

## RESEARCH ARTICLE

# Comparative and experimental studies on the relationship between body size and countershading in caterpillars

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## Abstract

Countershading is a gradient of colouration in which the illuminated dorsal surfaces are darker than the unilluminated ventral surface. It is widespread in the animal kingdom and endows the body with a more uniform colour to decrease the chance of detection by predators. Although recent empirical studies support the theory of survival advantage conferred by countershading, this camouflage strategy has evolved only in some of the cryptic animals, and our understanding of the factors that affect the evolution of countershading is limited. This study examined the association between body size and countershading using lepidopteran larvae (caterpillars) as a model system. Specifically, we predicted that countershading may have selectively evolved in large-sized species among cryptic caterpillars if (1) large size constrains camouflage which facilitates the evolution of a trait reinforcing their crypsis and (2) the survival advantage of countershading is size-dependent. Phylogenetic analyses of four different lepidopteran families (Saturniidae, Sphingidae, Erebidae, and Geometridae) suggest equivocal results: countershading was more likely to be found in larger species in Saturniidae but not in the other families. The field predation experiment assuming avian predators did not support size-dependent predation in countershaded prey. Collectively, we found only weak evidence that body size is associated with countershading in caterpillars. Our results suggest that body size is not a universal factor that has shaped the interspecific variation in countershading observed in caterpillars.

## KEYWORDS

background matching, camouflage, crypsis, lepidoptera, obliterative shading, self-shadow concealment

## 1 | INTRODUCTION

Countershading (also called obliterative shading) describes darker pigmentation on the surfaces exposed to the direction of illuminating light in camouflaged animals (Cott, 1940; Poulton, 1890). Natural illumination generates a luminance gradient across the animals' bodies due to the directional illumination from the sun, which consequently hinders effective camouflage. Countershading counteracts this luminance gradient and reduces the chance of detection. It has been described in various vertebrate and invertebrate animals (Caro et al., 2011; Ferguson & Messenger, 1991; Gomez & Théry, 2007;

Hamilton & Peterman, 1971; Stoner, 2003). Although most studies have explored countershading in relation to its camouflage function, other roles have also been postulated, including thermoregulation (Whitman, 1987), protection from ultraviolet (UV) damage and abrasion (Bonser, 1995; Braude et al., 2001).

Although countershading has been described for a long time, only recently has it undergone empirical investigation. Studies using various predators consistently support the concealment function of countershading (Donohue et al., 2020; Penacchio et al., 2018; Rowland et al., 2007, 2008). Light environments, especially illumination strength, affect the efficacy of countershading and determine

the optimal lightness gradient needed for effective camouflage (Allen et al., 2012; Cuthill et al., 2016; Penacchio et al., 2015, 2018). Indeed, previous comparative studies investigating the relationship between ecological/morphological variables and the degree of countershading reveal the association between light environment and countershading in mammals including pinnipeds and Sciuromorpha (Ancillotto & Mori, 2017; Caro et al., 2012). Although emerging studies have demonstrated the function and mechanisms of countershading, studies on actual biological systems are rare and biased to a few taxonomic groups (mostly mammals) (Allen et al., 2012; Caro et al., 2012; Caro & Koneru, 2021; Rowland, 2009). A central question that has been explored in natural systems is why we find countershading in only some of the camouflaged prey but not all of the camouflaged species. More specifically, what factors explain the variation in the presence of countershading? One possibility is that the efficacy of countershading depends on the interplay between other traits.

Naturally, countershading improves background matching (the degree of colour matching between animals and their backgrounds) by removing vertical gradient of body luminance (Allen et al., 2012; Poulton, 1890; Ruxton et al., 2004, 2018; Thayer, 1896). However, the camouflage effect of countershading depends on the degree of contrast against its focal background: countershading enhances camouflage more effectively against a low-contrast than high-contrast background (Donohue et al., 2020). Thus, one can predict that countershading has evolved in species that has low contrast against their natural background substrate. However, in natural system, the degree of contrast against background is largely context-dependent and is affected by various factors such as phenology and behaviours (Stevens & Ruxton, 2019); thus, testing this prediction in a large comparative study is challenging due to the difficulty of quantifying the degree of contrast against background for each species. Illumination strength also affects the efficacy of countershading (Allen et al., 2012; Cuthill et al., 2016; Penacchio et al., 2015, 2018); because stronger illumination generates a higher luminance contrast between dorsal and ventral bodies, the degree of countershading is predicted to be higher in open habitat species. Some comparative studies on mammal countershading support the following prediction: the increased use of open habitat is associated with the degree of countershading contrast (Allen et al., 2012; Ancillotto & Mori, 2017; Caro et al., 2012). Another hypothesis is that countershading is associated with body size.

One potential mechanism by which countershading coevolve with body size is that the effectiveness of countershading depends on prey size. Previous studies have shown that some insect colour defences (such as eyespots or deimatic displays) are more effective in larger species which has driven the selective evolution of such defences in larger species (Hossie et al., 2015; Kang et al., 2017). This relationship may also be present in countershading if the effectiveness of countershading depends on body size. In addition, comparative studies on mammals demonstrate the association between body size and the degree of countershading (Allen et al., 2012; Caro et al., 2011; Kamilar, 2009). These studies consistently suggest that

smaller mammals experience higher predation risk which could drive the evolution of stronger countershading in smaller mammals. In cryptically coloured insects, larger body size usually results in higher predation risk because either (or both) being large gives more energy reward to predators or larger size often impede camouflage (Hossie et al., 2015; Karpestam et al., 2014; Pembury Smith & Ruxton, 2021; Rimmel & Tammaru, 2009, but also see Mänd et al., 2007). In such circumstances, if stronger countershading is more likely to evolve in species with higher predation risk as in mammals, we predict the association between large size and stronger countershading in caterpillars.

In this study, we examined the relationship between body size and countershading using various groups of lepidopteran larvae as a model system. Caterpillars are renowned for their countershaded bodies and have been used in many empirical studies as a model for generating artificial prey (Cuthill et al., 2016; De Ruiter, 1956; Rowland et al., 2008; Thayer, 1918). However, surprisingly, no studies have quantitatively investigated the prevalence and evolution of caterpillar countershading in comparative contexts. We first examined the co-evolutionary patterns of body size and countershading using phylogenetic analyses. We further conducted field predation experiments to identify the interactive effect of body size and countershading.

## 2 | METHODS

### 2.1 | Data collection and species classification

We studied caterpillars of four different lepidopteran families (Erebidae, Geometridae, Saturniidae, and Sphingidae) for which comprehensive species-level phylogenetic trees were available (Barber et al., 2015; Kang et al., 2017; Kawahara et al., 2009; Murillo-Ramos et al., 2019). We obtained both body lengths of the final instar caterpillars and adult wingspans from either field guides or reliable online sources (see [Supporting Information](#)). Due to the limited availability of caterpillar size data, we mainly used adult wingspan as a proxy of the final instar caterpillar size after confirming a high correlation between the two variables (see below). We used the midrange (average of the largest and smallest number) when the size was indicated as a range. We collected online photos of the final instars to judge the presence of countershaded bodies. Whenever possible, we included at least two photos of caterpillars clearly showing the sideline of the body in which we could identify the colour gradient of dorsum-ventral lines. We also attempted to include photos showing caterpillars hanging (i) upside-down and (ii) upside-up to minimize the possibility of falsely judging countershaded caterpillars as non-countershaded due to the countershading effect present in the photos. We collected both adult wingspan data and photos for 392 species (59, 80, 74, and 179 species for Saturniidae, Sphingidae, Erebidae, and Geometridae, respectively).

Countershading is a camouflage strategy; thus, it is predicted to have evolved in non-aposematic species. Thus, we first removed

aposematic species from our analysis to examine whether body size is associated with countershading 'among camouflaged species'. Using the final instar photos, we first assigned species to one of the four conspicuous categories that were employed in previous studies on caterpillar colouration (Mappes et al., 2014). We assigned a 0 rating to species with no typical warning colour such as yellow, red, and orange, often combined with black, a 1 rating to species with some features of warning colouration in small patches on otherwise cryptic bodies, a 2 rating to species with moderate warning colouration that usually covers approximately half of the area of the bodies, and a 3 rating to species with strong warning colouration that covers the whole body. Two evaluators (YH and CK) independently conducted the classification. For those species where both evaluators rated as either 2 or 3, we considered these species potentially aposematic and did not use them for further analysis. For those species where both evaluators rated them either 0 or 1, we retained the species for further countershading classification (see below). The initial classification was in agreement between both evaluators for 86.5% of the species. For the species where disagreement exists (i.e., when the two classifications were not identical), we searched for additional photos of caterpillars and reclassified them together to reach a consensus. We failed to reach a final agreement for 13 species (3.3% of the total) and did not include these ambiguous species in further analysis. We also did not analyse the species when their bodies were entirely covered with either hairs or powders in such a way that having a countershaded body was physically constrained. When a species exhibited polymorphic forms, we classified each morph and determined whether all morphs were rated to the same conspicuous group. Otherwise, we removed the polymorphic species from further analysis.

Next, for those species classified as cryptic (rated either 0 or 1 in the previous classification), we further classified whether each species had countershaded bodies. We deemed a species countershaded when it exhibited (i) a ventral-dorsal boundary line with the dorsal and ventral parts differing in lightness and hue similarity or (ii) a ventral-dorsal lightness gradient with a similar hue. Hue similarity was determined subjectively by both evaluators. Although it has not yet been explored whether a combination of two chromatically different colours could function for countershading, we considered that considerably different hues are less likely to improve background matching through countershading. For example, when a species exhibited a white colour in either the ventral or dorsal body (found in some sphingid moths such as *Darapsa myron*), we did not consider the species to be countershaded due to clear hue differences. The same evaluators (YH and CK) independently classified each species. The results were in disagreement for 24.8% of species. We examined additional online photos together for those species and attempted to reach an agreement. After this process, we disagreed for 5.9% of the species, and removed these ambiguous species from the analysis. After all these processes, 252 cryptically coloured species were finally used for the phylogenetic analysis (Saturniidae: 24, Sphingidae: 47, Erebidae: 27, and Geometridae: 154 species).

In addition, there were clear differences in which substrate type each species resembles. One of the classifiers (YH) classified the resembling substrate (either leaf or twig) of each species. The substrate type was either leaves or twigs except for the two geometrid species that the resembling substrate was uncertain.

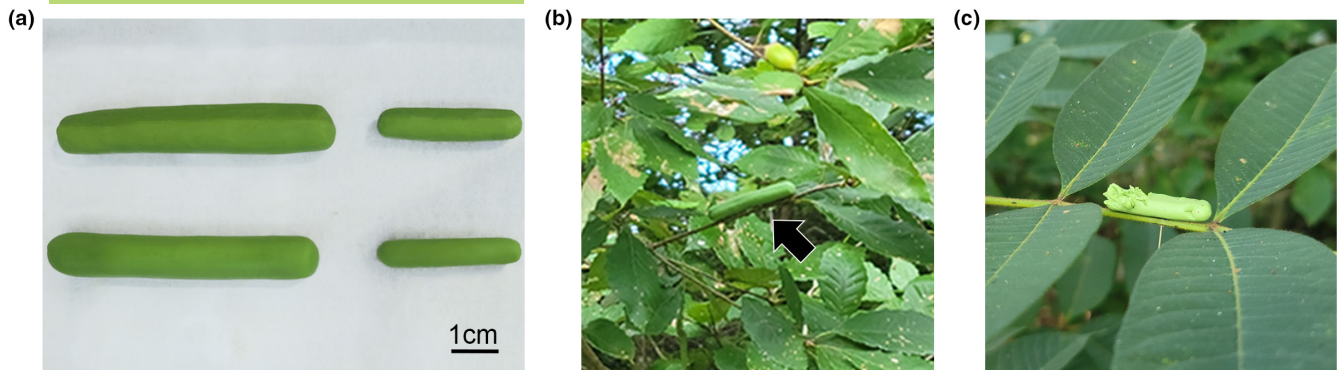
## 2.2 | Comparative data analysis

We employed phylogenetic generalized linear model (pGLZ) analysis to test whether there was an association between body size and countershading. Because the information on the final instar caterpillar size was limited (available for only 70 species), we instead used adult wingspans (available for 416 species including those without photos) as a proxy of caterpillar body size. Naturally, adult wingspan is highly correlated with final instar caterpillar size (Pearson's product-moment correlation,  $r = 0.76$ ,  $t_{69} = 9.62$ ,  $p < 0.001$ ). Using the published phylogenetic trees, we first ultrametricized each tree using the 'chronos' function in the 'ape' package to time-scale the tree relative to the genetic changes in the genes chosen for phylogenetic inference (Paradis et al., 2004). We assumed that rates of branch evolution are correlated (i.e., substitution rates on neighbouring branches are likely to be similar) (Sanderson, 2002). We used whether each caterpillar species exhibited countershaded bodies as a response variable and adult wingspan as an explanatory variable. We used the 'phyloglm' function implemented in the 'phyloglm' package (Tung Ho & Ané, 2014). To determine whether various frequency variables were related to each other, we performed the chi-square test of independence.

## 2.3 | Field predation experiments

We conducted field predation experiments assuming avian predators to examine the interactive effect of prey size and countershading on survival. There were four different caterpillar prey models made of plasticine clays (Van Aken, Van Aken International, Georgia, USA; Figure 1): small countershaded (SC), small uniform (SU), large countershaded (LC), and large uniform (LU). Small prey had a diameter of 5 mm and a length of 30 mm, whereas large prey had a diameter of 10 mm and a length of 60 mm. Both sizes were within the range of caterpillar length we investigated: 60 mm length was closer to the average length of fifth instar caterpillars we studied (mean length = 66 mm), and 30 mm was the first quantile of the caterpillar size distribution. Countershaded prey had two colour tones: dark and light green.

First, we determined (i) the ratio of differently coloured plasticine clay and (ii) the position of the boundary between dorsal and ventral parts that confer the optimal countershading effect under field conditions. We manufactured many two-tone models with various ratios of green, yellow, and white plasticine mixes. Then, we tested the countershading effect of each in the field conditions. We first put the testing models on tree branches and



**FIGURE 1** (a) Four types of artificial prey used in the field predation experiments. From top left and in a clockwise direction: large countershaded prey (LC), small countershaded prey (SC), small uniform prey (SU), and large uniform prey (LU). (b,c) Example photos of the pinned model (b) and a model with signs of avian attacks (c).

photographed them on a sunny day. Then, we checked for the presence of a linear lightness gradient from the top dorsal side to the bottom ventral side using the ImageJ 'Plot Profile' function. After these preliminary examinations, we used a dark green colour made with a 5:1 mixture of yellow and green plasticine clays and a light green colour made with a 5:1:1 mixture of yellow, green, and white clays, which exhibited a negligible lightness gradient (Figure S1). The boundary line between the dark and light green colours was placed at approximately two-thirds of the prey model (Figure 1). We made uniformly coloured prey by mixing yellow, green, and white plasticines at a 5:1:0.5 ratio.

The field predation experiments were conducted between June and September 2021. The experiment had a randomized block design with eight replicated blocks representing different hiking trails and dates in mixed forests at Mt. Seungdal, Jeollanamdo, South Korea (34.92 N, 126.43 E). We deployed 100 prey items (25 per treatment) along the hiking trail in random order in each block. There was at least a 10 m distance between each prey. We attached each prey model to a twig (diameter ~ 1 cm) at a height between 1.5 and 1.8 m using two metal pins for prey presentation (Figure 1b). We deployed prey at 8 am and checked the survival every 24 h for five consecutive days. We did not deploy the prey when heavy rain was predicted. There were occasional hikers along the trails (a few in an hour), but we found no evidence of human disturbance on our well-camouflaged prey models.

The disappearance of prey or clear beak marks on prey was considered as signs of predation (Figure 1c). Prey that survived 120 h were classed as censored values in the analysis (Klein & Moeschberger, 2003). The prey found on the ground without any signs of attack were also classed as censored at the time of discovery. We found no signs of any other predator types. We employed a mixed-effect Cox-proportional hazards model implemented in the 'coxme' package to compare the survivorship between different prey treatments (Therneau, 2020). We set prey size, whether prey had uniform or countershaded colour, and their interaction as explanatory variables. Specifically, we predicted a significant interaction effect between size and countershading if the protective effect of countershading is size-dependent: small-sized prey may achieve

higher survivorship without countershading which leaves less room for improvement (the ceiling effect), whereas large-sized prey may benefit more from countershaded bodies. Each block was set as a random factor.

### 3 | RESULTS

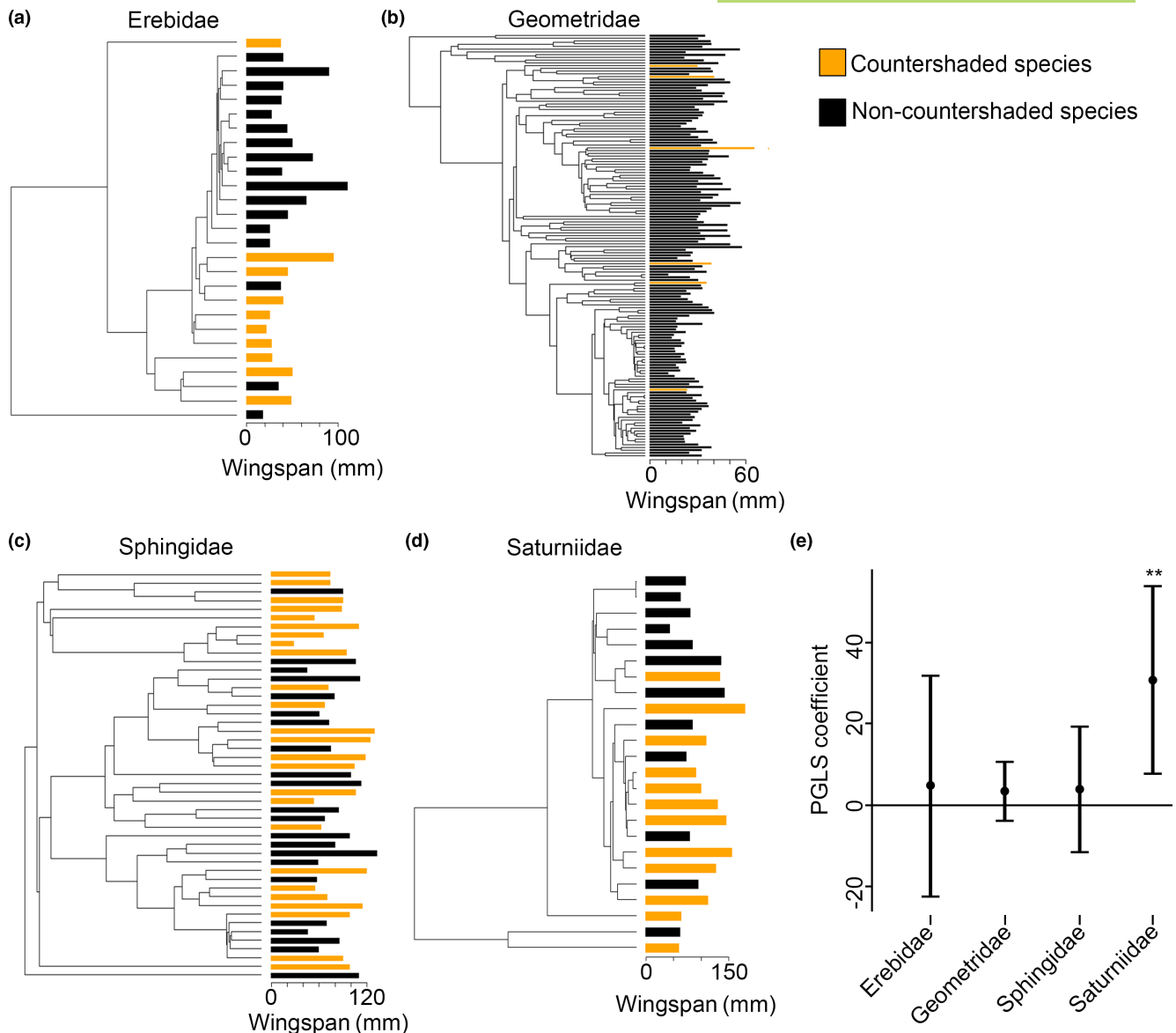
#### 3.1 | Comparative analysis

21% (53/252) of the analysed species were classified as having countershaded bodies. We found moderate evidence that the species with countershading are larger than those without in the Saturniidae family (Figure 2; Table 1). However, we found no evidence of an association between body size and countershading in the remainder of the families studied (all  $p > 0.05$ ; Figure 2; Table 1).

In addition, there was strong evidence in the difference in substrate types that species in each family resembled (Figure 3a;  $\chi^2_3 = 116.56$ ,  $p < 0.001$ ). Caterpillars in the family Saturniidae and Sphingidae primarily resemble leaves (88% and 94%, respectively), but less than half of the species resemble leaves in the Erebididae family (41%) and even less do in the Geometridae (17%). Generally, countershading was more highly associated with leaf-resembling than twig-resembling ( $\chi^2_1 = 58.13$ ,  $p < 0.001$ ; 58% in leaf-resembling species vs. 14% in twig-resembling species). When we analysed only leaf-resembling species separately, there were differences in the frequency of countershaded species among different families ( $\chi^2_3 = 22.55$ ,  $p < 0.001$ ). However, when we excluded species in the Geometridae family, in which only a few species exhibited countershading, we found no evidence that the frequency of countershaded species differed among different families (Figure 3b;  $\chi^2_2 = 1.26$ ,  $p = 0.53$ ).

#### 3.2 | Field predation experiment

In total, 30.25% of prey had signs of bird attacks. We found moderate evidence that prey survival was higher when prey had larger sizes (Figure 4;  $z = 2.01$ ,  $p = 0.045$ ). We also found strong evidence that



**FIGURE 2** Countershading classification and body size of each species mapped onto phylogenetic trees (a–d). The length of the bar represents each species' wingspan (see methods for the justification of using adult wingspan as a proxy of caterpillar size). The bar colour indicates whether each species was classified as having a countershaded body (yellow) or a non-countershaded body (black). (e) Coefficients and standard errors of the coefficients from the phylogenetic generalized least squares (PGLS) results. Positive values indicate that countershading was associated with large size after accounting for the phylogenetic relationship.

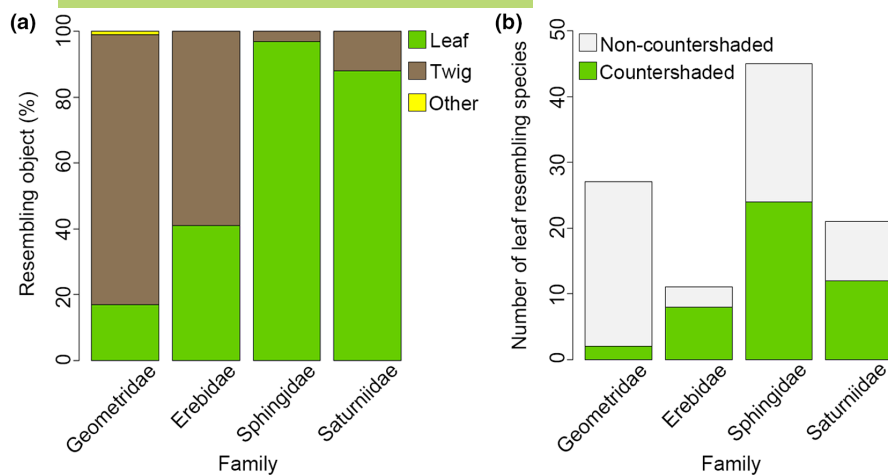
**TABLE 1** Summary of phylogenetic generalized linear model (pGLZ) results for each family.

	Saturniidae (N = 24)	Sphingidae (N = 47)	Erebidae (N = 26)	Geometridae (N = 154)
estimate	0.03	0.005	-0.003	0.08
s.e.	0.01	0.02	0.01	0.047
z	2.13	0.45	-0.03	1.74
P	0.03	0.65	0.97	0.08

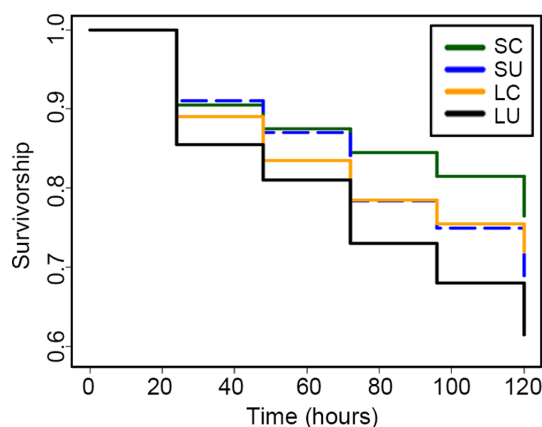
countershading increased survival ( $z = -2.62$ ,  $p = 0.009$ ). However, we found no evidence of an interaction effect between the two variables ( $z = 0.25$ ,  $p = 0.8$ ).

## 4 | DISCUSSION

Put together, we found no general relationship between body size and countershading in caterpillars. The association between large size and countershading was supported only in the family Saturniidae but not in the other families. While countershaded species were more common in generally large-sized families such as the Saturniidae and Sphingidae, this seems to be principally driven by a higher proportion of leaf-resembling species in these families. Countershaded bodies are more likely to be found in species resembling leaves than other substrates, suggesting that the adaptive benefits of countershading are stronger in leaf-resembling caterpillars than in twig-resembling species. This may



**FIGURE 3** (a) The proportions of species in each family that resemble either twig, leaf, or other substrates. (b) The number of countershaded vs. non-countershaded species among leaf-resembling species for each family.



**FIGURE 4** The survival curve of the four prey types in the field predation experiment. S: small, L: large, C: countershaded, U: uniformly coloured.

be related to the differences in protective mechanisms; countershading improves survival in leaf-resembling species mainly by removing the vertical luminance gradient, which hinders predator detection (Cuthill et al., 2016; Rowland et al., 2008), but twig-resembling species may rely on masquerade, which relies on predator object recognition error rather than detection (Skelhorn et al., 2010). Thus, the pressure for removing the luminance gradient may be weaker in twig-resembling than in leaf-resembling species. An interesting example is the polymorphic species *Pachylia ficus*. *P. ficus* caterpillars can take either twig- or leaf-resembling forms, but only the leaf-resembling form shows countershaded bodies (Figure S2).

Our results of the field predation experiment align well with previous findings in that countershading in cryptic prey provides a survival advantage against avian predators (Edmunds & Dewhirst, 1994; Rowland et al., 2008). Most field studies of countershading have used either pastry/clay prey or printed cylindrical papers to examine prey survival (Cuthill et al., 2016; Rowland et al., 2007; Rowland et al., 2008). Printed cylindrical papers have advantages in that the variation in countershaded patterns is negligible compared to handmade pastry/clay prey. However, their hollow inside may affect prey

detection which is not present in pastry/clay prey. Regardless of the methods adopted, the fact that all studies to date consistently found the survivorship advantage of countershading which strongly supports the concealment benefits of countershading. However, we found no interactive effect between countershading and body size. This suggests that the survival advantage of countershading does not depend on body size. While the families with generally large-sized species tend to have more countershaded species, when we selectively analysed leaf-resembling species which were more likely to have countershading than twig-resembling species, this proportion remained reasonably constant among the families (after excluding the Geometridae in which only a few species exhibited countershading). These results collectively imply that body size is not a primary factor that explains the interspecific variation in countershading.

By virtue of the increasing availability, online species images have been successfully used in many animal colouration studies (Loeffler-Henry et al., 2019). While this approach has clear advantages, especially in large-scale comparative analyses (Arbuckle & Speed, 2015; Penney et al., 2012), there are certain limitations. First, due to the heterogeneity in image quality and photographic conditions, the colours in images may not accurately represent the colours in natural conditions. In our study, we examined multiple images per species, which can help mitigate the potential errors induced by the lack of image standardizations. Second, because most commercial cameras only capture colours in human-visible wavelengths (400–700 nm), the colour information in the ultraviolet range (300–400 nm, where many predators of caterpillars can perceive) is missing (Endler, 1978; Osorio & Vorobyev, 2008). However, most camouflaged animals reflect a negligible amount of ultraviolet light, presumably because having ultraviolet colour is revealing against non-ultraviolet reflecting natural substrates (Eguchi & Meyer-Rochow, 1983; Kim et al., 2020). Since our main phylogenetic analyses were conducted among the camouflaged species, the inclusion of ultraviolet colour into the analysis should negligibly affect our classifications because most species are predicted to reflect a small amount of ultraviolet light.

Taken together, our results present only weak evidence that body size is associated with countershading in caterpillars. Although

countershading was more prevalent in the lepidopteran families containing species with large sizes, this seems to be driven by (i) the association between leaf resemblance and countershading and (ii) a higher proportion of leaf-resembling species in these families. Field predation experiments also do not support the size-dependent effectiveness in countershading. Thus, body size may not be a main factor that explains the interspecific variation in countershading in caterpillars. One alternative hypothesis is that interspecific variation in the light environment might have shaped the degree of countershading (Allen et al., 2012; Penacchio et al., 2018). This predicts stronger countershading in species experiencing a more open light environment. Our study highlights high interspecific variation in caterpillar countershading, and the ecological and evolutionary drivers that maintain such variation remain to be tested.

#### AUTHOR CONTRIBUTIONS

CK designed the study. YH collected the data. YH, SY and CP carried out the field experiment. YH and CK analysed the data and shaped the initial draft. All authors contributed to revising the text.

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#### CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

#### PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/jeb.14153>.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at <https://doi.org/10.5061/dryad.r7sqv9sgg>

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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