

1           **Comparative evidence supports a role for reproductive allocation in the**  
2                                 **evolution of female ornament diversity**

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10

11     **Abstract**

12             1. Sexually selected ornaments are highly variable, even among closely related  
13                 species, and the ultimate causes of variation in ornament evolution are unclear,  
14                 including in rare cases of female ornament expression. One hypothesis is that  
15                 differences across species in female reproductive allocation may help explain  
16                 patterns of female ornament expression among insects with nuptial gifts.

17

18             2. Dance flies (Diptera: Empididae: Empidinae) vary considerably among species  
19                 in the presence and extravagance of female ornaments, which probably evolved  
20                 through female contests for mates. In most dance flies, adult females appear to  
21                 acquire all their dietary protein from nuptial gifts provided by males during  
22                 mating. The importance of nuptial feeding on egg development is not yet  
23                 known.

24

25             3. To test the prediction that the presence of female ornaments reflects differences  
26                 in the degree to which females rely on nuptial feeding for egg development, we

27 examined egg development in wild females of two species, one ornamented  
28 and the other unornamented. We validated an ageing technique based on  
29 cuticular bands which permitted a regression of egg size on adult age.

30

31 4. We found that egg development depended on mating status in the ornamented  
32 species alone, meaning the eggs of unmated females of the ornamented species  
33 did not develop. This contrast across species is consistent with expectations that  
34 females of different species vary in their dependence on nuptial gifts for egg  
35 development.

36

37 5. Our findings provide preliminary support for the hypothesis that differences in  
38 reproductive allocation mediate the intensity of female contests for nuptial gifts.

39

40 Key words – courtship feeding, female ornaments, mate choice, reproductive  
41 allocation, anautogeny, sexual competition

42

### 43 **Introduction**

44 In some unusual mating systems, female fitness is limited by male monopolization of  
45 resources required for reproduction, and females may consequently compete for mates.

46 In such mating systems, females can evolve secondary sexual traits if the advantages of  
47 winning contests for mates is sufficiently large (Clutton-Brock 2009; Gwynne and

48 Simmons 1990; Herridge et al., 2016). However, while sexual selection and ornament

49 expression are common in males of many taxa (Janicke et al., 2016), even when females  
50 experience strong sexual selection they rarely have extravagant ornaments (Amundsen

51 2000). One possible explanation for this disparity is that female fitness tends to be

52 resource-limited to a greater degree than male fitness; by definition, females must make

53 large investments in eggs, which might trade-off with any investment in ornamentation

54 (Fitzpatrick et al., 1995). Male choice for adorned females is probably also constrained  
55 by trade-offs between ornaments and offspring, as males should prefer mates who  
56 invest in offspring rather than ornaments. Moreover, when females store sperm,  
57 attractive females may actually present a higher risk of sperm competition, such that  
58 males might avoid rather than prefer showy females (Herridge et al., 2016). Together,  
59 these arguments make rare species with male choice for showy female ornaments  
60 perplexing, and good candidates for testing theories about what regulates interspecific  
61 diversity in male choice and ornament expression.

62

63 Male insects often provide nutrition to females during courtship in the form of “nuptial  
64 gifts”, and these material donations are commonly used to initiate or accelerate egg  
65 production (Lewis et al., 2014). The degree to which females rely on nuptial gifts for  
66 egg production should covary with the sexual receptivity of females, because hungry  
67 females might use sex as a foraging technique. An increase in sexual receptivity can in  
68 turn lead to increased competition among females, especially if the preferred mating  
69 rate of females begins to exceed the rate at which males can provide gifts (Arnqvist and  
70 Nilsson 2000; Simmons and Gwynne 1993). Increased sexual selection on females  
71 arising from this enhanced competition could under some circumstances lead to the  
72 evolution of extravagant traits that improve female attractiveness and therefore female  
73 access to limiting nuptial gift nutrients.

74

75 While the role of nuptial gifts in promoting female contests and ornament evolution is  
76 relatively uncontroversial, the extravagance of ornaments often varies even among  
77 closely related species that share the same geographic distribution and mating behavior,  
78 which remains unexplained (Cumming 1994; Downes 1970). Cumming (1994) has  
79 hypothesized that the presence and level of expression of female ornaments may depend

80 on the intensity of female competition for nuptial gifts, which in turn might be  
81 determined by the degree to which females rely on male gifts for egg development. The  
82 allocation of resources to eggs prior to mating may therefore mediate the intensity of  
83 female competition for nuptial gifts, influencing the strength of selection on female  
84 investment into ornamental traits.

85

86 Female insects can allocate resources obtained during both larval and adult stages to  
87 reproduction, and the timing of acquisition and source of resources have important  
88 consequences on the reproductive and foraging strategies of animals (Boggs 1997a).  
89 Exclusively using larval-derived resources to invest into egg development is termed  
90 autogeny, and is common among Ephemeroptera and in many species of Lepidoptera  
91 and Diptera (Engelmann 1970). By contrast, anautogeny describes the condition in  
92 which females require some adult nutrients to mature eggs, e.g., as in mosquitoes that  
93 act as vectors for human diseases including malaria. The level of dependence on adult  
94 derived resources for egg production varies continuously across anautogenous species  
95 (Boggs 1981; Jervis 2012). While variation in anautogeny remains largely unexplained,  
96 the availability of resources at different stages in insect life cycles likely strongly  
97 influences the pattern of resource partitioning, and therefore resources available for  
98 reproductive allocation (Jervis et al., 2001). Nuptial gifts, being often of substantial  
99 size, may influence the partitioning of resources towards growth and reproduction in  
100 life stages prior to adult stages. This may occur because of female expectation of nuptial  
101 resources in the adult stage. However, to our knowledge the extent to which selection  
102 arising from the presence of nuptial gifts affects resource partitioning has not been  
103 investigated.

104

105 By definition anautogenous species should have females that depend more on adult  
106 derived resources for egg production when compared to others. In these species,  
107 therefore, nuptial gifts (should they be present) may represent essential resources for  
108 female reproductive success (Fritzsche et al., 2016). As such females of anautogenous  
109 species may experience greater competition for mating in order to acquire nuptial gifts,  
110 and subsequently have elevated selection for sexual trait investment (Cumming 1994).

111

112 One group of taxa with nuptial gift giving which exhibits extraordinary interspecific  
113 diversity in the extravagance of female ornaments are the dance flies (Diptera;  
114 Empididae; Empidinae) (Collin 1961; Cumming 1994; Downes 1970). Despite closely  
115 related species having similar courtship behaviour, females of different species display  
116 varying levels of ornamentation (Funk and Tallamy 2000; Murray 2015). Ornamental  
117 traits include pinnate leg scales, enlarged and or darkened wings, and inflatable  
118 abdominal sacs, although their presence and extravagance varies substantially among  
119 taxa (Collin 1961; Downes 1970). Within species variation in ornament size is known  
120 to influence male mate choice (Funk and Tallamy 2002; Murray et al 2018). Ornaments  
121 appear to have evolved independently multiple times across the dance fly phylogeny  
122 (Murray 2015). We do not yet know what regulates these many evolutionary transitions  
123 in ornament expression across dance flies, but we expect higher levels of ornament  
124 expression in species subject to more intense sexual selection on females. Therefore, it  
125 is reasonable that the fitness of females in species with substantial sexual ornaments is  
126 more strongly constrained by dietary protein than unadorned species. In such species,  
127 investment in ornaments may be justified because the returns on investment in  
128 ornaments (through the accrual of nuptial gifts) more than offset the cost of construction  
129 (Fitzpatrick et al., 1995).

130

131 In roughly a third of dance fly species of the subfamily Empidinae, females are more  
132 numerous than males in lek-like mating swarms (Cumming 1994; Downes 1970).  
133 Males approach swarms with prey items as nuptial gifts, typically another dipteran  
134 (Cumming 1994). Males typically assess females from below, apparently evaluating  
135 the gravidity of females (Funk and Tallamy 2000). In most species, females do not  
136 appear to hunt as adults, and therefore derive all of their dietary protein from nuptial  
137 gifts.

138

139 The strong link between mating and foraging in dance flies suggest that egg  
140 development may be copulation-dependent (Cumming, 1994), but this hypothesis has  
141 never been rigorously tested. One limitation has been an inability to rear dance flies in  
142 the lab, and thereby study ovarian physiology in individuals of known age and mating  
143 history. Additionally nuptial gifts vary in size and quality, and therefore females may  
144 not receive the same volume of resources in each mating (Svensson et al., 1990), which  
145 may cause fitness received by mating to depend on nuptial gift characteristics.

146

147 In this study we circumvent the inability to rear and manipulate flies by predicting that  
148 mating status (rather than mate number) covaries with egg development in ornamented  
149 and non-ornamented taxa. It is possible to distinguish mated from non-mated wild  
150 females by inspecting their sperm storage organs for the presence of sperm, and  
151 therefore to know whether a female has or has not received adult dietary protein, in the  
152 form of at least one nuptial gift. In order to assess the temporal dimension of ovarian  
153 maturation, we first adopt and validate an ageing method for wild caught dance flies.  
154 We compare two species that are locally abundant for long periods near our University  
155 in central Scotland, facilitating the collection of mated and non-mated individuals at a  
156 range of different ages. The two species are *Empis aestiva*, which has females with

157 extensive pennate scales on their mid and hind legs (see figure 1), and *Rhamphomyia*  
158 *crassirostris* with no obvious sexual ornaments on either sex (see figure 1). In both  
159 species, males obligately provide nutritious nuptial gifts to females (males are not  
160 known to provide non-nutritious “sham” gifts in these species). We predicted that when  
161 unmated, the eggs ornamented *E. aestiva* females would develop at a slower rate than  
162 the eggs of unadorned *R. crassirostris* females, whereas mated females with access to  
163 dietary protein might show no such differences.

164

## 165 **Materials and methods**

### 166 **Aim 1: Validating methods for ageing wild flies**

167 The thoracic apodemes of flies are known to continue growing even after eclosion,  
168 leaving evidence of time passed since eclosion (Schlein and Gratz 1973). A distinct line  
169 marks the extent of the apodeme structure upon eclosion (see supplementary Figure 1).  
170 After eclosion, the density of the cuticle deposited at the cortical part of apodemes is  
171 influenced by temperature. Diurnal temperature fluctuations cause banding to occur and  
172 therefore theoretically the number of apodeme bands covaries with the number of days  
173 since eclosion (Johnston and Ellison 1982; Schlein and Gratz 1973). However, it is  
174 likely that cuticle is not laid down on the apodemes indefinitely, and that the age at  
175 which the apodemes cease to grow varies among species and even individuals. Using  
176 apodeme bands to age flies has been validated for a number of Dipteran species, but  
177 not previously for any empid species (Neville 1983).

178

179 In order to test whether our focal species produce apodeme bands that reliably reflect  
180 adult age, individuals of known age were required. We were unable to collect a  
181 sufficient number of the two focal species (the precise habitats of larval empids remain  
182 unclear, and our sampling was unable to improve this knowledge, but it is likely that

183 larval empids are relatively well dispersed since they are thought to be predators). We  
184 therefore determine whether the thoracic apodemes of dance flies accumulate daily  
185 cortical growth bands using a range of Empidinae species caught in emergence traps.  
186 We also assessed which particular regions of the thoracic apodemes have the most  
187 distinguishable bands.

188

189 To ensure that the flies caught for this experiment were of known age, we used  
190 emergence traps (ground area 1 m. X 2 m., height 1 m.). The traps were deployed from  
191 the 13<sup>th</sup> of June till the 20<sup>th</sup> of July 2016 at three locations in central Scotland, UK:  
192 Stirling University campus, (56°14'81.10"N, 003°90'52.02"W), Kippenross forest,  
193 (56°17'04.07"N, 003°95'52.56"W) and a field near Enochdhu, (56°74'20.21"N,  
194 003°52'21.00"W). The traps were searched twice per day, once before 12pm and again  
195 after 6pm, which ensured that any flies found in the traps had emerged within the last  
196 24 hours. We aimed to collect as many flies as could be caught in the traps during the  
197 period. Flies in the dance fly sub-family Empidinae were retained and kept alive  
198 between zero and ten days, as experimentally determined after capture using a random  
199 number generator (pilot experiments suggested that individuals were unlikely to live in  
200 captivity beyond ten days). The captive flies were placed in individual plastic containers  
201 closed with cotton stoppers. Each container had two cuvettes placed inside, one with  
202 cotton wool soaked in water and another with sugar granules. The containers were kept  
203 sheltered, but out-of-doors so that the flies would experience natural diurnal  
204 temperature fluctuations, which are critical for the formation of the thoracic bands  
205 (Schlein and Gratz 1973; Johnston and Ellison 1982; Neville 1983). On the appointed  
206 day of sacrificing, the flies were killed by freezing ( $C^{\circ} \leq -12$ ).

207



208 The method used to dissect flies and count apodeme bands was modified based on the  
209 protocol published in Schlein and Gratz (1973). The head, abdomen, wings and legs of  
210 the flies were removed and the thorax bisected. The bisected thorax halves were placed  
211 in distilled water and, using a dissecting microscope (Lecia MZ12) and titanium  
212 forceps, the anterior and posterior thoracic apodemes extracted. The majority of the  
213 muscle tissue was removed from the apodemes with forceps, and the rest dissolved in  
214 potassium hydroxide (10%) for 150 minutes. The specimen was then rinsed in distilled  
215 water, stained in potassium permanganate (10%) for two minutes and rinsed again in  
216 distilled water. The anterior and posterior apodemes were bisected mid-sagittally with a  
217 razor blade to form four fragments. The two fragments of the anterior apodeme were  
218 bisected transversely into rostral and caudal halves. The resulting six fragments (two  
219 from the posterior and four from the anterior apodemes) were then mounted in DPX  
220 medium (Fisher Scientific, Loughborough, UK) on a microscope slide.

221

222 Taking care not to count the eclosion line, the bands on all six fragments of apodeme  
223 were counted using a light microscope (Olympus BX-41; see supplementary figure S1  
224 for the locations of bands on the three apodeme fragments). To prevent bias, we counted  
225 blind to the knowledge of how many days the fly had been kept alive. Recounting to  
226 quantify repeatability using intra-class correlation was performed blind to both the real  
227 adult age and the previous count.

228

229 Statistics were performed in R version 3.3.2 (R Core Team 2016). To validate the age  
230 estimates obtained from apodeme bands, we used major axis (model 2) linear modeling  
231 to regress the maximum number of bands counted from any apodeme per fly against  
232 known age. Major axis regression was used because we recognize the possible error in  
233 both the predictor and response, and because least squares regression is known to

234 negatively bias slope estimates in such cases. Because bands are often hard to  
235 distinguish, and their formation is unlikely to be as regular as theorized (especially in  
236 Scotland where daily temperature fluctuations are often modest), we minimized the  
237 resulting bias by using maximum band number instead of the average across apodemes.  
238 We used age as the y-axis in this case because we experimentally manipulated the age  
239 of these flies, having collected them on the day of eclosion; therefore the band number  
240 was the response variable of interest even though our motivation is to assess if band  
241 number reflects age. Models were validated by visually assessing diagnostic plots to  
242 confirm normality of residuals and homoscedascity, and to ensure that no records had  
243 unduly high influence. We used the ICC package for R to obtain repeatabilities (Wolak  
244 et al., 2012).

245

## 246 **Aim 2: The effect of age and mating on ovarian maturation**

247 In order to investigate the relationship between ovarian development and female  
248 resource allocation, we collected data on egg area (as a proxy for the stage of  
249 vitellogenesis), mating status (mated or unmated, based on the absence or presence of  
250 sperm in the spermatheca) and estimates of age using thoracic apodeme counts from  
251 wild females of unknown age captured in mating swarms or on vegetation using hand-  
252 held nets. We aimed to collect 30 unmated and mated females of both species. Females  
253 of *R. crassirostris* were caught from May to July in 2016 in central Scotland, on the  
254 University of Stirling campus, (56°14'88.01"N, 003°90'56.02"W,) and in Kippenross  
255 forest, (56°17'01.50"N, 003°95'51.22"W). Females of *E. aestiva* were caught in 2015  
256 in central Scotland, near the Scottish Centre for Ecology and Natural Environment  
257 (SCENE) (56°09'06.56"N, 004°38'36.16"W), and in 2016 in a forest near Enochdhu,  
258 (56°74'20.21"N, 003°52'21.00"W ). The flies were all frozen immediately on the day  
259 of capture ( $C^{\circ} \leq -12$ ).

260

261 Flies were dissected using a Leica MZ12 light microscope and titanium forceps. The  
262 abdomen was removed and placed in distilled water and opened. We photographed  
263 between three and five of the largest eggs (dance flies mature eggs in clutches, such  
264 that within female variation in egg size is negligible, pers. obs.) using a microscope  
265 mounted camera (Olympus SP-500UZ) and measured egg area using ImageJ version  
266 1.51h (Schindelin et al., 2015).

267

268 Like most other insects, female dance flies are able to store sperm, and eggs are  
269 fertilized only at the time of oviposition. We checked the mating status of females by  
270 splitting the spermatheca with forceps and visually assessing the presence of sperm  
271 (females captured while mating invariably contain sperm, suggesting that failures to  
272 transfer ejaculate are rare in these taxa, pers. obs.). Since females in the two species  
273 being investigated have never been seen mating without a nuptial gift (pers. obs.), by  
274 comparing ovarian development in mated and unmated females, we are able to  
275 distinguish the development of eggs in females with and without access to adult dietary  
276 protein. The thoracic apodemes of the species were dissected and stained using the  
277 method outlined above. We used the maximum number of bands counted on any of the  
278 apodeme types as our best estimate of age.

279

280 To test whether the two species of dance fly differ in the effect of mating status on egg  
281 development, we fit a multiple regression model including a three-way interaction  
282 between age (in days), mating status (mated or unmated as a categorical variable) and  
283 species (*E. aestiva* or *R. crassirostris*). The two species differed substantially in the size  
284 of mature eggs, so average egg sizes were standardized independently for each species,  
285 which facilitates interpretability of model coefficients (Schielzeth 2010) by providing

286 a strong test of whether the interaction between mating status, species and age  
287 represents a difference in the rate of maturation, rather than merely reflecting the  
288 different egg sizes across species. We assessed model quality by visually inspecting  
289 diagnostic plots. In order to achieve homoscedascity and improve fit, we used a natural-  
290 log-transformed average egg size for each female in our model.

291

## 292 **Results**

293 Among flies caught in emergence traps (see table 1) and reared in captivity for up to  
294 ten days, the number of bands counted on the rostral and caudal parts of their anterior  
295 apodemes ranged from zero to eight and zero to five, respectively. For the posterior  
296 apodemes the number of bands ranged from zero to nine.

297

298 The repeatabilities in table 2 show that estimates of age are most consistent across blind  
299 trials for the maximum band number, followed by the rostral fraction of anterior  
300 apodemes, then posterior apodemes and finally the caudal part of the anterior  
301 apodemes. We therefore estimated the age of field-caught dance flies using the  
302 maximum number of bands counted on any apodeme. This subset of counts produced  
303 an age predicting model with the lowest AIC and highest R squared when compared to  
304 models using counts from only one apodeme type (see figure 2).

305

306 As predicted, we found a strongly significant positive association between maximum  
307 band number and fly age (Intercept = 0.7478, 95% CI (lower = 0.0538, upper = 1.3341),  
308 Slope = 0.7788, 95% CI (lower = 0.627, upper = 0.9562), p-value <0.0001, R<sup>2</sup> adjusted  
309 = 0.7485, see figure 2).

310

311 The age estimates for *R. crassirostris* females ranged from zero to ten days, with a  
312 broadly Gaussian distribution notwithstanding the strict bound at zero, see figure 3.  
313 Nineteen of the 69 females were unmated, and average egg sizes ranged from  
314  $0.0092\text{mm}^2$  to  $0.1305\text{mm}^2$ , with mean egg size at  $0.0500 \pm \text{SE } 0.0036$ . The age  
315 estimates for *E. aestiva* ranged from zero to five days; see figure 3. Eighteen of 49  
316 females were unmated. Average egg sizes ranged from  $0.0068\text{mm}^2$  to  $0.0523\text{mm}^2$ , with  
317 mean egg size =  $0.0181 \pm 0.0014$ .

318

319 The effect of mating status on egg development differed between the two dance fly  
320 species. In the unadorned *R. crassirostris*, eggs increased gradually in size for both  
321 mated and unmated females (although the intercept for unmated females was lower, as  
322 might be expected if male nuptial gifts boosted vitellogenesis). In contrast and as  
323 predicted, unmated *E. aestiva* demonstrated no change in egg size with age (Partial F  
324 test of whether the removal of the 3-way interaction term results in a poorer model fit:  
325  $F = 4.1919$ ,  $P = 0.0430$ ; see Figure 4).

326

## 327 **Discussion**

328 We examined egg development in mated and unmated dance flies from two species that  
329 contrast sharply in female ornamentation, and found evidence consistent with an  
330 association between anautogeny and ornamentation, supporting the hypothesis that  
331 interspecific differences in dance fly sexual ornament expression derive at least in part  
332 from differences in ovarian physiology. Mindful of the limitations on inference that are  
333 inherent with two-species comparisons, we discuss the implications of our findings for  
334 mating system diversity in this group and female ornament expression in general, as  
335 well as the validated ageing method that we modified from previous work.

336

337 **How mate acquisition relates to investment in ornaments**

338 Sexually selected ornaments are highly variable, even among some closely related  
339 species (Murray 2015). Ornaments evolve as a consequence of competition for mates,  
340 but the underlying causes of competition are unclear in many cases. Female ornaments  
341 are particularly curious adaptations, because general conditions that favour their  
342 evolution appear to be exceedingly rare (Clutton-Brock 2009). In spite of this rarity,  
343 some taxa like the dance flies possess extravagant variation in female ornaments, which  
344 challenges our understanding of the general rules that are thought to regulate diversity  
345 in mating systems (Janicke et al 2016).

346

347 We tested Cumming's (1994) prediction that interspecific variation in how and when  
348 female dance flies allocated resources to eggs plays a key role in mediating the intensity  
349 of female contests for mates. Although we did not directly manipulate allocation (such  
350 experiments remain impossible given the current state of knowledge for culturing dance  
351 flies), we nevertheless generated a priori predictions for patterns of ovarian maturation  
352 in females of two different species that differed in ornament expression. We found that,  
353 as predicted, the eggs of unmated females of the ornamented species developed at much  
354 slower rates compared to the unornamented species. This pattern suggests that females  
355 of the ornamented species rely to a greater degree on male nuptial gifts than females  
356 from unadorned species (see table 3), which provides long-awaited support for  
357 Cumming's hypothesis, and can help explain the otherwise confounding diversity of  
358 sexual trait expression among dance flies (Herridge et al., 2016; Houslay and Bussière  
359 2012).

360

361 In light of the observational nature of our work, there are several factors in addition to  
362 the hypothesized mechanism which could be at play. For example, we cannot rule out

363 that females may receive other nutrition or simulants other than prey item nuptial gifts  
364 from mating, as is common in other insect species, which may also influence the  
365 development of eggs (Lewis et al., 2014). However, even if this were true it would not  
366 explain the difference in ovarian maturation seen among unmated females in the two  
367 focal species. Likewise, our estimates of egg size, mating status and age were all  
368 measured with error. While these sources of error do not lead to systematic biases in  
369 our estimates, they nevertheless make our central conclusions tentative, especially in  
370 light of the marginal significance of our key result. More information on how these  
371 errors contribute to patterns in the data would be welcome. For example, we did not  
372 know the number of times a female had mated, and the volume of resources acquired  
373 by the female during each mating. Some of these matings may have been too recent for  
374 the effective conversion of nuptial gifts to egg maturation, and others may have resulted  
375 in minimal resource intake. More detailed information on mating history rather than  
376 mere mating status (mated or unmated) would clearly provide more resolution for this  
377 kind of analysis.

378

379 Another possibility is that species differences in mating system caused differences in  
380 the representativeness of subsamples of unmated females. For example, if male choice  
381 is stronger in the ornamented *E. aestiva*, it might lead to a stronger difference in average  
382 condition between the mated and unmated fractions of females. The fact that low-  
383 condition females in that species are less likely to mature eggs over time could therefore  
384 be due to their lower condition rather than to species differences in reproductive  
385 allocation. Although we cannot rule out this possible alternative explanation, it is worth  
386 noting that the proportions of unmated females were similar in the two species,  
387 suggesting no large difference in the chances of mating (19/69 or 27.5% of females for

388 *R. crassirostris*, compared to 18/49 or 36% for *E. aestiva*). It seems unlikely that this  
389 small difference could explain our observed differences in ovarian physiology.

390

391 Another intriguing contrast across species involved the difference in female age range,  
392 as estimated by apodeme bands. *E. aestiva* ages ranged from zero to five days and *R.*  
393 *crassirostris*, zero to ten days. While these differences in apparent age structure are  
394 intriguing and unexplained, they may simply reflect differences across species in the  
395 deposition of cuticular bands. Once again, we think this is unlikely, because if many  
396 individuals were older than the maximum number of bands possible, we would expect  
397 a left-skewed distribution of apparent ages, which we did not observe in Figure 3.

398

399 In species with relatively brief adult life span, it is probably important for females to  
400 mate quickly in order to produce eggs and oviposit. Selection for females to mate  
401 quickly after emerging may act as an important factor leading to the expression of  
402 ornaments. In the apparently longer-lived *R. crassirostris*, females may be able to  
403 remain virgins for a longer period. Comprehensively disentangling the possible causes  
404 of species differences in sexual receptivity requires more information on the individual  
405 species in question as well as more comparative work on further species. Herridge  
406 (2016) found significant differences in the number of matings obtained by females of  
407 three nuptial gift giving species of dance fly, including our study species *E. aestiva*.  
408 Interestingly mate number in these species did not straightforwardly reflect ornament  
409 expression. While the most ornamented species (*R. longicauda*) does appear to have  
410 the most sexually receptive females, female *E. aestiva* appeared to mate less often than  
411 in a completely unornamented species (*E. tessellata*). The longevity of *R. longicauda*  
412 and *E. tessellata* are unknown, but it is possible that the combination of female size,



413 longevity and reproductive allocation all affect sexual receptivity and help further  
414 explain cross-species differences in female ornament expression.

415

416 Inferring adaptive patterns from two-species comparisons is difficult (Garland and  
417 Adolf 1994). We recognize that our study would benefit from comparing additional  
418 species, however the collection of sufficient numbers of mated and unmated females of  
419 even the two species used here was difficult, despite the fact that these species are  
420 abundant, and we know a reasonable amount about both their mating swarm locations  
421 and habitat use. Although we remain cautious in our conclusions, we agree with Cooper  
422 (1999) that two species comparisons help identify promising avenues of research.

423

424 Interspecific variation in anautogeny remains largely unexplained (Jervis et al., 2001).  
425 However resource allocation to reproduction is expected to relate to the nutritional  
426 ecology of the adult (Jervis et al., 2001). The quantity, quality and predictability of  
427 resources available to the adult in the environment are factors that likely influence the  
428 evolution of reproductive allocation across life history stages (Karlsson 1994). This fact  
429 supports the notion that nuptial gifts are in a unique position to influence both sexual  
430 selection and life history (Lewis et al., 2014).

431

#### 432 **Validation of an ageing method for wild dance flies**

433 A technique for ageing wild subjects is one of the prerequisites for measuring egg  
434 development rates in dance flies, which have heretofore been difficult to culture under  
435 lab conditions. We have demonstrated that using thoracic apodeme bands works at least  
436 as well for the Dipteran subfamily Empidinae as it has previously been shown for other  
437 groups of flies (Neville 1982), in spite of the relatively slight daily fluctuations in  
438 temperature that are routine in Scottish summers. We hope this demonstration will

439 stimulate more investigations of an ecological and evolutionary nature on the  
440 demographic patterns and life history traits of wild flies, which remain heavily reliant  
441 on lab studies with few exceptions (see Bonduriansky and Brassil 2002 for an  
442 exception).

443

444 In previous studies that evaluated the accuracy of apodeme bands to age other species  
445 of fly, flies were kept in controlled temperature regimes (Neville 1983); hence flies  
446 experienced constant temperature fluctuation between day and night for an extended  
447 period of time, which likely influenced how distinguishable the bands were (Johnson  
448 and Ellison 1982; Schlein and Gratz 1972). In our study, recently emerged flies were  
449 kept outside and experienced natural diurnal fluctuations in temperature. Although it  
450 was difficult to count bands in some specimens, which inflated uncertainty around age  
451 estimates, our estimates were nevertheless consistently close to the ages of known-age  
452 specimens (see figure 2). Regardless of the exact linear modeling approach (ordinary  
453 least squares, major axis, or forcing the intercept through the origin) our slope estimate  
454 was consistently more than one, indicating that band numbers are a good proxy for age  
455 but are likely to systematically underestimate it. This is not surprising since it is easier  
456 to imagine environmental conditions that obscure bands than those that might create  
457 additional bands.

458

459 Because of an inability to collect large numbers of individual species in emergence  
460 traps, we had to pool individuals of all dance fly species for our analysis. It is of course  
461 possible that species differ in the rate of deposition of cuticle, although the theoretical  
462 link between temperature and band deposition makes it unlikely that any such  
463 difference is a cause of bias in our study. Nevertheless, it would be useful to supplement  
464 our data with collections of more known age flies in the future to test this possibility.

465

466 It is also possible that the deposition of cuticle on apodemes ceases in the adult at a  
467 particular age, which is known to occur in several *Drosophila* species (Johnson and  
468 Ellison 1982), and that the age at which this occurs varies across species and  
469 individuals. Our data in figure 2 show a linear relationship of band number on age, with  
470 no identifiable upper limit to age resolution (if the number of bands were saturating, we  
471 might have expected a nonlinear relationship between age and band number). However  
472 only one fly was kept alive for ten days, and it exhibited nine bands on its posterior  
473 apodeme. Whether band deposition continues beyond the maximum of nine we  
474 observed is unclear. However, in the vast majority of flies used to measure egg  
475 development, individuals had fewer than nine bands and so were unlikely to be older  
476 than the maximum age that this method may provide. Furthermore, the histograms  
477 describing the distributions of bands in wild flies were not left-skewed (as might be  
478 expected if there were many individuals at or near an upper bound of band number),  
479 which suggests that most wild females were still accumulating bands at the time of  
480 capture (see figure 3).

481

482 The validation of an ageing technique for further groups of wild insects may be  
483 important to future investigations in evolutionary ecology. Estimates of age and  
484 longevity of wild populations are often the focus of studies of natural and sexual  
485 selection (Endler et al., 1986). The apodeme band ageing technique may successfully  
486 be applied in studies examining differences in the age structure of the sexes. For  
487 example, male dance flies are hypothesized to incur higher mortality rates than females  
488 due to the male sex-specific behavior of acquiring nuptial gifts (Murray 2015).  
489 However, the extent to which sexual differences may incur survival costs in other  
490 contexts remains unclear (e.g., due to the risk of entanglement for ornamented females

491 in spider webs, for example (Gwynne et al., 2015)). The consequences of sex  
492 differences in mortality could be evaluated by comparing the age profiles of the sexes  
493 in more wild populations.

494

495

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502

#### 503 **Supplementary Figures - S1**

504

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645 **Tables**

646 Table 1: The empidinae species and counts of individuals caught in the emergence traps.

Species	Number Caught
<i>Rhamphomyia longipes</i>	5
<i>Rhamphomyia flava</i>	1
<i>Hilara rejecta</i>	5
<i>Hilara manicata</i>	2
<i>Hilara litorea</i>	1
<i>Hilara intermedia</i>	2
<i>Hilara hirta</i>	1
<i>Hilara fulvibarba</i>	2
<i>Hilara clypeata</i>	7
<i>Hilara chorica</i>	1
<i>Hilara apta</i>	6
<i>Empis albinervis</i>	1

647

648 Table 2: The intra-class correlation (ICC) for thoracic apodeme band counts on three different apodemes  
649 types and for the maximum band number obtained from any apodeme type per fly.

Apodeme Type	ICC	Lower 95% CI	Upper 95% CI	N
Rostral Anterior Apodeme	0.8431	0.7104	0.9181	34
Caudal Anterior Apodeme	0.4344	0.1156	0.6730	33
Posterior Apodeme	0.4560	0.0.965	0.7118	26
Maximum Band Number	0.8468	0.6343	0.9411	17

650

651 Table 3: Parameter estimates describing the effect of age, mating status and species on egg development,  
652 X denotes interaction terms. The model uses treatment contrasts to compare categories; the reference  
653 levels are *Empis aestiva* and mated flies (see Schielzeth 2010). This model explained a significant  
654 fraction of the variation in natural log standardised egg sizes (mm<sup>2</sup>) (R square (adj) = 0.1756, p-value =  
655 0.0002 , n = 118).

Source	Estimate	SE	t-value	p-value
Intercept	-0.7761	0.4074	-1.905	0.0594
Species ( <i>R. crassirostris</i> )	-0.0262	0.5391	-0.049	0.9613
Age	0.4599	0.1565	2.938	0.0040
Age X Species ( <i>R. crassirostris</i> )	-0.2729	0.1708	-1.598	0.1129
Mating Status (Unmated)	0.3713	0.6185	0.600	0.5496
Mating Status (Unmated) X Species ( <i>R. crassirostris</i> )	-1.2807	1.1416	-1.122	0.2643
Age X Mating Status (Unmated)	-0.5239	0.2365	-2.215	0.0288
Age X Mating Status (Unmated) X Species ( <i>R. crassirostris</i> )	0.5946	0.2904	2.047	0.0430

656

657

658 **Figure Legends**

659

660 Figure 1: The sexual dimorphism of *Empis aestiva* (Top) and *Rhamphomyia crassirostris* (Bottom).

661 Males and females are shown in full, as are their disembodied legs (Photo credit Frederick Hunter).

662

663 Figure 2: The association between dance fly age and greatest apodeme band number. Lines represent

664 back transformed model predictions, and shaded area shows 95% confidence intervals. Transparency and

665 small random horizontal deviations have been added to the points to facilitate visualisation of

666 overlapping points.

667

668 Figure 3: The range and frequency of age estimates of wild caught *R. crassirostris* (left) and *E. aestiva*

669 (right).

670

671 Figure 4: The egg development of mated and unmated females of *E. aestiva* (left), and *R. crassirostris*

672 (right). Lines represent back-transformed model predictions, with shaded areas showing 95% CIs. Small

673 deviations have been added to data points to reduce overlap.

674







675 Figure S1: The location of the eclosion line and apodeme bands on (top) rostral anterior apodeme,

676 (middle) caudal anterior apodeme and (bottom) anterior apodeme.

677

678

679

<i>Empis aestiva</i>	♀	0.5cm		
	♂	0.5cm		
<i>Rhamphomyia crassirostris</i>	♀	1cm		
	♂	1cm	