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# Ultrastructure meets reproductive success: performance of a sphecid wasp is correlated with the fine structure of the flight-muscle mitochondria

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Organisms show a remarkable inter-individual variation in reproductive success. The proximate causes of this variation are not well understood. We hypothesized that the ultrastructure of costly or complex tissues or organelles might affect reproductive performance. We tested this hypothesis in females of a sphecid wasp, the European beewolf, *Philanthus triangulum* (Hymenoptera, Sphecidae), that show considerable variation in reproductive success. The most critical component of reproduction in beewolf females is flying with paralysed honeybees, which more than double their weight. Because of the high energetic requirements for flight, we predicted that the ultrastructure of the flight-muscle mitochondria might influence female success. We determined the density of mitochondria and the density of the inner mitochondrial membranes (DIMM) of the flight muscles as well as age, body size and fat content. Only DIMM had a significant influence on female reproductive success, which might be mediated by an elevated adenosine triphosphate (ATP) supply. The variation in DIMM might result from differences in larval provisions or from an accumulation of mutations in the mitochondrial genome. Our results support the hypothesis that the organization of complex structures contributes to inter-individual variation in reproductive success.

**Keywords:** density of inner mitochondrial membranes; variation in reproductive success; flight muscle; Sphecidae

## 1. INTRODUCTION

Organisms often show a huge variation in reproductive success (e.g. Clutton-Brock 1988; Klingenberg & Spence 1997). Morphological, behavioural, physiological and life-history traits have been shown to contribute to this variation (Endler 1986; Lessells 1991; Spicer & Gaston 1999). The trait that has been most frequently examined for an effect on reproductive success is body size (Blueweiss *et al.* 1978; Roff 1992; Stearns 1992; Honek 1993). However, body size often explains only a fraction of the variance (Leather 1988; Strohm & Linsenmair 1997*a*) or has no detectable influence on reproductive success, even in species that might be predisposed to show such an effect (e.g. Coltman *et al.* 1999; Strohm & Lechner 2000). Other studies revealed a large variation in reproductive success that could not be explained by any trait examined (Klingenberg & Spence 1997). Knowledge of the proximate causes of the unexplained variation in reproductive success would help to identify the target traits of natural selection (Clutton-Brock 1988).

Two lines of circumstantial evidence lead us to the hypothesis that differences in the ultrastructure of costly and/or complex structures that are closely related to reproductive performance might contribute to the variation among individuals. First, many dysfunctions of organs caused by mutations and/or diseases are associated with substantial changes in the ultrastructure of the affected tissue (e.g. Duyckaerts *et al.* 1999; Suomalainen & Kaukonen 2001; Fiala *et al.* 2002). Assuming a continuum between perfectly healthy and severely diseased individuals (Spicer & Gaston 1999), smaller changes in the ultrastructure of cellular structures of apparently healthy individuals might likewise influence performance and, eventually, reproductive success. Second, ageing is accompanied by changes in the ultrastructure of certain tissues or cell organelles (e.g. Collatz & Sohal 1986), which probably cause the observed loss of performance. Differences among individuals of the same age might be analogous to these age-related changes.

Mitochondria might be promising candidates to demonstrate the proposed association between ultrastructure and reproductive success. They play a central role in any biochemical process since they are the sites of adenosine triphosphate (ATP) production (Alberts *et al.* 1994). Thus, differences in the functioning of mitochondria will affect many aspects of an organism's performance. Mitochondria have their own genome and a complex structure. Their inner membranes are more or less strongly folded (so-called cristae) and carry the enzymes for ATP synthesis (Munn 1974). Among the processes that require the most ATP are probably extensive muscle activities such as fast running, swimming and, in particular, flying (Harrison & Roberts 2000). Thus, a test of the effect of the ultrastructure of mitochondria on reproductive success would be most promising in a model species that: (i) heavily depends on muscle functioning for reproduction; and (ii) shows considerable variation in reproductive success among individuals.

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Both of these preconditions are met in our study organisms, females of the European beewolf, *Philanthus triangulum* F (Hymenoptera, Sphecidae). First, females of this species hunt for honeybees (exclusively *Apis mellifera*) on flowers. They paralyse their prey by stinging, bring it to the nest, and provision brood cells with between one and five prey items. Females lay a single egg per brood cell and finally seal it. Nests contain up to 34 brood cells (Strohm & Linsenmair 1999, 2000). The most demanding component of reproduction in beewolves is the transportation, in flight, of the paralysed honeybees to the nest. The prey more than doubles the weight that a female has to carry (Strohm & Linsenmair 1997*a*). Since flying *per se* is energetically rather costly (e.g. Kammer & Heinrich 1978; Beenakkers *et al.* 1985; Wegener 1996; Marden 2000; Harrison & Roberts 2000), flying with such a heavy load is an extraordinary physiological effort. Several components of flight performance have been shown to depend on morphological and physiological traits in different insect species (e.g. Marden 1989, 2000; Coelho & Ladage 1999; Harrison & Roberts 2000; Coelho & Holliday 2001; Berwaerts *et al.* 2002). Thus, performance of the flight muscles is probably of prominent importance for reproductive success in the study species. Second, beewolf females show a considerable variation in reproductive success. In a sample of 21 females, the lifetime hunting success varied more than 20-fold (7–147 bees per lifetime, mean ± s.d. = 69.4 ± 41, coefficient of variation = 0.59; see also Strohm & Linsenmair (1997*a*)). Body size explained only 3–30% of the variance in female success in different samples (Strohm & Linsenmair 1997*a*; E. Strohm, unpublished data).

The density of mitochondria (DM) and the density of the inner mitochondrial membranes (DIMM) differ across animal taxa and between different types of tissues (e.g. Else & Hurlbert 1981, 1985; Eisenberg 1983). Generally, the DIMM increases with the metabolic demands of the species or tissue (Else & Hurlbert 1985; Hurlbert *et al.* 1991; Eisenberg 1983). Furthermore, the structure of the mitochondria of insect flight muscles change with age (e.g. Collatz & Sohal 1986). In particular, inner membranes of the mitochondria are disrupted, probably decreasing the enzymatic capacity of the mitochondria (e.g. Vann & Webster 1976; Collatz & Sohal 1986; McCarter 1995). This suggests that the DM and/or the DIMM might affect muscle performance.

Thus, we predicted that the hunting and provisioning success of European beewolf females is influenced by the DM or DIMM. To compare these potential effects with those of other traits, we also analysed the effects of age, body size and fat content on hunting success. Since we had to kill females to analyse the flight-muscle ultrastructure we could not determine their lifespan and lifetime reproductive success. Instead, we used the number of bees in a brood cell as a measure of a female's reproductive success. However, if lifespan is negatively (or positively) correlated with the number of bees per brood cell, the females' success would be overestimated (underestimated). In order to validate our measure of female success, we examined the relationship between the number of bees in a brood cell and the lifespan as well as the lifetime hunting success in a control group.

## 2. MATERIAL AND METHODS

All beewolf females were the daughters of females caught in the field. They were bred in the laboratory and individually colour-marked on the thorax after emergence. All females were allowed to mate by keeping them together with males for *ca.* 7 days in a large flight cage (Strohm & Linsenmair 1997*a*). Males showed regular territorial behaviour (Strohm & Lechner 2000). After this mating period, females were kept in a flight cage (4 m × 2 m × 2 m) in a greenhouse (temperature of 25–30 °C during the day and 20 °C at night, with a natural light : dark cycle). Honeybees were provided *ad libitum* as prey for the females. Honey was provided *ad libitum* as additional food.

We tested whether the hunting and provisioning activity affected the lifespan of beewolf females in a control group. After the mating period these females were kept individually in breeding cages (sand-filled box, length of 30 cm, width of 20 cm, height of 25 cm, with a similar-sized flight compartment). The number of bees brought into the nest per day was determined as the difference between the number of bees present on a day and the number of bees still present on the following day (dead or alive; Strohm & Linsenmair (1997*b*)). We recorded the females' lifespans and excavated their nests. The number of bees per brood cell could be reliably determined by the remains of the bees' cuticles (Strohm & Linsenmair 1997*b*, 1999, 2000).

The females whose muscles were to be examined were given the opportunity to establish a nest in a sand-filled box (length of 30 cm, width of 20 cm, height of 25 cm) with each box housing only one female. In contrast to those of the control group, these boxes had no individual flight compartment; thus, females could hunt for bees in the whole flight cage (4 m × 2 m × 2 m). Owing to the smooth side-walls of the boxes, these females invariably had to fly with a paralysed bee to bring it to the nest. Thus, flying capacity should have a stronger effect on these females' hunting success than in the individually housed control group. Once beewolf females have established nests, there is little nest switching; nevertheless, the identity of the nest owner was checked daily. The number of bees provisioned per brood cell was determined by nest excavations (see above).

At a mean ± s.d. age of 16.5 ± 5.5 days (which is approximately half the mean lifetime) females ($n = 32$) were killed, weighed on an electronic balance (to the nearest 0.1 mg, Mettler AE 163) and transferred to a Ringer solution on ice. Fat content was determined in the abdomen only and by an extraction method (Richards & Packer 1994). Fat content was calculated as the proportion of fat in the abdomen relative to the fresh weight of the abdomen.

Probably most relevant for flying performance are the dorsal longitudinal muscles, which are responsible for the lift-generating downstroke of the wings (Kammer 1985). These muscles are organized in several subunits of bundles of muscle fibres (Dettner & Peters 1999). The thorax of each female was dissected and five discernible subunits of the longitudinal muscles at equivalent positions were removed with tweezers and immediately transferred to a fixative (modified after Romeis (1989): 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 6% sucrose and 2% dimethyl sulphoxide (DMSO), for 2 h on ice). Specimens were rinsed in distilled water (3 × 15 min) and subjected to post-fixation (1% $OsO_4$ in cacodylate buffer, 30 min on ice and 30 min at room temperature, in the dark), rinsed in buffer (cacodylate, 3 × 15 min) and stored in a refrigerator overnight. Specimens were then dehydrated in graded alcohol series (on ice for 2 × 15 min in 30% ethanol, for

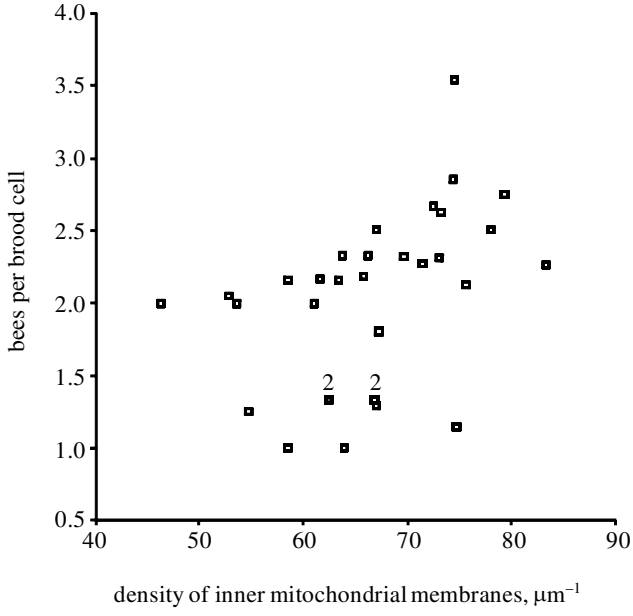


Figure 1. The mean number of bees per brood cell as a function of the mean density of inner mitochondrial membranes (number per micrometre). Numbers indicate the frequency of the respective data point.

20 min each in 50, 70, 90 and 100% ethanol and at room temperature for 20 min in 100% ethanol). Specimens were transferred to propylene oxide (2 × 30 min), to a mixture of propylene oxide and Epon (1 : 1 for 1.5 h, 1 : 2 for 2 h, and overnight) and embedded in pure Epon (5 h, polymerization at 65 °C for 2 days). Ultrathin cross-sections were cut on an ultramicrotome. Sections were stained with uranyl acetate and analysed using a Zeiss EM 10A.

Two sections of each muscle unit (minimum separation of 0.2 mm) were examined. For each section, one picture (8 cm × 10 cm sheet film, Agfa Scientia or Kodak SO 163) was taken at a magnification of ×6300 to determine the density of mitochondria (10 pictures for each of the 32 females). Since we chose the central area of the sections, the part that was analysed was a random fraction of the muscle. From a part of the section that contained some mitochondria, another picture was taken at a magnification of ×20 000 (10 pictures for each of the 32 females). Pictures were digitized (Polaroid Sprint Scan 45, resolution of 600 dpi) and analysed using the program Scion Image (Beta 4.02, Scion Corporation).

We did not use stereological methods to quantify the DM or the DIMM since no absolute measures were required and because of possible problems with the assumptions underlying the calculations (Eisenberg 1983). Instead, we chose a more straightforward and simple method. To quantify the DM, the perimeter of each mitochondrion (or parts of it) in the pictures was manually marked out (magnification of ×6300). These areas were determined by the program and totalled for all mitochondria on a picture. This value was divided by the total area of the picture to obtain the area DM. We used the mean of the different pictures as a measure of the DM of an individual.

The DIMM was determined by drawing two different transect lines (2.56 cm) through the mitochondria (magnification of ×20 000). The two lines did not overlap and were oriented perpendicular to each other, with one line oriented vertically and the other horizontally with regard to the observer's viewpoint. Thus, the orientation of the lines was random with respect to the orientation of the specimen and the mitochondria. The inner mitochondrial membranes along the transect line were visualized as peaks (procedure 'plot profile') that could be easily counted. The validity of this procedure was confirmed by direct counts on the picture. We calculated the mean number of membranes per micrometre as a measure of DIMM. Owing to the use of a number code (instead of the original colour marking), the identity of a female was not known during the determination of the parameters of the mitochondria.

All variables were normally distributed or could be adequately transformed to normality (checked by one-sample Kolmogoroff–Smirnov tests: $p > 0.05$ in all cases). Data are represented as the mean ± s.d. We used Pearson correlation analyses to assess the effect of hunting and provisioning activity on lifespan and on the females' total success in the control group, and to examine the association of DM as well as DIMM among different subunits of muscles of individuals. If the same dataset was subjected to several tests, either the $p$-levels of the individual tests were corrected using the sequential Bonferroni technique or the global significance of the correlation matrix was assessed using Fisher's method of combining probabilities (Legendre & Legendre 1998). We analysed the effect of the independent variables age, fresh mass, fat content, DM and DIMM on the number of bees in a brood cell by stepwise multiple regression analysis. We checked for collinearity among independent variables that could affect the multiple regression analysis by examination of the tolerance values (Norussis 1993). Calculations were conducted using SPSS 10.0 (SPSS Inc.).

## 3. RESULTS

In the control females, which were kept individually, the rate of bee hunting was significantly correlated with the mean number of bees per brood cell ($r = 0.64$, $n = 21$, $p = 0.002$; with Bonferroni correction $p = 0.006$). Furthermore, the lifetime hunting success was weakly correlated with the mean number of bees provisioned per brood cell ($r = 0.46$, $n = 21$, $p = 0.035$; no more significant with sequential Bonferroni correction $p = 0.07$). Lifespan was not significantly correlated with the mean number of bees per brood cell ($r = -0.003$, $n = 21$, $p = 0.99$). Thus, consistent with earlier results (Strohm & Linsenmair 1997*a*; Strohm & Marliani 2002), there was no effect of hunting and provisioning activity on the females' lifespan. Furthermore, the number of bees in a brood cell has a substantial effect on survival of offspring of both sexes (e.g. males that received two bees are twice as likely to survive until emergence as males that received only one bee; Strohm & Linsenmair (2000)). The reproductive success of adult daughters also increases with the amount of larval provisions (Strohm & Linsenmair 1997*a*). Thus, the number of bees in a brood cell indicates a female's hunting success as well as the survival probability and success of her progeny. Therefore, we consider the number of bees provisioned per brood cell as a valid measure of the reproductive success of beewolf females.

In the group of females whose flight muscles were examined, the independent variables (age, fat content, fresh mass, DM and DIMM) were not correlated with each other (tolerance > 0.947 in all cases). Thus, the multiple regression analysis is not affected by collinearity among variables. Age (16.5 ± 5.5 days), fresh mass (103.5 ± 30.3 mg), fat content (in the abdomen,

Table 1. Results of the stepwise multiple regression analysis with the number of bees per brood cell as the dependent variable and age, fresh mass, fat content, DM and DIMM as the independent variables. Given are $R^2$ for the variable included in the model as well as the standardized regression coefficient β, $t$ and the probability $p$ ($n = 32$).

| independent variables | $R^2$ | $\beta$ | $t$ | $p$ |
|---|---|---|---|---|
| DIMM | 0.18 | 0.43 | 2.5 | 0.02 |
| not included in final model: | | | | |
| age | | 0.1 | 0.56 | 0.58 |
| fresh mass | | 0.24 | 1.4 | 0.17 |
| fat content | | −0.28 | 1.6 | 0.12 |
| DM | | 0.04 | 0.2 | 0.83 |

Table 2. Correlation matrix for the association between pairs of different muscle subunits of an individual. For DIMM the results are shown above the diagonal, for DM below the diagonal. For each pair of variables first the correlation coefficient $r$ and second the probability $p$ are given. Sample size $n = 32$. A global test of significance of the correlation matrix (Legendre & Legendre 1998) is highly significant both for DM ($\chi^2 = 50.0$, d.f. = 20, $p < 0.001$) and for DIMM ($\chi^2 = 109$, d.f. = 20, $p < 0.001$).

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | | 0.47, 0.006 | 0.35, 0.046 | 0.40, 0.22 | 0.56, 0.001 |
| 2 | 0.49, 0.005 | | 0.65, < 0.001 | 0.51, 0.003 | 0.524, 0.002 |
| 3 | 0.48, 0.005 | 0.56, 0.001 | | 0.48, 0.005 | 0.45, 0.01 |
| 4 | 0.19, 0.33 | 0.08, 0.69 | 0.49, 0.10 | | 0.68, < 0.001 |
| 5 | 0.12, 0.58 | 0.09, 0.69 | 0.17, 0.44 | 0.23, 0.30 | |

16.4 ± 8.9%) and DM (15.7 ± 7.1%) were not included in the final model of the stepwise multiple regression (table 1). Thus, these factors had no significant effect on the number of bees provisioned per brood cell (2.03 ± 0.6 bees). However, there was a significant effect of DIMM (mean of 66.7 ± 8.3 membranes $\mu m^{-1}$) on the number of bees per brood cell (see figure 1). Forward and backward elimination of variables yielded the same result. DIMM varied moderately across the five muscle subunits of individuals (coefficient of variation = 0.10), whereas DM varied considerably (coefficient of variation = 0.46). Nevertheless, both the DMs and, in particular, the DIMMs of the five subunits were significantly correlated with each other (table 2). Thus, both DM and DIMM were similar in different subunits of the dorsal longitudinal flight muscles of beewolf females.

## 4. DISCUSSION

### (a) *Why is DIMM associated with reproductive success?*

Our results suggest that the ultrastructure of the mitochondria shows some meaningful variation: the DIMM correlated with the reproductive success of females of the European beewolf. This association might be either correlative or causal. A correlative association might result from an unknown underlying factor that represents an individual's overall quality and that has an analogous effect on both DIMM and reproductive success. In vertebrates, the ultrastructure of muscles (proportion of type I and type II muscle fibres, density of muscle mitochondria) changes considerably with exercise (e.g. Fridén *et al.* 1984; Howald *et al.* 1985). Accordingly, a beewolf female that is able to bring in many prey items per unit time as a result of unknown 'quality' effects might show higher DIMM owing to the training effect. There are no studies, to our knowledge, on the effect of training on the fine structure of muscles in insects. Thus, this explanation is difficult to evaluate but it cannot be ruled out.

Impairment of the function of mitochondria can be caused by mutations, infestation with pathogens or parasites, and ageing. First, mitochondria have their own genome (mt-DNA), which codes for a part of the mitochondrial proteins. The replication of the mt-DNA takes place at the inner mitochondrial membranes close to the site of synthesis of ATP. Owing to the concomitant generation of highly reactive oxygen species, mutations occur at a higher rate and are less efficiently repaired than in the nuclear DNA (Miquel 1998; Naviaux & McGowan 2000). Mutations of mt-DNA cause several diseases that result in a disorder of the ultrastructure of mitochondria and decreased performance (e.g. Wallace 2000; Larsson & Oldfors 2001; Suomalainen & Kaukonen 2001). McKenzie *et al.* (2002) have recently shown that such mutations accumulate during life and eventually cause a degradation of the function of the mitochondria and of the affected tissue. Second, pathogens and parasites have been shown to affect the organization of the inner membranes of mitochondria (Liu 1990). Third, ageing causes an increasing disruption of the inner mitochondrial membranes and a reduction in the functioning of the respiratory chain (e.g. Davies 1974; Collatz & Sohal 1986; Miquel 1998). Thus, degradation of the structure of mitochondria generally causes a loss of performance of the whole organism.

Furthermore, examination of different tissues and comparative studies of mammals and reptiles suggest that the level of oxidative metabolism depends on DIMM (Else & Hurlbert 1981; Eisenberg 1983). Accordingly, within a species, individuals with a higher DIMM might have a higher rate of ATP production and, as a result, an increased reproductive performance. In an ecological con-

text, this could also have negative consequences owing to increased energy consumption (Mueller & Diamond 2001). In beewolves, however, such an elevated demand for fuel might not generate problems since females feed on the nectar in the crop of the paralysed honeybees (Rathmayer 1962). Thus, based on current knowledge, a causal relationship mediated by the level of ATP production provides a plausible explanation for the association between DIMM and reproductive performance.

### (b) *Why does DIMM vary between individuals?*

Assuming that mitochondria are important for reproductive success, their formation should be strongly canalized (Stearns & Kawecki 1994; Debat & David 2001). Environmental effects, developmental errors and mutations might, nevertheless, generate variation (Debat & David 2001). First, as with many other traits (Schlichting & Pigliucci 1998), environmental conditions (quantity and quality of larval provisions, temperature, pathogens; Strohm 2000; Strohm & Linsenmair 2000, 2001) might influence growth and development of beewolf females and affect the ultrastructure of their mitochondria (e.g. Munn 1974). We tentatively suggest that differences in the availability of nutrients and trace elements are a major cause of the variation in DIMM among beewolf females. Thus, we predict that experimental manipulation of the quality and quantity of provisions should affect DIMM.

Second, the environmental factors mentioned previously might not operate directly but might cause developmental stress and affect the formation of certain structures (Debat & David 2001). The biogenesis of mitochondria is rather complex; for example, the nuclear and the mitochondrial genome are involved and have to be coordinated (Beattie 1976; Scheller *et al.* 2000). However, immaterial factors such as temperature seem less likely to affect the formation of mitochondria unless conditions become extreme. Since this was not the case in the study population, we do not consider developmental stress to be a major cause of the variation in DIMM in beewolf females.

Third, there is evidence that some ultrastructural characteristics of muscles are heritable (e.g. Simoneau & Bouchard 1995; Suwa *et al.* 1998). Likewise, DIMM might be influenced by the mitochondrial genome. As mentioned previously, mt-DNA shows a higher rate of mutations than nuclear DNA (Miquel 1991, 1998). This might lead to an accumulation of mutations (McKenzie *et al.* 2002) and different levels of heteroplasmy (i.e. the occurrence of both mutant and wild-type clones of mitochondria in a tissue; Brown *et al.* 1979; Clayton 1982; Palmer *et al.* 2000). Thus, lower mean DIMM and lower reproductive success might be the result of the mutation load of the mitochondria. This implies that DIMM should show some heritability.

## 5. CONCLUSION

Our results support the hypothesis that differences in the organization of complex structures between individuals might affect phenotypic performance and reproductive success. Thus, the ultrastructures of important tissues and organelles might be target traits of natural selection (Clutton-Brock 1988). Knowledge of the environmental and genetic causes of the variation of these traits would contribute to understanding how genotype and environment interact to build up a certain phenotype (Schlichting & Pigliucci 1998).

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