Phylogenetic clustering of wingbeat frequency and flight-associated morphometrics across insect orders

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1 **Title**

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5 **Running title**

6 Flight strategies between insect orders

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23   **Summary statement**

24   Insect flight strategy varies between orders but is generally well conserved within orders, this

25   has important evolutionary and ecological implications at high taxonomic levels.

26

27   **ABSTRACT**

28   Wingbeat frequency in insects is an important variable in aerodynamic and energetic

29   analyses of insect flight and has been studied previously on a family- or species-level basis.

30   Meta-analyses of these studies have found order-level patterns that suggests flight strategy

31   is moderately well conserved phylogenetically. Studies incorporated into these meta-

32   analyses, however, use variable methodologies across different temperatures that may

33   confound results and phylogenetic patterns. Here, a high-speed camera was used to

34   measure wingbeat frequency in a wide variety of species (n = 102) in controlled conditions to

35   determine the validity of previous meta-analyses that show phylogenetic clustering of flight

36   strategy and to identify new evolutionary patterns between wingbeat frequency, body mass,

37   wing area, wing length, and wing loading at the order level. All flight-associated

38   morphometrics significantly affected wingbeat frequency. Linear models show that wing area

39   explained the most amount of variation in wingbeat frequency (R^2 = 0.59, p = <0.001), whilst

40   body mass explained the least (R^2 = 0.09, p = <0.01). A multiple regression model

41   incorporating both body mass and wing area was the best overall predictor of wingbeat

42   frequency (R^2 = 0.84, p = <0.001). Order-level phylogenetic patterns across relationships

43   were consistent with previous studies. Thus, the present study provides experimental

44   validation of previous meta-analyses and provides new insights into phylogenetically

45   conserved flight strategies across insect orders.

46

47

INTRODUCTION

Wingbeat frequency in insects varies with body mass and wing area within and between species (Byrne et al., 1988; Dudley, 2000), from 5.5 Hz in the helicopter damselfly *Megaloprepus caerulatus* (Rüppell and Fincke, 1989) to over 1000 Hz in a ceratopogonid *Forcipomyia* sp. midge (Sotavalta, 1953). How frequently an insect beats its wings is an important variable when considering the biomechanics and physiology of insect flight (Ellington, 1984a-f; Dudley, 2000; Alexander, 2002; Vogel, 2013). For any given body mass, variables such as wing length, wing area, wing loading (body mass/wing area), wingbeat frequency and stroke amplitude can differ substantially and affect the energetics and biomechanics of insect flight, which is usually linked to evolutionary history (Byrne et al., 1988). Stroke amplitude, the angle between the points of wing reversal, has been shown to vary between taxa, from 66° in syrphids (Ellington, 1984c) to 180° in beetles (Atkins, 1960) and moths (Wilkins, 1991) and may vary significantly during a single flight as shown in dragonflies (Alexander, 1986), orchid bees (Dudley, 1995; Dillon and Dudley, 2004), and fruit flies (Lehmann and Dickinson, 1998; Fry *et al.*, 2003). Though undeniably important to understanding insect flight strategy and aerodynamics, stroke amplitude was not measured in the current study. This is because although both wingbeat frequency and stroke amplitude change during a single flight, wingbeat frequency is kept relatively constant because of the high energetic cost of deviating from the resonant frequency of the flight apparatus (Dudley, 2000). Conversely, stroke amplitude may be altered extremely rapidly to change direction (Fry *et al.*, 2003) or flight mode e.g. from hovering to forward flight (Dillon and Dudley, 2004). Because of this variability, stroke amplitude is likely to be a slightly less reliable indicator of flight strategy than wingbeat frequency.

The variables that influence the energetic and biomechanical aspects of flight could be used to broadly characterize flight strategies between different orders of insects. Typically, higher wingbeat frequencies are associated with insects of smaller size, to overcome the increasingly viscous forces of the air present at small spatial scales, represented by low Reynolds numbers

75   *Re* in the order of 10-100 in the smallest insects (Ellington, 1999; Wang, 2005), and to better

76   control their direction in a windswept world (Vogel, 2013). Furthermore, frequencies of >100

77   Hz are facilitated by asynchronous, or myogenic, flight muscle present in endopterygote

78   (Coleoptera, Diptera, Hymenoptera) and exopterygote (Thysanoptera and Hemiptera) groups

79   (Dudley, 2000) where one nerve impulse can initiate several wingbeats through stretch-

80   activation caused by mechanical loading on the wing (Pringle, 1967). Thus, the highest

81   wingbeat frequencies are found in smaller members of these groups (Byrne et al., 1988).

82   Members from other orders possess large wings that they beat at lower frequencies relative

83   to other insects of comparable body mass e.g. Lepidoptera and Neuroptera (Dudley, 2000)

84   and Orthoptera (Snelling *et al.*, 2012, 2017). Larger wings can produce more force per beat

85   than smaller wings, and therefore fewer beats are needed per unit time. Moreover, larger

86   wings afford lower wing loadings for insects of the same body mass, so wingbeat frequency

87   may be reduced further. It is possible then that flight-associated morphometrics, such as wing

88   area, can be used to predict wingbeat frequency and characterize flight for different groups of

89   insect using the same stroke strategy (i.e. conventional wingbeat or clap-fling).

90

91   Flight morphology and wingbeat frequency are dependent on the aerodynamic needs of the

92   insect according to their ecological niche and oxygen consumption increases with wingbeat

93   frequency (Bartholomew and Casey, 1978). Species with similar wing loadings may have

94   different wingbeat frequencies based on the flight velocity required to fulfil their ecological role.

95   Substantial variation in wingbeat frequency and flight morphology as a product of ecological

96   needs also exists within orders, such as the differences between Sphingidae and Nymphalidae

97   (Lepidoptera), where sphingids have small wings, rapid beat frequencies and very fast flight,

98   whilst nymphalids have much larger wings and lower wingbeat frequencies, usually flying at

99   overall slower speeds (Dudley, 2000). Such variation could conceal relationships between

100   flight-associated morphometrics and wingbeat frequency across higher taxonomic levels,

101   decreasing the overall level of phylogenetic grouping of flight strategy.

102

Order-level taxonomic relationships to these flight-associated morphometrics have been studied before (see Byrne et al., 1988; Dudley, 2000) but meta-analyses suffer from differences in both ambient conditions and methods of measuring wingbeat frequency between studies that may confuse relationships. For example, acoustic methods, stroboscopes, and high-speed cameras were used across studies incorporated into Dudley (2000) and Byrne et al.'s (1988) meta-analyses. Chadwick (1939) suggested stroboscopic methods are difficult to use effectively to glean kinematic data in insects because of the slight variations in wingbeat frequency and movements of the specimen during testing, making visualisation of the wing at the frequency of the strobe light challenging and Unwin and Ellington (1979) suggested picking up acoustic signals of smaller species difficult even with highly sensitive microphones. Both stroboscopic (e.g. Chen *et al.*, 2014) and acoustic (e.g. Raman *et al.*, 2007) methods have, however, been used successfully to measure wingbeat frequency in insects since advancement in the quality of measurement instruments (i,e, optical tachometers and microphones). Nevertheless, stroboscopic/optical and acoustic methods are not absolute measures of wingbeat frequency. High-speed cameras, in contrast, allow the recording of a temporally magnified visual depiction of the motion of insect wings. The reliability of the methods used in studies incorporated into important meta-analyses varies because of the problems faced when the technology was less well developed. Furthermore, temperatures vary from 7-25°C between studies used in previous meta-analyses. Insect wingbeat frequency has been shown to increase with higher temperatures (Unwin and Corbet, 1984; Oertli, 1989) and, therefore, meta-analyses of the relationships between measured characteristics may be confounded. An experimental approach using high-speed cameras in controlled conditions recording flight in species across several orders has not previously been done. Using common UK species of insect, relationships between body mass, wing length, wing area, wing loading and wingbeat frequency were investigated to determine if flight strategies could be broadly characterized between different orders of insect.

129

MATERIALS AND METHODS

131   Study specimens

132   Adult insects were caught using either sweep net (EFE & GB Nets, Totnes, Devon, UK –

133   handle length = 0.3 m; net diameter = 0.5 m; net depth = 0.7 m), pooter (NHBS, Totnes, Devon,

134   UK – barrel diameter = 30 mm, length = 55 mm, suction tube diameter = 5mm), or hand

135   collected into small sampling pots (varying sizes) within a 20 km radius of Harper Adams

136   University, Shropshire, UK (latitude ~52.772°N, longitude ~2.411°W) over the course of June

137   and July, 2017. In total, 112 specimens across 102 species in 10 orders were used in the

138   analysis.

139

140   Filming area and conditions

141   Filming took place inside a Fitotron® Standard Growth Room unit (Weiss Technik, Ebbw Vale,

142   UK) set to a constant 20°C and 60% relative humidity. This temperature was selected to film

143   flight behaviour of insects in standardised conditions and is unlikely to represent an extreme

144   for tested species, which were all collected during summer days and therefore active within

145   ~±5°C of the ambient temperature used. Ambient lighting intensity was 280 µmol m^{-2} s^{-1} inside

146   the Fitotron® unit and no other external light source was used. A flight box made of 6

147   transparent Perspex® panels, measuring 30x30x30 cm once constructed, was used to contain

148   flights of the specimens whilst filming. Study specimens were introduced to the flight box either

149   via a 2.5 cm diameter aperture made in the centre of one of the panels by offering up an open

150   test tube containing a specimen, or, for larger specimens, the entire panel could be removed

151   and the specimen introduced.

152

153   Filming procedure

154 Each specimen was filmed 2-5 times using an FPS1000HD monochromatic high-speed

155 camera (The Slow Motion Camera Company, London, UK). Specimens were filmed each time

156 during free flight. For each flight recorded, the camera was handheld in order to track insects

157 in free flight. This helped increase total length of each video and thus more reliably count

158 wingbeats. Across videos, insects were filmed from various angles, but this did not affect video

159 analysis. Sufficient video footage was gathered in <10 minutes for each specimen.

160

161 Morphological measurements

162 Specimens were killed in a killing jar (a jar with a base of plaster of Paris to which ethyl acetate

163 was intermittently added when needed) after the last video was recorded and immediately

164 weighed using a precision balance (Cahn C-33 Microbalance, Cerritos, California, USA). The

165 functional wing (in insects with only one pair of functional wings e.g. Diptera and Coleoptera)

166 or wing couple on the right side (i.e. the fore- and hindwing on the right side of the insect

167 viewed dorsally) was removed by dissection under a stereo microscope and forewing length

168 (henceforth wing length) was measured using a pair of digital calipers (0.01 mm precision),

169 measured from the base of the forewing to the most distal tip. A photo was taken of the

170 dissected wing couple using a microscope camera making sure the wings were perpendicular

171 to the camera lens. Wing area was measured in ImageJ version 1.49 (Schindelin et al., 2012)

172 by using the photo and following the ImageJ process for measuring leaf area (Reinking, 2007)

173 as in previous studies on insect wings (e.g. Outomuro *et al.*, 2013); the wing area value was

174 multiplied by 2 to quantify total wing area assuming symmetry. Wing loading was determined

175 by dividing body mass by total wing area.

176

177 Video analysis

178 Videos were first converted into a viewable format using ImageJ, where video frames-per-

179 second (FPS) was then altered to allow individual wingbeats to be clearly visible. A wingbeat

180   was judged to be both a full downstroke and full upstroke, terminating at pronation before the

181   next wingbeat (Fig. 1), and in all groups except for Odonata, fore- and hindwings beat at the

182   same time. For odonates, forewing and hindwing pairs were measured separately then the

183   mean was calculated; the difference between the wing pairs did not exceed 2 beats in any of

184   the odonate specimens. Sections of videos were carefully selected to represent free-flight,

185   omitting wingbeats immediately after take-off until a more regular rhythm was observed, which

186   was usually more rapid. The number of wingbeats n_v during free-flight was counted for each

187   video. Equation 1 was used to determine the wingbeat frequency n (Hz) from each video

188   where t_v is the length of the video in seconds, and f_m is the multiplication factor (the factor that

189   describes by how much time is magnified in each video), which is calculated by dividing filming

190   FPS by video playback FPS. All species were filmed at 1000 FPS except for 6 species of

191   nematoceran Diptera, which were filmed at 2000 FPS.

192

193   Statistical Analysis

194   Statistical analysis was conducted using R version 3.4.1. "Single Candle" (R Core Team,

195   2017) with packages MASS (Venebles and Ripley, 2002), ggplot2 (Wickham, 2009), caret

196   (Kuhn, 2017), hydroGOF (Zambrano-Bigiarini, 2014), relaimpo (Grömping, 2006), and

197   gridExtra (Auguie, 2016) used. Both simple and multiple linear regression analyses were

198   conducted to determine the relationships between morphological variables and wingbeat

199   frequency. Data were log-transformed to reduce skew and allow analysis by linear regression.

200   To better measure the level of phylogenetic clustering of flight strategy, a principal component

201   analysis (PCA) was conducted.

202

203   **RESULTS**

204   Morphometric data

205   Table 1 compiles the range and mean statistics for morphological measurements and

206   wingbeat frequency in each sampled order. Across all 112 specimens, wingbeat frequency

207   covered a range between 12.468 to 557.351 Hz ($\bar{x}$ = 121.588, sd = 92.679, se = 8.767), body

208   mass a range of 0.0003 to 2.245 g ($\bar{x}$ = 0.097, sd = 0.256, se = 0.024), wing length a range of

209   0.172 to 5.214 cm ($\bar{x}$ = 1.184, sd = 0.919, se = 0.087), wing area a range of 0.022 to 23.362

210   cm^2 ($\bar{x}$= 2.022, sd = 4.088, se = 0.386), and wing loading a range of 0.0028 to 0.245 g/cm^2

211   ($\bar{x}$= 0.061, sd = 0.059, se = 0.006).

212

213   These values show that some orders were better sampled than others and in some cases this

214   is reflected in the ranges of different variables recorded. Average values, however, are

215   generally in agreement with expected values for UK insects. Synchronous fliers

216   (Ephemeroptera, Lepidoptera, Mecoptera, Neuroptera, Odonata, Trichoptera) were overall

217   less well sampled than asynchronous fliers (Coleoptera, Diptera, Hemiptera, Hymenoptera)

218   and should be similarly taken into account when considering ranges of variables.

219

220   Relationships between morphometrics and wingbeat frequency

221   Figure 2 shows the relevant linear relationships between the log$_{10}$ transformed morphometric

222   data. Of these, wing area (cm^2) was the best predictor of wingbeat frequency (R^2 = 0.59, p =

223   <0.001). The strongest overall linear relationship between all morphometric measurements

224   was between wing length (cm) and wing area (R^2 = 0.93, p = <0.001). Body mass explained

225   only 9% of the variation in wingbeat frequency across specimens (R^2 = 0.09, p = <0.01) and

226   represented the poorest predictor of wingbeat frequency across the measured morphometrics.

227   Taxonomic distribution on the graphs (Fig. 2, especially A-D) sees a diffuse but identifiable

228   clustering of the orders most intensively sampled, suggesting that orders may broadly adhere

229   to a specific strategy and some new phylogenetic clustering between wingbeat frequency,

230   wing area, wing length, and wing loading have been revealed where previous meta-analyses

231 focussed solely on taxonomic grouping in relation to wingbeat frequency and body mass. For

232 example, looking at Figure 2D, Hymenoptera are quite closely clustered at the higher end of

233 the wing loading range and the upper-middle range of wingbeat frequency, denoting that most

234 hymenopterans sampled have small wings relative to their body mass, which they beat at

235 above average frequencies compared to other orders.

236

237 A multiple regression model using $\log_{10}$ values of wing area (β = -0.034, p = <0.001) and body

238 mass (β = 0.001, p = <0.001), with a fit of R^2 = 0.84 was the best overall model predicting

239 wingbeat frequency in insects: wingbeat frequency = (wing area * -0.77) + (body mass * 0.37)

240 + 5.56.

241

242 A dominance analysis (Azen and Budescu, 2003; Grömping, 2006) was conducted to

243 determine the relative importance of the explanatory variables to the response variable in the

244 model and showed that body mass and wing area explained 17.3% and 67.2% of the change

245 in wingbeat frequency, respectively.

246

247 Phylogenetic clustering of flight strategy

248 Wingbeat frequency and morphometric variables for all specimens were reduced to a dataset

249 summarising the variance and covariance between each using a Principle Component

250 Analysis (PCA). Initial eigenvalues indicated the first two principle components explained

251 64.039% and 33.291% of the data, respectively (97.331% cumulatively). Dimension 1 is

252 mainly loaded towards wing area (30.417%), wing length (30.073%), body mass (22.882%),

253 and wing loading (16.388%), whereas Dimension 2 is mainly loaded towards wing loading

254 (58.325%), wingbeat frequency (24.979%), and body mass (15.821%). Having determined the

255 loadings, a PCA biplot was produced to view the relationship between variables and whether

256 insect orders were clustered on the graph. Figure 3 reveals that most insect specimens are in

257 close proximity to their associated centroid (the mean value of the x and y coordinates for

258 each order), shown by the ellipses, which represent one standard deviation along each axis

259 and is rotated toward the direction of maximum spread of the point cloud. This strongly

260 suggests that flight strategy is well conserved at the order level.

261

262 **DISCUSSION**

263 Phylogenetic clustering apparent in this study broadly agrees with results from previous meta-

264 analyses (Byrne et al., 1988; Dudley, 2000). Past research looking at differences in wingbeat

265 frequency and flight-associated morphometrics are, therefore, experimentally validated by the

266 present study through the use of high-speed filming. However, although all measured

267 characteristics significantly affected wingbeat frequency, body mass did not show as clear a

268 relationship to it as in previous meta-analysis (Dudley, 2000). This is likely because of the lack

269 of specimen variation in the present study, compared to the very high number of different

270 specimens across a much broader body mass range in the meta-analysis (Dudley, 2000).

271 Indeed, previous meta-analyses included species from a much wider geographical range,

272 incorporating studies from many different countries and ecosystems, including those from

273 tropical forests.

274

275 Wing length and wing area are both able to predict wingbeat frequency moderately accurately,

276 explaining 42% and 59% of its variation, respectively. Wing length may affect wingbeat

277 frequency as a product of increasing body mass, where larger insects have slightly longer

278 wings to offset the lower wingbeat frequency and maintain good advance ratios (Vogel, 2013),

279 though this is also connected to wing area (Fig. 2E). Area of the wing generally increases with

280 body mass to accommodate the greater level of lift generation required and longer wings tend

281 to have a greater area than shorter ones. Thus, an increased area means fewer beats are

282   necessary per unit time to generate the same amount of lift. This is supported by the positive

283   relationship between wing loading and wingbeat frequency, where heavily loaded wings are

284   generally beaten more rapidly to generate enough lift. Relatively heavily loaded wings must

285   keep a weight aloft with a reduced area and are associated with larger insects (Fig. 2F)

286   because wing area, proportional to the square of body length, cannot keep pace with body

287   mass, proportional to the cube of body length, as insect size increases (Bartholomew and

288   Heinrich, 1973; Byrne et al., 1988; Ennos, 1989; Dudley, 2000; Vogel, 2013). Despite this,

289   heavier insects tended to also have lower wingbeat frequencies (Fig. 2C). Whilst initially

290   paradoxical that heavier insects with greater wing loading beat their wings relatively less

291   frequently, this is because smaller insects must overcome the increasingly viscous forces of

292   air at small scales, greater relative drag, and the greater effect of the wind on their direction

293   by beating their wings comparatively faster (Dudley, 2000; Alexander, 2002; Vogel, 2013) and

294   because the oscillatory frequency of the thorax is inversely dependent on its size, which

295   directly influences wingbeat frequency in asynchronous fliers (Pringle, 1949, 1967; Dickinson

296   and Tu, 1997; Dudley, 2000).

297

298   The best overall model explaining the variation in wingbeat frequency incorporated body mass

299   and wing area, the relative importances of which were 17.3% and 67.2%, respectively. This

300   suggests that despite the weak linear relationship between body mass and wingbeat

301   frequency, together with wing area the variables can explain 84% of the variation in wingbeat

302   frequency. These findings support previous agreement (Jensen, 1956; Ellington, 1984b-c,

303   1999; Dudley, 1990, 2000; Alexander, 2002) that wingbeat frequency is in large part

304   dependent on wing area and body mass.

305

306   Palaeopterous insects using direct flight muscles and neopterous insects using synchronous

307   flight muscles show generally lower wingbeat frequencies than insects with asynchronous

308   flight muscles (Figure 2) and these two groups are further clustered in Figure 3 (Neuroptera,

309   Lepidoptera, Odonata – bottom right; asynchronous fliers – middle/top left). The weak

310   relationship between wingbeat frequency and body mass in the present study as well as past

311   meta-analyses may arise because of the differences in scaling between these groups. Insects

312   with indirect synchronous flight muscles conduct wingbeats by single nerve impulses to the

313   tergosternal (wing depressor) and dorsal-longitudinal (wing elevator) muscles. Thus, the

314   wingbeat frequency of insects with synchronous musculature is determined by the frequency

315   of nervous stimulation to the muscles. In contrast, insects that possess asynchronous muscles

316   have essentially random nervous stimulation relative to the wingbeat frequency (Dickinson

317   and Tu, 1997). Wingbeat frequency in asynchronous fliers is determined primarily by the

318   resonant features of the pterothoracic apparatus to maximise efficiency of energy expenditure

319   (Pringle, 1949; Dickinson and Tu, 1997), as well as behavioural changes during rapid

320   manoeuvring (Nachtigall and Wilson, 1967). Asynchronous muscles are stretch-activated

321   (Pringle, 1949, 1967) by their antagonistic pair within the pterothorax and are therefore

322   dependent on mechanical loading. The inertial load of the whole thorax-wing system must

323   increase with body mass and wingbeat frequency has been shown to vary inversely with wing

324   inertia (Sotavalta, 1952). For asynchronous fliers, scaling of the resonant flight apparatus is

325   therefore especially important, as the oscillatory frequency of the pterothorax is inversely

326   dependent on its size, which directly influences wingbeat frequency (Pringle, 1949, 1967;

327   Dickinson and Tu, 1997; Dudley, 2000). In synchronous fliers, wing amputation experiments

328   to lower wing inertia results in only a relatively small increase in wingbeat frequency in

329   *Periplaneta* cockroaches and *Agrontia* moths compared to asynchronous fliers (Roeder,

330   1951), suggesting wingbeat frequency in synchronous fliers is independent of mechanical

331   load. Thus, asynchronous fliers are more likely to show a stronger scaling relationship

332   between wingbeat frequency and body mass than other insects. No strong inferences relating

333   to scaling differences between synchronous and asynchronous fliers can be made in the

334   present study because Lepidoptera encompassed the only well sampled synchronous fliers.

335

336   Orders are shown to be clustered when wingbeat frequency is viewed as a function of one of

337   the other measured morphometrics (Fig. 2A-C), supporting the idea that flight strategy can be

338   generally characterized based on evolutionary history. This may be because of a combination

339   of several factors: 1) species inherit a flight apparatus that can only be changed to a certain

340   extent in a given time to fit a new role/niche e.g. Coleoptera inherit heavy elytra, one pair of

341   functional wings, asynchronous flight muscles, and low flight muscle mass ratio relative to

342   body mass (Marden, 1987; Dudley, 2000) making it unlikely for them to be able to fill the role

343   of an aerial predator but well adapted to infrequent spells of sustained flight; 2) species may

344   need to fly in the same way even though they have different ecological niches, which may

345   increase the level of intra-order clustering because the existing flight apparatus can be used

346   to fulfil the same aerodynamic needs despite interacting with different organisms e.g.

347   Syrphidae and Tabanidae need to fly in similar ways – visiting flowers vs. visiting vertebrate

348   hosts (female tabanids), ability to hover above resources, ability to change direction rapidly to

349   regularly escape predators or swatting etc.; and 3) a specific goal may be achieved in more

350   than one way e.g. Diptera: Asilidae and Odonata are both aerial predators with a high

351   proportion of relative flight muscle mass (Marden, 1987), but likely utilise completely different

352   flight strategies because of their very different inherited flight apparatuses. Combined, these

353   factors suggest that although an inherited flight apparatus is predisposed to certain flight

354   strategies and precludes others, it can be somewhat modified in some instances to fit new

355   ecological niches or maintained if aerodynamic needs do not change with differing ecological

356   interactions. Ultimately, this may improve levels of flight strategy conservation at the order

357   level.

358

359   Order-level flight strategies may have interesting energetic, ecological, and evolutionary

360   implications though intra-order exceptions exist where some groups fly in unconventional

361   ways. For example, flies are very light to medium weight with high wingbeat frequencies,

362   medium to low wing area and wing length, and medium to high wing loading (Fig. 2A-D). These

363   attributes afford flies the ability to fly quickly, perform complex aerobatic manoeuvres and to

364   hover, conferring obvious ecological advantages to certain groups. Mosquitos and

365   chironomids, however, possess wingbeat frequencies that are unusually high, and wing

366   loadings that are unusually low relative to other Diptera (Table S1, Supplementary Information)

367   that likely increases energetic costs of flight substantially, and may be used for acoustic

368   communication during swarming and mating (Neems et al., 1992; Takken et al., 2006;

369   Bomphrey et al., 2017). One potential explanation of this presumably highly energetically

370   expensive trait uncharacteristic of most other members of the order may be related to sexual

371   selection, where males and females "duet" by reaching a common harmonic tone based on

372   their usually different wingbeat frequencies (Cator et al., 2009; Robert, 2009; Bomphrey et al.,

373   2017).

374

375   The variation between different clades within orders suggests broad categorization is possible,

376   with infrequent exceptions. For most orders, however, relationships between wingbeat

377   frequency and flight-associated morphometrics show moderately well conserved patterns

378   across the graphs. These align with previous meta-analyses (Byrne et al., 1988; Dudley, 2000)

379   looking at wingbeat frequency in relation to body mass, with the same orders covering the

380   same areas on the graphs (see Fig. 3.3B in Dudley, 2000). The present study therefore

381   provides strong experimental evidence that flight strategy is broadly conserved at the order

382   level, as specimens are generally clustered phylogenetically, and this validates previous meta-

383   analyses investigating wingbeat frequency and flight-associated morphometrics, although

384   there is evidence that some flight strategies show similarity between certain groups. The PCA

385   analysis could though be improved by incorporating other variables, such as relative flight

386   muscle mass, which is shown to be important when considering the ecology of different orders

387   (Marden, 1987; Dudley, 2000).

388

389 Energetic and ecological costs and benefits of differing flight behaviours are still poorly known

390 in most insect groups, though some have received attention e.g. Hymenoptera: Apidae:

391 Euglossini (see Casey et al., 1985; Dudley, 1995; Dillon and Dudley, 2004), Lepidoptera:

392 Sphingidae and Saturniidae (Bartholomew and Casey, 1978), Orthoptera: Acrididae (Snelling

393 et al., 2012), and Hymenoptera: Apidae: Bombini (Ellington et al., 1990). Elucidation of the

394 ecological pressures leading to adaptation of specific flight strategies and the energetic costs

395 associated may help illuminate evolutionary trade-offs. These trade-offs are likely to explain

396 the phylogenetic clustering found across flight-associated morphometrics and wingbeat

397 frequency in the present study. Studies that combine quantitative evaluation of insect flight

398 energetics with additional qualitative comparisons between orders can go some way in

399 revealing why different groups utilise different flight strategies (e.g. between bees, moths, and

400 locusts in Snelling *et al.*, 2012). Further work to reveal ecological pressures and energetic

401 costs of broad flight strategies in different orders is therefore required to infer why insect

402 groups fly the way they do.

403

409

410 **COMPETING INTERESTS**

411 The authors declare no competing or financial interests.

412

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553 **Figure 1. Images a-k show a complete wingbeat in the beetle** *Rutpela maculata*

554 **(Coleoptera: Cerambycidae)**; t is time in milliseconds from the start of the wingbeat. **a**. the

555 end of pronation; **b-e**. downstroke translation; **e-g**. supination; **h-j**. upstroke translation; **j-k**.

556 pronation.

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574 **Figure 2. Relationships between log$_{10}$ transformed morphometric variables. a**, wingbeat

575 frequency (Hz) as a function of wing length (cm): wingbeat frequency = -0.764 * wing length +

576 4.479, R^2 = 0.42, p = <0.001; **b**, wingbeat frequency as a function of wing area (cm^2): wingbeat

577 frequency = -0.413 * wing area + 4.345, R^2 = 0.59, p = <0.001; **c**, wingbeat frequency as a

578 function of body mass (g): wingbeat frequency = -0.129 * body mass + 4.04, R^2 = 0.09, p =

579 <0.01; **d**, wingbeat frequency as a function of wing loading (g/cm^2): wingbeat frequency =

580 0.385 * wing loading + 5.799, R^2 = 0.29, p = <0.001; **e**, wing area as a function of wing length:

581 wing area = 2.105 * wing length – 0.307, R^2 = 0.93, p = <0.001; **f**, wing loading as a function

582 of body mass: wing loading = 0.356 * body mass – 1.977, R^2 = 0.34, p = <0.001; **g**, wing area

583 as a function of body mass: wing area = 0.644 * body mass + 1.977, R^2 = 0.63, p = <0.001.

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Figure 3. Principle component data for Dimensions 1 and 2, categorised into different insect orders by symbol shape and colour. Small translucent symbols represent specimens and large opaque symbols represent the centroids for each order. The ellipses around each centroid represent one standard deviation along each axis of the associated order and are rotated in the direction of maximum spread. Trichoptera, Ephemeroptera, and Mecoptera lack ellipses because of an insufficient sample size. The Dimension scores show a moderate-high level of clustering of orders in relation to measured variables, as specimens are generally in close proximity to their associated centroid. The black point in the top-right quarter of the graph is the mean direction of the arrows and suggests the variables are on average positively correlated with dimensions 1 and 2.

619 **Table 1.** Range and mean of wingbeat frequency and associated morphological

620 measurements in each sampled order. Number of species are denoted in parentheses beside

621 sample size in the right-most column.

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640 **Supplementary Information**

641 **Table S1.** Wingbeat frequency and morphological measurements of all specimens. Lists

642 specimens by body mass in ascending order. Cells with a "-" denote the specimen failed to

643 be identified to the associated taxonomic rank.

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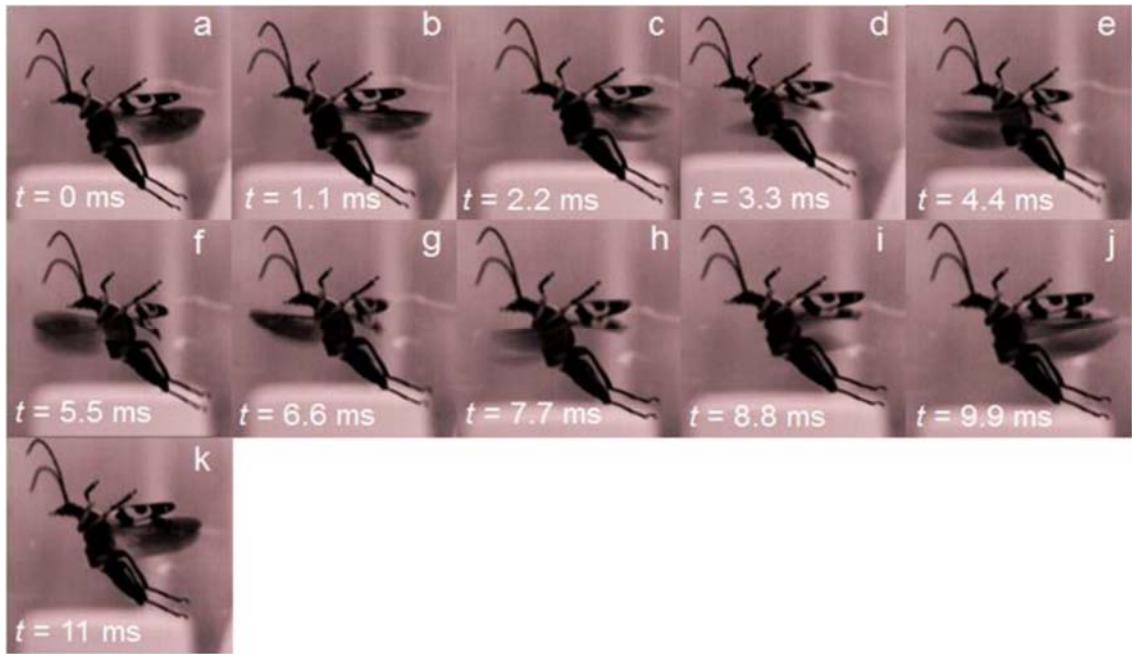654

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662 **Figure 1.**

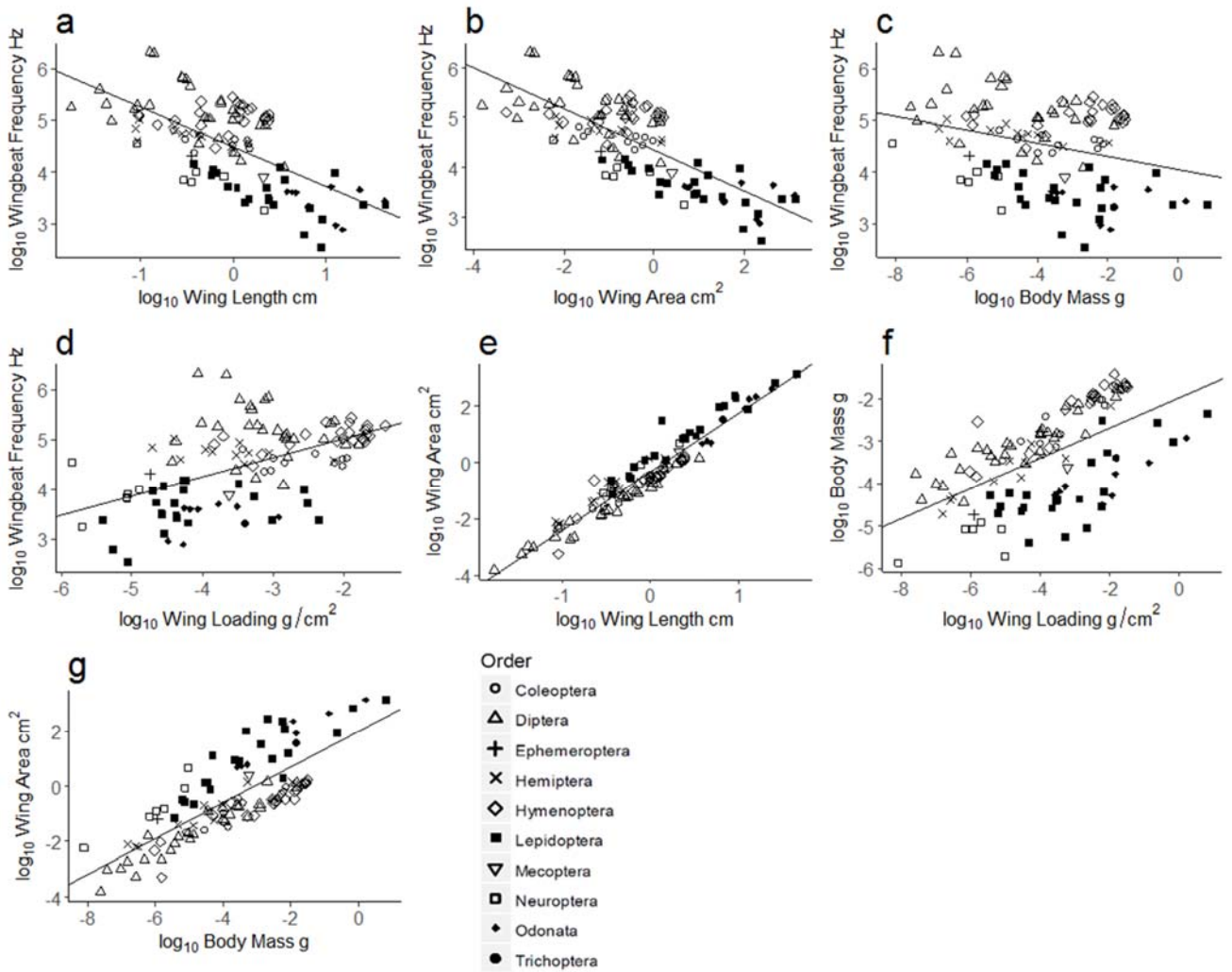663

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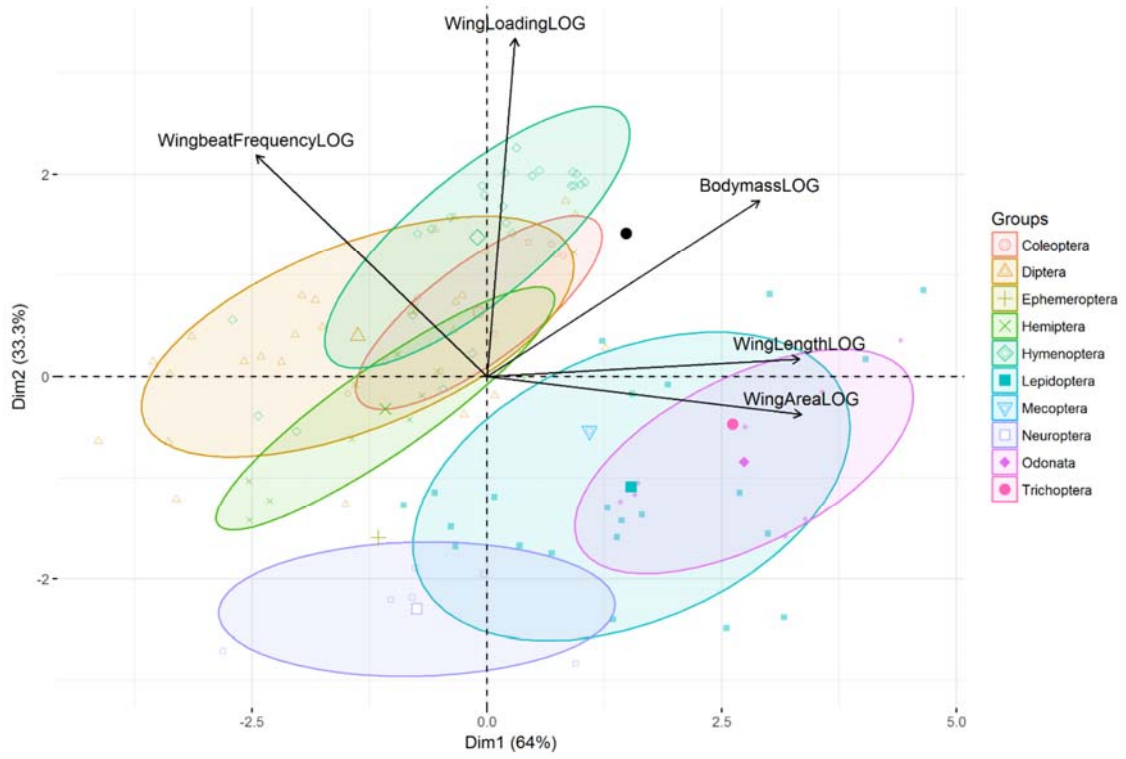675

676   **Figure 2.**

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697   **Table 1.**

`	Wingbeat frequency (mean Hz)		Bodymass (g)		Wing length (cm)		Wing area (cm^2)		Wing loading (g/cm^2)		Number of specimens
	range	mean	range	mean	range	mean	range	mean	range	mean	
Coleoptera	79 - 123.396	97.512	0.0061 - 0.117	0.0539	0.521 - 1.188	0.898	0.19 - 0.982	0.545	0.0321 - 0.141	0.085	10(10)
Diptera	59.567 - 557.351	208.244	0.0005 - 0.162	0.0268	0.172 - 1.739	0.729	0.022 - 1.17	0.327	0.0119 - 0.168	0.0554	28(28)
Ephemeroptera	n/a	75.0454	n/a	0.0027	n/a	0.634	n/a	0.306	n/a	0.00882	1(1)
Hemiptera	90.222 - 152.247	116.39	0.0011 - 0.14	0.0226	0.345 - 1.185	0.624	0.112 - 1.186	0.445	0.009 - 0.118	0.034	11(11)
Hymenoptera	87.129 - 230.987	163.89	0.0024 - 0.223	0.103	0.356 - 1.48	1.006	0.038 - 1.234	0.64	0.022 - 0.245	0.136	24(15)
Lepidoptera	12.468 - 64.566	39.606	0.0044 - 2.24	0.203	0.646 - 5.214	1.792	0.318 - 23.362	5.031	0.004 - 0.096	0.025	22(22)
Mecoptera	n/a	48.885	n/a	0.0398	n/a	1.387	n/a	1.492	n/a	0.027	1(1)
Neuroptera	25-923 - 94.413	52.801	0.0003 - 0.0065	0.0035	0.352 - 1.393	0.757	0.106 - 1.972	0.701	0.003 - 0.007	0.005	6(6)
Odonata	17.847 - 40.665	32.331	0.0278 - 1.23	0.27	1.795 - 5.158	3.002	1.964 - 22.784	8.768	0.0112 - 0.054	0.022	8(6)
Trichoptera	n/a	27.515	n/a	0.159	n/a	2.267	n/a	4.738	n/a	0.0336	1(1)

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711 **Supplementary Table.**

Species	Genus	Family	Order	Wingbeat frequency (mean Hz)	Bodymass (g)	Wing length (cm)	Wing area (cm²)	Wing loading (g/cm²)
-	*Micromus*	Hemerobiidae	Neuroptera	94.413	0.0003	0.352	0.106	0.003
-	*Syrphus.*	Syrphidae	Diptera	190.860	0.0005	0.172	0.022	0.023
-	-	Psychodidae	Diptera	144.611	0.0006	0.267	0.048	0.013
-	-	-	Diptera	204.355	0.0009	0.252	0.050	0.018
-	-	Chironomidae	Diptera	557.351	0.0011	0.406	0.064	0.017
-	-	Miridae	Hemiptera	127.872	0.0011	0.345	0.122	0.009
Thaumatomyia notata	*Thaumatomyia*	Chloropidae	Diptera	269.741	0.0014	0.234	0.038	0.037
-	-	-	Hemiptera	152.247	0.0014	0.358	0.114	0.012
Uroleucon cirsii	*Uroleucon*	Aphididae	Hemiptera	99.603	0.0015	0.353	0.112	0.013
-	-	Chironomidae	Diptera	544.494	0.0018	0.423	0.070	0.026
-	-	Tipulidae	Diptera	94.606	0.0020	0.687	0.168	0.012
Hemerobius humulinus	*Hemerobius*	Hemerobiidae	Neuroptera	46.583	0.0021	0.586	0.336	0.006
-	*Torymus*	Torymidae	Hymenoptera	160.011	0.0024	0.360	0.098	0.024
Wesmaelius subnebulosis	*Wesmaelius*	Hemerobiidae	Neuroptera	45.304	0.0026	0.640	0.416	0.006
Centroptilum luteolum	*Centroptilum*	Baetidae	Ephemeroptera	75.045	0.0027	0.634	0.306	0.009
-	-	Braconidae	Hymenoptera	136.261	0.0029	0.438	0.134	0.022
-	-	Chloropidae	Diptera	180.050	0.0030	0.342	0.070	0.043
-	-	Braconidae	Hymenoptera	164.443	0.0030	0.356	0.038	0.079
-	*Wesmaelius*	Hemerobiidae	Neuroptera	54.583	0.0033	0.666	0.444	0.007
-	-	-	Diptera	195.996	0.0039	0.354	0.098	0.040

-	-	Syrphidae	Diptera	198.890	0.0044	0.403	0.124	0.035
Pseudargyrotoza conwagana	*Pseudargyrotoza*	Tortricidae	Lepidoptera	64.246	0.0044	0.653	0.318	0.014
-	-	Miridae	Hemiptera	120.832	0.0048	0.530	0.250	0.019
Culex pipiens	*Culex*	Culicidae	Diptera	334.037	0.0049	0.578	0.158	0.031
-	-	Tortricidae	Lepidoptera	52.214	0.0055	0.785	0.612	0.009
-	-	Crambidae	Lepidoptera	57.948	0.0059	0.799	0.562	0.010
Micromus angulatus	*Micromus*	Hemerobiidae	Neuroptera	50.000	0.0059	0.904	0.932	0.006
Oulema melanopus	*Oulema*	Chrysomelidae	Coleoptera	123.398	0.0061	0.521	0.190	0.032
Chrysoperla carnea	*Chrysoperla*	Chrysopidae	Neuroptera	25.923	0.0065	1.393	1.972	0.003
Aedes cantans	*Aedes*	Culicidae	Diptera	286.949	0.0066	0.627	0.182	0.036
Culiseta annulata	*Culiseta*	Culicidae	Diptera	344.160	0.0070	0.572	0.150	0.047
Macrolophus sp.	*Macrolophus*	Miridae	Hemiptera	139.717	0.0076	0.515	0.244	0.031
Lobesia abscisana	*Lobesia*	Tortricidae	Lepidoptera	64.566	0.0076	0.646	0.526	0.014
Culiseta annulata	*Culiseta*	Culicidae	Diptera	331.157	0.0077	0.602	0.174	0.044
-	-	-	Hemiptera	116.865	0.0104	0.619	0.490	0.021
Propylea 14-punctata	*Propylea*	Coccinellidae	Coleoptera	102.427	0.0105	0.589	0.210	0.050
Pasiphila rectangulata	*Pasiphila*	Geometridae	Lepidoptera	41.358	0.0107	0.938	1.132	0.009
Pterophorus pentadactyla	*Pterophorus*	Pterophoridae	Lepidoptera	32.333	0.0114	1.192	1.120	0.010
Nephrotoma flavescens	*Nephrotoma*	Tipulidae	Diptera	79.470	0.0118	1.015	0.402	0.029
-	-	Miridae	Hemiptera	108.171	0.0119	0.710	0.402	0.030
Pandemis cerasana	*Pandemis*	Tortricidae	Lepidoptera	54.184	0.0124	0.835	0.890	0.014
Athalia scuttelariae	*Athalia*	Tenthredinidae	Hymenoptera	87.129	0.0132	0.826	0.352	0.038
Xanthorhoe montanata	*Xanthorhoe*	Geometridae	Lepidoptera	29.243	0.0133	1.561	2.980	0.004
Lygus rugulipennis	*Lygus*	Miridae	Hemiptera	115.183	0.0140	0.574	0.298	0.047

Nephrotoma quadrifaria	*Nephrotoma*	Tipulidae	Diptera	67.360	0.0181	1.084	0.464	0.039
Rhagonycha fulva	*Rhagonycha*	Catharidae	Coleoptera	79.712	0.0183	0.653	0.380	0.048
Chloromyia formosa	*Chloromyia*	Stratiomyidae	Diptera	156.043	0.0183	0.736	0.312	0.059
Haematopota pluvialis	*Haematopota*	Tabanidae	Diptera	151.568	0.0183	0.779	0.302	0.061
-	-	-	Hemiptera	112.917	0.0184	0.682	0.518	0.036
-	-	Vespidae: Eumeninae	Hymenoptera	135.597	0.0184	0.703	0.292	0.063
-	-	Empididae	Diptera	151.321	0.0193	0.759	0.288	0.067
Oedemera nobilis	*Oedemera*	Oedemeridae	Coleoptera	112.656	0.0210	0.698	0.232	0.091
Scathophaga stercoraria	*Scathophaga*	Scathophagidae	Diptera	104.015	0.0224	0.854	0.366	0.061
-	-	Ichneumonidae	Hymenoptera	110.116	0.0233	0.942	0.546	0.043
Manulea lurideola	*Manulea*	Erebidae	Lepidoptera	33.095	0.0258	1.470	2.542	0.010
Syrphus ribesii	*Syrphus*	Syrphidae	Diptera	177.908	0.0273	0.994	0.512	0.053
Coenagrion puella	*Coenagrion*	Coenagrionidae	Odonata	37.495	0.0277	1.795	1.964	0.014
Harmonia axyridis	*Harmonia*	Coccinellidae	Coleoptera	79.000	0.0283	0.993	0.644	0.044
Anania hortulata	*Anania*	Crambidae	Lepidoptera	40.996	0.0293	1.448	2.410	0.012
Episyrphus balteatus	*Episyrphus*	Syrphidae	Diptera	166.057	0.0294	1.025	0.488	0.060
Idaea aversata	*Idaea*	Geometridae	Lepidoptera	32.088	0.0303	1.471	2.420	0.013
Coenagrion puella	*Coenagrion*	Coenagrionidae	Odonata	36.691	0.0316	1.984	2.072	0.015
-	*Aphodius*	Scarabaeidae	Coleoptera	93.054	0.0327	0.987	0.560	0.058
Aphantopus hyperantus	*Aphantopus*	Nymphalidae	Lepidoptera	16.014	0.0373	2.168	7.262	0.005
Coenagrion puella	*Coenagrion*	Coenagrionidae	Odonata	36.839	0.0374	1.902	2.190	0.017
-	*Andrena*	Apidae	Hymenoptera	213.815	0.0376	0.703	0.352	0.107
-	-	-	Hemiptera	90.222	0.0383	0.989	1.164	0.033
-	-	Syrphidae	Diptera	208.540	0.0385	0.872	0.340	0.113

Panorpa communis	*Panorpa*	Panorpidae	Mecoptera	48.885	0.0398	1.387	1.492	0.027
-	*Andrena*	Apidae	Hymenoptera	172.581	0.0453	0.690	0.346	0.131
-	*Sarcophaga*	Sarcophagidae	Diptera	149.643	0.0540	0.995	0.526	0.103
Calliphora vomitoria	*Calliphora*	Calliforidae	Diptera	214.835	0.0549	0.874	0.460	0.119
Hypena proboscidalis	*Hypena*	Noctuidae	Lepidoptera	30.587	0.0565	1.137	4.496	0.013
-	*Tipula*	Tipulidae	Diptera	59.567	0.0676	1.739	1.170	0.058
Pieris brassicae	*Pieris*	Pieridae	Lepidoptera	12.468	0.0691	2.593	10.992	0.006
Vespula germanica	*Vespula*	Vespidae	Hymenoptera	145.156	0.0769	1.126	0.628	0.122
Ectemnius cavifrons	*Ectemnius*	Crabronidae	Hymenoptera	210.688	0.0800	1.037	0.542	0.148
-	*Zygaena*	Zygaenidae	Lepidoptera	60.595	0.0804	1.669	2.640	0.030
Vespula germanica	*Vespula*	Vespidae	Hymenoptera	152.006	0.0818	1.061	0.610	0.134
Vespula germanica	*Vespula*	Vespidae	Hymenoptera	146.908	0.0833	0.530	0.536	0.155
Vespula vulgaris	*Vespula*	Vespidae	Hymenoptera	173.277	0.0874	1.081	0.598	0.146
Apis mellifera	*Apis*	Apidae	Hymenoptera	230.987	0.0886	0.995	0.588	0.151
-	*Aphodius*	Scarabaeidae	Coleoptera	103.159	0.0929	1.018	0.658	0.141
Rutpela maculata	*Rutpela*	Cerambicidae	Coleoptera	86.840	0.1026	1.188	0.768	0.134
Geometra papilionaria	*Geometra*	Geometridae	Lepidoptera	22.023	0.1071	2.632	10.194	0.011
Chrysoteuchia culmella	*Chrysoteuchia*	Crambidae	Lepidoptera	40.626	0.1090	1.041	1.330	0.082
Calopteryx splendens	*Calopteryx*	Calopterygidae	Odonata	19.318	0.1092	3.054	9.760	0.011
-	*Aphodius*	Scarabaeidae	Coleoptera	101.111	0.1095	1.161	0.822	0.133
Polygonia c-album	*Polygonia*	Nymphalidae	Lepidoptera	27.501	0.1145	2.298	7.696	0.015
Bombus pascuorum	*Bombus*	Apidae	Hymenoptera	198.274	0.1166	1.074	0.614	0.190
Leptura quadrifasciata	*Leptura*	Cerambicidae	Coleoptera	93.768	0.1173	1.173	0.982	0.119
Orthosia gothica	*Orthosia*	Noctuidae	Lepidoptera	47.053	0.1253	1.753	3.260	0.038

Pentatoma rufipes	*Pentatoma*	Pentatomidae	Hemiptera	96.667	0.1397	1.185	1.186	0.118
Calopteryx virgo	*Calopteryx*	Calopterygidae	Odonata	17.847	0.1457	3.287	10.466	0.014
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	183.029	0.1504	1.171	0.818	0.184
Bombus lapidarius	*Bombus*	Apidae	Hymenoptera	199.547	0.1536	1.114	0.626	0.245
Phryganea grandis	*Phryganea*	Phryganeidae	Trichoptera	27.515	0.1590	2.267	4.738	0.034
Sympetrum striolatum	*Sympetrum*	Libellulidae	Odonata	40.665	0.1595	2.894	6.944	0.023
Volucella pellucens	*Volucella*	Syrphidae	Diptera	134.179	0.1613	1.418	1.152	0.140
Volucella bombylans	*Volucella*	Syrphidae	Diptera	133.078	0.1624	1.337	0.966	0.168
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	186.521	0.1641	1.229	0.864	0.190
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	152.625	0.1854	1.436	1.048	0.177
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	153.182	0.1972	1.422	1.128	0.175
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	144.712	0.2081	1.454	1.134	0.184
Bombus terrestris ♀	*Bombus*	Apidae	Hymenoptera	161.815	0.2125	1.425	1.086	0.196
Bombus terrestris ♂	*Bombus*	Apidae	Hymenoptera	165.085	0.2154	1.480	1.152	0.187
Bombus terrestris ♂	*Bombus*	Apidae	Hymenoptera	149.597	0.2227	1.480	1.234	0.180
Orthetrum cancellatum	*Orthetrum*	Libellulidae	Odonata	38.577	0.4176	3.944	13.960	0.030
Deilephila elpenor	*Deilephila*	Sphingidae	Lepidoptera	53.715	0.5281	3.026	6.792	0.078
Laothoe populi	*Laothoe*	Sphingidae	Lepidoptera	29.330	0.8449	4.085	17.152	0.049
Aeshna grandis	*Aeshna*	Aeshnidae	Odonata	31.214	1.2296	5.158	22.784	0.054
Acherontia atropos	*Acherontia*	Sphingidae	Lepidoptera	29.160	2.2403	5.214	23.362	0.096

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715   **Equation 1.**

716   Equation 1 $n = n_v \dfrac{1}{\left(t_v/f_m\right)}$

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