

Evolution of reproductive strategies in libellulid dragonflies (Odonata: Anisoptera)

Jessica Ware · Maria Karlsson · Göran Sahlén ·
Kamilla Koch

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Abstract In Libellulidae, oocyte production has been assumed to be continuous, with periods of egg-laying interspersed with periods of resting/eating; however, recent work suggests that two types of oocyte production are common: either (a) continuous or (b) step-wise. These are mirrored in the arrangement of the ovarioles in the ovaries. Likewise, two types of mate-guarding behavior have been observed in Libellulidae: (1) non-contact guarding and (2) tandem guarding in which the male either hovers above the female or is physically attached to her during oviposition. Using molecular (mitochondrial and nuclear) data we explored the evolution of female reproductive traits, focusing on ovariole

morphology, as well as guarding behavior, in Libellulidae. Continuous egg production appears to have evolved more than once, as have tandem and non-contact guarding. We discuss how the evolution of different ovariole types and guarding behavior may have been influenced by habitat instability, dispersal and crowded oviposition sites; thus, migratory behavior or habitat availability may have been the driving force of ovariole evolution.

Keywords Ovary type · Mate guarding · Outgroup selection · Phylogeny · Bayesian analyses · Trait correlation

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J. Ware (✉)
Department of Biology, Rutgers University,
Newark, NJ 07102, USA
e-mail: jware42@andromeda.rutgers.edu

M. Karlsson
Department of Biology, Ecology building, Lund University,
22362 Lund, Sweden

G. Sahlén
Ecology and Environmental Sciences, Halmstad University,
P.O. Box 823, 30118 Halmstad, Sweden

K. Koch
Department of Ecology, Johannes Gutenberg–University Mainz,
Becherweg 13,
55128 Mainz, Germany

J. Ware
Division of Invertebrate Zoology,
American Museum of Natural History,
79th and Central Park West,
New York, NY 10024, USA

Introduction

Understanding the evolution of reproductive systems in insects requires studies of many linked traits. From a male perspective, sperm competition is believed to play a key role during the evolution of mating systems and reproductive strategies (Danielsson 1998), as do parental investment and variance in mate quality (Bonduriansky 2001). From a female perspective, lifespan, age at first oviposition, mortality during maturation period, lifetime number of ovipositions, clutch size, egg fecundity and interclutch interval are important additional factors to consider (e.g., Arnqvist and Nilsson 2000; Fincke 1997; Koch et al. 2009; McPeck and Peckarsky 1998; Michiels and Dhondt 1991).

An insect group frequently used as a model organism for ecological and evolutionary questions are the dragonflies (Odonata) (Corbet 2004; Córdoba-Aguilar 2008). Male and female reproductive behavior differ greatly across the order, from the temporal investment in mating by males, to more time consuming egg-laying methods used by females.

There have been extensive studies of odonate reproductive behavior and morphology of genital structures (e.g., Bennett and Mill 1995; Corbet 1980; Cordero Rivera et al. 2004; Fincke et al. 1997; Matushkina 2011; Matushkina and Gorb 2001; Robertson 1982; Waage 1973, 1984) with the recognition of male sperm removal (Waage 1979) sparking a massive wave of behavioral and morphological studies (cf., below). However, in many cases the evolution of this reproductive behavior (and any corresponding morphology) has not been studied with reference to phylogenetic history. A few exceptions dealing with specific reproductive morphology highlighted against phylogeny exist, but rarely with detailed morphological evolution in mind. Some examples are the evolution of exophytic oviposition in Libelluloidea (Ware et al. 2007), which has been discussed with reference to phylogeny. Pfau examined the evolution of the *vesica spermalis* (i.e., sperm pump apparatus; Pfau 1971, 1991, 2005) in the suborder Anisoptera, and both Kennedy (1922) and Miller (1991) examined male penile morphology in detail with reference to the anisopteran phylogenetic history known at the time. Egg morphology was examined in Gomphidae (Andrew and Tembhare 1992; Sahlén 1995) and several other anisopteran taxa (Andrew et al. 2006; May 1995; Sahlén 1994a, 1994b; Trueman 1991). In general, however, our understanding of the evolution of morphology and reproductive behavior in this insect group is still poor.

Intra-ordinal odonate systematics have received a lot of attention of late, with anisopteran molecular studies in general agreement about interfamilial relationships (Bybee et al. 2008; Carle et al. 2008; Fleck et al. 2009; Letsch 2007), although some studies have suggested unique arrangements of anisopteran families (e.g., Dumont et al. 2010). The systematics of Libelluloidea, however—and especially Libellulidae—have been considered to be rather chaotic, with several subfamily level designations based on convergences and homoplasious traits (Pilgrim and von Dohlen 2008; Ware et al. 2007). Recent works by Letsch (2007), Ware et al. (2007), Pilgrim and von Dohlen (2008) and Bybee et al. (2008), however, have shown some consensus about interfamilial relationships within Libelluloidea and subfamily relationships within the family Libellulidae. With greater confidence in the arrangement of taxa within Libelluloidea, these phylogenies may be of great use when trying to understand the evolution of reproductive morphology and behavior. The evolution of male and female genitalia, egg formation, oviposition and mating habits, for example, may be better understood in a phylogenetic context.

Male dragonflies use their secondary genitalia to remove sperm from competing males and this behavior may have led to highly variable, often extremely elaborate penile structures, the homologies of which are

sometimes difficult to assess (e.g., Miller 1991; Waage 1984). Male–male competition also drives attempts to control female oviposition by mate guarding to ensure paternity (Waage 1984 and, e.g., Convey 1992; Fincke et al. 1997; Rehfeldt 1992).

Two types of mate-guarding behavior for the period of oviposition may be distinguished in the family Libellulidae: (1) non-contact guarding, with the male accompanying the ovipositing female and (2) tandem guarding, where the male is physically attached to the ovipositing female (cf., Conrad and Pritchard 1992). Note that the types of behavior within tandem guarding differ in some species where the tandem phase is short, the male leaves and the female continues to lay eggs on her own [e.g., in *Sympetrum sanguineum* (Sternberg and Buchwald 2000) and in *Libellula composita* (Beckemeyer 2004)]. We assume a certain amount of variation also exists within non-contact guarding. The size distribution of eggs within a clutch differs between guarding types (Koch and Suhling 2005; Schenk et al. 2004). Clutches laid by non-contact guarding species have egg sizes inversely proportional to laying order, whilst egg size is random in tandem guarding species (Schenk et al. 2004).

Ovaries of Odonata consist of numerous separate oocyte strings (ovarioles) (Gaino et al. 2008; Karlsson et al. 2010). These are longitudinally ordered and moniliform. In Libellulidae, oocyte production was, for a long time, assumed to be continuous, with periods of egg-laying interspersed with periods of resting/eating (e.g., Corbet 2004; Thompson 1990). Recent work examining ovary organization and oocyte development (Karlsson et al. 2010; Koch et al. 2009, 2011), however, suggests that libellulids have either (a) continuous or (b) step-wise oocyte production. In the latter type, the oocytes tend to mature in bursts, i.e., most eggs mature at the same time and are deposited, after which a long period of regrowth takes place. An understanding of the evolution of libellulid female reproduction is crucial to interpretations of the reproductive gestalt of Anisoptera, but, as mentioned above, most major phylogenetic assessments of odonate reproduction have focused on male structures, such as the penis (e.g., Kennedy 1922; Miller 1991; Pfau 2005). As natural selection should act as strongly on characteristics associated with egg-laying as on sexual selection (male–male and male–female interactions), we aim to evaluate the evolution of female reproductive traits using a phylogeny of Anisoptera mapping the relatively clear separation of non-contact versus tandem guarding and continuous versus step-wise egg production within Libellulidae on the tree. We examine the evolution of characters studies using Bayesian analyses and ancestral state reconstruction, searching for correlations between mate guarding and ovariole type. We believe that the divergence of these traits may have evolved in connection with major environmental events in the past and thus discuss

previous fossil and molecular estimates for the taxa in our phylogeny in order to possibly fit them into the context of geological history.

Materials and methods

Taxon sampling

We included sequences of 41 anisopteran specimens, newly extracted from GenBank (Table 1). Our analysis had a total of 2 aeshnid taxa, 2 gomphid taxa, 1 petalurid taxon, 1 neopetaliid taxon, 1 cordulegastrid taxon, 1 gomphomacromiinae syntemistinae idionychinae (GSI) taxon *sensu* (Ware et al. 2007), 1 macromiid taxon, 1 corduliid taxon and 23 libellulid taxa. Non-libellulid taxa served as outgroups, with the tree rooted using aeshnid taxa. We used taxa that were readily available to us, resulting in a bias towards European, North American and South African species. The non-Libellulid taxa were selected based on previous studies by JW.

Gene selection, DNA extraction and PCR amplification

We amplified the second, third and seventh hypervariable (divergent) regions (D2, D3 and D7) of the nuclear large subunit rDNA (28S), the third domain of the mitochondrial large subunit rDNA (16S) and a fragment of cytochrome oxidase one (COI). We downloaded COII, 12S, 18S, 28S and EF1- α sequences for our taxon sample. In all cases, we strove to have the most complete sequence data available for each taxon in the dataset.

Alignment

Initial sequence alignments were made using CLUSTAL-X (Thompson et al. 1997) and the resulting files were then aligned manually in Microsoft Word using the structural methods described in Kjer (1995) and Kjer et al. (2007), and secondary structure models based on Guttell et al. (1993). Ambiguously aligned regions were defined as single-stranded regions with multiple insertions and deletions (indels) of variable length (and thus unclear nucleotide homology) bounded by hydrogen-bonded base pairs. These regions were excluded from the dataset. Alignments are available upon request.

Analysis

The combined dataset was analyzed using Maximum Likelihood and Bayesian criteria. To estimate branch support 1,000 bootstrap pseudoreplicates (Felsenstein 1985) were performed.

We used MR BAYES version 3.1.2 to implement our Bayesian analyses (Huelsenbeck and Ronquist 2002). Four

Markov Chain Monte Carlo (MCMC) chains (1 cold and 3 hot) were run for 20 million generations. Each analysis had $\text{printfreq}=500$. Prior to Bayesian analyses we used JMODELTEST (Posada 2008) to select an appropriate model of evolution for each of the gene fragments. This suggested a GTR + I + Γ model (Yang 1994; Yang et al. 1994; Gu et al. 1995). We used GARLI version 0.951 with a GTR + I + Γ model for maximum likelihood analysis (bootstrap analysis was run with 1,000 pseudoreplicates). We did not run partitioned analyses in GARLI here. After the Bayesian analyses were completed, we verified that each run had stabilized using Tracer 1.4 (Rambaut and Drummond 2007), and calculated the burnin regions (i.e., we visually estimated the burnin by examining likelihood graphs to determine whether chains had converged/plateaued; in addition, we ran analyses until all effective sample size (ESS) values were over 200). Each analysis had a burnin of less than 10 % for both chains.

Morphology

Ovariole arrangement was determined as in Karlsson et al. (2010) using methods described in Koch et al. (2009) and Karlsson et al. (2010), from whose article we re-analyze part of the data. Drawings of the two ovariole arrangements are presented in Karlsson et al. (2010) and depicted schematically in Fig. 3 (see Results section).

Character mapping

We mapped guarding type (Type 1 = non-contact guarding, Type 2 = tandem guarding) and ovariole arrangement (Type a = step-wise, Type b = continuous) as derived from Schenk and Söndgerath (2005), Karlsson et al. (2010) and Koch et al. (2009) onto the consensus Bayesian topology using MESQUITE (Maddison and Maddison 2011). Behavioral and morphological observations were gathered from personal observations and literature (Table 1). For the purposes of our exploration of the evolution of ovariole arrangement, we examined the ages of Libellulidae in Ware et al. (2007; molecular) and Fleck et al. (2009; fossil), as well as other fossil publications (e.g., Grimaldi and Engel 2005). Note that we treat both ovariole arrangement and behavior as having strong genetic components based on the tentative phylogenies used in Koch et al. (2009) and Karlsson et al. (2010). We tested for a correlation between guarding type and ovariole arrangement using Pagel's correlation method in MESQUITE. Briefly, we used ovariole arrangement type = X, guarding type = Y, and tested a four and eight parameter model with 1,000 simulations. Because this test requires there to be no missing data, we used the reconstructed states estimated by MESQUITE for all taxa that were missing data.

Table 1 Taxon sample from present study in systematic (family) and alphabetical (species) order. Behavioral data (guarding type and ovariole arrangement) and GenBank numbers are listed for each species.

For some species guarding type and ovariole arrangement are unknown; missing information is listed here as a '?'. When written in italics it is known for another species within the same genus

Family	Taxon	Guarding type	Ovariole arrangement	GenBank Accession Number
Aeshnidae	<i>Aeshna canadensis</i> Walker 1908	<i>Non-contact</i> ^a	<i>Step-wise</i> ^r	EU183296, AF461230, AF461203
	<i>Anax junius</i> (Drury 1773)	<i>Tandem</i> ^b	<i>Step-wise</i> ^s	AY338676
Gomphidae	<i>Gomphus</i> sp. [species not given in GenBank]	<i>Non-contact</i> ^c	?	EU055237
	<i>Progomphus obscurus</i> (Rambur 1842)	<i>Tandem</i> ^c	?	EU477677, AY749909, AY750040
Petaluridae	<i>Phenes raptor</i> Gay 1854	?	?	EU055227
Neopetaliidae	<i>Neopetalia punctata</i> Hagen in Selys 1854	?	?	EF631563, EF631247, EF631452, EF631338, FJ596636, FJ010021
Cordulegastridae	<i>Cordulegaster erronea</i> Hagen in Selys, 1878	<i>Non-contact</i> ^d	?	EF631245, EF631450, EF631561
GSI	<i>Austrocordulia refracta</i> Theischinger, 1999	?	?	EF631243, EF631336, EF631448, EF631559
Macromiidae	<i>Macromia illinoiensis</i> Walsingham, 1862	<i>Non-contact</i> ^e	?	JN419957, FJ010027, FJ009971
Corduliidae	<i>Cordulia amurensis</i> Selys, 1887	<i>Non-contact</i> ^f	?	JQ964121, JQ964130
Libellulidae	<i>Celithemis elisa</i> (Hagen 1861)	<i>Tandem</i> ^g	?	EF631224, EF631320, EF631428, EF631541
	<i>Crocothemis erythraea</i> (Brullé, 1832)	<i>Non-contact</i> ^h	<i>Continuous</i> ^h	EF631225, EF631321, EF631429, EF631542
	<i>Crocothemis erythraea</i> (Brullé, 1832)	<i>Non-contact</i> ^h	<i>Continuous</i> ^h	JQ964118, JQ964127, JQ964135
	<i>Crocothemis sanguinolenta</i> (Burmeister, 1839)	<i>Non-contact</i> ⁱ	<i>Continuous</i> ⁱ	JQ964126
	<i>Diplacodes haematodes</i> (Burmeister, 1839)	<i>Tandem</i> ^j	?	EF631555, EF631443, EF631238
	<i>Diplacodes lefebvrei</i> (Rambur, 1842)	<i>Tandem</i> ^k	<i>Continuous</i> ^h	EF640419
	<i>Diplacodes lefebvrei</i> (Rambur, 1842)	<i>Tandem</i> ^k	<i>Continuous</i> ^h	EF640498, EF640419, FN356080
	<i>Ladona julia</i> (Uhler, 1857)	<i>Non-contact</i> ^l	<i>Step-wise</i> ^t	JN419856, EF631423, EF631315, EF631219, EF631536, AF037186
	<i>Leucorrhinia glacialis</i> Hagen, 1890	<i>Non-contact</i> ^m	<i>Continuous</i> ^u	EF631207, EF631304, EF631409, EF631523
	<i>Leucorrhinia intacta</i> (Hagen, 1861)	<i>Non-contact</i> ^m	<i>Continuous</i> ^u	EF640474, EF640396
	<i>Leucorrhinia orientalis</i> Selys, 1887	<i>Non-contact</i> ^m	<i>Continuous</i> ^u	EU477718, FJ596604
	<i>Libellula depressa</i> Linnaeus, 1758	<i>Non-contact</i> ^{h,i}	<i>Step-wise</i> ^h	JQ964136
	[<i>Plathemis d.</i> in GenBank]			
	<i>Libellula quadrimaculata</i> Linnaeus, 1758	<i>Non-contact</i> ⁱ	<i>Step-wise</i> ⁱ	EF631272, EF631363, EF631479
	<i>Libellula quadrimaculata</i> Linnaeus, 1758	<i>Non-contact</i> ⁱ	<i>Step-wise</i> ⁱ	EF631497, EF631589
	<i>Nesiothemis minor</i> Gambles, 1966	?	?	JQ964122
	<i>Orthetrum julia</i> Kirby, 1890	<i>Non-contact</i> ^h	<i>Step-wise</i> ^h	EF631285, EF631380, EF631498, EF631601
	<i>Pantala flavescens</i> (Fabricius, 1798)	<i>Tandem</i> ^h	<i>Continuous</i> ^h	EF631220, EF631316, EF631424, EF631537
	<i>Pantala flavescens</i> (Fabricius, 1798)	<i>Tandem</i> ^h	<i>Continuous</i> ^h	EF631280, EF631369, EF63148
	<i>Plathemis lydia</i> (Drury, 1773)	<i>Non-contact</i> ^l	<i>Step-wise</i> ^t	EF631234, EF631330, EF631438, EF631552
	<i>Plathemis subornata</i> (Hagen, 1861)	<i>Non-contact</i> ^l	<i>Step-wise</i> ^t	AF037185, EF640406, EF640486
	<i>Sympetrum corruptum</i> (Hagen, 1861)	<i>Tandem</i> ^{n,o}	<i>Step-wise</i> ^v	JQ964120, JQ964129
	<i>Sympetrum fonscolombii</i> (Selys, 1840)	<i>Tandem</i> ^h	<i>Step-wise</i> ^h	JQ964124, JQ964133
	[<i>S. fonscolombii</i> in GenBank]			
	<i>Sympetrum fonscolombii</i> (Selys, 1840)	<i>Tandem</i> ^h	<i>Step-wise</i> ^h	JQ964123, JQ964132
	[<i>S. fonscolombii</i> in GenBank]			
	<i>Sympetrum fonscolombii</i> (Selys, 1840)	<i>Tandem</i> ^h	<i>Step-wise</i> ^h	JQ964125, JQ964134
	[<i>S. fonscolombii</i> in GenBank]			
	<i>Sympetrum infuscatum</i> (Selys, 1883)	<i>Tandem</i> ^h	<i>Step-wise</i> ^h	AB127407, EF640442
	<i>Sympetrum infuscatum</i> (Selys, 1883)	<i>Tandem</i> ^h	<i>Step-wise</i> ^h	EF032726

Table 1 (continued)

Family	Taxon	Guarding type	Ovariole arrangement	GenBank Accession Number
	<i>Sympetrum vulgatum</i> (Linnaeus, 1758)	Tandem ^h	Step-wise ^h	JQ964131
	<i>Tholymis tillarga</i> (Fabricius, 1798)	Non-contact ^f	?	EF631202, EF631298 EF631403, EF631517
	<i>Tholymis tillarga</i> (Fabricius, 1798)	Non-contact ^f	?	JQ964119, JQ964128
	<i>Tramea lacerata</i> (Hagen, 1861)	Tandem ^a	Step-wise ^w	EF631221, EF631317, EF631425, EF631538
	<i>Trithemis arteriosa</i> (Burmeister, 1839)	Non-contact ⁱ	Continuous ^x	EF640535, EF640456, GU323109, FN356177

^a All *Aeshna* spp. oviposit in non-contact or unguarded, e.g. Sahlén (1996); ^b All *Anax* spp. are believed to oviposit in Tandem, e.g. Miller (1983); ³ All species in this family oviposit in non-contact or unguarded, e.g. Suhling & Müller (1996); ^d *Cordulegaster* females oviposit unguarded, e.g. Dunkle (2000); ^e Females oviposit unguarded in *M. splendens*, Rivera et al. (1999); ^f Females oviposit unguarded, Ubukata (1986); ^g *Celithemis* spp. oviposit in tandem, Dunkle (2000); ^h Karlsson et al. (2010); ⁱ Own observation; ^j Asian *Diplacodes* oviposit in tandem, Sakagami et al. (1974), Martens (2003); ^k *D. lefebvrei* and *D. luminans* oviposit in non-contact; Suhling & Martens (2007) and own observations; ^l Most species in *Libellula* s.lat. have non-contact or unguarded oviposition, e.g. Dunkle (2000); ^m own observations of *L. dubia* (van der Linden, 1825), *L. rubicunda* (Linnaeus, 1758) and *L. pectoralis* (Charpentier, 1825); ⁿ Dunkle (2000); ^o Females oviposit in tandem but unaccompanied at high temperatures, Ishizawa (2008); ^p Miller and Miller (1985); ^q Corbet (2004), *T. basilaris* (Beauvois, 1805) also oviposits in Tandem, Karlsson et al. (2010); ^r Own observation of *A. cyanea* Müller, 1764; ^s *A. imperator* Leach, 1815, own observation; ^t All *Libellula* s.lat. have probably step-wise oocyte production, based on own dissections of *L. depressa* and *L. quadrimaculata*; ^u Own observations of *L. dubia* (van der Linden, 1825), *L. rubicunda* (Linnaeus, 1758) and *L. pectoralis* (Charpentier, 1825); ^v Own observations of *Sympetrum fonscolombii*, *Sympetrum infuscatum* and *Sympetrum vulgatum*; ^w Own observations of *T. basilaris* (Beauvois, 1805), Karlsson et al. (2010); ^x Own observations of *T. kirbyi* (Gerstaecker, 1891), Karlsson et al. (2010).

Results

Our combined analysis included 9,901 characters; 8796 of which were constant and 705 parsimony informative.

Results from our GARLI analysis are shown in Figs 1 and 2, and our Bayesian analyses in Fig. 3, a majority rule consensus of 65,000 trees. Bootstrap support and posterior probability support are shown above the branches in Figs 2 and 3.

Topology

Our Bayesian analyses recovered a topology of Anisoptera that is in agreement with Letsch (2007), who recovered Aeshnidae as sister group to Gomphidae, Petaluridae and Libelluloidea. The support for the position of Petaluridae as a sister to Libelluloidea was high [100 % posterior probability (PP); 95 % bootstrap, B]. Within Libelluloidea, we recovered a topology that is largely congruent with analyses using larger libelluloid taxon samples (e.g., Carle and Kjer 2002; Letsch 2007; Pilgrim and von Dohlen 2008; Ware et al. 2007). *Celithemis* + *Leucorrhinia* + *Sympetrum* are weakly supported as a monophyletic group (64 % PP; 71 % B). The relationship of *Crocothemis* + *Diplacodes* is supported by 99 % PP, but less than 50 % B. Congruent with previous studies (Pilgrim and von Dohlen 2008; Ware et al. 2007) *Trithemis* + *Pantala* is recovered (73 % PP) as a sister to Libellulinae, but this is supported by <50 % B in the likelihood analysis. Libellulinae is well supported as monophyletic, with 98 % PP, although, again, the bootstrap support for this is <50 % B.

Character mapping

When we traced ovariole arrangement type onto the consensus topology in MESQUITE, the ancestral state for Anisoptera was parsimoniously reconstructed as step-wise. The ancestral state for the Libellulidae was reconstructed to be a step-wise ovariole arrangement. Continuous ovariole arrangement is present in taxa from two non-sister libellulid clades (Fig. 3), suggesting that this trait possibly evolved twice, independently. The ancestral state for guarding type was reconstructed to be non-contact (Fig. 3). Tandem guarding type evolved at least four times independently in the Anisoptera: tandem guarding is observed in some Aeshnidae, *Progomphus* (Gomphidae) and in taxa from at least three libellulid clades (Fig. 3). Within Libellulidae, the ancestral condition is tandem guarding. *Tramea* and *Tholymis* are recovered in a monophyletic clade and they are both known to have some variation from the ‘standard’ guarding types: *Tramea* uses a method of ‘interrupted tandem’ (Corbet 2004), whilst *Tholymis* employs a special type of non-contact guarding behavior where males hover closely by their ovipositing mates (Miller and Miller 1985). If the ancestral state for Libellulidae was tandem guarding, then several groups have reverted to non-contact guarding, including the Libellulinae, *Leucorrhinia*, *Crocothemis*, *Diplacodes* and *Trithemis*.

The Pagel’s 1994 Correlation test of trait correlation between guarding and ovariole type yielded a difference between likelihood scores for the eight- and four-parameter model of 0.89. After 1,000 simulations (including 272 sets

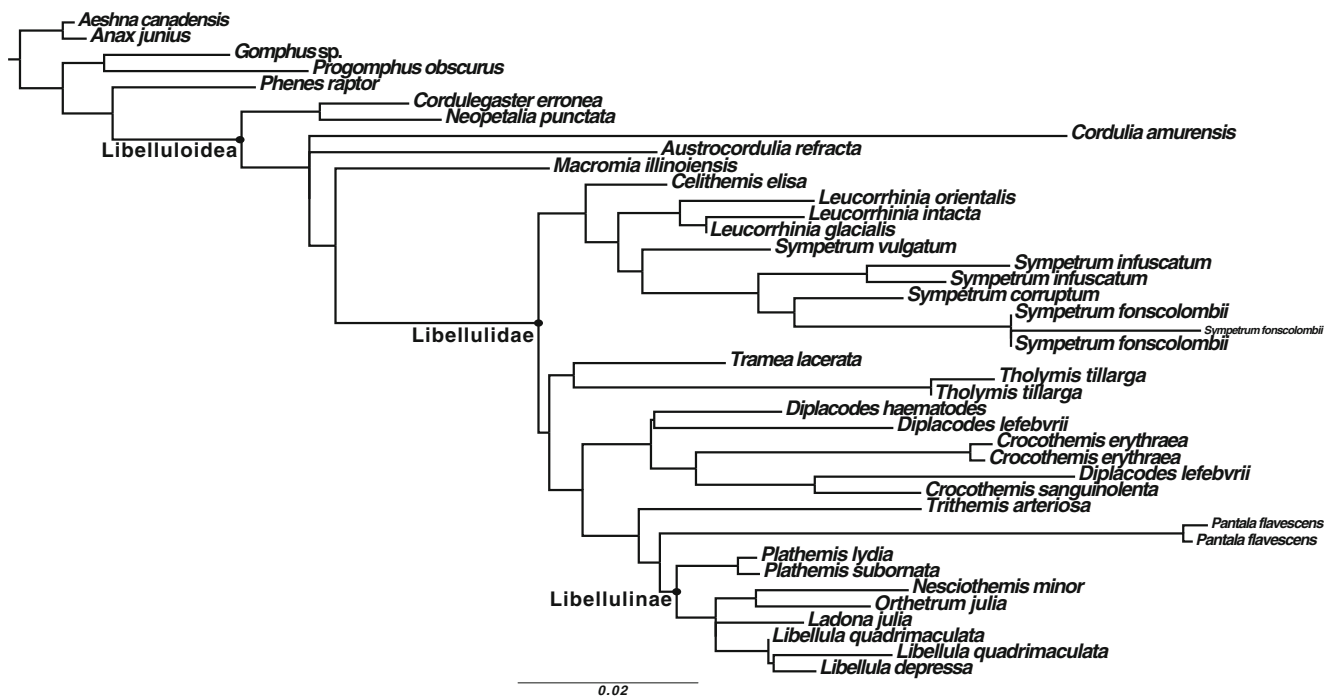


Fig. 1 Maximum Likelihood results; best tree. Scale bar show amount of character (nucleotide) change

with constant characters), $p=0.296$, suggesting the guarding type and ovariole traits are not significantly correlated.

Discussion

Using our rather limited phylogeny for Libellulidae we note that the ovariole arrangement appears to have changed more than once during the evolution of the family. In our phylogeny, continuous ovariole arrangement has evolved

independently at least twice. This result is largely dependent on the phylogenetic relationships in our tree, but our analyses recovered phylogenetic relationships among Libelluloidea that have been previously recovered by several studies (Letsch, pers. comm.; Pilgrim and von Dohlen 2008; Ware et al. 2007). Mate-guarding types are also apparently convergently acquired in Libellulidae, with tandem guarding arising several times.

What are the possible causes of this convergent and repeated evolution? Freshwater communities can be classified along

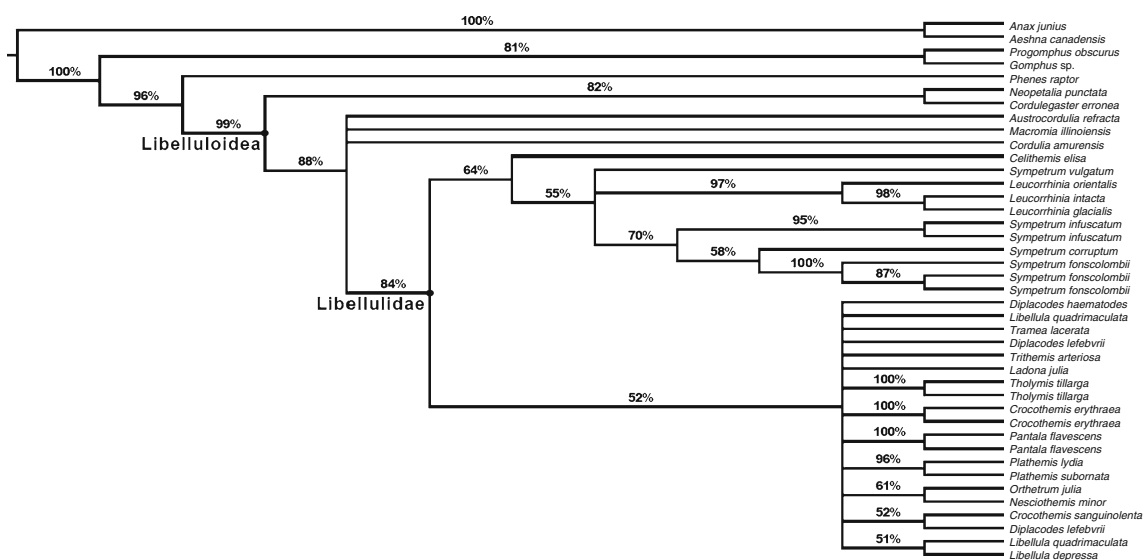


Fig. 2 Maximum Likelihood results; Bootstrap tree, with support values above branches

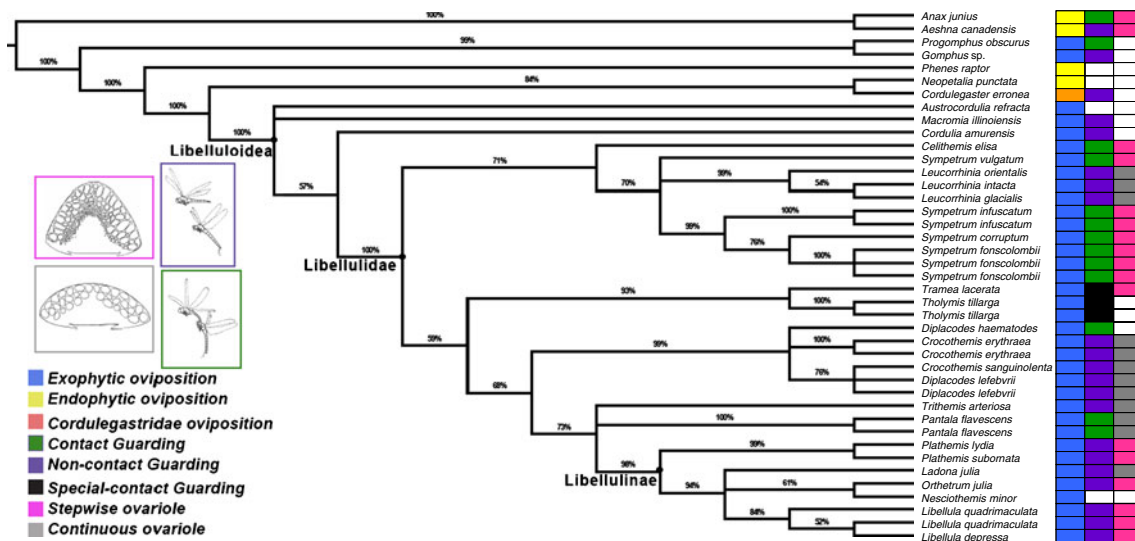


Fig. 3 Bayesian majority rule consensus tree with posterior probability values above branches. Ovariole and guarding type shown in inner figures: step-wise ovarioles outlined in pink, continuous ovarioles outlined in grey, non-contact guarding outlined in purple, contact guarding outlined in green. Exophytic oviposition type, ovariole type and guarding type indicated on right with solid rectangles: blue for

exophytic oviposition, yellow for endophytic oviposition, red for cordulegastrid type oviposition, green for contact guarding, purple for non-contact guarding, black for special interrupted tandem style guarding, pink for step-wise ovariole and grey for continuous ovariole arrangement

different gradients, for example with respect to the extent of disturbance (Peckarsky 1983) or drying (Stoks and McPeck 2003; Wellborn et al. 1996). Dragonflies that exhibit exophytic oviposition, Libelluloidea, Cordulegastridae and Gomphidae are able to complete egg-laying in water sources that are devoid of plant or other material, unlike those which oviposit endophytically (Corbet 2004) or endosubstratically (cf. e.g., Bechly et al. 2001). Endophytic or endosubstratic oviposition were the two egg-laying methods in use prior to the diversification of Anisoptera. Free from reliance on plant material, exophytically ovipositing libelluloid dragonflies are able to exploit smaller or temporary bodies of water. In addition, non-contact guarding species perform more habitat selection than tandem guarding species. Non-contact females actively seek out a favored location in the water body before depositing their egg clutches (e.g., Buskirk and Sherman 1985; Conrad and Pritchard 1992; Corbet 2004; Fincke 1992; Siva-Jothy and Hooper 1995). In tandem species, the males swiftly guide females to *several* locations at the water, resulting in numerous short and continuous egg-laying bursts (Conrad and Pritchard 1992; Hopper 1999; Koch and Suhling 2005; Schenk et al. 2004). Thus, both behaviors are favorable under certain conditions; non-contact species are well adapted to permanent waters, whilst tandem species are better adapted to temporary waters owing to their spatial risk-spreading [and their rapid larval development time (Schenk et al. 2004)]. In our analyses we found that the different reproductive strategies evolved several times and are convergent. Whether either of these were an adaptation to having sudden access to a wider range of reproductive habitats remains unclear. There are

many hypotheses that could be proposed to explain the evolution of different ovariole arrangement types; here, we will focus on habitat availability and dispersal.

Habitat instability and dispersal

Mate-guarding likely evolved as a result of male–male competition (Alcock 2001); non-contact and tandem guarding may increase the chances of paternity, but they also impose increased risks, such as that of predation and loss of potential mates. Organisms that breed in highly unpredictable habitats may be more likely to engage in risk-spreading (Kisdi 2002; Philippi and Seger 1989). In libellulid dragonflies, migratory *Pantala* inhabit highly unpredictable habitats and have been found ovipositing in temporary pools in desert environments (Johansson and Suhling 2004; Koch and Suhling 2005; Suhling et al. 2005, 2006); indeed, this taxon employs tandem guarding; however, *Urothemis* (Hassan 1981) tend to inhabit permanent, stable habitats and they also employ tandem guarding. Whilst it is possible that the ancestor to this taxon may have experienced great instability in its environment leading to the adoption of tandem guarding, it seems unlikely that these complex, risky behaviors would be maintained unless there was some current benefit to their reproductive success. Interestingly, species of *Diplacodes*, also found in permanent, stable habitats, have either continuous or step-wise ovariole production (*D. luminas* has step-wise ovarioles, while *D. lefebvrei* has continuous ovariole production; Karlsson et al. 2010). Thus, variation in strategy seems to occur between closely related species

pointing at a fairly simple regulating mechanism, possibly genetic. It is noteworthy that the guarding type also varies within this genus; the Asian species employs tandem guarding, whilst the African species uses non-contact guarding (Table 1). Thus, further investigation of the genus *Diplacodes* might provide a better insight into this.

Step-wise ovariole maturation may be similarly risky; whilst waiting for their eggs to mature, females risk the loss of a mate, suitable habitat or even their life. Continuous egg production may, however, lead to a potential waste of resources by using energy to create eggs during periods of time when a dragonfly may be, for example, in an inhospitable habitat. An ancestor to continuous egg-producing libellulid dragonflies may have benefited from this life history trait if they minimized the periods of time spent away from oviposition sites. With our phylogeny we have the ancestor to taxa with continuous ovariole arrangement appearing at the end of the Jurassic/beginning of the Cretaceous period, after the beginning of the break-up of Pangaea, during a period of time when there was, apparently, an increase in the volume of fresh water available (Stanley 2001). There are findings of lake sediments from various parts of Asia indicating that no species of Anisoptera inhabited lacustrine systems, at least in that part of the world at that time (Sinichenkova and Zherikhin 1996). Although speculative, we can assume that the number of lakes available on the continents would have increased in multitude following the break-up of Pangaea and the reversion into a moister climate situation on earth. Colonizing a mass of new, inland lake systems would explain the rapid speciation of Libellulidae. Dispersing libellulids, expanding their ranges and moving into newly available habitats may have benefited from both step-wise and continuous oocyte production. Many of the taxonomic groups within Libellulidae diverged in the same period and all are speciose (a measure of success). Today, obligate migratory or facultative migratory species (sensu Corbet 2004) have both kinds of ovariole arrangement. The best known migratory species within the Odonata, *Pantala flavescens*, has continuous ovarioles but *Sympetrum*, which has been considered to be a facultative migrant (Walker 1953), has step-wise egg production. Similarly, *Anax junius*, a well-known migratory species, has the ancestral, step-wise form of ovaries.

Even ‘permanent’ lakes and ponds may be considered to be, over geological time, transient, as they dry up or fill up (Hynes 1970; Sahlén 1995) because of changes in temperature and landmass. Perhaps the (re)evolution of step-wise ovarioles was in response to the drying out of temporary or transient bodies of water. Not maturing all eggs simultaneously might be advantageous when water sources are located long distances apart or when water sources are prone to drying up frequently within a single season and ‘residential’ females need to find other waters.

When did this occur?

Ware et al. (2007) estimated that the root of Anisoptera was approximately 250 million years old, similar to what some fossil records suggest (e.g., Grimaldi and Engel 2005), but this is debated [see, e.g., Bechly (2007) and Fleck et al. (2009) for estimates of a younger age]. Differences may be dependent on the definition of Anisoptera as a group. Libellulidae has been estimated to be roughly 100 million years old based on molecular ages (Ware et al. 2007) and fossil estimates (Fleck et al. 2009). Given these previous age estimates and evidence from the fossil record, it seems that libellulid clades with step-wise ovariole arrangement diverged during different time periods, but all after the Early Cretaceous. Apparently, Libellulidae with step-wise ovarioles diverged well after the break-up of Pangaea, when the continents were in roughly the same positions as they are today.

In our phylogeny, tandem and non-contact guarding types arose on several occasions, with the first documented instance of tandem guarding occurring in *Anax*. Tandem guarding next arose in the Gomphidae, in *Progomphus*. *Tramea* also use tandem guarding. Indeed, within Libellulidae, tandem guarding has apparently been lost and gained multiple times, arising again in *Sympetrum* (roughly Late Cretaceous) and then in *Pantala* in the Miocene.

Crowded oviposition sites

Step-wise ovariole arrangement is the ancestral state within Anisoptera and in Libellulidae. The evolution of continuous arrangement occurred after the major divergence of Libellulidae, i.e., more recently than 100 million years ago. The driving forces behind this and behind some clades in this family reverting to the ancestral state of the character may never be known, but competition and changing water levels may have been important factors. Libellulidae is the most speciose group of extant dragonflies with approximately 966 species and diversification estimates (Ware et al., 2007, 2009) suggest that they speciated relatively rapidly after their initial divergence from other Libelluloidea. Increased numbers of species, each exploiting slightly different niches, may have led to a larger number of individual dragonflies at oviposition sites. While Odonata may have evolved in rivers (Ward 1992), most Libellulidae are lake- and pond-dwellers. Perhaps a combination of continuous and step-wise ovariole arrangement was the key to the success of the group. Step-wise arrangement might be a better response to a reduction in the amount of available niche space due to increased competition for space. By maturing their eggs in batches, females may be able to forage during particularly crowded times without having had to expend their resources on egg maturation, and mature

and lay their eggs during less crowded periods. Likewise, continuous production might be advantageous under slightly less crowded conditions or when the number of adjacent water bodies is high.

Conclusions

We have presented a piece of the evolutionary puzzle concerning the rapid speciation of the libellulids beginning at the break-up of Pangaea. Although using female-specific structures, such as ovariole arrangement and egg-laying behavior, was not all-conclusive we could pinpoint a few possible evolutionary scenarios leading to the present status of the group. Hence, we argue that mapping a wide array of morphological and behavioral characters onto phylogenetic trees should be conducted more regularly, as all aspects of evolution are part of the process, not only a narrow selection of, for example, male-based data. A better understanding of the evolution of female-based systems will improve our interpretations of many aspects of dragonfly ecology and life history. Future work should expand on the present study to dissect and categorize the ovariole arrangement types of members of Macromiidae, Corduliidae, Synthemistidae and Cordulegastridae.

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