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Hairiness: the missing link between pollinators and pollination

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Functional traits are the primary biotic component driving organism influence on ecosystem functions; in consequence, traits are widely used in ecological research. However, most animal trait-based studies use easy-to-measure characteristics of species that are at best only weakly associated with functions. Animal-mediated pollination is a key ecosystem function and is likely to be influenced by pollinator traits, but to date no one has identified functional traits that are simple to measure and have good predictive power. Here, we show that a simple, easy to measure trait (hairiness) can predict pollinator effectiveness with high accuracy. We used a novel image analysis method to calculate entropy values for insect body surfaces as a measure of hairiness. We evaluated the power of our method for predicting pollinator effectiveness by regressing pollinator hairiness (entropy) against single visit pollen deposition (SVD) and pollen loads on insects. We used linear models and AIC_C model selection to determine which body regions were the best predictors of SVD and pollen load. We found that hairiness can be used as a robust proxy of SVD. The best models for predicting SVD for the flower species *Brassica rapa* and *Actinidia deliciosa* were hairiness on the face and thorax as predictors (R^2 = 0.98 and 0.91 respectively). The best model for predicting pollen load for *B*. *rapa* was hairiness on the face (R^2 = 0.81). Accordingly, we suggest that the match between pollinator body region hairiness and plant reproductive structure morphology is a powerful predictor of pollinator effectiveness. We show that pollinator hairiness is strongly linked to pollination – an important ecosystem function, and provide a rigorous and time-efficient method for measuring hairiness. Identifying and accurately measuring key traits that drive ecosystem processes is critical as global change increasingly alters ecological communities, and subsequently, ecosystem functions worldwide.

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Title

Hairiness: the missing link between pollinators and pollination

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Abstract

Functional traits are the primary biotic component driving organism influence on ecosystem functions; in consequence traits are widely used in ecological research. However, most animal trait-based studies use easy-to-measure characteristics of species that are at best only weakly associated with functions. Animal-mediated pollination is a key ecosystem function and is likely to be influenced by pollinator traits, but to date no one has identified functional traits that are simple to measure and have good predictive power. Here, we show that a simple, easy to measure trait (hairiness) can predict pollinator effectiveness with high accuracy. We used a novel image analysis method to calculate entropy values for insect body surfaces as a measure of hairiness. We evaluated the power of our method for predicting pollinator effectiveness by regressing pollinator hairiness (entropy) against single visit pollen deposition (SVD) and pollen loads on insects. We used linear models and AIC_C model selection to determine which body regions were the best predictors of SVD and pollen load. We found that hairiness can be used as a robust proxy of SVD. The best models for predicting SVD for the flower species *Brassica rapa* and *Actinidia deliciosa* were hairiness on the face and thorax as predictors ($R^2 = 0.98$ and 0.91 respectively). The best model for predicting pollen load for *B. rapa* was hairiness on the face ($R^2 = 0.81$). Accordingly, we suggest that the match between pollinator body region hairiness and plant reproductive structure morphology is a powerful predictor of pollinator effectiveness. We show that pollinator hairiness is strongly linked to pollination – an important ecosystem function, and provide a rigorous and time-efficient method for measuring hairiness. Identifying and accurately measuring key traits that drive ecosystem processes is critical as global change increasingly alters ecological communities, and subsequently, ecosystem functions worldwide.

Introduction

Trait-based approaches are now widely used in functional ecology, from the level of individual organisms to ecosystems (Cadotte et al. 2011). Functional traits are defined as the characteristics of an organism's phenotype that determine its effect on ecosystem level processes (Naeem & Wright 2003; Petchey & Gaston 2006). Accordingly, functional traits are recognised as the primary biotic component by which organisms influence ecosystem functions (Gagic et al. 2015; Hillebrand & Matthiessen 2009). Trait-based research is dominated by studies on plants and primary productivity, and little is known about key traits for animal-mediated and multi-trophic functions, particularly for terrestrial invertebrates (Didham et al. 2016; Gagic et al. 2015; Lavorel et al. 2013).

Most animal trait-based studies simply quantify easy-to-measure morphological characteristics, without a mechanistic underpinning to demonstrate these "traits" have any influence on the ecosystem function of interest (Didham et al. 2016). This results in low predictive power, particularly where trait selection lacks strong justification through explicit ecological questions (Gagic et al. 2015; Petchey & Gaston 2006). If the ultimate goal of trait-based ecology is to identify the mechanisms that drive biodiversity impacts on ecosystem function, then traits must be quantifiable at the level of the individual organism, and be inherently linked to an ecosystem function (Bolnick et al. 2011; Pasari et al. 2013; Violle et al. 2007).

Methodology that allows collection of trait data in a rigorous yet time-efficient manner and with direct functional interpretation will greatly enhance the power of trait-based studies. Instead of subjectively selecting a large number of traits with unspecified links to ecosystem functions, it would be better to identify fewer, uncorrelated traits, that have a strong bearing on the function of interest (Carmona et al. 2016). Selecting traits that are measurable on a continuous scale, would also improve predictive power of studies (McGill et al. 2006; Violle et al. 2012). However, far greater time and effort is required to measure such traits, exacerbating the already demanding nature of trait-based community ecology (Petchey & Gaston 2006).

Animal-mediated pollination is a multi-trophic function, driven by the interaction between animal pollinators and plants (Kremen et al. 2007). A majority of the world's wild plant species are pollinated by animals (Ollerton et al. 2011), and over a third of global crops are dependent on animal pollination (Klein et al. 2007). Understanding which pollinator traits determine the effectiveness of different pollinators is critical to understanding the mechanisms of pollination processes. However, current traits used in pollination studies often have weak associations with pollination function and/or have low predictive power. For example Larsen, Williams & Kremen (2005) used body mass to explain pollen deposition by solitary bees even when the relationship was weak and non-significant. Many trait-based pollination studies have subsequently used body mass or similar size measures, despite their low predictive power. Similarly, Hoehn et al (2008) used spatial and temporal visitation preferences of bees to explain differences in plants reproductive output. They found significant relationships (i.e. low *P* values) between spatial and temporal visitation preferences and seed set, but with small R^2 values, suggesting these traits have weak predictive power. To advance trait-based pollination research we require traits that are good predictors of pollination success.

Observational studies suggest that insect body hairs are important for collecting pollen that is used by insects for food and larval provisioning (Holloway 1976; Thorp 2000). Hairs facilitate active pollen collection e.g. many bees have specialised hair structures called scopae that are used to transport pollen to the nest for larval provisioning (Thorp 2000). Additionally, both bees and flies have hairs distributed across their body surfaces which act to passively collect pollen for adult feeding (Holloway 1976). Differences in the density and distribution of hairs on pollen feeding insects likely reflects their feeding behaviour, the types of flowers they visit, and whether they use pollen for adult feeding and/or larval provisioning (Thorp 2000). However, despite anecdotal evidence that insect body hairs are important for pollen collection and pollination, there is no proven method for measuring hairiness, nor is there evidence that hairier insects are more effective pollinators.

Here, we present a novel method based on image entropy analysis for quantifying pollinator hairiness. We define pollination effectiveness as single visit pollen deposition (SVD): the number of conspecific pollen grains deposited on a virgin stigma in a single visit (King et al. 2013; Ne'eman et al. 2010). SVD is a measure of an insects' ability to acquire free pollen grains on the body surface and accurately deposit them on a conspecific stigma. We predict that hairiness, specifically on the body parts that contact the stigma, will have a strong association with SVD. We show that the best model for predicting pollinator SVD for pak choi (*Brassica rapa*) is highly predictive and includes hairiness of the face and thorax dorsal regions as predictors, and the face region alone explains more than 90% of the variation. Our novel method for measuring hairiness is rigorous, time efficient and inherently linked to pollination function.

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Accordingly, this method could be applied in diverse trait-based pollination studies to progress understanding of the mechanisms that drive pollination processes.

Materials and Methods

Imaging for hairiness analysis

We photographed pinned insect specimens using the Visionary Digital Passport portable imaging system (Figure 1). Images were taken with a Canon EOS 5D Mark II digital camera (5616 x 3744 pix). The camera colour profile was sRGB IEC61966-2.1, focal length was 65mm and F-number was 4.5. We used ventral, dorsal and frontal shots with clear illumination to minimise reflection from shinny insect body surfaces. All photographs were taken on a plain white background. Raw images were exported to Helicon Focus 6 where they were stacked and stored in .jpg file format.

Image processing and analysis

We produced code to quantify insect pollinator hairiness using MATLAB (MathWorks, Natick, MA, USA), and functions from the MATLAB Image Processing ToolBox. We quantified relative hairiness by creating an entropy image for each insect photograph, and computed the average entropy within user-defined regions (Gonzales et al. 2004). To calculate entropy values for each image we designed three main functions. The first function allows the user to define up to four regions of interest (RoIs) within each image. The user can define regions by drawing contours as closed polygonal lines of any arbitrary number of vertexes. All information about regions (location, area and input image file name) is stored as a structure in a .mat file.

The second function executes image pre-processing. We found that some insects had pollen grains or other artefacts attached to their bodies, which would alter the entropy results. Our pre-processing function eliminates these objects from the image by running two filtering processes. First, the function eliminates small objects with an area less than the user definable threshold (8 pixels by default). For the first task, each marked region is segmented using an optimized threshold obtained by applying a spatially dependant thresholding technique. Once each region has been segmented, a labelling process is executed for all resulting objects and those with an area smaller than the minimum value defined by the user are removed. Secondly, as pollen grains are often round in shape, the function eliminates near-circular objects. The perimeter of each object is calculated and its similarity to a circle (S) id defined as:

$$S = \frac{4\pi \cdot Area}{Perimeter^2}$$

Objects with a similarity coefficient not within the bounds defined by the user (5% by default) are also removed from the image. Perimeter calculation is carried out by finding the object's boundary, and computing the accumulated distance from pixel centre to pixel centre across the border, rather than simply counting the number of pixels in the border. The entropy filter will not process objects that have been marked as "deleted" by the pre-processing function. This initial pre-processing provides flexibility by allowing users to define the minimum area threshold and the degree of similarity of objects to a circle. Users can also disable the image pre-processing by toggling a flag when running the entropy filter.

Once pre-processing is complete, each image is passed to the third function, which is the entropy filter calculation stage. The entropy filter produces an overall measure of randomness within each of the user defined regions on the image. In information theory, entropy (also expressed as

Shannon Entropy) is an indicator of the average amount of information contained in a message (Shannon 1948). Therefore, Shannon Entropy, H, of a discrete random variable X that can take n possible values $\{x_1,x_2,...,x_n\}$, with a probability mass function $P(X)$ is given by:

$$H(X) = -\sum_{i=1}^{n} P(x_i) \cdot Log_2(P(x_i))$$

When this definition is used in image processing, local entropy defines the degree of complexity (variability) within a given neighbourhood around a pixel. In our case, this neighbourhood (often referred to as the structuring element) is a disk with radius r (we call the radius of influence) that can be defined by the user (7 pixels by default). Thus for a given pixel in position (*i,j*) in the input image, the entropy filter computes the histogram G_{ij} (using 256 bins) of all pixels within its radius of influence, and returns its entropy value H_{ij} as:

$$H_{ij} = -G_{ij} \cdot log_2(G_{ij})$$

where G_{ij} is a vector containing the histogram results for pixel (*i,j*) and ($\cdot$) is the dot product operator. Using default parameters, our entropy filter employs a 7 pixel (13 x 13 neighbourhood) radius of influence, and a disk-shaped structuring element, which we determined based on the size of hairs. Therefore, in the entropy image, each pixel takes a value of entropy when considering 160 pixels around it (by default). However, the definition of the optimum radius of influence depends on the size of the morphological responsible for the complexity in the RoI. This is defined not only by the physical size of these features but also by the pixel-to-millimetre scaling factor (i.e. number of pixels in the sensor plane per mm in the scene plane). Thus, although 7 pixels is the optimum in our case to detect hairs, the entropy filter function takes this radius as an external parameter which can be adjusted by the user to meet their needs.

The entropy filter function is a process that runs over three different entropy layers (E_R, E_G, E_B), one for each of the camera's colour channels (Red, Green, and Blue), for each input image. These three images are combined into a final combined entropy image E_S, where each pixel in position (i,j) takes the value $E_{S(i,j)}$:

$$E_{S(i,j)} = E_{R(i,j)} \cdot E_{G(i,j)} \cdot E_{G(i,j)}$$

Once entropy calculations are complete, our function computes averages and standard deviations of E_S within each of the regions previously defined by the user, and writes the results into a .csv file (one row per image). Entropy values produced by this function are consistent for different photos of the same region on the same specimen (Supporting Information 6; Table S2). The scripts for the image pre-processing, region marking and entropy analysis functions are provided, along with a MATLAB tutorial (Supporting Information 1-4).

Case study: Hairiness as a predictor of SVD and pollen load

Model flower floral biology

We used *Brassica rapa* var. *chinensis* (Brassicaceae) or pak choi as our model flower to determine if our measurement of insect hairiness is a good predictor of pollinator effectiveness. *B. rapa* is a mass flowering global food crop (Rader et al. 2009). It has an actinomorphic open pollinated yellow flower with four sepals, four petals, and six stamens (four long and two short) (Walker et al. 1999). The nectaries are located in the centre of the flower, between the stamens and the petals, forcing pollinators to introduce their head between the petals. *B. rapa* shows increased seed set in the presence of insect pollinators and the flowers are visited by a diverse assemblage of insects that differ in their ability to transfer pollen (Rader et al. 2013).

Insect pollinator collection for entropy analysis

We collected pollinating insects from *B. rapa* crops for image analysis during the summer of December 2014 – January 2015. Insects were chilled immediately and then killed by freezing within 1 day and stored at -18°C in individual vials. All insects were identified to species level with assistance from expert taxonomists.

Image processing

We measured the hairiness of 10 insect pollinator species (*n*=8–10 individuals per species), across five families and two orders. This included social, semi-social and solitary bees and pollinating flies. Regions marked included: 1) face; 2) head dorsal; 3) head ventral; 4) front leg; 5) thorax dorsal; 6) thorax ventral; 7) abdomen dorsal and 8) abdomen ventral. All entropy analysis was carried out using our image processing method outlined above.

Single visit pollen deposition (SVD) and pollen load

We used SVD data for insect pollinators presented in Rader et al. (2009) and Howlett et al. (2011); a brief description of their methods follows.

Pollen deposition on stigmatic surfaces (SVD) was estimated using manipulation experiments. Virgin *B. rapa* inflorescences were bagged to exclude all pollinators. Once flowers had opened, the bag was removed, and flowers were observed until an insect visited and contacted the stigma in a single visit. The stigma was then removed and stored in gelatine-fuchsin and the insect was captured for later identification. SVD was quantified by counting all *B*. *rapa* pollen grains on the stigma. Mean values of SVD for each species are used in our regression models.

To quantify the number of pollen grains carried (pollen load) Howlett et al. (2011) collected insects while foraging on *B*. *rapa* flowers. Insects were captured using plastic vials containing a rapid killing agent (ethyl acetate). Once dead, a cube of gelatine-fuchsin was used to remove all pollen from the insect's body surface. Pollen collecting structures (e.g. corbiculae, scopae) were not included in analyses because pollen from these structures is not available for pollination. Slides were prepared in the field by melting the gelatine-fuchsin cubes containing pollen samples onto microscope slides. *B. rapa* pollen grains from each sample were then quantified by counting pollen grains in an equal-area subset from the sample and multiplying this by the number of equivalent sized subset areas within the total sample.

Statistical analyses

We used linear regression models and AIC_C (small sample corrected Akaike information criteria) model selection to determine if our measure of pollinator hairiness is a good predictor of SVD and pollen load. We constructed global models with SVD or pollen load as the response variable, body region as predictors and body length as an interaction i.e. SVD or pollen load ~ body length * entropy face + entropy head dorsal + entropy head ventral + front leg + entropy thorax dorsal + entropy thorax ventral + entropy abdomen dorsal + entropy abdomen ventral. Global linear models were constructed using the lm(stats) function. We excluded other body size measurements from models as they had high correlation coefficients (Pearson's $r > 0.7$) with body length. AIC_C model selection was carried out on the global models using the function glmulti() with fitfunction = “lm” in the package *glmulti*. We examined heteroscedasticity and normality of errors of models by visually inspecting diagnostic plots using the *glmulti* package (Crawley 2002). Variance inflation factors (VIF) of predictor variables were checked for the best models using the vif() function in the *car* package. All analyses were done in R version 3.2.4 (R Development Core Team 2014).

Results

Body hairiness as a predictor of SVD

For SVD, the face and thorax dorsal regions were retained in the best model selected by AIC_C, which had an adjusted R^2 value of 0.98. The subsequent top models within 10 AIC_C points all retained the face and thorax dorsal regions and additionally included the abdomen ventral (adjusted $R^2 = 0.98$), head dorsal (adjusted $R^2 = 0.98$), and thorax ventral (adjusted $R^2 = 0.97$) and front leg (adjusted $R^2 = 0.97$) regions respectively (Table 1). The model with the face region included as a single predictor had an adjusted R^2 value of 0.88, indicating that this region alone explained a majority of the variation in the top SVD models (Figure 2).

Body hairiness as a predictor of pollen load

The best model for pollen load retained the face region only and had an adjusted R^2 value of 0.81 (Figure 3; Table 1). The subsequent best models retained the abdomen dorsal (adjusted R^2 value of 0.73), the face and head dorsal (adjusted $R^2 = 0.83$), the face and abdomen dorsal (adjusted $R^2 = 0.82$) and the abdomen dorsal and front leg (adjusted $R^2 = 0.8$) regions respectively.

Discussion

Here we present a rigorous and time-efficient method for quantifying hairiness, and demonstrate that this measure is an important pollinator functional trait. We show that insect pollinator hairiness is a strong predictor of SVD for the open-pollinated flower *Brassica rapa*. Linear models that included multiple body regions as predictors had the highest predictive power; the top model for SVD retained the face and thorax dorsal regions. However, the face region was retained in all of the top models, and when included as a single predictor, had a very strong positive association with SVD. In addition, we show that hairiness, particularly on the face and ventral regions, is a good predictor of SVD for a plant with a different floral morphology, kiwifruit (*Actinidia deliciosa*) (Supporting Information 5; Figure S1; Table S1), indicating our method is likely to be suitable for a range of flower types. Hairiness was also a good predictor for pollen load, and the face region was again retained in the top model for *B. rapa*. The abdomen dorsal, head dorsal and front leg regions were also good predictors of pollen load and were retained in the subsequent top models. Our results validate the importance of insect body hairs for transporting and depositing pollen. Surprisingly, we did not find strong associations between SVD and body size, and top models did not contain the body length interaction. Similarly, body length was not retained in the top models for pollen load. This indicates that our measure of hairiness has far greater predictive power than body size for both SVD and pollen load.

When deciding on which body regions to measure hairiness, researchers may first need to assess additional pollinator traits, such as flower visiting behaviour. This is because the way in which insects interact with flowers influences what body parts most frequently contact the floral reproductive structures (Roubik 2000). For some open pollinated flowers, such as *B. rapa*, facial hairs are probably the most important for pollen deposition because the face is the most likely region to contact the anthers and stigma. However, for flowers with different floral morphologies, facial hairs may not be as important because the floral reproductive structures have different positions relative to the insect's body structures. For example, disc-shaped flowers tend to deposit their pollen on the ventral regions of pollinators, while labiate flowers deposit their pollen on the dorsal regions (Bartomeus et al. 2008). We found that hairiness on the face and ventral regions of pollinators was most important for pollen deposition on *A. deliciosa* flowers. The reproductive parts of *A. deliciosa* form a brush shaped structure and therefore are most likely to contact the face and ventral surfaces of pollinators. Accordingly, where studies focus on a single plant species i.e. crop based studies, it is important to consider trait matching when selecting pollinator body region(s) to analyse (Butterfield & Suding 2013; Garibaldi et al. 2015). It is also important to consider that pollinator performance is a function of both SVD and visitation frequency, and these two components operate independently (Kremen et al. 2002; Mayfield et al. 2001). Pollinator performance can also be influenced by pollinator activity patterns relative to the timing of stigma receptivity (Potts et al. 2001) and pollinator foraging behaviour (Herrera 1987; Rathcke 1983). Therefore, SVD should not be taken as the only measure of pollinator performance.

For community-level studies that use functional diversity approaches, our method could be used to quantify hairiness for several body regions and weighted to give better representation of trait diversity within the pollinator community. This is necessary where plant communities contain diverse floral traits i.e. open-pollinated vs. closed-tubular flowers (Fontaine et al. 2006). Hairs on different areas of the insect body are likely to vary in relative importance for pollen deposition depending on trait matching (Bartomeus et al. 2016). Our method requires hairiness to be measured at the individual-level (Figure S2), which makes it an ideal trait to use in new functional diversity frameworks that use trait probabilistic densities rather than trait averages (Carmona et al. 2016; Fontana et al. 2016). Combining predictive traits, such as pollinator hairiness, with new methods that amalgamate intraspecific trait variation with multidimensional functional diversity, will greatly improve the explanatory power of trait-based pollination studies.

One of the greatest constraints to advancing trait-based ecology is the time-demanding nature of collecting trait data. This is because ecological communities typically contain many species, which have multiple traits that need to be measured and replicated (Petchey & Gaston 2006). To improve the predictive power of trait-based ecology and streamline the data collection process we must firstly identify traits that are strongly linked to ecosystem functions and secondly, develop rigorous and time-efficient methodologies to measure traits at the individual level. We achieve this by providing a method for quantifying a highly predictive trait at the individual-level, in a time-efficient manner. Our method also complements other recently developed predictive methods for estimating difficult-to-measure traits that are important for pollination processes i.e. bee tongue length; Cariveau et al. (2016).

Predicating the functional importance of organisms is critical in a rapidly changing environment where accelerating biodiversity loss threatens ecosystem functions (McGill et al. 2015). Our novel method for measuring pollinator hairiness could be used in any studies that require quantification of hairiness, such as understanding adhesion in insects (Bullock et al. 2008;

Clemente et al. 2010) or epizoochory (Albert et al. 2015; Sorensen 1986). It is also a much needed addition to the pollination biologist's toolbox, and will progress the endeavour to standardise trait-based approaches in pollination research. This is a crucial step towards developing a strong mechanistic underpinning for trait-based pollination research.

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Tables

Table 1

Top regression models examining the effect of insect body region entropy on single visit pollen deposition (SVD) and pollen load for *Brassica rapa*. Models are presented in ascending order based on AIC_C values. Δi is the difference in the AIC_C value of each model compared with the AIC_C value for the top model. w_i is the Akaike weight for each model and acc w_i is the cumulative Akaike weight. Top models for each response variable are highlighted in bold.

Response variable	**Model**	**Adj R^2**	**AIC_c**	**Δi**	**w_i**	**acc w_i**
SVD	**Face + Thorax dorsal**	**0.98**	**88.29**	**0.00**	**0.82**	**0.82**
	Face + Thorax dorsal + Abdomen ventral	0.98	93.09	4.80	0.07	0.89
	Face + Head dorsal + Thorax dorsal	0.98	93.81	5.52	0.05	0.94
	Face + Thorax ventral + Thorax dorsal	0.97	96.59	8.29	0.01	0.96
	Face + Thorax dorsal + Front leg	0.97	97.02	8.72	0.01	0.97
Pollen load	**Face**	**0.81**	**168.47**	**0.00**	**0.64**	**0.64**
	Abdomen dorsal	0.73	171.59	3.12	0.13	0.78
	Face + Head dorsal	0.83	173.59	5.12	0.05	0.83
	Face + Abdomen dorsal	0.82	173.76	5.29	0.05	0.87
	Abdomen dorsal + Front leg	0.80	174.86	6.39	0.03	0.90

Figure 1

Entropy image of the face of a native New Zealand solitary bee *Leioproctus paahaumaa.*

Image of the face of a native New Zealand solitary bee *Leioproctus paahaumaa* (a) and the corresponding entropy image (b). Warmer colours on the entropy image represent higher entropy values (shown by the scale bar on the right). Black dots on the entropy image are near-round and small objects that have been removed from the analysis by the pre-processing function.

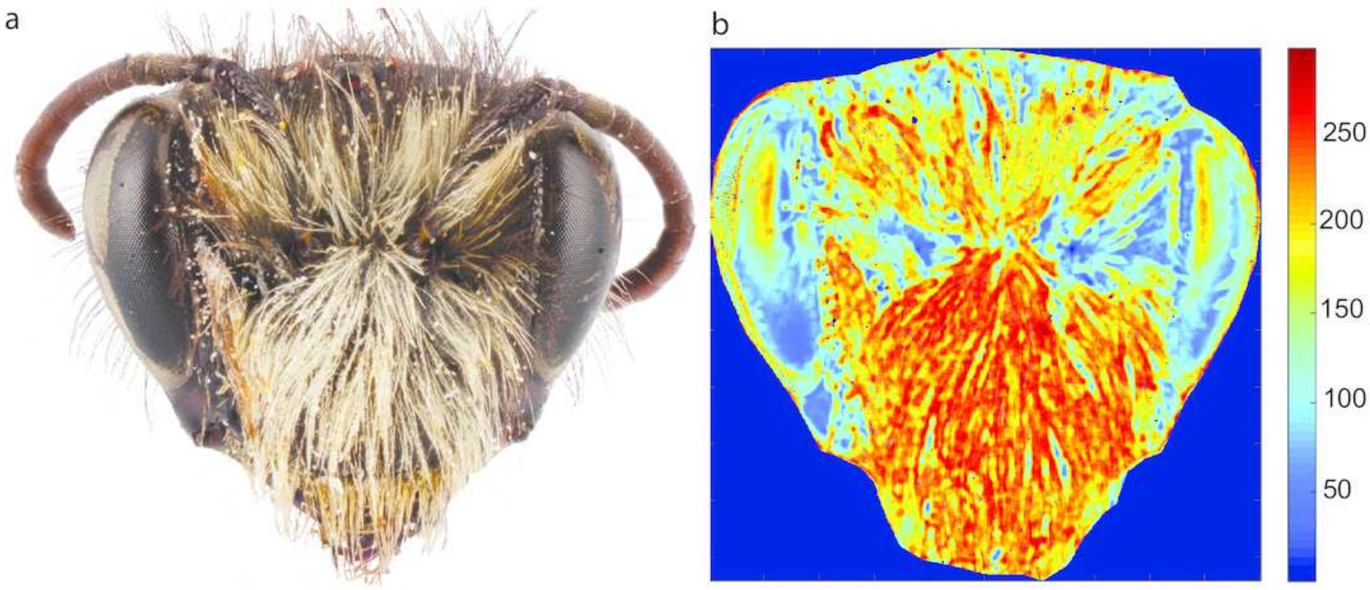

Figure 2

Relationships between mean entropy for each body region and mean single visit pollen deposition on *Brassica rapa*

Relationships between mean entropy for each body region and mean single visit pollen deposition (SVD) on *Brassica rapa* for 10 different insect pollinator species. Black lines are regressions for simple linear models.

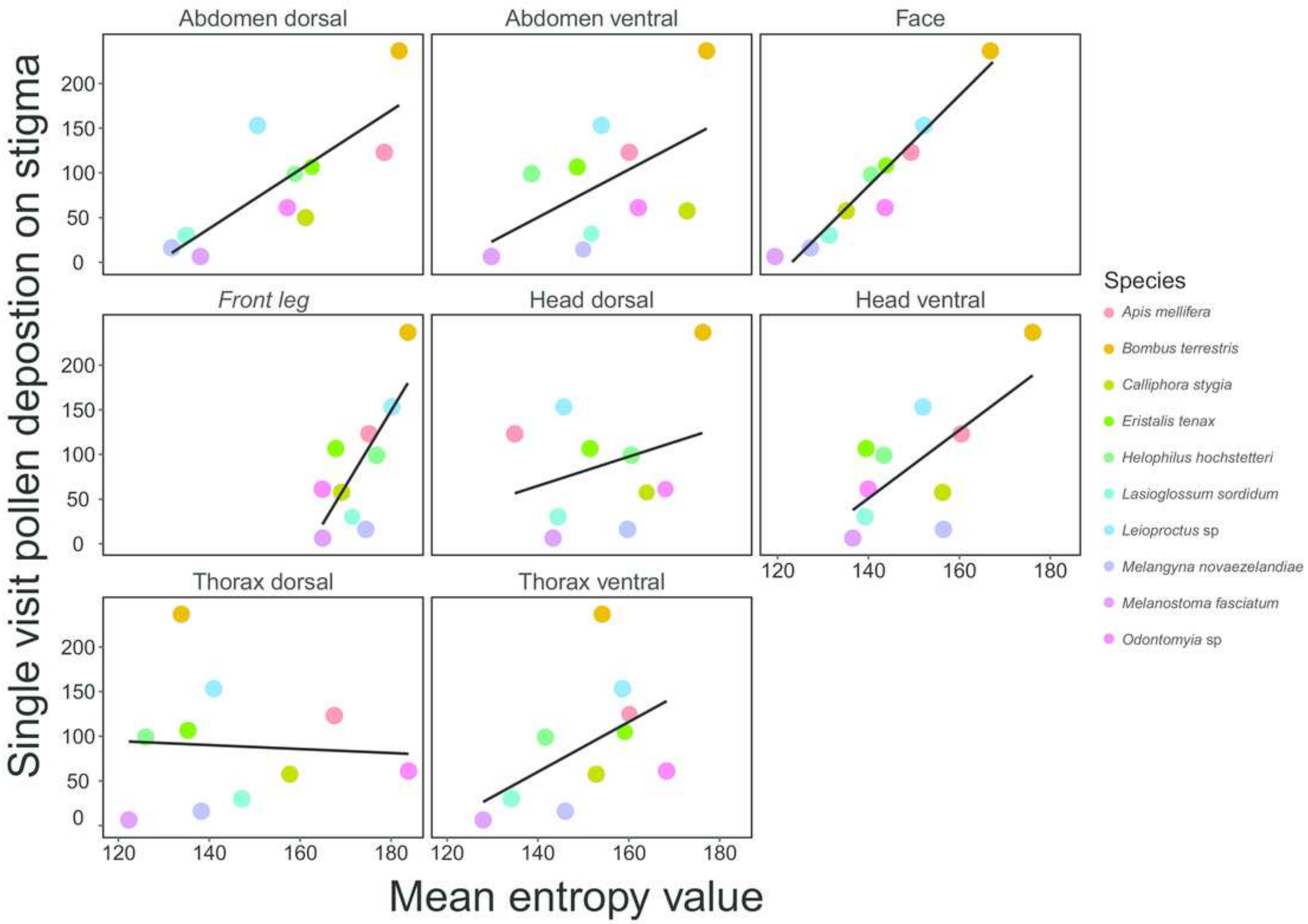

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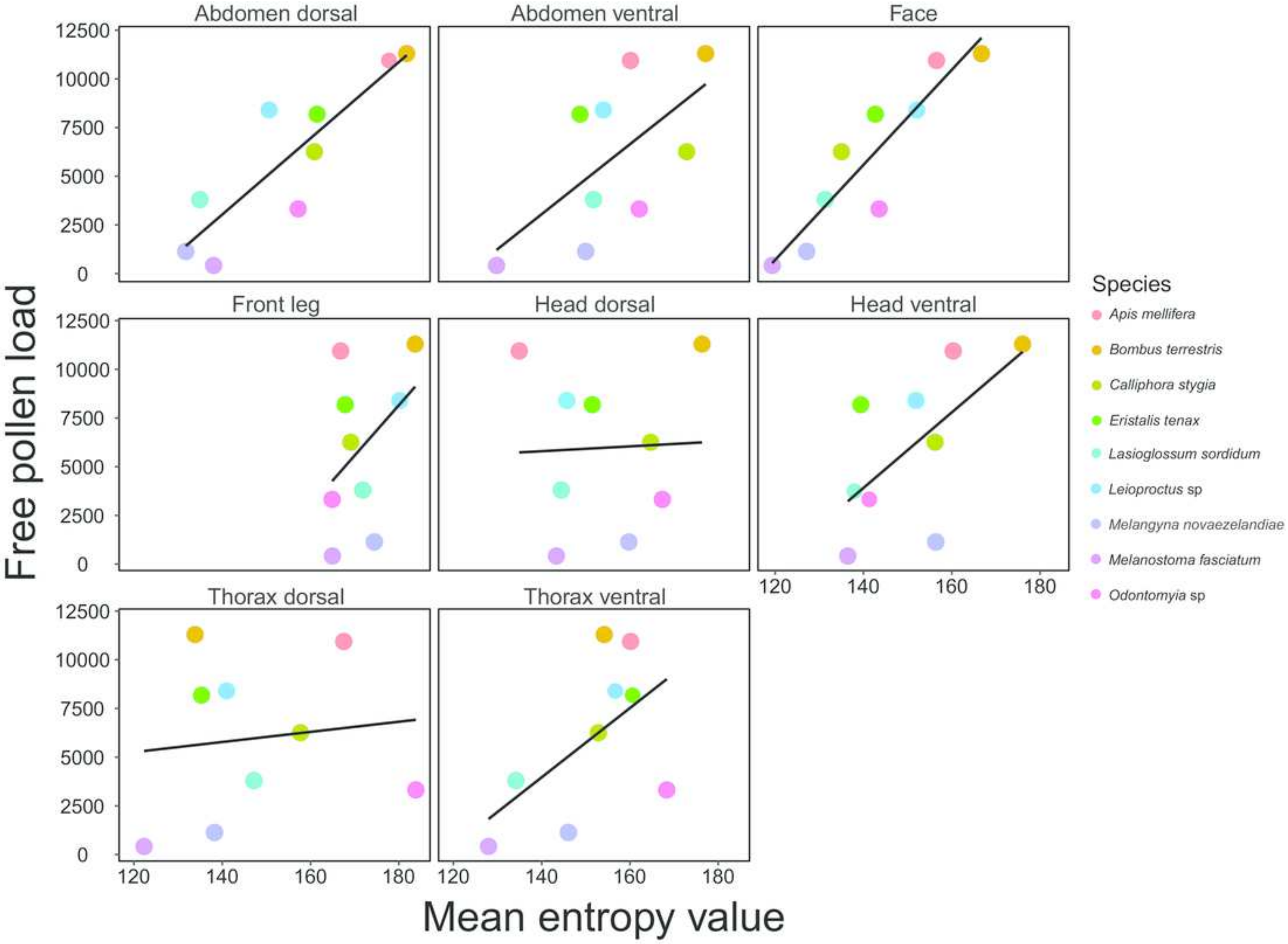
Figure 3

Relationships between mean entropy for each body region and the mean number of *Brassica rapa* pollen grains

Relationships between mean entropy for each body region and the mean number of *Brassica rapa* pollen grains carried by 9 different insect pollinator species. Black lines are regressions for simple linear models.



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