**Hemolymph viscosity in hawkmoths and its implications for hovering flight**

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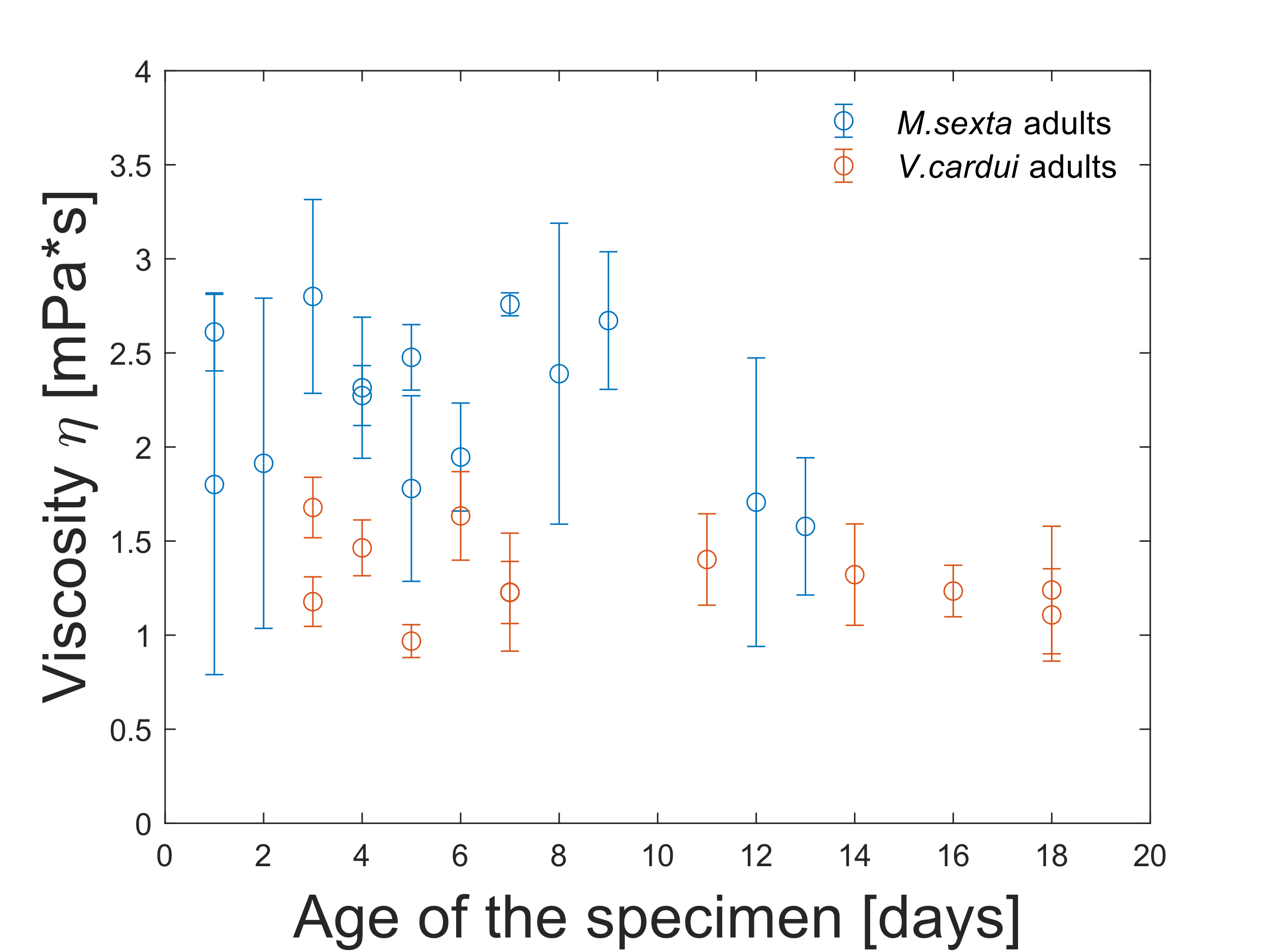
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**Supplementary Material**

**Table S1.** Hemolymph viscosity, body length, body width, and sample size for adults of 14 species of Lepidoptera.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Species | Hemolymph viscosity, mPas | Body length, cm | Body width, cm | Forewing length, cm | Number of individuals | Number of nanorods | Number of measurements |
| *Vanessa cardui* | 1.34 ± 0.24 | 2.11 ± 0.08 | 0.50 ± 0.10 | 3.01 ± 0.10 | 14 | 43 | 190 |
| *Danaus plexippus* | 1.44 ± 0.39 | 3.18 ± 0.13 | 0.57 ± 0.11 | 5.16 ± 0.10 | 9 | 16 | 106 |
| *Dolba hyloeus* | 1.48 ± 0.11 | 3.40 ± 0.14 | 0.96 ± 0.08 | 2.83 ± 0.29 | 5 | 15 | 85 |
| *Paratrea plebeja* | 1.51 ± 0.17 | 3.43 ± 0.35 | 0.99 ± 0.10 | 3.13 ± 0.24 | 5 | 11 | 63 |
| *Manduca rustica* | 1.47 ± 0.16 | 5.47 ± 0.27 | 1.55 ± 0.15 | 5.99 ± 0.10 | 5 | 16 | 95 |
| *Manduca quinquemaculata* | 1.70 ± 0.24 | 5.31 ± 0.44 | 1.23 ± 0.13 | 5.40 ± 0.30 | 7 | 26 | 168 |
| *Manduca sexta* | 2.17 ± 0.50 | 4.55 ± 0.49 | 1.10 ± 0.06 | 5.28 ± 0.52 | 19 | 49 | 315 |
| *Ceratomia catalpae* | 2.07 ± 0.33 | 3.40 ± 0.40 | 0.91 ± 0.10 | 4.38 ± 0.48 | 6 | 13 | 72 |
| *Agrius cingulata* | 1.85 ± 0.29 | 5.37 ± 0.50 | 1.28 ± 0.07 | 4.80 ± 0.40 | 5 | 21 | 110 |
| *Xylophanes tersa* | 1.77 ± 0.29 | 4.01 ± 0.21 | 0.85 ± 0.06 | 3.40 ± 0.20 | 4 | 18 | 99 |
| *Eumorpha pandorus* | 1.61 ± 0.14 | 4.86 ± 0.28 | 1.10 ± 0.09 | 4.60 ± 0.50 | 5 | 19 | 123 |
| *Hyles lineata* | 1.55 ± 0.28 | 3.87 ± 0.66 | 1.02 ± 0.17 | 3.10 ± 0.20 | 6 | 31 | 174 |
| *Hemaris diffinis* | 1.81 ± 0.12 | 2.72 ± 0.30 | 1.04 ± 0.18 | 2.95 ± 0.06 | 5 | 19 | 104 |
| *Enyo lugubris* | 1.83 ± 0.22 | 3.45 ± 0.19 | 0.96 ± 0.11 | 3.67 ± 0.10 | 5 | 18 | 103 |
| **AVERAGE** | 1.69 ± 0.25 | 3.94 ± 0.32 | 1.01 ± 0.11 | 4.12 ± 0.26 | 7.14 | 22.5 | 129.07 |
| **TOTAL** | - | - | - | - | 100 | 315 | 1,807 |



**Fig S1.** Hemolymph viscosity of *Manduca sexta* and *Vanessa cardui* does not depend on the age of the insect. Specimens were held in a refrigerator at 40C and fed every 2 - 4 days. One-way ANOVA test was performed and there was no significant difference among ages within species, suggesting that the conditions were suitable for the insects and the insects were not dehydrated.

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**Fig S2.** Hemolymph collection. A–E. *Manduca sexta*. (A) The moth was restrained with its wings folded. (B) The tegula (small, scale-like sclerite) at the wing base was removed with microscissors, exposing (C) the thorax. (D) An incision was made in the membrane between the thorax and the wing base, using a scalpel. (E) Hemolymph was collected by placing the tip of the capillary tube at the incision. (F, G) *Danaus plexippus*. (F) The butterfly was restrained with its wings held dorsally, exposing the wing base where an incision was made. (G) Hemolymph was collected in a capillary tube, and (H) delivered from the tube to the experimental setup, using a micropipette. (I) Hemolymph of *M. sexta* in a 5-µl capillary tube.

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**Fig S3.** Time dependence of relative viscosity (relative to the viscosity of DI water) of Lepidoptera adults. The data within ~15 minutes remain nearly constant, providing an experimental window for analysis of viscosity that excludes any effects of the environment and of clotting.



**Fig S4.** Average distance between the longitudinal muscle fibers in relation to the forewing length in 12 species of hawkmoths. Grey dots represent the subfamily Macroglossinae, whereas orange dots represent Sphinginae. Whiskers represent the standard deviation. Vertical dashed line represents the cutoff between small-winged species (less than 4 cm) and large-winged species (more than 4 cm).

**Table S2.** Distance between the longitudinal muscle fibers measured at five equidistant points along the thorax of a hawkmoth. We measured one individual per species.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Species | Average distance, mm | Standard deviation, mm | Maximum distance, mm | Minimum distance, mm | Forewing length, cm |
| *Agrius cingulata* | 0.17664 | 0.018785 | 0.2064 | 0.1487 | 4.8 |
| *Ceratomia catalpae* | 0.1105 | 0.031154 | 0.1644 | 0.0706 | 4.38 |
| *Dolba hyloeus* | 0.055884 | 0.011287 | 0.07171 | 0.03959 | 2.83 |
| *Enyo lugubris* | 0.104408 | 0.016872 | 0.1301 | 0.07924 | 3.67 |
| *Eumorpha pandorus* | 0.144416 | 0.037314 | 0.1773 | 0.08248 | 4.6 |
| *Hemaris diffinis* | 0.40396 | 0.044677 | 0.4751 | 0.3438 | 2.95 |
| *Hyles lineata* | 0.091354 | 0.012669 | 0.1107 | 0.07216 | 3.1 |
| *Manduca quinquemaculata* | 0.1298 | 0.018618 | 0.1542 | 0.1039 | 5.4 |
| *Manduca rustica* | 0.08687 | 0.009792 | 0.0983 | 0.07293 | 5.99 |
| *Manduca sexta* | 0.109416 | 0.028463 | 0.1532 | 0.07128 | 5.28 |
| *Paratrea plebeja* | 0.25914 | 0.077201 | 0.4044 | 0.182 | 3.13 |
| *Xylophanes tersa* | 0.15458 | 0.034772 | 0.1943 | 0.1025 | 3.4 |

**Table S3.** Regressions between hemolymph viscosity and (a) body volume and (b) forewing length, using different evolutionary models. We built full models (i.e., with all the parameters) and compared among evolutionary models using AIC. Whenever ∆AIC ≥ 2, the model was considered a poor fit. If models tied with the same AIC value, we chose the evolutionary model that added the fewest parameters to the data (i.e., Brownian motion).

|  |  |  |
| --- | --- | --- |
| **Model** | **AIC** | **∆AIC** |
| 1. *Body volume* |  |  |
| Brownian motion | 5.887188 | - |
| Lambda | 5.887188 | - |
| Ornstein-Uhlenbeck | 7.887188 | 2 |
| 1. *Forewing length* |  |  |
| Brownian motion | 1.593441 | - |
| Lambda | 1.593441 | - |
| Ornstein-Uhlenbeck | 3.593441 | 2 |

**Estimates of flow characteristics through dorsal vessel and thorax**

The dorsal vessel consists of an anterior portion, the aorta, which doesn’t pump, and a posterior heart, which pumps. The contribution of the insect circulatory system driven by the dorsal vessel is defined through the cardiac output providing hemolymph volume per stroke, , where is the heartbeat frequency. This hemolymph is discharged through a cylindrical aorta of radius with cross-section . Therefore, the flow rate balance reads , where is the mean velocity of hemolymph in the aorta.

To the best of our knowledge, the volume has never been documented in the literature. We, therefore, estimate the volume of the dorsal vessel from our measurements of the hawkmoth characters, Table S1 and Table S2, modeling it as a cylindrical tube of radius and length . The body width remains about the same for all studied hawkmoths, therefore, taking , and using two extreme examples for the dorsal vessel lengths and , we have . For an order of magnitude estimate of , we take With these parameters, the mean velocity of hemolymph in the dorsal vessel is estimated as /( . This estimate of velocity seems reasonable and close to the measured velocity in the dorsal vessel of the grasshopper (*Schistocerca americana*), 9.5 mm/s (Lee & Socha, 2009, full reference below).

From our micro-CT scans, we estimate the aorta radius as . Therefore, we find for small hawkmoths /( and for large hawkmoths . The associated Reynolds number in the aorta for small hawkmoths is and for large hawkmoths . These estimates suggest that hemolymph flow through the aorta is mostly controlled by inertial forces.

Typically, the cardiac velocity through the thorax is much slower than the mean velocity . The estimate of the ratio / is obtained from the balance of cardiac output, passing through a cylindrical aorta of radius , and then through the thorax of cross-sectional area and porosity Thus, velocity in each pore of a complex intermuscle pore network is directly proportional to the velocity in aorta, with a factor defining how small this velocity is. For our species this ratio is about 0.01, making We estimate the Reynolds number for cardiac flow through the thorax as , where is the average thickness of the intermuscle gap. Taking for small hawkmoths and for large hawkmoths , we find for small hawkmoths and for large hawkmoths . These estimates show that the cardiac flow regime in thoraxes of large and small hawkmoths is similar and mostly controlled by inertial forces.

Lee, W. K., & Socha, J. J. (2009). Direct visualization of hemolymph flow in the heart of a grasshopper (*Schistocerca americana*). *BMC physiology*, *9*(1), 1-11.

**Sensitivity analyses**

As described in the Methods section of the main paper, we performed a sensitivity analyses. Our goal was to understand how much impact large species had in the quadratic relationship we found between hemolymph viscosity and forewing length.

The first goal of our sensitivity analysis was to evaluate the effect of the two largest species in the set, *Manduca rustica* and *Manduca quinquemaculata*, on the behavior of viscosity on the forewing length without consideration of phylogenetic correlation between species. By fitting the data with a second order polynomial using the Least Squares Method, we obtained two trend lines including and excluding *M. rustica*, and a linear fit excluding the two largest species *M. rustica* and *M. quinquemaculata* (Table S4). The best fits for quadratic relationships were relatively similar (Fig. S5) with R-squared values equally small for both, and linear fit with a better R-square value (Table S5). The Chi-square statistical analysis showed that for predicting viscosity for the data set without the two largest species, all fits were equally good (Table S6). Both parabolic fits predict viscosity well without the single largest species. The red and green lines in Fig. S5 have increased difficulty extrapolating for larger species without either the largest or both largest species. This suggests that not just *M. rustica*, but also *M. quinquemaculata* are important indicators of the viscosity behavior as a function of the forewing size. Increasing the number of large hawkmoths would indicate if the the observed trend is consistent.

Gráfico

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**Fig S5.** Mean hemolymph viscosity versus mean forewing length. The error bars indicate the intra-individual variation of each datum. To fit the data, only average viscosity and average forewing length were used. The data were fitted with *Manduca rustica* (the black parabola), without *Manduca rustica* (the red parabola), and without two largest species, *M. rustica* and *M. quinquemaculata* (the green line).

**Table S4.** Parameters estimated with the Least Squares Method for the second and first order polynomial , where is viscosity and is the forewing length. The data are shown in Fig S5. The species removed were *Manduca rustica* and *Manduca quinquemaculata*.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Quadratic parameter, | Linear parameter, | Intercept, |
| With *M. rustica* | -0.1503 | 1.3377 | -1.0608 |
| Without *M. rustica* | -0.0774 | 0.7619 | 0.0231 |
| Without *M. rustica* and *M. quinquemaculata* | 0 | 0.1856 | 1.0598 |

**Table S5.** Comparison of goodness of fit metrics between the red (without *M. rustica*)*,* the black (with *Manduca rustica*) parabolas, and the green (without *M. rustica* and *M. quinquemaculata*) line shown in Fig. S5.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | R-squared | Sum of Squares Total (Without *M. rustica* and *M. quinquemaculata*) | Sum of Squares Total (Without *M. rustica*) | Sum of Squares Total (All data) | Residual Standard Error |
| Black quadratic model | 0.39 | 0.33 | 0.34 | 0.35 | 0.15 |
| Red quadratic model | 0.36 | 0.29 | 0.32 | 0.43 | 0.15 |
| Green linear model | 0.49 | 0.25 | 0.38 | 0.87 | 0.13 |

**Table S6.** Observed chi-square values obtained from calculating the difference in the goodness of fit of each model we performed.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Chi-square goodness of fit test | | |
| Fit with *M. rustica* | Fit without *M. rustica* | Fit without *M. rustica* and *M. quinquemaculata* |
| Without two largest species | 0.19 | 0.16 | 0.14 |
| Without  *M. rustica* | 0.19 | 0.18 | 0.20 |
| With  *M. rustica* | 0.20 | 0.24 | 0.43 |

Second, we also evaluated the effect of *M. rustica* by running the same analysis contained in the main paper while removing *M. rustica* from the sample. Therefore, the same R package (*phylolm*) and the same procedure were adopted (using *phylostep* to determine the best fit model).

Contrary to the full dataset, the best fitted model was a linear monotonic relation between hemolymph viscosity and forewing length (Table S7). However, the difference between the model with only the linear parameter and the quadratic model does not allow us to differentiate between these two models.

**Table S7**. Regression between hemolymph viscosity and forewing length, using different evolutionary models. We built full models (i.e., with all the parameters) and used stepwise selection to find the best model. When ∆AIC > 2, the model was considered a poor fit. If models tied with the same AIC value, we chose the model that added the fewest parameters (fewest degrees of freedom, DF) to the data. If models also tied in the number of degrees of freedom, models were considered indistinguishable.

|  |  |  |  |
| --- | --- | --- | --- |
| **MODEL** | **AIC** | **∆AIC** | **DF** |
| Intercept + linear parameter | 0.90936 | - | 4 |
| Intercept + quadratic parameter | 1.20613 | 0.296 | 4 |
| Intercept + linear + quadratic parameter | 2.20855 | 1.299 | 5 |
| Intercept only | 3.05318 | 2.143 | 3 |

To further test the fit of this linear model to the data, we calculated three goodness of fit metrics for the linear model without *M. rustica* and for our quadratic model with the full dataset: R², sum of squares total, and residual standard error. In the three metrics, the quadratic model was better fitted by ~10% when compared to the linear model (Table S8). In summary, the evidence gathered from this sensitivity analysis suggests that the quadratic model provides the best fit to the data (Table S8); and even though *M. rustica* lends further support to the quadratic dependence, without this species the quadratic parameter still has a similar fit to the data when compared to the linear fit (Table S7; Fig. S6).

**Table S8.** Comparison of goodness of fit metrics between the parabolic fit (with *Manduca rustica*) and the linear fit (without *M. rustica*)shown in Fig. S6.

|  |  |  |  |
| --- | --- | --- | --- |
|  | R-squared | Sum of Squares Total | Residual Standard Error |
| Quadratic fit | 0.39 | 0.564 | 0.330 |
| Linear fit | 0.31 | 0.667 | 0.395 |

Diagrama

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**Fig S6.** Phylogenetic linear models between mean hemolymph viscosity and mean forewing length calculated with (solid line) and without (dashed line) *Manduca rustica*. While the model with *M. rustica* (solid line) yielded a quadratic model as the best fit, the model without *M. rustica* (dashed line) yielded a monotonic linear relationship. However, the monotonic linear relationship fits the data worse than the quadratic model (see Table S8).