Report

Current Biology

An Insect with Selective Control of Egg Coloration

Highlights

- A predatory stink bug can selectively control the coloration of its eggs
- Dark eggs tend to be laid on leaf tops and light eggs on leaf undersides
- The egg pigment protects developing embryos from UV radiation
- Surprisingly, the egg pigment is not melanin

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In Brief

Abram et al. describe the first example of an animal able to selectively control the color of its eggs. They found that a predatory stink bug tends to lay dark eggs on leaf tops and light eggs on leaf undersides. Darker eggs are more resistant to UV radiation, although surprisingly, the pigment conferring this benefit is not melanin.

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An Insect with Selective Control of Egg Coloration

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SUMMARY

The color and patterning of animal eggs has important consequences for offspring survival. There are examples of between-species and polymorphic differences in egg coloration in birds and amphibians [1–3], as well as cases of birds and insects whose nutritional status or age can cause within-individual variation in egg pigmentation [4–6]. However, no studies to date have demonstrated that individual animals can selectively control the color of their eggs. Here, we show that individual females of the predatory stink bug *Podisus maculiventris* can control the pigmentation of their eggs during oviposition, as a response to environmental conditions. The color of egg masses produced by individual females can range from pale yellow to dark black/brown. Females tend to lay darker eggs, which are more resistant to UV radiation, on the upper surface of leaves where UV exposure is highest in nature. Conversely, they lay lighter eggs on the undersides of leaves. However, egg color is not determined by the intensity of UV radiation falling on the surface where they are laid. Rather, female stink bugs appear to use a visual assessment of oviposition substrate reflectance to determine egg color. Unexpectedly, biochemical analyses revealed that the egg pigment is not melanin, the most ubiquitous light-absorbing pigment in animals. Our study offers the first example of an animal able to selectively control the color of its eggs.

RESULTS AND DISCUSSION

Description and Quantification of Egg Pigmentation

First, we undertook a descriptive evaluation of *Podisus maculiventris* egg pigmentation (Figure S1). The eggs' chorion (shell) was always pale white immediately after laying, reaching its final pigmentation level within an hour. The dark pigment, when present, was contained in the outermost layer of the chorion, especially concentrated in chorionic spines (which were also present in eggs with little or no pigment). In most cases, pigmentation was homogeneous on individual eggs and within the same egg mass. In contrast to some other descriptions of stink bug eggs [7–9], the variation in egg color described here is not due to the age of eggs, although egg contents do darken slightly in the later stages of embryonic development.

Next, we developed a pigmentation index (PI) to quantify variation in egg pigmentation. First, we took standardized photos (white balance corrected) of egg brightness under controlled lighting conditions. We then plotted the average brightness measurements of pooled groups of differently pigmented eggs against their spectral absorbance when solubilized in Soluene-350 [10], subtracting out the absorbance of the unpigmented egg shell (Figure 1). This calibration curve corrected for the non-linearity of the photographic measurements with regards to light intensity [11] and allowed us to approximate the relative amount of pigment in eggs in subsequent experiments simply by taking photographs and converting the resulting brightness measurements to PI.

Demonstration of Within-Individual Conditional Plasticity in Egg Coloration

We then tested whether individual *P. maculiventris* are able to lay eggs of different pigmentation levels and whether females modify egg pigmentation in response to the color (reflectance) of the substrate on which they are laying. Individual females were monitored, over the course of their lives, in Petri dishes painted black, white, or half black/half white. Females were supplied with a mate, insect prey, and plant material. Eggs were collected from the dishes every 2–3 days to measure their PI. Individual females were able to lay eggs spanning the full range of pigmentation levels; the average difference in PI between the lightest and darkest egg laid was 15.97 ± 1.93 (mean ± 95% confidence interval [CI]), and 20 out of 35 individuals laid both heavily pigmented (PI > 15) and lightly pigmented (PI < 5) eggs during their lives. Eggs tended to be more pigmented in black petri dishes than white dishes and more pigmented when laid on the bottom of dishes than when laid on the side of the dish or the underside of the lid (Figure 2). In half black/half white dishes, there was a tendency toward less pigmented eggs on the white half of the dish, although it was not significant when controlling for the more important effect of laying position (Figure 2). These results provided evidence of context-dependent plasticity in egg pigmentation in *P. maculiventris*, supported the hypothesis that substrate reflectance plays a role in determining egg pigmentation, and revealed the previously unexpected importance of laying position.

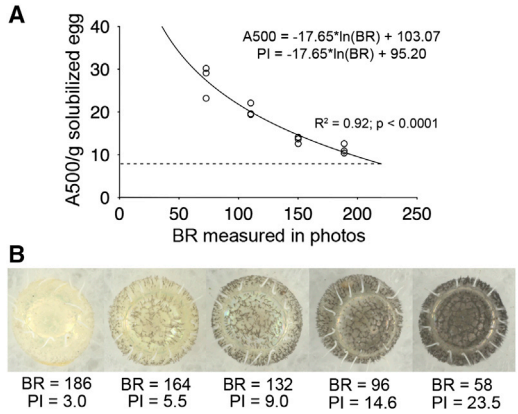

Figure 1. Quantification of *P. maculiventris* Egg Pigmentation

(A and B) A calibration curve (A) of the absorbance of 500 nm light (A500) by solubilized *P. maculiventris* eggs versus their brightness (BR) measured in photographs, after subtracting out the background A500 of unpigmented eggs (7.87/g), was used to assign a pigmentation index (PI) to eggs of different pigmentation levels; examples shown in (B). See Figures S1 and S2 for detailed descriptions of egg pigmentation, spectral measurements, and biochemical analyses.

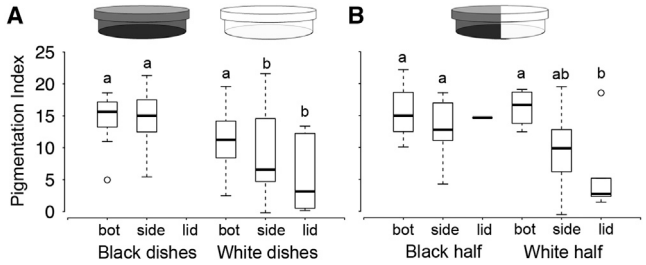

Figure 2. The Pigmentation of Eggs Laid by *Podisus maculiventris* in Painted Petri Dishes, Depending on whether Eggs Were Laid on the Bottom, Side, or Inside Lid of the Dish

(A) Egg PI differed significantly between black and white petri dishes (linear mixed model with female ID as random factor; $\chi^2 = 11.58$, $p < 0.001$) and varied among laying positions ($\chi^2 = 11.37$, $p = 0.0034$). bot, bottom.

(B) Egg pigmentation was only marginally different between the two sides of half black and half white petri dishes ($\chi^2 = 3.39$, $p = 0.066$) but varied among positions ($\chi^2 = 9.09$, $p = 0.011$). Different letters indicate differences among categories ($p < 0.05$; Tukey contrasts following linear mixed model analysis). Categories missing or with single data points were excluded from analysis. Total n in (A)/(B) = 25/14 females; 128/58 egg masses. bot, bottom.

Egg Pigmentation Is Correlated with Plant Structure and Luminosity Levels

We reasoned that the response of egg pigmentation to laying location in the previous experiment could be the expression of an evolved response to plant structure, which can modulate exposure to biotic and abiotic mortality factors [12–15]. Furthermore, although black and white oviposition substrates are not present in nature, the direction and intensity of light could change the apparent reflectance of plant leaf surfaces and elicit differences in egg pigmentation. For example, sunlight passing through leaves from above illuminates their lower surfaces and increases their reflectance levels relative to leaf tops. Thus, one would expect lighter eggs to be laid on leaf undersides if egg pigmentation level is positively correlated with substrate reflectance. Furthermore, reduced lighting levels could cause the reflectance of all leaf surfaces to be lower overall, increasing egg pigmentation levels. To test these predictions, we placed groups of female *P. maculiventris* in cages containing soybean (*Glycine max*) plants and measured the PI of egg masses laid on leaf tops and undersides. Cages were either exposed to full ambient lighting conditions or shaded to reduce luminosity levels more than 50-fold. We found that *P. maculiventris* laid eggs that were on average 2.1 times more pigmented on the upper surface of leaves compared to those laid on leaf undersides (Figure 3). Bugs laid slightly more pigmented eggs (+17%) in the shaded cages, but the large difference between the pigmentation of eggs on leaf tops versus leaf undersides was maintained (Figure 3). Overall, 47.3% of eggs were laid on leaf tops in the fully lit cages, compared to 44% in the shaded cages; these proportions did not differ significantly between treatments (Fisher's exact test; $p = 0.84$). We reasoned that the strong correspondence between laying position on leaves and egg pigmentation could be the key to understanding the adaptive significance of *P. maculiventris* egg coloration.

Egg Pigmentation Protects Developing Embryos against Ultraviolet Radiation

The peculiar tendency of some predatory stink bugs, including *P. maculiventris*, to lay many of their egg masses on the upper surface of leaves has been previously noted [16]. In contrast, most plant-dwelling arthropods, including many species of stink bugs, tend to lay their eggs on the undersides of plant leaves [17–20]. Laying eggs on the undersides of leaves is generally considered to provide a sheltered microclimate for developing embryos, offering protection against wind, rain, overheating, and desiccation. Perhaps most significantly, leaves block the passage of UV radiation [13], which could otherwise cause embryonic mortality by damaging cellular machinery and causing DNA replication errors [21, 22]. In other animals, including humans, pigments such as melanin can act as sunscreen by absorbing UV radiation [22, 23]. Thus, applying pigment to eggs laid on the tops of leaves could protect developing embryos from exposure to UV radiation. To test this hypothesis, we exposed egg masses of different pigmentation levels to four different doses of UV radiation during the 16-hr light period for each day of their development. The different doses were administered by varying the distance of egg masses from a UVA/B lamp, and for the lowest-intensity treatment, attenuating UV wavelengths (<390 nm) using a filter. The probability of *P. maculiventris* embryonic survival decreased as the dose rate of UVA/B radiation administered to eggs increased (Figure 4). Furthermore, embryos were much more likely to survive at a given dose rate of UVA/B radiation when eggs were more pigmented (Figure 4). To our knowledge, this is the first convincing evidence of a pigment protecting insect eggs from UV radiation damage.

Determination of Egg Pigmentation by Females during Oviposition

We next asked how egg pigmentation is controlled, and what cues could be involved. Eggs themselves could accumulate pigment in response to ambient levels of UV radiation, as has

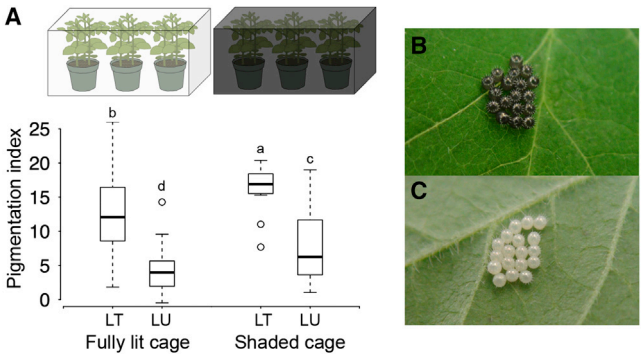

Figure 3. The Effect of Leaf Position and Luminosity Level on Egg Pigmentation

(A) The pigmentation of eggs laid by *P. maculiventris* on soybean leaf tops (LT) or leaf undersides (LU) in cages that were either fully lit (12,000–13,000 lux) or shaded (200–300 lux). Different letters indicate statistically significant differences (adjusted $p < 0.05$; Tukey contrasts following linear mixed model analysis). Eggs were more pigmented on leaf tops (linear mixed model with experiment block as random factor; $\chi^2 = 72.04$, $p < 0.0001$) and in the shaded cage treatment ($\chi^2 = 14.18$, $p < 0.001$); there was not a significant interaction between leaf position and luminosity ($\chi^2 = 0.066$ $p = 0.80$). Total n = 110 egg masses.

(B) A heavily pigmented egg mass (PI > 20) laid on the top of a leaf.

(C) A lightly pigmented egg mass (PI < 5) laid on the underside of a leaf.

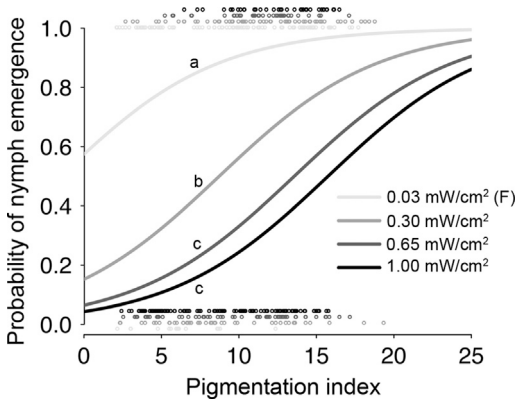

Figure 4. The Protective Effect of Egg Pigmentation against Ultraviolet Radiation

The probability of *P. maculiventris* nymphs successfully developing and emerging when developing in eggs of different pigmentation levels and exposed to four different constant intensities of UV radiation emanating from a UVA/B lamp (300–390 nm; applied during 16-hr light period of each day during development). F indicates that a UV-filtering lens was placed over the eggs to achieve the given intensity. Points show the successes and failures of individual eggs (displaced vertically for clarity). Lines show predictions from a logistic regression model fitted to the data; both UV treatment ($\chi^2 = 129.07$, $p < 0.0001$) and PI ($\chi^2 = 46.61$, $p < 0.0001$) were significant predictors of emergence probability. Different letters indicate significant differences between UV treatments (Tukey contrasts; $p < 0.05$). Total n = 460 eggs. For the response of ovipositing females to UV radiation, see Figure S3.

been observed in various life stages of other animals [23, 24]. Alternatively, female stink bugs may be able to detect the intensity of incident UV radiation (or visual wavelengths of light correlated with the presence of UV) and use this information to adjust the application of pigment to eggs. Another possibility is that *P. maculiventris* utilizes indirect gravitational or visual information to adjust pigment application to eggs. We attempted to distinguish between these hypotheses by conducting an experiment where individual stink bugs, contained in petri dishes, laid inside on the underside of white fabric illuminated from above, where we knew that they would tend to lay lightly pigmented eggs. To test whether egg pigmentation is influenced by the presence of UV light falling on the oviposition surface, we applied UVA/B radiation, filtered UVA/B radiation (wavelengths below 390 nm attenuated), or no light from below. In a fourth treatment, petri dishes were kept in complete darkness inside a closed box. Female *P. maculiventris* laid lightly pigmented eggs regardless of the type of radiation falling on the oviposition substrate (Figure S3). Furthermore, in the complete absence of any light, females tended to lay dark eggs on the underside of the white surface (Figure S3).

These results demonstrated that egg pigmentation (1) is not determined by the intensity of ultraviolet or visual light falling on the oviposition substrate, (2) is not due to pigment accumulation by the egg in response to UV radiation, and (3) is not determined by gravity. Rather, they support the idea that females evaluate visual characteristics of the substrate to determine egg pigmentation. Integrating the results of the previous experiment conducted on soybean plants (Figure 2), slightly more pigmented eggs may be laid in shaded environments because leaf surfaces appear overall darker, although extreme differences in luminosity were needed to produce such an effect. The fact that leaf position was a much more important determinant of egg pigmentation than luminosity suggests that ovipositing females may partially overcome this constraint by using a relative, rather than absolute, visual assessment of the substrate. The mechanism for this assessment could be an evaluation of the ratio of incident light (hitting the oviposition substrate) to reflected light (coming from the oviposition substrate). This ratio would be lower on the undersides of leaves, whose surface reflectance is increased by light passing through them from above. This proposed mechanism is similar to that suggested for crabs, prawns, and flatfish that dynamically modify their own pigmentation to match background brightness [25]. Given that temporal patterns of luminosity and UV radiation vary widely in nature (due to cloud cover, time of day, etc.), this kind of indirect, relative visual assessment could actually be a more reliable indicator of eggs' future cumulative UV exposure than direct measurement of light levels at the time of oviposition. The visual assessment of oviposition surfaces by *P. maculiventris* deserves further investigation, as it could reflect a general mechanism by which insects, including those that do not pigment their eggs, select oviposition sites.

The Egg Pigment Is Not Melanin

Most dark pigmentation in insects is attributable to melanin, a pigment composed of monomer units connected by strong carbon-carbon bonds, conferring a strong capacity to absorb UV radiation [21, 26]. Expecting to confirm our suspicion that the *P. maculiventris* egg pigment is melanin, we conducted standard biochemical analyses to detect markers of the two known groups of animal melanins: eumelanins and pheomelanins [10, 27–29]. Surprisingly, the amount of pigment in eggs (i.e., their

spectral absorbance) was not correlated with the concentration of markers for either type of melanin, and the concentration of markers in the samples analyzed was extremely low overall (Figure S2). However, the spectral absorbance of heavily pigmented eggs was similar to that of sepia melanin (Figure S2). Thus, the egg pigment is not melanin but appears to have a similar biological activity. Future work will focus on identifying the chemical composition and structure of this potentially novel pigment.

The Evolution of Selective Egg Pigmentation

For oviparous animals, being able to selectively apply pigment to eggs would presumably widen the range of potential environments available for oviposition, while minimizing the costs of pigment production. Our study raises the question of why the egg color of most animals is fixed, and, by extension, what set of conditions would be needed to favor the evolution of selective egg pigmentation. First, a physiological mechanism would have to evolve by which mothers can selectively apply pigment to eggs; this mechanism is as yet unknown for *P. maculiventris* (or any other organism). Additionally, for selective egg pigmentation to be evolutionarily stable, at least two conditions would have to be met: (1) the ability to deposit eggs in locations where pigmentation is needed, resulting in a net increase in offspring survival, and (2) laying non-pigmented eggs in locations where pigment is unneeded being advantageous in some situations. For *P. maculiventris*, the first condition could be met if predation pressure is higher on the undersides of leaves, as has been observed in other plant-dwelling arthropod systems [12, 15]. The upper surface of leaves would then represent "enemy free space" [30, 31], and applying the pigment when eggs are laid there would minimize the cost of the resulting tradeoff in terms of higher UV radiation exposure. The second condition could be met if there is a significant physiological cost of pigment production, as demonstrated in many other systems [24, 32, 33]. Laying eggs on the underside of leaves without having to pay the cost for applying pigment—while accepting the risk that eggs could be killed by predators—could be adaptive in some situations, especially if predators are uncommon or females are nutritionally stressed and have less resources to allocate to pigment production. Even if females are unable to adjust their oviposition behavior to match predation pressure in the environment, spreading lifetime egg production over leaf tops and leaf undersides, minimizing the relevant costs in each case, could be a "bet hedging" strategy [34] that ensures that at least some offspring survive in the face of environmental unpredictability.

The possibility remains that the *P. maculiventris* egg pigment could provide additional, secondary benefits not explored in the current study. For example, plasticity in egg pigmentation could camouflage eggs, if the matching of egg pigmentation with substrate reflectance decreases the contrast between eggs and leaf surfaces with respect to the visual systems of predators and parasitoids. Indeed, some egg parasitoids of stink bugs have visual biases toward certain colors [35], although the extent to which these visual biases are important for short-range host localization is unknown. Additionally, dark egg pigmentation could potentially allow eggs to collect more radiative heat and develop more rapidly [36], though we suspect that temperature differences between leaf tops and undersides may not be enough to select for this adaptation. The possibility that egg pigmentation could have one or more secondary functions for *P. maculiventris* is currently under evaluation (unpublished data).

Conclusions

Although seldom studied to date, the pigmentation of insect eggs could have a wide variety of ecological roles and may explain much of the variation in oviposition site selection and habitat use by insects in natural settings. Even though *P. maculiventris* is the first animal found to have selective control of egg pigmentation, we suspect that it is far from the only species with this adaptation. Indeed, the example described here occurs in an extremely well-studied and economically important insect species that is reared in laboratories around the world and was thus hiding in plain sight. A diverse array of similar adaptations and a multitude of evolutionary variations on this theme could be waiting to be discovered.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.06.010.

AUTHOR CONTRIBUTIONS

Conceptualization, P.K.A., E.G.-G., G.B., and J.B.; Methodology, P.K.A., E.G.-G., M.-L.D.-E., S.I., and K.W.; Investigation, P.K.A., E.G.-G., M.-L.D.-E., S.I., and K.W.; Formal Analysis, P.K.A, E.G.-G., and M.-L.D.-E.; Writing – Original Draft, P.K.A.; Writing – Review & Editing, P.K.A., S.I., K.W., G.B., and J.B.; Supervision, J.B. and G.B.; Funding Acquisition, J.B., S.I., and K.W.

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Supplemental Information

An Insect with Selective Control of Egg Coloration

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Supplemental Figures and Legends

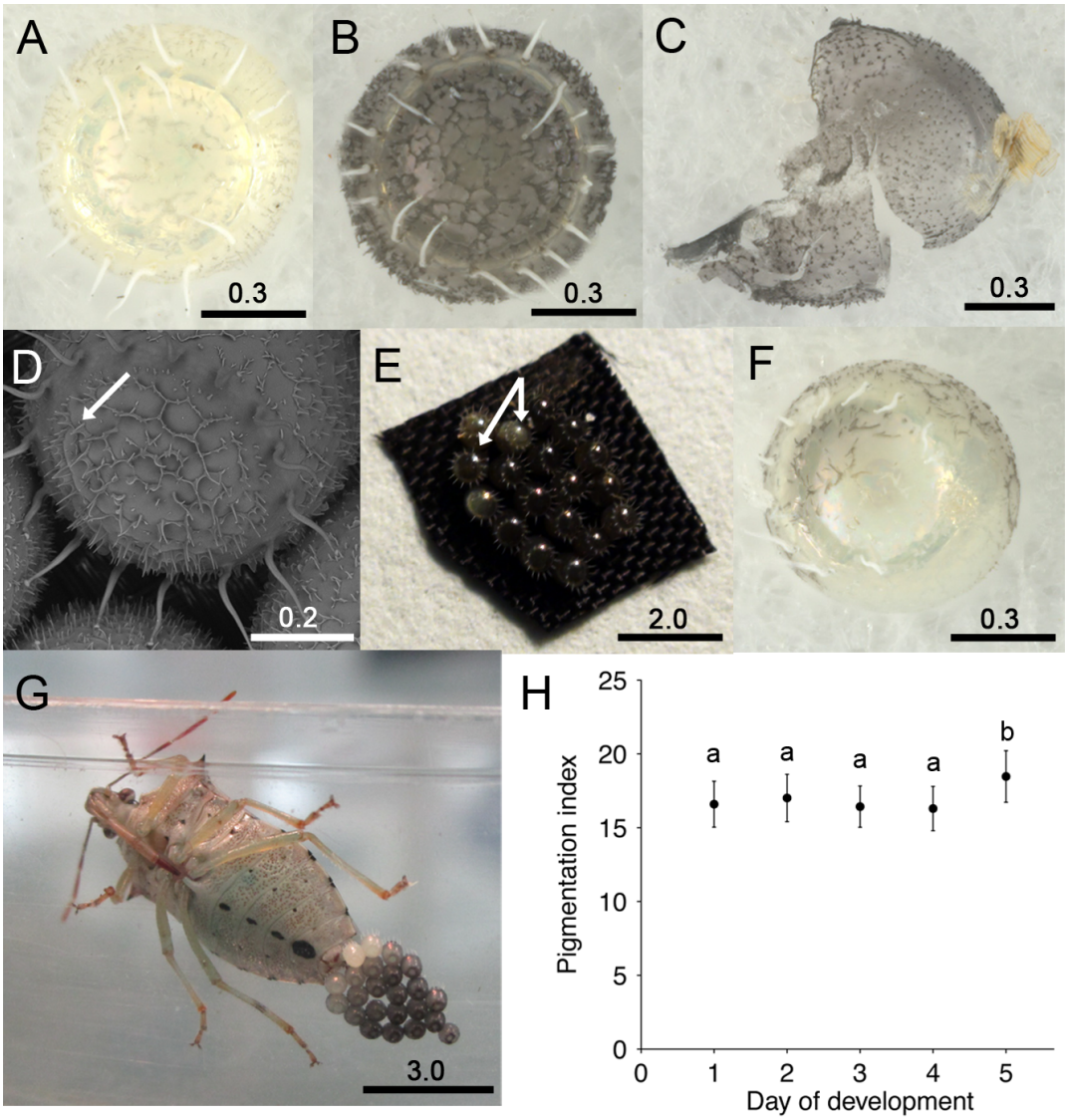

Figure S1 related to Figure 1. Description of *Podisus maculiventris* egg pigmentation. Overhead photos of (*A*) lightly (Pigmentation Index = 4.3) and (*B*) heavily (Pigmentation Index = 21.5) pigmented eggs are shown. Pigment is contained within the exochorion, seen removed in (*C*), particularly concentrated in exochorionic spines (*D*), which are also present on lightly pigmented eggs. Pigmentation rarely shows noticeable variability (*E*) within the same egg mass or (*F*) on the same egg. (*G*) The chorion is pale white immediately after laying, reaching its final pigmentation within an hour. (*H*) The apparent darkness of the egg varies slightly over a 5-day

period due to embryonic development (linear mixed model with individual egg identity as a random factor; day of development $\chi^2 = 22.58$, $p < 0.001$, total N = 25 eggs), but not significantly so until the 5th day, soon before emergence of the nymph (different letters indicate significant differences between categories; Tukey contrasts, $p < 0.05$). All scale bar measurements are in millimeters.

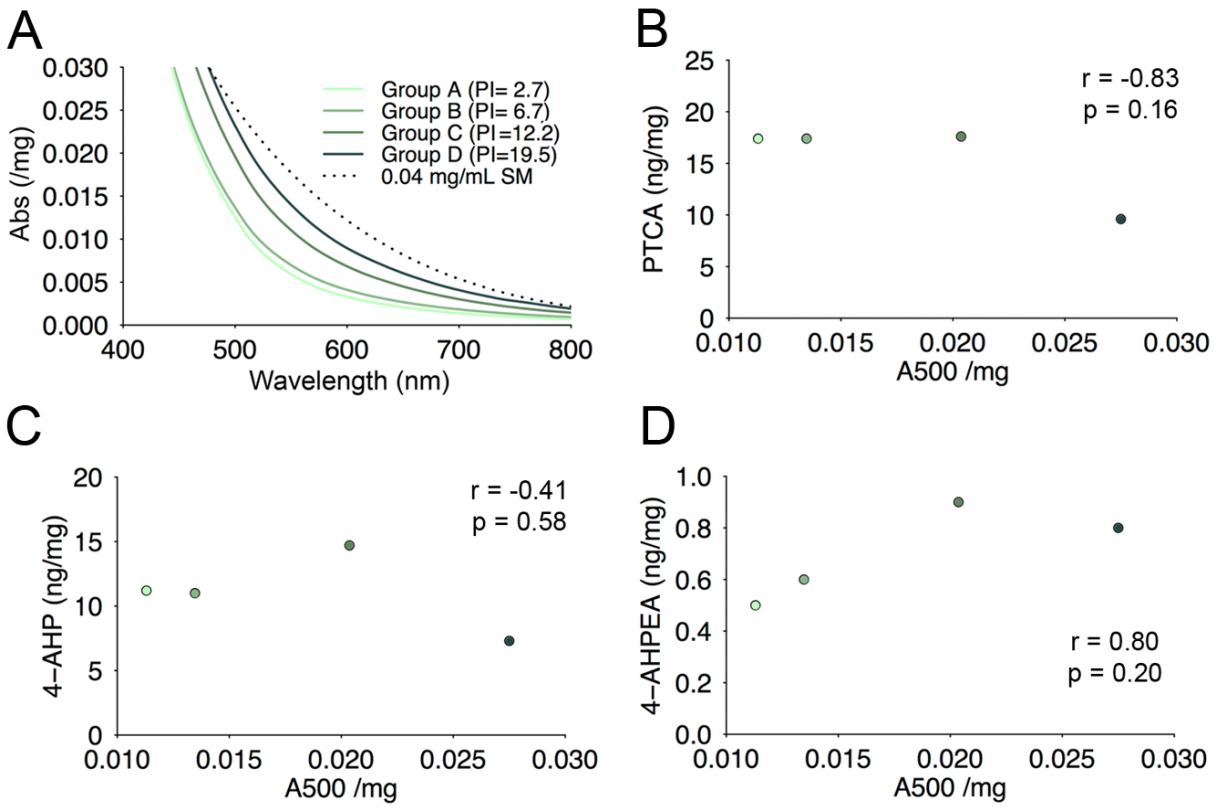

Figure S2 related to Figure 1. Spectral absorbance and biochemical analysis of *Podisus maculiventris* eggs. (*A*) Absorbance spectra of pooled groups of eggs with different pigmentation levels compared to 0.04 mg/mL sepia melanin (SM); Relationships between spectral absorbance at 500 nm (A500, marker for total melanin) and (*B*) PTCA (marker for eumelanin), (*C*) 4-AHP (marker for pheomelanin), and (*D*) 4-AHPEA (marker for cysteinyl dopamine-derived units). In (*B*)-(*D*) each point is the average of a duplicate assay.

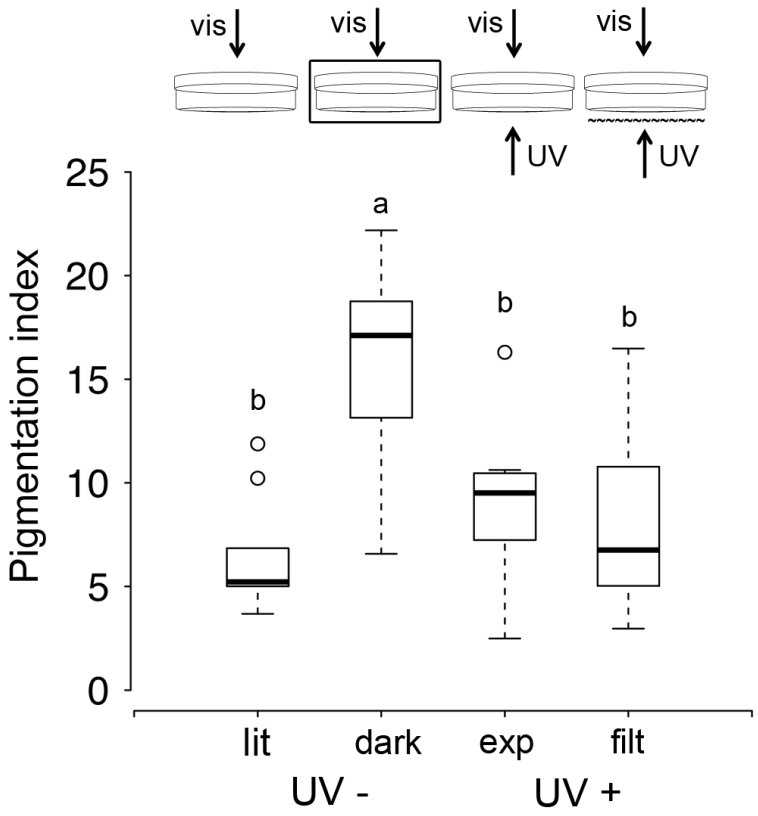

Figure S3 related to Figure 4. The pigmentation of eggs laid by *Podisus maculiventris* females on the underside of a white substrate, inside Petri dishes. Treatments: *lit* – visual light from above, no UV from below; *dark*: visual light from all directions blocked, no UV from below; *exp* – visual light from above, UV from below; *filt* – visual light from above, filtered UV from below. Pigmentation index varied significantly among treatments (linear mixed model with individual as random factor; $\chi^2 = 23.90$, $p < 0.0001$). Different letters indicate significant differences between treatments (Tukey contrasts; $p < 0.05$). Total N = 50 egg masses.

Supplemental Experimental Procedures

Study system

Podisus maculiventris Say (Hemiptera: Pentatomidae) is a predatory stink bug indigenous to North America that has also been introduced to other regions of the world as a part of classical biological control programs. Nymphs and adults feed on a wide variety of arthropods in diverse habitat types [S1]. The general biology and ecology of *P. maculiventris* has been extensively studied [S2-S5], in part due to its frequent use as a biological control agent of arthropod pests in agroecosystems and mass production by the biological control industry. Eggs are barrel-shaped and metallic, typically laid in tight-fitting clusters of up to 50 (but typically between 8 and 20) eggs on the upper and lower surfaces of the leaves of many different plant species [S1, S6].

Insect colonies

Colonies of *P. maculiventris* were established from individuals (~200) collected from several locations in the London and Ottawa (Ontario, Canada) regions in 2011 and 2012. Colonies were maintained continuously thereafter in ventilated cages 30 cm^3 (BugDorm, Taiwan) for late-instar (IV-V) nymphs, and ventilated plastic cylinders (diameter: 11.0 cm, height: 15.5 cm) for early-instar (I-III) nymphs. Nymphs and adults were fed with the larvae and pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and fresh green beans. Upon molting, adults were transferred to plastic bins (l: 29.0 cm, w: 17.0 cm, h: 10 cm) lined with green polyester fabric (Fabricville, Montréal, Canada) for oviposition substrate, from which egg masses were collected daily for experiments. When cohorts of adults of known ages were needed, newly-molted (<48 h) adults were separated into plastic cylinders in groups of 10-20 individuals until they were used for experiments, following sexual maturation and mating (ca. 7-10 days after molting). Unless

stated otherwise, insects were kept at 24 ± 1°C, 50 ± 5% RH, and a 16L:8D photoperiod, at an illumination of 9000 ± 1000 lux, produced by linear fluorescent lights (Philips 86W F96T8/TL841/H0/Plus).

Quantification of egg pigmentation

To measure their pigmentation, eggs were first removed from the oviposition substrate and glued upright on white filter paper with non-toxic white glue (LePage©, Canada). Photographs of the eggs were then taken under standardized lighting conditions (9000 ± 1000 lux) under 14x magnification with a digital microscope camera (Dino-Lite AM-4012NZT, London, Canada) connected to a digital recording device (MSD09, Dino-Lite, London, Canada). Each photograph included a square of filter paper painted white on one side and black on the other, to subsequently allow the correction of white balance using ImageJ software version 1.48 [S7] and its "Chart White Balance" macro. Next, in ImageJ, the circular area on the top of each egg, bounded by the egg's chorionic processes, was selected and its brightness (=[image values of red channels + green channels + blue channels]/3) was measured as a proxy for egg pigmentation. These measurements corresponded well to qualitative visual assessments of egg pigmentation (see Figure 1), and were highly repeatable when the same eggs were photographed and analyzed separately by two different experimenters ($R^2 = 0.95$, $y = 1.04x + 1.1324$, $n = 50$ eggs). Whenever eggs were photographed on green fabric instead of white filter paper, a correction (filter paper brightness = 0.9401*fabric brightness + 28.27) obtained by measuring the brightness of the same eggs on fabric and filter paper ($R^2 = 0.91$, n=47), was applied. Measurements of egg brightness were always taken between 2-72 hours after eggs were laid.

Next, we developed a calibration curve to relate the brightness measurements taken in photographs to the actual quantity of pigment in eggs (measured via spectral absorbance). This was necessary to correct for the fact that measurements of brightness by imaging devices are non-linear with regards to light intensity, which can cause over-estimation of low reflectance values and under-estimation of high values [S8]. We collected pooled groups of 300-400 eggs that belonged to four brightness categories (i) 60-80 (mean = 72), (ii) 100-120 (mean = 110), (iii) 140-160 (mean = 150), (iv) 180-200 (mean = 189). Samples of eggs (11-12 mg) from each category were directly subjected to Soluene-350 solubilization [S9], with three replicates performed for each brightness category. The absorbance spectrum of each sample was measured in the visual light range (400-800 nm) and compared to a standard sample of 1 mg/mL sepia-melanin (compressed by a factor of 0.04). Spectral measurements below 400 nm were not possible because of strong absorption by Soluene-350 at lower wavelengths, although absorbance of melanin is known to increase exponentially into the UV range [S10]. To calculate pigmentation index (PI), the absorbance of 500 nm light for each egg category was plotted against its average brightness, a regression curve was fitted to the data, and the predicted background absorbance of eggs with the highest possible brightness (220; i.e., the absorbance of background egg constituents in the absence of pigment) was subtracted out (see Figure 1).

Embryonic development and egg color

To characterize how embryonic development (i.e., the darkening of egg contents) affected measurements of egg pigmentation, we measured the brightness of the same 25 eggs on each of five consecutive days after they were laid (nymphs emerge after 6 days). Brightness measurements were then converted to PIs for analysis. Eggs spanned a wide range of PIs (min:

6.4, max: 30.75, median: 16.6) on the first day of measurement (photos taken between 2 and 16 h after laying). To test whether egg pigmentation measurements varied significantly over the development period, we ran linear mixed models with PI as the dependent variable, day of development as a categorical fixed factor, and egg ID as a random factor.

Oviposition substrate reflectance

The goal of the first experiment was to determine the effect of substrate reflectance on the pigmentation of eggs laid by *P. maculiventris*, and to confirm that a single individual can produce eggs of different levels of pigmentation. Three types of Petri dish arenas (d: 9.0 cm, h: 1.8 cm) were prepared, differing with respect to the water-based acrylic paint (DecoArt, United Kingdom, DCA47-black and DCA01-white) applied to their entire outside surface: (i) B: black (n=14), (ii) W: white (n=11), and (iii) BW (n=14): painted black on one side and white on the other (i.e., half of the dishes' lid and half of the bottom were painted with each shade of paint). The paint did not completely block the transmission of light through the lids of the dishes; lids were somewhat illuminated from light passing through them from above. Mating couples of *P. maculiventris* (7-10 days old) were placed in the arenas and provided with two *T. molitor* larvae and a small (4-6 cm) piece of green bean. Food was replaced and eggs were collected every 2-3 days, until the death of the female. Upon collection of eggs, their location within the Petri dish (top, side, or bottom; white side or black side in the BW treatment) was noted, and their brightness was measured, converted to PI and averaged for each egg mass. To compare the PI of eggs laid in B and W dishes, we fitted linear mixed models with PI as the dependent variable, dish color and laying position as fixed categorical factors, and female ID as a random factor.

The same analysis was run to compare the PI of eggs laid on the white and black half of the BW dishes.

Laying position on leaves and luminosity level

Next, an experiment was conducted to evaluate the effect of lighting level on the pigmentation of eggs laid by *P. maculiventris*, and to investigate how plasticity in egg pigmentation manifests itself when a natural oviposition substrate (i.e., plant leaves) is available. Ten female and five male *P. maculiventris* (10-20 days old) were placed in ventilated plexiglass cages (51.0 x 35.5 x 30.5 cm) containing three pots (d: 15.2 cm, h: 10.7 cm) with five soybean (*Glycine max* (L.)) cultivar BeSweet, 2001-11C, Stokes, Canada) plants (stage V2) each for 72h. Each of five full blocks contained two cages set up at the same time, one exposed to ambient lighting ("Fully lit": 12000-13000 lux) and the other completely covered with a single sheet of black polyester fabric (Fabricville, Montréal, Canada), which reduced luminosity levels inside the cage more than 50-fold ("Shaded": 200-300 lux). Eggs were collected, noting their position (leaf top, leaf underside). Egg masses laid on the plexiglass cage or on the pot were excluded from the analysis. For each egg mass collected, the brightness of a subset of five randomly selected eggs was measured, converted to PI, and averaged. With egg mass PI as the dependent variable, we then fitted linear mixed models with shading treatment and laying position as fixed categorical factors, and block as a random factor.

Effects of UV radiation and egg pigmentation on embryonic developmental success

To test whether egg pigmentation affects the developmental success of eggs under different doses of UV radiation, egg masses were first collected from the *P. maculiventris* colony, left on

small (~1-2 cm^2) pieces of the green polyester fabric, and photographed to measure and correct the brightness of each egg (later converted to PI). They were then placed at one of four different UV intensities by varying their distances from a UV lamp (Exo-Terra Repti Glo 15W linear desert bulb, 41.5 cm long), which was resting on the top of an open glass frame (40.8 x 20.3 x 25.9 cm), under standard lighting conditions, producing an overall illumination of 8000 ± 400 lux inside the frame. The UV lamp simulated natural sunlight by emitting both visual and UVA/B-spectrum light, with UV emission ranging between 290 and 400 nm, peaking in the UV-A at 330-370 nm. Although other studies sometimes administer UV-A and UV-B radiation to arthropods separately [S11, S12], we considered a full-spectrum treatment more representative of natural conditions, especially since there could be an interactive effect of UV-A and UV-B wavelengths [S13]. UV intensity (mW/cm^2) was measured with a UVA/B light meter (model 850009, Sper Scientific, Scottsdale, AZ), whose responsivity curve ranged between 290 and 375 nm, peaking between 310-360 nm, and thus closely matched the output of the UV lamp. Egg masses assigned to the lowest intensity-treatment, 0.03 mW/cm^2, were at the same distance (22.1 cm) from the lamp as the 0.30 mW/cm^2 treatment, but a UV lens filter (Polaroid Pro Series 86mm Super Slim L39 MC UV Filter) was suspended 2 cm above the egg masses to attenuate the passage of UV light below 390 nm. The two remaining treatments of 0.65 mW/cm^2 and 1.0 mW/cm^2 were 14.1 and 9.3 cm from the UV lamp, respectively, and did not have a UV filter placed over them. The four intensities of UV radiation resulted in cumulative doses that are within the range present in exposed locations outdoors, based on measurements performed in Montréal, Canada in the summer of 2014 with the same UV-meter (PKA, unpublished data). A household fan blowing under the UV lamp was used to equalize temperature at 25 ± 1°C throughout the vertical column of the setup. When the emergence of nymphs commenced, egg

masses were removed from the setup and placed under standard rearing conditions inside glass tubes (d: 2.2 cm, h: 5.2 cm) noting the number of individuals emerging twice daily thereafter. Egg masses with no emergence were removed after 8 days, ~24-48 h after expected emergence (there was no subsequent emergence from these masses). The egg masses were inspected under a dissection microscope (40 x magnification) and the state of each egg (emerged or not) was recorded. Eggs that did not emerge contained embryos along the spectrum of maturation, although they typically died in the early stages of development. With emergence probability as the dependent variable, we then fitted a logistic regression with egg PI as a continuous factor and UV treatment as a categorical factor.

Oviposition under different UV exposure and lighting conditions

We next tested whether pigmentation of eggs is directly related to the presence of UV radiation and/or visible light. Adults were placed in transparent plastic Petri dishes (d: 9.0 cm, h: 1.3 cm) with white polyester fabric on inside of the upper surface, to create conditions where females would normally lay relatively lightly-colored eggs, as determined by previous experiments (i.e., mimicking 'leaf underside conditions'). They were provided with two *T. molitor* larvae and three to four green bean seeds (instead of pods) to minimize the amount of light blocked from below. These arenas were placed under standard rearing conditions (9000 ± 1000 lux of illumination from above) at one of four conditions with respect to UV exposure and illumination: (i) *lit* – suspended 16.0 cm over a plastic tray, with no UV from below; (ii) *dark*: inside a black metal box (18.8 x 21.2 x 7.5 cm) lined with black foam, with 0 mW/cm^2 of UV radiation and 0 lux of illumination; (iii) *exp* – suspended 16.0 cm above a UV lamp (see above), with UV intensity from below ranging from 0.30 to 0.70 mW/cm^2, depending on location within the Petri

dish; (iv) *filt* – placed 16.0 cm above the UV lamp and fitted with a UV filter (see above), with the resulting attenuated UV intensity from below ranging from 0.01 to 0.03 mW/cm^2. All aforementioned UV intensities were measured through the surfaces of the Petri dishes to account for the fact that the plastic attenuated UV intensity by ~3-5%. The experiment lasted for 48 h, after which eggs were collected and their position in the Petri dish was noted. Any eggs not laid on the white fabric were excluded from the analysis. The brightness of all eggs was measured, converted to PI, and averaged for each egg mass. We then fitted a linear mixed model to the data, with egg mass PI as the dependent variable, light treatment and laying position as fixed factors, and female ID as a random factor.

Biochemical analyses of the egg pigment

We conducted biochemical analyses to test for markers of eumelanin (pyrrole-2,3,5-tricarboxylic acid; PTCA), pheomelanin (4-amino-3-hydroxyphenylalanine; 4-AHP), and cysteinyl-dopamine derived units (4-amino-3-hydroxyphenylethylamine; 4-AHPEA), the last of which is a chemical precursor to pheomelanin. Egg samples of a known brightness (see above) were homogenized with Ten-Broeck glass homogenizer at a concentration of 10 mg/mL H_2O, and 100 μL (1 mg) aliquots were subjected to alkaline hydrogen peroxide oxidation [S14] and hydroiodic acid hydrolysis [S15]. 4-AHPEA was analyzed as described previously [S16]. In these previous studies, the concentrations of the aforementioned markers correlate well with the amount of melanin (and thus spectral absorbance) in a sample. We conducted Pearson's correlation analyses to test whether the mean pigmentation index of a sample (i.e., its absorbance of 500 nm light) was correlated with the concentration of each marker after chemical analysis.

Statistical analyses

All statistical analyses were conducted with the R software package, version 2.15.1 [S17]. For linear mixed models, assumptions of error normality and homoscedasticity were verified via evaluation of residual and quantile-quantile plots. We also made sure that logistic regression fits showed no signs of overdispersion [S18]. Tukey contrasts among levels of categorical factors were performed with the "glht" function in the "multcomp" package of R software.

Supplemental References

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