then divided this value by the standard deviation in body sizes for that sample day. The standard scores for body sizes of mating animals could then be pooled across all sample days and compared with the standardized population mean (set to 0) with one-sample *t* tests.

Tagging study

To collect data on individual movement and multiple mating, we tagged 56 *A. californica* with number-coded transponder tags injected inside the left parapodium that could be detected with a Trovan LID-500 handheld reader (InfoPet Identification Systems; Angeloni et al., 1999). The location of each tagged individual was mapped within the study site by taking angle and distance measures from the southeast corner of the site. To recapture tagged individuals, all animals within the study site or within 5 m of it were scanned on 10 subsequent sampling days. We noted the identity, mating status, mass, and location of tagged animals when recaptured. Distances were calculated between subsequent capture locations to determine minimum estimates of movement rates. These are underestimates of movement because they assume a straight line of travel between two locations and do not include the many animals that traveled long distances out of the study site and therefore were not recaptured.

Molecular paternity analysis

A 2-mm × 1-cm section of parapodial tissue was cut from 77 *A. californica* at False Point Tide Pools in Bird Rock, San Diego County, California, USA, in June 2000. Removal of this tissue was found to have no evident adverse affects on individuals monitored in the lab. This tissue was used to genotype individuals and determine population allele frequencies at a microsatellite locus. To extract DNA from parapodial tissue, we boiled a small portion (approximately 0.5 mm^3) in 200 μl of 10% chelating resin (Sigma Chemical Co., St. Louis) for 8 min, vortexed the sample for 10 s, and then centrifuged it for 2 min at 4°C. Three μl of the supernatant were used as template in a 25 μl polymerase chain reaction (PCR). We designed PCR primers to amplify a (approximately 210-bp) microsatellite locus with an AAT-repeat region within a previously published sequence of a neuropeptide Y gene from *A. californica* (Rajpara et al., 1992). Primers used were AplF (5′-CTTCTTACCATATCGTTTGGGA-3′, position 885–906 in the published sequence) end-labeled with [γ-^{32}P]-dATP, and AplR (5′-CAAAATCCTACCCAAGAGTAAG-3′, position 1075–1096). We used the following PCR thermocycle profile: 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s for 35 cycles followed by 7 min at 72°C. PCR products were run on 5% denaturing polyacrylamide sequencing gels and dried and scored by autoradiography. Alleles were scored against a standard allele that was arbitrarily designated allele 0, so that all other alleles were assigned a number based on the relative number of repeats more or less than allele 0.

We collected 20 animals found mating as sperm recipients during low tide periods at False Point Tide Pools (May–August 2000), in many cases interrupting the mating event; parapodial samples from the sperm donor and sperm recipient were saved for later genotyping. Each sperm recipient was maintained individually in 20 L of flow-through seawater at 17°C–20°C on a 12-h/12-h light/dark cycle and fed one head of romaine lettuce. Collected animals produced a single egg clutch within 1 week of collection. Because *A. californica* egg masses are large and typically made up of several hundred thousand to millions of eggs (Kandel and Capo, 1979; MacGinitie, 1934), we saved only a portion of each egg mass

to raise offspring for genotyping. Each egg mass was weighed and separated into the half deposited first and the half deposited last; one segment of egg strand was saved from each half and allowed to develop separately in beakers with 300 ml of fresh seawater. After larvae hatched (within 2 weeks), a sample of 20 larvae were frozen individually from each egg mass half (40 larvae per egg clutch). To extract DNA, each single larva was incubated at 65°C in 10 µl extraction buffer (50 mM KCl, 10 mM Tris–HCl at pH 8.3, 0.5% Tween 20, and 20 mg/ml proteinase K) for 60 min, and then denatured at 95°C for 15 min (Hoelzel and Green, 1992; Simpson et al., 1999). Three µl of template were used in 25 µl PCR as described above. Larvae from an egg clutch were run together on the same gel, along with the mother and the last individual to donate sperm to the mother. We calculated the paternity of the observed sperm donor as the proportion of offspring that shared a paternal allele with that individual. Because there were no statistical differences between the paternity measured in the first and second halves of egg masses, we pooled those data for analysis. In some cases, alleles from an additional sperm donor may be the same as those of the observed sperm donor, so we used population allele frequencies to calculate the probability of detecting genes contributed by additional sperm donors, or the probability of excluding the observed sperm donor (following the method of Chakravarti and Li, 1983; Westneat et al., 1987).

In August 1999 and August 2000, 13 additional individuals that were not found mating were collected from Bird Rock and False Point Tide Pools and maintained as above. After depositing an egg mass, a sample of parapodial tissue and offspring from both first and last portions of egg masses (between nine and 28 offspring) were genotyped from each individual as described above. Data on number of paternal alleles in these egg clutches were combined with the similar data for egg clutches in which the last sperm donor was known to calculate an overall measure of multiple paternity in egg clutches. Number of paternal alleles was used to calculate a minimum estimate for the number of fathers per egg clutch.

All data were compared to normal distributions, transformed when appropriate, and analyzed statistically by using JMP, version 3.0. Last-mate paternity proportions were arcsine-transformed before statistical analyses. Throughout this article, values are mean ± SE. For results that were not statistically significant, power analysis was conducted to determine the probability of rejecting the null hypothesis given our sample size, an α level of 0.05, and the expected magnitude of effect of the independent variable on the dependent variable (Cohen, 1988). Because expected effect sizes were unknown, we calculated the power to detect a hypothetical "medium" effect, following conventions of Cohen (1988), with an operational definition specific to each test (e.g., a medium effect for a t test, or medium difference between two groups scaled by the within-group SD, is defined as 0.5). In addition, we calculated the minimum sample size needed for a future study to achieve power of 0.80 with the actual effect size observed from the data, reported as n_{min}.

RESULTS

Mating patterns

As the population within the 30 × 30-m study site ranged from 48 to 176 animals, the density ranged from 0.05 to 0.20 sea slugs/m^2 (mean ± SE: 0.12 ± 0.02 sea slugs/m^2, $n = 7$). Between 0 and 41% of the population was found mating during sampling periods (18 ± 4%, $n = 8$). A total of 108 mating pairs were found during sampling periods, as well as 22 chains of three mating animals, four chains of four mating animals, and one chain of five mating animals. Three of the mating pairs were observed to be simultaneously and reciprocally inseminating each other; insemination was unilateral in all other pairs. We detected a slight trend toward size-assortative mating in pairs ($r_p = .19$, $n = 108$, $p = .05$) (Figure 1).

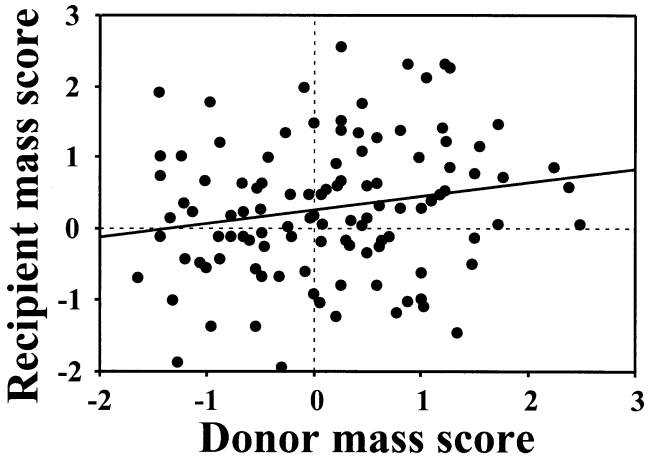

Figure 1
Regression of sperm recipient body mass on sperm donor mass for mating pairs ($r = .19$, $n = 108$, $p = .05$). Mass score was calculated as SDs from the mean for that day to correct for growth in body size throughout the study.

Body masses within the population ranged from 110 to 1320 g. Those individuals found mating as sperm donors ranged in body mass from 180 to 1180 g, and those mating as sperm recipients ranged from 170 g to 1320 g. In mating pairs, sperm recipients were significantly larger than the mean of the population (mean mass = 0.30 SD larger than the mean of the population ± 0.09 SE; one-sample t test, $t_{104} = 3.2$, $p < .01$), whereas sperm donors were not significantly different from the mean (mean mass score = 0.14 ± 0.09; $t_{104} = 1.6$, $p > .1$; power = 0.99, $n_{min} = 354$). However, within mating pairs, sperm recipients were not significantly different in size from their sperm donors (mean difference = 0.16; paired t test: $t_{104} = 1.3$, $p > .1$; power = 0.98, $n_{min} = 1299$). In chains of three to five mating animals, the mean mass of individuals in the middle positions (mating in both roles) was significantly greater than the population mean (mean mass score = 0.32 ± 0.14, $t_{33} = 2.3$, $p < .05$), whereas terminal sperm recipients and terminal sperm donors were not different from the population mean (recipient mean mass score = 0.10 ± 0.24; $t_{26} = 0.44$, $p > .1$; power = 0.71, $n_{min} = 1092$; donor mean mass score = 0.31 ± 0.21; $t_{26} = 1.48$, $p > .1$; power = 0.71, $n_{min} = 98$).

Tagging study

Of 56 tagged animals, 40 were recaptured at some later date (10 animals once, 13 animals twice, nine animals four times, seven animals five times, and one animal six times). Tagged animals mated multiply; six animals were detected mating twice, and two animals were detected mating three times. Of the eight tagged animals that we observed mating multiple times, five mated in both roles, one was detected mating twice as a sperm recipient, and two mated twice as a sperm donor.

The minimum estimate of movement per day by tagged animals between recapture events, which occurred from 1–43 days apart (mean = 7 days), was 2.4 ± 0.3 m/day ($n = 94$). This underestimates actual movement by dividing straight-line distances between recapture locations (confined to the study

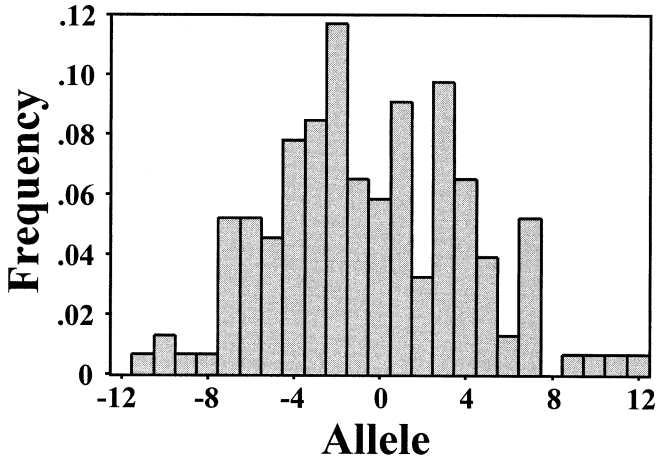

Figure 2
Frequency of Apl microsatellite alleles ($n = 154$). One allele was arbitrarily assigned a value of zero, and all other alleles were recorded in terms of the number of repeats more or less than allele 0.

site) by days between recapture, which were often many, and does not include the additional distance likely traveled between two capture events. By using only recaptures separated by 1 day, the minimum estimate of distance traveled is 5.0 ± 0.5 m/day ($n = 33$).

Molecular paternity analysis

The Apl microsatellite locus was found to be highly polymorphic with 23 alleles, all at frequencies less than 0.12 (mean ± SE = 0.04 ± 0.01) (Figure 2). The probability of detecting alleles contributed by a sperm donor other than the observed donor is 0.86 (Chakravarti and Li, 1983; Westneat et al., 1987). Thus, this single microsatellite locus proved adequate for estimating the proportion of offspring fathered by the known sperm donor, as the probability of detection of genes contributed by an alternative sperm donor was high. Our measures of multiple paternity likely reflect most (86%) but not all of the actual levels of multiple paternity in the field.

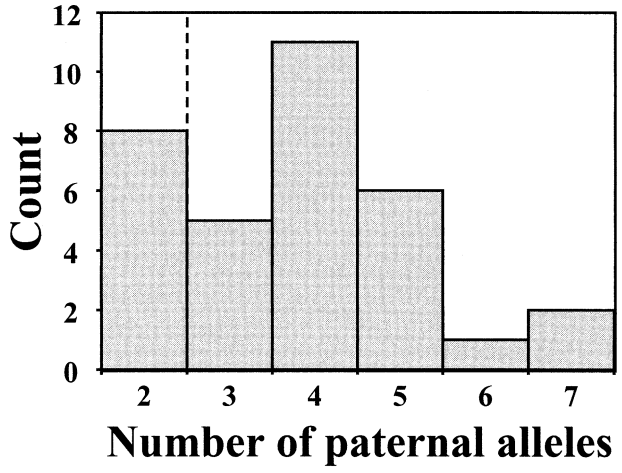

Figure 3
Frequency distribution of the number of paternal alleles detected in each egg clutch genotyped. Offspring from egg clutches above the dotted line had more than two paternal alleles, indicating multiple paternity.

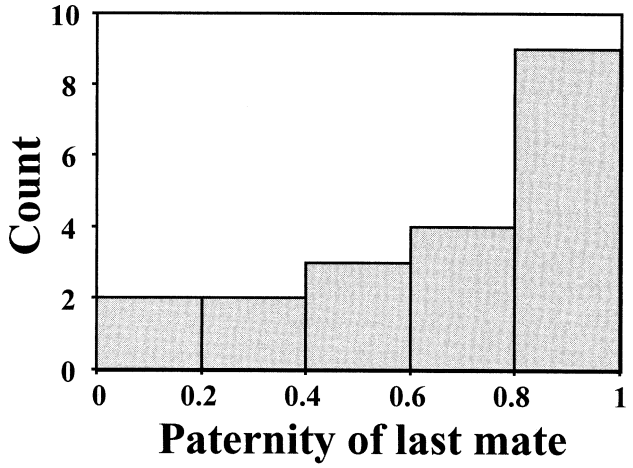

Figure 4
Frequency distribution of the proportion of paternity achieved by the observed sperm donor in the first egg clutch produced by the sperm recipient after the mating event. In four cases, the sperm donor fathered 100% of the offspring genotyped.

Multiple mating in the field resulted in high levels of multiple paternity in egg clutches. Of 33 egg masses sampled, 25 (76%) had more than two paternal alleles, indicating at least two fathers, and nine of those (27%) had at least three fathers (Figure 3). Based on number of paternal alleles, the mean estimate for number of fathers per egg clutch was 2.1 ± 0.15. The number of alleles detected did not depend on the day within the breeding season ($F = 2.4$, $r = -.27$, $n = 33$, $p > .1$, power = 0.53, $n_{min} = 68$). Our method of interrupting some mating events to sample tissue from the last sperm donor did not affect the level of multiple paternity; there was no difference in the number of alleles detected in egg masses produced by individuals whose last mating event was interrupted ($n = 16$; mean ± SE = 4.0 ± 0.38) and whose last mating event was not interrupted or not observed at all ($n = 17$; mean ± SE = 3.6 ± 0.32; mean difference = 0.41; t test: $t_{31} = 0.84$, $p > .1$, power = 0.29, $n_{min} = 175$ each group).

In cases in which the genotype of the last sperm donor was known, the known sperm donor fathered significantly more than half of the offspring sampled, suggesting last-mate sperm precedence (mean = 0.73 ± 0.09; one-sample t test: $t_{19} = 2.29$, $p < .05$); the known sperm donor fathered more than half of the offspring in 15 of 20 clutches sampled (Figure 4). However, in four cases, the alleles of the known sperm donor were the only alleles present among the offspring, and thus, those high measures of paternity (100%) may reflect only one sperm donor rather than last-mate sperm precedence. Excluding those four cases from the analysis, the last-mate paternity did not differ significantly from 0.5 (mean = 0.60 ± 0.10; one-sample t test: $t_{15} = 0.96$, $p > .1$; power = 0.48, $n_{min} = 137$).

There was no effect of the body sizes of the observed sperm donor ($F = 0.12$, $r = .081$, $p = .73$; power = 0.25, $n_{min} = 966$) or sperm recipient ($F = 0.024$, $r = -.037$, $p = .88$; power = 0.25, $n_{min} = 3858$) on the level of paternity achieved by the sperm donor in regression analyses ($n = 20$). Nor did paternity depend on the day of the mating event within the breeding season ($F = 0.41$, $r = .15$, $p = .53$; power = 0.25, $n_{min} = 276$), the size of the egg mass ($F = 1.01$, $r = -.29$, $p = .34$; power = 0.17, $n_{min} = 71$), or the number of days between when the animal was collected and the egg mass was deposited ($F = 0.96$, $r = -.23$, $p = .34$; power = 0.25, $n_{min} = 120$). None of these variables were significant predictors of paternity in multiple regressions, including all combinations of variables (all, $p > .3$).

DISCUSSION

We found evidence for limited effects of body size on mating patterns in *A. californica*, with size affecting the tendency to mate in the female role but not in the male role. High movement rates (confirming those found by Kupferman and Carew, 1974) and population densities suggest that individuals encountered many potential mates, and that size-based mating patterns likely reflect active choices. Individuals mated multiply in both sexual roles, and multiple mating resulted in multiple paternity within egg masses. We did not detect any evidence for an effect of body size on sperm precedence patterns.

Although sperm recipients were not larger than sperm donors, they were larger than the mean for the population, suggesting that larger-than-average animals engaged in mating events as females more frequently or for longer duration and, thus, spent more time mating in the female role. This finding indirectly supports theoretical predictions that relatively larger animals should emphasize female function in populations of sperm-storing hermaphrodites and/or that more time and energy should be invested to inseminate a large animal than a small one (Angeloni et al., 2002). However, contrary to our predictions, we did not find any evidence that sperm recipients were larger than their respective sperm donors, or that smaller than average animals emphasized male function; the mean mass of sperm donors was intermediate between that of the entire population and that of sperm recipients and was not significantly different from either.

Support for an effect of body size on mating patterns or sex allocation has been found in other species in the same genus (e.g., *A. vaccaria*, Angeloni and Bradbury, 1999; *A. punctata*, Otsuka et al., 1980; *A. kurodai*, Yusa, 1996), and in other simultaneous hermaphrodites (DeWitt, 1996; Klinkhamer et al., 1997; Michiels et al., 2001; Petersen 1995; Petersen and Fischer, 1996; Schärer et al., 2001; St. Mary, 1997; Vreys and Michiels, 1997). Several studies have not found such an effect (Baur, 1992; Leonard and Lukowiak, 1985; Pennings, 1991a; Peters and Michiels, 1996), including one study on *A. californica* (Pennings, 1991a). In some cases, this may reflect differences in sample sizes; detection of the apparently subtle body size effects requires large sample sizes (see Angeloni and Bradbury, 1999).

However, it remains unclear why theoretical predictions are only inconsistently supported by size-dependent mating patterns in this and other studies. Sex allocation predictions for size-dependent gamete investment may not translate into size-dependent mating patterns, and direct measures of sperm versus egg production could more consistently support theory in *A. californica* and other species. In addition, size-dependent sex allocation is predicted to be most extreme in populations or species with costly sperm transfer. When hermaphrodites can efficiently displace sperm and fill storage organs by using a small fraction reproductive resources, body size should have a reduced, and perhaps undetectable, effect on sex allocation, with all individuals investing little in male function (Angeloni et al., 2002). The cost of filling a sperm storage organ is unknown in *A. californica* and may represent a small fraction of an individual's total reproductive resources.

Alternatively, theoretical models could be too simple to predict actual mating patterns because they make a number of assumptions, such as perfect size assessment, equal mating opportunities independent of body size, and constant reproductive resources for each mating event (see Angeloni et al. 2002; Charnov, 1996). If mating opportunities change with body size, or if animals adjust the resources they invest in reproduction depending on qualities of the current mate, sex allocation predictions may differ. Additional theoretical work to incorporate these complexities, direct measures of sperm and egg production, and behavioral experiments are needed to understand how body size affects hermaphroditic mating strategies and the extent to which individuals can assess their own and their mate's body sizes. To date, only a few studies have shown that hermaphrodites use tactile and chemical cues to assess mate size (Lüscher and Wedekind, 2002; Vreys and Michiels, 1997).

Individuals mated multiply in the field and in both sexual roles, confirming previous findings by Pennings (1991a) that *A. californica* does not specialize in one particular sexual role to the exclusion of the other. Multiple mating leads to multiple paternity in egg masses, with an estimate of 2.1 fathers per egg clutch. This is a minimum value, as our molecular marker could detect 86% of genes contributed by additional sperm donors, and we could only genotype a small fraction of the hundreds of thousands of eggs in a typical egg mass. Multiple paternity appears to be common in hermaphroditic broods (Baur, 1994; Gaffney and McGee, 1992; Landolfa et al., 2001; Murray, 1964; Rogers and Chase, 2002; Rollinson et al., 1989; Todd et al., 1997; Vianey-Liaud, 1995; Wethington and Dillon, 1991).

Although sperm precedence was variable, last-sperm donors were capable of achieving high levels of paternity; we found significant levels of last mate sperm precedence when we included four measures of 100% paternity by the observed sperm donor. It is possible that instead of being successful sperm competitors, those four individuals were the only sperm donors to inseminate their recipients. This may be unlikely, however, given the high level of multiple mating in this population, our personal observation of never having collected a virgin, and previous accounts that *A. californica* begins breeding and producing fertilized eggs at small sizes (1 g) and stores sperm throughout the breeding season (Kandel and Capo, 1979; MacGinitie, 1934; Pennings 1991b). In addition, the cases of 100% paternity did not consistently occur early in the breeding season; last-male paternity and the level of multiple paternity did not depend on sampling day. Some paternity estimates may be low owing to interruption of mating events during sampling. However, interruption did not affect the number of alleles detected, suggesting that its effect on paternity of the known sperm donor was probably minimal.

Widely varying, but reduced, last-mate paternity patterns are consistently found in the other hermaphrodites that have been studied, but are likely explained by very different mechanisms, depending on the behavior and anatomy of the species. For example, among gastropods, variable sperm precedence has been explained by sperm storage organs with blind sacs that may allow recipients to sort and select sperm (*Arianta arbustorum*, Baur, 1994), hypodermic sperm transfer to any location on a mate's body that may reduce the ability to displace sperm (*Alderia modesta*; Angeloni, in press), and varying levels of successful penetration of a love dart into a mate (*Helix aspersa*, Landolfa et al., 2001; Rogers and Chase, 2002). Even a hermaphroditic ascidian that passively disperses sperm exhibits variable sperm precedence with reduced last-mate paternity (Bishop et al., 2000).

Currently, the mechanisms of sperm precedence patterns are unknown in *A. californica*, warranting further studies to directly look at the fate of labeled sperm, as well as paternity experiments with multiple known sperm donors. Last-mate sperm precedence can be owing to any number of mechanisms in *A. californica*: active sperm displacement or removal, passive loss or digestion of sperm from earlier mating events, sperm layering so that the last sperm is closest to the site of fertilization, greater sperm numbers transferred by later sperm donors, and recipient selection of sperm from later higher-quality donors (Birkhead and Parker, 1997). In the

genus *Aplysia*, sperm are transferred to a blind-ended seminal receptacle where sperm displacement or layering can occur (Ghiselin, 1965; Thompson and Bebbington, 1969). Sperm are stored there throughout the breeding season and go on to fertilize eggs just outside of that sac or can be moved to a nearby organ where sperm digestion may occur. Mating events last several hours, and it is unknown whether sperm transfer occurs continuously, intermittently, or only at the beginning or end of the mating event. Long mating events may reflect a mate-guarding strategy or some other method for sperm donors to increase paternity.

We did not detect any effect of the size of the sperm donor or sperm recipient on paternity patterns. Theory for hermaphrodites and animals with separate sexes does not predict an effect of body size on paternity; small animals should invest a greater proportion of resources in sperm than do large animals, allowing them to transfer similar levels of sperm despite reduced overall resource levels (Angeloni et al., 2002; Birkhead and Parker, 1997; Parker and Simmons, 1994). However, other possibilities might also predict paternity patterns independent of body size, such as selective sperm use by recipients based on other traits or incomplete sperm mixing. The lack of relationship between body size and paternity could also be owing to the low statistical power (0.25) of our study to detect a medium effect (i.e., $r = .3$; Cohen, 1988). On the other hand, the small effect of body size on paternity estimated from our data suggests that an increase in power (to 0.80) would require a prohibitively large sample size, close to 1000. Additional paternity studies in combination with methods to directly measure sperm transfer would clarify the effects of sex allocation and complex reproductive anatomy and behavior on sperm precedence patterns in hermaphrodites.

Many thanks to K. R. Crooks for time spent in the field and valuable discussions of the project. M. Medina helped with locating paternity markers. T. Ballard, T. J. Case, and N. D. Tsutsui provided assistance with microsatellite analysis. N. D. Holland and J. R. Kohn provided advice and comments on the manuscript. The manuscript was improved by D. F. Westneat and two anonymous reviewers. Funding for this research was provided by a Graduate Research Award from the Animal Behavior Society, an Eloise Gerry Fellowship from Sigma Delta Epsilon Graduate Women in Science, and a Dissertation Improvement Grant from the National Science Foundation (IBN-9902307). During preparation of the manuscript, L.A. was supported by a Ford Foundation Dissertation Fellowship.

REFERENCES

Adiyodi KG, Adiyodi RG, 1988. Reproductive biology of invertebrates: accessory sex glands, vol. 3. Chichester: Wiley.

Angeloni L, in press. Sexual selection in a simultaneous hermaphrodite with hypodermic insemination: body size, allocation to sexual roles, and paternity. Anim Behav.

Angeloni L, Bradbury J, 1999. Body size influences mating strategies in a simultaneously hermaphroditic sea slug, *Aplysia vaccaria*. Ethol Ecol Evol 11:187–195.

Angeloni L, Bradbury J, Chaine A, 1999. Growth, seasonality, and spatial dispersion in a population of the black sea hare, *Aplysia vaccaria* Winkler 1955. Veliger 42:1–9.

Angeloni L, Bradbury JW, Charnov EL, 2002. Body size and sex allocation in simultaneously hermaphroditic animals. Behav Ecol 13:419–426.

Arnold SJ, 1994. Bateman's principles and the measurements of sexual selection in plants and animals. Am Nat 144:S127–S149.

Audesirk TE, 1979. A field study of growth and reproduction in *Aplysia californica*. Biol Bull 157:407–421.

Baur B, 1992. Random mating by size in the simultaneous hermaphroditic land snail *Arianta arbustorum*: experiments and an explanation. Anim Behav 43:511–518.

Baur B, 1994. Multiple paternity and individual variation in sperm precedence in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Behav Ecol Sociobiol 35:413–421.

Beeman RD, 1970. An autoradiographic study of sperm exchange and storage in a sea hare, *Phyllaplysia taylori*, a hermaphroditic gastropod (Opisthobranchia: Anaspidea). J Exp Zool 175:125–132.

Birkhead TR, Parker GA, 1997. Sperm competition and mating systems. In: Behavioural ecology: an evolutionary approach, 4th ed (Krebs JR, Davies NB, eds). Oxford: Blackwell; 121–145.

Bishop JDD, Pemberton AJ, Noble LR, 2000. Sperm precedence in a novel context: mating in a sessile marine invertebrate with dispersing sperm. Proc R Soc Lond B 267:1107–1113.

Carefoot TH, 1987. *Aplysia*: its biology and ecology. Ann Rev Oceanogr Mar Biol 25:167–284.

Chakravarti A, Li CC, 1983. The effect of linkage on paternity calculations. In: Inclusion probabilities in parentage testing (Walker RH, ed). Arlington, Virginia: American Association of Blood Banks; 411–422.

Charlesworth D, Charlesworth B, 1987. The effect of investment in attractive structures on allocation to male and female functions in plants. Evolution 41:948–968.

Charnov EL, 1979. Simultaneous hermaphroditism and sexual selection. Proc Natl Acad Sci USA 76:2480–2484.

Charnov EL, 1996. Sperm competition and sex allocation in simultaneous hermaphrodites. Evol Ecol 10:457–462.

Cohen, J. 1988. Statistical power analysis for the behavioral sciences, 2nd ed. Hillsdale, New Jersey: Erlbaum Associates.

Darwin C, 1874. The descent of man, and sexual selection in relation to sex, 2nd ed. London: J. Murray.

DeWitt TJ, 1996. Gender contests in a simultaneous hermaphrodite snail: a size advantage model for behavior. Anim Behav 51:345–351.

Gaffney PM, McGee B, 1992. Multiple paternity in *Crepidula fornicata* (Linnaeus). Veliger 35:12–15.

Ghiselin MT, 1965. Reproductive function and the phylogeny of opisthobranch gastropods. Malacologia 3:327–378.

Greeff JM, Michiels NK, 1999. Low potential for sexual selection in simultaneously hermaphroditic animals. Proc R Soc Lond B 266:1671–1676.

Greeff JM, Nason JD, Compton SG, 2001. Skewed paternity and sex allocation in hermaphroditic plants and animals. Proc R Soc Lond B 268:2143–2147.

Haase M, Baur B, 1995. Variation in spermathecal morphology and storage of spermatozoa in the simultaneously hermaphroditic land snail *Arianta arbustorum* (Gastropoda: Pulmonata: Stylommatophora). Invert Reprod Develop 28:33–41.

Hoelzel AR, Green A, 1992. Analysis of population-level variation by sequencing PCR-amplified DNA. In:, Molecular genetic analysis of populations: a practical approach (Hoelzel AR, ed). Oxford: Oxford University Press.

Kandel ER, 1979. Behavioral biology of *Aplysia*. San Francisco: Freeman.

Kandel P, Capo TR, 1979. The packaging of ova in the egg cases of *Aplysia californica*. Veliger 22:194–198.

Klinkhamer PGL, de Jong TJ, Metz H, 1997. Sex and size in cosexual plants. Trends Ecol Evol 12:260–265.

Kupferman I, Carew TJ, 1974. Behavior patterns of *Aplysia californica* in its natural environment. Behav Biol 12:317–337.

Landolfa MA, Green DM, Chase R, 2001. Dart shooting influences paternal reproductive success in the snail *Helix aspersa* (Pulmonata, Stylommatophora). Behav Ecol 12:773–777.

Leonard JL, Lukowiak K, 1985. Courtship, copulation, and sperm trading in the sea slug, *Navanax inermis* (Opisthobranchia: Cephalaspidea). Can J Zool 63:2719–2729.

Leonard JL, Lukowiak K, 1986. The behavior of *Aplysia californica* Cooper (Gastropoda: Opisthobranchia), I: ethogram. Behavior 98:320–360.

Lüscher A, Wedekind C, 2002. Size-dependent discrimination of mating partners in the simultaneous hermaphroditic cestode *Schistocephalus solidus*. Behav Ecol 13:254–259.

MacGinitie GE, 1934. The egg laying activities of the sea hare, *Tethys californicus* (Cooper). Biol Bull 67:300–303.

Michiels NK, 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. In: Sperm competition and sexual selection (Birkhead TR, Moller AP, eds). London: Academic Press; 219–254.

Michiels NK, Hohner A, Vorndran IC, 2001. Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? Behav Ecol 12:612–618.

Morgan MT, 1994. Models of sexual selection in hermaphrodites, especially plants. Am Nat 144:S100–S125.

Murray J, 1964. Multiple mating and effective population size in *Cepaea nemoralis*. Evolution 18:283–291.

Otsuka C, Rouger Y, Tobach E, 1980. A possible relationship between size and reproductive behavior in a population of *Aplysia punctata* Cuvier, 1803. Veliger 23:159–162.

Parker GA, Simmons LW, 1994. Evolution of phenotypic optima and copula duration in dungflies. Nature 370:53–56.

Pen I, Weissing FJ, 1999. Sperm competition and sex allocation in simultaneous hermaphrodites: a new look at Charnov's invariance principle. Evol Ecol Res 1:517–525.

Pennings SC, 1990. Size related shifts in herbivory: specialization in the sea hare *Aplysia californica* Cooper. J Exp Mar Biol Ecol 142:43–61.

Pennings SC, 1991a. Reproductive behavior of *Aplysia californica* Cooper: diel patterns, sexual roles and mating aggregations. J Exp Mar Biol Ecol 149:249–266.

Pennings SC, 1991b. Spatial and temporal variation in recruitment of *Aplysia californica* Cooper: patterns, mechanisms and consequences. J Exp Mar Biol Ecol 146:253–274.

Peters A, Michiels NK, 1996. Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. Fresh Biol 36:623–630.

Petersen CW, 1991. Sex allocation in hermaphroditic sea basses. Am Nat 138:650–667.

Petersen CW, 1995. Reproductive behavior, egg trading, and correlates of male mating success in the simultaneous hermaphrodite, *Serranus tabacarius*. Environ Biol Fish 43:351–361.

Petersen CW, Fischer EA, 1996. Intraspecific variation in sex allocation in a simultaneous hermaphrodite: the effect of individual size. Evolution 50:636–645.

Rajpara SM, Garcia PD, Roberts R, Eliassen JC, Owens DF, Maltby D, Myers RM, Mayeri E, 1992. Identification and molecular cloning of a neuropeptide Y homolog that produces prolonged inhibition in *Aplysia* neurons. Neuron 9:505–513.

Rogers DW, Chase R, 2001. Dart receipt promotes sperm storage in the garden snail *Helix aspersa*. Behav Ecol Sociobiol 50:122–127.

Rogers DW, Chase R, 2002. Determinants of paternity in the garden snail *Helix aspersa*. Behav Ecol Sociobiol 52:289–295.

Rollinson D, Kane RA, Lines JRL, 1989. An analysis of fertilization in *Bulinus cernicus* (Gastropoda: Planorbidae). J Zool 217:295–310.

Schärer L, Karlsson LM, Christen M, Wedekind C, 2001. Size-dependent sex allocation in a simultaneous hermaphrodite parasite. J Evol Biol 14:55–67.

Simpson RJ, Wilding CS, Grahame J, 1999. Simple protocol for extracting nuclear DNA from single embryos of a marine snail. BioTechniques 26:1050–1052.

St. Mary CM, 1997. Sequential patterns of sex allocation in simultaneous hermaphrodites: do we need models that specifically incorporate this complexity? Am Nat 150:73–97.

Thompson TE, 1976. Biology of opisthobranch molluscs. London: Stonebridge Press.

Thompson TE, Bebbington A, 1969. Structure and function of the reproductive organs of three species of *Aplysia* (Gastropoda: Opisthobranchia). Malacologia 7:347–380.

Todd CD, Hadfield MG, Snedden WA, 1997. Juvenile mating and sperm storage in the tropical corallivorous nudibranch *Phestilla sibogae*. Invert Biol 116:322–330.

Tompa AS, Verdonk NH, van den Biggelaar JAM, 1984. The mollusca: reproduction, vol. 7. London: Academic Press.

Vianey-Liaud M, 1995. Bias in the production of heterozygous pigmented embryos from successively mated *Biomphalaria glabrata* (Gastropoda: Planorbidae) albino snails. Malacol Rev 28:97–106.

Vreys C, Michiels NK, 1997. Flatworms flatten to size up each other. Proc R Soc Lond B 264:1559–1564.

Westneat DF, Frederick PC, Haven Wiley R, 1987. The use of genetic markers to estimate the frequency of successful alternative reproductive tactics. Behav Ecol Sociobiol 21:35–45.

Wethington AR, Dillon RT Jr, 1991. Sperm storage and evidence for multiple insemination in a natural population of the freshwater snail, *Physa*. Am Malacol Bull 9:99–102.

Winkler LR, Dawson EY, 1963. Observations and experiments on the food habits of California sea hares of the genus *Aplysia*. Pacific Sci 17:102–105.

Yusa Y, 1996. The effects of body size on mating features in a field population of the hermaphroditic sea hare *Aplysia kurodai* Baba 1937 (Gastropoda: Opisthobranchia). J Moll Stud 62:381–386.