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

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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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(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