Differential nymphal development of *Blattella asahinai* and *B. germanica* and their hybridization potential (Blattodea: Ectobiidae)

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 8, 2020

Key words: peridomestic pest, life cycle, reproduction, instar, clustering, development

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Abstract

The Asian cockroach, *Blattella asahinai* (Blattodea: Ectobiidae), is the closest relative of the German cockroach, *B. germanica* (L.), and has become a peridomestic nuisance pest in the southeastern United States. However, many basics aspects of the biology of *B. asahinai*, such as nymphal development and temperature thresholds have not yet been studied. The objectives of this project were to detail the nymphal development and developmental responses to temperature of *B. asahinai* as compared to *B. germanica* and examine the hybridization potential of these two species, as well as behavioral and morphological characteristics of the hybrids. In the following experiments, through measurements of nymphs, *B. asahinai* was estimated to have six or seven instars during nymphal development, and these results were subsequently confirmed through direct observation. Across six constant temperatures ranging from 10°C to 35°C, *B. germanica* had shorter nymphal development periods, fewer instars, and higher survivorship than *B. asahinai*. These developmental differences illuminated possible advantages that *B. germanica* and *B. asahinai* have in their respective environments. Both hybrid crosses were successfully performed, and the resulting hybrids appeared to have inherited intermediate wing morphology, allowing for flight in some hybrids. The results from these experiments will be useful in creating targeted management methods for *B. asahinai* based on unique biological traits that allow it to thrive in its outdoor habitat.

Acknowledgments

I am grateful to my advisers Dr. Hu and Dr. Appel for their support during my time at Auburn. Their advice and encouragement have allowed me to persevere in the midst of difficulties. I am grateful to my committee member Dr. Kesheimer not only for her help in the preparation of this thesis, but also for the valuable extension opportunities that she has coordinated that I have been able to partake in. I also thank Mrs. Marla Eva for her invaluable assistance with supplies and colony maintenance. I am grateful to Dr. Snoddy and Dr. Wu for their generous provision of materials that contributed to this research. I thank my lab mates Gökhan Benk, Seun Oladipupo, Patrick Thompson, and Richard Murphy for their support, encouragement, and advice. I also thank the Department of Entomology and Plant Pathology faculty and staff for their support. I thank my parents, Nancy Dunlap and Michael Dunlap, for their dedication to my academics and career goals, as well as their sound guidance. Most importantly, I thank my Creator, who is always good, and who has always directed my paths.

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CHAPTER 1

Introduction and literature review

Cockroaches and Humans

Cockroaches have the public perception of being survivors, pests, nuisances, and threats to public health. The few species of cockroaches that are indoor and outdoor pests are usually easily recognizable and as easily reviled. Most research has been concentrated on the control of cockroaches by examining their behavior, physiology, and life history. These traits are important factors that contribute to the ability of pest cockroaches to infest and proliferate in urban environments. Due to their relatively large size, ease of rearing, and locomotion ability, cockroaches are also valuable model organisms in invertebrate biology, especially chemical ecology, neurobiology, and toxicology (Gemeno and Schal 2004, Stankiewisz et al. 2012, Adedara et al. 2015).

There are 69 species of cockroaches across 31 genera and five families in North America (Pratt 1988, Atkinson et al. 1990). Of these, only about nine are consistently regarded as domestic or peridomestic pests, while the remaining species serve a variety of beneficial ecological functions, including decomposition, pollination, and food for insectivores, and are found in an even wider variety of habitats, including forests, deserts, caves, and quasi-aquatic habitats (Roth and Willis 1960, Nagamitsu and Inoue 1997). The two most common and important domiciliary cockroach species in the United States are the American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae), and the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae) (Schal 2011, Nasirian 2017). *Blattella germanica* is a true domestic pest with a cosmopolitan range, and it is especially problematic in low-income apartments (Koehler et al. 1987). Worldwide, the domestic brown-banded cockroach, *Supella longipalpa* (Serville) (Blattodea: Ectobiidae), and peridomestic oriental cockroach, *Blatta orientalis* L. (Blattodea: Blattidae), are also important pests, but their specific temperature requirements limit their ranges (Schal 2011, Nasirian 2017). In the southeastern United States and Asia particularly, the smokybrown cockroach, *Periplaneta fuliginosa* (Serville), has become a common urban pest (Appel and Smith 2002).

Cockroaches are omnivorous, which contributes to many species' close association with humans and our food (Schal 2011). Multiple cockroach proteins are human allergens that induce asthma; this is especially a problem in inner-city areas (Bernton and Brown 1964, Rosenstreich et al. 1997). Two major allergens are *Periplaneta americana* allergen 1 (Per a 1) from the American cockroach and *Blattella germanica* allergen 1 (Bla g 1) from the German cockroach, which are antigenically cross-reactive (Gore and Schal 2007). *Periplaneta americana* tropomyosin (Per a 7) is also antigenically cross-reactive with other allergenic invertebrate tropomyosins, such as those of dust mites and shrimp (Santos et al. 1999).

Pest cockroaches have also long been suspected disease-vectors, and bacterial contamination of food is an increasing concern, as the rate of cockroach infestation in human dwellings is rising with globalization and anthropization (Nasirian 2017, Schapheer et al. 2018, Nasirian 2019, Okon et al. 2020). Much of the evidence for cockroaches as disease-vectors has been circumstantial and correlative, and studies that have induced infection and tracked transmission did not accurately mimic field conditions (Schal 2011). Still, cockroaches in hospitals are mechanical vectors of many medically important bacterial, fungal, and parasite pathogens, and their possible role in nosocomial infections has been studied extensively (Fotedar et al. 1991, Pai et al. 2004, Fakoorziba et al. 2010). Although only about 1% of cockroach species worldwide are pests in association with humans (Rehn 1945), the public health impact of pest cockroaches, along with public perception and reaction to them, underscore the importance of developing effective and cost-efficient management strategies.

External Anatomy and Functional Roles

Most domestic and peridomestic cockroaches are quite recognizable in appearance, with long antennae, dark coloration, and often, wings. Their antennae contain many sensilla housing sensory neurons, including olfactory receptors, contact chemosensory receptors, tactile sensory receptors, thermoreceptors, and hygroreceptors, which are used to detect food odors, pheromones, temperature, and humidity (Schafer and Sanchez 1973, Block and Bell 1974, Sass 1978, Yokohari 1981). Cockroach antennae are generally long, aiding in the location of odor sources; the antennae of *P. americana* are 4 cm long, approximately the length of the body (Lockey and Willis 2015). The number of divisions, commonly called “annuli,” on the antennal flagellum increases each molt (Campbell and Priestley 1970). Cockroaches have asymmetrical dicondylic mandibles with both distal incisivi used to cut food and proximal grinding areas called mola used to grind food; this reflects the omnivorous diet of most cockroaches (Weihmann et al. 2015). In addition to compound eyes, cockroaches have lateral ocelli, but interestingly, they lost the median ocellus, which was retained by the rest of the lower Neoptera (Wipfler et al. 2016). The thorax consists of a pronotum that partially covers the head, the coloration of which is often used for sight identification (Triplehorn and Johnson 2005). The abdomen has ten sclerotized ventral sternites and dorsal tergites (Guthrie and Tindall 1968). Cockroaches have a pair of cerci on the end of the abdomen, which, like the antennal flagellum, usually add segments each molt and are used in the detection of air currents and gravity orientation (Murray 1967, Fraser 1977).

Most pest cockroaches are winged, even if they are not capable of sustained flight. Of the most common pest species worldwide, only *Periplaneta* spp. (both sexes) and male *S. longipalpa* can fly; in temperate climates, however, *P. americana* is usually restricted to gliding (Farnworth 1972). When a quick escape is needed, cockroaches are more adapted for running, though, with long legs and five-segmented tarsi (Triplehorn and Johnson 2005). The average running speed of adult *P. americana* at 30˚C is 91 cm/s (McConnell and Richards 1955). Tarsal arolia help many species of cockroaches climb a variety of surfaces (Arnold 1974). Cockroach cerci are very sensitive to wind acceleration, allowing them to detect the movement of predators and stimulating a running response (Camhi et al. 1978, Plummer and Camhi 1981). Cerci also aid in gravity orientation in some species, as in the case of the sand-burrowing *Arenivaga* spp. Rehn (Blattodea: Corydiidae) (Walthall and Hartman 1981)

Courtship and Mating

Courtship rituals are similar for most species of cockroaches, involving the male tergal glands, which produce pheromones that attract the female and often place it in mating position (Roth and Willis 1952, 1954). In *B. germanica*, the male tergal glands on the 7th and 8th tergites produce secretions containing sex pheromones, which the females eat during courtship (Roth and Willis 1952, Nojima et al. 1999). The morphology of the male tergal glands on the 8th tergite can also be used diagnostically to differentiate species (Roth 2003), as in the case of distinguishing between the closely related German and Asian cockroaches, *B. germanica* and *Blattella asahinai* Mizukubo, respectively (Roth 1986).

Courtship is usually stimulated by antennal sparring between a male and a female, after which the male turns and raises its wings, if present, with its abdomen facing the female (Roth and Willis 1952). Wings are an important part of cockroach courtship and mating rituals, and they likely have not been lost in the males of many species due to benefit during courtship (Kotyk and Varadínová 2017). If the female is sexually receptive, in species with male tergal glands, the female will place its mouthparts on the glands and, in some species such as *B. germanica*, will eat the secretion (Roth and Willis 1954). If the female is in the correct position, the male will extend its abdomen and grasp the female's genitalia to initiate copulation, which will usually last at least one hour (Roth and Willis 1954).

Life History and Reproduction

The Blattodea are hemimetabolous and have the basic life stages of egg, nymph, and adult. After hatching from the egg, the nymph grows through a series of molts, which varies in number depending on the species (Willis et al. 1958). The nymph attains sexual maturity after a final molt to become an adult, during which the individual will also develop wings, if present in the species (Belles 2019). Female cockroaches produce oothecae, which contain the developing eggs; in some species, where the oothecae develop externally, the walls of the oothecae are sclerotized, and, in other species, where the oothecae develop internally, the walls are thin membranes (Roth and Willis 1954). The oothecae of *B. germanica* can contain 30-50 eggs each, the most of any pest cockroach species, and each female produces an average of 4-6 oothecae per lifetime, making *B. germanica* one of the cockroach species with the highest rates of reproduction, which allows for rapid infestation of structures and selection of genes that confer insecticide-resistance (Willis et al. 1958, Ross and Mullins 1988, Schal 2011).

An interesting illustration of life history traits allowing an invasive species to replace an established species is displayed by the Turkestan cockroach, *Blatta lateralis* (Walker), that replaced *B. orientalis*, in the Southwest, described by Kim and Rust (2013). They reported that *B. lateralis* does not experience fewer eggs with successive oothecae, contributing to its ability to outcompete *B. orientalis*. *Blatta lateralis* also has a shorter period of nymphal development, and females produce more oothecae in their lifetime than *B. orientalis* (Kim and Rust 2013).

Roth and Willis (1954) noted the importance of cockroach reproductive diversity among different communities. Cockroaches may be oviparous, ovoviviparous, or, in a few species, viviparous; the latter two have also been described as "false ovoviviparity" and "false viviparity," since egg production is not physiologically distinct between the three classifications (Roth 1970). In some oviparous cockroaches, such as *Periplaneta* spp., females deposit well-sclerotized oothecae in the environment a few days after oothecal formation, often by attaching it to a surface with secretions from the mouthparts (Roth and Willis 1954, Schal 2011). In others, such as *Blattella* spp., females externally carry their oothecae at posterior end of their abdomen until eclosion; the anterior end of these oothecae are less sclerotized and are permeable to water from the female (Roth and Willis 1955). External retention of the oothecae until eclosion is an adaptation that aids the ability of *B. germanica* to thrive indoors, as the egg case is more protected from desiccation and parasitism (Schal 2011). "False" ovoviviparous species, such as many Blaberidae, have oothecae that are first extruded from the body then retracted, with the oothecae hatching within the body (Roth and Willis 1954). The only known "false" viviparous species are in the genus *Diploptera* spp. de Saussure (Blattodea: Blaberidae), in which the oothecae is formed within the body and is not extruded, and the developing eggs are nourished directly by the female rather than by the yolk (Roth and Willis 1955).

While the majority of cockroach species reproduce sexually, *P. americana* has been observed to have facultative thelytokous parthenogenesis, and a few species reproduce exclusively by thelytokous parthenogenesis, such as the Surinam cockroach, *Pycnoscelus surinamensis* (L.) (Blattodea: Blaberidae) (Roth and Willis 1956).Some cockroach species offer extensive care to young, such as subsocial *Cryptocercus* spp. Scudder (Blattodea: Cryptocercidae), the closest cockroach relative to termites. *Cryptocercus* spp. offer biparental care to young, feeding nymphs through trophallaxis and thereby supplying first and second instars the necessary gut microbiota necessary for cellulose digestion (Nalepa 1990). Even though third instar nymphs and onward have established gut protozoa and can live independently, continued biparental care increases the body size and weight of nymphs, as well as the nymphal growth rate (Park and Choe 2003).

In pest cockroaches, parental care of young does not exist or is limited, because many species abandon their oothecae after oviposition (Nalepa and Bell 1997). In *B. germanica*, social interactions and nymphal aggregation are essential for optimal development and sexual maturation (Izutsu et al. 1970, Uszák and Schal 2013), but true post-embryonic parental care is not observed; instead, biparental investment, in the form of urate transfer to females at the time of mating by males and uric acid provision to oothecae by females ensure adequate nutrition for the developing progeny (Mullins and Keil 1980, Mullins et al. 1992).

Factors Affecting Nymphal Development

As with most exothermic animals, temperature and diet account for much of the variability in the cockroach life cycle. The length of the life cycle from egg to adult is widely variable among species and is extremely dependent on temperature (Stejskal et al. 2003, Tsai and Chi 2007, Wu et al. 2017). Incubation time, egg to adult development period, and instar number are all affected by temperature and nutrients. At 25˚C, the German cockroach has been reported to have 6-7 instars and an egg to adult development range of 54-215 days (Schal 2011). In the laboratory, temperature and diet are variable factors that should be selected carefully in order to directly compare species from different studies.

Diet

Pest cockroaches are generally omnivorous scavengers; domestic species, like *B. germanica*, will eat nearly anything they find indoors and are especially attracted to starchy and sugary foods, while peridomestic species, like *P. americana* can eat decaying organic matter outdoors and, similarly to *B. germanica*, will eat nearly anything indoors, including glue, hair, and paper (Bell and Adiyodi 1981). When suboptimal nutritional conditions are experienced, such as a low protein availability, *B. germanica* compensates by foraging more to increase food intake (Hamilton and Schal 1988).

Cooper and Schal (1992) compared the development and reproduction of *B. germanica* on three different diets. Female *B. germanica* that were fed rat food gained mass quicker, and a higher percentage of nymphs reached adulthood than those fed Purina dog food. They reported that while newly eclosed adults on both diets had similar masses, the effect of food is primarily on the rate of development. They also found that grinding the dog food led to quicker gains in body mass, but there was no significant effect for ground rat food versus whole pellets. *Blattella germanica* nymphs are able to regulate growth throughout nymphal development to achieve standard adult body size despite adverse or suboptimal conditions (Tanaka 1982). This regulation mechanism causes variable development periods and number of instars, but it ensures that *B. germanica* nymphs that experience developmental delays can still reach standard adult size.

Temperature

As exotherms, insect's metabolic rates are affected by the environmental temperature. Insects require a certain number of accumulated "degree-days" to pass to the next development stage. The lower development threshold is the minimum temperature that an organism will continue to develop (Wilson and Barnett 1983). In cockroaches, similar to other insects, the number and length of each instar stage is highly dependent on temperature, and longer development periods with more instars are more likely to occur in colder-than-optimal temperatures. Wu et al. (2017) noted a lack of degree-day studies on cockroaches, presumably because it would not be used for rearing the most commonly studied species. In the field, however, knowing the number of degree days required for a pest insect to complete a life stage allows control methods to be synchronized and strategically initiated (Wu et al. 2017).

Environment and Social Interactions

Cockroaches are paraphyletic with respect to termites, with Cryptocercidae as a sister group to the former order of Isoptera (Inward et al. 2007). Cryptocercids are similar morphologically to termites and have common gut flagellate endosymbiont species, reinforcing their close relation (Thorne 1990). Nearly all other cockroach families also harbor a variety of gut microbiota that have been implicated in a variety of beneficial roles for the host, including nutritional supplementation, insecticide resistance, and, in *B. germanica*, mediation of aggregation (Wada-Katsumata et al. 2015, Ayayee et al. 2018, Pietri et al. 2018). There are likely further undiscovered benefits that cockroach gut microbiota serve, as axenically-reared *P. americana* often not able to complete nymphal development (Jahnes et al. 2019). All cockroaches, except *Nocticola* spp. Bolívar (Blattodea: Nocticolidae), also have *Blattabacterium*, an endosymbiont residing in the fat body that is maternally transmitted and likely to play a role in crucial nitrogen recycling from uric acid stored in the fat body (Lo et al. 2007, Sabree et al. 2009).

For most pest cockroaches, group living and social interactions are essential for optimal development and reproductive processes. In many domestic pest species, such as *B. germanica* and *S. longipalpa*, nymphs develop faster when reared in groups due to the tactile stimuli normally provided by aggregation (Willis et al. 1958, Izutsu et al. 1970, Lihoreau and Rivault 2008). Additionally, in *B. germanica*, group living stimulates juvenile hormone biosynthesis in adults (Gadot et al. 1989, Uzsák and Schal 2013). The aggregation of *B. germanica* first and second instar nymphs is particularly strong and has been widely studied (e.g., Ishii and Kuwahara 1968, Rust and Appel 1985). However, the chemical identity and production site of the "aggregation pheromone" have not been confirmed yet. Rivault et al. (1998) found evidence that cuticular hydrocarbons induced aggregation. However, Hamilton et al. (2019) recently challenged this finding and presented evidence that fatty acids in the feces were a more important component of the pheromone than cuticular hydrocarbons.

Discovery and Invasion of the Asian Cockroach

In 1981, Mizukubo published the first description of a newly discovered species of cockroach found in Okinawa Island, Japan, *Blattella asahinai*, named after S. Asahina, who did extensive work with *Blattella* taxonomy (Mizukubo 1981). The new species' physical characteristics were compared to the German cockroach, *B. germanica*, and to the false German cockroach, *Blattella lituricollis* (Walker) (Mizukubo 1981). In 1985, Roth also described a new species distributed throughout South Asia, naming it *B. beybienkoi* (Roth 1985). In 1986, Roth synonymized the names after Mizukubo found no differences between samples of two species (Roth 1986). It is likely that South Asia is the native of range of *B. asahinai*, and it was invasive to Japan. *Blattella asahinai* is speculated to have been introduced into the United States through Florida from imports from Japan (Roth 1986, Richman 2020). Since then, it has been recorded throughout Florida and in southern areas of Alabama, Georgia, Texas, North Carolina, and South Carolina and has been given the common name "Asian cockroach" (Brenner et al. 1988, Sitthicharoenchai 2002, Snoddy and Appel 2008, Austin et al. 2007, Matos and Schal 2015). Its outdoor habitat and capability of sustained flight are the most striking differences that separate it from the German cockroach, an entirely domestic species that cannot fly (Roth 1986). It appears to have been expanding its range through human transportation along major highways (Snoddy and Appel 2008).

Morphological Comparisons Between *B. asahinai* and *B. germanica*

The Asian cockroach has not been widely studied since its introduction, and most research has focused on its distribution and on morphological and genetic comparisons with *B. germanica*. Accurate identification of a species is the key basis for control. Morphologically, the two species are similar, but many studies have proposed reliable differentiating traits. In adults, Mizukubo (1981) noted that the interocular distance of *B. asahinai* was narrower than *B. germanica*, and morphology of the male tergites differed. Asian cockroach wings are longer, so less of the oothecae is seen dorsally past the wings than the German cockroach (Richman 2020). Comparisons of *B. asahinai* nymphs and oothecae with *B. germanica* have also been made. Ross and Mullins (1988) proposed that the patterns and colors of late-instar Asian and German cockroach nymphs were a reliable diagnostic trait. They observed the late-instar *B. asahinai* nymphs were smaller and had light spots on the dorsal tergites, appearing close together as two stripes down the dorsal side of the abdomen. The oothecae and eggs of *B. asahinai* were also smaller than *B. germanica*.

Outward morphological characteristics may not be readily distinguishable, and other diagnostic traits have been reported that allow for more confident species identification. Roth (1986) reported that the male tergal glands on the 8th abdominal segment can be used to differentiate *B. asahinai* from *B. germanica*. In the Asian cockroach, the posterior margins of the tergal glands are not curved anteriorly as in the German cockroach (Roth 1986; Snoddy 2007). Most morphological distinctions between the two species require adults for examination, but Carlson (1988) and Carlson and Brenner (1988) found that the cuticular hydrocarbon profiles, analyzed by gas chromatography mass spectrometry, were consistent within species, even among different sexes, strains, and life stages. Both studies compared *B. asahinai* to *B. germanica* and determined that this method was a reliable distinguishing trait for diagnostics. Carlson and Brenner (1988) also found that the cuticular hydrocarbon composition of the field cockroach, *Blattella vaga* Hebard, was very different from either *B. asahinai* and *B. germanica*. This is consistent with both phylogeny and morphology, as the Asian and German cockroaches are more closely related to each other than either is to the field cockroach, which is more easily distinguishable from the other two species (Carlson and Brenner 1988).

Behavioral differences between the two species are mostly related to differences in habitat and ecological niche. Because *B. germanica* has become a highly domesticized pest, unique behavioral adaptations, such as aggregation and avoidance of cannibalism on nymphs by adults, allow it to thrive indoors (Faulde et al. 1990, Tang et al. 2019). The behavioral traits of *B. asahinai* have not been characterized to the extent of *B. germanica*. Given that *B. germanica* nymphs are attracted to the feces of *B. lituricollis* and *B. vaga*, two outdoor species, but the nymphs of the *B. lituricollis* and *B. vaga* are either repelled by or not attracted to the feces of *B. germanica* or each other, it can be assumed that the domestication of *B. germanica* has led to the adaptation of certain aggregation tendencies that are not present in peridomestic species (Rust and Appel 1985).

Biological Comparisons of *B. asahinai* and *B. germanica*

Atkinson et al. (1991) reported that at25˚C, female *B. asahinai* produced an average of 3.7 oothecae in a lifetime, each with an average of 37.5 eggs. Ross and Cochran reported that when reared at 24°C-27°C, the average number of eggs per ootheca of *B. asahinai* was 43.0, only slightly less than the average 46.1 eggs per ootheca for *B. germanica*. For both female *B. germanica* and *B. asahinai*, there are fewer eggs per ootheca after a certain number of oothecae produced, and this decline is species-specific (Atkinson et al. 1991). When reared individually at 25°C, *B. asahinai* males had a mean nymphal development period of 65.7 d, and females had a mean nymphal development period of 67.8 d (Atkinson et al. 1991). In comparison, at 25°C, *B. germanica* require about 50 d (Tanaka and Hasegawa 1979). Using the number of viable oothecae per female, number of eggs per oothecae, and hatch rate, Atkinson et al. (1991) estimated that *B. germanica* had almost double the potential lifetime fecundity of both *B. asahinai*. However, the only development study conducted on *B. asahinai* was at 25°C, restricting the number of comparable *B. germanica* studies.

While *B. germanica* are usually able to breed continuously throughout the year, the population structure of *B. asahinai* is highly affected by seasonal conditions. Nymphs constitute a larger proportion of the population during June and July, and females are more prevalent than males during the winter months (Brenner 1991). Both habitat and seasonal age structure differences are important differences between the Asian and German cockroach that provide specific control techniques for *B. asahinai*. Asian cockroaches are largely insecticide susceptible compared to their German cockroach relatives (Snoddy and Appel 2014; Ross and Cochran

1995). More details about the life history and population dynamics of the Asian cockroach can help create a more targeted and robust integrated pest management (IPM) strategy.

A large percentage of studies with *B. asahinai* have examined its genetic relationship with *B. germanica*. There is strong evidence to conclude that *B. asahinai* and *B. germanica* are phylogenetic sisters, making them each other's closest extant relative (Roth 1986; Mukha et al. 2002). Many *B. germanica* and *B. asahinai* hybridization and cytological studies have been performed, examining the X chromosome (Ross 1992) and chiasma (Ross and Liu 1995) as factors influencing species divergence. *B. asahinai* also responds to blattellaquinone, the sex pheromone of *B. germanica* (Matos and Schal 2015). These data give more support to the close relationship between the species. Ross and Cochran (1995) found that F_1 *B. asahinai* and *B. germanica* hybrids inherited some insecticide resistance from their German cockroach parent, resulting in higher LT_{50} values than the Asian cockroach parent, but the values were still more similar to the Asian cockroach parent than to the German. Although hybridization between the two species has been successful in the laboratory, it is unlikely to occur in nature, due to the distinct habitats of each species. However, hybridization studies have illuminated the close relationship between the two species and have contributed to knowledge on interspecific divergence and inheritance of traits important to cockroach control.

Blattella germanica are domestic; although in cases of extreme indoor infestation, some individuals can be found outdoors (Appel and Tucker 1986). Conversely, *B. asahinai* are peridomestic, and no evidence exists that they can survive indoors for extended periods. One biological factor that would restrict them to a moister outdoor habitat is their cuticular permeability (CP). The link between cuticular permeability and the abundance, composition, and structure of epicuticular lipids in insects is not clearly understood. Hadley (1978) studied this correlation in desert tenebrionid beetles and found a loose correlation between CP and composition and structure of the hydrocarbons, which accounted for most of the lipids. Appel et al. (1983) noted that the hydrocarbon profile of *B. orientalis* and *Rhyparobia maderae* (Fabricius) (Blattodea: Blaberidae) were almost identical but resulted in very different CP values. Other factors, like body fat, likely influence water loss and can possibly mitigate effects of hydrocarbon composition and abundance (Appel et al. 1983). Appel (1993) tested the cuticular permeabilities of four strains of German cockroaches, two insecticide resistant and two susceptible, and found that CP could not be correlated with insecticide resistance. This result is consistent with Carlson's (1988) findings that hydrocarbon profile did not vary significantly among insecticide resistant and susceptible strains of a species. *Blattella germanica* has a reported CP value of 19.9 ± 0.80 (Appel et al. 1983). *Blattella asahinai* has been reported to have a greater CP value than this, likely a key factor in restricting its habitat and range, since it is more prone to desiccation (unpublished data).

Range Expansion of *B. asahinai*

Blattella asahinai appears to be expanding its range in the southern states. It was likely introduced in Florida and has been spreading northward and westward (Roth 1986). Snoddy and Appel's (2008) distribution map that describes *B. asahinai* as present throughout Florida and in certain southern counties in Alabama and Georgia is likely already outdated, as the Asian cockroach has been found in counties where they were not present 10 years ago when the study was conducted (personal observation). *Blattella asahinai* was found in Weslaco, Texas, close to the border of Mexico (Pfannenstiel et al. 2008), and in southeast Texas, in Harris County (Austin et al. 2007). The main factors that would affect its range limits are temperature and humidity, the latter especially in its westward expansion. The critical thermal minima (CTMin) and maxima

(CTMax) were determined by Snoddy (2007), with the lowest critical thermal mimima (CTMin) among all life stages to be 0.8˚C ± 0.60˚C for large nymphs. CTMin, along with the lower development threshold (LDT) are important temperature parameters that would govern the northward expansion of *B. asahinai*. Snoddy (2007) theorized that *B. asahinai* had expanded more northward in Georgia than it had in Alabama, because of coastal Georgia's more humid climate. More information about *B. asahinai* temperature tolerances and desiccation would help predict specific areas that could facilitate or hinder expansion.

Agricultural Interactions of *B. asahinai*

An interesting observation of *B. asahinai* is its consumption of pest eggs and parasitized aphids in agricultural settings. Pfannenstiel et al. (2008) placed sentinel eggs of two species of pest lepidopterans on soybean, where *B. asahinai* was observed at night, and found that *B. asahinai* accounted for 53.7% of nocturnal predators observed and captured feeding on the eggs. Persad and Hoy (2004) investigated puzzling low densities of an aphid parasitoid in citrus groves and tested the possibility of consumption of parasitized aphids by both *Solenopsis invicta*, the red imported fire ant, and *B. asahinai*. They concluded that *S. invicta* was a more significant threat parasitoid emergence, but starved *B. asahinai* would consume parasitized aphids, preventing adult parasitoids from emerging. While *B. asahinai* may not significantly affect current biological control methods, attention and observation should be given to the role of *B. asahinai* in agricultural settings, especially as it has been reported to infest and consume a variety of agricultural products, including sweet corn, cabbage, strawberries, and nursery plants, causing both economic and aesthetic injury (Brenner 1991).

Current Control of *B. asahinai*

Since *B. asahinai* have not been extensively and historically treated with insecticides in the same manner as *B. germanica* have, the lack of this selective pressure has resulted in a general susceptibility to insecticides for *B. asahinai* (Snoddy 2012). *Blattella asahinai* can infest outdoor habitats in large numbers of up to nearly 200,000 individuals per acre and become a major nuisance pest for homeowners (Brenner 1991). Current control methods include baiting, as well as environmental modification (Snoddy 2012, Matos and Schal 2016). Because *B. asahinai* have specific mulch preferences and cannot see red, yellow, or LED light, a few different cultural control options exist to lower attraction for *B. asahinai* to lawns and gardens (Snoddy 2012). Snoddy (2012) found that cypress mulch was toxic and repellant to *B. asahinai*, recommending it be incorporated into an IPM program for *B. asahinai*.

Literature Summary

Cockroaches are among the most reviled home invaders, despite the fact that most species are beneficial decomposers that are not associated with humans. However, the few species that do impact humans negatively can be serious health hazards and cause significant aesthetic and economic damage. Many biological and life history traits are responsible for the success of these pest species, such as temperature, social interactions, and diet, and these specific traits can be exploited for management and control. *Blattella asahinai* is a relatively recent invasive species that can infest yards and agricultural fields in high numbers, annoying homeowners and causing damage to commodities. Information on its basic biology is limited, and physiological and behavioral differences between *B. asahinai* and *B. germanica* are important to determine the best control methods for *B. asahinai*, since control methods are most effective when they target a species' life cycle and utilize their unique behaviors.

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CHAPTER 2

Instar determination of *Blattella asahinai* (Blattodea: Ectobiidae) from digital measurements of the pronotum using gaussian mixture modeling and the number of cercal annuli

Abstract

The Asian cockroach, *Blattella asahinai* Mizukubo, has expanded its range throughout the southeastern United States since its introduction into Florida. Unlike its closest relative, the German cockroach, *B. germanica* (L.), *B. asahinai* lives outdoors and can fly. There is little information on the biology and development of *B. asahinai*, including the number of instars during nymphal development. To estimate the number of instars of *B. asahinai*, nymphs were photographed, sexed, and the lengths and widths of their pronota were measured digitally. The number of instars of *B. asahinai* was estimated using Gaussian mixture models with the pronotal data. The most probable model and its clusters were selected to assign individuals to an instar. Instars were also determined by counting the number of cercal annuli of nymphs. Both clustering and cercal annuli indicated that *B. asahinai* most frequently had six instars when reared at 30˚C. Growth did not strictly follow the Brooks-Dyar Rule, because nymphs had different numbers of instars and different growth patterns. Although Gaussian mixture models are not efficient for field sampling experiments, digital measurements may provide a way to estimate instars with live specimens in development studies without handling the animals in a way that may alter growth.

Introduction

The Asian cockroach, *Blattella asahinai* Mizukubo, is a relatively new invasive species from South Asia and a peridomestic pest around residences and in crop fields in the southeastern United States (Mizukubo 1981, Roth 1986, Brenner et al. 1988). Since its likely initial introduction into Florida, *B. asahinai* has expanded its range and has been confirmed in Alabama, Georgia, Texas, North Carolina, and South Carolina (Brenner et al. 1988, Sitthicharoenchai 2002, Austin et al. 2007, Snoddy and Appel 2008, Matos and Schal 2015). This range expansion is likely facilitated by human transportation along major highways (Snoddy and Appel 2008). The closest relative of *B. asahinai* is one of the most prevalent cockroach pests worldwide, *Blattella germanica* (L.), the German cockroach (Nasirian 2017). The two species are morphologically similar but can be positively identified using the morphology of the adult male tergal glands and cuticular hydrocarbon profiles (Roth 1986, Carlson and Brenner 1988). The outdoor habitat and capability of sustained flight are the most striking differences between *B. asahinai* and *B. germanica*, an entirely domestic species that cannot fly (Roth 1986, Brenner et al. 1988, Brenner 1991).

Information on the life history and biology of pest insects is critical to develop control methods based on predictions of pest population dynamics. In. *B. germanica*, different instars have different tolerances to temperature and insect growth regulators, and relative food consumption rates (Das and Gupta 1977, Valles et al. 1996, Snoddy 2007). There is little information on details of the life cycle of *B. asahinai*, including a confirmation of the number of instars during nymphal development. The presence of *B. asahinai* in agricultural settings and observation of both pestiferous and beneficial behavior on crops (Brenner 1991, Persad and Hoy 2004, Pfannenstiel et al. 2008), in addition to its close relationship to a major domestic pest, warrant more investigation into the biology, development, and seasonal patterns of this species. The identification of nymphal instars is indispensable from both ecological and taxonomic perspectives.

Several studies with *B. germanica* report most nymphs undergo six instars (Seamans and Woodruff 1939, Koehler et al. 1994). In other studies, where the growth of each sex was independently observed, males had five instars and females had five or six (Tanaka and Hasegawa 1979, Keil 1981, Kunkel 1981). Keil (1981) hypothesized that selection favors 6-instar-type females that are larger as adults and have more metabolic resources for egg production, while males achieve sexual maturity faster when the adult molt occurs after the fifth instar, allowing them more mating opportunities. The specific environmental pressures on *B. germanica* may select for an instar number in each sex that produces maximum reproductive capability. While the number of molts is determined primarily by genetics, external factors such as temperature, diet, injury, anesthesia, and other adverse rearing conditions may cause additional instars (Seamans and Woodruff 1939, Wigglesworth 1972, Kunkel 1981, Tanaka 1982).

In indirect instar determination, where nymphs are not observed throughout their development, measurements of the head capsule are traditionally used with the Brooks-Dyar Rule, which states that any highly sclerotized structure increases in size in each subsequent instar by a constant growth ratio (Brooks 1886, Dyar 1890). Growth ratios are calculated by dividing the post-molt size by the pre-molt size (Wu et al. 2013), representing an increase in size (Gaines and Campbell 1935). Since the ratio is theoretically constant, with growth increasing by a geometric progression between each instar, Dyar (1890) proposed that inconsistencies between the observed measurements of each theoretical instar and the measurements expected by the growth ratio can signify that an instar has not been accounted for. This rule, however, has been questioned as a reliable method to detect missing instars from measurements, since the growth “constant” is not invariable within all species, as males and females may have different growth ratios, and the ratios may be affected by environmental conditions (Gaines and Campbell 1935, Seamans and Woodruff 1939, Floater 1996).

The length and width of the pronotum are two characteristics that have been used in a multivariate approach to determine the number of instars with Gaussian mixture models, a model-based clustering method that assumes normal distributions for each component and uses Bayesian inference to select the most probable model of the data (Wu et al. 2013). Using these models, individuals can be assigned to an instar cluster based on their pronotal lengths and widths. Wu et al. (2013) used the package “mclust” in R software (R Core Team 2019) to determine the number of nymphal instars of *Blaptica dubia* Serville (Blattodea: Blaberidae) and corroborated their results with the Brooks-Dyar Rule. For “mclust,” the optimal number of groups is determined with the Bayesian Information Criterion (BIC), a modified maximum likelihood approach developed by Schwarz (1978).

The shafts of the cerci of some insects have external indentations in the cuticle that form rings, or annuli, which result from the division of the basal segment (Murray 1967, Chapman 1998). In cockroaches, the number of annuli on the cerci can also be used to determine instars, as nymphs normally gain a set number of annulations on each cercus in each successive molt (Murray 1967, Chapman 1998). First instar *B. germanica* nymphs have three dorsal cercal annuli, second instar nymphs have six, and then one annulation is normally gained in each molt after the second instar (Murray 1967). The number of cercal annuli is usually used in conjunction with other morphological features to determine developmental stage. Measuring the head capsule of the shed exuviae is a common method of obtaining measurements without handling the insect (Moser et al. 1991, Chen and Seybold 2013). However, some insects eat their exuviae after molting (Mira 2000, Raubenheimer and Barton-Browne 2000); this behavior has been observed in the American cockroach, *Periplaneta americana* (L.), as a factor hindering instar determination (Gould and Deay 1938, Griffiths and Tauber 1942). In development experiments, insects generally should not be handled or anesthetized to avoid altering growth (Tanaka 1982). Digital measurements, then, for some live insects, may be a solution for future studies. Although head width has the least variation for *B. germanica* (Tanaka and Hasegawa 1979), pronotal measurements are often easier to obtain digitally with live insects, particularly since the cockroach head is partially concealed beneath a pronotum.

The purpose of this study is to determine the number of instars of *B. asahinai* using Gaussian mixture models, based on digital measurements of the length and width of the pronotum, and to investigate the use of this process to improve the efficiency of instar identification. We compare the estimated number of instars of *B. asahinai* to that of *B. germanica*, and we analyze sex-specific growth patterns revealed by clustering and the Brooks-Dyar Rule. We also compare the digital measurement method of instar determination to traditional methods and assess it as a viable option for live insects in ongoing development studies and for possible sampling of field populations.

Materials and Methods

Collection and rearing

Blattella asahinai were collected from two locations around Auburn University (The Old Rotation: 32.5934˚, -85.4857˚; AU Medical Clinic: 32.5931˚, -85.4880˚), in Auburn (Lee

County), Alabama, USA, between August and October 2018. They were identified as *B. asahinai* at the time of collection by their outdoor habitat and flight capability. The male tergal glands on the 8th abdominal segment were also examined for positive identification as *B. asahinai* (Roth 1986, Snoddy 2007). Cockroaches were reared at 30 ± 2˚C with photoperiod of 12:12 (L:D) h in 3.8 L (1 gallon) glass jars with a cardboard harborage and were provided rat chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and water *ad libitum.*

Imaging and measurements

Nymphs were removed from colony jars by tapping the harborage on the side of a plastic bin near the top, with CO_2 anesthesia flowing at the bottom of the bin. Nymphs that landed in the bin were collected after being anesthetized and killed by freezing. Thawed nymphs of similar sizes were arranged in groups of 10-15 on a Petri dish lined with filter paper at the bottom with their pronota clearly visible. Nymphs were photographed using a 12-megapixel Olympus TG-5 camera (IM005, Olympus Corporation, Tokyo, Japan) attached to a tripod. An extra light was pointed at the dish. Photographs were often taken using both the fill-in flash setting and the LED light setting; the better photograph of the two was selected for analysis. All images were analyzed with ImageJ software (Schneider et al. 2012), a popular choice for image processing (Mutanen and Pretorius 2007, Teale et al. 2009, Seiter et al. 2010, Wieferich et al. 2013). A pair of calipers with millimeter marks (Model 62379-531, VWR International Inc., West Chester, PA) were included in each photograph to calibrate pixels/mm. The pronotal length and width of each nymph was measured by drawing a line and using the measure function. After each nymph was measured, it was sexed according to the methods of Ross and Cochran (1960).

Cercal annuli

The cerci of the photographed nymphs were examined under a dissecting microscope (EMZ-TR, Meiji Techno, Tokyo, Japan) at approximately 45x. The number of dorsal annulations were counted using the methods of Murray (1967). When necessary, the last abdominal segments with cerci were removed from nymphs and mounted on glass microscope slides with water to aid annuli counting. An instar number was assigned to each nymph based on the number of cercal annuli, following the designations of Murray (1967) and Tanaka and Hasegawa (1979). If an annulus was partially segmented as described by Murray (1967), it was not included in the total annuli count for that nymph. Deformed cerci were not recorded, since the number of annuli was reduced, but the nymphs to which they belonged were still measured and included in cluster analyses.

Gaussian mixture models

Finite mixture models are used to analyze a population containing a mixture of subpopulations, or components, each with different parameters but all assumed to be from the same parametric family (Gelman et al. 2013). The distribution of each observation is described by a probability density function $f(x_i;\Psi) = \sum_{k=1}^{G} \pi_k f_k(x_i;\theta_k)$, where Ψ are the estimated parameters (π_k, θ_k) of the mixture model; G is the number of components in the mixture; π_k are the mixing probabilities, where $\pi > 0$ and $\sum_{k=1}^{G} \pi_k = 1$; and $f_k(x_i;\theta_i)$ is the density of the kth component for each observation x_i with parameter vector θ_k (Scrucca et al. 2016). Bayesian Gaussian mixture models assume all components are from multivariate normal distributions and use posterior probabilities to assess and update the model (Scrucca et al. 2016). Each component, or cluster, is normally distributed, so $f_k(x_i;\theta_i) \sim N(\mu_k, \Sigma_k)$, where μ_k is the mean vector and center of each cluster and Σ_k is the covariance matrix that determines cluster characteristics, such as shape and volume (Scrucca et al. 2016).

The Bayesian Information Criterion (BIC) uses a modified maximum likelihood approach to assess each model (Schwarz 1978). The BIC is calculated $\mathrm{BIC}_{M,G} = -2\ell_{M,G} + K \log n$, where $\ell_{M,G}$ is the maximized log-likelihood for the model with G components, K is the number of estimated parameters, and n is the sample size (Posada and Buckley 2004, Scrucca et al. 2016). The number of components corresponds to the number of clusters. Added parameters (explanatory variables) can increase the log-likelihood but will reduce the model's BIC value (Scrucca et al. 2016). Because "mclust" returns negative BIC values, the model with the highest BIC value is the most probable model (and most parsimonious) given the data (Chapman and Feit 2015).

Data analysis

Data were analyzed with R software (R Core Team 2019) using package "mclust" (Scrucca et al. 2016). From previous unpublished use of "mclust" in determining the number of instars, sorting the data by any measurement either in ascending or descending order produced more accurate results than "unsorted" data (MKP, unpublished). Since sorting the data produced more accurate or equivalent results to the unsorted data, data were sorted using length or width before analysis. The default parameters for "mclust" were used, where G = 1:9 (BIC calculated for one to nine clusters). A 95% prediction interval was calculated by linear regression of the lengths and widths of the pronotum from all nymphs. Due to potential measurement errors caused by suboptimal nymph arrangement and picture clarity, individuals outside the 95% prediction interval were identified as outliers and removed. The natural logarithms of pronotal lengths and widths were used to approximate normality for each instar cluster. This step was taken to test whether log transformation affected clustering and whether the log-transformed model fit better with the results from the number of annuli.

The untransformed and natural log-transformed pronotal length and width data from combined sexes were each analyzed with “mclust.” Extreme values within the 95% prediction interval that caused skewness were removed from some clusters prior to analyses of means to help approximate normal length and width distributions. For comparisons between instars, mean pronotal lengths rather than widths were used, since lengths were normally distributed more often than widths were. Mean pronotal lengths were compared between identified clusters using the nonparametric Kruskal-Wallis test to account for non-normal distributions with Dunn’s multiple comparison test using the Benjamini-Hochburg false-discovery rate *P*-value adjustment method (Kruskal and Wallis 1952, Dunn 1964, Benjamini and Hochberg 1995). Within each instar, means of pronotal length and width were compared between sexes with Welch’s *t*-tests with a Games-Howell *post hoc* test (Games and Howell 1976). “Mclust” was used to separately cluster female and male untransformed and log-transformed data to further analyze growth patterns between sexes.

To determine if the results would be similar with a smaller sample size, the data set without outliers was reduced to one-fourth (357), one-third (476), half (714), two-thirds (952), and three-fourths (1071) of its original size. For each size, three different data sets were randomly generated by sampling the original data set (excluding outliers) without replacement using package “dplyr” (Wickham et al. 2018) in R software (R Core Team 2019). All data sets were sorted according to pronotal length. Pronotal lengths and widths were natural log-transformed. Both the untransformed and transformed data were then analyzed with “mclust,” and models with the highest BIC values were selected for each permutation of data. All figures were created using package "ggplot2" (Wickham 2016) in R software (R Core Team 2019).

Pronotal length and width means were compared between instars determined by cercal annuli using Welch's ANOVA with a Games-Howell *post hoc* test (Games and Howell 1976). Within each sex, pronotal length and width means were compared between instars with a Kruskal-Wallis test and Dunn's multiple comparison test with the Benjamini-Hochburg false-discovery rate *P*-value adjustment method (Kruskal and Wallis 1952, Dunn 1964, Benjamini and Hochberg 1995). For each instar, means of pronotal length and width were compared between sexes using Welch's *t*-tests with a Games-Howell *post hoc* test (Games and Howell 1976). To compare our results with traditional methods of instar determination, frequency distributions were created using the pronotal width of male and female nymphs. Instars were identified within those distributions using cercal annuli data.

To calculate the growth ratio using the Brooks-Dyar Rule, the formula $\frac{premolt\ size}{postmolt\ size} = growth\ constant$ was used (Wu et al. 2013). For each sex, linear regression was also performed for the natural log-transformed pronotal length and width means by the instars identified by "mclust." The growth ratios for pronotal length and width for each sex were calculated as e^{slope} (Wu et al. 2013). To determine if the sex ratios in each instar deviated from 1:1, two-sided binomial tests were used for each instar identified by both clustering and annuli. All analyses were conducted at $\alpha = 0.05$, and all clustering and statistical analyses were performed using R software (R Core Team 2019).

Measurement testing

To test the precision between direct measurements with caliper and digital measurements from photographs using ImageJ, five nymphs from each of the six instars (determined by counting cercal annuli) were collected and killed as above and the lengths and widths of their pronota were measured using the digital measurement method and then using a pair of digital calipers (Model 62379-531, VWR International Inc., West Chester, PA). One picture was used for each instar group. Paired *t*-tests were performed on the caliper lengths and digital lengths and on the caliper widths and digital widths. Linear regression with 95% confidence and prediction intervals were used to compare the two methods for pronotal length and width. Pearson correlation coefficients were calculated for caliper and digital measurements for pronotal length and width. Data were analyzed using R software (R Core Team 2019)

Results

Measurement comparisons

There were no significant differences between measurements made from digital photographs and those made using a pair of calipers for each instar for either pronotal length or width (Table 1). The correlation coefficient for pronotal length between the two methods was 0.9943. The correlation coefficient for pronotal width was 0.9981. Fig. 1 shows the 95% confidence and prediction intervals between the two methods for pronotal length (A) and width (B).

Gaussian mixture models

With outliers removed, 1,428 nymphs were included in the clustering analyses. Using the pronotal length and width data, the model with the highest BIC value of 1,653.57 and a log-likelihood of 935.74 had six clusters (Fig. 2A). Because this model had some clusters that severely overlapped, the natural logarithms of pronotal length and width were analyzed with “mclust.” The log-transformed model with the highest BIC value of 3,028.50 and a log-likelihood of 1,623.21 had six clusters, and the clusters did not strongly overlap (Fig. 2B).

Although the selected model was generated from log-transformed data (Fig. 2B), there were still some "mclust”-identified clusters without normal distribution of pronotal lengths and widths. Removing six extreme values from the first and sixth clusters resulted in normal distribution of the natural-log transformed pronotal lengths for most instar clusters. The fifth instar cluster was not normally distributed for either length or width when the sexes were combined. Natural log-transformed fifth instar pronotal lengths had a platykurtic distribution. Since pronotal length was normally distributed more often than pronotal width, pronotal length was used to compare the six clusters with a non-parametric Kruskal-Wallis test. All cluster means were significantly different for combined sexes (Table 2).

Within each sex, pronotal lengths of the six clusters were significantly different (Table 3). Within each instar, second and sixth instar pronotal lengths were significantly different between males and females (Second: $t_{0.05} = 2.7$, $df = 320$, $P = 0.009$; Sixth: $t_{0.05} = -5.8$, $df = 115.5$, $P < 0.001$). Sixth instar pronotal widths were signficantly different between sexes ($t_{0.05} = -8.2$, $df = 112.9$, $P < 0.001$). Pronotal length and width were not significantly different between sexes for all other instars ($P > 0.2$). Pronotal length and width means for each sex for each instar cluster are reported in Table 3.

Both the untransformed and log-transformed pronotal lengths and widths for males and females were analyzed separately with “mclust.” The untransformed data for males resulted in four clusters (Fig. 3A); however, the transformed data for males resulted in five clusters (Fig.

3B). A small group of possible 5-molt-type males is seen in Figs. 3A and 3B. The untransformed data for females resulted in seven clusters (Fig. 3C), whereas the transformed data resulted in eight clusters. However, one of these eight clusters was a "side cluster" assigned to a group of first instar outliers (Fig. 3D).

The smallest data set that produced six clusters similar to the whole data set for all three random data sets was 714 (half of the original data set). With the three half-data sets, all three models with transformed pronotal lengths and widths produced six clusters. Untransformed pronotal lengths and widths produced six clusters for only two of the three randomly sampled half-data sets. The untransformed model from the third half data set had only three clusters and is shown in Fig. 4A, with the transformed model from the same data set shown in Fig. 4B. "Mclust" models from the three untransformed two-thirds data sets (952) either had fewer than six clusters or had six clusters with some clusters overlapping. An example is shown in Figs. 4C and 4D. The untransformed three-fourths data sets (1071) all produced models with only five clusters. For the same transformed data sets, two of the three models had six clusters, but one had five. An example of one of the untransformed models with five clusters and its transformed model with six clusters is shown in Figs. 4E and 4F. From all fifteen untransformed data sets, only two generated models with six clusters that did not significantly overlap. From all fifteen transformed data sets, ten generated models with six clusters that did not significantly overlap.

Cercal annuli

The distribution of annuli-identified instars using their pronotal lengths and widths is shown in Fig. 5. Using annuli, six distinct instar groups were seen. Possible seventh instar nymphs with 11 dorsal cercal annuli were also identified. The seven instars determined from cercal annuli were approximately normally distributed after outliers were removed. The means for each instar are reported in Table 4. All instars were signficantly different by their pronotal lengths (Table 4).

Table 5 reports the mean pronotal length and width for each instar by sex. Using pronotal length, male instars five, six, and seven were not significantly different from each other ($P >$ 0.05). Female instars five and seven and female instars six and seven were also not significantly different ($P > 0.05$). Within the fifth instar, pronotal lengths were significantly different between males and females ($t_{0.05} = 2.2$, $df = 164.58$, $P = 0.03$). Within all other instars, pronotal length and width were not significantly different between males and females ($P > 0.05$).

Pronotal lengths were plotted against widths for males and females (Figs. 5B and 5C, respectively), showing the distribution of instar by sex determined by annuli. For males, a small group of nymphs was seen separated from the rest of the fifth instar group (Fig. 5B). For females, the fifth and sixth instars separated into three groups (Fig. 5C). Frequency distributions of pronotal length and width were similar for both males and females. Frequency distributions using pronotal width resulted in clearer peaks (Fig. 6). Both male and female pronotal widths had approximately seven main peaks, but the instar compositions of the sixth peaks differed by sex when annuli data were used with the frequency distributions (Figs. 6A and 6B). Fifth and sixth instars were bimodal for female pronotal width (Fig. 6B).

Growth ratio

Growth ratios, representing the relative increase in size between instars, calculated by the Brooks-Dyar Rule from the mean pronotal measurements for each instar for each sex and for each method of instar determination, are presented in Table 6. The growth ratio between the fourth and fifth instar for both the pronotal length and width and for both sexes was noticeably greater than the other ratios for both methods of instar assignment. The general pattern observed was an increase in ratio between the third and fourth instars (average 0.06 greater) and a greater increase in ratio (average 0.08 greater) between the fourth and fifth instars All average ratios were higher for females than males (0.01 – 0.02 greater). Clustering produced the same or slightly higher (0.00 – 0.01 greater) ratios for both sexes for both pronotal length and width. The growth ratio between the sixth and possible seventh instar (with 11 dorsal annuli) was 1.03 for males and 1.05 for females for pronotal length and was 1.04 for males and 1.06 for females for pronotal width.

Using the slopes of the regression lines of the natural log-transformed means of measurements for instars one through six by sex, the growth constants for male were 1.28 for pronotal length and 1.30 for pronotal width (Fig. 7A). The correlation coefficient between both male pronotal length and instar and between male pronotal width and instar was 0.997. For females, the growth constants were 1.29 for pronotal length and 1.31 for pronotal width (Fig. 7B). The correlation coefficient between both pronotal length and instar and between pronotal width and instar for females was 0.996.

Sex ratio

Based on either instars by clustering or instars by annuli, the overall male:female ratio did not significantly differ from 1:1. The only exception occurred in the possible 7th instar (with 11 annuli), which had significantly more females (22) than males (7) ($P = 0.008$).

Discussion

One goal of this study was to assess the use of digital measurements of the pronotum with Gaussian mixture models for efficient nymphal instar identification. A group of 10-15 *B. asahinai* nymphs took approximately 40-60 minutes to photograph, measure digitally, sex, and assign to instar based on annuli. The nymphs had to be arranged in a way where all pronotal boundaries could be clearly seen, and multiple attempts at an acceptable photograph for each group were needed. The estimated time required in our study to obtain digital measurements is likely not shorter than the time needed to collect the same data with direct measurements using a pair of calipers or an optical micrometer. However, nondestructive sampling may be necessary for species that are not abundant or not in culture and to prevent potential effects on normal behavior and development. Smiley and Wisdom (1982) also recommended using photography to estimate the weight of live insects, especially in the field, to assess growth under natural conditions. The equipment and software used in our study are inexpensive or open-source and do not require special training to use. The ImageJ software was only used for basic measuring and occasionally image sharpening to clarify pronotal borders, but there are a wide variety of image analysis software features that can be used creatively to reduce estimation and time for processing.

Wu et al. (2013) successfully used Gaussian mixture models to determine the number of instars of *B. dubia* from 1,925 nymphs. The present study only included 1,428 nymphs in the clustering analysis. The model with six clusters generated from untransformed pronotal length and width measurements was clearly erroneous for the third instar cluster, as it overlapped both the first and second clusters (Fig. 2A). Because clustering with log-transformed measurements produced more discrete clusters that did not strongly overlap, the clusters assigned to the model fitted to log-transformed data were used for all calculations (Fig. 2B).

Since some insects lack a method of instar determination apart from measurements, such as the number of cercal annuli, the use of clustering with measurements may be the only indirect method available to determine the number of instars (Frątczak and Matuszewski 2014). In cases with non-overlapping instar measurements where data are normally distributed, clustering alone will likely produce accurate results, especially when the sample size is large, similarly to what frequency distributions would provide. The minimum sample size needed for accurate clustering will likely depend on the exact data and degree of overlap of groups. In our study, the majority of models with six clusters generated from both transformed and untransformed data came from sample sizes of ≥ 714. Since Gaussian mixture modeling assumes that each cluster comes from a normal distribution, transforming the data helped cluster assignment in nearly all cases with a sample size of ≥ 714, evidenced by the log-transformed half, two-thirds, and three-fourths data sets that produced similar clusters to the full data set (Figs. 4B, 4D, and 4F), compared to the untransformed data from the same number of nymphs (Fig. 4A, 4C, and 4E). Fink (1984) determined that counting the number of peaks of simple frequency distribution of measurements led to the detection of "false" instars for mayflies, since growth was not homogenous for all individuals. This problem extends to many insects and to any measurement method. Use of cluster analysis alone to determine the number of instars may lead to false conclusions.

For instar determination through Gaussian mixture models, Wu et al. (2013) used measurements of *B. dubia*, which did not have significant differences in body mass or development periods between sexes (Wu et al. 2017). *Blaptica dubia* may have a constant instar number between sexes, simplifying clustering. For *B. asahinai*, however, clustering males and females separately better revealed sex-specific growth patterns. Each cluster represented a size class, rather than an instar. Fifth instar *B. germanica* females tend to have pronotal widths that separate into two distinct groups, indicating the separation of sizes of 5- and 6-instar types (Tanaka and Hasegawa 1979). Using instars determined by annuli, the distributions of female fifth and sixth instar widths were bimodal, revealing size separations for different instar types in the fifth and sixth instars (Fig. 6). When clustering using just female pronotal length and width, "mclust" identified a total of seven clusters with three clusters containing the fifth and sixth instars (Fig. 3C). Using the annuli data allowed the approximate proportion of fifth and sixth instars in each cluster to be seen (Fig. 5C). The cluster with the smallest pronota represents the small fifth instars, some likely requiring a seventh instar to reach adult size. The intermediate cluster contained mixed fifth and sixth instars representing the large fifth instars and small sixth instars, both in their penultimate instars. The cluster with the largest pronota contained large sixth instars in their final instar with possible seventh instar individuals. Although clustering by sex was revealing of sex-specific growth patterns, it could lead to erroneous conclusions if used without another index of development to corroborate results, if each cluster were assumed to be an instar. Similarly, a frequency distribution of female pronotal width had seven peaks, but each peak did not represent an instar (Fig. 6B).

The cluster model for the male nymphs did not identify all instars present when untransformed data were used (Fig. 3A); the model generated using transformed data identified a higher number of clusters (Fig. 3B). Male pronotal data may not been normally distributed enough for all instar clusters to be identified. Although using "mclust" alone for males was not very revealing, using both pronotal length and width with the annuli data was. A small number of individuals grouped separately ahead from the main fifth instar group (noted in Figs. 3A, 3B, and 5B). This small group likely indicates 5-instar-type males in their final instar with body sizes close to the sixth instar nymphs. The annuli data also showed that a few fifth instar males had smaller pronotum sizes similar to the fourth instar. These likely represent the few 7-instar-type males with 11 annuli. The results from analyzing males and females separately indicated that some males had five or seven instars, but most had six; females had six or seven instars, with six being more common.

Male and female *B. asahinai* nymphs were not significantly different in their pronotal dimensions for most instars. In only the sixth instar determined by “mclust” were females significantly larger than males in both pronotal length and width. Male pronotal lengths were significantly larger in the second instar according to “mclust” and in the fifth instar according to annuli (Tables 3 and 5). From Mizukubo’s (1981) original description of adult *B. asahinai*, pronotal length ranges overlapped heavily for males and females, but pronotal width was more distinct between sexes, with a range of 3.0 – 3.5 mm for males and 3.5 – 3.7 mm for females. However, there were no consistently significant differences between the pronotal sizes of male and female *B. asahinai* instars for both clustering and annuli. *Blattella asahinai* nymphs may be similar to *B. germanica* nymphs, then, in that pronotal size differences between sexes are only slight (Woodruff 1939).

Since handling insects should be avoided to prevent injury and potential alteration of insect behavior and development, use of digital measurements with Gaussian mixture models is a possible solution for efficient instar determination. However, this approach may not be more efficient and accurate than traditional methods when measuring dead nymphs, because cluster analysis often requires large sample sizes, and digital photography for measurements can be time consuming. Neither growth experiments nor sampling field populations would likely produce enough individuals for accurate and useful clustering, even with a data transformation. However, if the pronotum size ranges for each instar and sex are previously known, measurements made on digital photographs can aid in instar determination in conjunction with other indices of instar number, if available. In this study, annuli data were very useful to determine the approximate instar composition of each cluster, but annuli cannot be counted on live nymphs easily without possibly injuring them.

Atkinson et al. (1991) found that *B. asahinai* females on average required slightly longer to develop than males when reared in isolation at 25˚C, but the difference was only 2.1 days, unlikely to be caused by an extra instar in the majority of females. This small increase in average development period may have been caused by a small number of females with an extra instar (Table 5, Fig. 5C). Tanaka (1982) found that *B. germanica* nymphs with more instars consistently required a longer development period than nymphs with fewer instars. To separate immature development period by sex, Atkinson et al. (1991) did not rear the nymphs in conditions optimal for most cockroaches, as each nymph was isolated. This likely prolonged the mean development period, as chemical stimuli induce aggregation of nymphs, which provides tactile stimuli that increase the development rate in *B. germanica* (Lihoreau and Rivault 2008).

The number of males and females in the sixth instar cluster was not significantly different. This, along with the clustering and annuli data, demonstrates that most male and female *B. asahinai* had six instars. This result contrasts with many reports of *B. germanica*, in which male nymphs commonly had five instars, while females either had six instars (Keil 1981) or five or six instars in approximately a 1:1 ratio (Tanaka and Hasegawa 1979, Kunkel 1981, Tanaka 1982). Laboratory rearing conditions might have increased the number of instars in our study. Asian cockroaches experience more stable temperatures in laboratory culture than when outside, as well as constant access to food and water, but the stress of enclosed containers or a suboptimal diet may delay growth. Atkinson et al. (1991) noted the possibility of using larger rearing containers and diets with more sugar to more closely mimic field conditions. In *B. germanica*, both sixth and seventh instar nymphs may have 11 dorsal cercal annuli, and the entire range of head width of seventh instars is included in the range of sixth instar head width (Murray 1967). Since there was a small sample size for nymphs with 11 annuli, the Kruskal-Wallis test had very low power to distinguish them from other instars. The ratios between the sixth and possible seventh instars for both sexes were lower than the other ratios, indicating a seventh instar was only added to achieve the adult size to compensate for delayed growth. Because female sixth instar widths separated into groups, the presence of a seventh instar is likely, even though specific seventh instar individuals were not able to be identified with certainty.

Carbon dioxide anesthesia was used to collect nymphs that were then killed by freezing. Adult *B. asahinai* can readily fly, especially when newly collected from the field. From personal observations, they attempted flight less after a few months in enclosed containers, but any disturbance often still prompted them to do so. This makes collection of nymphs from a mixed stage colony more difficult without anesthesia, since adults need to be kept alive to propagate the colony. Anesthesia was used to separate nymphs from adults, and an effort was made so no CO_2-exposed nymphs were returned to the colony. However, it is possible that some young nymphs were exposed to CO_2 during the beginning of collection and allowed to grow in culture. Since the number of molts needed to attain adult size is determined before the third instar, anesthetized first through third instar *B. germanica* tend to have one more molt than unanesthetized controls, while nymphs older than the third instar do not usually add molts (Tanaka 1982). If a large number of 5-molt-type males were present in our colony, anesthesia or poor rearing conditions may have forced them to become 6-molt-type males, since measurements indicative of 5-molt-type males were not frequent in our data (Figs. 3A, 3B, and 5B).

Even when both *B. germanica* and *B. asahinai* are in their natural environments without delayed growth, both sexes of *B. germanica* may simply have a lower instar number than those of *B. asahinai*. The possible lack of a lower instar number for *B. asahinai* males may be correlated with lower rates of reproduction compared to *B. germanica* (Atkinson et al. 1991), if a different instar number between sexes is a reproductive strategy (Keil 1981). *Blattella asahinai* seem to demonstrate plasticity in instar number, like *B. germanica* do, meaning individuals can alter the number of instars in response to environmental conditions to meet the minimum adult body size. Since instar number can vary by season in some insects, overwintering *B. asahinai* nymphs could have more instars and longer nymphal periods that allow them to accumulate more energy reserves (Peterson and Haeussler 1928, Snoddy 2007).

Since pronotal length and width were more variable after the third instar, the time when growth regulation occurs, growth ratios were higher between the fourth and fifth and between the fifth and sixth instars (Table 6). Tanaka and Hasegawa (1979) found that *B. germanica* nymphs may add an instar due to poor environmental conditions before the third instar, but this extra instar did not significantly increase the nymph's size past the standard adult range, even when those conditions improved after the third instar. This apparently was true in *B. asahinai*, as the sixth cluster was a fairly homogenous mixture of nymphs with 10 and 11 annuli, corresponding to sixth and possible seventh instar nymphs. The ratio between the sixth and possible seventh instars for both sexes were lower than the average ratio for instars one through six (Table 6). Although female *B. germanica* do not prefer slightly larger males over smaller males when choosing a mate, within sexes, individual *B. germanica* vary little in body size (Lihoreau et al.

2007). This narrow range of adult body size, achieved through regulation by nymphs during development, may have been selected for in *B. germanica* and *B. asahinai* to optimize mating success (Tanaka 1982).

Dyar (1890) did not specifically extend his observations on head width to species outside Lepidoptera, but his “rule” has been, perhaps erroneously, extended to all insects (Gaines and Campbell 1935). Our data suggest growth ratios were not consistent throughout development due to the presence of different instar types, even within sexes. This means that Brooks-Dyar Rule likely cannot be broadly applied to cockroaches that can regulate body size. Despite variable growth ratios between sexes and instars, the average ratios for *B. asahinai* instars one through six from both direct calculation and regression were similar to the median growth ratio for hemimetabolous insects of 1.27 (Cole 1980), indicating when growth is “normal,” it generally follows the Brooks-Dyar Rule. More research is needed on seasonal development patterns to determine how *B. asahinai* specifically utilize growth regulation in the field.

Number of instars and developmental time may change seasonally with temperature (Peterson and Haeussler 1928). The plasticity in instar number of *B. asahinai* warrants investigation into its seasonal developmental patterns to better determine the impact of specific populations in the field. A combination of phenological and physiological data may help predict when populations peak, when nymphal development is shortest, and possibly when mating strategies are maximized if males do have a lower instar number in certain conditions. Knowledge of the number of instars of *B. asahinai* may be used in assessing field populations and determining the best rearing conditions for this species. Since instars react differentially to temperature and insecticides (Das and Gupta 1977, Snoddy 2007), the timing of pesticide treatments or release of biological control agents depends on population structure (McClellan and Logan 1994). *Blattella asahinai* exhibit feeding behaviors that are both beneficial (lepidopteran eggs) and harmful (strawberry) (Brenner 1991, Pfannenstiel et al. 2008), and instars and instar-types may have differential relative food consumption and feeding preferences (Satterthwait 1933). Since diet is a significant variable affecting the number and period of instars (Seamans and Woodruff 1939, Cooper and Schal 1992), differences in growth by diet may inform specific treatment strategies for populations around different field crops. Esperk et al. (2007) noted that many species with instar number plasticity in response to environmental variables are important pests. Since *B. asahinai* are abundant around residences, with reported populations as high as 30,000 to 250,000 individuals per acre, it has become an important peridomestic pest requiring management (Brenner et al. 1988, Richman 2020). While *B. asahinai* may not have the reproductive capabilities of *B. germanica*, they are able to respond to delays in growth caused by variable environmental factors and reach adult size nonetheless.

For future studies, the most frequent number of male and female molts of *B. asahinai* should be determined for populations collected from the field, as well as by direct observation for *B. asahinai* in culture. Imaging live nymphs is possible as an aid in instar determination when nymphs cannot be handled, if clear photographs can be obtained. Without a secondary index, such as cercal annuli in this study, to corroborate results, the use of measurements with the Brooks-Dyar Rule should be cautioned against. For certain species, there may be possible undiscovered indices of instar other than measurements of body structures. Sardesai (1969) correlated fecal pellet size to instar in Lepidoptera, for example.

In conclusion, from both cluster analysis with digital measurements of the pronotum and from counting dorsal cercal annuli, male and female *B. asahinai* most commonly had six instars. We found probable seventh instar females and 5-molt-type males, as well, based on the number of annuli and the separation of instars into size groups. Laboratory rearing conditions may have caused nymphs used in our study to have longer development periods and more instars than those in the field. Our results should be confirmed with direct observation using the pronotum sizes from this study to corroborate molts. Digitally measuring nymphs for instar determination can be recommended to limit harming live nymphs, but because of the inability to manually position live nymphs, as well as the overlap in sizes, the accuracy and precision of this method may depend heavily on the procedures and technological resources used. Clustering with "mclust" often requires a large sample size, preventing it from being extremely useful in most experiments. Using solely measurements to determine instars cannot be relied upon in insects that can regulate growth and do not adhere to the Brooks-Dyar Rule. In future studies, males and females should always be separated for analysis, as growth patterns may be different and can be indicators of underlying mating strategies and behavior.

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Table 1. Mean digital and caliper pronotal length and width for each of six *B. asahinai* mixed sex instars determined using cercal annuli with results from paired *t*-tests.

Pronotal Measurement	Instar	Digital Mean (mm)	Caliper Mean (mm)	*df*	$t_{0.05}$	*P*
Length	1	0.570 ± 0.013	0.59 ± 0.015	4	-2.2	0.088
	2	0.713 ± 0.023	0.73 ± 0.023	4	-1.0	0.37
	3	0.865 ± 0.017	0.94 ± 0.036	4	-2.7	0.052
	4	1.057 ± 0.017	1.11 ± 0.017	4	-2.3	0.081
	5	1.622 ± 0.033	1.59 ± 0.045	4	1.2	0.31
	6	2.010 ± 0.111	2.05 ± 0.094	4	-1.2	0.29
Width	1	0.858 ± 0.014	0.89 ± 0.014	4	-2.5	0.065
	2	1.068 ± 0.037	1.08 ± 0.045	4	-0.84	0.45
	3	1.363 ± 0.027	1.39 ± 0.042	4	-1.4	0.23
	4	1.618 ± 0.046	1.61 ± 0.046	4	0.67	0.54
	5	2.460 ± 0.065	2.46 ± 0.034	4	0.010	0.99
	6	3.184 ± 0.132	3.14 ± 0.148	4	1.6	0.20

Table 2. Mean pronotal length and width for six instar clusters identified by “mclust” analysis of 1,422 mixed sex *B. asahinai* nymphs. Clustering analysis used natural log-transformed length and width. Reported values are untransformed length and width means of assigned clusters. All groups are significantly different using pronotal length (Kruskal-Wallis: $\chi^2 = 1352.3$, $df = 5$, $P < 0.001$).

Instar (by “mclust”)	n	Mean Length ± SE (mm)*	Mean Width ± SE (mm)
1	185	0.6077 ± 0.0029 a	0.8857 ± 0.0044
2	322	0.7561 ± 0.0028 b	1.1108 ± 0.0035
3	243	0.9284 ± 0.0041 c	1.3729 ± 0.0051
4	327	1.1846 ± 0.0075 d	1.8172 ± 0.0101
5	227	1.6540 ± 0.0108 e	2.5654 ± 0.0151
6	118	2.1268 ± 0.0106 f	3.3155 ± 0.0142

*Means followed by different letters are significantly different ($P < 0.001$) using Dunn’s *post hoc* test.

Table 3. Mean pronotal length and width by sex for the six instar clusters of 734 male and 688 female *B. asahinai* nymphs from "mclust," using natural log-transformed pronotal data. Reported means are untransformed pronotal length and width data. For each sex, pronotal lengths of each cluster are significantly different (Kruskal-Wallis: Male: $\chi^2 = 696.5$, $df = 5$, $P < 0.001$; Female: $\chi^2 = 654.5$, $df = 5$, $P < 0.001$).

Instar (by "mclust")	Sex	n	Mean Length ± SE (mm)*	Mean Width ± SE (mm)
1	M	96	0.6096 ± 0.0039 A	0.8903 ± 0.0062
	F	89	0.6057 ± 0.0042 a	0.8807 ± 0.0062
2	M	157	0.7635 ± 0.0039 B	1.1118 ± 0.0052
	F	165	0.7490 ± 0.0039 b	1.1098 ± 0.0045
3	M	134	0.9283 ± 0.0055 C	1.3758 ± 0.0072
	F	109	0.9286 ± 0.0063 c	1.3693 ± 0.0073
4	M	175	1.1890 ± 0.0102 D	1.8254 ± 0.0142
	F	152	1.1796 ± 0.0110 d	1.8078 ± 0.0144
5	M	115	1.6431 ± 0.0142 E	2.5597 ± 0.0204
	F	112	1.6652 ± 0.0162 e	2.5713 ± 0.0225
6	M	57	2.0705 ± 0.0131 F	3.2199 ± 0.0139
	F	61	2.1794 ± 0.0133 f	3.4049 ± 0.0178

*Within each sex, means followed by different letters are significantly different ($P < 0.05$) using Dunn's *post hoc* test.

Table 4. Pronotal length and width means for six instars of 1,282 mixed sex *B. asahinai* nymphs based on dorsal cercal annuli. Asterick represents possible seventh instar nymphs with 11 annuli. All instars are signficantly different using pronotal length (Welch's ANOVA: $P < 0.001$).

Instar (by Annuli)	Annuli	n	Mean Length ± SE (mm)*	Mean Width ± SE (mm)
1	3	170	0.6061 ± 0.0035 a	0.8801 ± 0.0046
2	6	291	0.7460 ± 0.0030 b	1.1056 ± 0.0040
3	7	278	0.9268 ± 0.0045 c	1.3783 ± 0.0072
4	8	256	1.1900 ± 0.0076 d	1.8253 ± 0.0112
5	9	167	1.6023 ± 0.0135 e	2.4824 ± 0.0204
6	10	91	2.0487 ± 0.0174 f	3.1820 ± 0.0256
7*	11	29	2.1490 ± 0.0248 g	3.3607 ± 0.0331

*Means followed by different letters are significantly different ($P \leq 0.001$) using a Games-Howell *post hoc* test.

Table 5. Pronotal length and width means of instars by sex from 650 male and 632 female *B. asahinai* nymphs determined from counting dorsal cercal annuli. For both males and females, there are significant differences between at least two instars (Kruskal-Wallis: Male: $\chi^2 = 614$, *df* = 6, $P < 0.001$; Female: $\chi^2 = 599.3$, *df* = 6, $P < 0.001$).

Instar (by Annuli)	Annuli	Sex	n	Length ± SE (mm)*	Width ± SE (mm)
1	3	M	87	0.6082 ± 0.0050 A	0.8867 ± 0.0067
		F	83	0.6040 ± 0.0049 a	0.8732 ± 0.0062
2	6	M	136	0.7480 ± 0.0043 B	1.1042 ± 0.0062
		F	155	0.7442 ± 0.0041 b	1.1068 ± 0.0051
3	7	M	154	0.9251 ± 0.0058 C	1.3775 ± 0.0102
		F	124	0.9288 ± 0.0069 c	1.3792 ± 0.0100
4	8	M	136	1.2025 ± 0.0106 D	1.8454 ± 0.0168
		F	120	1.1759 ± 0.0107 d	1.8025 ± 0.0142
5	9	M	89	1.6298 ± 0.0190 E	2.5151 ± 0.0288
		F	78	1.5709 ± 0.0187 eg	2.4449 ± 0.0285
6	10	M	41	2.0179 ± 0.0214 E	3.1496 ± 0.0239
		F	50	2.0740 ± 0.0260 fh	3.2085 ± 0.0421
7**	11	M	7	2.0694 ± 0.0444 E	3.2523 ± 0.0544
		F	22	2.1743 ± 0.0278 gh	3.3952 ± 0.0377

*Within each sex, different letters are significantly different ($P < 0.05$) using Dunn's *post hoc* test.

**possible 7th instar with 11 dorsal cercal annuli

Table 6. Brooks-Dyar growth ratios for the six instars by sex calculated by dividing the mean length or width of each instar by the preceding instar. Ratios for instars determined by both clustering and annuli are presented.

Method	Pronotum Characteristic	Sex	Ratios (for 6 instars)	Average Ratio
Clustering	Length	Male	1.25, 1.22, 1.28, 1.40, 1.24	1.28
Clustering	Length	Female	1.23, 1.24, 1.27, 1.42, 1.31	1.29
Annuli	Length	Male	1.23, 1.24, 1.30, 1.36, 1.24	1.27
Annuli	Length	Female	1.23, 1.25, 1.27, 1.34, 1.32	1.28
Clustering	Width	Male	1.25, 1.23, 1.33, 1.42, 1.24	1.29
Clustering	Width	Female	1.26, 1.23, 1.32, 1.43, 1.31	1.31
Annuli	Width	Male	1.25, 1.25, 1.34, 1.36, 1.25	1.29
Annuli	Width	Female	1.27, 1.25, 1.31, 1.36, 1.31	1.30

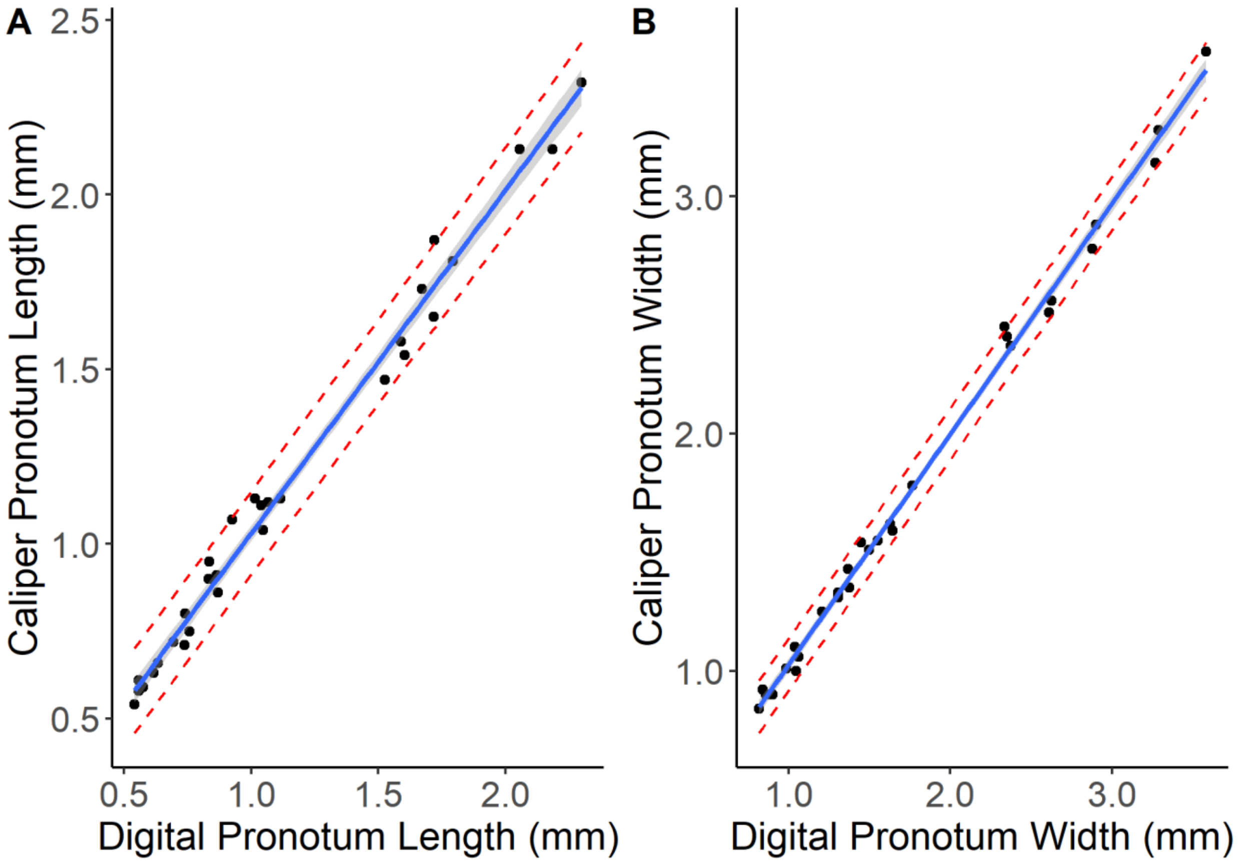

Fig. 1. Linear regression with 95% confidence (gray) and prediction (red dashed lines) intervals for digital and caliper pronotal measurements for 30 mixed sex *B. asahinai* nymphs. (A) Digital and caliper pronotal lengths (mm). Equation of line is $y = 0.98256x + 0.04837$. (B) Digital and caliper pronotal widths (mm). Equation of line is $y = 0.97120x + 0.05472$.

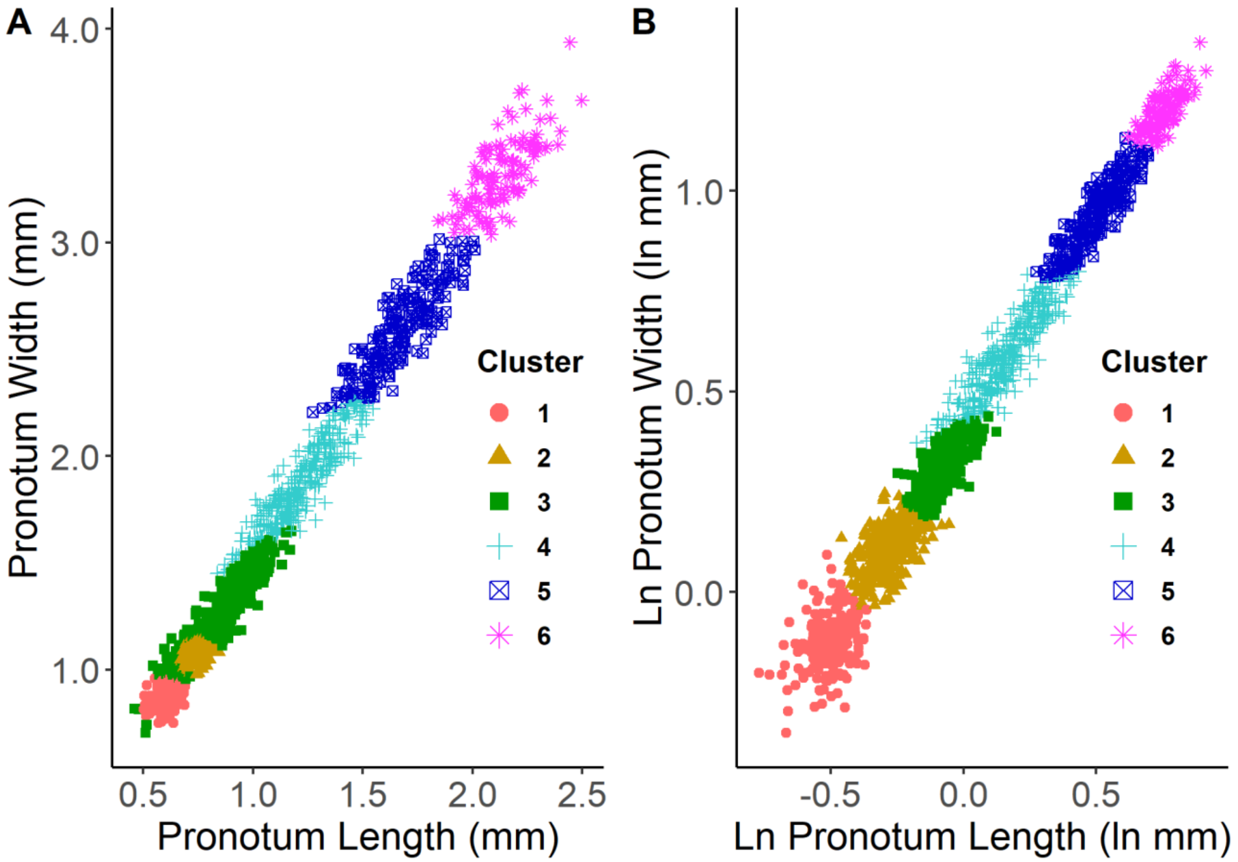

Fig. 2. "Mclust" clusters from 1,428 mixed sex *B. asahinai* nymphs based on pronotal length and width. (A) Clusters from untransformed pronotal length and width (mm). Cluster "3" (green) severely overlaps clusters "1" and "2." (B) Clusters from natural log-transformed pronotal length and width (ln mm). Clusters are more logically distributed with less overlap.

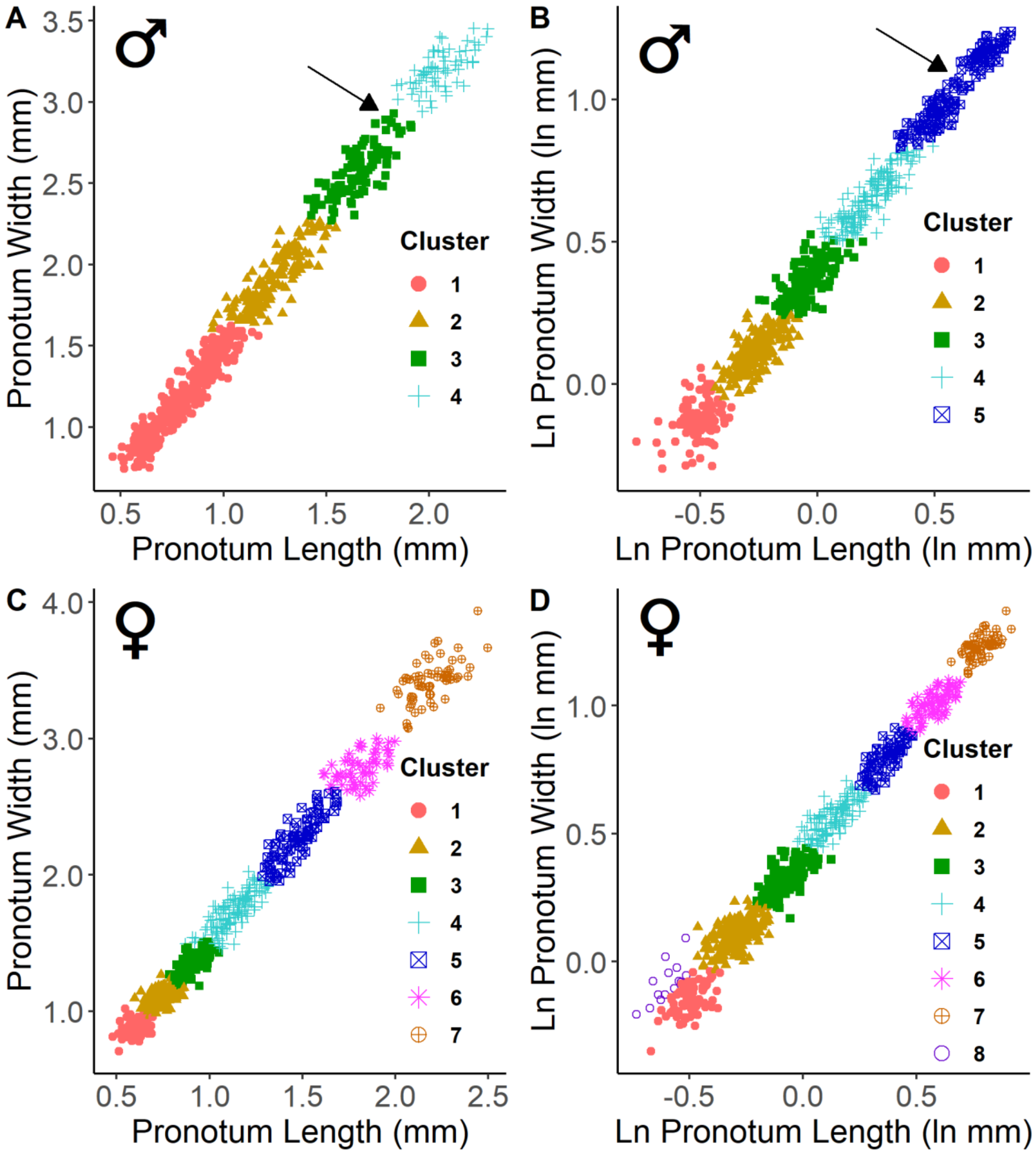

Fig. 3. (A) "Mclust" clusters from 736 male *B. asahinai* nymphs using pronotal length and width (mm). The small group of 5-molt males is identified. (B) "Mclust" clusters from 736 male *B. asahinai* nymphs using natural log-transformed pronotal length and width (ln mm). The small group of 5-molt males is identified. (C) "Mclust" clusters from 692 female *B. asahinai* nymphs using pronotal length and width (mm). (D) "Mclust" clusters from 692 female *B. asahinai* nymphs using pronotal length and width (ln mm).

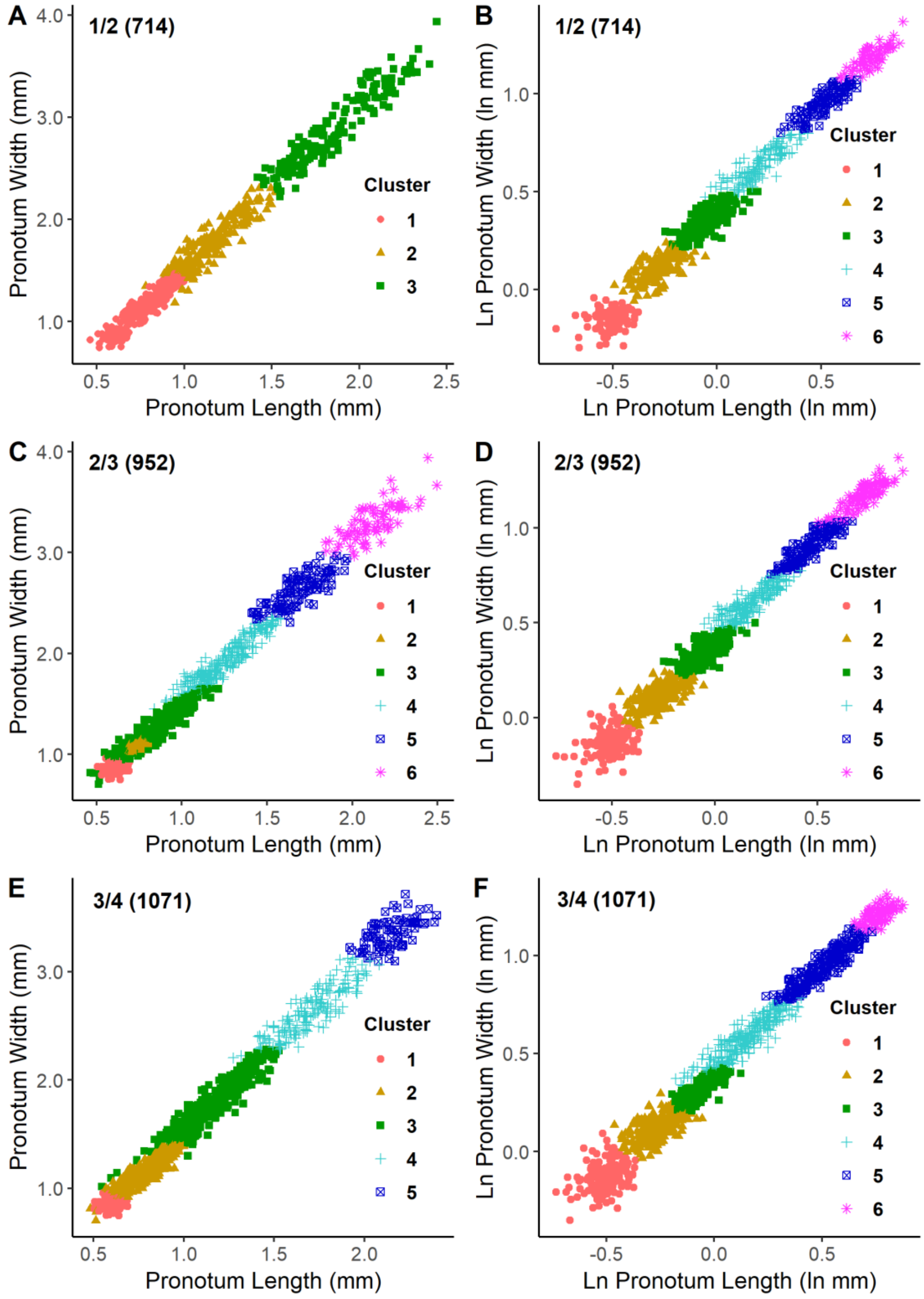

Fig. 4. (A) "Mclust" clusters from untransformed 1/2 data set (714 nymphs). (B) "Mclust" clusters from transformed 1/2 data set (714 nymphs). (C) "Mclust" clusters from untransformed 2/3 data set (952 nymphs). (D) "Mclust" clusters from transformed 2/3 data set (952 nymphs). (E) "Mclust" clusters from untransformed 3/4 data set (1071 nymphs). F. "Mclust" clusters from transformed 3/4 data set (1071 nymphs).

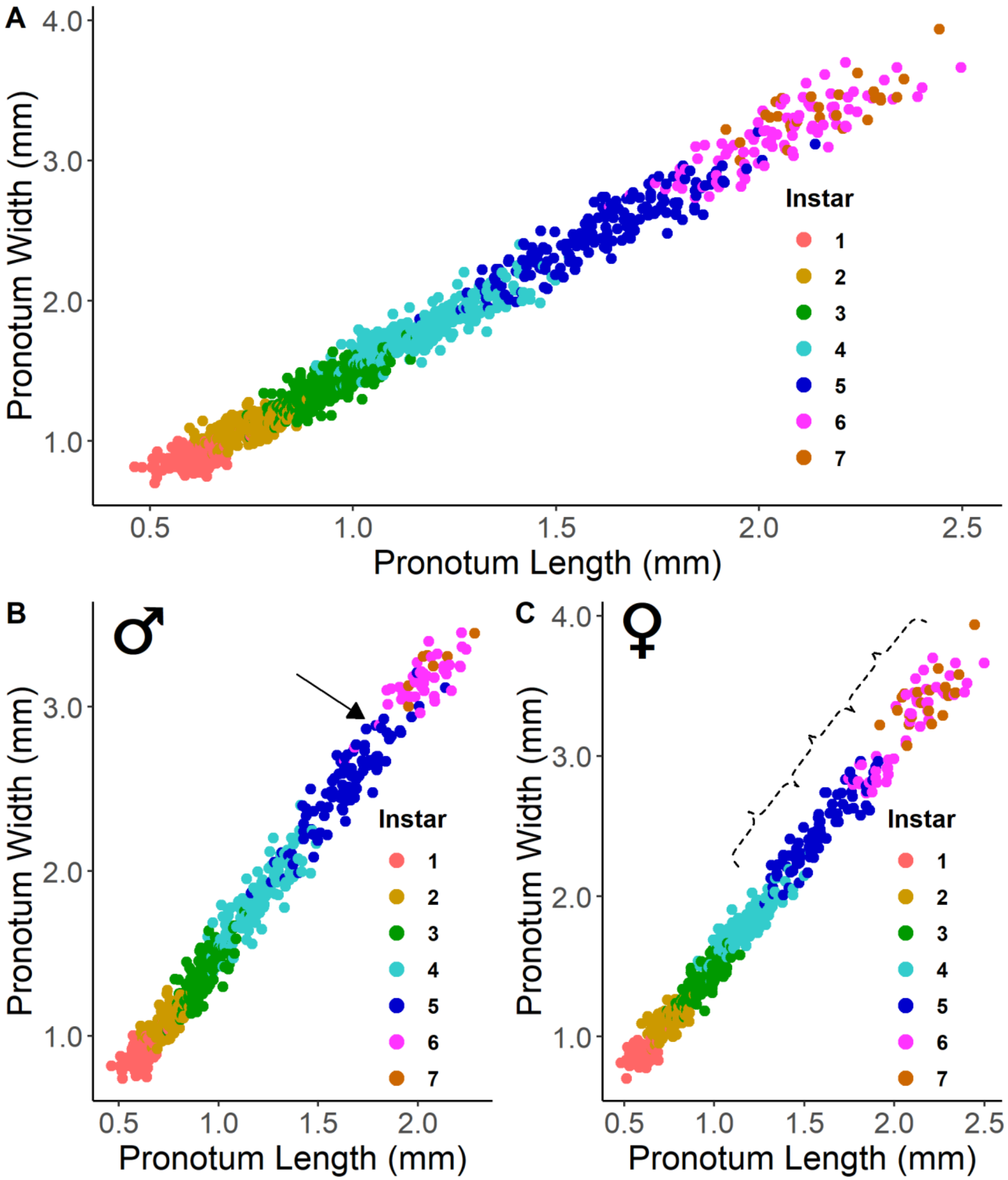

Fig. 5. Scatterplots of pronotal length and width for *B. asahinai* nymphs with instars determined by dorsal cercal annuli. A possible seventh instar with 11 dorsal annuli is included. (A) Scatterplot of 1,282 mixed sex *B. asahinai* nymphs and instars. (B) Scatterplot of 650 *B. asahinai* male nymphs and instars. The small group of 5-molt males is identified. (C) Scatterplot of 632 *B. asahinai* female nymphs and instars. The three clusters that "mclust" had identified for females (Fig. 4C) are identified with braces.

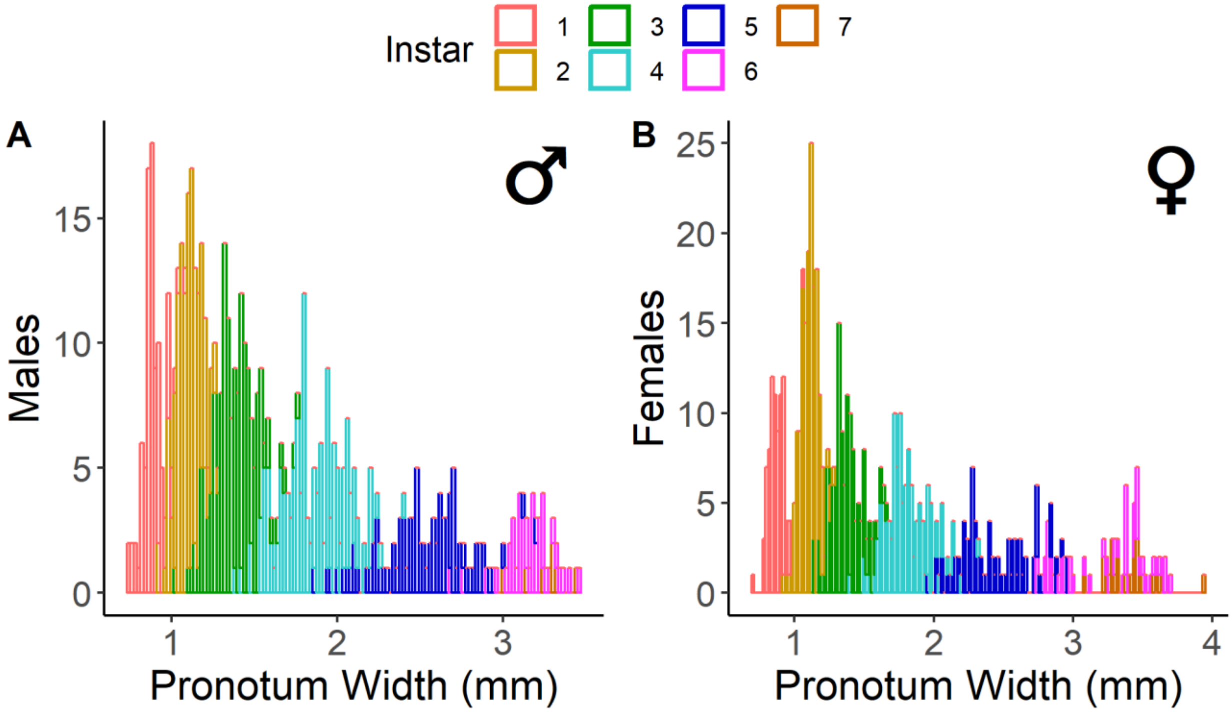

Fig. 6. Frequency distribution of the pronotal width of male and female *B. asahinai* instars determined by dorsal cercal annuli. A possible seventh instar with 11 dorsal annuli is included. (A) Pronotal width of 650 male *B. asahinai* instars, with approximately seven peaks. (B) Pronotal width of 632 female *B. asahinai* instars, with seven clear peaks. Female fifth and sixth instars overlap for pronotal width, creating the sixth peak.

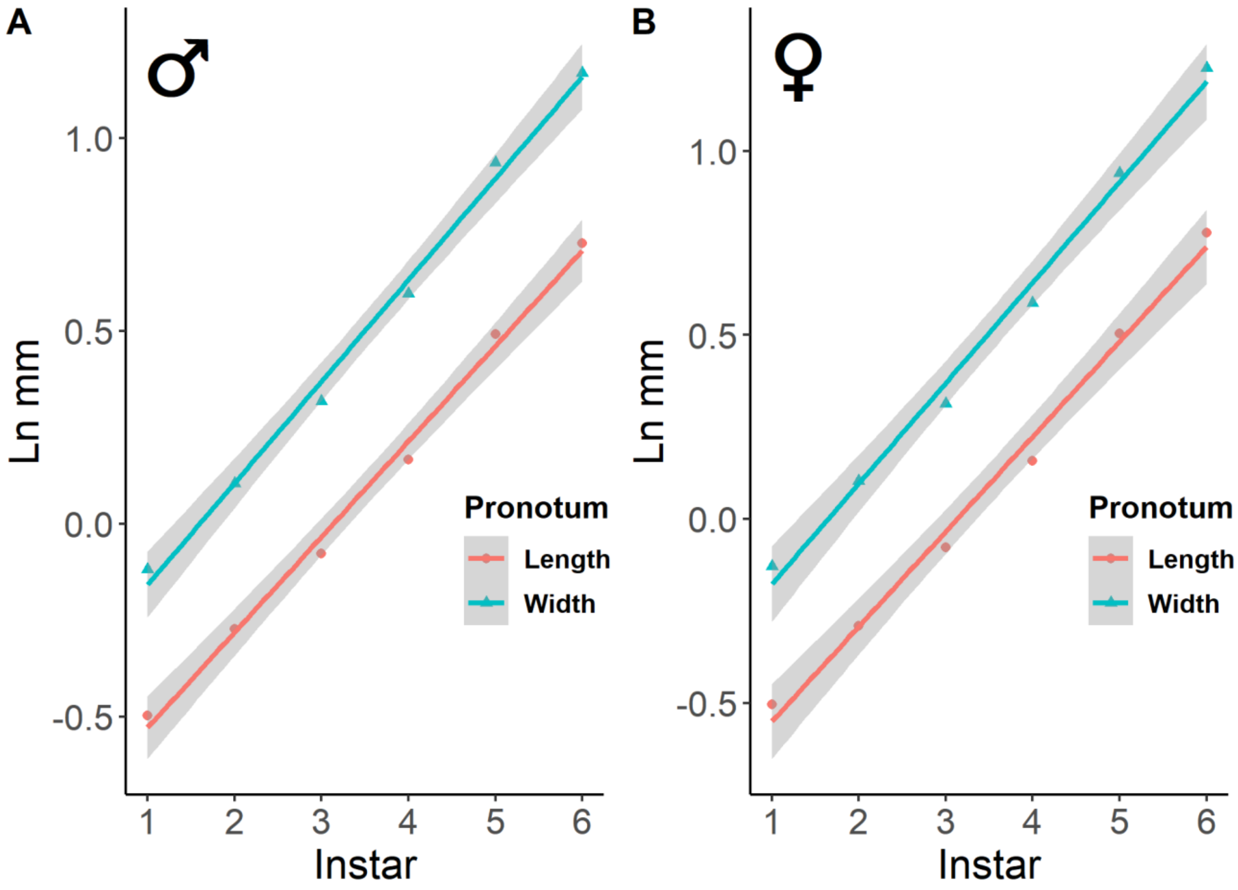

Fig. 7. Linear regression of *B. asahinai* means of natural log-transformed pronotal length and width by instar. Instars were determined by "mclust." (A) Male pronotal length and width regression. (B) Female pronotal length and width regression.

CHAPTER 3

Differential survival and development of *Blattella asahinai* and *B. germanica* (Blattodea: Ectobiidae) at six constant temperatures

Abstract

The Asian cockroach, *Blattella asahinai*, is a peridomestic nuisance pest in the southeastern United States. *Blattella asahinai* is the closest relative to *Blattella germanica*, the German cockroach, one of the most prolific domestic pests. Since *B. asahinai* lives outdoors and is subject to variable climatic factors, and *B. germanica* is a domestic pest that has evolved to thrive in the indoor biome, these two species are expected to have differential development patterns reflecting environmental adaptations. The development of *B. asahinai* and *B. germanica* cockroach nymphs were observed at six constant temperatures ranging from 10˚C to 35˚C. At 10˚C and 15˚C, all nymphs died in the first instar, but *B. germanica* nymphs survived longer (10˚C: 13.07 d; 15˚C: 43.31 d) than *B. asahinai* nymphs (10˚C 8.2 d; 15˚C 17.52 d) at both temperatures. At 20˚C, 25˚C, and 30˚C, *B. asahinai* consistently had more instars and a longer period for each stadium than *B. germanica*. At 35˚C, only *B. germanica* was able to complete nymphal development. The results for *B. asahinai* corroborated previously estimated growth patterns. The lower development threshold for nymphal development was 14.06˚C for *B. germanica* and 13.73˚C for *B. asahinai*. Cannibalism of molting *B. asahinai* nymphs was frequently observed, but the specific causes of this phenomenon could not be determined. Comparing the development of *B. germanica* directly with its closest relative reveals specific physiological adaptations that *B. germanica* has developed for the indoor biome.

Introduction

The Asian cockroach, *Blattella asahinai* Mizukubo, is a peridomestic nuisance pest first identified in the United States in the 1980's (Roth 1986). Native to South Asia, *B. asahinai* was introduced into Florida and has spread throughout the southeastern United States, including into Alabama, Georgia, South Carolina, North Carolina, and Texas using major highways (Mizukubo 1981, Roth 1986, Brenner et al. 1988, Sitthicharoenchai 2002, Austin et al. 2007, Snoddy and Appel 2008, Matos and Schal 2015). The closest relative to *B. asahinai* is the German cockroach, *Blattella germanica* (L.) (Roth 1985), one of the most prevalent domestic pests worldwide (Nasirian 2017). In contrast to the flightless *B. germanica*, *B. asahinai* can readily fly and lives outdoors (Brenner et al. 1988). *Blattella asahinai* and *B. germanica* appear similar, but they can be positively identified morphologically using the male tergal glands on the 8th abdominal segment and distinguished chemically from their cuticular hydrocarbon profiles (Roth 1986, Carlson and Brenner 1988, Snoddy 2007).

Blattella asahinai mainly inhabit shady areas with abundant leaf litter and are frequently found in abandoned citrus groves, crop fields, and yards around domestic structures (Roth 1986, Brenner et al. 1988, Pfannenstiel et al. 2008). Unlike *B. germanica*, *B. asahinai* are attracted to light and will readily fly into lit homes at night (Brenner et al. 1988). Homeowners are no more tolerant of *B. asahinai* than *B. germanica* when seen in the house (Brenner et al. 1988). *Blattella asahinai* have been reported to play both harmful and beneficial roles in agricultural pest communities, such as feeding on parasitized aphids and preventing parasitoid emergence (Persad and Hoy 2004), as well as preying on pest lepidopteran eggs on soybean (Pfannenstiel et al. 2008). *Blattella asahinai* can also cause significant damage to strawberry, establishing them as a minor crop pest (Brenner 1991). Due to the close relationship between *B. asahinai* and *B. germanica*, the low tolerance for *B. asahinai* in and around homes, and the current and potential pest status of this species, more information about factors affecting range and seasonal habits of *B. asahinai* is needed to aid in potential management strategies.

Because *B. asahinai* are peridomestic and subject to climatic and seasonal changes, predicting emergence and population peaks is critical to developing control techniques. Snoddy (2007) sampled field populations of *B. asahinai* in Alabama over two years and found that *B. asahinai* likely have three generations annually, with nymphs constituting a larger proportion of the population from February to May before the adult molts occur. Populations then build throughout the summer and peak at the beginning of September before low temperatures cause populations to decline. Because of decreases in visual counts and an increase in Berlese sample counts from leaf litter during adverse climatic conditions, Snoddy (2007, personal communication) concluded that *B. asahinai* utilized the leaf litter to escape unfavorable temperature and humidity levels. Although broad annual population patterns are known, details of development and thermal development thresholds have not been determined for this species. Habitat and seasonal age structure are important differences between *B. asahinai* and *B. germanica* that could help develop specific control techniques for *B. asahinai*.

The development of insects, like other poikilotherms, depends on heat input. Degree-day models were developed to describe the relationship between development and temperature and can be utilized to predict important life cycle events of a pest species, like egg hatch, and inform growers when to apply insecticides (Rice et al. 1984, Zou et al. 2004). Degree days are heat units that accumulate for a certain period, usually one calendar day, based on daily temperatures (Wilson and Barnett 1983). Degree days accumulate above the lower development threshold (LDT), which is the temperature under which no development occurs (Wilson and Barnett 1983). One method to experimentally determine the number of degree days required for each life stage, as well as estimate the lower threshold, is to record development at a series of constant temperatures and calculate the number of degree-days required for each life stage (Marco et al. 1997, Wu et al. 2017). This information can be used to create simple linear models through linear regression of days and experimental temperatures (Stejskal et al. 2003, Wu et al. 2017). The resulting models can be applied in the field by tracking the number of degree days accumulated since a certain event or day (e.g., January 1, biofix) to predict the date of important life cycle events, such as spring emergence or generational egg hatch (Marco et al. 1997, Liu et al. 2002).

One limitation to models developed from laboratory data is that the actual temperatures experienced by the organism in the field may be different than the reported air temperature, as poikilotherms can seek microhabitats to regulate body temperature (May 1979). *Blattella asahinai* can seek conditions more favorable to their survival and development by burrowing into the leaf litter; reported daily air temperatures cannot account for this behavior (Snoddy 2007). Development is not solely dependent on temperature, either; photoperiod, humidity, and diet also influence the duration of life cycle stages (Hagstrum and Milliken 1988, Cooper and Schal 1992, Zhu and Tanaka 2004). Although simple degree-day models have many limitations and assumptions, they have been applied successfully for a variety of organisms and can be used in conjunction with trap catch and host phenology data to improve the accuracy of predictions (Pitcairn et al. 1992, Kumral et al. 2005).

Since temperature is a major limiting factor in the range expansion of invasive species and seasonal activity, development thresholds and degree days are important in predicting *B. asahinai* expansion northward, as well as seasonal population dynamics. Additionally, the differential developmental responses of each species to high and low temperatures can provide details on possible physiological adaptations to each species' unique habitat. The purpose of this study was to evaluate the nymphal development *B. asahinai* and *B. germanica* across a series of constant temperatures. Development for each species was compared across a range of temperatures between two species, and the lower development threshold for each species was estimated through linear models. These simple models, along with field observations, can provide a basis for further studies on developmental and behavioral responses to temperature in *B. asahinai* that can help predict range limits and aid in efficient timing of pest management implementation.

Materials and Methods

Cockroach Rearing

Blattella asahinai were collected from two locations in Auburn, AL, between June and October 2019 (backyard: 32.6151˚ N, 85.4825˚ W; Davis Arboretum: 32.5952 N˚, 85.4830˚ W). Individuals were identified as *B. asahinai* at the time of collection by their outdoor habitat and flight capability and were subsequently confirmed in the laboratory by morphology of their tergal glands (Roth 1986, Snoddy 2007). The collected individuals were reared at 30˚C in 3.8-liter (1-gallon) glass jars with a photoperiod of 12:12 (L:D) and provided rat food (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. *Blattella germanica* stock were received from American Cyanamid Co. in Clifton, NJ, in 1985, and were reared in 121-liter (32-gallon) trashcans at 27˚C ± 2˚C and provided rat chow, and water *ad libitum*.

A small group of adult male and gravid female *B. asahinai* were kept in a 1.9-liter (0.5-gallon) glass jar and given rat food and water *ad libitum*. Jars were checked for neonates daily. Neonates were removed within 24 hours of eclosion and separated into groups of 15-20 nymphs.

Each group of nymphs was put into a 1.9-liter (0.5-gallon) glass jar and provided with rat food and water *ad libitum*. A ruled 7.62 cm x 12.7 cm index card was folded and placed in each jar as a harborage. A layer of petroleum jelly and mineral oil was applied near the mouth of the jar, and the mouth was covered with mesh and a paper towel, which were secured with rubber bands. This same process was employed for *B. germanica* to obtain newly emerged nymphs.

Experimental Design

The six constant temperatures used were 10, 15, 20, 25, 30, and 35˚C, each with a photoperiod of 12:12 (L:D). This temperature range was selected because, in Alabama, the hottest month is July, with average high temperatures of 32.6°C (National Weather Service Forecast Office 2018). The lower development threshold for *B. germanica* was reported to be 16.2°C (Stejskal et al. 2003), but because Alabama can experience average low temperatures of -1.39°C in January (National Weather Service Forecast Office 2018), 10°C was used as the lowest temperature for this experiment. Six different incubators were used for each of the six temperatures. Humidity was unregulated inside of the incubator but stayed within the range of. All replicate jars contained identical water jars, and humidity was likely similar within each jar. For each combination of temperature and species, there were six replicates, for a total of 108 jars, each with approximately 20 nymphs. Since hatching was not synchronized, jars were staggered, and not all replicates for the same temperature were present in the same incubator at the same time. Jars with neonates were placed in their assigned incubators. The jars were checked nearly every day, and the number of new instars and dead nymphs was recorded. Dead nymphs and shed exuviae were removed.

To examine the possible effects of the presence of leaves on *B. asahinai* survival during molting at 35°C, six extra *B. asahinai* replicates were conducted at 35°C, and 3-4 dead leaves were placed the bottom of the jar. The leaves were a mixture of Southern magnolia (*Magnolia grandiflora*) and white oak (*Quercus alba*) that were collected from the ground on the Auburn University campus. An attempt to collect information on mortality and survival to the second instar was made, but due to the extra shelter provided, the development and mortality of the nymphs were not able to be recorded in detail without causing possible injury to the nymphs.

Instar Determination

When necessary, instars of live nymphs were confirmed by taking photographs with an Olympus TG-5 digital camera (IM005, Olympus Corporation, Tokyo, Japan). Pictures were analyzed with ImageJ (Schneider et al. 2012), using the ruled lines on the harborage for scale to measure the pronotal length, pronotal width, and, when possible, head width, to help determine instar. Measurement data from Tanaka and Hasegawa (1979) were used for *B. germanica*, and data from Peterson et al. (2019) were used for *B. asahinai* for instar determination. Instars of dead nymphs could be confirmed by counting cercus annuli (Murray 1967, Tanaka and Hasegawa 1979). Jars were rotated inside the incubators almost daily. Individuals were removed from the jars after they molted to adults, unless a single nymph remained, in which case an adult was left in the jar to retain tactile stimuli needed for normal development.

Adults that were removed from the jar were killed by freezing. The length and width of the pronota of each adult were measured using a pair of digital calipers (Model 62379–531, VWR International Inc., West Chester, PA). The number of cercal annuli were counted according to Murray (1967) and Tanaka and Hasegawa (1979). The number of molts each individual went through during nymphal development was estimated using a combination of pronotal measurements, the number of cercal annuli, notes from direct observation, and the date that the adult molt occurred. In a replicate that appeared to have a mixture of instar types, individuals that molted earlier, had smaller pronota, or had one fewer cercal annulus were considered to have one fewer molt than the individuals that molted later, had larger pronota, or had one more cercal annulus.

Data Analysis

The mean number of days required for each instar and total first instar to adult development period for each replicate was calculated. To calculate the mean number of days required for a developmental stage for all individuals of one species at one temperature, individuals were combined across replicate jars. For temperatures where no development occurred, the average lifespan of nymphs was calculated. Nymphs that appeared to die from causes other than temperature, such as being crushed by the water jar, were not included in the calculation of average lifespan. Due to non-normal distribution of data, Mann-Whitney U Tests (Wilcoxan 1945, Mann and Whitney 1947) were performed to test for differences between species for total first instar to adult development period and average lifespan at one temperature.

Linear regression was performed for each instar and for average nymphal development period using the mean number of days required for completion and the temperature within each species. To calculate degree days and the lower threshold temperature for each life stage, the formula $y = ax + b$ or $\frac{1}{D} = aT + b$ was used, where $1/D$, the reciprocal of the number of days required to complete the life stage, is the development rate, and T is the temperature (Stejskal et al. 2003, Wu et al. 2017). The lower development threshold was calculated as the temperature

(T) at which the development rate ($1/D$) is 0, or $-b/a$. Degree days were calculated as the reciprocal of the slope, $1/a$, which is the number of days required for development (D) multiplied by the temperature (T) above the development threshold ($-b/a$), or $\frac{1}{a} = D(T + \frac{b}{a})$, also written as $\frac{1}{a} = D(T - T_0)$ (Wu et al. 2017).

The pronotal measurements were separated by species, temperature, and sex and analyzed using the package "mclust" (Scrucca et al. 2016) in R (R Core Team 2020) using the same method as Peterson et al. (2019). Scatter plots of pronotal length and width with the number of instars each individual identified were also analyzed. All statistical analyses were conducted at $\alpha < 0.05$ using R software (R Core Team 2020), and all plots were created using the R package "ggplot2" (Wickham 2016).

Results

10°C and 15°C

At 10°C and 15°C, no nymphs of either species molted, and all died in the first instar. At 10°C, the average lifespan *B. germanica* (13.07 d) was significantly greater than that of *B. asahinai* (8.2 d) ($W = 9497.5$, $P < 0.001$). At 15°C, the average lifespan of *B. germanica* (43.31 d) was also significantly greater than that of *B. asahinai* (17.52 d) ($W = 9623$, $P < 0.001$). Likely fluctuations in temperature occurred in the 15°C incubator, as an independent measure of temperature recorded 11°C as the temperature rather than 15°C after the replicates of that temperature were completed. The temperatures of all other incubators were determined to be accurate.

20°C

At 20°C, *B. asahinai* had significantly greater average nymphal development period than *B. germanica* (Table 1). For *B. germanica,* nymphal development periods ranged from 71 d to 119 d, and, for *B. asahinai,* they ranged from 94 d to 143 d. Of 122 *B. germanica* nymphs, 106 (86.89 %) survived to adulthood. Of 58 female nymphs, six (10.34%) were estimated to have gone through five instars and 51 (87.93%) through six instars before molting to an adult. One *B. germanica* female also was estimated to have gone through seven instars before molting to an adult. Of 48 males, 19 (39.58%) were estimated to have five instars, and 29 (60.42%) were estimated to have six instars.

For *B. asahinai,* only 24 of 110 (21.82%) nymphs developed to adulthood. Of ten females, nine (90%) were estimated to have gone through seven instars. Only one female was estimated to have had six instars. Of 14 males, 12 (85.71%) were estimated to have gone through seven instars and two through six instars before molting to an adult. For both sexes, the most common number of instars for *B. germanica* was six, and the most common number of instars for *B. asahinai* was seven. The average development periods of each instar and the total nymphal development periods of *B. germanica* and *B. asahinai* at 20°C are presented in Table 1.

25°C

At 25°C, the average period of nymphal development was significantly greater for *B. asahinai* than *B. germanica* (Table 2). For *B. germanica*, nymphal development periods ranted from 37 d to 51 d, and, for *B. asahinai*, they ranged from 48 d to 93 d. For *B. germanica*, 108 of 120 (90%) nymphs developed to adulthood. Of 61 females, 52 (85.25%) were estimated to have gone through five instars, and nine (14.75%) went through six instars before molting to an adult.

Of 47 *B. germanica* males, 46 (97.87%) were estimated to have five instars, and only one appeared to go through six instars.

For *B. asahinai*, of 57 adults, 33 (57.89%) of those were females. Of those, 23 (69.70%) were estimated to have gone through six instars, and ten (30.30%) of those were estimated to have gone through seven instars. Of 24 males, an estimated 19 (79.17%) went through six instars, and five (20.83%) went through seven instars. The most common number of instars for both sexes was five for *B. germanica* and six for *B. asahinai*. The average development periods of each instar and the total nymphal development periods of *B. germanica* and *B. asahinai* at 25°C are presented in Table 2

30°C

At 30°C, the total nymphal development period was significantly greater for *B. asahinai* than *B. germanica* (Table 3). For *B. germanica*, nymphal development periods ranged from 28 d to 49 d, and, for *B. asahinai*, they ranged from 37 d to 60 d. For *B. germanica*, 105 of 121 (86.78%) nymphs survived to adulthood. Of 52 females, 48 (92.31%) were estimated to have gone through five instars and four (7.69%) through six instars before molting to an adult. Of 51 males, 50 (98%) were estimated to have five instars, and only one appeared to have gone through six instars

For *B. asahinai*, 43 of 119 (36.13%) developed to adulthood. Of 22 females, 11 (50%) were estimated to have gone through six instars, and 11 were estimated to have gone through seven instars. Of 21 males, 19 (90.48%) were estimated to have gone through six instars and two through seven instars before molting to adults. The most common number of instars for both sexes of *B. germanica* was five. The most common number of instars for male *B. asahinai* was six, and an equal number of female *B. asahinai* had six and seven instars. The average development periods of each instar and the total nymphal development periods of *B. germanica* and *B. asahinai* at 30°C are presented in Table 3.

35°C

At 35°C, only one of 120 (0.08%) *B. asahinai* survived to adulthood, while 76 of 115 (66.09%) *B. germanica* survived to adulthood. The lifespans of *B. germanica* that did not survive to adulthood ranged from 4 d to 45 d with an average of 20.68 d and standard deviation of 12.04 d. The lifespans of *B. asahinai* that did not survive to adulthood ranged from 1 d to 27 d with an average of 6.98 d and a standard deviation of 4.90 d. The average lifespan of *B. germanica* was significantly greater than that of *B. asahinai* ($W = 4018$, $P < 0.001$). Of 29 female *B. germanica*, five (17.24%) were estimated to have gone through five instars and 24 (82.76%) through six instars before molting to an adult. Of 47 *B. germanica* males, 28 (59.6%) were estimated to have gone through five instars and 18 (38.3%) through six instars. One *B. germanica* male likely had seven instars. The lone *B. asahinai* adult that survived to adulthood was a male that went through six instars. The development periods of each instar and average nymphal development period of *B. germanica* at 35°C are presented in Table 4.

During evaluation of *B. asahinai* replicates at 35°C, many deceased nymphs had been cannibalized, and this occurred most often to teneral nymphs. Because of these observations, the most common cause of death for *B. asahinai* nymphs could not be attributed solely to temperature with certainty, since cannibalism seemed to affect the survival of *B. asahinai* at this temperature, especially during molting. Although the effects of the presence of leaves on cannibalism were investigated through an addition experiment, because of the inability to observe the nymphs in detail among the leaves, the results were not conclusive.

Adults

The pronotal length and width and the number of cercal annuli were able to be measured for 378 *B. germanica* and 121 *B. asahinai* adults. Any individuals with deformed pronota were not recorded. Using a combination of pronotal measurements with the number of cercal annuli was more useful than using cercal annuli alone in estimating the number of instars for each adult, since counting cercal annuli to determine instar is only reliable from the until the fifth instar (Murray 1967). For both sexes and species, both “large” and “small” adults were observed, but not all adults were able to be visually distinguished according to the number of estimated instars. Figure 1 provides a clear example of a separation in size in *B. germanica* with a female and male thought to have five molts with a female and male thought to have six molts. The only set of measurements that were able to be separated by clustering into distinct groups based on pronotal size was *B. germanica* males from 20°C (Figure 2A). The instar assignments of each adult based on molt date, pronotal size, and cercal annuli was similar to the clustering assignment of the same set of measurements (Figure 2B).

Degree days

The range of temperatures at which more than one individual of both species were able to develop from first instar to adult was 20°C to 30°C, and those temperatures were used in the calculation of degree days and LDT. Table 5 and Table 6 present the estimations of lower development thresholds and degree days required for each instar and for total development of *B. asahinai* and *B. germanica* using the method of linear regression. The LDTs for each of the instars of *B. asahinai* ranged from 8.81°C to 14.26°C, and the LDTs for each of the instars of *B. germanica* ranged from 10.95°C to 12.85°C. The LDT of *B. asahinai* for total nymphal development was 13.73°C, and the LDT for *B. germanica* nymphal development was 14.06°C. The slopes of the regressed total nymphal development rates for *B. asahinai* and *B. germanica* were not significantly different ($F = 0.926$, $P = 0.39$).

Discussion

Blattella asahinai is a peridomestic species that typically inhabits leaf litter in shady areas (Brenner et al. 1988). Snoddy (2007, personal communication) found that during drought conditions and winter months in Dothan, AL, most *B. asahinai* adults and nymphs were only able to be collected from the leaf litter using Berlese traps, indicating that individuals moved downward into the leaf litter to escape dry and cold conditions. The current experiment was conducted in a laboratory with temperature as the main environmental variable. No substrate was provided at the bottom for either *B. germanica* or *B. asahinai*, which allowed for more consistent temperatures and easier observation, but it also removed the benefits of natural harborage that *B. asahinai* has in nature. Humidity was also unregulated throughout the experiment, and the relative humidity in the incubators was likely different than what *B. asahinai* experience in the field. Because the present study was conducted in a laboratory setting, its results can only provide a baseline for predicting *B. asahinai* development and population peaks in nature.

Temporal fluctuations in the 15°C incubator during the experiment are likely, since the first *B. asahinai* replicate at 15°C had an average lifespan of 42.4 d and was completed a month prior to the remaining replicates, which did not survive past 17.06 d. Stejskal et al. (2003) calculated the lower development threshold for the first instar of *B. germanica* to be 16.2°C, which is higher than 11.75°C, the LDT for the first instar of *B. germanica* determined in the present experiment. In one study by Wu et al. (2017), the LDT values for each of the seven instars of *Blaptica dubia* Serville (Blattodea: Blaberidae), a tropical cockroach, ranged from 7.02°C to 16.48°C. The LDT for the first instar was estimated to be 7.02°C by linear regression, but only one *B. dubia* nymph was able to molt to a second instar at 15°C, demonstrating that using linear regression can only provide an estimation of LDT, which may be different than experiment results (Wu et al. 2017). Using linear regression, Tsai and Chi (2007) reported an LDT of 16.96°C for *Supella longipalpa* (F.) (Blattodea: Ectobiidae), but this was not tested experimentally. Although linear methods of calculating degree days and thresholds are simple and widely used, determining the lower development threshold by extrapolation tends to overestimate the lower development threshold, since growth is not linear toward extreme temperatures (Campbell et al. 1974). The LDT values for *B. germanica* and *B. asahinai* from the current experiment are comparable to those previously estimated for cockroaches, even though LDT values determined through linear regression are only estimations, and, ultimately, strain and specific rearing conditions will affect development periods, resulting in variability among LDT estimations.

The number of degree days required for each instar tended to increase over development, because later instars required more days for completion. The number of degree days required for the sixth instar of *B. germanica* and the seventh instar of *B. asahinai* both required fewer degree days than that of the previous instar, but calculations of the period of the final instar of both species were likely less accurate than the earlier instars due to increased difficulty in distinguishing between later instars. The total number of degree days required was nymphal development was 482.88 d for *B. germanica* and 719.69 for *B. asahinai*. In comparison, *S.*

longipalpa had an average nymphal development period 106.4 d at 29°C and was estimated to require 1,289.6 degree days for nymphal development (Tsai and Chi 2007). *Blaptica dubia* required an average of 149.20 d and 2,071 degree days to complete nymphal development (Wu et al. 2017). Considering that *S. longipalpa* requires approximately twice the number of days and *B. dubia* approximately thrice the number of days to complete nymphal development as *B. asahinai* (44.95 d), the number of degree days estimated for *B. germanica* and *B. asahinai* in the current study are consistent with previous results.

Near Auburn, Alabama, where *B. asahinai* are consistently abundant every summer, average low temperatures in January are -1.39°C, and average high temperatures in July are 32.6°C (National Weather Service Forecast Office 2018). Given Snoddy's (2007, personal communication) observation of the utilization of leaf litter during dry and cold conditions, in order to develop a more accurate model of *B. asahinai* development in the field, further studies on *B. asahinai* responses to humidity and temperature in nature would be required, as well as seasonal information on leaf litter temperatures in *B. asahinai* habitats.

The temperature with the most drastic differences in development and survival between the two species was 35°C. Most *B. germanica* developed to adulthood normally in this temperature, while nearly all *B. asahinai* nymphs died before completing development. However, the cause of death was uncertain for most *B. asahinai* nymphs at 35°C. The dead individuals that were found each day had often been partially eaten, usually while molting or while teneral. In many cases, the nymphs were in the process of emerging from their exuviae and were found with a hole had been eaten out of the top of the thorax. To investigate further whether the primary cause of death was cannibalism or temperature, another set of replicates was conducted at 35°C with leaves serving as extra harborages. The results were inconclusive because of the lack of visibility of nymphs the leaves conferred. Leaf litter has been observed to shelter *B. asahinai* from cold or dry conditions (Snoddy 2007, personal communication), and it likely also plays an important role in protection during ecdysis. The lack of a complex harborage in the present experiment may have increased mortality due to cannibalism. An interesting observation is that cannibalism in *B. germanica* was almost never observed, which may indicate that nymph-on-nymph cannibalism is a trait selected against in the domestication of *B. germanica*. Cannibalism of nymphs by adults has been frequently observed in *B. germanica*, which is useful in pest control, as cannibalism of nymphs fed toxic baits can induce secondary kill in adults (Gahlhoff et al. 1999). However, nymphs have many defense mechanisms against adults, such as foraging during the day when adults are resting, utilizing protective harborage, and having a repellant compound in their saliva (Woodland et al. 1968, Faulde et al. 1990).

In the present experiment, *B. germanica* was only provided a single harborage, yet the survival rate during molting was extremely high, indicating that adults rather than nymphs are usually the perpetrators of cannibalism in *B. germanica*. Izutsu et al. (1970) similarly observed that even when rearing 100 nymphs together, cannibalism between nymphs was not observed frequently, except for occasionally during periods of ecdysis. Considering that *B. germanica* live together in aggregations, and individuals likely molt near each other, frequent cannibalism between nymphs during molting could greatly affect the ability of this species to proliferate in human dwellings. Conversely, although *B. asahinai* can occur in high numbers outdoors, they generally are found scattered throughout the leaf litter and are not as densely distributed as in the laboratory environment. Therefore, cannibalism between nymphs is unlikely to occur as often in nature as in the laboratory. Given that abundant leaf litter likely offers protection while molting, in addition to a stable microhabitat, limiting watering and shade in yards, as well as limiting leaf litter coverage or changing the ground covering to a material that offers less protection, would be possible cultural controls that homeowners could implement for *B. asahinai*.

Willis et al. (1958) reported an average total nymphal development period for *B. germanica* at 30°C of 41.3 d for males and 40.1 d for females when reared in groups, which is longer than 31.08 d, the average period recorded for *B. germanica* at the same temperature in the current study. This difference is likely caused by the strain and specific rearing conditions used, since strain, container size, and diet are all factors that can affect the number of instars of *B. germanica*, which consequently affects the period nymphal development (Kunkel 1981).

Although the exact development periods of the laboratory-reared *B. asahinai* in this experiment are likely to be different than in the field, the results from this experiment revealed core differences in development between *B. asahinai* and *B. germanica*. At every temperature, *B. germanica* had shorter development periods and lower mortality than *B. asahinai*. For both species, 30°C was the temperature that produced the shortest development periods. At this temperature, the highest percentage of *B. germanica* molted to an adult after only five instars, and fewer *B. asahinai* had seven instars. However, mortality was lowest for both species at 25°C, and *B. asahinai* had seven instars less often at 25°C than 30°C. Given the relatively high mortality of and greater number of instars of *B. asahinai* at 30°C, it is likely that *B. asahinai* experienced suboptimal conditions at 30°C, which, similarly to 35°C, may be related to the lack of complex harborage at this temperature. In laboratory cultures, *B. asahinai* has been observed to be more successful at 30°C when leaf litter is provided (personal observation). At 35°C, *B. germanicai* had six instars more often in both males and females than at 30°C, indicating that 35°C impeded the growth of some individuals, even though instar development periods were shorter at 35°C for many nymphs.

Male *B. germanica* often have one fewer instar than female *B. germanica* (Willis et al. 1958, Keil 1981, Tanaka 1982). Keil (1981) speculated that males have a reproductive advantage by earlier sexual maturity, because they are able to mate as soon as females reach sexual maturity after their adult molt. Since males tend to have longer final instars than females, males with five instars are able to mate with six-instar females sooner than males with six instars. This pattern, present in many cockroaches, was also seen in *B. asahinai* from this experiment. These results also confirm the estimation for *B. asahinai* made by Peterson et al. (2019), which used cercal annuli from nymphs along with clustering with pronotal measurements to determine the number of instars. Peterson et al. (2019) estimated that *B. asahinai* males most often had six instars and females either had six or seven instars. This was confirmed in our study, as half of *B. asahinai* females molted to adults after six instars and half molted after seven instars at 30°C, while all except two males molted to adults after six instars.

In general, the range of pronotal sizes for adults was continuous, and only for *B. germanica* males at 20°C were the adults able to be clearly separated into instar groups by pronotal size (Figure 2). The likely reason for this is that this was one of the only set of measurements that had an adequate number of individuals in both instar groups for the groups to be identified by clustering (19 adults with 5 instars and 29 adults with 6 instars). In most other cases, a single instar type was the majority for each temperature and species, such as nearly all *B. germanica* females having five instars at 25°C, which prevented distinct size classes from being identified. Tanaka (1982) also found that easily distinguishing instar types from adult measurements was not possible without having the date of the adult molt. Tanaka (1982) also found that although individuals with more molts had larger head widths, the difference was small. In the present experiment, although individuals of distinctly different sizes were observed

(Figure 1), most were not able to be separated easily by size. Considering that extra instars may be added to compensate for delays in growth, extra instars may only result in larger adult body sizes in certain cases (Tanaka 1982). Keil (1981) theorized that differences in the number of instars between sexes in *B. germanica* provided an advantage for both sexes, as males could mate earlier, and females would be larger and would gain extra resources for reproduction. Since body size is not an influential factor in male mating success (Lihoreau et al. 2007), the possibility of gaining a larger body size with an extra molt would only advantageous for females. Therefore, fewer molts is preferable for males, because males with fewer molts are able to mate earlier with females than males with the same number of molts as females (Tanaka 1982).

Since *B. asahinai* is estimated to be the closest extant relative of *B. germanica*, directly comparing the development of these two species reveals possible adaptations *B. germanica* has made in the process of domestication. Shorter development periods, fewer instars, tolerance to a wider range of temperatures, and possible avoidance of cannibalism are all adaptations that would aid *B. germanica* survive in the indoor environment and successfully colonize it. Similar to Keil's (1981) theory for the higher number of female instars, the longer development periods, and higher number of instars of *B. asahinai* compared to *B. germanica* could possibly help outdoor survival with more stored resources. Although the nymphal survival of *B. asahinai* was somewhat low for this experiment, this was mostly attributed to laboratory conditions, since *B. asahinai* tend to be numerous in outdoor settings. Although ground covering seems to be crucial for mitigating the effects of extreme temperatures on *B. asahinai* survival, *B. asahinai* is still limited in its distribution in the United States to regions where leaf litter can provide a microclimate suitable enough for shelter during overwintering and during dry and hot conditions. For future research, more studies on the microclimates of *B. asahinai* in the southeastern United

States could be conducted to further understand the seasonal behaviors and survival strategies of this species.

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Table 1. Cumulative mean time (d) and standard deviation to complete each instar of *B. germanica* and *B. asahinai*, as well as total nymphal development, at 20°C, with *n* in parentheses. The average nymphal development periods of each species are significantly different ($W = 39$, $P < 0.001$).

	Instar							
	1	2	3	4	5	6	7	Total
B. germanica	11.49 ± 1.11 (115)	22.84 ± 1.36 (114)	35.78 ± 2.18 (110)	50.79 ± 4.62 (107)	70.29 ± 7.39 (107)	89.52 ± 7.36 (81)	119.00 (1)	86.98 ± 8.75 (106) a
B. asahinai	17.43 ± 5.32 (46)	32.59 ± 7.62 (32)	48.39 ± 8.95 (28)	64.04 ± 9.94 (23)	80.30 ± 10.77 (23)	97.82 ± 9.15 (22)	122.84 ± 9.28 (19)	119.73 ± 11.87 (22) b

Table 2. Cumulative mean time (d) and standard deviation to complete each instar for six replicates of *B. germanica* and *B. asahinai*, as well as total nymphal development, at 25°C, with *n* in parentheses. The average nymphal development periods of each species are significantly different ($W = 6.5$, $P < 0.001$).

	Instar							
	1	2	3	4	5	6	7	Total
B. germanica	6.80 ± 0.60 (115)	13.15 ± 0.81 (114)	20.49 ± 1.01 (113)	28.83 ± 1.62 (112)	40.23 ± 2.47 (108)	46.40 ± 2.01 (10)	n/a	41.24 ± 2.59 (108) a
B. asahinai	10.04 ± 5.63 (82)	18.05 ± 7.11 (65)*	26.18 ± 6.95 (66)	35.36 ± 8.88 (61)	44.97 ± 9.15 (59)	57.25 ± 9.82 (57)	64.93 ± 5.35 (15)	60.91 ± 9.40 (57) b

*one molt was not recorded

Table 3. Cumulative mean time (d) and standard deviation to complete each instar for six replicates of *B. germanica* and *B. asahinai*, as well as total nymphal development, at 30°C, with n in parentheses. The average nymphal development periods of each species are significantly different ($W = 54.5$, $P < 0.001$).

	Instar						
	1	2	3	4	5	6	7	Total
B. germanica	5.13 ± 0.39 (119)	9.99 ± 0.49 (115)	15.39 ± 0.70 (114)	21.54 ± 1.06 (103)	30.59 ± 2.42 (105)	38.00 ± 2.35 (5)	n/a	31.05 ± 2.86 (105) a
B. asahinai	6.49 ± 1.76 (91)	12.75 ± 2.93 (77)	19.04 ± 3.44 (68)	25.52 ± 3.76 (56)	32.27 ± 3.32 (49)	41.74 ± 4.30 (43)	50.15 ± 3.83 (13)	44.95 ± 5.49 (43) b

Table 4. Cumulative mean time (d) and standard deviation to complete each instar and total nymphal development for six replicates of *B. germanica* at 35°C, with *n* in parentheses.

	Instar							
	1	2	3	4	5	6	7	Total
B. germanica	4.34 ± 1.14 (113)	8.71 ± 1.47 (107)	13.76 ± 1.59 (103)	20.64 ± 3.93 (95)	28.50 ± 5.44 (84)	35.98 ± 5.28 (43)	43.00 (1)	34.29 ± 5.91 (76)

Table 5. Linear regression models, results of ANOVA, lower developmental thresholds (°C), and degree days required for completion of each instar 1-7, as well as total nymphal development of *B. asahinai*, based on development at 20°C, 25°C, and 30°C.

Instar	Equation	*F*	*P*	LDT	Degree days
1	Y=0.0096X-0.1376	193.41	0.0457	14.26	103.66
2	Y=0.0094X-0.1176	45.88	0.0933	12.54	106.63
3	Y=0.0096X-0.1242	50.06	0.0894	12.98	104.50
4	Y=0.0091X-0.1175	70278	0.0024	12.96	110.30
5	Y=0.0087X-0.1121	9593.8	0.0065	12.93	115.41
6	Y=0.0048X-0.0396	95596	0.0021	8.18	206.72
7	Y=0.0079X-0.1012	1.8256	0.4056	12.81	126.60
Total Development	Y=0.0014X-0.01907	115.82	0.0590	13.73	719.69

Table 6. Linear regression models, results of ANOVA, lower developmental thresholds (°C), and degree days required for completion of each instar 1-6, as well as total nymphal development of *B. germanica*, based on development at 20°C, 25°C, and 30°C.

Instar	Equation	*F*	*P*	LDT	Degree days
1	Y=0.0108-0.1268	236.45	0.0413	11.75	92.68
2	Y=0.0118X-0.1439	92.775	0.0659	12.23	84.94
3	Y=0.0108X-0.1376	375.86	0.0328	12.72	92.38
4	Y=0.0096X-0.1230	233.54	0.0416	12.85	104.48
5	Y=0.0059X-0.0649	53.381	0.08429	10.95	168.88
6	Y=0.0083X-0.0910	1.0967	0.4853	10.97	120.55
Total Development	Y=0.0021X-0.0291	55.992	0.08458	14.06	482.88

Fig. 1. In the top row, a *B. germanica* female that had 5 instars (left) and a female that had 6 instars (right), and in the bottom row, a *B. germanica* male that had 5 instars (left) and a male that had 6 instars (right).

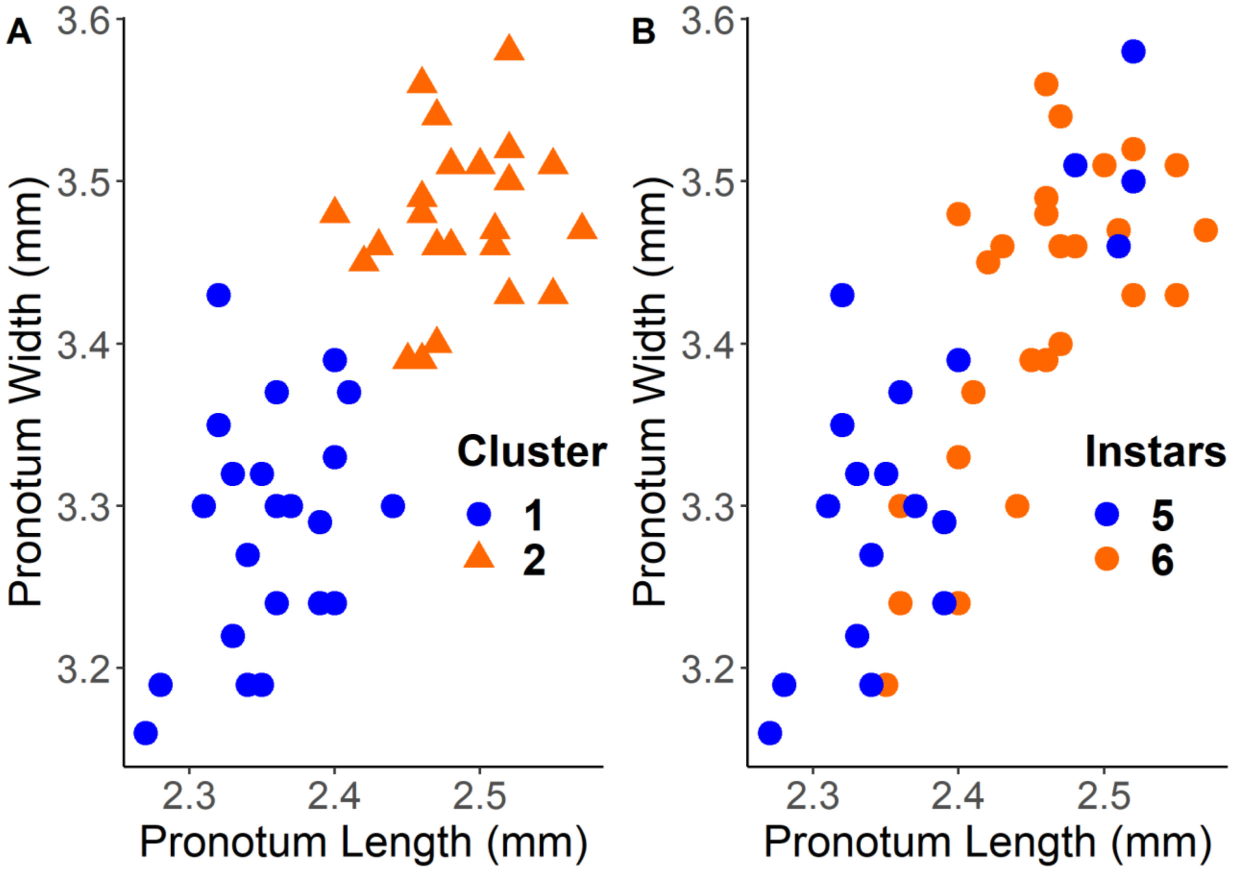

Fig. 2. (A) Clusters identified by mclust from pronotal length and width of 48 *B. germanica* males reared at 20°C. (B) Pronotal length and width of the same 48 *B. germanica* males reared at 20°C with the estimated number of instars for each individual.

CHAPTER 4

When German cockroaches fly?: investigating the interspecific hybrids of *Blattella asahinai* and *B. germanica* (Blattodea: Ectobiidae)

Abstract

The Asian cockroach, *Blattella asahinai*, is a peridomestic nuisance pest in the southeastern United States. It is the closest relative of the German cockroach, *B. germanica*, which is a notorious domestic health pest. The two species have successfully hybridized in the laboratory. Unlike flightless *B. germanica*, *B. asahinai* are capable of flight, which contributes to their ability to enter homes at night when attracted by lights. Although *B. germanica* are primarily domestic, they have been observed living in a mixed-species group in an outdoor shelter in the case of a large infestation. Although *B. asahinai* cannot survive long periods indoors and *B. germanica* rarely are found outside, it is possible the two species may come into contact under certain conditions in nature. To investigate the biological properties of German-Asian cockroach hybrids, both interspecific crosses were made in the laboratory. The wings from adults of each species and hybrid cross were examined for venation differences. The behavior and general laboratory success of each species and hybrid cross were also observed. Our findings indicated that the hybrids have wing characteristics intermediate of the two parent species, which allow them minimal flight ability. Although hybrid crosses were not achieved consistently, the resulting individuals of successful crosses thrived in laboratory conditions, indicating domestic pest potential. Although hybridization of *B. asahinai* and *B. germanica* is unlikely to occur in nature, investigating the traits of the hybrids allows for an interesting examination of the traits of German cockroaches that contribute to domestic success.

Introduction

The Asian cockroach, *Blattella asahinai* Mizukubo (Blattodea: Ectobiidae), was discovered in the United States in Florida in 1986 (Roth 1986). Since then, it has spread throughout the southeastern United States, where it is a peridomestic nuisance pest and minor agricultural pest (Brenner et al. 1988, Brenner 1991, Sitthicharoenchai 2002, Austin et al. 2007, Snoddy and Appel 2008, Matos and Schal 2015). Its closest relative is *Blattella germanica* (L.), the German cockroach (Roth 1985), a notorious cosmopolitan pest, especially in low-income housing (Wang et al. 2008, Zha et al. 2018). Unlike the flightless *B. germanica*, *B. asahinai* is capable of flying distances over 30 meters (Brenner et al. 1988). The enhanced mobility of *B. asahinai* allows it to easily enter homes at night, attracted by the lights, which makes it an undesirable resident of yards and gardens (Brenner et al. 1988, Snoddy 2012). Its flight capability also has likely contributed to its range expansion via major highways (Snoddy and Appel 2008).

Blattella germanica is almost exclusively an indoor pest. In cases of extreme indoor infestation, *B. germanica* may migrate outside (Cornwell 1968, Appel and Tucker 1986). In the southeastern United States, outdoor infestations of *B. germanica* have been observed, as well as a mixed population infestation of German, American, and smokybrown cockroaches in a telephone pole (Appel and Tucker 1986). With increased urbanization and globalization, human settlements are creating more suitable habitats for pest insects even in previously unfavorable environments (Schapheer et al. 2018). *Blattella germanica* may find suitable outdoor habitats when overpopulation forces individuals outside, which could cause habitat overlap with *B. asahinai*.

Males of *B. asahinai* respond to blattellaquinone, the female sex pheromone of *B. germanica* (Matos and Schal 2015), and in the laboratory, these two species have successfully hybridized. Roth (1986) reported *B. asahinai* males would mate with *B. germanica* females and produce offspring, but the reciprocal cross did not result in any progeny. Both reciprocal crosses, as well as backcrosses, in Ross (1992), however, successfully resulted in F_1 offspring. Unequal success with reciprocal crosses during interspecific hybridization studies is not uncommon (e.g. Laster 1972, Leslie and Dingle 1983). The inconsistency in success of reciprocal crosses may have been caused by differences in strain and rearing conditions.

Interspecific hybrids may exhibit an intermediate phenotype or may resemble one parent more closely (Leslie and Dingle 1983). Ross and Cochran (1995) found that F_1 hybrids of *B. asahinai* and *B. germanica* inherited some insecticide resistance from their German cockroach parent, resulting in higher LT_{50} values than the Asian cockroach parent, but the values were still more similar to the susceptible Asian cockroach parent than to the resistant German cockroach parent. Since *B. asahinai* and *B. germanica* live in very different biomes to which they are specifically adapted, further investigation into the morphological, physiological, and genetic characteristics of *B. asahinai-B. germanica* hybrids is necessary to assess their habitat preferences and further reproduction potential.

The objective of this study was to characterize important biological traits of the interspecific hybrids of *B. asahinai* and *B. germanica*, such as morphology, flight capability, behavior, and reproductive fitness. These findings will be useful to estimate the success of the hybrids in the field. Even if interspecific hybridization of these two species is unlikely in nature, these studies can provide valuable insights into the traits that contribute to the success of *B. asahinai* and *B. germanica* in their respective natural habitats.

Materials and Methods

Cockroach Rearing and Hybridization

Blattella asahinai nymphs and adults were collected from two locations in Auburn, AL between June and October 2019 (Davis Arboretum: 32.5952° N, 85.4830° W; Private Residence: 32.6152˚ N, 85.4825˚ W). Individuals were identified as *B. asahinai* at the time of collection by their outdoor habitat and flight capability and were subsequently confirmed in the laboratory by morphology of their tergal glands (Roth 1986, Snoddy 2007). The collected individuals were reared at 30˚C in either 3.8-liter (1-gallon) or 1.9-liter (0.5 gallon) glass jars with a photoperiod of 12:12 (L:D) and given rat food (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. Pine straw and leaves were gathered from the collection sites and used as substrate for *B. asahinai* during rearing. *Blattella germanica* stock were received from American Cyanamid Co. in Clifton, NJ, in 1985 and were reared under the same conditions as *B. asahinai*, except a paper harborage was substituted for substrate.

Hybrids of *B. asahinai* and *B. germanica* were obtained by pairing virgin females of one species with virgin males of the other species in 1.9-liter (0.5-gallon) glass jars in the same conditions as were used for *B. germanica* rearing. Multiple males and females of the opposite species were often paired together in one jar and were regularly replaced or added to when more virgins became available. Jars were checked often for oothecae formation and neonates. When interspecific hybrid nymphs were obtained, they were isolated from their parents in a separate 1.9-liter (0.5-gallon) glass jar in the aforementioned conditions.

Wing Venation

Adults and nymphs of various instars of *B. asahinai*, *B. germanica*, and both interspecific hybrids were collected and killed by freezing. Hind wings were obtained by floating adult cockroaches in hot water and gently spreading the hind wings out from the body. The right hind wing was used for all observations, since the hind wings are the only pair of wings used to power flight. The cockroach was removed from the water using a paper towel, so that the wing remained spread on the wet paper. The body was separated from the wing, and the wing was dried underneath a glass slide using a light. Once dried, the wing retained its spread position, and it was mounted onto a glass slide under a cover slip using either Permount (Fisher Scientific Company, Fair Lawn, NJ) diluted with xylenes or Euparal (Hempstead Halide, Galveston, TX). The venation of the wings was examined using the terminology for cockroach wing venation from Rehn (1951). For *B. asahinai,* two female wings and three male wings were examined; for B. germanica, three female wings and one male wing were examined; for the hybrids of the male *B. asahinai* and female *B. germanica* cross, three female and three male wings were examined; and for the hybrids of the male *B. germanica* and female *B. asahinai* cross, one female wing was examined. The variable number of replicates for each species was caused by difficulties in mounting the wings and low numbers of hybrids.

Hybrid Flight Capability

To quantify the flight capability of each species and their interspecific hybrids, adult males and females from each species or hybrid cross were gently tossed upwards into the air at approximately breast height (1.37 m) in a 21˚C room. The method of arrival towards the ground was observed and recorded. Ten replicates, consisting of five males and five females, were evaluated each for *B. germanica* and *B. asahinai*. Due to the inconsistent timing in the generation of hybrid offspring, only five males of the cross of female *B. germanica* and male *B. asahinai* were able to be evaluated.

Results

Hybridization

Both hybrid crosses were achieved from the pairing of male *B. germanica* with virgin female *B. asahinai* and male *B. asahinai* with virgin female *B. germanica*. However, the crosses were not achieved with equal ease or frequency. The first successful hybrids produced were from the cross between male *B. germanica* and female *B. asahinai*. However, this cross was more difficult to achieve later in the time period of the experiment, resulting in overall low numbers of F_1 hybrids from this cross. Hybrids produced from the cross between female *B. germanica* and male *B. asahinai* were achieved months later in the time period of the experiment. However, after the first cohort of hybrid nymphs was eventually produced, the successive cohorts were produced more easily, resulting in more abundant hybrids from the cross between female *B. germanica* and male *B. asahinai*. The hybrids were similar in general appearance to both *B. germanica* and *B. asahinai*, but the length of the wings of the hybrids were similar to that of *B. asahinai* (Figure 1).

Behavior and Reproduction

Both hybrid crosses were able to successfully produce their respective F_2 generations, as well as successive generations. The behavior of the hybrids from both crosses was similar to that of *B. germanica*. Reproduction was not closely monitored, but the F_2 and successive generations were rapidly produced and became abundant quickly. Both hybrid crosses thrived in the laboratory setting. Unlike *B. asahinai*, which seems to thrive best in the laboratory in contains with leaf litter (personal observation), both hybrid crosses thrived in the same conditions as *B. germanica*, with a normal paper harborage.

Wing Venation

Wing venation varied between species and hybrid crosses. Using the wing venation terminology from Rehn (1951), *B. asahinai* tend to have two to three subcosta, three to four radia 1, three to five anterior radial rami, six apical radial rami, a branched apical posterior branch of the radius, an unbranched medial vein, two cubital veins, and 18-21 branched axillary veins (Figure 2). *B. germanica* tend to have two to three subcostal, two to three radia 1, six anterior radial rami, six apical radial rami, either a branched or unbranched apical posterior branch of the radius, an unbranched medial vein, one to three cubital veins, and 14-16 branched axillary veins (Figure 3).

Hybrids from a cross of female *B. germanica* and male *B. asahinai* tend to have two to three subcosta, three radia 1, four to six anterior radial rami, five apical radial rami, a branched apical posterior branch of the radius, an unbranched medial vein, one or two cubital veins, and 16-17 branched axillary veins (Figure 4). Only one wing of a hybrid from a cross of female *B. asahinai* and male *B. germanica* was examined due to the low numbers of this hybrid cross. This wing had two to three subcosta, three radia 1, five anterior radial rami, four apical radial rami, a branched apical posterior branch of the radius, an unbranched medial vein, two cubital veins, and 17 branched axillary veins (Figure 5). The most reliable characteristic for distinguishing wings between *B. germanica*, *B. asahinai*, and hybrids was the number of branched axillary veins in the lower portion of the wing, as *B. asahinai* had 18-21, *B. germanica* had 14-16, and hybrids had 16-17.

Flight Capability

Ten *B. asahinai* were evaluated for flight ability, five of each sex. Of five males, three flew upwards and had sustained flight until landing on the wall or ceiling. One glided downwards towards the ground, and one fell without flying. Of five females, four flew in the same manner as the males, and one glided downwards. Ten *B. germanica* were evaluated in the same way as *B. asahinai*. Of five males, three glided towards the ground with some minimal wing movements, one fell downwards with wings outstretched, and one fell downwards without wing activity. Of the five male hybrids from the cross of female *B. germanica* and male *B. asahinai*, two had sustained flight, two glided with some wing movement, and one fell downwards.

Discussion

The hybridization of *B. asahinai* and *B. germanica* in nature has never been confirmed likely due to the usually well-separated habitats of each. *Blattella germanica* has strong aggregation pheromones, and group living is very important in this species for normal development (Izutsu et al. 1970, Lihoreau and Rivault 2008). Because German cockroaches are so adapted to the indoor biome, they are rarely found outdoors. Conversely, *B. asahinai* cannot survive indoors for long periods of time likely due to desiccation and higher cuticular permeability (CP) values than *B. germanica* (unpublished data). However, in the southeastern United States, *B. germanica* have been observed sheltering with other cockroaches in a telephone pole near sites of extreme indoor infestation (Appel and Tucker 1986). Although the likelihood of these species to have enough contact for hybridization is low, certain conditions may allow for these two species to cohabit. The results from this study demonstrate that reciprocal crosses between *B. germanica* and *B. asahinai* result in offspring, but that hybrid production is likely not as consistently successful as conspecific production of young.

The resulting hybrids appeared to be intermediate between *B. germanica* and *B. asahinai* in their capability of flight. Although only a few hybrids could be tested, the results demonstrate that at least some hybrids can fly in a manner similar to *B. asahinai*, while it is well known that *B. germanica* cannot fly. The *B. asahinai* in this experiment had been laboratory-reared over several generations and do not have the tendency to fly as readily as freshly-collected *B. asahinai*, although the ability to fly is not lost. Most *B. asahinai* were still able to fly after being tosses into the air, though. No *B. germanica* were able to fly when tossed, but some did appear to glide towards the ground.

The hind wings of *B. asahinai* had the most veins in the lower part of the wing, *B. germanica* had the fewest veins, and hybrids had an intermediate number of veins. Individual wing venation varied somewhat between individuals even within species, but the number of veins in the lower section of the wing was the most consistent. Lawless (1999) found that the number of veins in the lower section of the wing ranged from 16-18 in *B. germanica* and from 20-22 in *B. asahinai*, making this a reliable method of distinguishing wings of the two species. The number of veins in the lower part of the wing were consistently intermediate for hybrids. The range of veins of the hybrid wings overlaps that of *B. germanica*, but hybrid wings are larger than *B. germanica* wings, meaning that a hybrid wing could likely be distinguished from wings of both *B. germanica* and *B. asahinai*. However, it is unlikely that one hybrid cross could be distinguished from a hybrid from the reciprocal cross easily by wing venation.

Although the probability of hybridization in nature is low, if it did occur, the resulting hybrids would have beneficial traits from both parents, some flight ability from the *B. asahinai* parent and hardiness and reproduction potential from the *B. germanica* parent. However, the habits of the hybrids in the laboratory were similar to *B. germanica*, meaning that they likely would have more of a domestic pest potential than a peridomestic pest potential. The power of flight would likely be lost with successive generations, and the hybrids would likely eventually be indistinguishable from *B. germanica*.

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Fig. 1. Pairs of adult females and males of (from left to right) *B. germanica*, hybrids (from cross of male *B. germanica* and female *B. asahinai*), and *B. asahinai*.

Fig. 2. Right hind wing from a female *B. germanica* with approximately 16 veins in the lower section.

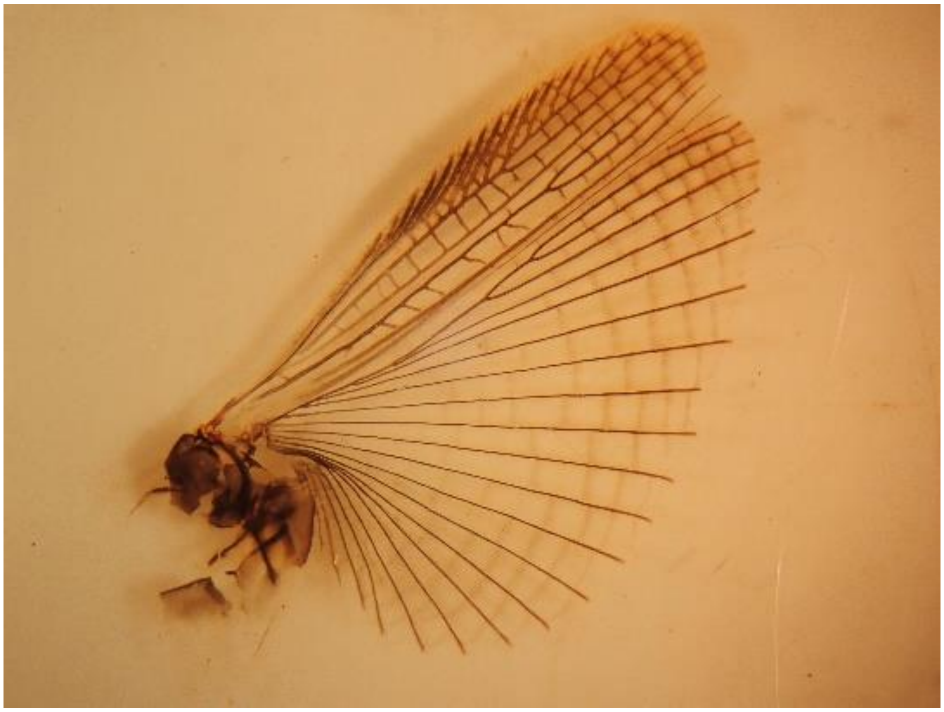

Fig. 3. Right hind wing from a female *B. asahinai* with approximately 21 veins in the lower section.

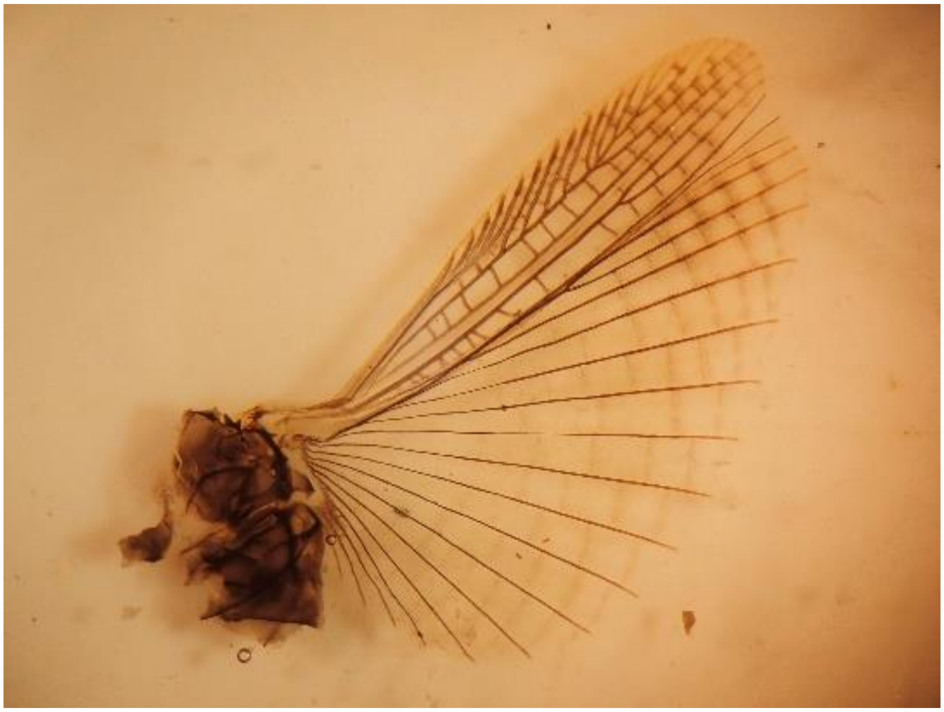

Fig. 4. Right hind wing from a female hybrid of a male *B. germanica* and female *B. asahinai* cross with approximately 17 veins in the lower section.

Fig. 5. Right hind wing of a female hybrid of a male *B. asahinai* and female *B. germanica* cross with approximately 17 veins in the lower section.