

Mating Behavior and Reproductive Biology of Emerald Ash Borer (Coleoptera: Buprestidae) and Two of Its Native Congeners, the Twolined Chestnut Borer and the Bronze Birch Borer

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Abstract

We studied the mating behavior and reproductive biology of three members of the genus *Agrilus*: the bronze birch borer, *Agrilus anxius* Gory; the twolined chestnut borer, *Agrilus bilineatus* (Weber); and the emerald ash borer, *Agrilus planipennis* Fairmaire. All three species share a highly stereotyped mating behavior. However, the copulation duration of *A. planipennis* was 90% longer than that of its two congeners. Female reproductive tracts of the three species were anatomically similar, as were the spermatophores. Within the spermatophores, sperm were single in *A. anxius* and *A. bilineatus*, while in *A. planipennis*, sperm were bundled in groups of approximately 20 in a hyaline sheath. We found that field-caught *A. anxius* and *A. bilineatus* had higher rates of female insemination than *A. planipennis*. In additional studies with *A. planipennis* and *A. anxius*, we found that mating duration was related to mating success, and fecundity for *A. planipennis*, but not for *A. anxius*. For both *A. planipennis* and *A. anxius*, the spermatophore was passed to the female toward the end of the copulatory period. Sperm were found in the spermatheca immediately after copulation ended in *A. planipennis* and 30 min after copulation ended in *A. anxius*. We present possible explanations for these differences.

Key words: *Agrilus*, reproductive biology, mating behavior

We examined the mating behavior of three species of the genus *Agrilus*. Two of the species are native to North America: the bronze birch borer, *Agrilus anxius* Gory and the twolined chestnut borer *A. bilineatus* (Weber). Although they generally are considered secondary pests (Johnson and Lyon 1991), both species occasionally cause large-scale mortality events for their host trees, *Betula* species (Betulaceae) and *Quercus* species (Fagaceae), respectively. These events are often correlated with large-scale stressors for their hosts such as cohort senescence (Muilenburg and Herms 2012), drought (Nash 1943, Anderson 1944, Barter 1957) or, defoliation (Chapman 1915, Dunbar 1975, Dunn et al. 1986a, Haack and Acciavatti 1992, Muzika et al. 2000). *Agrilus bilineatus* recently seems to have established in Turkey (Hızal and Arslangündoğdu 2018). Although it is known to be able to feed on at least one species of European oak, *Quercus robur* (Petrice and Haack 2014), it is still unknown how much of an impact it will have on *Quercus* in Eurasia. The third species we studied was emerald ash borer, *A. planipennis* Fairmaire, which is native to Far Eastern Asia and is a highly destructive, invasive pest of *Fraxinus* species (Oleaceae) in North America and Eastern

Europe (Herms and McCullough 2014, Orlova-Bienkowskaja and Bieńkowski 2015).

The three species share many aspects of their ecology and reproductive biology. Mating takes place on the larval host plants, and males locate the females primarily by sight (Lelito et al. 2007, 2011; Rodriguez-Saona et al. 2007; Domingue et al. 2013, 2016). There is some evidence of a longer-range sex pheromone in *A. bilineatus* (Dunn et al. 1986b), and contact and short-range volatile sex pheromones have been identified for *A. planipennis* (Lelito et al. 2009, Silk et al. 2009, Ryall et al. 2012, Domingue et al. 2016). *Agrilus* species have an ‘ambush habit’ and males either land on, or jump onto the females without any pre-copulatory courtship, nor is there any evidence of post-copulatory associations (Chapman 1915; Akers 1985; Lelito et al. 2007, 2011; Rodriguez-Saona et al. 2007). They all have a sex ratio of roughly 1:1 and all are protandrous. All three species require an adult maturation feeding period, and the females are synovigenic, requiring 5–10 d before mating. Both sexes mate multiple times in all three species (Chapman 1915; Muilenburg and Herms 2012; Rutledge and Keena 2012a,b). Eggs are laid in

cracks and crevices of the bark in small groups. Previous work on *A. planipennis* showed that females are more likely to produce any eggs if they are allowed to mate multiple times (Rutledge and Keena 2012b), while *A. anxius* was equally likely to lay eggs with one mating or many (Rutledge and Keena 2012a). This was likely due to the higher rate of successful spermatophore transfer in *A. anxius*.

We examined several aspects of the mating behavior and reproductive morphology of *A. anxius*, *A. bilineatus*, and *A. planipennis*. Casual observation in earlier studies showed that *A. planipennis* mated for a longer duration than either *A. anxius* or *A. bilineatus*. An extended copulation, such as seen in *A. planipennis*, comes with potential costs for both partners (Simmons 2001). During mating, the couple is more exposed to the risk of predation (Jersabek et al. 2007). For example, *Cerceris fumipennis* Say (Hymenoptera: Crabronidae), a wasp that uses adult Buprestidae to provision their brood, will capture and paralyze a mating female buprestid, and the un-paralyzed male will remain *in copula* as the pair is brought back to the wasp's burrow (C.E.R., personal observation). A longer period of time engaged in mating will use more energy for both participants (Brown and Baer 2005), and it reduces time available for other activities such as eating, resting, or oviposition. Time spent mating with one partner is time spent not mating with another, so by necessity both partners are losing mating opportunities (Thornhill and Alcock 1983).

However, there are other factors that encourage a longer copulation duration. An environment with fewer predators can allow for more leisurely mating. This has been shown to be the case for copepods that spend more time mating in lakes that are fish free, than in lakes with fish (Rehfeldt 1996, Jersabek et al. 2007). Another potential explanation is a differential level of sperm competition among the three species. Sperm competition can be an important driver of evolution (Thornhill and Alcock 1983, Reinhardt et al. 2015). Such competition increases as the ratio of sexually active females to sexually active males (i.e., the operational sex ratio decreases). Higher sperm competition has been shown to increase copulation duration within a species, individual males will adjust copulation time in accordance to perceived competition (Sakaluk and Muller 2008, Wang et al. 2008). This effect has also been shown across species within a taxon, with species experiencing greater sperm competition exhibiting longer copulation durations than species in the same taxon that experience less sperm competition (Takami and Sota 2007a,b).

There are a number of mechanisms by which copulation duration can impact sperm competition. A longer mating duration could improve a male's potential to inseminate a female's eggs by acting as *in copula* mate guarding (Schoff and Taborsky 2002, Linn et al. 2007, Chaudhary et al. 2017). Alternatively, prolonged copulation may act as an 'honest signal' of the male's quality (Brown and Baer 2005). To the extent that cryptic female choice exists, such as favorable placement of sperm in the reproductive tract, a male that has shown his fitness by an extended copulation duration may be favored (Eberhard 2002). There may also be physiological mechanisms by which a prolonged copulation increases a male's chance to inseminate the female's eggs. A longer copulation may allow for a greater quantity of sperm to be transferred, thus boosting the statistical chances that that male's sperm will be used to fertilize the female's eggs (Simmons 2001, Garcia-Gonzalez and Gomendio 2004, Sziranyi et al. 2005, Gao and Kang 2006). It may allow for the passing of more, or additional, accessory gland fluids that could have a number of physiological impacts such as improving female longevity (Reinhardt et al. 2009), inhibiting female mating receptivity (Jang et al. 1998), or inducing oviposition so that eggs are

fertilized before the female has a chance to mate again (Yamane and Miyatake 2010). Alternatively, a longer mating duration may allow the male to either place a mating plug (Avila et al. 2011), or to remove sperm (Siva-Jothy 1987) or the mating plug of another male (Eberhard 1994).

Other characteristics not related to copulation duration may indicate higher levels of sperm competition in one species than in another species. For example, variation in the morphology of sperm, such as longer sperm, sperm bundles or even hooks may be indicative of a higher level of sperm competition (Takami and Sota 2007b, Firman and Simmons 2009, Fitzpatrick and Baer 2011).

To explore the hypotheses that might best explain the difference in copulation duration among the three species, we used a multi-pronged approach. First, we made video recordings of mating behavior and compiled ethograms and a behavioral flow chart to understand the mating behaviors of each species and how they compare to one another. We also examined the morphology of the female reproductive tract, the spermatophore, and the sperm of each species to see if any of these features differed. We dissected wild-caught females of each of the three species to ascertain actual rates of mating success for females in the wild (Rhains 2010).

In a series of additional experiments with *A. planipennis* and *A. anxius*, we then experimentally determined the timing of sperm transfer during copulation and the position of sperm in the female's reproductive tract at different time periods after mating by dissection. We also examined the relationship between mating duration and fecundity for *A. planipennis* and *A. anxius*.

Materials and Methods

Insects

Agrilus planipennis

Adults emerged from infested ash bolts (up to 30 cm diameter and 70–72 cm long) collected in the Brighton, MI area by USDA APHIS PPQ personnel in 2008 and 2009. Shipments were 100–200 beetles each and beetles that died during shipment or within 3 wk of initial mating were not used in the studies. Voucher specimens were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

To collect these beetles, field collected bolts were held in a refrigerated box ($4.0 \pm 0.1^\circ\text{C}$) until they were needed and then put at room temperature ($20\text{--}23^\circ\text{C}$) in rolled cardboard tubes with a clear plastic cup attached to one end (see Myers et al. 2009 for photograph and Barak et al. 2010 for more detailed description of emergence tubes). Adults were attracted to the light and collected from the cups daily and housed 10 per petri dish ($90\text{ d} \times 15\text{ h mm}$) with two to three clipped ash leaves until shipped under a valid permit to the USDA Forest Service quarantine facility in Ansonia, CT. The petri dishes were placed inside a Tyvek envelope (Dupont, Wilmington, DE) in a small cooler ($25\text{ l} \times 20\text{ h} \times 20\text{ d cm}$) for shipment. Upon arrival, beetles were sexed by examining them for the 'beard' of short golden setae that is only present on the prosternum of the male (Fairmaire 1888).

Before beetles were used in experiments, they were placed in single-sex, 7.6 L containers for at least 6 d of maturation feeding and were supplied hardened-off foliage of *Fraxinus uhdei* (Wenzig) Lingelsh and water (for details see, Rutledge and Keena 2012b). Beetles were held at $25 \pm 2^\circ\text{C}$, 16:8 L:D and $65 \pm 5\%$ RH. These conditions have been shown to be ideal for *A. planipennis* (Keena et al. 2009). Foliage was changed twice a week.

Agrilus bilineatus

Four 2 m lengths of *Quercus alba* L. infested with *A. bilineatus* larvae were obtained from Freetown State Forest, Fall River, MA in spring 2007 and brought to Hamden, CT where they were housed in an unheated screen house. Once emergence began, beetles were collected daily before 10:00 h. Males and females were sexed in the screen house by ascertaining the presence or absence of a vertical groove on the first and second abdominal sternite, which is found only on the male (Bright 1987). They were then housed by emergence date in single-sex cages constructed from nesting, clear 236 ml plastic cups (Solo Cup Company, Urbana, IL). The top cup had the top cut out and a circular piece of aluminum mesh fitted inside to cover the hole. Beetles were supplied with hardened-off oak foliage and water. The petioles of the oak leaves and dental wick (Patterson Brand, Minneapolis, MN) were inserted into a slit made in the top of the lid of a 30 ml plastic cup filled with water. Beetles were held at ambient laboratory temperatures, which ranged from 20 to 25°C. Foliage was obtained from oak seedlings reared from locally sourced red oak, *Quercus rubra* L., acorns in the greenhouse.

Agrilus anxius

Birch trees infested with *A. anxius* were collected locally. *Agrilus anxius* used in the mating observation, mating disruption, and morphology studies were reared from *Betula utilis* variety *jacquemontii* collected from a private home in Bloomfield, CT in 2008. Beetles used in experiments examining the relationship between mating duration and fecundity, as well as information on rates of successful insemination were reared from two heavily infested specimens of *B. utilis* var. *jacquemontii* were collected in February 2011. Beetles that were used in the experiments examining sperm dynamics were reared from *B. utilis* var. *jacquemontii* collected in April 2014 from a farm in Hamden, CT.

In each year, the trees were cut into bolts and brought back to Hamden, CT where they were stored in an unheated screened shed.

We began checking for emergence in the third week of May, and once emergence started, beetles were collected daily before 10:00 h. Collected beetles were sexed on site by examining the color of the frons (female beetles have a copper colored frons, while the males are green) and by looking for a vertical groove on the first and second abdominal sternite, which is found only on the male (Bright 1987). In 2008, beetles were brought back to the Connecticut Agricultural Experiment Station in New Haven, CT where they were held in cages as described above for *A. bilineatus*. In subsequent years, they were then placed in single-sex vials and transported directly to the USDA Forest Service quarantine facility in Ansonia, CT, where they were housed as described for *A. planipennis*. Beetles were supplied with field-collected, hardened-off foliage of *B. utilis* var. *jacquemontii* and water as described above.

Mating Behavior Observations

Mating observations were conducted in arenas consisting of 1 oz clear plastic cups placed upside down on clean filter paper during the hours of 10:00–15:00. For *A. anxius* and *A. bilineatus*, natural light from laboratory windows was supplemented by a 100 W incandescent bulb. Temperature was ambient lab temperature (20–25°C). As *A. planipennis* observations took place inside a rearing chamber in quarantine, the majority of observations took place at 25°C and under fluorescent lighting supplemented by incandescent bulbs. Although the lighting and temperature did influence how readily mating occurred, once mating began, it was highly stereotyped for all species. A pair of randomly selected, appropriately aged, virgin beetles were placed in an arena, and filmed for at least ½ h or until mating ended. Filming was done using a Sony HD digital camcorder (Sony, Tokyo, Japan). A total of 58 successfully mated pairs were observed for *A. planipennis*, 42 for *A. bilineatus*, and 20 for *A. anxius*.

Films of mating pairs were observed and a list of mating behaviors (an ethogram) was constructed. Only behaviors directly

Table 1. Ethogram for mating behavior of *Agrilus planipennis*, *A. anxius*, and *A. bilineatus*

Behavior code	Description
Groom	Cleaned body, including opening wings and raising abdomen
Walk	Female walked with male mounted on back
Mount	Male rapidly climbed onto female's back, can be from front, side or back of female.
Unsheath aedeagus	Aedeagus is rotated to the side, fully extended, then brought around under the male's body
Insert	Inserted aedeagus into female's valvulae
Pat	Mounted male gripped female with middle-legs. Pro-legs performed a rapid series of five to seven pats to the females pronotum/elytra starting at the front of their reach and moving backwards to just before the female's middle-legs. The male's head bobbed in and out during the sequence, and the palpi brushed the surface of the elytra. This behavior was always accompanied by antennation of the elytra (see below for description). Simultaneously, the hind legs, which were held out to the side during the rest of copulation, moved up and inwards in a stroke resembling a whip-kick in swimming. During this stroke, the tibia stroked the side of the female in an upward motion. The whole sequence took about 1 s to perform and was usually performed several times in succession (up to 90×) at 1–2 s intervals
Pump	Aedeagus moved in and out within vulva, the visible displacement varied from small to large
Wing buzz ^a	Opened elytra and rapidly fanned wings for up to 5 s
Antennate	Male moved antennae alternately touching female's elytra
Withdraw	Male withdrew aedeagus from female
Dismount	Male climbed off of female's back
Sheath aedeagus	Aedeagus drawn back into body
Raise/lower abdomen	Female raised or lowered abdomen while male is mounted
Wag	Female threw whole body from one side to the other
Leg wave	Female lifted single leg, held it in the air, placed it back down
Rest	Remained motionless

All three species shared a highly conserved, stereotypical pattern of behavior.

^aOnly displayed by *A. bilineatus*.

Table 2. A typical transition matrix

<i>A. planipennis</i>	Preceding					
	Mount	Unsheath aedeagus	Insert	Attempt insert	Pat pump buzz	Wag wave walk
Following	Mount	Unsheath aedeagus	Insert	Attempt insert	Pat pump buzz	Wag wave walk
Mount	1					
Unsheath aedeagus						1
Insert						
Attempt insert						
Pat/pump/buzz			1			
Wag/wave/walk		1			38	35
Withdraw					1	1
Dismount					1	

Each cell shows the number of times the behavior in that column heading preceded the behavior in that row (e.g., pats by the male were followed by wags from the female 38 times during this mating). The behaviors are as described in the ethogram in Fig. 1. The most common in copula male behaviors, pat, pump, and buzz were combined, as were wag, wave, and walk for the female.

involved in mating were included. The behaviors in the ethogram were then used to score observations of 10 successfully mated pairs of each species. Common transitions were included in a flow diagram showing the most probable sequences of acts in a successful mating. Mating behaviors for successful mating sequences were transcribed into a first-order preceding-following transition matrix. Several behaviors were pooled to facilitate analysis. We did not include self-transitions (i.e., consecutive repetitions of the same behavior). Chi-square statistic was used to determine the presence of a first-order Markov Chain (Bishop 1975, Fagan and Young 1978). Descriptive statistics were calculated for various aspects of courtship and copulation, including durations (timed to the nearest second), proportion of mating duration spent in various behaviors, and the number of repetitions of behaviors. Means were compared among the three species using analysis of variance tests, with species as the independent variable, and the duration or number of repetitions as the dependent variable. Post hoc testing among species was done using Least Significant Difference. Means are reported with their SEs. All tests were conducted in Systat (Systat Software, Inc., v.9, San Jose, CA).

Morphology of Female Reproductive Tract, Spermatophores, and Sperm

Mated females of all three species were frozen at -20°C shortly after completing copulation. After the females were frozen, the reproductive tract was removed by gently pulling on the last abdominal segment. We observed and photographed the genital tract, spermatophore, and sperm using light microscopy. For a smaller number of individuals, the spermatophore was stained with Giemsa (Fisher Scientific, Pittsburgh, PA) and the sperm was observed using light microscopy.

Mating Success in the Field

Adult female *A. anxius*, *A. bilineatus*, and *A. planipennis* were collected using *C. fumipennis* (Hymenoptera: Crabronidae). These wasps use adult buprestid beetles to provision their brood, and captured beetles are paralyzed, but not otherwise damaged. Intercepting wasps returning to nests from hunts allows sampling of otherwise difficult to collect individuals. Females were collected over the course of two hunting seasons, 16 June–31 July 2015 and 21 June–23 July 2016. *Agrilus anxius*, *A. bilineatus*, and *A. planipennis* begin to emerge in early June in Connecticut and *C. fumipennis* emerges in mid-June (Rutledge et al. 2013). Collected beetles were frozen at -20°C , and then the reproductive tract was removed and examined for the presence of sperm. Proportion of females that were mated was compared among the three species using a chi-square test in Excel (Microsoft 2010).

Mating Disruption Experiment

Mating pairs of *A. anxius* and *A. planipennis* were disrupted at different points during copulation to ascertain when insemination occurred. There were four to five pairs of beetles that had completed maturation feeding for each time point. Mating arenas were placed on a table top with two incandescent lamps supplementing the room's fluorescent lighting (*A. planipennis*) or natural lighting (*A. anxius*). Temperature varied between 21 and 25°C . Virgin beetles that had maturation fed for at least 5 d (*A. anxius*) or 6 d (*A. planipennis*) were placed in 1 oz clear cup arenas either as one pair, or with another pair to allow greater choice of mating partners to beetles. Pairs were allowed 1 h together. If the pairs had not started mating at that time, males were moved to new cup with a

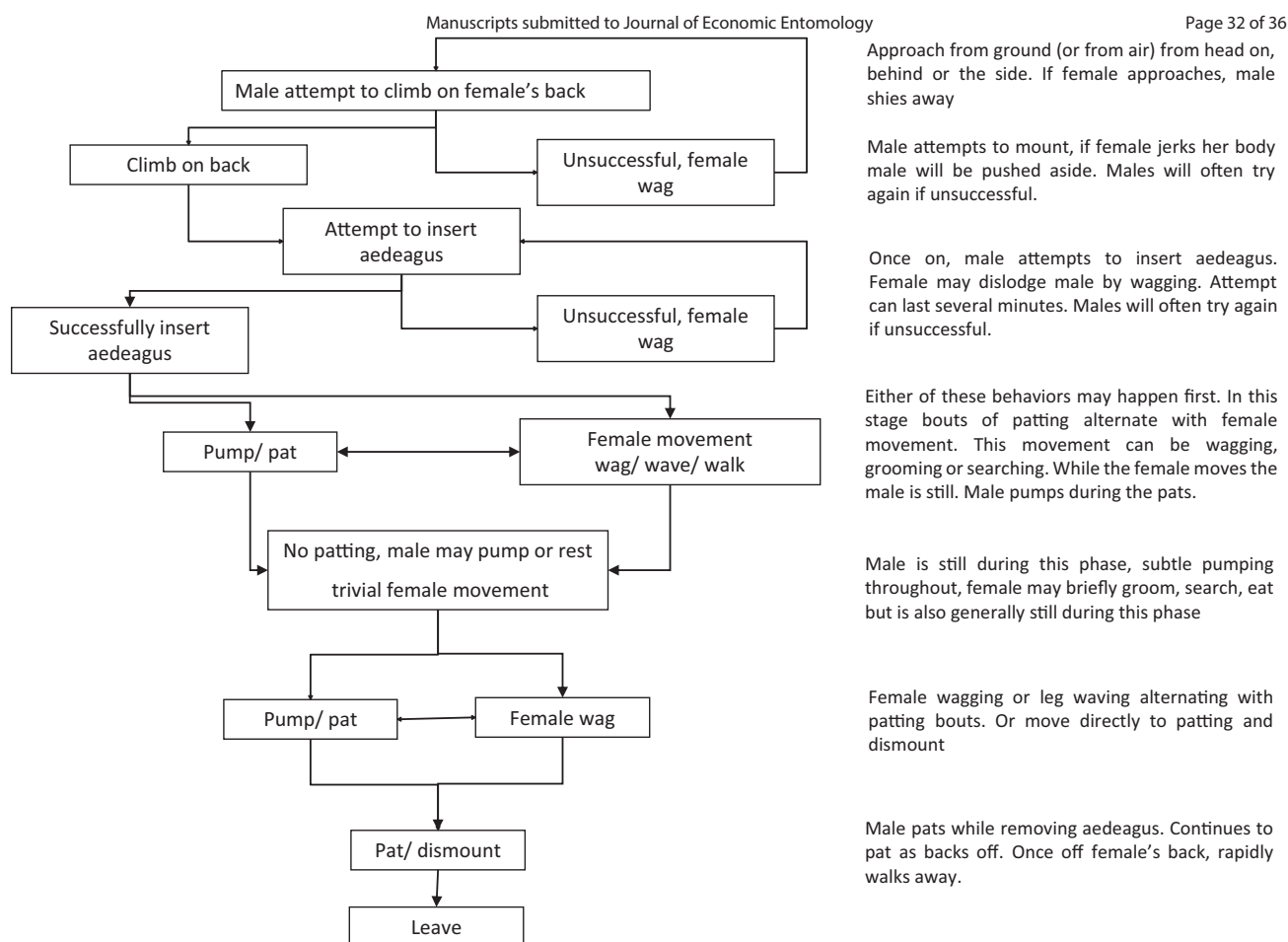


Fig. 1. Flow chart of the most common sequence of events in *Agrilus anxius*, *A. bilineatus*, and *A. planipennis* mating. Terms in boxes are explained in the ethogram in Table 1.

new female partner and waiting resumed. Mating was considered to start when a male inserted his aedeagus into the female's valvulae. Mating pairs of *A. planipennis* were separated manually at 5, 15, 30, and 40 min after the start of mating (10, 20, 40, and 60% of average copulation duration, respectively) or were allowed to mate until completion. Mating pairs of *A. anxius* were separated manually at 1, 3, and 5 min after the start of mating (10, 40, and 60% of average copulation duration, respectively) or were allowed to mate until completion. Immediately after they were separated, beetles were placed in individual, labeled vials and frozen at -20°C until dissection. Mating success was ascertained as described above.

Sperm Dynamics Within the Female Reproductive Track

Virgin *A. anxius* that had been maturation feeding in single-sex cages for at least 5 d were placed together into a rearing cage to allow choice of partners. Beetles were observed until copulation began, at which point the couple were gently removed and placed in an individual petri dish to complete mating. All copulations were timed. After copulation, males were weighed, then returned to the mating cage. Females were also weighed, and then frozen at -20°C at intervals of 0, 15, 30, 60, 90, 120, 150, 180, and 240 min, as well as 1 and 2 d after mating. There were three to five mated females in each category. Females that were not frozen within 30 min of copulation were provided with water in a dram vial plugged with a cotton dental wick, and fresh

foliage in a 7 cm diameter petri dish with a filter paper to absorb excess moisture. The same procedure was followed for *A. planipennis*, although due to longer maturation feeding period, we waited for 6 d after eclosion to experiment. After the females were frozen, the reproductive tract was removed by gently pulling on the last abdominal segment. The genital tract was examined under a dissecting scope for the presence of a spermatophore. Position of sperm within the tract was scored in one of four categories: absent, common oviduct, lower spermatophore, and bulb of spermatophore (Fig. 3F). The presence or absence of the spermatheca was also recorded. For *A. planipennis*, we noted whether the sperm was still bundled or was individual.

Mating Duration, Success, and Fecundity

Using data from the sperm dynamics experiment described above, we examined the relationship between copulation duration and mating success. As described above, pairs of *A. anxius* and *A. planipennis* were allowed to mate until they separated naturally, and were considered to be behaviorally complete. We then compared the copulation duration of successful and unsuccessful copulations (i.e., whether or not a spermatophore had been transferred to the female) using a Student's *t*-test for both *A. anxius* and *A. planipennis*.

To ascertain the relationship between fecundity (number of eggs laid) and mating duration, we used data from earlier experiments in which one subset of female *A. anxius* and *A. planipennis* were observed mating one time, and fecundity was recorded (Rutledge and

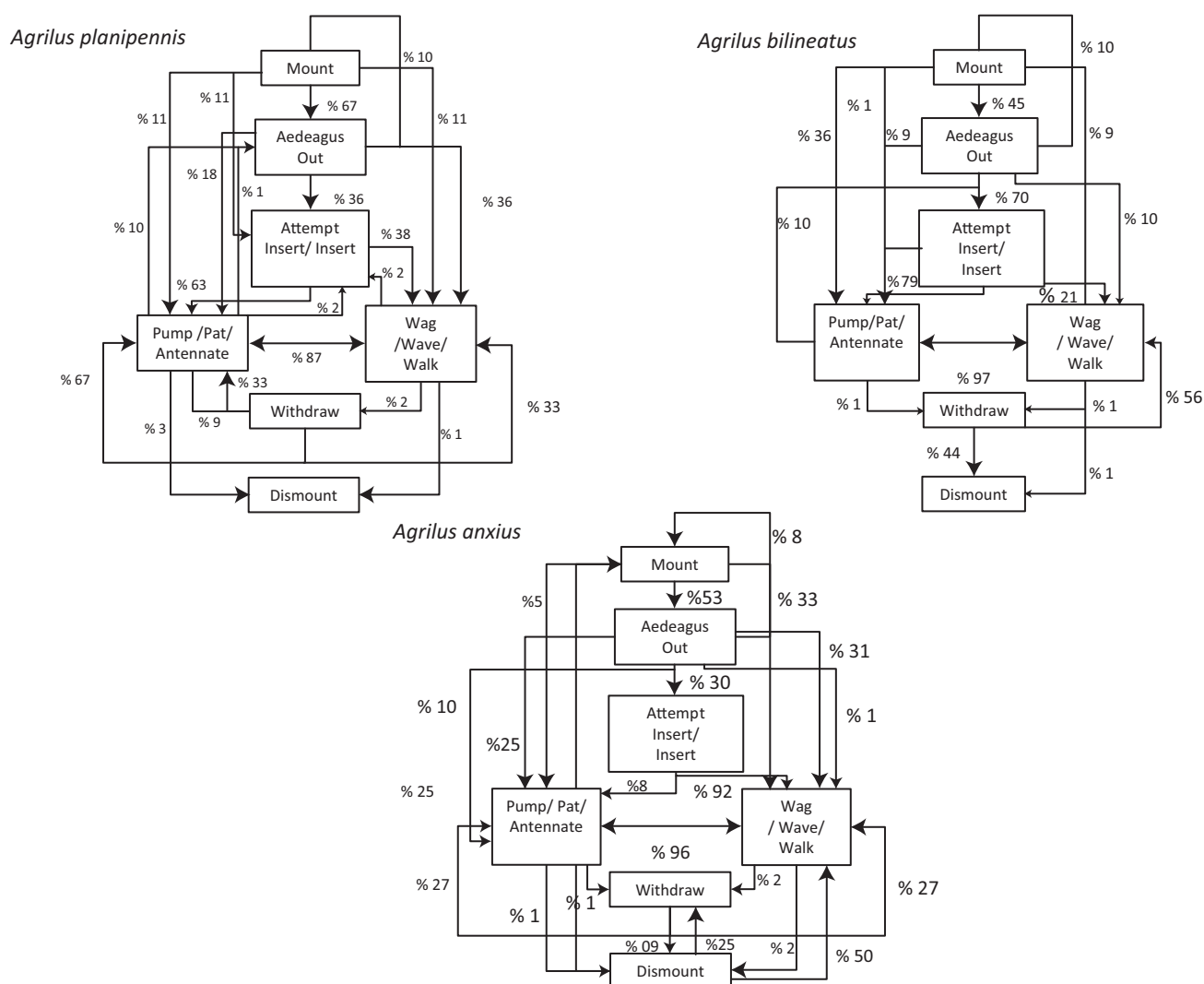


Fig. 2. Simplified ethogram diagram for the three species showing relative probability of transitions between the various behaviors. Line thickness is relative to transition frequency.

Keena 2012a,b). All copulations were timed for these experiments, but the data had not been analyzed or published. Data from females that survived for less than 21 d after mating were excluded from the analysis. We used the data to perform a linear regression of copulation duration on fecundity with an alpha level of 0.05. All statistical calculations were performed in Systat (Systat Software, Inc., v.9).

Results

Mating Behavior

An ethogram of behaviors displayed is listed in Table 1. The behaviors observed were highly stereotyped, and strongly conserved among all three species. The wing buzz behavior of *A. bilineatus* stood out as the only behavior unique to one of the three species. In this behavior, the males opened their elytra and rapidly fanned their wings while on the dorsum of the female. It often occurred immediately before the patting behavior (see Table 1), and males often were buzzing throughout mounting until they had successfully coupled their genitalia with the female beetle's. Buzzing was also seen during uncoupling of the genitalia and dismount from the female's dorsum. The other two species rarely exhibited wing buzzing.

All three species showed a significant first-order Markov Chain process, in that certain preceding behaviors were more likely to be followed by specific behaviors than others (*A. anxius*: $\chi^2 = 1328.34$, $df = 49$, $P < 0.001$; *A. bilineatus*: $\chi^2 = 1713.1$, $df = 49$, $P < 0.001$; *A. planipennis*: $\chi^2 = 386.53$, $df = 25$, $P < 0.001$). An example of a typical transition matrix is shown in Table 2. The flow diagram (Fig. 1) shows the most typical sequence of events during mating. All three species showed limited pre-copulatory courtship, with the male sometimes patting the female before inserting his aedeagus. The beginning of copulation was characterized by alternating bouts of patting by the males and wagging by the females. After an active period, both males and females remained quiescent for the next 20–50% of the copulation period. Finally, there was another flurry of activity, males patting and females wagging, and then the male withdrew, dismounted, and rapidly left the female. Simplified flow diagrams show the relative frequency of behavioral transitions for each species (Fig. 2).

While the overall pattern of the copulation was very similar among the three species, as were the stereotyped behaviors themselves, there were some differences among the species in terms of the relative proportions of different behaviors. The duration of the mating was strikingly and significantly longer for *A. planipennis*

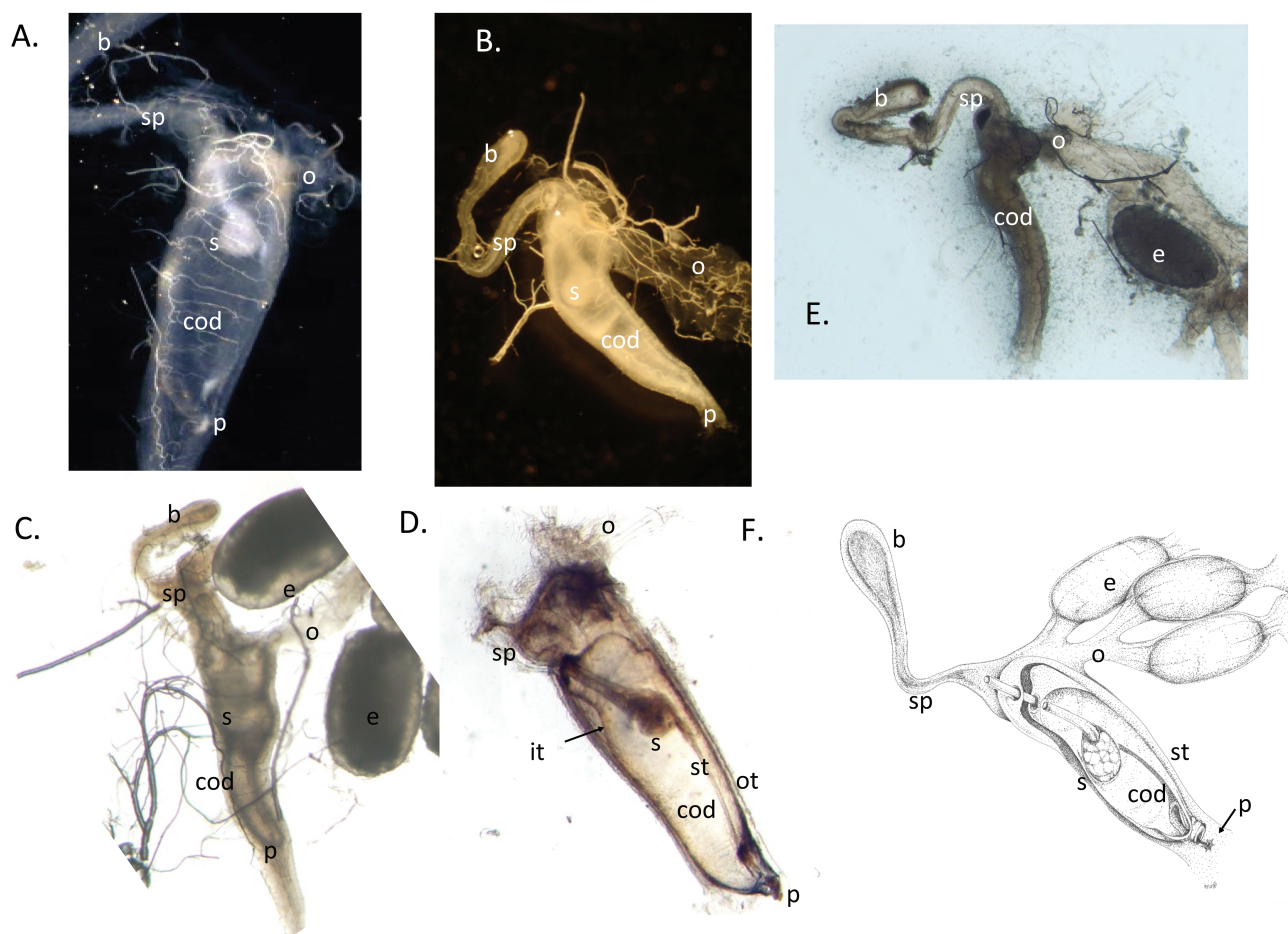


Fig. 3. Reproductive tract with spermatophore: (A) *Agrilus planipennis*, (B) *A. anxius*, (C) *A. bilineatus*. (D) Spermatophore of *A. planipennis*, (E) reproductive tract of *A. anxius*, failed mating, and (F) idealized *Agrilus* reproductive tract. Note in the picture of the failed mating that the common oviduct was not expanded as it was when mating was successful and that the bulb of the spermatophore was empty. S = spermatophore; b = bulb of spermatheca; Sp = spermatheca; COD = common oviduct; E = ovum; O = ovary; St = strap of spermatophore; p = putative mating plug; IT = inner tunic; OT = outer tunic.

Table 3. Comparison of mating behaviors of *A. anxius*, *A. bilineatus*, and *A. planipennis*

	<i>A. anxius</i>	<i>A. bilineatus</i>	<i>A. planipennis</i>
Total length of mating (s)	514.1 ± 8.6 ^A	482.2 ± 8.0 ^A	3926.8 ± 65.4 ^B
Average number of patting bouts	9.8 ± 0.91 ^{AB}	6.9 ± 1.9 ^A	15.5 ± 4.8 ^B
Average number of pats per bout	8.0 ± 1.2 ^A	5.3 ± 1.0 ^A	9.8 ± 3.1 ^A
Average number of wagging bouts	3.5 ± 1.6 ^A	10.8 ± 2.9 ^A	10 ± 2.4 ^A
Average number of wags per bout	3.5 ± 1.6 ^A	8.4 ± 3.3 ^A	11.5 ± 2.7 ^A
Average proportion of quiescent period to length of mating	0.22 ± 0.03 ^A	0.51 ± 0.08 ^B	0.62 ± 0.10 ^B

Numbers shown are averages and SEs for 10 scored behaviorally complete matings per species. Values followed by the same letter are not significantly different within the row ($\alpha = 0.05$).

than for the two native species ($F = 38.06$, $df = 2, 25$, $P < 0.001$; Table 3). The other differences among the species were less predictable. The quiescent period in the middle period of the copulation was a significantly shorter proportion of the total mating period for *A. anxius* than for *A. planipennis* or *A. bilineatus* ($F = 9.70$, $df = 2, 25$, $P = 0.001$). *Agrilus bilineatus* had significantly fewer bouts of patting than *A. planipennis*, while *A. anxius* was intermediate ($F = 5.56$, $df = 2, 25$, $P = 0.010$; Table 3). There was a borderline significant difference among the species in the number of pats per bout ($F = 3.32$, $df = 2, 25$, $P = 0.052$), but individual males ranged considerably with averages of 1–28 pats per bout. Females did not differ among the three species in the number of bouts of the wagging

behavior they performed ($F = 1.3$, $df = 2, 25$, $P = 0.287$; Table 3). There was a borderline significance in the number of wags per bout ($F = 3.29$, $df = 2, 25$, $P = 0.054$; Table 3), with *A. anxius* females performing fewer wags per bout than the other two species. Across all species, there was a significant correlation between the number of wags and the number of pats, showing the reciprocal nature of the two behaviors ($F = 11.8$, $df = 1, 26$, $P = 0.002$; Table 3).

Reproductive Anatomy

The reproductive tracts of female *A. anxius*, *A. bilineatus*, and *A. planipennis* were strikingly similar (Fig. 3A–C and F). The spermatophores were also shaped in a similar fashion and shared the

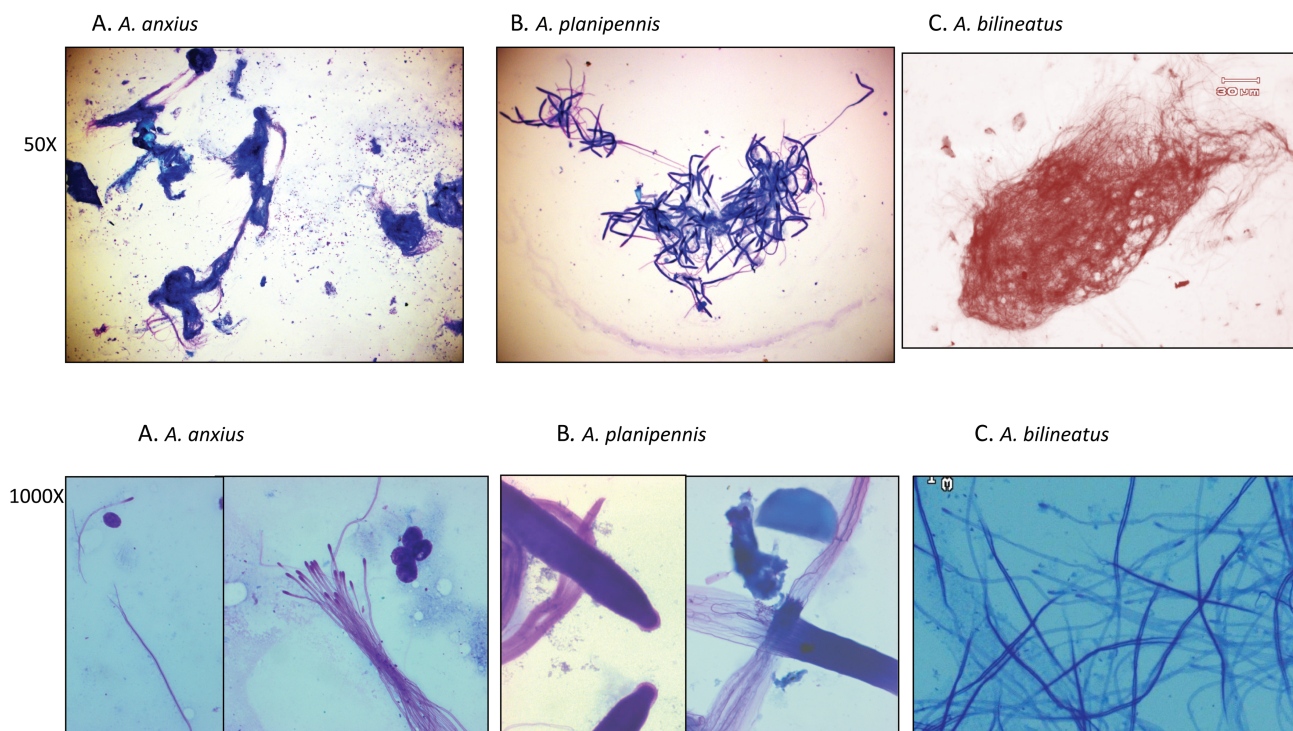


Fig. 4. Sperm, at different magnifications, for (A) *Agrilus anxius*, (B) *A. planipennis*, and (C) *A. bilineatus*. From top to bottom for each species is the spermatophore, somewhat pulled apart, at 50 $\times$ magnification, and individual or groups of sperm at 1,000 $\times$. Note double flagella of *A. anxius* and *A. bilineatus* sperm. Also note the bundled structure of the *A. planipennis* sperm.

Table 4. Results of mating disruption experiment

% normal mating period	<i>A. anxius</i>		<i>A. planipennis</i>	
	Minutes mating	Females with spermatophores	Minutes mating	Females with spermatophores
%10	1 min	0/5	5 min	0/4
%20	NA	NA	15 min	0/4
%40	3 min	0/5	30 min	0/4
%60	5 min	2/5	40 min	1/5
%100 ^a	avg = 8.5 min	5/5	avg = 65 min	6/6

Numbers show number of matings in each time category for which the spermatophore had been successfully transferred to the female over the total number of matings. Percentages refer to the time during mating at which we manually separated the couple.

^aMating allowed to finish.

same position in the common oviduct. Their spermatophores were complex structures, with a conduit at the distal end leading to the sperm storage area of the spermatheca, and a 'strap'-like extension that tethered the spermatophore to the wall of the common oviduct, and extended through the mouth of the oviduct (Fig. 3D). This strap appeared to plug the mouth of the oviduct, but this has not been confirmed. The sperm themselves were distinct. The *A. planipennis* sperm were contained within a hyaline sheath with approximately 20 sperm to a bundle and each had only one flagella (Fig. 4B). Those of *A. anxius* and *A. bilineatus* by contrast were single and each had two flagella (Fig. 4A and C).

Mating Success in the Wild

We dissected 51 *A. bilineatus*, 62 *A. anxius*, and 159 *A. planipennis* females. We found that wild-caught female *A. planipennis* with 102 out of 159 females inseminated (64%) had a lower rate of

insemination than wild-caught *A. anxius* (52 out of 62, 84%) and *A. bilineatus* (36 of 51, 70%) ($\chi^2 = 10.6$, $df = 1$, $P = 0.005$).

Mating Disruption Experiment

Sperm was always passed in a spermatophore and at no time were sperm seen outside of a spermatophore. We also did not see any spermatophore without sperm (although we have seen this in *A. planipennis* in other experimental settings; C.E.R., personal observation). Spermatophores were seen in all of the females that had completed copulation naturally. They were also seen in two of the five *A. anxius* females and one of the five *A. planipennis* females who had been allowed to mate for 60% of the average copulation time (5 min *A. anxius* and 40 min *A. planipennis*). Given that 60% of the average mating time falls within the range of completed copulation times for each species, it seemed likely that the females with spermatophores were in copulations that would have been

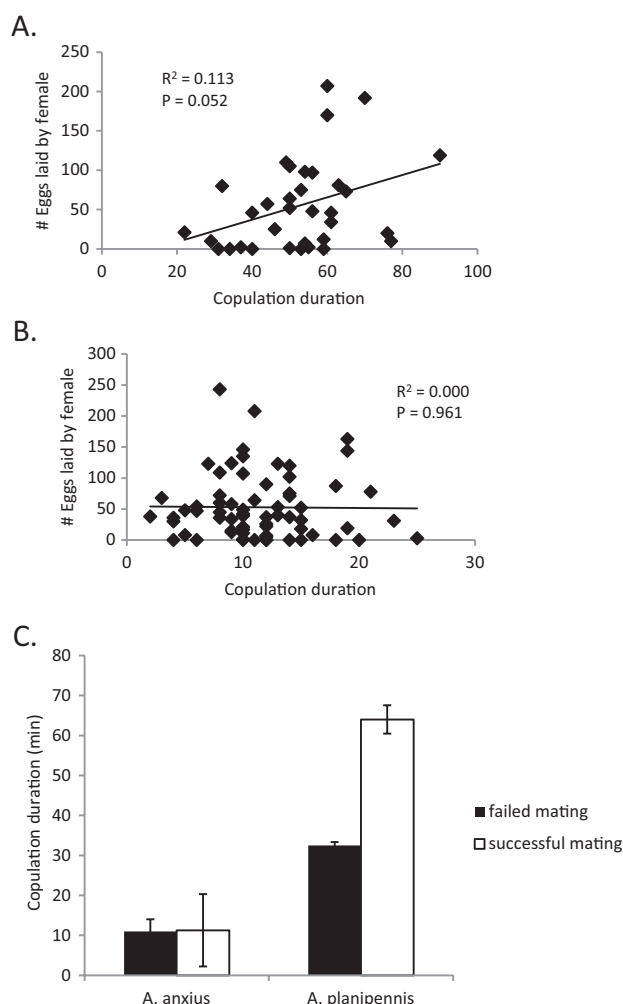


Fig. 5. Correlation between copulation duration and fecundity for (A) *Agrilus planipennis* and (B) *A. anxius*. (C) Average copulation duration and mating success (defined by successful transfer of a spermatophore and sperm) for *A. planipennis* and *A. anxius*.

completed sooner than the average copulation time. No spermatophores were seen in females that had mated for less than 60% of the average time of copulation (Table 4). Given these results, it would seem that the spermatophore is transferred toward the end of the copulation.

Sperm Dynamics Within the Reproductive Tract

Sperm was found in the bulb of the spermatheca of all female *A. planipennis* at all time periods, from immediately after mating to 7 d after mating had ended (Table 5). For *A. anxius*, sperm was not found in the spermatheca consistently until 30 min after mating had ended. Both immediately and at 15 min after mating, only one of four, and one of five *A. anxius* females, respectively, had any sperm in the lower segment of the spermatheca. Sperm was not found in the bulb of the spermatheca in any female until 30 min after mating, and it took 1 h for all to have sperm in the bulb of the spermatheca. In *A. planipennis*, one female of three had a visible spermatophore at 3 h. The spermatophore in *A. anxius* lasted a similar amount of time, but there were still two of three females who had a spermatophore at 3 h, and two of four that had one at 4 h. No female of either species had a spermatophore after 24 h.

Table 5. Movement of sperm within the female reproductive tract after mating

Hours after mating	Spermatophore present		Sperm in common oviduct		Sperm in lower spermatheca		Sperm in bulb of spermatheca	
	<i>A. anxius</i>	<i>A. planipennis</i>	<i>A. anxius</i>	<i>A. planipennis</i>	<i>A. anxius</i>	<i>A. planipennis</i>	<i>A. anxius</i>	<i>A. planipennis</i>
0	4/4	4/4	4/4	4/4	1/4	1/4	0/4	4/4
0.25	5/5	—	5/5	—	1/5	—	0/5	—
0.5	6/6	3/3	6/6	3/3	6/6	3/3	5/6	3/3
1	6/6	4/4	6/6	4/4	6/6	3/4	6/6	4/4
1.5	4/4	3/3	4/4	3/3	4/4	2/3	4/4	3/3
2.0	4/4	4/4	4/4	4/4	4/4	3/4	4/4	4/4
2.5	2/4	3/3	2/2	3/3	2/4	1/3	4/4	3/3
3.0	2/3	1/3	2/3	3/3	3/3	3/3	3/3	3/3
4.0	2/4	—	1/4	—	4/4	—	4/4	—
24.0	0/3	0/4	0/3	3/4	2/4	3/4	3/3	4/4
48.0	0/5	0/3	0/5	1/3	4/5	1/3	5/5	3/3

Couples were allowed to finish mating and then female beetles were frozen at a set period after mating was completed. Dissections were conducted to determine the presence of absence of a spermatophore, if there was sperm in the spermatheca, and whether the sperm had reached lower and/or upper end (the bulb) of the spermatheca. Numbers show how many females met the condition per number of females dissected (there were no *A. planipennis* females frozen 0.25 or 4 h after mating).

Copulation Duration and Fecundity

Agrilus planipennis matings that resulted in a successful transfer of a spermatophore to the female as determined by post-copulation dissection were significantly longer in duration than those that were not successful (Successful: $N = 44$, mean = $64.0 \text{ m} \pm 3.56$, Unsuccessful: $N = 9$, mean = $32.5 \text{ m} \pm 9.0$; $P = 0.021$). For *A. anxius*, there were too few unsuccessful matings for any meaningful statistical analysis (Successful: $N = 56$, mean = $11.27 \text{ m} \pm 0.08$, Unsuccessful: $N = 2$, mean = $11 \text{ m} \pm 3.0$). Copulation duration was not significantly correlated with fecundity for *A. anxius* ($F = 0.011$, $df = 1, 65$, $P = 0.916$, $R^2 = 0.0001$). In contrast, there was a marginally significant positive correlation between copulation duration and fecundity for *A. planipennis* ($F = 4.080$, $df = 1, 32$, $P = 0.052$, $R^2 = 0.113$) (Fig. 5).

Discussion

The invasive *A. planipennis* and its native congeners *A. bilineatus* and *A. anxius* share a stereotyped copulation behavioral pattern. The behaviors and the sequence of the behaviors were conserved across the three species, as was the proportion of time spent in each behavior during the copulatory period. The most striking difference among the three species was the longer copulation time of *A. planipennis*. While the average *A. planipennis* mating duration of 1 h is not remarkable in Buprestidae (Pinkaew 2001), it was eight times longer than the average 8.5- and 8-min mating durations of *A. anxius* and *A. bilineatus*, respectively.

Both the success rate of passing spermatophores and the fecundity of females were related to the duration of mating in *A. planipennis*. Unsuccessful matings for *A. planipennis* averaged about half the length of successful ones, even though males performed the complete sequence of mating behaviors during unsuccessful matings. There were too few unsuccessful matings in *A. anxius* to statistically address whether or not the duration of copulation had an impact on success. Fecundity also varied with mating length for *A. planipennis* but not *A. anxius*. Given these results, it appears that the duration of mating was very important for *A. planipennis* mating success, but not for *A. anxius*.

We tested some of the hypotheses for the difference in copulatory period between *A. anxius* and *A. planipennis* by examining when the spermatophore was passed to the female during copulation. For both species, the timing of the spermatophore passage was similar; it was not passed until the end of the copulatory period. This suggests that *A. planipennis* males are not gaining sperm precedence by swamping the female's reproductive tract with extra-amounts of sperm. However, it is possible that additional accessory fluids leading to increased sperm viability are being passed during the extended copulation. Another hypothesis is that the extended mating may act as 'copulatory mate guarding' in *A. planipennis*. Mate guarding is favored when females mate more than once. But all three species mate repeatedly, so that is not a unique condition for *A. planipennis*. Another reason for mate guarding is a low availability of fecund females, a higher operational sex ratio. Perhaps the ecological conditions of *A. planipennis* in its native habitat are sufficiently different from those of *A. anxius* and *A. bilineatus*, that they have evolved a form of mate guarding available within the behavioral repertoire of the genus.

While the timing of the spermatophore transfer and courtship behaviors is similar among the species, sperm morphology differs between the native species and *A. planipennis*, with *A. planipennis* sperm being bundled and *A. anxius* and *A. bilineatus* sperm being single. Bundled sperm has been correlated with higher sperm

competition in a genus of Carabidae (Coleoptera) (Takami and Sota 2007b). The purpose of the bundling is unclear, but it is suggested that the bundled sperm may move more quickly to the spermatheca. Our results seem to support this explanation. In our post-copulation dissections of the *A. planipennis* and *A. anxius* females, we saw a difference in how quickly the sperm reached the spermatheca. In all successfully mated *A. planipennis* females, there were sperm present in the spermatheca immediately after mating was terminated. While for *A. anxius*, sperm was not found in the spermatheca of any females until 30 min after mating, and it was not until an hour after mating that all females examined had sperm in the spermatheca.

The longer copulatory period, presence of the sperm bundles, and faster rate of sperm migration to the spermatheca post-mating all support the hypothesis that *A. planipennis* is under greater sperm competition pressure than is *A. anxius*. In an apparent contradiction, *A. planipennis* has a lower rate of mating success than *A. anxius* by several measures. In the laboratory, a greater proportion of behaviorally complete matings were unsuccessful at transferring a spermatophore from the male to the female. In addition, fewer of the eggs laid by female *A. planipennis* were fertilized than those laid by *A. anxius* (Rutledge and Keena 2012a,b). Fertility rates vary greatly in laboratory rearing, often depending on the quality of host foliage provided to adults (Akers and Nielsen 1990; D. Juan and L. Bauer, personal communication). However, in wild-caught beetles, *A. planipennis* females were inseminated at a lower rate than either *A. anxius* or *A. bilineatus* females.

Why would a species under high sperm competition be worse at mating? Perhaps there is an Allee effect; *A. planipennis* females are not able to access sufficient males to mate in this new environment (Stephens et al. 1999, Taylor and Hastings 2005). However, given their relative rarity in their native Eastern Asian range, and their outbreak status in North America, this seems unlikely. Another possibility is that the North American *A. planipennis* population is suffering from a founder effect. Genetic work by Bray et al. (2011) showed that the North American population of *A. planipennis* was much less genetically diverse than populations of *A. planipennis* in their native range of Eastern Asia. Alternatively, perhaps there is a trade-off between the production of the more complicated bundled sperm which ensures quicker passage to the spermatheca, and the rate of spermatophore transfer or even the ability to transfer the spermatophore. The benefit of the former would need to be great enough to outweigh the costs of the latter. Similar work looking at insemination and fertility rates of *A. planipennis* populations in Asia, as well as exploration of other species of *Agrilus* with single and bundled sperm, would be necessary to understand if our observed results are a result of a homogeneous gene pool or are typical for the species.

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