

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

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Abstract

1. Sexually selected ornaments are highly variable, even among closely related species, and the ultimate causes of variation in ornament evolution are unclear, including in rare cases of female ornament expression. One hypothesis is that differences across species in female reproductive allocation may help explain patterns of female ornament expression among insects with nuptial gifts.
2. Dance flies (Diptera: Empididae: Empidinae) vary considerably among species in the presence and extravagance of female ornaments, which probably evolved through female contests for mates. In most dance flies, adult females appear to acquire all their dietary protein from nuptial gifts provided by males during mating. The importance of nuptial feeding on egg development is not yet known.
3. To test the prediction that the presence of female ornaments reflects differences in the degree to which females rely on nuptial feeding for egg development, we

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

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

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

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

Introduction

In some unusual mating systems, female fitness is limited by male monopolization of resources required for reproduction, and females may consequently compete for mates. In such mating systems, females can evolve secondary sexual traits if the advantages of winning contests for mates is sufficiently large (Clutton-Brock 2009; Gwynne and Simmons 1990; Herridge et al., 2016). However, while sexual selection and ornament expression are common in males of many taxa (Janicke et al., 2016), even when females experience strong sexual selection they rarely have extravagant ornaments (Amundsen 2000). One possible explanation for this disparity is that female fitness tends to be resource-limited to a greater degree than male fitness; by definition, females must make large investments in eggs, which might trade-off with any investment in ornamentation

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Aim 1: Validating methods for ageing wild flies

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

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

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

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

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

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

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

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

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

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

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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

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

Results

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

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

As predicted, we found a strongly significant positive association between maximum band number and fly age (Intercept = 0.7478, 95% CI (lower = 0.0538, upper = 1.3341), Slope = 0.7788, 95% CI (lower = 0.627, upper = 0.9562), p-value <0.0001, R² adjusted = 0.7485, see figure 2).

The age estimates for *R. crassirostris* females ranged from zero to ten days, with a broadly Gaussian distribution notwithstanding the strict bound at zero, see figure 3. Nineteen of the 69 females were unmated, and average egg sizes ranged from 0.0092mm² to 0.1305mm², with mean egg size at $0.0500 \pm \text{SE } 0.0036$. The age estimates for *E. aestiva* ranged from zero to five days; see figure 3. Eighteen of 49 females were unmated. Average egg sizes ranged from 0.0068mm² to 0.0523mm², with mean egg size = 0.0181 ± 0.0014 .

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

Discussion

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

How mate acquisition relates to investment in ornaments

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

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

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

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

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

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

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

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

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

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

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

Validation of an ageing method for wild dance flies

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

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

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

Because of an inability to collect large numbers of individual species in emergence traps, we had to pool individuals of all dance fly species for our analysis. It is of course possible that species differ in the rate of deposition of cuticle, although the theoretical link between temperature and band deposition makes it unlikely that any such difference is a cause of bias in our study. Nevertheless, it would be useful to supplement 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

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

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Supplementary Figures - S1

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Tables

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

Table 2: The intra-class correlation (ICC) for thoracic apodeme band counts on three different apodemes 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

Table 3: Parameter estimates describing the effect of age, mating status and species on egg development, X denotes interaction terms. The model uses treatment contrasts to compare categories; the reference levels are *Empis aestiva* and mated flies (see Schielzeth 2010). This model explained a significant fraction of the variation in natural log standardised egg sizes (mm²) (R square (adj) = 0.1756, p-value = 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

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	