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TECHNICAL NOTE

Sexing live mountain pine beetles *Dendroctonus ponderosae*: refinement of a behavioral method for *Dendroctonus* spp.

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Introduction

Members of the genus *Dendroctonus* (Coleoptera: Curculionidae, Scolytinae) are some of the most aggressive tree-killing bark beetles in the world. As such, much research on this genus has been undertaken to understand the factors that affect the population dynamics of these insects (Six & Bracewell, 2015; Aukema et al., 2016). Despite biome-level ecological impacts of the most aggressive members of this genus when at outbreak levels, the flight periods of many temperate species are constrained to just a few weeks of peak emergence during which beetles locate and procure hosts via pheromone-mediated mass attacks (Rudinsky, 1962; Raffa, 2001; Bentz et al., 2014). Females initiate boring into a host. Thus, for many manipulative laboratory and field experiments assessing reproduction or host selection, the ability to quickly and accurately determine the sex of live insects is required.

Sexual dimorphism on the frons and pronotum is present in some species of *Dendroctonus*, most prominently in those species closely related to the southern pine beetle (*Dendroctonus frontalis* Zimmermann) (Wood, 1982), and provide varying degrees of accuracy in determination (Osgood & Clark, 1963; Tate & Bedard, 1967). The only consistently 100% accurate method of sex determination via secondary characters for *Dendroctonus* spp. requires examination of adults for the presence of a highly sclerotized plectrum on the seventh abdominal tergite (Lyon, 1958; Safranyik & Carroll, 2006). This plectrum is used for stridulation by males but absent in females. This morphological character is highly accurate (Lyon, 1958; Jantz & Johnsey, 1964; Godbee & Franklin, 1978), but can pose challenges when working with live insects (Tate & Bedard, 1967). For example, female mountain pine beetles (*Dendroctonus ponderosae* Hopkins) tend to draw their abdomens tight against the elytra when prodded. Squeezing the abdomen or manipulating its position with a metal probe under a dissecting microscope (McCambridge, 1962) can extend handling times and result in harm to the insect (Godbee & Franklin, 1978). Morphological examinations remain a popular technique, however, and work reliably when executed properly.

Several authors have tested the efficacy of stridulatory behavior as a potential method for sex determination of live *Dendroctonus* beetles (Table 1). When disturbed, males will use stridulation to produce predominantly simple 'stress' chirps that are characterized by rapid short bursts (McCambridge, 1962; Michael & Rudinsky, 1972; Fleming et al., 2013). Chirps are produced as males move the plectrum against the pars stridens on the underside of the elytra (Hopkins, 1909; Michael & Rudinsky, 1972). Female *Dendroctonus* spp. beetles are also able to stridulate, using a different stridulatory apparatus, but their short, simple chirps, characterized by a low sound pulse rate, are easily differentiated from the rapid chirping and higher sound pulse rates of males (Barr, 1969; Rudinsky & Michael, 1973; Yturralde & Hofstetter, 2015). Sonic emissions of female *Dendroctonus* spp. are typically restricted to courtship behaviors, though a stress response has been detected in the red turpentine beetle (*Dendroctonus valens* LeConte) and the larger Mexican pine beetle (*Dendroctonus approximatus* Dietz) (Ryker & Rudinsky, 1976a; Yturralde & Hofstetter, 2015). The ability to chirp likely confers reproductive advantage to the joining sex, as this trait has been independently gained in both *Dendroctonus* and *Ips* (Barr, 1969; Lewis & Cane, 1990), and is conserved among males across *Dendroctonus* spp. (Ryker, 1988).

Though audible observations of stridulation can be useful for sexing adults of *Dendroctonus* spp., some error is

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Table 1 Results of this and other studies assessing accuracies of sex determination in *Dendroctonus* spp. by a single assessment of audible stridulation

Dendroctonus species	Mean (± SEM) % of total beetles correct[1]	n	Reference
D. brevicomis	87.5 ± 1.2	741	Tate & Bedard (1967)
D. frontalis	82.0 ± 3.8	100	Osgood & Clark (1963)
D. ponderosae	97.7 ± 0.6	663	This study
D. pseudotsugae	97.5 ± 1.6	100	Jantz & Johnsey (1964)
D. terebrans	94.7 ± 1.3	300	Godbee & Franklin (1978)

[1]To standardize reporting, we report means of the datasets utilized. For example, if the paper reports results of experienced vs. inexperienced worker, we report the mean of the results.

common, which has reduced its use among researchers. Reported accuracy has varied between species. For example, up to 97.5% accuracy in sex determination has been obtained by listening for male stress chirps in Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) (Jantz & Johnsey, 1964). Up to 92% accuracy has been achieved in identifying female black turpentine beetles [*Dendroctonus terebrans* (Olivier)] based on females being silent (43%) or producing a low-pitch rasping sound (57%), with error occurring due to silent males (Godbee & Franklin, 1978). This 'low-pitch rasping' may be similar to the stress response reported in the closely related female red turpentine beetles (Ryker & Rudinsky, 1976a). For the western pine beetle (*Dendroctonus brevicomis* LeConte), 85–90% of males and <1% of females stridulate (Tate & Bedard, 1967). Only 82% accuracy was reported for *D. frontalis*, although misidentifications may have been exacerbated by experimental design issues acknowledged by the authors (Osgood & Clark, 1963). Inaccuracies most frequently result from some males remaining silent and not due to females chirping (Tate & Bedard, 1967; Godbee & Franklin, 1978). In summary, although this method can be fairly accurate, improvements to audible examination of insects would likely enhance further employment of this technique.

A related approach to determine the sex of adult bark beetles is to look at, not listen to, stridulatory movements from males under low magnification. Specifically, the abdomen will make rapid stridulatory movements when the insect is disturbed by touching (McCambridge, 1962) or dropping (Lyons, 1982). This method has been used most successfully in the native elm bark beetle, *Hylurgopinus rufipes* (Eichhoff), with accuracies of 99.5% (Lyons, 1982). A similar method has been used with mountain pine beetles, for which observation of these movements has proven to be 95.9% accurate at distinguishing adult males from females (McCambridge, 1962). However, observation of stridulatory movements is less accurate than listening for audible chirps in other *Dendroctonus* spp. (Tate & Bedard, 1967; Godbee & Franklin, 1978). To our knowledge, although many investigators familiar with mountain pine beetle have used the audible method for sexing beetles (Hynum & Berryman, 1980; Raffa & Berryman, 1982; Fleming et al., 2013), no studies have explicitly examined the accuracy of using auditory male stress chirps for sexing mountain pine beetles.

Materials and methods

We have found in our work with mountain pine beetle that auditory examination can identify males and females with >99% accuracy, especially when subjecting initial cohorts to a second or third assessment that reveals males which previously remained silent. This repetition can be integrated easily into previously designed protocols to reduce the time needed for sexing relative to morphological examinations (Lyon, 1958). For example, beetles can be sexed initially during collections and placement in storage containers. A second assessment of purported females can be quickly completed during handlings for experiments. Alternatively, beetles can be sexed in a production line scenario with multiple workers sexing the same beetles.

Table 2 Classification accuracy of the sex of adult mountain pine beetles according to the number of times beetles were assessed for stridulatory chirping

		First assessment		Second assessment		Improvement
Source	n	% correct males (n)	% correct females (n)	% correct males (n)	% correct females (n)	% reclassified females (n)
Field-captured	292	100 (139)	95.4 (153)	100 (142)	97.3 (150)	2.0 (3)
Laboratory-reared	371	100 (117)	96.9 (254)	100 (121)	98.4 (250)	1.6 (4)

To determine the sex of mountain pine beetles using audible chirping signals, a beetle is held gently by the margins of the pronotum between thumb and forefinger, with hindwings fully folded, the abdomen facing up, and the ventral side facing out. Holding them in this way will elicit stress chirps from males (Fleming et al., 2013). These chirps can be detected by holding the insect within 10 cm of the worker's ear (Fleming et al., 2013) especially when tapping the side of the thumb holding the beetle with the forefinger of the other hand 5–10×. This additional stimulation will often elicit chirps from initially silent beetles. We have found that sex can be determined at a rate of ca. seven beetles per minute, or 400–500 beetles per h, though additional time to allow for delayed response may increase accuracy.

To investigate the accuracy of audible sexing, mountain pine beetles were captured in the Black Hills of South Dakota, USA (44°7′N, 103°34′W). Lindgren funnel traps were baited with mountain pine beetle lure (Contech Enterprises, Delta, BC, Canada) in ponderosa pine (*Pinus ponderosa* Douglas ex C Lawson) forests during peak flight in mid-August 2013. Beetles were also sourced from infested logs in 2015. Beetles emerged naturally or were removed from the logs once peak emergence had passed. If beetles had been stored in a refrigerator, they were allowed to warm at room temperature before being processed. Sexing was accomplished by listening 2–3× for stridulatory chirps in all beetles tested, as described above. Males and females were placed in separate containers each time they were assessed. A minimum of 3 min was allotted between each assessment of the same beetle. After 2–3 consecutive assessments, beetles were moved to a freezer to be killed for later sex verification using the morphology of the seventh abdominal tergite (Lyon, 1958). To determine whether our method improves upon previous reports on stridulation in mountain pine beetles, we compared our audible stridulatory results with the visual stridulatory movement results reported by McCambridge (1962). For this analysis, we used a generalized linear logistic model in R v. 3.1.0 (R Development Core Team, Vienna, Austria) with the 'lme4' package for binomial data. The bimodal response variable was the accurate or inaccurate identification of sex based on the presence or absence of chirps (our data) or abdominal movements (data from McCambridge, 1962).

Results and discussion

In total, 1 095 male and female mountain pine beetles were used to evaluate the accuracy of audible sexing with repeated assessments. All beetles that produced rapid chirps were accurately classified as males during the initial assessment with no false positives (Table 2). Initial accuracy of sex classification was 97–98% for both field and laboratory-sourced individuals, and was not significantly different from results reported by McCambridge (1962), who used a visual stridulatory technique (field-captured: $\chi^2 = 1.82$, $P = 0.18$; laboratory-reared: $\chi^2 = 2.87$, $P = 0.09$, both d.f. = 1). The proportion of beetles accurately identified as females improved with an additional assessment as previously silent males were identified. The accuracy of sexing after two assessments using an auditory technique was significantly greater than the 95.9% accuracy of a single visual identification reported by McCambridge (1962) for both our field-captured (98.6%; $\chi^2 = 5.46$, d.f. = 1, $P = 0.019$) and laboratory-emerged (98.9%; $\chi^2 = 8.43$, d.f. = 1, $P = 0.0037$) beetles.

Using a separate cohort of 432 field-captured beetles, we found that accuracy of female identification was 99.1% after three assessments. As we did not measure results after one or two assessments for this group, we exclude this cohort from Table 2.

We suspect the initial or total silence of some males may have been due to a combination of obstructions to the stridulatory apparatus, e.g., incomplete folding of the hindwings, malformed stridulatory structures, or other physical damage. Indeed, a damaged or malformed male, unable to stridulate, would unlikely be accepted by a female, as stridulation is required for mate acceptance (Ryker & Rudinsky, 1976b). Thus, female rejection of silent males may serve as a means of ensuring male physical or genetic fitness and ability to assist in gallery formation. This study suggests that such reduced fitness may be present in as many as 2–3% of males.

Further research should examine whether repetitive assessments can increase accuracies in sex determination among other *Dendroctonus* spp. (Table 1), and whether male production of rapid stress chirps can be used for sex differentiation in other groups of bark beetles. The morphology of the pars stridens and plectrum are similar across the tribe Hylurgini, to which the genus *Dendroctonus* belongs, and stress signals have been observed in members of other groups within the tribe (Rudinsky & Vallo, 1979; Oester et al., 1981; Lyons, 1982; Swedenborg et al., 1989). The male stress chirp may be a basal characteristic across *Dendroctonus* spp., as this behavior has been observed in all *Dendroctonus* spp. tested to date (Table 1; Ryker, 1988; Yturralde & Hofstetter, 2015), spanning most of the clades within the genus (Reeve et al., 2012).

Although researchers have been aware of the audible approach to sexing adult mountain pine beetles for some time, our study is the first to experimentally demonstrate the reliability of this method and demonstrate how audible sexing can be improved. Our results indicate that beetles sorted as males can be 100% accurate after just one assessment, and female accuracy can approach 100% after 2–3 assessments. We show that inaccuracy in sexing by listening to stridulatory chirping is due to males that remain silent and thus are wrongfully identified as females. However, assuming they are in good condition, silent males will not necessarily remain silent in additional assessments.

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