

**Ecological studies on dispersal flight and host selection of
the ambrosia beetle *Platypus quercivorus* (Murayama)**

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2020

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Chapter 1

General introduction: Dispersal flight and host selection of bark and ambrosia beetles

Worldwide forests have provided human societies with essential ecosystem services and great economic values for centuries (Ritter and Dauksta 2013). Some of these values are now increasingly threatened by the extensive outbreaks of bark and ambrosia beetles (Coleoptera: Scolytidae and Platypodidae) (Morris et al. 2018), regarded as one of the most destructive groups of forest insects (Ciesla 2011). Some bark and ambrosia beetles vector pathogens (Holmes 1980; Hoover et al. 1996; Kolarik et al. 2011) and cause considerable mortality in natural and planted forests of large areas (Ploetz et al. 2013). For example, bark beetles of the genus *Dendroctonus* are mortality agents to conifer trees in United State of America (Ciesla 2011; Fernando and Richard 2015). Similarly, ambrosia beetles of the genus *Platypus* are mortality agents to oak trees in Japan, Korea and Europe (Henriques et al. 2006; Hong et al. 2006; Kinuura and Kobayashi 2006; Ciesla 2011). In recent years, global warming temperatures have created optional conditions for the expansion of the beetles beyond their original habitats (Kamata et al. 2002; Taylor et al. 2006; Martini et al. 2012). Hence, development of effective management practices is an exciting subject of many studies which require our deep understanding of the bark and ambrosia beetle ecology.

The bark beetles live inside and consume the tree's phloem, a thin plant tissue between outer bark and wood, while ambrosia beetles live inside the tree's wood and do not directly feed on the plant tissue as they feed on symbiotic fungi (Farrell et al. 2001; Fernando and Richard 2015). Despite these differences, the overwhelming majority of the beetles have very similar life history strategies. In general, they spend their whole of life inside a host tree, but at each generation, developing broods must emerge from the breeding galleries where they are born or used as overwintering sites and fly in search of suitable host trees for colonization, mating and reproduction (Fernando and Richard 2015). At first, beetles of only one sex which may be female or male depending on the species, initiate dispersal flight process. Once an attacking beetle finds a susceptible host

which is weakened in physiology by multiple elements such as disease, drought, injures and fire (Wood 1982), it releases aggregation pheromones that attract potential conspecifics to increase attack density, which overcomes the defenses of healthy trees (Rudinsky 1962; Raffa 2001).

Bark and ambrosia beetles contain at least 6000 species from 181 genera worldwide (Wood 1982). The genus *Dendroctonus* is the most studied, e.g. *Dendroctonus ponderosae*, *D. frontalis*, *D. pseudotsugae* and *D. brevicomis*. Other well studied genera were *Ips*, *Scolytus*, *Xyleborus* and *Trypodendron*. Thus, most understanding of bark and ambrosia beetles is derived from studies on relatively few pest species, and many species remain poorly studied.

Little can be done to control these beetles once trees have been infested because beetles live beneath the bark. Therefore, we must clarify the beetle colonization process to make the management plans of bark and ambrosia beetles. The host colonization is a primary component of the life history of bark and ambrosia beetles, which can be divided into four sequential phases including dispersal flight, host selection, concentration and reproduction (Wood 1982). Numerous studies have been done with regard to these phases, both in the laboratory and the field (Sheperd 1966; McCambridge 1971). In this chapter, I selectively focus on papers in regard with the two initial phases of host colonization, dispersal flight and host selection.

Flight during dispersal phase

Brood adults emerge when they are fully developed and when their habitat conditions are becoming unsuitable (Raffa et al. 2015), which are probably resulted from either degradation in food sources or increase in population density (Beaver 1989). Adults usually disperse in late spring and early summer, but it can vary depending on temperature (Lifeutier 2004). New adult beetles tend to disperse to attack new host elsewhere; however, in species having several generations, adults are not necessary to fly away from former host if they re-infest the same host (Raffa et al. 2015). After emergence, adults begin a dispersal flight that can ranges from few meters to hundred kilometers (Atkins 1961; Beckwith 1972). Laboratory based flight studies have revealed that 50% of the *Ips sexentatus* fly over 20 km, and 10% fly over 45 km (Jactel and Gaillard 1991). *Dendroctonus pseudotsugae*, on average, can fly continuously 2 h in flight mill with some individuals fly up to 8 h after resting (Jactel and Gaillard 1991). Beetles can end dispersal flight early if an attractant presents nearby host sources (Chase et al. 2017). Bark and ambrosia beetle behavior during dispersal is poorly understood. Some studies indicate that in the absence of host cues, flight direction of beetles is mainly affected by wind at the beginning of flight, and then they fly against the wind in response to attractive cues (Chapman 1962; McMullen and Atkins 1962; Gray et al. 1972; Safranyik et al. 1992). In addition, flight direction is also influenced by the light condition (Igeta et al. 2003). There are believed to be two main modes of the beetle dispersal: long-range and short-range (Jones et al. 2019). Long-range dispersal often occurs when beetles fly between stands through flying above the forest canopy and are carried by wind. For example, *D. ponderosae* can fly at a range between 30 and 110 km per day above forest canopy with aid of the wind (Jackson et al. 2008). Long distance dispersal has been observed in some species of *Dendroctonus*, *Ips* and *Scolytus* (Furniss and Furniss 1972; Forsse and Solbreck 1985; Jackson et al. 2008). This kind of dispersal enable beetles to increase chance of finding suitable mates as well as host trees and thereby increase fitness; however, long distance dispersal is costly and risky for the beetle: it requires increased energy expenditures which potentially cause greater mortality during flight or decreased reproduction success (Elkin and Reid 2005). The dominant dispersal approach is short-

range in which beetles fly within stand under the forest canopy through its own flight ability to find nearby trees, so called within-stand dispersal (Safranyik et al. 1989). The average flight distance of most bark beetles is less than 5 km in laboratory flight mills (Kinn 1986; Williams and Robertson 2008; Evenden et al. 2014). Lee et al. (2019) points out that more than 85% of *P. koryoensis* is caught within 25 m in a field observation. Other studies have shown *I. typographus* beetles are captured at flight distance ranges of 120 to 1000 m (Botterweg 1982; Zumr 1992; Zolubas and Byers 1995; Duelli et al. 1997). The ambrosia beetle, *Trypodendron lineatum* is recaptured at 500 m (Salom and McLean 1989).

Measurement of dispersal flight

Despite the importance of bark and ambrosia beetle dispersal in their life history, little is known about their dispersal capacity and behavior, owing to small body size which leads to the difficulties in making observations and experiments. Several techniques have been used to study flight capacity and behavior of these beetles including flight mill, capture-mark-recapture, semiochemical-baited traps, sticky traps, aerial survey via geographic information system, individual-based simulation model and genetic markers (Anderbrant 1985; Wichman and Rawn 2001; Mock et al. 2007; Robertson et al. 2007; Evenden et al. 2014; Okada et al. 2018; Lee et al. 2019). Each approach has associated advantages and disadvantages. The use of flight mill has provided us novel opportunities to understand flight potential in the laboratory (Jactel and Gaillard 1991; Jactel 1993; Minter et al. 2018; Okada et al. 2018) where beetles are tethered on an flight arm with the use of quickly dry glues and beetles can freely turn around an axis (Evenden et al. 2014; Okada et al. 2018). This method is applied under constant environmental conditions of humidity, temperature and light intensity during flight experiments (Jactel and Gaillard 1991). The beetle flight is detected by highly sensitive sensors and flight data are stored in a personal computer (Jactel and Gaillard 1991; Okada et al. 2018). Flight mills allow the determination of maximum flight capacity (Evenden et al. 2014). Flight mills also allow the understanding of behavior that occurs during the dispersal phase of host selection (Minter et al. 2018). For example, we can test the effect of olfactory cues on its

flight characteristics by exposing beetles to chemical volatiles during flight (Bennett and Borden 1971). Dispersal of bark and ambrosia beetle in the forest is difficult to determine because of their high speed and small size, so mark-recapture experiments are sometime used as indirect methods (Anderbrant 1985; Lee et al. 2019), which give information on flight distance of the recaptured beetles. This method, however, requires large numbers of beetles for release into flight, but few numbers of beetles can be recaptured, and no information is obtained for their flight behavior. Moreover, it sometimes does not reflect the true distribution of flight distance because beetles captured in the trap nearby the release site are suspected to have further flight (Yamamura et al. 2003). Mark recapture studies have been applied for a range of bark and ambrosia beetle species (Turchin and Thoeny 1993; Meurisse and Pawson 2017; Lee et al. 2019), and the results showed that beetles can fly from a few meters to over at least several kilometers (Botterweg 1982; Jactel 1991; Zumr 1992; Zolubas and Byers 1995). Semiochemical-baited traps, sticky traps and aerial survey via geographic information system techniques sometime can be used to predict beetle spatial-temporal dynamic patterns such as flight direction and flight height as well as the timing of flight (Wichman and Rawn 2001; Zolubas and Byers 1995; Igeta et al. 2004). Because the limitations in direct measurement of flight capacity, much effort has poured into developing a local-scaled individual-based simulation model to indirectly predict potential dispersal and infestation patterns across the landscape (Robertson et al. 2007; Lundquist and Reich 2014; Kautz et al. 2016; Liang et al. 2017; Powell et al. 2018). More recently, several studies have developed genetic markers to infer gene flow and deduce dispersal patterns based on within and among population genetic differences (Mock et al. 2007; Cullingham et al. 2012; Gayathri Samarasekera et al. 2012). Of these methods, the use of flight mills is the most prevalent despite experimental environment is artificial.

Both flight mill and mark-release-recapture experiments revealed that flight capacity considerably varies among species of bark and ambrosia beetles (Atkins 1960; Gara 1963; Kinn 1986; Jactel and Gaillard 1991; Kinn et al. 1994; Williams and Robertson 2008; Chen et al. 2010; Costa et al. 2013; Evenden et al. 2014; Kees et al. 2017; Meurisse and Pawson 2017). In particular, even within a population, these beetles display wide variations in flight capacity. For example, average flight distance of *D.*

ponderosae ranges from 2.12 to 5.95 km, but longest flight distance of an individual is over 24 km (Evenden et al. 2014). Average flight distance of *Pityophthorus juglandis* is 372 m; however, maximum flight distance is 3.5 km (Kees et al. 2017). Similarly, average flight distance of *D. armandi* is 275.1 m, but its distance range of flight is from 0.3 to 48921.4 m (Chen et al. 2010). In *D. frontalis*, average flight distance is 0.98 km and range of flight distance is 0.3 to 2.6 km (Kinn 1986).

Factors affecting dispersal flight

Multiple factors can influence flight capacity and dispersal of bark and ambrosia beetles. These factors are categorized into three main groups: physiology, morphology and environment (Jones et al. 2019). Flight is an ecological process that expends a great amount of energy to initiate and sustain. Physiological factors such as beetle lipid content, carbohydrates and protein and amino acid could explain individual flight capacity variation in flight mills (Lieutier 1984; Chen et al. 2011; Evenden et al. 2014). Many studies have proved that body lipid is the major energy sources used during flight of bark and ambrosia beetles (Kinn et al. 1994; Chen et al. 2011; Evenden et al. 2014). However, lipid content level that beetles allocate to flight depend on the conditions in the brood tree during larvae development. Lipid content is found to be lower in beetles flown than beetles that did not fly (Atkins 1969; Thompson and Bennett 1971; Kinn et al. 1994; Chen et al. 2011; Evenden et al. 2014). In general, beetles with high lipids fly further distance than beetles with low lipids (Kinn et al. 1994; Williams and Robertson 2008; Chen et al. 2011; Evenden et al. 2014). *Dendroctonus pseudotsugae* beetles containing less than 10% lipids do not initiate fly, whereas those with more than 20% lipids initiate flight (Atkins 1966a). Lipid content decreases during flight (Botterweg 1982; Kinn et al. 1994). The lipid content of male *D. pseudotsugae* decreases from 14.79 to 5.21% after 5 h of flight (Thompson and Bennett 1971). The relation between lipid content and flight duration is not significant in some beetles (Botterweg 1982; Forse and Solbreck 1985; Jactel 1993). *D. armandi* uses carbohydrates for survival but uses lipids for sustained flight (Chen et al. 2011).

The relation between morphological factors such as body weight, wing size and

shape, and flight muscle and beetle flight capacity have been studied (Marden 2000; Evenden et al. 2014; Shegelski et al. 2019). Among these factors, body weight is a general measure to explain individual variation in flight capacity because body weight is thought to directly relate to lipid content (Thompson and Bennet 1971; Anderbrant and Schlyter 1989; Chen et al. 2011), which is an important energy that beetles allocate to flight (Thompson and Bennet 1971, Chen et al. 2011, Evenden et al. 2014). In general, big beetles fly farther and longer than small beetles (Atkins 1975; Slansky and Haack 1986; Evenden et al. 2014; Shegelski et al. 2019). Body weight has positive impact on flight propensity of *D. ponderosae* with bigger beetles more ready to fly than smaller beetles (Evenden et al. 2014). In addition, flight distance and flight time of *D. ponderosae* is significantly correlated to beetle body weight (Evenden et al. 2014), while only flight time of *D. armandi* is significantly related to body weight, but not flight distance (Chen et al. 2011).

However, factors determining individual variations in flight capacity remain unclear. Despite relative importance of lipid content and body weight in beetle flight capacity, there is not always sexual difference in flight capacity. Although females are bigger and have higher lipid content than males, flight distance and duration is similar between females and males of *D. ponderosae* and *D. armandi* (Chen et al. 2011; Evenden et al. 2014). Similar results are also observed in *Ips calligraphus* in which males are bigger than females (Kinn 1986). Shegelski et al. (2019) has suggested that the body weight variable explains less than 20% of the variation in flight capacity. Therefore, further investigation into other intrinsic factors influencing on individual variability of flight capacity is needed.

Environmental factors, such as temperature, humidity, wind and light conditions have been documented as influencing factors on the flight patterns of bark and ambrosia beetles (Rudinsky and Vité 1956; Atkins 1960; Atkins 1961; McMullen and Atkins 1962; Igeta et al. 2003; Safranyik et al. 2010). A limit of temperature required for flight vary among beetle species (McMullen and Atkins 1962; Gray et al. 1972; Botterweg 1982; Thompson and Moser 1986; Wermelinger 2004; Gaylord et al. 2008). The temperature limits required for flight initiation is higher than that required for sustainability of flight. For example, *D. ponderosae* begins flight initiation when the temperature reaches 17.8°C;

however, flight is maintained stable if the temperature reduced until 6.7°C (Atkins 1961). In addition, *D. pseudotsugae* could not initiate fly when the temperature is less than 18°C but can sustain flight at 13°C of temperature (Rudinsky and Vité 1956). Although there is negative correlation between the temperature and humidity, but the influence of humidity on bark and ambrosia beetle flight is less documented well. When temperature is lower than limit required for initiation of flight in *D. pseudotsugae*, high humidity increases the frequency of wing beat (Atkins 1960). There is a positive effect of relative humidity on flight time and speed of *D. pseudotsugae*, while no influence is observed on flight distance (Atkins 1961). In the presence of wind, bark beetles can fly up to hundreds of kilometers (Safranyik et al. 2010). Igeta et al. (2003) suggested that positive phototaxis is proximate causes for high distribution of *P. quercivorus* flying beetles near the forest edge. Similarly, Nam and Choi (2014) reported that over 77% of *P. koryoensis* flying beetles are trapped between 11:00 and 14:00, suggesting that flight behavior of the beetle can affected by the light.

In a forest, as a consequence of attack by bark and ambrosia beetles on living healthy trees, especially when pathogenic fungi is introduced into hardwood, dieback of the trees occurs subsequently. Such deaths create the openings in the forest canopy which increase in amount of sunlight around dead trees. Canopy reduction and changes in sunlight can impact beetle dispersal direction. Phototactic behavior has been observed in many species of bark and ambrosia beetles in the laboratory and the field conditions (Graham 1959; Shepherd 1966; Choudhury and Kemedly 1980; Chen et al. 2010; Igeta et al. 2013; Nam and Choi 2014; Wertman et al. 2018). It is not known whether new brood adults make a migration flight after emergence, or they simply disperse to forage or mates, and food.

Host selection phase

After dispersal phase, the adults of bark and ambrosia beetles attempt to locate suitable host trees from among many non-host and unsuitable host trees (Rudinsky 1962), however, these trees are likely scattered in the forest which is generally unpredictable for the beetles. Host selection mistakes can raise adverse consequences on survival chance and reproductive success because of high expenditures of energy (Raffa et al. 2016). Bark and ambrosia beetles are thus believed to develop elaborate mechanisms by integrating information from multiple senses (Campbell and Borden 2006), mainly based on use of visual and olfactory cues (Wood 1982) to make correct decisions as to which hosts to select. There is evidence for the attraction of *Xyleborus glabratus* to tree stem silhouette (Mayfield and Brownie 2013). Similarly, it is found that the dark colored silhouettes are the best for capturing *D. frontalis* as compared to white and yellow (Strom and Goyer 2001). Sometime, an integration of both olfactory and visual cues can promote beetle host selection behavior (Campbell and Borden 2006).

In this section, I focus on the olfactory cues, which is of extremely importance for long range attraction of beetles to hosts. Olfactory attractants of bark and ambrosia beetles involve chemical volatiles released by host trees and aggregation pheromones released from beetles (Byers 1995, 2007; Byers and Zhang 2011). Typical example for the use of these olfactory cues in host selection is exemplified by *D. ponderosae* (Pureswaran and Borden 2005). When a suitable host tree is located by female beetles, they release an aggregation pheromone, namely frontalin, which, in combination with chemical volatiles produced by conifer trees, α -pinene, attract a male for mating and additional males and females for mass attack of the tree (Pitman et al. 1968).

Primary attraction

There are two alternative hypotheses regarding behavioral mechanism of the initial host selection: random landing on host or non-host trees (Wood 1982; Byers 1995; Saint-Germain et al. 2007), and primary attraction to host tree volatiles in flight (Tunset et al. 1993; Brattli et al. 1998). A field study suggested that the two hypotheses are not

mutually exclusive but scale-dependence (Saint-Germain et al. 2007). It is well known that ethanol released from stressed or dying trees (Moeck 1970; Gara et al. 1993; Kelsey and Joseph 2001; Ranger et al. 2013) is attractive to a wide variety of bark and ambrosia beetle species (Cade et al. 1970; Moeck 1970; Montgomery and Wargo 1983; Schroeder 1988). Additionally, host specific chemical volatiles are important for attraction of beetles to the suitable host species (Montgomery and Wargo 1983). Various host monoterpenes such as α -pinene, myrcene, and terpinolene are attractive to large numbers of conifer attacking bark beetle species (Byers et al. 1985; Witcosky et al. 1987; Schroeder 1988; Phillips 1990). Synergism between ethanol and various monoterpenes has been also widely reported (Tilles et al. 1986; Schroeder 1988; Schroeder and Lindelow 1989; Phillips 1990). Ethanol and the host monoterpenes are widely used as the standard lure in traps for the monitoring of bark and ambrosia beetles in the field (Miller and Rabaglia 2009). The attraction to ethanol in traps away from trees is negligible (Byers 1995; Hanula et al. 2008), while the attraction to monoterpenes in these traps is significant (Schroeder 1988).

Secondary attraction

In addition to the primary attraction to host chemical volatiles, some aggregation pheromones produced by attacking bark and ambrosia beetles are also attractive cues which inform a message to other attacking beetles that a suitable tree have been successfully located (Byers 1989). However, after successful colonization and reproduction, pheromone production is stopped in order to inhibit further competition (Byers et al. 1985). So-called "secondary" bark beetle species, those that colonize dying or decaying trees, less often use an aggregation pheromone, but generally are strongly attracted to host volatiles and ethanol (Klimetzek et al. 1986; Schroeder 1988; Schroeder and Lindelow 1989). Evidence for secondary attraction in the western balsam bark beetle, *Dryocoetes confusus*, was obtained in laboratory bioassays and field experiments where pheromone production was induced by exposing males to host resin volatiles (Stock and Borden 1983). Chemical components of aggregation pheromones have been identified in specific species of bark and ambrosia beetles, and these pheromones can

comprise a sole compound or multiple compounds (Kashiwagi et al. 2006; Byers et al. 2013; Rahmani et al. 2019). Furthermore, host derived volatiles are not only important for primary attraction to host trees but also may play a role in enhancing the bark beetles' response to aggregation pheromone (Bedard et al. 1980; Pitman et al. 1975; Paiva and Kiesel 1985). Larger numbers of *D. pseudotsugae* beetles are captured by pheromone-baited traps when the host volatiles were added (Pureswaran and Borden 2005). Miller and Rabaglia (2009) found evidence that ethanol enhanced the attraction of 49 species of bark and ambrosia beetles.

Factors affecting primary and secondary attraction

Behavioral responses of bark and ambrosia beetles to the host chemical volatiles and the pheromone can be influenced by several factors. Previous studies showed that continued flight exercise causes an increase in olfactory responses of *Trypodendron*, *Dendroctonus*, *Scolytus*, and *Ips* to olfactory cues of the host and pheromones (Graham 1959; Choudhury and Kennedy 1980). *Trypodendron lineatum* requires 30 min of flight activity, while *D. pseudotsugae* requires 90 min of flight activity, before they responded to the pheromone (Bennet and Borden 1971). The requirement of flight before responding to attractants in these beetles is considered to relate to lipid content reserves which may be higher in newly emerged beetles so that they have ability for long flight dispersal before responding to the hosts (Anderbrant 1985). Atkins (1966a) reported that *D. pseudotsugae* containing more than 20% of lipid content are not responsive to the host volatiles, while those containing less than 20% of lipid content are responsive to the host volatiles. In contrast, some bark beetles are found to immediately respond to pheromones after emergence (Gara 1963) and when beginning of flight (Wood and Bushing 1963; Botterweg 1982).

Japanese oak wilt and the ambrosia beetle *Platypus quercivorus* (Murayama)

Japanese oak wilt (JOW) is a fungal disease caused by the pathogen *Raffaelea quercivora* Kubono et Shin Ito (Ito et al. 1998; Kubono and Ito 2002) (Fig. 1.1). The ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) (Fig. 1.2) is regarded as a vector transporting this disease among trees (Saito et al. 2001; Kubono and Ito 2002; Kinuura and Kobayashi 2006) via excavating a gallery system inside the trunk of oak trees (Esaki et al. 2009). Several preventive methods have been currently deployed for JOW. The protection of susceptible trees of *Quercus crispula* Blume and *Quercus serrata* Thumb from *P. quercivorus* boring into trees by binding the trunks of individual trees with vinyl sheets (Kobayashi et al. 2001a) is frequently applied to some urban forests and botanical gardens. However, this method is very costly and difficult to implement in large areas. Another method is to reduce the number of brood adults emerging from infested trees by harvesting dead trees and then treat them with insecticides, but it requires extensive investment of labors and prices. In addition, the capture of flying *P. quercivorus* adults through pheromone-baited traps has been used, however, the effectiveness of beetle capturing was very low (Kamata et al. 2008). Additionally, the injection of NCS (N-methyl dithiocarbamic acid ammonium salt) into the trunk of attacked oak trees (Saito et al. 1999), and spray of fenitrothion on the bark surface of intact oak trees have been performed (Esaki 2011). Overall, it is now expected to find more efficacious ways to prevent and control JOW since current management systems are not sufficiently effective (Saito 2002).

Flight and dispersal behavior of *P. quercivorus* has been studied in the field and in the laboratory (Esaki et al. 2002, 2004; Igeta et al. 2003, 2004; Okada et al. 2018). *P. quercivorus* brood adults disperse in summer (Soné 1998). Most emerged adults were captured by the sticky screen traps in the morning (Igeta et al. 2002). A previous field study has showed a high probability of attack by *P. quercivorus* for distant trees rather than for those nearby the natal tree (Yamasaki et al. 2016) which suggests that this beetle might use long-range dispersal in search of new host trees. However, the study used the distribution data of trees killed by *P. quercivorus* to predict beetle dispersal, and not estimated actual flight behavior of the beetle. Previous studies also reported that dispersal

direction of *P. quercivorus* is mainly influenced by light condition with large number of flying adults were captured near the upper forest edge (Igeta et al. 2003; Esaki et al. 2004). Moreover, flight height of *P. quercivorus* is observed in range of 0.5 to 2.5 m, but several individuals extend up to 16 m (Igeta et al. 2004). However, the actual flight behavior of *P. quercivorus* at its dispersal phase remains unclear. For example, which direction that the beetle flies after emergence from breeding galleries or at what height the beetle flies at the time of seeking for a new host tree are not clarified yet. At initial stage of dispersal, *P. quercivorus* emerge from breeding galleries and it seek and find new host trees at later stage of dispersal. Therefore, behavioral responses of *P. quercivorus* to environmental stimulus may be different between before and after flight. Thus, studies are necessary to clarify this point. Recently, Okada et al. (2018) have developed a flight mill system which allows us to understand flight capacity of *P. quercivorus* in laboratory conditions. It was found that its maximum flight distance and duration of *P. quercivorus* was 27.1 km and 7.5 h, respectively, and there was no sexual difference in flight distance and duration (Okada et al. 2018). However, details of flight characteristics and factors affecting on flight capacity of *P. quercivorus* are still undiscovered.

Several studies have been done to understand characteristics of *P. quercivorus* host selection. Previous studies have indicated that *P. quercivorus* selects suitable host trees without previous infestation history (Yamasaki and Futai 2008). Yamasaki and Futai (2012) studied the infestation patterns of *P. quercivorus* on various host tree species. They have shown that *P. quercivorus* exhibits more preference to *Q. crispula* over *Q. serrata* and *C. crenata* (Yamasaki and Futai 2012). It is generally thought that *P. quercivorus* is a secondary pest, i.e. it prefers weakened or stressed trees (Ito and Yamada 1998). However, there is opinion that *P. quercivorus* turns into primary pest attacking apparently healthy trees (Ito and Yamada 1998). It now remains unclear that which status of trees is the most preferable to *P. quercivorus*. Effectiveness of ethanol to attraction of *P. quercivorus* has been tested in the field study; however, flying *P. quercivorus* adults are weakly attracted to ethanol-baited traps (Iidzuka et al. 2016), which suggests that ethanol, signal of weakened trees, is not effective primary attractant for *P. quercivorus*. In addition, effectiveness of aggregation pheromone to *P. quercivorus* antennal response has been confirmed in the laboratory (Tokoro et al. 2007); however, it was failed to attract *P.*

quercivorus in the field tests when the pheromone-baited traps were placed solely in the forest (Kamata et al. 2007). These pheromone traps strongly attracted *P. quercivorus* when they were placed on the trunk of oak trees (Ohashi 2013), therefore, some kind of host tree information (e.g. chemical volatiles) is necessary to attract the beetle. *Platypus quercivorus* is also known to show preference for size of host trees: it prefers large diameter hosts rather than small diameter hosts (Yamasaki and Futai 2008). Furthermore, *P. quercivorus* prefers attacking clump of host trees over single tree (Yamasaki and Sakimoto 2009). These results indicate that size-dependent information is important for host selection of *P. quercivorus*. One possibility is that big trees and dense trees produce and release higher amount of leaf chemical volatiles than small and single trees, and that *P. quercivorus* detect these volatiles. Therefore, studies on the chemical interaction between *P. quercivorus* and their host trees should be carried out since these studies have important implications in the development of strategies against *P. quercivorus*. An intensive understanding of ecological features of the dispersal flight and host selection of *P. quercivorus* and links between them may enable us to make correct approaches.

Aim of this study

The overall objective of this study is to clarify the dispersal flight and the host selection process of *P. quercivorus*. Specifically, the study aimed to address four main questions: (1) What factors affect flight distance of *P. quercivorus*; (2) How phototactic behavior of *P. quercivorus* changes after flight; (3) Do *P. quercivorus* prefer declined or healthy trees; and (4) Can *P. quercivorus* discriminate hosts from non-hosts by chemical volatiles. The results of this study are expected to contribute to understandings of characteristics of flight and strategies and mechanism of host selection of *P. quercivorus* whereby those findings may be useful for controlling Japanese oak wilt in future.

This thesis is composed of six chapters including this chapter. In chapter 2, I investigated factors that affect flight distance of *P. quercivorus*.

In chapter 3, I examined effects of flight on phototactic behavior of *P. quercivorus*.

In chapter 4, I investigated effects of flight and leaf conditions on the olfactory responses of *P. quercivorus* to leaf volatiles.

In chapter 5, I examined difference in the olfactory responses of *P. quercivorus* to chemical volatiles emitted from leaves of host and non-host, and identified the chemical profile of leaf volatiles from host and non-host trees.

At last, in the general discussion of chapter 6, I connected the results of my series of studies and discussed issues in relation to dispersal flight and host location of *P. quercivorus*.



Fig. 1.1 Wilt disease of Japanese oak trees observed in a secondary forest of Kyoto Prefecture, Japan.



Fig. 1.2 The ambrosia beetle *Platypus quercivorus* (Murayama), a primary vector for Japanese oak wilt. Male (left) and female (right).

Chapter 2

Factors affecting flight distance of *Platypus quercivorus*

Introduction

In general, bark and ambrosia beetles (Coleoptera: Scolytidae: Platypodidae) are specialized in living and reproducing inside host trees through feeding on plant tissues and symbiotic fungi (Wood 1982; Fernando and Richard 2015). Emerging adults of these beetles often have a short phase of dispersal, achieved through flight, to locate new host trees where they mate and reproduce (Wood 1982). Within-stand dispersal and long-range dispersal are the main mechanisms used to move within and between infested patches and new un-infested patches (Nilssen 1984; Safranyik et al. 1992; Robertson et al. 2007; Jackson et al. 2008).

Previous studies showed that flight distance is highly variable among individual beetles (Atkins 1961; Kinn 1986; Jactel 1991; Jactel and Gaillard 1991; Chen et al. 2010; Costa et al. 2013; Evenden et al. 2014; Kees et al. 2017; Lee et al. 2018; Shegelski et al. 2019), indicating that energy is differentially allocated to flight among individuals. Common approaches used to evaluate the energetic state of beetles for flight include lipid content and body weight (Atkins 1966b, 1969; Bennett and Borden 1971; Thompson and Bennett 1971; Jactel 1993; Kinn et al. 1994; Chen et al. 2011; Evenden et al. 2014). Previous laboratory-based studies showed that flight reduced the lipid content of beetles compared to those that did not fly (Atkins 1969; Thompson and Bennet 1971; Kinn 1994; Chen et al. 2011; Evenden et al. 2014); however, lipid measurements require the destruction of beetles, preventing the same individuals from being tested twice. In addition, body weight was found to be positively correlated with flight distance (Evenden et al. 2014). However, other studies on bark beetles found no clear relationship between body weight and flight distance (Botterweg 1982; Chen et al. 2011; Shegelski et al. 2019). Consequently, further approaches are required to improve our understanding of the factors driving large variation in the flight distance of beetles.

Studies of bark beetles have shown that their energy reserves influence how they

locate hosts (Gast et al. 1993; Wallin and Raffa 2000; Chubaty et al. 2009; Elkin and Reid 2010; Latty and Reid 2010; Chubaty et al. 2014) and behave during reproduction (Elkin and Reid 2005). When energy is constrained, beetles should allocate energy appropriately to multiple tasks. Pioneer bark and ambrosia beetles are vital for locating suitable hosts, initiating breeding galleries, and releasing aggregation pheromones, which facilitate the mass attack of trees. In comparison, follower beetles are recruited by pioneers via aggregation pheromones (Ciesla 2011). Therefore, the pattern of energy allocation might differ between pioneers and followers. The females of monogamous *Dendroctonus* species are pioneers that initiate the attack (Fernando and Richard 2015). In comparison, the males of polygamous *Ips* species are the pioneers that initiate attack (Fernando and Richard 2015). Thus, sexual differences in the pattern of energy allocation could be driven by differences in the ecological roles of the sexes.

The ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae), is an important pest of Fagaceae trees in Japan. This beetle is a vector of the fungus *Raffaelea quercivora* Kubono et Shin-Ito, which is a causal agent for Japanese oak wilt (Ito et al. 1998; Kinuura and Kobayashi 2006; Kubono and Ito 2002; Kobayashi and Ueda 2005). *Platypus quercivorus* is a monogamous species, in which the males initiate attack on oak trees (Kobayashi et al. 2001). Once attack is initiated, males release an aggregation pheromone (Kashiwagi et al. 2006) to call other males and females to attack oak trees en masse. A previous study using a flight mill reported that the flight distance of *P. quercivorus* largely varies among individual beetles (max distance: 27.1 km), with no significant sexual differences (Okada et al. 2018). Even if males and females have the same flight distance, factors causing individual variation in flight distance might differ between sexes. Thus, it is necessary to clarify the flight characteristics of this beetle to control Japanese oak wilt in the field.

Flight distance is determined by flight duration and flight speed. Assuming that active beetles fly faster than inactive beetles, measurements of flight speed could provide more accurate estimates on energy allocated to flight. This study aimed to clarify factors causing variation in the flight distance of *P. quercivorus*, and to estimate sex-based differences for each influencing factor. Specifically, flight distance, flight duration, and initial and terminal flight speed were measured using flight mills. In the experiments of

my previous studies using a flight mill (Pham et al. 2017, 2019), the flight speed of the beetle was observed to decline during flight, but was not examined (Pham et al. personal observation). Thus, I proposed using flight speed measured at the beginning of flight (initial flight speed) as an indicator of energy allocated to flight, and flight speed measured at the end of flight (terminal flight speed) as an indicator of energy saved for other tasks. Both initial flight speed and terminal flight speed influence flight distance, but males and females might invest differently in these two components. The results are expected to improve current knowledge on the hidden ecology of beetle flight in forests.

Materials and methods

Beetle collection

Platypus quercivorus adults used in the flight experiments were collected from the stumps of dead trees of *Quercus crispula* Blume and *Quercus serrata* Thumb (Table 2.1). The attacked host-trees were cut down, sawn into logs, and transferred to Kyoto University, Japan (35°01.44'N, 135°47.15'E) in 2015, 2016, 2018 and 2019. These logs were then sealed with silicon at the cut ends and placed outdoors. Emergence traps made of polyethylene plastic tubes (16 mm in diameter and 100 mm long) (Iidzuka et al. 2013) were attached to the entry holes of beetles. When adult beetles emerged and entered the traps, they were immediately transferred to the laboratory for use in the experiments.

Flight mill

A flight mill system developed by Okada et al. (2018) was used to study the flight of *P. quercivorus* adults (Fig. 2.1). The main part of the mill was composed of two flight arms, which were made of two thin needles with plastic handle (0.25 mm in diameter and 40 mm long for the needle part) that connected perpendicularly. The beetles were tethered to the end of the horizontal arm via the insect's pronotum with a small amount of quick-drying adhesive (Aron Alpha Super Jelly, Konishi Co. Ltd., Osaka, Japan). Each rotation of the flight arm and the beetle was detected by an infrared LED and photodiode sensor. The sensor was connected to an A/D converter (U3HV-LJ, LabJack Co., Lakewood, CO, USA), and the data were logged in a personal computer. The experiments were conducted at room temperature (25 °C) and under light illumination (100 lux).

Flight data measurement

In total, 323 beetles (135 males and 188 females) were used in the flight experiments. Initially, these experiments were completed to simulate the status of beetles before and after flight, and to compare behavioral responses between each status. Data

logged for each beetle were processed, and flight speed was calculated for each rotation, as follows:

$$\text{Flight speed of each rotation (km/h)} = \frac{0.00004 (\text{radius of horizontal arm}) \times 2 \times 3.14}{\text{rotation time in seconds} / 3600}$$

Total flight distance was estimated using the number of rotations of the flight mill arm using the following equation:

$$\text{Flight distance (km)} = 0.00004 \times 2 \times 3.14 \times \text{number of rotations}$$

Because there were two flight patterns (continuous and segmented flight), flight duration was calculated by summing flight time, excluding flight intervals (Fig. 2.2). Initial flight speed was calculated by averaging the speed of the 31st to 40th rotations from the first rotation (circle at the start of flight in Fig. 2.2). Terminal flight speed was calculated by averaging the speed of the 31st to 40th rotations from the last rotation (triangle at the end of flight in Fig. 2.2). The first 30 rotations and last 30 rotations were excluded from the calculation above to reflect the initial and terminal speed of flight under stable conditions. For segmented flight, the initial speed of the first segment and terminal speed of the last segment were used to represent initial and terminal speed, respectively.

Because the initial objectives of these experiments were not to estimate the flight capacity of beetles, I did not set a time limit on when beetles were removed from the flight mill. Beetles were only removed from the flight mill when they stopped flying during the period of observation. Therefore, there was high variation in the time that beetles were kept attached after their last flight segment (attached time). For cases where the attached time was relatively short, data on flight distance and duration might not show the capacity of the beetle. After processing 4 years data, 626 intervals were observed in the flights of 323 beetles. Intervals ranged from 10 sec to 78 min, and were shorter than 20 min on 95% occasions (0.95 quantile of the data was 19.34129 min). Therefore, I set the threshold of attached time as 20 min, and excluded all data of beetles with attached times of <20 min. Thus, data on 139 beetles (57 males and 82 females) were used for the analyses (Table 2.2).

Statistical analyses

A general additive mixed model was used to predict the flight distance of *P. quercivorus* beetles. Flight duration, initial flight speed, terminal flight speed, beetle sex, and their interaction terms were specified as candidate explanatory variables. Flight duration was assumed to have a linear effect on flight distance, while initial and terminal flight speed were assumed to have non-linear effects on flight distance. Therefore, the model consisted of a linear part and non-linear part. The linear part of the model first used a full model that included flight duration, beetle sex, and their interaction as explanatory variables. The non-linear part of the model assumed a different two-dimensional smooth effect of initial and terminal flight speeds for each sex. The year of the experiments were treated as the random intercept in the model to account for any correlation among data from the same year. Model selection was completed by removing the variables one at a time, and comparing the Akaike Information Criterion (AIC) between models. The model with lowest AIC was selected as a best-fit model. Model construction and selection were completed in the *gam4* package of R, version 3.5.3 (R Core Team 2019).

Results

The average flight distance of *P. quercivorus* measured in flight mill was 3.01 km in males (range: 0.08 to 24.70 km) and 3.75 km in females (range: 0.04 to 28.81 km).

The selection process of the model predicting the flight distance of *P. quercivorus* is shown in Table 2.3. AIC decreased when the interaction term between flight duration and sex was excluded from the full model, and it further decreased when the variable sex was excluded (Table 2.3). Further exclusion of the two-dimensional smooth effect of initial speed and terminal speed and the linear effect of flight duration caused AIC to increase (Table 2.3). Therefore, the model with the linear effect of flight duration and two-dimensional smooth effect of initial speed and terminal speed was selected as the best-fit model to predict flight distance.

Results of the best-fit models for the linear and non-linear parts are shown separately for males and females in Fig 2.3. Beetle sex was not included as an explanatory variable for the linear part; therefore, linear regression for the effect of flight duration was the same for males and females (Fig. 2.3a, b). Estimated coefficient for flight duration showed that average flight speed was 4.22 km/h. Predictions of the non-linear part of the model are shown in Fig. 2.3c and d, with information on the residuals of the linear part. Most predicted values ranged from -1 to 0.5 for males (Fig. 2.3c), and from -1 to 1 for females (Fig. 2.3d). Predictions for males increased from a simultaneous increase in initial flight speed and terminal flight speed (Fig. 2.3c). Predictions for males decreased from a simultaneous decrease in initial flight speed and terminal flight speed (Fig. 2.3c). Predictions for females increased from 0.5 to 1 or higher from an increase in initial flight speed (Fig. 2.3d). Predictions for females decreased from -0.5 to -1 (or lower) from a decrease in terminal flight speed (Fig. 2.3d).

Discussion

The estimated average flight speed of *P. quercivorus* (4.22 km/h) was faster than that observed for bark beetles of the genus *Dendroctonus*, including the mountain pine beetle *D. ponderosae* Hopkins (1.55–1.93 km/h) (Evenden et al. 2014), Chinese white pine beetle *D. armandi* Tsai & Li (2.38 km/h) (Chen et al. 2010), Douglas-fir beetle *D. pseudotsugae* Hopkins (1.22–1.36 km/h) (Williams and Robertson 2008), and the Southern pine beetle *D. frontalis* Zimmerman (1.13–1.16 km/h) (Kinn et al. 1994). These previous studies used flight mills with different structure; therefore, the difference in flight speed might be caused by differences to the structure of the mill. Alternatively, differences in flight speed might be attributed to differences in the height of flight among species. Wind speed increases higher in the forest canopy (Baynton et al. 1965; Oliver 1971), with wind likely being stronger above the canopy compared to below it. Thus, beetles might not need to fly fast under the canopy but would need to fly fast above the canopy to achieve upwind motion, which is evidenced by beetles in the presence of host chemicals and pheromones (Chapman 1962; Gray et al. 1972; Byers 1988; Salom and McLean 1991). I hypothesized that *P. quercivorus* flies above the forest canopy because they use leaf volatiles to select suitable hosts (Pham et al. 2019). Further studies are necessary to measure the height at which *P. quercivorus* fly in the forest, including above the canopy.

While males and females exhibited similar initial and terminal flight speeds, they adjusted these speeds differently (Fig. 2.3c, d). Figure 2.4 shows the representative patterns of beetle flight estimated by this study. Compared to the average flight pattern, a simultaneous increase in initial and terminal speed enabled males to fly longer distances (Fig. 2.4a, which corresponds to changes from the center to top right in Fig. 2.3c). In comparison, compared to the average flight pattern, a simultaneous decrease in initial and terminal speed resulted in males flying shorter distances (Fig. 2.4b, which corresponds to changes from the center to bottom left in Fig. 2.3c). I hypothesized that initial flight speed is a relative measure of energy allocated to flight, while terminal speed is a relative measure of energy saved for other tasks. Thus, flight and other tasks were equally important for males. An additional increase in initial flight speed enabled females to fly

greater distances (Fig. 2.4d). In comparison, an additional decrease in terminal flight speed and a negligible decrease in initial flight speed resulted in shorter flight distances (Fig. 2.4e). Thus, flight was more important than other tasks for females.

Flight mill experiments were conducted in a laboratory where information on host trees (such as chemical volatiles) was not available. Therefore, the flight patterns observed in this study could be interpreted as the behaviors of beetles in situations where they could not locate host trees. Males are the pioneering sex (Kobayashi et al. 2001) and females are the following sex (Ueda and Kobayashi 2001) in the host location and colonization process for *P. quercivorus*. After dispersal, pioneer males should carefully select suitable host trees to increase their fitness. In comparison, it might be easier for females to select hosts because they can use the aggregation pheromone emitted by pioneer males, which ensures they reach suitable hosts. If males cannot find host trees, they might stop flying earlier to save energy for other tasks, whereas females might invest more energy in flight to continue searching for a find host. These suggestions are based on the hypothesis that the energetic status of beetles is represented by flight speed. Additional studies on the relationship between the flight speed of beetles and energy status are required to verify this suggestion. Body weight (Chen et al. 2011; Evenden et al. 2014) and lipid content (Atkins 1969; Thompson and Bennett 1971; Williams and Robertson 2008; Chen et al. 2011; Evenden et al. 2014) were typically used to measure the energy status of bark and ambrosia beetles in previous studies. The relationship between these measures and flight speed should be tested for *P. quercivorus*, at least.

In conclusion, the results confirm that sexual differences exist to increase flight distance in the life history strategy of *P. quercivorus*. Studying the flight ecology of beetles in the field is difficult; however, this information could be used to interpret the results of the current study more appropriately.

Table 2.1 Tree species from which the ambrosia beetle *Platypus quercivorus* was collected and the sampling locations.

Year	City	Latitude	Longitude	Tree species
2015	Toyooka, Hyogo Pref.	35°30.44'N	134°37.58'E	<i>Quercus crispula</i>
2016	Joyo, Kyoto Pref.	34°51.34'N	135°47.50'E	<i>Quercus serrata</i>
2016, 2018	Sanda, Hyogo Pref.	34°54.72'N	135°16.45'E	<i>Quercus serrata</i>
2019	Akashi, Hyogo Pref.	34°41.98'N	134°55.62'E	<i>Quercus serrata</i>

Table 2.2 Number of ambrosia beetle *Platypus quercivorus* individuals used for the flight measurements. In 2015, the flight properties of beetles were measured after testing their response to light. In 2016, 2018 and 2019, flight measurements were completed after testing their response to leaf volatiles.

Year	Males	Females	Total	Test before flight	Data source
2015	12	12	24	Response to light	Pham et al. (2017)
2016	35	48	83	Response to volatiles	Pham et al. (2019)
2018	8	18	26	Response to volatiles	Pham et al. (unpublished)
2019	2	4	6	Response to volatiles	Pham et al. (unpublished)
Total	57	82	139		

Table 2.3 Model selection process for predicting the flight distance of *Platypus quercivorus*. A general additive mixed model was constructed using flight duration, initial flight speed, terminal flight speed, and beetle sex as candidate explanatory variables. Model composed of a linear part and non-linear part, and two-dimensional smooth effect of initial flight and terminal flight speed was assumed for non-linear part. The year of experiments, 2015, 2016, 2018 and 2019, was included in the model as random intercepts. Terms were dropped from the model, once at a time, to identify the lowest AIC (Akaike Information Criterion). Bold values of the AIC show the lowest value.

Linear part	Non-linear part	AIC
FlightDur+Sex+FlightDur:Sex	s(InitSpeed,TermSpeed,by=Sex) ^{*1}	410.3
FlightDur+Sex	s(InitSpeed,TermSpeed,by=Sex)	406.3
FlightDur	s(InitSpeed,TermSpeed,by=Sex)	402.5
FlightDur	-	409.5
-	-	871.0

FlightDur: Flight duration (hours) of beetles in the flight mill

Sex: Beetle sex, male or female

InitSpeed: Initial flight speed of beetles in the flight mill

TermSpeed: Terminal flight speed of beetles in the flight mill

*1: Two-dimensional smooth effect of initial speed and terminal speed was estimated for each sex separately

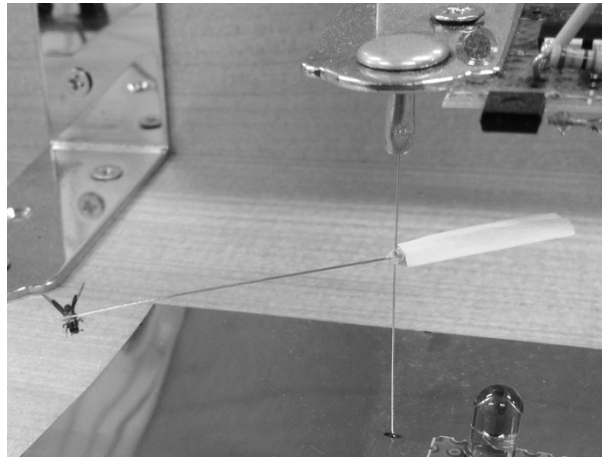


Fig. 2.1 Flight mill used in this study. Ambrosia beetles *Platypus quercivorus* were attached to the tip of the thin needle, and the rotation of the needle caused by flight was detected by an infrared LED and photodiode sensor. See Okada et al. (2018) for details.

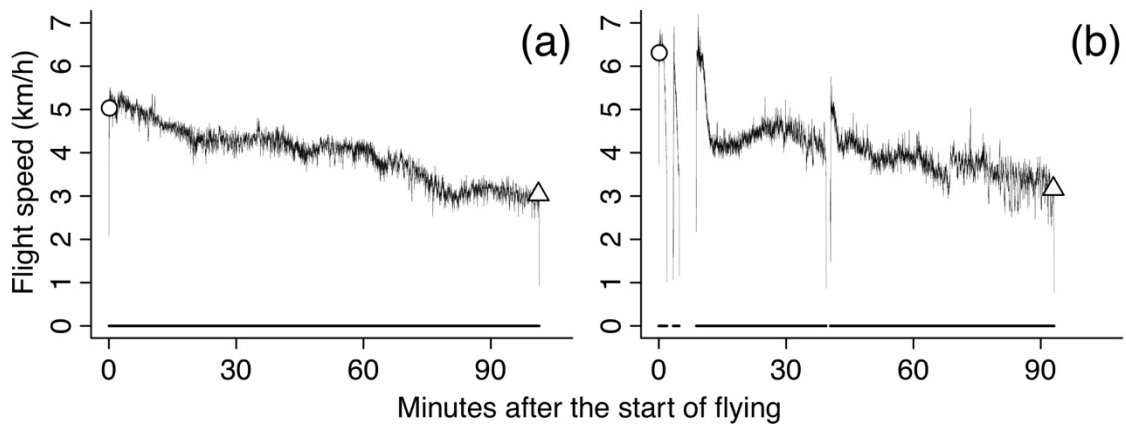


Fig. 2.2 Changes to the flight speed of *Platypus quercivorus* during flight in the flight mill. Data on individuals that showed continuous flight (a) and segmented flight (b) are shown. Flight speed was calculated for each rotation of a beetle in the flight mill, and the initial speed of flight was calculated by averaging the speed of the 31st to 40th rotations from the first rotation (circle at the start of flight). Terminal speed of flight was calculated by averaging the speed of the 31st to 40th rotations from the last rotation (triangle at the end of flight). Flight time was defined as the time from the start to the end of flight (bars at the bottom of the graph). For segmented flight (b), flight time was calculated by summing the time of each segment, while the initial speed of the first segment and terminal speed of the last segment were used as the initial and terminal speed, respectively.

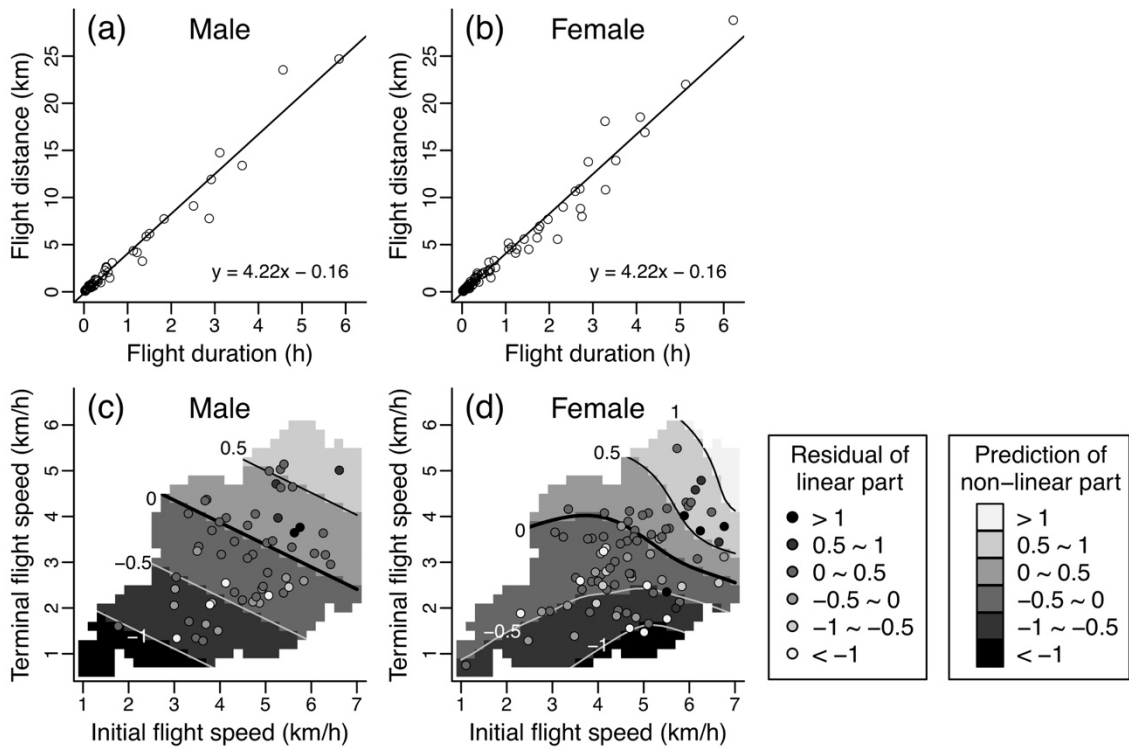


Fig. 2.3 Results of a general additive mixed model predicting the flight distance of *Platypus quercivorus*. Relationship between flight duration and flight distance for males (a) and females (b). The same regression lines, which correspond to the linear part of the model, were added to these slides. Relationships between initial flight speed and terminal flight speed for males (c) and females (d). In (c) and (d), each point was colored a different shade of gray based on the residuals of the linear part of the model (a and b). Background color shows the prediction of the non-linear part of the model, in which changes from dark to light grey correspond to an increase in predicted values. Dark color points on a light background, and light color points on a dark background show good fit of the model.

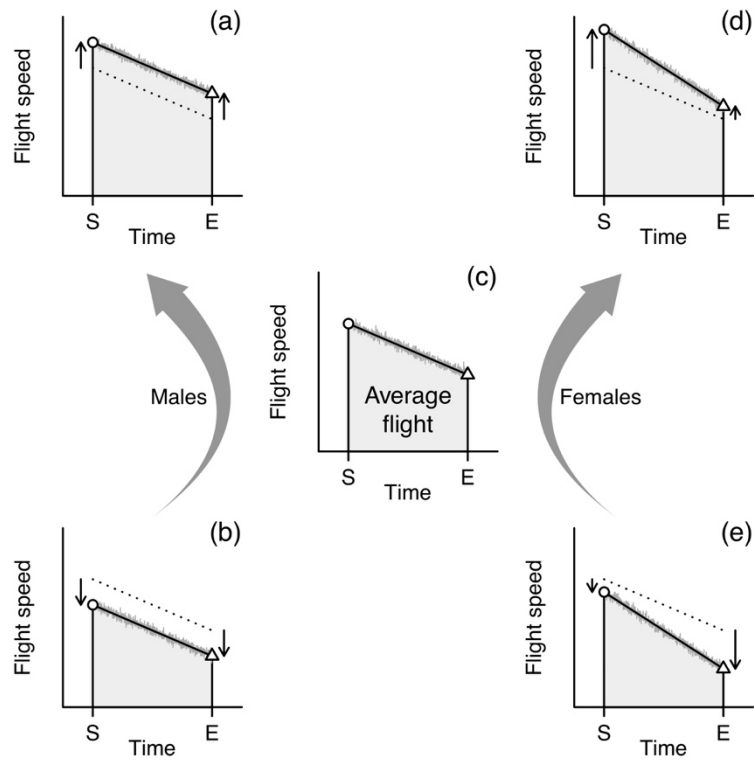


Fig. 2.4 Suggested representative patterns of flight of *Platypus quercivorus*, showing changes to flight speed from the start (“S” in X axis) to end (“E” in X axis) of flight. Flight duration was fixed for all patterns. The area of each polygon corresponded to flight distance. (c) Typical average flight. For males, (a) a longer flight distance compared to average flight was yielded by a simultaneous increase in initial and terminal flight speed, and (b) a shorter flight distance compared to average flight was yielded by a simultaneous decrease in initial and terminal flight speed. For females, (d) an increase in distance was yielded by increasing the initial flight speed relatively more as compared to terminal flight speed, and (e) a decrease in distance was yielded by decreasing the terminal flight speed relatively more as compared to initial flight speed. Dotted lines in (a), (b), (d), (e) show the pattern of average flight.

Chapter 3

Effects of flight on phototactic behavior of *Platypus quercivorus*

Introduction

Light affects the behavior and development of insect species in a variety of ways (Shimoda and Honda 2013). Phototaxis is one of the most common behavioral responses observed in various insect groups including Coleoptera (Perttunen 1958; Graham 1959; Henson 1962; Atkins 1966b; Francia and Graham 1967; Chen et al. 2013; Jiuxuan et al. 2013; Kim et al. 2013; Jiuxuan et al. 2015), Hymenoptera (Menzel and Greggers 1985; Chen et al. 2012; Symonowicz et al. 2015; Chen et al. 2016), Hemiptera (Butler 1938; Coombe 1981; Summers 1997), Lepidoptera (Yang et al. 2012; Sun et al. 2014). The movement of insects toward and away from a light source is defined as positive and negative phototactic behavior, respectively (Jander 1963). The phototactic behavior of insects is influenced by multiple factors, including light intensity (Igeta et al. 2003; Chen et al. 2012; Chen et al. 2016), wavelength (Coombe 1981; Antignus 2000; Yang et al. 2003; Chen et al. 2012), and color (Depickère et al. 2004, Werle et al. 2014), or a combination of light color and intensity (Chen et al. 2013; Chen et al. 2016; Luo and Chen 2016). The ultimate factors driving phototactic behavior include finding suitable food sources (Ruxton et al. 2004; Nissinen et al. 2008; Sétamou et al. 2012), finding mates (Lum and Flaherty 1970; Leonard and Córdoba-Aguilar 2010), and dispersal (Doukas and Payne 2007; Chen et al. 2010).

Phototactic behavior has been confirmed in bark beetles (Choudhury and Kennedy 1980; Chen et al. 2010) and ambrosia beetles (Graham 1959; Igeta et al. 2003; Nam and Choi 2014), which are two large groups of forest insects that bore holes into trees throughout the world (Ciesla 2011). They generally exploit weakened or dead trees (Wood 1982; Ranger et al. 2010) and occasionally attack living, apparently healthy trees as their hosts (Rudinsky 1962). These insects may cause severe mass mortality of host trees by introducing pathogenic fungi (Paine et al. 1997). In some beetles, phototactic behavior is not constant but varies depending on whether it occurs before or after dispersal. For example, the bark beetle *Scolytus multistriatus* (Marsham) (Coleoptera: Scolytidae)

exhibits a reduced response to light after flight and begins instead to respond to pheromones (Choudhury and Kennedy 1980).

The ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodinae) is a known vector of the fungal pathogen, *Raffaelea quercivora* Kubono et Shin-Ito (Ophiostomatales: Ophiostomataceae), which causes Japanese oak wilt (JOW) (Kubono and Ito 2002; Kobayashi and Ueda 2005). In Japan, JOW has caused high levels of oak mortality (Kubono and Ito 2002; Kinuura and Kobayashi 2006; Hamaguchi and Goto 2010). This disease has expanded steadily throughout Japan since 1980, as *P. quercivorus* has become a primary pest that uses healthy trees as hosts (Ito and Yamada 1998; Kobayashi and Ueda 2005). Adult male and female *P. quercivorus* leave their galleries to find new host trees between June and October. Males are the pioneering sex in locating hosts and undertaking the colonization process for *P. quercivorus*. Upon reaching a suitable host tree, males release an aggregation pheromone that attracts both sexes of conspecifics, leading to mass attack of fagaceous trees (Kashiwagi et al. 2006). Following tunnel excavation, adult females deposit eggs at the terminal ends of the galleries. The larvae feed on the symbiotic fungus carried by adult females. They spend their life cycle within the gallery system that originates from the entrance holes bored by male beetles. Dispersal of new adults from infested trees begins in June (Soné et al. 1998).

The phototactic behavior of *Platypus* spp. has been studied previously. Igeta et al. (2003) reported that *P. quercivorus* expresses positive phototactic behavior in both field and laboratory experiments. The ambrosia beetle *Platypus koryoensis* (Murayama) (Coleoptera: Platypodidae) exhibits positive phototactic behavior during flight (Nam and Choi 2014). However, it is not clear whether this taxis has an ecologically adaptive significance for the *Platypus* beetles. When trees are attacked *en masse* by *P. quercivorus*, they die, leaving gaps in the forest canopy. I assumed that when a new generation of beetles emerges from source trees, positive phototaxis is necessary for *P. quercivorus* to fly directly up to the canopy gap as the first step of their dispersal. Additionally, I assumed that positive phototaxis may decrease after flight because it is not necessary for the detection of host trees. The first hypothesis was supported by a previous field study that showed low frequency of attack by *P. quercivorus* in close vicinity to their dispersal source (Yamasaki et al. 2016). In this study, I tested the second hypothesis by conducting

a laboratory experiment.

The objective of this study was to test the effect of flight status (before or after flight) on the phototactic behavior of *P. quercivorus* under laboratory conditions. Among factors other than flight status that may affect the phototactic behavior of *P. quercivorus*, we focused on beetle sensitivity to light, sex, time beetles were stored in spitz tubes before experiments, and flight duration. Flight activity has been thought to cause some physiological changes in bark and ambrosia beetles, for example, changes in responsiveness to chemical or other types of stimuli (Graham 1959, Atkins 1969, Bennett and Borden 1971). I assumed that the time it takes beetles to respond to light stimulus and arrive at the light or dark side of the phototactic apparatus is a measure of beetle light sensitivity status, which may change after flight activity. Differences between the sexes in phototactic behavior have not previously been observed in *P. quercivorus* (Igeta et al. 2003). However, there may be sexual differences in how this behavior changes before and after flight, since the ecological role of adult beetles differs between the sexes: males of *P. quercivorus* fly first to the host tree, while females arrive later (Kobayashi et al. 2001b). Additionally, in the present study, I stored emerged adults in spitz tubes under laboratory conditions until they were used for phototactic experiments, which is unlike natural conditions. Beetle flight was simulated using a flight mill and changes in phototaxis may depend on flight duration. It is important to take possible sources of variation in phototactic behavior into account in the model to predict the probability of positive phototaxis. The findings of this study would aid in the understanding of the dispersal ecology of *P. quercivorus* in the field.

Materials and methods

Source tree and beetles

Platypus quercivorus adults were collected from a dead *Quercus crispula* Blume (Fagales: Fagaceae) tree attacked by the beetles in 2014 and located in a secondary forest of Toyooka City, Hyogo Prefecture, Japan (35°30.44'N, 134°37.58'E), on 14 June 2015. The tree was cut down, and the stump was dug up and transported to Kitashirawa Experimental Station, Field Science Education and Research Center, Kyoto University (35°01.44'N, 135°47.15'E). The stump, 80 cm in diameter at aboveground height, was sawn into three logs, and spitz tubes (polyethylene, 16 mm in diameter and 100 mm in length) were attached to all beetle entrance holes found on the bark surface. The three logs were then placed at the side of the nursery in the experimental station. Adult emergence was monitored daily from 25 June to 8 August 2015, with individual adults were collected every morning. On the day of the phototactic experiment, the beetles that emerged in the morning of that day were brought to the laboratory and maintained in the spitz tubes until being used for phototactic tests at room temperature (25°C). The time spent in the spitz tube prior to phototactic bioassays was calculated for each beetle, assuming that all adults emerged from entrance holes at 7:00 am on the day of the experiment.

Phototactic apparatus

Phototactic behavior of *P. quercivorus* was examined using a light-dark alternative apparatus constructed according to Igeta et al. (2003) (Fig. 3.1). The apparatus consisted of a 120-mm-long transparent plastic tube. The basal portion of a pipette tip (5 mm in diameter at entrance, 20 mm long) was attached to a round hole bored at the midpoint of the plastic tube to enable insertion the beetle. The diameter of the plastic tube (9.6 mm) was large enough for an adult beetle to freely change direction. Each end of the plastic tube was connected to a glass bottle (14 mm in diameter at the entrance). One bottle was covered with black adhesive tape and the bottom of the other bottle was illuminated from the side by a halogen lamp (Luminar Ace LA-100 USW) using a fiber optic light guide.

Light intensities were measured at the midpoint of the plastic tube, where the pipette tip was attached, with the light sensor aimed towards the dark and light sides of the phototactic apparatus, and were 0.84 and 41.6 lx, respectively. These intensities were maintained consistently throughout the experiments. The experimental apparatus was shaded with black cloth to simulate a constantly dark environment.

Phototactic experiments

The degree of sensitivity to light may differ among individuals of *P. quercivorus*, which are probably habituated by continuous light stimulation. To obtain reliable measurements of and prevent any influence of light adaptation on beetle phototactic behavior, each phototactic testing regime was repeated ten times in succession, both before and after flight, for a total of 20 repetitions for each individual.

On the day of the experiment, *P. quercivorus* beetles that had emerged in the morning of that day were carried to the laboratory and their phototactic behavioral responses were observed. The beetles that actively moved in the spitz tubes were selected for phototactic tests. The light was turned on immediately after a single *P. quercivorus* beetle was released into a pipette tip. The phototactic behavioral response was determined based on which end of the plastic tube pathway, the light or dark side, the beetle finally reached. The distance from the midpoint to each end of the plastic tube pathway was 60 mm (Fig. 3.1). The time it took for a beetle to respond to the stimulus after release into the apparatus from the midpoint of the plastic tube and to arrive at the light or dark side of the phototactic apparatus was recorded for each trial (Fig. 3.1).

Flight mill and flight bioassays

To test the phototactic behavior of *P. quercivorus* adults after flight, a flight mill was used (Fig. 3.2). After ten sequential repetitions of the light-dark choice experiment, beetles were cooled on ice for 30 seconds, and then, the top of the beetle's pronotum was carefully tethered to the straight tip of a thin needle (0.25 mm in diameter, 40 mm long) using instant adhesive glue (Aron Alpha Super Jelly, Konishi Co. Ltd., Japan), so as to

not impede elytral movement (Fig. 3.2a). The needle was affixed perpendicular to another similarly sized mill arm. The needle, with the beetle, was attached to the apparatus. Each rotation of the beetle was detected by an infrared LED and a sensor (Fig. 3.2b). The sensor was connected to an AD converter with data logger (U3HV-LJ Sumitomo Precision Product. Co. Ltd.). The logged data were processed to calculate flight time. The voltage value of the sensor was transmitted to the computer in 1/1000 second intervals. The beetle's rotations—passings of the beetle between the LED and the sensor—were detected using the pulsed voltage, and the flight duration was calculated by summation of the interval time of each pulsed voltage. Sequential rotations of more than 60 seconds were regarded a flight. At the end of each flight, beetles were removed from the flight mill, the tether was detached, and phototactic behavior of *P. quercivorus* was immediately re-measured. In total, 66 *P. quercivorus* individuals were tested, 6 of which did not fly in the flight mill. Re-measurements of phototactic behavior were also done for these 6 individuals after being removed from the flight mill. Both phototactic and flight experiments were performed in the laboratory at a mean temperature of 25°C and under illumination with a fluorescent light source, though the phototactic experiments were conducted in the dark as described above.

Model construction and selection

We modeled the probability of positive phototaxis of *P. quercivorus* by constructing a generalized linear mixed model (GLMM) with the *lme4* package in R, version 3.3.1 (R Core Team 2016). The response variable used in this model was the side that beetles selected at each test (“1” for light side and “0” for dark side). As the response variable follows a binomial distribution, I set the link function of the model as a logit link. Flight status (before or after flight), response time (the time it took for a beetle to respond to the stimulus after introduction into the apparatus from the midpoint of the plastic tube and to arrive at either side of the phototactic apparatus), beetle sex (male or female), storage time (time that a beetle was kept in the spitz tube prior to the phototactic experiment), flight duration (hours that a beetle flew in the flight mill), and the interaction term of beetle sex and flight status were specified as candidate explanatory variables.

Individual beetle number nested within the experiment date was incorporated into the model as a random factor because the probability of positive phototaxis of the same individual, or beetles that emerged on the same date, were likely to be more correlated to each other than to data taken from different individuals or beetles that emerged on different dates. Models were constructed using data from the 60 individuals (25 male and 35 female beetles) that flew in the flight mill. Pearson's correlation coefficients among continuous variables were calculated to identify and exclude highly correlated ($|r| \geq 0.6$) variables. No variable was highly correlated, and thus none was excluded from candidate explanatory variables. Using different combinations of the 6 candidate explanatory variables, 40 candidate models were constructed. Among these, the model with the lowest Akaike's Information Criterion (AIC) was selected as the best-fit model. In addition, the relative variable importance (RVI) of each candidate explanatory variable was calculated using Akaike weights for the 40 models. Comparisons of AIC and calculations of RVI were carried out using the *MuMIn* package in R, version 3.3.1 (R Core Team 2016).

Results

The time that the *P. quercivorus* beetles were stored in spitz tubes until phototactic experiments were conducted was 179.48 ± 17.53 minutes (mean $\pm$ SE). The flight duration of 60 individuals in the flight mill was 106.41 ± 17.53 minutes. The time it took for the beetles to respond to the stimulus after release into the apparatus from the midpoint of the plastic tube and to arrive at the light or dark side of the phototactic apparatus before and after simulated flight in the flight mill was 24.66 ± 5.37 and 30.83 ± 7.09 seconds, respectively.

Changes in proportions of trials where male and female *P. quercivorus* selected the light side of the phototactic apparatus before and after flight are shown in Fig. 3.3. In general, the majority of beetles selected the light side more frequently than the dark side regardless of their flight status: the proportion of trials in which beetles selected the light side of the phototactic apparatus was more than 0.5 in most cases (Fig. 3.3). However, the proportion of trials in which beetles selected the light side decreased from 0.92 ± 0.02 (mean $\pm$ SE) to 0.85 ± 0.03 after flight. The number of beetles that showed decreased light-side selection after flight (13 males and 14 females, Fig. 3.3a, d) was greater than that of beetles that showed increased light-side selection after flight (5 males and 7 females, Fig. 3.3b, e), while no change in light-side selection after flight was observed in 21 beetles (7 males and 14 females, Fig. 3.3c, f). Sexual differences were not observed in the pattern of changes in phototactic behavior.

Six individuals of *P. quercivorus* did not fly in the flight mill, and the change in proportions of trials where these 6 individuals selected the light side of the phototactic apparatus before and after being attached to the flight mill is shown in Fig. 3.4. Three males were attached to the flight mill for 29, 100, and 109 minutes, respectively, and they always selected the light side regardless of whether or not they had been attached to the flight mill (Fig. 3.4a). Three females were attached to the flight mill for 51, 57, and 194 minutes, respectively. Though the pattern of change in proportions of trials in which the female beetles selected the light side before and after being attached to the flight mill differed among the 3 individuals, the proportions were always equal to or higher than 0.8, regardless of whether or not they had been attached to the flight mill (Fig. 3.4b).

Relationships between the proportion of trials in which beetles selected the light

side and 3 candidate explanatory variables, response time, storage time, and flight duration, are shown in Fig. 3.5. No clear increasing or decreasing trends were observed in the proportions with increases in these variables.

Among the 40 candidate models, the model including only flight status as an explanatory variable was considered the best-fit model to predict the probability of positive phototaxis (Table 3.1). The other 5 variables, response time, beetle sex, storage time, flight duration, and the interaction term of beetle sex and flight status, were dropped from the model to predict the probability of positive phototaxis. RVIs of these 5 explanatory variables were lower than 0.4, while that of beetle flight status was 1.00 (Table 3.1).

Discussion

The results of the present study confirmed the positive phototactic behavior of *P. quercivorus*, supporting previous work (Igeta et al. 2003): the proportion of trials in which beetles selected the light side was more than 0.5 (Fig. 3.3). The results also showed that the probability of positive phototaxis was significantly lower after flight than before: in the model to predict the probability of positive phototaxis, the coefficient estimated for post-flight was significantly lower than zero ($Z = -4.21$, $P < 0.001$, Table 3.1). The RVI of flight status was relatively high (Table 3.1). Negligible changes in positive phototactic behavior in beetles that did not fly in the flight mill (Fig. 3.4) showed that the decrease in positive phototaxis observed in beetles that flew in the flight mill (Fig. 3.3) was caused by the flight, not by the stress of handling. These results support the hypothesis that the positive phototaxis of *P. quercivorus* decreases after flight. Positive phototaxis is expected to be more important for the dispersal of *P. quercivorus* than for host selection.

Choudhury and Kennedy (1980) reported that the behavior of the bark beetle, *S. multistriatus*, was affected by both phototactic and chemical cues, but that their response to a light source was reduced after flight; at this point, the bark beetle starts to respond to pheromone cues. Similarly, Blackmer and Phelan (1991) demonstrated that, after a period of vertical flight, the phototactic behavior of the dried fruit beetle *Carpophilus hemipterus* (Linnaeus) (Coleoptera: Nitidulidae), declined and shifted to a vegetative response. The physiological cause of these changes in response to light is thought to flight performance (Graham 1959), which is of great importance to the decline in positive phototactic response of the ambrosia beetle *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae). The proximate cause of changes may be a reduction in lipid content, which was reported in the mountain pine beetle *Dendroctonus ponderosa* Hopkins (Coleoptera: Scolytidae) (Evenden et al. 2014), and the Douglas-fir beetle *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Scolytidae) (Atkins 1969), flown in a flight mill, as compared to beetles not given the opportunity to fly.

On average, the response time of beetles after flight was 5.17 seconds longer than that before flight. This result suggests that the change in light sensitivity in *P. quercivorus* resulted from flight activity. However, this decrease in sensitivity may not be a factor leading to the change in phototactic behavior of *P. quercivorus*, because this parameter

was not included as an explanatory variable in the model to predict the probability of positive phototaxis of *P. quercivorus* (Table 3.1). In fact, the beetles continued to show a high proportion of light-side selection, even when they took more than 2 minutes to respond to light (Fig. 3.5a).

Evenden et al. (2014) showed a decrease in body lipid content in *D. ponderosa* with an increase in flight distance, suggesting a gradual decrease in energy during flight. Therefore, I assumed a negative effect of flight duration on the phototactic behavior of *P. quercivorus*. This assumption was not supported in this study, because no clear relationship was observed between flight duration and phototactic behavior (Fig. 3.5c), and flight time was not selected as an explanatory variable to predict the probability of positive phototaxis of *P. quercivorus* (Table 3.1). A threshold value of lipid content may exist, below which the phototactic behavior of *P. quercivorus* may change, without showing a gradual change. Future studies are necessary to test this hypothesis.

In the present study, there was a wide variation in the time that individual *P. quercivorus* beetles were confined to spitz tubes in laboratory conditions, between being collected from logs until the start of phototactic experiments (over 8 hours at maximum, Fig. 3.5b). This treatment is completely unnatural for the beetles, and was hypothesized to have had a negative effect on the phototactic behavior of *P. quercivorus*. However, this parameter was excluded from the best-fit model to predict the probability of positive phototaxis (Table 3.1). Insects are known to possess a remarkable capacity to adapt rapidly to suboptimal conditions owing to their complex neural system, which carries information from head sensors to motor organs to deal with situations such as gaps, barriers, escape from enemies, and movement towards an attractant source (Ritzmann and Buschges 2007). Therefore, the phototactic behavior of *P. quercivorus* may be robust and not easily altered by containment in spitz tubes.

Beetle sex and the interaction term of beetle sex and flight status were not selected as explanatory variables for predicting probability of positive phototaxis of *P. quercivorus* (Table 3.1). This result showed (1) positive phototactic behavior is not different between male and female beetles, and (2) positive phototaxis decreases in the same way for both sexes of *P. quercivorus* after flight. The first finding confirms the results of a previous study that reported a non-significant difference in positive phototactic behavior between

P. quercivorus males and females (Igeta et al. 2003). The second finding suggests that the light sensitivity decreases after flight for both sexes, though it is equally important for the two sexes immediately after emergence.

My present study reveals that positive phototaxis decreases after flight in *P. quercivorus*. The results suggest that this taxis has an adaptive significance in the dispersal of the beetle immediately after emergence. The findings of this study lay the foundation for future investigations of host selection behavior by *P. quercivorus* under more natural conditions.

Table 3.1 Results of a generalized linear mixed model (GLMM) that predicts the probability of positive phototaxis of the ambrosia beetle *Platypus quercivorus* (n = 1200, AIC = 740.7). Estimated coefficients, standard errors (SE), Z-statistics, and P-values are shown. Significance of differences from 0 was tested using Z-statistics. RVI (relative variable importance) was calculated using Akaike weights of all candidate models. Individual beetle numbers nested within the date of experiment were incorporated into the model as random intercepts. Variables without estimates were not selected as explanatory variables for the best-fit model.

Predictor variable	Estimate	SE	Z	P	RVI
Flight status	-0.887	0.211	-4.21	< 0.001	1.00
Response time	-	-	-	-	0.28
Beetle sex	-	-	-	-	0.34
Storage time	-	-	-	-	0.28
Flight duration	-	-	-	-	0.28
Beetle sex × flight status	-	-	-	-	0.09

Flight status: before or after beetles flew in the flight mill. A negative estimate shows that the probability of positive phototaxis of beetles after flight was lower than that of beetles before flight.

Response time: the time it took for beetles to respond to stimulus after being introduced into the apparatus from the midpoint of the plastic tube and to arrive at the light or dark side of the phototactic apparatus

Beetle sex: male or female

Storage time: time that beetles were kept in spitz tubes prior to phototactic experiments

Flight duration: hours that beetles flew in the flight mill

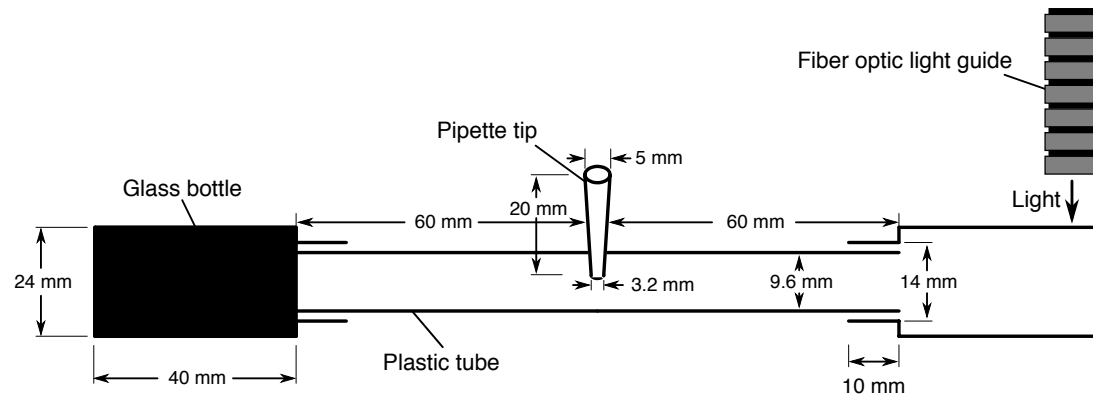


Fig. 3.1 Schematic representation of the apparatus used in this study to test the phototactic behavior of the ambrosia beetle *Platypus quercivorus*. The light (right) side of the apparatus was illuminated with a halogen lamp using a fiber optic light guide, whilst the dark (left) side of the apparatus was covered by black tape. An individual beetle was inserted into the apparatus through a pipette tip located at the midpoint of a plastic tube.

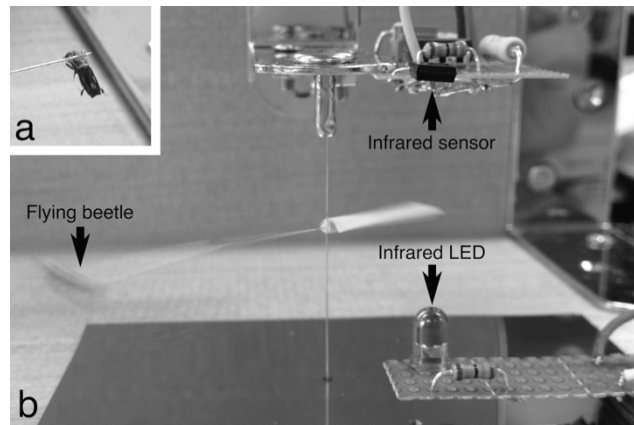


Fig. 3.2 Flight mill apparatus. a An individual beetle was attached to the needle tip of the flight mill apparatus. b The needle with beetle was attached to the apparatus. The rotation of the beetle was detected with an infrared LED and infrared sensor.

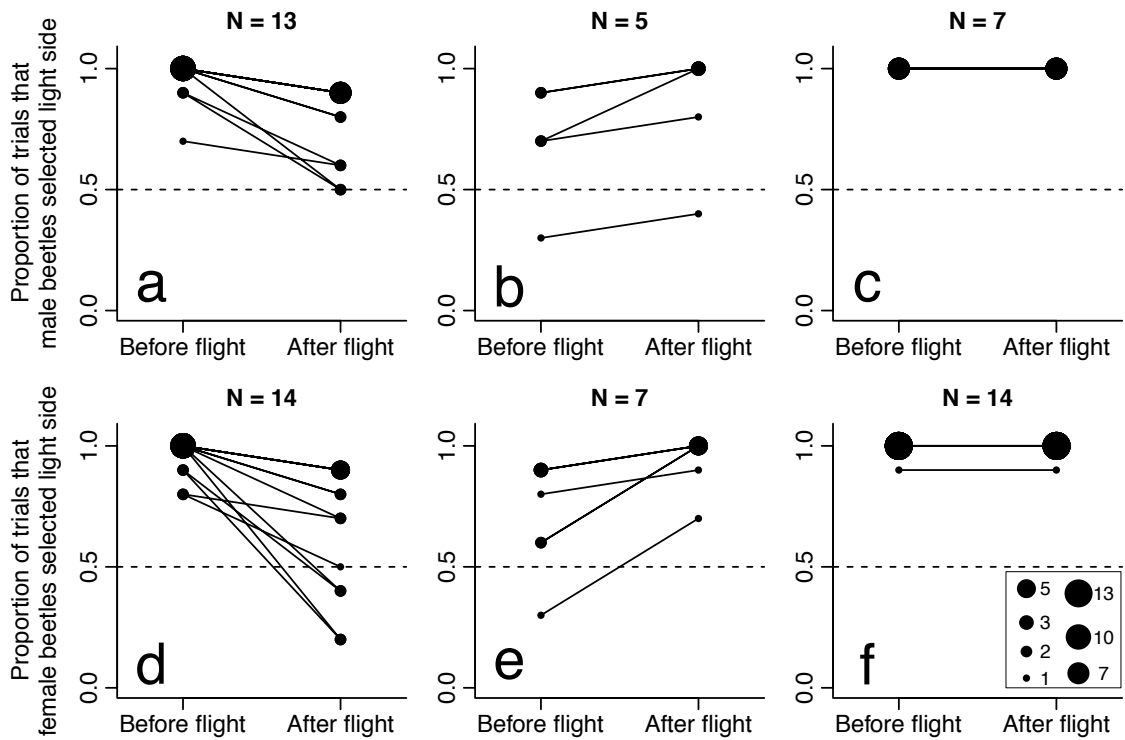


Fig. 3.3 Proportion of trials in which individual beetles selected the light side of the phototactic apparatus in repeated runs, in testing of their phototactic behavior. Tests were conducted 20 times for 60 beetles (25 males and 35 females), 10 times per individual, before and after flight in the flight mill apparatus. Values greater than 0.5 (dashed lines) show positive phototaxis. The size of the symbol represents the number of beetles on a logarithmic scale. Data for the same beetles are connected by lines. a, b, c Data for male beetles that showed a decrease, increase, or no change in their proportions of light-side selection, respectively. d, e, f Data for female beetles that showed a decrease, increase, or no change in their proportions of light-side selection, respectively.

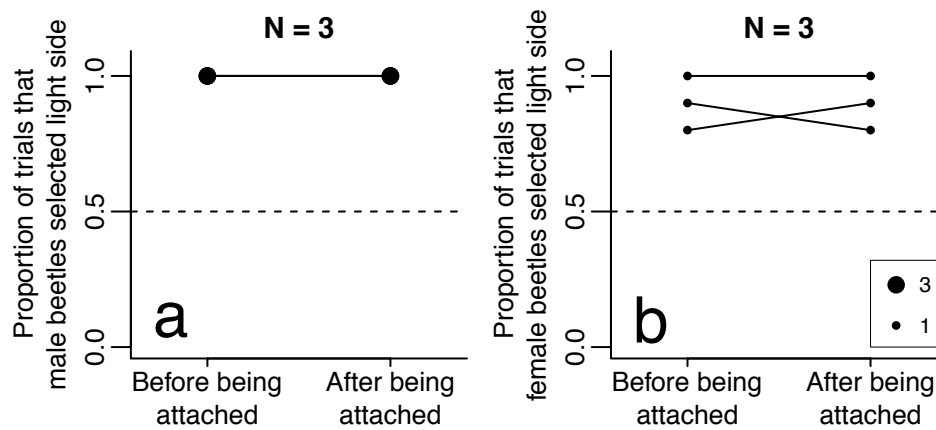


Fig. 3.4 Proportion of trials in which individual beetles selected the light side of the phototactic apparatus in repeated runs, in testing of their phototactic behavior. Tests were conducted 20 times for 6 beetles (3 males and 3 females), 10 times per individual, before and after being attached to the flight mill. Values greater than 0.5 (dashed lines) show positive phototaxis. The size of the symbol indicates the number of beetles on a logarithmic scale. Data for the same beetles were connected by lines. a Data for male beetles. b Data for female beetles.

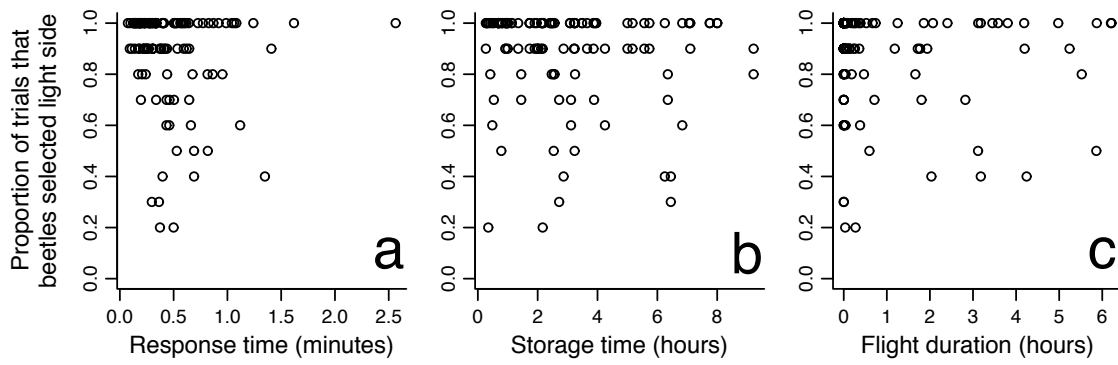


Fig. 3.5 Proportion of trials that individual beetles selected the light side of the phototactic apparatus, in testing of their phototactic behavior. Sixty individuals (25 males and 35 females) were used. In all, 120 points of data (proportions calculated for each of 10 trials, before and after flight, for 60 beetles) were plotted for the purpose of data presentation, while the raw data (1200 trials) were used for data analysis. See text for details. a Relationship between the time it took for beetles to respond to the stimulus after being released into the phototactic apparatus from the midpoint of the plastic tube and to arrive at the light or dark side of the phototactic apparatus and proportions of light-side selection. The response time of 10 trials was averaged. b Relationship between the time that *Platypus quercivorus* beetles were stored in spitz tubes and proportions of light-side selection. c Relationship between flight duration of *P. quercivorus* beetles in the flight mill and proportions of light-side selection.

Chapter 4

Effects of leaf conditions on the olfactory response of *Platypus quercivorus* to leaf volatiles

Introduction

The bark and ambrosia beetles are a diverse group of forest insects that spend most of their lives underneath the bark and in the xylem of plants, respectively (Rudinsky 1962), and sometimes they cause severe damage to forest trees (Ciesla 2011). Generally, these beetles feed on either the plant phloem tissues or symbiotic ambrosia fungi, which are the sole source of nutrition for the larvae and adults (e.g., Baker and Norris, 1968; Fernando and Richard 2015). However, their habitats are often temporarily available, and the emerging adults must locate new hosts for breeding, egg laying, and introducing the fungus into host trees. Chemical volatiles are key cues that a variety of scolytid and platypodid beetles use to locate their hosts. For example, ethanol, produced from the deterioration of plants by environmental stresses, is considered to be a primary attractant (Galko et al. 2014; Moeck 1970; Ranger et al. 2010; Ruling and Kearby 1975). In addition, secondary attractants, which are insect-produced pheromones, also mediate host detection (Wood 1982). For instance, aggregation pheromones (Blomquist et al. 2010; Pitman and Vité 1970) and sex pheromones (Wood et al. 1966) have been identified in several bark and ambrosia beetles.

The ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) causes widespread severe wilt disease in trees—especially damaging some *Quercus* spp. in Japan—via the dissemination of the pathogenic fungi *Raffaelea quercivora* Kubono et Shin-Ito (Ophiostomatales: Ophiostomataceae) (Ito et al. 1998; Ito and Yamada 1998; Kubono and Ito 2002) among trees (Kinnura and Kobayashi 2006; Saito et al. 2001). The beetle was thought to be a secondary pest, which attacked only weakened host trees; however, it appears to have become a primary pest capable of attacking apparently healthy trees since the 1980s (Ito and Yamada 1998). *Platypus quercivorus* eggs hatch in approximately one week and the larvae grow in the wood

gallery by feeding on yeast fungi (Endoh et al. 2008a, b, c; Endoh et al. 2011). From June to October, *P. quercivorus* adults disperse from a maternal tree to find new host trees (Soné et al. 1998). The aggregation pheromone [(1S,4R)-4-isopropyl-1-methyl-2-cyclohexen-1-ol] ((-)-IMCH), called quercivorol (Kashiwagi et al. 2006), is produced by the males that initiate host attacks. Synthetic quercivorol has been confirmed to induce antennal responses in *P. quercivorus* under laboratory conditions (Tokoro et al. 2007). However, synthetic quercivorol-baited traps exhibited a low ability to capture beetles when they were away from host trees in a forest (Kamata et al. 2008; Tokoro et al. 2007), but a high ability to attract beetles when placed on the trunks of host trees (Ohashi 2013). Therefore, information about the host tree is necessary for the beetle to detect their host.

A previous study extracted volatile compounds from the wood tissues of *Quercus crispula* Blume (Fagales: Fagaceae) and examined their ability to attract *P. quercivorus*. However, wood volatiles did not attract this beetle (Takemoto et al. 2006). A recent study has reported that the redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), is attracted to the volatiles emitted from the leaves of its host plant (Martini et al. 2015). Therefore, the ambrosia beetle *P. quercivorus* might also be attracted to the leaf volatiles of its host tree. In addition to attacking trees that are already weakened, dying, or recently dead as preferable hosts (Wood 1982), the bark and ambrosia beetles are also known to damage apparently healthy living trees (Mayfield et al. 2008). However, the possible roles of host leaves and their chemical volatiles as attractants of *P. quercivorus* have not been well studied.

The males of *P. quercivorus* are pioneers in host location and colonization, during which they fly to a new host tree where they bore entrance holes into the basal area of the trunk (Kobayashi et al. 2001b). Simultaneously an aggregation pheromone is generated to call other males and females to establish a mass attack (Tokoro et al. 2007; Ueda and Kobayashi 2001). Females are followers in the arrival sequence of *P. quercivorus* (Kobayashi et al. 2001b). It is possible that conditions of leaves from attacked trees differ between when males arrive and when females arrive, but no studies have yet investigated this possibility. In this study, we hypothesized that differences in the timing of male and female arrival to trees could be due to different preferences for leaf volatiles from fresh or deteriorating leaves. Specifically, males may be more attracted to volatiles of fresh

leaves than deteriorating leaves, whereas females may not be as sensitive to fresh leaves because they are attracted to host trees after the males have landed. Moreover, we hypothesized that these preferences may change before and after migration. Males may increase their sensitivity to leaves as flight time increases and energy reserves are reduced. In contrast, females may lower their sensitivity to leaves as flight time increases, in order to locate suitable host trees by increasing relative sensitivity to the aggregation pheromone over leaf volatiles.

The objectives of this study were to clarify differences in the olfactory responses of male and female *P. quercivorus* (1) to fresh and dried leaves and (2) before and after flight. The results of the present study are expected to provide novel insights into the function of leaf volatiles in the host selection behavior of *P. quercivorus*.

Materials and methods

Plants

Leaves were obtained from a healthy *Q. crispula* tree of 39 cm diameter at breast height (DBH) planted in the Kitashirakawa Experimental Station, Field Science Education and Research Center, Kyoto University (35°01.44'N, 135°47.15'E). The leaves of *Q. crispula* were used as a source of volatiles for olfactory bioassays as this tree species is a favored host of *P. quercivorus* (Yamasaki and Futai 2012). Different deterioration statuses were simulated by drying leaves for different amounts of time after cutting (cut days). Fresh leaves (cut days = 0) were obtained by cutting the branches of *Q. crispula*, at 7 a.m. on the day of the experiment. Dry leaves (cut days = 1–9) were obtained by maintaining the branches with leaves under laboratory conditions (25°C and 70%–80% RH) for 1 to 9 d after cutting.

Beetles

Platypus quercivorus adults were obtained from two dead trees of *Q. serrata* Thumb (Fagales: Fagaceae) attacked by the beetle in 2015. One tree (63.8 cm DBH) was in an urban forest of Joyo City, Kyoto Prefecture, Japan (34°51.34'N, 135°47.50'E). The other (59.5 cm in DBH) was in an urban forest of Sanda City, Hyogo Prefecture, Japan (34°54.72'N, 135°16.45'E). The stumps of the two trees were dug up and subsequently transported to Kyoto University on May 5 and 23, 2016, respectively. Six logs were sawn from the two stumps and cross sections of each log were coated with massive glass silicone sealant, which was placed inside a net-shade house to reduce desiccation from sunlight. Emergence traps (polyethylene, 16 mm in diameter and 100 mm in length) for collecting adults were carefully attached to holes of inner diameter > 1.5 mm, reported to be entry holes bored by *P. quercivorus* (Inoue et al. 2003). The emergence of beetles was observed every morning (around 7 a.m.) from June 1 to July 17, 2016. The beetles that occurred in the traps in the morning of experimental days were immediately transferred to the laboratory for olfactory choice tests.

Experiment 1: Olfactory responses of beetles to leaf volatiles

A two-choice Y-tube olfactometer was designed to determine the olfactory responses of *P. quercivorus* to volatiles emitted from the leaves of *Q. crispula* (Fig. 4.1). The olfactometer consisted of two 250 mL fluoroplastic gas wash bottles (AS ONE Corporation, Osaka City, Japan). The bottle had an inlet for clean air and an outlet connected to either arm of the polypropylene Y-shaped tube. Air was filtered using activated charcoal, and then humidified with distilled water before introduction into the olfactometer. The water was replaced daily to exclude non-volatile particulates. The air was circulated within the olfactometer using silicon tubes (6 mm in diameter) equipped with an electric pump (MP-2N; Code: 8086-2, Sibata Scientific Technology Ltd., Kusaka city, Japan). The flow of air was calibrated before and after each experiment using a flow meter (RK-1350V; KOFLOC Co. Ltd., Kyotanabe city, Japan) to ensure that the air velocity did not adversely affect the movement of beetles. Air flow rates through each arm of Y-tubes ranged from 55–99 mL/min, with an average of 67 mL/min.

At the beginning of the bioassay, seven leaves were placed in one bottle (bioassay treatment), whereas the other bottle was empty (bioassay control). The insects were individually released into the olfactometer through the polypropylene Y-shaped tube at a 60° angle (5 mm in diameter, 27 mm in length from the point of release to the intersection of the Y-tube) (Fig. 4.1). Once inside the Y-tube, the olfactory responses of the insects were observed. An insect was considered to have made a choice between the two arms when it passed the edge of each arm (27mm from the intersection of the Y-tube). In contrast, when an insect remained at the basal part of the Y-tube and had not passed the edge of either arm within 5 min, they were considered not to have made a choice.

In this experiment, differences in the olfactory responses of *P. quercivorus* to fresh leaf volatiles (cut days = 0) and dry leaf volatiles (cut days = 1–4) were tested. Throughout the experiments, when a *P. quercivorus* individual was tested first (10 repetitions) with fresh leaf volatiles and the control, the individual was then tested (10 repetitions) with dry leaf volatiles and the control. Subsequent individuals alternated between starting with fresh or dry leaves. The type of leaves (fresh or dry) that were used first was randomly

selected on each day of the experiment. Overall, 20 trials were done for each of the 60 *P. quercivorus* individuals (32 males, 28 females) using leaves of different deterioration status (14, 14, 9, and 23 beetles for 0–1, 0–2, 0–3, and 0–4 cut days, respectively). As a consequence of the experimental sequence described above, 29 beetles were first tested using fresh leaves, and 31 beetles were first tested using dry leaves. Subsequently, the proportion of trials in which an individual beetle selected fresh leaf volatiles was compared with that in which the beetle selected dry leaf volatiles with a different deterioration status (0–1, 0–2, 0–3, and 0–4 cut days).

The location of the leaf and control bottles connected to each arm of the Y-tube was alternated after every 5 (of 10) trials to minimize potential direction bias. After the experiment, all parts of the Y-tube olfactometer were washed daily with distilled water and dried at 25°C for at least 24 h.

Experiment 2: Changes in the olfactory responses of beetles to leaf volatiles caused by flight

This experiment was conducted to evaluate the effect of previous flight activity on the olfactory response of *P. quercivorus* to host leaf volatiles (Fig. 2). Flight of *P. quercivorus* adults was simulated by gluing the beetles to a flight mill, which was developed for measuring the flight ability of this beetle (Okada et al. 2018). The main component parts of the flight mill were two thin needles attached perpendicular to each other as flight mill arms. The rotation of the tip of a horizontal arm was recorded when the arm passed through an infrared LED and photo sensor. On each day of the experiments, beetle adults were allowed to fly on a flight mill after 10 choice test trials between leaf volatiles with a different deterioration status (cut days = 0–9) and the control. Eight flight mills were used to measure the exact flight duration for each beetle. Our objective was not to estimate the flight capacity, therefore, there was no clear definition of the termination of flight. Flight experiments and the following 10 choice trials were processed simultaneously. Beetles were removed from the flight mill if they had stopped flying when we observed them at intervals during the olfactory experiments.

Among 141 *P. quercivorus* individuals (58 males and 83 females) that were

attached to the flight mill, 107 individuals (38 males and 69 females) flew for more than 1 minute and 34 individuals (20 males and 14 females) did not fly or flew for less than 1 minute. After being removed from the flight mill, olfactory behaviors were measured 10 times for all beetles, regardless of their flight duration. In summary, 20 trials of choice tests were carried out with each beetle. The bottle rotation and the Y-tube olfactometer purification were also conducted in the same way as in Experiment 1.

Some beetles showed a single long flight, and others showed multiple short flights. Flight duration was calculated from the logged data by summing the flight time for each beetle. Some beetles were left attached to the flight mill for a long time after stopping their flight. This variable, time that beetles were left on the flight mill before being removed, was also calculated from the logged data for each beetle.

Statistical analyses

We constructed a generalized linear mixed model (GLMM) to predict the probability of olfactory response of *P. quercivorus* to volatiles emitted from fresh leaves compared to those from dried leaves, using the data obtained from Experiment 1. The response variable used in this model was the side of the Y-tube olfactometer that a beetle selected at each trial with “1” for the leaf side and “0” for the control. Explanatory variables were leaf (deterioration status of leaves, represented by days after cutting), sex (male or female), and interaction between leaf and sex.

A generalized linear mixed model was also used to clarify the factors affecting the probability of olfactory response of *P. quercivorus* to leaf volatiles with different deterioration status and changes in the olfactory responses of a beetle due to flight activity, using the data obtained from Experiment 2. The response variable used for model construction was the side of the Y-tube olfactometer that a beetle selected at each trial with “1” for the leaf side and “0” for the control. Leaf (deterioration status of leaves, represented by days after cutting), sex (male or female), interaction between leaf and sex, flight (hours that beetles flew in the flight mill), and interaction between flight and sex, attached-time (the time that beetles were left on the flight mill before being removed), and interaction between attached-time and sex were specified as explanatory variables in

the model predicting the probability of leaf side selection of 107 beetles that flew in the flight experiments. Leaf, sex, interaction between leaf and sex, attachment (before or after attachment of beetles to the flight mill), interaction between attachment and sex were included as explanatory variables in the model predicting the probability of leaf side selection of 34 beetles that did not fly in the flight experiments.

The GLMM model construction was carried out using the package *lme4* in R, version 3.5.2 (R Core Team 2018). As the response variable followed a binomial distribution, we set the link function of the model as a logit link. The number of individual beetles and the date of experiment were added to the model as random intercepts to account for correlations among response variables taken from the same individual or on the same date. Pearson's correlation coefficients of the candidate explanatory variables were calculated to exclude highly correlated variables ($|r| \geq 0.6$) from the models.

Given a set of candidate models with various combinations of potential explanatory variables, the best-fit model was the one with the lowest Akaike information criterion (AIC) value. The relative variable importance (RVI) was calculated for each explanatory variable using Akaike weights. Comparison of AIC values and calculation of RVI were conducted using the package *MuMIn* in R, version 3.5.2 (R Core Team 2018).

Results

Experiment 1: Olfactory responses of beetles to leaf volatiles

In general, the proportion of trials in which each of the 60 *P. quercivorus* individuals selected fresh leaf volatiles was higher than those where the beetles selected volatiles from dry leaves with different deterioration statuses (Fig. 4.3). This trend was consistent for both males and females (Fig. 4.3).

The results of model selection are presented in Table 4.1. It shows that the leaf was always selected as an explanatory variable in the best-fit model for predicting the probability of olfactory response of *P. quercivorus* to volatiles from fresh and dry leaves. The other two variables, sex and interaction term between leaf and sex, were excluded from the model. Coefficients estimated for dried leaves compared with those estimated for fresh leaves were always significantly less than zero ($P < 0.001$) (Table 4.1). The RVI of this variable was relatively high (Table 4.1).

The probabilities of leaf side selection predicted by the model are shown in Fig. 4.4, with 95% confidence intervals. Predicted probabilities for fresh leaves were always higher than 0.5, showing that *P. quercivorus* showed significant attraction to the volatiles emitted from fresh leaves. On the contrary, predicted probabilities for dry leaves were lower than 0.5 in general, showing that beetles were not attracted to the volatiles emitted from dry leaves from the host tree (Fig. 4.4).

Experiment 2: Changes in the olfactory responses of beetles to leaf volatiles caused by flight

Flight durations of 107 *P. quercivorus* individuals on the flight mill ranged from 1.27 to 384.50 min, with an average of 86.63 min. The time that these 107 individuals were left on the flight mill before the tether was detached ranged from 2.16 to 258.50 min, with an average of 77.08 min.

The relationship between the flight duration of the 107 *P. quercivorus* individuals that flew and their olfactory responses to leaf volatiles are presented in Fig. 4.5. Two

different trends in the olfactory behavior of *P. quercivorus* were observed based on sex. A gradual increase was found in the proportion of trials in which *P. quercivorus* males selected leaf volatiles with an increase in their flight duration (Fig. 4.5a). However, a considerable decrease in this proportion was observed in *P. quercivorus* females with an increase in their flight duration (Fig. 4.5b).

Among the seven candidate explanatory variables proposed for the model to predict the probability of olfactory responses of *P. quercivorus* before and after flight experience, flight, leaf, sex, and interaction between flight and sex were adopted in the best-fit model (Table 4.2). The RVI values for these 4 variables were 0.97, 0.93, 0.90, and 0.58, respectively. Attached-time, interaction between leaf and sex, and interaction between attached-time and sex were excluded from the model (Table 4.2). The RVI values for these 3 variables were 0.36, 0.29, and 0.12, respectively.

To confirm that changes in olfactory responses were caused by flight activity, olfactory responses before and after being attached to the flight mill were compared with regard to beetles that did not fly in the flight mill. In general, no consistent trends in changes were seen in the olfactory responses of 34 non-flying individuals of *P. quercivorus* to leaf volatiles, before and after attachment to the flight mill (Fig. 4.6). Differences between sexes were not observed in the patterns of change in olfactory behavior (Fig. 4.6).

The best-fit model to predict the probability of olfactory response of non-flying *P. quercivorus* included only leaf as explanatory variable (Table 4.3). The RVI of leaf was 0.60. Sex, interaction between leaf and sex, attachment, and interaction between attachment and sex were not selected in the model (Table 4.3). The RVI values for these 4 variables were 0.44, 0.20, 0.37, and 0.06, respectively.

Discussion

The results of our experiments demonstrated that *P. quercivorus* was highly attracted to the volatiles from fresh leaves, but not to those from dry leaves (Fig. 4.4). This suggests that *P. quercivorus* is attracted to leaf volatiles from healthy host trees. Similar results have been reported for the ambrosia beetle *X. glabratus*, which was significantly attracted to leaf volatiles from intact host trees (Martini et al. 2015). Moreover, Yamasaki et al. (2014) indicated that a high host tree crown density increased the probability of attack by *P. quercivorus*. Thus, the volatiles emitted from the leaves of healthy trees might act as a guiding cue for *P. quercivorus* to locate host trees.

Different responses to ethanol between *P. quercivorus* and other bark and ambrosia beetles (Iidzuka et al. 2016; Miller and Rabaglia 2009; Montgomery and Wargo 1983) also suggest that the former may be attracted to healthy host trees. Host trees that are weakened by various factors emit large amounts of ethanol (Kelsey and Joseph 2003; Ranger et al. 2013; Robert et al. 1993), which attracts bark and ambrosia beetles (Miller and Rabaglia 2009; Montgomery and Wargo 1983). However, this chemical is present at low concentrations in healthy trees (Klimetzek et al. 1986; Ranger et al. 2010). A recent study reported that only a small number of flying *P. quercivorus* were captured by ethanol-baited traps in a forest, which was being damaged by this beetle (Iidzuka et al. 2016), suggesting that ethanol might not be an effective attractant for *P. quercivorus*. In this milieu, it is noteworthy that the fresh leaf volatiles are an important alternative that can be considered as a host kairomone for *P. quercivorus*. In this study, I used leaves detached from the branches as odor sources, therefore, we cannot exclude the possibility for potential changes in the volatile profiles or release rates of volatiles as compared to intact leaves. The attraction of beetles to fresh leaf volatiles may have been different if intact plants had been used as the odor sources. Further study using intact plants is necessary to verify these results. Additionally, studies are needed to identify the candidate leaf volatiles that account for the olfactory behaviors of this ambrosia beetle.

One of my hypotheses was that *P. quercivorus* females prefer dry leaves because they arrive at the new host trees after the males. However, this hypothesis was not supported by the results of the present study; male and female *P. quercivorus* exhibited

relatively similar patterns of attraction to fresh leaf volatiles (Fig. 4.3). The results of the best-fit model further validated this finding as the interaction between leaf status and sex was dropped from the best-fit model predicting the probability of olfactory response of *P. quercivorus* to leaf volatiles (Table 4.1). The period from the initiation of boring entry holes on the trunk of a healthy tree by pioneer males to the later arrival of females may be too short to display physiological changes in leaves and, thus, both sexes might prefer fresh leaves.

During the initial period of flight, both sexes showed a similar degree of attraction to leaf volatiles (Fig. 4.5). The estimated intercept for males compared with that of females (0.214) was not significantly different from zero when the flight duration was equal to 0 ($P = 0.260$). These results indicate that males and females were equally attracted to leaf volatiles from host trees immediately after their emergence from inhabited galleries. However, different patterns between the sexes in the response to leaf volatiles emerged when their flight duration increased (Fig. 4.5). Males increased their olfactory responses to leaf volatiles (Fig. 4.5a), whereas the opposite was observed in females (Fig. 4.5b). No clear trends were observed for changes in olfactory responses to leaf volatiles in beetles that did not fly in the flight mill (Fig. 4.6), which suggests that the changes in behaviors of the beetles to host leaf volatiles (Fig. 4.5) resulted from the flight, and were not due to a decline in the condition of beetles while attached to the flight mill. Exclusion of attached-time (i.e., the time that beetles were left on the flight mill before being removed) from the best-fit model (Table 4.2) further supports this claim.

Differences in olfactory responses between sexes may be due to the difference in ecological roles, where males undertake the key task of locating new host trees at the dispersal phase. Therefore, males should be much more responsive to the leaf volatiles. In addition, flight consumes a lot of energy, and is thought to be one of the potential causes leading to changes in the behaviors of many bark and ambrosia beetles (Atkins 1969; Bennett and Borden 1971; Evenden et al. 2014; Graham 1959; Pham et al. 2017). Under conditions of low energy reserves after a relatively long period of flight in search of a new host tree, males should be immediately responsive to host leaf volatiles. In the case of females, the aggregation pheromone (Tokoro et al. 2007) deposited by the pioneer males may function as a guiding attractant that these followers use to approach the host

trees. Therefore, the females might lower their olfactory response to leaf volatiles in order to detect the pheromone cue during migration. In this context, males may decrease their response to aggregation pheromones, and females may increase their response to aggregation pheromones after flight. This should be tested in future studies using the same experimental design as this study.

A field study conducted in a forest showed that aggregation pheromone alone is not sufficient to attract *P. quercivorus* (Kamata et al. 2008), suggesting that other information on trees is necessary for beetles to locate their host. Results of this study showed that male beetles increased their sensitivity to leaf volatiles after longer duration flights (Fig. 4.5a), suggesting that leaf volatiles provide information used by beetles to locate hosts. After approaching a host tree, however, the olfactory response of *P. quercivorus* may be affected by the combination of leaf volatiles with aggregation pheromone. At this stage, beetles could be able to quickly respond to the aggregation pheromone by lowering their sensitivity to leaf volatiles, allowing them to locate suitable positions in the tree for reproduction. Therefore, future studies should test for a possible interaction between beetle pheromones with leaf volatiles.

The results of the present study revealed that the volatiles emitted from fresh leaves of *Q. crispula* play significant roles in the host-seeking behavior of *P. quercivorus*. In addition to their role as attractants, the volatiles emitted from dry leaves function as an effective repellent of the beetle, suggesting that these volatiles could be beneficial in field trials to control *P. quercivorus*.

Table 4.1 Results of a generalized linear mixed model (GLMM) predicting the probability of leaf-side selection by the ambrosia beetle *Platypus quercivorus* (n = 1200). Estimated coefficients, standard errors (SE), Z-statistics, and P-values are shown. Significance of differences from 0 was determined using Z-statistics. The relative variable importance (RVI) was calculated using Akaike weights of all the candidate models generated. Number of individual beetles nested within the experimental day was incorporated into the model as a random intercept. Variables without estimates were not included as explanatory variables in the best-fit model.

Leaf status Comparison	Explanatory variable	Estimate	SE	Z	P	RVI
0–1	Leaf	-0.901	0.264	-3.409	< 0.001	1.00
	Sex	-	-	-	-	0.49
	Leaf:Sex	-	-	-	-	0.26
0–2	Leaf	-2.628	0.329	-7.986	< 0.001	1.00
	Sex	-	-	-	-	0.40
	Leaf:Sex	-	-	-	-	0.17
0–3	Leaf	-1.784	0.342	-5.213	< 0.001	1.00
	Sex	-	-	-	-	0.45
	Leaf:Sex	-	-	-	-	0.20
0–4	Leaf	-2.046	0.218	-9.379	< 0.001	1.00
	Sex	-	-	-	-	0.48
	Leaf:Sex	-	-	-	-	0.29

Leaf: the deterioration status of leaves, reflected by number of days after cutting. 0 indicates fresh leaves that were cut on the day of experiment. 1, 2, 3, and 4 indicate dry leaves that were cut 1 to 4 days before the experiment, respectively

Sex: male or female

Table 4.2 Results of a generalized linear mixed model (GLMM) predicting the probability of leaf-side selection by the ambrosia beetle *Platypus quercivorus* before and after flight in a flight mill (n = 2140). Thirty-five candidate models were constructed using different combinations of the seven candidate explanatory variables: flight, leaf, sex, attached-time, interaction between flight and sex, interaction between leaf and sex, and interaction between attached-time and sex. Best model with the lowest AIC is listed first, and models with delta AIC < 2 are shown below.

Generalized linear mixed model	AIC	delta AIC
Flight + Leaf + Sex + Flight:Sex	2536.98	0.00
Flight + Leaf + Sex + Flight:Sex + Leaf:Sex	2538.34	1.36
Flight + Leaf + Sex	2538.36	1.38
Flight + Leaf + Sex + Attached-time + Flight:Sex	2538.97	2.00

Flight: hours that the beetles flew in a flight mill

Leaf: the deterioration status of leaves, reflected by different number of days after cutting

Sex: male or female

Attached-time: the time that beetles were left on the flight mill before being removed

Delta AIC: difference between AIC of each candidate model and the minimum AIC of all the models

Table 4.3 Results of a generalized linear mixed model (GLMM) predicting the probability of leaf-side selection by the ambrosia beetle *Platypus quercivorus* before and after attachment to a flight mill (n = 680). Thirteen candidate models were constructed using different combinations of the five candidate explanatory variables: leaf, sex, attachment, interaction between leaf and sex, and interaction between attachment and sex. Best model with the lowest AIC is listed first, and models with delta AIC < 2 are shown below.

Generalized linear mixed model	AIC	delta AIC
Leaf	696.18	0.00
Null model	696.20	0.02
Leaf + Sex + Leaf:Sex	697.15	0.97
Leaf + Attachment	697.57	1.39
Attachment	697.59	1.41
Leaf + Sex	698.17	1.99

Leaf: the deterioration status of leaves, reflected by different number of days after cutting

Sex: male or female

Attachment: before or after attachment of beetles to the flight mill

Delta AIC: difference between AIC of each candidate model and the minimum AIC of all the models

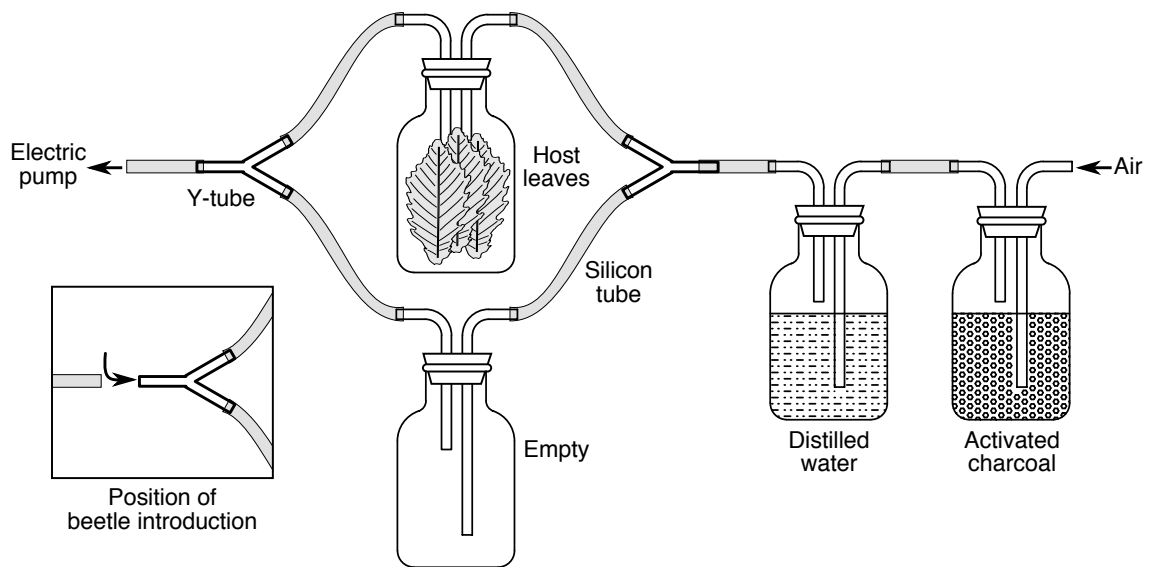


Fig. 4.1 Y-tube olfactometer used to investigate the olfactory responses of the ambrosia beetle *Platypus quercivorus* to leaf volatiles. One of the two bottles contains leaves with different deterioration statuses. Before introduction into the olfactometer, the air inside was cleaned using activated charcoal and distilled water. *P. quercivorus* adults are released into the olfactometer through a Y-tube connected to the bottles.

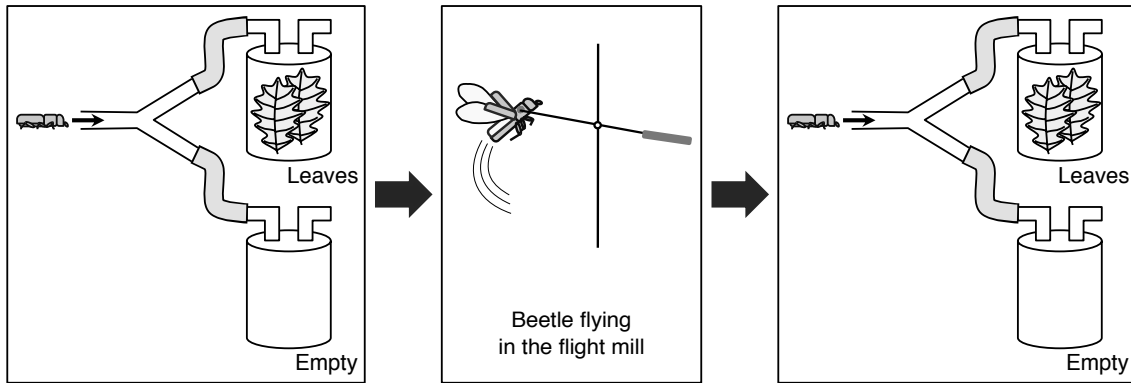


Fig. 4.2 Procedure of Experiment 2. *Platypus quercivorus* individuals were tested continuously 10 times. Beetles chose between volatiles from leaves with different deterioration status (days after cutting leaves = 0–9) and a control (no leaves), respectively. The beetle was then tethered to a flight mill to fly. Lastly, the beetle was detached from the flight mill at the end of flight and was tested 10 times in the same way as before flight.

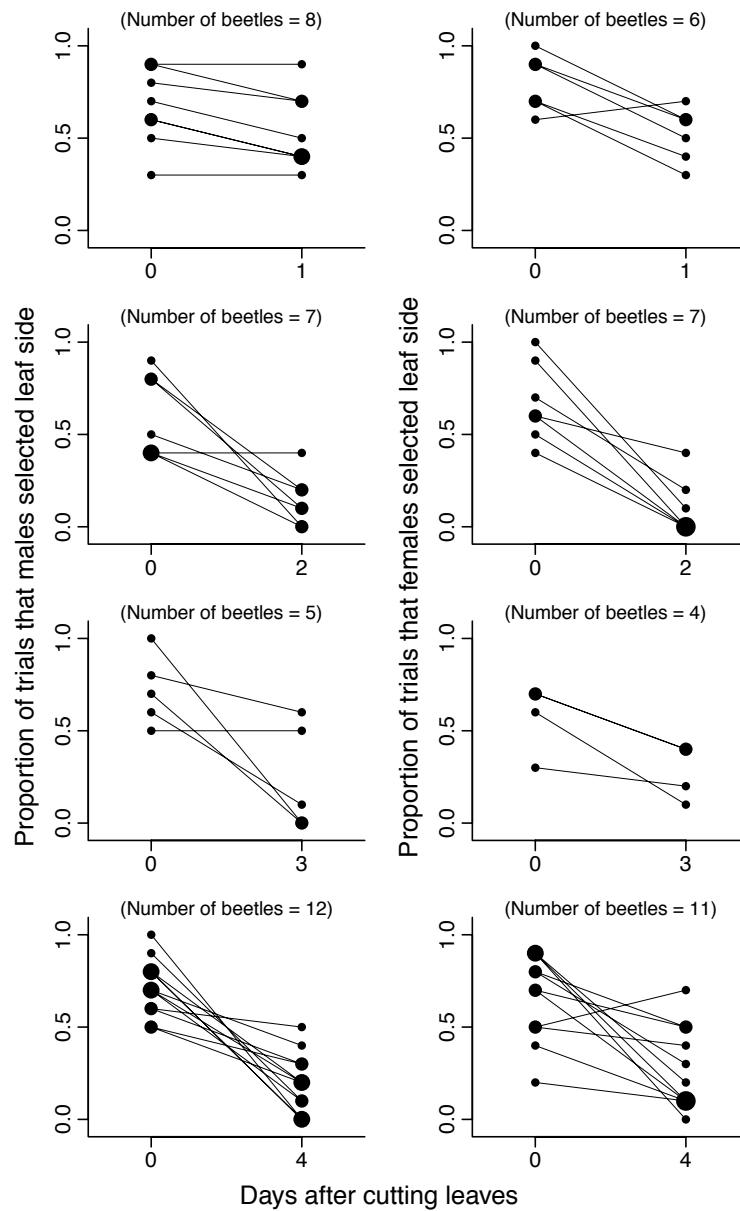


Fig. 4.3 Proportion of trials in which the ambrosia beetle *Platypus quercivorus* selected the leaf side of a Y-tube olfactometer. Individual beetles were each tested 10 times with a choice between fresh leaf volatiles (days after cutting leaves = 0) or the control (no leaves), and were tested 10 times with a choice between volatiles from dry leaves (days after cutting leaves = 1–4) or the control. Males (left side) and females (right side). The size of the symbols represents the number of individual beetles on a logarithmic scale. Data from the same beetles are connected by lines.

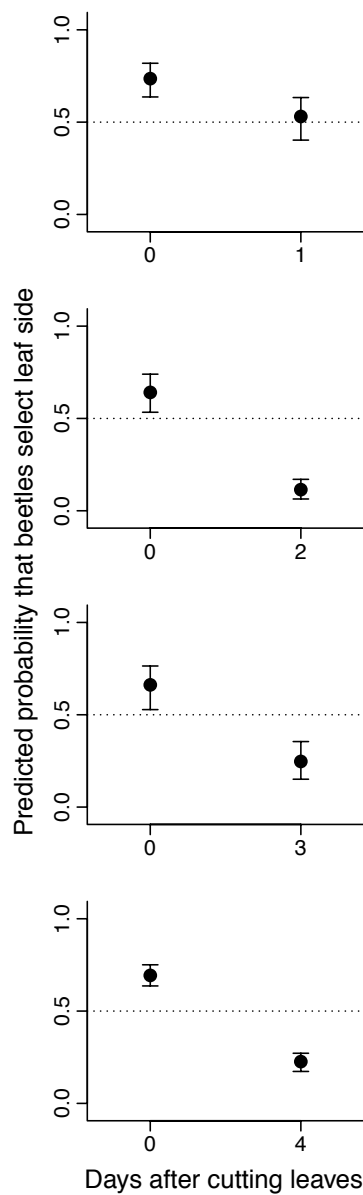


Fig. 4.4 Predicted probability of leaf-side selection by the ambrosia beetle *Platypus quercivorus* when their preference was tested between fresh leaf volatiles and a control, and dry leaf volatiles (days after cutting leaves = 1–4) and a control, respectively. Predictions were made using generalized linear mixed models. The bars represent 95% confidence intervals of the predicted probability.

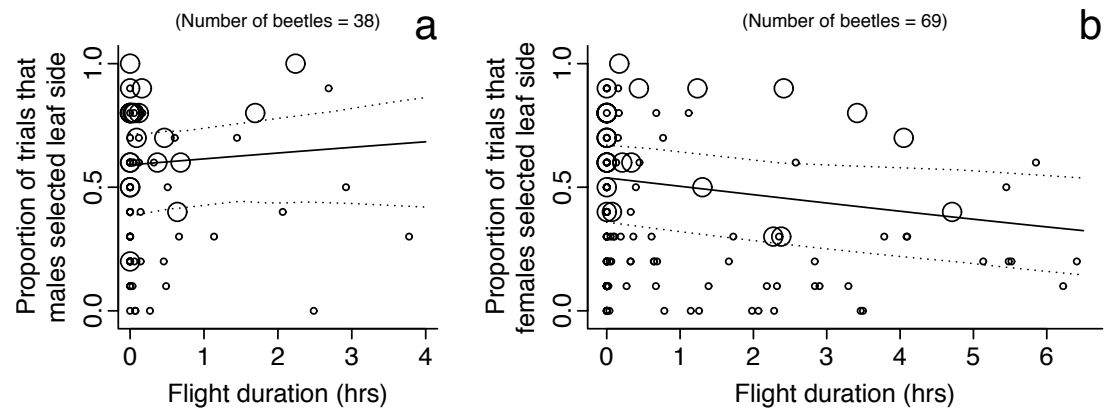


Fig. 4.5 Effect of flight duration on leaf-side selection by the ambrosia beetle *Platypus quercivorus*, while testing olfactory responses to leaf volatiles with different deterioration status (days after cutting leaves = 0–9) in males (a) and females (b). The large circles represent fresh leaves (days after cutting leaves = 0). The small circles represent dry leaves (days after cutting leaves = 1–9). Solid lines show the probability of leaf-side selection, which was predicted by a generalized linear mixed model. Dashed lines demonstrate 95% confidence intervals of the predicted probability.

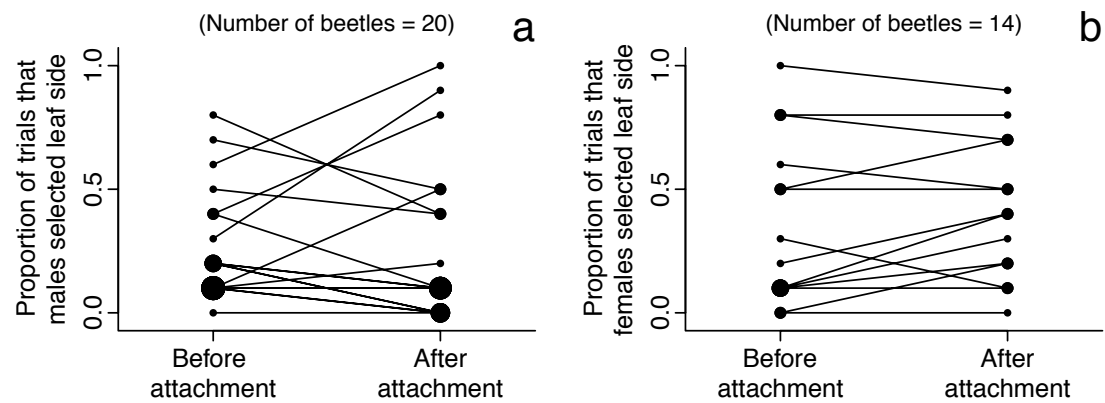


Fig. 4.6 Changes in proportion of trials in which the ambrosia beetle *Platypus quercivorus* selected the leaf side of a Y-tube olfactometer, before and after attachment to a flight mill, in males (a) and females (b). Individual beetles were tested 10 times before and 10 times after attachment to a flight mill, with a choice between volatiles from leaves that differed in deterioration status (days after cutting leaves = 0–9) or the control (no leaves). The size of the symbol indicates the number of beetles on a logarithmic scale. Data for the same beetles are connected by the lines.

Chapter 5

Difference in the olfactory response of *Platypus quercivorus* to leaf volatiles from host and non-host trees

Introduction

Ambrosia and bark beetles (Coleoptera: Scolytinae and Platypodinae) form diverse groups that play different roles in forest ecosystems (Fernando and Richard 2015), for instance in nutrient cycling and woody biomass decomposition (Edmonds and Eglitis 1989). Most of the beetles are secondary pests that attack trees that are either already dead, weakened by disease or drought, or are in physical decline (Wood 1982; Klepzig et al. 1991). Several species are primary pests, capable of attacking and killing healthy trees in natural and planted forests (Paine et al. 1997; Lieutier et al. 2004), occasionally causing significant damage to the local biodiversity, landscape, and economy (Orbay et al. 1994; Ciesla 2011). Some beetles use a broad range of trees in many families as their hosts, whereas others use only a single tree species or a few species in one family (Kelly and Farrel 1998).

In Japan, an ambrosia beetle, *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae), is a vector of the pathogenic fungus, *Raffaelea quercivora* Kubono & Shin-Ito (Ophiostomatales: Ophiostomataceae), which causes extensive wilt disease of oak trees (Ito and Yamada 1998; Ito et al. 1998; Kubono and Ito 2002). This beetle was first regarded as a secondary pest, but was considered a primary pest in the 1980s (Ito and Yamada 1998). Expansion of *P. quercivorus*, together with its associated wilt disease, remains a problem in Japan. This beetle is monogamous; male adults first fly to and land on a tree, then release the aggregation pheromone [(1S,4R)-p-menth-2-en-1-ol] (Kashiwagi et al. 2006) that attracts other beetles, leading to mass attacks on oak trees. Females arrive on the trees later and mate with males at the entrances to their burrows; they lay their eggs inside the galleries, then inoculate the walls of the galleries with the spores of the ambrosia fungi. The offspring reach adulthood in their natal host tree, then emerge usually between June and October, and fly to new host trees (Soné et al. 1998).

Typically, newly emerged adults of bark and ambrosia beetles leave their natal trees and seek other trees to find mates and food; accordingly, the adults' ability to locate a suitable host tree and bore into it is crucial for their fitness and survival (Raffa 2001). During host location and selection, they are thought to use several senses, including olfaction, vision, touch, and gustation (Wood and Bushing 1963; Raffa and Berryman 1982; Campbell and Borden 2006; Pureswaran et al. 2006; Mayfield and Brownie 2013; Gray et al. 2015). Olfaction is recognized as the primary sense in most species: the beetles rely on olfactory cues to distinguish hosts from non-hosts (Rudinsky 1966; Werner 1972; Heikkinen 1977; Wood 1982; Byer et al. 2004; Pureswaran et al. 2004; Zhang and Schlyter 2004; Pureswaran and Borden 2005; Martini et al. 2015). For example, many bark beetle species infesting conifers use α -pinene, one of the volatiles produced by coniferous trees, to locate suitable host trees (Schroeder 1988; Miller 2006; Gallego et al. 2008).

Using olfactometer bioassays, it has been shown that the ambrosia beetle *Xyleborus glabratus* Eichhoff (Coleoptera: Platypodidae) is highly attracted to the chemical volatiles emitted by the intact leaves of their host trees, but weakly attracted to those of the non-host trees (Martini et al. 2015). We have reported that *P. quercivorus* was significantly attracted to the volatiles emitted from leaves of *Quercus crispula* Blume (Fagales: Fagaceae), one of the species most susceptible to infestation by this species (Pham et al. 2019). However, in that study, we used leaves detached from trees as volatile sources, and cannot eliminate the possibility that beetles were attracted to volatiles emitted from cut ends of the leaves. Therefore, it is necessary to confirm the response of the beetle to volatiles from intact leaves. *Platypus quercivorus* has a relatively large range of hosts, and the probability of attack differs in the field among their host trees species (Inoue et al. 2000; Kobayashi and Shibata 2001; Yamasaki et al. 2007; Yamasaki and Futai 2012; Yamasaki et al. 2014). Therefore, their responses to leaf volatiles may differ even among their host-tree species. In addition, the responses of *P. quercivorus* to non-host leaf volatiles have not yet been tested.

In the laboratory conditions, after flight, the responses of bark beetles to light decrease, and they subsequently respond strongly to pheromones and host-derived volatiles (Graham 1959; Atkins 1966a; Bennett and Borden 1971; Choudhury and

Kennedy 1980). This suggests that the response to light is important in the early phase of dispersal, and that the response to olfactory attractants is important in the late phase of dispersal. Positive phototaxis, which declines after flight, has been confirmed in this species (Pham et al. 2017). In this context, it is possible that the olfactory responses of *P. quercivorus* to leaf volatiles are relatively weak immediately after emergence. A study using *Q. crispula* leaves has shown that *P. quercivorus* males increase, whereas females reduce, their olfactory response to host leaf volatiles during flight; this was explained by the difference in the host-locating process between males and females in which the former are pioneers and the latter are followers (Pham et al. 2019). It is possible that non-host leaf volatiles could also elicit this kind of sexual difference, but with a different pattern. It is also important to examine whether the species' olfactory responses differ among host species that experience different levels of attack in the field.

Therefore, I tested the hypotheses that the olfactory responses of *P. quercivorus* to leaf volatiles (i) differ between host and non-host trees and among host species; (ii) are influenced by flight; (iii) differ between males and females; and (iv) can be explained by the chemical profiles of the leaf volatiles. I aimed to provide new insights into chemical ecology of *P. quercivorus*.

Materials and Methods

Insects

Adult *P. quercivorus* beetles were collected from two dead *Q. serrata* trees that were infested and killed by this species in 2018, in an urban forest in Akashi City, Hyogo Prefecture, Japan (34°41.98'N, 134°55.62'E). The diameters at breast height of these two trees were 47.9 cm and 38.4 cm. The stumps (1.5 m in length) of these trees were excavated and placed outdoors at Kyoto University (35°01.44'N, 135°47.15'E) in May 2019. Collection traps were attached to the entrance holes, and the emergence of adult beetles was monitored daily from 06:00 to 07:00. From June 13 to July 13, beetles that emerged into the traps were collected and taken straight to the laboratory, where the bioassays were immediately conducted.

Plants

Three-year old seedlings of six tree species (Table 5.1) were used as odor sources in the olfaction bioassays. Four Fagaceae species were selected as the hosts (positive treatment). The probability of trees dying from ambrosia beetle infestation differs between tree species. For example, *Quercus crispula* and *Q. serrata* are attacked at high frequency (Inoue et al. 2000; Kobayashi and Shibata 2001). In contrast, *Q. salicina* and *Castanea crenata* are less likely than *Q. crispula* to become infested (Yamasaki et al. 2007, 2014). *Fagus crenata* and *Cryptomeria japonica* were the dominant species in a forest where a severe attack of *P. quercivorus* on *Q. crispula* was observed (Yamasaki and Sakimoto 2009). Because there were no records of beetle attack on these species, they were selected as the non-hosts (negative treatment). The seedling heights ranged from 40 to 60 cm. The seedlings were purchased from a local nursery in Kyoto Prefecture, Japan, two weeks before the olfaction bioassays. They were maintained inside a green house located outdoor at the Kitashirakawa Experimental Station, Field Science Education and Research Center, Kyoto University (35°01.44'N, 135°47.15'E). Water was supplied to the seedlings as needed.

Y-tube olfactometer system

In order to determine the responses of adult beetles to leaf volatiles, I constructed a two-choice Y-tube olfactometer. The Y-shaped tube, with a 12 cm stem and two 2 cm arms at a 60° angle, was prepared from acrylic plastic tubing (1 cm inner diameter). Filter paper was inserted inside to facilitate the beetle's movement. The end of each arm of the Y-tube was connected to two 10 L fluoro-resin bags (CC-10; GL Science, Tokyo, Japan): one contained the entire canopy of the seedling being tested (treatment), the other was empty (control). Hereafter, we refer to volatiles emitted from the seedling canopy as "leaf volatiles". Two 160 lux halogen lamps were suspended 40 cm above the fluoro-resin bags and seedlings to induce transpiration. The air was cleaned by circulating it through bottles filled with activated charcoal and distilled water before entering each fluoro-resin bag. The water in the bottles was replaced daily. The clean air was drawn through the fluoro-resin bags using an electric suction pump (MAS-1; AS ONE, Osaka, Japan). The airflow at the base of the Y-tube was 80 mL/min, and it was constantly monitored using a flow meter (RK-1350V; KOFLOC, Kyoto, Japan). The different parts of the olfactometer were connected using polytetrafluoroethylene and vinyl tubing, and the apparatus was cleaned with distilled water between experiments.

Olfactory responses of beetles to leaf volatiles

The seedlings were transferred from the green house to the laboratory on the morning the olfaction bioassay commenced, and they were placed in the fluoro-resin bags as described. The lights above the treatment bags were turned on, and water was supplied to the seedlings one hour before the bioassay. The base of the Y-tube stem was connected to the electric pump using vinyl tubing. At the start of the bioassay, this tubing was temporarily disconnected, and *P. quercivorus* adults were released one at a time into the base of the Y-tube stem, 12 cm from the intersection, where they were exposed to the air from the treatment and control arms. The vinyl tubing was then reconnected to the Y-tube apparatus, and the behavioral response of the beetles was observed. We timed the beetles' walk from its start and until they crossed the edge of the arm and entered into either the

seedling or control arm (hereafter “selection time”), and recorded the arm that the beetles selected. A single beetle was assayed 10 times. If a beetle remained at the basal area of the Y-tube and did not pass the edge of the arm within 5 min, it was removed and a different beetle was used. The number of times each beetle selected the treatment arm was divided by 10 to express olfactory response as a proportion. I tested both males and females to evaluate sexual differences in olfactory response to leaf volatiles.

Once a beetle had completed 10 olfactory trials, it was immediately transferred to the flight mill, where it flew without being exposed to any leaf volatiles. Some beetles flew continuously, whereas others exhibited short repeated periods of flight. The total flight time until the beetle finally stopped flying was calculated, excluding the intervals between flights. After the beetle stopped flying, it was removed from the flight mill, and subjected to another 10 olfactory trials. The experimental procedures are described in Fig 5.1. The olfactory and flight experiments were conducted at 25 °C. Newly emerged beetles were used on each day of the experiment, and different beetles were used to test responses to each of the different tree species.

Flight mill

A flight mill system, described by Okada et al. (2018), was used to test the effect of flying on the olfactory response of *P. quercivorus* to leaf volatiles. The primary structure consists of two similarly sized flight arms (0.25 mm in diameter, 40 mm long), each comprising two thin needles connected perpendicularly, forming a cross. A beetle is tethered to one end of the horizontal arm using instant adhesive glue. The beetle then flies in circles, and the flight rotations are detected and counted by a photo sensor, when the horizontal flight arm and the beetle pass over an infrared LED. The data were logged on a personal computer, and exact flight times were calculated for each beetle individual.

Identification of leaf volatiles

In the morning, on the day of the experiment, the seedlings were transported from the vinyl house to the laboratory and enclosed in the fluororesin bags. A HayeSep Q trap

was connected directly to the outlet portion of the fluororesin bag. In addition to the activated charcoal and distilled water already in place, we connected an absorption trap to the inlet portion of the fluororesin bag to draw clean air into the bag. Air was pulled through the absorption trap (to collect the leaf volatiles) at 0.1 L/min. Water provision and lighting conditions were the same as for the olfaction bioassays. The volatiles were sampled for 4 h at 25 °C. This was repeated four times for each plant species using the same individual to measure variation in the composition of the leaf compounds caused by the time of collection.

After collection, the trapped volatiles were rapidly extracted using 100 μ L of dichloromethane/hexane (50:50). Nonyl acetate (5 μ L) was added to the extracts at a diluted concentration of 0.5 ng/ μ L as an internal standard. A 1 μ L portion of each sample was manually injected into a gas chromatography–mass spectrometry (GC–MS) system (QP2010 Ultra; Shimadzu, Kyoto, Japan). We used a DB-5ms + DG capillary column (30 m $\times$ 0.25 mm, 0.25 μ m in thickness; Agilent Technologies, Santa Clara, CA, US). Helium was used as the carrier gas. The temperature of the injection port was 240 °C. The temperature of the column oven was programmed at 40 °C for 2 min, then increased at 10 °C/min to 290 °C, and maintained at 290 °C for 5 min. Compound identification was achieved by matching the experimental mass spectra to those from the National Institute of Standards and Technology (NIST). Compounds with >90% mass-spectrum similarity to those in the NIST library were regarded as candidate compounds. Of these candidates, several were further examined by comparing their mass spectra and retention times with those of the authentic compounds, where available. Chemical contaminants dodecamethylcyclhexasiloxane, ethylbenzene, 1,3-dimethylbenzene, 1,2-dimethylbenzene, and 1,3,5-trimethylbenzene were occasionally observed among the candidate compounds. We calculated the relative percentages of the peak areas in the chromatograph and used those of the candidate compounds (excluding the contaminants) to compare leaf volatile composition among the six tree species.

Statistical analysis

All data analyses were performed in R version 3.5.3 (R Core Team 2019). Models

were constructed using the *lme4* package (Bates et al. 2015) and compared and selected using *MuMIn* package (Barton 2019). To obtain the predicted selection probabilities and their confidence intervals (CIs), and to perform multiple comparisons among groups, we used the *emmeans* package (Lenth 2020). To predict the probabilities of the olfactory responses of *P. quercivorus* to the leaf volatiles, I constructed a generalized linear mixed model using the arm an individual beetle selected in each trial as the response variable. Selection of the seedling arm was assigned a value of 1, and selection of the control arm was assigned a value of zero. The selection variable follows a binomial distribution; therefore, I used a logit link function. The candidate explanatory variables of the model were tree species (*Q. crispula*, *Q. serrata*, *Q. salicina*, *Castanea crenata*, *Fagus crenata*, and *Cryptomeria japonica*), flight (trial was conducted before or after flight in the flight mill), and sex (male or female). The interaction terms among tree species, flight, and sex were included. The individual number of each beetle used in the bioassays was treated as a random factor. Models were constructed using the data of individuals that flew for at least 1 min during the flight experiments. For model selection, I compared the Akaike Information Criterion (AIC) values of each candidate model. The model with the lowest AIC was considered the best-fit model. Variables included in the best-fit model are discussed as influential factors. The 95% CIs of the predicted probability of seedling-arm selection were calculated; attraction to the test species was considered significant if the lower confidence interval was >0.5. Differences in *P. quercivorus* responses to the leaf volatiles among the six tree species were tested using Tukey's multiple comparison tests.

Differences in the composition of leaf volatiles among the six tree species were visualized based on relative percentages of peak areas of the candidate compounds using non-metric multidimensional scaling (NMDS). NMDS analysis was conducted using the *vegan* package (Oksanen et al. 2019).

Results

The responses to leaf volatiles were tested in 30 females and 24 males. The selection times for females and males were 67.3 ± 2.0 and 69.5 ± 2.1 s (mean $\pm$ SE), respectively. Flight times for females and males were 1.64 ± 0.34 and 1.37 ± 0.32 h (mean $\pm$ SE), respectively. The respective numbers of olfaction trials for females and males were 100 and 120 (*Q. crispula*), 160 and 120 (*Q. serrata*), 100 and 80 (*Q. salicina*), 80 and 60 (*Castanea crenata*), 80 and 40 (*F. crenata*), and 80 and 60 (*Cryptomeria japonica*).

The proportion of trials in which leaf volatiles were selected was relatively high and mostly >0.5 in the three host *Quercus* species (Fig 5.2). In contrast, the proportion was relatively low in the two non-host species and <0.5 in most cases (Fig 5.2). This proportion tended to be higher after flight, and there was no marked sexual difference (Fig 5.2).

Tree species, flight, and sex were selected as explanatory variables in the best-fit model; the interactions among the terms were not selected (Table 5.2). The predicted selection probabilities were high for hosts (*Q. crispula*, *Q. serrata*, *Q. salicina*, and *Castanea crenata*) and low for non-hosts (*F. crenata*, *Cryptomeria japonica*) (Fig 5.3). In particular, the lower limits of the confidence intervals were >0.5 for the four host species. Multiple comparison tests showed significant differences in *P. quercivorus* responses to leaf volatiles between the tree species, except between *Castanea crenata* and *Cryptomeria japonica* (Fig 5.3). Predicted selection probabilities were higher after flight than before flight, and higher in males than in females (Fig 5.3).

The leaf volatiles identified by GC–MS are shown in Table 5.3. Some compounds in the leaf volatiles of the non-host *F. crenata*, such as 2-ethyl-1-hexanol, nonanal, decanal, dodecane, and tetradecane, also occurred in the four host species (Table 5.3). *Cryptomeria japonica* shared few compounds with the other five species (Table 5.3) and was therefore clearly separated in the NMDS plot from the other species; the other species were not separated from each other (Fig 5.4a). When *Cryptomeria japonica* was excluded from the NMDS analysis, the remaining species were separated (Fig 5.4b): the host *Quercus* species and non-host *F. crenata* were clustered near one another, and the host *Castanea crenata* was separated (Fig 5.4b).

Discussion

Platypus quercivorus was attracted to the leaf volatiles of its host trees, but not to those of the non-hosts, supporting our hypothesis that the olfactory responses of this beetle to leaf volatiles differ between host and non-host trees. This corroborated the findings by Pham et al. (2019) who reported a significant attraction of the beetle to volatiles emitted from detached leaves of the host *Q. crispula*. Our study is also consistent with the findings of Yamasaki et al. (2014), who conducted a field study and found that the probability of attack by *P. quercivorus* was higher under a dense canopy of host trees, concluding that *P. quercivorus* uses leaf volatiles as host-location cues. Similarly, the ambrosia beetle *X. glabratus* is attracted to host leaf volatiles, but not to those of non-hosts (Martini et al. 2015).

The four host species that we studied experience different rates of attack by *P. quercivorus* in the field (Inoue et al. 2000; Kobayashi and Shibata 2001; Yamasaki et al. 2007; Yamasaki et al. 2014). Nonetheless, *P. quercivorus* responses to the leaf volatiles did not differ significantly between hosts (Fig 5.3). Yamasaki and Futai (2012) reported similar numbers of *P. quercivorus* males flying around *Q. crispula*, *Q. serrata*, and *Castanea crenata* trees in the period before hole boring, and were corroborated by our findings (Fig 5.3). Therefore, the different rates of attack in the field may be caused by other discriminatory behaviors after approaching the tree or after landing.

Consistent with our second hypothesis, *P. quercivorus* responded more strongly to leaf volatiles after flight (Fig 5.3). In contrast, positive phototaxis is weaker after flight for this species (Pham et al. 2017), suggesting that the relative importance of light level and leaf volatiles differs before and after flight. After emergence, *P. quercivorus* may be attracted to light as a dispersal-flight mechanism; thereafter, it may be attracted to leaf volatiles for host location. Yamasaki et al. (2016) showed a high probability of attack by *P. quercivorus* for distant trees rather than for those near the natal tree. This suggests that *P. quercivorus* may ignore its host trees immediately after emergence, even though host leaf volatiles are released around the tree. The flight phase is considered a life history phase with the highest survival risk for bark and ambrosia beetles (Atkins 1966a), because they face many challenges, including limited energy reserves (Atkins 1969), predation

(Dahlsten 1982; Fernando and Richard 2015), and weather conditions (McMullen and Atkins 1962). In this context, it would be advantageous for *P. quercivorus* to minimize its host search time. An immediate response to light following emergence and the later response to leaf volatiles during flight may be an optimal host-location strategy for *P. quercivorus*.

The olfactory response of *P. quercivorus* to leaf volatiles increased after flight, and this response did not differ between host and non-host trees (Fig 5.2). The adaptive significance of this increase in response to non-host leaf volatiles is unclear. However, after flight, the predicted selection probability was lower for non-hosts (about 0.5) than for hosts (Fig 5.3). Therefore, we conclude that *P. quercivorus* can distinguish non-host from host even in the situation when the response to non-host is enhanced by flight.

Males were more responsive than females to leaf volatiles both before and after flight, although the estimated sexual difference in response was small (males – females = 0.064; Fig 5.3). The different roles of males and females in host-tree location may explain this difference. Specifically, males select the host trees (Kobayashi et al. 2001b); hence, they should be more sensitive than females to leaf volatiles. Females follow the males (Ueda and Kobayashi 2001) and therefore should also be somewhat sensitive to leaf volatiles. Nonetheless, sensitivity to the aggregation pheromone may be more important for females in locating suitable trees for reproduction. However, no clear sexual differences were observed in the raw data (Fig 5.2). Further, sex had less explanatory power than tree species and flight for olfactory response: it was not included in the top four models (Table 5.2). Therefore, further research is necessary to evaluate the role of sexual difference.

The response of *P. quercivorus* individuals was stronger to the leaf volatiles of their host trees than to those of the non-hosts *Cryptomeria japonica* and *F. crenata* (Fig 5.3). For *Cryptomeria japonica*, this difference can be explained by differences in leaf volatile chemical profiles between this and the host species (Table 5.3; Fig 5.4a). However, that does not apply to *F. crenata*, which shared five compounds with the host species (Table 5.3). Further, the chemical profiles of *F. crenata* and the host *Quercus* species were more similar than those of the host *Castanea crenata* and host *Quercus* species (Fig 5.4b). It is likely that other compounds specific to the host species are responsible for these

differences. For instance, isoprene, which we could not detect by GC–MS under given conditions, was reported to be emitted in high rates in *Quercus* species (David et al. 1979; Bao et al. 2008; Tani and Kawawata 2008; Gil-Pelegrín et al. 2017), the primary hosts of *P. quercivorus*. Further analysis using other methods is necessary to clarify the chemical profiles of leaf volatiles produced by the host trees of *P. quercivorus*.

These findings show that *P. quercivorus* beetles use leaf volatiles as olfactory cues to discriminate host from non-host trees. Beetles may initially locate the appropriate habitat by orienting toward common leaf volatiles found in broadleaved trees. Thereafter, they may avoid the leaf volatiles of non-host conifers to save energy. Identifying the leaf volatile compounds specific to the host trees that are target for *P. quercivorus* remains to be elucidated in order to develop effective control methods for this species and consequently for Japanese oak wilt.

Table 5.1 Tree species used to test the olfactory response of the ambrosia beetle *Platypus quercivorus* to leaf volatiles.

Tree species	Family	Category
<i>Quercus crispula</i> Blume	Fagaceae	Host
<i>Quercus serrata</i> Murray	Fagaceae	Host
<i>Quercus salicina</i> Blume	Fagaceae	Host
<i>Castanea crenata</i> Sieb. & Zucc.	Fagaceae	Host
<i>Fagus crenata</i> Blume	Fagaceae	Non-host
<i>Cryptomeria japonica</i> D. Don	Cupressaceae	Non-host

Table 5.2 Results of a generalized linear mixed model predicting the probability of olfactory response of *Platypus quercivorus* individuals to leaf volatiles present in the olfactometer. Movement toward the tree, rather than the empty control (no tree), was taken as a positive response. The model with lowest Akaike information criterion (AIC) is listed first, followed by those with delta AIC < 2.

Generalized linear mixed model	AIC	delta AIC
Tree + Flight + Sex	1376.9	0.00
Tree + Flight + Sex + Tree × Sex	1377.8	0.90
Tree + Flight	1377.9	0.98
Tree + Flight + Sex + Flight × Sex	1378.9	1.99

Trees: *Quercus crispula*, *Q. serrata*, *Q. salicina*, *Castanea crenata*, *Fagus crenata*, *Cryptomeria japonica*.

Flight: trial was conducted before or after the beetle flew in the flight mill.

Sex: male or female.

Additive effects are indicated by +. Interactions are indicated by ×

Table 5.3 Peak area percentage ($\pm$ SE) of the 18 compounds identified by gas chromatography–mass spectrometry in leaf volatiles of the six tree species. Compound identification was performed by matching the experimental mass spectra to the mass spectra in the National Institute of Standards and Technology (NIST) database. Compounds with $>90\%$ mass-spectrum similarity to those in the NIST database are shown. The sum for each species is not 100% because the unidentified compounds and chemical contaminants are excluded from the table.

Compound name	Abbreviation	Tree species							
		<i>Quercus crispula</i>	<i>Quercus serrata</i>	<i>Quercus salicina</i>	<i>Castanea crenata</i>	<i>Fagus crenata</i>	<i>Cryptomeria japonica</i>		
2-ethyl-1-hexanol	Hex *	21.66 $\pm$ 4.12 (96)	18.37 $\pm$ 0.69 (97)	13.16 $\pm$ 5.47 (97)	24.03 $\pm$ 1.43 (97)	24.81 $\pm$ 2.14 (97)			
Dodecane	Do *	11.75 $\pm$ 5.00 (97)	23.38 $\pm$ 4.56 (97)	4.81 $\pm$ 0.81 (95)	23.7 $\pm$ 0.64 (98)	11.99 $\pm$ 4.01 (97)			
Nonanal	No *	3.68 $\pm$ 0.91 (94)	7.83 $\pm$ 0.51 (95)	7.64 $\pm$ 1.42 (96)	2.67 $\pm$ 0.17 (95)	3.17 $\pm$ 0.22 (93)			
Decanal	De *	3.97 $\pm$ 1.48 (95)	5.87 $\pm$ 2.33 (95)	5.71 $\pm$ 2.11 (95)	1.11 $\pm$ 0.38 (95)	2.08 $\pm$ 0.19 (95)			
Tetradecane	Tet *	1.52 $\pm$ 0.13 (95)	2.53 $\pm$ 0.54 (97)	3.82 $\pm$ 0.85 (94)	1.62 $\pm$ 0.06 (95)	4.21 $\pm$ 1.03 (96)			
Tridecane	Tri	1.73 $\pm$ 0.63 (95)	2.19 $\pm$ 0.91 (96)		1.59 $\pm$ 0.04 (97)				
α -Farnesene	Fa	6.38 $\pm$ 2.90 (93)							
3,7-Dimethyl-1,3,6-octatriene,	DiO	4.24 $\pm$ 2.44 (94)							
6-Methyl-5-hepten-2-one,	Hep	3.12 $\pm$ 1.29 (94)							
Octanal	Oc		1.99 $\pm$ 0.14 (95)	1.58 $\pm$ 0.55 (95)					
α -Pinene	aP			2.01 $\pm$ 0.93 (96)			0.05 $\pm$ 0.008 (94)		
β -Phellandrene	Phe				4.20 $\pm$ 0.23 (95)		0.12 $\pm$ 0.001 (94)		
β -Pinene	bP				3.34 $\pm$ 0.32 (93)				
trans- β -Ocimene	tbO				2.23 $\pm$ 0.11 (95)				
β -Myrcene	My						4.39 $\pm$ 4.36 (94)		
3-Carene	Ca						4.39 $\pm$ 4.36 (95)		
Kaur-16-Ene	Ka						0.29 $\pm$ 0.05 (92)		
D-Limonene	Li						0.12 $\pm$ 0.02 (96)		

The values in parentheses indicate percentage mass-spectrum similarity to the compounds in the NIST database.

* Determined by comparison with authentic compounds

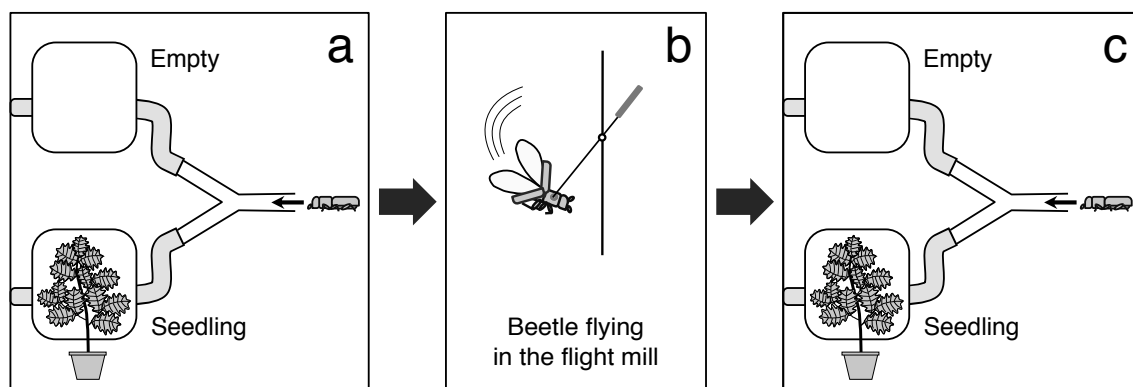


Fig. 5.1 Graphic representation of experimental procedures. (a) The olfactory response of the ambrosia beetle *Platypus quercivorus* to leaf volatiles was tested 10 times in a Y-tube olfactometer. (b) The beetle was attached to a flight mill, and its flight duration was recorded until it stopped flying. (c) Another 10 olfactory response trials were conducted after the flight in flight mill.

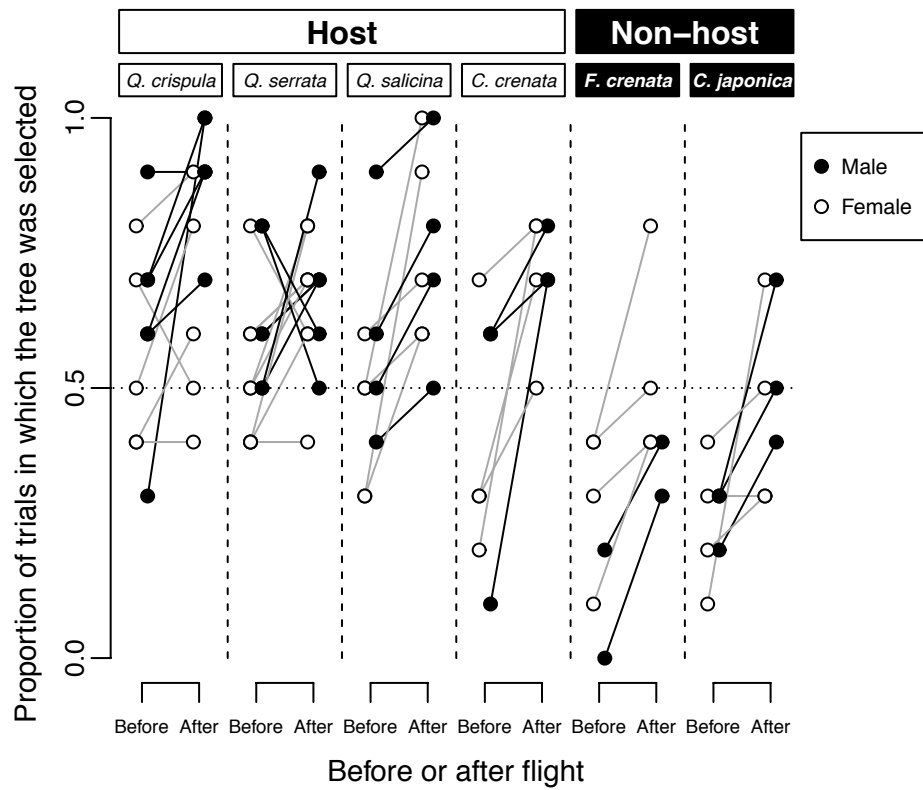


Fig. 5.2 The proportion of trials in which *Platypus quercivorus* beetles (30 females and 24 males) selected the tree, indicating their preference for the leaf volatiles emitted from host (*Quercus crispula*, *Q. serrata*, *Q. salicina*, and *Castanea crenata*) and non-host (*Fagus crenata* and *Cryptomeria japonica*) seedlings in a Y-tube olfactometer. The olfactory experiment was conducted 20 times for each individual beetle, 10 before and 10 after flight in a flight mill. Points for the same individuals are connected by lines.

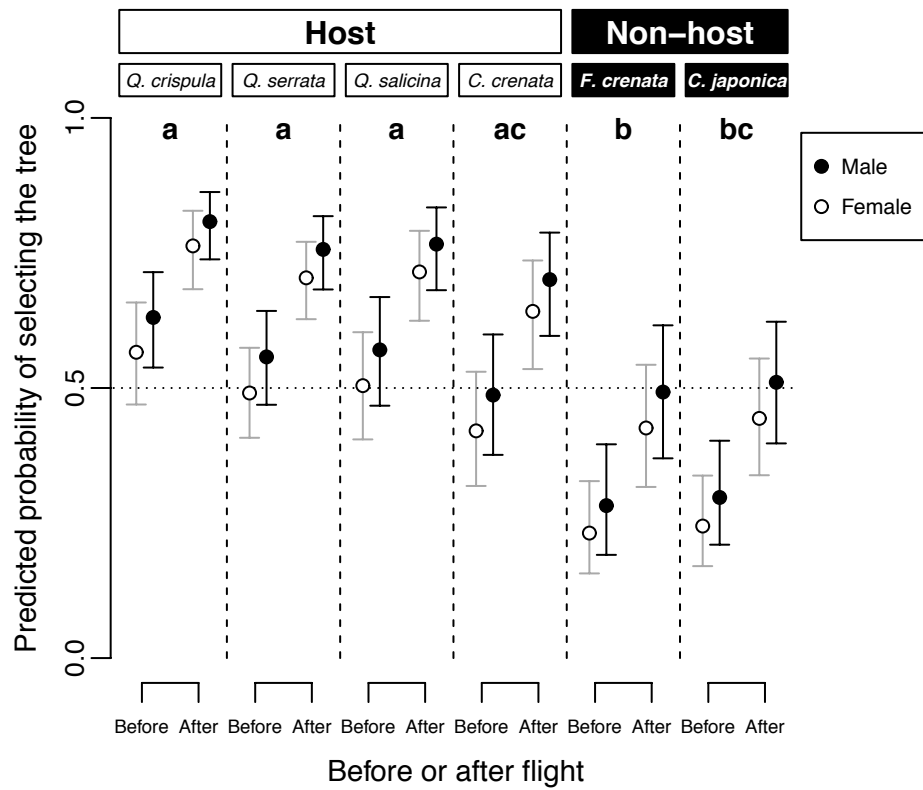


Fig. 5.3 Predicted probabilities (and 95% confidence intervals) of *Platypus quercivorus* beetles (30 females and 24 males) choosing the seedling arm of the olfactometer. Olfaction trials were conducted before and after flight in the flight mill. Probabilities were predicted using a generalized linear mixed model. Host trees: *Quercus crispula*, *Q. serrata*, *Q. salicina*, and *Castanea crenata*. Non-host trees: *Fagus crenata* and *Cryptomeria japonica*. Lowercase letters below the tree species names identify groups of species with significantly different predictions ($P < 0.05$; Tukey's multiple comparison tests).

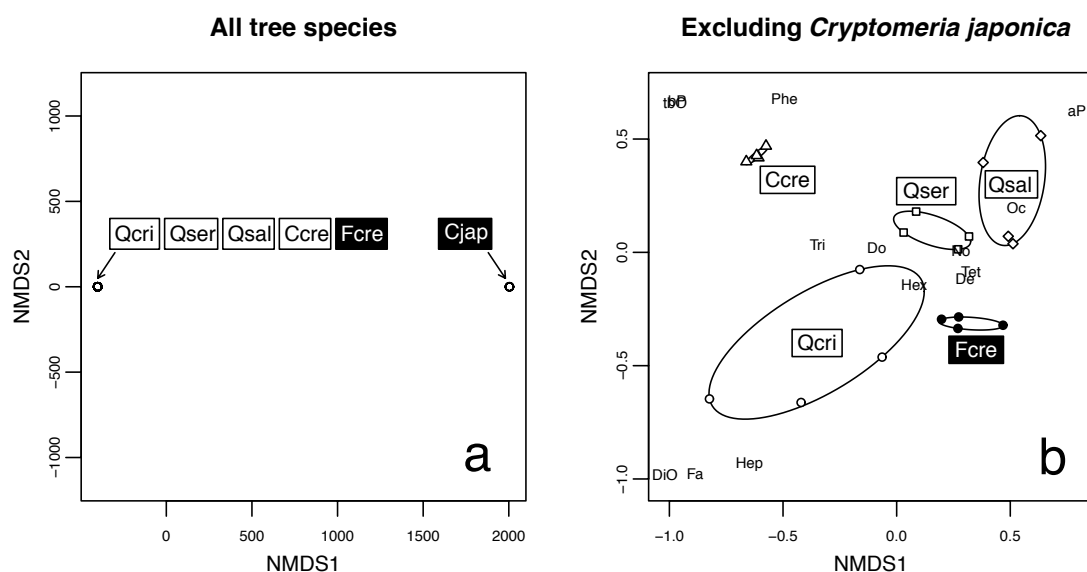


Fig. 5.4 Non-metric multidimensional scaling (NMDS) plot where each point represents the chemical composition of the leaf volatiles of (a) all six tree species and (b) the species excluding *Cryptomeria japonica*. Black letters in white squares: host species; white letters in black squares: non-host species of the ambrosia beetle *Platypus quercivorus*. Small letters in (b) show the abbreviations of the chemical compounds identified (see Table 3 for details). (a) Twenty points for five species are concentrated to the left, and four points for *Cryptomeria japonica* to the right. Qcri: *Quercus crispula*; Qser: *Quercus serrata*; Qsal: *Quercus salicina*; Ccre: *Castanea crenata*; Fcre: *Fagus crenata*; Cjap: *Cryptomeria japonica*.

Chapter 6

General discussion: Suggested process of dispersal flight and host selection in *Platypus quercivorus*

The present thesis elucidates characteristics of dispersal flight and host selection of *P. quercivorus*. I revealed sexual difference in the influence of flight speed, which is measured by initial and terminal flight speed, on flight distance of *P. quercivorus* (Chapter 2), and olfactory responses to leaf volatiles of its host and non-host trees (Chapter 4, 5). Additionally, I also revealed no sexual difference in the influence of flight duration on flight distance (Chapter 2), and phototactic response of the beetle before and after flight (Chapter 3). Furthermore, I suggested that flight activity appeared to weaken phototactic response of *P. quercivorus* to light, but intensify its olfactory responses to leaf volatiles (Chapter 3, 4, 5).

Reduced phototactic responses of *P. quercivorus* to light after flight and its enhanced responses to host leaf volatiles after flight are consistent with the behavioral responses of other bark and ambrosia beetles (Graham 1959; Choudhury and Kennedy 1980). In the laboratory, *T. lineatum* beetles took flight toward light, regardless of presence of attractive wood volatiles emitted from host trees. When light disappeared, beetles bored into wood of host trees (Graham et al. 1959). Similarly, after continued flight exercise, positive phototaxis of *S. multistriatus* is reduced and its olfactory response to the pheromone is increased (Choudhury and Kennedy 1980). These results suggest that *P. quercivorus*, *T. lineatum* and *S. multistriatus* requires a dispersal flight exercise before responding to host materials. In the present study, I did two separate experiments to obtain the shift from positive phototaxis to olfactory responses of *P. quercivorus* (Chapter 3 and Chapter 4 and Chapter 5), while previous study examined this points by the single experiment (Graham 1959; Choudhury and Kennedy 1980). My study showed that the decreased response to light is the same for the sexes of *P. quercivorus* (Chapter 3), which is similar to that indicated by *T. lineatum* (Graham 1959). These results suggest that response to light immediately after their emergence is important for both sexes. Furthermore, different responses to leaf volatiles between the sexes of *P. quercivorus*

(Chapter 4, 5) suggest sexual difference in life history strategies of host selection.

Martini et al. (2015) was the first that reported that an ambrosia beetle *X. glabratus* is attracted to the leaf volatiles of its host trees, and but not attracted to the leaf volatiles non-host trees. The findings from my current study reinforce previous study and pay the pathway for critical studies on the potential use of leaf volatiles as an additional cue for host selection of ambrosia beetles. Previous study has identified candidate leaf compounds that attracted *X. glabratus* and confirmed their effectiveness in the field (Martini et al. 2015), but my study has not yet clarified specific chemical volatiles which are candidate for attraction of *P. quercivorus*.

Based on my findings from this thesis, possible process of dispersal flight and host selection for *P. quercivorus* can be summarized as follows: Upon emergence from breeding galleries, new brood beetles of *P. quercivorus* initiate dispersal phase by flying upward to the canopy openings created by the wilt of infested oak trees, and then fly above tree canopy surface in search of new host trees. After a certain threshold of flight exercise, beetles begin seeking for a suitable host through avoiding leaf volatiles released from non-host conifers as an adaptive mechanism of minimizing energy expenditure. At the same time, beetles also follow common leaf volatiles released from broad leaved trees to stay flying in a correct habitat. Finally, beetles narrow the target down to their host by detecting leaf volatiles specific to oak trees.

The attractive responses of *P. quercivorus* to leaf chemical volatiles released from its host trees in this study imply that these leaf volatiles can be used as lures in developing a novel approach that reduce population density of *P. quercivorus* beetles in the field. For example, we can simulate the host tree status by setting up host specific volatile lures at the canopy top of non-host trees. Beetles may be captured on these non-host trees and their surroundings when sticky traps were attached to trunk surface of these trees.

My present study indicates that *P. quercivorus* flies upward toward canopy gaps in the course of dispersal; however, field studies are necessary to confirm this result. Olfactory responses of *P. quercivorus* to leaf volatiles were tested in a Y-tube olfactometer system where the beetle walked in a rough surface to select leaf volatiles; however, in nature *P. quercivorus* beetles find and locate host in flight. In future research, I will test olfactory responses of *P. quercivorus* to host leaf volatiles in free flight where beetles

respond to leaf volatiles in flight. Despite that *P. quercivorus* was found to be highly attracted to host leaf volatiles, none of chemicals were identified as potential cues for *P. quercivorus*. It is important to determine specific chemicals responsible for attractiveness to *P. quercivorus*.

Summary

Chapter 1

Forests provide multiple values that are critical to humans. However, forest values are now being decreased by widespread outbreaks of bark and ambrosia beetles (Coleoptera: Scolytidae: Platypodidae), which are primarily responsible for the large areas of tree mortality. Development of effective methods for prevention of bark and ambrosia beetles is an exciting subject of studies, which require our deep understanding of the ecology. This chapter reviewed published studies in relation to characteristics of dispersal flight and host selection of bark and ambrosia beetles. I also introduced known and unknown aspects of dispersal flight and host selection for the ambrosia beetle *Platypus quercivorus* (Murayama), which is the pathogen vector of Japanese oak wilt disease. The chapter also presented the objective of this thesis.

Chapter 2

Dispersal flight is an essential component of the life history of bark and ambrosia beetles. Previous studies showed high variation in flight distance among individual beetles; however, different factors drive this variation among species. Furthermore, when the ecological role of dispersal flight differs between sexes (i.e., males locate hosts and females follow), influencing factors might also vary. Here, we evaluated the factors driving variation in the dispersal flight distance of the ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae), which is the causal agent of Japanese oak wilt, and we estimated sex-based differences for each influencing factor. A flight mill was used to measure flight distance, flight duration, and initial and terminal flight speed. Because flight speed was not constant during beetle flight, we estimated both initial and terminal flight speed and assumed that they represent relative measures of energy allocated to flight and the energy saved for other tasks, respectively. Flight distance was mainly determined by flight duration, with an average flight speed of 4.22 km/h for both sexes. Initial and terminal flight speed affected flight distance; however,

these factors had contrasting effects between sexes. Specifically, a simultaneous increase in initial and terminal flight speed by males increased flight distance. In comparison, increased flight distance by females was only achieved by increasing the initial flight speed. These different patterns in increased flight speed likely reflect differences in the relative importance of flight and other tasks in the life history of the two sexes.

Chapter 3

The ambrosia beetle *Platypus quercivorus* (Coleoptera: Platypodidae) is a vector of the pathogenic fungi, *Raffaelea quercivora* (Ophiostomatales: Ophiostomataceae), which causes Japanese oak wilt disease. Previous studies have shown that the beetle displays positive phototactic behavior; however, the adaptive significance of this behavior remains unclear. I postulated that positive phototaxis is necessary to allow the beetle to fly skyward immediately after emergence from a tree, and that this taxis changes following a certain period of flight. The present study aimed to clarify the changes in phototactic behavior of *P. quercivorus* before and after flight by using individual beetles emerging from the trunk of a *Quercus crispula* (Fagales: Fagaceae) tree that was attacked in 2014. The response of 60 beetles to light was tested ten times each, before and after flight in a flight mill. A generalized linear mixed model was constructed to predict the probability of positive phototaxis of *P. quercivorus* before and after flight. A best-fit model showed that the probability of positive phototaxis was lower after flight than before. The results suggest that positive phototaxis of *P. quercivorus* is decreased after flight.

Chapter 4

The important role of semiochemicals in the interactions between plants and insects has been extensively investigated. The volatiles produced by oak trees are thought to attract the ambrosia beetle *Platypus quercivorus* (Murayama), which causes wilt disease in *Quercus* trees, resulting in widespread damage. In the present study, I performed olfactory bioassays to survey the preferences of both sexes of *P. quercivorus* for the leaf volatiles of *Quercus crispula* Blume at various stages of leaf deterioration,

represented by different number of days after cutting. Additionally, the effect of flight on both sexes was evaluated by testing the beetle flight on a flight mill. The results showed that *P. quercivorus* was attracted to the volatiles emitted from fresh leaves and was not attracted to those emitted from dry leaves. This suggests that leaf volatiles from healthy hosts are primary attractants for *P. quercivorus*. Further, males exhibited increased olfactory responses to leaf volatiles as their flight duration increased, whereas the opposite pattern was observed in females. These results suggest that the different ecological roles of the sexes contribute to differences in olfactory responses.

Chapter 5

Although chemical volatiles emitted from host and non-host trees have been suggested as important cues for beetles, the response of the beetles to leaf volatiles is poorly understood. The ambrosia beetle *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae) is a vector for the fungus that causes Japanese oak wilt. Using a Y-tube olfactometer, we tested the olfactory responses of *P. quercivorus* to leaf volatiles emitted from host trees (*Quercus crispula* Blume, *Q. serrata* Murray, *Q. salicina* Blume, *Castanea crenata* Sieb. & Zucc.), and non-host trees (*Fagus crenata* Blume and *Cryptomeria japonica* D. Don). A flight mill was used to evaluate the effect of flight on the olfactory response to leaf volatiles. The bioassays were repeated 10 times before and 10 times after flight in the flight mill for each of the 54 individual beetles. Leaf volatile components were analyzed using gas chromatography–mass spectrometry. The bioassay results supported our hypothesis: *P. quercivorus* was attracted to the leaf volatiles of hosts and avoided the leaf volatiles of non-hosts, and the olfactory responses of *P. quercivorus* to the leaf volatiles were stronger after flight. Males had a stronger olfactory response than females to leaf volatiles. The leaf volatile chemical profile of the non-host *Cryptomeria japonica* differed from those of the hosts. However, that of the non-host *F. crenata* was similar to those of the hosts. Our findings provide evidence that will help to improve the control of *P. quercivorus* and therefore the control of Japanese oak wilt.

Chapter 6

This conclusion chapter summarizes key findings of this thesis. Sexual difference was observed in the influence of flight speed on flight distance of *P. quercivorus* and olfactory responses to leaf volatiles of its host and non-host trees. Sexual difference was not observed in the influence of flight duration on flight distance and phototactic response of the beetle. Changes in phototactic and olfactory responses of *P. quercivorus* caused by flight were compared to those of other bark ambrosia beetles. The results of this thesis suggested possible process of dispersal flight and host selection for *P. quercivorus*. Upon emergence from breeding galleries, new brood beetles of *P. quercivorus* initiate dispersal phase by flying upward to the canopy openings created by the wilt of infested oak trees, and then fly above tree canopy surface in search of new host trees. After a certain flight exercise, beetles begin seeking for a suitable host through avoiding leaf volatiles released from non-host conifers. At the same time, beetles also follow common leaf volatiles released from broad leaved trees to stay flying in a correct habitat. Finally, beetles narrow the target down to their host by detecting leaf volatiles specific to oak trees.

Acknowledgements

I would like to thank my supervisor Dr. YUJI ISAGI for various supports to conduct this study. I also would like to thank Dr. NAOYA OSAWA and Dr. NAOKI MORI for acting as my committee members, letting my dissertation defense to be an unforgettable experience through various insightful comments and suggestions.

I would like to thank Dr. ATSUSHI TAKAYANAGI for multiple advices on the study.

Special thanks to Dr. MICHIMASA YAMASAKI. You have been a tremendous mentor for me. I would like to thank you for encouraging my research and for supporting budget and all difficulties I faced in Japan.

I would like to thank the following collaborate researchers: Dr. HIDETOSHI IKENO, Dr. RYUCHI OKADA, and Mr. YASUTO ITO for various supports on this study. I would like to extend my thankfulness to Dr. HARUNA KAZAMA for supporting me chemical analyses. I would like to thank Mr. HIDEKI OGATA (The Daichi Day Care Center) for helping us in the field works.

I would like to thank members of Laboratory of Forest Biology for offering me assistance and advice during this work.

Words cannot express how grateful I am to my paternal uncle PHAM DUY HIEN who offered me a chance to grow as a scientist. Your support and advice on both career as well as on my life have been priceless.

Special thanks to my family, my parents, parents-in-law and other natives for all of the sacrifices that they have made on my behalf. At the end, I would like to express my appreciation to my beloved wife TRIEU THI LUONG and my daughter PHAM KIEU ANH who have always been the major inspiration in every single day of my life.

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