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MULTIVARIATE SEXUAL SELECTION ON PERFORMANCE-RELATED TRAITS IN SCRAMBLING AND TERRITORIAL DAMSELFLIES

Doctoral (PhD) dissertation / Egyetemi doktori (PhD) értekezés

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Uitgegeven in eigen beheer, Hajnalka Anna Gyulavári

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Inset of the doctoral thesis / A doktori értekezés betétlapja

MULTIVARIATE SEXUAL SELECTION ON PERFORMANCE-RELATED TRAITS IN SCRAMBLING AND TERRITORIAL DAMSELFLIES

TÖBBVÁLTOZÓS SZEXUÁLIS SZELEKCIÓ ÉRVÉNYESÜLÉSE TÜLEKEDŐ VERSENGÉST ÉS TERRITORIÁLIS VISELKEDÉST FOLYTATÓ KISSZITAKÖTŐK REPÜLÉSI SAJÁTOSSÁGAINÁL

Dissertation to obtain the double doctoral (PhD) degree
/ Értekezés a kettős doktori (PhD) fokozat megszerzése érdekében
Doctor in Science (Biology) (from the KU Leuven) and Doctor in Environmental Sciences
(from the University of Debrecen)
/ Biológia (KU Leuven) és Környezettudomány (Debreceni Egyetem) tudományágakban

Written by / Írta: **Gyulavári Hajnalka Anna**
Environmental Scientist **/** okleveles környezetkutató

Made within the framework of a co-operation agreement between the KU Leuven**, Arenberg Doctoral School (Programme of Biology)** and the University of Debrecen, **Pál Juhász-Nagy Doctoral School (Programme of Hydrobiology)**.
/ Készült a Leuveni Katolikus Egyetem **Arenberg Doktori Iskolája** (**Biológia Doktori program**ja) valamint a Debreceni Egyetem **Juhász-Nagy Pál Doktori Iskolája** (**Hidrobiológia Doktori programja**) keretében.

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I made this thesis within the framework of a co-operation agreement between the KU Leuven, **Arenberg Doctoral School, Programme of Biology** and the University of Debrecen, Natural Sciences Doctoral Council, **Pál Juhász-Nagy Doctoral School, Programme of Hydrobiology** to obtain a double doctoral degree: Doctor in Science (Biology) from the KU Leuven and Doctor in Environmental Sciences from the University of Debrecen.

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Leuven/Debrecen, 2016.

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signature of the candidate /a jelölt aláírása

Hereby I testify that the PhD candidate **Hajnalka Anna Gyulavári** has worked under my supervision between **2010-2016** in the Arenberg Doctoral School in the **Programme of Biology**. The candidate decisively contributed with her individual work to the results of the thesis. I recommend the thesis to be accepted.

Tanúsítom, hogy **Gyulavári Hajnalka Anna** doktorjelölt **2010-2016** között az Arenberg Doktori Iskola **Biológia doktori** programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.
Leuven/Debrecen, 2016.

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signature of the promotor / a témavezető aláírása

Hereby I testify that the PhD candidate **Hajnalka Anna Gyulavári** has worked under my supervision between **2008-2016** in the Pál Juhász-Nagy Doctoral School in the **Programme of Hydrobiology**. The candidate decisively contributed with her individual work to the results of the thesis. I recommend the thesis to be accepted.

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……………………….……………………………
signature of the promotor / a témavezető aláírása

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Summary

Sexual selection is a powerful force directing evolution which can vary in strength (strong or weak), direction (positive or negative) and form (linear or nonlinear). Despite the many studies on sexual selection, three aspects remain understudied, yet very relevant to fully understand sexual selection on the traits of a given species. (1) While most of the studies on sexual selection focused on a single or few phenotypic traits, a minority of studies followed a multivariate approach. This is important to disentangle direct selection versus indirect sexual selection on a trait. (2) Most studies focused on morphological traits (mainly body size) and ignored physiological traits, for example related to energy reserves, and locomotor performance. (3) Finally, recent studies suggest that sexual selection may vary both among populations and among time periods within a given population. Paying attention to such spatiotemporal variation in sexual selection will learn whether the same traits are preferred across populations and seasons which is directly relevant to understand the evolutionary dynamics of these traits.

To study these aspects in-depth we used damselflies, upcoming model systems in ecology and evolution. In this thesis we combined field and laboratory studies to obtain a multivariate picture of sexual selection on whole-organism performance and the underlying morphological and physiological traits in the scrambling damselfly *Coenagrion puella* and the territorial damselfly *Chalcolestes viridis*. In addition, we performed a detailed morphometric analysis and reconstructed phylogenetic trees to resolve the position of *C. viridis* and its eastern form, *C. parvidens*.

1. *The taxonomic position of C. viridis and C. parvidens*

Our genetic data supported the genus status of *Chalcolestes*. We found that the two *Chalcolestes* taxa formed a monophyletic group separated from other investigated European *Lestes* species based on both the mitochondrial COI gene and the nuclear ribosomal ITS region. The morphometric study showed that *C. viridis* and *C. parvidens* were differentiated in morphometric space, even though there were no single morphometric characters that could conclusively distinguish the two taxa. Our genetic analysis further supported the species status of *C. parvidens.* The two *Chalcolestes* taxa do not share haplotypes, suggesting that lineage sorting was complete. Although we find the data supportive of recognizing *C. parvidens* as a distinct species, further detailed morphologic and genetic analyses are needed to shed further light on how both species are separated reproductively.

2. *Spatial and temporal variation of sexual selection on whole-organism performance and the underlying morphological and physiological traits*

Although the two study species have a contrasting mating system, we found that flight endurance had a key role determining mating success in both species. In contrast, we could not detect selection on flight speed in neither of the species. This indicates that flight speed is not as relevant as flight endurance neither for territorial defence nor for scrambling in the study species. This is intriguing as speed is likely to play a role in capturing the female, especially in scrambling species. More likely, speed is already maximized due to strong selection by other agents such as predators leaving less variation to detect selection on this performance trait, while endurance is less relevant for damselflies to escape predators. From the six key phenotypic traits, four were found to be under sexual selection. The detected selection patterns that could not be explained via their effect on flight performance are generating novel testable hypotheses about how the covariation between these traits and mating success was generated. This urges caution when using different traits as proxies for the two studied aspects of flight performance. We provided support that not only spatial but also temporal variation occurs within a single reproductive season for sexual selection on phenotypic traits. Furthermore, we provided the first test for spatially and temporally varying sexual selection on performance traits, which confirmed our novel hypothesis that selection on performance (here flight endurance) is more stable through space and time than the observed selection on the underlying traits. We hypothesize this to be a general pattern given that, compared to morphological and physiological traits, performance is the direct target of selection. In contrast, selection on phenotypic traits may be more variable as the contribution of phenotypic traits to performance may depend on environmental conditions. Additionally, different combinations of underlying morphological and physiological traits may result in similar performance.

The here reported studies are the first investigating sexual selection on flight performance and the underlying physiological and morphological traits in an aerial scrambling and territorial species. The PhD thesis adds to the increasing number of studies finding that the strength, direction, and form of selection are often spatially and temporally dynamic. This thesis helps to refine our knowledge of the spatial and temporal dynamics of selection patterns in nature, which is of paramount importance to arrive at a better understanding of adaptive evolutionary dynamics of traits in natural populations.

Samenvatting

Seksuele selectie kan evolutie in belangrijke mate sturen en is een kracht die kan variëren in sterkte (sterk of zwak), richting (positief of negatief) en vorm (lineair of niet-lineair). Ondanks de vele studies op seksuele selectie, is de kennis van drie cruciale aspecten van seksuele selectie nog steeds beperkt. (1) De meeste studies bestuderen seksuele selectie op één of een beperkt aantal fenotypische kenmerken, terwijl weinig studies een multivariate aanpak volgen. Een multivariate aanpak is belangrijk om directe selectie van indirecte selectie op een bepaald kenmerk te kunnen onderscheiden. (2) De meeste studies focussen uitsluitend op morfologische kenmerken (voornamelijk lichaamsgrootte), terwijl fysiologische kenmerken (bijvoorbeeld energiereserve) en locomotorisch vermogen (bijvoorbeeld vliegvermogen) genegeerd wordt. (3) Ten laatste, recente studies suggereren dat seksuele selectie kan variëren tussen populaties en dat seksuele selectie in dezelfde populatie kan variëren in de tijd. Door het bestuderen van de ruimtelijke en temporele variatie in seksuele selectie zal duidelijk worden of seksuele selectie op een bepaald kenmerk gelijk is tussen populaties en constant blijft in de tijd; kennis hierover is belangrijk om evolutionaire dynamieken in deze kenmerken te begrijpen.

We gebruiken waterjuffers – opkomende modelsystemen in ecologie en evolutie – om voorgaande aspecten grondig te onderzoeken. In deze thesis combineren we studies in het veld en in het laboratorium om een multivariaat beeld te verkrijgen van seksuele selectie op de performanties van het volledige organisme en de onderliggende morfologische en fysiologische kenmerken die de performanties van het organisme bepalen. In deze thesis voeren we onderzoek uit op twee soorten met een verschillend paarsysteem: *Coenagrion puella* waarbij mannetjes via ‘scrambling’ competitie concurreren om paring met vrouwtjes te bekomen; en *Chalcolestes viridis* waarbij mannetjes concurreren voor een territorium waardoor de kansen tot voortplanting verhogen. Daarnaast voerden we een gedetailleerde morfometrische analyse uit en reconstrueerden een fylogenetische boom om duidelijkheid te krijgen in de taxonomische positie van *C. viridis* en de oosterse vorm *C. parvidens*.

1. *De taxonomische positie van C. viridis en C. parvidens*

De genus status van *Chalcolestes* wordt ondersteund door onze genetische studie. Gebaseerd op het mitochondriaal COI gen en de nucleaire ribosomale ITS regio vonden we dat de twee taxa van *Chalcolestes* een monofyletische groep vormen, afgescheiden van andere bestudeerde

Europese *Lestes* soorten. Gebaseerd op de combinatie van de opgemeten morfologische kenmerken (morfometric space) toonden we een afscheiding tussen *C. viridis* en *C. parvidens* aan. De twee taxa konden echter nooit met zekerheid onderscheiden worden door één enkel kenmerk. De status van de soort *C. parvidens* werd verder ondersteund door onze genetische analyse. De twee *Chalcolestes* taxa hebben geen gemeenschappelijke haplotypes, wat een volledig uitsplitsen van genetische lijnen tussen de taxa suggereert. Onze data suggereert dus dat *C. parvidens* voldoende verschilt om als afzonderlijke soort te worden beschouwd. Verder morfologisch en genetisch onderzoek zijn nodig om duidelijkheid te brengen in de reproductieve scheiding van beide soorten.

2. *Ruimtelijke en temporele variatie in seksuele selectie op de performantie van het hele organisme en de onderliggende morfologische en fysiologische kenmerken*

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De uitgevoerde studies in het kader van mijn doctoraat zijn de eerste waarbij seksuele selectie op vliegvermogen in combinatie met de onderliggende morfologische en fysiologische kenmerken worden bestudeerd in een ‘scrambling’ en territoriale vliegende soort. Deze doctoraatsthesis levert een bijdrage aan het groeiend aantal studies die de ruimtelijke en temporele variatie in de sterkte en vorm van seksuele selectie documenteren. De resultaten van deze thesis verfijnen onze kennis van de ruimtelijke en temporele dynamieken aan selectiepatronen in de natuur; dit is van doorslaggevend belang voor het beter begrijpen van adaptieve evolutionaire dynamieken in natuurlijke populaties.

Összefoglalás

A szexuális szelekció az evolúció egyik erőteljes mozgatórugója, melynek érvényesülése (erős vagy gyenge), iránya (pozitív vagy negatív) és a formája (lineáris vagy nemlineáris) is változhat. Annak ellenére, hogy számos tanulmány született a szexuális szelekció témakörében, három szempont kevésbé tanulmányozott maradt. Ezek viszont nagyon fontosak ahhoz, hogy teljes mértékben megértsük, hogyan hat a szexuális szelekció egy adott faj jellegeire. (1) A legtöbb szexuális szelekciós tanulmány középpontjában egy vagy néhány fenotípusos jelleg áll, de csak néhány tanulmány alkalmaz többváltozós megközelítést. A többváltozós megközelítés azért fontos, hogy megkülönböztethető legyen az adott jellegre ható közvetlen és közvetett szexuális szelekciót. (2) A legtöbb tanulmány középpontjában morfológiai jellegek (főleg testméretek) állnak, és például az energiatartalékokkal kapcsolatos fiziológiai jellegek, vagy a mozgással kapcsolatos teljesítőképesség figyelmen kívül marad. (3) A legutóbbi vizsgálatok azt sugallják, hogy a szexuális szelekció nemcsak egyes populációk között, hanem egy adott populáción belül és bizonyos időszakok között is változhat. Ha figyelmet fordítunk a szexuális szelekció ilyen tér- és időbeli változására, megtudhatjuk, hogy különböző populációknál és különböző időszakokban vajon ugyanazok a jellegek részesülnek-e előnyben. Ennek jelentősége abban áll, hogy ezáltal megérthetjük e jellegek evolúciós dinamikáját.

Az előbbi szempontok alapos tanulmányozása kisszitakötőkön történt, melyek mindinkább közkedvelt modellek az ökológia és az evolúcióbiológia területén. A dolgozatban terepi és laboratóriumi vizsgálatokat végeztünk, hogy többváltozós képet nyerhessünk a kisszitakötők repülési teljesítőképességére, továbbá a teljesítőképességük alapjául szolgáló morfológiai és fiziológiai jellegeikre ható szexuális szelekcióról. Vizsgálatainkat tülekedő versengést (*Coenagrion puella*) és territoriális viselkedést mutató (*Chalcolestes viridis*) kisszitakötőkön végeztük. Emellett a *C. viridis* és keleti formája, a *C. parvidens* taxonómia helyzetének tisztázására részletes morfometriai elemzést és filogenetikai vizsgálatokat is folytattunk.

1. *A C. viridis és C. parvidens taxonómia helyzete*

Genetikai vizsgálataink eredményei alátámasztották a *Chalcolestes* nemzetség érvényességét. Azt találtuk, hogy a két *Chalcolestes* taxon monofiletikus csoportot alkotott, mely elkülönült a többi vizsgált európai *Lestes* fajtól, mind a mitokondriális COI gén, mind a nukleáris riboszomális ITS régió alapján. A morfometriai vizsgálat azt mutatta, hogy a *C. viridis* és a *C.*

parvidens elkülönültek egymástól a morfometrikus térben, annak ellenére, hogy nem volt egyetlen olyan morfológiai jellemző sem, ami meggyőzően megkülönböztetné a két taxont. A genetikai vizsgálatok a *C. parvidens* faji státuszát sugallták. A két *Chalcolestes* taxon eltérő haplotípusú, ami arra utal, hogy különböző leszármazási vonalakba tartoznak. Bár adataink a *C. parvidens* önálló faji státuszára engedtek következtetni, további részletes morfológiai és genetikai vizsgálatok szükségesek ahhoz, hogy megtudjuk, miként különül el a két taxon egymástól szaporodásbiológiájuk tekintetében.

2. *Az élőlények teljesítőképességére és a teljesítőképességük alapjául szolgáló morfológiai és fiziológiai jellegeikre ható szexuális szelekció tér- és időbeli változása*

Bár a két vizsgált fajnak eltérő a párzási rendszere, azt találtuk, hogy a repülés maximális időtartamának mindkét fajnál kulcsszerepe volt a párzási siker meghatározásában. Ezzel szemben a repülési sebességre ható szelekciót egyik fajnál sem tudtuk kimutatni. Ez azt mutatja, hogy a tanulmányozott fajoknál a repülési sebesség kevésbé lényeges a repülés maximális időtartamához képest. Ez igaz mind a territoriális viselkedés esetében, mind a tülekedő versengésnél. Ez azért érdekes, mert a repülési sebesség a kisszitakötőknél valószínűleg szerepet játszik a nőstények elfogásában, különösen a tülekedő versengést folytató fajoknál. Sokkal valószínűbb, hogy a repülési sebesség már eleve maximalizált egyéb tényezők, mint például a ragadozók általi erős szelekció miatt. Ezáltal kisebb a lehetőség a teljesítőképesség e tulajdonságára ható szelekció kimutatására, míg a kisszitakötők esetében a ragadozók előli menekülésnél a repülés időtartama kevésbé lényeges. A vizsgált hat fő fenotípusos jelleg közül négy szexuális szelekció alatt állt. Észleltünk olyan szelekciós mintázatokat, melyek nem magyarázhatóak meg a fenotípusos jellegek repülési teljesítőképességre gyakorolt hatásával. Ezek újfajta, tesztelhető hipotéziseket vetnek fel arra vonatkozóan, hogy az adott jellegek és a párzási siker közötti kapcsolat miként jött létre. Ugyanakkor ez óvatosságra is int, amikor a különböző jellegeket a repülési teljesítőképesség két vizsgált jellegének (sebesség és maximális időtartam) helyettesítésére szeretnénk használni. Alátámasztottuk továbbá, hogy a fenotípusos jellegekre ható szexuális szelekció egyetlen szaporodási időszakon belül nemcsak térbeli, hanem időbeli változáson is keresztül megy. Az elsők között vizsgáltuk a repülési teljesítőképességre ható szexuális szelekció térbeli és időbeli változását, melynek eredménye megerősítette azt az új feltételezést, hogy a teljesítőképességre (itt a repülés maximális időtartamára) ható szelekció sokkal állandóbb térben és időben, mint a teljesítőképesség alapjául szolgáló jellegekre ható szelekció. Azt feltételezzük, hogy ez a mintázat általános lehet, hiszen a morfológiai és fiziológiai jellegekkel összehasonlítva a teljesítőképesség a szexuális szelekció közvetlen célpontja. Ezzel szemben a fenotípusos jellegekre ható szelekció jóval változóbb lehet, mivel a fenotípusos jellegek teljesítőképességhez való hozzájárulása függhet a környezeti feltételektől. A teljesítőképesség alapjául szolgáló morfológiai és fiziológiai jellegek különböző kombinációja hasonló teljesítőképességet eredményezhet.

Az itt bemutatott tanulmányok az elsők között vizsgálják a repülési teljesítőképességre és az annak alapjául szolgáló fiziológiai és morfológiai jellegekre ható szexuális szelekciót repülő tülekedő versengést és territoriális viselkedést folytató kisszitakötőknél. A doktori értekezés hozzájárul azoknak a tanulmányoknak az egyre növekvő számához, amelyek megállapítják, hogy a szelekció ereje, iránya és formája gyakran térben és időben változik. A dolgozat segít elmélyíteni a szelekciós mintázatok természetben előforduló térbeli és időbeli dinamikájára vonatkozó tudásunkat, amely rendkívül fontos, hogy jobban megértésük a különböző jellegek adaptív evolúciós dinamikáját a természetes populációkban.

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CHAPTER I

General Introduction

1. *The order Odonata*

1.1 *Taxonomy of Odonata*

Odonata are among the most ancient winged insects, dating back from the Lower Permian around 250 million years ago. The order Odonata is divided into three suborders including the Zygoptera or damselflies with 2941 described species (18 families, 308 genera), the Anisoptera or true dragonflies with 3011 described species (11 families, 344 genera), and a third suborder, the Anisozygoptera or damseldragons with only four described species (Dijkstra et al. 2013). Zygoptera are smaller, have a broad head with eyes separated and the size of their fore and hind wings is almost similar. Most species rest with wings closed. The larvae are slender and rely mainly on three caudal gills for respiration. Anisoptera are on average larger and more robust than Zygoptera. Their hind wings are distinctly broader at their base than the fore wings and in most families the eyes are touching on the top of the head. Most species spread their wings during resting. The larvae are typically more robust than those of Zygoptera and lack caudal gills: they absorb oxygen through gills in the rectum. At first sight Anisozygoptera are similar to the Anisoptera, but their wings have narrow bases (a characteristic feature of Zygoptera). The larvae also show features that are typical for both other suborders. According to rough estimations between 1000 and 1500 species are still waiting for description (Kalkman et al. 2008).

Traditionally, taxonomy in general is based on phenotypic analyses. Morphology-based methodologies for taxonomy developed strongly in the last decades, new analysis methods were included into the taxonomic toolkit such as multivariate morphometrics and landmark-based geometric morphometrics (Adams et al. 2004). These developments lead, for example, to the discovery of several cryptic species (e.g. Klimov et al. 2004; Hernández-Ortiz et al. 2012). While morphological studies are highly valuable and necessary, in many taxa this approach is impossible due to the lack of sufficient morphological characters (such as e.g. in prokaryotes: Oren 2004; or in nematodes: Chilton et al. 1995; Floyd et al. 2002). For classifying Odonata in the past, patterns in wing venation were a leading tool, but as similar characters evolved multiple times (see e.g. Carle et al. 2008), they are often not a reliably indication for close relationships. Studies including other morphological characters of the genitalia or the larvae may succeed in dealing with this problem (e.g. Fleck et al. 2008) but are not always feasible. As old approaches reached their boundaries, new methods such as molecular genetic techniques have become widespread in taxonomic studies. Molecular data can serve different purposes at different levels. For example, the mitochondrial cytochrome c oxidase subunit I gene (COI) is proposed as the barcode source to identify and delimit animal species (Herbert et al. 2003). Mitochondrial genes evolve too rapidly to provide resolution of deeper divergences, therefore the use of COI sequences in phylogenetics, especially without additional genetic data is controversial (Mitchell 2008). However, at shallower divergences (genus level) COI signal was comparable with the nuclear genes (Wilson 2010). It is clear that life's diversity needs to be studied from multiple and complementary perspectives, therefore the different morphological and molecular approaches should ideally be combined (Dayrat 2005). Although the number of studies combining DNA sequences and morphology are increasing, relatively few studies have focused on odonates (but see e.g. Pilgrim et al. 2002; Stoks et al. 2005; Pilgrim and Von Dohlen 2007). As a result, many taxonomic affiliations still remain debated in this order (Schmidt 2001; Dijkstra 2003; Dijkstra and Lewington 2006). The increasing interest toward odonates as bioindicators for environmental health and conservation management, model organisms in scientific research and primary invertebrates in freshwater conservation in the European Union's Habitat Directive calls for resolving questions on the European species' status, names and phylogenetic relationships.

1.1.1 *The taxonomic position of C. viridis and C. parvidens*

One of the debated issues concerns the status and phylogenetic position of *Chalcolestes viridis* (Vander Linden, 1825) and *C. parvidens* Artobolevskii, 1929 (Jödicke 1997; Schmidt 2001). Traditionally, both taxa were placed in the genus *Lestes* Leach, 1815, a heterogeneous genus with over 80 species occurring in all continents except Antarctica. However, given that their larvae differ from other *Lestes* species by having a broad instead of a greatly narrowed (spoon-shaped) prementum, Kennedy elevated them to a separate genus *Chalcolestes* in 1920. Nevertheless, this new genus name has not been generally accepted. Furthermore, *C. parvidens* was for a long time considered to be a subspecies of *C. viridis*. Later, this consideration was questioned based on phenotypic traits and electrophoretic analyses (Cobolli et al. 1994).

1.2 *Relevant biology of Odonata*

The general biology, ecology and behaviour of Odonata are broadly discussed in Corbet (1999), Askew (2004) and Suhling et al. (2015). Here, we give a short overview about the relevant features in connection with the topic of the PhD thesis, based on these standard works.

Members of the order Odonata have an incomplete metamorphosis: their life cycle includes egg, larval and adult stages but no pupa (Fig. 1). Metamorphosis consists of irreversible changes in morphology, physiology, and behaviour that take place between the final larval instar and emergence. The molt that produces the adult odonate is called the emergence. The larvae are completely aquatic. The adults are associated with still or running fresh water. Both the larvae and adults are predators. Odonata are excellent fliers, mostly have bright colouration and show a complex behaviour.

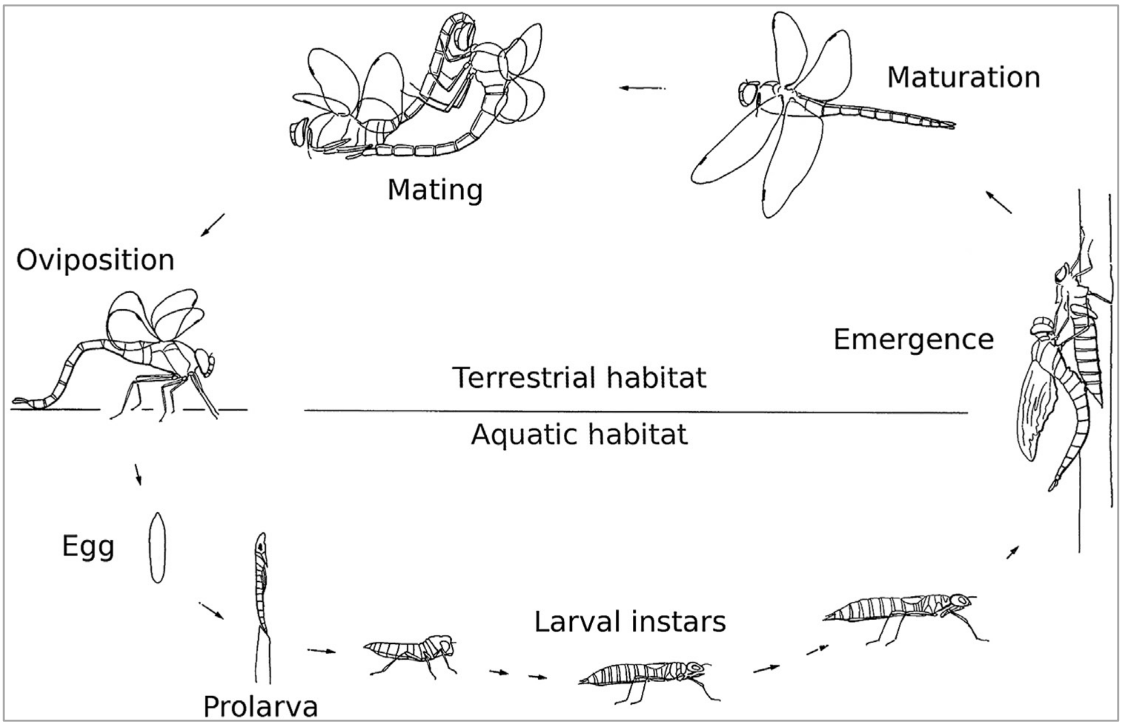

Figure 1. The complex life cycle of Odonata. Figure from Suhling et al. 2015, original by H. Wildermuth.

Odonates have a complex and unique reproductive behaviour (Fig. 2). At first, the male grabs the female’s thorax with his legs, and then grasps her on the prothorax (Zygoptera) or on the head (Anisoptera) with his anal appendages. Then they continue flying in the so-called tandem position. After forming the tandem position the male transfers sperm in an indirect way to the female’s genitalia. Sperm is produced in the testes situated at the tip of the abdomen, but the secondary copulatory organs (penis) that transfer sperm to the female lie on the ventral side of the second abdomen segment. Males transfer sperm externally to this organ before copulation. The female then curls her abdomen to pick up the sperm from the male, a position known as the mating wheel. The mating wheel is unique and allows the couple to fly in copula.

Female odonates store sperm in specialized organs, delay oviposition and may mate multiply. Unlike anisopterans, in Zygoptera copulation can clearly be divided into two or three behavioural stages: in the first stage the present male removes the rivals’s sperm from the bursa copulatrix. In the second stage, the new male’s sperm is transferred to the female, while in the third stage the new sperm is brought with active collaboration of the female to the spermatheca. During copulation, males use the hamulus, the secondary genitalia to remove, squash or push out any sperm that the female may still be carrying from prior matings with other males to prevent sperm competition. Other parts of the hamulus are then used by the male to fertilize the female with his own sperm. This process ensures his genetic investment in the next clutch of eggs that the female will lay. The time needed to complete fertilization ranges from 15 seconds to well over an hour. Territorial males often copulate for a shorter time than non-territorial males, apparently to reduce their risk of losing their territory to intruders.

After copulation, males engage in different sorts of female-guarding behaviour during oviposition that prevents females from re-mating. They either return to the tandem position (contact guarding) or the male remains close to the female to prevent other males from mating with her as she lays eggs alone (non-contact guarding). This extended association and the sperm removal increase the male’s reproductive success but it is also reduces the amount of time that is available to mate with other females. Studies of paternity expectations among odonates have reported nearly 100% paternity for the last male, although with some intraspecific variation. Cooper et al. (1996) studied more than 3000 larvae from both wild and captively bred *Ischnura elegans* females. Microsatellite typing showed that most wild-caught females had mated with several males. Analysis of offspring from females which mated in captivity showed that multiple-matings resulted in a large proportion of last-male sperm precedence.

There was appreciable variation in the extent and patterns of immediate and longer-term precedence, which could reflect differences in male sperm removal ability or selective use of sperm by females. In the damselfly *Enallagma hageni* the last male to mate was found to fertilize up to 95% of the eggs of the first clutch laid after mating. Dissection of females collected before, during, and after copula showed that a male removes a maximum of 87% of the sperm by volume of a previous mate (Fincke 1984). In the damselfly *Calopteryx splendens* the last male sperm precedence was also supported in the bout of ovipositions immediately following copulation, despite the fact that the males of this species do not completely remove the sperm of previous males (Hooper and Siva-Jothy 1996). These evidences so far suggest that in damselflies the last male sperm precedence is typically high and that most offspring of a given clutch are fathered by the male that last mated the females before oviposition.

In territorial species, the average number of matings by a male per day may range from 0 to >3. In nonterritorial species with contact guarding, the daily mating frequency is much lower and males typically do not mate with more than one female in a given day. For example, a study on the scrambling damselfly *Lestes sponsa* showed that in detailed lifetime study of 326 males, only six males were observed to mate twice on the same day (Stoks 2000). With regard to mating frequency in females, on overview study of 22 species in 17 genera found that females of only 4 (18%) typically mated more than once on a given day (Fincke 1997). Females are receptive and willing to mate only on days when they have mature eggs to lay, and become unreceptive for several days after having laid an egg clutch (Fincke 1986).

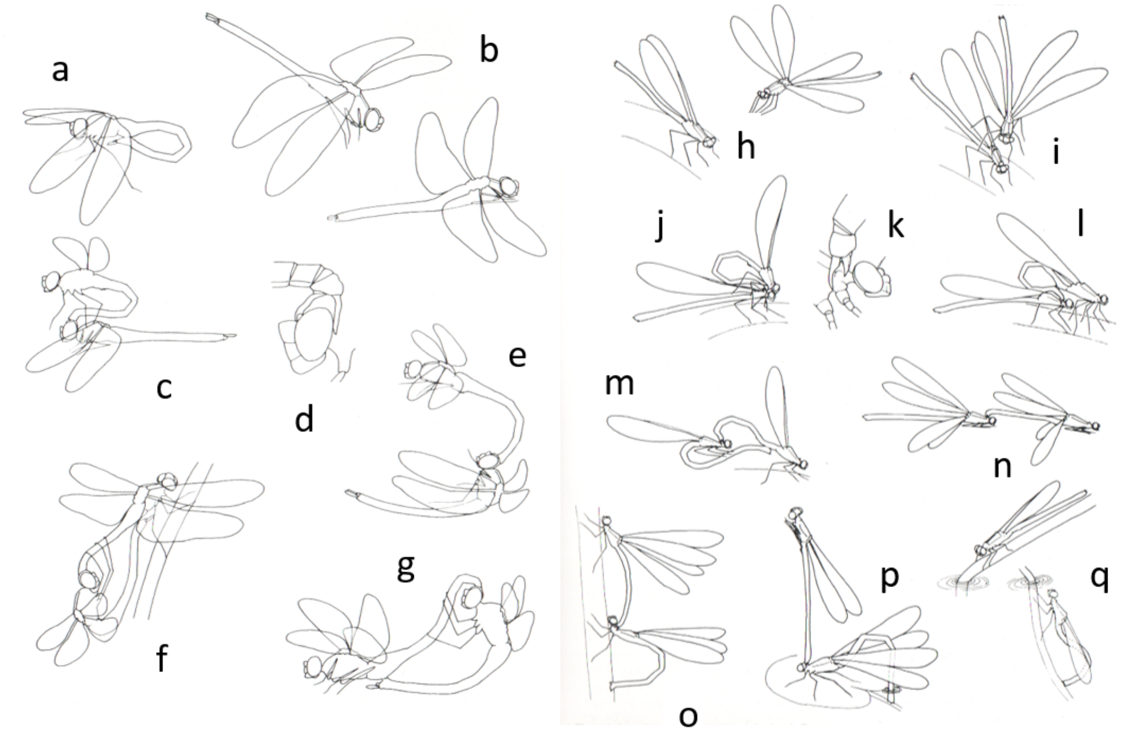

Figure 2. Reproductive behavior of Anisoptera (left) and Zygoptera (right). Before mate acquisition, a male anisopteran transfers sperm to his secondary copulatory organ (a). The male flies to the female (b) and grabs her through the head (c-d). In tandem position, the female curls up her abdomen to pick up the sperm (e). Copulation in a mating wheel can occur on a perch (f) or in flight (g). A Zygoptera male flies above the female (h) and grabs her on the thorax with his legs (i). The male grabs the pronotum of the female (j-k). and transfers sperm to his secondary copulatory organ whilst in tandem (l). The pair forms a mating wheel (m) and flies in tandem (n). Oviposition can take place in plant stems above the water (o) or in submerged plant stems (p-q). Figure from Askew (2004).

1.3 *Classification of odonate mating systems*

Odonata display a large variation in mating behavior, even among morphologically similar species, and are therefore useful for studying the relationship between mating behavior and selection. In most odonate mating systems, sexual selection on male traits results from male-male competition for access to mates. Sexual selection via female choice is relatively less important or operates indirectly through females' choices of times or places to mate (Conrad and Prichard 1992). Odonates exhibit territorial and non-territorial mating strategies. In the former case males establish territories at suitable oviposition sites and actively defend them against conspecific males. Alternative mating tactics are found in some species.

Several males can coexist in the same territory if a dominance hierarchy is present. Satellite/sneaker males are found in many species, where mating can occur inside or outside the territory of the defending male when he is occupied elsewhere. In contrast, males of scrambling species gather at sites where encounters with females are likely and engage in scramble competition for females.

Traditionally, reviews classify odonate mating systems according to several criteria: whether they are resource or non-resource based, the frequency of male-female encounters, the ability of males to monopolize resources that they control and the predictability of female occurrence. Odonate mating systems have been reviewed by Campanella (1975), Waage (1984), Conrad and Pritchard (1992), Fincke (1997) and Corbet (1999). Most classification systems draw heavily on ideas proposed by Emlen and Oring (1977). Here we present a general, ecological classification of mating systems of animals based on Emlen and Oring (1977) with special emphasis of the systems for the studied species:

1. Monogamy: Neither sex has the opportunity of monopolizing additional members of the opposite sex. Fitness often maximized through shared parental care.

2. Polygamy: Three types are recognized, most animals opt for only one of these strategies, some exhibit hybrid strategies.

2.1 Polygyny: Individual males frequently control or gain access to multiple females. Different types of polygyny exist. Here, we focus only the ones that are relevant for the studied species.

2.1.1 Scramble competition: Here resources are accessible to all competitors and are not monopolizable by an individual or group. In scramble competition males try to outrace other males to locate and copulate with females before other males can mate with them. Males focus on mate location rather than defending territories or engaging in prolonged contests over access to females. Males can increase the chance of encounters with females by remaining at the pond as long as possible and being more active flyers. Mating success goes to the males that are the most persistent, durable, and perceptive searchers, not the most aggressive ones (Andersson 1994). A typical example is the study species *Coenagrion puella* (Banks and Thompson 1985).

2.1.2 Resource defense or territoriality: If the resources females need are clumped spatially, males may defend these clumped resources and thereby can gain access to multiple fertile females attracted to the resources. Males actively establish and defend such territories against aggressive conspecifics by fights or aggressive displays (Askew 2004; Suhonen et al. 2008). In many insects, this is a common reproductive tactic.

In Odonata, males generally interact by fighting but in some species physical conflict is replaced by a ritualized aggressive display for example in species with colored wings or broad tibia. Males often perch on a visible location within their territory, from where they can observe the arrival of females as well as competitors. Other males of some territorial species perform patrol flights, which enable several males to coexist within a limited area, territorial disputes arise when males meet each other. The study species *Chalcolestes viridis* belongs to this mating system (Dreyer 1978).

2.2 Polyandry: Individual females frequently control or gain access to multiple males.

2.3 Polygynandry: Two or more males have an exclusive relationship with two or more females; the numbers of males and females do not have to be equal, and in vertebrate species studied so far, the number of males is usually less.

3. Promiscuity: A member of one sex within the social group mates with any member of the opposite sex.

2. *Sexual selection*

Sexual selection is a powerful force directing evolution. Since Charles Darwin, sexual selection has been known as a factor that often seems to be in conflict with adaptation via survival selection, and that can result in elaborate secondary sexual traits designed to maximize the success in competition over mates. In case of sexual selection, organisms are competing not for food or other resources in the environment but for mates (Andersson 1994). The opportunity for sexual selection is usually thought to be greater in males compared to females because the reproductive success of males is limited by access to females (as sperm production is typically cheap). In contrast, the reproductive success of females is limited by the number of offspring they can carry or raise, or by food, shelter and other resources required for care of the offspring (Trivers 1972). When individuals of the same sex differ in their mating success and that difference is correlated to the expression of a particular trait, that trait is said to be under sexual selection. Sexual selection has two major forms: intersexual selection in which members of the less limited sex (typically males) compete with each other to be chosen by the limited sex (typically females); and intrasexual selection in which members of the less limited sex compete among themselves for access to the other sex. Physiological and behavioral adaptations such as sperm competition, mate guarding and territoriality have evolved as a consequence of intrasexual selection. Sexual selection is not only a major force directing evolution but it is also a dynamic one.

It can vary in strength (strong or weak), direction (positive or negative) and form (linear or nonlinear). Considering spatial and temporal variation in selection is a major focus of interest of modern evolutionary and ecological studies (Miller and Svensson 2014). The traits targeted by sexual selection (the so-called selective targets) can be manifold and include both performance traits, physiological traits and morphological traits.

2.1 *Performance as selective target*

In the scope of this thesis we define performance as the ability of an animal to conduct a physically challenging, ecologically relevant task (Irschick et al. 2008). Studies of whole-organism performance form a cornerstone of evolutionary and physiological ecology (Lailvaux and Husak 2014). Despite the many studies on sexual selection, the real target of selection namely the performance of the animals has largely been ignored. This is surprising as selection is expected to directly act on organismal performance traits, such as locomotor ability, rather than on the underlying morphological and physiological traits (Irschick et al. 2007; Irschick et al. 2008).

Locomotion is a key performance trait that has long been considered as an important contributor to survival and reproductive success in a variety of animal taxa (Dickinson et al. 2000). In line with this, recent studies showed that maximal locomotor ability plays a central role as a target for sexual selection (e.g. Lailvaux and Irschick 2006; Irschick et al. 2007; Husak and Fox 2008).

Adults of Odonata are aerial insects mating on the wing. Despite the general belief that flight performance is important for sexual selection in this insect order (Marden and Cobb 2004; De Block and Stoks 2007), the many sexual selection studies have not directly quantified flight performance. Sexual selection should be acting on different performance traits depending on the mating system. In flying territorial species territory ownership is settled by means of aerial male-male contests, in which the male that endures longer is usually the winner (Marden and Waage 1990; Suhonen et al. 2008). In addition, previous studies have shown the importance of speed for obtaining territories (Kodric-Brown and Nicoletto 1993) and mates (Husak et al. 2008) in territorial species. In contrast, males in aerial scramble competition can increase the chance of encounters with females by active searching, for this endurance is likely more important than speed (Andersson 1994).

2.2 *Physiological traits as selective targets*

Among physiological traits there could also be important selective targets such as immunity, energetic reserves (fat content), and flight muscle mass (which is linked to the flight ability). In invertebrate immunity, melanogenesis is an important component of host defense. It is responsible for encapsulating multicellular pathogens, repairing tissues, and defending against other pathogens. Phenoloxidase (PO) is responsible for the activation of melanogenesis. PO is a costly enzyme, whose production and maintenance have fitness costs for hosts. Therefore, it seems to be a reliable indicator of host condition (González-Santoyo and Córdoba-Aguilar 2011).

Fat content is the main energy storage in insects (Rolff and Joop 2002). Territorial males with a higher fat content have been shown to win more territorial fights in damselflies (Marden and Waage 1990; Fitzstephens and Getty 2000). Fat content has been related to body condition, so we expected higher levels in mated than in unmated males not only in territorial but in the scrambling species as well.

To establish and defend territories against aggressive conspecifics requires not only energy in terms of fat reserves but also flight muscles (Marden and Waage 1990). A high ratio of flight muscle mass to total body mass has been shown to increase flight performance and success in competitive interactions in flying insects (Marden 1989; Berwaerts et al. 2002) thus we expected this trait to show higher values in mated males than in unmated males both in territorial and in scrambling species.

2.3 *Morphological traits as selective targets*

A multitude of studies documented sexual selection on phenotypic, mainly morphological, traits (Kingsolver et al. 2012). Phenotypic traits are, however, mostly not the direct target of selection and serve as a proxy for the performance trait under selection. Yet, morphology alone is often a poor predictor of performance (Wainwright et al. 2005; Hendry et al. 2011). As a morphological selective target, body size has often been cited as an important trait in determining mating success in odonates (e.g. Anholt 1991, Stoks 2000). Large size can be important for males of territorial species because they must actively establish and defend territories against aggressive conspecifics. Yet, some studies (including two studies on the here studied species *C. viridis*: De Block and Stoks 2007; Swillen et al. 2009) showed a small-male mating advantage potentially caused by a higher flight manoeuvrability of smaller males (e.g. Crompton et al. 2003).

Alternatively, no effect of size on mating success was also found (e.g. Gribbin and Thompson 1991). Non-territorial males, by contrast, achieve mating success mainly by chance. They can increase the chance of encounters with females by remaining at the pond as long as possible and being more active flyers. In this case agility is more advantageous and smaller males might be expected to have higher success (Crompton et al. 2003).

Finally, it is shown that a higher wing loading (the ratio of total body mass to wing area) and a higher wing aspect ratio (the squared wing span divided by the wing area, where wing span is two times the wing length) increase flight performance and success in competitive interactions in flying insects (Marden 1989; Berwaerts et al. 2002). Therefore higher values for these traits in mated males than in unmated males are to be expected.

2.4 *Spatial variation in sexual selection*

Our current knowledge about the spatial variation in sexual selection and its ecological causes is limited (but see Gosden and Svensson 2008; Monteiro and Lyons 2012; Johnson et al. 2013). Geographic patterns in sexual selection may be expected if (i) sexual selection patterns are driven by geographic variation in the phenotypic traits as selection on a trait depends on the mean phenotypic trait value and its variation present in the population (Conner and Hartl 2004; Steele et al. 2011). Or if (ii) sexual selection is directly driven by geographically structured environmental conditions, as these can modify the covariation pattern between a given trait and mating success (Twiss et al. 2007; Moya-Laraño et al. 2007). For instance, Arnqvist (1992) compared the sexual selection regimes in three natural populations of the water strider *Gerris odontogaster*. In this species matings are initiated by males that pounce on females and attempt forced copulation. Females are reluctant to mate and try to dislodge males. Males are having a grasping apparatus with two abdominal processes that are essential for mating success. Males with long abdominal processes are more difficult for females to dislodge. Females fight less intensely to dislodge males attempting copulation as the sex ratio becomes male biased and/or the population density increases. The author found relatively strong selection for longer abdominal processes (hence grasping ability) in two population with low densities. In the third population, where population density was the highest, sexual selection was the weakest. Spatial variation is caused most likely by density-related differences in female mating behavior between the different populations. Forsgren et al. (1996) showed that sexual selection in two populations of sand gobies, *Pomatoschistus minutus*, was affected by differences in resource availability.

In one population nest sites were scarce, leading to stronger male-male competition over nests, a higher nest site colonization rate and reduced potential for female choice compared with the other population that had a surplus of nests. Thus, spatial distribution of resources and resource availability influenced the relative contribution of intrasexual competition and mate choice in a population.

2.5 *Temporal variation in sexual selection*

Selection can show considerable temporal variation not only in strength but also in direction and form (Kingsolver et al. 2001; Kingsolver and Diamond 2011; Siepielski et al. 2009, 2011). The temporal dynamics in selection may be caused by several factors. One of the major factors are changes in the ecological conditions encountered (e.g. sex ratio: Punzalan et al. 2010; weather: Purse and Thompson 2005), thereby causing changing relationships between phenotypes and fitness. For example, the across-year variation in sexual selection on body size (going from stabilizing to disruptive) in the damselfly *Ischnura elegans* appeared to be strongly influenced by highly localized ecological factors, such as female densities and female body sizes (Gosden and Svensson 2008). *I. elegans* is sexually dimorphic, males are smaller than females, moreover females occur in three different colour morphs: androchromes (male mimic: blue coloration and male-like melanin patterning), infuscans (brown coloration and male-like melanin patterning) and infuscans-obsoleta (pinkish to reddish coloration and weak melanin patterning). Small male body size was selected for when the density of androchrome females was high whereas high density of infuscans females selected for larger males. This suggests that at high androchrome densities larger males might suffer more male harassment due to mistaken gender identity as other males may identify them as females. In case of high density of infuscan females larger males may have advantage through male-male competition. Sexual selection on male body size was stronger in populations with larger infuscans females relative to populations with smaller infuscans females, possibly because male-male competition over these highly fecund infuscans females intensifies the strength of sexual selection on male body size. Another related factor is a change in the phenotypic distribution of the population through time. For example, female mate choice in damselflies is partly determined by the fit between male (cerci) and female (mesostigmal plates) reproductive structures (e.g. Paulson 1974; Robertson and Paterson 1982), which scale with body size (McPeek et al. 2011), thus the distribution of female sizes at any given time may largely shape the fitness surface experienced by males.

Indeed, Steele et al. (2011) found that within a year, when the average female body size differed substantially from the average male body size, male body size experienced directional selection. In contrast, when male and female body size distributions overlapped, male body size experienced stabilizing selection when variances in body size were large, but no appreciable selection when the variances in body size were small.

Given the generality of these ecological factors, temporal variation in selection is likely widespread and crucial for a better understanding of the adaptive evolutionary dynamics of the targeted traits (Siepielski et al. 2009). Our understanding of the dynamics of temporal variation in sexual selection still remains limited. The relatively few studies on sexual selection indicate that sexual selection on males may vary more over time than survival selection (Siepielski et al. 2011). Furthermore, studies considering variation in selection at a smaller temporal scale than the annual scale are scarce (Siepielski et al. 2009; Miller and Svensson 2014). The few studies that looked at within year temporal variation in sexual selection showed that the strength of selection may change across the season (McLain 1992; Blanckenhorn et al. 1999; Kasumovic et al. 2008; Punzalan et al. 2010; Steele et al. 2011).

2.6 *Studying sexual selection*

The phenotype-performance-fitness paradigm (Arnold 1983) has dominated the field of ecological and evolutionary physiology for decades. According to this paradigm, phenotypic traits influence (directly and indirectly) performance traits, which in turn influence (directly or indirectly) fitness. Arnold (1983) used the term phenotype as a shorthand for biochemical, physiological, behavioral and morphological attributes that affect performance. He argued that selection can be studied directly by statistically characterizing the relationships between phenotype, performance and fitness. The statistical methodology for this characterization was based on regression techniques and advances in multivariate selection theory, which deals with the effects of selection acting simultaneously on multiple characters (Lande 1982). Before this approach, selection has usually been treated as if it is acted only on single characters. This is a huge oversimplification, since selection acts on many characters simultaneously and correlation between traits may be present (Lande and Arnold 1983). Lande and Arnold’s (1983) approach provided a tool for distinguishing the direct and indirect components (due to selection on correlated traits) of selection on a set of correlated traits. While this approach has long been advocated, it is still considerably underutilized in studies of sexual selection.

Variation in mating success is the basis of sexual selection. Mating success is the number of mates obtained by an individual. Comparing field-collected mated and unmated males is a well-established method to study sexual selection (e.g. Blanckenhorn et al. 2003, 2004; Gosden and Svensson 2008; Steele et al. 2011). Such cross-sectional approach, contrasts with the study of lifetime mating success. While the latter obviously gives a more realistic estimate of fitness (Grafen 1982; Clutton-Brock 1983), it may confound survival and sexual selection. Indeed, variation in lifetime mating success can be caused by variation resulting from variation in survivorship (hence survival selection) as well as by variation in the number of matings per day alive resulting from sexual selection (McCauley 1983).

2.7 *Quantifying and visualizing sexual selection*

The approach of Lande and Arnold (1983) based on selection gradients and differentials is suggested to be one of the best available methods for analysing selection by using observational data (Andersson 1994). When selection is acting on a trait it can be formally expressed as the statistical covariation between the given trait and relative fitness (Arnold 1983). Multivariate selection theory deals with the effects of selection acting simultaneously on multiple characters. Given that selection may act only indirectly on phenotypic traits, through their link with other traits that directly influence mating success it is important to distinguish between direct and indirect selection. Direct selection occurs when a trait is causally linked to mating success and indirect selection is present if a trait is indirectly linked to mating success because it correlated with a trait under direct selection. In order to distinguish the two scenarios one must perform not only univariate but also multivariate regressions of the studied traits against relative fitness.

Selection coefficients are of two basic types. The selection differential reflects the impact of total selection: the sum of indirect selection on correlated traits as well as direct selection on the trait in question. The combined effect of direct and indirect selection on a trait, the total selection, was estimated by univariate selection differentials, which were quantified by performing univariate regressions of each trait separately against relative fitness. The following equations can be used to estimate the univariate linear selection differential (S) on a trait z: $w = a + S z$ and the univariate quadratic selection differential (C): $w = a + S z + 0.5\, C z^2$ which includes the squared trait values in the regression. The selection gradients on the other hand correct for selection on correlated traits and estimate only the direct effects of selection on a trait (Lande and Arnold 1983). They can be quantified by multiple regression of all traits under study against relative fitness.

A linear selection gradient is the slope (the regression coefficient) of the standardized trait against relative fitness (Lande and Arnold 1983). A quadratic selection gradient is 2× the regression coefficient of the squared term of the standardized trait against relative fitness (Stinchcombe et al. 2008). The equation to estimate the multivariate linear selection gradient (β) is: $w= a + \Sigma(\beta_i)z_i$ and the multivariate quadratic selection gradient (γ) is: $w= a + \Sigma(\beta_i)z_i + 0.5\ \Sigma(\gamma_i)z_i^2$ which includes the squared trait values in the regressions. Therefore, traits that are only significant in the univariate regression are under indirect selection and traits that are also significant in the multivariate regression are under direct selection. For cross-sectional studies, relative fitness (w) is calculated by dividing individual fitness (0 for unmated, 1 for mated males) by the mean fitness of the males in a given sampling day.

Note that several traits, such as flight endurance, cannot only be under sexual selection but also under survival selection. This, however, is no problem for cross-sectional studies because sexual selection is studied by comparing trait values between samples of mated and unmated males alive at a given day. By doing so, it avoids any problem of males with a certain trait value having more matings in their life because they live longer (hence perform better in survival selection) rather than because they have higher mating rates (hence perform better in sexual selection). The strength of selection is defined by the magnitude of the gradients. The sign of the linear selection gradient (β) defines the direction of selection. The sign of the quadratic selection gradient (γ) defines whether stabilizing ($\gamma < 0$, where intermediate trait values give the highest mating success) or disruptive ($\gamma > 0$, where extreme trait values give the highest mating success) selection is at work. Quadratic selection gradients thus measure the curvature of the selection surface [the relationship between the relative fitness of individuals in a population and the values of a phenotypic trait (Pearson 1903)].

When one uses simple linear regression to estimate the selection coefficients, significance values are not reliable because the dependent variable (in this case mating status: 0/1) is binomially distributed rather than normally distributed. Therefore tests of statistical significance have to be performed using logistic regression (Janzen and Stern 1998).

To visualise and estimate selection surfaces, the cubic spline is a powerful tool. It is a method to smoothen the relationship of a trait against relative fitness. Its goal is to accurately describe the selection surface without making any prior assumptions about its shape (Schluter 1988).

2.8 *Concluding remarks*

In summary, despite the many studies on sexual selection, three aspects remain understudied, yet very relevant to fully understand sexual selection on the traits of a given species. (1) While most of the studies investigating sexual selection focused on a single or few phenotypic traits (e.g. Gosden and Svensson 2008; Steele et al. 2011), a minority of studies followed a multivariate approach (e.g. Blanckenhorn et al. 2003; Swillen et al. 2009; Punzalan et al. 2010). This is important to disentangle direct selection versus indirect sexual selection on a trait. (2) Most studies focused on morphological traits (mainly body size) and ignored physiological traits, for example related to energy reserves (but see e.g. Blanckenhorn et al. 2003, 2004; Swillen et al. 2009), and locomotor performance (but see e.g. Kelly et al. 2008). (3) Finally, recent studies suggest that sexual selection may vary both among populations and among time periods within a given population (e.g. Gosden and Svensson 2008; Steele et al. 2011). Paying attention to such spatiotemporal variation in sexual selection may for example learn whether the same trait is preferred across populations and seasons.

3. ***Study system***

Insects in general are suggested to be ideal model organisms to investigate spatiotemporal variation (and the ecological influences) on sexual selection (Miller and Svensson, 2014). Odonates are especially good models because they are often abundant, relatively easy to catch and mark, readily observed, and geographically widespread yet generally concentrated on and near water sources (Corbet 1999; Córdoba-Aguilar 2008). The thesis is focusing on damselflies, upcoming model systems in ecology and evolution (Córdoba-Aguilar 2008). Given that selection should be acting on different performance traits depending on the mating system, more on endurance in scrambling species and more on speed in territorial species (Husak et al. 2006; Husak and Fox 2008), we studied both scrambling and territorial species.

One of the study species is *Chalcolestes viridis* (Vander Linden, 1825), a widespread territorial damselfly in Europe (Dijkstra and Lewington 2006) (Fig. 3). The species is univoltine, overwinters in the egg stage, and has a short aquatic larval stage of ca. three months in spring (De Block and Stoks 2004). Adults emerge over a long period, which may take >2 months in natural populations (Agüero-Pelegrin et al. 1999). Males reach reproductive maturity after ca. 5 weeks and have a mean reproductive lifespan of ca. 3 weeks (Cordero 1988). Reproduction in our study region occurs from late July through October (R Stoks, unpublished data).

Males defend small territories in oviposition trees by aggressively chasing away other males and use a sit-and-wait strategy in these trees to capture passing females in flight (Dreyer 1978). Males of *C. viridis* stay connected to females throughout the entire oviposition bout (duration: 1.43 ± 0.08 h, mean ± 1 SE, n = 42; Fig. lb in De Block and Stoks 2005). Females oviposit in overhanging branches of trees at the waterside and mate up to seven times during their reproductive life (De Block and Stoks 2005). The eastern form of *C. viridis*, which was long considered as a subspecies, is *C. parvidens*. In Chapter II, we compared these two taxa in order to clarify their taxonomic status.

The other study species is *Coenagrion puella* (Linnaeus, 1758), one of the most common damselflies in Central Europe typically found at ponds (Fig. 4). Females are described to be polymorphic in colour while males are always blue (Dijkstra and Lewington 2006). Males search for females by patrolling low over the breeding pond and display scramble competition for mates. The species shows no female behaviour to assess and select potential mates indicating there is no female choice (Banks and Thompson 1985). *C. puella* pairs remain in wheel position for an average of 28 min and pairs remain in tandem for an average of 111 min (Banks and Thompson 1985). Males contact-guard after mating and accompany the females during egg laying in tandem position (Anholt et al. 2001). When oviposition has finished the male releases the female, who flies rapidly away from the pond. *C. puella* females mated only once on a given day (Banks and Thompson 1985).

Figure 3. Pairs of C. viridis (left) and C. parvidens (right). Photo credit: HAGY

Figure 4. Copulating pair of Coenagrion puella. (Photo credit: Ambrus András)

4. *General methods and traits under investigation*

In Chapter II we reconstructed phylogenetic trees to resolve the taxonomic position of *C. viridis* and its eastern form. We compared morphometry of body, head, wings and cerci of males from several populations to determine if the two taxa can be unequivocally distinguished using simple measurements. We also included males with intermediate traits (based on appendage colour and shape) assumed to be hybrids (according to Olias et al. 2007) to test whether these traits show intermediate morphometry. Additionally, we analysed DNA sequences from nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase I (COI) regions to test whether these taxa represent separate monophyletic groups. These DNA sequences were also used to compare the genetic distance between the two taxa with those found among well-accepted European *Lestes* species.

To study multivariate sexual selection on whole-organism performance and the underlying morphological and physiological traits we used the cross-sectional sampling method where mated and unmated males were collected with hand-held nets in the field. Their flight performance (speed and endurance) were tested in a 2 m high plexiglass flight tube (Fig. 5) in situ or in the laboratory among controlled conditions. In case of *C. viridis* flight performance was tested in the laboratory. At the start of a trial, males were gently placed at the bottom of the flight tube with their head facing the same direction. Then, they were gently touched in a standardized way with a blunt object to initiate a flight bout. Each flight bout consisted of a fast linear upward trajectory during which the highest vertical distance of the flight trajectory was attained, whereafter males kept flying until they finally reached the bottom of the flight tube again. The flight tube was graded every 5 cm to allow estimating the maximum height of the flight bout. Using the grid lines on the tube and a chronometer we quantified three variables for each flight bout: the time needed to reach the maximum height, the maximum height and the total duration of the flight bout (time until the animal reached the bottom again). We estimated the flight speed as the maximum height divided by the time to reach it, and flight endurance as the total flight time. *C. puella* males in contrast were tested in the flight tube *in situ*. Flight trials were executed in the afternoon at the day of capture. All males took off spontaneously within a few seconds from their cup. Here, we also estimated the flight speed as the height reached during the first linear part of the flight divided by the time needed to reach that height. Furthermore, key flight-related morphological and physiological traits were also quantified for both species (see below).

Figure 5. Experimental set-up to quantify flight performance of damselflies in the field. The transparent Plexiglas flight tube (height: 2 m, diameter: 20 cm) was placed vertically, connected with a metal ring to a wooden mainstay and was fixed on a wooden ground plate for stabilisation. The top of the tube was covered with a grey non-transparent lid. The cup containing the animal was placed at the bottom of the tube and the lid of the cup was gently removed at the start of the flight test. (Photo credit: Lieven Therry)

In Chapter III, we applied a multivariate approach to study temporal variation in sexual selection on two key performance traits, flight speed and flight endurance, and a set of underlying morphological and physiological traits throughout an entire reproductive season in a population of the territorial damselfly *Chalcolestes viridis* (Vander Linden, 1825). In addition, we considered selection on four flight-related traits: fat content, flight muscle ratio, wing loading and wing length.

In Chapter IV, we applied a functional approach to explore the phenotype-performance-fitness axis in the scrambling damselfly *Coenagrion puella* by studying the links between flight performance, the underlying morphological and physiological traits and short-term mating success. We quantified a set of physiological traits related to body condition: a measure of investment in immune function (the activity of the enzyme phenoloxidase), energy storage (fat content) and relative flight muscle mass (flight muscle ratio), and a set of morphological traits related to flight in damselflies: wing length, aspect ratio and wing loading. To assess consistency of selection patterns we collected mated and unmated males from six populations in Central Europe.

5. *Research aims*

The goal of the thesis is to study multivariate sexual selection on whole-organism performance and the underlying morphological and physiological traits and contrast this between species with a scrambling and territorial mating system. As the taxonomic position of one study species is debated, an additional objective is to reconstruct phylogenetic trees to resolve the position of the species.

In **Chapter II**, our aim is to clarify the taxonomic position of the study species *C. viridis*. More specifically, we investigate whether it belongs to the genus *Lestes* or *Chalcolestes* and study its relation to *C. parvidens*. We test whether these taxa represent separate monophyletic groups as well as compare the genetic distance with those that are found between well-accepted European *Lestes* species.

In **Chaper III**, we apply a multivariate approach to study how temporal variation in different morphological and physiological traits may generate temporal variation in whole-organism performance traits (flight speed and flight endurance) and in sexual selection throughout the entire reproductive season in one population of the territorial damselfly *C. viridis*. Here, we analyse the effects of the mating status on flight performance and the underlying morphological and physiological traits along the temporal gradient.

In **Chapter IV**, we explore the phenotype-performance-fitness axis in the scrambling damselfly *C. puella* by studying the links between a set of physiological and morphological traits, flight performance, and short-term mating success.

We quantified a set of physiological traits related to body condition: a measure of investment in immune function (the activity of the enzyme phenoloxidase), energy storage (fat content) and relative flight muscle mass (flight muscle ratio), and a set of morphological traits related to flight in damselflies: wing length, aspect ratio and wing loading. To test for the consistency of the results across populations we include several populations of *C. puella* from Central Europe.

The here presented study has four key strengths: (i) With the so called functional approach (Lailvaux and Irschick 2006) we integrate phenotypic traits, performance traits and the fitness component in a single study which reveals insights that cannot be reached otherwise. (ii) The direct quantification of traits from a wide range provides a multivariate picture of trait differentiation between mated and unmated males in terms of sexual selection. (iii) Including understudied traits such as performance traits and physiological traits helps to refine our current insights on spatial and temporal dynamics of selection. (iv) We provide the first test for temporally varying sexual selection on performance traits and the covariation between flight performance and the underlying morphological and physiological traits.

The different chapters of this thesis are presented as manuscripts and can be read separately. This may result in some overlap among chapters with regard to literature, methods and results.

CHAPTER II

Morphometric and molecular studies on the populations of the damselflies **Chalcolestes viridis** *and* **C. parvidens** *(Odonata, Lestidae)*

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Modified version

Abstract

Morphometric and genetic differences were analysed for two closely related damselflies, *Chalcolestes viridis* and *C. parvidens*. A total of 305 male individuals were collected from six European countries (Austria, Croatia, Germany, Greece, Hungary and Portugal). Measurements from a total of 28 populations of *C. viridis* and *C. parvidens* and several intermediate forms were collected to determine if they can be definitely distinguished using simple morphometric characters. DNA sequences from two independent loci (nuclear ribosomal ITS region and mitochondrial cytochrome oxidase I gene) were analysed to test whether these taxa represent separate monophyletic groups as well as to compare the genetic distance with those found between well-accepted European *Lestes* species. Discriminant analysis revealed that *C. viridis* and *C. parvidens* are differentiated in morphometric space. Individuals with intermediate anal appendage traits overlapped with both *C. viridis* and *C. parvidens* which raised the possibility that they are merely subspecies of a single species. However, genetic analysis of both investigated DNA regions showed that the two *Chalcolestes* taxa did not share haplotypes, indicating their status as true species. Furthermore, they formed a monophyletic group separated from the investigated *Lestes* species, supporting the recognition of the genus *Chalcolestes*. The two *Chalcolestes* species are very closely related compared with European *Lestes* species, suggesting that their divergence occurred relatively recently.

1. Introduction

Traditionally, taxonomy is based on phenotypic analyses; although in many taxa this approach is impossible due to the lack of sufficient morphological characters (Wilkerson et al. 1993; Chilton et al. 1995; Floyd et al. 2002). For many aquatic insect orders such as Ephemeroptera (Ball et al. 2005; Williams et al. 2006; Alexander et al. 2009), Diptera (Pfenninger et al. 2007), Coleoptera (Balke et al. 2007; Dutton and Angus 2007) and Trichoptera (Pauls et al. 2010) morphological characters alone do not allow reliable distinction. Hence molecular genetic techniques have become widespread in taxonomic studies. Although the number of studies combining DNA sequences and morphology are increasing, relatively few studies have focused on odonates (e.g. Pilgrim et al. 2002; Stoks et al. 2005; Pilgrim and von Dohlen 2007). Not surprisingly, many debated taxonomic affiliations still remain in this order (Schmidt 2001; Dijkstra 2003; Dijkstra and Lewington 2006).

One of these issues concerns *Chalcolestes viridis* (Vander Linden, 1825) and *C. parvidens* Artobolevskii, 1929 (Jödicke 1997; Schmidt 2001). Traditionally, both taxa were placed in the genus *Lestes* Leach, 1815, because adults are similar to other European *Lestes* species. However, given that their larvae differ by having a broad instead of a greatly narrowed (spoon-shaped) prementum, Kennedy elevated them to a separate genus *Chalcolestes* in 1920. Nevertheless this new genus name has not been generally accepted but seems well supported based on DNA-based phylogeny of two separate genes (Dumont et al. 2010). Another debated issue is the rank of the taxon parvidens which was originally described by Artobolevskii in 1929 as a subspecies of *C. viridis* but later has been considered to be a valid species based on phenotypic traits and electrophoretic analyses (Cobolli et al. 1994). Both taxa live in similar habitats, including ponds, lakes, canals and slow-flowing rivers, with overhanging bushes required for oviposition (Askew 2004). Where the two co-exist they segregate in their daily timing of the reproductive periods, indicating some level of reproductive isolation (Dell'Anna et al. 1996). Yet, the species status of *C. parvidens* has not been widely accepted (Jödicke 1997; Sternberg and Buchwald 1999; Wildermuth et al. 2005) although some authors have treated it as a good species (Askew 2004; Dijkstra and Lewington 2006; Olias et al. 2007).

The size and the general appearance of the two taxa are very similar. Size differences have been described but the literature is equivocal (e.g. Utzeri et al. 1995; Marinov 2000). The most important discriminatory traits for males are found on the anal appendages: *C. parvidens* has a smaller tooth on the inner border of the cerci, which is placed slightly more dorsal than in *C. viridis*, and its paraprocts also have more slender and strongly up-curved tips (Askew 2004; Dijkstra and Lewington 2006). Furthermore, the anal appendages differ in colour pattern: the cerci of male *C. parvidens* are yellow with a sharply defined black tip, while those of *C. viridis* are more diffusely darkened at the apex and also at the base (Askew 2004). These differences may, however, represent intraspecific variation instead of stable species-level differences (figure 54 in Jödicke 1997) and may also be affected by factors such as the age of the specimen. Overlapping characters (Matushkina 2006; Olias et al. 2007) and hybrids with intermediate male traits have been reported between these two taxa (Cobolli et al. 1994; Dell'Anna et al. 1996; Olias et al. 2007).

We compared morphometry of body, head, wings and cerci of males from several populations to determine if the two taxa can be unequivocally distinguished using simple measurements. We also included males with intermediate traits (based on appendage colour and shape) assumed to be hybrids (according to Olias et al. 2007) to test whether these parameters show intermediate morphometry. Additionally, we analysed DNA sequences from nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase I (COI) regions to test whether these taxa represent separate monophyletic groups. These DNA sequences were also used to compare the genetic distance between the two taxa with those found among well-accepted European *Lestes* species.

2. Material and methods

For morphometric comparison, 305 adult male damselflies were collected from Austria (AT), Croatia (HR), Germany (DE), Greece (GR), Hungary (HU) and Portugal (PT). A subset of 30 individuals was used for DNA analyses, including the following outgroup taxa: *Lestes barbarus* (Fabricius, 1798), *Lestes dryas* Kirby, 1890, *Lestes macrostigma* (Eversmann, 1836), *Lestes sponsa* (Hansemann, 1823), *Lestes virens vestalis* Rambur, 1842, *Lestes virens virens* (Charpentier, 1825) and *Sympecma fusca* (Vander Linden, 1820) (see Table 1 for details). At four of the 28 sampling localities both taxa were present. Here, we collected males with intermediate traits which may represent hybrids based on the colour and shape of the anal appendages (according to figure 1 in Olias et al. 2007). Specimens were collected and preserved in 70% ethanol for measurements and absolute ethanol for DNA isolation. Taxa were identified and classified in three groups a priori using Askew (2004), Dijkstra and Lewington (2006) and Olias et al. (2007).

Table 1. Collection information of the investigated specimens. Country codes: AT – Austria, DE – Germany, GR – Greece, HR – Croatia, HU – Hungary, PT – Portugal. Locality codes are given [in square brackets] for the specimens that were subjected to molecular biological analysis. Collector codes:[1] Attila Ferenc Kalmár,[2] György Dévai,[3] Hajnalka Anna Gyulavári,[4] Henrietta Beáta Nagy,[5] Margit Miskolczi,[6] Theodor Benken,[7] Tibor Jakab,[8] Valér Horvai,[9] Zoltán Varga. n1: number of individuals used for morphometric analysis, n2: number of individuals used for genetic analysis.

Specimens	Locality	Country	Coordinates	Date and collector	n1	n2
Chalcolestes parvidens	Apetloner Wäldchen	AT	N47°44',E16°48'	16 September 2009[6]	1	0
	Kiesgrube, N Illmitz	AT	N47°46'',E16°48'	15 September 2009[6]	4	0
	Drava floodplain, N Botovo [B]	HR	N46°14',E16°56'	28 October 2009[8]	2	1
	Stream, E Tavronitis, Crete [T]	GR	N35°31',E23°49'	28 May 1994[2,5]	2	1
	Bédai-Holt-Duna, Erdőfű (Kölked) [BK]	HU	N45°54',E18°45'	30 August 2009[3,8]	0	1
	Diás-sziget, Kis-Balaton (Keszthely)	HU	N46°40',E17°13'	12 August 2009[3]	10	0
	Fancsikai-mocsár (Debrecen)	HU	N47°30',E21°44'	29 August 2008[2,5]	17	0
	Fenyves-tömpöly (Debrecen) [FeD]	HU	N47°30',E21°45'	07 August 2009[1,3]	8	1
	Fertő (Fertőboz) [FF]	HU	N47°38',E16°42'	02 September 2009[2,5]	6	1
	Halápi-tározó (Debrecen)	HU	N47°30',E21°47'	25 September 2006[4,9]	33	0
	Halápi-tározó (Debrecen)	HU	N47°30',E21°47'	29 August 2008[2,5]	63	0
	Kis-mező -szegi-Holt-Tisza (Kisar) [KK]	HU	N48°03',E22°26'	16 August 2007[2,5]	18	1
	Marázs (Egyek)	HU	N47°40',E20°51'	09 August 2007[7]	6	0
	Mentett-rét (Tiszaalpár)	HU	N46°49',E19°59'	31 July 2008[2,5]	37	0
	Mentett-rét (Tiszaalpár)	HU	N46°49',E19°59'	02 August 2008[2,5]	4	0
	Mentett-rét (Tiszaalpár) [MT]	HU	N46°49',E19°59'	03 July 2009[2]	11	1
	Nagy-berek (Darány) [ND]	HU	N45°59',E17°33'	27 August 2009[3,8]	11	1
	Suhonya (Pörböly) [SP]	HU	N46°11',E18°50'	15 August 2009[3]	10	2

Continuation of Table 1.

Specimens	Locality	Country	Coordinates	Date and collector	n1	n2
Chalcolestes viridis	Kiesgrube, N Illmitz	AT	N47°46',E16°48'	15 September 2009[6]	1	0
	Kiesgrube, NE Wallern	AT	N47°44',E16°57'	19 September 2009[6]	1	0
	Rosalia Kapelle	AT	N47°46',E16°50'	15 September 2009[6]	1	0
	Schwarzseelacke	AT	N47°44',E16°53'	19 September 2009[6]	1	0
	Backwater, NW Rheinbischofsheim [R]	DE	N48°39',E07°54'	21 August 2009[3,6]	11	2
	Bacsó-nyak-alji-mocsár (Aggtelek) [BA]	HU	N48°28',E20°29'	06 September 2009[2,5]	3	1
	Barbacsi-tó (Barbacs) [BB]	HU	N47°37',E17°19'	02 September 2009[2,5]	0	1
	Kőzet-parki-tanösvény (Ipolytarnóc) [KI]	HU	N48°13',E19°39'	19 August 2009[1]	1	1
	Vörös-tó (Aggtelek)	HU	N48°28',E20°32'	06 September 2009[2,5]	6	1
	Rio Anqueira, Algoso	PT	N41°27',W06°35'	08 July 2010[3]	7	0
	Rio Sabor, Gimonde	PT	N41°48',W06°41'	07 July 2010[1]	1	0
	Rio Anqueira, Uva	PT	N41°29',W06°31'	08 July 2010[3]	3	0
	Vila Chã de Braciosa	PT	N41°26',W06°19'	11 July 2010[3]	1	0
Intermediate forms	Graben, W Pamhagen	AT	N47°42',E16°52'	16 September 2009[6]	5	0
	Kiesgrube, N Illmitz [I]	AT	N47°46',E16°48'	15 September 2009[6]	2	1
	Kiesgrube, NE Wallern	AT	N47°44',E16°57'	19 September 2009[6]	2	0
	Fertő (Fertőboz) [FF]	HU	N47°38',E16°42'	02 September 2009[2,5]	4	1
Lestes barbarus	Fancsikai-mocsár (Debrecen)[FD]	HU	N47°30',E21°44'	13 July 2010[2]	0	2
Lestes dryas	Nagy-szik (Balmazújváros) [NB]	HU	N47°35',E21°20'	12 July 2010[2]	0	2
Lestes macrostigma	Kelemen-szék (Fülöpszállás) [KF]	HU	N46°47',E19°10'	22 July 2010[2,7]	0	2
Lestes sponsa	Halápi-tározó (Debrecen) [HD]	HU	N47°30',E21°47'	14 July 2010[5]	0	2
Lestes virens vestalis	Nagy-szik (Balmazújváros) [NB]	HU	N47°36',E21°22'	15 July 2010[2]	0	2
Lestes virens virens	Pond, SW Duas Igrejas [D]	PT	N41°27',W06°22'	11 July 2010[3]	0	1
Sympecma fusca	Halápi-tározó (Debrecen) [HD]	HU	N47°30',E21°47'	14 July 2010[2]	0	1

Sixteen continuous characters were measured with digital caliper (DC) and a stereomicroscope using an ocular micrometer (SM) (Figure 1). These characters (Figure 1) were: the total length of the body (BL, from the frons to the tip of the cerci), the abdomen (AL), both pairs of fore and hind wings (RFw, RHw, LFw, LHw) and the width of the head (HW) and labrum (LW); the distance between the compound eyes (CED), the antenna scapes (ASD), and the nodus and the pterostigma on the right wings (RFwNP, RHwNP); and the characteristic dimensions of the cerci (C1–C4; Figure 1d). To allow accurate measurements, the head, wings and abdomen were separated for each individual with scissors after measurement of BL and AL, and body parts were placed at the same fixed position. To identify traits that separate the taxa and to evaluate the position of intermediate forms we performed a forward stepwise discriminant function analysis using Statistica version 9.1. (StatSoft, Tulsa, OK, USA). In each step of such an analysis all variables are evaluated to determine which contributes most to the discrimination of groups.

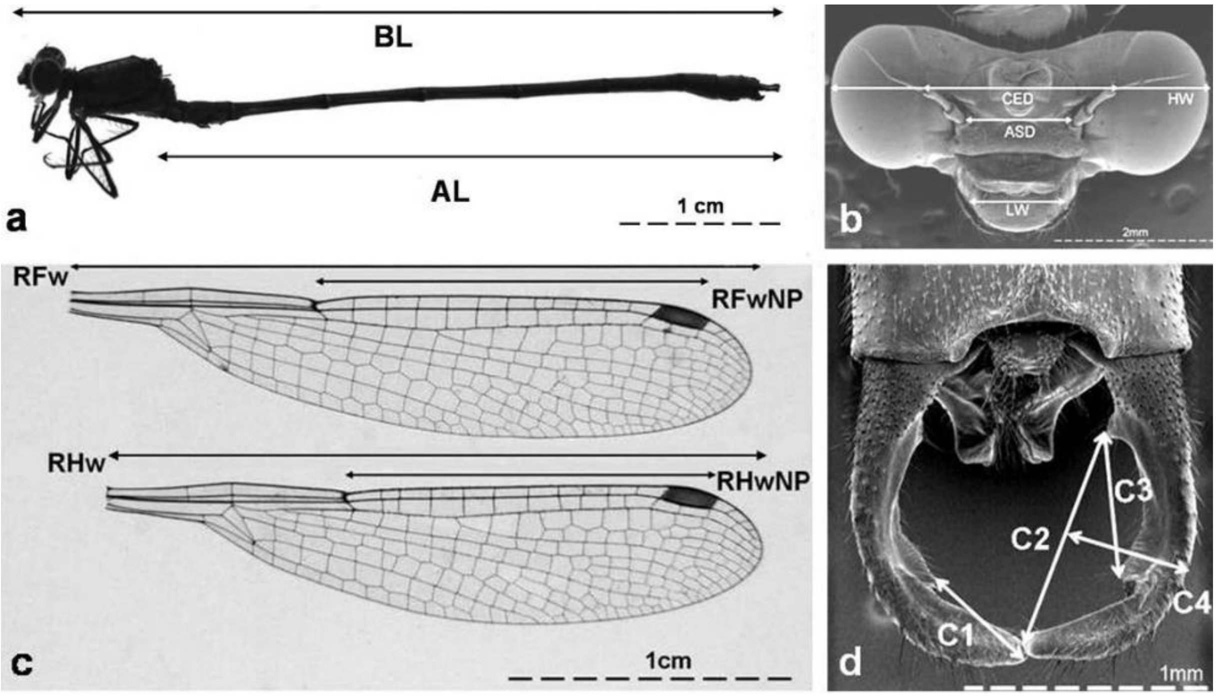

Figure 1. Characters used in the morphometric analysis of the two Chalcolestes taxa. (a) Total length of the body and the abdomen of a male C. viridis. (B) Measurements of the head of a male C. viridis. (C) Right wing measures of a male C. parvidens. (D) Anal appendages of a male C. viridis (dorsal view). See Material and methods for definition of the morphometric characters.

For genetic analysis muscular tissue was excised from the thoracic muscle and total DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was applied to amplify the nuclear ribosomal ITS region (containing the ITS1, 5.8S rRNA gene and ITS2) with the primers ETTS1 [5'-TGC TTA AGT TCA GCG GGT-3'] and ETTS2 [5'-TAA CAA GGT TTC CGT AGG TGA A-3'] (Kane and Rollinson, 1994). To amplify the mitochondrial COI gene we used the primers CW-3031 [5'-TTT GC(A/C) CT(A/T) ATC TGC C(A/C)T ATT-3'] (Heinze et al. 2005) and C1- J-2195 [5'-TTG AAT TTT TGG TCA TCC AGA AGT-3'] (Simon et al. 1994). Reactions were performed in a final volume of 50 µl using approximately 2 µl of purified genomic DNA, 0.2 mM of each deoxynucleotide, 2 mM MgCl2 , 1 U LC *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1× PCR buffer (Fermentas), 0.325 µM of each primers. The thermal profile consisted of 5 min at 96ºC for initial denaturation, followed by 35 cycles of 1 min at 48ºC in case of COI PCR or at 54ºC in case of the ITS PCR for annealing, 1 min at 72ºC for extension and 1 min at 94ºC for denaturation and with a final step of 10 min at 72ºC for final extension. Amplified products were visualised on agarose gel stained with ethidium bromide. PCR products were purified using PCR-M™ Clean Up System (Viogene, Sijhih, Taiwan). DNA fragments were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The Chromas software v1.45 (Technelysium, Brisbane, QLD, Australia) was used for the manual correction of automatic base calling on chromatograms and for the removal of primer sequences. Sequence alignments were generated with ClustalW (Thompson et al. 1994). Alignments were corrected manually using the MEGA5 software (Tamura et al. 2011), and only unambiguously aligned positions were retained for subsequent phlyogenetic analyses. Maximum likelihood (ML) and maximum parsimony (MP) analyses were performed with the MEGA5 software. Nucleotide substitution models were selected based on the results calculated with Modeltest version 3.7 (Posada and Crandall, 1998). For COI sequences the Hasegawa-Kishino-Yano model using gamma distribution with invariable sites (HKY+I+G) and for ITS sequences the Kimura 2-parameter nucleotide substitution model using gamma distribution (K80+G) was applied. Bayesian analysis was performed with MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) applying the settings nst = 6 and rates = invgamma for 500,000 (ITS) and 1,000,000 generations (COI). Pairwise distances were calculated using the Kimura 2-parameter model in MEGA5. Sequences determined in this study were deposited in GenBank (accession numbers for ITS: HQ830270-HQ830298; for COI: HQ830299-HQ830321).

3. Results

Discriminant function analysis showed that traits contributing significantly to the overall discrimination were measurements of the cerci (C3, C2 and C1), sizes of the head (CED, LW and HW), the distances between the nodus and pterostigma on the forewing (RFwNP) and the total body length (BL). Overall, Wilks' lambda was low (0.339), which means that most of the total variability was attributable to differences among the means of the groups [F (32, 550) = 12.312, $p < 0.001$].

Different independent discriminant functions were computed in a canonical analysis to see how the variables discriminate between the different groups (Figure 2). The first discriminant function represented most (96.51%) of the discriminatory power and was weighted most heavily by some wing measurements (RFwNP and LFw), body length (BL) and one dimension of the cerci (C3). This function was marked by positive coefficients for the body length and one cerci size and negative coefficients for the wing measurements (Table 2). Thus, the longer the body and the cerci, and the shorter the wings, the more likely it is that the individual is *C. parvidens*. The second function accounted for a minor (3.49%) portion of the variance and seemed to be marked mostly also by wing sizes (RHw, RFw and LHw), the labrum width (LW) and the length of the abdomen (AL). The first discriminant function discriminated mostly between *C. viridis* and the other two groups. The second function was most useful to separate the intermediate forms; however, the magnitude of the discrimination was much smaller. Squared Mahalanobis distances (D^2) between *C. viridis* and *C. parvidens* were more than two times larger than distances between the intermediate forms and the other two taxa. The Mahalanobis distance is the distance of a case from the centroid in the multidimensional space, defined by the correlated independent variables. The distance matrix also showed that intermediate forms are almost as close to *C. viridis* as *C. parvidens* ($D^2_{\textit{C. viridis, C. parvidens}} = 15.21$; $D^2_{\textit{C. viridis}, \text{intermediate forms}} = 5.62$; $D^2_{\textit{C. parvidens}, \text{intermediate forms}} = 5.13$). In general, males of *C. viridis* and *C. parvidens* are separated well from each other whilst intermediate forms were not differentiated well from *C. viridis* and *C. parvidens.*

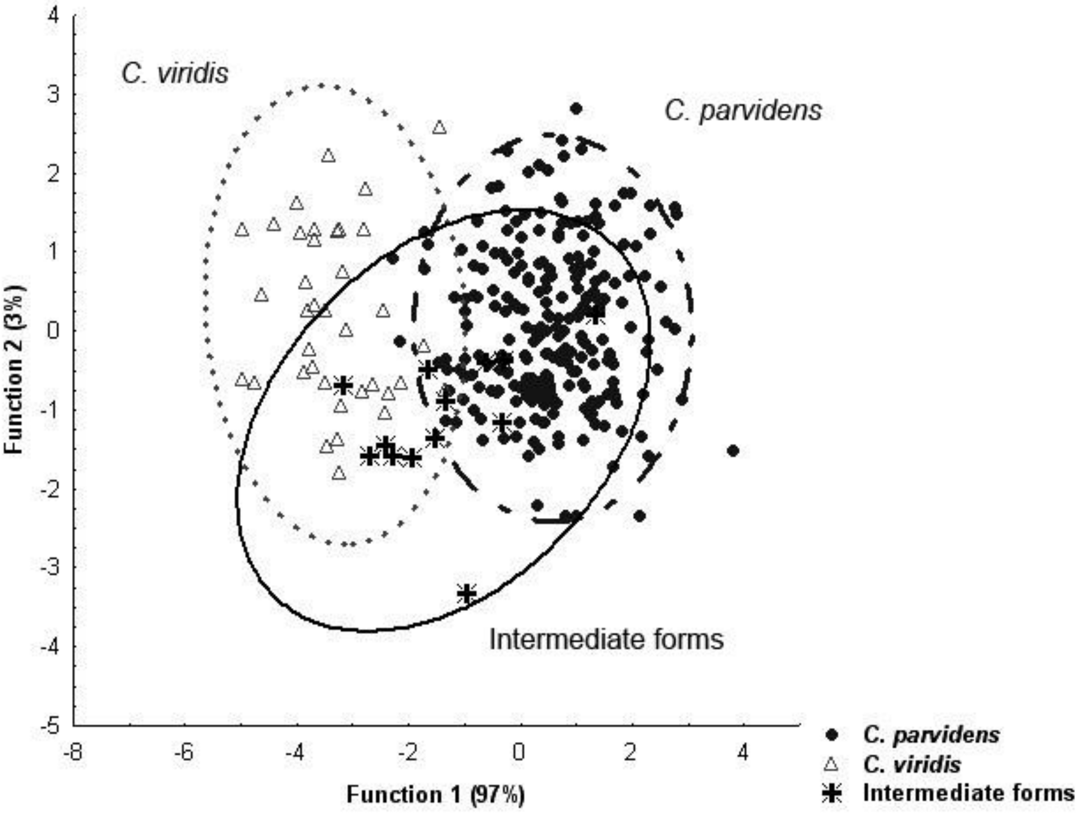

Figure 2. Plot of the discriminant analysis based on the morphometric measurements of C. viridis, C. parvidens and the intermediate forms.

Table 2. Results of the forward stepwise discriminant function analysis. Canonical vector coefficients for the two first axis (CV1 and CV2) and correlations between the values of the discriminant function and the values of variables (r1 and r2) are given.

Character	CV1	CV2	*r*1	*r*2
BL	0.592	0.559	0.108	0.076
AL	0.303	-0.802	0.124	0.000
RFw	0.188	-1.197	-0.490	-0.066
LFw	-0.727	0.533	-0.502	-0.026
RHw	-0.184	1.429	-0.458	-0.001
LHw	0.043	-0.689	-0.471	-0.024
RFwNP	-0.744	-0.138	-0.484	-0.029
RHwNP	0.548	0.155	-0.395	0.062
HW	0.249	-0.400	-0.254	-0.247
CED	-0.461	-0.245	-0.454	-0.080
ASD	0.307	-0.419	-0.117	-0.020
LW	-0.119	1.071	-0.237	0.484
C1	-0.227	-0.419	-0.391	-0.329
C2	-0.342	0.172	-0.119	-0.206
C3	0.615	-0.430	0.234	-0.247
C4	0.110	-0.084	0.066	-0.114

An assignment test based on the squared Mahalanobis distances between each sample and the center of each group (the group centroids defined by the respective group means for each variable) showed that in total, 13 cases were misclassified. The percentage of cases that were correctly classified for *C. parvidens* was 99.18% and for *C. viridis* was 91.89%. From the 13 intermediate forms six males were classified as *C. parvidens* and two males as *C. viridis* (only 38.46% of the total cases were correctly classified) which demonstrated that there were no obvious differences to separate these intermediate forms from the supposed parental taxa. Males with intermediate traits of the anal appendages had intermediate sizes (Table 3). The group of intermediate forms also showed higher standard errors for most measured characters and possessed remarkable morphometric variability compared with the two groups formed by the unequivocally identified *C. viridis* and *C. parvidens* specimens.

Table 3. Results of the measurements of the two Chalcolestes taxa and the intermediate forms. Mean and standard error (SE) are given. See Material and methods for definition of morphometric characters and measurement technique (Mt) abbreviations. Sizes are given in millimetres.

	C. parvidens		*C. viridis*		Intermediate forms		Mt
Character	Mean	SE	Mean	SE	Mean	SE	
BL	45.410	0.097	44.797	0.223	44.962	0.410	DC
AL	36.796	0.081	36.203	0.169	36.500	0.354	DC
RFw	24.156	0.049	25.649	0.147	24.965	0.253	DC
LFw	24.120	0.048	25.648	0.149	24.907	0.276	DC
RHw	23.336	0.046	24.677	0.142	24.005	0.244	DC
LHw	23.274	0.046	24.648	0.144	23.981	0.256	DC
RFwNP	14.126	0.030	15.053	0.091	14.606	0.183	SM
RHwNP	13.496	0.029	14.237	0.091	13.828	0.173	SM
HW	5.033	0.011	5.182	0.020	5.156	0.029	SM
CED	2.421	0.003	2.519	0.011	2.476	0.020	SM
ASD	1.356	0.005	1.391	0.006	1.375	0.009	SM
LW	1.293	0.002	1.332	0.008	1.288	0.008	SM
C1	0.414	0.002	0.455	0.004	0.446	0.005	SM
C2	0.987	0.002	1.003	0.007	1.005	0.013	SM
C3	0.644	0.003	0.605	0.005	0.637	0.008	SM
C4	0.470	0.001	0.464	0.004	0.470	0.005	SM

The phylogenetic relationships of the investigated damselflies were recovered using two different DNA regions: COI and ITS (Figure 3). All examined specimens of *C. parvidens* and of *C. viridis* formed separate monophyletic clades with high bootstrap support for both independent loci (>95 in the case of COI and >80 in the case of ITS based on three different analyses). Maximal pairwise sequence divergences for COI and ITS within taxa were 0.8% and zero among *C. parvidens* individuals, 0.6% and 0.2% among *C. viridis* individuals, respectively. In contrast, divergences between the two taxa for these DNA regions were approximately an order of magnitude higher: 12.4–13.8% and 1.0–1.2%. *Chalcolestes parvidens* and *C. viridis* together formed a monophyletic group with high bootstrap support (≥99) clearly separated from the investigated *Lestes* species. In the case of the *Lestes* species, within-species dissimilarity values ranged between zero (*L. barbarus*) and 1.3% (*L. sponsa*) for COI sequences and between zero (*L. dryas* and *L. macrostigma*) and 0.3% (*L. sponsa* and *L. virens*) for ITS sequences. These divergence values were similar to those observed in the *Chalcolestes* species. Between *Lestes* species nucleotide sequence divergence ranged from 7.3% (*L. dryas* and *L. sponsa*) to 16.9% (*L. virens virens* and *L. barbarus*) based on COI and from 1.8% (*L. dryas* and *L. sponsa*) to 12.9% (*L. sponsa* and *L. virens*) based on ITS data. These dissimilarity values are also very similar to those in *Chalcolestes* (~13% and ~1.1%, respectively). Divergence in the ITS region between the two subspecies *L. v. virens* and *L. v. vestalis* only ranged from 0.2% to 0.3%; similar to the variation within the *Chalcolestes* taxa. On the whole, sequence dissimilarity between the two *Chalcolestes* species was similar to those of the investigated *Lestes* species based on COI while in the ITS region interspecies sequence dissimilarity was slightly lower than among *Lestes* species, although considerably higher than among the studied *Lestes* subspecies. Furthermore, all species and genus level categories investigated in this study were supported with high bootstrap values (usually >95). Finally, *Sympecma*, rather than *Lestes*, was recovered as sister taxon to *Chalcolestes* in the ITS tree and as sister to *Lestes* in the COI tree, i.e. in neither analysis were *Chalcolestes* and *Lestes* sister taxa to one another.

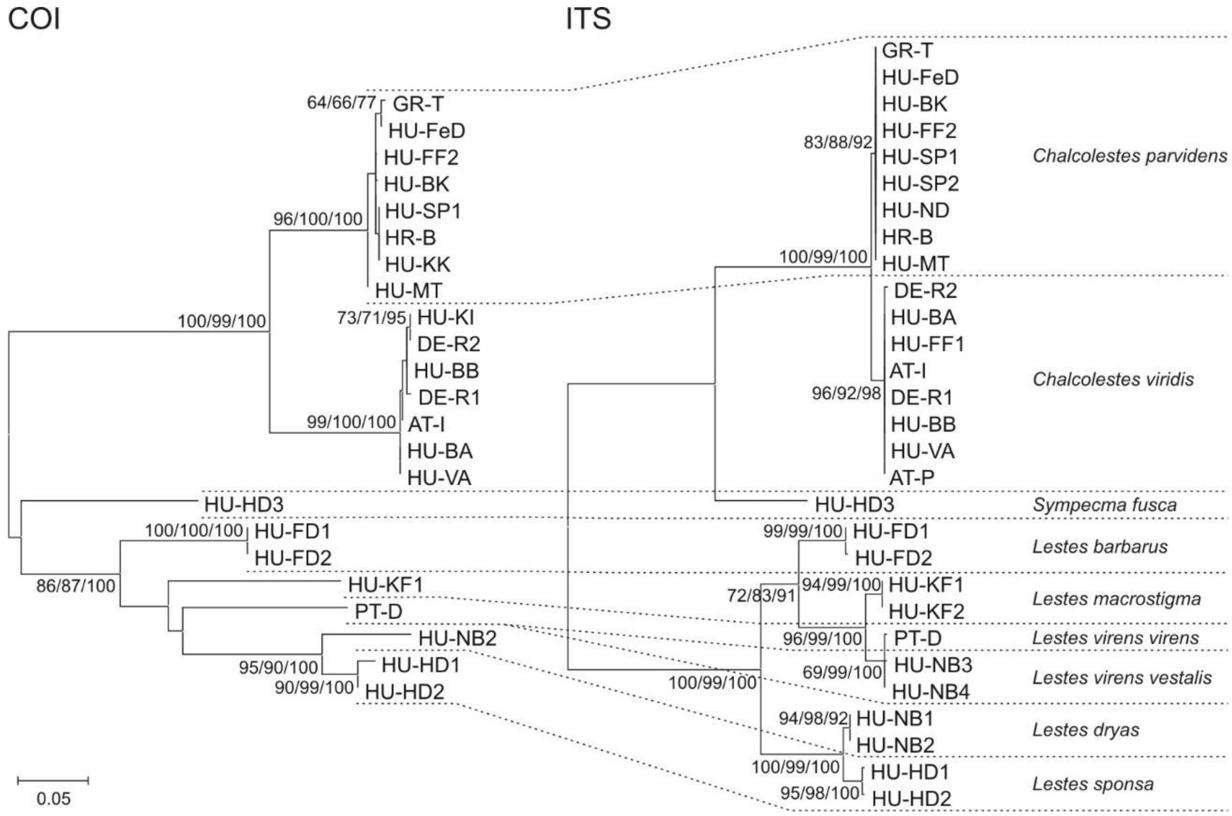

Figure 3. Phylogenetic relationship of Chalcolestes and Lestes taxa investigated in this study using mitochondrial cytochrome oxidase subunit I (COI) gene and genomic ribosomal ITS sequences. Sequence analyses were performed based on 534 and 643 unambiguously aligned nucleotide positions, respectively. Phylogenetic trees were constructed according to the maximum likelihood (ML) method. Bayesian (B) posterior probabilities and bootstrap values greater than 50 [based on 500 and 1000 replicates for ML and maximum parsimony (MP), respectively] are shown (order: ML/MP/B). Specimen codes were generated from the abbreviations of sampling sites: the first two letters are the country code and the additional letters indicate sampling locality, and numbers are serial numbers (if at least two specimens were sequenced from the same locality). See country and locality codes in Table 1.

4. Discussion

Our genetic data support the generic status of *Chalcolestes*. The two *Chalcolestes* taxa formed a monophyletic group separated from other investigated *Lestes* species based on both the mitochondrial COI gene and the nuclear ribosomal ITS region. Dumont et al. (2010) analysed the 5.8S and 18S rRNA genes augmented with ITS1 and ITS2 sequences and concluded that *Chalcolestes* formed a monophyletic group with *Sympecma* and *Indolestes* distantly from the true *Lestes* species. In the present study, similar grouping patterns were observable in the phylogenetic trees; however, present sampling is far from exhaustive. Because *Lestes* is a large, cosmopolitan and heterogeneous genus with 84 species (Schorr et al. 2011), further study is needed from the family *Lestidae* to establish the exact position of *Chalcolestes*. Even though we were unable to find morphometric characters that would always conclusively distinguish *C. viridis* from *C. parvidens* they were differentiated in morphometric space. Our quantitative analysis of specimens from widely different regions showed that *C. parvidens* males usually had a longer body and smaller wings than *C. viridis* males. Contrary to this, Utzeri et al. (1995) reported not only smaller wings but also a shorter body in two Italian populations of *C. parvidens*, while Marinov (2000) reported equal body lengths but smaller wings. Because we did not include Italian and Bulgarian specimens in our study it remains to be tested whether animals from these regions differ morphometrically.

Female damselflies have mechanoreceptors on the mesostigmal plate that are stimulated by male cerci during tandem linkage (Robertson and Paterson 1982). Smaller males may have difficulties grasping the thorax of a larger female with the cerci, while males that are too large may not be able to position their cerci correctly on the thoracic plates of the females. Loibl (1958) provided evidence that females of *Lestes* recognize conspecific males due to the tactile stimulation of the pronotum by the male appendages which are distinctively shaped. Females refused to copulate with conspecific males having experimentally altered inferior appendages, indicating mechanical or tactile isolation. Therefore further studies are recommended that focus not only on the cerci but also on the paraprocts.

McPeek et al. (2011) tested species identity traits in mate choice among six species of *Enallagma* damselflies (Coenagrionidae). They concluded that cerci sizes overlapped among species. In contrast, cerci shapes were non-overlapping among species, and five of six *Enallagma* species showed no population variation across their entire range. This suggests that cerci shape is the primary feature to discriminate conspecifics from heterospecifics during mating. This reflects the view that anal appendages are determinative for species recognition, hence reproductive isolation, in some odonates (Loibl 1958; Paulson, 1974; McPeek et al. 2008).

Therefore, the differences in size and shape may reduce the probability of heterospecific copulation, but this mechanism apparently does not completely separate the two *Chalcolestes* taxa. This was supported with another study, in which we examined 20 pairs in copula in NW Hungary (where both species live syntopically), and found two pairs of male *C. viridis* with female *C. parvidens* and one reverse combination and some individuals with intermediate traits (Gyulavári et al. personal observation).

Probably after the Würm and earlier glaciations, the two *Chalcolestes* taxa re-advanced into Europe from different refugia in the western (*C. viridis*) and eastern Mediterranean (*C. parvidens*) (Dévai 1976; Samraoui et al. 2003). In the Carpathian basin and in the Mediterranean region, the western and eastern invaders presumably mixed (Stewart and Lister 2001), which resulted in a contact zone with intermediate forms. The intermediate forms suggest that the two *Chalcolestes* taxa are capable of interbreeding, but further studies are needed to determine the fertility of such offspring.

Genetic analysis showed that the two *Chalcolestes* taxa are closely related compared to other European Lestes species, but also that they form two monophyletic groups without shared haplotypes, suggesting that lineage sorting was complete and supporting the species status of *C. parvidens*. However, interspecies sequence dissimilarity values suggest a close relationship and a relatively recent divergence of the two taxa. This may also explain the occurrence of individuals with intermediate morphological traits presumed to be hybrids. Although we find the data supportive of recognizing *C. parvidens* as a distinct species, detailed morphometric (including shape analysis of the cerci and paraprocts) and genetic analysis of individuals of possibly hybridizing populations in the contact zone, and fertility studies on potential hybrids are recommended.

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Author contributions: HAGY, TF, KM and GYD conceived and designed the experiments. HAGY, TB, MM, VH and GYD collected the samples. CSCS was involved in the SE microscope photo documentation. HAGY and TF performed the laboratory work. HAGY, LJSZ and TF analyzed the data. HAGY, TF and GYD contributed to the manuscript.

CHAPTER III

Sexual selection is more stable on flight performance than on morphology and physiology

Hajnalka Anna Gyulavári, Lieven Therry, György Dévai and Robby Stoks

Abstract

While selection is a major evolutionary force, our understanding of the temporal variation in selection patterns remains limited. This is because studies are strongly biased toward survival selection, temporal variation among years, and typically only consider single, mostly morphological, traits. Here, we studied fine-scaled sexual selection patterns on two key performance traits, flight speed and flight endurance, and a set of underlying morphological and physiological traits throughout an entire reproductive season in a natural population of the territorial damselfly *Chalcolestes viridis*. While some traits had nearly stable trait values (flight speed and wing length), others showed considerable temporal variation (flight endurance, fat content, flight muscle ratio and wing loading). Despite the fine-scaled temporal dynamics in flight endurance, sexual selection consistently favoured males with a higher flight endurance. In contrast, selection on two flight-related traits (body size and wing loading) varied strongly in time. We hypothesize that selection on performance traits which are direct targets of selection will in general be more stable through time than selection on the underlying phenotypic traits. Our results indicate that current insights on temporal dynamics of selection that are mainly based on morphological traits may not hold across trait types. In addition, more studies focusing on understudied traits such as performance traits and physiological traits are needed to refine our knowledge of the temporal dynamics of selection patterns in nature. This is of paramount importance as the inclusion of the temporal dynamics of selection is highly needed to arrive at a better understanding of adaptive evolutionary dynamics of traits in natural populations.

1. Introduction

Selection can show considerable temporal variation not only in strength but also in direction and form (Kingsolver et al. 2001; Kingsolver and Diamond 2011; Siepielski et al. 2009, 2011). Several factors may cause temporal dynamics in selection. One major factor is the changes in the ecological conditions encountered (e.g. sex ratio: Gosden and Svensson 2009; weather: Purse and Thompson 2005), thereby causing changing relationships between phenotypes and fitness. Another related factor is a change in the phenotypic distribution of the population through time (Steele et al. 2011). Given the generality of these factors, temporal variation in selection is likely widespread and crucial for a better understanding of the adaptive evolutionary dynamics of phenotypic traits (Siepielski et al. 2009). For example, fluctuating selection may maintain genetic variation in the targeted traits (Bell 2010).

Our understanding of the dynamics of temporal variation in selection still remains limited. First, most of what we know is based on studies dealing with survival selection while much less studies focused on sexual selection. Yet, the relatively few studies on sexual selection indicate that sexual selection may vary more over time than survival selection in males (Siepielski et al. 2011). Second, survival and sexual selection studies mainly considered variation across years, while we know much less about variation in selection at smaller temporal scales (Siepielski et al. 2009; Miller and Svensson 2014). The few studies that looked at within year temporal variation in sexual selection showed that the strength of selection may change across the season (McLain 1992; Blanckenhorn et al. 1999; Kasumovic et al. 2008; Punzalan et al. 2010; Steele et al. 2011). Third, our knowledge on temporal variation in both survival and sexual selection is largely based on studies focusing on morphology, life history and to a much lesser extent behaviour, while studies on physiology are virtually absent (Siepielski et al. 2009). Moreover, while selection is expected to directly act on organismal performance traits, such as locomotor ability, rather than on the underlying morphological and physiological traits (Irschick et al. 2007, 2008), no studies considered temporal variation in selection on whole-organism performance (the maximal ability of an animal to conduct an ecologically relevant, physically challenging task, Irschick et al. 2008). Fourth, most studies looking at temporal variation in both survival and sexual selection only considered a single trait, so we do not know whether selection patterns on different traits show similar fluctuations through time. Given that different combinations of underlying morphological and physiological traits may contribute to similar performance (many-to-one mapping, Strobbe et al. 2009), and that performance is more directly linked to fitness it may be expected that selection on performance is more stable through time than selection on the underlying traits (for a similar pattern at a spatial scale, see Therry et al. 2014).

Here, we applied a multivariate approach to study temporal variation in sexual selection on two key performance traits, flight speed and flight endurance, and a set of underlying morphological and physiological traits throughout an entire reproductive season in a population of the territorial damselfly *Chalcolestes viridis* (Vander Linden, 1825). As in flying territorial species ownership of mating territories is decided by aerial male-male contests, we expected consistent sexual selection on flight speed and flight endurance (Lailvaux and Irschick 2006; Husak et al. 2008). In addition, we considered selection on four flight-related traits: fat content, flight muscle ratio, wing loading and wing length. Territorial males with a higher fat content have been shown to win more territorial fights in damselflies (Marden and Waage 1990; Fitzstephens and Getty 2000). A high ratio of flight muscle mass to total body mass and a higher wing loading increases flight performance and success in competitive interactions in flying insects (Marden 1989; Berwaerts et al. 2002). Finally, also size (including wing length) has been associated with territorial success, yet the pattern is not consistent. In most territorial species larger males have a higher mating success (Andersson 1994). Yet, some studies (including two studies on the here studied species: De Block and Stoks 2007; Swillen et al. 2009) showed a small-male mating advantage potentially caused by a higher flight manoeuvrability of smaller males (e.g. Crompton et al. 2003), or no effect of size on mating success (e.g. Gribbin and Thompson 1991). We expected both flight performance traits and the underlying flight-related traits to show temporal variation but, given their closer link to mating success, sexual selection on flight performance traits to be more stable through time compared to selection on flight-related traits.

2. Materials and methods

2.1 Study species and collection

Chalcolestes viridis is a widespread territorial damselfly in Europe (Dijkstra and Lewington 2006). The species is univoltine, overwinters in the egg stage, and has a short aquatic larval stage of ca. three months in spring (De Block and Stoks 2004). Adults emerge over a long period, which may take >2 months in natural populations (Agüero-Pelegrin et al. 1999). Males have a mean reproductive lifespan of ca. 3 weeks (Cordero 1988). As a result, there is a mixture of age classes in the sexually mature part of the population throughout most of the reproductive season. Following emergence, the exoskeleton and wings harden and reach their final size.

Reproduction in our study region occurs from late July through October (R Stoks, unpublished data). Females oviposit in overhanging branches of trees at the waterside. Males defend small territories in oviposition trees by aggressively chasing away other males and use a sit-and-wait strategy in these trees to capture passing females in flight (Dreyer 1978).

We quantified sexual selection by comparing field-collected mated and unmated males, a well-established method to study sexual selection in natural populations (e.g. Blanckenhorn et al. 1999; Gosden and Svensson 2008; Steele et al. 2011). We collected a total of 282 mated and 238 unmated males throughout the entire 12-weeks reproductive season in 2011 at a large pond (perimeter: ca. 2210 m) in Tervuren, Belgium. To study temporal variation in sexual selection, we divided the reproductive season in six 2-week periods with (except for the last period) similar sample sizes (Table 1). We kept the sample sizes low per sampling day to assure a short interval between capture and the flight performance test (see below). Therefore, and because we only sampled at sunny days, it was not meaningful to define shorter time periods.

All males were caught on sunny days between 12:30 and 15:30 hours, when sexual activity was observed. We considered a male as mated when caught in tandem or copulation wheel. Unmated males were those not associated with a female but that were active at the reproduction site. Note that we cannot fully exclude that males captured singly may have mated before capture or would have mated after capture (Steele et al. 2011). If so, this would have introduced noise in our dataset and therefore make our results conservative (i.e. the real covariation patterns between a trait and relative fitness would have been stronger if we had classified these males correctly as mated).

Each collected male was stored in a separate opaque cup (diameter: 7.5 cm; height 4.5 cm) placed in a dark and cooled box (15 °C). Each time a mated male was caught, the associated female was kept separately in another cup and not directly released to avoid that other, uncaptured males from the population would start mating with one of these females. Most (2/3) of the females were released after the sampling on a given day had ended, yet 106 females (ca. 1/3) were kept for another project. Although we removed in total many males from the study population across the 12 weeks (n = 520), we only removed ca. 25 males per sampling day (Table 1). This likely only had a slight effect on the sex ratio, given the large male population size that was estimated (based on a transect counts of 100m shoreline the total male population size on a given day could reach >2,000 males). Note that in damselfly populations there is a large turnover because the relatively short lifespan compared to the length of the adult flight period.

2.2 Flight performance test

At the day of capture, each male was tested for its flight performance (flight speed and flight endurance) in the laboratory. This was done in a Plexiglas flight tube (2 meter high, 20 cm diameter) which stood in a 43° angle using the methodology described in Therry et al. (2014) that was adjusted for the laboratory. Given the absence of sun light in the laboratory, we placed a strong 500 W halogen lamp (type: EP 202) above the transparent Plexiglas lid of the flight tube. Before the trials, males were allowed to acclimate for ca. 10 minutes at room temperature (23°C). At the start of a trial, males were gently placed at the bottom of the flight tube with their head facing the same direction. Then, they were gently touched in a standardized way with a blunt object to initiate a flight bout. A single observer (HAGY) conducted all flight trials.

Each flight bout consisted of a fast linear upward trajectory during which the highest vertical distance of the flight trajectory was attained, where after males kept flying until they finally reached the bottom of the flight tube again. The flight tube was graded every 5 cm to allow estimating the maximum height of the flight bout. Using the grid lines on the tube and a chronometer (accuracy 0.01s) we quantified three variables for each flight bout: the time needed to reach the maximum height, the maximum height and the total duration of the flight bout (time until the animal reached the bottom again). We estimated the flight speed as the maximum height divided by the time to reach it, and flight endurance as the total flight time. Three flight bouts were obtained for each individual, and the maximum values for flight speed and flight endurance per male were retained for analysis (for justification of the use of maximum values in performance studies, see e.g. Adolph and Pickering 2008). Both flight performance traits showed significant repeatability (following Lessells and Boag 1987) with intraclass correlation coefficients being 0.17 for endurance ($F_{1,458} = 1.60$, $p < 0.0001$) and 0.11 for speed ($F_{1,458} = 1.36$, $p < 0.0001$). Afterwards, each male was placed in a separate Eppendorf tube and flash-frozen at -80 °C for later analyses.

We additionally quantified two variables that may potentially interfere with the measured flight performance traits. First, because temperature may influence insect flight performance (Samejima and Tsubaki 2010), we tried to minimize and correct for temperature differences among males. Although the room where the flight test was done was temperature-controlled, the heat of the halogen lamp induced gradual warming of the flight tube. At the end of each flight test, air temperature was measured to the nearest 0.1 °C with a thermometer at a height of 70 cm in the tube. In addition, on a subset of males the thorax temperature was measured to the nearest 0.1 °C with a micro-thermocouple (BAT-12 type, company Physitemp

Instruments, Inc., Clifton, NJ, USA). Given that thorax temperatures and air temperatures were correlated ($r = 0.38$, $N = 121$, $p < 0.001$) we included only the latter in the statistical analyses. Replacing thorax temperature with air temperature did not change the conclusions. Second, we also quantified the time that each male was stored in the cooled box between capture and the moment its flight test began (interval length).

2.3 Physiological and morphological flight-related traits

For each male we quantified a set of four physiological and morphological flight-related traits in damselflies: energy storage (fat content), flight muscle ratio, wing length and wing loading. Sample sizes differed slightly per trait because some wings were accidentally damaged after the flight test and some samples accidentally thawed. Exact sample sizes per response variable are given in Table S1.

Males were dissected while keeping them on dry ice. Body parts were thawed on ice and the thorax and the abdomen were weighed separately to obtain the total wet body mass. We then measured fat content in the thorax. The thorax was 15 times diluted, homogenized in ice-cold homogenization buffer [25 mM Hepes, pH 7.4, 2 mM EDTA, 10 mM β-mercaptoethanol (βME)] using a pestle and centrifuged (10,000 g) at 4 ºC. The resulting supernatant was used for further analysis. Fat content was quantified based on the protocol of Bligh and Dyer (1959). We filled 2 ml glass bottles with 20 µl of the homogenates and 140 µl sulfuric acid (100%). The bottles were heated for 20 minutes at 150 ºC. Afterwards, 160 µl mili-Q water was added. We filled a transparent 384 well microtiter plate with 30 µl of the sample and measured absorbance at 340 nm. Based on a standard curve of glyceryl tripalmitate we converted absorbance into fat content. Relative fat content was calculated as the ratio of the thoracic fat content to total wet body mass and expressed as mg fat per mg wet mass.

As the flight muscles of damselflies make up most of the thorax (Marden 1989), we estimated the amount of flight muscle by quantifying the protein content of the thorax (see e.g. Snell-Rood et al. 2014). For this, we used the protocol of Bradford (1976). We mixed 1 µl homogenate, 160 µl mili-Q water and 40 µl Bio-Rad Protein Dye in a 96 well plate. After an incubation of 5 minutes at 30 ºC, we measured the absorbance at 595 nm. We estimated the flight muscle mass as the protein content of the thorax based on a standard curve of known concentrations of bovine serum albumin. The flight muscle ratio (FMR) was calculated as the ratio of the estimated flight muscle mass to total wet body mass and expressed as mg per mg wet mass.

Wing length was used as a proxy for body size (see e.g. also Plaistow and Siva-Jothy 1999; Carchini et al. 2000). It has been explicitly shown in other damselflies that when taking other body size measures such as body length and abdomen length, these all group together on a single PC with wing length, hence all reflect body size (e.g. Gosden and Svensson 2008; Steele et al. 2011). Wing length and wing area were quantified on the digital pictures of the left hind wing using the software TpsDig 2.16 (Rohlf 2010). Wing length was calculated as the distance between the proximal apex of the quadrilateral cell and the proximal tip of the pterostigma (see Fig. S1). To quantify wing area we digitized 17 landmarks along the wing margin (Fig. S1) thereby obtaining a polygon spanning the wing surface. Wing loading was measured as the ratio of total wet body mass to wing area.

2.4 Statistical analyses

All statistical analyses were carried out with STATISTICA v.12. To meet ANOVA assumptions we log-transformed flight speed, flight endurance and the relative fat content. To test whether the flight performance traits and the physiological and morphological flight-related traits changed through the reproductive season, we ran separate general linear models per trait that included period as response variable and mating status as categorical predictor variable. In the case of the flight performance traits, temperature and the interval length between capture and flight test were included as continuous predictor variables in the model.

When testing for selection, all traits were standardized to a mean of zero and a standard deviation of one. To test for total sexual selection on flight performance, we ran univariate generalized linear models (separately per flight performance trait) with the flight performance traits as the predictor variables and the mating status (0 for unmated, 1 for mated males) as binary response variable (Lande and Arnold 1983). To generate p-values, we used a binomial error structure and the logit link function (Janzen and Stern 1998). When testing for quadratic selection, we also included the squared terms of the flight performance traits. We explicitly took into account that males were collected at different time periods by including period as a categorical factor in the models. We also included interactions with period and the flight performance traits to check for consistency of sexual selection on flight performance across the reproductive season. In case of significant period-by-trait interactions, we ran separate analyses per period. Furthermore, we added the following covariates to the model: air temperature in the flight tube and the interval length that each male had spent in the cooled box before the flight test began. To test for direct sexual selection on a given flight performance trait, we ran multivariate generalized linear models using a similar statistical model as described above but that included both flight performance traits as independent variables.

Whenever directional sexual selection on a trait was significant we report the univariate linear selection differential (*S*) and the multivariate linear selection gradient (β) as the regression coefficients of the standardized trait against relative fitness (Lande and Arnold 1983). Whenever quadratic sexual selection on a trait was significant we report the univariate quadratic selection differential (*C*) and the multivariate quadratic selection gradient (γ) as 2× the regression coefficients of the squared term of the standardized trait against relative fitness (Stinchcombe et al. 2008). Regression coefficients were extracted from the same model as explained above, yet using a normal error distribution (Janzen and Stern 1998). Univariate and multivariate linear and quadratic selection gradients are reported per trait and per period in Table S1.

We similarly evaluated sexual selection on the underlying physiological and morphological flight-related traits. To explicitly test whether these traits contributed to flight performance, we ran separate general linear models per flight performance trait with all four phenotypic traits and their quadratic terms as continuous predictor variables and the flight performance traits as dependent variables. We also included period and interactions between period and the phenotypic traits and their quadratic terms.

We visualised selection on the flight performance traits and on the four flight-related traits using cubic splines with the software glms 4.0 (Schluter 1988). When no significant period-by-trait interaction was present, indicating consistent selection patterns across time, we presented an overall fitness function across the reproductive season. Otherwise, separate fitness functions per period are shown.

3. Results

While flight speed did not show a clear temporal pattern, flight endurance showed a hump-back shape with lower values at the start and at the end of the reproductive season (ANOVA, Period effect, flight speed: $F_{5,486} = 2.08$, $p = 0.067$, flight endurance: $F_{5,487} = 5.17$, $p < 0.0001$, Figure 1a,b). Of the four flight-related traits, relative fat content ($F_{5,499} = 122.55$, $p < 0.0001$), flight muscle ratio ($F_{5,497} = 21.65$, $p < 0.0001$) and wing loading ($F_{5,486} = 7.95$, $p < 0.0001$) showed clear temporal variation with higher values in period 2 and a tendency for lower values in period 3. Wing length, instead, showed no obvious temporal pattern ($F_{5,466} = 1.95$, $p = 0.085$, Fig. 1c-f).

Table 1. Sampling scheme across the six periods during the 2011 reproductive season of the damselfly C. viridis at the Tervuren study pond.

Period	Dates	# Mated males	# Unmated males	# Subtotal mated males	# Subtotal unmated males
1	01 August	9	10	61	71
1	02 August	14	15		
1	04 August	24	24		
1	10 August	6	8		
1	11 August	8	14		
2	16 August	15	15	45	45
2	17 August	16	16		
2	25 August	14	14		
3	01 September	14	14	54	48
3	02 September	17	13		
3	10 September	15	11		
3	15 September	8	10		
4	24 September	15	11	50	38
4	25 September	8	7		
4	28 September	10	10		
4	29 September	7	7		
4	30 September	10	3		
5	01 October	22	9	50	22
5	02 October	16	5		
5	15 October	12	8		
6	16 October	20	10	22	14
6	24 October	2	4		
Total				282	238

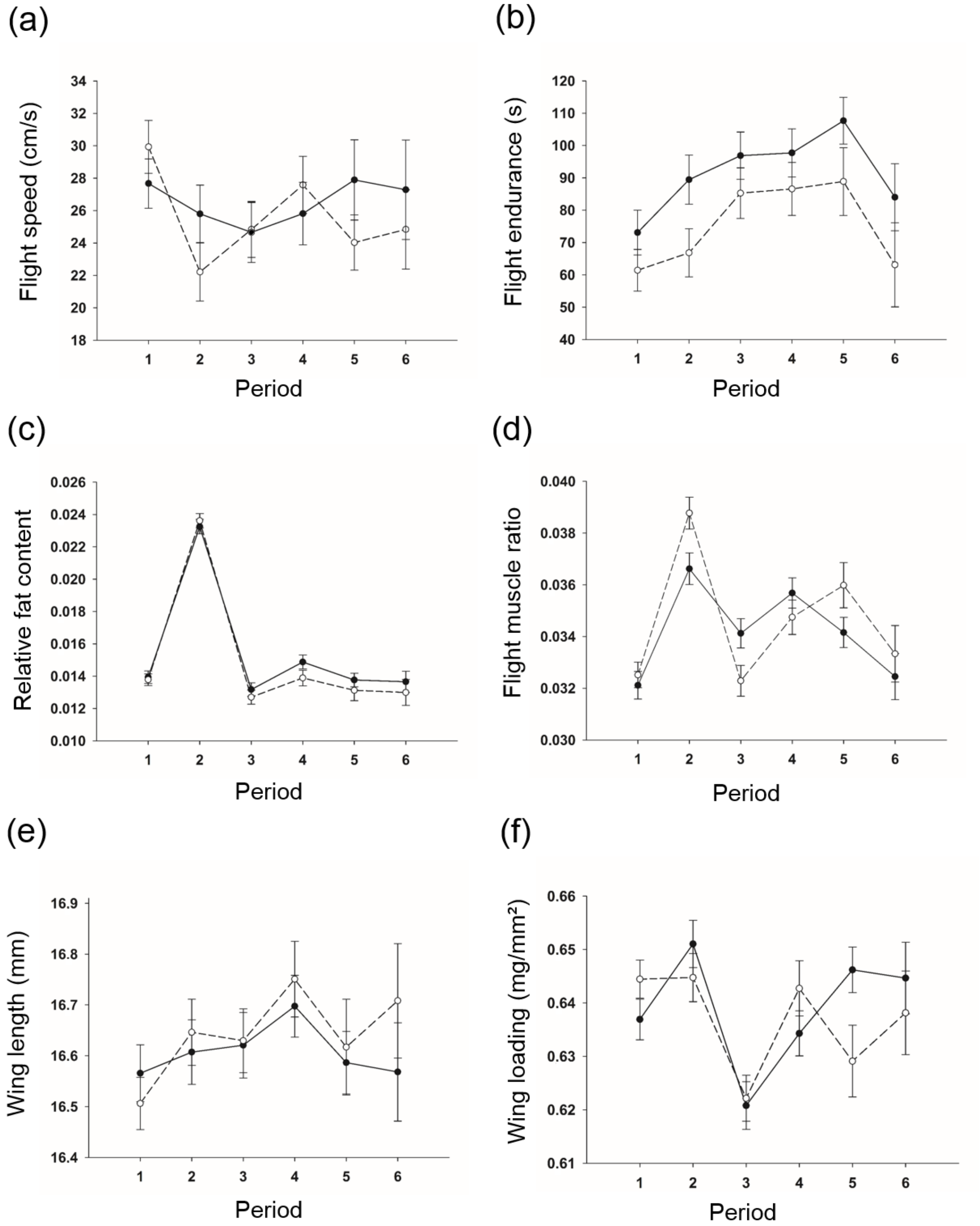

Figure 1. Temporal variation in the flight performance traits: (a) flight speed and (b) flight endurance, and the four flight-related traits: (c) relative fat content, (d) flight muscle ratio, (e) wing length, and (f) wing loading in mated males (solid line and solid circles) and unmated males (dashed line and open circles) of C. viridis across the entire reproductive season.

Both the univariate and the multivariate analyses did not detect a clear pattern of selection on flight speed (Tables 2-3, Fig. 2a). The multivariate analysis did show a Flight speed × Period interaction indicating that the direction of selection was not consistent across the periods (Table 3, Fig. 2a). However, in no single period, was the selection on flight speed significant (all $p > 0.066$). Flight endurance was under positive directional selection with mated males having a higher flight endurance than unmated males, this was upheld in both the univariate and multivariate analyses ($S = 0.12$, $\beta = 0.12$; Tables 2-3, Fig. 2b). Directional sexual selection for a higher endurance was consistent across periods as indicated by the absence of Flight endurance × Period interactions (Tables 2-3).

Table 2. Univariate results of the generalized linear models examining sexual selection on the two flight performance traits across the reproductive season of the damselfly C. viridis. The response variable is the mating status (0 = unmated, 1 = mated). Significant p-values are indicated in bold. Interval length refers to the duration between capture and the flight test.

		Trait shaping mating success			
		Flight speed		Flight endurance	
	Df	χ^2	p	χ^2	p
Period	5	111.038	**0.049**	42.546	0.51
Trait	1	20.325	0.15	191.192	**< 0.0001**
Trait^2	1	0.3433	0.56	15.087	0.22
Trait × period	5	99.836	0.08	58.333	0.32
Trait^2 × period	5	45.095	0.48	61.950	0.29
Temperature	1	73.960	**0.007**	33.274	0.07
Interval length	1	88.105	**0.003**	71.893	**0.007**

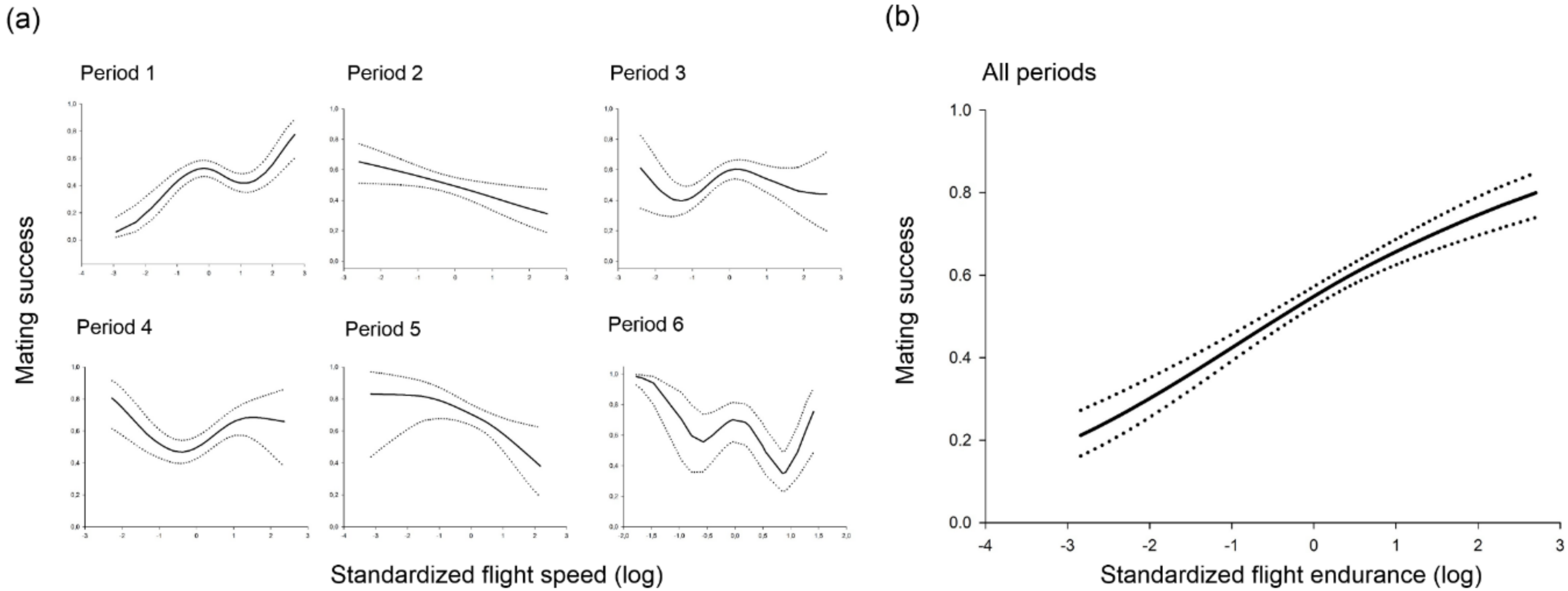

Figure 2. Fitness functions (solid lines) relating mating success of the damselfly C. viridis to flight performance: (a) flight speed and (b) flight endurance. Dotted lines delineate 1 SE bands (based on 999 bootstrap replicates) around the fitness function which was visualized using cubic splines.

Table 3. Multivariate results of the generalized linear models examining sexual selection on the two flight performance traits across the reproductive season of the damselfly C. viridis. Significant p-values are indicated in bold. Interval length refers to the duration between capture and the flight test.

	Mating success		
	df	χ^2	p
Period	5	7.5780	0.18
Flight speed	1	0.2802	0.60
Flight speed^2	1	1.7471	0.19
Flight speed × Period	5	13.8804	**0.016**
Flight speed^2 × Period	5	10.8515	0.05
Flight endurance	1	22.4775	**< 0.0001**
Flight endurance^2	1	2.7308	0.10
Flight endurance × Period	5	4.1777	0.52
Flight endurance^2 × Period	5	10.3518	0.07
Temperature	1	4.4373	**0.035**
Interval length	1	7.8062	**0.005**

Of the four flight-related traits, the relative fat content and the flight muscle ratio explained variation in flight speed (Table 4). Males with a higher relative fat content had a lower flight speed (Fig. 3a). Males with a higher flight muscle ratio had a higher flight speed but only in period 4 as indicated by the Flight muscle ratio × Period interaction (Table 4, Fig. 3b). Except for the flight muscle ratio, the three other traits significantly contributed to flight endurance (Table 4). Males with intermediate values for relative fat content, wing length and wing loading had a higher flight endurance as indicated by the significant quadratic terms (Table 4, Fig. 3c-e). In case of wing length this pattern was not consistent across periods and not formally significant in any of the periods (Wing length^2 × Period, Table 4, Fig. 3d).

Table 4. Results of the general linear models testing for the contribution of the four flight-related traits to both flight performance traits (flight speed and flight endurance) across the reproductive season of the damselfly C. viridis. Significant p-values are indicated in bold. Interval length refers to the duration between capture and the flight test.

	Flight speed			Flight endurance		
	df	F	p	df	F	p
Period (Per)	5, 395	0.53	0.75	5, 411	2.40	**0.037**
Relative fat content	1, 395	5.01	**0.026**	1, 411	0.03	0.87
Relative fat content^2	1, 395	2.11	0.15	1, 411	5.24	**0.023**
Relative fat content × Per	5, 395	0.67	0.64	5, 411	1.80	0.11
Relative fat content ^{2} × Per	5, 395	0.39	0.86	5, 395	0.81	0.54
Flight muscle ratio	1, 395	1.11	0.29	1, 411	0.25	0.62
Flight muscle ratio^2	1, 395	1.21	0.27	1, 410	2.21	0.14
Flight muscle ratio × Per	5, 395	3.14	**0.009**	5, 411	1.26	0.28
Flight muscle ratio^2 × Per	5, 395	1.34	0.24	5, 400	1.15	0.33
Wing length	1, 395	1.24	0.27	1, 411	1.32	0.25
Wing length^2	1, 395	0.14	0.71	1, 411	0.18	0.67
Wing length × Per	5, 395	1.28	0.27	5, 411	0.66	0.65
Wing length^2 × Per	5, 395	1.19	0.31	5, 411	2.42	**0.035**
Wing loading	1, 395	1.58	0.21	1, 411	2.60	0.11
Wing loading^2	1, 395	0.03	0.87	1, 411	4.29	**0.039**
Wing loading × Per	5, 395	0.55	0.74	5, 411	0.70	0.62
Wing loading^2 × Per	5, 395	0.30	0.91	5, 405	1.79	0.11
Temperature	1, 395	0.16	0.68	1, 411	13.05	**0.0003**
Interval length	1, 395	0.92	0.34	1, 411	3.03	0.08

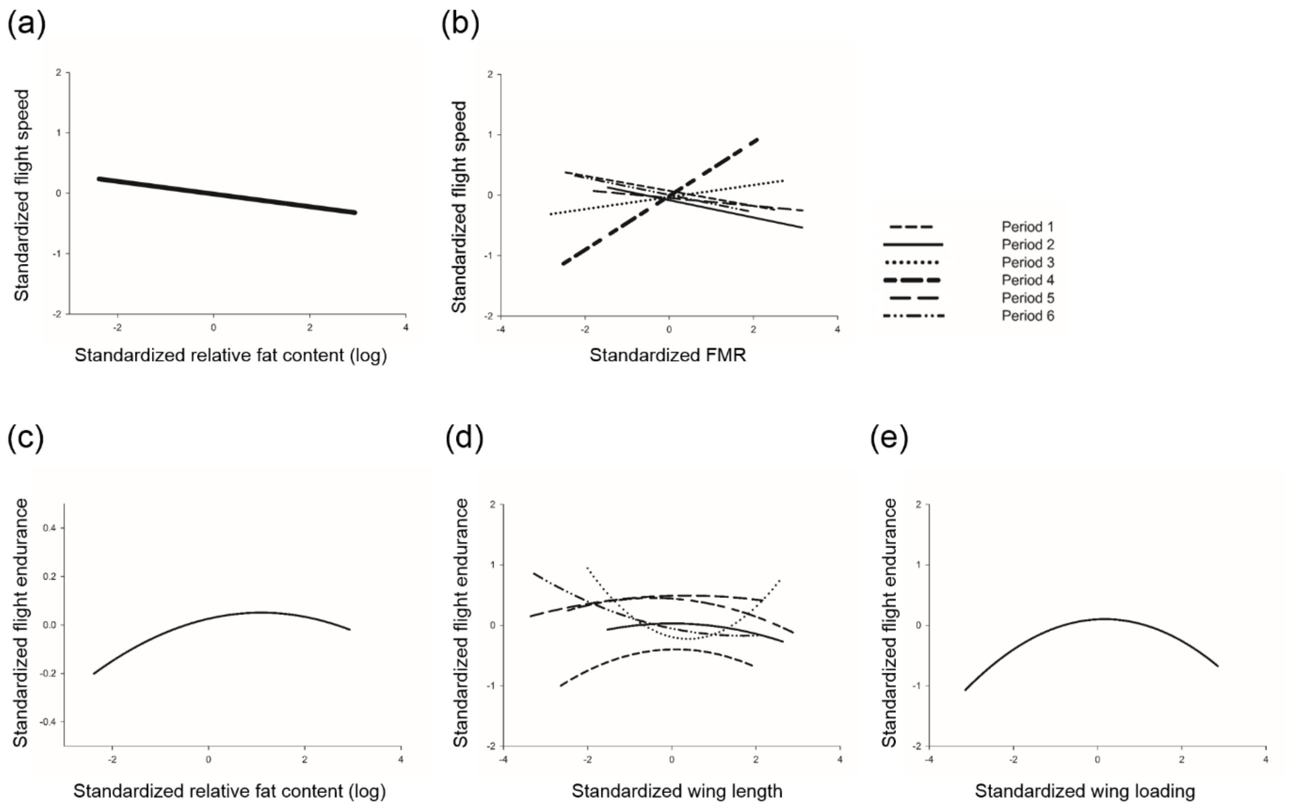

Figure 3. Relationships between the standardized flight-related traits and flight performance traits of the damselfly C. viridis: (a) relative fat content and flight speed, (b) flight muscle ratio and flight speed per period, (c) relative fat content and flight endurance, (d) wing length and flight endurance per periods, and (e) wing loading and flight endurance. Regression lines with a slope significantly different from zero are shown in bold; this was the case for relative fat content and flight speed in plot (a) and flight muscle ratio and flight speed for period 4 in plot (b).

Both the univariate and the multivariate analyses suggested quadratic sexual selection on wing size that differed among periods (Wing length^2 and Wing length^2 × Period, Tables 5-6). The cubic splines indeed suggested stabilizing selection to occur only in periods 5 and 6, with only in period 6 this being formally significant ($\chi^2 = 5.81$, df = 1, $p = 0.016$, $C = -0.25$, $\gamma = -0.21$, Fig. 4a). Both the univariate and multivariate analyses indicated period-dependent linear selection on wing loading. Moreover the multivariate analyses also indicated period-dependent quadratic selection (Wing loading × Period, Wing loading^2 × Period, Tables 5-6). The cubic splines showed quadratic selection to occur only in period 5 and suggested this was driven by the fact that wing loading positively affected mating success at lower values and then reached a plateau ($S = 0.13$, $\beta = 0.11$; $C = -0.21$, $\gamma = -0.22$, Fig. 4b). We did not detect selection on relative fat content and flight muscle ratio (Tables 5-6).

Table 5. Univariate results of the generalized linear models examining sexual selection on the four flight-related traits across the reproductive season of the damselfly C. viridis. The response variable is the mating status (0 = unmated, 1 = mated). Significant p-values are indicated in bold. FMR refers to the flight muscle ratio, see text.

	Trait shaping mating success											
	Relative fat content			FMR			Wing length			Wing loading		
	df	χ2	P	df	χ2	p	df	χ2	p	df	χ2	p
Period	5	12.5638	**0.028**	5	12.7577	**0.026**	5	12.7998	**0.025**	5	14.8829	**0.011**
Trait	1	1.3621	0.24	1	0.3636	0.55	1	2.0211	0.16	1	0.0294	0.86
Trait^2	1	1.4173	0.23	1	0.8510	0.36	1	3.5661	0.06	1	0.0207	0.89
Trait × period	5	2.9711	0.70	5	8.4245	0.13	5	2.7817	0.73	5	10.8574	0.054
Trait^2 × period	5	2.7356	0.74	5	3.4239	0.64	5	9.2292	0.10	5	7.2561	0.20

Table 6. Multivariate results of the generalized linear models examining sexual selection on the set of four flight-related traits across the reproductive season of the damselfly C. viridis. Significant p-values are indicated in bold.

	Mating success		
	df	χ^2	p
Period (Per)	5	20.0798	**0.001**
Relative fat content	1	1.3624	0.24
Relative fat content^2	1	1.5224	0.22
Relative fat content × Per	5	2.6445	0.75
Relative fat content ^{2} × Per	5	0.8717	0.97
Flight muscle ratio	1	0.7914	0.37
Flight muscle ratio^2	1	0.8307	0.36
Flight muscle ratio × Per	5	7.4262	0.19
Flight muscle ratio^2 × Per	5	9.5824	0.09
Wing length	1	3.5657	0.06
Wing length^2	1	3.4577	0.06
Wing length × Per	5	5.4172	0.37
Wing length^2 × Per	5	13.1644	**0.022**
Wing loading	1	0.4011	0.53
Wing loading^2	1	1.3040	0.25
Wing loading × Per	5	12.6786	**0.027**
Wing loading^2 × Per	5	12.1904	**0.032**

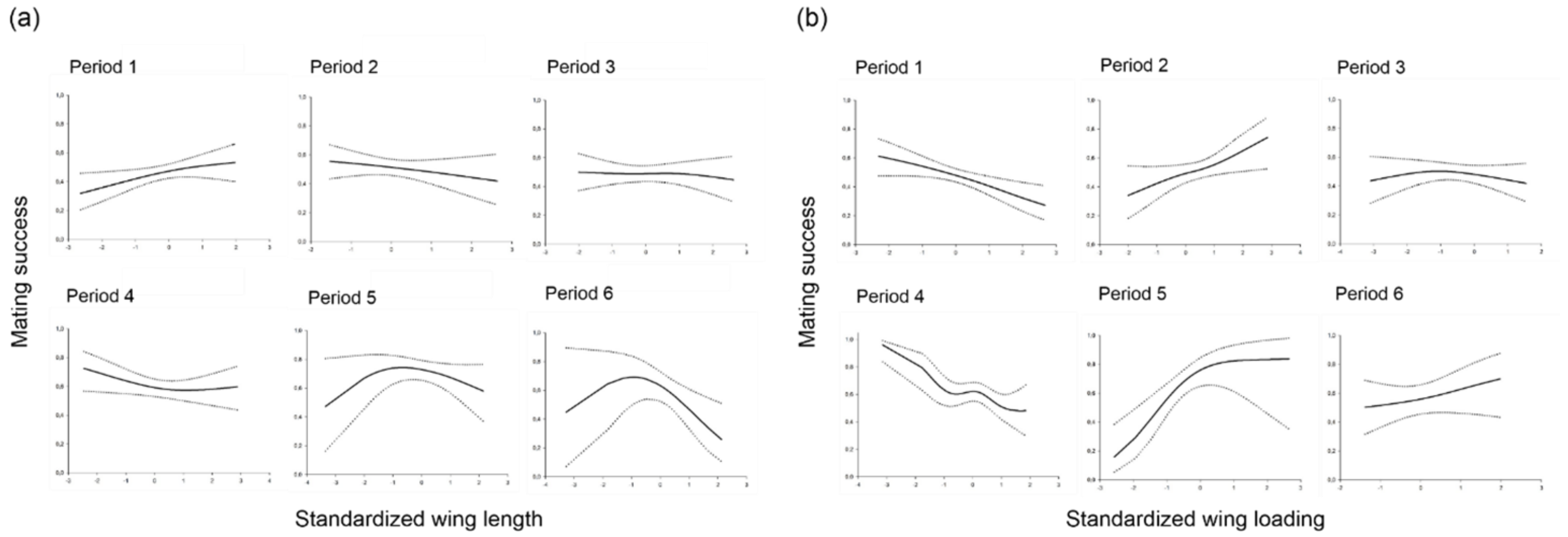

Figure 4. Fitness functions (solid lines) per period relating mating success to (a) standardized wing length, and (b) standardized wing loading. Dotted lines delineate 1 SE bands (based on 999 bootstrap replicates) around the fitness function which was visualized using cubic splines.

4. Discussion

Our results showed a mosaic of temporal patterns within a single reproductive season in the values of both flight performance traits and the four underlying flight-related traits, with some traits being rather stable (flight speed and wing length) and others showing considerable temporal variation (flight endurance, fat content, flight muscle ratio and wing loading). A key finding, in line with our expectations, was that despite the temporal dynamics in flight endurance values we found persistent sexual selection for an increased endurance, while the selection on two flight-related traits (body size and wing loading) that also contributed to flight endurance, differed strongly through time.

4.1 Temporal variation in flight performance and flight-related traits

While the three flight-related traits that showed temporal variation changed rather synchronously, their temporal dynamics did not match the temporal pattern in flight performance. This suggests that temporally varying ecological factors differentially affected performance and the flight-related traits. The rather similar temporal changes in fat content, flight muscle ratio and wing loading may have been caused by, for example, temporal changes in food levels experienced during the final larval stage (for the study species: De Block and Stoks 2005; Stoks et al. 2006a) or in the adult stage (Fitzstephens and Getty 2000). The temporal changes in fat content, flight muscle ratio and wing loading may have not been reflected in flight endurance because of many-to-one mapping (Strobbe et al. 2009) whereby other unmeasured underlying traits may have resulted in another temporal pattern in this performance trait.

In contrast, flight speed and wing length were relatively stable through time. For flight speed, this may reflect strong survival selection by predators (Marden and Chai 1991). The pattern of a stable wing length through time may be explained by the fact that wing length is fixed at emergence and not affected by conditions during the adult stage. Furthermore, it cannot be compensated during the adult stage causing stronger selection to buffer size at emergence against larval stressors (Strobbe and Stoks 2004).

4.2 Sexual selection on flight performance and its temporal dynamics

In line with theory (Husak and Fox 2008) and the very few empirical studies (Sinervo et al. 2000; Lailvaux and Irschick 2006), locomotor endurance was under positive directional sexual selection. This is in line with the belief that territorial fights in damselflies can be described as wars of attrition, in which the male that endures longer is usually the winner (Marden and Waage 1990; Suhonen et al. 2008). *C. viridis* performs an aggressive aerial territorial defence which contains repeated short bursts of rapid flights against territorial intruders (Dreyer 1978; HA Gyulavári and R Stoks, personal observations). The form of sexual selection was consistent through the entire reproductive season with mated males having higher flight endurance compared with unmated ones.

Despite the importance of speed for obtaining territories (Kodric-Brown and Nicoletto 1993) and mates (Husak et al. 2008) in territorial species we could not detect sexual selection for a higher locomotor speed. This may suggest that flight speed is not relevant for territorial defence and mate acquisition in the study species, or that speed is already maximized due to strong selection by other agents such as predators (Marden and Chai 1991; Almbro and Kullberg 2008). Moreover, in some periods flight speed was lower (although not significantly) in mated males compared to unmated males. This could reflect the well-known trade-off between speed and endurance (see e.g. Wilson et al. 2002). In line with this, we found a significant (although weak) negative correlation between endurance and speed ($r = -0.19$, $N = 519$, $p < 0.0001$).

4.3 Sexual selection on flight-related traits and its temporal dynamics

We could detect sexual selection on two of the four flight-related traits, wing length and wing loading; in both cases the selection patterns differed among periods and when significant (in period 6) took the form of quadratic selection. Quadratic sexual selection on size has been shown before in the territorial dragonfly *Libellula luctuosa* where it was explained as a result of selection against extremes (Moore 1990). The temporally changing selection on wing length, our measure of total body size, within a single reproductive season extends the observation of among-year variation in sexual selection on body size (going from stabilizing to disruptive) in the damselfly *Ischnura elegans* (Gosden and Svensson 2008). The across-year variation in the latter study could be explained by changes in ecological factors such as female densities (Gosden and Svensson 2008). Beside density, sex ratio can also account for temporal variation in sexual selection on body size in insects (Kasumovic et al. 2008; Punzalan et al. 2010). These ecological factors are known to vary in nature at much smaller temporal scales within a single season (e.g. McLain 1992; Stoks et al. 1997; Van Gossum et al. 2007), as has been documented in the study species (Westermann 2008). Moreover, temporally varying conditions during the larval stage, such as food quality, have been shown to shape sexual selection on size in the adult stage (Gillespie et al. 2014). This suggests that ecological factors likely also contributed to our observed within-season dynamics in sexual selection. Furthermore, small-scale temporal dynamics in the relative size of males and females may have contributed to changes in selection pressure on body size as shown in the damselfly *Enallagma aspersum* (Steele et al. 2011).

Also for wing loading period-specific quadratic selection was detected. For this trait, this reflected the positive effect on mating success at lower values following by a levelling off of the fitness function at higher values. This selection pattern on wing loading can be explained via its observed non-linear positive effect on flight endurance, which was under positive directional selection. The disappearance of a positive effect of wing loading on endurance and mating success at higher values may be driven by wing loading increasing flight costs due to higher wing beat frequencies to maintain a given level of flight performance (Byrne 1988). Besides its effect on flight endurance, wing loading may also have influenced mating success through positive effects on aerial manoeuvrability (Marden 1989; Marden and Chai 1991).

Although males with higher fat content tended to win more fights in other territorial damselfly species (Marden and Waage 1990; Fitzstephens and Getty 2000; but see Plaistow and Tsubaki 2000) and sexual selection for a higher fat content has been previously found for the study species (Swillen et al. 2009), we were not able to detect sexual selection on fat content. Flight endurance did increase with fat content up to intermediate fat levels, while this was no longer true at higher fat levels. This levelling off may be explained by the cost of increased wing loading at high levels of fat storage (Blanckenhorn and Hosken 2003). We could also not link the flight muscle ratio to mating success, and in line with this the flight muscle ratio did also not explain variation in flight endurance. While flight muscle mass has been linked to territory holding success (Marden 1989), other studies on *Calopteryx* species also failed to find difference in the flight muscle ratio between winners and losers in territorial fights (Plaistow and Siva-Jothy 1996; Marden and Waage 1990).

4.4 Conclusions

There is a growing insight that natural selection can show considerable temporal variation among years (Kingsolver et al. 2001; Kingsolver and Diamond 2011; Siepielski et al. 2009, 2011). Our results add two important insights to this with regard to sexual selection. First, we provide support that this temporal variation also occurs within a single reproductive season for sexual selection on morphological traits (see also McLain 1992; Kasumovic et al. 2008; Punzalan et al. 2010; Steele et al. 2011). This indicates that deciding about the presence and form of selection based on pooling samples across the season and based on samples limited to restricted parts of the reproductive season (often done for logistic reasons when comparing populations) may be misleading. By ignoring within season dynamics in sexual selection on morphological traits, one may indeed confound patterns of among-year or among-site variation with fine-scale temporal patterns of within-site variation. Second, we provided the first test for temporally varying sexual selection on performance traits, which confirmed our hypothesis that selection on performance (here flight endurance) is more stable through time than the observed selection on the underlying traits (here size and wing loading). We hypothesize this to be a general pattern (also for survival selection) given that, compared to morphological and physiological traits, performance is the direct target of selection (Irschick et al. 2007, 2008). In contrast, selection on phenotypic traits may be more variable as the contribution of phenotypic traits to performance may depend on environmental conditions (e.g. Moya-Laraño et al. 2007). Additionally, different combinations of underlying morphological and physiological traits may result in similar performance (Strobbe et al. 2009). This indicates that current insights on temporal dynamics of selection that are mainly based on morphological traits may not hold across trait types. In addition, more studies focusing on understudied traits such as performance traits and physiological traits are needed to refine our knowledge of the temporal dynamics of selection patterns in nature. This is of paramount importance as the inclusion of the temporal dynamics of selection is highly needed to arrive at a better understanding of adaptive evolutionary dynamics of traits in natural populations (Siepielski et al. 2009; Bell 2010; Miller and Svensson 2014).

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Author contributions: HAGY and RS conceived and designed the experiments. HAGY and LT collected the samples. HAGY performed the laboratory work. HAGY analyzed the data. HAGY, LT, GYD and RS contributed to the manuscript.

APPENDICES

Table A1. Univariate linear (S) and quadratic (C) selection differentials, and multivariate linear (β) and quadratic (γ) selection gradients for all traits per period together with the sample sizes of mated and of unmated males.

		S	*C*	β	γ	Sample size	
	Period					mated	unmated
Flight speed	1	0.05	-0.04	0.07	-0.05	61	71
	2	-0.08	-0.04	-0.06	-0.01	45	44
	3	0.02	-0.04	0.07	-0.02	54	48
	4	0.04	0.12	0.07	0.25	50	38
	5	-0.10	-0.08	-0.10	-0.09	50	22
	6	-0.14	0.21	-0.12	0.21	22	14
Flight endurance	1	0.10	0.04	0.11	0.04	61	71
	2	0.23	-0.11	0.22	-0.11	45	45
	3	0.10	-0.03	0.12	-0.03	54	48
	4	0.11	-0.07	0.13	-0.19	50	38
	5	0.13	-0.09	0.13	-0.10	50	22
	6	0.10	0.05	0.08	0.08	22	14
Relative fat content	1	0.01	0.16	0.02	0.21	61	69
	2	-0.04	0.09	0.06	0.21	45	45
	3	0.08	0.20	-0.02	0.43	52	47
	4	0.18	0.32	0.17	0.07	48	38
	5	0.12	-0.27	0.18	0.09	49	22
	6	0.10	0.14	0.11	-0.02	21	14

Continuation of Table A1.

		S	C	β	γ	Sample size	
	Period					mated	unmated
Flight muscle ratio	1	-0.04	-0.05	-0.06	-0.05	59	69
	2	-0.17	-0.03	-0.19	-0.22	45	44
	3	0.12	0.03	0.10	0.09	52	47
	4	0.09	0.07	0.01	0.19	49	38
	5	-0.10	-0.12	-0.17	-0.02	49	22
	6	-0.07	-0.16	-0.01	-0.24	21	14
Wing length	1	0.06	-0.10	0.08	-0.12	57	67
	2	-0.04	-0.07	-0.04	0.04	44	42
	3	-0.01	-0.04	-0.02	-0.02	43	45
	4	-0.03	0.12	-0.02	0.18	48	32
	5	-0.02	-0.14	-0.01	-0.19	47	20
	6	-0.08	-0.25	-0.09	-0.21	19	14
Wing loading	1	-0.08	-0.06	-0.10	-0.03	59	67
	2	0.08	0.02	0.11	-0.02	44	42
	3	-0.01	-0.11	-0.02	-0.07	43	46
	4	-0.09	0.04	-0.09	0.13	48	32
	5	0.13	-0.21	0.11	-0.22	47	19
	6	0.07	0.28	0.09	0.54	19	14

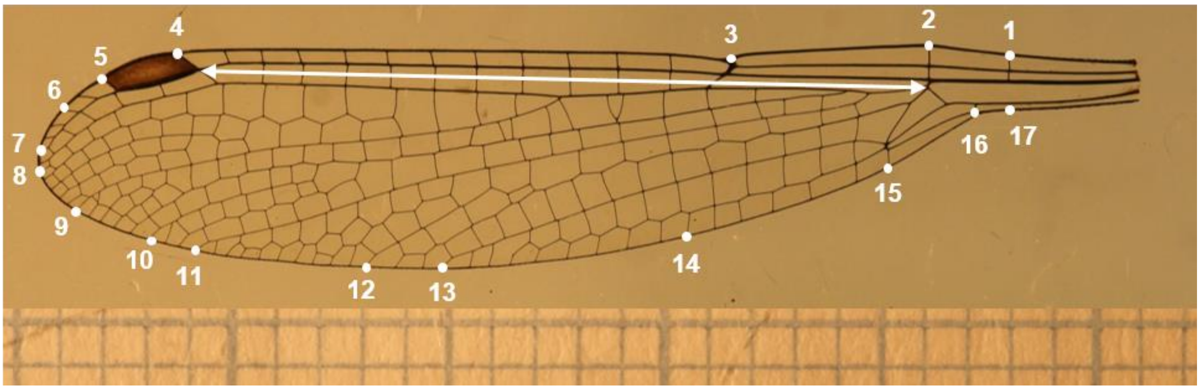

Figure A1. The left hind wing of a C. viridis male with the 17 digitalized landmarks. Wing size was measured as the distance between the proximal apex of the quadrilateral cell and the proximal tip of the pterostigma.

CHAPTER IV

Sexual selection on flight endurance, flight-related morphology and physiology in a scrambling damselfly

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Abstract

We have limited knowledge on the mechanistic base of sexual selection, especially in scrambling species. This asks for a functional approach that explores the link between each component of the phenotype-performance-fitness axis and that includes both morphological and physiological traits. We explored the phenotype-performance-fitness axis in the scrambling damselfly *Coenagrion puella* by studying the links between a set of physiological and morphological traits, flight performance (flight speed and flight endurance), and short-term mating success. As expected for scrambling competition, there was sexual selection for increased flight endurance rather than for increased flight speed. For fat content, we could demonstrate the full phenotype-performance-fitness axis, where selection for a higher fat content could be explained by the sexual selection for a higher flight endurance and the positive covariation between fat content and flight endurance. For three other traits (size, relative flight muscle mass and wing loading), however, we detected selection that could not be explained via their effect on flight performance, generating novel testable hypotheses about how the covariation between these traits and mating success is generated. This also urges caution when using morphological traits as proxies for flight speed and flight endurance in phenotypic selection studies.

1. Introduction

Phenotypic selection, covariation between fitness components and phenotypic traits, is a major force driving evolutionary change (Endler 1986). Given that sexual selection is stronger (Endler 1986; Hoekstra et al. 2001; Kingsolver et al. 2001) and more consistent in direction (Siepielski et al. 2011) than survival selection, it may cause more persistent directional evolution. A multitude of studies documented sexual selection on phenotypic, mainly morphological, traits (Kingsolver et al. 2012). These phenotypic traits are, however, mostly not the direct target of selection and serve as a proxy for the performance trait under selection. Far fewer studies have investigated how selection directly acts on performance, and the small subset of these that also included the phenotypic traits underlying performance are strongly biased toward morphology (Moore and Hopkins 2009). Yet, morphology alone is often a poor predictor of performance (Wainwright et al. 2005; Hendry et al. 2011) and physiology is another important predictor (e.g. Marden et al. 1999). As a result, we have limited knowledge on the mechanistic base of sexual selection (Irschick et al. 2007). This asks for a functional approach that explores the link between each component of the phenotype-performance-fitness axis (Arnold 1983; Kingsolver and Huey 2003) whereby the phenotype is any physiological and morphological trait underlying performance, and performance is the maximal ability of an animal to conduct an ecologically relevant, physically challenging task (Irschick et al. 2007).

Female choice and contest competition are the most widely studied types of sexual selection, while scrambling competition is much less investigated (Lailvaux and Irschick 2006; Irschick et al. 2007). This is especially true for studies investigating sexual selection on performance. Scramble competition for mates is, however, a wide-spread mating tactic where locomotor performance is assumed to be of key importance (Ghiselin 1974; Andersson 1994). In scramble competition location of the mate is crucial for mating success and for this endurance is likely more important than speed (Andersson 1994; Husak and Fox 2008). Several phenotypic traits have been associated with mating success in scrambling species, mainly through their assumed effect on locomotor performance. These include a small body size (Blanckenhorn et al. 1995) and a higher fat content (Blanckenhorn et al. 2004). So far, no functional approach has been applied to study sexual selection via aerial scrambling competition that fully explored the phenotype-performance-fitness axis (see Kelly et al. 2008 for the only example for terrestrial scramble competition).

We applied a functional approach to sexual selection in the scrambling damselfly *Coenagrion puella* (Linnaeus, 1758) by studying the links between flight performance, the underlying morphological and physiological traits and short-term mating success. We did so by comparing field-collected mated and unmated males, a well-established method to study sexual selection (e.g. Blanckenhorn et al. 2003, 2004; Gosden and Svensson 2008; Steele et al. 2011). Odonates are important model organisms for the study of sexual selection (Córdoba-Aguilar 2008). Yet, despite the general belief that flight performance is important for sexual selection in this insect order (Marden and Cobb 2004; De Block and Stoks 2007), the many sexual selection studies have not directly quantified flight performance. In general, the study of aerial scrambling species has been identified as an important future direction to improve our understanding of how sexual selection might operate on locomotor performance and its underlying traits (Husak and Fox 2008). Specifically, we studied sexual selection on two flight performance traits (flight speed and flight endurance). Because in scrambling competition encounter rate is crucial for mating success and males which can fly for longer durations at the breeding site are more likely to encounter females we expected the mating success of the scrambling males to be mainly driven by flight endurance and less by flight speed (Andersson 1994; Husak and Fox 2008). We additionally studied a set of three physiological and three morphological traits that have been associated with flight performance. For the two physiological traits that have been related to body condition, the activity of the enzyme phenoloxidase that plays a key role in insect immune function (González-Santoyo and Córdoba-Aguilar 2012), and the fat content, the main energy storage in insects (Rolff and Joop 2002), we expected higher levels in mated than in unmated males. Given that the third physiological trait, relative flight muscle mass, and two morphological traits, wing aspect ratio and wing loading relate positively to flight performance (Berwaerts et al. 2002) we expected these traits to show higher values in mated males than in unmated males. Finally, for the third morphological trait, wing length, we expected a small-male mating advantage because small size is more advantageous in scramble competition (Blanckenhorn et al. 1995; Moya-Laraño et al. 2002; Crompton et al. 2003). To assess consistency of selection patterns we collected mated and unmated males from six populations in Central Europe.

2. Materials and methods

2.1 Study species and collection

Coenagrion puella is one of the most common damselflies in Central Europe typically found at ponds (Dijkstra and Lewington 2006). Males search for females by patrolling low over the breeding pond and display scramble competition for mates. The species shows no female behaviour to assess and select potential mates indicating there is no female choice (Banks and Thompson 1985). In 2010 we sampled mated and unmated males in six populations in Central Europe (Table 1). In total 337 males (182 unmated, 155 mated) were collected during the peak of the mating season (12 June-22 July). All males were caught on sunny days between 10:30 and 16:00 hours, when sexual activity peaks, near the border of their reproduction pond. Only males that were collected during copulation were categorized as mated. Unmated males were those not associated with a female but that were active at the reproduction site. Males were kept in separated opaque cups (diameter: 7.5 cm; height 4.5 cm) in a dark and cooled box (15 °C) until the start of the flight performance test. Each time a mated male was caught, the female was kept separately in another cup to avoid that unmated males would start a mating with one of these females. Females were released after the sampling on a given day was finished.

2.2 Flight performance test

Males were tested for their flight performance (flight speed and flight endurance) *in situ*. Each male was gently placed in his opaque cup at the bottom of a 2 meter high, 20 cm diameter Plexiglas flight tube which was placed vertically. Flight trials were executed in the afternoon at the day of capture. All males took off spontaneously within a few seconds from their cup. Each flight bout consisted of a fast linear upward trajectory followed by a slower trajectory where the male moved up in steps and eventually reached the highest vertical distance of its flight trajectory whereafter it kept flying until it finally reached the bottom of the flight tube again. The flight tube was graded every 10 cm to allow estimating the height of the male at different parts of the flight trajectory. Using the grid lines on the tube and a chronometer we quantified four variables for each flight bout: the height reached (height 1) and total duration (time 1) of the first linear part of the flight trajectory, the maximum height (height 2) reached during the flight bout and the total duration (time 2) of the flight bout (time until the animal reached the bottom again). We estimated the flight speed as height 1 divided by time 1. Note that there was no covariation between height 1 and speed (ANCOVA with population as categorical variable, height1 as covariate and speed as dependent variable, effect of height 1:

F = 2.275; df = 1, 195; p = 0.133; slope ± SE= 0.102 ± 0.068). Estimating endurance under laboratory conditions may be confounded with motivation unless care is taken to maximally motivate the animals (Careau and Garland 2012). Horizontal surfaces are avoided by *C. puella* in nature, this was here reflected by the quick spontaneous departure of the males. As the males were reluctant to land at the horizontal surface, they were highly motivated to keep flying. Although we cannot fully exclude a behavioural component we therefore interpret the total duration of the flight as a measure of endurance.

Each male was tested only once. Afterwards, each male was placed in a separate Eppendorf tube and flash-frozen at -80 °C for later analyses. Due to time constraints, the flight performance test was carried out only on a subset of the collected males (see Table S1 for exact sample sizes). Only males with intact wings were tested. Males whose flight performance was not quantified were manipulated similarly and also stored in cups for a comparable time before being frozen.

We additionally quantified three variables that may potentially interfere with the measured flight variables. First, as thorax temperature may influence insect flight performance (Berwaerts and Van Dyck 2004; Samejima and Tsubaki 2010) we tried to minimize and correct for temperature differences among males and populations. Therefore, before each flight test, a male was placed in an incubator at 30 °C for 5 minutes to minimize potential initial differences between individual’s thorax temperatures. Preliminary thorax temperature measurements of damselflies in the field revealed that this is a typical body temperature for active *Coenagrion* males (L Therry, unpublished data). At the end of each flight test, the thorax temperature was measured to the nearest 0.1 °C with a micro-thermocouple (BAT-12 type, company Physitemp Instruments, Inc., Clifton, NJ, USA). Additionally, air temperature was measured to the nearest 0.1 °C with a thermometer at a height of 70 cm in the tube at the end of each flight test. Given that thorax temperatures and air temperatures were highly correlated ($r = 0.89$, $N = 179$, $p < 0.001$) we included only the former in the statistical analyses. Replacing air temperature with thorax temperature did not change the conclusions. Secondly, the number of attached ectoparasitic water mites may negatively influence flight ability (e.g. Nagel et al. 2010) and mating success in male damselflies (Forbes and Robb 2008), including the study species (Thompson et al. 2011). To take this potential effect into account the number of attached mites was counted after the flight test using a magnifying glass. Thirdly, we also quantified the time that each male spent in the cooled box between capture and the moment its flight test began.

Table 1. The study populations of the damselfly Coenagrion puella and the number of collected mated and unmated males.

Country	Code	Locality	Latitude	Longitude	Mated males	Unmated males
France	CA	La Ville-aux-Bois, Champagne-Ardenne	48°24'37.52"N	04°40'10.94"E	29	33
France	PR	Roussent, Picardie	50°21'52.42"N	01°47'21.02"E	13	25
France	PM	Merlimont, Picardie	50°26'24.50"N	01°34'58.87"E	33	27
Germany	DNZ	Zülpich, Nordrhein-Westfalen	50°42'07.00"N	06°38'43.00"E	28	35
Belgium	BKP	Zwin, Knokke	51°21'29.93"N	03°20'36.13"E	13	19
The Netherlands	HC	Cadzand	51°22'50.19"N	03°23'58.61"E	39	43

2.3 Physiological and morphological measurements

For each male we quantified (i) a set of physiological traits related to body condition: a measure of investment in immune function (the activity of the enzyme phenoloxidase), energy storage (fat content) and relative flight muscle mass (flight muscle ratio), and (ii) a set of morphological traits related to flight in damselflies: wing length, aspect ratio and wing loading. Wing length was used as a size measure (e.g. Carchini et al. 2000). Sample sizes differ slightly per trait because some wings were damaged after the flight test and some samples accidentally thawed. Exact sample sizes per dependent variable are given in the figures.

The activity of phenoloxidase (PO) was measured in hemolymph samples closely following the protocol of Stoks et al. (2006b). Shortly, we measured the total activity of PO, including the pro-enzyme proPO, by monitoring the transition from l-DOPA to dopachrome at 490 nm in a spectrophotometer at 30 °C. Enzyme activity was measured as the slope during the linear phase of the reaction. To adjust PO activity we quantified the protein concentration of the haemolymph sample using a calibration curve based on bovine serum albumin.

To obtain fat content and muscle mass we followed the protocol by Swillen et al. (2009). Thoraces and abdomens were placed individually in Eppendorf tubes and weighed to the nearest 0.01 mg using a microbalance after being dried for 48 h at 60 °C. Subsequently, fat was extracted by adding 1.5 ml dichloromethane (99 %) to the Eppendorf tubes which were placed for 24 h on an automatic shaker. Thereafter, the dichloromethane with the dissolved fat was removed and the body parts were dried for another 48 h at 60 °C and weighed. Fat content was calculated by subtracting the dry mass after extraction from the dry mass before extraction. To obtain muscle mass, we added 1.5 ml NaOH (0.35 M) to the Eppendorf tubes to break down all muscle tissue, and placed the tubes on a shaker for 24h. Flight muscle mass was quantified as the difference in dry thorax mass before and after this procedure. Relative fat content was calculated as the ratio of total body fat to total body mass. The flight muscle ratio (FMR) was calculated as the ratio of flight muscle mass to total dry body mass (Marden 1989).

Wing length and wing area were quantified on the digital pictures of the left hind wing using the software TpsDig 2.16 (Rohlf 2010). Wing length was calculated as the distance between the landmarks 1 and 9: the point where the third and fourth longitudinal vein (radius and media; R+M) meet the second cross vein and where the R+M reach the wing margin (see Fig. S1). Aspect ratio was calculated as the squared wing span divided by the wing area, where wing span is two times the wing length. To quantify wing area we digitized 18 landmarks along the wing margin (Fig. S1) thereby obtaining a polygon spanning the wing surface. Wing loading was measured as the ratio of total body mass to wing area.

2.4 Statistical analyses

All statistical analyses were carried out with STATISTICA v.11 on standardized trait values as variables were measured at different scales. Prior to statistical testing a principal components (PC) analysis was performed on the flight variables to obtain two independent performance measurements. To maximize the interpretation of the axes we applied a varimax normalized rotation. Further analyses were performed on the scores of each male on the two extracted flight performance PC axes.

To test for univariate (total) linear sexual selection on flight performance, we ran univariate generalized linear models (separately per flight performance PC) with the PC score as independent variable and the mating status (0 for unmated, 1 for mated males) as binary dependent variable (Lande and Arnold 1983). We used a binomial error structure and the logit link function (Janzen and Stern 1998). When testing for quadratic selection, we also included the squared terms of the PC scores. We explicitly took into account that males were collected at different sites by including population as a categorical factor in the models. We also included interactions with population and the flight performance PC scores to check for consistency of sexual selection on flight performance across populations. Furthermore, we added following covariates to the model: thorax temperature, mite number and the time that each male had spent in the cooled box before the flight test began. As none of these covariates were significant, nor their interactions with population (all $p > 0.21$) they were removed from the final model.

To test for multivariate (direct) linear selection, we ran multivariate generalized linear models with the above described design that included both flight performance PC scores as independent variables. Whenever directional sexual selection on a trait was significant we report in the results section the univariate linear selection differential (S) and the multivariate linear selection gradient (β) as the regression coefficients of the standardized trait against relative fitness. Whenever quadratic sexual selection on a trait was significant we report the univariate quadratic selection differential (C) and the multivariate quadratic selection gradient (γ) as 2× the regression coefficients of the squared term of the standardized trait against relative fitness (Stinchcombe et al. 2008). Regression coefficients were extracted from the same model as explained above, yet using a normal error distribution (Janzen and Stern 1998). All selection gradients for each trait and population together with the sample sizes are reported in Table S1 in the appendix. Note that our aim is to test for sexual selection on flight performance and that we included several populations to test for the consistency of the documented patterns, yet our focus was not on quantifying selection differentials and gradients in separate populations (see e.g. also Blanckenhorn et al. 2003, 2004). Because the multivariate analyses gave very similar results to the univariate analyses we present the results of the multivariate analyses in an appendix (Table S2, Table S3).

We similarly evaluated sexual selection on the underlying physiological and morphological traits. We initially added protein concentration to correct for PO activity; yet, since it had no significant effect ($p = 0.07$) it was removed from the final model; statistical models with and without protein concentration gave qualitatively similar results. Besides formal statistical testing, we also visualised selection when we detected consistent (across populations) selection on a given trait using cubic splines with the software glms 4.0 (Schluter 1988).

To explicitly test whether the measured physiological and morphological traits contributed to flight performance, we ran separate general linear models per flight performance PCs with all six phenotypic traits and their quadratic terms as continuous predictor variables and the flight performance PCs as dependent variable. We also included population and interactions between population and the phenotypic traits and their quadratic terms; but as none were significant (all $p > 0.13$), these were removed from the final model. For these variables, we report in the statistical tables the p-values at the time of removal from the final model.

3. Results

The first two PCs extracted from the set of flight variables summarized ca. 77 % of the variation in the dataset. Flight performance PC1, which explained 43 % of the variation, was interpreted as a metric of flight speed as it had high loadings for time 1 (-0.89) and speed (0.92) and low loadings for time 2 (-0.16) and height 2 (0.08). Males with more positive scores for PC1 had a higher flight speed. Flight performance PC2, explained 34 % of the variation, and reflected flight endurance as it was strongly positively associated with the total flight time (0.79) and the maximal height obtained (0.85) and had lower loadings for time 1 (0.19) and speed (0.10). Males with higher scores for PC2 had a higher flight endurance and therefore also attained a higher height in the flight tube.

We could not detect selection on flight speed (PC1): neither its linear term nor its quadratic term affected a male's mating status (Tables 2, S2; Fig. 1a). This was consistently so across populations as indicated by the absence of interactions with population (Tables 2, S2). Flight endurance (PC2) was under positive directional selection with mated males having a higher flight endurance than unmated males (S = 0.62, β = 0.62; Tables 2, S2; Fig. 1b). This was consistently so across populations as indicated by the absence of interactions with population (Tables 2, S2). Although the quadratic term was not significant, the cubic spline suggested that flight endurance only positively influenced mating success above a threshold value (Fig. 1b).

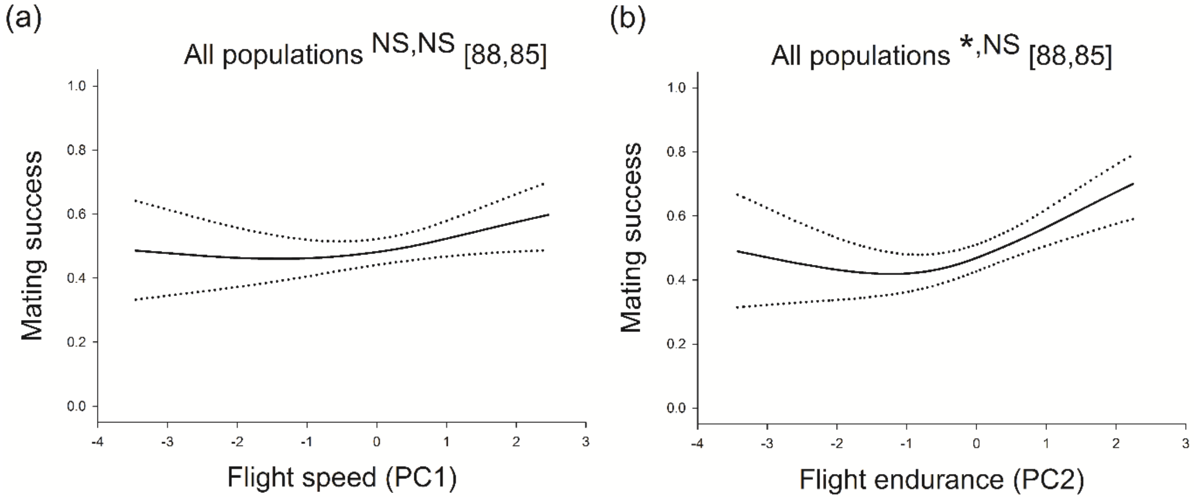

Figure 1. Cubic splines of the fitness functions (solid line) relating mating success to flight performance: ***a*** *flight speed (PC1) and* ***b*** *flight endurance (PC2). Dotted lines delineate regression bands of ±1 SE (based on 999 bootstrap replicates) around the fitness function. The significance code represents the p-value for the linear and quadratic terms, respectively (* $p < 0.05$). Numbers between square brackets represent the numbers of unmated and mated males, respectively.*

Table 2. Univariate results of the generalized linear models examining sexual selection on the two flight performance traits in the set of six study populations of the damselfly C. puella. Significant p-values are indicated in bold.

		Flight performance PC shaping mating success			
		Flight speed (PC1)		Flight endurance (PC2)	
	df	χ^2	p	χ^2	p
Population	5	3.0670	0.69	1.1846	0.95
Trait	1	0.8983	0.34	6.2455	**0.012**
Trait^2	1	0.1988	0.66	0.0014	0.97
Trait × population	5	4.4800	0.48	7.1279	0.21
Trait^2 × population	5	5.7571	0.33	2.1099	0.83

Of the six studied phenotypic traits only the wing loading significantly contributed to explaining variation in flight speed (PC1, Table 3). Males with a higher wing loading had a higher flight speed (Fig. 2a). Besides wing loading also the relative fat content contributed to flight endurance (PC2, Table 3). Males with a lower wing loading and males with a higher relative fat content had a higher flight endurance (Fig. 2b,c). All these patterns were consistent across populations as indicated by the non-significant trait-by-population interactions (Table 3).

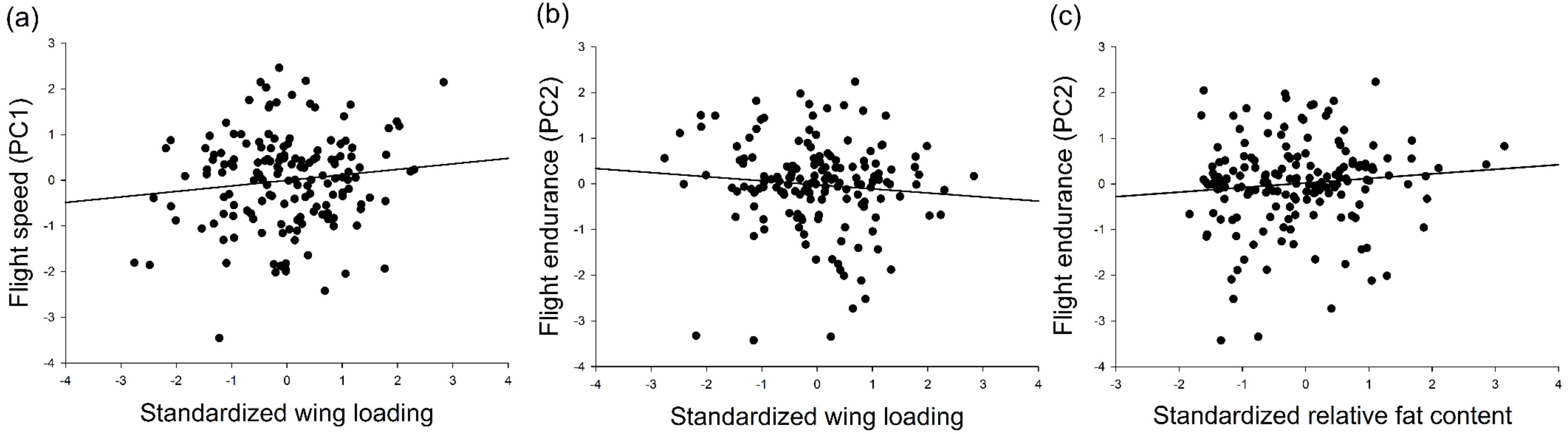

Figure 2. Relationships between the standardized phenotypic traits and the flight performance PCs: ***a*** *wing loading and flight speed (PC1),* ***b*** *wing loading and flight endurance (PC2), and* ***c*** *relative fat content and flight endurance.*

Table 3. Multivariate results of the general linear models testing for the contribution of the three physiological traits (phenoloxidase activity, relative fat content and FMR) and the three morphological traits (wing length, aspect ratio and wing loading) to both flight performance traits: flight speed (PC1) and flight endurance (PC2). Significant p-values are indicated in bold.

	Flight speed (PC1)			Flight endurance (PC2)		
	df	F	p	df	F	p
Population (Pop)	5, 144	**4.28**	**0.001**	5, 143	2.11	0.07
Phenoloxidase activity	1, 141	1.41	0.24	1, 141	0.41	0.52
Phenoloxidase activity^2	1, 103	0.02	0.88	1, 98	1.07	0.30
Phenoloxidase activity × Pop	5, 109	0.91	0.48	5, 109	1.26	0.28
Phenoloxidase activity^2 × Pop	4, 78	0.46	0.76	4, 78	1.95	0.11
Relative fat content	1, 140	0.67	0.41	1, 148	**4.18**	**0.043**
Relative fat content^2	1, 103	0.55	0.46	1, 98	0.39	0.53
Relative fat content × Pop	5, 109	0.48	0.79	5, 109	0.42	0.83
Relative fat content ^{2} × Pop	4, 78	0.11	0.98	4, 78	1.12	0.36
Flight muscle ratio	1, 139	0.10	0.75	1, 139	0.04	0.84
Flight muscle ratio^2	1, 103	1.36	0.25	1, 98	0.53	0.47
Flight muscle ratio × Pop	5, 109	0.09	0.99	5, 109	0.96	0.45
Flight muscle ratio^2 × Pop	4, 78	0.63	0.64	5, 98	1.63	0.16
Wing length	1, 142	1.91	0.17	1, 142	2.39	0.12
Wing length^2	1, 103	0.36	0.55	1, 98	0.07	0.79
Wing length × Pop	5, 109	1.03	0.41	5, 109	0.67	0.65
Wing length^2 × Pop	4, 78	0.41	0.80	4, 78	0.41	0.80
Aspect ratio	1, 143	1.61	0.21	1, 140	0.12	0.73
Aspect ratio^2	1, 103	0.19	0.67	1, 98	0.74	0.39
Aspect ratio × Pop	5, 109	1.76	0.13	5, 109	0.47	0.80
Aspect ratio^2 × Pop	4, 78	0.49	0.75	4, 78	0.39	0.81
Wing loading	1, 144	**4.68**	**0.032**	1, 148	**5.64**	**0.019**
Wing loading^2	1, 103	2.42	0.12	1, 98	0.24	0.62
Wing loading × Pop	5, 109	1.51	0.19	5, 109	0.29	0.92
Wing loading^2 × Pop	4, 78	0.36	0.84	4, 78	0.98	0.42

Of the three physiological traits, PO, relative fat content and FMR, only relative fat content was under directional sexual selection (Tables 4, S3; Fig. 3a,b). Across populations, mated males had a higher relative fat content (S = 0.28, β = 0.30) as confirmed by the cubic spline (Fig. 3b). There was quadratic selection on FMR, yet the pattern differed among populations (Tables 4, S3). In only one population (BKP) there was significant quadratic selection with males with an intermediate FMR having a higher mating success (C = -1.31, γ = -1.30, Table S1).

Except for aspect ratio, all morphological traits were under sexual selection, although the type of selection differed among traits and sometimes among populations. Selection on wing length was always directional but the sign differed among populations (wing length × population, Tables 4, S3). While significant negative directional selection was observed in three populations (CA: S = -0.54, β = -0.44; HC: S = -0.42, β = -0.40 and PM: S = -0.63, β = -0.54), there was marginally significant positive selection in one population (PR) (S = 0.67, β = 0.61; Tables 4, S3; Table S1). We could not detect selection on aspect ratio: neither its linear term nor its quadratic term affected a male's mating status. This was consistently so across populations as indicated by the absence of interactions with population (Tables 4, S3, Fig. 3c). The selection on wing loading was quadratic (note in the multivariate analysis there was only a trend, P = 0.096) and this was consistently so across populations (C = 0.47, γ = 0.28; Tables 4, S3; Fig. 3c). The cubic spline confirmed the non-linear selection pattern and suggested this was driven by the fact that wing loading did not affect mating success except at higher values where it increased mating success.

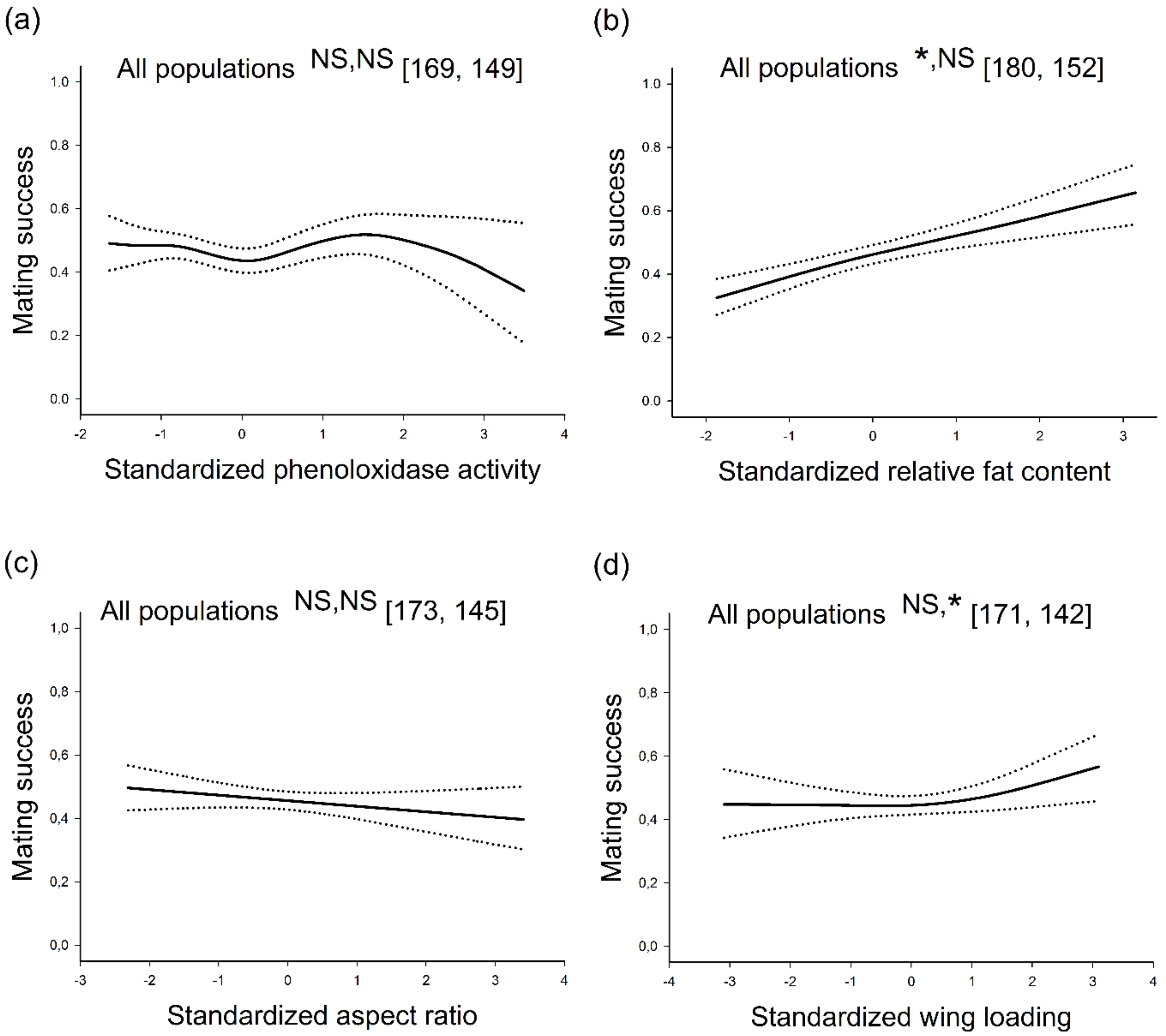

*Figure 3. Cubic splines of the fitness functions (solid line) relating mating success to **a** standardized phenoloxidase activity, **b** standardized relative fat content, **c** standardized aspect ratio and **d** standardized wing loading. Dotted lines delineate regression bands of ±1 SE (based on 999 bootstrap replicates) around the fitness function. The significance code represents the p-value for the linear and quadratic terms, respectively (* $p < 0.05$). Numbers between square brackets represent the number of unmated and mated males, respectively.*

Table 4. Univariate results of the generalized linear models examining sexual selection on each of the six phenotypic traits (the three physiological and three morphological traits) in the set of six study populations of the damselfly C. puella. Significant p-values are indicated in bold. FMR refers to the flight-muscle ratio, see text.

		Trait shaping mating success											
		Phenoloxidase activity		Relative fat content		FMR		Wing length		Aspect ratio		Wing loading	
	df	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Population	5	7.0916	0.21	3.7971	0.58	6.2127	0.29	8.9764	0.11	4.0670	0.54	9.2482	0.10
Trait	1	0.0059	0.94	6.5671	**0.010**	0.1506	0.70	1.9172	0.17	0.9471	0.33	0.8467	0.36
Trait^2	1	0.4184	0.52	0.3802	0.54	1.3886	0.24	0.4079	0.52	4.8709	0.43	5.5255	**0.019**
Trait × population	5	3.8833	0.57	2.4022	0.79	2.6585	0.75	14.1947	**0.014**	4.6753	0.46	3.5251	0.62
Trait^2 × population	5	4.7313	0.45	5.0342	0.41	13.5215	**0.019**	0.7934	0.98	5.0173	0.41	4.7067	0.45

4. Discussion

4.1 Sexual selection on flight performance

Sexual selection studies on locomotor performance are few especially in scrambling species (Husak and Fox 2008; but see Kelly et al. 2008). Males in aerial scramble competition can increase the chance of encounters with females by active searching (Andersson 1994). In line with this, we could demonstrate consistent positive sexual selection for flight endurance. This matches indirect evidence obtained in a comparative study by Wickman (1992) where patrolling butterfly species (in which males actively search in flight for females) had morphological traits that were assumed to be more favourable for flight endurance compared to the traits shown by perching species. We did not detect sexual selection for higher flight speeds. This supports the suggestion by Husak and Fox (2008) that endurance is likely more important than maximum speed in scramble competitors.

4.2 Sexual selection on physiological traits

Of the three studied physiological traits, fat content was under positive directional sexual selection. As was the case for all other traits, the multivariate selection analysis confirmed the results of the univariate selection analysis, indicating the presence of direct selection on the trait under study. This can be explained by our observations that fat contributed to flight endurance and males with a higher flight endurance had a higher short-term mating success. This is to be expected given that flight is the most energetically demanding form of locomotion and lipids make up the largest part of energy reserves in insects (Klowden 2002). Moreover, the maximum duration of flight in insects depends directly on the amount of energy stores (Kaufmann et al. 2013). The finding of a higher mating probability in males with more fat has been observed before in other scrambling insect taxa (Otronen 1995; Blanckenhorn et al. 2003, 2004). Yet, these studies did not explicitly evaluate the performance link with flight endurance.

Although we expected PO activity, just as fat content, to reflect body condition (González-Santoyo and Córdoba-Aguilar 2012), PO activity was not related to endurance and mating success. Because body fat is involved in the synthesis of immunoproteins (e.g. Boman 1986), previous studies suggested a positive link between fat reserves and immune ability (for example, Contreras-Garduño et al. 2006). Yet, this pattern is not general. Indeed, fat content and PO activity did not covary in our study ($r = 0.05$, $N = 313$, $p = 0.39$) suggesting that PO activity may not always indicate body condition (for another example in damselflies see Rolff et al. 2004). Alternatively, mating activity may have reduced PO levels (Siva-Jothy et al. 1998) which may have masked a positive covariation between PO activity and fat content. In the related scrambling damselfly *C. armatum*, immune defense (measured as encapsulation response) was even observed to be lower in mated compared to unmated males (Honkavaara et al. 2009).

We detected no general selection across populations on FMR. In the only population where we could detect selection on FMR this took the form of stabilizing selection. An advantage of an intermediate FMR for mating success may not be that unexpected. The initial increase of mating success with FMR may be due to a higher acceleration ability (Marden 1989), and the decrease at higher values may be due to a lowered aerial agility (Samejima and Tsubaki 2010).

4.3 Sexual selection on morphological traits

Of the three studied morphological traits, we detected directional selection on wing length, quadratic selection on wing loading but aspect ratio was not under sexual selection. Despite it has been suggested that a high aspect ratio is associated with extended flight (Betts and Wootton 1988) we did not detect covariaton between the aspect ratio and flight endurance nor selection on aspect ratio. Similarly, Johansson et al. (2009) failed to find differences in aspect ratio among dragonfly genera differing in the occurrence of long-distance migration (thus endured flight). This may suggest that the aspect ratio might not fully capture wing shape in this order.

We expected a small-male mating advantage because small size is more advantageous in scramble competition (Blanckenhorn et al. 1995; Moya-Laraño et al. 2002; Crompton et al. 2003). Crompton et al. (2003) showed that smaller midges outperformed larger ones with respect to acceleration and maneuverability, parameters relevant for male mate acquisition in flight. Moreover, smaller males might need less food to support themselves; resulting in more energy and time available to search females (Blanckenhorn et al. 1995). In line with this in three populations we detected directional sexual selection for smaller males.

Yet, the selection pattern on size we observed was not consistent across populations, and there was even a trend in one population for sexual selection to favour large males. This resembles the study by Gosden and Svensson (2008) where in the scrambling damselfly *Ischnura elegans* sexual selection favoured small males in some populations and large males in other ones, with in some populations sexual selection favouring males of intermediate size (as observed in other scrambling species, see Stoks 2000; Thompson and Fincke 2002). This was explained by sexual selection regimes being driven by a combination of the densities of female colour morphs (that also occur in the study species) and local female body sizes. As we were not interested in among population variation in sexual selection *per se*, we have no data to verify whether similar density and frequency dependent selection may also explain the here documented spatial variation in selection on body size.

We found consistent quadratic selection on wing loading across populations: wing loading did not affect mating success except at higher values where it had a positive effect on mating success. This selection pattern on wing loading cannot be explained through its observed contributions to the here scored performance measures, flight speed and flight endurance. While, as expected from previous studies (Dudley and Srygley 1994), males with a higher wing loading had a higher flight speed, we did not detect sexual selection on flight speed. Further, while wing loading negatively affected flight endurance, flight endurance was under positive directional sexual selection. Such negative covariation pattern between wing loading and flight endurance may be explained by the demand of a higher wing loading for higher wing beat frequencies to maintain a given level of flight performance (Byrne 1988) thereby increasing flight costs (Bartholomew and Casey 1978). Taken together, this indicates that males with a higher wing loading scored better for other aspects of flight performance or achieved a mating advantage for other reasons. In support of the former, a higher wing loading has been suggested to be associated with a higher turning agility (Norberg and Rayner 1987), which may be favourable during scrambling competition.

We have limited knowledge on the mechanistic base of sexual selection (Irschick et al. 2007), this is especially true for scrambling competition. Our study is the first investigating sexual selection on flight performance and the underlying physiological and morphological traits in an aerial scrambling species (Husak and Fox 2008). As expected, the mating success of the scrambling males was driven by flight endurance rather than by flight speed (Husak and Fox 2008). The functional approach (*sensu* Irschirck et al. 2007) taken here whereby phenotypic traits, performance traits and the fitness component were all integrated in a single study revealed insights that could not have been reached otherwise. For one trait, fat content, we could demonstrate the full phenotype-performance-fitness axis, where selection for a higher fat content could be explained by the sexual selection for a higher flight endurance and the positive covariation between fat content and flight endurance. For three other traits (size, FMR and wing loading), however, we detected selection that could not be explained via their effect on flight performance, generating novel testable hypotheses about how the covariation between these traits and mating success was generated. This also urges caution when using these traits as proxies for the two studied aspects of flight performance, flight speed and flight endurance (e.g. De Block and Stoks 2007). Because locomotor performance has been shown to exhibit genetic variation in other insects (e.g. Gilchrist 1996; Berwaerts et al. 2008), sexual selection on performance has the potential to be translated in evolutionary change. Given that sexual selection is typically stronger (Endler 1986; Hoekstra et al. 2001; Kingsolver et al. 2001) and more consistent in direction (Siepielski et al. 2011) than survival selection, the here documented sexual selection should therefore be considered when studying the evolutionary trajectories of locomotor performance. Another interesting avenue for further research would be to test to what extent the here documented differences in phenotypic traits between mated and unmated males that are indicative of sexual selection, are also under survival selection. This would provide insight whether these traits also determine lifetime mating success.

Acknowledgments

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Author contributions: RS and LT conceived and designed the experiments. HAGY and LT collected the samples. HAGY performed the laboratory work. HAGY analyzed the data. HAGY, LT, GYD and RS contributed to the manuscript.

APPENDICES

Table A1. Univariate linear (S) and quadratic (C) selection differentials, and multivariate linear (β) and quadratic (γ) selection gradients for all traits and populations together with the sample sizes of mated males (m) and of unmated males (um). Note that for some populations sample sizes are lower than the others. With the inclusion of these populations our aim was to test for the consistency of the documented patterns rather than quantifying selection gradients in separate populations.

		S	*C*	β	γ	Sample size	
						m	um
Flight speed	CA	-0.53	-0.61	-0.52	-0.54	12	12
	BKP	0.87	0.86	-0.12	-3.01	3	6
	HC	0.24	0.79	0.29	0.77	19	19
	PM	0.06	-0.12	0.03	-0.17	24	18
	DNZ	0.28	0.15	0.26	0.15	19	21
	PR	-0.06	-0.49	-0.07	0.13	8	12
Flight endurance	CA	0.21	-2.39	0.13	-1.95	12	12
	BKP	1.74	2.34	1.78	3.78	3	6
	HC	0.46	0.10	0.50	0.22	19	19
	PM	-0.12	0.23	-0.11	0.27	24	18
	DNZ	0.26	-0.14	0.21	-0.13	19	21
	PR	1.19	0.11	1.19	0.09	8	12
Phenoloxidase activity	CA	-0.03	0.24	0.08	0.27	26	31
	BKP	-0.35	-0.56	-0.84	-0.39	13	19
	HC	-0.15	-0.04	-0.11	0.22	36	40
	PM	0.14	0.31	-0.04	0.06	33	23
	DNZ	-0.03	-0.46	-0.25	0.09	28	34
	PR	0.46	-0.16	0.45	-0.68	13	22
Relative fat content	CA	0.33	-0.03	-0.07	-0.19	28	32
	BKP	0.27	-0.02	0.32	-0.04	13	19
	HC	0.53	-0.14	0.34	-0.12	38	43
	PM	0.05	-0.63	0.25	-1.44	32	27
	DNZ	0.27	-0.36	0.71	-0.51	28	35
	PR	0.20	0.63	0.14	1.06	13	24

Continuation of Table A1.

		S	*C*	β	γ	Sample size	
						m	um
Flight muscle ratio	CA	-0.23	0.53	-0.24	0.81	29	33
	BKP	-0.31	-1.31	-0.16	-1.23	13	19
	HC	0.08	0.56	0.33	0.61	38	42
	PM	0.30	0.06	0.60	0.10	33	27
	DNZ	0.26	-0.55	0.43	-0.33	28	34
	PR	-0.31	-0.19	-0.06	-0.65	13	24
Wing length	CA	-0.54	-0.21	-0.54	-0.54	29	33
	BKP	-0.48	0.27	-0.19	-0.47	13	19
	HC	-0.42	0.12	-0.42	0.25	36	43
	PM	-0.63	0.04	-0.70	0.05	31	27
	DNZ	0.30	0.38	0.17	0.25	28	32
	PR	0.67	0.33	0.81	-1.01	13	24
Aspect ratio	CA	0.11	-1.08	0.51	-1.36	28	33
	BKP	-0.78	0.28	-0.80	-0.90	13	18
	HC	-0.08	0.16	0.15	0.14	36	41
	PM	0.14	0.04	0.48	-0.47	29	26
	DNZ	0.01	-0.17	-0.17	-0.13	27	31
	PR	-0.02	0.48	-0.26	0.54	12	24
Wing loading	CA	0.25	0.68	0.15	0.26	26	32
	BKP	0.09	1.20	0.37	0.86	13	18
	HC	0.39	-0.14	0.25	-0.38	35	41
	PM	-0.51	0.03	-0.55	-0.02	29	26
	DNZ	0.00	0.45	-0.18	0.25	27	31
	PR	0.06	0.60	-0.04	0.45	12	23

Table A2. Multivariate results of the generalized linear model analyses examining sexual selection on the two flight performance traits in the set of six study populations of the damselfly C. puella. Significant p-values are indicated in bold.

	Mating success		
	df	χ^2	p
Population (Pop)	5	3.9641	0.55
Flight speed (PC1)	1	0.6614	0.42
Flight speed $(PC1)^2$	1	0.2878	0.59
Flight speed (PC1) × Pop	5	3.9546	0.56
Flight speed $(PC1)^2$ × Pop	5	6.6027	0.25
Flight endurance (PC2)	1	5.8360	**0.016**
Flight endurance $(PC2)^2$	1	0.1924	0.66
Flight endurance (PC2)× Pop	5	6.3597	0.27
Flight endurance $(PC2)^2$ × Pop	5	1.6723	0.89

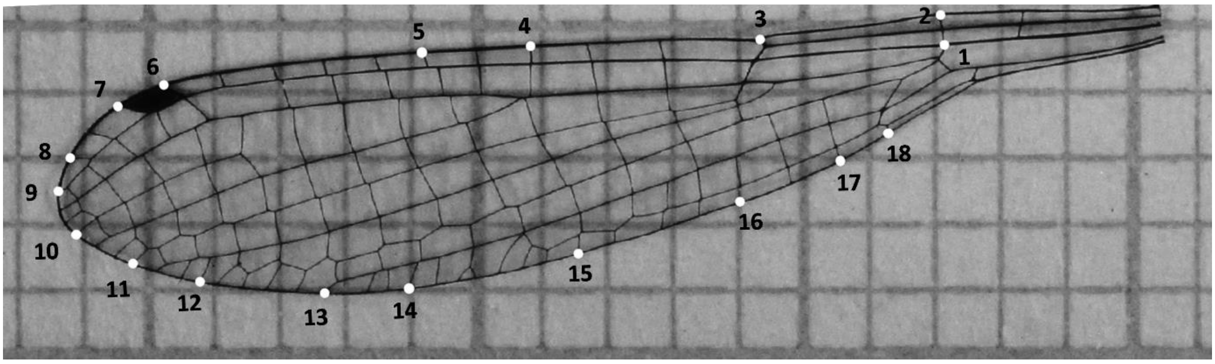

Figure A1. The left hind wing of a C. puella male with the 18 digitalized landmarks. Wing size was measured as the distance between landmark 1 and 9.

Table A3. Multivariate results of the generalized linear models examining sexual selection on the set of six phenotypic traits in the set of six study populations of the damselfly C. puella. Significant p-values are indicated in bold.

	Mating success		
	df	χ^2	p
Population (Pop)	5	11.1845	**0.048**
Phenoloxidase activity	1	0.0886	0.77
Phenoloxidase activity^2	1	0.0341	0.85
Phenoloxidase activity × Pop	5	6.5812	0.25
Phenoloxidase activity^2 × Pop	5	5.9323	0.31
Relative fat content	1	4.0091	**0.045**
Relative fat content^2	1	0.4040	0.53
Relative fat content × Pop	5	2.8340	0.73
Relative fat content ^{2} × Pop	5	7.7972	0.17
Flight muscle ratio	1	1.9615	0.16
Flight muscle ratio^2	1	0.3459	0.56
Flight muscle ratio × Pop	5	6.3463	0.27
Flight muscle ratio^2 × Pop	5	13.8766	**0.016**
Wing length	1	1.0029	0.32
Wing length^2	1	0.2425	0.62
Wing length × Pop	5	12.1824	**0.032**
Wing length^2 × Pop	5	2.1499	0.83
Aspect ratio	1	0.1330	0.72
Aspect ratio^2	1	0.5591	0.45
Aspect ratio × Pop	5	8.3618	0.14
Aspect ratio^2 × Pop	5	4.0946	0.54
Wing loading	1	0.9412	0.33
Wing loading^2	1	2.7655	0.096
Wing loading × Pop	5	7.0017	0.22
Wing loading^2 × Pop	5	6.6951	0.24

CHAPTER V

General Discussion

In this thesis we combined field and laboratory studies to obtain a multivariate picture of sexual selection on whole-organism performance and the underlying morphological and physiological traits in the territorial damselfly *Chalcolestes viridis* and the scrambling damselfly *Coenagrion puella*. In addition, as the taxonomic position of *C. viridis* and its eastern form *C. parvidens* was debated, we reconstructed phylogenetic trees to resolve the position of these two taxa. In this general discussion, we first discuss the taxonomic position of *C. viridis* and *C. parvidens.* Afterwards, we compare the multivariate sexual selection in the territorial *C. viridis* and the scrambling *C. puella*. Finally, we conclude with highlighting new insights and provide suggestions for further research.

1. ***The taxonomic position of C. viridis and C. parvidens***

1.1 *The genus status*

Dumont et al. (2010) analysed the 5.8S and 18S rRNA genes augmented with nuclear ITS1 and ITS2 sequences and concluded that *Chalcolestes* formed a monophyletic group with *Sympecma* and *Indolestes* clearly separated from the true *Lestes* species. In a review about European Odonata Dijkstra and Kalkman (2012) also recommended to recognise *Chalcolestes* as a genus. This is in line with our genetic data which further supported the genus status of *Chalcolestes*. We found that the two *Chalcolestes* taxa formed a monophyletic group separated from other investigated European *Lestes* species based on both the mitochondrial COI gene and the nuclear ribosomal ITS region.

1.2 *The species status*

We found that *C. viridis* and *C. parvidens* were differentiated in morphometric space, even though there were no single morphometric characters that could conclusively distinguish the two taxa. Our quantitative analysis of specimens from widely different regions showed that *C. parvidens* males usually had a longer body and smaller wings than *C. viridis* males. The size differences may serve as a pre-mating isolation mechanism.

Female damselflies have mechanoreceptors on the mesostigmal plate that are stimulated by male cerci during tandem linkage (Robertson and Paterson 1982). Smaller males may have difficulties grasping the thorax of a larger female with the cerci, while males that are too large may not be able to position their cerci correctly on the thoracic plates of the females.

Besides size differences also shape differences of the anal appendages [which were previously observed in a preliminary study (HA Gyulavári, unpublished data)] may reduce the probability of heterospecific copulation. McPeek et al. (2011) tested species identity traits in mate choice among six species of *Enallagma* damselflies (Coenagrionidae). They concluded that cerci sizes overlapped among species. In contrast, cerci shapes were non-overlapping among species, and five of six *Enallagma* species showed no population variation across their entire range. This suggests that cerci shape is the primary feature to discriminate conspecifics from heterospecifics during mating. This reflects the view that anal appendages are determinative for species recognition, hence reproductive isolation, in some odonates (Loibl 1958; Paulson 1974; McPeek et al. 2008).

The present pre-mating isolation mechanisms did not completely exclude mating between the two *Chalcolestes* species. Indeed, when examining 20 copulating pairs in northwest Hungary (where both species live syntopically), two pairs with a male *C. viridis* mating with a female *C. parvidens* and one reverse combination were found (Gyulavári et al. personal observation). Moreover, some individuals showed intermediate traits, suggesting that the two *Chalcolestes* taxa are capable of interbreeding. Further studies are needed to determine the fertility of such offspring.

Our genetic analysis further supported the species status of *C. parvidens.* It showed that the two *Chalcolestes* taxa are closely related compared to other European *Lestes* species, but form two monophyletic groups without shared haplotypes, suggesting that lineage sorting was complete. However, interspecies sequence dissimilarity values suggest a close relationship and a relatively recent divergence of the two taxa. This may also explain the occurrence of individuals with intermediate morphological traits presumed to be hybrids.

2. *Sexual selection*

To summarize our results of the sexual selection studies we provide an overview of the sexual selection patterns on the performance traits and the underlying morphological and physiological traits in Table 1. In addition we present for both study species a schematic figure representing the phenotype-performance-fitness axis with regard to sexual selection (Figures 1-2).

Table 1. Overview table of the detected sexual selection patterns on the key performance and phenotypic traits in the two studied species, C. viridis and C. puella.

Key traits under study		**Sexual selection pattern**	
		Territorial species ***C. viridis***	**Scrambling species** ***C. puella***
Performance traits	Flight speed	• Negative (marginally significant) • Linear • Not consistent	• Not detected
	Flight endurance	• Positive • Linear • Consistent	• Positive • Linear • Consistent
Physiological traits	Phenoloxidase activity	• Not measured	• Not detected
	Relative fat content	• Not detected	• Positive • Linear • Consistent
	Flight muscle ratio	• Not detected	• Positive • Stabilizing • Not consistent
Morphological traits	Wing length	• Positive • Stabilizing • Not consistent	• Not consistent: sometimes positive, sometimes negative (marginally significant) • Directional
	Aspect ratio	• Not measured	• Not detected
	Wing loading	• Positive • Stabilizing • Not consistent	• Positive • Stabilizing • Consistent

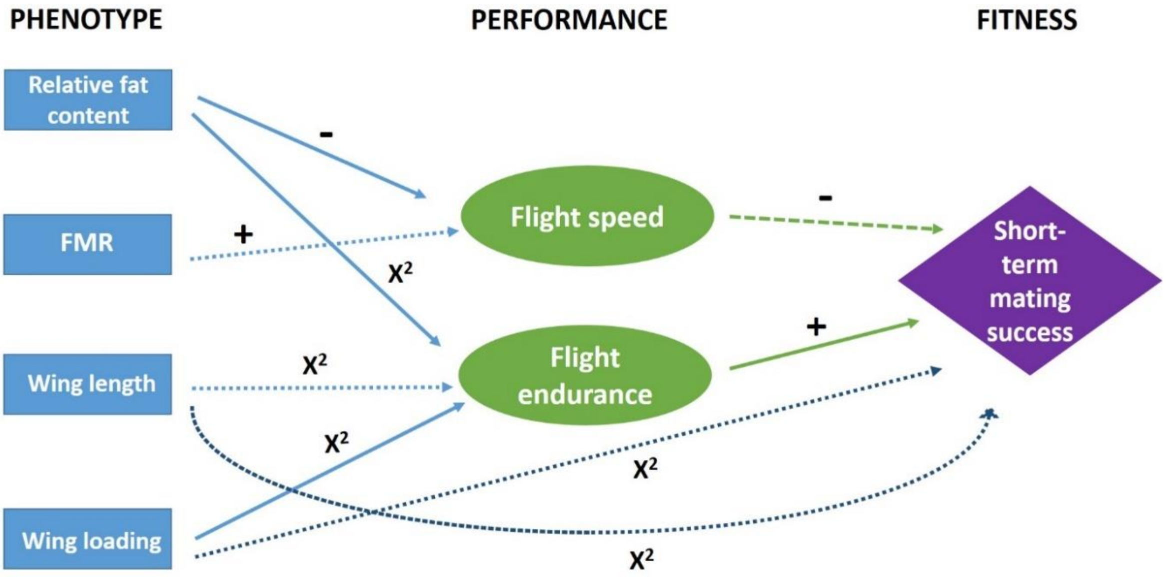

Figure 1. Schematic overview of the detected relationships along the phenotypic-performance-fitness axis for short-term mating success in the territorial damselfly C. viridis. Solid (dashed) lines represent significant (marginally significant) relationships. Dotted lines represent non-consistent relationships. X² indicate the presence of quadratic relationships; FMR = Flight muscle ratio.

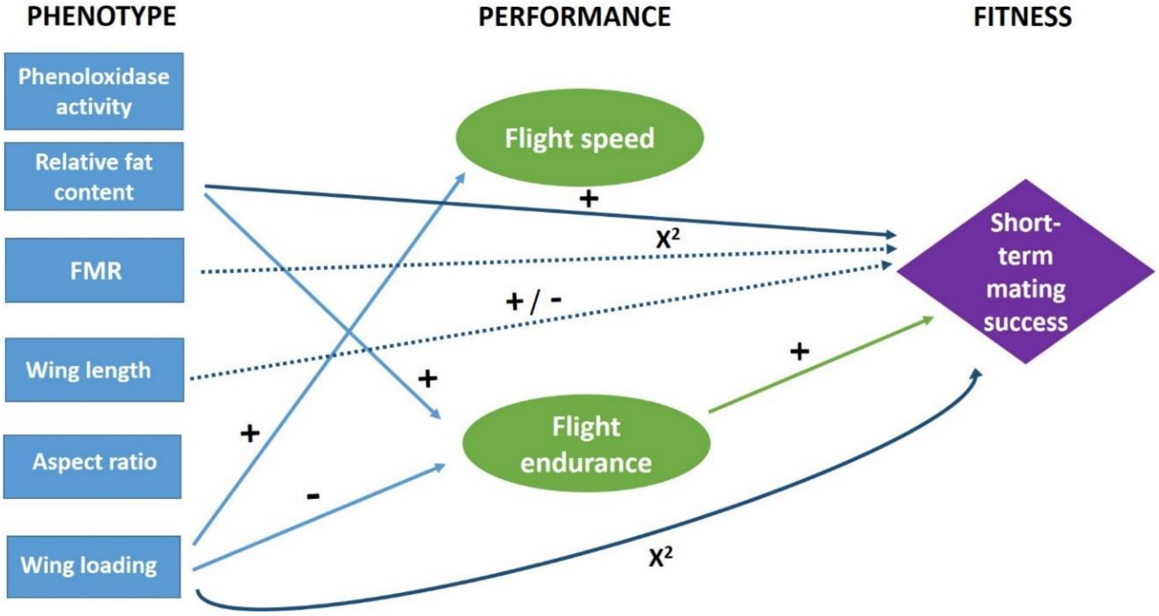

Figure 2. Schematic overview of the detected relationships along the phenotypic-performance-fitness axis for short-term mating success in the scrambling damselfly C. puella. Solid lines represent significant relationships. Dotted lines represent non-consistent relationships. X² indicate the presence of quadratic relationships; FMR = Flight muscle ratio.

2.1 *Sexual selection on flight performance*

Flight performance in aerial species is suggested to be an important selection target, yet rarely directly measured in sexual selection studies (Husak and Fox 2008). *C. viridis* performs an aggressive aerial territorial defence which contains repeated short bursts of rapid flights against territorial intruders (Dreyer 1978; HA Gyulavári and R Stoks, personal observations). In territorial species territory ownership is settled by means of aerial male-male contests, in which the male that endures longer is usually the winner (Marden and Waage 1990; Suhonen et al. 2008). Furthermore, the importance of speed for obtaining territories (Kodric-Brown and Nicoletto 1993) and mates (Husak et al. 2008) in territorial species was also reported. In line with our expectation, we found that locomotor endurance was under positive directional sexual selection with mated males having a higher flight endurance compared to unmated males. Although we expected mating males having a higher speed, in some periods flight speed was lower (although not significantly) in mated males compared to unmated males. This could reflect the well-known trade-off between speed and endurance (see e.g. Wilson et al. 2002). In line with this, we found a significant (although weak) negative correlation between endurance and speed. This contradicts another rare study on a territorial species. Faster males of the territorial collared lizard (*Crotaphytus collaris*) had larger territories that contained more females (Peterson and Husak 2006), resulting in the siring of more offspring thus higher reproductive success (Husak et al. 2006). Faster-sprinting individuals were able to translate their higher speeds into a greater share of the limited, suitable habitat and mated with more females that were overlapped by their territories. The direct link between whole-animal performance and reproductive success suggesting that speed contributes directly to effective defense of a territory with the result of excluding other males and reducing their relative fitness.

In contrast, the outcome of sexual selection in scrambling competitors, such as *C. puella*, is expected to be mainly determined by the ability to active search for females in flight, hence flight endurance (Andersson 1994). Indeed, we documented consistent positive directional sexual selection on flight endurance but not on flight speed. This finding matches with the indirect evidence obtained in a comparative study by Wickman (1992) where patrolling butterfly species (in which males actively search in flight for females) had morphological traits that were assumed to be more favourable for flight endurance compared to the traits shown by perching species. Our results concur with another recent study of the scrambling damselfly *Coenagrion scitulum*, where we found positive directional sexual selection for a longer flight duration (Therry et al. 2014) across a set of populations. In the latter study we also did not detect sexual selection for higher flight speed which is in line with the current results.

Although the two study species had a contrasting mating system, we found that flight endurance had a key role determining mating success in both species. In contrast, we could not detect selection on flight speed in neither of the species, even when it was expected to be important in the mating system. This indicates that flight speed is not as relevant as flight endurance neither for territorial defence nor for scrambling in the study species. This is intriguing as speed is likely to play a role in capturing the female, especially in scrambling species. More likely, speed is already maximized due to strong selection by other agents such as predators (Marden and Chai 1991; Almbro and Kullberg 2008) leaving less variation to detect selection on this performance trait, while endurance is less relevant for damselflies to escape predators.

2.2 *Sexual selection on physiological traits*

We investigated three physiological traits assumed to be related to body condition: a measure of investment in immune function (the activity of the enzyme phenoloxidase), energy storage (relative fat content) and relative flight muscle mass (flight muscle ratio).

The activity of the enzyme phenoloxidase was quantified only in the scrambling species, *C. puella*. Although we expected PO activity to reflect body condition (González-Santoyo and Córdoba-Aguilar 2012), PO activity was not related to endurance and mating success and was not under sexual selection. Because body fat is involved in the synthesis of immunoproteins (e.g. Boman 1986), previous studies suggested a positive link between fat reserves and immune ability (for example, Contreras-Garduño et al. 2006). Yet, this pattern does not seem to be general. The absence of covariation between fat content and PO activity in our study suggests that PO activity may not always indicate body condition (for another example in damselflies see Rolff et al. 2004). Alternatively, mating activity may have masked a positive covariation between PO activity and fat content as a result of reduced PO levels due to mating (Siva-Jothy et al. 1998). In the related scrambling damselfly *C. armatum*, immune defense (measured as encapsulation response) was even observed to be lower in mated compared to unmated males (Honkavaara et al. 2009). In insects the decreased immune function associated with increased reproductive effort might be mediated by juvenile hormone as it both inhibits PO-activity (Rolff and Siva-Jothy 2002) and stimulates gamete production (Wigglesworth 1965).

Flight is the most energetically demanding form of locomotion and lipids make up the largest part of energy reserves in insects (Klowden 2002). Moreover, the maximum duration of flight in insects depends directly on the amount of energy stores (Kaufmann et al. 2013). In addition, males of territorial species with higher fat content tend to win more fights (Marden and Waage 1990; Fitzstephens and Getty 2000; but see Plaistow and Tsubaki 2000) and sexual selection for a higher fat content has been previously found for the territorial *C. viridis* (Swillen et al. 2009). Nonetheless, we were not able to detect sexual selection on fat content in the studied population of *C. viridis*. Flight endurance did increase with fat content up to intermediate fat levels, while this was no longer true at higher fat levels. This levelling off may be explained by the cost of increased wing loading at high levels of fat storage (Blanckenhorn and Hosken 2003). On the other hand, we detected fat content to be under positive directional sexual selection in the scrambling species, *C. puella*. The multivariate selection analysis confirmed the results of the univariate selection analysis, indicating the presence of direct selection on the trait under study. This can be explained by our observation that fat contributed to flight endurance and males with a higher flight endurance had a higher short-term mating success. The finding of a higher mating probability in males with more fat has been observed before in other scrambling insect taxa, namely the flies *Scathophaga stercoraria* and *Sepsis cynipsea* (Otronen 1995; Blanckenhorn et al. 2003, 2004). Yet, these studies did not explicitly evaluate the performance link with flight endurance.

For the third physiological trait, flight muscle ratio (FMR), there was no general pattern observable. For the territorial *C. viridis* we could not link FMR ratio to mating success, and in line with this FMR did also not explain variation in flight endurance. While flight muscle mass has been linked to territory holding success (Marden 1989), other studies on *Calopteryx* species also failed to find difference in FMR between winners and losers in territorial fights (Plaistow and Siva-Jothy 1996; Marden and Waage 1990). In one population of *C. puella* we could detect selection on FMR which took the form of stabilizing selection. An advantage of an intermediate FMR for mating success may not be that unexpected. The initial increase of mating success with FMR may be due to a higher acceleration ability (Marden 1989), and the decrease at higher values may be due to a lowered aerial agility (Samejima and Tsubaki 2010) which can be an important trait for mating acquisition in scrambling species. On the contrary, we found that flight muscle mass did not differ between mated and unmated males in another scrambling damselfly *Coenagrion scitulum* (Therry et al. 2014) across a set of populations.

2.3 *Sexual selection on morphological traits*

We could detect sexual selection on two flight-related morphological traits studied: wing length (as a measure of body size) and wing loading. The third trait, aspect ratio was measured only in *C. puella* and not found to be under selection.

Large size can be important for males of territorial species because they must actively establish and defend territories against aggressive conspecifics. Yet, some studies (including two studies on the here studied *C. viridis*: De Block and Stoks 2007; Swillen et al. 2009) showed a small-male mating advantage potentially caused by a higher flight manoeuvrability of smaller males. Crompton et al. (2003) showed that smaller midges outperformed larger ones with respect to acceleration and maneuverability, parameters relevant for male mate acquisition in flight. In *C. viridis* the selection patterns on wing length differed among periods and when it was significant it took the form of quadratic selection. Quadratic sexual selection on size has been shown before in the territorial dragonfly *Libellula luctuosa* where it was explained as a result of selection against extremes (Moore 1990). In the scrambling *C. puella* the chance of encounters with females can be increased by remaining and actively patrolling at the pond as long as possible. Smaller males might need less food to support themselves; resulting in more energy and time available to search females resulting in a small-male mating advantage. (Blanckenhorn et al. 1995). In line with this we detected directional sexual selection for smaller males in three populations, but in one population there was a trend for sexual selection to favour large males. These results suggest that body size is indeed an important trait in determining mating success in odonates but the selection pattern may differ, probably depending on ecological factors such as male density and the operational sex ratio.

Higher wing loading increases flight performance and success in competitive interactions in flying insects (Marden 1989; Berwaerts et al. 2002). In *C. viridis* selection on wing loading was, however, not entirely directional but quadratic in some periods. This reflected the positive effect on mating success at lower trait values followed by a levelling off of the fitness function at higher values. This selection pattern on wing loading can be explained via its observed non-linear positive effect on flight endurance, which was under positive directional selection. The disappearance of a positive effect of wing loading on endurance and mating success at higher values may be driven by wing loading increasing flight costs due to higher wing beat frequencies to maintain a given level of flight performance (Byrne 1988). Besides its effect on flight endurance, wing loading may also have influenced mating success through positive effects on aerial manoeuvrability (Marden 1989; Marden and Chai 1991). For

C. puella also quadratic selection on wing loading was found but the pattern was different compared to *C. viridis*: wing loading did not affect mating success except at higher values where it had a positive effect on mating success. This selection pattern on wing loading cannot be explained through its observed contributions to the here scored performance measures, flight speed and flight endurance. While, as expected from previous studies (e.g. Dudley and Srygley 1994), males with a higher wing loading had a higher flight speed, we did not detect sexual selection on flight speed. Further, while wing loading negatively affected flight endurance, flight endurance was under positive directional sexual selection. Such negative covariation pattern between wing loading and flight endurance may be explained by the demand of a higher wing loading for higher wing beat frequencies to maintain a given level of flight performance (Byrne 1988) thereby increasing flight costs (Bartholomew and Casey 1978). This indicates that males with a higher wing loading scored better for other aspects of flight performance or achieved a mating advantage for other reasons. In support of the former, a higher wing loading has been suggested to be associated with a higher turning agility (Norberg and Rayner 1987), which may be favourable during scrambling competition.

The third morphological trait, aspect ratio, that was only measured in *C. puella* was not under sexual selection. Although it has been suggested that a high aspect ratio is associated with extended flight (Betts and Wootton 1988) we did not detect covariaton between the aspect ratio and flight endurance nor selection on aspect ratio. Similarly, Johansson et al. (2009) failed to find differences in aspect ratio among dragonfly genera differing in the occurrence of long-distance migration (thus endured flight). This may suggest that the aspect ratio might not fully capture wing shape in this order.

2.4 *Spatial variation in sexual selection*

Two mechanisms may cause spatial variation in sexual selection. (i) Sexual selection patterns can be driven by geographic variation in the phenotypic traits as selection on a trait depends on the mean phenotypic trait value and its variation present in the population (Conner and Hartl 2004; Steele et al. 2011). (ii) Sexual selection patterns may be directly driven by geographically structured environmental conditions, as these can modify the covariation pattern between a given trait and mating success (Moya-Laraño et al. 2007; Twiss et al. 2007). Of the two performance and six phenotypic traits of *C. puella* (the species where we studied spatial variation in sexual selection) we detected constant selection patterns for three traits: flight endurance, relative fat content and wing loading. The other traits were not under selection or the selection pattern differed across populations. The latter was the case for FMR where quadratic selection was detected, yet only in one population with males with an intermediate FMR having a higher mating success.

In wing length (our measure of body size) we detected directional sexual selection for smaller males in three populations but in one population there was a trend for sexual selection to favour large males. This resembles the study by Gosden and Svensson (2008) where in the scrambling damselfly *Ischnura elegans* sexual selection favoured small males in some populations and large males in other ones, with in some populations sexual selection favouring males of intermediate size (as observed in other scrambling species, see Stoks 2000; Thompson and Fincke 2002). This was explained by sexual selection regimes being driven by a combination of the densities of female colour morphs (that also occur in the study species) and local female body sizes. However, we have no data to verify whether similar density and frequency dependent selection may also explain the here documented spatial variation in selection on body size.

2.5 *Temporal variation in sexual selection*

We studied temporal variation in sexual selection throughout a reproductive season in *C. viridis*. For flight endurance the form of sexual selection was consistent through the entire reproductive season with mated males having higher flight endurance compared with unmated ones. Furthermore, we could detect sexual selection on two of the four flight-related traits, wing length and wing loading; in both cases the selection patterns differed among periods. For wing loading period-specific quadratic selection was detected. For the periods where selection on wing length could be detected it also took the form of quadratic selection. The temporally changing selection on wing length, our measure of total body size, within a single reproductive season extends the observation of among-year variation in sexual selection on body size (going from stabilizing to disruptive) in the damselfly *Ischnura elegans* (Gosden and Svensson 2008). The across-year variation in the latter study could be explained by changes in ecological factors such as female densities (Gosden and Svensson 2008). Beside density, sex ratio can also account for temporal variation in sexual selection on body size in insects (Kasumovic et al. 2008; Punzalan et al. 2010). These ecological factors are known to vary in nature at much smaller temporal scales within a single season (e.g. McLain 1992; Stoks et al. 1997; Van Gossum et al. 2007), as has been documented in the study species (Westermann 2008). Moreover, also temporally varying conditions during the larval stage, such as food quality, have been shown to shape sexual selection on size in the adult stage (Gillespie et al. 2014). This suggests that ecological factors likely contributed to our observed within-season dynamics in sexual selection. Furthermore, small-scale temporal dynamics in the relative size of males and females may have contributed to changes in selection pressure on body size as shown in the damselfly *Enallagma aspersum* (Steele et al. 2011).

A key finding, in line with our expectations, was that despite the temporal dynamics in flight endurance values we found persistent selection for an increased endurance, while the selection on two flight-related traits (size and wing loading) that also contributed to flight endurance, differed strongly through time. This was expected as whole-animal performance traits are the manifestation of underlying variation in morphology and physiology and different combinations of these underlying traits may result in similar performance values (many-to-one mapping, Strobbe et al. 2009). Furthermore, performance is more directly linked to fitness due to being the direct target of selection (Irschick et al. 2007, 2008). A similar pattern at a spatial scale was observed in the scrambling, poleward range-expanding damselfly, *Coenagrion scitulum,* where the selection pattern on flight duration was consistent across edge and core populations and along the thermal regimes which parallels the observation that flight duration itself did not show a geographic signal (Therry et al. 2014).

3. ***General conclusions and perspectives***

Reliable identification is still difficult for some Odonata species because of the existence of complexes of morphologically indistinguishable or hard to distinguish species. If a sample of sympatric organisms falls into two discrete clusters that differ in two or more characters, it is likely to represent two species. Although we find the data supportive of recognizing *C. parvidens* as a distinct species, further detailed morphological and genetic analysis may shed further light on how both species are separated reproductively (with special emphasis on individuals of possibly hybridizing populations). Loibl (1958) provided evidence that females of *Lestes* recognize conspecific males due to the tactile stimulation of the pronotum by the male appendages which are distinctively shaped. Females refused to copulate with conspecific males having experimentally altered inferior appendages, indicating mechanical or tactile isolation. Thus the shape analysis of the cerci and paraprocts could be the first step for future research. Another interesting scope for further studies would be to directly test the propensity to interbreed or the ability to produce offspring between the two taxa in the contact zone. A mating experiment could be only feasible under controlled conditions like in a previous study where authors used outdoor insectaries for obtaining matings in *C. viridis* (De Block and Stoks 2005). Because *Lestes* is a large, cosmopolitan and heterogeneous genus with 84 species (Schorr et al. 2011), further studies are needed including samples from the genus and also from other members of the family Lestidae to establish the exact position of *Chalcolestes*.

Our knowledge on the mechanistic base of sexual selection is still limited (Irschick et al. 2007), this is especially true for scrambling competition. The here reported studies are the first investigating sexual selection on flight performance and the underlying physiological and morphological traits in an aerial scrambling and territorial species (Husak and Fox 2008). As expected, the mating success of both species was driven by flight endurance. On the contrary, flight speed was expected to play an important role rather in territorial species than in scrambling species (Husak and Fox 2008). The functional approach (*sensu* Irschirck et al. 2007) taken whereby phenotypic traits, performance traits and the fitness component were all combined revealed insights that integrated along the phenotype-performance-fitness axis. In the scrambling *C. puella*, we could demonstrate the full phenotype-performance-fitness axis, where selection for a higher fat content could be explained by the sexual selection for a higher flight endurance and the positive covariation between fat content and flight endurance. In the territorial species *C. viridis* we could also find the link between flight performance and short-term mating success, but the relationship between the traits that contributed to performance (wing length and wing loading) did not match entirely with the observed selection pattern. The detected selection patterns that could not be explained via their effect on flight performance are generating novel testable hypotheses about how the covariation between these traits and mating success was generated. This also urges caution when using different traits as proxies for the two studied aspects of flight performance, flight speed and flight endurance (e.g. De Block and Stoks 2007). Thus we encourage researchers in general to quantify locomotor performance and include it in selection studies. Because locomotor performance has been shown to exhibit genetic variation in other insects (e.g. Gilchrist 1996, Berwaerts et al. 2008), sexual selection on performance has the potential to be translated in evolutionary change. Given that sexual selection is typically stronger (Endler 1986, Hoekstra et al. 2001, Kingsolver et al. 2001) and more consistent in direction (Siepielski et al. 2011) than survival selection, the here documented sexual selection patterns should therefore be considered when studying the evolutionary trajectories of locomotor performance. Another interesting avenue for further research would be to test to what extent the here documented differences in phenotypic traits between mated and unmated males that are indicative of sexual selection, are also under survival selection. This would provide insight whether these traits also determine lifetime mating success.

Furthermore, this PhD thesis adds to the increasing number of studies finding that the strength, direction, and form of selection are often spatially and temporally dynamic (e.g. Gosden and Svensson 2008; Siepeilski et al. 2009). We provided support that not only spatial but also temporal variation occurs within a single reproductive season for sexual selection on morphological traits (see also McLain 1992; Kasumovic et al. 2008; Punzalan et al. 2010; Steele et al. 2011). This indicates that deciding about the presence and form of selection based on pooling samples across the season and based on samples limited to restricted parts of the reproductive season (often done for logistic reasons when comparing populations) may be misleading. By ignoring the here documented within-season and among-population dynamics in sexual selection on morphological traits, one may indeed confound patterns of among-year or among-site variation with fine-scale temporal patterns within sites. In addition, we provided the first test for spatially and temporally varying sexual selection on performance traits, which confirmed our novel hypothesis that selection on performance (here flight endurance) is more stable through space and time than the observed selection on the underlying traits. We hypothesize this to be a general pattern (also for survival selection) given that, compared to morphological and physiological traits, performance is the direct target of selection (Irschick et al. 2007, 2008). In contrast, selection on phenotypic traits may be more variable as the contribution of phenotypic traits to performance may depend on environmental conditions (e.g. Moya-Laraño et al. 2007). Additionally, different combinations of underlying morphological and physiological traits may result in similar performance (Strobbe et al. 2009). This indicates that current insights on spatial and temporal dynamics of selection that are mainly based on morphological traits may not hold across trait types. In addition, more studies focusing on understudied traits such as performance traits and physiological traits are needed to refine our knowledge of the spatial and temporal dynamics of selection patterns in nature. This is of paramount importance to arrive at a better understanding of adaptive evolutionary dynamics of traits in natural populations (Siepielski et al. 2009; Bell 2010; Miller and Svensson 2014).

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Publications

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List of publications related to the dissertation

Foreign language scientific article(s) in international journal(s) (2)

1. **Gyulavári, H.A.**, Therry, L., Dévai, G., Stoks, R.: Sexual selection on flight endurance, flight-related morphology and physiology in a scrambling damselfly.
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2. **Gyulavári, H.A.**, Felföldi, T., Benken, T., Szabó, L.J., Miskolczi, M., Cserháti, C., Horvai, V., Márialigeti, K., Dévai, G.: Morphometric and molecular studies on the populations of the damselflies Chalcolestes viridis and C. parvidens (Odonata, Lestidae).
   Int. J. of Odonatology. 14 (4), 329-339, 2011. ISSN: 1388-7890.
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Hungarian conference proceeding(s) (4)

3. **Gyulavári H.**, Miskolczi M., Dévai G.: A zöld rabló [Chalcolestes viridis (VAN DER LINDEN, 1825)] három populációjának morfometriai vizsgálata.
   In: VI. Makroszkopikus Vízi Gerinctelenek Kutatási Konferencia Program és összefoglalók Villány, 2009. április 16-18. Szerk.: Csabai Zoltán, [s.n.], [S.l.], 29, 2009.

4. **Gyulavári H.A.**, Nagy H.B., Cserháti C., Schnitchen C., Grigorszky I., Miskolczi M., Dévai G.: A Chalcolestes viridis (van der Linden, 1825) két keletmagyarországi populációjából származó hímek összehasonlító jellemzése.
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5. **Gyulavári H.A.**, Nagy H.B., Cserháti C., Grigorszky I., Miskolczi M., Dévai G.: A vitatott taxonómiai helyzetű Chalcolestes Viridis (Van der Linden, 1825) egyik magyarországi populációjának jellemzése.
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Foreign language conference proceeding(s) (5)

7. **Gyulavári, H.A.**, Therry, L., Dévai, G., Stoks, R.: Sexual selection on flight endurance and flight-related morphology and physiology in a scrambling damselfly.
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8. **Gyulavári H.A.**, Felföldi T., Benken T., Szabó L.J., Miskolczi M., Cserháti C., Horvai V., Márialigeti K., Dévai G.: Comparative analysis of the species-group taxa in genus Chalcolestes based on different DNA sequences.
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10. **Gyulavári, H.A.**, Benken, T., Szabó, L.J., Cserháti, C., Horvai, V., Miskolczi, M., Dévai, G.: Comparative morphometrical analysis of Lestes (Chalcolestes) parvidens and L. viridis populations.
In: 29. Jahrestagung der Gesellschaft deutschsprachiger Odonatologen (GdO), Rothenburg ob der Tauber, 19.-21. März 2010. Ed.: Florian Weihrauch, GdO, Börnsen, 53-54, 2010.

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List of other publications

Hungarian scientific article(s) in Hungarian journal(s) (8)

12. Kis O., Vajda C., **Gyulavári H.A.**, Szabó L.J., Miskolczi M., Cserháti C., Dévai G.: A nyugati zöld rabló [Chalcolestes viridis (Vander Linden, 1825)] egy észak-magyarországi imágópopulációjának morfológiai jellemzése.
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Foreign language scientific article(s) in international journal(s) (2)

20. van Dinh, K., Janssens, L., Therry, L., **Gyulavári, H.A.**, Bervoets, L., Stoks, R.: Rapid evolution of increased vulnerability to an insecticide at the expansion front in a poleward moving damselfly.
Evolutionary Applications. "Accepted by Publisher" (2015) ISSN: 1752-4571.
IF:3.896 (2014)

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Ecography. 37 (10), 1012-1021, 2014. ISSN: 0906-7590.
DOI: http://dx.doi.org/10.1111/ecog.00630
IF:4.774

Hungarian conference proceeding(s) (10)

22. Vajda C., **Gyulavári H.A.**: Előtanulmány a Lestes- és a Chalcolestes-fajok összehasonlítására hím imágók ivarkészüléke alapján [Preliminary studies on comparison of male Lestes and Chalcolestes adult damselfly species based on their sexual organs].
In: Akvatikus és terresztris kutatások kapcsolata : Hidrobiológus Napok programfüzete. Szerk.: Bíró Péter, Reskóné Nagy Mária, Kiss Keve Tihamér. Magyar Hidrológiai Társaság Limnológiai Szakosztálya, Budapest, 49, 2013.

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In: VII. Makroszkopikus Vízi Gerinctelenek Kutatási Konferencia Program és összefoglalók Sümeg, 2010. április 15-17. Szerk.: Bereczki Csaba, [s.n.], [S.l.], 28, 2010.

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In: VI. Makroszkopikus Vízi Gerinctelenek Kutatási Konferencia Program és összefoglalók Villány, 2009. április 16-18. Szerk.: Csabai Zoltán, [s.n.], [S.l.], 30, 2009.

29. Czirok A., Horvai V., **Gyulavári H.A.**: Egyes környezeti változók hatása a makrogerinctelen közösség összetételére a Völgységi-patak hosszszelvényében.
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30. Prill É., **Gyulavári H.**, Jakab T., Miskolczi M., Dévai G.: Az érces szitakötő [cordulia aenea (linné, 1758)] egyik magyarországi populációjának átfogó jellemzése a morfológiai jellemzők, a testtömeg és az energiatartalom alapján.
    Hidrol. Közlöny. 89 (6), 49-52, 2009. ISSN: 0018-1323.

31. Prill É., **Gyulavári H.**, Jakab T., Miskolczi M., Dévai G.: Az érces szitakötő [Cordulia aenea (Linné, 1758)] imágójának átfogó jellemzése egy magyarországi populáció testméret-, testtömeg és energiatartalom-adatai alapján.
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Foreign language conference proceeding(s) (2)

32. Kis, O., **Gyulavári, H.A.**, Szabó, L.J., Cserháti, C., Miskolczi, M., Dévai, G.: Comparative morphometric description of two Hungarian adult populations of the genus Chalcolestes.
    In: 31. Jahrestagung der Gesellschat deutschsprachiger Odonatologen (GdO) e. V. 9. bis 11. März 2012 in Freiberg. Ed.: Marko Olias, André Günther, GdO, Freiberg, 63-64, 2012.

33. Kézér, K., **Gyulavári, H.A.**, Szabó, L.J., Cserháti, C., Miskolczi, M., Dévai, G.: Morphometrical characterization of the Dark Spreadwing, Lestes macrostigma, based on the adult population from a typical Hungarian alkaline pond.
    In: 29. Jahrestagung der Gesellschaft deutschsprachiger Odonatologen (GdO), Rothenburg ob der Tauber, 19.-21. März 2010. Ed.: Florian Weihrauch, GdO, Börnsen, 59-60, 2010.

Total IF of journals (all publications): 11,801
Total IF of journals (publications related to the dissertation): 3,131

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

24 November, 2015

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR

Nyilvántartási szám: DEENK/243/2015.PL
Tárgy: PhD Publikációs Lista

Jelölt: Gyulavári Hajnalka Anna
Neptun kód: RJEIGO
Doktori Iskola: Juhász-Nagy Pál Doktori Iskola
MTMT azonosító: 10039976

A PhD értekezés alapjául szolgáló közlemények

Idegen nyelvű tudományos közlemény(ek) külföldi folyóiratban (2)

1. **Gyulavári, H.A.**, Therry, L., Dévai, G., Stoks, R.: Sexual selection on flight endurance, flight-related morphology and physiology in a scrambling damselfly.
   Evol. Ecol. 28 (4), 639-654, 2014. ISSN: 0269-7653.
   DOI: http://dx.doi.org/10.1007/s10682-014-9703-1
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2. **Gyulavári, H.A.**, Felföldi, T., Benken, T., Szabó, L.J., Miskolczi, M., Cserháti, C., Horvai, V., Márialigeti, K., Dévai, G.: Morphometric and molecular studies on the populations of the damselflies Chalcolestes viridis and C. parvidens (Odonata, Lestidae).
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   DOI: http://dx.doi.org/10.1080/13887890.2011.651983
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Magyar nyelvű konferencia közlemény(ek) (4)

3. **Gyulavári H.**, Miskolczi M., Dévai G.: A zöld rabló [Chalcolestes viridis (VAN DER LINDEN, 1825)] három populációjának morfometriai vizsgálata.
   In: VI. Makroszkopikus Vízi Gerinctelenek Kutatási Konferencia Program és összefoglalók Villány, 2009. április 16-18. Szerk.: Csabai Zoltán, [s.n.], [S.l.], 29, 2009.

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5. **Gyulavári H.A.**, Nagy H.B., Cserháti C., Grigorszky I., Miskolczi M., Dévai G.: A vitatott taxonómiai helyzetű Chalcolestes Viridis (Van der Linden, 1825) egyik magyarországi populációjának jellemzése.
Hidrol. Közlöny. 88 (6), 66-69, 2008. ISSN: 0018-1323.

6. **Gyulavári H.A.**, Nagy H.B.: A vitatott taxonómiai helyzetű Chalcolestes Viridis (Van der Linden, 1825; Odonata : Lestidae) halápi populációjának jellemzése.
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Idegen nyelvű konferencia közlemény(ek) (5)

7. **Gyulavári, H.A.**, Therry, L., Dévai, G., Stoks, R.: Sexual selection on flight endurance and flight-related morphology and physiology in a scrambling damselfly.
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8. **Gyulavári H.A.**, Felföldi T., Benken T., Szabó L.J., Miskolczi M., Cserháti C., Horvai V., Márialigeti K., Dévai G.: Comparative analysis of the species-group taxa in genus Chalcolestes based on different DNA sequences.
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9. **Gyulavári, H.A.**, Felföldi, T., Benken, T., Szabó, L.J., Miskolczi, M., Cserháti, C., Horvai, V., Márialigeti, K., Dévai, G.: Preliminary morphometric and molecular invest igations on adult specimens of two Lestes (Chalcolestes) taxa.
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További Közlemények

Magyar nyelvű közlemény(ek) hazai folyóiratban (8)

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