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Title

Mating behavior between alien Asian longhorned beetle *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and a native related species *Anoplophora chinensis* in Japan

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Abstract

A highly invasive alien species in North America and Europe, the Asian longhorned

beetle *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae), has been detected in various parts of Japan since 2020. In addition to damaging host trees, *A. glabripennis* may severely impact the Japanese ecosystem by interfering with *A. chinensis* (Foster) (formerly called *A. malasiaca*), a common and related native species with a similar niche. To assess the potential risk of reproductive interference between the two species, mating behavior between interspecific females and males was examined in the laboratory using field-collected sample individuals. Males of both species mounted on female *A. glabripennis* and attempted to insert genitalia at similar frequencies (approximately 50%), but *A. chinensis* males always failed to copulate. In contrast, male *A. glabripennis* did not have sexual behavior with female *A. chinensis*. The results show that mating between the two species is typically unsuccessful, although recent research showed that the two species could produce a hybrid when paired for four weeks. The erroneous interspecific mating attempts may reduce both species' fitness. Therefore, the population-level impact of this reproductive interference should be assessed. ~~Additionally, reproductive interference may be used to control the *Anoplophora* pests.~~

Keywords *Anoplophora malasiaca* · biological invasions · mate recognition · pheromone · wood-boring pest

Introduction

The Asian longhorned beetle *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae) is native to China and the Korean Peninsula and has become a highly invasive alien species in North America and Europe since the late 1990s (Haack et al. 2010). This species uses maples of the genus *Acer* and various other hardwood trees as its hosts and kills them through mass infestation. It is listed among 100 of the world's worst invasive species owing to its severe damage (Lowe et al. 2000). Since 2020, *A. glabripennis* establishment and associated tree damage have been detected in several locations in Japan: Hyogo, Aichi, Toyama, Saitama, Ibaraki, Fukushima, and Miyagi Prefectures (Akita et al. 2021; Iwata and Kiriyaama 2022; Kanada and Kidono 2021; Nishiura et al. 2021; Saitama Prefecture 2021; Sato and Nishiura 2021; Yanagi et al. 2021).

There are native *Anoplophora* species closely related to *A. glabripennis* in Japan, unlike in North America and Europe (Makihara 2007). Among them, *A. chinensis* (Foster) [formerly called *A. malasiaca* (Thomson); detailed later] is commonly found in Japan except for some remote islands. Like *A. glabripennis*, it uses various hardwood trees as its hosts, such as maple, citrus, and willow (Fukaya 2003). Additionally, the following ecological traits of Japanese *A. chinensis* overlap those of *A. glabripennis*: host tree, body size, and adult occurrence period (most abundant in June and July) (Akita et al. 2021). Therefore, in addition to tree damage already reported from invaded countries (Haack et al. 2010), *A. glabripennis* may severely impact the native Japanese ecosystem by interacting with *A. chinensis*, a related species with a similar niche.

Reproductive interference, a possible interaction between the two *Anoplophora*

species, is defined as any interspecific interaction during reproduction that reduces the fitness of one or both species (Gröning and Hochkirch 2008). It can strongly affect species distribution, including the displacement of native species by alien species (e.g., Nishida et al. 2014). For example, misdirected courtship and mating attempts among closely related insect species can lead to species exclusion (Fea et al. 2013; Kishi et al. 2009). If such reproductive interference occurs between *A. glabripennis* and Japanese *A. chinensis*, it negatively affects *A. chinensis*. The displacement of *A. chinensis* concerns the Japanese ecosystem, even though *A. chinensis* is an agricultural pest in Japan (Fukaya 2003). Alternatively, if such reproductive interference adversely affects *A. glabripennis*, *A. chinensis* may be used as a biological control agent of *A. glabripennis* (e.g., Honma et al. 2018). Therefore, investigating the reproductive interaction between *A. chinensis* and *A. glabripennis* is crucial for assessing the potential impact of *A. glabripennis* in Japan.

Anoplophora mating begins from the attraction of males and females to host trees via host tree volatiles (Fukaya 2003; Xu and Teale 2021). In the host trees, males and females seek mates, in which males are more active than females (Hoover et al. 2014; Yasui 2009) using male/female volatile pheromones, female trail pheromones, and visual cues (Fukaya 2003; Xu and Teale 2021). Primarily, males palpate around the tree surface to detect and follow the female trail pheromone laid on the female's path (Hoover et al. 2014). When the two sexes come together, the male, with his antennae, tarsi, or palpi, senses the female contact pheromone on the female's body (Fukaya 2003; Zhang et al. 2003). The male then quickly mounts the female without any prior courtship behavior, rapidly and actively turning his abdomen to seek out the end of the female abdomen and attempting to insert his genitalia (Fukaya 2003; Keena and

Sánchez 2018a; Zhang et al. 2003). The male may mount the female for a long time (1–2 h) and copulate several times (5–10 min each) while protecting his mate (Lingafelter and Hoebeke 2002). The females lay eggs during or after this mating, with lifetime fecundity of >50 eggs (Adachi 1988; Keena 2002). In *A. glabripennis*, a single pair-bond is sufficient to achieve approximately 60% fertility during the female's lifetime (Keena and Sánchez 2018a). Both sexes of *A. glabripennis* and Japanese *A. chinensis* copulate multiple times (Fukaya 2003; Morewood et al. 2004). *Anoplophora glabripennis* and Japanese *A. chinensis* have some common female and male volatile pheromone components but no common contact pheromones (Fukaya 2003; Xu and Teale 2021; Yasui et al. 2019) (Online Resource 1).

This study examined the interspecific mating behavior between *A. glabripennis* and Japanese *A. chinensis* to assess the potential risk of reproductive interference. The focus was placed on the last steps of mating behavior, a laboratory situation where a male approaches a conspecific or heterospecific female on a branch was created, and the male responses to females between the two *Anoplophora* species were compared. The hybridization potential between *A. glabripennis* and Japanese *A. chinensis* was recently studied by four-week laboratory crossing experiments (Wang and Keena 2021). Egg laying, hatching, and development were observed by crossing *A. glabripennis* and Japanese *A. chinensis*, although less than conspecific pairings. However, mating behavior was not recorded in detail. Thus, the likelihood of mating at brief encounters in the field is unclear. Additionally, reproductive interference during the mating behavior might have been overlooked. Furthermore, Wang and Keena (2021) used laboratory strains of *Anoplophora* maintained for multiple generations. Using individuals collected in the field could produce results closer to what is in nature. *Anoplophora glabripennis*

and *A. malasiaca* collected in the field were observed in detail to determine reproductive interference in this study.

Importantly, researchers have not agreed on the taxonomy of Japanese *A. chinensis*. Previously, the Japanese and South Korean populations of *A. chinensis* were called *A. malasiaca* (Thomson) and considered an independent species (Makihara 2000). Meanwhile, *A. chinensis* (~~Thompson~~Foster) naturally occurred in China, North Korea, and Myanmar but was not found in Japan (Makihara 2000). They are distinguished by their external morphology (Makihara 2000) and are genetically different (Muraji et al. 2011; Ohbayashi and Ogawa 2009). Lingafelter and Hoebeke (2002) placed *A. malasiaca* as a synonym of *A. chinensis* because they found no consistent differences in the genitalia of the two species. However, Makihara (2007) stated that there was confusion in the specimens Lingafelter and Hoebeke (2002) examined and did not adopt synonymization. Recently, Wang and Keena (2021) showed that *A. chinensis* and *A. malasiaca* are reproductively compatible and have been suggested to be subspecies. Therefore, following Lingafelter and Hoebeke (2002) and Wang ~~&~~and Keena (2021), this study regards *A. malasiaca* as a junior synonym of *A. chinensis*, and refers to the former as Japanese *A. chinensis*.

Materials and methods

Insects

The tested insects were collected in July and August 2021. Adults of *A. glabripennis*

were collected from elm *Ulmus parvifolia*, katsura *Cercidiphyllum japonicum*, and willow *Salix chaenomeloides* trees in Tsukuba City, Ibaraki Prefecture (17 males and 20 females; mean \pm SD body length was 25.1 ± 2.1 mm for males and 27.2 ± 4.2 mm for females). In Tsukuba, *A. glabripennis* establishment was detected in June 2021 (Nishiura et al. 2021). Adults of Japanese *A. chinensis* were collected from maple *Acer buergerianum* and *Acer palmatum* trees (11 males and four females) in Tsukuba City, Yotsukaido City, Chiba Prefecture (one female, host tree not recorded), Asaka City, Saitama Prefecture (one female, host tree not recorded), and Hachijo Town, Tokyo Metropolis (four males and two females from alder *Alnus sieboldiana* trees) (body length 28.0 ± 2.4 mm for males and 30.0 ± 3.5 mm for females). The adults were individually maintained in plastic containers (10 cm in diameter and 5.5 cm in height) at 25°C and 14L:10D in the laboratory. They were fed cupped jelly for beetle rearing (Pro Jelly, Wraios Corporation, Japan) *ad libitum*. All individuals were used for testing within 30 d after collection.

Laboratory mating experiments

The behavioral tests were carried out following the procedure of Keena and Sánchez (2018a, b). A fresh branch of cherry tree *Cerasus* \times *yedoensis* ‘Somei-yoshino’ (2–3 cm in diameter; 20–25 cm in length) was placed in the center of the bottom of the plastic cover (22 \times 30 \times 6 cm). Cherry trees attract both *A. glabripennis* and Japanese *A. chinensis* in the field (Yanagi et al. 2021). First, a female of *A. glabripennis* or *A. chinensis* was released on the branch. One minute later, a male of *A. glabripennis* or *A. chinensis* was released on the branch, and the actions of the two were recorded for 15

min. In particular, the presence or absence of 1) male mounting on female, 2) male genital insertion attempt into the female, and 3) successful copulation (connection of male and female genitalia) were recorded as the indicators of mating behavior. The plastic cover and branch were renewed for each trial. The experiments were performed at 25°C–28°C during the daytime (around 11 am to 5 pm).

The number of tested pairs was 10 for *Ag* ♀ × *Ag* ♂, 10 for *Ag* ♀ × *Ac* ♂, eight for *Ac* ♀ × *Ag* ♂, and five for *Ac* ♀ × *Ac* ♂ (*Ag*: *A. glabripennis*, *Ac*: Japanese *A. chinensis*). Mean ± SD female/male body length ratio was 1.1 ± 0.18 , 0.99 ± 0.13 , 1.2 ± 0.20 , and 1.2 ± 0.23 for the respective combinations. Because of the small number of insects tested, some individuals were used for more than one experiment (5/60 individuals).

Fisher's exact tests were conducted for *A. glabripennis* and *A. chinensis* females separately, using the occurrence and absence of male mounting on the female as the variables to test whether the frequency of mating attempts toward females differed between conspecific and heterospecific males.

Results

The mounting frequency on *A. glabripennis* females did not differ between *A. glabripennis* and *A. chinensis* males (Fisher's exact test, $p = 1$) and was approximately 50% (Fig. 1). Mounting occurred at first contact between *A. glabripennis* females and *A. glabripennis* males in 5/10 observed trials. Similarly, *A. chinensis* males mounted *A. glabripennis* females at the first or second contact in 4/10 trials. Where mounting did

not occur, the number of contacts was 8.4 ± 10.7 (1–27 times) in *Ag* ♀ × *Ag* ♂ experiments, and 3.2 ± 1.7 (1–6 times) in *Ag* ♀ × *Ac* ♂ experiments. After mounting, males of both species attempted to insert their genitalia into *A. glabripennis* females. The males continued to mount until the end of the 15-min trials, spending most of the time bending their abdomen and trying to insert their genitalia into females, and copulating (Fig. 2). Time spent by males just resting on females was short. In the longest case, it was 92 s of 892 s mounting. *Anoplophora glabripennis* males achieved copulation in many cases (Online Resource 2). It took 366 ± 341 s (range: 115–860 s) for *A. glabripennis* males to achieve copulation after mounting (Fig. 2). In contrast, *A. chinensis* males always failed to copulate (Online Resource 2, 3).

Fig. 1

The mounting frequency on *A. chinensis* females was higher in *A. chinensis* males than in *A. glabripennis* males (Fisher's exact test, $p < 0.001$). *Anoplophora glabripennis* males showed no mounting or mating behavior toward *A. chinensis* females [number of contacts was 3.4 ± 2.6 (1–9 times)] (Fig. 1). *Anoplophora glabripennis* males tried to repel *A. chinensis* females by opening mandibles and menacing them in three trials (Fig. 2). In contrast, *A. chinensis* males mounted on *A. chinensis* females in all five trials. Mounting occurred at the first contact in four trials, and the second contact in one trial. Males copulated successfully in two trials (Figs. 1 and 2; Online Resource 2). The time required from mounting to successful copulation was 163 and 671 s, respectively.

Generally, individuals showed no interest in other conspecific or heterospecific individuals unless they came in direct contact. In one *Ag* ♀ × *Ag* ♂ trial and two *Ag* ♀ × *Ac* ♂ experiments, the males approached the females by following the female paths (Fig. 2). When doing so, the males palpated the branch and container surface with their mouthparts. This behavior is known as trail pheromone following (Hoover et al. 2014).

Fig. 2

Another notable behavior observed was that some females resisted male mounting by kicking the males by hind legs or trying to run away, as reported by Keena and Sánchez (2018a, b). This behavior was observed in both conspecific and heterospecific pairs (Fig. 2).

Discussion

Anoplophora glabripennis females attracted *A. glabripennis* males and Japanese *A. chinensis* males at similar frequencies on contact. This finding contradicts the previous findings that *A. glabripennis* female contact pheromone does not have a common component to Japanese *A. chinensis* female contact pheromone (Fukaya 2003; Zhang et al. 2003) (Online Resource 1). There may be unidentified common or similar components. Another possibility is that female visual cues play a significant role in mate recognition by Japanese *A. chinensis* males, and *A. glabripennis* and *A. chinensis* females have similar visual cues. *Anoplophora glabripennis* males' indifference toward Japanese *A. chinensis* females suggests that species recognition ability is more accurate in *A. glabripennis* males than in Japanese *A. chinensis* males.

Wang and Keena (2021) briefly reported the results of 30-min observation of their cross-breeding experiments between *A. glabripennis* and Japanese *A. chinensis*; Mating behavior was observed in 4/25 *Ag* ♀ × *Ac* ♂ pairs (16%) and none of the 25 *Ac* ♀ × *Ag* ♂ pairs. In this study, however, mating behavior was observed more frequently in *Ag* ♀ × *Ac* ♂ pairs (40%). The experimental settings were similar between the two studies, including the container size. However, two factors could have reduced the possibility of

detecting interspecific mating attempts in this study. First, the duration of observation was shorter (15 min). Second, *A. glabripennis* and Japanese *A. chinensis* used in this study were collected from different host plants. Prior host usage by *Anoplophora* beetles may alter their sexual attractiveness because some host compounds are acquired in their elytra as attractants or repellents (Fujiwara-Tsujii et al. 2013; Yasui et al. 2007; Yasui and Fujiwara-Tsujii 2016). Therefore, if the beetles feed on different hosts, they may not recognize each other. Despite these factors, Japanese *A. chinensis* males' mating attempts on *A. glabripennis* females were more frequent than those in Wang and Keena (2021). The results suggested that field populations of Japanese *A. chinensis* males attempt copulation with *A. glabripennis* females more often than reported from laboratory strains. Therefore, field-collected individuals may be useful in predicting the reproductive interference level between the two species on the field.

The observation that copulation between *A. glabripennis* females and Japanese *A. chinensis* males failed suggests that the morphology of their genitalia does not fit each other. Lingafelter and Hoebeke (2002) recognized qualitative differences between the genital morphology of the *A. chinensis* group (including Japanese samples) and *A. glabripennis*. However, the morphological differences may not be sufficient to achieve complete reproductive isolation, because the females produced eggs in the four-week crossing experiments between *A. glabripennis* and Japanese *A. chinensis* in Wang and Keena (2021).

Hybridization is a harmful form of reproductive interference that native species can receive from related invasive species (Gröning and Hochkirch 2008). Wang and Keena (2021) showed that hybridization could occur between *A. glabripennis* and Japanese *A. chinensis* by pairing them for four weeks. However, this research showed that Japanese

A. chinensis males' mating attempts on *A. glabripennis* females are typically unsuccessful. Besides, *A. glabripennis* males are usually indifferent to Japanese *A. chinensis* females. Furthermore, the hybrid's fitness appears low (Wang and Keena 2021). Therefore, the risk of hybridization between the two *Anoplophora* species may not be high in the field, although further field survey is necessary.

However, Japanese *A. chinensis* males' mating attempts on *A. glabripennis* females may reduce the fitness of both species. In particular, both species may waste their time and energy in attempting or refusing copulation and may lose chances to mate normally with conspecific partners. Therefore, erroneous interspecific mating can be regarded as reproductive interference. The population-wide impact of this reproductive interference deserves further investigation. Additionally, the possibility of interspecific attraction on a medium to long-distance scale should be evaluated because the two *Anoplophora* species have several common volatile attractants (Xu et al. 2020; Yasui et al. 2007) (Online Resource 1).

Reproductive interference found in this research may be used to control *A. glabripennis* and Japanese *A. chinensis*. Recently, a new pest management technique incorporating reproductive interference into a sterile insect was proposed by Honma et al. (2018): mass release of a sterile insect pest can control field populations of the pest species and related pest species through reproductive interference. In this case, mass release of sterile Japanese *A. chinensis* males may effectively suppress the reproduction of the alien *A. glabripennis*. In *A. glabripennis*, female fertility decreases with age (Keena and Sánchez 2018a). Therefore, delayed mating and reduced fertility in *A. glabripennis* females can be expected in the reproductive interference from sterile beetles. Additionally, sterile *A. chinensis* males can effectively suppress wild *A.*

chinensis in orchards where it is a serious pest (Fukaya 2003).

Supplementary Information The online version contains supplementary material available at XXX.

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Figure legends

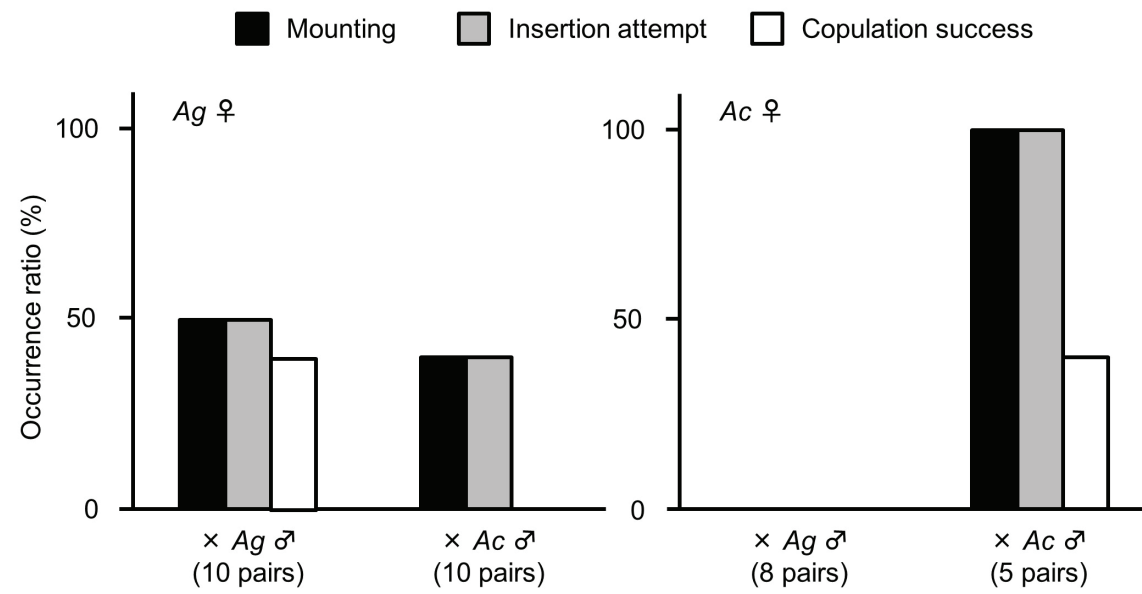
Fig. 1 Results of intraspecific and interspecific mating experiments using *Anoplophora glabripennis* (*Ag*) and Japanese *A. chinensis* (*Ac*). Shown are the occurrence ratios of mounting, genital insertion attempt, and copulation success (connection of genitalia) for female *A. glabripennis* (left) and female *A. chinensis* (right)

Fig. 2 Sexual interaction between different combinations of *Anoplophora glabripennis* (*Ag*) and Japanese *A. chinensis* (*Ac*). The behaviors observed between each individual pair over 15 min are shown as follows. Light gray column: male mounting on female, dark gray column: copulation success (connection of genitalia), diagonal line column: male following of female path, black diamond: contact, white diamond with M: male menacing female with open mandibles, two-headed arrow with R: female resisting to male by trying to shake off mounting male or escape. n = 10 for *Ag* ♀ × *Ag* ♂, 10 for *Ag* ♀ × *Ac* ♂, 8 for *Ac* ♀ × *Ag* ♂, and 5 for *Ac* ♀ × *Ac* ♂

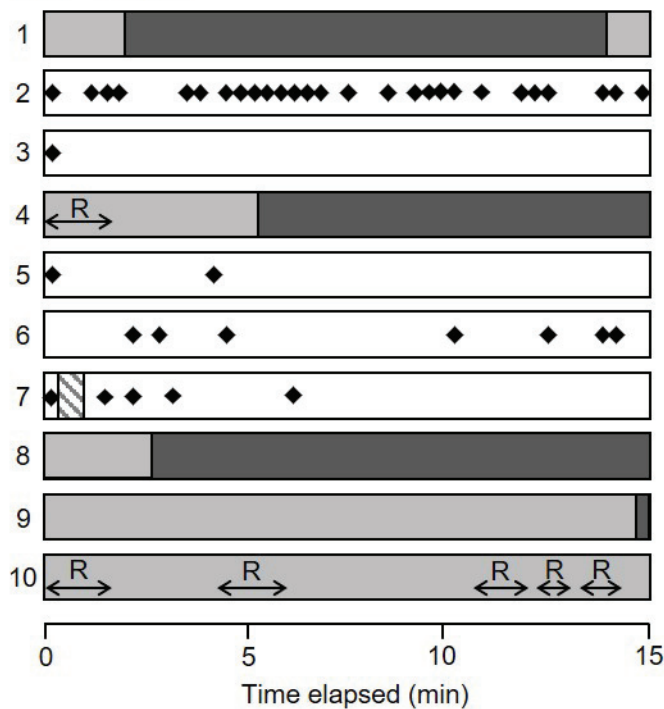
430 **Legends for Electronic Supplementary Material**

431

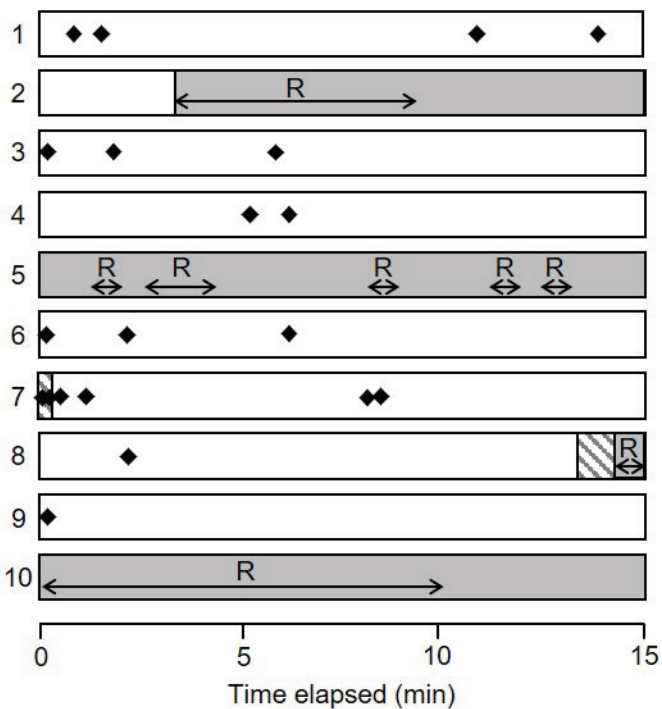
432 **Online Resource 3** A Japanese *Anoplophora chinensis* male failing to insert genitalia
433 into an female *A. glabripennis*. Here the male individual failed to open the female
434 terminal abdominal tergite and sternite. However, in some other cases, the tergite and
435 sternite were open, but males still failed to copulate



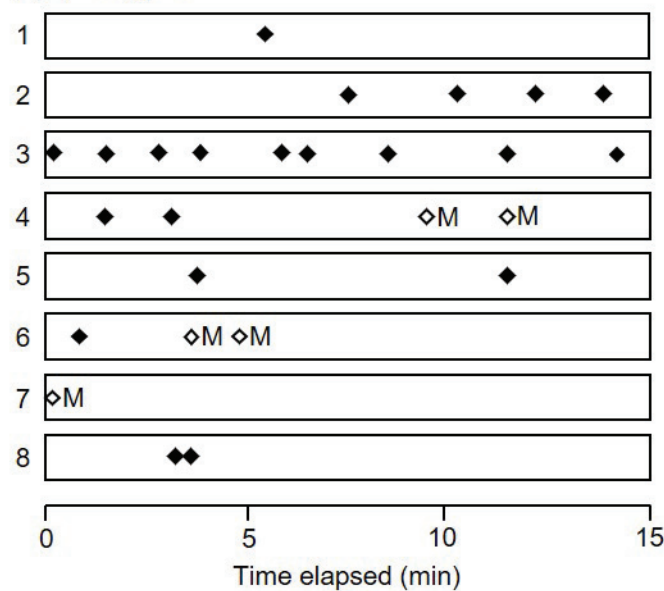
$Ag \text{ } \text{♀} \times Ag \text{ } \text{♂}$



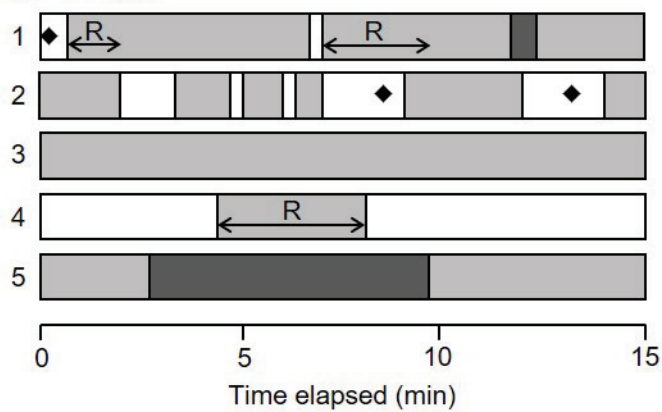
$Ag \text{ } \text{♀} \times Ac \text{ } \text{♂}$



$Ac \text{ } \text{♀} \times Ag \text{ } \text{♂}$



$Ac \text{ } \text{♀} \times Ac \text{ } \text{♂}$



Online Resource 1 List of known pheromone compounds [and attractants](#) associated with mating behavior of *Anoplophora glabripennis* and [Japanese A. chinensis](#) ~~malasiaca~~

	<i>Anoplophora glabripennis</i>	Japanese <i>Anoplophora</i> chinensis malasiaca
Female volatile sex pheromone and attractants *	hexadecanal ¹	β -elemene ^{7*}
	heptanal ¹	β -caryophyllene ^{7*}
	nonanal ¹	α -humulene ^{7*}
	α -longipinene ²	α -farnesene ^{7*}
	α -cubebene ²	
	α -ylangene ²	
	(-)- α -copaene ²	
	α -bergamotene ²	
	<u>β-caryophyllene²</u>	
	<u>α-farnesene²</u>	
Male volatile sex pheromone and attractants *	<u>4-(n-heptyloxy)butanal³</u>	β -elemene ^{7*}
	<u>4-(n-heptyloxy)butan-1-ol³</u>	β -caryophyllene ^{7*}
	<u>(3E,6E)-α-farnesene⁴</u>	α -humulene ^{7*}
		α -farnesene ^{7*}
		<u>4-(n-heptyloxy)butan-1-ol⁸</u>
		<u>4-(n-heptyloxy)butanal⁸</u>
		nonanal ⁸
Female trail pheromone	2-methyldocosane ⁵	No study
	(Z)-9-tricosene ⁵	
	(Z)-9-pentacosene ⁵	
	(Z)-7-pentacosene ⁵	
Female contact sex pheromone	(Z)-9-tricosene ⁶	n-heptacosane ⁹
	(Z)-7-pentacosene ⁶	n-nonacosane ⁹
	(Z)-9-pentacosene ⁶	4-methylhexacosane ⁹
	(Z)-7-heptacosene ⁶	4-methyloctacosane ⁹
	(Z)-9-heptacosene ⁶	9-methylheptacosane ⁹
		9-methylnonacosane ⁹
		15-methylhentriacontane ⁹
		15-methyltritriacontane ⁹
		10-heptacosanone ¹⁰
		(Z)-18-heptacosen-10-one ¹⁰
		(18Z,21Z)-heptacos-18,21-dien-10-one ¹⁰
		(18Z,21Z,24Z)-heptacos-18,21,24-trien-10-one ¹⁰
		gomadalactones A, B, and C ¹¹

Compounds commonly used by the two species are underlined.

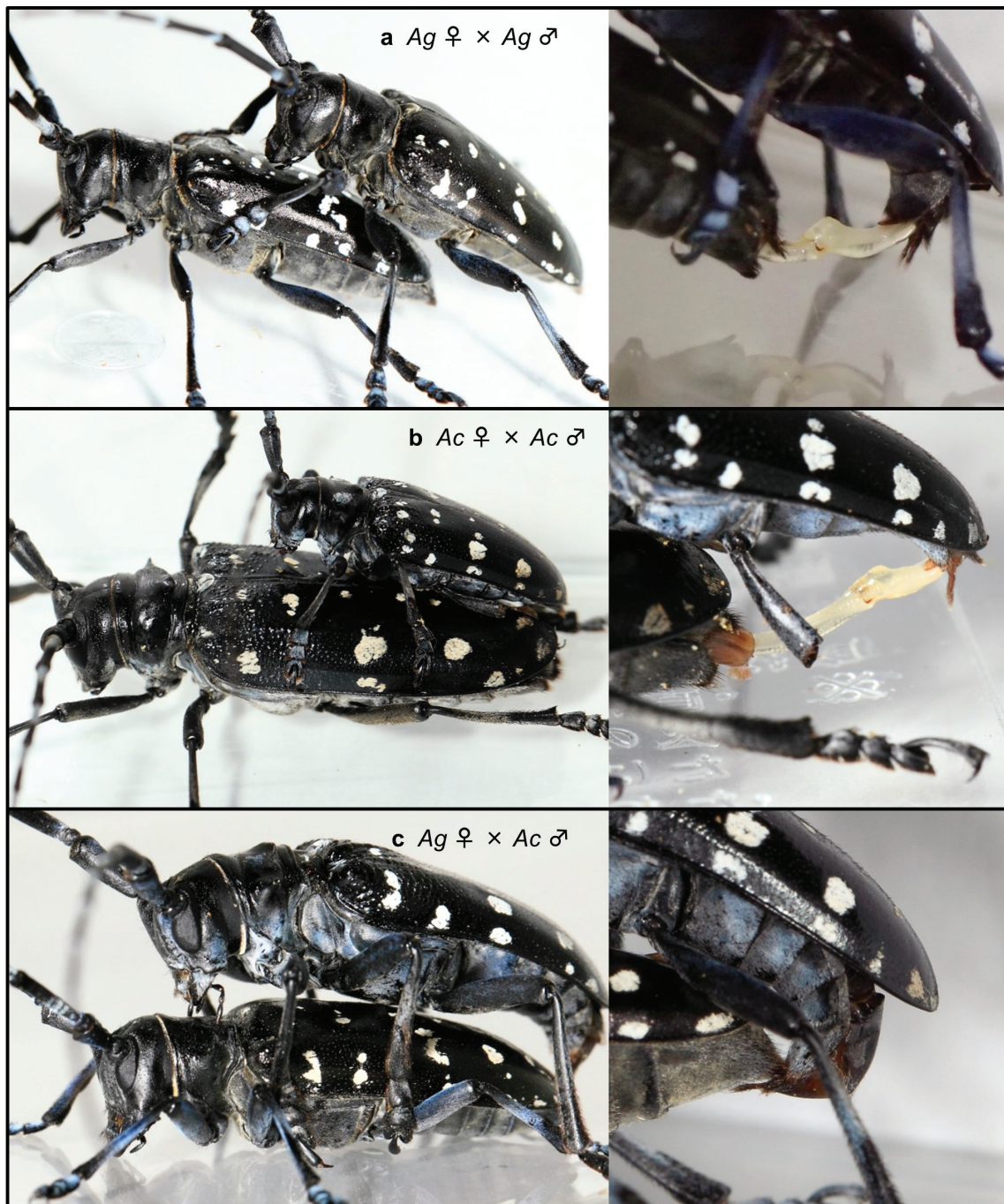
References which identified respective compounds are:

¹Wickham et al. 2012, ²Xu et al. 2020, ³Zhang et al. 2002, ⁴Crook et al. 2014, ⁵Hoover et al. 2014, ⁶Zhang et al. 2003, ⁷Yasui et al. 2007a, ⁸Yasui et al. 2019, ⁹Fukaya et al. 2000, ¹⁰Yasui et al. 2003, ¹¹Yasui et al. 2007b

[*Attractants: unlike pheromones, the attractants are not biosynthesized by the insects but are acquired from their host plants](#)

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Online Resource 2 Intraspecific and interspecific mating behavior of *Anoplophora glabripennis* (Ag) and Japanese *A. chinensis* (Ac). Male mounting on female was observed in pairs of: **a** Ag ♀ × Ag ♂; **b** Ac ♀ × Ac ♂; and **c** Ag ♀ × Ac ♂. Conspecific pairs achieved copulation (**a** and **b**). In contrast, heterospecific pairs always failed, although Ac ♂ bent their abdomen, searched for Ag ♀ abdominal tip, and tried to insert genitalia repeatedly (**c**). Ac can be distinguished from Ag by its granulated elytra base, blue-white spots on pronotum, and blue hairs on the side