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# Morphological differences in the ovary of Libellulidae (Odonata)

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## Abstract

All female Odonata have been assumed to produce oocytes continuously during their mature life span. However, a recent study of ovariole orientation and development led to the suggestion that Libellulidae are divided into two groups of species, one with continuous, the other with stepwise oocyte production. To find more evidence of this division, we compared the size variation and growth within the vitellarium of the ovary, studying oocytes, and follicle cells. We found that morphological characters discriminate between the two ovary types in eight of the 10 investigated species. In both types we found an increase in all measurements from the anterior to the posterior end of the vitellarium. The increase in oocyte width and follicle cell length was significantly higher in species with a continuous oocyte production. We also noted that follicle cells may have more than one nucleus and that their number can vary during vitellogenesis. Our study confirmed the hypotheses that two different ovary types exist in Libellulidae. The two species not fitting into this grouping could be an artefact of small sample size due to intraspecific phenotypic plasticity, or else there might be more than two ovary groups, or even a continuum. We could not offer an explanation as to how the process of stepwise oocyte production differs from continuous based production on morphological characters.

## Introduction

There have been numerous studies on lifetime reproductive success in recent decades, ranging across many animal groups which have served as model organisms, e.g. primates (reviewed by Ellis 1995), fish (Barbosa & Magurran 2006) and insects (Jarvis & Ferns 2004). Lifetime reproductive success is the product of reproductive lifespan, fecundity, and offspring survival of an individual (Clutton-Brock 1988; but see also Brommer et al. 2002). In insects, female lifetime reproductive success is determined by several parameters, e.g. lifespan, mortality rate, dispersal rate, age at first oviposition, lifetime number of ovipositions, clutch size, fecundity and interclutch interval amongst others. In Odonata, lifetime

mating success has been used to estimate lifetime reproductive success (for Zygoptera: Fincke 1982, 1986, 1988; Banks & Thompson 1985; Hafernik & Garrison 1986; Bennett & Mill 1995; for Anisoptera: Koenig & Albano 1987; Michiel & Dhondt 1991; Kasuya et al. 1997). However, lifetime mating success cannot be translated directly into lifetime reproductive success in Odonata as each observed mating would then be equated with an oviposition bout. As libellulid clutch size is very variable (e.g. Sahlén & Suhling 2002) simply multiplying the average lifetime number of ovipositions by the average clutch size might yield a strongly over- or underestimated value. In addition, what happens in the female's body during the interclutch interval is not well known. Although the interclutch interval often corresponds to the time required for maturation of the next clutch of eggs, this is not necessarily the case (Corbet 1999: 38-40). High clutch sizes may not necessarily represent the number of eggs laid in one bout – they may result from females having been unable to oviposit, while follicles continuing to mature in their ovaries (Corbet 1999: 38-40). Hence, studying the oogenesis and clutch formation in Odonata might cast new light on the mechanisms regulating their lifetime reproductive success.

Ovaries of Odonata consist of numerous separate oocyte strings (ovarioles). These are longitudinally ordered, moniliform, and can be subdivided into three sections: terminal end-filament, germinal area, and vitellarium area (Tillyard 1917). The vitellarium area constitutes the major part of the ovary (Tillyard 1917). It is known that in the vitellarium area, the volume of oocyte plasma increases significantly (Büning 1994) as the oocytes grow, reaching mature egg size. Consequently, both, maturing oocytes and the follicle cells (nurse cells) covering their surface will change their size and/or shape when they pass from the anterior to the posterior end of the vitellarium area.

So far all odonates have been assumed to have a continuous oocyte production during their whole mature life span, with egg-laying periods interspersed with resting (i.e. eating) periods away from the oviposition sites (e.g. Thompson 1990; Corbet 1999: 37-38). However, looking at ovariole organisation and oocyte development within the ovaries Karlsson et al. (2009) proposed a division into species with (1) a continuous vs (2) a stepwise egg production in Libellulidae. The authors found that in ovary type 1, which occurred in five species studied, namely *Crocothemis erythraea* (Brullé), *Diplacodes lefebvrii* (Rambur), *Leucorrhinia dubia* (Vander Linden), *Pantala flavescens* (Fabricius), and *Trithemis kirbyi ardens* Gerstäcker, there was a gradual maturation process of the oocytes within the ovarioles with only ca 15-25% of them containing mature oocytes at any time. These mature oocytes were found towards the outer perimeter of the female body with the less mature oocytes in ovarioles on their inside. The youngest, most immature, oocytes were found in ovarioles at the centre of the female body close to the rectum. They (Karlsson et al. 2009) assumed that this arrangement makes it possible for the species to have a more or less continuous egg production as the oocytes in the ovarioles mature in succession, which enables the females to lay a few mature eggs at all times, thus reducing the length of any interclutch intervals. By contrast, they (Karlsson et al. 2009) found that in ovary type 2, occurring in 10 species, all developing oocytes were more or less equal in size, maturing at the same time. The ovaries of an egg-laying female are thus filled with ready-to-lay oocytes, sometimes – but not always – separated from one another by connective tissue (Karlsson et al. 2009). This group encompasses species which seem to be adapted to deposit a large number of eggs during a short time span, namely *Diplacodes luminans* (Karsch), *Libellula depressa* Linnaeus, *Orthetrum brachiale* (P. de Beauvois), *O. julia* Kirby, *Tramea basilaris* (P. de Beauvois), *Urothemis edwardsii* (Selys), *Sympetrum fonscolombii* (Selys),

*S. danae* (Sulzer), *S. frequens* (Selys), and *S. vulgatum* (Linnaeus). As no immature ovarioles were found along with mature ones, Karlsson et al. (2009) assumed that these species should lay one or more egg clutches, after which a period (interclutch interval) of ovariole regrowth should follow.

The aim of this study was to find more evidence that libellulids can be separated into distinct groups of species with continuous and stepwise oocyte production. We focused on the oocytes and the follicle cells within the vitellarium area and investigated (1) whether oocyte and follicle cell characters differ with different ovary types and (2) whether follicle cell characters change from the anterior to the posterior end of the vitellarium area.

## Methods

### Measurements and calculations

Females of 10 species of Libellulidae were used. Four of them, *Crocothemis erythraea*, *Leucorrhinia dubia*, *Pantala flavescens*, and *Trithemis kirbyi*, have ovaries with continuous oocyte production while three, *Libellula depressa*, *Sympetrum fonscolombii*, and *S. vulgatum*, have ovaries with stepwise oocyte production (Karlsson et al. 2009). From three further species we had no prior information of the ovary type: *Orthetrum cancellatum* (Linnaeus), *S. sanguineum* (O.F. Müller), and *T. stictica* (Burmeister). Females were captured immediately after copulation and stored in 70% alcohol.

We cut three sectors of the vitellarium area of the ovaries of each individual: the anterior end (sector 1); part of the middle section (sector 2) and the posterior end (sector 3) (Fig 1). We took the sectors through a normal dehydration protocol with 80-99.5% alcohol and xylene for paraffin sectioning. Microtome sections of 6 µm thickness were cut and stained with haematoxylin. For all measurements we used the Easy Image™ analysis program from Bergström Instruments AB, Lund, Sweden, in combination with a Nikon Labophot 2 microscope and a Sony Exwave-HAD-Digital camera. Only mature oocytes with a visible eggshell (vitelline envelope and chorion developing) were measured. The oocytes were studied at a magnification of 400x and the follicle cells at 1,000x, which in the image analysis program gives a resolution better than 0.1 µm.

First we examined the oocyte surface and the follicle cells as seen externally in order to obtain an overview of oocyte and follicle cell shape. Then, mean oocyte length (OL) and width (OW) per female were calculated by measuring four to 10 oocytes per sector from each female. From each oocyte between three and 10 follicle cells were measured, all of them close to the oocyte equator. Mean follicle cell length was given as an average of inside (the side of the cell directed towards the egg centre; L1) and outside (L2) length. To estimate the number of follicle cells per oocyte (FN), the following approximation formula for ellipse perimeter was used: $FN = \pi * (1.5 * [a+b] - \sqrt{a*b}) / (L1+L2/2)$, with $a = 0.5 *$ OL and $b = 0.5 *$ OW, assuming the oocytes have the same ellipsoid shape as the eggs (e.g. Sahlén 1994, 1995). Additionally, we measured follicle cell thickness (FT).

### Statistical analysis

To investigate whether the morphological characters differed between the two ovary types, we conducted two discriminant analyses, the first one with ovary type and the second one with species as grouping variables. After studying the oocyte sections we made the

assumption that *T. stictica* belongs to the group with a continuous oocyte production whereas *O. cancellatum* and *S. sanguineum* belong to the group with stepwise oocyte production. Subsequently, we used regression analyses for each species separately to see if and how the morphological characters varied from the anterior to the posterior end of the vitellarium area (sector 1 to sector 3) (cf. Fig. 1) within the two ovary types, using sector as independent variable and the morphological characters as dependent. Owing to the results of the discriminant analysis and the small sample size we did no regression analyses for *O. cancellatum*, *S. sanguineum*, and *T. stictica*. To test for further differences between the ovary types, we used Mann Whitney *U*-tests with the morphological characters as independent variable and the slopes derived from the regression analyses above from all seven species as the response variables. All analyses were performed in SPSS version 15.0.

## Results

Oocytes within the vitellarium area had an ellipsoid shape and the follicle cells, when viewed from the outside, seemed to have a more or less balanced hexagonal shape (Fig. 2). Follicle cells often contained more than one nucleus. In cross section the shape of the follicle cells were variable, from quadrate and trapezoid to triangular; the shorter side of the cells often alternating between the inside and the outside when several adjacent cells were observed.

The discriminant analysis with ovary type as grouping variable confirmed that 97.9% ($n = 95$) of all oocytes in the ovary type 1 and 84.9% ($n = 93$) of all oocytes in the ovary type 2 were classified in the predicted group. In the second discriminant analysis with species as grouping variable it turned out that only 60% of the *Orthetrum cancellatum* and *Sympetrum sanguineum* were correctly classified compared to 90.9% of *Leucorrhinia dubia* and 91.8% of *Trithemis kirbyi*. All other species were 100% correctly classified. In conclusion, in both discriminant analyses 91.5% of all oocyte samples were correctly classified.

We found that all morphological characters (Table 1) differed significantly between the two ovary types. Within both ovary types all morphological characters were strongly correlated with the sectors in the vitellarium (Table 2). OL, OW, FL, and FT increased in size

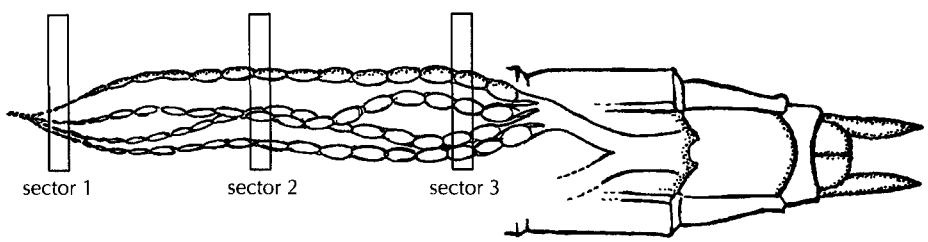


Figure 1: A panoistic dragonfly ovary — schematic drawing of *Sympetrum vulgatum*, showing the three sectors; the anterior end (sector 1), the middle section (sector 2), and the posterior end (sector 3) of the vitellarium area, which were sectioned and analysed (cf. Methods). For simplicity, only one ovary with four ovarioles is depicted; the ovariole length shortened by 50%.

Table 1. Five morphological characters of 10 libellulid species — descriptive statistics (mean ± s.d.) of oocyte length (OL) and width (OW), follicle cell length (FL) and thickness (FT), and calculated number of follicle cells per oocyte (FN). Type 1: *Crocothemis erythraea* = Ce, *Leucorrinia dubia* = Ld, *Pantala flavescens* = Pf, *T. kirbyi* = Tk; type 2: *Libellula depressa* = Lde, *Sympetrum fonscolombii* = Sf, *S. vulgatum* = Sv; type 1?: *T. stictica* = Ts; type 2?: *Orthetrum cancellatum* = Oc, *Sympetrum sanguineum* = Ss). Two to seven females (*n*F) were used and 13 to 187 oocytes examined (*n*O).

| **Ovary tpye** | | ***n*F** | ***n*O** | **Oocyte characters** | | **Follicle cell characters** | |
|---|---|---|---|---|---|---|---|
| | Species | | | | Mean ± s.d. | | Mean ± s.d. |
| 1 | Ce | 4 | 34 | OL (µm) | 234.67 ± 51.58 | FL (µm) | 7.31 ± 0.64 |
| | | | | OW (µm) | 112.56 ± 21.71 | FT (µm) | 7.15 ± 1.54 |
| | | | | | | FN | 142.93 ± 24.89 |
| | Ld | 5 | 60 | OL (µm) | 174.67 ± 90.60 | FL (µm) | 9.68 ± 1.16 |
| | | | | OW (µm) | 98.68 ± 18.88 | FT (µm) | 10.95 ± 1.95 |
| | | | | | | FN | 110.34 ± 18.43 |
| | Pf | 6 | 72 | OL (µm) | 188.88 ± 27.99 | FL (µm) | 7.41 ± 2.93 |
| | | | | OW (µm) | 86.02 ± 22.18 | FT (µm) | 8.14 ± 1.23 |
| | | | | | | FN | 128.23 ± 24.45 |
| | Tk | 6 | 187 | OL (µm) | 229.28 ± 18.91 | FL (µm) | 10.12 ± 1.41 |
| | | | | OW (µm) | 94.03 ± 14.12 | FT (µm) | 7.35 ± 0.50 |
| | | | | | | FN | 107.89 ± 12.80 |
| 2 | Lde | 7 | 136 | OL (µm) | 341.21 ± 40.65 | FL (µm) | 12.39 ± 2.46 |
| | | | | OW (µm) | 170.97 ± 34.15 | FT (µm) | 15.81 ± 3.55 |
| | | | | | | FN | 141.55 ± 36.39 |
| | Sf | 6 | 148 | OL (µm) | 309.94 ± 21.85 | FL (µm) | 13.58 ± 2.24 |
| | | | | OW (µm) | 137.91 ± 7.44 | FT (µm) | 9.85 ± 0.40 |
| | | | | | | FN | 112.09 ± 25.56 |
| | Sv | 5 | 31 | OL (µm) | 314.04 ± 40.44 | FL (µm) | 9.56 ± 0.98 |
| | | | | OW (µm) | 146.30 ± 23.44 | FT (µm) | 8.95 ± 1.44 |
| | | | | | | FN | 163.60 ± 14.21 |
| 1? | Ts | 2 | 16 | OL (µm) | 245.78 ± 13.56 | FL (µm) | 9.06 ± 1.24 |
| | | | | OW (µm) | 109.37 ± 18.72 | FT (µm) | 6.22 ± 0.16 |
| | | | | | | FN | 138.54 ± 37.28 |
| 2? | Oc | 6 | 74 | OL (µm) | 267.47 ± 54.08 | FL (µm) | 10.08 ± 1.55 |
| | | | | OW (µm) | 124.56 ± 9.62 | FT (µm) | 8.46 ± 0.76 |
| | | | | | | FN | 127.55 ± 16.73 |
| | Ss | 4 | 13 | OL (µm) | 172.69 ± 30.56 | FL (µm) | 4.76 ± 1.33 |
| | | | | OW (µm) | 70.23 ± 27.98 | FT (µm) | 8.21 ± 1.45 |
| | | | | | | FN | 152.94 ± 15.73 |

and mean FN increased when going from the anterior to the posterior end of the vitellarium area (Table 2). The *U*-tests showed that the slopes derived from the regression analyses were significantly higher for OW ($Z = -2.12$, $p = 0.034$) and FL ($Z = -2.12$, $p = 0.034$) in species with a continuous oocyte production. The OL ($Z = -0.71$, $p = 0.480$), FT ($Z = -1.77$, $p = 0.077$), and FN ($Z = -1.05$, $p = 0.289$) did not differ between the ovary types.

## Discussion

In all species oocytes had the elliptic shape typical of eggs after oviposition (e.g. Ando 1962) already at the anterior end of the vitellarium. However, newly laid eggs, at least in some of the species, are bigger than the oocytes still in the vitellarium (cf. Koch & Suhling 2005; Schenk & Söndgerath 2005). This size difference might at least be partly due to the uptake of water causing an increase in volume which takes place in all insect eggs laid in a wet or damp environment (e.g. Chapman 2004).

All five morphological characters clearly discriminated between the two ovary types in eight of the 10 investigated species. Within both groups the size or number of the characters increased from the anterior to the posterior end of the vitellarium. The resulting regression slopes for oocyte width and follicle cell length were higher in species with a continuous oocyte production. In those species the youngest oocytes should be in the anterior part of the ovaries, so the observation is in line with the mechanisms suggested by Karlsson et al. (2009). But we also expected that the size of the follicle cells should increase during the maturation of the oocytes, based on the assumption that the number of follicle cells is more or less constant during oogenesis (Büning 1994) and that they flatten themselves during oogenesis. But instead we found that their thickness increased, implying a high activity in synthesis of vitellin etc. in these cells, also in the posterior part of the ovaries. Further, we observed that the number of follicle cells increased slightly during oogenesis. This increase in numbers can, naturally, be an artefact caused by us calculating

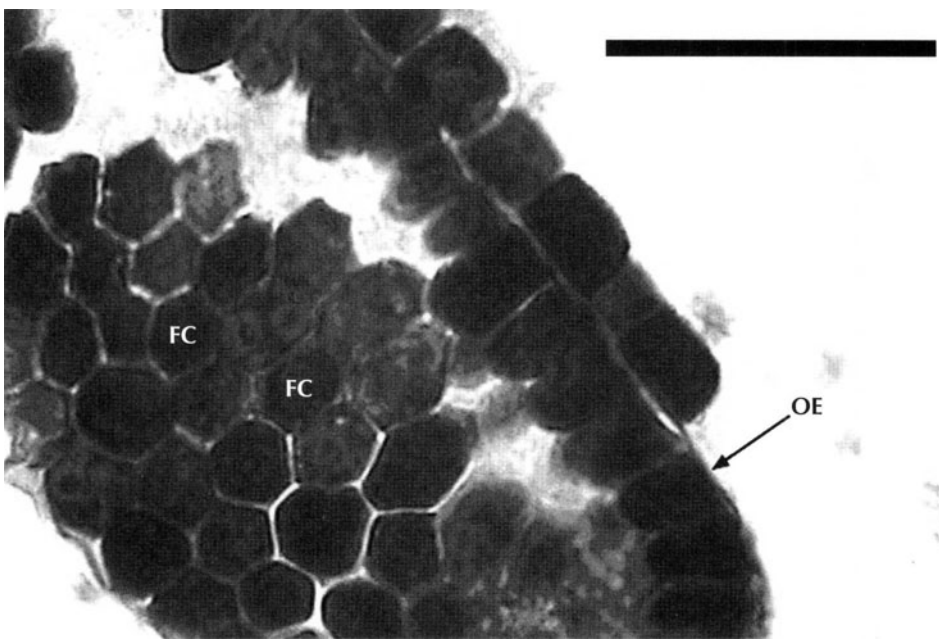


Figure 2: Surface of an oocyte of *Trithemis kirbyi* — light microscope image with several polygonal follicle cells (FC) within the vitellarium area (sector 3). OE: outer margin of the follicle cell clad oocyte; cells on the outside belong to an oocyte in the next ovariole which has been removed during dissection. Scale bar 40 µ.

Table 2. Morphological characters of seven libellulids correlated with sector in the vitellarium — regression analysis for the three sectors within the vitellarium area against the five different morphological characters. For abbreviations, see captions of Table 1. The results of all tests are highly significant (all $p < 0.0001$).

| Ovary tpye | Species | Morphological character | $R^2$ | slope |
|---|---|---|---|---|
| 1 | Ce | OL | 0.71 | 4.3 |
| | | OW | 0.62 | 55.51 |
| | | FL | 0.61 | 2.37 |
| | | FT | 0.74 | 35.03 |
| | | FN | 0.70 | 4.61 |
| | Ld | OL | 0.83 | 80.74 |
| | | OW | 0.78 | 36.84 |
| | | FL | 0.81 | 57.68 |
| | | FT | 0.86 | 7.28 |
| | | FN | 0.85 | 50.23 |
| | Pf | OL | 0.84 | 74.36 |
| | | OW | 0.83 | 35.58 |
| | | FL | 0.76 | 7.35 |
| | | FT | 0.86 | 43.99 |
| | | FN | 0.88 | 3.44 |
| | Tk | OL | 0.86 | 121.36 |
| | | OW | 0.82 | 58.93 |
| | | FL | 0.82 | 3.67 |
| | | FT | 0.86 | 3.06 |
| | | FN | 0.83 | 97.43 |
| 2 | Lde | OL | 0.77 | 78.42 |
| | | OW | 0.68 | 41.42 |
| | | FL | 0.76 | 4.08 |
| | | FT | 0.81 | 6.76 |
| | | FN | 0.76 | 144.44 |
| | Sf | OL | 0.82 | 64.68 |
| | | OW | 0.81 | 3.83 |
| | | FL | 0.85 | 3.26 |
| | | FT | 0.85 | 4.22 |
| | | FN | 0.77 | 53.38 |
| | Sv | OL | 0.82 | 56.68 |
| | | OW | 0.76 | 5.25 |
| | | FL | 0.85 | 59.98 |
| | | FT | 0.88 | 44.51 |
| | | FN | 0.77 | 39.61 |

and not counting the numbers. The number of follicle cells around a developing oocyte has been studied in detail in *Drosophila* (Koch & King 1966). They found that the number of follicle cells around an oocyte started with ca 80 and increased to around 1,200 before yolk production started. In the vitellarium area the cells did not increase in numbers and

only flattened out and stretched laterally (Koch & King 1966). In Odonata, Ando (1962: 12-13) reported mitosis in the follicle cells of *Sieboldius albardae* (Selys), not however commenting on this further. It thus seems as if follicle cells in the panoistic ovarioles of the Odonata can also divide during vitellogenesis.

Returning to the second ovary type, which was interpreted by Karlsson et al. (2009) as species with a stepwise oocyte production, we expected other patterns. Here oocyte size should be more uniform throughout the vitellarium area, mirroring a stepwise egg production where most of the oocytes in the ovaries should be mature at the same time as suggested by Karlsson et al. (2009). But we found that also these species had increasingly bigger oocytes towards the posterior part of the abdomen. However, their oocyte width and follicle cell length increased less than in the first ovary type.

We suggest that females with ovaries type 2 might lay their eggs only when all oocytes are mature. After egg-laying they would leave the water and go into a clutch interval with ovariole regrowth (cf. Büning 1994). During such a period of regeneration the form of the oocytes within the ovaries might have a similar to those of type 1 (above). We studied a limited number of females of each species and might not yet be able to see the whole picture, but as we caught all females at water sites it does not seem likely that all specimens were within their interclutch period with regenerating ovaries. The similar size of the oocytes (and eggs; Schenk & Söndgerath 2005) of type 2 must therefore have another explanation. Follicle cells with more than one nucleus are known from *Sieboldius*, *Ictinogomphus*, and from early stages of oocyte development in *Tanypteryx*, but not in *Anotogaster* (Ando 1962: 14-15). This character seems to vary between genera and might have a phylogenetic basis. In all our studied Libellulidae, however, more than one nucleus was found.

With the current data we can support the hypothesis of two different oocyte maturation processes (Karlsson et al. 2009). However, so far it is unclear why two species of the 10 investigated did not fit into this grouping. Interestingly, both species were assumed to belong to the group with stepwise oocyte production. Within this group Karlsson et al. (2009) found two subgroups of species. Perhaps we cannot yet see the whole complex picture of the oocyte production in Libellulidae, and there might be more than two ovary types or even a continuum. Therefore, a study including more species is necessary. It might also be possible that there are only these two ovary types, but with some species exhibiting a high phenotypic plasticity.

In our study the number of specimens examined per species was rather low. Measuring only mature oocytes might also obscure the picture, as the ratio of mature to immature oocytes per sector per female could give additional information. Thus, to get a better picture of the two oocyte types we recommend measuring actual oocyte production rates and interclutch intervals on individual living females under semi-controlled conditions. In this way a measurement of lifetime egg production could be obtained. This, combined with phylogenetic studies might provide an answer to whether Libellulidae can be divided into clades containing species with continuous and stepwise oocyte production, thus revealing patterns of evolution of ovarian dynamics within the family.

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