Application of Pheromone Lures to Study Mechanisms of Reproductive Isolation and to Develop Cost-efficient Population Assessments of Click Beetles in North America

by

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Abstract

I tested the effects of synthetic (*E*)-4-ethyloct-4-enoic acid ('limoniic acid'; sex pheromone component of the click beetles *Limonius canus* and *L. californicus*) and of (*E*)-5-ethyloct-4-enoic acid (analog of limoniic acid) as trap lures on captures of *L. canus, L. californicus, L. infuscatus* and *L. agonus* across North America. Males of all four species were attracted to both limoniic acid and the analog irrespective of lure dose (0.4 or 4 mg). Exploring mechanisms that underlie species-specificity of sexual communication in *Limonius* congeners, I discovered that *L. canus* and *L. californicus* have seasonally distinct communication periods but that captures of *L. infuscatus* overlap with those of *L. canus* and *L. californicus.* Investigating whether mixed pheromone lures attract elaterid heterogeners (*Agriotes* spp. and *Limonius* spp.), I found that mixed lures did not reduce captures of target species, suggesting that these lures can be used to effectively monitor, or possibly control, select elaterid heterogeners.

Keywords: Click beetle; wireworm; pheromone-based monitoring; field-testing; integrated pest management; *Limonius*

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Chapter 1. Distribution, basic biology, and pest status of the pacific coast wireworm, western field wireworm, sugarbeet wireworm, and eastern field wireworm

The taxonomic family of click beetles (Coleoptera: Elateridae) comprises an extensive 10,000 species worldwide, approximately 100 of which are classified as pests (Johnson et al. 2002; Traugott et al. 2015; Vernon and van Herk 2022). Wireworms, the larvae of many species of click beetles, are serious pests of many agricultural crops including vegetables, cereals, forage, sugar cane, and strawberries. *Limonius* species – particularly the sugarbeet wireworm, *Limonius californicus* Mannerheim, the western field wireworm, *Limonius infuscatus* Motschulsky, and the Pacific Coast wireworm, *Limonius canus* Leconte, in western regions as well as the eastern field wireworm, *Limonius agonus* Say, in eastern regions – are some of the most predominant and detrimental agricultural pests in North America (Toba and Campbell 1992; Andrews et al. 2008; Rashed et al. 2015; Milosavljevic et al. 2016; van Herk et al. 2021b). Yield reductions in cereal crops of up to 70% have been reported for these species in the Pacific Northwest of the USA, and complete stand destruction of spring wheat has been reported in fields with high *L. californicus* populations in Southern Alberta, Canada (Reddy et al. 2014; van Herk et al. 2018a). Since the early 1900s, growers have experienced the severe economic damage that *Limonius* wireworms can cause to potatoes and sugar beets (Essig 1915; Hyslop 1915). Since the 1940s, insecticides including several organochlorines, carbamates and organophosphates have effectively controlled wireworms and reduced their importance as pests in North America. However, due to the gradual deregistration of most of these insecticides concurrent in recent decades with farming practices favourable to wireworms (i.e., crop rotations which include grasses or cereal crops and minimum tillage practices), wireworm populations are again increasing (Vernon and van Herk 2022). As a result, wireworms are once again regaining their importance as primary pests throughout North America, and the need to find alternate means for wireworm control have stimulated renewed scientific activities into the biology and management of

elaterids since the turn of the current century. Understanding the general biology, sexual communication system, mating behaviour, feeding preference, geographic distribution as well as seasonal and diel activity pattern of key pest species is essential in designing appropriate pest management programs. This is complicated, since endemic or invasive pest elaterid species occupy delimited or expanding geographical regions across North America, generally have lengthy (3-5 years is common) but variable life histories, often overlap in arable fields and their surrounding headlands, and not all species respond equally to the currently available arsenal of control methods. For instance, certain species are known to respond differently to various insecticides, biocontrols, rotational crops and soil amendments (Lange et al. 1949; van Herk et al. 2007; Traugott et al. 2015; Vernon and van Herk 2022), and therefore it is becoming more imperative to design customized control tactics at a local level. Based on the acquired knowledge to date, multi-tactical integrated pest management (IPM) programs can now be considered that target the adult, click beetle stage, and might include novel pheromone-based monitoring approaches, mass trapping, mating disruption and attract-and-kill methods involving microbial biological control agents (Vernon and van Herk 2022).

1.1. Distribution and biology of *Limonius* species pests

The distributions of *L. canus, L. californicus* and *L. infuscatus* range from British Columbia (BC), Canada south to the US states of Washington, Oregon and California (Stone 1941; Vernon and van Herk 2013; Milosavjlevic et al. 2016; Andrews et al. 2020; E.L. pers. obs.). *Limonius californicus* and *L. infuscatus* are also present in Idaho and Montana, where *L. canus* was historically found (Mail 1932; Etzler et al. 2014; Morales-Rodriguez et al. 2014; Milosavjlevic et al. 2016). *Limonius californicus* has a larger distribution than *L. canus* and *L. infuscatus,* also being present on the Canadian prairies in Alberta and Saskatchewan (van Herk et al. 2021). The eastern field wireworm, *L. agonus,* as its name implies is present in eastern regions from southern Ontario and Quebec (Canada) throughout Maine, New York, Pennsylvania, and New Jersey in the Northeastern USA (Horsfall and Thomas 1926; Hawkins 1930; Macleod and Rawlins 1935; Pepper et al. 1947; Vernon and van Herk 2013; Vernon and van Herk 2022).

Regular sampling for species presence is crucial for effective IPM because species distributions and community compositions are continuously changing due, in part, to the influx of invasive species and climate change (Mainka and Howard 2010).

Based on the literature to date, pest *Limonius* species have a long-life history, with larvae spending 1–6 years in the soil before pupating and emerging as adults in late summer (Stone 1941; Lanchester 1946). Newly formed beetles remain in their overwintering cells approximately 5 – 15 cm below the soil surface until the following spring and typically emerge when the soil reaches a threshold average temperature of 10 °C (LaFrance 1963; van Herk and Vernon 2014). Males usually emerge before females (Stone 1941) but the average lifespan of male and female adults is still unknown. Mated females lay 100–200 eggs and die shortly after, and unmated females produce infertile eggs (Jones 1951; Woodworth 1942). Male *L. canus* may mate more than once (Woodworth 1942), but as noted by Zacharuk (1958), it is not known if female *Limonius* beetles will mate more than once, or if the second matings by males result in the fertilization of eggs. Site selection for oviposition differs among species, with female *L. canus* and *L. californicus* preferring bare soil and vegetated cover, respectively (Gibson et al. 1958). Larvae of *L. agonus, L. californicus* and *L. canus,* but not as much *L. infuscatus,* prefer sandy soil, specifically finer, sandy soils for *L. agonus* (Stone 1941; Rawlins 1943; Kring 1957; Milosavljevic et al. 2016a).

Studying the biology and life history traits of pest click beetle species will help inform the development of new management tools. For instance, the development and implementation of control measures for adult click beetles requires a sound understanding of the beetles' life span and their temperature-dependent seasonal and diel activity periods.

1.2. Synthetic sex pheromones as management tools for click beetles

Sex pheromones have been identified for more than 30 – mainly Palaeartic – elaterid species, primarily in the genera *Agriotes, Elater* and *Melanotus* (Toth 2013). Pheromones of most Nearctic species remain unknown. For *L. californicus* and *L. canus*, pheromone

research began in the early 1930s, and as of the 1970s, the sex pheromones of these species were believed to consist of pentanoic and hexanoic acids (Lehman 1932; Lilly 1959; Jacobson et al. 1968; Onsager et al. 1968). When further field and laboratory experiments were conducted in the 1970s, a revised conclusion was that the sex pheromone was likely a 2-component blend consisting of a carboxylic acid and a "branched 10-carbon unsaturated acid" (Onsager et al. 1968; Butler et al. 1975), but when pentanoic acid was later tested in field trials in British Columbia it was found not to be attractive to male *L. canus* (Vernon, unpubl. data; Gries et al. 2021). The inconclusive results of pentanoic acid as well as the suspected 2nd component hypothesized by Butler et al. 1975, warranted further investigation for these four *Limonius* species. Sex pheromones of *L. infuscatus* and *L. agonus* were not investigated in the past largely because most studies were directed toward developing insecticidal control methods. Recently, the major sex pheromone component of female *L. canus, L. californicus* and *L. agonus,* has been determined to be, (*E*)-4-ethyloct-4-enoic acid ('limoniic acid', Gries et al. 2021; van Herk et al., unpubl.), which also serves as a sex attractant for male *L. infuscatus* (Gries et al. 2021). Other sex pheromones recently identified for common elaterid pest genera in North America include *Agriotes ferrugineipennis* LeConte, *Agriotes mancus* Say, *Cardiophorus tenebrosus* LeConte, *Cardiophorus edwardsi* Horn, *Idolus californicus* Schaeffer, *Melanotus communis* Gyllenhal, *Parallelostethus attenuatus* Say and *Selatosomus aeripennis destructor* Brown (Gries et al. 2022; Millar et al. 2022; Serrano et al. 2018, 2022; Singleton et al. 2022a, 2023; van Herk et al. 2021c; Williams et al. 2019).

The development of synthetic click beetle pheromones will provide the opportunity to (*i*) curtail populations of adult beetles via mating disruption and mass trapping, (*ii*) delineate the geographic distribution of target species, (*iii*) predict crop damage, and (*iv*) help time and assess the deployment of insecticidal and biological control measures (Reddy and Tangtrakulwanich 2014; Furlan et al. 2020; Vernon and van Herk 2022). Effective pheromone-based monitoring and control measures for click beetle populations are contingent upon the determination of optimal pheromone blends and dosages, dispensers and trap types, and inter-trap or inter-dispenser distance.

1.3. Co-occurrence of pest elaterids in North America

In North America, multiple elaterid pests frequently co-occur in agricultural fields. Palaearctic and Nearctic *Agriotes* congeners, such as *A. mancus, A. obscurus* and *A. lineatus*, are major agricultural pests that commonly co-occur with *Limonius* species in the same geographic locations (Table 1.1). *Agriotes obscurus* and *A. lineatus*, two European invasive species, were first reported in British Columbia in the 1950s, are now rapidly spreading throughout North America and are likely to affect the formerly predominant species in the PNW (Vernon and Pats 1997; Andrews et al. 2008; Vernon and van Herk 2022). *Agriotes* species follow the typical elaterid life cycle of *Limonius* species, requiring several years to complete their life cycle as wireworms, pupating into adults in late summer, overwintering and then emerging from the soil in spring (Miles 1942). The female sex pheromones for *A. lineatus* and *A. obscurus* have been identified as geranyl octanoate + geranyl butanoate and geranyl octanoate + geranyl hexanoate, respectively, and have been used for monitoring in several countries in Europe since the 1990s and in the PNW since the early 2000s (Furlan 2001; Vernon and Toth 2007). The female sex pheromone for *Agriotes mancus* has also recently been identified as geranyl butanoate + geranyl hexanoate and is just beginning to be used as a monitoring tool (Singleton et al. 2023). As *Agriotes* and *Limonius* heterogeners often overlap in their geographic distribution and seasonal activity patterns, synthetic trap lures containing pheromones of both *Agriotes* and *Limonius* species without compromising the lure's optimal attractiveness to all species would be a novel and cost-effective monitoring and mass trapping tool for click beetle populations.

Table 1.1. List of elaterids commonly co-occuring with *L. agonus, L. canus, L. californicus* or *L. infuscatus* in North America. References are reported below table.

***Limonius* species**	**Elaterid species co-inhabiting the same field**	**Geographic location (US state/ Canadian province)**
Limonius agonus	*Agriotes mancus*	ON[1,2,3], QC[1,2,3]
	Agriotes pubescens	QC[2]
	Agriotes sputator	QC[2]
	Hypnoidus abbreviatus	ON[1], QC[1]
	Melanotus similis	ON[1], QC[1]
Limonius canus	*Aeolus mellilus*	BC[4]
	Agriotes ferrugineipennis	BC[2,4,5]
	Agriotes lineatus	BC[2,3,4,5,6]
	Agriotes obscurus	BC[2,5,6]
	Cardiophorus montanus	OR[7]
	Cardiophorus spp.	BC[4]
	Corymbitodes moerens	BC[3]
	Corymbitodes lobata	BC[2]
	Ctenicera pruinina	OR[7]
	Dalopius spp.	BC[2,4], OR[7]
	Limonius californicus	BC[2,3,4], OR[3]
	Limonius infuscatus	BC[2,3,4], WA[3]
	Melanotus spp.	BC[4], OR[7]
Limonius californicus	*Aeolus mellillus*	BC[4], AB[8], SK[8], MB[8], MT[9]
	Agriotes criddlei	AB[8]
	Agriotes ferrugineipennis	BC[4]
	Agriotes lineatus	BC[2,3,4]
	Agriotes obscurus	BC[2]
	Agriotes stabilis	SK[8], MB[8]
	Ampedus behrensi	BC[4]
	Cardiophorus spp.	BC[4]
	Dalopius spp.	BC[4]
	Gambrinus ursinus	AB[3]
	Hadromorphus glaucus	WA[10]

	Hypnoidus bicolor	AB[3,8], SK[8], MB[11], MT[9]
	Hypnoidus abbreviatus	MB[11]
	Limonius canus	BC[2,3,4], WA[3], OR[3]
	Limonius infuscatus	BC[2,3,4], WA[10], OR[10], ID[3,10], MT[3,9]
	Melanotus spp.	BC[4]
	Selatosomus aeripennis	MT[9]
	Selatosomus a. destructor	AB[3,8], SK[8]
	Selatosomus pruininus	WA[10]
Limonius infuscatus	*Aeolus mellillus*	BC[4]
	Agriotes ferrugineipennis	BC[4]
	Agriotes lineatus	BC[2,3,4,6]
	Agriotes obscurus	BC[2,6]
	Ampedus behrensi	BC[4]
	Cardiophorus spp.	BC[4]
	Dalopius spp.	BC[4]
	Gambrinus ursinus	AB[3]
	Hadromorphus glaucus	WA[10]
	Hypnoidus bicolor	AB[3], MT[9]
	Limonius californicus	BC[2,3,4], WA[3,10], OR[10], ID[3,10], MT[3,9]
	Limonius canus	BC[2,3,4], WA[3]
	Melanotus spp.	BC[4]
	Selatosomus a. destructor	AB[3]
	Selatosomus pruininus	WA[10]

References

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* = obtained from supplementary data.

1.4. Overview of research chapters

In Chapter 2 (Research Chapter 1), I tested the effects of (*E*)-4-ethyloct-4-enoic acid (‘limoniic acid) and (*E*)-5-ethyloct-4-enoic acid (an analog of limoniic acid) as trap lures on captures of *Limonius* spp. at 27 sites across North America. Moreover, I tested the ability of six carboxylic acids – that are present in headspace volatiles of *Limonius* females and that elicit responses from male antennae – to enhance the attractiveness of limoniic acid or its analog. I show that all four *Limonius* species are attracted to limoniic acid and to the analog but not to the carboxylic acids. Adding the carboxylic acids to limoniic acid, or to the analog, reduced their attractiveness. In dose–response studies, I further show that trap lures containing either 0.4 mg or 4 mg of limoniic acid afford large captures of *L. californicus* and *L. infuscatus*. Considering that limoniic acid is very attractive to *Limonius* spp. and not deterrent to other elaterid pest species, I conclude that the development of generic pheromone-based monitoring and management of multiple click beetle species seems feasible.

In Chapter 3, I tested the hypothesis that sympatric *L. canus, L. californicus*, and *L. infuscatus* – which use limoniic acid as a sex attractant pheromone component (*L. canus, L. californicus*) or respond to it as a sex attractant (*L. infuscatus*) – maintain species-specificity of sexual communication through nonoverlapping seasonal occurrence and/or contrasting diel periodicity of sexual communication. Using capture times of beetles in pheromone-baited traps as a proxy for sexual communication periods, I show that *L. canus* and *L. californicus* have seasonally distinct communication periods, with most *L. canus* males (>90%) captured in April and most *L. californicus* males (>95%) captured in May/June/July. In two separate 24-hr trapping studies, with data recordings every hour, I captured almost exclusively *L. infuscatus* males, rendering it inconclusive as to whether the three *Limonius* congeners communicate at different times of the day. Nonetheless, I show that males of *L. infuscatus* respond to pheromone lures only during daytime hours and during the warmest period each day. As captures of male *L. infuscatus* overlap with those of male *L. canus* in April and those of male *L. californicus* in May/June, I conclude that reproductive isolating mechanisms other than seasonal separation of sexual communication periods must exist.

In Chapter 4, I investigated whether trap lures effective for multiple elaterid genera can be developed, improving cost efficiency in elaterid pest management programs. Specifically, I investigated whether the sex pheromones of sympatric *Agriotes* spp. and *Limonius* spp. can be combined in a mixed lure without reducing its attractiveness to all target species. In western Canada, I show that the pheromones of *A. lineatus* (geranyl butanoate & geranyl octanoate) and *Limonius* spp. (limoniic acid) can be combined without significantly reducing captures of male *A. lineatus*, *L. canus*, *L. californicus* and *L. infuscatus* relative to traps baited with species-specific lures for *A. lineatus* and *Limonius* spp.. Similarly, I show that the sex pheromone of *A. obscurus* (geranyl hexanoate & geranyl octanoate) and limoniic acid can be combined without significantly reducing trap captures of male *L. canus*, *L. infuscatus* and *L. californicus* but that the mixed lure reduced *A. obscurus* captures relative to traps baited only with the *A. obscurus* pheromone. In eastern Canada, I show that combining pheromones for *Agriotes mancus* (geranyl butanoate & geranyl hexanoate) and limoniic acid reduced captures of *A. mancus* but not *A. pubescens* and *A. sputator*. Based on all these data, I conclude that pheromones of select elaterid heterogeners can be combined in a 'catch-more' pheromone lure to effectively monitor for, or possibly control, multiple elaterid pests, but that such mixed lures must be evaluated for each species combination.

Chapter 2.

Limoniic acid and its analog as trap lures for pest *Limonius* species (Coleoptera: Elateridae) in North America*

*A near identical version of this chapter has been published: Willem G. van Herk, Emily Lemke, Gerhard Gries, Regine Gries, Jacqueline M. Serrano, Haley Catton, Kevin Wanner, Peter J. Landolt, W. Rodney Cooper, Scott Meers, Atoosa Nikoukar, Jocelyn L. Smith, Santosh K. Alamsetti, and Frank E. Etzler. *Journal of Economic Entomology,* 114(5), 2108-2120 (2021). https://doi.org/10.1093/jee/toab154.

WvH, GG, and EL conceived the study; WvH and EL conducted field experiments in BC; HC and SM conducted experiments in AB; JLS conducted experiments in ON; JS, PJL, and RC conducted experiments in WA and OR; KW conducted experiments in MT; AR conducted experiments in ID; RG captured headspace odorants, and analyzed odorant extract as well as model compounds by GCEAD and GC-MS; SA synthesized chemicals; EL and FE identified beetles captured in traps; WvH analyzed capture data statistically; WvH and GG wrote the first draft, and all authors reviewed and approved of the final draft.

2.1. Abstract

Four species of *Limonius* wireworms (Coleoptera: Elateridae), *L. californicus*, *L. canus*, *L. infuscatus* and *L. agonus,* are serious crop pests in North America. Limoniic acid, (*E*)-4-ethyloct-4-enoic acid, has been reported as a sex pheromone component of female *L. californicus* and *L. canus*, and a sex attractant for male *L. infuscatus*. In the same study, both limoniic acid and the analog (*E*)-5-ethyloct-4-enoic acid were highly attractive in field experiments. Moreover, six carboxylic acids in headspace volatiles of *Limonius* females elicited responses from male antennae but were not tested for behavioral activity. Here, we report trap catch data of *Limonius* spp. obtained in field experiments at 27 sites across North America. All four *Limonius* species were attracted to limoniic acid and to the analog but not to the carboxylic acids. Adding these carboxylic acids to limoniic acid, or to the analog, reduced its attractiveness. In dose–response studies, trap lures containing 0.4 mg or 4 mg of limoniic acid afforded large captures of *L. californicus* and *L. infuscatus*. Neither limoniic acid nor the analog were deterrent to other elaterid pest species. The broad attractiveness of limoniic acid to *Limonius* spp., and

its non-deterrent effect on heterogeners, may facilitate the development of generic pheromone-based monitoring and management tools for multiple click beetle species.

Key words: click beetle, wireworm, monitoring, pheromone, integrated pest management

2.2. Introduction

Synthetic sex pheromone lures of click beetles (Coleoptera: Elateridae) are powerful tools to determine the presence and relative abundance of pest species, map their distributions and assess the persistence of populations over time (Blackshaw and Vernon 2008; Tóth 2013; Traugott et al. 2015). Pheromone lures also have the potential to reduce larval populations by preventing beetle reproduction through mass trapping of males, mating disruption, or luring of beetles to entomopathogens (e.g., Vernon and van Herk 2022; Reddy and Tangtrakulwanich 2014).

Until recently, sex pheromones were known for only a few click beetle pests, most of which are native to Europe. Research in the 1950s and 1960s to identify the pheromones of click beetle pests native to North America [i.e., *Limonius californicus* (Mann.) and *L. canus* (LeConte)] was terminated when interest in click beetles waned in the 1970s and 1980s. Only recently has pheromone research on North American native click beetle species resumed. Female-produced sex pheromones are now known for some of the key pest species, including *Melanotus communis* (Gyll.) (Williams et al. 2019), *Cardiophorus tenebrosus* L. and *C. edwardsi* Horn (Serrano et al. 2018), *L. californicus* (Mannerheim) and *L. canus* LeConte (Gries et al. 2021), and *Selatosomus aeripennis destructor* (Brown) (GG and WvH, unpubl. data).

In North America, four species of *Limonius* are serious crop pests, including *L. agonus* (Say) in northeastern regions, and *L. californicus, L. canus* and *L. infuscatus* (Mots.) in western regions (Wilkinson 1963; Milosavljevic et al. 2016; Saguez et al. 2017; Andrews et al. 2020). Populations of these species are increasing, likely due to a lack of effective insecticides available to producers following the de-registration of

lindane (Vernon and van Herk 2022). Limoniic acid, (*E*)-4-ethyloct-4-enoic acid (Fig. 2.1A), was shown to be the major sex pheromone component of both female *L. canus* and female *L. californicus,* and a sex attractant for male *L. infuscatus* (Gries et al. 2021). In the same study, a pheromone analog, (*E*)-5-ethyloct-4-enoic acid, was also highly attractive but six aliphatic carboxylic acids (pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic), which occur in the headspace of *Limonius* females and elicit responses from male antennae, were not tested for pheromonal activity.

Our objectives in this study were to (1) test limoniic acid and its analog as trap lures for capturing *L. canus, L. californicus* and *L. infuscatus*; (2) assess the effect of pheromone dose on beetle captures, and (3) determine whether aliphatic fatty acids alone or in combination with either limoniic acid or the analog affect captures.

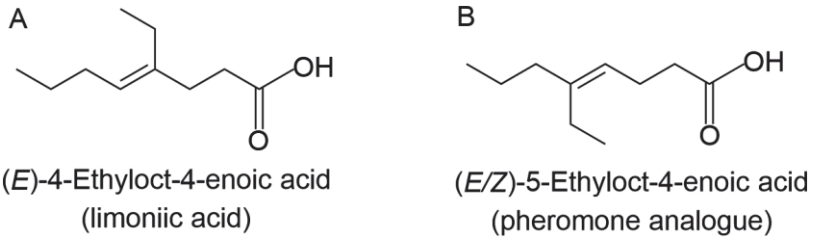

Figure 2.1. Molecular stuctures of (A) limoniic acid, a sex pheromone component of *Limonius californicus* and *L. canus*, and (B) an analog [blend (80:20) of E- and Z-isomers; the E-isomer is shown].

2.3. Materials and Methods

2.3.1. Traps and Lures

All experiments (Table 2.1) used Vernon Pitfall Traps ® (VPT, Intko Supply Ltd., Chilliwack, BC; Fig. 2.2A and B) (van Herk et al. 2018b). Test stimuli were dispensed from a cotton pellet (Richmond Dental #0, Charlotte, NC) inside a low-density-polyethylene receptacle (1 ml, diameter: 8 mm, wall thickness: 0.98 mm; Kartell Labware, Noviglio, IT) (van Herk et al. 2021a). For field deployment, baited receptacles

were removed from refrigeration (~4°C), inserted into the center of VPT trap lids, and receptacle lids were opened for pheromone release. Lures were not replaced during the course of a study. Whenever a trap was checked, its 180-ml insert cup was refilled with propylene glycol preservative fluid (100 ml) both to prevent beetle escape and to preserve captured beetles. Limoniic acid and the pheromone analog were synthesized by SKA (Simon Fraser University, Burnaby, BC), and aliphatic fatty acids were purchased from Sigma–Aldrich (Oakville, ON, Canada). The pheromone analog consisted of a blend of *E*- and *Z*-isomers (80:20).

2.3.2. Field Sites and Trap Placement

Study sites and trap deployment periods are listed in Table 2.1. Unless noted otherwise, experiments were set up using a randomized complete block design (RCBD). Traps were placed in a continuous line along grassy edges of agricultural fields, which are preferred oviposition sites of beetles (Traugott et al. 2015), with an intertrap spacing of 10 m and 20 m within and between replicates, respectively. Traps were installed such that the trap lip resided on the soil surface (Fig. 2.2B), thereby preventing beetles from crawling under traps. Studies were initiated at the time of beetle emergence and sites with beetle populations potentially depleted from previous trapping studies (van Herk and Vernon 2020) were not used.

2.3.3. Beetle Identification

Captured click beetles were identified using taxonomic keys (Johnson 2002; Etzler 2013), and an identification guide for northwestern *Limonius* species found on agricultural land (FEE, unpubl. field guide). All *Limonius* beetles were identified to species. Specimens that were damaged or had ambiguous species characteristics were identified based on genitalic morphology (Al Dhafer 2009) and advice from expert taxonomists. For studies with low beetle captures, all specimens were identified to species. For all other studies, sub- samples of 100–330 *Limonius* beetles per treatment were identified to species, using 2–9 samples per treatment, and 10–50 beetles per

sample. Voucher specimens have been retained at the Agassiz Research and Development Centre (Agassiz, BC, Canada).

2.3.4. Beetle Captures in Response to the Pheromone Analog Tested at Low and Medium Dose

Experiments 1–4 (Table 2.1) were run in 2019 in Kelowna (BC), Cawston (BC), Granum (AB), and Hermiston (OR). All experiments tested three treatments: (1, 2) the pheromone analog at 4 mg (1) or 40 mg (2), and (3) an unbaited control. Traps were placed and checked as follows: Kelowna: placed 16 April and checked 23 April and 3 May; Cawston: placed 3 May, checked 9 and 30 May; Granum: placed 3 May, checked 6, 13, 20, 27 May, and 3 and 10 June; and Hermiston: placed 16 April, checked 24 April and 1 May. Study sites in Kelowna and Cawston were located on small (3–5 ha) organic fruit and vegetable farms with a history of wireworm infestation. The Granum site was infested with *L. californicus* and had high populations of *S. a. destructor* and *Hypnoidus bicolor* (Esch.), the two predominant pest wireworm species of the Prairie Provinces (van Herk et al. 2021b). Trap captures of these beetles revealed their seasonal emergence relative to the mean daily air temperature (recorded at the Environment and Climate Change Canada weather station at Claresholm, AB; 50.003631, −113.638636). The study site at Hermiston was located on the campus of Oregon State University.

2.3.5. Beetle Captures in Response to Limoniic Acid, Its Analog, or Both

Experiments 5–16 (Table 2.1) were run in 2020 in Kelowna, Summerland (BC), Granum, Ridgetown (ON), Pasco (WA), George (WA), Hermiston, Manhattan (MT), Radersburg (MT), Moscow (ID), Lenville (ID), and Sandpoint (ID). Each experiment tested four treatments per replicate: (1) limoniic acid (4 mg), (2) pheromone analog (4 mg), (3) limoniic acid (2 mg) and analog (2 mg), and (4) an unbaited control. Traps were placed and checked on the following dates: Kelowna and Summerland: placed 11 April, checked 18 April (Kelowna only), 25 April and 2 May; Granum: placed 3 May, checked 10 May; Ridgetown: placed 30 April, checked 7, 14, 21, 28 May, and 5 June; Pasco and

George: placed 23 April, checked 22, 29 April, 6 May (Pasco), and 30 April, 7 May (George); Hermiston: placed 15 April, checked 22, 29 April, and 6 May. Traps in Kelowna and Summerland were placed in uniform grassy strips between rows of fruit trees and along field perimeters. Traps in Ridgetown were placed along field borders of the Ridgetown campus of the University of Guelph, to determine the response of *L. agonus* to limoniic acid.

Experiments 11–12 in Montana (Manhattan, Radersburg) followed a complete randomized design with all traps (8 per treatment) placed 10 m apart in a single line; a 9th trap was included for limoniic acid in Manhattan. Traps were placed and checked the following dates: Manhattan: placed 14 May, checked 27 May and 12 June; Radersburg: placed 16 May, checked 20 and 27 May.

Experiments 13–15 in Idaho (Moscow, Lenville, Sandpoint) were replicated in time and consisted of one trap per treatment. Traps were placed and checked on the following dates: Moscow: placed 10 April, checked 17 April, 1 May, 12 June, and 24 July; Lenville: placed 15 April, checked 20 April, 7 and 21 May, and 3 June; and Sandpoint: placed 21 April, checked 29 April, 5 and 19 May, 5 and 18 June, and 8 July. On each date, the four traps deployed in these studies were relocated to sites that had not previously been used for beetle trapping.

2.3.6. Beetle Captures in Response to Aliphatic Fatty Acids, the Pheromone Analog, or Both

Experiments 17–19 (Table 2.1) were conducted in 2019 in Kelowna, Granum, and at the USDA-ARS experimental farm near Moxee (WA). Each experiment tested four treatments per replicate: (1) a blend (4 mg) of aliphatic fatty acids (hexanoic, heptanoic, octanoic, nonanoic, decanoic acid; 0.8 mg each), (2) the pheromone analog (4 mg), (3) a binary blend of the aliphatic fatty acids (2 mg) and the analog (2 mg), and (4) an unbaited control. Traps were placed and checked on the following dates: Kelowna: placed 3 May, checked 12, 17, and 30 May; Granum: placed 10 June, checked 17 and 24 June, and 3, 10, 17, and 29 July; and Moxee: placed 5 May, checked 12, 19 and 24 May.

2.3.7. Beetle Captures in Response to Aliphatic Fatty Acids, Limoniic Acid, or Both

Experiments 20–23 (Table 2.1) were conducted in 2020 in Kelowna, Granum, George, and Radersburg. All experiments tested four treatments in each replicate: (1) a blend of the aliphatic fatty acids (as above) (4 mg), (2) limoniic acid (4 mg), (3) a binary blend of the aliphatic fatty acids (2 mg) and limoniic acid (2 mg), and (4) an unbaited control. Traps were placed and checked on the following dates: Kelowna: placed 6 May, checked 16 May; Granum: placed 10 May, checked 17 and 24 May; George: placed 8 May checked 15, 22, and 29 May; and Radersburg: placed 16 May, checked 20 May and 5 June.

2.3.8. Beetle Captures in Response to Four Doses of Limoniic Acid

Experiments 24–26 (Table 2.1) were conducted in 2020 in Kelowna, Granum, and George. All experiments tested five treatments in each replicate: (1–4) limoniic acid at 0.04, 0.4, 4, and 40 mg, and (5) an unbaited control. Traps were placed and checked on the following dates: Kelowna: placed 25 April, checked 2 and 6 May; Granum: placed 24 May, checked 30 May and 7 June; and George: placed 8 May, checked 15, 21, and 29 May.

2.3.9. Additional Trapping for (Non-Pest) *Limonius* spp.

Upon incidental observations that analog-baited traps captured non-pest *Limonius* spp., five analog-baited traps and corresponding control traps were deployed in the Ahtanum State Forest along the South Fork of Ahtanum Creek (46.505833, −120.926389) near Tampico (WA), to determine their attractiveness to *Gambrinus seminudus* Van Dyke, a species inhabiting riparian zones of oak groves and until recently thought to be a *Limonius* congener (Etzler 2019). Traps were placed on 20 June and checked on 27 June and 2 July 2019.

2.4. Statistical Analyses

The relative proportion of the three *Limonius* species per treatment was compared using generalized linear models with a binomial distribution and a logit link function (Proc GENMOD, SAS 9.2, SAS Institute, Cary, NC). Where the proportion of each species in subsamples did not differ significantly between treatments (Table 2.2), the mean proportion for all samples in the study was used to calculate the number of beetles collected per species per trap. The mean proportion for all samples was also used to estimate proportions for the control and other treatments with <10 beetles per trap. Where significant differences in the proportion of species were observed between treatments, beetle numbers were calculated per treatment using mean ratios for each treatment. Differences between treatments in the actual or interpolated number of beetles collected were then analyzed with generalized linear models using a negative binomial distribution and a log link function. No analyses were conducted for species that comprised a low proportion (i.e., <0.1) of the total collected in the study. All analyses for RCBD studies included a factor for treatment replicate and used the total number of beetles collected over the course of the study (collection dates combined). Studies conducted in Idaho, which were moved to different locations at each collection date, were considered to be replicated over time, and collection date was included as a factor. Due to differences in the timing and duration of the trapping periods at study sites (Table 2.1), direct between-site comparisons for similar studies were not performed.

2.5. Results

2.5.1. General Observations

In 14 out of 25 studies run on farmland in western Canada and the USA, at least two of the three target *Limonius* pest species were collected, with all three species co-occurring in high numbers in some locations (e.g., Cawston, Kelowna; Tables 2.1 and 2.2). Species ratios per treatment differed significantly in (1) the pheromone analog studies in Kelowna (Exp. 4: *L. canus*, *L. infuscatus*: $\chi 2 = 16.16$, df = 1, $P < 0.0001$; Exp.

17: *L. californicus:* $\chi 2 = 41.33$, df = 1, $P < 0.0001$; *L. canus*: $\chi 2 = 37.14$, df = 1, $P < 0.0001$; *L. infuscatus*: $\chi 2 = 92.40$, df = 1, $P < 0.0001$), (2) the limoniic acid vs. analog studies in (i) Kelowna (Exp. 9: *L. californicus*: $\chi 2 = 4.41$, df = 2, $P < 0.0001$; *L. canus*: $\chi 2 = 122.13$, df = 2, $P < 0.0001$; *L. infuscatus*: $\chi 2 = 115.79$, df = 2, $P < 0.0001$), (ii) Lenville (Exp. 13: *L. californicus, L. infuscatus:* $\chi 2 = 27.84$, df = 2, $P < 0.0001$), (iii) Moscow (Exp. 14: *L. californicus, L. infuscatus*: $\chi 2 = 6.65$, df = 2, $P = 0.036$), and (3) the lure-load study in Kelowna (Exp. 26: *L. californicus*: $\chi 2 = 13.76$, df = 3, $P = 0.0033$; *L. canus*: $\chi 2 = 3.08$, df = 3, $P = 0.38$; *L. infuscatus*: $\chi 2 = 15.41$, df = 3, $P = 0.0015$; Table 2.2). For these studies, beetle numbers of each species collected were calculated using treatment specific ratios (Table 2.2), and the mean proportion of all samples in the study was used to calculate the number of beetles collected per species in the control and in other treatments with few beetles (Table 2.2). Data for species that occurred in low numbers, or that comprised less than 10% of the total collected in a study, are not reported.

In all limoniic acid and analog studies conducted in 2019 and 2020, *Limonius* beetles were collected in large numbers during the first weeks that traps were deployed (i.e., mid-April in BC, WA, OR; first week of May in AB). Captures of *L. californicus* in Alberta peaked when the mean daily temperature reached ~10°C, and diminished rapidly thereafter (Fig. 2.3). Based on these data, *L. californicus* emerges as early as *Agriotes obscurus* L., which appears between mid-April and early-May in southwestern BC, with both pest species co-inhabiting many locations in southern BC (van Herk 2021c). The ~1-month seasonal occurrence of *Limonius* spp. is considerably shorter than the 3-months occurrence of *A. obscurus*. There is some indication that *L. canus* may emerge before *L. californicus* in areas inhabited by both. According to trapping data in Kelowna, *L. californicus* was absent in April, and fewer *L. canus* than *L. infuscatus* were present in May than in April (Table 2.1).

Two studies conducted in Granum (2019) afforded large captures of male and female *S. a. destructor*. In the first study, weekly checks of 36 traps from 6 May to 10 June (Fig. 2.3) collected 0, 40, 34, 54, 190, and 210 *S. a. destructor*, with relatively similar numbers in analog-baited and unbaited control traps (total captures: 157, 134 and 109 beetles, in control, low-, and medium-dose treatments, respectively, of which 73, 64

and 57% were males). In the second study, weekly checks of 60 traps between 17 June and 29 July, collected 651, 255, 234, 75, 94, and 52 *S. a. destructor*, again with similar numbers of beetles (range: 169–254) and sex ratios (range of proportion males: 0.24–0.37) per treatment. In addition, >400 specimens of *H. bicolor*, a predominantly parthenogenetic species, were collected in the second study, also with similar beetle numbers per treatment (range: 60–139). Captures of both *S. a. destructor* and *H. bicolor* peaked around mid-June, but almost no *L. californicus* were captured after 27 May (Fig. 2.3).

2.5.2. Beetle Captures in Response to the Pheromone Analog at Low and Medium Doses

Irrespective of lure dose, the analog was highly attractive to all three *Limonius* species. Over the study period, traps baited with low and medium lure doses of the analog (4 mg vs. 40 mg) afforded comparable mean total captures of *L. californicus* in Cawston (21.0 vs. 28.6) and Granum (1,210.7 vs. 1,113.0) (Fig. 2.4A and C), of *L. canus* in Cawston (22.6 vs. 30.8), Hermiston (147.8 vs. 152.4) and Kelowna (162.3 vs. 98.5) (Fig. 2.4B, D, and E), and of *L. infuscatus* in Kelowna (82.1 vs. 98.8) (Fig. 2.4F). Lure dose had no statistical effect on captures in all locations except Kelowna, where 4-mg lures attracted more *L. canus* and fewer *L. infuscatus* than 40-mg lures (Fig. 2.4E and F).

2.5.3. Beetle Captures in Response to Limoniic Acid, Its Analog, or Both

In most trapping locations, limoniic acid as a trap lure afforded larger captures of *Limonius* spp. than its analog (Fig. 2.5), except in Kelowna where *L. canus* was more strongly attracted to the analog (Fig. 2.5E). Compared to analog-baited traps, pheromone-baited traps captured 7.1×, 11.4×, 3.7×, and 3.2× more *L. californicus* in George, Granum, Manhattan, and Radersburg, respectively (Fig. 2.5A, B, G, and H), 2.6× more *L. canus* in Hermiston (Fig. 2.5C), and 7.0× more *L. infuscatus* in Kelowna (Fig. 2.5C). For the time-replicated studies conducted in Idaho (Fig. 2.6), this trend was evident only for *L. infuscatus* (Fig. 2.6B and D), and statistically significant only in Sandpoint (Fig. 2.6D)

(99.4× more beetles in pheromone-baited traps). Adding the analog to limoniic acid reduced captures of *L. infuscatus* 3.3× in Summerland (Fig. 2.5D), and of *L. californicus* 1.2× in George (Fig. 2.5A), 1.7× in Granum (Fig. 2.5B), 2.4× in Manhatten (Fig. 2.5G), and 1.9× in Radersburg (Fig. 2.5H). The reduction in captures was statistically significant only in George and Granum. Conversely, the same two-component blend afforded higher captures of *L. canus* in Hermiston (1.3×; Fig. 2.5C) and Kelowna (2.6×; Fig. 2.5E), and similar captures of *L. infuscatus* in Kelowna (0.9×; Fig. 2.5F). In the time-replicated studies in Idaho (Fig. 2.6), limoniic acid alone or in combination with the analog was statistically equally attractive to *L. californicus* and *L. infuscatus* in Lenville and Sandpoint, respectively (Fig. 2.6A and D), whereas limoniic acid alone was more attractive to *L. infuscatus* in Lenville (Fig. 2.6B), and the combination more attractive to *L. infuscatus* in Moscow (Fig. 2.6C).

2.5.4. Beetle Captures in Response to Aliphatic Fatty Acids, the Pheromone Analog, or Both

To all three *Limonius* species, aliphatic fatty acids were as unattractive as unbaited controls (Fig. 2.7). Adding aliphatic fatty acids to the analog reduced its attractiveness to *L. californicus* 2.6× (Kelowna) and 4.3× (Moxee) (Fig. 2.7A and D), and to *L. infuscatus* 19.0× (Kelowna) and 4.7× (Moxee) (Fig. 2.7C and E). In contrast, the effect was reversed (but statistically not significant) in Kelowna in 2019, where traps baited with the fatty acid/ analog blend captured 4.3× more *L. canus* than traps baited with the analog alone (Fig. 2.7B).

2.5.5. Beetle Captures in Response to Aliphatic Fatty Acids, Limoniic Acid, or Both

As in previous experiments (Fig. 2.7), traps baited with aliphatic fatty acids captured no more males than unbaited controls (Fig. 2.8). When aliphatic fatty acids were added to limoniic acid, attractiveness to *L. californicus* was reduced 1.4× (George), 3.1× (Granum) and 5.1× (Radersburg) (Fig. 2.8A, B and D), and to *L. infuscatus* by 10.0×

(Kelowna) (Fig. 2.8C). These reductions in beetle captures were statistically significant except for the study in George (Fig. 2.8A).

2.5.6. Beetle Captures in Response to Four Doses of Limoniic Acid

In all three study sites, each of the four lure doses tested (0.04, 0.4, 4.0, or 40 mg) attracted more *Limonius* males than unbaited controls (Fig. 2.9). Only in George did increasingly higher lure doses produce progressively larger beetle captures, with similar captures of *L. californicus* in traps baited with a 4- or 40-mg lure, significantly fewer captures in traps baited with a 0.4-mg lure, and significantly fewer captures again in traps with a 0.04-mg lure (Fig. 2.9A).

2.5.7. Bycatches of Related Species

In Ahtanum State Forest (Tampico), several *G. seminudus* males [mean: 2.20 (SEM: 0.20)] were captured in each of the analog-baited traps, but none in unbaited control traps. In southern Ontario, significantly more *L. agonus* males were captured in traps baited with the analog [mean total capture over trapping period: 3.4 (SEM: 0.60)], or the blend of analog and limoniic acid [3.0 (2.04)], than in unbaited control traps [0.13 (0.13)] ($\chi 2 = 9.41$, df = 3, $P = 0.024$), with captures in limoniic acid-baited traps [1.6 (0.60)] statistically not different from those in control traps. These data are complemented by electrophysiological recordings, showing that antennae of male *G. seminudus* and male *L. agonus* strongly respond to the analog (limoniic acid was not tested; RG, unpubl. data). In Granum (2020), a few *G. ursinus* (Van Dyke) – another species previously considered a *Limonius* congener (Etzler 2019) – were also collected in limoniic acid-baited traps, suggesting that both *Limonius* and *Gambrinus* spp. may be attracted to this compound.

2.6. Discussion

This comprehensive field study has considerably advanced our current understanding of the sexual communication systems of *L. californicus*, *L. canus* and *L.*

infuscatus, and has revealed the utility of limoniic acid and its analog for monitoring *Limonius* click beetle populations. Below, we discuss the main findings of our study, their implications for click beetle management, and future research needs.

1. As all three pest *Limonius* species frequently co-occur in farmland in western North America (Andrews et al. 2008; Milosavljević et al. 2017; van Herk et al. 2021a), a tactic for monitoring these species at any site is needed. Development of this tactic should address compatibility (or not) of pheromone lures and seasonal activity periods of all species in all their geographic locations, as well as interactions between species, and differences in damage potential and response to conventional management approaches (e.g., insecticides) (Vernon and van Herk 2022; Milosavljević et al. 2017).

2. In western Canada and the Pacific Northwest, *L. californicus, L. canus* and *L. infuscatus* swarm earlier (April–early May), and for a shorter period (approx. 4–6 wk), than co-occurring pest species such as *S. a. destructor*, *H. bicolor*, and *Aeolus mellillus* Say, which are active for >8 wk (van Herk et al. 2021b). Hence, traps for monitoring populations and for pheromone-based mass trapping should be deployed early in the season, possibly as early as the beginning of March for species with southern distribution ranges (e.g., California), where the trapping season might also be short. In field sites co-inhabited by several *Limonius* congeners, *L. canus* and *L. infuscatus* appear to emerge earlier than *L. californicus* (cf. relative species ratios from the Kelowna studies, Table 2.2), complementing findings that larvae of *L. californicus* seem to be active later in the season than those of *L. infuscatus* (Milosavljević et al. 2017). Further studies in various geographic ranges need to substantiate the seasonal occurrence of *Limonius* congeners.

3. Both limoniic acid and its analog are highly attractive to all three *Limonius* species. However, the pheromone is more attractive than the analog in nearly every study, except for *L. canus* in Kelowna, and possibly *L. infuscatus* in Moscow. The differential attractiveness of these compounds was most and least pronounced in studies with *L. californicus* and *L. canus,* respectively, suggesting that *L. canus* is less specific in its response than *L. californicus*. If true, this would corroborate previous reports (Lilly

and McGinnis 1968) that male *L. canus* responded to extracts of female *L. californicus*, but that male *L. californicus* did not respond to extracts of female *L. canus*.

4. Both limoniic acid and its analog are attractive to the closely related pest species *L. agonus* and to the non-pests *Gambrinus seminudus* and *G. ursinus*, raising the question as to how all these species are able to maintain their specificity of sexual communication, particularly as *L. californicus* and *L. canus* appear not to produce additional pheromone components (RG, unpubl. data). However, the attractiveness of limoniic acid to all three western pest species (*L. californicus, L. canus*, and *L. infuscatus*) may facilitate the development of generic pheromone-based monitoring and management tools effective for all species. Such monitoring tools should consider that catch rates of these three species may vary due to differences in beetle activity levels, as has been observed for *A. obscurus*, *A. lineatus* L., and *A. sputator* L. (Hicks and Blackshaw 2008), and that the presence of some species in specific collections may be of greater economic importance than others.

5. The carboxylic acids tested in our study were not attractive on their own, and when added to limoniic acid or its analog reduced their attractiveness, except for studies with *L. canus* in Kelowna and Hermiston, and possibly *L. infuscatus* in Moscow and Sandpoint. This reduction in lure effectiveness could not be attributed to a 2× reduction (2 mg vs. 4 mg) of limoniic acid or its analog, as evidenced in the dose–response studies where 0.4-mg and 4.0-mg trap lures afforded similar beetle captures. It seems possible, however, that only one or two of the carboxylic acids are pheromone components and that their pheromonal activity is linked to release rate. This inference is based on observations that the amount of pentanoic acid varies with the mating status of *L. californicus* females, being most abundant in virgin females and decreasing quickly in mated females (Lilly and McGinnis 1968). It is conceivable then, that limoniic acid functions as the major sex pheromone component of *Limonius* congeners, and that the particular ratio and/or emission rate of some carboxylic acids impart specificity to the sexual communication system of each congener.

6. Even at the low 0.4-mg dose, both limoniic acid and its analog are highly attractive to *Limonius* spp., and comparably effective as 40-mg pheromone lures for *A.*

obscurus, A. lineatus, and *A. sputator* (van Herk et al. 2021a). These lure effectiveness data corroborate findings that *L. californicus* males exhibited strong sexual responses to virgin female extracts at only 0.008 female equivalents (Lilly and McGinnis 1968). With this level of pheromonal potency, synthetic limoniic acid should be explored for the management of *Limonius* populations, and 0.4–4 mg may be a sufficient dose for monitoring beetle populations.

7. As a trap lure, limoniic acid or its analog does not prevent or reduce captures of other click beetle taxa such as *S. a. destructor, H. bicolor, Aeolus mellillus* and *Agriotes lineatus* that co-occur with *Limonius* spp. (data above, and WvH pers. obs.). In contrast, some sympatric *Agriotes* spp. are repelled by pheromone components of congeners. For example, females of *A. obscurus* and *A. lineatus* produce a two-component pheromone blend with geranyl octanoate as a shared component, and geranyl hexanoate and geranyl butanoate, respectively, as species-specific components (Tóth et al. 2003). Combining these three esters in a single trap lure with the intention to capture males of both species slightly increased lure attractiveness to *A. lineatus* males, but reduced lure attractiveness to *A. obscurus* males 5× relative to the *A. obscurus* specific lure (Vernon et al. 2014). While it may not be possible to combine pheromones of several *Agriotes* spp. in a single pheromone lure, ongoing studies reveal that pheromones of distantly related taxa (e.g., *A. lineatus, L. canus*) can be combined without adversely affecting lure attractiveness to males of either species (EL and WvH, unpubl. data). This may permit the development of monitoring, mass trapping or mating disruption tactics that target co-occurring and generally similar pest species such as *A. lineatus* and *Limonius* spp. in southern BC.

8. In all field experiments, the lid of pheromone receptacles (Fig. 2.2) was opened prior to baiting traps, but concurrent studies in 2020 (data not shown) indicate that even closed pheromone receptacles are highly effective, as also shown for e.g., *A. obscurus, A. lineatus*, and *A. sputator* (van Herk et al. 2018b; van Herk and Vernon 2020). Keeping the pheromone receptacle closed could potentially increase the longevity of lures in the field. Both closed and opened lures were highly effective immediately after preparation and deployment and did not require a conditioning period. In some trials (e.g., Kelowna, 2019), beetles were captured within 1 h of lure deployment, even though

these lures had been cold stored (~4°C) for extended periods of time (WvH, pers. obs.). In this regard, *Limonius* lures differ from *Agriotes* lures (*A. obscurus, A. lineatus, A. sputator*) which – after lure preparation – require aging at room temperature for 2–3 wk prior to deployment in the field (van Herk et al. 2021a).

2.6.1. Future Directions and Research Needs

Development of effective pheromone-based monitoring and management programs for *L. californicus, L. canus*, and *L. infuscatus* requires a sound understanding of their life histories, which currently is lacking. For each of these three species and their entire distribution range, we need to determine the length of the larval stage, the annual activity periods of larvae, the time larvae pupate and adult beetles emerge, and the duration of the beetles' swarming period. The obtained information will then inform decisions as to when pheromone-baited traps should be deployed, whether lures need to be exchanged to remain effective during the swarming periods, and how to test for potential correlations between larval populations, beetle captures, and crop damage.

As multiple species are attracted to limoniic acid, further research is needed to determine the mechanisms that impart species-specificity of sexual communication. For control tactics such as mating disruption, it would be important to know whether females exhibit diel periodicity of pheromone release ('calling behavior'), sense their own pheromone, mate once or multiple times, and cease pheromone emission after mating. It would be equally important to know whether the males' responsiveness to sex pheromone is confined to the calling period of females. If calling periods were known, synthetic pheromone could be dispensed through metered timed-release systems only during calling periods, thus accruing savings for pheromone purchase. Pest insects with polyandrous mating systems are typically more difficult to control through pheromone-based mating disruption than pest insects with monogamous mating systems (Waterworth et al. 2011; Lee et al. 2014) but little is known about the mating systems of *Limonius* spp. other than that male *L. canus* may be able to mate more than once and that unmated conspecific females produce infertile eggs (Woodworth 1942). Reports that sexual responses of *L. californicus* males declined and eventually ceased after repetitive

exposure to abdomen extracts of females (Lilly and McGinnis 1968) imply that broadcast field applications of synthetic pheromone have the potential to disorient mate-seeking males.

Reports that *L. californicus* males were attracted to abdomen extracts of virgin (but not gravid) females over a distance of >12 m (Lilly 1959) reveals information on the active range over which females attract males. Knowledge of the active range of limoniic acid-baited traps would inform decisions on trap spacing for mass trapping males, a tactic that curtails mating opportunities for females.

Table 2.1. Locations and trap deployment periods of field experiments (Exps.) conducted to test the effect of pheromonal limoniic acid [(*E*)-4-ethyloct-4-enoic acid], a pheromone analog [(*E*)-5-ethyloct-4-enoic acid], and a blend of aliphatic fatty acids (all presented singly or in combinations as trap lures) on captures of three pest *Limonius* species in western Canada and the United States

Exp. #	Study location	Latitude, Longitude	Trapping period (days)	Traps (*n*)	Total no. beetles
Pheromone analog: 2019					
1	Cawston, BC	49.171714, −119.742517	3 May–30 May (27)	36 (*12*)	1,461
2	Granum, AB	49.903135, −113.560967	3 May–10 June (38)	36 (*12*)	27,897
3	Hermiston, OR	45.820681, −119.285069	16 April–1 May (15)	36 (*12*)	3,604
4	Kelowna, BC	49.822189, −119.437123	16 April–3 May (17)	36 (*12*)	5,307
Limoniic acid and analog: 2020					
5	George, WA	47.062288, −119.859185	23 April–7 May (14)	32 (*8*)	4,672
6	Granum, AB	49.903135, −113.560967	3–10 May (7)	48 (*12*)	16,210
7	Hermiston, OR	45.845192, −111.419939	15 April–6 May (21)	32 (*8*)	844
8	Summerland, BC	49.567699, −119.689813	11 April–2 May (21)	32 (*8*)	152
9	Kelowna, BC	49.822189, −119.437123	11–25 April (14)	32 (*8*)	8,321
10	Pasco, WA	46.221979, −119.071012	15 April–6 May (21)	32 (*8*)	15
11	Manhatten, MT	45.845192, −111.419939	14 May–12 June (29)	33	1,671
12	Radersburg, MT	46.109794, −111.597242	16 May–27 June (42)	32	4,758
13	Lenville, ID [a]	46.688744, −116.873974	15 April–3 June (18)	4 (*4*)	10,308
14	Moscow, ID [a]	46.807387, −117.008715	10 April–24 July (105)	4 (*4*)	621
15	Sandpoint, ID [a]	48.369222, −116.401278	21 April–8 July (78)	4 (6)	10,706
16	Ridgetown, ON	42.447288, −81.889115	30 April–5 June (36)	32 (*8*)	65
Analog and fatty acids: 2019					
17	Kelowna, BC	49.822189, −119.437123	3–30 May (27)	48 (*12*)	4,813
18	Granum, AB	49.903135, −113.560967	10 June–29 July (59)	60 (*12*)	16
19	Moxee, WA	46.500222, −120.171056	5–24 May (19)	48 (*12*)	202
Limoniic acid and fatty acids: 2020					
20	George, WA	47.059584, −119.861112	8–29 May (21)	32 (*8*)	2,844
21	Granum, AB	49.903135, −113.560967	10–24 May (14)	48 (*12*)	43,308
22	Kelowna, BC	49.822189, −119.437123	6–16 May (10)	32 (*8*)	1,959
23	Radersburg, MT	46.109794, −111.597242	16 May–5 June (20)	32 (*8*)	2,007
Dose of limoniic acid: 2020					
24	George, WA	47.062288, −119.859185	8–29 May (21)	40 (*8*)	1,469
25	Granum, AB	49.903135, −113.560967	24 May–7 June (14)	40 (*8*)	7,216
26	Kelowna, BC	49.822189, −119.437123	25 April–6 May (11)	40 (*8*)	3,637

Shown are also the numbers of traps and replicates (*n*) used in each experiment, and the total number of *Limonius* beetles captured. [a] Treatments were replicated over time.

Table 2.2. Mean proportions of *Limonius californicus, L. canus* and *L. infuscatus* captured overall in each of field experiments (Exp.) 1-26.

Exp. #	Study location	Mean (SE) proportion of beetles captured			Figures
		L. californicus	*L. canus*	*L. infuscatus*	
Pheromone analog: 2019					
1	Cawston, BC	0.41 (0.204)*	0.44 (0.153)*	0.15 (0.150)	2.4A, B
2	Granum, AB	1.0 (0.0)	0	0	2.4C
3	Hermiston, OR	0	1.0 (0.0)*	0	2.4D
4	Kelowna, BC	0	0.57 (0.055)*	0.43 (0.055)*	2.4E, F
Limoniic acid and analog: 2020					
5	George, WA	0.98 (0.004)*	0.02 (0.004)	0	2.5A
6	Granum, AB	1.0 (0.0)*	0	0	2.5B
7	Hermiston, OR	0	1.0 (0.0)*	0	2.5C
8	Summerland, BC	0	0	1.0 (0.0)*	2.5D
9	Kelowna, BC	0.002 (0.001)	0.144 (0.045)*	0.854 (0.044)*	2.5E, F
10	Pasco, WA	0.07	0.93	0	
11	Manhatten, MT	0.93 (0.023)*	0	0.07 (0.023)	2.5G
12	Radersburg, MT	0.98 (0.007)*	0	0.02 (0.007)	2.5H
13	Lenville, ID [a]	0.82 (0.037)*	0	0.18 (0.037)*	2.6A, B
14	Moscow, ID [a]	0.12 (0.056)	0	0.88 (0.056)*	2.6C
15	Sandpoint, ID [a]	0	0	1.0 (0.0)*	2.6D
16	Ridgetown, ON	0	0	0	
Analog and fatty acids: 2019					
17	Kelowna, BC	0.29 (0.109)*	0.12 (0.073)*	0.59 (0.121)*	2.7A, B, C
18	Granum, AB	1.0	0	0	
19	Moxee, WA	0.43 (0.175)*	0.08 (0.083)	0.49 (0.092)*	2.7D, E
Limoniic acid and fatty acids: 2020					
20	George, WA	1.0 (0.0)*	0	0	2.8A
21	Granum, AB	1.0 (0.0)*	0	0	2.8B
22	Kelowna, BC	0.029 (0.009)	0.002 (0.002)	0.969 (0.009)*	2.8C
23	Radersburg, MT	0.99 (0.011)*	0	0.01 (0.011)	2.8D
Dose of limoniic acid: 2020					
24	George, WA	1.0 (0.0)*	0	0	2.9A
25	Granum, AB	1.0 (0.0)*	0	0	2.9B
26	Kelowna, BC	0.08 (0.023)	0.02 (0.021)	0.89 (0.037)*	2.9C

Figure 2.2. (A, B) Photographic illustrations of a Vernon Pitfall Trap ® [(A) trap parts: 1-trap bottom, 2-trap lid, 3-180 ml insert cup, 4-pheromone receptacle; (B) a trap bottom installed in the field for testing limoniic acid or its analog in field experiments; and (C) a representative example of *Limonius californicus* males captured in a single trap over the course of one week during the beetles' peak swarming period in Granum, Alberta. Photo credit (B): David Shack

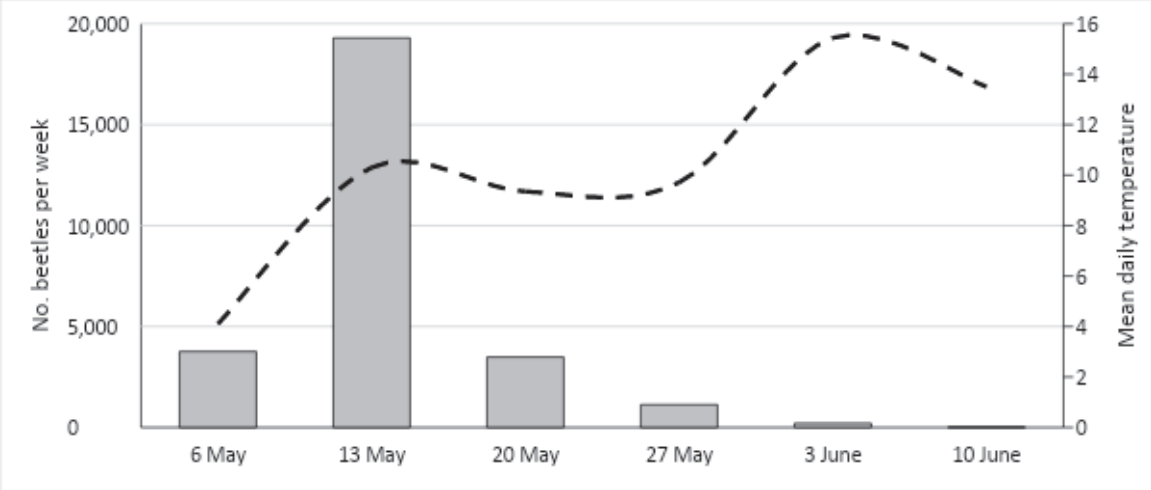

Figure 2.3. Mean daily temperature (°C) and mean (SE) weekly captures of *Limonius californicus* males in traps baited with the pheromone analog (*E*)- 5-ethyloct-4-enoic acid; Granum (Alberta), 2019.

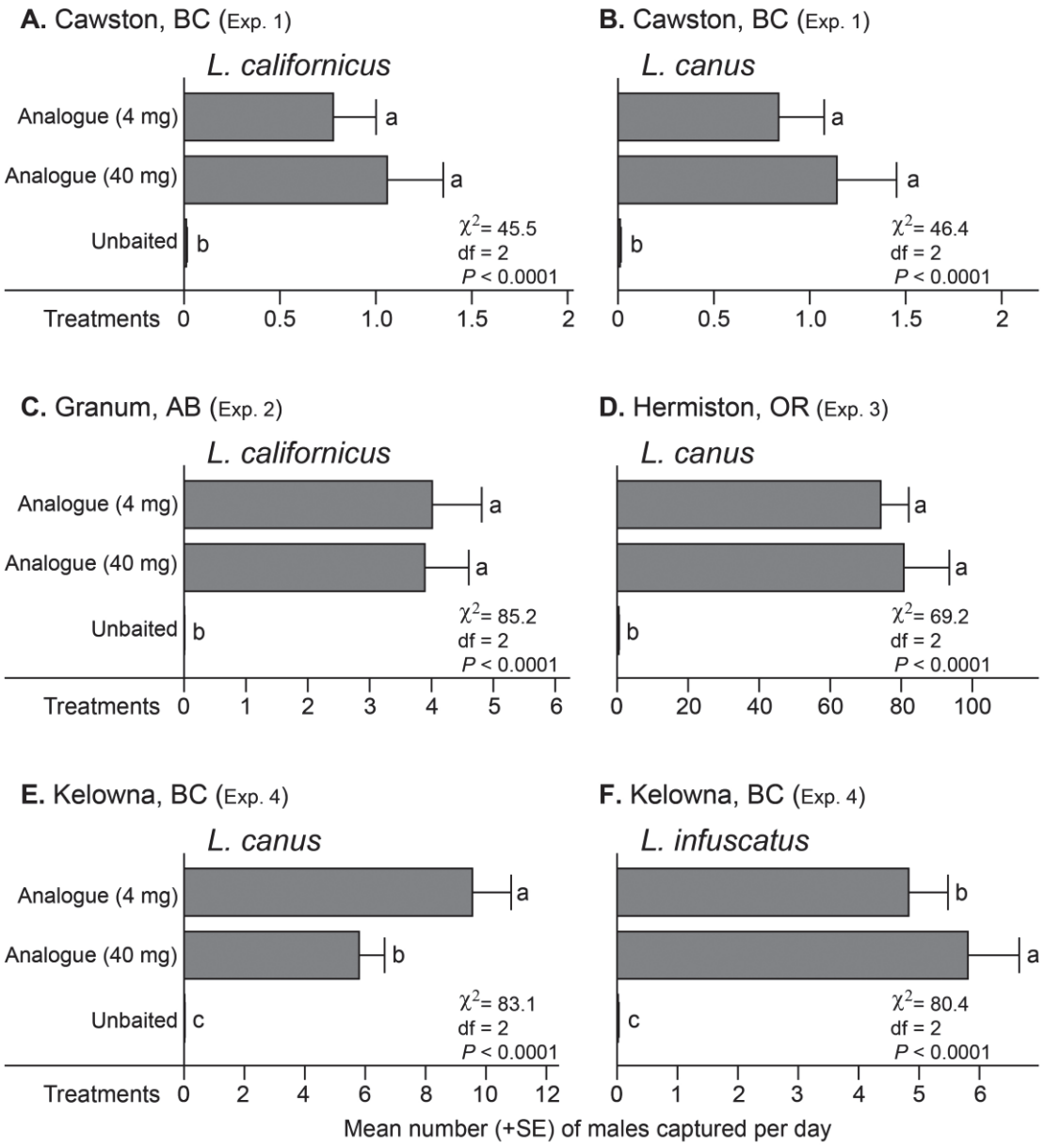

Figure 2.4. Mean (+SE) captures of three *Limonius* spp. in traps baited with the pheromone analog (*E*)-5-ethyloct-4-enoic acid at 4 mg (low dose) and 40 mg (medium dose). For each of subpanels A-F, bars with different letters indicate statistically significant differences in trap captures (χ^2 tests).

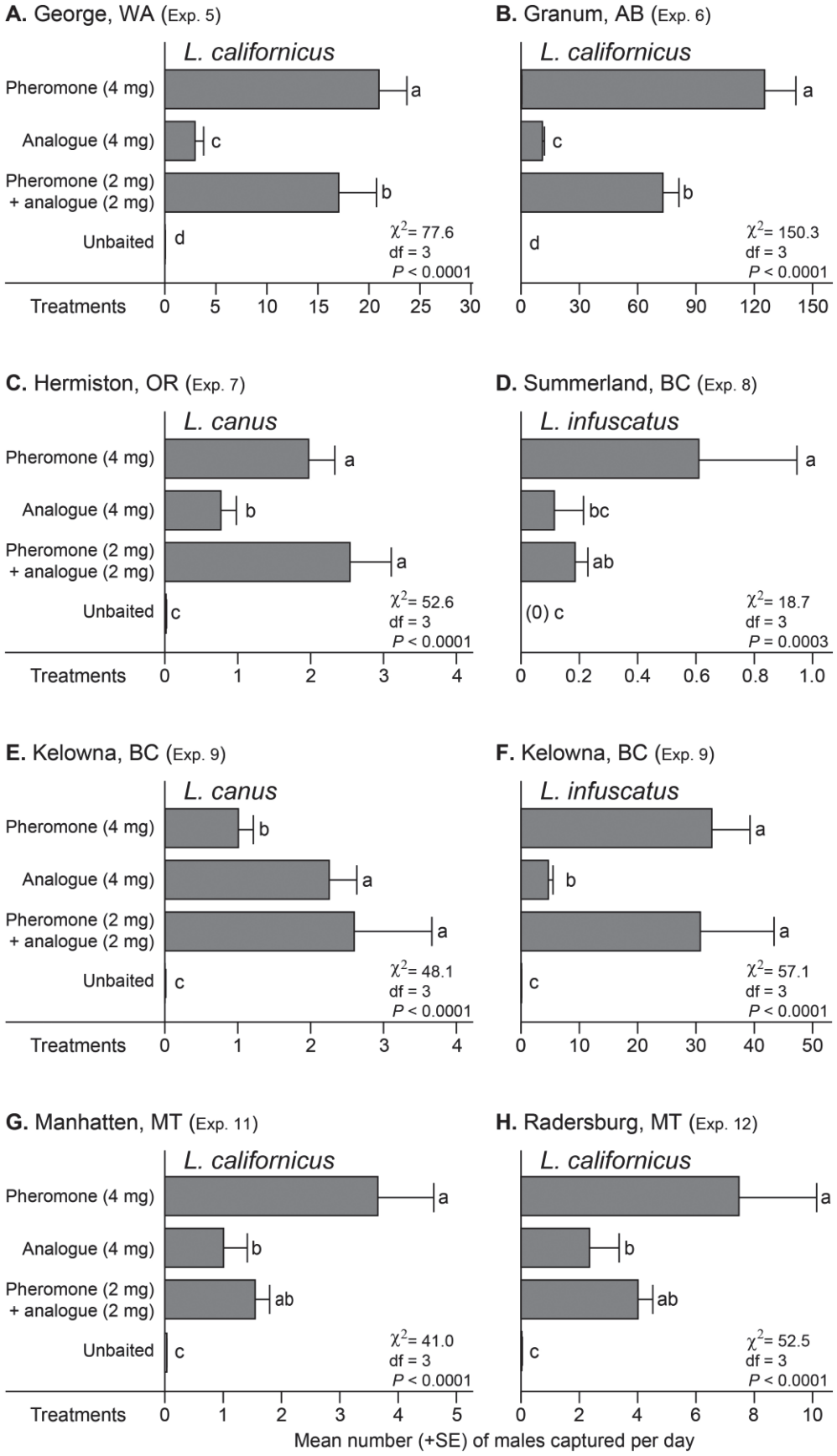
A. George, WA (Exp. 5)
L. californicus
Pheromone (4 mg)
Analogue (4 mg)
Pheromone (2 mg) + analogue (2 mg)
Unbaited
a
c
b
d
χ2= 77.6
df = 3
P < 0.0001
Treatments
0 5 10 15 20 25 30
B. Granum, AB (Exp. 6)
L. californicus
a
c
b
d
χ2= 150.3
df = 3
P < 0.0001
0 30 60 90 120 150
C. Hermiston, OR (Exp. 7)
L. canus
Pheromone (4 mg)
Analogue (4 mg)
Pheromone (2 mg) + analogue (2 mg)
Unbaited
a
b
a
c
χ2= 52.6
df = 3
P < 0.0001
Treatments
0 1 2 3 4
D. Summerland, BC (Exp. 8)
L. infuscatus
a
bc
ab
(0) c
χ2= 18.7
df = 3
P = 0.0003
0 0.2 0.4 0.6 0.8 1.0
E. Kelowna, BC (Exp. 9)
L. canus
Pheromone (4 mg)
Analogue (4 mg)
Pheromone (2 mg) + analogue (2 mg)
Unbaited
b
a
a
c
χ2= 48.1
df = 3
P < 0.0001
Treatments
0 1 2 3 4
F. Kelowna, BC (Exp. 9)
L. infuscatus
a
b
a
c
χ2= 57.1
df = 3
P < 0.0001
0 10 20 30 40 50
G. Manhatten, MT (Exp. 11)
L. californicus
Pheromone (4 mg)
Analogue (4 mg)
Pheromone (2 mg) + analogue (2 mg)
Unbaited
a
b
ab
c
χ2= 41.0
df = 3
P < 0.0001
Treatments
0 1 2 3 4 5
H. Radersburg, MT (Exp. 12)
L. californicus
a
b
ab
c
χ2= 52.5
df = 3
P < 0.0001
0 2 4 6 8 10
Mean number (+SE) of males captured per day

Figure 2.5. Mean (+SE) captures of three *Limonius* spp. in traps baited with the pheromone component limoniic acid [(*E*)-4-ethyloct-4-enoic acid; 4 mg], its analog [(*E*)-5-ethyloct-4-enoic acid; 4mg], or both (2 mg each). For each of subpanels A-H, bars with different letters indicate statistically significant differences in trap captures (χ^2 tests); note: captures of only 15 beetles in experiment 10 (Table 2.1) did not warrant graphical illustrations.

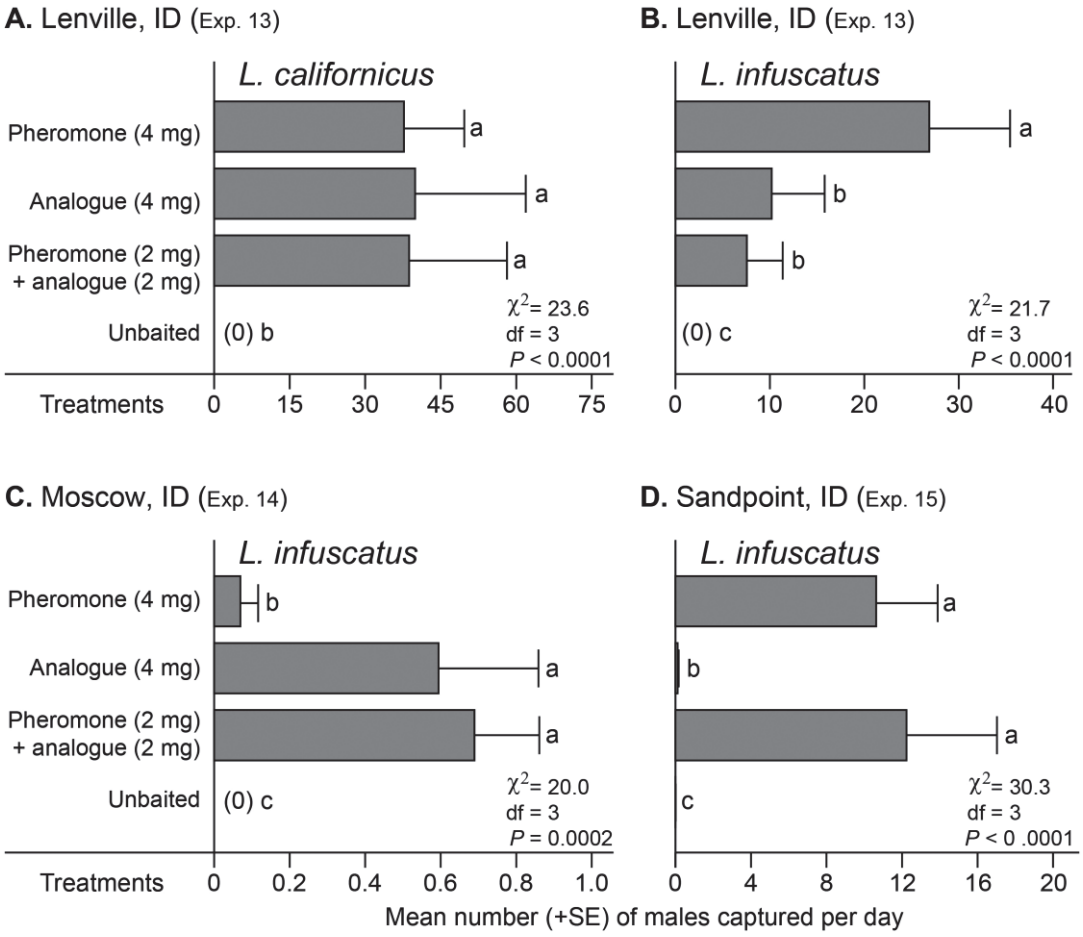

Figure 2.6. Mean (+SE) captures of two *Limonius* spp. in traps baited with the pheromone component limoniic acid [(E)-4-ethyloct-4-enoic acid; 4 mg], its analog [(E)-5-ethyloct-4-enoic acid; 4mg], or both (2 mg each). For each of subpanels A-D, bars with different letters indicate statistically significant differences in trap captures (χ^2 tests; treatments were replicated over time, and traps placed in new locations each time after they were checked).

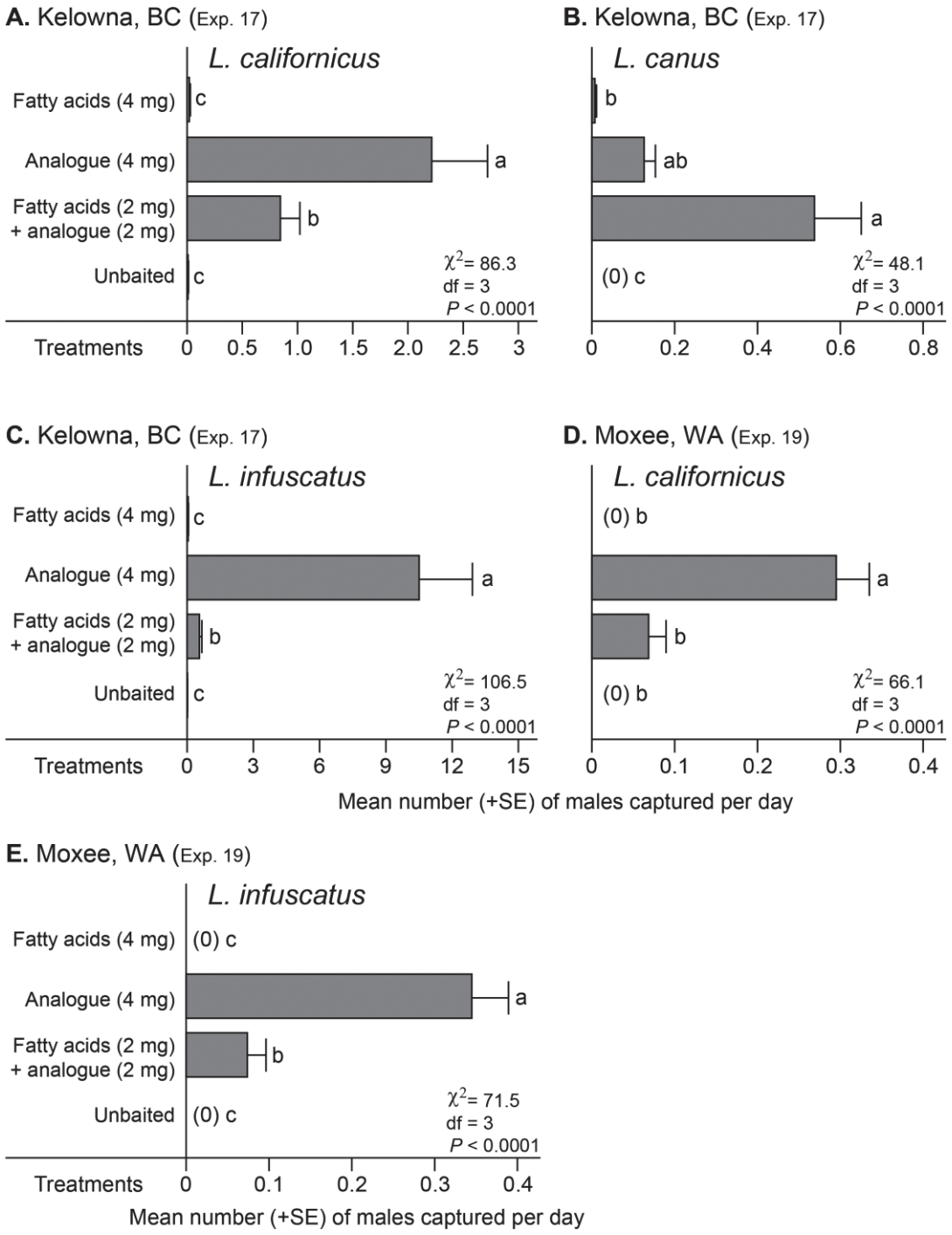

Figure 2.7. Mean (+SE) captures of three *Limonius* spp. in traps baited with a blend of five carboxylic acids (hexanoic, heptanoic, octanoic, nonanoic, decanoic; 4 mg total, equal ratios), the pheromone analog [(*E*)-5-ethyloct-4-enoic acid; 4 mg], or both [carboxylic acids (2 mg) and analog (2 mg)]. For each of subpanels A-E, bars with different letters indicate statistically significant differences in trap captures (χ^2 tests).

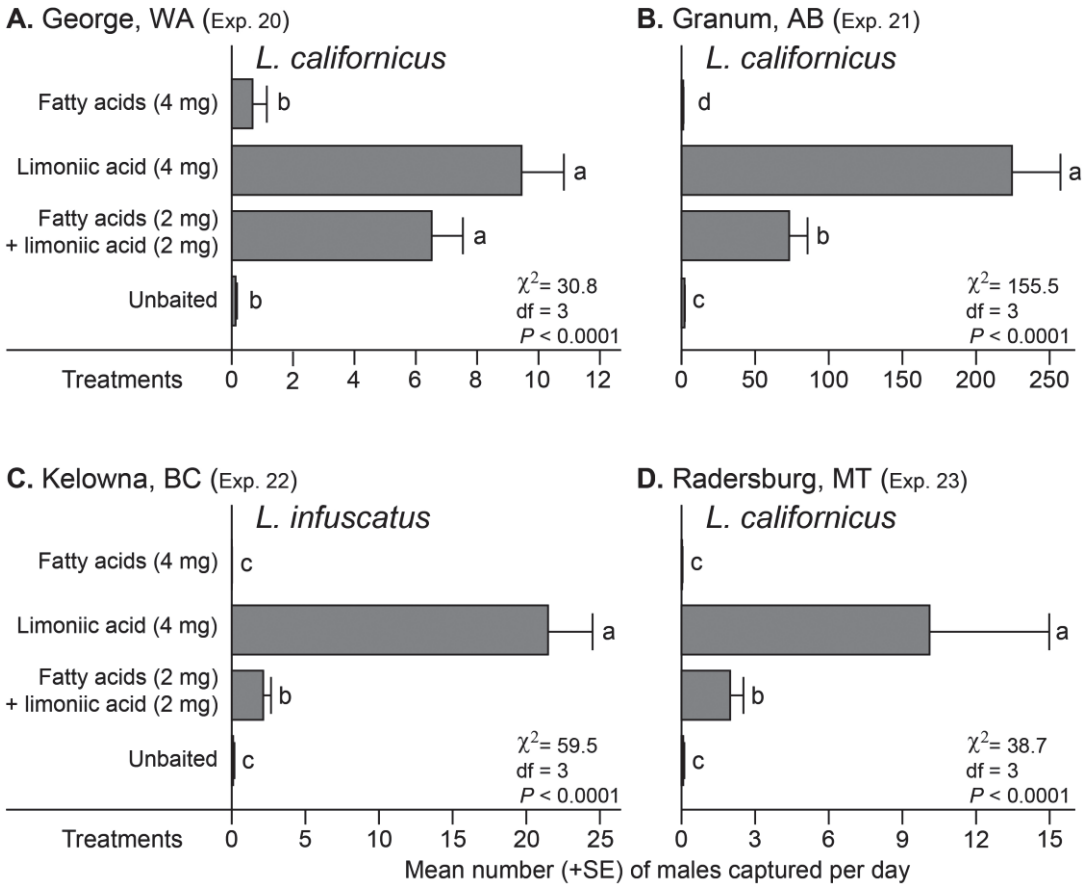

Figure 2.8. Mean (+SE) captures of two *Limonius* spp. in traps baited with a blend of five carboxylic acids (hexanoic, heptanoic, octanoic, nonanoic, decanoic; 4 mg total, equal ratios), the pheromone component limoniic acid [(*E*)-4-ethyloct-4-enoic acid; 4 mg], or both [carboxylic acids (2 mg) and limoniic acid (2 mg)]. For each of subpanels A-D, bars with different letters indicate statistically significant differences in trap captures (χ^2 tests).

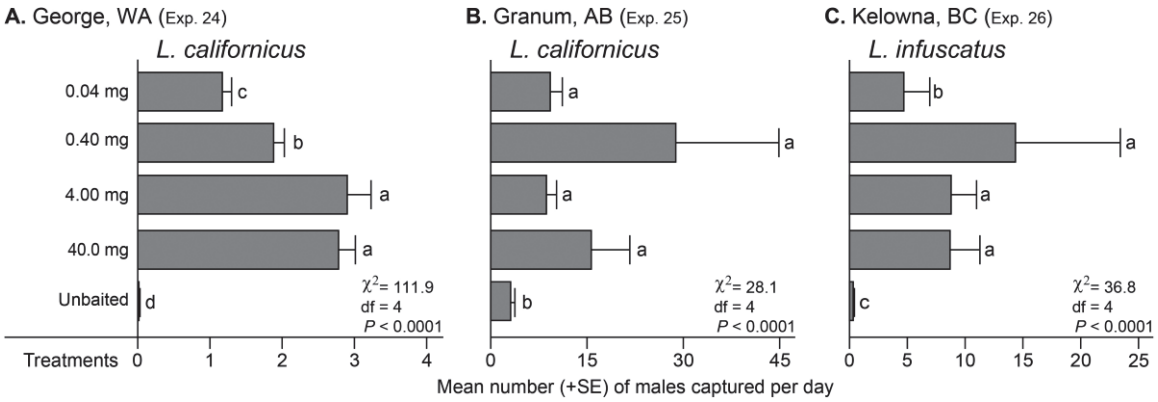

Figure 2.9. Mean (+SE) captures of two *Limonius* spp. in traps baited with different doses of the pheromone component limoniic acid [(*E*)-4-ethyloct-4-enoic acid]. For each of subpanels A-C, bars with different letters indicate statistically significant differences in trap captures (χ^2 test).

Chapter 3.

Seasonal and diel communication periods of sympatric pest *Limonius* click beetle species (Coleoptera: Elateridae) in western Canada*

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EL, WvH & GG conceived the study; EL, KS & WvH ran field experiments; EL counted captured beetles, identified them to species, and determined their sex; EL analyzed diel periodicity capture data statistically and with the help of Adam Blake statistically analyzed seasonal periodicity capture data; EL and GG wrote the first draft, and all authors reviewed and approved of the final draft.

3.1. Abstract

In western North America, sympatric *Limonius* click beetle species produce limoniic acid [(E)-4-ethyloct-4- enoic acid] as a sex pheromone component (*L. canus* (LeConte), *L. californicus* (Mannerheim)) or respond to it as a sex attractant (*L. infuscatus* (Motschulsky)). We tested the hypothesis that these three congeners maintain species-specificity of sexual communication through nonoverlapping seasonal occurrence and/or contrasting diel periodicity of sexual communication. Using capture times of beetles in pheromone-baited traps as a proxy for sexual communication periods, our data show that *L. canus* and *L. californicus* have seasonally distinct communication periods. Most *L. canus* males (>90%) were captured in April and most *L. californicus* males (>95%) were captured in May/June/July. As almost exclusively *L. infuscatus* males were captured in two separate 24-hr trapping studies, with data recordings every hour, it remains inconclusive whether the three *Limonius* congeners communicate at different times of the day. Males of *L. infuscatus* responded to pheromone lures only during daytime hours and during the warmest period each day. Captures of *L. infuscatus*

overlapping with those of *L. canus* in April and those of *L. californicus* in May/June imply the presence of reproductive isolating mechanisms other than seasonal separation of sexual communication periods.

Key words: click beetle, wireworm, pheromone, reproductive isolation, monitoring

3.2. Introduction

Closely related sympatric insect species maintain reproductive isolation through various prezygotic reproductive isolation mechanisms including the specificity of their pheromonal communication signals, divergent signaling times, and/or different micro-locations sought for signaling (Roelofs and Cardé 1974). For examples, the sympatric moths *Archips mortuana* (Kearfott) and *A. argyrospila* (Walker) (Lepidoptera: Tortricidae) employ species-specific pheromone blends (Roelofs and Comeau 1969). The cabbage looper *Trichoplusia ni* (Hübner) and soybean looper *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), produce pheromone blends with components that suppress cross-attraction of heterospecifics (Linn et al. 1988), as does the nun moth *Lymantria monacha* (Linnaeus), (Lepidoptera: Erebidae), repelling co-seasonal spongy moth, *L. dispar* (Linnaeus) (Lepidoptera: Erebidae) (Grant et al. 1996, Gries et al. 1996). The artichoke plume moths *Platyptilia carduidactyla* (Riley) and *P. williamsii* (Riley) (Lepidoptera: Pterophoridae) produce identical sex pheromones but emit them during different times at night (Haynes and Birch 1986). Finally, females of eight species of neotropical cockroaches seek height-specific micro-locations in the vegetation to attract males (Schal 1982).

Several *Limonius* click beetle species (Coleoptera: Elateridae) co-occur in various geographic locations in North America, and their larvae (‘wireworms’) are severe pests of agricultural crops (Andrews et al. 2020, van Herk et al. 2021c, Vernon and van Herk 2022). Traps baited with synthetic limoniic acid [(E)-4-ethyloct-4-enoic acid] – a sex pheromone component of *L. californicus* (Mannerheim) and *L. canus* (LeConte) (Gries et al. 2021) – and deployed in multiple geographic locations across North America captured

L. californicus, *L. canus* and *L. infuscatus* (Motschulsky) in western regions (Fig. 3.1), and *L. agonus* in eastern regions (van Herk et al. 2021a). With two or more species co-occurring in some geographic locations, and with all congeners responding to limoniic acid, the question arose as to how these *Limonius* species maintain reproductive isolation. With no evidence of hybridization between sympatric *Limonius* species (EL, pers. obs.), and no evidence that haplotypes within *L. californicus* and *L. infuscatus* species complexes interbreed (Andrews et al. 2020), some reproductive isolating barriers must exist (Coyne and Orr 2004).

Divergent communication signals and/or signaling times generally contribute to reproductive isolation. As *L. canus* and *L. californicus* apparently do not produce additional pheromone components that may impart specificity to their communication signal (R. Gries, unpubl. data), these species may have unique communication periods. Temporal separation of sexual communication could entail both species-specific seasonal and diel signaling patterns, as reported for various insects (Sasaerilla et al. 2000, Gries et al. 2001, Birge et al. 2007, DeVries et al. 2008). Signaling at different times of the day or night would allow potentially hybridizing species to coexist in the same geographical location (Samudra et al. 2002, Birge et al. 2007, DeVries et al. 2008, Gill et al. 2012). Based on preliminary data in previous reports (Horton and Landolt 2001, Milosavljević et al. 2016, van Herk et al. 2021a), *L. californicus* beetles and larvae seem to be active later in the season than *L. infuscatus*, and *L. canus* may be active earlier in the season than *L. californicus* and *L. infuscatus*. However, definitive seasonal and diel activity periods of *Limonius* species have not yet been described and potential adjustments of activity patterns in geographical locations inhabited by multiple congeners have not yet been studied. Here, we tested the hypothesis that sympatric *Limonius* congeners maintain species-specificity of sexual communication through nonoverlapping seasonal occurrence and/or contrasting diel periodicity of communication.

3.3. Methods

3.3.1. General Methods

Vernon Pitfall Traps ® (VPT, Intko Supply Ltd., Chilliwack, BC) (van Herk et al. 2018b) baited with 4 mg of limoniic acid (Gries et al. 2021) were deployed in two studies to investigate the seasonal and diel communication periods of *Limonius* spp. click beetles. Limoniic acid was dispensed from a cotton pellet (Richmond Dental #0, Charlotte, NC) inside a 1-ml low-density-polyethylene receptacle (Kartell Labware, Noviglio, IT) (van Herk et al. 2021b) which was kept open for pheromone release and inserted into VPT trap lids. Trap lures were not replaced during seasonal beetle activity studies because lures remained attractive for more than 90 d (van Herk et al. 2021a; WvH, EL, pers. obs.). Trap lures used in the diel beetle activity study were removed in between trapping dates and stored at ~4°C. Study locations were chosen based on historical wireworm damage and organic farm status.

3.3.2. Study 1: Seasonal Periodicity of Communication

In the Okanagan region of British Columbia (BC), eight VPT traps were installed at each of locations 1–10 (Table 3.1). Traps were placed at 10-m intervals in the grassy headlands of fields (Traugott et al. 2015) which are less likely subject to disturbances such as farming operations, foot traffic, and flooding. Traps were installed on 30 March 2021 at locations 1–2 and 4–6 in Cawston and Oliver, and on 06 April at location 3 in Cawston and locations 8–10 in Kelowna. In Cawston and Oliver, traps were checked weekly from 06 May to 22 June. In Kelowna, traps were checked weekly from 14 April to 22 June. During weekly trap checks, trap insert cups (140 ml; Fisher Scientific, Ottawa, ON, CA) with beetle captures were replaced with new insert cups filled with 100 ml of WinterProof Water System Antifreeze (5% propylene glycol, Canadian Tire, BC, CA) to prevent beetle escape and preserve beetle captures. All samples were temporarily kept at 4°C and later stored in 70% ethanol for counting. In Oliver and Kelowna, low beetle captures by 22 June prompted the termination of trapping, whereas steady beetle

captures in Cawston justified an additional trap check on 06 July. The decision to terminate the study at this time in Cawston was prompted by heat wave temperatures and nearby wildfires.

3.3.3. Study 2: Diel Periodicity of Communication

On 20 April 2021, 24 VPT traps were installed at a former Christmas tree farm in Kelowna (location 11, Table 3.1), where *L. canus*, *L. infuscatus*, and *L. californicus* had been collected in previous years. For ease of trap checks, traps were spaced 8 m apart in three parallel rows. Traps were checked every hour for 24 hr, starting at 12:00 hr on 20 April. Trap checks at night were completed using headlamps and flashlights. During each trap check, insert trap cups were replaced and captured beetles were counted. As the first trapping date yielded captures of only *L. infuscatus*, trapping was repeated on 28–29 April 2021, following the same protocol as described above. During both trapping dates, mean hourly temperature data were obtained from the Kelowna UBCO weather station (Latitude, longitude: 49.9408, −119.4002) (Government of Canada, Historical Weather, 2021).

3.3.4. Identification of Captured Beetles in Studies 1 and 2

In the seasonal-periodicity-of-communication study (Study 1), captures of many beetles made it necessary to identify only up to 100 beetles per trapping location and date. At each location per date, five of eight replicates (~20 beetles per replicate, captures permitting) were randomly selected for beetle identification. When captures were low (i.e., <100 beetles) at collection dates and locations, all beetles were identified. The proportion of each of the three species was calculated per date to estimate the total beetle number of each species collected per site and per date (Table 3.2). In the diel-periodicity-of-communication study (Study 2), all beetles were identified.

3.4. Statistical Analyses

3.4.1. Study 1: Seasonal Periodicity of Communication

Data were analyzed using the R software environment (4.1.2, R Core Team 2022) with RStudio (1.4, RStudio Team 2021). Species proportions over time were compared and analyzed using generalized linear mixed models with a beta-binomial distribution and a logit link function, with the experimental site as a random factor (Brooks et al. 2017). These models were used to account for potential overdispersion. To test for changes in species composition over time, models including a slope term for time were compared using a likelihood ratio test. Each binary species combination was analyzed in this manner. Peak emergence times between species were analyzed using a linear mixed effects model, again including site as a random factor (Pinheiro et al. 2022). Tukey multiple comparison tests were run for post-hoc multiple comparisons among species (Lenth 2022).

As traps in location 1 in Cawston captured less than 50 beetles total, these data were excluded from analyses. Also, trap catch data of 06 July were excluded from analysis because traps were checked after a 2- instead of 1-wk period. Species proportions were calculated from subsamples, unless all beetles could be identified (i.e., total captures of <100 beetles per site/collection date).

3.4.2. Study 2: Diel Periodicity of Communication

Data were analyzed using the R software environment with RStudio. Data were checked for normal distribution using a Shapiro-Wilk normality test. Mean beetle captures during daytime (06:00 – 18:00 hr) and nighttime (18:00 – 06:00 hr) were compared using an unpaired two-sample Wilcoxon rank sum test. A Kruskal-Wallis rank sum test was used to analyze beetle captures at three temperature thresholds: >15°C, 10–15°C, and <10°C. A pairwise comparison Wilcoxon rank sum test with continuity correction was used to compare captures between the three temperature threshold groups.

3.5. Results

3.5.1. Study 1: Seasonal Periodicity of Communication

During 12 weeks at trapping locations 2–10, >45,100 beetles were captured. Sufficient captures (i.e., more than five beetles on average per trap) were obtained for (i) *L. canus* at locations 2 and 4–7; (ii) *L. infuscatus* at locations 4–9, and (iii) *L. californicus* at locations 2, 3, 5–7, and 9. All three *Limonius* congeners co-occurred at six of the nine locations. At these locations, analyses of weekly trapping data by likelihood ratio tests revealed significant differences between proportions of species captured over time (*L. californicus* vs. *L. infuscatus*: deviance = 287.01, df = 4, $P < 0.0001$; *L. canus* vs. *L. californicus*: deviance = 196.15, df = 4, $P < 0.0001$; *L. canus* vs. *L. infuscatus*: deviance = 256.17, df = 4, $P < 0.0001$). Captures of *L. canus* peaked on 13 April [Location (Loc.) 2], 20 April (Locs. 4, 6, 7), and 28 April (Loc. 5); captures of *L. infuscatus* peaked on 20 April (Locs. 4, 6–9) and 28 April (Loc. 5); and captures of *L. californicus* peaked on 28 April (Loc. 9), 4 May (Loc. 5), 11 May (Locs. 6–9), 18 May (Loc. 2), 01 June (Locs. 2, 3) and 22 June (Loc. 2). Peak capture times of *L. canus* and *L. infuscatus* differed significantly from those of *L. californicus* (*L. canus* vs. *L. californicus*: Tukey test; df = 7, $P = 0.0149$; *L. infuscatus* vs. *L. californicus*: Tukey test; df = 7, $P = 0.0145$), but peak capture times of *L. canus* and *L. infuscatus* were not significantly different (Tukey test; df = 7, $P = 0.9854$).

3.5.2. Study 2: Diel Periodicity of Communication

Over the course of two 24-hr trapping periods, 477 beetles (99% *Limonius* infuscatus) were captured in limoniic acid-baited traps. On 20–21 April, males of *L. infuscatus* were captured between 09:00 and 19:00 hr (Fig. 3.4A), and on 28–29 April between 08:00 and 19:00 hr (Fig. 3.4B). A single male was also captured at 04:00 hr. Significantly more beetles were captured during daytime hours (06:00–18:00 hr) than during nighttime hours (18:00–06:00 hr) (Wilcoxon rank sum test: W = 120.5, P <

0.0036). One male of *L. californicus* was captured at each of 12:00 hr and 17:00 hr on 20 April, and at 11:00 hr on 29 April. One female *L. infuscatus* was captured at 11:00 hr and 16:00 hr on 21 and 28 April, respectively. Beetle captures were air temperature-dependent (Kruskal- Wallis rank sum test: <10°C; 10–15°C; >15°C: $\chi 2 = 18.904$, df = 2, $P < 0.0001$) and peaked when temperatures exceeded 15°C (pairwise comparisons Wilcoxon rank sum test: >15°C vs. 10–15°C: $P < 0.00354$; 10–15°C vs. < 10 °C: $P < 0.02101$; >15°C vs. <10°C: $P < 0.00053$). No beetles were captured when temperatures were below 10°C.

3.6. Discussion

We tested the hypothesis that sympatric *Limonius* congeners maintain species-specificity of sexual communication through nonoverlapping seasonal occurrence and/or contrasting diel periodicity of communication. Our trapping data show that *L. canus* and *L. californicus* indeed have nonoverlapping, seasonally distinct communication periods, whereas communication periods of *L. canus* and *L. infuscatus* and of *L. infuscatus* and *L. californicus* overlap, even though their peak seasonal activity times differ. Captures of almost exclusively *L. infuscatus* males in the diel-periodicity-of-communication study made it impossible to assess whether *L. canus*, *L. californicus*, and *L. infuscatus* communicate, species-specifically, at different times of the day.

With up to three sympatric *Limonius* congeners emitting communication signals in the same geographic location, habitats become 'noisy,' with selection pressure to improve the signal-to-noise ratio of communication channels (Cardé and Baker 1984, Baker 1985). Divergent communication signals, times of signaling, and micro-locations sought for signaling, would all help improve the signal-to-noise ratio of *Limonius* communication channels. *Limonius* canus and *L. californicus* do not seem to produce additional sex attractant pheromone components that may impart specificity to their communication signals (R. Gries et al. unpubl. data) but are active at different times in Spring. *Limonius* canus was captured in pheromone-baited traps mainly in early April, whereas *L. californicus* was captured in early May to mid-June (Figs. 3.2 and 3.3). This temporal separation of seasonal occurrence may have been caused by competition-

induced reproductive character displacement sensu Butlin (1987) (divergence of traits that are coincidentally shared by sympatric species after speciation has occurred, so that hybridization is impossible) but the evolution of such displacement is difficult to prove. In *L. canus* and *L. californicus*, narrower communication periods in sympatric than in allopatric populations would support the concept of reproductive character displacement. Close examination of communication periods in sympatric populations of *L. canus* and *L. californicus* (panels A, D & E in Figs. 3.2 and 3.3) indeed reveals emergence of *L. californicus* in appreciable numbers not before late April/early May, but emergence as early as 13 April in a Kelowna study site (panel H, Fig. 3.3) where *L. canus* is absent. Emergence of *L. californicus* not before mid-May in a Cawston study site (panel B, Fig. 3.2) would suggest that *L. canus* may appear only in some years or was formerly present in that site, as it is present in another study site in Cawston (panel A, Fig. 3.2) that is separated by only 1.4 km. The seasonal emergence of *L. californicus* seems to be affected by the presence or absence of congener competitors. Late emergence of *L. californicus* in the Okanagan may help avoid competition with *L. canus* and *L. infuscatus*, whereas early emergence in southern Alberta (van Herk et al. 2021a) may occur because *L. canus* and *L. infuscatus* are absent or rare (van Herk et al. 2021c). At a particular location, the presence and annual relative abundance of sympatric species are likely affected by the long-life history of these *Limonius* species, with larvae spending 1–6 yr in the soil (Stone 1941, Lanchester 1946), a time frame that is modulated by the location's cropping history (Vernon and van Herk 2022). More studies in sites with sympatric and allopatric populations of *L. canus* and *L. californicus* are needed to rigorously assess whether character displacement may have contributed to the temporal separation of *L. canus* and *L. californicus* sexual communication periods.

Incomplete temporal separation between *L. canus* and *L. infuscatus* and between *L. infuscatus* and *L. californicus* implies that other reproductive isolating mechanisms are in place that prevent hybridization. The sex pheromone blend of *L. infuscatus* has not yet been analyzed and may contain not only limoniic acid but also additional sex attractant pheromone components. Alternatively, it may be incompatible courtship behavior or species-specific contact sex pheromone components, that provide reproductive barriers.

Contact sex pheromone components play key roles in mate recognition and speciation in Timema walking sticks (Riesch et al. 2017) and could also play a role in click beetles.

Selection of spatially distinct micro-locations for signaling is yet another conceivable reproductive isolating mechanism between *L. canus* and *L. infuscatus*, and *L. infuscatus* and *L. californicus*. *Limonius* canus and *L. californicus* are thought to select different oviposition sites based on soil characteristics and type of vegetation (Gibson et al. 1958) and may also select different sites for sexual signaling and mating. Here, we commonly observed more captures of *L. canus* in study sites with open areas and drier soil, and more captures of *L. californicus* and *L. infuscatus* in moist, grassy areas with mixed vegetation. Without temporal isolation, spatial isolation on a habitat scale could be an important prezygotic reproductive isolating barrier (Coyne and Orr 2004).

The near-exclusive captures of *L. infuscatus* males in the diel-periodicity-of-communication study were surprising because several *Limonius* species were present in the same field site in previous years. As a result of this disproportionate representation of *Limonius* congeners in our field site, we could not definitively determine whether they communicate at different times of the day. Species-specific communication times do not seem likely, though, based on concurrent captures of many *L. infuscatus* males and three *L. californicus* males. Regardless, the study revealed interesting new results. Even though synthetic pheromone lures release pheromone at all times, males responded, and trap captures materialized, mainly in the afternoon hours likely due to warmer temperatures. If we accept the premises that trap capture periods reflect mate-seeking periods of males, and that mate-seeking periods of males largely overlap with mate-calling (pheromone-emitting) periods of conspecific females, as shown in other insects (Morgan 1987, Bell 1990, Levi-Zada and Byers 2021), then female *L. infuscatus*, and possibly other click beetle females, obviously emitted pheromone exclusively during day- time hours (08:00–19:00 hr) (Fig. 3.4), and during the warmest times of day. This was not previously known. Indeed, captures of click beetles in light traps at night (Maclean 1977, Morrill 1978, Manole 1999) could have meant that beetles are typically active, and pos- sibly engage in sexual communication, during nighttime hours.

Some of our data also reveal the usefulness of pheromone technology for integrated pest click beetle management. Deployment of synthetic pheromone lures will help gather information on the geographic distribution of species. Tracking trap captures of target species every year and throughout the season will reveal seasonal activity patterns and changes in population size over time which, in turn, will inform decisions whether beetle control measures are required and when to apply them. These control measures may also include pheromone-based technologies such as mass trapping of males, mating disruption, and attract & kill tactics (Vernon et al. 2014, Reddy and Tangtrakulwanich 2014, Kabaluk et al. 2015, Vernon and van Herk 2022).

In conclusion, we investigated how three sympatric *Limonius* congeners that respond to limoniic acid as a sex pheromone component (*L. canus*, *L. californicus*) or as a sex attractant (*L. infuscatus*) maintain reproductive isolation. Our data show that *L. canus* and *L. californicus* have seasonally distinct communication periods. The seasonal occurrence of *L. infuscatus* overlaps with that of its two congeners, implying the presence of reproductive isolating mechanisms other than temporal separation of sexual communication periods.

Table 3.1 – List of study location numbers (Loc. #), names of study locations (all in British Columbia, Canada) and their geographic coordinates (latitude, longitude), trapping durations (in 2021), and total numbers of three *Limonius* click beetles congeners captured in Vernon Pitfall Traps ® baited with synthetic limoniic acid pheromone lures. Experiments were designed to determine both the seasonal and the diel periodicity of the beetles' sexual communication periods.

Loc. #	Location	Latitude, longitude	Trapping period (days)	Traps	Beetles captured
Seasonal periodicity					
1	Cawston, BC	49.189237, -119.767038	30 Mar – 6 Jul (98)	8	40
2	Cawston, BC	49.172004, -119.743985	30 Mar – 6 Jul (98)	8	1,316
3	Cawston, BC	49.170512, -119.741892	6 Apr – 6 Jul (91)	8	2,220
4	Cawston, BC	49.184321, -119.745588	30 Mar – 6 Jul (98)	8	8,134
5	Oliver, BC	49.241220, -119.573272	30 Mar – 22 Jun (84)	8	7,460
6	Oliver, BC	49.245886, -119.562828	30 Mar – 22 Jun (84)	8	3,135
7	Oliver, BC	49.247359, -119.555761	30 Mar – 22 Jun (84)	8	2,705
8	Kelowna, BC	49.821058, -119.440338	6 Apr – 22 Jun (77)	8	7,347
9	Kelowna, BC	49.826998, -119.437391	6 Apr – 22 Jun (77)	8	2,906
10	Kelowna, BC	49.868444, -119.441274	6 Apr – 22 Jun (77)	8	9,848
Diel periodicity					
11	Kelowna, BC	49.822336, -119.440524	20-21 & 28-29 Apr (2)	24	477

Table 3.2. Proportions of male click beetles *Limonius canus, L. infuscatus* and *L. californicus* captured per collection date and study site [Location numbers (Loc.) 1-10; Table 3.1]; Mean = mean ratio of all traps per date and per location.

		Mean (SE) species proportions captured per collection date											
Loc. #	Species	06 April	13 April	20 April	27 April	04 May	11 May	18 May	25 May	01 June	08 June	15 June	22 June
1	*L. canus*	1.0 (0.0)	0	1.0 (0.0)	1.0 (0.0)	0	0	0	0	0	0	0	0
	L. infuscatus	0	0	0	0	0	1.0 (0.0)	1.0 (0.0)	0	0	0.17 (0.14)	0	0
	L. californicus	0	0	0	0	0	0	0	0	1.0 (0.0)	0.83 (0.14)	1.0 (0.0)	1.0 (0.0)
2	*L. canus*	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	0.97 (0.01)	1.0 (0.0)	0.94 (0.06)	0.62 (0.17)	0.50 (0.20)	0	0.95 (0.04)	0	0.36 (0.12)
	L. infuscatus	0	0	0	0.03 (0.01)	0	0.06 (0.06)	0	0	0	0.05 (0.04)	0	0.03 (0.03)
	L. californicus	0	0	0	0	0	0	0.38 (0.17)	0.50 (0.20)	1.0 (0.0)	0	1.0 (0.0)	0.61 (0.12)
3	*L. canus*		0.52 (0.19)	0.62 (0.18)	0.75 (0.16)	0.02 (0.0)	0	0	0	0	0	0	0
	L. infuscatus		0.04 (0.04)	0.01 (0.01)	0	0	0	0.02 (0.01)	0	0	0	0	0
	L. californicus		0.44 (0.17)	0.37 (0.18)	0.25 (0.16)	0.98 (0.0)	1.0 (0.0)	0.98 (0.01)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)
4	*L. infuscatus*	0	0	0.93 (0.05)	0.40 (0.16)	0.52 (0.13)	0.03 (0.01)	0.01 (0.0)	0.01 (0.0)	0	0.01 (0.0)	0	0
	L. californicus	0	0	0.07 (0.05)	0.60 (0.16)	0.48 (0.13)	0.97 (0.01)	0.99 (0.0)	0.99 (0.0)	1.0 (0.0)	0.99 (0.0)	1.0 (0.0)	1.0 (0.0)
5	*L. canus*	0.05 (0.0)	0.10 (0.0)	0.23 (0.0)	0.15 (0.0)	0.16 (0.0)	0.09 (0.0)	0.01 (0.0)	0.04 (0.0)	0.08 (0.02)	0	0	0
	L. infuscatus	0.95 (0.0)	0.90 (0.0)	0.77 (0.0)	0.85 (0.0)	0.84 (0.0)	0.91 (0.0)	0.96 (0.0)	0.96 (0.0)	0.88 (0.02)	0.91 (0.02)	0.93 (0.03)	1.0 (0.0)
	L. californicus	0	0	0	0	0	0	0.03 (0.0)	0	0.04 (0.01)	0.09 (0.02)	0.07 (0.03)	0
6	*L. canus*	0.26 (0.12)	0.49 (0.10)	0.49 (0.0)	0.42 (0.0)	0.14 (0.0)	0.16 (0.02)	0.07 (0.04)	0.13 (0.02)	0.06 (0.04)	0.20 (0.14)	0.12 (0.09)	0
	L. infuscatus	0.74 (0.12)	0.51 (0.10)	0.47 (0.0)	0.45 (0.0)	0.39 (0.0)	0.32 (0.02)	0.49 (0.07)	0.64 (0.04)	0.56 (0.13)	0.47 (0.11)	0.58 (0.09)	0.10 (0.06)
	L. californicus	0	0	0.04 (0.0)	0.13 (0.0)	0.47 (0.0)	0.51 (0.01)	0.44 (0.04)	0.23 (0.05)	0.38 (0.12)	0.34 (0.09)	0.30 (0.11)	0.90 (0.06)
7	*L. canus*	0.92 (0.01)	0.87 (0.0)	0.66 (0.0)	0.37 (0.0)	0.31 (0.03)	0.20 (0.01)	0.05 (0.01)	0.37 (0.04)	0.01 (0.01)	0.04 (0.02)	0.29 (0.17)	0.21 (0.14)
	L. infuscatus	0.08 (0.01)	0.13 (0.0)	0.34 (0.0)	0.63 (0.0)	0.52 (0.02)	0.46 (0.01)	0.65 (0.0)	0.59 (0.03)	0.57 (0.06)	0.47 (0.03)	0.71 (0.17)	0.75 (0.15)
	L. californicus	0	0	0	0	0.17 (0.01)	0.34 (0.0)	0.30 (0.01)	0.04 (0.01)	0.42 (0.06)	0.48 (0.03)	0	0.04 (0.04)
8	*L. canus*		0.05 (0.0)	0.04 (0.0)	0	0	0	0	0	0	0.01 (0.01)	0	0
	L. infuscatus		0.95 (0.0)	0.96 (0.0)	0.97 (0.0)	0.94 (0.0)	0.77 (0.0)	0.88 (0.0)	1.0 (0.0)	0.95 (0.0)	0.91 (0.02)	0.97 (0.03)	1.0 (0.0)
	L. californicus		0	0	0.03 (0.0)	0.06 (0.0)	0.23 (0.0)	0.12 (0.0)	0	0.05 (0.0)	0.08 (0.02)	0.03 (0.03)	0
9	*L. infuscatus*		1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)
10	*L. infuscatus*		0.96 (0.0)	0.95 (0.0)	0.68 (0.0)	0.83 (0.0)	0.70 (0.0)	0.76 (0.0)	0.77 (0.0)	0.67 (0.01)	0.62 (0.08)	0.84 (0.08)	0.80 (0.10)
	L. californicus		0.04 (0.0)	0.05 (0.0)	0.32 (0.0)	0.17 (0.0)	0.30 (0.0)	0.24 (0.0)	0.23 (0.0)	0.33 (0.01)	0.38 (0.08)	0.16 (0.08)	0.20 (0.10)

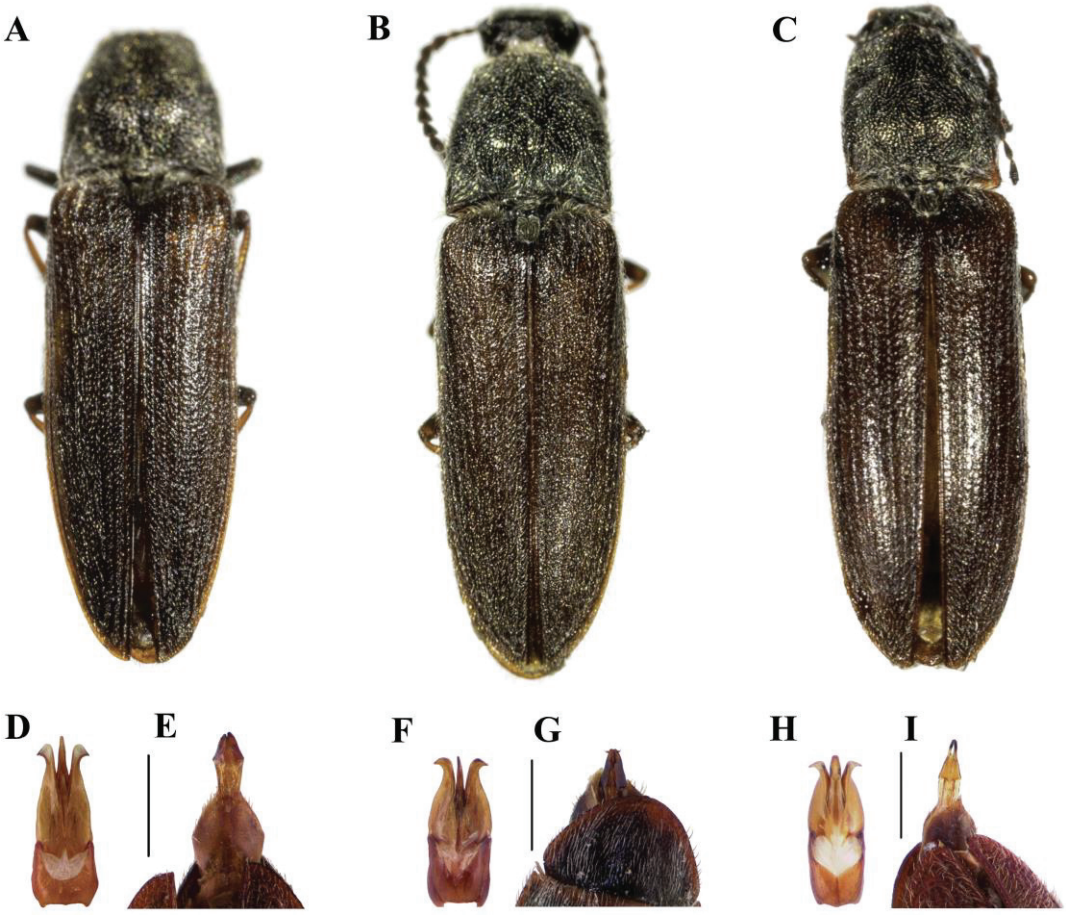

Figure 3.1 - Images of (i) dorsal *Limonius canus* (A), *L. californicus* (B) and *L. infuscatus* (C), and (ii) genitalia of male and female *L. canus* (D, E), male and female *L. californicus* (F, G), and male and female *L. infuscatus* (H, I). Image credits: A-C: Warren Wong; D-I: Frank Etzler; scale bars: 1mm.

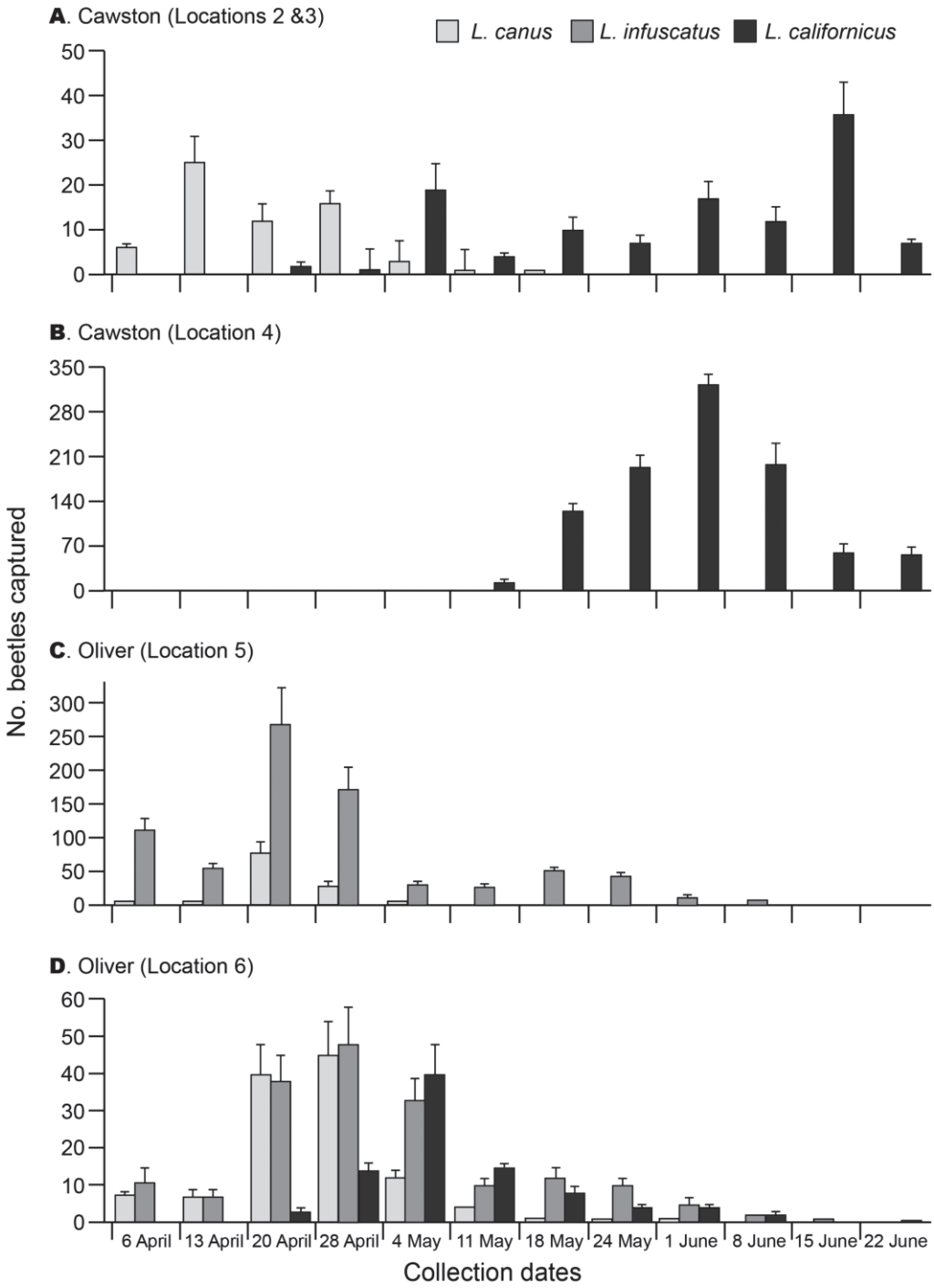

Figure 3.2. Mean (+SE) weekly captures of male Limonius click beetles in Study 1 in Vernon Pitfall Traps ® baited with synthetic limoniic acid pheromone lures and deployed in various study sites (Table 3.1) in the Okanagan region of British Columbia between 06 April and 22 June 2021. Mean numbers (+SE) of male captures were extrapolated by identifying beetles in subsamples of weekly captures (see methods for details).

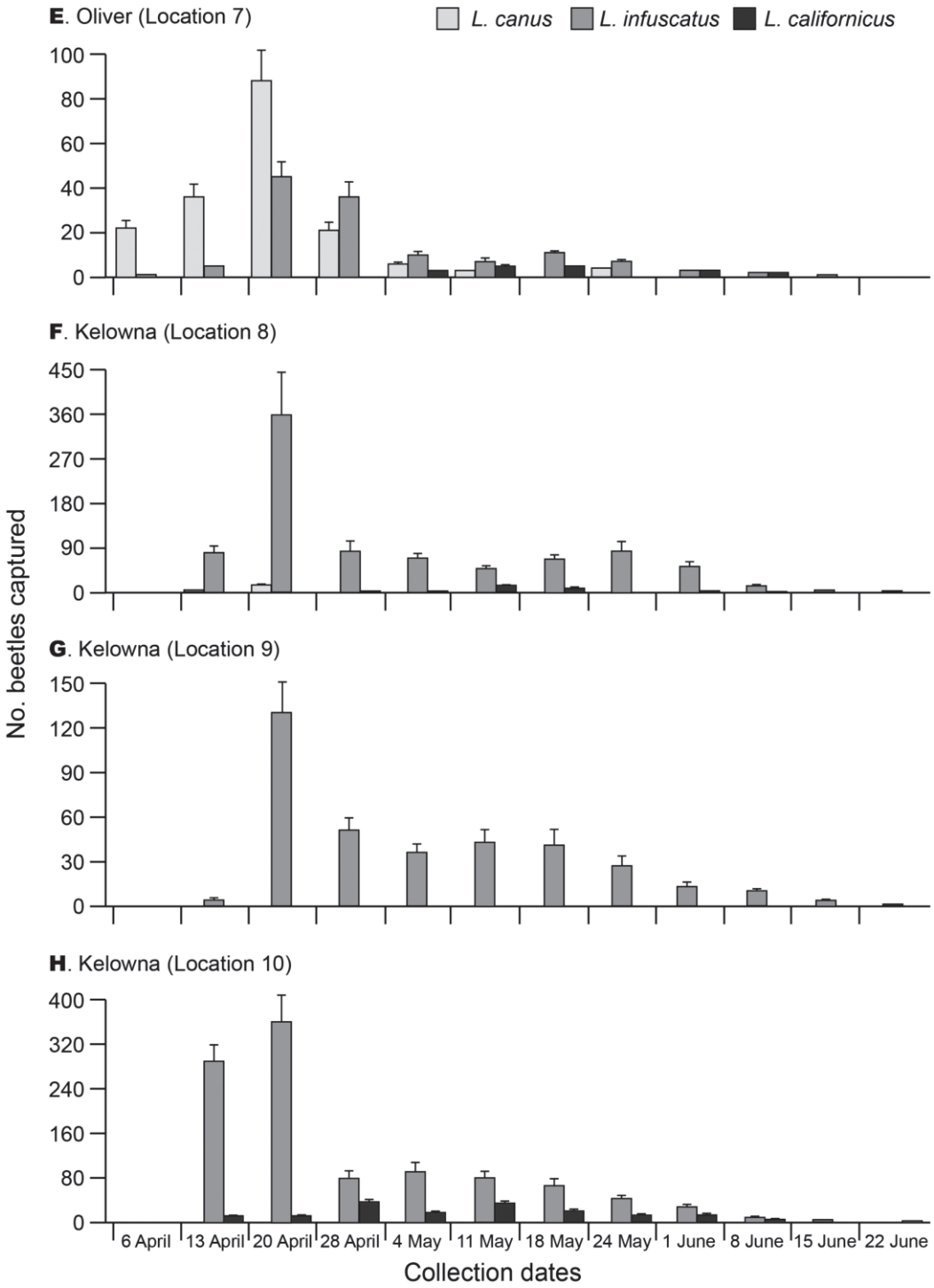

Figure 3.3. Mean (+SE) weekly captures of male *Limonius* click beetles in Study 1 in Vernon Pitfall Traps ® baited with synthetic limoniic acid pheromone lures and deployed in various study sites (Table 3.1) in the Okanagan region of British Columbia between 06 April and 22 June 2021. Mean numbers (+SE) of male captures were extrapolated by identifying beetles in subsamples of weekly captures (see methods for details).

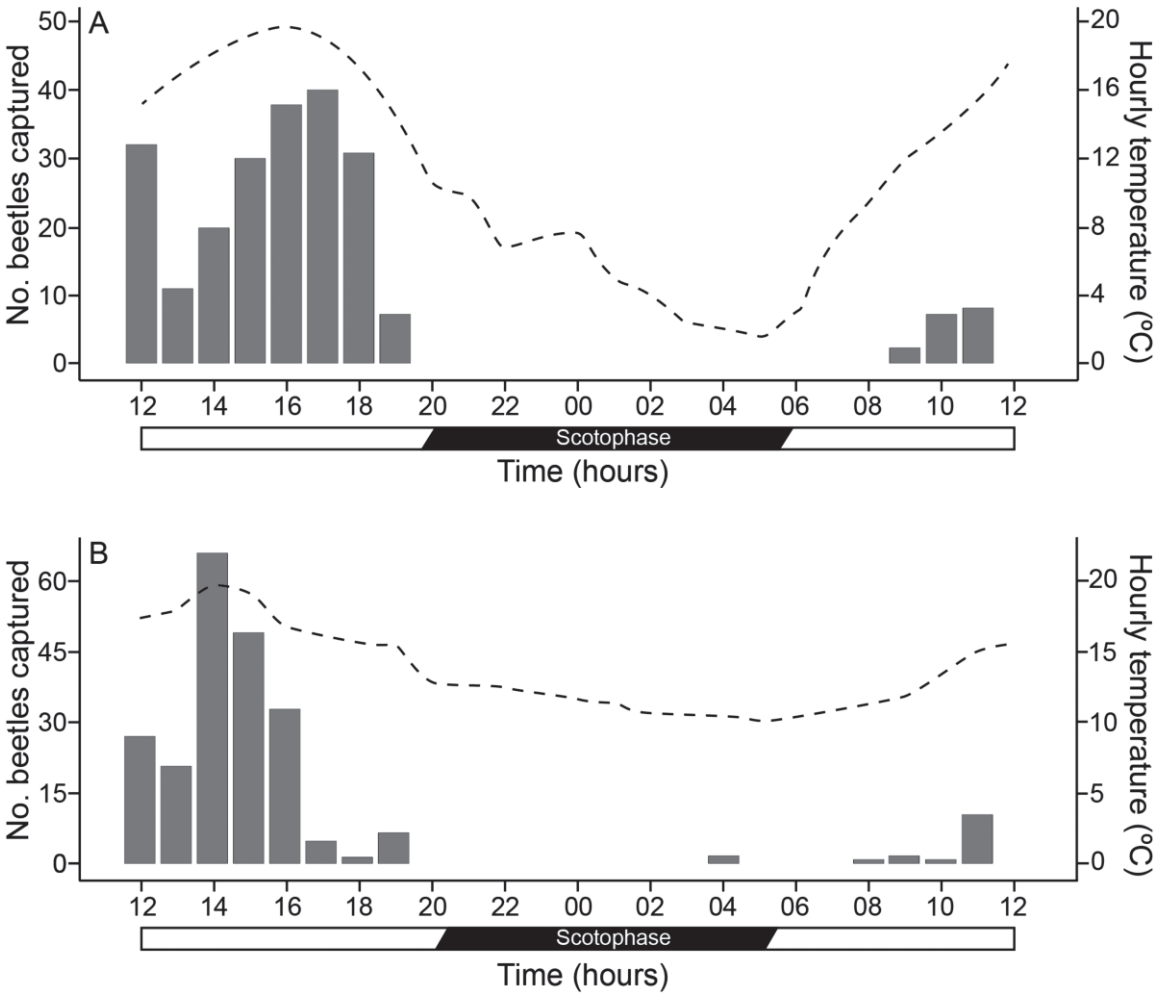

Figure 3.4. Total captures per hour of *Limonius infuscatus* males in Study 2 in Vernon Pitfall Traps ® baited with synthetic limoniic acid pheromone lures and deployed at a study site neat Kelowna, British Columbia, on 20-21 April 2022 (A) and 28-29 April 2021 (B). Trap captures occurred only during daytime hours and were likely affected by temperature.

Chapter 4. Mixed sex pheromone lures for combined captures of *Agriotes* and *Limonius* pest click beetles in North America

*A near identical version of this chapter has been accepted for publication pending revisions: Emily Lemke, Willem G. van Herk, Kendal Singleton, Julien Saguez, Graeme Fowler, Doug Pepper, Kathleen Furtado and Gerhard Gries. *Submitted to the Journal of Applied Entomology*.

EL, WvH & GG conceived the study; EL, KS, WvH, JS, GF, & DP ran field experiments; EL & KF identified the species and sex of beetles captured in traps; WvH analyzed capture data statistically; EL and WvH wrote the first draft, and all authors reviewed and approved of the final draft.

4.1. Abstract

Sex pheromone lures are effective tools for monitoring and potentially controlling populations of pest click beetles (Coleoptera: Elateridae). To date, these lures are genus-specific (e.g., *Limonius* spp.) or species-specific (e.g., *Agriotes lineatus* Linnaeus). However, if sympatric heterogeners were not to be repelled by each other's pheromones, trap lures effective for multiple elaterid genera could be developed, improving cost efficiency in elaterid pest management programs. In both western and eastern North America, several species of *Agriotes* spp. and *Limonius* spp. co-occur and inflict similar crop damage. We investigated whether the sex pheromones of these species can be combined in a mixed lure without reducing its attractiveness to all target species. In western Canada, we show that the pheromones of *A. lineatus* (geranyl butanoate & geranyl octanoate) and *Limonius* spp. [(*E*)-4-ethyloct-4-enoic acid (limoniic acid)] can be combined without significantly reducing captures of male *A. lineatus*, *L. canus* (LeConte), *L. californicus* (Mannerheim) and *L. infuscatus* (Motschulsky) relative to traps baited with species-specific lures for *A. lineatus* and *Limonius* spp.. Similarly, the pheromone of *A. obscurus* (Linnaeus) (geranyl hexanoate & geranyl octanoate) and limoniic acid can be combined without significantly reducing trap captures of male *L. canus, L. infuscatus* and *L. californicus* but reduced *A. obscurus* captures relative to traps

baited only with the *A. obscurus* pheromone. In eastern Canada, combining pheromones for *Agriotes mancus* (Say) (geranyl butanoate & geranyl hexanoate) and limoniic acid reduced captures of *A. mancus* but not *A. pubescens* (Melsheimer) and *A. sputator* (Linnaeus). These data imply that pheromones of select elaterid heterogeners can be combined in a 'catch-more' pheromone lure to effectively monitor for, or possibly control, multiple elaterid pests, but that such mixed lures should be evaluated for each species combination.

Keywords: Wireworms, Elateridae, pheromone-based monitoring, integrated pest management, insect chemical communication, species detection

4.2. Introduction

Pheromone-based trapping is an effective tool in integrated pest management (IPM) for monitoring and estimating population densities, detecting endangered or pest species, mapping species distributions, and timing pest management tactics (Howse et al. 1998; Vernon and van Herk 2022). Synthetic sex pheromones of click beetles (Coleoptera: Elateridae) are deployed to monitor the presence and abundance of pest species (Furlan et al. 2020), and to curtail pest populations by mass trapping (Arakaki et al. 2008; Vernon et al. 2014a) and mating disruption (Vernon and van Herk 2022; Reddy and Tangtrakulwanich 2014). In North America, sex pheromones are now identified for multiple pest click beetles, including *Agriotes ferrugineipennis* and *A. mancus* (Singleton et al. 2022a, 2023), *Limonius canus* and *L. californicus* (Gries et al. 2021; van Herk et al. 2021a), *Selatosomus aeripennis destructor* (Gries et al. 2022), *Cardiophorus tenebrosus* and *C. edwardsi* (Serrano et al. 2018), and *Melanotus communis* (Williams et al. 2019).

In agricultural fields in North America, multiple elaterid pest species and genera commonly co-occur, such as *Glyphonyx recticollis* (Say) and *Melanotus communis* (Gyllenhal) in the southeastern USA (Vernon and van Herk 2022), *Limonius infuscatus, L. californicus* and *Selatosomus pruininus* in the Pacific Northwestern USA (Milosavljević, Esser and Crowder 2016), *Selatosomus aeripennis destructor* (Brown) and *Hypnoidus bicolor* (Eschscholtz) on the Canadian Prairies (van Herk et al. 2021c), *A. obscurus* and *A. lineatus* in coastal British Columbia (BC), and *L. canus*, *L. infuscatus*, and *A. lineatus* in central BC (Vernon and van Herk 2022). In the province of Quebec, the abbreviated wireworm, *Hypnoidus abbreviatus* (Say), often co-occurs with *Melanotus, Agriotes* and *Limonius* species (Saguez et al. 2017). In Canada to date, populations of elaterid species are monitored with species-specific pheromone lures, which makes pheromone-based monitoring and mass trapping cumbersome and expensive (Vernon and van Herk 2022). With sex pheromones of key elaterid pests becoming known and commercially available, pheromone lures that attract more than one elaterid species or genus would improve cost efficiency and practicality of pheromone-based tactics for elaterid pest management. As previously shown, (*E*)-4-ethyloct-4-enoic acid (limoniic

acid) as a single-component sex pheromone is highly attractive to each of the four major North American *Limonius* pests (*L. californicus*, *L. canus, L. infuscatus* and *L. agonus*) (Gries et al. 2021; van Herk et al. 2021a; Lemke et al. 2022; van Herk et al. 2023). Conversely, the sex pheromones of *A. obscurus* (geranyl hexanoate & geranyl octanoate) and *A. lineatus* (geranyl butanoate & geranyl octanoate) could not be combined in coastal BC without reducing the lure's attractiveness to *A. obscurus*. When synthetic sex pheromones of *A. lineatus* and *A. obscurus* were combined in a mixed lure, captures of *A. lineatus* remained unaffected but captures of *A. obscurus* decreased by 76-77% (Vernon et al. 2014b; van Herk et al. 2022a), indicating a deterrent effect of the *A. lineatus* pheromone on attraction and capture of male *A. obscurus*. Responses of male *A. lineatus* and *A. obscurus* to limoniic acid, and responses of male *Limonius* spp. to pheromones of *Agriotes* heterogeners, have not yet been tested. With the distinctively different types of pheromone produced by *A. lineatus* and *A. obscurus* (terpenoid esters), and by *Limonius* spp. (ethyl-branched aliphatic acid), we predicted that a mixed ester/acid lure would still be attractive to both *Agriotes* spp. and *Limonius* spp.

The concept of combining synthetic pheromones of multiple pest species in a mixed pheromone lure has previously been explored with varying degrees of success in several integrated pest management programs. The concept was tested with mealybugs (Waterworth et al. 2011), longhorn beetles (Nakamuta et al. 1997; Wong et al. 2012; Fan et al. 2019; Rice et al. 2020), moths (Jones et al. 2009; Brockerhoff et al. 2013; Preti et al. 2020) and true bugs (Yasuda et al. 2010; Kim et al. 2015). Mixed pheromone lures are effective if no pheromone in that lure adversely affects attraction and capture of all target species.

Here, we investigated whether synthetic sex pheromones of *Agriotes* spp. and *Limonius* spp. (Fig. 4.1) can be combined in a mixed lure without affecting its attractiveness to any *Agriotes* and *Limonius* species. Mixed lures were designed to capture *Limonius* spp. and *A. lineatus* and *A. obscurus* in western Canada, and *L. agonus* and *A. mancus* in eastern Canada. These species were selected because *Limonius* spp. and *Agriotes* spp. commonly co-occur in Canada with generally overlapping seasonal swarming periods (Begg 1959; Levesque and Levesque 1993; Jakubowska et al. 2018;

van Herk et al. 2021a; van Herk et al. 2021b; Vernon and van Herk 2022; Lemke et al. 2022). As *A. mancus* may also co-occur with *A. pubescens* and *A. sputator*, the effect of mixed lures on captures of *A. pubescens* and *A. sputator* was also of interest. The biology and life history of *Limonius* spp. and native *Agriotes* spp., such as *A. pubescens,* remain hardly studied, but these species inhabit the same microhabitat (i.e., unfarmed grassy margins surrounding crops), making them ideal heterogenera to study together (Traugott et al. 2015).

4.3. Materials and Methods

4.3.1. General Methods

Five experiments were conducted with *L. canus*, *L. californicus*, *L. infuscatus* and *A. lineatus* or *A. obscurus* in western Canada, and one experiment with *L. agonus* and *A. mancus* in eastern Canada. All six experiments (n = 8 each) used Vernon Pitfall Traps ® (VPT) (van Herk et al. 2022b) and tested four or six treatments in a randomized complete block design. Traps were placed at 10-m and 20-m spacing between treatments and blocks (replicates), respectively, in grassy field edges, following a general protocol previously detailed (Gries et al. 2021). Trap lures consisted of a closed, 1-mL low-density-polyethylene receptacle (Kartell Labware, Noviglio, IT) containing a cotton pellet (Richmond Dental #0, Charlotte, NC), onto which the pheromones were dispensed. Lures were suspended from trap roofs and not replaced over the trapping period of each study (van Herk et al. 2021b). Studies were placed in locations with historic wireworm damage and were terminated once total beetle captures declined.

4.3.2. Sources of Pheromones

Limoniic acid was synthesized from a previous study (detailed in Gries et al. 2021), and geranyl octanoate, geranyl hexanoate and geranyl butanoate were purchased from Penta Manufacturing Corporation (Fairfield, NJ, USA).

4.3.3. Trapping in Western Canada

Three experiments were run for captures of *Limonius* spp. and *A. lineatus*, and two experiments for captures of *Limonius* spp. and either *A. lineatus* or *A. obscurus*. Experiment 1 was run at an organic farm in Kelowna, BC (Table 4.1), where three *Limonius* spp. and *A. lineatus* had been found in abundance in previous years (van Herk et al. 2021a,b; Lemke et al. 2022). Traps were installed on 25 April 2020 and checked on 02, 06 and 16 May 2020. Experiments 2 & 3 were run in Courtenay and Duncan, BC (Table 4.1) on farms historically infested with *L. infuscatus* and *A. lineatus* (van Herk, personal obs.). In Courtenay, traps were installed on 04 May 2021 and checked on 10, 17, 24 May and 01 June. In Duncan, traps were installed on 03 May 2021 and checked on 12, 19, 26 May and 02 June. Experiment 4 was run in Kelowna, BC (Table 4.1) on two organic vegetable farms with historical presence of *L. infuscatus, L. canus, L. californicus* and *A. lineatus,* and a low population of *A. obscurus* (van Herk, personal obs). Experiment-4 traps were installed at two locations (less than 5 km apart) on 14 April and checked on 20, 28 April and on 04, 11, 18 May 2021. Experiment 5 was run in Pemberton, BC (Table 4.1) in a fallow field near a potato farm where *L. canus, A. lineatus* and *A. obscurus* had previously been found (van Herk et al. 2021b). Experiment-5 traps were installed 12 April and checked on 19, 26 April and on 06, 10, 13 May 2021.

Experiments 1–3 tested four treatments: (1) an unbaited control; (2) *A. lineatus* pheromone (geranyl octanoate & geranyl butanoate at a 1:1 ratio; 40 mg); (3) *Limonius* spp. pheromone (limoniic acid; 4 mg); and (4) *A. lineatus* pheromone (20 mg) *plus* limoniic acid (2 mg). Experiments 4 and 5 also tested treatments 1–4 as well as (5) *A. obscurus* pheromone (geranyl hexanoate & geranyl octanoate at a 1:1 ratio; 40 mg), and (6) *A. obscurus* pheromone (20 mg) *plus* limoniic acid (2 mg).

4.3.4. Trapping in Eastern Canada

One experiment (Exp. 6) was run at the grain research centre (CEROM) in Saint-Mathieu-de-Beloeil, Quebec (Table 4.1), where *L. agonus, A. mancus, A. pubescens* and *A. sputator* had previously been found (Singleton et al. 2022b, 2023). Traps were

installed on 03 May and checked on 07, 11, 13, 17, 19, 21, 25, 27, 31 May and on 04, 07, 10, 14, 18 June 2021. Each experimental replicate consisted of four treatments: (1) an unbaited control; (2) *A. mancus* pheromone (geranyl hexanoate & geranyl butanoate at a 1:1 ratio; 40 mg); (3) limoniic acid (4 mg); and (4) *A. mancus* pheromone (20 mg) *plus* limoniic acid (2 mg).

The dose of esters and of limoniic acid in all mixed lures (compared to ester- or acid-only lures) was erroneously halved. This error, however, is deemed to have little bearing on beetle captures and the interpretation of experimental results. As limoniic acid is similarly attractive at 0.4 mg, 4 mg and 40 mg (van Herk et al. 2021), testing limoniic acid here at 2 mg or 4 mg would not have significantly affected trap captures. Similarly, although the ester dose in mixed and ester-only lures (20 mg and 40 mg, respectively) for attraction of *Agriotes* spp. differed by 2-fold, ester release rates from both types of lures were virtually identical (as determined by laboratory release rate studies over seven days), suggesting no effect of ester dose on trap captures, at least not in the first one or two weeks of the trapping period.

4.3.5. Identification of Captured Beetles

In experiments 1 & 6, all captured beetles were identified to species and their sex was determined using taxonomic keys (Johnson 2002; Al Dhafer 2009; Etzler 2013) and an identification guide for northwestern *Limonius* species found on agricultural land (Frank Etzler, unpublished). In experiments 2–5, all beetles were counted and sorted by genus until further species identification. In experiments 2, 3 and 5, large beetle captures made it necessary to identify beetles in only subsamples. At each location per collection date, up to 50 *Limonius* and 50 *Agriotes* beetles per treatment were identified to species and their sex determined from two of eight randomly selected replicates (Exps. 2, 3) and from all eight replicates (Exp. 5; up to 800 beetles per collection date). In experiment 4 with a greater diversity of captured species, at each collection date up to ~100 *Limonius* and 100 *Agriotes* beetles per treatment were identified and their sex determined from all eight experimental replicates (up to 1,400 beetles per collection date).

4.3.6. Statistical Analyses

All data were analyzed using SAS Enterprise Guide v.7.1 (SAS Institute, Cary, NC, USA). The mean proportion of species per treatment and replicate was used to calculate the number of each species collected. These numbers were then analyzed with a two-factor generalized linear model (Proc GENMOD), using a log-link function and a negative binomial distribution. Model factors were 'replicate' and 'treatment'. Pairwise comparisons between treatments used the 'lsmeans' statement with Tukey's adjustment. Data were not analyzed for species with low captures (i.e., < 0.1 proportion of total captures), and for the few female beetles that were captured in traps (i.e., < 0.02 proportion of total captures). For experiment 4, initial analyses indicated that data from the two study sites were not significantly different, and these data were therefore combined for final analysis.

4.4. Results

4.4.1. Trapping in Western Canada

More than 145,000 click beetle males were collected in Exps. 1–5, with the proportion of target species ranging from 0.00 to 0.75 (Table 4.2). Captures of *L. canus, L. infuscatus, L. californicus* and *A. lineatus* were not significantly reduced (P >0.05) when limoniic acid was mixed with the *A. lineatus* pheromone [capture ratio of limoniic acid to mixed lure: *L. canus* = 0.59 and 1.24 in experiments 1 and 4, respectively (Figs. 4.2A & 4.3I); *L. infuscatus* = 1.39, 1.38, 1.08 and 1.17 in experiments 1, 2, 3 and 4, respectively (Figs. 4.2B, E, G & 3J); *L. californicus* = 1.54 in experiment 4 (Fig. 4.3K); capture ratio *A. lineatus* pheromone to mixed lure: *A. lineatus* = 1.17, 0.85, 0.83, 1.23 and 1.18 in experiments 1, 2, 3, 4 and 5, respectively (Figs. 4.2D, F, H, 3L & N). Similarly, when limoniic acid was mixed with the *A. obscurus* pheromone, captures of *L. canus, L. infuscatus, L. californicus* and *A. lineatus* were not significantly reduced (capture ratio of limoniic acid to mixed lure: *L. canus* = 1.07 and 2.11 in experiments 4 and 5, respectively (Figs. 4.3I & M); *L. infuscatus* = 1.03 in experiment 4 (Fig. 4.3J); *L. californicus* = 1.54

in experiment 4 (Fig. 4.3K)]. Male *A. obscurus* were captured only in traps baited with either the *A. obscurus* pheromone alone or in combination with limoniic acid but capture data were too low to warrant analysis.

Overall, the data reveal no significant differences in captures of *L. infuscatus* or *A. lineatus* when traps were baited with either the species-specific pheromone alone or in combination with the heterogeneric pheromone (Figs. 4.2B & D-H). Trap captures of *A. lineatus* increased >15% when limoniic acid was added to the *A. lineatus* pheromone (Fig. 4.2, Exps. 2 & 3). Significantly more *L. infuscatus* males and *A. lineatus* males were captured in traps baited with the *A. lineatus* pheromone and with limoniic acid, respectively, than in unbaited control traps (Fig. 4.2; Exp. 2).

4.4.2. Trapping in Eastern Canada

Predominantly *A. mancus* males but also some *A. pubescens* and *A. sputator* males were captured in traps baited with the *A. mancus* pheromone (Fig. 4.4; Exp. 6). Mixing the *A. mancus* pheromone with limoniic acid significantly reduced captures of *A. mancus* males (capture ratio of *A. mancus* pheromone to mixed lure: 1.70 (Fig. 4.4P) but not of *A. pubescens* and *A. sputator* males (capture ratio: 1.30 and 1.33 for *A. pubescens* and *A. sputator* (Fig. 4.4Q & R), respectively. Captures of the three *Agriotes* species in traps baited with limoniic acid and left unbaited did not differ (Fig. 4.4). Captures of *L. agonus* beetles were too low for analysis.

4.5. Discussion

In western Canada, mixed trap lures containing both *Agriotes* and *Limonius* pheromones were as effective as species-specific lures in attracting and capturing male *Limonius canus, L. californicus, L. infuscatus,* and either *Agriotes lineatus* or *A. obscurus.* In eastern Canada, mixed trap lures containing both *Limonius and Agriotes mancus* pheromones attracted 40% fewer male *A. mancus* than the *A. mancus* specific lure, whereas lure type (mixed or species-specific) did not alter captures of *A. pubescens.*

As *L. agonus* males were nearly absent from the trapping location in eastern Canada, the effect of lure type on attraction and capture of *L. agonus* males could not be determined. The effect of mixed lures containing (*i*) the pheromones of all three eastern *Agriotes* spp. and limoniic acid, or (*ii*) the pheromones of *A. pubescens* and *A. sputator* and limoniic acid on attraction and captures of all target species including *L. agonus*, has yet to be determined. Overall, our data suggest that mixed pheromone lures can be developed for monitoring or mass trapping sympatric elaterid heterogeners, such as *A. lineatus* and *L. infuscatus* in the Interior of BC or on Vancouver Island. Compared to species-specific lures, mixed pheromone lures would require fewer traps and reduce the costs for lure preparation and trap deployment.

Prior to deploying mixed pheromone lures, multiple interrelated factors must be considered to optimize trapping efficiency, including dispenser type (e.g.; rubber septa, bubble caps, cotton pellet), lure dose, lure longevity and attractive range, as well as the mobility and activity period of target elaterid pests. The optimal dose for attraction of beetles would need to be determined for each specific dispenser type. Limoniic acid dispensed at a dose of 0.4–4 mg from a cotton pellet inside a low-density-polyethylene receptacle is highly attractive to *Limonius* species (van Herk et al. 2021a), whereas a 40-mg lure dose optimally attracts *A. lineatus* and *A. obscurus* (van Herk unpublished). Both dispenser type and lure dose affect lure longevity which is particularly important for trapping beetles with separate, non-overlapping seasonal activity periods. The attractiveness range for *A. lineatus* lures is 5–20 m (Traugott et al. 2015) but is still unknown for *Limonius* spp. The distance males cover in search for mates is still largely unknown but could be determined in mark-release-recapture studies.

Low captures of male *A. obscurus* and *L. agonus* in various experiments did not allow us to assess whether lure type (mixed or species-specific) affects beetle capture rates. The lack of *A. obscurus* captures at two study sites was likely due to low abundance of *A. obscurus* rather than repellency caused by nearby *A. lineatus* lures, because traps baited with *A. obscurus* or *A. lineatus* pheromone lures can be placed as little as 3 m apart without compromising the attractiveness of either lure (Vernon et al. 2014b). Low captures of male *L. agonus* could be attributed to underestimated beetle abundance in the

particular trapping site, or to traps being deployed too late in the season. The onset and duration of seasonal swarming by *L. agonus* are still not known but may be short and take place as soon as soil temperatures rise above 10 °C (van Herk et al., unpublished), as shown for western *Limonius* spp. (van Herk et al. 2021a; Lemke et al. 2022).

Understanding lure dose-dependent attraction of target elaterids is essential for the preparation of optimally effective mixed pheromone lures. Although a 40-mg lure dose is currently used to delineate the distribution range of *A. mancus* in eastern Canada (van Herk unpubl.), the optimal lure dose has yet to be determined. Our findings that mixed lures containing both limoniic acid and *A. mancus* pheromone attracted 40% fewer male *A. mancus* than the *A. mancus* specific lure could be due to the lower amount of *A. mancus* pheromone in the mixed lure which may have reduced lure longevity, even though ester release rates from both types of lures were identical for at least the first seven days. Follow-up studies should compare the attractiveness of mixed and species-specific lures when the amount of *A. mancus* pheromone is kept identical in either lure type. But even if mixed-pheromone lures for concurrent attraction of multiple species are somewhat less attractive for some target species than single-species pheromone lures (e.g., *L. californicus* in Kelowna; Fig. 4.1; *A. mancus* in Quebec; Fig. 4.4), mixed-pheromone lures could still be operationally viable. Diminished attractiveness of mixed-pheromone lures may be tolerable as long as trap captures reliably indicate species presence and threshold numbers that would trigger control measures.

Attraction of both male *A. pubescens* and male *A. sputator* to the *A. mancus* pheromone blend can be attributed to geranyl butanoate in that blend which is a pheromone component of both *A. sputator* (Toth 2013; Singleton et al. 2022b) and *A. pubescens* (van Herk, unpubl), and of several other *Agriotes* species (Toth et al. 2008; Vuts et al. 2018).

In conclusion, our study provides proof of concept that mixed pheromone lures containing synthetic pheromones of elaterid heterogeners can be as effective as species-specific lures in attracting target species. While here we tested mixed pheromone lures comprising binary combinations of *Agriotes* and *Limonius* pheromones, many other binary or even ternary combinations of heterogeneric elaterid pheromones are

conceivable for testing in future studies. If successful, mixed pheromone lures could be developed for cost-effective integrated elaterid pest management.

Table 4.1. List of experiment numbers (Exp. #), cities near study locations, geographic coordinates (latitude, longitude) of study sites in British Columbia (BC) and Quebec (QC), trapping durations (in 2020* and 2021), numbers of Vernon pitfall traps ® deployed, and total numbers of *Limonius* spp. and *Agriotes* spp. captured in all traps.

Exp. #	Cities	Geographic coordinates	Trapping period (days)	Traps	Beetles captured
Study 1					
1	Kelowna, BC	49.821284, -119.441011	25 April – 16 May (21)*	16	10,063
2	Courtenay, BC	49.732937, -125.018113	04 May – 01 June (28)	32	67,982
3	Duncan, BC	48.735409, -123.669593	03 May – 02 June (30)	32	31,567
Study 2					
4	Kelowna, BC	49.868836, -119.443256	14 April – 18 May (34)	42	29,627
5	Pemberton, BC	50.429281, -122.914445	12 April – 13 May (31)	48	6,506
Study 3					
6	Saint-Mathieu-de-Beloeil, QC	45.585074, -73.2464447	3 May – 18 June (46)	32	365

Table 4.2. Mean proportion of male *Limonius canus, L. californicus, L. infuscatus, Agriotes lineatus, A. obscurus, A. mancus, A. pubescens,* and *A. sputator* captured per pheromone treatment and control in each study and experimental location in British Columbia (BC) and Quebec (QC) (Exps. 1–6).

			Mean proportion of species captured per control and pheromone treatment					
Exp. #	Location	Species captured	Control	*A. lineatus* pheromone	*Limonius* spp. pheromone	*A. lineatus* + *Limonius* spp. pheromone	*A. obscurus* pheromone	*A. obscurus* + *Limonius* spp. pheromone
Study 1								
1	Kelowna, BC	*L. canus*	0.03	0.43	0.20	0.34		
		L. californicus	0.00	0.03	0.65	0.32		
		L. infuscatus	0.00	0.02	0.57	0.41		
		A. lineatus	0.00	0.54	0.00	0.46		
		A. obscurus	0.00	0.25	0.00	0.75		
2	Courtenay, BC	*L. infuscatus*	0.00	0.00	0.58	0.42		
		A. lineatus	0.01	0.45	0.01	0.53		
3	Duncan, BC	*L. infuscatus*	0.00	0.00	0.52	0.48		
		A. lineatus	0.02	0.44	0.01	0.53		
Study 2								
4	Kelowna, BC	*L. canus*	0.06	0.07	0.31	0.25	0.02	0.29
		L. californicus	0.01	0.00	0.43	0.28	0.00	0.28
		L. infuscatus	0.00	0.01	0.35	0.30	0.00	0.34
		A. lineatus	0.00	0.54	0.02	0.44	0.00	0.00
		A. obscurus	0.00	0.00	0.00	0.00	0.71	0.29
5	Pemberton, BC	*L. canus*	0.05	0.05	0.19	0.53	0.09	0.09
		A. lineatus	0.00	0.53	0.00	0.45	0.01	0.01
		A. obscurus	0.00	0.00	0.01	0.00	0.61	0.37
Study 3			Control	*A. mancus* pheromone	*Limonius* spp. pheromone	*A. mancus* + *Limonius* spp. pheromone		
6	Saint-Mathieu-de-Beloeil, QC	*A. mancus*	0.00	0.63	0.00	0.37		
		A. pubescens	0.00	0.56	0.01	0.43		
		A. sputator	0.00	0.57	0.00	0.43		

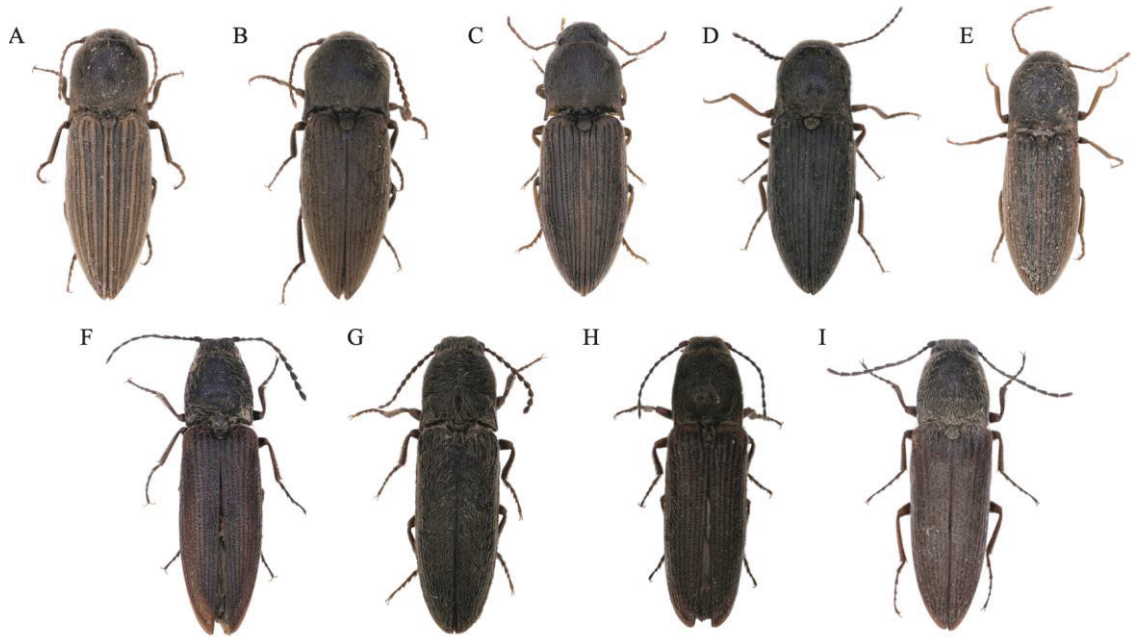

Figure 4.1. Photographs of (A) *Agriotes lineatus,* (B) *A. obscurus,* (C) *A. mancus,* (D) *A. pubescens,* (E) *A. sputator,* (F) *Limonius canus,* (G) *L. californicus,* (H) *L. infuscatus,* (I) *L. agonus.* Photo credits: Julien Saguez

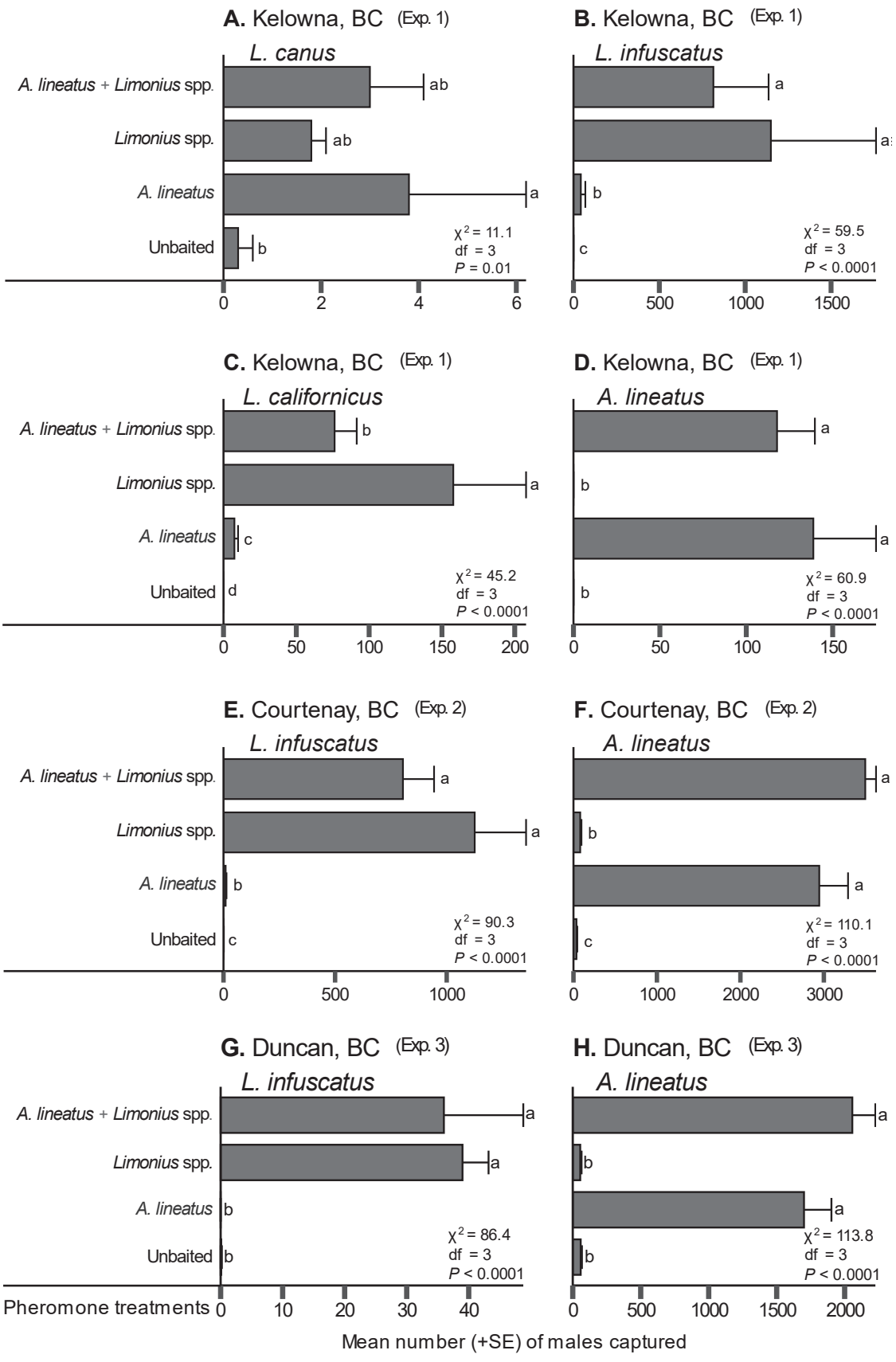
A. Kelowna, BC (Exp. 1)
L. canus
A. lineatus + Limonius spp.
Limonius spp.
A. lineatus
Unbaited
ab
ab
a
b
χ2 = 11.1
df = 3
P = 0.01
0
2
4
6
B. Kelowna, BC (Exp. 1)
L. infuscatus
a
a
b
c
χ2 = 59.5
df = 3
P < 0.0001
0
500
1000
1500
C. Kelowna, BC (Exp. 1)
L. californicus
b
a
c
d
χ2 = 45.2
df = 3
P < 0.0001
0
50
100
150
200
D. Kelowna, BC (Exp. 1)
A. lineatus
a
b
a
b
χ2 = 60.9
df = 3
P < 0.0001
0
50
100
150
E. Courtenay, BC (Exp. 2)
L. infuscatus
a
a
b
c
χ2 = 90.3
df = 3
P < 0.0001
0
500
1000
F. Courtenay, BC (Exp. 2)
A. lineatus
a
b
a
c
χ2 = 110.1
df = 3
P < 0.0001
0
1000
2000
3000
G. Duncan, BC (Exp. 3)
L. infuscatus
a
a
b
b
χ2 = 86.4
df = 3
P < 0.0001
0
10
20
30
40
H. Duncan, BC (Exp. 3)
A. lineatus
a
b
a
b
χ2 = 113.8
df = 3
P < 0.0001
0
500
1000
1500
2000
Pheromone treatments
Mean number (+SE) of males captured

Figure 4.2. Mean (+ SE) captures of three *Limonius* spp. and *Agriotes lineatus* in traps baited with *Limonius* spp. pheromone ((*E*)-4-ethyloct-4-enoic acid (limoniic acid); 4 mg), *A. lineatus* pheromone (geranyl octanoate & geranyl butanoate at a 1:1 ratio; 40 mg) or both (2 mg limoniic acid + 20 mg *A. lineatus* pheromone). For each of subpanels A–H, bars with different letters indicate statistically significant differences between in trap captures (χ^2 tests); low capture data of *A. obscurus* in Kelowna, BC (< 2 mean beetle captures per treatment) were not statistically analysed and did not warrant graphical illustrations.

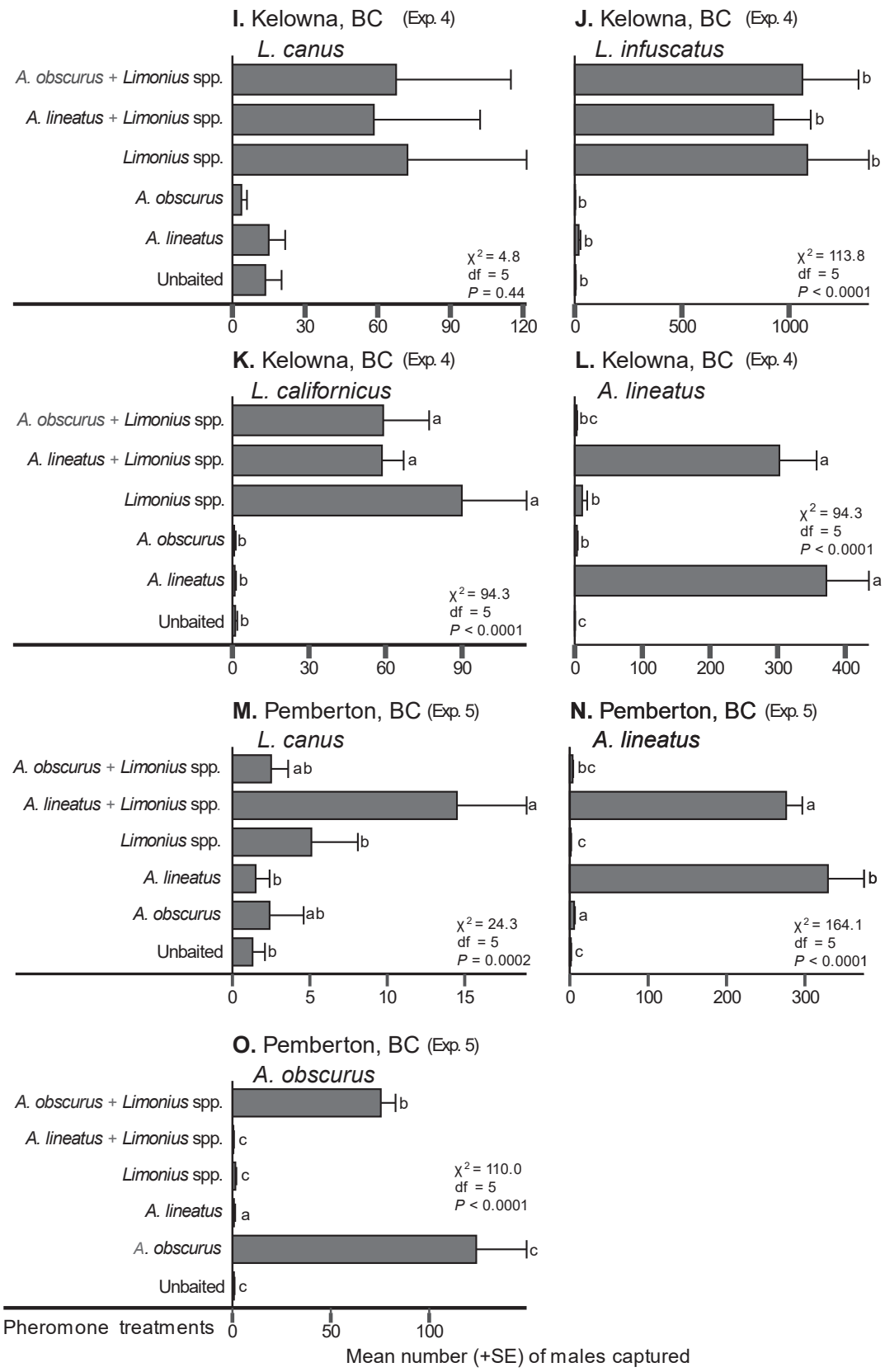
I. Kelowna, BC (Exp. 4)
L. canus
A. obscurus + Limonius spp.
A. lineatus + Limonius spp.
Limonius spp.
A. obscurus
A. lineatus
Unbaited
χ2 = 4.8
df = 5
P = 0.44
0 30 60 90 120
J. Kelowna, BC (Exp. 4)
L. infuscatus
b b b b b b
χ2 = 113.8
df = 5
P < 0.0001
0 500 1000
K. Kelowna, BC (Exp. 4)
L. californicus
a a a b b b
χ2 = 94.3
df = 5
P < 0.0001
0 30 60 90
L. Kelowna, BC (Exp. 4)
A. lineatus
bc a b b a c
χ2 = 94.3
df = 5
P < 0.0001
0 100 200 300 400
M. Pemberton, BC (Exp. 5)
L. canus
ab a b b ab b
χ2 = 24.3
df = 5
P = 0.0002
0 5 10 15
N. Pemberton, BC (Exp. 5)
A. lineatus
bc a c b a c
χ2 = 164.1
df = 5
P < 0.0001
0 100 200 300
O. Pemberton, BC (Exp. 5)
A. obscurus
b c c a c c
χ2 = 110.0
df = 5
P < 0.0001
0 50 100
Pheromone treatments
Mean number (+SE) of males captured

Figure 4.3. Mean (+ SE) captures of three *Limonius* spp. and two *Agriotes* spp. in traps baited with *Limonius* spp. pheromone ((*E*)-4-ethyloct-4-enoic acid (limoniic acid); 4 mg), *Agriotes lineatus* pheromone (geranyl octanoate & geranyl butanoate at a 1:1 ratio; 40 mg), *A. obscurus* pheromone (geranyl hexanoate & geranyl octanoate at a 1:1 ratio; 40 mg), and binary combinations of limoniic acid (2 mg) and either *A. lineatus* pheromone (20 mg) or *A. obscurus* pheromone (20 mg). For each of subpanels I–O, bars with different letters indicate statistically significant differences between trap captures (χ^2 tests); low capture data of *A. obscurus* in Kelowna, BC (< 2 mean beetle captures per treatment) were not statistically analysed and did not warrant graphical illustrations.

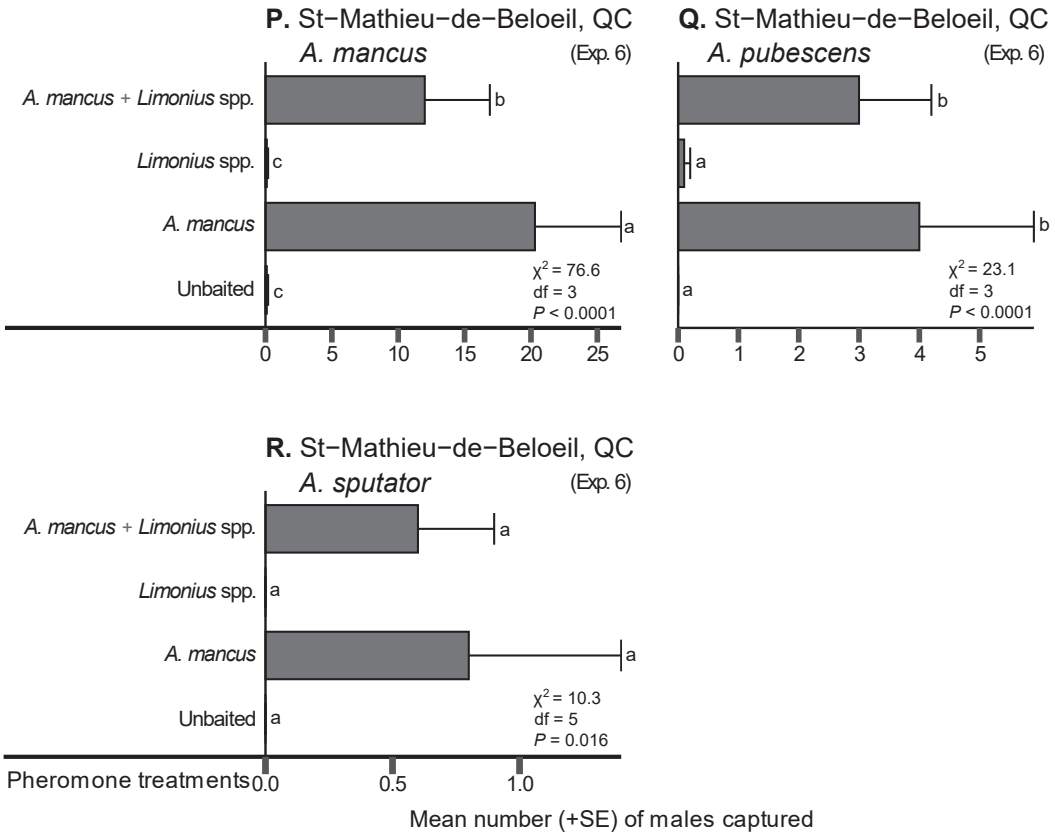

Figure 4.4. Mean (+SE) captures of two *Agriotes* spp. in traps baited with *Limonius* spp. pheromone ((*E*)-4-ethyloct-4-enoic acid (limoniic acid); 4 mg), *Agriotes mancus* pheromone (geranyl hexanoate & geranyl butanoate at a 1:1 ratio; 40 mg) or both (2 mg limoniic acid + 20 mg *A. mancus* pheromone). For each of subpanels P–R, bars with different letters indicate statistically significant differences between in trap captures (χ^2 tests); low capture data of *Limonius agonus* (< 2 mean beetle captures per treatment) were not statistically analysed and did not warrant a graphical illustration.

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