Animal Behaviour 84 (2012) 975–981

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

The role of age on sperm traits in the American horseshoe crab, *Limulus polyphemus*

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ARTICLE INFO

Article history:
Received 16 May 2012
Initial acceptance 28 June 2012
Final acceptance 10 July 2012
Available online 13 August 2012
MS. number: A12-00366

Keywords:
age-related sperm trait
horseshoe crab
Limulus polyphemus
postcopulatory sexual selection
senescence
sperm competition

Sperm competition is an important component of postcopulatory sexual selection. Despite the fact that sperm traits may be important in determining fitness and thus may be under directional selection, they are often highly variable in mating systems with intense sperm competition. One possible explanation for this variation is that sperm traits vary with age. Age affects the expression of many life-history traits and sexual selection signals, but its influence on sperm traits is not well understood. In this study, we examined the correlation between individual age and sperm traits in a natural population of the American horseshoe crab. We compared five sperm traits (ejaculate size, concentration, total sperm ejaculated, velocity and viability) between males of three age categories. Young males ejaculated more total sperm and had significantly more concentrated sperm than old males. Males of different ages did not differ in sperm velocity or viability. Our results suggest that age influences traits associated with sperm quantity more than it does traits associated with sperm quality. Our results also suggest that individual age may be an important, but often overlooked, factor in studies examining sperm traits in natural populations.

Sperm competition has long been recognized as an important component of postcopulatory sexual selection. In systems where females mate multiply and sperm competition occurs, sperm traits can play a role in determining successful fertilization for an individual (Snook 2005; Stoltz & Neff 2006). Thus, sperm traits might be expected to be under directional selection in many, if not most, species. Contrary to this expectation, however, sperm traits often show high variation in natural populations (Moore et al. 2004; Snook 2005). One factor that may be related to this variation in sperm traits across males is individual age. Age influences the expression of a large suite of traits, including the ability to win fights (Hu & Morse 2004), to gain a high-quality territory (Holmes et al. 1996), obtain mates (Perez-Staples et al. 2010; Prokop et al. 2012) and to have a high reproductive output (Robertson & Rendell 2001; Broussard et al. 2003). Despite the recognition that a male's sperm traits can have profound fitness implications (e.g. Gage et al. 2004; Dziminski et al. 2009), the influence of age on sperm traits is less well understood.

Age may affect sperm quantity, most likely as a result of senescence, when older animals have a reduced ability to forage and acquire resources (Catry et al. 2006). Trade-offs exist between allocating resources to gametes and reproduction and allocating resources to the maintenance of somatic tissues and survival (Stearns 1989), and resources are often assumed to be used first for maintenance over other needs (Roff 1983; Heino & Kaitala 1999). Since sperm production can be energetically costly (Pitnick et al. 1995; Olsson et al. 1997), older animals may not possess or be able to afford the resources to produce the same numbers of sperm as younger animals.

Male age may also affect sperm quality, defined as any trait other than sperm quantity that can affect fertilization success, such as sperm velocity or viability (Snook 2005). As animals age, they may experience an increase in germ-line mutations, either due to changes in the mutation rate, or due to a decreased ability to repair damaged DNA (Crow 1997; Agrawal & Wang 2008). Male-biased germ-line mutations accumulate with age because of the increased number of cell divisions and chromosome replications that occur during spermatogenesis (Crow 2000). Mutations in the germ-line are thus more likely to occur the longer an animal lives and the more sperm it produces over its lifetime. Thus, germ-line mutations are predicted to affect sperm particularly in animals with long life spans, iteroparity and high levels of sperm competition (Pizzari et al. 2008). High numbers of germ-line mutations coupled with the failure to repair DNA accurately may increase the proportion of abnormal sperm (Crow 2000), as has been seen in at least one study

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0003-3472/$38.00
http://dx.doi.org/10.1016/j.anbehav.2012.07.023

(Velando et al. 2011). Additionally, germ-line mutations or excessive oxidative stress in sperm may result in decreased sperm velocity, viability and motility (Agarwal et al. 1994; Møller et al. 2009).

While it has been established that senescence is correlated with a decline in sperm traits in humans (Kidd et al. 2001; Kühnert & Nieschlag 2004), similar patterns in natural systems have rarely been investigated (Pizzari et al. 2008). In the handful of studies that investigated the effect of age on sperm in animals, senescence has often been correlated with less competitive reproductive traits such as diminished sperm motility (Wolf et al. 2000), decreased sperm velocity (Møller et al. 2009; Gasparini et al. 2010), smaller ejaculates (Vuthiphandchai & Zohar 1999) and reduced sperm transfer (Hale et al. 2008; Dean et al. 2010). If older males have fewer or lower-quality sperm than younger males, they may be at a disadvantage in competitive situations. In this study, we assess how age correlates with sperm traits in a long-lived, iteroparous species with high levels of sperm competition, the American horseshoe crab, *Limulus polyphemus*.

Male horseshoe crabs have conditional alternative reproductive tactics (Brockmann & Penn 1992). During the high tide, younger males generally come ashore attached to a female, whereas older males often arrive at the spawning beach unattached (Brockmann 2002). The attached male normally stays with the female throughout the spawning event as the eggs are laid and fertilized in the sand, and he leaves the beach with the female when she has completed spawning for that day (Brockmann 1990). During and throughout a spawning event, unattached males roam the beach and join some attached pairs as satellites (Brockmann 1996). When no satellites are present, the attached male fertilizes all the female's eggs, but when satellites occupy the most favourable position around the female (over the female's incurrent canal), they fertilize many of the eggs that the female lays, on average, 41% when a single satellite male is present, and 74% when multiple satellite males are present (Brockmann et al. 1994, 2000). Satellite males in less favourable positions average only 3–26% paternity (Brockmann et al. 2000). In this system, the older, unattached males always face sperm competition with attached males and sometimes with additional satellites, whereas younger males face competition only when densities are high and satellites are present. Fertilization takes place externally (in the sand under the female), and thus, sperm traits are probably particularly important in determining fertilization success in competitive situations (Stoltz & Neff 2006). Horseshoe crabs have a terminal moult (Shuster & Sekiguchi 2003; Smith et al. 2010) and probably live as adults for 6–8 years (Botton & Ropes 1987; Brockmann & Johnson 2011). As they age, the dorsal surface of their shells deteriorates (Brockmann 1996). Males switch from the attached reproductive tactic to the satellite tactic as they age and their physical condition declines (Brockmann & Penn 1992; Brockmann 1996; Duffy et al. 2006), although sometimes young males can be satellites and old males can attach (D. A. Sasson, personal observation).

In this study we evaluate the hypothesis that increased age is correlated with a decline in sperm quantity and quality in horseshoe crabs. We assayed five sperm traits (ejaculate size, sperm concentration, total sperm ejaculated, sperm velocity and sperm viability) for horseshoe crabs that differed in age. If age is correlated with sperm attributes, then older males should have fewer and/or slower and less viable sperm than younger males.

METHODS

We conducted this study in the spring and autumn reproductive seasons during 2008–2010 at the University of Florida Marine Laboratory at Seahorse Key, which is located on a small island in the Gulf of Mexico near Cedar Key, Florida (29°5′47″N, 83°3′55″W). The island is part of the Lower Suwannee National Wildlife Refuge, and the undisturbed south beach of the island attracts many spawning horseshoe crabs (Brockmann & Johnson 2011). During high tides, when males and females migrate to shore for spawning, we collected one attached male and one unattached male, without regard to age, that were spawning with the same female, and gave each a unique tag. These tags consisted of a thumbtack with an attached plastic label, upon which we printed a unique number. The thumbtacks were placed through a thin part of the carapace along the side of the horseshoe crab. We held the marked individuals in a flow-through water table for 1–12 h, until after the high tide, when we collected their sperm, measured them and estimated their age. We kept a subset of males for up to 1 week to measure individual variability in male ejaculate size and sperm viability. These males were fed shrimp every other day. All animals were returned to the beach from which they had been collected. No mortality occurred and we often saw marked animals returning to the beach on subsequent high tides. We never collected animals on subsequent tides that had already been tagged.

Measurements

We measured crab size in two ways: (1) carapace width (CW, measured ventrally at the widest point) and (2) crab mass (we placed each crab in a Styrofoam bucket and measured mass with a hand-held Pesola scale). At maturation, horseshoe crabs have a terminal moult (Shuster & Sekiguchi 2003; Smith et al. 2010), and as they age their carapace degrades. For our purposes, condition and age were generally synonymous, so we calculated relative crab age based on four categorical measures of carapace condition: (1) carapace colour, from light to dark (the carapace of horseshoe crabs darkens over time, possibly due to exposure to UV light, or abrasions; Brockmann & Penn 1992); (2) carapace pitting, an estimate of the proportion of the carapace pitted with holes and indentations (due to chitinoclastic bacteria and abrasions); (3) the condition of the lateral eyes, from perfect to soft, or covered with epibionts (Duffy et al. 2006); and (4) carapace mucus, from entirely covered to none. Horseshoe crabs produce a mucus film over their carapace that inhibits epibiotic organisms (Harrington et al. 2008), but as they age the amount of mucus declines (Brockmann 1996). Although our measurements of condition differ from those used in other studies (see Jakob et al. 1996), previous work has shown that these measures of condition are correlated with age (Brockmann 1996). In addition, similar measures of carapace condition are correlated with physical performance in horseshoe crabs (Penn & Brockmann 1995; D. A. Sasson, unpublished data), as would be expected if declining condition is due to senescence. Older crabs pair more slowly (Brockmann & Penn 1992), take longer to right themselves on the beach (Penn & Brockmann 1995) and move more slowly from the shore to the ocean (H. J. Brockmann, unpublished data) than younger males. We scored each categorical variable on a scale from 1 to 3, with a score of 3 indicating the best condition for that measure. We then added metrics to an index to assess each animal's relative age. The oldest crabs received a rank of 4, and the youngest received a rank of 12. We placed crabs into one of three age classes: young, middle-aged and old. This categorical approach is more conservative than analysing each value of the index. Males in the upper quartile of condition scores (11–12) were classified as young males, and males in the bottom quartile of condition scores (4–8) were classified as old males. All other males were categorized as middle-aged (9–10).

Sperm Collection

We removed each crab from its holding tank and placed it dorsal side down on a table with a rubber mat. We lifted the opercular flap to find the two gonopores and wiped away any remaining water with

a Kimwipe. We obtained sperm by using an electro-stimulator applying 10 V and 0.5 A of electricity for 20 s to the area just below and around the gonopores. This impulse resulted in a contraction of the muscles around the gonopores and sperm release. We collected ejaculate using a micropipette as it flowed from the gonopores, placed the samples in marked microcentrifuge tubes and kept them cool until assayed.

Sperm Measurements

Ejaculate size

For each crab, we stimulated each gonopore separately for 10 s and measured the amount of ejaculate released (μl) using micropipettes. We then averaged the output from the two gonopores as our measure of ejaculate size. This measurement probably does not quantify the total ejaculate produced by the animal, but rather gives a relative estimate of ejaculate available to the male during his recent mating opportunity. To test whether our measure of ejaculate size was consistent within males, 19 males were maintained in the flow-through water table for 5 days and retested. We log transformed all ejaculate size data before analysis.

Sperm concentration

Sperm concentration measures the number of spermatozoa/ml of ejaculate. To measure sperm concentration, we took 1 μl of each ejaculate and serially diluted it to obtain a centrifuge tube containing 100 μl of ejaculate at a 1/1000 dilution. We then fixed this sample with 33 μl of 2% glutaraldehyde. We counted the number of spermatozoa in a given area on an improved Neubauer haemocytometer slide and, using this number, calculated the number of spermatozoa/ml for each male. We did this twice for each sample and averaged the two measures. We used square-root transformation on these data before analysis.

Total sperm ejaculated

Both ejaculate size and sperm concentration are measures of sperm quantity. While ejaculate size measures the amount of ejaculate produced by a horseshoe crab, sperm concentration measures the number of spermatozoa/ml of ejaculate. Thus, these two measures can be combined (ejaculate size $\times$ sperm concentration) to provide an overall metric of the total number of spermatozoa ejaculated by a male during stimulation. We log transformed these data before analysis.

Sperm velocity

We measured sperm velocity using a subset of the original collected sperm. We diluted 1 μl of sperm in 99 μl of sea water, kept at room temperature, and kept the sample cool by placing the samples on an icepack until activated (<1 h after collection). Sperm were collected and measured at the same time without regard to male age, so any potential effect of temperature on sperm velocity should not have been biased in any one direction. Since horseshoe crab sperm only swim in the presence of compounds associated with eggs (Brown 1976), we activated the sperm by adding 100 μl of a 195 mM concentration of $MgCl_2$ to the sample and shook it vigorously (Clapper & Brown 1980a). We then placed 10 μl of the sample on a haemocytometer slide with a coverslip and recorded 25 s of sperm movement with a black and white high-speed camera (Prosilica EC650) mounted on a phase-contrast microscope (mean $\pm$ SD rate: 83.2 $\pm$ 4.1 frames/s). We used the computer-assisted sperm analysis (CASA) add-on developed by Wilson-Leedy & Ingermann (2007) for ImageJ (v1.42d, National Institutes of Health, Bethesda, MD, U.S.A.) to measure sperm velocity from the first 10 s of the videos. To eliminate nonsperm items from the count, only sperm with a minimum size of five pixels that appeared on the video for at least 0.5 s were included as part of the analysis. Since no egg was present to guide the sperm, sperm paths were not expected to be linear. Thus, all sperm velocity measurements are given as mean curvilinear velocity (VCL), a measurement of the point-to-point path of the sperm (Rurangwa et al. 2004). Only sperm travelling a minimum VCL of 25 μm/s were included in the analysis to avoid counting sperm that were moving across the slide due to drift of the solution. Any sample with fewer than 25 swimming sperm ($N = 7$) was excluded from analysis. We used a log transformation on measures of sperm velocity to increase normality.

Sperm viability

Sperm viability measures the percentage of viable, or live, sperm present in a sample. Since this work requires nonportable equipment, we brought a subset of the horseshoe crabs to our laboratory on the main campus of the University of Florida where they were kept in a recirculating salt water tank for 3–5 days. Any horseshoe crabs that could not be returned to their nesting sites within 3 days were fed shrimp. We collected sperm and diluted 2 μl of the ejaculate in 98 μl of HEPES buffer. We then added 0.5 μl of a 50-fold dilution of SYBR 14 dye (Live/Dead Sperm Viability assay, Molecular Probes, Inc., Eugene, OR, U.S.A.) to the diluted sperm and allowed the sample to sit in darkness for 5 min (García-González & Simmons 2005). SYBR 14 stains DNA so that live cells with intact membranes appear green when viewed with a fluorescence microscope. We photographed the sample under a fluorescence microscope and used ImageJ to count the number of cells both fluorescently labelled green and not coloured green. We then calculated the percentage of viable sperm in the sample (green sperm cells/total sperm cells). The sperm assay kit also contains propidium iodine that can be used to stain dead sperm cells red. In our initial trials, we could not get the propidium iodine stain to work with the horseshoe crab cells. Therefore, we used only the SYBR 14 stain in our samples. This provided us with a conservative estimate of the live sperm cells in the sample. We counted an average $\pm$ SD of 179 $\pm$ 130 spermatozoa/individual (range 50–555). Individuals with fewer than 50 total sperm counted ($N = 2$) were removed from the analysis. Sperm viability data were arcsine transformed before analysis to increase normality.

Statistical Analyses

All statistical analyses were calculated with JMP v8.0 (SAS Institute, Cary, NC, U.S.A.). We combined horseshoe crabs from across multiple breeding seasons into our analyses. However, we found an effect of mating season on sperm concentration, sperm velocity and total sperm ejaculated. To account for this breeding season effect, we used a general linear model (GLM) with season as a random effect to test for an effect of age on sperm concentration, velocity and total quantity. We used a one-way ANOVA to compare sperm viability across ages (we took viability measurements only during one season). Tukey's honest significant difference (HSD) test was used for all post hoc analyses. Body size and mass were linearly correlated with ejaculate size but were not perfectly correlated with each other ($r^2 = 0.62$, $P < 0.0001$). Therefore, in calculating the effect of age on ejaculate size, we used an ANCOVA with body size and mass as covariates. Sample sizes differed across sperm trait measurements because of differences in the availability of equipment and supplies (see Table 1).

RESULTS

Ejaculate Size

Ejaculate size was highly repeatable within individuals when we stimulated the same crab 5 days after the original data were taken

Table 1
Sample sizes for each sperm trait measurement, by age category of horseshoe crab

Age category	Concentration	Ejaculate size	Total sperm	Velocity	Viability
Young	85	36	35	26	11
Middle-aged	119	47	47	40	20
Old	104	37	38	37	20

(Spearman rank correlation: $r_S = 0.85$, $N = 19$, $P < 0.001$; Fig. 1). Ejaculate size was also weakly positively correlated with carapace width (CW) ($r^2 = 0.04$, $N = 121$, $F_{1,120} = 5.53$, $P = 0.02$) and nonsignificantly correlated with mass ($r^2 = 0.03$, $N = 121$, $F_{1,121} = 3.47$, $P = 0.07$), but males across ages did not differ in carapace width (ANOVA: $F_{2,321} = 0.27$, $P = 0.76$) or mass ($F_{2,291} = 1.4$, $P = 0.25$). Although the relationships between CW, mass and ejaculate size were significant across all males, when each age category was analysed separately, only young males showed a significant relationship between CW and ejaculate size ($r^2 = 0.12$, $N = 36$, $F_{1,34} = 4.5$, $P = 0.04$); CW was not significantly correlated with ejaculate size for middle-aged or old males. While the ANCOVA (CW and mass as covariates) investigating differences in ejaculate size across ages was significant (ANCOVA: $F_{4,115} = 2.6$, $P = 0.04$), age showed a strong, but nonsignificant, effect on ejaculate size ($F_{2,117} = 3.0$, $P = 0.052$; Fig. 2a), with young males ($N = 36$) producing larger ejaculates than middle-aged ($N = 47$) or old ($N = 37$) males.

Sperm Concentration

We found a strong effect of season on sperm concentration; horseshoe crabs sampled in spring 2010 ($N = 89$) had less concentrated sperm than males sampled in autumn and spring of 2008 and 2009 (ANOVA: $F_{4,301} = 5.1$, $P < 0.001$). With season as a random effect, the overall model was significant (GLM: $F_{2,302} = 6.4$, $P < 0.01$) and young ($N = 85$) and middle-aged ($N = 119$) males had significantly more concentrated sperm than old males (Tukey HSD: $P < 0.001$ for both comparisons; Fig. 2b). We found no difference in sperm concentration between young and middle-aged males (Tukey's HSD test: $P = 0.92$).

Total Sperm Ejaculated

Males sampled in autumn 2009 had more total sperm than males sampled in spring 2010 season (t test: $t_{118} = -2.7$, $P < 0.01$).

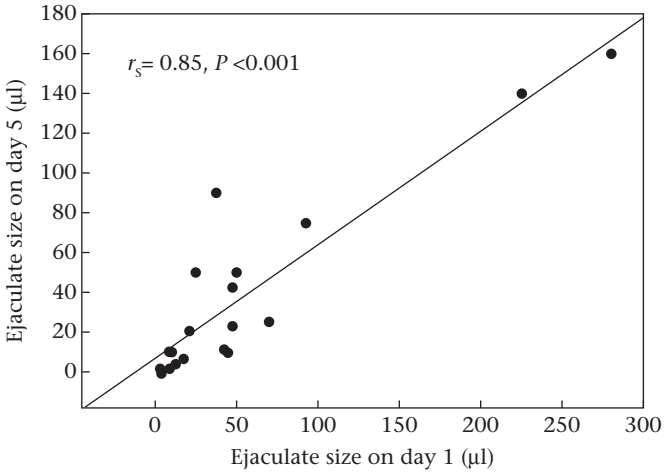

Figure 1. Ejaculate size of horseshoe crabs on day 1 and day 5.

With season as a random effect in our model, we found that age did affect total sperm ejaculated (GLM: $F_{2,117} = 4.3$, $P = 0.02$; Fig. 2c). Younger males had significantly more total sperm than old males (Tukey's HSD test: $P = 0.01$). Middle-aged males did not significantly differ in total sperm ejaculated from either young (Tukey's HSD test: $P = 0.14$) or old males ($P = 0.43$).

Sperm Velocity

We measured sperm velocity of 103 horseshoe crabs. The number of swimming spermatozoa tracked per sample ranged from 25 to 1251 (mean ± SD: 199 ± 264). We found a significant season effect on sperm velocity, with horseshoe crabs sampled in spring 2010 having slower sperm than those sampled in spring and autumn 2009 (ANOVA: $F_{2,100} = 5.0$, $P < 0.01$). Using season as a random effect we found no effect of age on sperm velocity (GLM: $F_{2,\ 99} = 0.7$, $P = 0.48$; Fig. 2d).

Sperm Viability

Horseshoe crabs from every age category showed high levels of viable sperm across all seasons (mean ± SD viability: young males: 88.9 ± 11%, $N = 11$; middle-aged males: 85.8 ± 13%, $N = 20$; old males: 86.5 ± 12%, $N = 20$). We found no differences in sperm viability across ages (ANOVA: $F_{2,48} = 0.22$, $P = 0.80$; Fig. 2e).

DISCUSSION

Our results demonstrate that age is correlated with sperm traits in horseshoe crabs. The oldest males had less concentrated sperm, smaller ejaculates (although this result was marginally nonsignificant) and fewer overall total sperm than young males. However, we found no correlation between age and sperm velocity or viability. This result indicates that sperm quantity, but not sperm quality, may be associated with age in horseshoe crabs. This result conforms to the few other studies of sperm traits that indicate age is correlated with sperm quantity (Vuthiphandchai & Zohar 1999; Kidd et al. 2001; Hale et al. 2008; Dean et al. 2010).

Older horseshoe crabs had smaller ejaculate sizes than younger males, and fewer spermatozoa were present, both absolutely and proportionally, in the ejaculate. At least one previous work has found that males in poor condition shift their ejaculate strategies to produce smaller ejaculates that contain more sperm (Perry & Rowe 2010). We found no evidence for such a shift with age in ejaculate composition in horseshoe crabs. This result suggests that both spermatozoa and other seminal fluids are energetically costly for horseshoe crabs. Older males may not possess the resources necessary to produce sperm at the same level, or may be required to devote a higher proportion of their resources to maintenance of somatic tissues (Stearns 1989) than do younger males. One possible alternative explanation for the correlation between age and sperm quantity is that older males may mate more frequently than younger males, since older males are more likely to be satellites or unattached males. Thus, these males may have used a larger proportion of their ejaculate stores prior to capture. However, we tested males of all ages again 5 days after being confined in tanks where no mating took place. We found that, regardless of age, ejaculate size was generally consistent across these 5 days. We also found no difference in ejaculate size or sperm concentration when comparing old attached males to old satellite males. This suggests that an individual's ejaculate size is not simply a function of mating history but rather a difference in sperm production. However, if high levels of sperm production are costly, then males that spend energy on producing large amounts of sperm may not live as long as males that produce smaller amounts. Such an outcome might be

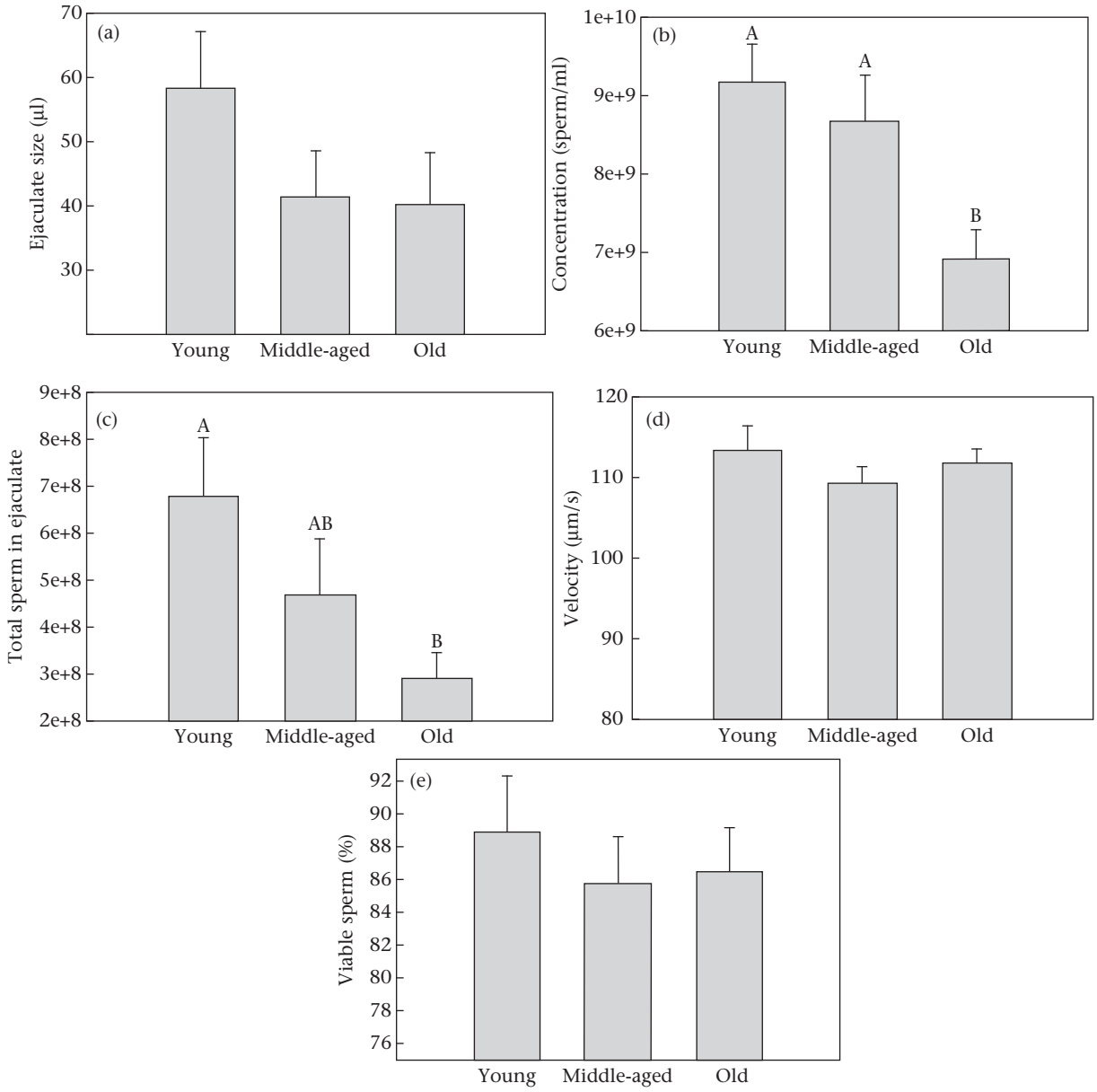

Figure 2. Effect of condition on sperm traits of horseshoe crabs. Nontransformed means ± SE are shown. Letters indicate significant differences across condition categories: (a) ejaculate size; (b) sperm concentration; (c) sperm/ejaculate; (d) sperm velocity and (e) sperm viability.

expected if fertility selection is stronger on younger animals than on older animals (Hansen & Price 1995). A test of this hypothesis would require a longitudinal study on the effect of ageing on sperm traits, which would be difficult in a natural population (Johnson & Gemmell 2012).

Body size may also affect sperm quantity. Within taxa, larger males often have larger absolute testes size than smaller males (Gage et al. 1995; Neff et al. 2003). For example, a study in honeybees found that body size was positively correlated with high sperm concentration (Schlüns et al. 2003). While we found no effect of body size on sperm concentration in horseshoe crabs, ejaculate size did weakly increase with both carapace width and body mass. This result suggests that larger males have more absolute testicular tissue than smaller males. However, horseshoe crabs do not increase in body size with age after they reach adulthood (Shuster & Sekiguchi 2003; Smith et al. 2010), and in our study, we found no significant difference in body size across age categories. Thus, at any age, larger males may have an advantage over smaller males in sperm quantity. Interestingly, the relationship between body size and ejaculate size was strongest for the young males. This may indicate that any advantage of body size in horseshoe crabs is most pronounced only when males have the available resources to produce large quantities of sperm. Old horseshoe crabs may also shift from producing many small sperm to producing fewer large sperm. Large sperm may have an advantage if they can swim faster than small sperm (Gomendio & Roldan 1991) or if sperm size is positively correlated with sperm longevity (Ball & Parker 1996). Since we did not measure sperm size, we cannot completely rule out this possibility. However, we saw no difference in sperm velocity across age classes, so this explanation seems unlikely.

Contrary to other studies in which sperm quality declined with senescence (Wolf et al. 2000; Møller et al. 2009; Gasparini et al. 2010), we found no significant difference in sperm quality across

horseshoe crabs of different age classes. While sperm velocity is an important factor affecting fertilization success in numerous systems with external fertilization (Gage et al. 2004; Casselman et al. 2006; Dziminski et al. 2009), this may not hold true for horseshoe crabs. Horseshoe crab sperm remain quiescent until activated by chemical cues exuded from eggs (Clapper & Brown 1980b). This activation does not occur until sperm are in close proximity to the egg (Brown 1976). If sperm only activate when near an egg, sperm velocity may not have as strong an effect on fertilization success as sperm number.

In some species, ovarian fluid affects sperm velocity (e.g. Turner & Montgomerie 2002; Rosengrave et al. 2008, 2009a; Møller et al. 2009; Simmons et al. 2009), but in our study, no ovarian fluid was present since we conducted all of our sperm velocity trials in sea water without eggs. Under natural conditions, horseshoe crabs spawn into the sand as waves wash over and around them, which could potentially dilute any ovarian fluid present and may increase variation in sperm velocity across individuals.

Our measure of velocity assumes that $MgCl_2$ activation of sperm results in normal swimming behaviour. In salmon, $MgCl_2$ affects sperm motility and duration, but not velocity (Rosengrave et al. 2009b). Horseshoe crab eggs contain multiple compounds that activate sperm (Clapper & Brown 1980a). If the presence of multiple compounds is required for normal swimming behaviour, our measure of velocity may not be an accurate reflection of sperm quality. We are currently conducting competitive in vitro fertilization assays to ascertain whether our measure of sperm velocity is correlated with fertilization success in horseshoe crabs.

We found that sperm viability, another measure of sperm quality, did not vary significantly between males that differed in age. The majority of horseshoe crabs had over 90% viable sperm ($N = 51$, median = 91%, mean = 87%). Given our small sample size, especially for young males ($N = 11$), this low level of variability makes it difficult to detect an effect of age on sperm viability. Like other systems with intense sperm competition, horseshoe crabs have high levels of viable sperm (Hunter & Birkhead 2002; García-Gonzalez & Simmons 2005; Thomas & Simmons 2009). This result suggests that the high level of sperm viability we found may be an adaptation to the high level of sperm competition that American horseshoe crabs face, particularly for the older, unattached males.

Both sperm velocity and sperm viability are potentially affected by mutations in the germ-line (Radwan 2003; Møller et al. 2009). The fact that sperm quality is not correlated with age may indicate that older horseshoe crabs do not have higher rates of germ-line mutations, or that their ability to repair damaged DNA does not significantly decrease with age.

Our findings indicate that future studies of sperm competition, especially when conducted in the field, should measure individual age. This may be especially important in systems with age-related alternative reproductive tactics. Numerous studies have found that males that adopt a parasitic tactic to gain access to mates or eggs have faster or more concentrated sperm than males that invest in primary access to mates or eggs (i.e. adopt a bourgeois tactic; Marconato & Shapiro 1996; Simmons et al. 1999; Neff et al. 2003). However, in at least a few systems, the ages of males differ across tactics. This is the case in the bluegill sunfish, *Lepomis macrochirus*, for example, where bourgeois males are many years older and larger than parasitic males (Gross & Charnov 1980). Furthermore, in this system the parasitic males switch from a sneaker tactic to a female-mimic tactic as they age and increase in body size (Gross & Charnov 1980). These two parasitic tactics also differ in their sperm competitive ability (Fu et al. 2001), with the larger female-mimic males having more concentrated sperm than sneaker males (Neff et al. 2003). Disentangling the effects of size, age and tactic on sperm traits in these systems may prove difficult. Additionally, if not controlled for, individual age may help to explain the often large variation in sperm investment and quality within tactics (Simmons et al. 1999; Neff et al. 2003; Byrne 2004) as well as the large variation in tactic-specific paternity in a number of systems, including horseshoe crabs (Brockmann et al. 2000; Fu et al. 2001; Rudolfsen et al. 2008).

Our results indicate that age influences sperm quantity, but not sperm quality, in horseshoe crabs. In contrast to a number of other systems (Møller et al. 2009; Velando et al. 2011), we found no evidence that age resulted in decreased sperm velocity or viability. Our results are similar to those of Hale et al. (2008), who found that sperm quantity but not quality declined with age in hide beetles. It may be that the costs of producing high numbers of sperm are higher than are the costs associated with producing viable or fast-swimming sperm in this system. Older males may not be able to afford these greater costs. In addition, if sperm velocity does not greatly influence fertilization success, and sperm viability is not highly variable across males, then the most effective and cost-efficient way for younger males to increase their fertilization success in this system may be by allocating resources towards increasing the number of sperm in an ejaculate.

Acknowledgments

We thank our field assistants, Melissa Clark, Jessica Diller, Mariantu Robles Zarate and Natalie Williams, other members of the Brockmann lab, Matthew Smith and Katie Saunders, and numerous undergraduate volunteers who helped with fieldwork and data collection. We conducted our research under a permit from the Lower Suwannee National Wildlife Refuge. We thank Harvey Lillywhite, Henry Coulter, Al Dinsmore, and Bronko Gukanovich of the University of Florida Marine Laboratory at Seahore Key for their help. This research was supported by the National Science Foundation (IOB 06-41750), the University of Florida Division of Sponsored Research, the University of Florida Marine Laboratory at Seahorse Key, The PADI Foundation, and the Department of Biology, University of Florida (Frank Maturo Award).

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