

Body size affects the evolution of eyespots in caterpillars

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Many caterpillars have conspicuous eye-like markings, called eyespots. Despite recent work demonstrating the efficacy of eyespots in deterring predator attack, a fundamental question remains: Given their protective benefits, why have eyespots not evolved in more caterpillars? Using a phylogenetically controlled analysis of hawkmoth caterpillars, we show that eyespots are associated with large body size. This relationship could arise because (i) large prey are innately conspicuous; (ii) large prey are more profitable, and thus face stronger selection to evolve such defenses; and/or (iii) eyespots are more effective on large-bodied prey. To evaluate these hypotheses, we exposed small and large caterpillar models with and without eyespots in a 2 × 2 factorial design to avian predators in the field. Overall, eyespots increased prey mortality, but the effect was particularly marked in small prey, and eyespots decreased mortality of large prey in some microhabitats. We then exposed artificial prey to naïve domestic chicks in a laboratory setting following a 2 × 3 design (small or large size × no, small, or large eyespots). Predators attacked small prey with eyespots more quickly, but were more wary of large caterpillars with large eyespots than those without eyespots or with small eyespots. Taken together, these data suggest that eyespots are effective deterrents only when both prey and eyespots are large, and that innate aversion toward eyespots is conditional. We conclude that the distribution of eyespots in nature likely results from selection against eyespots in small caterpillars and selection for eyespots in large caterpillars (at least in some microhabitats).

antipredator defense | caterpillars | Lepidoptera | predator-prey | mimicry

Predation is a strong selective force acting to shape animal phenotypes, and numerous traits have evolved to reduce the probability of detection and attack by potential predators (1, 2). One important and taxonomically widespread adaptation is the possession of conspicuous eyespots (3–6). These markings on lepidopteran caterpillars have long captured the imagination of many eminent evolutionary biologists (7–9), each of whom understood eyespots to be defensive features mimicking the eyes of a dangerous vertebrate and thereby dissuading would-be predators from attack. This idea was explored more formally by Pough (10) and again more recently by Janzen et al. (4), who updated and expanded this hypothesis, arguing that the aversion to eye- or face-like stimuli is likely innate. Subsequent work has now empirically confirmed the protective value of eyespots for caterpillars (11, 12), and has shown that the mimetic fidelity of many caterpillars with eyespots to dangerous snakes increases when they adopt their defensive posture (13). Although some research has questioned the degree to which eyespots confer protection through eye mimicry in adult Lepidoptera (14, 15), other work has convincingly supported the original hypothesis of eye mimicry (16–18); however, there remain fundamental gaps in our understanding of the function and evolution of eyespots. In particular, it remains unclear why, if they are so beneficial, caterpillar eyespots are not more widespread across Lepidoptera?

In caterpillars that live fully exposed to potential predators, eyespots seem to occur more frequently in species of large body size (although eyespots also occur in many small caterpillars that feed in leaf shelters) (4). Anecdotally, many of the most impressive examples of putative snake mimic caterpillars are hawkmoths (family Sphingidae), which are comparatively large (7, 8, 19). Indeed, even in most hawkmoth species, eyespots are substantially reduced or absent altogether during early instars and tend to not become prominent until late instars, when the caterpillars are much larger (8, 20, 21). Taken together, these observations suggest that the selective advantage of eyespots to caterpillars might depend on their body size.

There are three nonmutually exclusive reasons why selection might be expected to favor eyespots more strongly in large caterpillars. First, large animals are intrinsically more conspicuous, and thus might face relatively strong selection favoring the evolution of defenses that operate after detection. On the other hand, small animals are better able to avoid predators; their small body allows them to go undetected, and they either do not experience strong selection for secondary defenses or face selection against conspicuous markings that break crypsis. Similar arguments have been used to explain why other forms of mimicry are more likely to evolve in species that are already moderately conspicuous (22–24). Second, selection might favor eyespots more strongly in large caterpillars, because large prey can be more profitable prey items. Both the incentive to locate a given prey item and to then discriminate on discovery edible prey from genuine threats to their

Significance

Eyespots are a widespread form of antipredator defense that have long captured the imagination of evolutionary biologists, geneticists, psychologists, and artists. These markings are particularly common within Lepidoptera, and eyespots on caterpillars have been shown to deter avian predators; however, why eyespots have evolved in particular caterpillar species, and why they are not even more widespread, remain unclear. Here we answer this question using a powerful three-pronged approach. Our phylogenetically controlled analysis of hawkmoths demonstrates that eyespots are typically restricted to large caterpillars, and our field and laboratory experiments provide an explanation for this. Eyespots are costly to small caterpillars because they enhance detectability without providing a protective advantage, but they are beneficial to large caterpillars because they deter predators.

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safety could increase with prey size. Consequently, prey with increasingly large body size might experience progressively stronger selection for effective secondary defenses (25). Finally, eyespots might confer a greater advantage to large caterpillars because their size allows them to convincingly resemble something that poses a legitimate threat, either because large bodies per se allow the caterpillars to more accurately resemble a threat (e.g., a snake) or because it allows the caterpillars to have eyespots large enough to deter predators, for example, by being more conspicuous (5, 26) or by matching the eye size of an animal that poses a legitimate threat.

To examine the relationship between the possession of eyespots and body size in extant species, a phylogenetically controlled analysis is essential to conservatively control for any lack of independence of species data arising through shared ancestry (27, 28). Here we conducted (i) the first phylogenetically controlled assessment of the association between larval body size (final instars) and the possession of eyespots in caterpillars; (ii) a field experiment using caterpillar models of different sizes exposed to wild bird predation that directly tested whether the protective value of eyespots is greater in large caterpillars compared with small caterpillars when eyespot size is proportional to body size; and (iii) a laboratory experiment in which similar caterpillar models with and without eyespots were presented to naive domestic chicks, with body size and eyespot size manipulated independently to quantify which of these factors influences prey detection and predator hesitation. Through this combination of phylogenetic, field, and laboratory approaches, we comprehensively investigated whether the evolution of eyespots in caterpillars is size-dependent, and evaluated hypotheses as to why this might be so.

Results

Phylogenetic Analyses. We conducted phylogenetic analyses of hawkmoths in MacroGLOSSINAE, the primary subfamily, with extraordinary caterpillar eyespot morphology (20, 29), and the most speciose, with more than 800 described species (30). The hawkmoth phylogenies inferred from both maximum likelihood (ML) and Bayesian analyses of five nuclear genes were largely congruent with previous hypotheses on hawkmoth relationships (31, 32). Trees differed only in the placement of two taxa, which was not well supported in either analysis (Figs. S1 and S2). Their placement did not affect the outcome of the trait analyses; below we present the results using the ML phylogeny.

We examined the distribution of eyespots among final instar caterpillars of known size. The phylogenetic generalized least squares (PGLS) using an Ornstein–Uhlenbeck model of evolutionary change ($\alpha = 11.41$) was a better fit than the model assuming Brownian motion ($\Delta AIC = 2.88$). There was a significant relationship between maximum body size in the final larval instar and the presence of eyespots in macroglossine caterpillars ($t_{63} = 2.14$, $P = 0.036$) (Fig. 1). The coefficient of determination for this relationship was 0.254, and the phylogenetic signal in the residuals (Pagel's λ) was estimated as 0.72.

Field Presentations. The survival of artificial caterpillars arranged in a 2×2 design (small vs. large caterpillars, with or without eyespots scaled to body size) and pinned to tree branches in the field was monitored, in person, at regular intervals for evidence of attack by wild birds. The 90-h mortality rate of artificial caterpillars ranged from 24% to 97% per location (mean \pm SE, $68.5 \pm 4.52\%$). Hazard (i.e., the instantaneous mortality rate) was significantly influenced by the main effects of both body size ($z = 3.04$, $P = 0.002$) and eyespots ($z = 2.24$, $P = 0.025$), but no significant interaction was detected ($z = -1.04$, $P = 0.30$) (Fig. 2). Having a larger body size increased mortality by $37 \pm 14.8\%$, and having eyespots also appeared to increase overall mortality by an estimated $23.7 \pm 14.9\%$ (Table 1). Although the eyespot-induced increase in risk appeared to be qualitatively stronger in small prey than in large prey (26.3% vs. 5.61%), without a significant

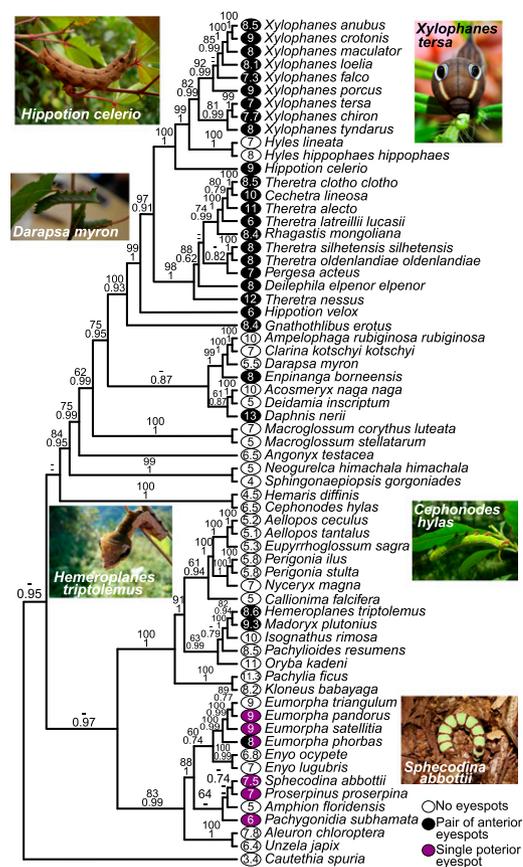


Fig. 1. Phylogeny of MacroGLOSSINAE hawkmoths (Sphingidae) showing the evolution of larval eyespots and body size. Filled symbols indicate the caterpillar species with eyespots, and the numbers inside the symbols represent the maximum body length (cm) at final instar. Note that *Eumorpha phorbis* has both anterior and posterior eyespots. Bootstrap values $> 60\%$ from ML, placed above posterior probabilities > 0.6 from the Bayesian analyses, are plotted on branches. (Photo credits: *Hippotion celerio* and *Cephonodes hylas*, T. Pittaway; *Xylophanes tersa*, M. Sullivan; *Hemeroplanes triptolemus*, S. Chaparro Herrera; *Sphecocodina abbottii*, K. Stohlgren; *Darapsa myron*, T. Hossie.)

interaction, such results should be interpreted carefully (Fig. 2). Interestingly, the strength and direction of the eyespot \times body size interaction appeared to differ among microhabitats (Table S1 and Fig. S3), perhaps indicating that the protective value of eyespots is habitat-specific. Classifying only prey recovered with peck marks as killed (i.e., considering all missing prey as censored) produced qualitatively similar results (SI Materials and Methods).

Laboratory Presentations. Artificial caterpillars arranged in a 2×3 factorial design (small, large body size \times no eyespots, small eyespots, large eyespots) were presented singly to domestic chicks, which were monitored until the prey item was attacked. Main effects of prey body size, eyespot size and presence, and the body size \times eyespot interaction significantly affected the latency to attack (i.e., total time from introduction to attack) (Table 1). Eyespots had opposite effects on small prey and large prey (Fig. 3). Chicks attacked small-bodied prey with small and large eyespots more quickly than small-bodied no-eyespot prey [no eyespots: 148.8 ± 16.74 s; small eyespots: 40.6 ± 5.37 s; large eyespots: 17.2 ± 3.52 s; all $P < 0.001$, Tukey's honest significant difference (HSD) test]. Latency to attack did not differ significantly between small-bodied prey with small eyespots and those with large eyespots ($P = 0.086$). In contrast, chicks delayed attacking large prey with large eyespots (378.1 ± 29.43 s) compared with large-bodied prey lacking eyespots or with small

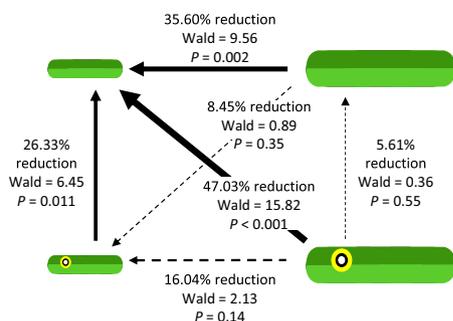


Fig. 2. Pairwise Cox proportional hazard regression comparisons of pastry caterpillar mortality in the field experiment. Eyespot (i.e., no eyespots vs. eyespots present) and body size (i.e., 2 cm vs. 4 cm long) treatments were arranged in a 2×2 factorial design ($n = 576$). Arrow width is proportional to the percent reduction in mortality and point toward the treatment with lower mortality rate. Solid arrows are significant at $P < 0.05$.

eyespots (no eyespots: 154.2 ± 17.20 s; small eyespots: 187.0 ± 14.48 s; all $P < 0.001$, Tukey's HSD). The latency to attack large-bodied no-eyespot prey was not significantly increased by the addition of small eyespots ($P = 0.60$) (Fig. 3).

The main effects of eyespots and body size, as well as the body size \times eyespot interaction, also affected the time to first inspection (a proxy for detection time and/or reluctance to closely approach stimuli) (Table 1). For small prey, time to first inspection decreased with the addition of eyespots (no eyespots: 146.1 ± 16.76 s; small eyespots: 38.1 ± 5.67 s; large eyespots: 16.2 ± 3.34 s; all $P < 0.01$, Tukey's HSD), but increasing eyespot size did not significantly further reduce the time to first inspection ($P = 0.13$). In contrast, for large-bodied prey, the presence or size of eyespots did not significantly affect the time to first inspection (no eyespots: 111.6 ± 16.14 s; small eyespots: 153.0 ± 17.08 s; large eyespots: 218.9 ± 45.64 s; all $P > 0.094$, Tukey's HSD).

Body size, eyespots, and their interaction significantly affected inspection time (Table 1). Chicks spent very little time inspecting small prey (no eyespots: 2.7 ± 1.50 s; small eyespots: 2.5 ± 1.75 s; large eyespots: 1.0 ± 0.73 s), and the inspection time of small prey was not affected by the presence or size of eyespots (all $P > 0.95$, Tukey's HSD) (Fig. 3). All large prey were inspected longer before an attack compared with small prey (all $P < 0.001$), and large prey with large eyespots were inspected longer than any other treatment (large-large eyespots: 159.2 ± 32.40 s; all $P < 0.001$, Tukey's HSD) (Fig. 3). Interestingly, large prey lacking eyespots were inspected for approximately the same amount of time as large prey with small eyespots (no eyespots: 42.6 ± 4.96 s; small eyespots: 34.0 ± 4.88 s).

Finally, the frequency of approach-retreat behavior was associated with both body size and eyespots ($\chi^2 = 10.130$, $df = 2$, $P = 0.006$, log-linear analysis). In particular, approach-retreat behavior was recorded only once in trials with small prey (in a small-small eyespots prey), but was observed in 90% (9 of 10) of the trials with large-large eyespot prey, only once in large-no eyespot prey, and never in large-small eyespot prey.

Discussion

Here we provide a thorough analysis of the phylogenetic distribution of eyespots in hawkmoth caterpillars, and a clear answer as to why eyespots are not ubiquitous in lepidopteran larvae more generally. Our phylogenetic analysis clearly demonstrates that the evolution of eyespots is associated with large body size, and our field and laboratory experiments provide an explanation for this; eyespots increase predation rates on small caterpillars by making them easier to detect, but—at least as far as our laboratory data and previous studies (11, 12) indicate—large eyespots decrease predation rates on large caterpillars, likely

because they intimidate predators (see also ref. 33). Consequently, our work suggests that eyespots tend to be selected for in large species and against in small species. In addition, our laboratory experiment with naïve domestic chicks confirms the suggestion that predators' wariness of eyespots is effectively innate (4, 10), yet this unlearned wariness is conditional on both caterpillars' bodies and eyespots being large. More broadly, our results provide important support for the growing body of evidence suggesting that the evolution of antipredator adaptations is closely tied to other prey traits, such as body size and habitat use (25, 34–39).

Large prey had substantially higher predation risk in the field than small prey, possibly resulting from increased detectability associated with large size (40), although also possibly from predator preference for larger, more profitable prey on encounter (25). As a key life-history trait influencing a range of characters from developmental time to fecundity, body size in caterpillars is itself subject to selection. However, if large body size is advantageous overall, then there naturally will be subsequent selection to mitigate its disadvantages, which include increased exposure to predators.

Both our field and laboratory experiments show that eyespots do not effectively deter predators when possessed by small prey. In the laboratory, small prey without eyespots took the longest to find, and adding eyespots to these prey significantly reduced the time to first inspection (concurrently reducing the overall latency to attack). Likewise, the survival rate of small prey in the field was reduced by the addition of eyespots. We propose that when prey are small, eyespots should be selected against, because conspicuous markings substantially enhance detectability without providing a benefit through intimidation.

The effect of eyespots on large prey was very different, at least in the laboratory. Under laboratory conditions, domestic chicks clearly refrained from attacking large prey with large eyespots. Even without eyespots, chicks showed substantial hesitation to attack large prey, but adding large eyespots to these prey further delayed attack, with a ~ 3.7 -fold increase in time from detection to attack. In addition, chicks consistently exhibited approach-retreat behavior when presented with large prey possessing large eyespots (and never in small prey with large eyespots), emphasizing that the ability of eyespots to deter predators is dependent on prey body size. Assuming that our large-bodied prey were easier to detect than small prey, our overall laboratory results are consistent with those of Stevens et al. (41), who found that

Table 1. Results from factorial ANOVA analyzing the results from the laboratory experiment in which naïve domestic chicks were presented with small or large artificial caterpillars with or without eyespots (no eyespots, small eyespots, large eyespots)

Term	df	F value	P value
Latency to attack			
Body size	1	246.37	<0.001
Eyespots	2	7.62	0.001
Body size \times eyespots	2	81.11	<0.001
Error	54		
Time to first inspection			
Body size	1	43.36	<0.001
Eyespots	2	4.29	0.019
Body size \times eyespots	2	22.51	<0.001
Error	54		
Inspection time			
Body size	1	217.66	<0.001
Eyespots	2	7.65	0.001
Body size \times eyespots	2	10.52	<0.001
Error	54		

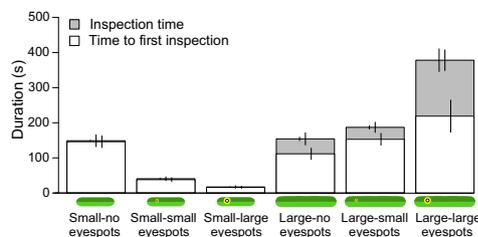


Fig. 3. Results from the laboratory experiment in which naïve domestic chicks were presented with artificial caterpillars. Bars represent the latency to attack, divided into time to first monocular inspection and inspection time (i.e., time from first inspection to attack). Ten chicks were exposed to each treatment. Vertical lines on each bar represent the estimated SEM. The thickened central vertical lines represent SEM for overall latency to attack; vertical lines left of center, SEM for inspection time; lines right of center, SEM for time to first inspection.

eyesspots decreased survival in hard-to-detect prey, but increased the survival of conspicuous prey.

Importantly, the foregoing experiment with naïve predators demonstrates that their reluctance to attack objects with eyespots is innate, and also highlights the fact that eyespots alone are insufficient to generate this aversion; only when sufficiently large eyespots were on large-bodied prey was effective deterrence achieved. At the same time, our work fails to support the idea that small caterpillars are constrained to having eyespots too small to be effective, but instead is consistent with the idea that effective mimicry of snakes or other dangerous vertebrates depends on prey appearing large enough to pose a legitimate threat to attackers (4, 10, 13).

Previous field experiments using similar model caterpillars have shown a protective effect of eyespots (11, 12), yet large prey with and without eyespots had approximately equal mortality in our current field experiment. Indeed, although there was significant (overall deleterious) effect of eyespots in our present field experiment, there was no evidence of a significant eyespots \times body size interaction. Further inspection of the data suggest that the strength of the eyespots \times body size interaction in the field experiment varied considerably among microhabitats in which the caterpillar models were placed (*SI Materials and Methods*). In particular, restricting the analysis to prey pinned to *Populus deltoides* trees revealed a significant interactive effect analogous to the laboratory data (Table S1 and Fig. S3), suggesting variation in the efficacy of eyespots among microhabitats. We suspect that the wider array of avian predators in the field, a subset of which is less fearful of eyespots or prioritize different features as cues of danger [e.g., “head” shape (11, 13), sinuous movements (4)], renders the protective value of eyespots more variable or moderate in natural settings compared with the laboratory (42). In addition, experiments using stationary models are likely to underestimate the protective value of eyespots to live caterpillars, which enhance these signals through startle and/or behavioral mimicry. We also note that the time to first inspection in our laboratory experiment represents not only the difference in detection time, but also any reluctance to closely approach the prey item in question. Thus, the time to first inspection was longer for large prey than for small prey with eyespots, probably because chicks were reluctant to closely approach large, intimidating stimuli, not because large prey items were hard to detect (Fig. 3).

Our examination of hawkmoth caterpillar traits revealed that eyespots generally are more common in species whose larvae attain relatively large body size; however, an important exception to this relationship is the frangipani hornworm (*Pseudosphinx tetrio*). This caterpillar can attain a larval body length of 11.3 cm (20), well within the size range of other putative snake mimics, but lacks eyespots. Instead, this caterpillar appears to be aposematic

(43–46), and might even mimic coral snakes (47), clearly having found a distinct way to solve the problem of being a large-bodied prey item. Curiously, within Sphingidae, larval eyespots seem to be restricted to Macroglossinae and are not observed in the related Smerinthinae and Sphinginae subfamilies. It is not immediately apparent what might constrain the evolution of eyespots in other related subfamilies, given that many larvae of Smerinthinae and Sphinginae are large (20) and eyespots appear to have evolved multiple times within Macroglossinae (Fig. 1). It remains to be seen whether a body size–eyespot relationship exists in other groups (e.g., adult Lepidoptera, fish), but such a study would help broaden our understanding of why these markings evolve in the species they do.

Although eyespots occur in large species that live fully exposed (primarily during the larger, late instars of those species), in other lepidopteran families (e.g., Hesperiiidae), they also occasionally occur in small species whose larvae feed inside leaf shelters (4). Our work shows that eyespots actually would be detrimental to small prey that live fully exposed, because eyespots increase conspicuousness without making the prey intimidating to predators. However, by inhabiting leaf shelters, small prey would be able to minimize the cost of conspicuousness while maximizing the “startle” effect of the markings and making it more difficult for a bird to estimate their body size. Eyespots did not seem to increase the conspicuousness of large prey, yet some large species with eyespots conceal their eyespots within folds of the cuticle [e.g., *Madoryx plutonius* (4)]. This indicates that under natural conditions, for some species there is still strong selection for large prey to minimize the conspicuousness of these markings.

Our findings add to the mounting body of research suggesting that antipredator adaptations are closely linked to other aspects of prey life history and habitat use. There is now abundant empirical evidence indicating that the efficacy of various antipredator adaptations is influenced by both prey size (35, 48) and the habitats in which prey are found (49, 50). A number of comparative phylogenetic studies also have established links between prey ecology and defensive strategy; for example, conspicuous warning signals are selected in *Papilio* caterpillars only if they inhabit narrow-leaved plants in which it is difficult to hide (51), whereas the possession of weaponry (i.e., horns) in female bovids is related to large body size and living in open habitats, both of which increase conspicuousness (52). Similar analyses have shown that small species tend to invest more in camouflage compared with large species (53), and that selection for mimetic fidelity increases with body size (25). Combining comparative and empirical approaches allows researchers to identify the phylogenetic trends and, more importantly, the selective pressures responsible for generating them.

Eyespots have attracted attention from naturalists and evolutionary biologists for more than 150 y, and here we provide an explanation for why eyespots in caterpillars are often restricted to late instars of large species and to small species inhabiting leaf shelters. Consistent with previous suggestions (4, 10), our experiment with naïve chicks indicates that indeed innate processes likely have played key roles in the evolution of caterpillar eyespots. Avoidance of predators is a life-or-death decision, often made at great speed, so it is not surprising that receivers have evolved innate avoidance. The extent to which such innate aversion is held more widely by birds across natural settings, and how innate processes have shaped antipredator defenses more generally, remain to be discovered.

Materials and Methods

Phylogenetic Comparison. We collected data on maximum body size (final instar body length, in cm) and the presence or absence of eyespots in final instar hawkmoth caterpillars from a range of reliable online databases, field guides, direct observations, and other publications (detailed in Table S2). Following Weismann (8), larvae with repeated concentric circular markings

along the full length of the body (e.g., *Hyles lineata*) were not considered to have eyespots. We tested the hypothesis that larger caterpillars are more likely to have eyespots using a new molecular phylogeny constructed in both an ML and a Bayesian framework (*SI Materials and Methods*). The data matrix has a significantly expanded taxon set from a previously published study (31) (*Dataset S1*). We conducted a PGLS analysis on the ML tree with body size as the response variable and the presence/absence of eyespots as a binary predictor (factors are not permitted as response variables in PGLS). We ran PGLS models assuming either a Brownian motion or an Ornstein–Uhlenbeck (54) model of evolution, and then compared their fits using a likelihood ratio test (55). To test whether accounting for phylogenetic autocorrelation was necessary, we tested whether the residuals from a linear model without phylogenetic control showed a phylogenetic signal (56) using the “phylosig” function in the R package “phytools” (57). We estimated the strength of the association by comparing the residual variance of the model and the null model (assuming the same correlation structure; i.e., $1 - \sigma_M^2/\sigma_0^2$), as outlined by Paradis (58).

Artificial Prey. Artificial caterpillars were designed to quantify the selective advantage of eyespots on large and small prey. Construction of artificial caterpillars followed a methodology similar to that used in previous studies (11, 12). The caterpillar models in our field experiment comprised four treatments: small-no eyespots, large-no eyespots, small-eyespots, and large-eyespots. All treatments were similarly countershaded, because Hossie and Sherratt (12) revealed that eyespots effectively protected these artificial caterpillars from birds only when the caterpillars were countershaded as well.

Pastry caterpillars were manufactured with a 3:1 mixture of white flour and lard. Light-green pastry was produced by adding three drops of Leaf Green AmeriColor Soft Gel Paste (AmeriColor) and 20 mL of water to 40 mL of lard and 118 mL flour; dark-green pastry was produced by adding six drops of gel paste and 15 mL of water to the same volumes of flour and lard. The dark and light pastries were rolled into strips of 3.5 mm diameter for small prey and 5 mm diameter for large prey, and then cut into lengths of 20 mm for small prey and 40 mm for large prey. Two-toned (i.e., countershaded) prey were then created by pressing together one piece of dark pastry and one piece of light pastry, such that the final artificial prey were cylinders 20 mm long \times 5 mm diameter for small prey and 40 mm \times 7 mm for large prey. For our purposes, the dark side was considered dorsal, because prey were presented on the upper side of branches.

We used the same eyespot design as used by Hossie and Sherratt (11). Eyespots were hand-painted onto pastry caterpillars using Elmer’s Nontoxic Tempera Paint (Elmer’s Products). For the large prey, eyespots were 4.5 mm in diameter, 4 mm apart, and 7 mm from the anterior tip of the prey. The eyespots had a 3-mm black “pupil,” with a 1-mm white “sparkle.” For the small prey, eyespots were sized proportional to body size; specifically, eyespots were 2.2 mm in diameter, 2 mm apart, and 3.5 mm from the anterior tip, with a 1.5-mm black pupil and a 0.5-mm white sparkle. Finished pastry caterpillars were checked against a standard model prey for each treatment to ensure uniformity of size and shape of the body, as well as of the size and position of eyespots.

The design of artificial prey for the laboratory presentations to domestic chicks was slightly modified to allow the inclusion of two additional treatments to account for a possible confounding effect of eyespot size. Specifically, small prey were 25 mm long and 6 mm in diameter, and large prey were 40 mm long and 7 mm in diameter. For each prey body size, either small or large eyespots (2.2 mm or 4.5 mm diameter, respectively) were added such that six treatments were produced: small-no eyespots, small-small eyespots, small-large eyespots, large-no eyespots, large-small eyespots, and large-large eyespots.

Field Presentations. Field trials were conducted at three sites in Ontario, Canada (site 1: near the Ferguson Forest Centre, Kemptville, 45°03′00″N, 75°39′44″W; site 2: University of Guelph, Kemptville campus, 44°59′48″N, 75°38′22″W; site 3: Oxford Mills, 44°56′27″N, 75°38′56″W). Experiments were run between May 7 and September 7, 2013. All three sites were composed of secondary growth mixed-deciduous forest, and no site was closer than 5.5 km to any other site. Insectivorous bird species observed in each of the field locations are detailed in *Table S3*.

Pastry caterpillars were deployed between 1600 and 1900 h on the first day of a replicate site. Specifically, 24 trees were selected along a linear transect (~10 m apart), and to each tree a single artificial prey was pinned on the upper side of a live branch in each cardinal zone (north, south, east, and west) of a tree. Trees were restricted to four species: *Acer rubrum*, *Betula papyrifera*, *Populus deltoides*, and *Populus tremuloides*. Each tree received a single pastry caterpillar from each treatment and cardinal zone, allocated at random. Selected branches were 1–2 m from the ground and were

roughly 0.5–1 cm in diameter. Pastry caterpillars were considered killed if they were no longer present or had been attacked by a bird (i.e., peck marks present). “Survival” was checked at 14, 19, 24, 38, 43, 48, 62, 67, 74, and 90 h from deployment. In all cases of missing baits, the pin was found, and the ground underneath was searched for remains. All attacked prey were photographed and removed. Artificial prey damaged by rain, slugs, or ants were removed and considered censored (i.e., considered to have survived only to that time period, but not considered killed) in the final analysis. After 90 h, any remaining prey were removed.

The entire process was repeated six times, for a total of 576 artificial prey deployed. Each consecutive replicate was conducted in an entirely new location within one of the three larger sites and was at least 200 m from any previous location within that site. To avoid temporal confounding among sites, consecutive replicates were always conducted at a different site in a systematic order (i.e., site 1, location 1; site 2, location 1; site 3, location 1; site 1, location 2, etc.).

Laboratory Presentations. Seventy-two female domestic chicks of the Novogen Brown strain (*Gallus gallus domesticus*) were purchased from a commercial hatchery on the day of hatching. Sixty served as experimental chicks, and 12 served as buddies. During training and testing, two “buddy” chicks were placed in a “buddy arena” adjacent to the experimental arena to prevent experimental chicks being visually isolated from conspecifics. All chicks were fed a diet of starter crumbs, and experimental chicks were trained for 2 d to acclimate them to the experimental laboratory setting (*SI Materials and Methods*). On day 3, experimental birds were randomly assigned to one of six groups ($n = 10$ per group). Birds in all groups received a single test trial, in which they were placed in the experimental arena individually after 30 min of food deprivation (*SI Materials and Methods*).

In the experimental arena, each experimental chick encountered a 50-cm-long branch of sycamore (*Acer pseudoplatanus*) containing a single pastry caterpillar. This branch was placed in the center of the experimental arena with one end 25 cm from the buddy arena and the other end 25 cm from the rear wall of the arena. Chicks were placed in a central position immediately in front of the rear wall of the experimental arena. Caterpillars were attached to the branches using pins pushed into the branches in random positions. The tops of the pins were then cut off, leaving ~3 mm protruding from the branches: pastry caterpillars were impaled on these. The type of caterpillar that each bird encountered differed among experimental groups; individual chicks received one of the following: a small caterpillar without eyespots, a small caterpillar with small eyespots, a small caterpillar with large eyespots, a large caterpillar without eyespots, a large caterpillar with small eyespots, or a large caterpillar with large eyespots. Birds were left in the arena until they attacked the caterpillar.

We recorded the latency to first inspect the caterpillar (i.e., tilt the head and view the caterpillar monocularly) as a crude measure of search time (although it also incorporates latency to closely approach prey). We also recorded the latency to attack the caterpillar outright, and the presence/absence of approach-retreat behavior (i.e., repeatedly approaching and retreating from the caterpillar). We calculated “inspection time” as the total latency to attack minus search time. In this way, we were able to separate the effects of our treatments on the processes of prey detection (which should affect search time) and predator hesitation (which should affect the time from prey detection to attack).

Statistical Analyses of Field and Laboratory Experiments. We analyzed the “survival” of artificial prey in the field using Cox proportional hazards regression (59), which can handle both censored data and nonuniform changes in predation risk. We controlled for the possible lack of independence between baits on the same tree by clustering the baits by tree in our analysis. We assessed the overall significance of the model using the Wald test, which does not assume independence of observations within a cluster. Factors in our model included body size (i.e., small vs. large), eyespot (i.e., eyespots vs. no eyespots), and body size \times eyespot interaction. The analysis was stratified to permit separate baseline hazard rates for location and tree species. This analysis was conducted in R (60) using the survival library (61), and the assumption of proportionality was tested using the *cox.zph* function.

Latency to attack, search time, and inspection time in the chick experiment were each analyzed using separate factorial ANOVAs in R (60), with body size, eyespots, and body size \times eyespot interaction as factors. Latency to attack and inspection time were square root-transformed to meet ANOVA assumptions. The data were analyzed separately (i.e., search time and inspection time) and in combination (i.e., latency to attack) to help reveal the mechanisms driving the change in overall latency to attack, although by definition these are not independent measures. Associations among body size, eyespots, and the presence/absence of approach-retreat behavior were

analyzed by fitting a log-linear model using the `loglm` function of the MASS package. The importance of the three-way interaction was evaluated by comparing the fit of the full model (size \times eyespot \times approach) with and without the three-way interaction, using the log-likelihood ratio test.

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Supporting Information

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SI Materials and Methods

Molecular Dataset Construction. We compiled a molecular dataset of variation at five nuclear genes in 71 hawkmoth species. Sixty-five of these species were in the subfamily Macroglossinae, and the other taxa were included as outgroups. The five genes chosen for this study fully corresponded to the loci used by Kawahara et al. (1): *CAD* (2), *DDC* (3), *EF-1 α* (4), *period* (5), and *wingless* (6). Taxa not included in the study of Kawahara et al. (1) were sequenced following the methodology outlined in Kawahara et al. (7). A list of taxa with GenBank accession numbers is provided in Dataset S1.

Each new sequence was profile-aligned in MAFFT 6.857b (8) to the dataset of Kawahara et al. (1) and checked for possible contamination following the protocol outlined by Kawahara et al. (9). The five genes were concatenated into a single matrix, and PartitionFinderV1.0.1 (10) was used to determine the best substitution model, with the Bayesian information criterion (11) applied to determine an optimal partitioning strategy for each gene and codon position. Optimal trees were estimated with ML in RAXML 7.3.2 (12) and in a Bayesian framework with MrBayes 3.2 (13). In RAXML, we applied a GTRGAMMA model and executed 200 ML tree searches with a random starting parsimony tree with the “-f d” option and a combined 1,000 bootstrap and likelihood search using the “-f a” option. In MrBayes, we sampled every 1,000th generation for 10 million generations using default flat priors, with unlinked *statefreq*, *revmat*, *shape*, and *pinvar* parameters. Chain convergence was determined by whether split frequencies fell below 0.01. After exclusion of a burn-in of 5,000 trees, MrBayes runs were combined, and a majority rule consensus tree with posterior probabilities was created.

Housing and Training of Domestic Chicks. Domestic chicken chicks have long been used as models for investigating the responses of birds to objects with eyespot-like markings. As ground-dwelling predators, the wild ancestors of domestic chickens (red junglefowl, *Gallus gallus*) are omnivorous, feeding on insects, seeds, and fruits. Junglefowl are found in a range of tropical habitats in southeast Asia and are sympatric with a range of predators, including snakes.

Our domestic chicks were housed in floor pens measuring 120 cm \times 70 cm and subjected to a 14-h light/10-h dark daily cycle using fluorescent lights with full daylight spectrum. The temperature of the laboratory was maintained at 24–27 °C using room heaters. Water was provided ad libitum, as were chick starter crumbs, except during training and experiments, when brief periods of food deprivation were necessary to ensure that the chicks were motivated to forage. At the end of the experiments, the chicks were rehomed to free-range smallholdings. The laboratory experiment was conducted under local Ethical Committee approval from Newcastle University (ERC Project ID 340) and in accordance with both UK Home Office regulations and the Association for the Study of Animal Behavior’s Guidelines for the Treatment of Animals in Research and Teaching.

On posthatch day 1, all chicks were left to acclimatize to the laboratory for 1 h. Afterward, 60 experimental chicks were trained to eat chick starter crumbs from the white paper floor of an experimental arena. Two identical arenas were used, each consisting of a cage measuring 120 cm \times 50 cm \times 50 cm, with a section measuring 20 cm \times 50 cm \times 50 cm partitioned off using wire mesh to create a separate buddy arena. In all training and experimental trials, two chicks were placed in the buddy arena to reduce any potential distress from placing experimental chicks alone in the

arena. These buddy chicks were selected from a stock of 12 individuals and were changed every three trials. These chicks were not given access to artificial prey at any point during the experiment.

Chicks underwent six training trials at regular intervals, three on posthatch day 1 and three on day 2. In all trials, chick crumbs were scattered over the floor of the experimental arena, and chicks were then placed in the arena for an interval of 2–3 min. In the first two trials, chicks were placed in the arena in groups of three; in the next two trials, chicks were placed in the arena in groups of two; and in the final two trials, chicks were placed in the arena individually. In the final trial, a 50-cm-long branch of sycamore (*A. pseudoplatanus*), complete with leaves, was placed in the center of the arena to familiarize chicks with branches, so that the chicks did not show a neophobic response to the branch in the subsequent test trial (*Materials and Methods*). Chicks were food-deprived for 30 min before each training trial on day 2 to ensure that they were motivated to forage. During all training trials, the two arenas were used simultaneously, but during the test trial, only one arena was used, so that chicks could be observed individually. Chicks were trained and tested in a random order; by the end of trial 6, all chicks had pecked the branch and were eating crumbs in the experimental arena.

Results from Phylogenetic Analyses Using the Bayesian Tree. We reran our phylogenetic analysis using the Bayesian tree (Fig. S1), and found qualitatively similar results to those reported in the main text. Specifically, the PGLS using an Ornstein–Uhlenbeck model of evolutionary change ($\alpha = 11.74$) provided a significantly better fit than the model assuming Brownian motion ($\Delta\text{AIC} = 2.27$; $\chi^2 = 4.27$, $P = 0.039$, likelihood ratio test). There was a significant relationship between maximum body size in the final larval instar and the presence of eyespots in macroglossine caterpillars ($t_{63} = 2.23$, $P = 0.03$). The phylogenetic signal (Pagel’s λ) in the residuals was estimated as 0.77.

Results from the Field Experiment Treating Only Pecked Prey as Killed. Classifying only prey recovered with peck marks as killed (i.e., considering all missing prey as censored) produced qualitatively similar results as treating both pecked and fully removed prey as killed. Slight differences included a stronger effect of body size ($251.6 \pm 28.26\%$ increase in hazard; $z = 4.45$, $P < 0.001$) and a nonsignificant effect of eyespots ($z = 0.93$, $P = 0.35$). The interaction was nonsignificant ($z = -0.74$, $P = 0.46$). This difference from the original model, which treated both pecked and fully removed prey as killed, resulted from a greater proportion of small prey being completely removed on attack (small-no eyespots: $83.2 \pm 1.85\%$ removed completely; small-eyespots: $80.8 \pm 2.19\%$; large-no eyespots: $48.7 \pm 5.00\%$, large-eyespots: $49.5 \pm 4.60\%$; $F_{3,20} = 4.44$, $P = 0.015$).

Results from the Field Experiment Partitioned by Tree Species. Our previous work using pastry caterpillars revealed that treatment efficacy can differ among tree species (14, 15). To examine such effects, we reanalyzed our data treating tree species as a predictor as opposed to a stratifying factor. Here we present the results from Cox proportional hazards regression models, identical to those presented in the main text, but examining prey pinned to each of the four tree species separately (see also refs. 14, 15). Qualitatively similar results were obtained when we reanalyzed our complete data set treating tree species as a predictor, opposed to a stratifying factor. Note the highly significant body size \times eyespots interaction for prey pinned to *P. deltoides*. This interaction indicates

that the effect of eyespots on survival differed between small prey and large prey pinned to *P. deltooides*. Subsequent pairwise analyses revealed that eyespots significantly reduced survival in small prey, but significantly increased survival in large prey (Fig. S3). These results are consistent with those of the laboratory experiment with domestic chicks (Fig. 3), suggesting that such patterns are at least plausibly observed in field settings as well.

Despite the foregoing observations, there was no evidence of a size \times eyespot interaction for artificial caterpillars pinned on the most commonly sampled tree, *P. tremuloides*. We also note that whereas the size \times eyespot interaction was highly significant for

caterpillars pinned on *P. deltooides* ($P = 0.007$), the same interaction on *B. papyrifera* was in the opposite direction—although here it was not only of lower estimated magnitude, but also nonsignificant ($P = 0.06$). Taken together, these findings suggest that the influence of microhabitat is complex and warrants further study. The extent to which these tree-specific results differ from the overall analysis including all trees likely reflects variation in the efficacy of constantly displayed eyespots across different microhabitats (14, 15). The extent to which the physical environment mediates the strength of selection for eyespots remains to be studied.

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Table S2. Data and sources for body size (final instar body length in cm) and eyespot presence/absence in Macroglossinae caterpillars

Latin name	Subfamily	Eyespot	Maximum body size	Source	Voucher
<i>Cautethia spuria</i>	Macroglossinae	0	3.4	(1)	99-SRNP-1121
<i>Unzela japix</i>	Macroglossinae	0	6.4	(1)	01-SRNP-2399
<i>Aleuron chloroptera</i>	Macroglossinae	0	7.8	(2)	
<i>Pachygonidia subhamata</i>	Macroglossinae	1	6	(1)	08-SRNP-32172
<i>Amphion floridensis</i>	Macroglossinae	0	5	(3)	
<i>Proserpinus proserpina</i>	Macroglossinae	1	7	(4)	
<i>Sphecodina abbottii</i>	Macroglossinae	1	7.5	(3)	
<i>Enyo lugubris</i>	Macroglossinae	0	7	(1)	05-SRNP-31417
<i>Enyo ocypete</i>	Macroglossinae	0	6.8	(1)	05-SRNP-56550
<i>Eumorpha phorbas</i>	Macroglossinae	1	8	(5)	
<i>Eumorpha satellitia</i>	Macroglossinae	1	9	(1)	09-SRNP-56221
<i>Eumorpha pandorus</i>	Macroglossinae	1	9	(3)	
<i>Eumorpha triangulum</i>	Macroglossinae	0	9	(1)	99-SRNP-283
<i>Kloneus babayaga</i>	Macroglossinae	0	8.2	(1)	04-SRNP-60316
<i>Pachylia ficus</i>	Macroglossinae	0	11.3	(1)	08-SRNP-70715
<i>Oryba kadeni</i>	Macroglossinae	0	11	(1)	07-SRNP-32563
<i>Pachylioides resumens</i>	Macroglossinae	0	8.5	(1)	78-SRNP-51
<i>Isognathus rimosa</i>	Macroglossinae	0	10	(1)	78-SRNP-116
<i>Madoryx plutonius</i>	Macroglossinae	1	9.3	(1)	97-SRNP-32926
<i>Hemeroplanes triptolemus</i>	Macroglossinae	1	8.6	(1)	03-SRNP-11365
<i>Callionima falcifera</i>	Macroglossinae	0	5	(1)	11-SRNP-12976
<i>Nyceryx magna</i>	Macroglossinae	0	7	(1)	09-SRNP-2633
<i>Perigonia stulta</i>	Macroglossinae	0	5.8	(1)	09-SRNP-36431
<i>Perigonia ilus</i>	Macroglossinae	0	5.8	(1)	01-SRNP-14555
<i>Eupyrrhoglossum sagra</i>	Macroglossinae	0	5.3	(1)	06-SRNP-30742
<i>Aellopos tantalus</i>	Macroglossinae	0	5.1	(6)	
<i>Aellopos ceculus</i>	Macroglossinae	0	5.2	(1)	02-SRNP-7920
<i>Cephonodes hylas</i>	Macroglossinae	0	6.5	(4)	
<i>Hemaris diffinis</i>	Macroglossinae	0	4.5	(3)	
<i>Sphingonaepiopsis gorgoniades</i>	Macroglossinae	0	4	(7)	
<i>Neogurelca himachala</i>	Macroglossinae	0	5	(4)	
<i>Angonyx testacea</i>	Macroglossinae	0	6.5	(4)	
<i>Macroglossum stellatarum</i>	Macroglossinae	0	5	(4)	
<i>Macroglossum corythus luteata</i>	Macroglossinae	0	7	(4)	
<i>Daphnis nerii</i>	Macroglossinae	1	13	(4)	
<i>Deidamia inscriptum</i>	Macroglossinae	0	5	(3)	
<i>Acosmeryx naga naga</i>	Macroglossinae	0	10	(4)	
<i>Enpinanga borneensis</i>	Macroglossinae	1	8	(4)	
<i>Darapsa myron</i>	Macroglossinae	0	5.5	(3)	
<i>Clarina kotschyi kotschyi</i>	Macroglossinae	0	7	(7)	
<i>Ampelophaga rubiginosa</i>	Macroglossinae	0	10	(4)	
<i>Gnathothlibus erotus</i>	Macroglossinae	1	8.4	(8)	
<i>Hippotion velox</i>	Macroglossinae	1	6	(4)	
<i>Theretra nessus</i>	Macroglossinae	1	12	(4)	
<i>Deilephila elpenor elpenor</i>	Macroglossinae	1	8	(4)	
<i>Pergesa acteus</i>	Macroglossinae	1	7	(4)	
<i>Theretra oldenlandiae</i>	Macroglossinae	1	8	(4)	
<i>Theretra silhetensis</i>	Macroglossinae	1	8	(4)	
<i>Rhagastis mongoliana</i>	Macroglossinae	1	8.4	(4)	
<i>Theretra latreillii lucasii</i>	Macroglossinae	1	6	(4)	
<i>Theretra alecto</i>	Macroglossinae	1	11	(7)	
<i>Cechetra lineosa</i>	Macroglossinae	1	10	(4)	
<i>Theretra clotho clotho</i>	Macroglossinae	1	8.5	(4)	
<i>Hippotion celerio</i>	Macroglossinae	1	9	(7)	
<i>Hyles hippophaes hippophaes</i>	Macroglossinae	0	8	(7)	
<i>Hyles lineata</i>	Macroglossinae	0	7	(3)	
<i>Xylophanes tyndarus</i>	Macroglossinae	1	8	(1)	09-SRNP-56411
<i>Xylophanes chiron</i>	Macroglossinae	1	7.7	(1)	04-SRNP-1496
<i>Xylophanes tersa</i>	Macroglossinae	1	7	(1)	05-SRNP-34374
<i>Xylophanes porcus</i>	Macroglossinae	1	9	(1)	06-SRNP-21542
<i>Xylophanes falco</i>	Macroglossinae	1	7.3	Kawahara, unpublished	
<i>Xylophanes loelia</i>	Macroglossinae	1	8.1	(1)	04-SRNP-41581

Table S2. Cont.

Latin name	Subfamily	Eyespot	Maximum body size	Source	Voucher
<i>Xylophanes maculator</i>	Macroglossinae	1	8	(1)	05-SRNP-30849
<i>Xylophanes crotonis</i>	Macroglossinae	1	9	(1)	97-SRNP-1321
<i>Xylophanes anubus</i>	Macroglossinae	1	8.5	(1)	01-SRNP-6893

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Table S3. Insectivorous birds observed in each location during the associated field trail conducted over the summer of 2013 near Kemptville (sites 1 and 2) and Oxford Mills (site 3)

Bird species	Site 1		Site 2		Site 3	
	May 8	June 19	May 25	July 21	June 3	September 4
<i>Bombycilla cedrorum</i>						x
<i>Cardinalis cardinalis</i>			x			
<i>Carduelis tristis</i>			x		X	
<i>Contopus virens</i>						x
<i>Cyanocitta cristata</i>			x	x	X	x
<i>Dendroica Petechia</i>					X	
<i>Dumetella carolinensis</i>			x		x	
<i>Geothlypis trichas</i>		x	x		x	
<i>Melospiza melodia</i>		x				x
<i>Passerculus sandwichensis</i>					x	
<i>Pheucticus ludovicianus</i>		x	x	x		
<i>Poecile atricapilla</i>	X	x	x	x	x	x
<i>Quiscalus quiscula</i>					x	
<i>Sitta carolinensis</i>				x		x
<i>Turdus migratorius</i>	X	x	x	x	x	x
<i>Vireo solitarius</i>						
<i>Zonotrichia albicollis</i>	X	x				
<i>Passerina cyanea</i>					x	
<i>Setophaga pensylvanica</i>					x	x
<i>Agelaius phoeniceus</i>					x	
<i>Catharus fuscescens</i>		x				
<i>Setophaga virens</i>				x		
<i>Myiarchus crinitus</i>				x		
<i>Troglodytes hiemalis</i>				x		
<i>Eremophila alpestris</i>						x
<i>Vireo olivaceus</i>						x

Other Supporting Information Files

[Dataset S1 \(XLSX\)](#)