**Abstract**

Ants and other insects are often an overlooked source of localised secondary dispersal for a variety of plants and play an important ecological role in the dispersal of plant propagules. While there is an increasing amount of literature on insect-mediated secondary dispersal, there is limited information regarding how climate change will affect it, as quantifying this dispersal can be quite challenging in the first place. Here, we seek to investigate the effects of climate change on insect-mediated secondary dispersal by examining the effects of increased growing temperatures on how attractive seeds from the invasive thistles *Carduus nutans* and *Carduus acanthoides* are to secondary insect dispersers. To accomplish this, we use “cafeteria” experiments to observe seed removal events from controlled locations and estimate the effects of warming treatments on rates at which seeds are removed. Our results indicate that seeds from maternal plants grown at temperatures 0.6 °C higher than normal are more attractive to insect dispersers than their unwarmed counterparts, indicating that warming treatment on individuals affected the rates at which their seeds were removed. We also observe that seed elaiosomes in these two species play an important role in dispersal, as seeds that had their elaiosomes removed were significantly less likely to be taken by insects. These results provide evidence that climate change may alter aspects of the dispersal process such as seed removal, which could possibly affect how plants disperse their seeds in a changing world.

**Introduction**

For sedentary organisms such as plants, seed dispersal is essential to the movement of populations because it is the only stage in the life cycle that movement can occur. While plant propagules are often dispersed by abiotic vectors such as wind, water, and gravity, they are often dispersed biotically by organisms such as insects, birds, and mammals. For example, the seeds of the mahaleb cherry *Prunus mahaleb* are ingested, moved, and defecated by a variety of mammals such as foxes and badgers as well as birds such as crows, thrushes, warblers, and robins (Herrera and Jordano 1981; Guitián *et al*. 1992; Jordano *et al*. 2007). The acorns of various oak trees (*Quercus* sp.) are often dispersed and cached by a variety of rodents such as mice and squirrels (Jensen and Nielsen 1986; Vander Wall 2001; Gómez *et al*. 2008). Some species can even be dispersed by carnivores when they prey on frugivores or granivores with seeds in their digestive tract (Hämäläinen *et al*. 2017). Humans are even involved in the process of dispersing plant propagules, as numerous plants can be dispersed when propagules attach to clothing (Wichmann *et al*. 2009; Ansong and Pickering 2014), are caught in air currents generated by passing vehicles (Von Der Lippe *et al*. 2013) or on the vehicles themselves (Veldman and Putz 2010; Taylor *et al*. 2012), as a contaminant of horticultural stock (Hodkinson and Thompson 1997), and as impurities in agricultural produce such as grains (Shimono and Konuma 2008; Michael *et al*. 2010; Wilson *et al*. 2016). The prevalence in ecological literature of studies regarding these sorts of dispersal indicates a growing interest in identifying and quantifying dispersal by organisms such as insects, animals, and humans.

Such dispersal of seeds by biotic vectors typically occurs not in isolation, but as part of a larger network of parallel and serial dispersal events for any given species. In the *Prunus mahaleb* example listed earlier, while seeds are often consumed directly from the tree by birds before primary dispersal via gravity, they may also be consumed off the ground and undergo secondary dispersal by birds and mammals after primary dispersal via gravity (SOURCE). Integration of these primary and secondary pathways and the vectors responsible into a total dispersal kernel, or probability distribution of how far seeds are dispersed when accounting for all possible dispersal vectors, can help us quantify how plant populations expand at a variety of different spatial scales (Nathan 2007). However significant challenges in identifying these biotic dispersers and quantifying how they disperse seeds make it challenging to fully understand their role in seed dispersal, especially for smaller seeds that cannot be easily tracked through empirical means (Rogers *et al*. 2019).

For these smaller seeds, ants and other insects are often an overlooked source of localised secondary dispersal and are responsible for moving seeds after they have been dispersed by wind, water, gravity, or another biotic dispersal vector (Vander Wall and Longland 2004). In particular, ant-mediated seed dispersal, or myrmecochory, has been shown to be a common occurrence in many plant species. In more than 80 plant families across the world, ant-dispersed plant species contain seeds that bear an elaiosome, a small structure on the seed achene that facilitates dispersal by ants (Edwards *et al*. 2006). The elaiosome has a high lipid content and likely serves as a reward for the ant dispersing the seed (Brew *et al*. 1989; Hughes and Westoby 1990, 1992), as ants will typically take the seeds back to their nest and consume the elaiosome, then store the seed achene in a midden (Berg 1975; Culver and Beattie 1978). This dispersal can be advantageous for a variety of different reasons, including moving propagules to areas with better germination and growing conditions as well as less competition from other plants (Handel and Beattie 1990).

Investigations of such ant-mediated secondary dispersal, as well as the primary dispersal that precedes it, can be possibly complicated by climate change, as climate change may affect various aspects and characteristics of these dispersal processes. For example, changes in the number of seeds that plant can produce may result from elevated growing temperatures (Sato *et al*. 2006, Hedhly *et al*. 2009, Caignard *et al*. 2017) and increased CO2 levels (Edwards *et al*. 2001, Thürig *et al*. 2003, Way *et al.* 2010). The dynamics of seed release can also be affected by increased growing temperatures (Teller *et al*. 2016). Seed shape and size can also be affected (SOURCES), which can have implications for both abiotic and biotic dispersal distances. Climate change may also cause shifts in the range or abundance of species that disperse a certain species’ seeds, as well as shifts in the traits that attract animal dispersers, and may thus affect its dispersal capabilities (Ruxton and Schaefer 2012, Mokany *et al*. 2014, Sales *et al*. 2021). Climate change also has the potential to affect seed nutrient content and a variety of studies, especially on agricultural crops, have shown changes in nutrient allocations and chemical makeup due to factors such as increased temperatures or elevated CO2 (Thomas *et al*. 2003, Caldwell *et al*. 2005, Singh *et al*. 2013). However, there is very little research on how these changes in seed shape, size, or nutrient allocations affect seed attractiveness to dispersers; more of the research addressing the effects of climate change on dispersal is focused on seed production and abiotic vectors of dispersal, with less known about how climate change may affect the dispersal of seeds by insects, animals, and humans.

Given the role that ants and other insects play in seed dispersal, a better understanding of how they move seeds and how far they do it can help us better understand how plant species dispersed by these insects spread. Such an understanding starts with quantifying seed removal, as this is the first step of dispersal via ants and other insects. A commonly used and straightforward experimental setup to quantify seed removal by secondary dispersers consists of so-called “cafeterias” (SOURCES), where seeds are aggregated in various locations and the number of seeds removed is closely monitored.

Here, we seek to develop a better understanding of how seeds are moved by secondary dispersal vectors, namely ants, with a focus on investigating the first step of this secondary dispersal process: seed removal. We also seek to explore the role of the elaiosome, as well as whether increased growing temperatures on maternal plants, in seed removal rates and overall attractiveness to insect dispersers. We thus investigate three questions. First, what are the approximate rates of seed removal via insect after a seed has experienced primary dispersal? Second, does the seed elaiosome affect the rate of removal? And third, are seeds from maternal plants that experienced increased growing temperatures any more or less likely to be removed by insects than their unwarmed counterparts?

**Materials and Methods**

***Study Species***

*Carduus nutans* L. (“musk thistle” or “nodding thistle”) and *Carduus acanthoides* L. (“plumeless thistle”) are two closely-related invasive thistles in the Asteraceae family. Both species germinate in autumn or spring and bolt in the early summer (Zhang *et al*. 2012), reproduce exclusively by seed, and have monocarpic perennial life cycles that have been demonstrated to shift from biennial towards annual under warming conditions (Keller and Shea, *in press*). While sharing similar life histories, the two species display significant morphological differences in dispersal-related characteristics such as number of flower heads, flower head size, number of seeds produced per flower head, and distribution of flower heads across an individual (Desrochers *et al*. 1988). These invasive thistles have high reproductive potential and are a considerable agricultural pest since they thrive in pastures, are unpalatable to most grazers, and decrease pasture productivity (Trumble and Kok 1982). In addition to pastures, these thistles occur in other highly disturbed areas such as drainages and roadsides. Both species can be found across the U.S. and often co-occur (Allen and Shea 2006), and are listed as noxious weeds in several states (Skinner *et al.* 2000).

Wind serves as the primary dispersal vector in both *C. nutans* and *C. acanthoides*, as seeds of both species display a prominent pappus that increases hang time and makes it possible for them to be carried at long distances. However, dispersal of these seeds also extends beyond wind, with paths of secondary dispersal possible after seeds have hit the ground. Seeds from both species contain elaiosomes that are thought to play a role in ant-mediated dispersal (Pemberton and Irving 1990), and have been documented to be moved by insects and small mammals, with insects such as ants, crickets, and grasshoppers likely playing a significant role in the movement of seeds (Jongejans et al. 2015).

***Seed collection and preparation***

*C. nutans* and *C. acanthoides* from which seeds were harvested were grown under control and ambient warming treatments as part of an experiment in Drees and Shea (*in prep.*) to estimate the effects of warming on the distribution of flower heights; this publication discusses the methods and experimental setup in greater detail. In this experiment, individuals of each species were grown in a grid-like arrangement, and a subset of individuals within each species were randomly assigned a fibreglass open-top chamber. These chambers, built to specifications of the International Tundra Experiment Manual (Molau and Mølgaard 1996), have been demonstrated to increase the ambient temperature by approximately 0.6 °C (Zhang *et al*. 2011) without significantly affecting other possible growth factors such as soil moisture and snow depth. When flowers set seed, mesh pollen bags were wrapped around the flowers endure that seeds from these invasive thistles did not escape and contaminate other parts of the study area, as seeds can stay dormant for years and could pose problems for future experiments once germination occurs.

Once individuals completed their life cycle or collapsed under their own weight, they were cut down, and bagged seed heads were collected from all individuals that produced five or more viable flowers. During this process, seed heads from all individuals of the same species were mixed together to randomise possible differences in seed size, shape, nutrient content, and any other factors that vary between individuals and may affect seed attractiveness to insects. The mesh pollen bags were then removed from the decapitated seed heads, and the heads were placed in a large paper bag and allowed to desiccate in a dry, low-light environment at approximately 24 °C. After approximately a month of desiccation, the bags were sealed and shaken vigorously to separate the seeds from the seed heads and pappi from the seeds; separated seeds were then sifted to remove spines, pappi, and other plant debris, and were then stored in an airtight container.

After seeds were successfully extracted, they were irradiated to ensure they could be freely dispersed by ants and other insects without spreading these invasive thistles and contaminating study site. Seeds from *C. nutans* and *C. acanthoides* were irradiated using a 1000 KR dosage of gamma radiation; this dosage was demonstrated by Jongejans *et al*. (2014) to prevent any germination without affecting how attractive seeds from either of these species are to insects.

Once seeds were irradiated, a subset of seeds from each species and warming/ambient treatment were assigned an elaoisome removal treatment. Elaiosomes were removed my holding the seed with a pair of forceps, aligning the flattest side of the seed parallel to the workbench, and then using a teasing needle to press the elaiosome against the workbench until it was severed from the seed achene. In instances where the elaiosome did not cleanly separate, the teasing needle was used to gently dig out the remainder of the elaiosome from the achene. Seeds not receiving the elaiosome removal treatment were handled with forceps in the same manner mentioned above, for approximately the same duration as the seeds whose elaiosomes were removed; this was done so that possible differences in experimental outcomes could not be attributed to differences in the way the seeds were handled. After seeds had their elaiosomes removed and the control seeds were handled appropriately, seeds were again placed in airtight containers based on treatment type.

Seed depots were then constructed to serve as points where insects could easily access and remove seeds. All depots were constructed in a similar manner to those in Jongejans *et al*. (2014), using 95 mm diameter petri dishes with a 15 cm tall edge and placing black sandpaper at the bottom to mimic the appearance of soil. Sandpaper was adhered to the bottom of the petri dish in order to negate any warping or movement of the paper due to wind, moisture, sunlight exposure, or insect activity. Note that unlike in Jongejans *et al*. (2014), we do not seek to exclude specific types of insects as a treatment, so Tanglefoot was not used when constructing these seed depots. Once the depots were constructed, each one was loaded with 25 seeds placed near the centre of the petri dish, though spread out enough so that seeds were not piled on top of each other. All depots were prepared in a controlled environment and were transported to the field site, rather than being constructed *in situ*.

***Experimental setup***

The seed removal experiment was conducted at the Russell E. Larson Agricultural Research Farm in Rock Springs, Pennsylvania, which is the same study site at which the experiment from which the seeds were derived was conducted. All seed depots were arranged over an 8 x 10 grid in a small field approximately 50 m from the location of the warming experiment, with individual seed depots spaced 1 m apart. Seeds within a particular seed depot represented one of eight unique combinations of species (*C. nutans* vs *C. acanthoides*), warming treatment (warmed vs unwarmed), and elaiosome treatment (present vs absent), and each row of eight depots contained a randomised arrangement of these eight unique treatment combinations. Before placing each depot, the small patch of vegetation below was trimmed and lightly compacted so that the petri dish could be snugly fit in the grass canopy, reducing the chance of depots being disturbed by wind and ensuring that insects could access them.

Once all seed depots were placed, each seed depot was photographed from directly above by an observer in 30-minute intervals for the first 12 hours, from 09:00 to 21:00 on 07 September 2020; after 12 hours had elapsed, depots were then photographed once at 24 hours, again at 36 hours, and then once more at 48 hours. In instances where the sandpaper bottom of the dish was disturbed, it was fixed to the bottom of the petri dish again, and the remaining seeds gently placed back around the centre of the seed depot; such instances were extremely uncommon, though. No rain was observed over the duration of the experiment, and temperatures ranged between approximately 7°C and 27°C.

***Image processing***

All images were processed manually by counting the number of seeds for each seed depot at each recorded time; this was made easy by the contrast in colour between the seeds and the black sandpaper on the seed depots. Seeds were counted as removed only if they were completely removed from the seed depot; thus, even in instances where seeds or disturbed or scattered across the depot, they were still marked as present. For each image, the number of seeds was scored twice to ensure accuracy of the data.

***Statistical Analyses***

All statistical analyses were conducted in R (R Development Core Team 2009). To assess significance of the three treatment variables (species, warming, and elaiosome removal), we used the **glmer** function from the package **lme4** (Bates *et al*. 2012) to fit a generalised linear mixed-effects model to the data, using a logit link and treating the number of seeds as a binomial response. Each of the three treatment variables were encoded as a two-level factor and treated as a fixed effect; interactions between treatments were treated as fixed effects as well, and treatment block was treated as a random effect. To examine significance of treatment at different points in time, the model was fit separately at the 6, 12, 24, and 48 hour marks. The **lrtest** function from the package **lmtest** (Zeileis and Hothorn 2002) was used for conducting likelihood ratio tests to examine whether removal of interaction terms from each model was significant; only significant interaction terms were retained in the final models.

**Results**

Over the course of the 48 hours that seed removal was documented, both photographs and direct observation suggest that insects were responsible for the majority of seed removal, as no birds or mammals were documented in the vicinity of the study area or were observed removing any of the seeds. Seed removal by ants was observed and made up the vast majority of direct observations of seed removal, with the ants dragging individual seeds across the flat surface of the seed depot before carrying the seed over the outer walls; these observations were especially common during daytime hours, but also occurred at night as well. Crickets were also observed on the seed depots, though mostly at night, where they would consume the seeds on the depot rather than carry them away and consume elsewhere. Grasshoppers frequently visited the depots during the daytime hours but were not observed consuming or removing seeds, though they consumed and attempted to remove the adhesive tape used to mark the depots. A variety of other insects were observed at the depots as well, though none of them were observed removing any seeds.

Overall rates of seed removal were high; 48.4% of seeds were removed after 12 hours, 85.5% after 24 hours, and 94.6% after 48 hours. Out of the eight possible treatment combinations of species, warming, and elaiosome presence/absence, warmed *C. nutans* seeds with elaiosomes experienced the highest rates of removal, with 79.6% of seeds removed after 12 hours, 98.0% after 24 hours, and 98.4% after 48 hours; these observed values differ only slightly from the respective model estimates of 76.3%, 97.5%, and 99.1%.

Coefficient estimates from Table 1 suggest that both warming treatment and elaiosome removal significantly impacted the proportion of seeds removed, as the coefficient estimates for these treatments were both highly significant at the 6, 12, 24, and 48 hour marks. At these times, seeds from warmed maternal plants were more likely to have been removed than those from warmed maternal plants, and seeds with their elaiosomes intact were more likely to have been removed than those without their elaiosomes. Over the course of the experiment, seeds with their elaiosomes intact were generally removed at a faster rate than seeds without their elaiosomes for all combinations of warming and species, except for warmed *C. nutans* (Figure 1). Seeds from warmed maternal were removed at a faster rate than seeds from unwarmed maternal plants for all combinations of elaiosome presence/absence and species, except for *C. nutans* with intact elaiosomes (Figure 2). There is not substantial evidence that seed removal rates differed between species (Figure 3), as coefficient estimates for the species term in the mixed-effects model were not significant for three of the four time points at which the model was fit.

Interactions between species and warming treatment, between species and elaiosome treatment, and between warming treatment and elaiosome treatment were observed in the mixed effects models, though their direction and significance varied between the time marks. For example, while the interaction between species and elaiosome treatment was significant for the 6, 12, 24, and 48 hour marks, it was negative at the 48-hour mark and positive at the others. The interactions between species and warming treatment and between warming treatment and elaiosome treatment were consistent in direction between hour marks, but were only significant at a subset of those hour marks.

**Discussion**

Our results indicate that the rate of post-dispersal seed removal is quite high, with more than 85.5% of all seeds removed over the course of only 24 hours. These results are similar to observed removal rates of *C. nutans* and *C. acanthoides* in a similar study by Jongejans *et al*. (2014), where also demonstrate that up to 88% of seeds are removed in 24 hours when there is no impediment to the movement of insect dispersers. These extremely high rates of seed removal suggest that the majority of *C. nutans* and *C. acanthoides* seeds dispersed via wind also undergo some sort of secondary dispersal via insects or animals. Jongejans *et al*. (2014) also demonstrate that rates of seed removal were significantly lower when ant access to seed depots was restricted, indicating that the majority of seed removers were ants; this supports our observations of a disproportionately large number of ant visits to seed depots.

We also demonstrate that the elaiosome plays an important role in attracting seed removers, as seeds of both species had significantly higher rates of removal when the elaiosome was left intact compared to when it was removed. This provides evidence to previous suggestions (Pemberton and Irving 1990, Alba-Lynn and Henk 2010) that elaiosomes in *C. nutans* and *C. acanthoides* that elaiosomes on these seeds attract ants and help promote dispersal.

Paragraph about warming. Surface warming did not change seed removal rates. (Stuble et al 2014)

One of the limiting factors of our study is a lack of knowledge regarding how far seeds travel after removal; while we can easily observe seeds being removed from a controlled experimental area, it is significantly more challenging to find where dispersers move seeds after removing them from the seed depots. While not used in our study here, there are a variety of techniques to assess where animal-dispersed seeds are moved, with each having its own set of strengths and weaknesses. Some seeds are large enough to store electronic devices and can be tracked using telemetry (e.g. Pons and Pausas 2007, Hirsch *et al*. 2012), and can thus be found without visual cues, which can be particularly useful in areas with dense vegetation or when seeds are cached underground. Seeds of various sizes can be dyed with a fluorescent material or pigment that makes visual location easier (e.g. Levey and Sargent 2000, Reiter *et al*. 2006), but addition of pigment to seeds can significantly change their mass depending on the size of the seed and amount of pigment applied (Lemke *et al*. 2009), which could possibly alter the dynamics of seed removal or dispersal. For ant-dispersed seeds such as *C. nutans* and *C. acanthoides*, fluorescent pigment may not be helpful in tracking seeds if they are taken underground to ant nests, where they are then no longer visible from the surface. Mapping emerging seedlings within the vicinity of a seed depot can provide some information on secondary dispersal distances, though for invasive species such as *C. nutans* and *C. acanthoides*, there are ethical concerns about allowing dispersal of viable seeds from an experiment into the environment (Jongejans *et al*. 2014). Also, this method would only provide dispersal distance information for only a fraction of dispersed seeds because it only examines the seeds that germinate, and would present the added challenge of ensuring that a particular seedling can be traced back to its source so that dispersal distance can be accurately quantified.

Thus, while our study helps shed light on secondary dispersal by quantifying seed removal, which is the first part of the secondary dispersal process, it does not document the fate of the seeds after they have been removed. While some seeds are consumed after removal, others can be left intact by ants and scatter-hoarding animals (SOURCES), indicating that removed seeds are exposed to some mixture of dispersal and predation. However, many studies often conflate seed removal with predation, and often treat removed seeds as if they are consumed without any evidence indicating so (Vander Wall *et al*. 2005). Doing so can not only overestimate actual rates of seed predation, but also vastly underestimate secondary dispersal of seeds, as seed removal may not necessarily entail destruction but could instead be the first part of a multi-step secondary dispersal process (SOURCES). As such, we discuss our results in terms of seed removal, as our uncertainty in the proportions of removed seeds that are consumed or dispersed limits us from making any claims about post-removal seed fate.

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**Table 1.** Estimates ( 1 standard error), -scores, and -values for the coefficient estimates for the mixed-effects GLM with logit link fit to the proportion of seeds removed at 6, 12, 24, and 48 hours. The intercept (baseline) represents *C. acanthoides* seeds from unwarmed maternal plants and without elaiosomes. Coefficient estimates must be transformed using the inverse logit function to yield the proportion of seeds removed.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **6 Hours** | | | **12 Hours** | | |
|  | **Estimate** | **z** | **p** | **Estimate** | **z** | **p** |
| **Intercept** | -3.220 0.426 | -7.569 | <0.001 | -1.859 0.325 | -5.712 | <0.001 |
| **Species** | -0.365 0.290 | -1.259 | 0.208 | 0.282 0.159 | 1.770 | 0.077 |
| **Warming** | 1.767 0.275 | 6.426 | <0.001 | 1.888 0.171 | 11.053 | <0.001 |
| **Elaiosome** | 1.564 0.276 | 5.674 | <0.001 | 1.869 0.204 | 9.153 | <0.001 |
| **Species:Warming** | 1.170 0.270 | 4.338 | <0.001 |  |  |  |
| **Species:Elaiosome** | 1.242 0.270 | 4.593 | <0.001 | 0.738 0.213 | 3.459 | <0.001 |
| **Warming:Elaiosome** | -1.921 0.294 | -6.542 | <0.001 | -1.747 0.222 | -7.876 | <0.001 |
|  | | | | | | |
|  | **24 Hours** | | | **48 Hours** | | |
|  | **Estimate** | **z** | **p** | **Estimate** | **z** | **p** |
| **Intercept** | 1.339 0.343 | 3.901 | <0.001 | 2.666 0.456 | 5.853 | <0.001 |
| **Species** | -0.738 0.222 | -3.324 | <0.001 | -0.300 0.275 | -1.090 | 0.276 |
| **Warming** | 0.946 0.254 | 3.732 | <0.001 | 1.149 0.244 | 4.707 | <0.001 |
| **Elaiosome** | 1.124 0.260 | 4.328 | <0.001 | 2.406 0.547 | 4.400 | <0.001 |
| **Species:Warming** | 1.082 0.311 | 3.483 | <0.001 |  |  |  |
| **Species:Elaiosome** | 1.006 0.313 | 3.217 | 0.001 | -1.227 0.622 | -1.972 | 0.049 |
| **Warming:Elaiosome** | -1.115 0.317 | -3.521 | <0.001 |  |  |  |

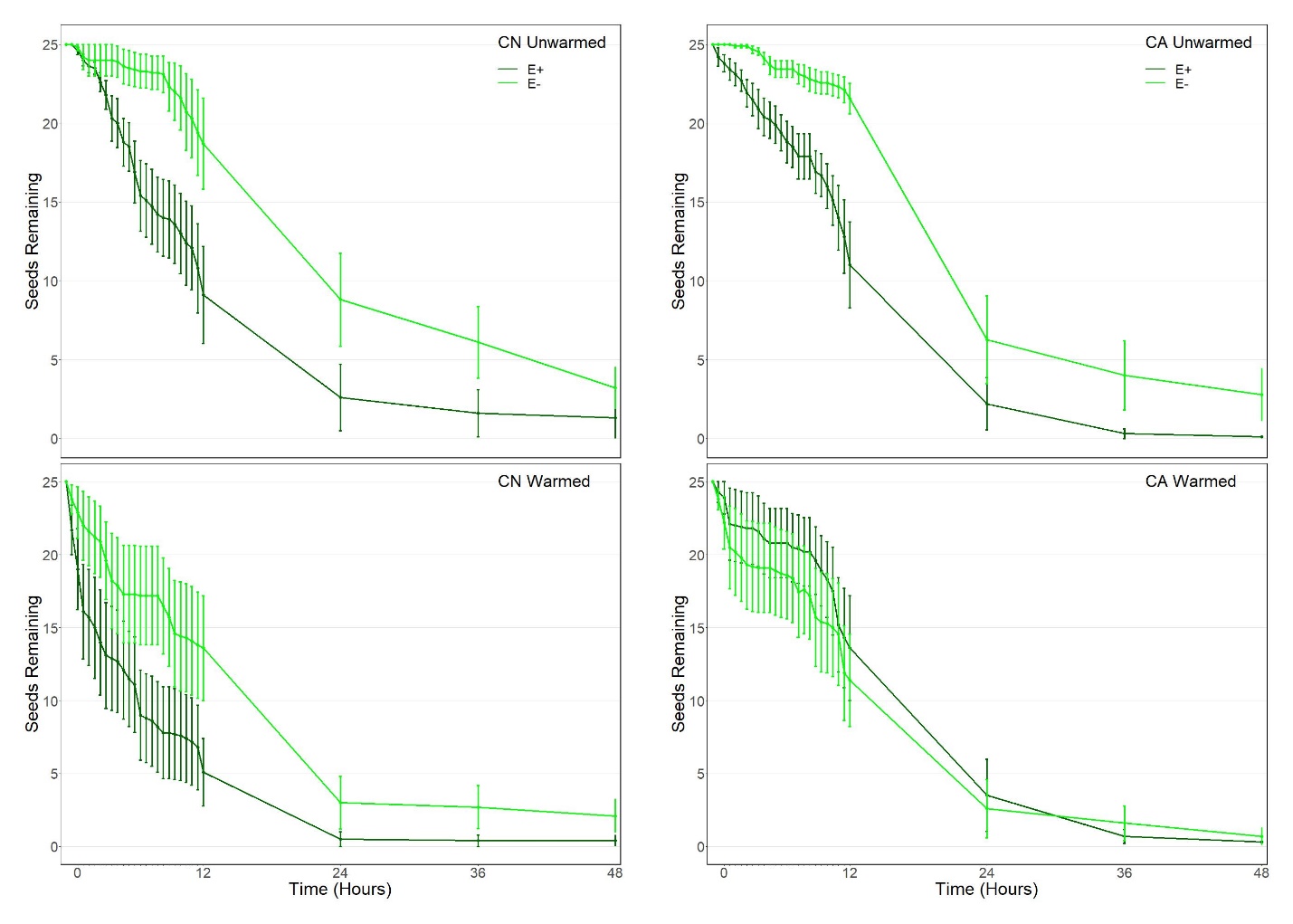
**Figure 1.** Ant removing a *C. acanthoides* seed with an elaiosome (11:30).



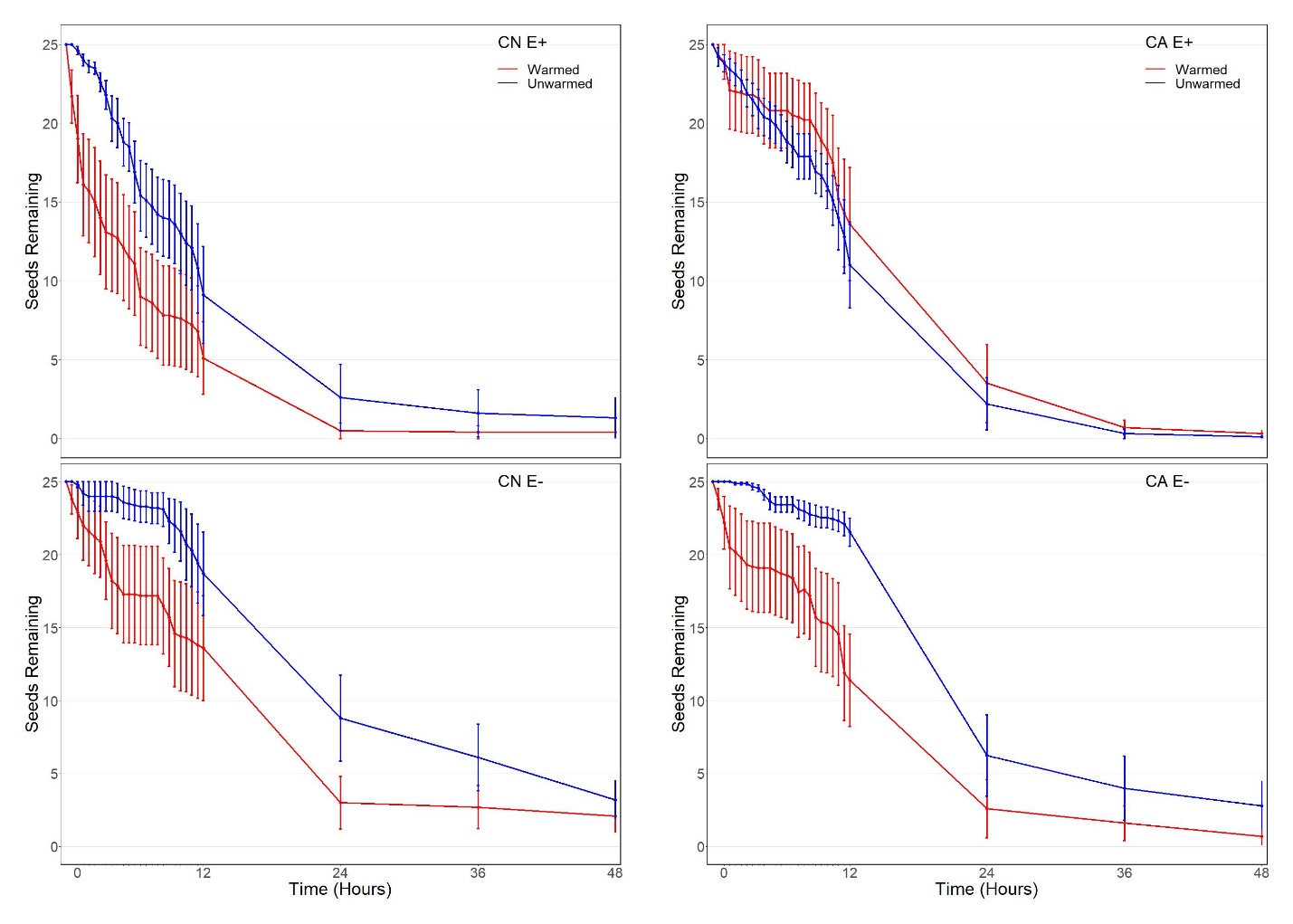
**Figure 2.** Cricket removing a *C. acanthoides* seed without an elaiosome (20:30).



**Figure 3.** Observed number of seeds remaining when elaiosome is present (E+) or absent (E-), conditioned on species (CN *Carduus nutans*, CA *Carduus acanthoides*) and warming treatment applied to the maternal plant.



**Figure 4.** Observed number of seeds remaining when maternal plant is warmed or unwarmed, conditioned on species (CN *Carduus nutans*, CA *Carduus acanthoides*) and elaiosome presence (E+ present, E- absent).



**Figure 5.** Observed number of seeds remaining between species species (CN *Carduus nutans*, CA *Carduus acanthoides*), conditioned on warming treatment applied to the maternal plant and elaiosome presence (E+ present, E- absent).

