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BDS

The aims of the **British Dragonfly Society** (BDS) are to promote and encourage the study and conservation of Odonata and their natural habitats, especially in the United Kingdom.

The Journal of the British Dragonfly Society, published twice a year, contains articles on Odonata that have been recorded from the United Kingdom and articles on European Odonata written by members of the Society.

Articles for publication should be sent to the Editor. Instructions for authors appear inside the back cover.

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Cover illustration: Male *Leucorrhinia dubia* at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve. Photograph by Greg Osborn.

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- Manuscripts should be one and a half spaced, on one side of the page only and with margins at least 25mm on both sides and top and bottom. Footnotes should be avoided.
- Use of these terms is acceptable: 'exuvia' for cast skin (plural: 'exuviae'); 'larva' (instead of 'naiad' or 'nymph'); 'prolarva' to designate the first larval instar.
- Dates in the text should be expressed in the form: 24 July 2010.
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  Hammond, C.O. 1983. *The Dragonflies of Great Britain and Ireland* 2nd Edition (revised by R. Merritt), Harley Books, Colchester, 116pp.

  Longfield, C. 1949. The Dragonflies of the London area. *The London Naturalist* **28**: 90-98.
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Please refer to a recent issue of the journal for further style details.

SCIENTIFIC AND ENGLISH NAMES OF BRITISH ODONATA

ZYGOPTERA	DAMSELFLIES
Calopteryx splendens	Banded Demoiselle
Calopteryx virgo	Beautiful Demoiselle
Ceriagrion tenellum	Small Red Damselfly
Chalcolestes viridis	Willow Emerald Damselfly
Coenagrion armatum	Norfolk Damselfly
Coenagrion hastulatum	Northern Damselfly
Coenagrion lunulatum	Irish Damselfly
Coenagrion mercuriale	Southern Damselfly
Coenagrion puella	Azure Damselfly
Coenagrion pulchellum	Variable Damselfly
Coenagrion scitulum	Dainty Damselfly
Enallagma cyathigerum	Common Blue Damselfly
Erythromma najas	Red-eyed Damselfly
Erythromma viridulum	Small Red-eyed Damselfly
Ischnura elegans	Blue-tailed Damselfly
Ischnura pumilio	Scarce Blue-tailed Damselfly
Lestes barbarus	Southern Emerald Damselfly
Lestes dryas	Scarce Emerald Damselfly
Lestes sponsa	Emerald Damselfly
Platycnemis pennipes	White-legged Damselfly
Pyrrhosoma nymphula	Large Red Damselfly
Sympecma fusca	Winter Damselfly
ANISOPTERA	DRAGONFLIES
Aeshna affinis	Southern Migrant Hawker
Aeshna caerulea	Azure Hawker
Aeshna cyanea	Southern Hawker
Aeshna grandis	Brown Hawker
Aeshna juncea	Common Hawker
Aeshna mixta	Migrant Hawker
Anaciaeshna isoceles	Norfolk Hawker
Anax ephippiger	Vagrant Emperor
Anax imperator	Emperor Dragonfly
Anax junius	Green Darner
Anax parthenope	Lesser Emperor
Brachytron pratense	Hairy Dragonfly
Cordulegaster boltonii	Golden-ringed Dragonfly
Cordulia aenea	Downy Emerald
Crocothemis erythraea	Scarlet Darter
Gomphus flavipes	Yellow-legged Club-tail
Gomphus vulgatissimus	Common Club-tail
Leucorrhinia dubia	White-faced Darter
Leucorrhinia pectoralis	Large White-faced Darter
Libellula depressa	Broad-bodied Chaser
Libellula fulva	Scarce Chaser
Libellula quadrimaculata	Four-spotted Chaser
Orthetrum cancellatum	Black-tailed Skimmer
Orthetrum coerulescens	Keeled Skimmer
Oxygastra curtisii	Orange-spotted Emerald
Pantala flavescens	Wandering Glider
Somatochlora arctica	Northern Emerald
Somatochlora metallica	Brilliant Emerald
Sympetrum danae	Black Darter
Sympetrum flaveolum	Yellow-winged Darter
Sympetrum fonscolombii	Red-veined Darter
Sympetrum pedemontanum	Banded Darter
Sympetrum sanguineum	Ruddy Darter
Sympetrum striolatum *	Common. Darter *
Sympetrum vulgatum	Vagrant Darter

* Includes dark specimens in the north-west formerly treated as a separate species, *Sympetrum nigrescens* Highland Darter

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Identification of the exuviae of Lestidae (Emerald Damselflies) in Britain

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Summary

This study investigates those features that can be used to separate the final instar exuviae of the four lestid species currently breeding in Britain. A binocular microscope was used to look at the finer details and allow close-up photographs to be taken. Important features relate to the labium, the caudal lamellae, the ovipositor and the spines/hairs on the abdomen.

Introduction

There are eight species of emerald damselflies in Europe (Askew, 1988), four of which at present occur in Britain: *Lestes dryas* Scarce Emerald Damselfly, *Lestes sponsa* Emerald Damselfly, *Chalcolestes viridis* Willow Emerald Damselfly (an exuvia of which was first found in Britain in 1992 (Brook & Brook 2003)) and *Lestes barbarus* Southern Emerald Damselfly. Until *L. barbarus* arrived in the country in 2002 (Parr, 2003), the exuviae of the other three were relatively easy to separate. The exuviae of *L. barbarus* are very similar to those of *L. dryas* and, where both of these occur at the same site, identification can sometimes be difficult.

Material and Methods

All samples of *Lestes dryas* exuviae were taken from a site in Kent where no *Lestes. barbarus have been recorded.* Samples of *L. barbarus* exuviae were supplied by Dave Chelmick from a site in Spain where *L. dryas* has never been found. This eliminates any confusion between the identification of the exuviae of *L. dryas* and *L. barbarus* when comparing the two species, which can be found in similar habitats which tend to dry out, or nearly so, in summer. A small sample of *L. barbarus* was also obtained from a site in Kent. The following account is based on last instar exuviae. A binocular microscope was used to examine the fine details and to take close-up photographs.

Results

The main features to look at to separate the four lestids found in Britain are the labium, the caudal lamellae, the female ovipositor and the abdomen. High magnification is necessary when looking at the labial palps and at the sensilla (spines and hairs) on the abdomen.

The Labium

In Europe there are eight lestids, five of which have a "racket-shaped" labium, while the remaining three have a labium more like that of an aeshnid dragonfly (Gerken & Sternberg, 1999). Only one of the latter, *Chalcolestes viridis*, occurs in Britain at present, the other three in Britain, *Lestes dryas, Lestes sponsa* and *Lestes barbarus,* having a 'racket-shaped' labium (Plate 1, Table 1). Thus, at present, the shape of the labium of *C. viridis* readily separates it from the remaining three species, though new species coming into the country must never be ruled out, the most likely of which would be *Sympecma fusca* Winter Damselfly. The labial palps of *L. sponsa* have two setae on the movable hook

Plate 1. The 'Racket-shaped' labium typical of *Lestes sponsa*, *Lestes dryas* and *Lestes barbarus* (left) and the 'Aeshnid-shaped' labium of *Chalcolestes viridis* (right).

Table 1. Summary of the features that can be used to separate the four lestid species currently breeding in Britain.

	Lestes dryas	*Lestes barbarus*	*Lestes sponsa*	*Chalcolestes viridis*
Labium	'Racket-shaped'			'Aeshnid shaped'
	Labial palps with 3 setae on moveable hook	Labial palps with 3 or 4 setae on moveable hook	Labial palps with 2 setae on moveable hook	
Caudal lamellae	Tips pointed		Tips rounded	
	2 distinct bands. (sometime a pale 3rd)	2 bands (sometime a pale 3rd) but bands may be missing	3 distinct dark bands	2 distinct pale bands (sometime a pale 3rd)
	Distal two-thirds of ventral edge of median lamella with distinct concave curve	Distal two-thirds of ventral edge of median lamella almost straight	Edges of outer lamellae almost parallel	
	8.5-11.0 mm long	7.5-10.75 mm long	8.5-10.0 mm long	7.0-9.0 mm long
Ovipositor	Extends well beyond segment 10	Extends just beyond segment 10	Extends to or just beyond segment 10.	
Abdominal segments 7-9	Short spines	Long, fine hairs		

(Fig. 1) while those of *L. dryas* have three (Fig. 2). Those of *L. barbarus* have three (Fig. 2) or four (Askew, 1988) (Table 1). The majority of the samples of *L. barbarus* which we collected in Kent had three setae on each movable hook, whereas, of the 26 *L. barbarus* from Spain, 21 had three on each movable hook, four had three on one movable hook and four on the other, and just one had four on each movable hook.

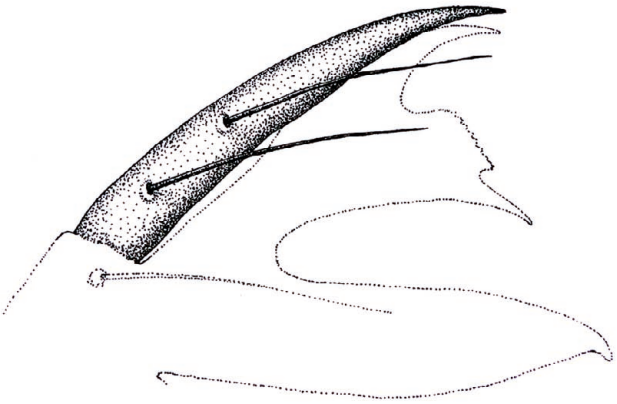

Fig. 1. A labial palp with two setae on the moveable hook, which is typical of *Lestes sponsa*.

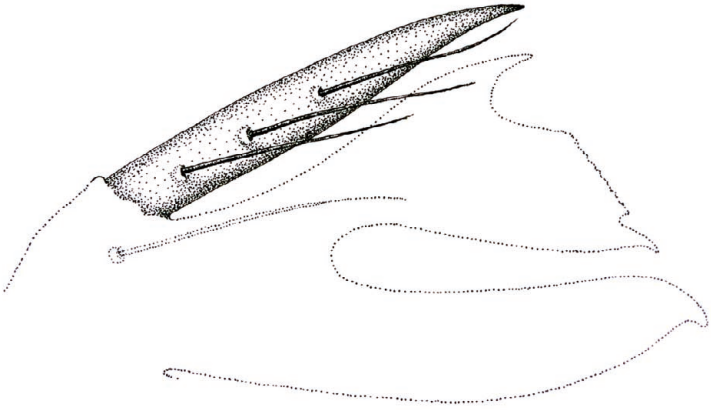

Fig. 2. A labial palp with three setae on the moveable hook, which is typical of *Lestes dryas* and most *Lestes barbarus*.

The Caudal Lamellae

The caudal lamellae are another important identification feature (Plates 2, 3, Table 1). It is necessary to moisten the lamellae in order to float them off separately onto a slide to see the distinguishing features. The edges of the outer lamellae of *Lestes sponsa* and *Chalcolestes viridis* are more or less parallel and fairly strongly rounded at the distal end, whereas the median lamella is slightly wider at the basal end. The lamellae of *L. sponsa* have three distinct dark, transverse bands, with usually a small dark area at the distal end. *Chalcolestes viridis* has only two distinct paler bands with sometimes a very pale third band but the lamellae of *C. viridis* are generally shorter (7 - 9mm) (Cham, 2012) than those of *L. sponsa* (8.5 – 10mm).

The lamellae of *Lestes dryas* and *Lestes barbarus* are both pointed at the distal end and both have two distinct transverse bands, which vary from dark to pale, and sometimes a very pale third band. In the case of *L. barbarus* the bands may be missing completely (Brochard, pers. com.). In Kent the lamellae of *L. barbarus* have generally been shorter than those of *L. dryas* and the shape of the median lamella is different. The length of the lamellae of 91 specimens of *L. dryas* were measured and ranged from 8.5 – 11mm. Those of *L. barbarus* from Spain ranged from 8.0 – 10.75mm, although those of the few *L. barbarus* found in Kent were between 7.5 – 9.5mm (Fig. 3). As there is an overlap in their lengths, this feature was found to be unreliable for identification, even though the lamellae of *L. dryas* are most often longer (Fig. 3). The ventral edge of the median lamella of *L. dryas* has a distinct concave curve in its distal two thirds, whereas the curve in *L. barbarus* is far less pronounced and the distal two thirds of the lamella is almost straight (Plate 3).

The Ovipositor

The females of *Lestes dryas* and *Lestes barbarus* are easier to identify than the males. The ovipositor of *L. dryas* extends well beyond the tenth abdominal segment whereas that of *L. barbarus* extends only a short way beyond the tenth abdominal segment (Plate 4, Table 1). The ovipositor of *L. sponsa* reaches to, or just beyond, the tenth abdominal segment (Hammond, 1983) (Plate 4, Table 1).

The abdomen

The most useful character to separate the males of *Lestes dryas* and *Lestes barbarus* is only revealed with the use of a microscope. Especially noticeable on segments 7 to 9*, L. dryas* has very short spines (Plate 5, Table 1) whereas *L. barbarus* has longer, finer hairs (Brochard *et. al*., 2012) (Plate 6, Table 1). This

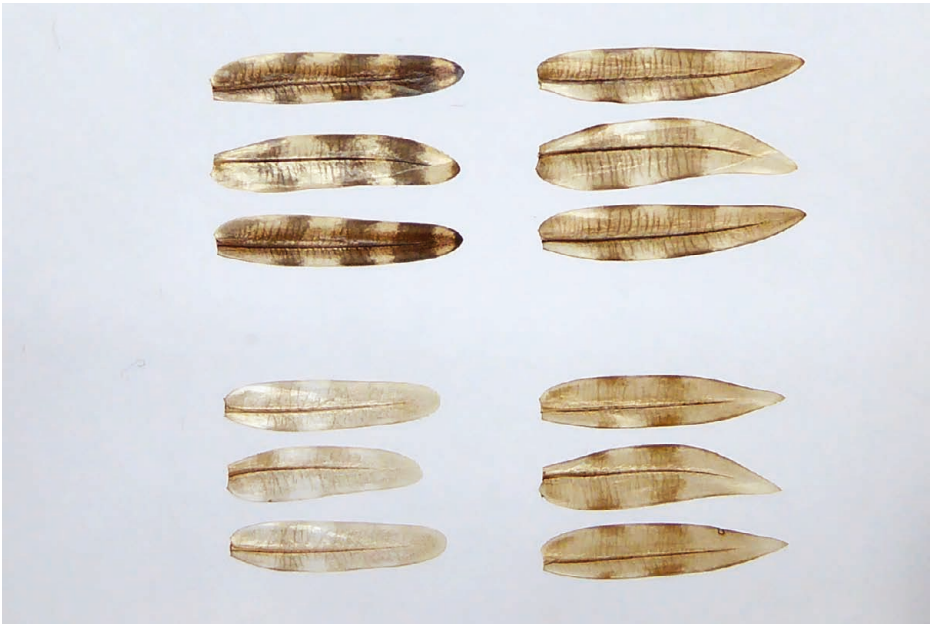

Plate 2. The caudal lamellae of *Lestes sponsa* (top left), *Lestes dryas* (top right), *Chalcolestes viridis* (bottom left) and *Lestes barbarus* (bottom right).

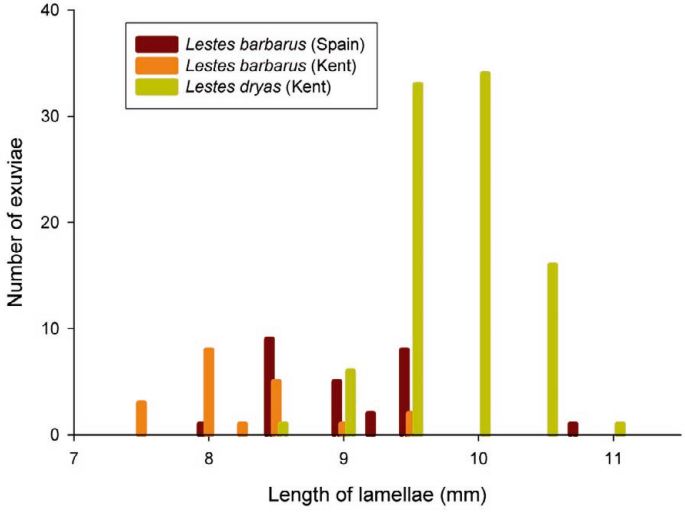

Fig. 3. The length of the lamellae of *Lestes barbarus* from Spain and Kent and *Lestes dryas* from Kent.

Plate 3. The median caudal lamellae of (from top to bottom) *Lestes sponsa*, C*halcolestes viridis*, *Lestes dryas* and *Lestes barbarus*.

Plate 4. The ovipositors of (from top to bottom) *Lestes dryas*, *Lestes barbarus* and *Lestes sponsa*.

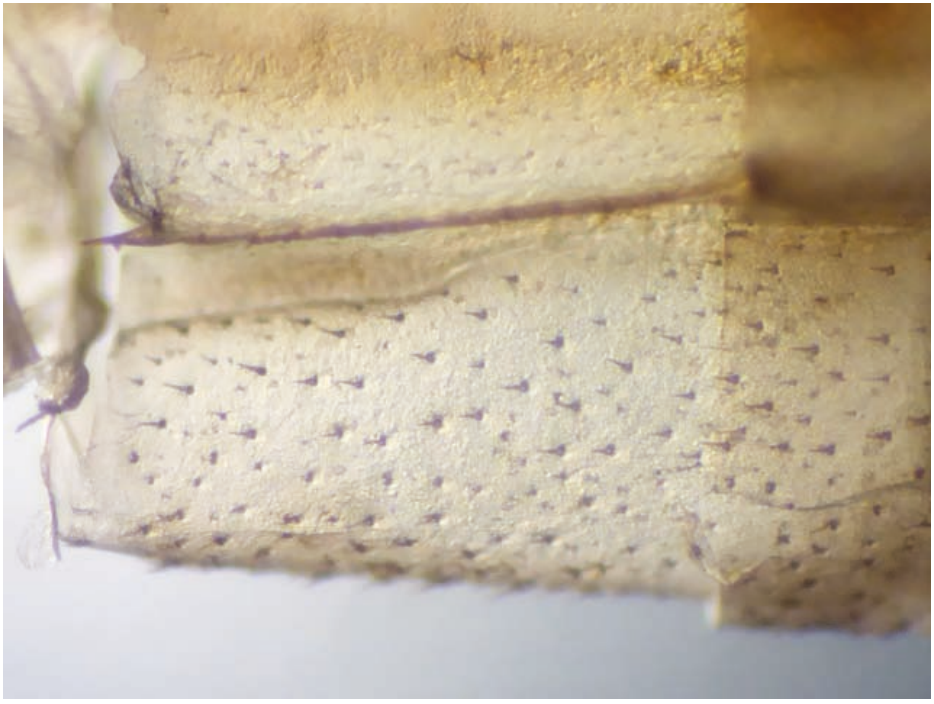

Plate 5. Lateral view of abdominal segment 8 of *Lestes dryas* to show the short spines on the abdomen.

feature can also be seen on the females.

Summary

The characters described above have been summarised (Table 1) and a key is presented to separate the final stage exuviae of the four lestid species that currently are known to breed in Britain.

Verification

Due to its rarity, if exuviae of *Lestes barbarus* are found it is important to have them checked by an acknowledged expert for confirmation.

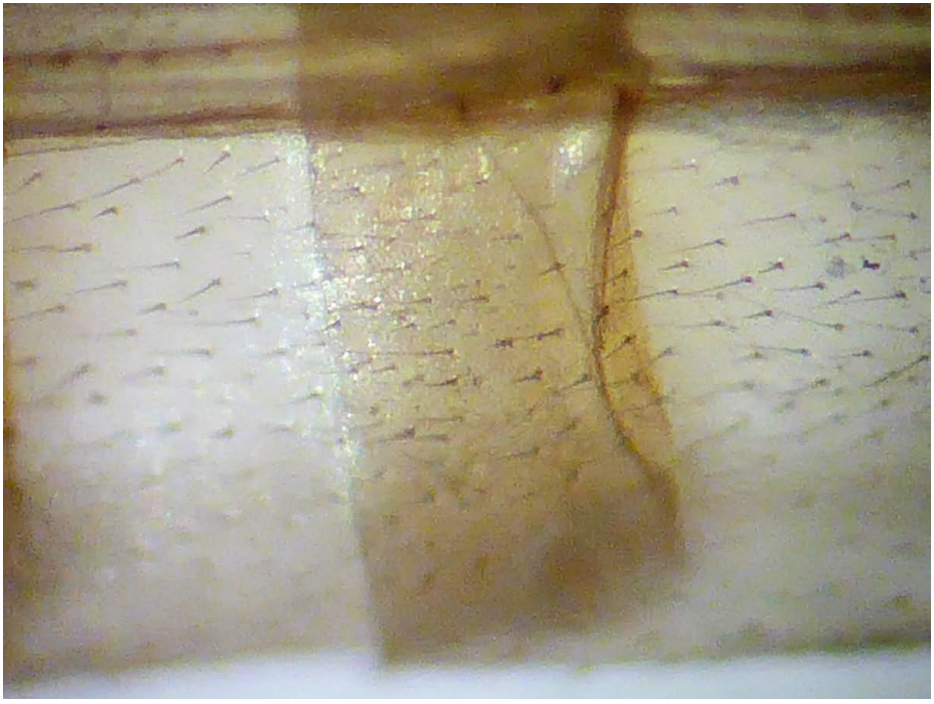

Plate 6. Lateral view of abdominal segments 7 and 8 of *Lestes barbarus* to show the long fine hairs.

Key to the four lestids currently breeding in Britain.

1.	Labium 'Racket-shaped'	2
	Labium 'Aeshnid-shaped'	*Chalcolestes viridis*
2.	Tips of caudal lamellae pointed	3
	Tips of caudal lamellae rounded	*Lestes sponsa*
3.	Distal two-thirds of ventral edge of median lamella with distinct concave curve; ovipositor extends well beyond segment 10; short spines on abdominal segments 7-9	*Lestes dryas*
	Distal two-thirds of ventral edge of median lamella almost straight; ovipositor extends just beyond segment 10; long, fine hairs on abdominal segments 7-9	*Lestes barbarus*

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A brief review of research on dragonflies involving molecular techniques

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Summary

A review is presented of those molecular techniques that have been applied to Odonata, illustrated with examples of recent research. Developments in molecular biology have increased our knowledge of the taxonomy of odonate species, how they are related to each other and their evolution. This is especially relevant with species that share a similar morphology and habitat and consequently are difficult to distinguish from each other. The techniques mentioned include Fluorescent and Genomic in Situ Hybridisation (FISH and GISH respectively), Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment length Polymorphism (AFLP). Nuclear, mitochondrial and ribosomal DNA have all been used. The examples include insights into the frequency of colour morphs in populations of dragonflies, the correlation between the abundance of transcripts of a flight gene with wingbeat frequency and amplitude, the correlation between parasitic susceptibility and genetic variation within a species of *Calopteryx*, the effects of population and habitat fragmentation, and the evolutionary proximity of various species of Odonata.

Introduction

The disciplines of molecular biology and genetics have progressed very rapidly in recent years. The completion of fast, accurate sequencing techniques has enabled the genetic structure of many species to be determined. The development of techniques for the analysis of DNA, not merely from the nucleus of a cell but also from the ribosomes and mitochondria, have revolutionised our knowledge concerning the classification of organisms. In addition, developments in the fields of understanding whole cell metabolism and how genetic factors are mediated have transformed our understanding of the function and co-dependency of many cellular processes. These have helped to provide further insights into the complex processes controlling the development of organisms, their behaviour, structure and function. The development of genetic markers has provided efficient, powerful and flexible methods for investigating fundamental

ecological and environmental questions. Consequently, it is now possible to determine patterns of parentage and reproductive success, in addition to population diversity and evolutionary relationships.

The earliest use of molecular technologies reviewed here mostly date from the 1990s and the aim of this article is to provide a brief overview of some of the more important research techniques where they have been used in relation to Odonata; not to cover the whole literature on the subject. There are reviews on molecular techniques used in insects but with little or no mention of odonates (Behura, 2006; Jain *et al*., 2010).

Cytogenetics

Before the development of molecular techniques there was already a considerable volume of work based on studies of the karyotypes of odonate species. For example, Kiauta (1979) and Mola & Agopian (1985) used chromosome squashes to examine the karyoptypes of anisopteran species from Surinam and South America respectively.

It is possible to extract DNA from a dragonfly non-destructively, using a single leg, so the insect is relatively unharmed. The cells are placed on a slide and denatured to break down the cell walls and nuclear membranes so that the chromosomes can be stained and hence visualised. Small fragments of DNA are produced artificially and used as probes which bind to the corresponding regions on the chromosomes. The first probes were radioactive and the labelled regions appeared much lighter in photographs (Gall & Pardue, 1969). Subsequently, fluorescent labelled probes were used, which provide a safer and more stable alternative to the use of radioactive probes (Rudkin & Stollar, 1977). Those regions of the chromosomes that are complementary to the probes glow a different colour. This technique was developed further by Speel *et al*. (1992).

The two techniques based on this technology are Fluorescent In Situ Hybridisation (FISH) and Genomic In Situ Hybridisation (GISH).

Fluorescent In Situ Hybridisation (FISH)

This technique is used to detect and locate specific DNA sequences or a gene on a chromosome. It uses a small fluorescent DNA sequence as a probe. The DNA in the probe binds (anneals) to the complementary part of the organism's DNA which then fluoresces. Fluorescent microscopy is used to visualise where the probe is bound (Fig. 1). Grozeva & Marinov (2007) used this technique to study the number and type of chromosomes and the degree of homogeneity in four corduliid dragonflies (*Somatochlora borisi*, *S. metallica*, *S. meridionalis*

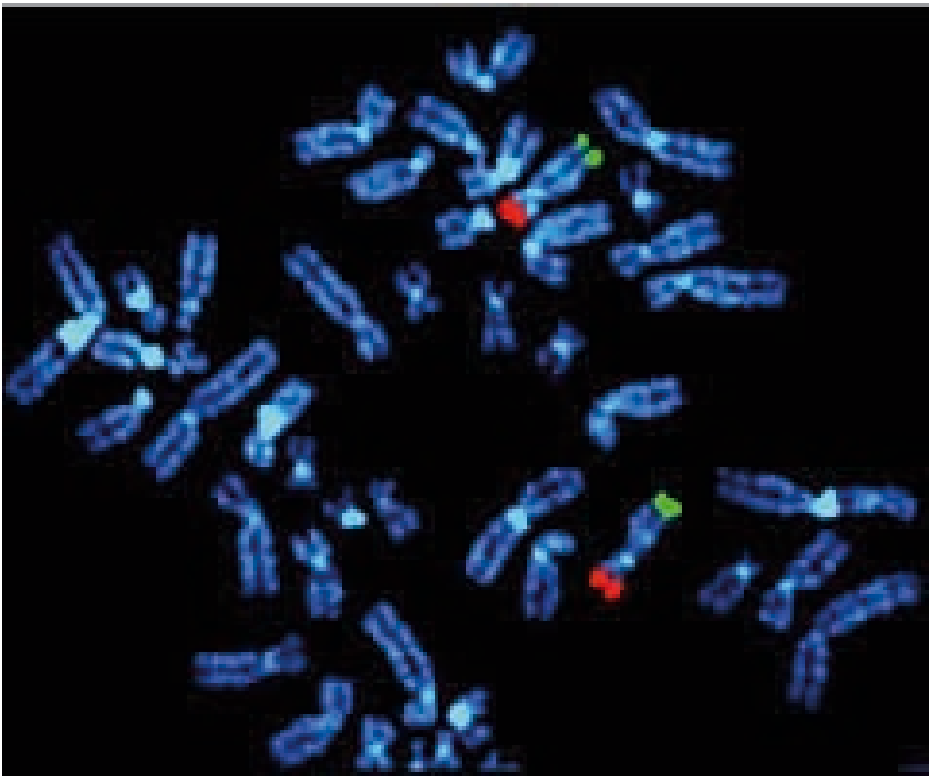

Figure 1. Fluorescent In Situ Hybridisation (FISH). A chromosome preparation of a human cell in metaphase. Note the red and green fluorescent markers. From TheBestMedic (2014).

and *Cordulia aenea*). *Somatochlora borisi*, which was only described in 2001 (Marinov, 2001) and is endemic to the eastern Balkans, combines morphological characters from both *Somatochlora* and *Cordulia* (Marinov & Seidenbusch, 2007). Based on the morphology and the argument that the more homology that is present the closer the relationship between different species, Grozeva & Marinov (2007) confirmed that *Somatochlora borisi* deviated not only from *C. aenea* but also from the other two species of *Somatochlora*. It has thus now been placed in a new genus, *Corduliochlora* (Marinov & Seidenbusch, 2007).

Genomic In Situ Hybridisation (GISH)

GISH is a similar technique that enables scientists to label parts of the genome within the cells. It is a modification of FISH in which the whole genomic DNA is used as a probe, rather than the small sequences used in FISH. This technique is used to study phylogenetic relationships between species. The

total genomic DNA of one of the species being studied is extracted to use as a probe. Chromosome preparations are then made of both species and the probe applied. Any sequences that are repeated in both species anneal faster than unique sequences and hence fluoresce. It has been used to determine to what degree a population is sedentary. GISH has not been widely used (Markova & Vyskot, 2009) and I have not found any reference to it being used on odonates.

Molecular Genetics

Random Amplified Polymorphic DNA (RAPD).

Another technique that has been used to determine relatedness between dragonflies is Random Amplified Polymorphic DNA (RAPD). This analyses the genetic diversity of an individual by using random primers and only requires one primer for amplification. It has been used for gene mapping, population genetics and evolutionary genetics and has the advantage that no previous knowledge of the genome is required (e.g. Kumar & Gurusubramanian, 2011).

DNA from a dragonfly specimen is extracted, again non-destructively if possible, and subjected to a Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction PCR This is a technique for amplifying (producing numerous copies) of one or more small sections (sequences) of DNA. Using PCR the DNA strands are broken apart and then short chains (about 10 base pairs long) of artificial, carefully selected DNA (primers) anneal (bind) to the genomic DNA. The synthetic short chains of DNA are, as noted above, random and therefore they attach in a number of random positions depending solely on their match to the DNA strand (Fig. 2). The enzyme Taq polymerase is used to produce more copies of the part of the DNA it has attached to and makes the copies longer. Therefore, a number of lengths of DNA are present in the mixture. This process is repeated many times, increasing the numbers of copies at each repeat (Fig. 3). No knowledge of the DNA sequence of the targeted genome is required. Although it is not certain exactly where, the primers will bind somewhere in the sequence.

At the end of the PCR process the mixture may contain thousands of strands of DNA of different lengths, depending on where the random DNA bound and how much the enzyme extended the section to which the primers bound before reaching a stop code. For example, suppose the random DNA bound to three sites, producing strands of four hundred, three hundred and two hundred base pairs (bp) long respectively. After the PCR process there would be a mixture of DNA strands of these lengths (Fig. 3).

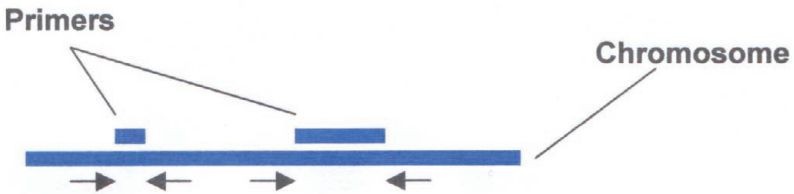

Figure 2. Random amplified polymorphic DNA (RAPD). Arrows indicate the location and orientation of PCR primer sequences.

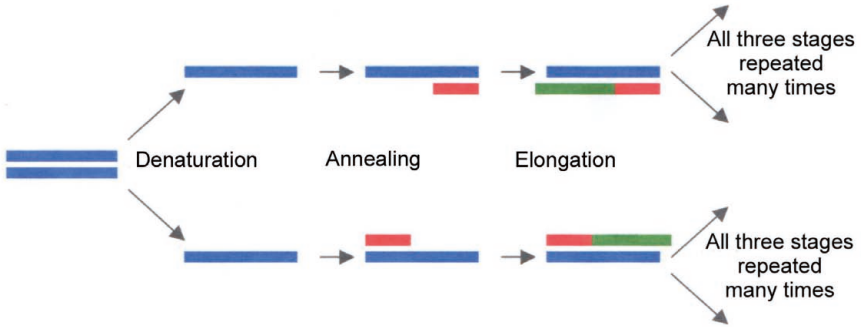

Figure 3. Polymerase Chain Reaction (PCR) showing the three stages of denaturation (to produce separate strands), annealing and elongation.

Agarose Gel Electrophoresis The next stage is to use gel electrophoresis, a technique that is used to separate a mixed population of DNA or proteins in a matrix of agarose. It is based on the observation that DNA carries an electrical charge and consequently will be attracted towards an opposite charge. In order to observe the DNA, a gel similar to that used in bacterial plates is used, suspended within a solution (Fig. 4). Once a charge is applied the negatively charged DNA travels through the pores of the gel towards the positive pole.

The rate at which the DNA travels through the gel is inversely proportional to the size of the DNA fragment and to the concentration of the gel and directly proportional to the strength of the applied current. Thus short DNA strands travel faster than longer strands and therefore, in a given time, travel further through

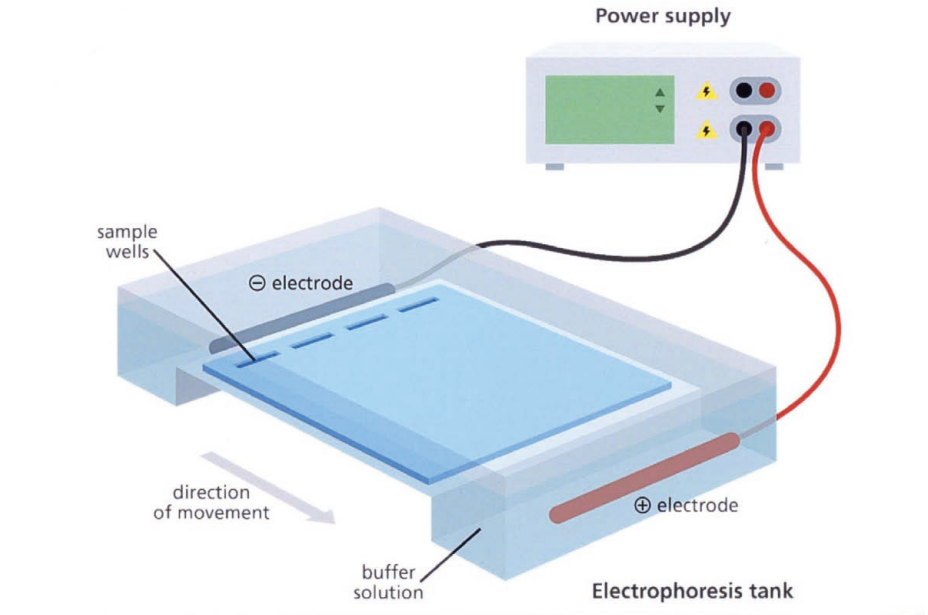

Figure 4. Electrophoresis equipment used to separate DNA fragments by size. A gel sits in a tank of buffer. A mixture of DNA with sections of known length is placed in a well at one side of the gel. The DNA samples under investigation are placed in the other wells. When an electric current is passed across the gel the negatively-charged fragments of DNA move towards the positive electrode, producing a pattern, e.g. as in Fig. 5. From Genome Research Limited (2016).

the gel. It is possible, by using an artificial mixture of DNA of known lengths at one edge of the gel (ladder), to determine how long each strand of DNA is by how far it has travelled through the gel (Fig. 5). In order to see the patterns of the bands, ethidium bromide or a Fast Blast ™ DNA stain is used, which binds to the DNA strands and either fluoresces under ultraviolet light (ethidium bromide) or is visible without ultraviolet illumination (Fast Blast™).

Returning to the simplified example above, the gel would now show a series of three bands corresponding to the three sites where the artificial DNA originally bound. The length of the bands can be determined by matching them with those of the artificial DNA markers of known length in the ladder at the side of the gel.

Using this technique with DNA of offspring from an individual it is possible to determine whether the offspring are the product of just two individuals or whether they are a result of multiple matings with the female prior to her ovipositing. Thus Hadrys *et al*. (1993) used RAPD fingerprinting to determine

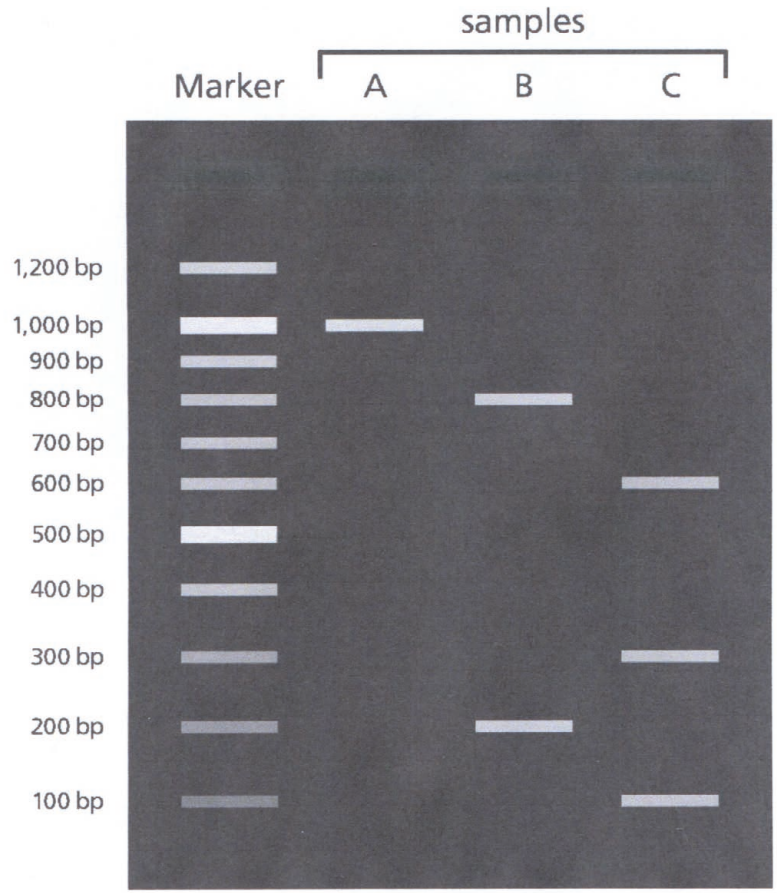

Figure 5. DNA bands separated on a gel. The lengths of the DNA fragments in the samples are compared to the 'ladder' on the left which contains DNA fragments of known length. From Genome Research Limited (2016).

paternity in *Anax parthenope* and *Orthetrum coerulescens*. For the former they found "strong evidence for complete paternity success for the contact guarding male". However, in the case of *C. coerulescens*, which is highly polygamous, they found mixed paternity in sequential clutches, with fertilization success positively correlated with the duration of copulation.

Also, in a species where a number of colour morphs are present, the genetic differentiation observed can be compared to the differentiation of colour morphs in the population. Thus Andrés *et. al.* (2000) studied whether the female colour polymorphism in *Ischnura graellsii* is under selection in the wild, possibly from

predation or from sexual selection. They compared the degree of genetic differentiation based on RAPD markers with that based on colour alleles. They found that both showed a significant degree of population differentiation but that it was significantly greater for the set of RAPD loci. They used these data, combined with field studies, to argue that, in *I. graellsii* populations, density-dependent mechanisms (predation, disease and parasites) might help to prevent the loss of this polymorphism but concluded that this could not explain the similarity in morph frequencies among populations.

Amplified Fragment Length Polymorphism (AFLP)

This technique is used, for example, in DNA fingerprinting. As with RAPD it necessitates extraction of the DNA from cells of the organism. However, once extracted the samples are mixed with (restriction) enzymes that cut the DNA whenever they encounter a specific pattern of DNA bases. Each enzyme is specific to a particular sequence of DNA bases (Fig. 6). The resultant, incubated mixture thus contains many fragments of DNA with the appropriate sequences at their ends. In order to stop the sequences rejoining, small sequences of artificial DNA (linkers) are stuck to the ends. Once the artificial DNA sequences have adhered to the cut ends of the DNA (a process called ligation) the DNA sections are replicated using PCR. The primers used in the PCR (see above) contain the sequences from the ends of the DNA with the addition of a random

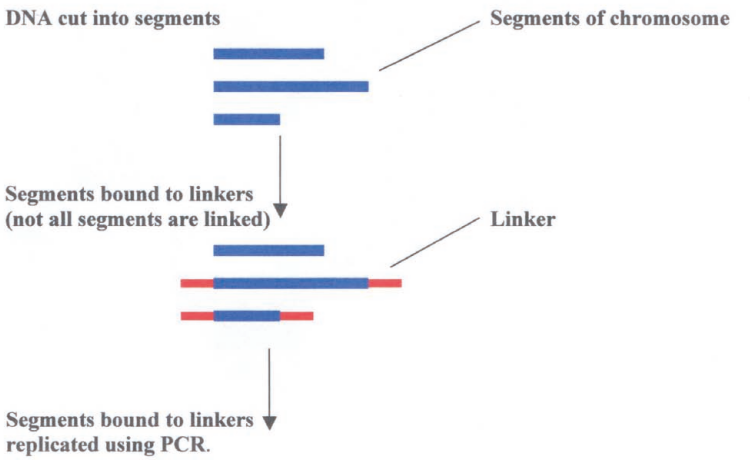

Figure 6. Amplified Fragment Length Polymorphism (AFLP). The DNA is extracted and cut into sections using restriction enzymes which cut where they encounter a specific DNA sequence. The ends of the DNA segments are attached to linkers, providing the linkers match the ends of the segments. PCR is then carried out using primers that bind to the linkers. The multiplied DNA segments are then separated using agarose gel electrophoresis.

base and bind to the linkers (Fig. 6).

As noted above, once the PCR has finished, a number of lengths of DNA are present in the mixture and many replicates of each of these fragments are present. In order to see these fragments the mixture is subjected to gel electrophoresis, which separates the fragments according to the relative speeds at which they travel through the gel (see above) (Fig. 5). Staining and visualizing these bands produces a genetic profile of an individual, which can be compared to the profiles from other individuals.

Kaunisto *et al*. (2013) used AFLP to determine whether genetic variation within 11 populations of *Calopteryx splendens* was correlated to susceptibility to parasite infections. The percentage of males parasitized by endoparasitic gregarines (Apicomplexa: Actinocephalidae) varied between 5% and 92% while the numbers of parasites within each individual varied between zero and 47, with the parasites being aggregated in just a few hosts. A positive association was found between the inbreeding coefficient of an individual and its parasite burden; the more inbred (homozygous) the individual, the higher its parasite load and consequently the less fit the individual was. Therefore, these parasites provide a strong selection pressure against inbreeding within this species. These results support the heterozygosity-fitness correlation hypothesis, suggesting that heterozygosity is important for an individual's resistance to pathogens.

Marden *et al.* (2001) investigated the transcription of a single troponin gene in the flight muscles of *Libellula pulchella.* This gene controls the sensitivity of muscle fibres to calcium ions and thereby the performance of muscles during flight. They found that variation in the relative abundance of different troponin transcripts was positively correlated with the sensitivity of muscle fibres to calcium ions. Using these results to reanalyse their previously published data, on wing kinematics (Marden *et al.,* 1999,) they demonstrated that the relative abundance of the transcripts was positively correlated with wingbeat frequency and amplitude. It was not possible to determine whether this variation alone was responsible for these effects, or whether the variation was "a marker for changes in a suite of co-regulated molecules" (Marden *et al*., 2001).

Microsatellite Loci

Microsatellites are short, tandem repeats in DNA sequences and are of between two and six base pairs in length. They are also called Simple Sequence Repeats (SSRs), Variable Number Tandem Repeats (VNTRs) and Short Tandem Repeats (STRs). They are typically abundant in the genomes of many species. Mutations at these loci result from slippage and proofreading errors during DNA

replication. These primarily change the number of repeats and therefore the length of the microsatellite array (Ellergen, 2004). Sometimes a single repeat motif is lost or gained. These changes in length of microsatellites can easily be distinguished by gel electrophoresis. It is this characteristic that makes them the genetic markers of choice for molecular ecological studies at the level of populations. The drawback to using microsatellites is that they must be isolated from the target species each time before they can be used. Despite this, panels of microsatellites have been developed for thousands of species.

In a review, Watts (2009) noted that, by 2009, 116 microsatellite loci had been isolated from 11 species of odonate. He noted that odonate microsatellites usually have fewer than 10 core motifs and thus are relatively short; also there is a low level of heterozygosity (gene diversity). Although the lengths of microsatellite arrays vary considerably within and among species, he found no obvious contrast between anisopterans and zygopterans. The mean microsatellite array length was positively correlated with heterozygosity, although the correlation was weak.

da Silva-Méndez *et al.* (2013) used microsatellites to produce microsatellite loci for two threatened dragonfly species *Macromia splendens* (8 loci) and *Oxygastra curtisii* (13 loci) in order to assess the spatial genetic structure in these species. Hadrys *et al.* (2004) used recombination analysis to isolate DNA from a leg of three unrelated individuals of the neo-tropical dragonfly species *Megloprepus caerulatus*, which lives in tree holes. Using RAPD analysis they discovered 36 RAPD fragments containing microsatellite motifs, including five polymorphic microsatellite loci. *Megloprepus caerulatus* is a bio-indicator for primary rainforests and Hadrys *et al.* (2004) suggested that these microsatellites could be used to study the effects of forest fragmentation on population viability.

Sánchez-Guillén *et al*. (2011) studied population divergence of colour polymorphism in seven species of *Ischnura*, with particular emphasis on *Ischnura elegans* and *I. graellsii*. For *I. elegans* they used eight Spanish and five eastern European populations; for *I. graellsii* three Iberian populations and one from northern Africa, all outside the range of *I. elegans* to avoid any possibility of using hybrids. Six microsatellite loci were used for *I. elegans* and six for *I. graellsii*. Their results showed that there was considerable variation in the proportions of colour morphs in the Spanish populations of *I. elegans* and that there was a strong deviation from neutral expectations, implying the operation of divergent selection on the colour locus in these populations. In contrast, there was little variation in colour morph frequencies in the eastern European populations of *I. elegans* and in the populations of *I. graellsii*, with no divergence from neutral expectations, implying genetic drift. Thus they conclude that the degree of importance of different factors varies in different

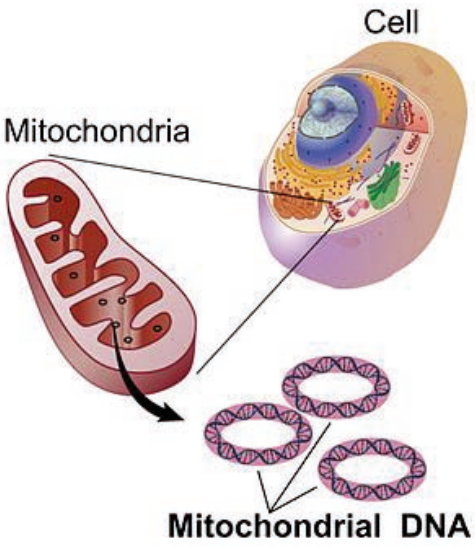

Figure 7. The location of mitochondrial DNA in the cell. The mitochondrial DNA comprises the small circular chromosomes found inside the mitochondria. National Institutes of Health. National Human Genome Research Institute. "Talking Glossary of Genetic Terms." (2017).

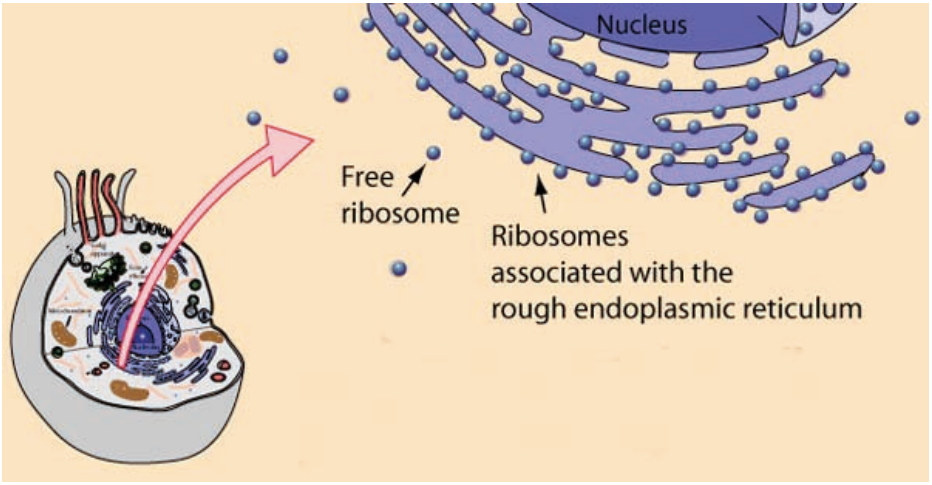

Figure 8. Ribosomal DNA. The location of the ribosomes in a cell. Note that some are free in the cytoplasm (free ribosomes); others are attached to the rough endoplasmic reticulum (bound ribosomes), From Nave (2005).

geographical regions.

Using Mark-Release-Recapture (MRR), Watts *et al*. (2004) found that, at a site in southern England, adults of the endangered *Coenagrion mercuriale* (Southern Damselfly) are highly sedentary, with little movement to neighbouring sites. Using a microsatellite-based genetic analysis, they found that this low frequency of movement was reflected in significant genetic variation between populations.

Non-genomic DNA

Much of the research involving molecular techniques has been for taxonomic purposes. In addition to classification based on morphology, it is now possible to determine taxonomy at the genetic level, including how close closely related species are to each other, whether they derive from a common ancestor and how early in the evolutionary process they diverged.

The problem with genomic DNA for such studies is that sexual reproduction involves combination of DNA from both parents and thus results in changes to the genome with every subsequent generation. These rapid changes hinder the process of tracking genetic variation and derivation over many generations. However, genomic DNA is not the sole source of DNA in an organism. The mitochondria (Fig. 7), ribosomes (Fig. 8) and, in plants, chloroplasts, also contain DNA. The presence of separate DNA in these structures is hypothesized to result from their initial role as separate symbiotic structures within ancient organisms. The mitochondrial and ribosomal DNA are transferred to the next generation only through the female ova. Consequently mitochondrial and ribosomal DNA are not mixed at fertilization, as is genomic DNA. The slow changes that mitochondrial and ribosomal DNA undergo through mutations over many generations make them an ideal tool for determining the derivation and phylogenetic relationships of species and for discriminating between closely related species.

The mitochondrial genome has been described in a number of zygopterans and anisopterans, for example in the zygopteran *Mnais costalis* (Lorenzo-Carballa *et al*., 2016). In this species it comprises 15,487 base pairs and is similar to that of other odonates, with 13 protein-coding genes, large and small rRNA genes, 22 tRNA genes and three intergenic spacers. It has also been described in an anisozygopteran (a group in which the species contain a mixture of morphological features of both anisopterans and zygopterans), *Epiophlebia superstes* (Wang *et al*., 2014). They found it to be 15,435 base pairs long and comprise a set of 37 genes and an A+T-rich control region with the gene arrangement identical to that of other odonates. It contains three non-coding intergenic spacers

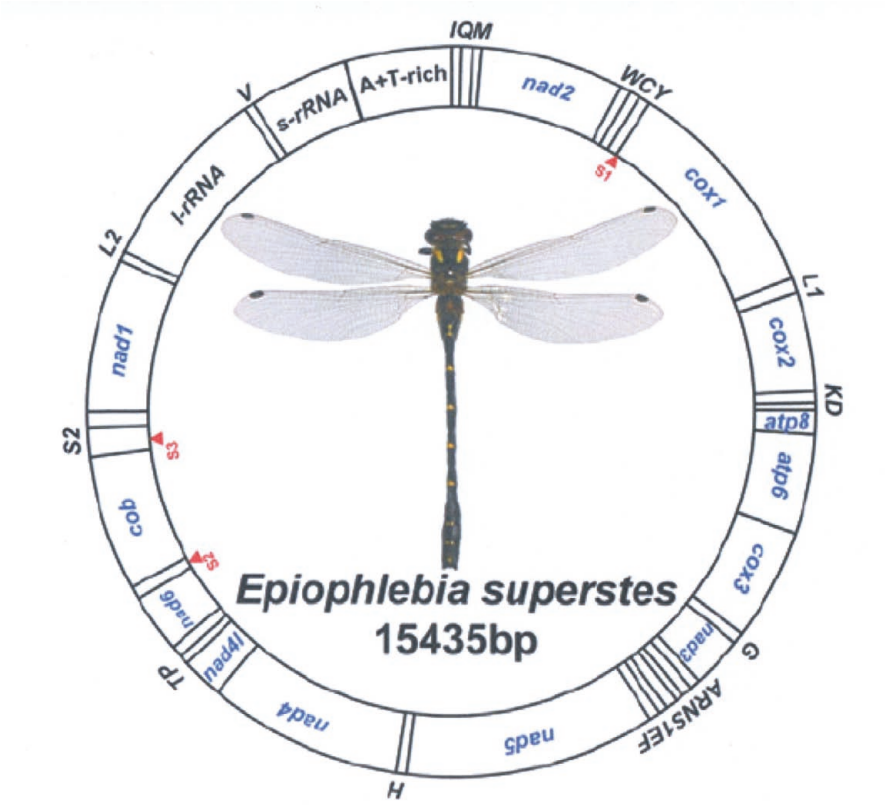

Figure 9. The structure of the mitochondrial DNA of the anisozygopteran dragonfly *Epiophlebia superstes*. It has a total length of 15,435 bp and contains 37 genes. .Blue, protein-coding genes; red, s1–s3 intergenic spacers; transfer RNA genes are labelled by the one-letter amino acid code. From Wang et al. (2014).

(s1–s3), which also occur in all other odonates analysed (Fig. 9). However, it lacks the intergenic spacer s5 typically found in the Anisoptera, suggesting a mitochondrial genome organization more closely related to the Zygoptera than to the Anisoptera.

Ribosomal DNA (rDNA) has a similar pattern of rRNA gene organisation in all eukaryotes. It comprises a number of gene clusters. with three sets of genes (18S, 5.8S and 28S) in each cluster, separated from each other by internal transcribed spacers (ITS-1, ITS-2). Adjacent clusters are separated by external transcribed spacers (ETS) (Fig. 10).

Chippindale *et al*. (1999) used portions of two mitochondrial genes (cytochrome

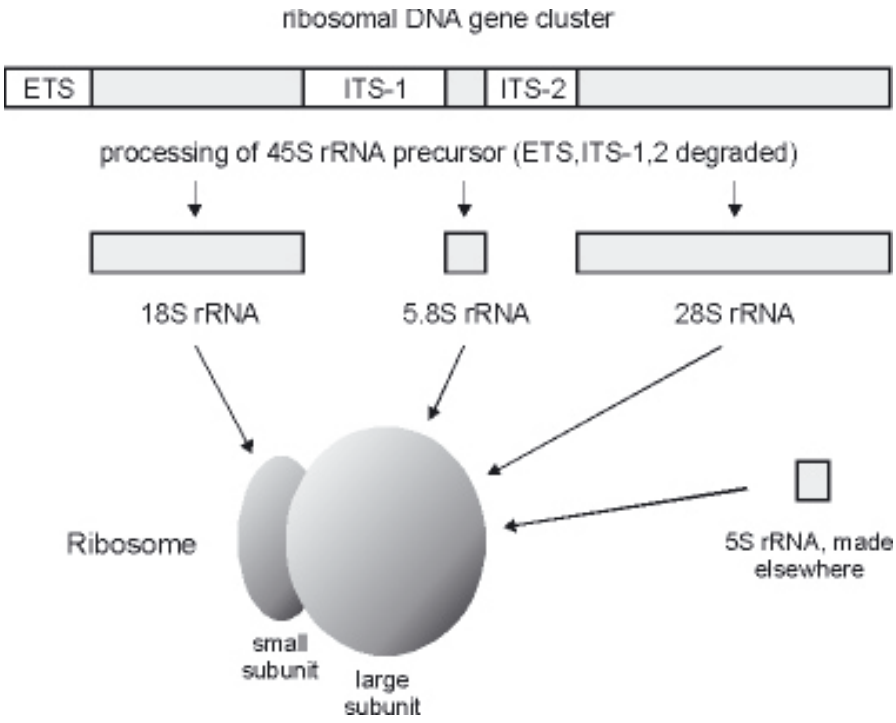

Figure 10. The structure of a ribosomal DNA gene cluster. Note the three sets of genes (!8S, 5.8S and 28S), internal transcribed spacers (ITS-1, ITS-2) and external transcribed spacer (ETS). From Seifarth (2008).

b and cytochrome oxidase II) and a ribosomal gene (12S) to investigate the relationships between North American species of *Ischnura*. The phylogenetic trees that they obtained from a combined analysis of these three genes produced evidence that *Ischnura kellicotti, I. barberi, I. prognata, I. hastata, I. ramburii* and *I. capreola* appear to represent an early divergence in the group, whereas *Ishnura damula, I. demorsa, I. perparva, I. posita posita, I. posita atezca, I. verticalis,* and probably I. *denticollis*, form a monophyletic group that diverged more recently.

Misof *et al.* (2000) used ribosomal DNA markers to reconstruct the evolutionary history of the genus *Calopterix*. They concentrated on establishing phylogenetic information using a 570 bp fragment of the 16S rDNA gene, which they sequenced for nine species of *Calopteryx* and five outgroup species (*Neoneura esthera*, *Palaemnema* sp., *Hetaerina americana*, *Mnais costalis* and *Matrona basilaris*) (outgroup species are distantly related to the species under study and are used to provide a common ancestry). They produced a consensus phylogenetic tree,

which supported the case for *Mnais*, *Matrona* and *Calopteryx* being monophyletic genera. However *Calopteryx atrata* and *Matrona basilaris* are sister taxa, indicating that *Calopteryx* may not be a monophyletic genus. Nevertheless, the three North American species of *Calopteryx* (*C. maculata*, *C. dimidiata* and *C. aequabilis*) do form a monophyletic clade, as do the four European species (*C. xanthostoma*, *C splendens*, *C. virgo* and *C. haemorrhoidalis*). However, the two Asian species, *Calopteryx atrata* and *C. cornelia* are not monophyletic. Misof *et al.* (2000) suggested that the extant european and asian species may have been affected by glacial periods, whereas the extant North American species groups were not.

Turgeon & McPeek (2002), analysed the geographical pattern of genetic variability of 868 bp of mitochondrial DNA (mtDNA) among 283 individuals of five North American species of *Enallagma* that displayed little ecological differentiation. The data were used to identify the ancestral lineage of these species. They showed that *Enallagma hageni* has two races, Atlantic and Continental, that hardly overlap and probably resulted from a past event of range fractionation in the species, the two races having distinct dispersal histories. The other four *Enallagma* species were shown to be derived from the continental race of *E. hageni*. The three species that are endemic to the Atlantic coastal plain show little genetic variation. However, *Enallagma ebrium* shared several haplotypes (sets of DNA variations that are inherited together) with the Continental race of *E. hageni*. The data support a recent origin for the latter.

Kiyoshi & Sota (2006) used both mitochondrial and nuclear ribosomal gene sequences to determine the evolutionary proximity of three species of *Davidus* (*Davidus nanus*, *D. fujiama* and *D. moiwanus*) endemic to Japan and *Davidus lunatus* from the Korean peninsula. The genes they used were the mitochondrial gene COI (cytochrome oxidase subunit I) and the nuclear ribosomal gene region encompassing 18S, ITS1, 5.8S, and ITS2 sequences. *Davidus nanus* and *D. moiwanus* were shown to be closely related and are a sister group to the Korean species *D. lunatus*, whereas *D. fujiama* separated from an ancestor of the other three species. There are three subspecies of *D. moiwanus* and the data from the nuclear ribosomal DNA (but not the mitochondrial DNA) indicated that they are a monophyletic group, while the mitochondria DNA data showed that the divergence in local populations was much greater than in *D. nanus*, possibly resulting from differences in their respective dispersal ranges.

Conclusions

As noted in the introduction, this review provides an overview of the varieties of genetic analyses that have been used on odonates. The examples given

illustrate the use of these various techniques and are not, in any way, meant to be comprehensive.

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Wing size variation in *Calopteryx virgo* (L.) (Beautiful Demoiselle), in response to geographic and climatic variables, using museum collections

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Abstract

Museum specimens of *Calopteryx virgo* the Beautiful Damselfly collected in the UK, Ireland and the Channel Islands, were measured to explore the relationship between wing length and latitude, longitude, average seasonal temperatures and date of collection. The results show some differences in male and female wing shape, but both sexes increase in size with an increase in latitude, were significantly smaller later in the season and became smaller with increasing spring temperatures in the year of collection. These results are broadly in line with the temperature size rule.

Introduction

The body size of an animal plays a key role in longevity (Sokolovska *et al*., 2000), predator-prey interactions (Kuchta & Svensson, 2014), sexual selection (Tynkkynen *et al*., 2004) and fitness (Sokolovska *et al*., 2000), and varies significantly between species, across geographical regions, as well as within species (Hassall, 2013; Hassall *et al*., 2014).

Several theories have been proposed to explain the relationship between body size and temperature. Bergmann's rule (Bergmann, 1847) states that body size will increase with increasing latitude, presumably because a lower surface area to volume ratio in larger animals means that the individual stores more body heat than it radiates. This rule holds for the majority of vertebrate species (Millien *et al*., 2006). However, a review of body size in insects by Chown & Gaston (2010) has shown that, out of 58 studies, 28 followed Bergmann's rule but 19 showed a negative relationship (termed "Bergmann clines") (Blanckenhorn & Demont, 2004). Some species showed U-shaped relationships between size and latitude (Elmes *et al*., 1999; Johansson, 2003) and some authors have concluded that such relationships are due to genetic factors (De Block & Stoks, 2007), while

others have suggested that temperature and climate mostly affected the growth rates of their study organism (e.g. *Orthetrum cancellatum*) and in turn its size (Flenner *et al*., 2010). Nevertheless, the underlying mechanisms of these responses have been criticised since there are better adaptations than size to retain body heat (Scholander, 1955).

Another explanation for body size variation is the "temperature-size rule" (TSR) (Atkinson *et al*., 2006) which predicts that lower temperatures will cause individuals to grow larger, whereas individuals that experience higher temperatures will be smaller. This is because individuals, especially ectotherms like dragonflies, can reach maturity more quickly when temperatures are high since they can increase their ontogenetic development as temperatures increase (Zuo *et al*., 2012). Another reason proposed is that increasing temperatures make it harder for an invertebrate's gas exchange mechanisms to function properly and thus it struggles to acquire enough oxygen. This consequently causes the organisms not to reach their potential full size (von Bertalanffy, 1960). Furthermore, other studies have shown that increasing temperature increases the number of generations in a given period (per year in Odonata and Lepidoptera), which in turn results in smaller adults as there is less time for growth (Zeuss *et al*., 2016). It has been suggested that the TSR might be more relevant to aquatic insects than terrestrial insects (Atkinson *et al*., 2006; Forster *et al*., 2012; Horne *et al*., 2015) as it is more difficult for them to acquire sufficient oxygen when temperatures increase, due to the lower solubility of oxygen in warmer water relative to cold (Woods, 1999; Hassall *et al*., 2014).

On the other hand, other natural processes, such as photoperiod, could potentially produce more complex relationships between body size, temperature and the environment as these can be the main cues regarding the progress of the growing season (De Block & Stoks, 2003), potentially exerting a time stress. As a result, the organism's development rate can accelerate as the season progresses, resulting in an earlier emergence (Johansson *et al*., 2001) and a smaller adult size. Time stress has been studied recently in the damselfly *Lestes viridis* by Stoks *et al*. (2006) who showed that the larvae exhibited higher metabolic rates, faster energy depletion and lower energy storage investments when faced by time stress, explaining smaller adult sizes at emergence.

The present study the relationship between temperature and wing length (as a proxy for body size) in British populations of *Calopteryx virgo* is investigated and the results are compared and contrasted with a similar study on *Calopteryx splendens* (Upton *et al*., 2016).

Materials and Methods

A total of 836 specimens of *Calopteryx virgo* (363 females, 473 males) were used for this study, from 199 locations in the United Kingdom, the Channel Islands and Ireland, covering much of the species distribution in the British Isles and Ireland (Fig.1). The specimens used were collected between 1874 and 1999. Specimens were accessed from the following museums: 23 from the National Museum, Cardiff, 46 from the National Museum of Scotland, Edinburgh, 72 from the Oxford University Museum of Natural History and 695 from the Natural History Museum, London (NHM).

Wing Imaging

Specimens were photographed by placing them in a tray with a scale, a colour checker and an elevated platform for the labels (Fig. 2). The colour checker was added to make the digitised specimens more useful to other researchers studying wing pigmentation in Odonata species.

Images were captured following the method outlined in Upton *et al*. (2016). A Nikon D5300 camera was secured on a tripod and placed above the tray. A light box fitted with a daylight fluorescent ring-light was placed around the tray to ensure that the specimen received consistent lighting. The camera was operated on manual mode (ISO 100, 1/50sec speed, f11 aperture) to ensure exposures were consistent between photographs.

Both pinned and papered specimens were photographed in the same way but grey paper was placed between the wings of papered material which was then imaged twice (left side and right side) so that both left and right wings were visible and thus could be measured.

A unique identifier was provided for each specimen. The specimens at the NHM were provided with a unique bar code [NHMUK0102…] and specimens from all the other museums were given a unique code ranging from AC001-AC400. Label data on each specimen were transcribed and georeferenced using the website LatLong.net (2016) and placed on the NHM data portal (http://dx.doi.org/10.5519/0012616).

Wing Measurements

Only hind wings (both left and right) were analysed in this study since forewings were sometimes obscured in papered material and therefore accurate measurements were not possible. Hind wings are commonly used as a proxy for body size in dragonflies (Rantala *et al*., 2001; Tynkkynen *et al*., 2004). Wing

Figure 1. Distribution of *Calopteryx virgo* specimens used in this study.

Figure 2. The tray used to photograph each specimen of *Calopteryx virgo* in this study (female specimen shown).

landmarks were used to measure wing length and width following Upton *et al*. (2016) (Fig. 3). Wing measurements were carried out in Image J version 1.50.

Wing Size Analysis

ANOVA was used to determine whether wing size differed significantly between the sexes.

In order to test whether there was asymmetry in the wings of both sexes, simple Pearson correlation tests were used to compare left and right hind wing length, as well as left and right hind wing width.

Gaussian, family-generalised, linear models were used to compare seven variables (Longitude, Latitude, Average seasonal summer, autumn, winter and spring temperatures, and Day of collection) with wing length. Wing length was always used as the dependent variable and a combination of the variables listed above were chosen randomly as the independent variables. To assess the importance of each independent variable, and to choose the best model, we considered both the Akaike Information Criterion, corrected for sample size (AICc), and the Bayesian Information Criterion (BIC) (Bolker *et al*., 2009;

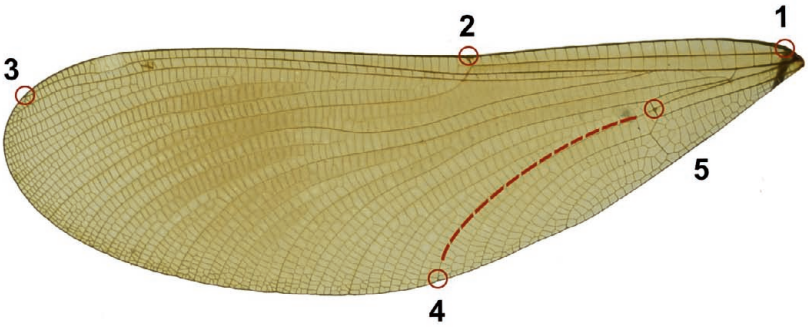

Figure 3. Wing "landmarks" (shown by red circles) were used to calculate wing length and width of *Calopteryx virgo*. The distance between points 1 and 3 was used to calculate wing length and the distance between 2 and 4 was used for wing width. Point 5 was used to locate the cubitus posterior (CuP) vein that was used for width measurements.

Wagenmakers & Farrell, 2004).

Mean summer, autumn, winter and spring temperatures were obtained from the Central England Temperature (CET) record (Parker *et. al*., 1992). Average seasonal temperatures were calculated using both the year of collection and the year before collection, since *Calopteryx virgo* is semi-voltine and the larva develops over two years before it emerges (Brooks *et al*., 2014). Each season's temperatures were calculated as follows: Spring (average of March, April, May), summer (average of June, July, August), autumn (average of September, October, November) and winter (average of December, January and February).

To test within- and between-population differences in wing length, six different localities were selected which included many male and female specimens collected on the same day (Fig. 4). 'One-sample one-tailed' t-tests were carried out for each location, with wing length as the variable for each site. An ANOVA on the wing length was then used to test for between-site variation. Where there was a significant difference, a Tukey's 'Honest Significant Difference' method (HSD test) was used to test if those differences were homogenous between the populations, i.e. whether all locations varied from each other or if some populations were significantly different from the rest.

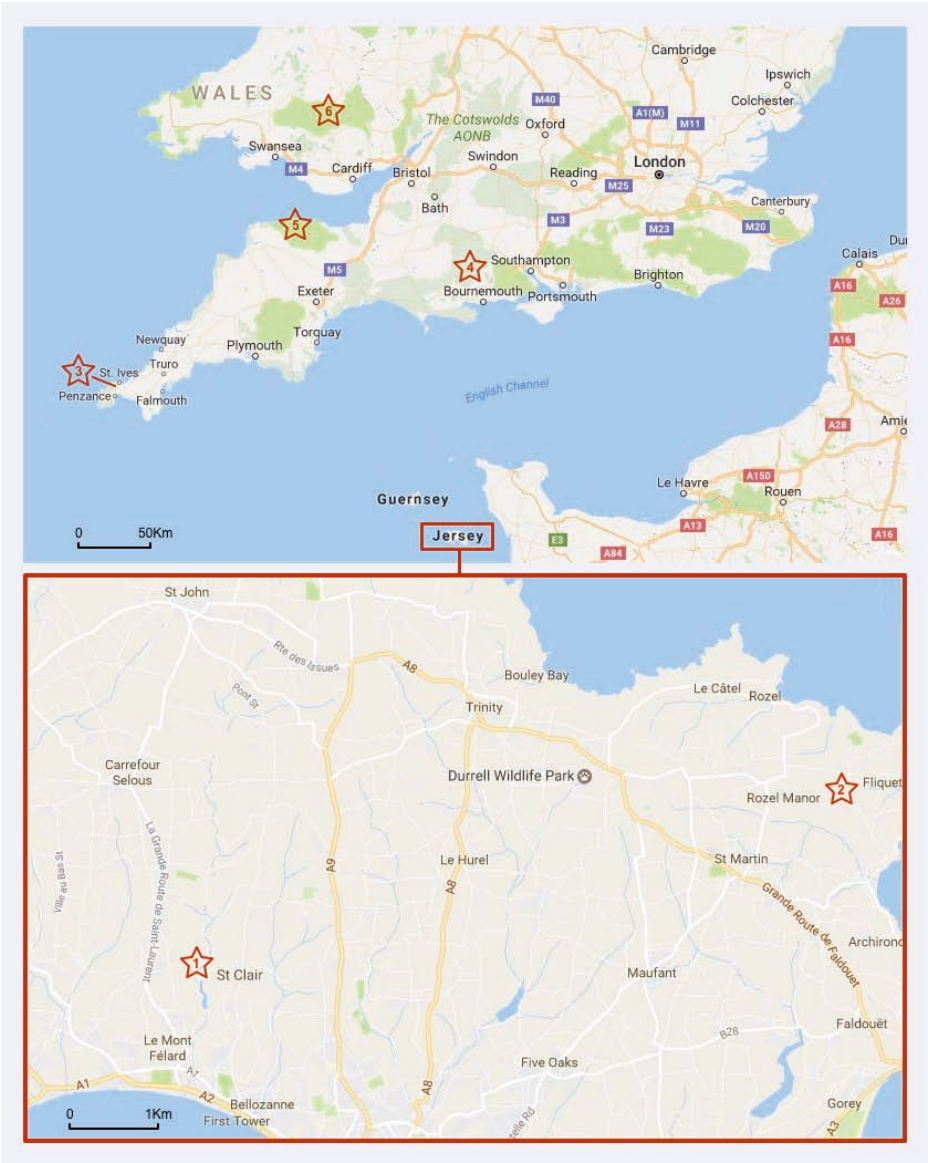

Figure 4. Locations of the six populations of *C. virgo* analysed. Numbers were assigned according to latitude (i.e. Number 1 shows the lowest latitude value). 1, St Lawrence, Jersey; 2, Rozel Manor, Jersey; 3, St Just, Cornwall; 4, Furzehill, Dorset; 5, Lynton, Devon; 6, Llanfrynach, Powys. Map data© 2016 Google Maps

All the analyses were carried out using the “stats”, “ggplot”, “AICcmodavg” and “agricolae” packages in R version 3.1.1.

Results

An ANOVA test, comparing male and female wings, confirmed that males and females differed significantly from each other, both in terms of wing length ($F=362.8$, $df=1$, $p<0.0001$) and wing width ($F= 188.2$, $df=1$, $p<0.0001$). Females were significantly larger than males in terms of wing length (Fig. 5), but males were significantly larger in terms of wing width (Fig. 5), implying a difference in wing shapes between the sexes. Therefore, males and females were analysed separately in all subsequent analyses.

Correlation values were high between male left and right hind wing length ($r=0.7852$, $p<0.0001$) and width ($r=0.\ 8770$, $p<0.0001$). Similarly, female left and right hind wing lengths were correlated, both in respect to length ($r=0.7974$, $p<0.0001$) and width ($r=0.8643$, $p<0.0001$). Therefore, in all subsequent analyses right and left hind wing lengths were averaged and termed ‘wing length’. We decided that only one measure of wing size was needed for this study and wing length was chosen so we could compare our results with the studies of Hassall & Thompson (2009) and Upton *et al*. (2016) .

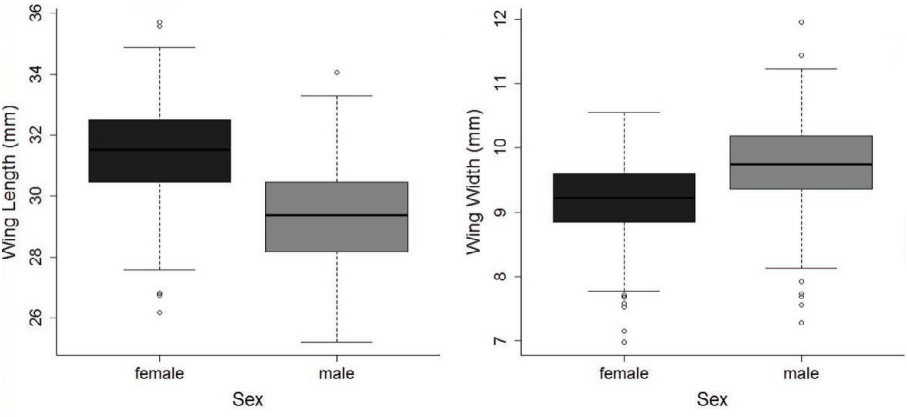

Figure 5. Boxplots showing the difference between males and females in terms of wing length and width. The median value is represented by the bold line in the centre of each box. The whiskers show the interquartile range, representing the top and bottom 25% of the data. Outliers are shown as circles.

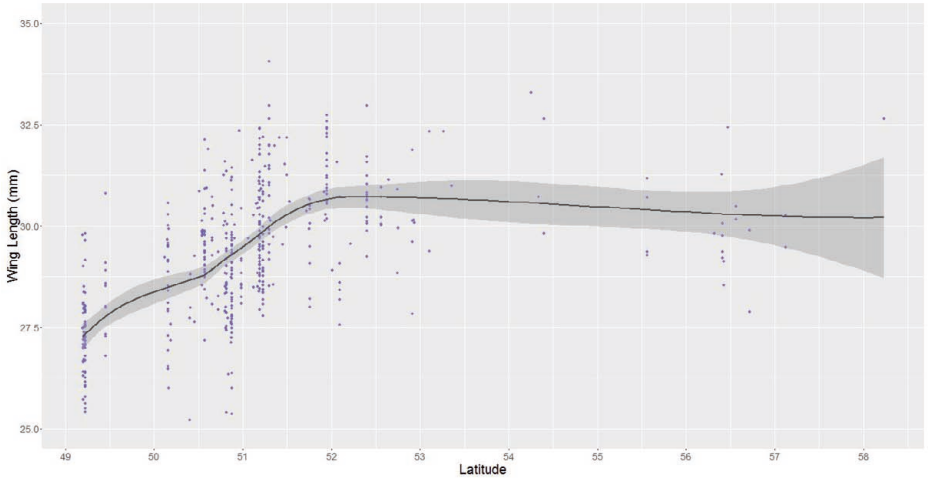

Figure 6. Plot of wing length against latitude (degrees north) in male specimens of *Calopteryx virgo.* The grey shaded area is the 95% confidence interval on the fitted values.

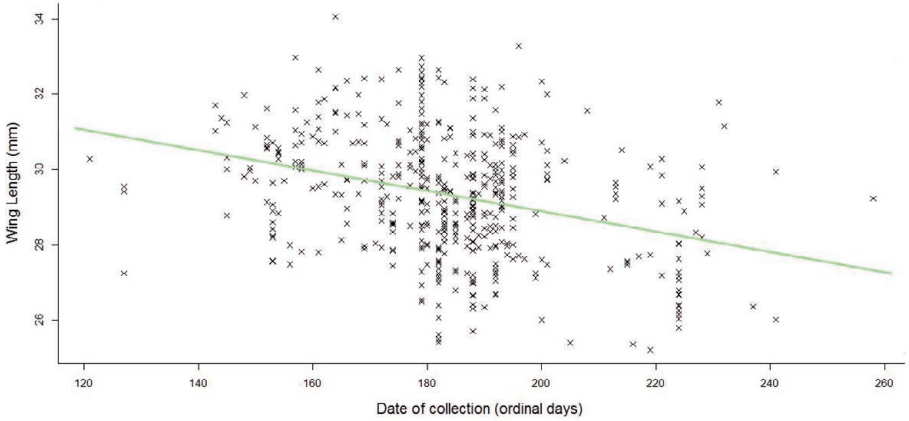

Figure 7. Wing length of female specimens of *Calopteryx virgo* plotted against day of collection. Ordinal day number 120 is 30 April and 260 is 17 September. Green, linear regression line.

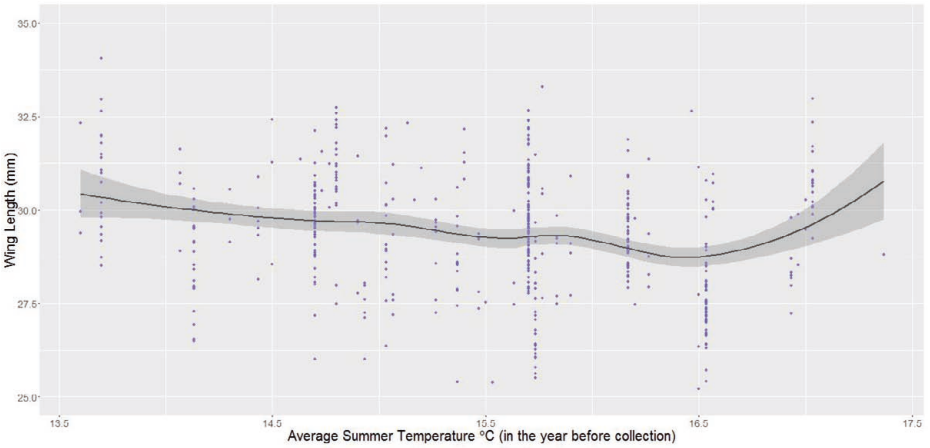

Figure 8. Relationship between wing length and average summer temperatures in year before collection of male specimens of *Calopteryx virgo*. The grey shaded area is the 95% confidence interval on the fitted values.

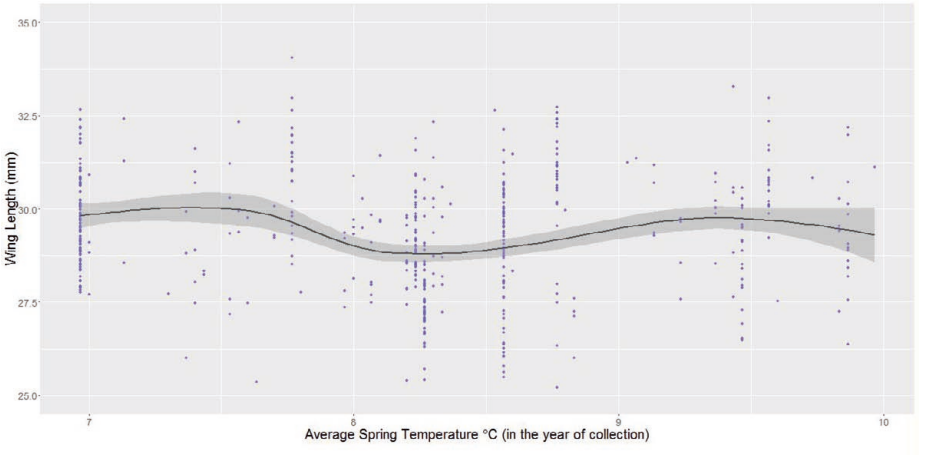

Figure 9. Relationship between wing length and average spring temperatures in year of collection of male specimens of *Calopteryx virgo*. The grey shaded area is the 95% confidence interval on the fitted values.

Table 1. Variables having a significant relationship with wing length in males of *Calopteryx virgo*.

Variable	t-value	p-value
Latitude	8.955	<0.0001
Spring in year of collection	-2.468	<0.01
Summer in year before collection	-3.639	<0.0001
Day of collection (ordinal days)	-6.122	<0.0001

Table 2. Variables having a significant relationship with wing length of female *Calopteryx virgo*.

Variable	t-value	p-value
Longitude	-3.974	<0.0001
Latitude	2.866	<0.001
Spring in year of collection	-3.193	<0.001
Day of collection (ordinal days)	-6.248	<0.0001

For males, the most significant variables affecting wing length were latitude (Fig. 6), day of collection (Fig. 7), average summer temperatures in the year before collection (Fig. 8) and, slightly less significant, average spring temperatures in the year of collection (Fig. 9). Latitude affected wing length positively, primarily between 49° 12'N and about 52° N. The other three variables all had a negative effect on wing length (Table 1).

For females, the most significant variables affecting wing length were longitude (Fig. 10) and day of collection (Fig. 11) and, slightly less significant, latitude (Fig. 12) and average spring temperatures in the year of collection (Fig. 13). Only latitude had a positive effect, again primarily between 49° 12' N and about 52° N. Day of collection and the average spring temperatures in the year of collection had negative effects. As far as lonitude was concerned there was a negative correlation from west to east (Table 2).

The variation of wing length between individuals collected on the same day at the same place revealed that there were significant differences within all the sub populations when examined with one-sample t-tests (Table 3; see Fig. 4 for locations).

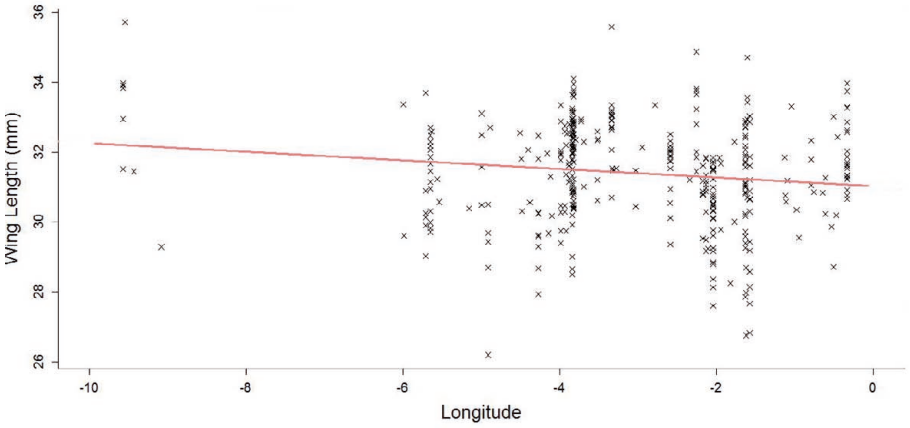

Figure 10. Wing length of female *Calopteryx virgo* plotted against longitude (0 is the Greenwich Meridian). Red, linear regression line.

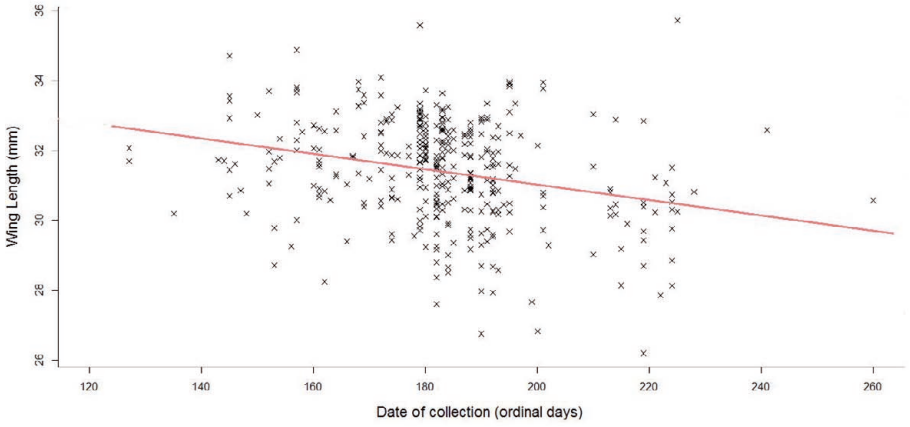

Figure 11. Wing length of female specimens of *Calopteryx virgo* plotted against day of collection. Ordinal day number 120 is 30 April and 260 is 17 September. Red, linear regression line.

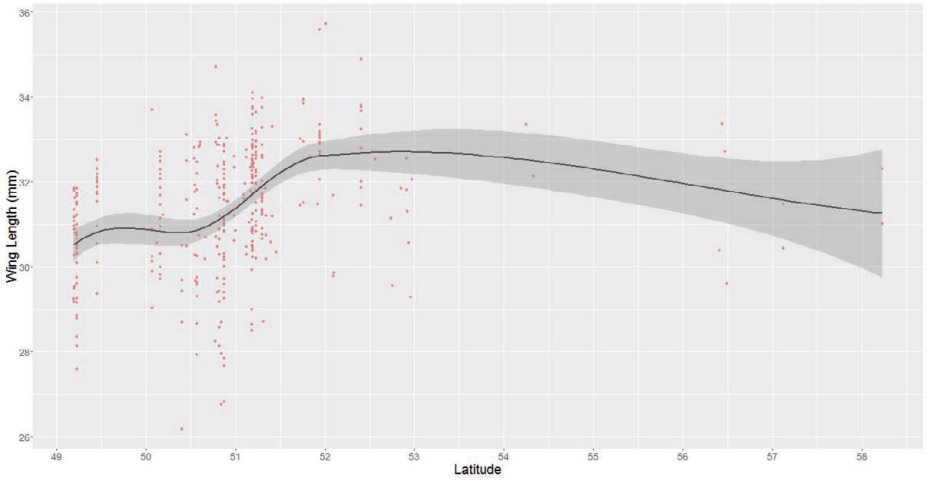

Figure 12. Relationship between wing length and latitude (degrees north) in female specimens of *Caloptyeryx virgo*. The grey shaded area is the 95% confidence interval on the fitted values.

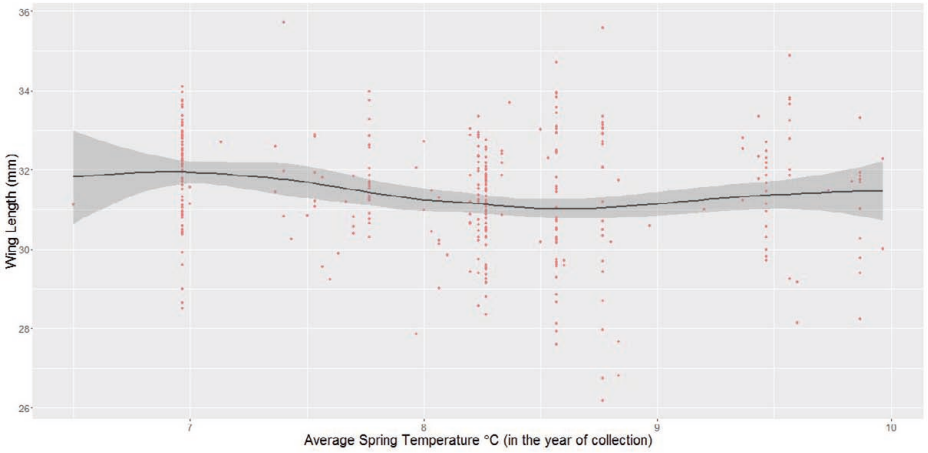

Figure 13. Relationship between wing length and average spring temperatures in year of collection in female specimens of *Calopteryx virgo*. The grey shaded area is the 95% confidence interval on the fitted values.

Table 3. Summary of wing length variation in males and females of *Calopteryx virgo* collected on the same day at each of six localities (see Fig 4 for location of sites).

Location (lowest latitude first)	Date of Collection	Males			Females		
		Degrees of Freedom (df)	t-value	p-value	Degrees of Freedom (df)	t-value	p-value
1. St. Lawrence	06/07/1956	13	115.85	<0.0001	9	108.54	<0.0001
2. Rozel Manor	30/06/1956	13	104.54	<0.0001	21	144.92	<0.0001
3. St. Just	28/06/1957	19	106.94	<0.0001	13	114.03	<0.0001
4. Furzehill	21/06/1941	10	77.62	<0.0001	8	93.841	<0.0001
5. Lynton	02/07/1941	17	170.79	<0.0001	18	175.32	<0.0001
6. Llanfrynach	27/06/1964	19	169.98	<0.0001	11	135.27	<0.0001

There was a significant difference in the wing length between the selected six locations for both males ($F=41.66$, $df=5$, $p<0.0001$) and females ($F=21.93$, $df=5$, $p<0.0001$), although populations located at similar latitudes tended to be less different than those located further away. There were greater wing length differences between female (Fig. 14) than male populations (Fig. 15). The results reflect the generally increasing wing length with latitude.

Discussion

Wing asymmetry

Our results indicated that there was no asymmetry in the wing lengths and widths of either males or females of *Calopteryx virgo*, in agreement with other recent studies on *Calopteryx splendens* (Upton *et al*., 2016; Hassall & Thompson, 2009).

Wing length and latitude

There was a significant positive correlation between wing length and latitude in both males and females, in accordance with Bergmann's Rule. The results of our study are in agreement with a similar study using museum specimens of

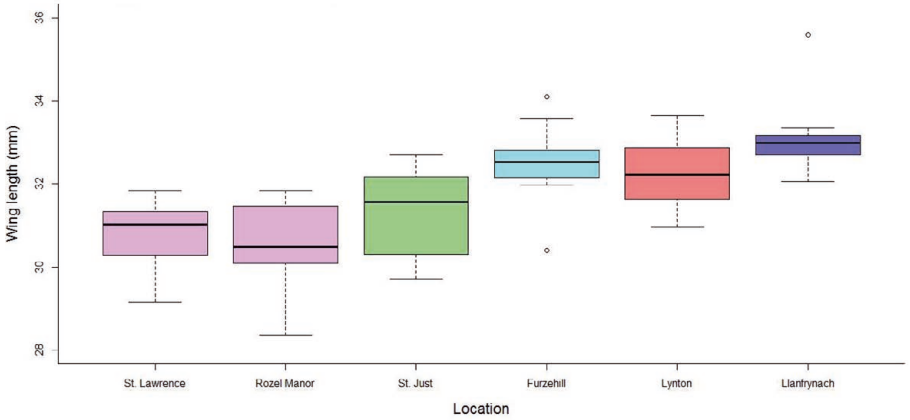

Figure 14. Boxplots comparing female *Calopteryx virgo* wing length within and between different locations. Different colours are used to indicate when the size differences are significant at $p<0.05$ calculated using the HSD test (i.e. the same colour indicates no significant difference at that level). The whiskers indicate wing length range and the bold line within each boxplot shows the median value. Outliers are shown in circles. Locations are presented in latitudinal order (lower latitudes on the left and higher on the right).

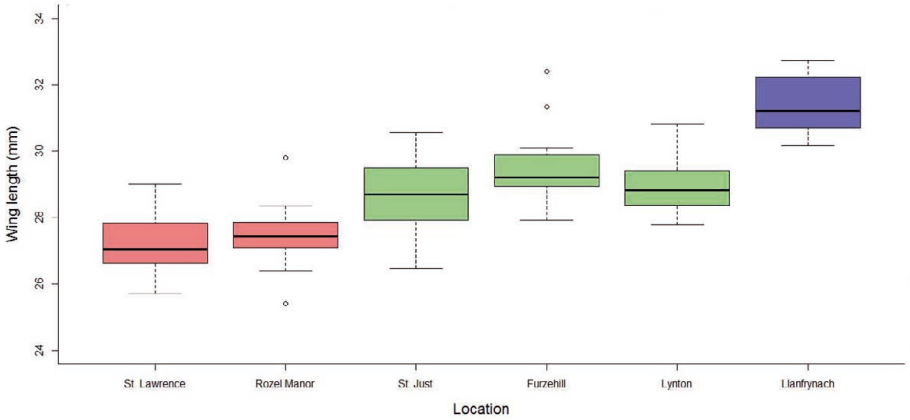

Figure 15. Boxplots comparing male *Calopteryx virgo* wing length within and between different locations. Different colours are used to indicate when the size differences are significant at $p<0.05$ calculated using the HSD test (i.e. the same colour indicates no significant difference at that level). The whiskers indicate wing length range and the bold line within each boxplot shows the median value. Outliers are shown in circles. Locations are presented in latitudinal order (lower latitudes on the left and higher on the right).

the congener *C. splendens* (Upton *et al*., 2016) but contradicts the findings of Hassall & Thompson (2009), who compared two populations of *C. splendens*, one in the north (Northumberland) and one in the south (Hampshire) of England and showed that neither wing length nor wing area varied with latitude. In the present study, while there is substantial variation in wing length between specimens collected at the same latitude (Figs. 6, 12), the underlying relationship is clear. In addition, the results show that, above 52 ° N, wing length in both sexes reaches a plateau, and even slightly decreases in females. This result is interesting as it contradicts Bergmann's rule (increased size at higher latitudes), and may reflect the size constraints imposed by the shorter growing season in northern populations. In addition, our results might also be affected by the smaller sample size of northern specimens.

Wing length and longitude

Females displayed a significant wing length decrease from west to east (Fig. 10). However, this relationship may be confounded by the response to latitude. Moreover, the fact much of the eastern part of the UK is not suitable for the species (Brooks *et al*., 2014) makes this relationship difficult to understand.

Wing length and average seasonal temperatures

Male wing length was negatively correlated with temperature in the summer before collection (Fig. 8). In addition, wing lengths of both sexes were negatively correlated with temperature in spring of the year of collection (Figs 9, 13). These results therefore follow Bergmann's Rule (Bergmann, 1847) and the Temperature Size Rule (TSR) (Atkinson *et al*., 2006).

The fact that female wing length was not correlated with the previous mean summer temperature suggests differences between male and female sensitivities to temperature. Female and male wing size may be subject to different natural pressures (Outomuro & Johansson, 2011). A possible explanation for this could be that females and males differ in their optimum size and, regardless of temperature, females do not emerge until they have reached a certain size (De Block & Stoks, 2003). However, the reasons for differences in growth responses between the sexes are still poorly understood (De Block & Stoks, 2003).

Winter and autumn temperatures in the year prior to collection were not significantly correlated with wing length in either males or females. However, a similar study on *Calopteryx splendens* has shown that both males and females showed an increase in wing length with increasing winter temperatures, suggesting that larvae continue to grow during mild winters (Upton *et al*., 2016). Nevertheless, these differences could have resulted from differences in larval

habitat between the two species, or be due to the differences between our sample sizes.

Wing length and date of collection (ordinal days)

Date of collection was strongly positively correlated to wing length in both males and females. However, care is needed when interpreting collection date results because collection date does not equate to emergence date and the adults can survive for several weeks (Daguet, 2007).

Interestingly, in another study on *Calopteryx virgo*, collected in the field in Finland, no such relationship was found, although this could be explained by the difference in latitude and climate, as well as shorter flight seasons in Finland than in Britain (Rantala *et al*., 2001). Another study, using museum specimens of *Calopteryx splendens,* did not find a decrease in wing length later in the season in either males or females (Upton *et al*., 2016). Nevertheless, this could be explained by the smaller sample sizes in their study (n=94 females; n=176 males).

Our results (Figs 7, 11) suggest that there might be an environmental cue, such as photoperiod (De Block & Stoks, 2003), signalling that flight season is almost over. Larvae respond to photoperiod by accelerating development and adults emerge regardless of the fact that they might not have attained their maximum size (Stoks *et al*., 2006). However, other factors might cause this decline in size. For example, maternal nutritional provision in the egg might decrease as the season progresses, thus not allowing the adults to reach their full potential size (Rantala *et al*., 2001). Another theory is that larval competition may increase as the season progresses, leading to wing size decrease (Atkinson & Begon, 1988). Temperature variation may explain smaller size and late emergence, since an increase in water temperatures can increase the metabolic costs and thus result in a smaller adult (Stoks *et al*., 2006). This last theory is also supported by our results, since smaller wing length in both sexes was significantly correlated with increasing spring temperatures during the year of collection.

Wing length variation within and between locations

Analysis of specimens collected on the same day from the same location, confirmed that there is large variation in wing length within populations. This suggests that wing length within *Calopteryx virgo* could be affected by environmental factors other than temperature (Angilletta & Dunham, 2003), such as intraspecific competition (Tynkkynen *et al*., 2004) or predator-prey interactions (Kuchta & Svensson, 2014). Microhabitats (e.g., patchy tree shade across a site) could also affect the development rate of larvae through a change

in water temperature, affecting both sexes and causing these size variations (Fenberg *et al*., 2016).

Populations located close to each other showed less difference in wing length than populations situated further away. Also (Figs 14, 15) female populations differ from each other in wing size more than males. This suggests that female wing length may be more affected by local geographical and physical aspects of the environment (e.g., vegetation cover) than that of males.

Conclusions

Our results support Bergmann's Rule that wing length increases with increasing latitude. Furthermore, they suggest differences in response by males and females to longitude and summer temperatures. Unlike males, female wing length was strongly correlated with longitude whereas, unlike females, male wing length was significantly correlated with summer temperatures in the year before collection. This study also shows that wing length in both sexes was significantly correlated with latitude, mean spring temperature in the year of collection and the date of collection. However, it is difficult to state with confidence which is the most important factor, since they often co-vary (Millien *et al*., 2006).

This study highlights the value of natural history museum collections. By analysing a large sample, mostly collected before the 1970s and the onset of current rapid climate change, we can observe the differences in wing length across most of the geographical range of *Calopteryx virgo* in Britain and Ireland, as well as being able to construct a baseline of the responses of this species to temperature. The study has also shown how much variability there is in wing length, in both males and females, within and between populations. The fact that wing length in both males and females was positively correlated with latitude, and the fact that wing length in both sexes was negatively correlated with spring temperatures, shows that the *C. virgo* data support both Bergmann's Rule and the Temperature-Size Rule. Another important influence on wing length is timing of the flight season, which has been found in other Odonata species (Corbet, 2004). Wing length tends to be shorter in individuals flying later in the summer than those flying in the spring. The influence of all these factors on wing length might be common for *Calopteryx* species with semi-voltine life cycles and could potentially enable us to make better predictions of their possible response to climate change.

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Reintroduction of *Leucorrhinia dubia* (Vander Linden) (White-faced Darter) to Delamere Forest, Cheshire

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Abstract

Leucorrhinia dubia (The White-faced Darter) has shown a marked reduction in its distribution in recent years. A project led by Cheshire Wildlife Trust began in 2013 to reintroduce the species to Delamere Forest, Cheshire, where the species was last recorded in 2003 and was subsequently declared extinct. *Leucorrhinia dubia* was lost from Delamere through habitat destruction, due to a history of drainage and plantation forestry dating back to 1812. Since 1994 work has been underway, in partnership with the Forestry Commission, Cheshire West and Chester Council and Natural England, to restore some of the mossland habitats that are scattered throughout the 972 hectare forest. Suitable habitat has now been restored for *L. dubia*, making reintroduction a viable prospect. The reintroduction project was carried out by translocating larvae from the two nearest breeding populations, i.e. Fenn's, Whixall and Bettisfield Mosses National Nature Reserve (NNR) on the Shropshire/Wrexham border and Chartley Moss NNR in Staffordshire (all managed by Natural England). Exuviae recording was used to monitor both donor sites and the reintroduction site during the period of the project (2013-2016); also recording the conditions under which *L. dubia* emerged to provide an insight into the species' preferences. The translocation of mature larvae and, later in the year, *L. dubia* eggs and young larvae, was found to have no immediate impact on the breeding populations at either donor site. The genetic diversity of *L. dubia* across Europe is being studied by students at Manchester Metropolitan University, who have been provided with exuviae collected over the course of the reintroduction project.

Introduction

Leucorrhinia dubia (The White-faced Darter) (Plate 1) is a species adapted for survival on lowland peat bogs and is found throughout northern and north-central Europe (Dijkstra & Lewington, 2006). Following dramatic habitat loss over the past 100 years, as lowland peat bogs have been drained for conversion to agriculture and forestry or for development, the species has been lost from many of its breeding sites. Indeed, *L. dubia* now has only three strong breeding

Plate 1. Male *Leucorrhinia dubia* at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve. Photograph by Greg Osborn.

sites in England, with a fourth site having been recently established through a reintroduction in Cumbria (Clarke, 2014). The present study concerns a reintroduction into Delamere Forest in Cheshire (Fig. 1), where the species was last recorded in 2003, in a bid to reverse the decline it has suffered over recent decades. It is listed as endangered on the UK Red List due to its severely fragmented, declining population (Daguet *et al.*, 2008). A small, dark dragonfly of acidic peat bogs, *L. dubia* can be found emerging from May until July, after a larval stage lasting an average of two years. In Britain *L. dubia* is restricted to populations in the north-west with a stronghold for the species across the Scottish Highlands. The current range of *L. dubia* in England is restricted to four large (over 60 hectares) mossland sites, two in Cumbria, one on the Shropshire/ Wrexham border and one in Staffordshire. *Leucorrhinia dubia* is a species well adapted to its northerly distribution and, while some odonates are experiencing range expansion in response to climate change, there are concerns that the

Fig. 1. Project sites for the Delamere Forest *Leucorrhinia dubia* reintroduction. © Crown copyright and database rights 2016. Ordnance Survey Licence number 100030835.

range of *L. dubia* is contracting (Corbet & Brooks, 2008).

In 2010 a Steering Group was enlisted to begin the preliminary stages of a reintroduction attempt into Delamere Forest, the last breeding location of *L. dubia* in Cheshire. Over 100 basin mires formed in Delamere around the same time as the mosslands of north-west England, about 20,000 years ago, but are significantly smaller, ranging from less than 0.1 to 40 hectares, with many being under one hectare in size. The drainage of Delamere's mosslands began in 1812 when Forest Law was removed from the area. Prior to this, *L. dubia* would probably have been widespread across the landscape, although records only date back to 1882 when many suitable sites would have already been lost due to drainage (Latham, 1991).

The Steering Group comprises members of Natural England, the Forestry Commission, Cheshire Wildlife Trust and the British Dragonfly Society, plus the County Dragonfly Recorder and two independent ecologists. A feasibility study was commissioned by the Steering Group to assess several sites within Delamere Forest for suitability for a *L. dubia* reintroduction attempt. Through this work Doolittle Moss was selected as it possessed a mixture of open water and aquatic sphagnum cover, emergent vegetation and good cover for sheltering

dragonflies. In addition to good *L. dubia* habitat the water level and its quality were stable with good connectivity to other restoration sites, enabling possible future dispersal through the forest. Before the reintroduction programme began an action plan was drawn up, ensuring that the IUCN guidelines for conservation translocations (IUCN/SSC, 2013) were followed throughout. Two donor sites were chosen: Fenn's, Whixall and Bettisfield Mosses National Nature Reserve and Chartley Moss National Nature Reserve.

It is hoped that the restoration of *L. dubia* to Doolittle Moss will eventually prove successful enough to encourage dispersal to other suitable sites throughout Delamere Forest, creating a metapopulation that will be more resistant than isolated populations to localised extinction events.

Donor sites

Fenn's, Whixall and Bettisfield Mosses National Nature Reserve (NNR). (Fig. 1, Plate 2). These form the third largest lowland raised bog complex in Britain at nearly 1000 hectares. Spanning the Shropshire/Wrexham border, the site is managed by Natural England in partnership with Natural Resources Wales. Drainage was carried out throughout the 19th century, with the complete collapse of the saturated raised domes occurring in the 1920s when large scale drainage was carried out to prepare for commercial peat cutting. Peat was cut from all three Mosses for 500 years, initially by small scale hand-cutting before mechanised cutting was introduced in 1968. By 1989 the peat harvest was four times what it was in 1968. This rapid increase in production prompted a campaign to protect the Moss and the central area was acquired as an NNR in 1990, bringing an end to large-scale peat extraction (Natural England, 2010). Widespread restoration for over 25 years has allowed mossland species to once again spread, with *Leucorrhinia dubia* expanding from a small relict population to now cover most of the Moss. The many steep-sided, hand cut pools are perfect for *L. dubia* breeding, their depth preventing rapid plant succession. Three pools (13.3, 15.1 and 31.1) were chosen as donor pools.

Chartley Moss National Nature Reserve (NNR). (Fig. 1, Plate 3). This is Britain's largest schwingmoor, or quaking bog, although at 105 hectares it is considerably smaller than Fenn's, Whixall and Bettisfield Mosses (FWBM). Located in Staffordshire, the site is managed by Natural England for nature conservation. A raft of peat and Sphagnum moss up to 4 metres thick covers a 10 metre deep lake. The lake has been deepened by the subsidence of solid ground beneath, due to high concentrations of salt in the lake bed dissolving in the presence of water. This slows the rate at which the peat basin is infilled and means maintenance of schwingmoor is possible over a longer time than would otherwise be expected. This schwingmoor development through subsidence

Plate 2. Searching for *Leucorrhinia dubia* larvae in Pool 13.3 at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve.

is an exceptionally rare occurrence throughout the world, occurring at only one other moss in Britain, Wybunbury Moss NNR in Cheshire (Beynon, 1995). There has been some past drainage at Chartley Moss and Pine trees were planted historically, although many have been cleared from the mire expanse. Peat has not been extracted from the site and so *L. dubia* survives in a small number of natural pools and dammed, man-made ditches. Following the loss of *Leucorrhinia dubia* from Thursley Common, Surrey, in the late 1990s, Chartley Moss became the species most southerly breeding site in Britain (Cham *et al.*, 2014). Two pools (Europa and Shooters) were chosen as donor pools.

Receiving site

Doolittle Moss. (Fig. 1, Plate 4). This Moss is approximately 1 hectare in size and located within Delamere Forest, Cheshire. It is connected to the largest Moss in the forest, Great Blakemere, by an historic drainage ditch. The pool is well vegetated with Common Cotton-grass, Soft Rush and White Sedge while the banks are covered by trees, largely comprising planted Pines, Sweet Chestnut and regenerating Birch. To the north of the pool is a section of wet woodland including Birch, Willow and Alder. There is a small amount of water that flows through this woodland into Doolittle Moss, maintaining water levels

Plate 3. Europa Pool at Chartley Moss National Nature Reserve.

throughout the year. Doolittle Moss was one of the first mosses in Delamere to be restored following the flagship restoration of Great Blakemere in 1998. The main outflow ditch from the Moss was blocked and, over 10 years later, in 2012, regenerating scrub was removed. Water levels were raised once more before the *Leucorrhinia dubia* reintroduction attempt, in order to prevent the encroachment of scrub and to encourage the continued establishment of the Sphagnum raft.

Material and Methods

Translocation

The *Leucorrhinia dubia* reintroduction programme, adopted from the Cumbrian reintroduction, took place in two stages each year, in late April, before the start of their flight season, and in August following the cessation of breeding (Clarke, 2014).

The first translocation of *L. dubia* into Doolittle Moss took place in 2013. Permission was originally granted for the removal of 50 final instar *L. dubia*

Plate 4. Doolittle Moss in Delamere Forest, the receiving pool for the *Leucorrhinia dubia* reintroduction. Photograph by Colin Hayes.

larvae from each of the two donor sites, Fenn's, Whixall and Bettisfield Mosses NNR and Chartley Moss NNR. The same number of larvae were translocated in 2014. Intensive monitoring of the pools from which the donor larvae were collected was carried out each year (2013-2016). Following this monitoring in 2013 and 2014, which showed large populations to be present, it was agreed that from 2015 the number of larvae taken could be increased to 100 from each donor site. Cold weather at the end of April 2015 made obtaining the larvae difficult and only 75 were removed from each site. However, in 2016, 100 larvae were collected from each site and transported to Doolittle Moss (Table 1). Each year of the translocations, following the end of the *L. dubia* flight season, further collections were made from the donor sites of Sphagnum moss containing *L. dubia* eggs and hatchlings.

Final instar larvae were collected using pond dipping nets and trays at the end of April or the beginning of May, before mass emergence began, so as to prevent disturbing the fragile emergent dragonflies. The comparative length of larval wing buds was used to identify the final larval instar, the wing buds extending past the fourth abdominal segment at this stage; less mature individuals were

Table 1. The number of larvae of Leucorrhinia dubia released at the reintroduction site, Doolittle Moss in each year (2013-2016) and details of recorded emergence and adults.

	2013	2014	2015	2016
Larvae released	100	100	150	200
Exuviae counted	4 (4%)	28 (28%)	18 (12%)	51 (26%)
Emergents	1	5	1	7
Male holding territory	2	4	4	0
Ovipositing	1	0	1	0

put back into the pools. The final instar larvae were placed in sealed tubs along with bog water and some Sphagnum to be transferred to the reintroduction site in cool boxes. The use of cool boxes prevented the rapid warming of water in which the larvae were transported, which would lead to a reduction in available dissolved oxygen, an important measure of water quality (Corbet & Brooks 2008). The larvae were then placed into submerged Sphagnum at Doolittle Moss after a maximum of four hours since removal from the donor site pools. This limit was set to keep larval stress to a minimum.

Sixty litres of Sphagnum were removed at the end of the flight season in all four years. This had little effect on the cover in the pools at the donor sites but would have contained enough *L. dubia* stock to offer a significant population boost at the receptor site. The methodology for this stage of the translocation process was discussed against the alternative of collecting mating pairs of *L. dubia* and separating the female, encouraging her to lay eggs in a collection pot. This method would reduce the risk of accidental introduction of other species but the Cumbrian reintroduction found it to be very time consuming and the viability of the eggs was not known. In addition, the use of a few females from which to obtain eggs could greatly reduce the genetic variation in the reintroduced population, leading to a potential genetic bottleneck and hence to inbreeding depression. To reduce the number of non-target species moved between sites, visible invertebrates were removed from the Sphagnum before it was transported. The material was moved to Doolittle Moss as quickly as possible and placed into the water where it was hoped that the eggs and hatchling larvae would continue development.

Monitoring

Following the translocation of final instar larvae into Doolittle Moss, weekly exuviae searches were used to monitor the populations of *Leucorrhinia dubia* at both donor sites, while the reintroduction site was monitored at least once a week using exuviae searches; also transects were followed to monitor adult dragonflies. The vegetation was searched within and around the pools from which larvae were taken to locate *L. dubia* exuviae, which are easily identified by three dark stripes on their underside. Each year monitoring of the donor sites ceased when the number of exuviae recorded dropped below 50 at each pool, although this figure varied a little depending on weather conditions. Exuviae searches took place in most weather conditions but three visits were missed over the four year study due to thunderstorms.

When *L. dubia* exuviae were found, data were collected to enhance our understanding of emergence preferences by the species. Originally the data collected consisted of the species of vegetation on which the exuvia was found, its height above the water, distance from the bank and proximity to other *L. dubia* exuviae. With teams of volunteers helping, the proximity of exuviae to one another was difficult to measure accurately so, for 2014, data collection was altered by creating grid squares across all donor site pools so that the location of each exuvia could be recorded, allowing the study of microhabitat preference as well as grouping tendencies during emergence

Also, from 2014 to 2016, some of the *L. dubia* exuviae found were collected to be sexed and measured. They were collected in individual, labelled sample tubes before being examined under a microscope. High quality digital photos were taken of each exuvia and the image measured using Image J software. Three measurements were taken, where possible, for each exuvia, i.e. total body length, head width and wing bud length (Fig. 2). Some of the exuviae collected were sent to Manchester Metropolitan University for morphological and genetic analysis. Any exuviae that were not collected for these analyses were removed from their emergence support to prevent them being counted more than once.

In addition to monitoring the number of *L. dubia* at both donor sites and at Doolittle Moss, other dragonfly species were also monitored around Doolittle Moss. Monthly measurements of conductivity and pH were taken from six locations across Doolittle Moss to assess water quality. Vegetation surveys were carried out at both donor sites to identify microhabitat preferences of *L. dubia.* Also predation of *L. dubia* and other dragonfly species was noted at the donor sites and at Doolittle Moss.

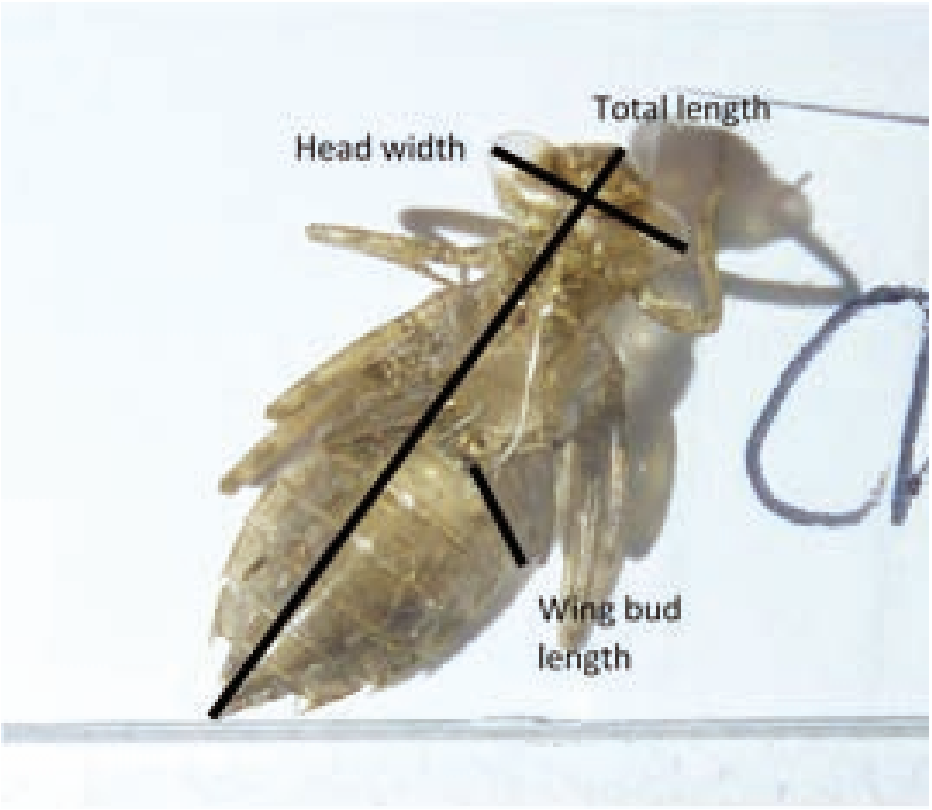

Fig. 2. Exuvia of *Leucorrhinia dubia* showing how total length, head width and wing bud length were measured.

Results

Donor sites

The weekly exuviae searches following the translocation of *Leucorrhinia dubia* showed that, at Chartley Moss, there was an upwards trend in the total number of *L. dubia* exuviae counted over the four year period, despite fewer exuviae being found around Shooters pool in 2016 than in 2015. The variation in numbers of *L. dubia* exuviae between years was high at Chartley Moss, with nearly three times as many found at Europa pool in 2016 than in 2013 (Table 2). The story was very similar at Fenn's, Whixall and Bettisfield Mosses, although even more dramatic in one pool (pool 15.1) which produced 58 exuviae in 2014 and 425 the following year. Over the whole monitoring period a total of 11,157 exuviae

Table 2. The number of exuviae of *Leucorrhinia dubia* recovered from the donor site pools at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve and Chartley Moss National Nature Reserve over the four year monitoring period, 2013 – 2016.

Site	Pool	2013	2014	2015	2016
Chartley Moss	Europa	688	1292	1710	2007
	Shooters	-	310	651	568
	Total	688	1602	2361	2575
Fenn's, Whixall and Bettisfield Mosses	13.3	888	336	356	762
	15.1	73	58	425	421
	31.1	-	297	103	212
	Total	961	691	884	1395

were found at the donor sites (Table 2).

From the number of *L. dubia* exuviae collected weekly from both donor sites over the four year monitoring period it can be seen that the emergence period spanned between seven and ten weeks over the four years, with the emergence peak being between weeks 22 and 25, the largest difference falling between 2015 and 2016 (Fig. 3). As an early emerging dragonfly species it would be expected that most of the emergence would occur in the early part of the emergence period and, indeed, this was what occurred in 2016 (Fig. 3). However most years did not show this, highlighting variation in the species' emergence patterns. *Leucorrhinia dubia* showed quite dramatic responses to weather conditions and temperature, their emergence timings being quite plastic over the years of this study. These cues enabled the cohort to emerge in synchrony when the conditions were favourable, maximising emergence success. The emergence of *L. dubia* showed a close correlation to the prevailing weather conditions. In 2015, when the emergence peak was particularly late and the emergence period drawn out, a warm April soon turned cold from the 24th, slowing the start of emergence. May remained cool and wet until the weather improved in the second week of June (week 24), when a sharp increase in the number of emerging *L. dubia* was observed. May 2014 was similarly cold and unsettled, again pushing the majority of the emergence into June. 2016 showed

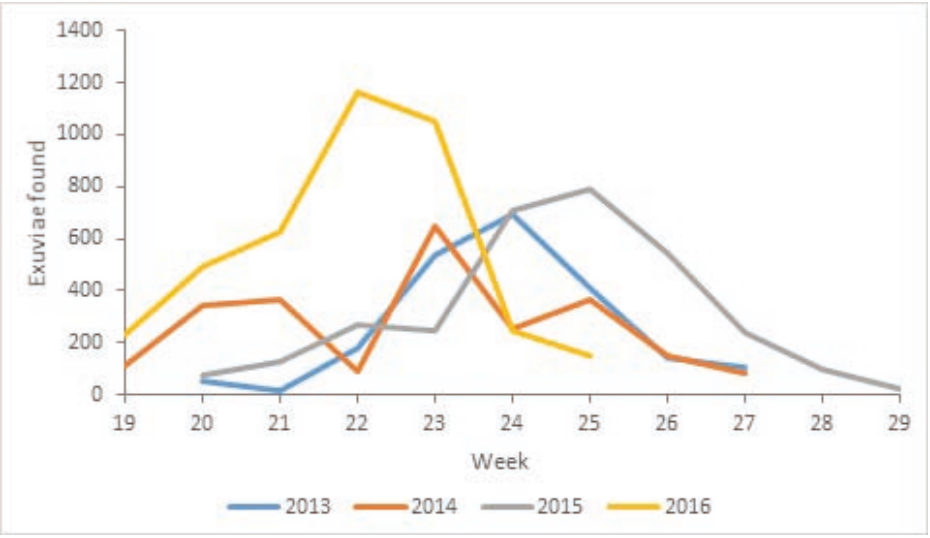

Fig. 3. *Leucorrhinia dubia* emergence recorded at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve and Chartley Moss National Nature Reserve over the period 2013 to 2016.

the opposite to these previous years when, despite a fairly cool April, plenty of sunshine warmed the peat stained water of the donor site pools, and a bright and mild May encouraged emergence, which not only peaked early but finished around the same time as the 2015 emergence peak.

In total, 886 exuviae were collected from the donor sites for further examination over the three year period from 2014 to 2016, although not all of these could be sexed or measured due to damage. Of those that could be sexed, 392 were males and 437 females, a sex ratio of 1:1.11, which was not found to be significantly different from 1:1 (Chi^2 = 2.443; P = 0.12). However, the number of exuviae sexed was relatively small (n = 829) compared to the total number of exuviae recorded (11,157). Throughout the emergence period male and female *L. dubia* were found to emerge at roughly the same frequency (Fig. 4). Although in 2014 the exuviae were all collected at the beginning of the survey season, not spread out across it, in 2015 and 2016 there appeared to be more females emerging later in the season, A Komolgorov-Smirnov test did not find the difference in male and female emergence curves significant (D_{stat} = 0.0945, D_{crit} = 0.0946, P = 0.05).

The majority of *L. dubia* exuviae were found on Common Cotton-grass (*Eriophorum angustifolium*) (Table 3), most of which was growing within the water

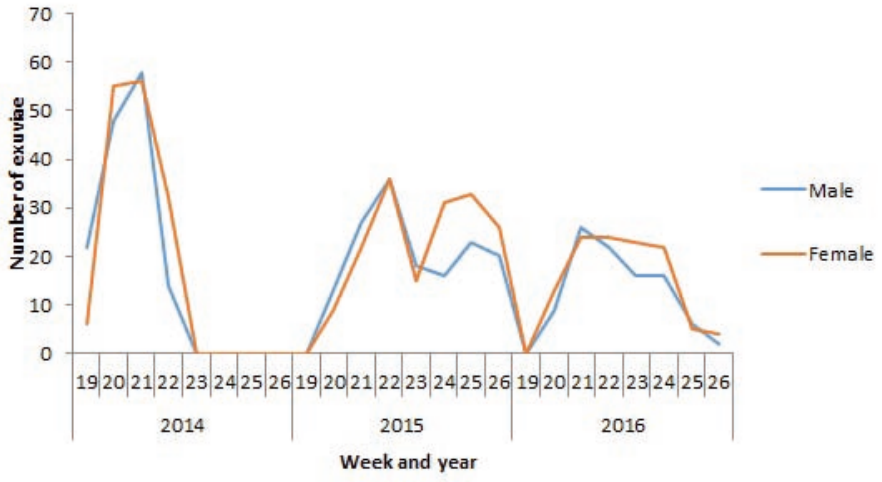

Fig. 4. The frequency of male and female *Leucorrhinia dubia* exuviae found over three years (2014-2016), including exuviae from Fenn's, Whixall and Bettisfield Mosses National Nature Reserve and Chartley Moss National Nature Reserve.

of the donor site pools. The average height climbed by the larvae was 4.47cm, although exuviae were found from 1cm to 80cm above the water. Those exuviae found on Sphagnum moss were checked to ensure they had not been blown there but had emerged on the Sphagnum. Exuviae appear to be found higher up on sturdier species, e.g. Heather (*Calluna vulgaris*), or tussocky species, e.g. Purple Moor Grass (*Molinia caerulea*), compared to Common Cotton-grass. The differences between the heights climbed up different plant species was found to be significant using an analysis of variance on the log transformed data ($F = 121.1$, $P < 0.001$); this test included all species of vegetation in Table 3 apart from Sphagnum and Cranberry so as not to skew the result as a result of larvae being restricted to the total height of the plant. Some exuviae were also found on the carnivorous Sundew (*Drosera rotundifolia*), where many of the individuals managed to emerge, leaving only their exuviae stuck to the sticky leaves (although not all adults survived). Those exuviae on Sundew were found to be 5.13cm above the water on average ($n = 30$). A large number of exuviae found had been dislodged ($n = 1546$), defined as when an exuviae was found floating in the water, lying on Sphagnum without their legs gripping the moss or when they were found on emergent vegetation but at the water's surface. The average heights should be taken as a minimum as it is likely that some exuviae may have slipped down the emergent vegetation.

Table 3. The ten most commonly used plants that provided emergence supports for the larvae of *Leucorrhinia dubia*, along with records for each plant species of the number of exuviae found, the percentage of the total number of emergences and the mean height climbed by larvae at the donor site pools at Fenn's, Whixall and Bettisfield Mosses National Nature Reserve and Chartley Moss National Nature Reserve.

Species	Exuviae found	Percentage of total	Mean height climbed (cm)
Sphagnum	402	3.55	3.40
Common Cotton-grass	6741	59.58	4.47
Soft Rush	49	0.43	6.16
Hare's-tail Cotton-grass	1262	11.15	6.18
Cranberry	68	0.60	6.99
Bog Rosemary	50	0.44	7.24
Cross-leaved Heath	66	0.58	7.26
Woody spp.	63	0.56	8.38
Purple Moor Grass	791	6.99	8.72
Heather	130	1.15	8.80

The vegetation species used for emergence differed quite markedly between the donor sites themselves and also between pools at the same sites (Fig. 5). Although Common Cotton-grass was the most commonly used species at both donor sites, the second most widely used species differed: at FWBM this was Purple Moor Grass but at Chartley Moss Hare's-tail Cotton-grass (*Eriophorum vaginatum*) was favoured. Pool 15.1 at FWBM was the obvious exception to this general rule, with Purple Moor Grass used most extensively for emergence with Hare's-tail Cotton-grass the second most used.

The majority of *L. dubia* exuviae recovered were found on emergent vegetation (n = 7621, 81.3%) as opposed to on the bank (n = 1751, 18.7%). However, this summary does not accurately describe the picture at each pool. For example, pool 15.1 at FWBM had little emergent vegetation and a long, easily searched

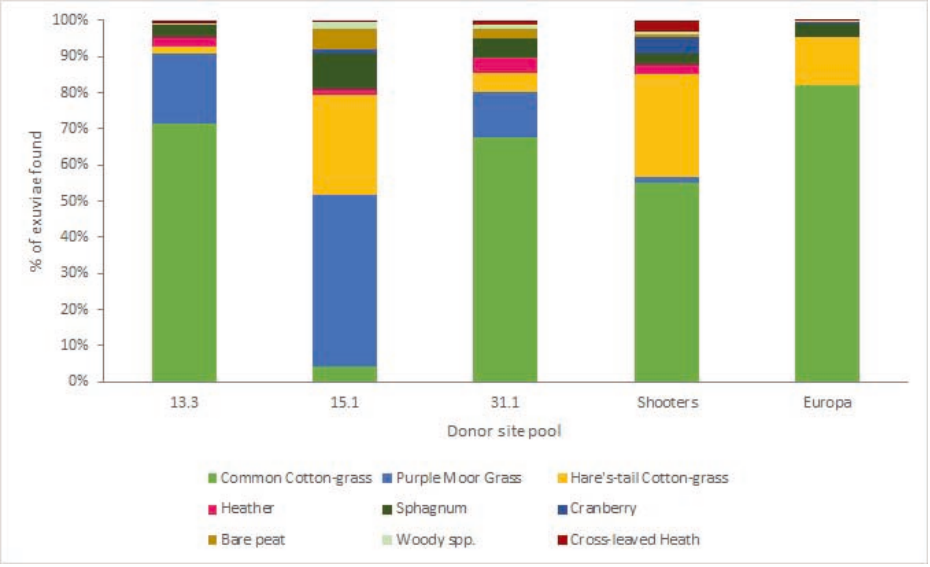

Fig. 5. The relative use of different vegetation supports by *Leucorrhinia dubia* for emergence from each donor site pool.

and well vegetated bank, and 56% of all exuviae at this pool were found on the bank. The opposite was true at Europa pool on Chartley Moss where there are no obvious banks, just a gently sloping Sphagnum raft. Here only 8.9% of exuviae were found out of the water.

Exuviae recorded as found on the bank were often found close to the water's edge (mean distance from the edge = 12.6cm), whereas those found on emergent vegetation were further away from the bank (mean = 145.6cm). The mean height climbed by larvae emerging on the bank was 7.2cm; this was found to be significantly higher than those emerging on emergent vegetation (4.7cm), using a t-test ($t = 15.7$, $P < 0.001$).

In 2014 each pool at the donor sites was divided into a series of 2 metre grid squares. Over any one season the most *L. dubia* exuviae that any grid square contained was 148, at Europa pool on Chartley Moss. The mean number of exuviae found per 'occupied' grid square ranged from 2 to 20 at the different donor pools. Despite the occurrence of some heavily used grid squares around certain pools, no correlation was found between the emergence location of *L. dubia* and emergent vegetation type, Sphagnum density or aspect.

Size. Of the exuviae that were measured, the body length, head width and

wing bud length were recorded for 756, 779 and 872 individuals respectively. The wing bud length is not discussed further as the measurement technique did not account for the three dimensionality of the exuviae, which drastically affects the perceived length of the wing bud. The average length of the *L. dubia* exuviae was 17.95cm and there was no significant difference between the size of exuviae from FWBM and Chartley Moss, as shown using t-tests ($t = 0.24$, $P = 0.81$; head width $t = 0.95$, $P = 0.34$). There was found to be a significant difference between the size of male and female *L. dubia* exuviae, the mean female length being 17.73cm and head width 4.80cm, while the average male measurements were 18.22cm and 4.90cm respectively (body length, $t = 6.32$, $P < 0.001$; head width, $t = 2.34$, $P = 0.02$). No correlation was found between the size of the exuviae and their height up an emergence support or their time of emergence.

Predation. *Leucorrhinia dubia* at the donor sites suffer some losses through avian predation, with both male and female Reed Bunting having been seen at FWBM picking off emergent *L. dubia*. Several Hobbies (*Falco subbuteo*) also spend the summer on FWBM and undoubtedly take large numbers of *L. dubia* during their time there, although their predation of dragonflies is more specialised than that shown by more generalist birds, which are most able to catch odonates at, or shortly after, emergence. However, the pools studied on the donor sites are a considerable distance away from the nearest mature trees, meaning the maiden flights of teneral *L. dubia* tend to be into the relative safety of the surrounding Heather or scrub.

Receiving site

Over the reintroduction period (2013-2016) 550 last instar larvae of *Leucorrhinia dubia* were released at Doolittle Moss together with an unknown number of young larvae in the Sphagnum moss introductions. Over this period, a total of 101 exuviae were recorded, including 14 individuals seen emerging (Plates 5, 6). The best year for exuviae was 2016 when 51 were found. However, few mature adults were seen at Doolittle Moss over the four year period of the reintroduction programme. A total of 10 males were observed holding territory, the maximum in any one year being four, and only two female *L. dubia* were seen ovipositing over the four years. 2016 was the only year in which no mature adults were seen, although more exuviae were recovered and more teneral or emergent dragonflies observed that year.

The monthly water quality measurements, taken from the six locations across the Moss, remaining fairly constant throughout, with an average pH of 4.00 and an average electrical conductivity of 252.79µS/m.

Plate 5. Emerging *Leucorrhinia dubia* at Doolittle Moss.

Predation. From the first year of the reintroduction attempt avian predation of *Leucorrhinia dubia* has been a concern, after the first emerging *L. dubia* took its maiden flight only to be intercepted by a House Sparrow. Various species were seen taking teneral dragonflies and damselflies over the monitoring period, although only House Sparrow and Crow were confirmed to have taken *L. dubia.* The other species emerging from Doolittle Moss at the same time as *L. dubia* were mainly *Libellula quadrimaculata* (Four-spotted Chaser) and *Pyrrhosoma nymphula* (Large Red Damselfly), and both these species suffered heavy predation by Nuthatch, Robin, Great Tit and Crow (Plate 7). The smaller bird species had a tendency to sit and wait on trees surrounding the moss, catching teneral odonates as they headed for the cover of the trees. However, Nuthatches focussed heavily on the larger bodied *Libellula quadrimaculata*. They have perfected the skill of waiting for *Libellula quadrimaculata* to settle on

Plate 6. Teneral L*eucorrhinia dubia* resting on a Pine tree by Doolittle Moss. Photograph by Janette Renshaw.

the trunk of a large Pine tree before swooping in to pin them against the tree. Crows were seen regularly collecting emerging odonates by walking over the Sphagnum rafts of the developing schwingmoor, returning to their nest with a beakful of high protein dragonflies (Plate 7). There is no doubt that this predation has put some pressure on the *L. dubia* reintroduction at Doolittle Moss, but it has been hard to quantify this as only two *L. dubia* have been seen taken by birds.

Discussion

One of the major difficulties with assessing the success of the *Leucorrhinia dubia* reintroduction programme was monitoring emergence at the receptor site. In stark contrast to the small pools at both donor sites, where there is a clear view of all vegetation within them, Doolittle Moss covers 1 hectare and is well vegetated. Also the surface of Doolittle Moss is uneven, with historic ditches and planting furrows meaning that changes in water depth from 0.3 metres to over 2 metres are possible in a single step, which makes exuviae searches, even in chest waders, difficult. The maximum number of exuviae was found in 2014. Since any small larvae introduced in 2013 would not have matured by this time, this represents 28% of the final instar larvae translocated

Plate 7. A crow with a beakful of odonates on Doolittle Moss. Photograph by Janette Renshaw.

in 2014 and thus represents a good proportion considering the above factors. DuBois (2015) found that the detection probability of dragonfly exuviae along a riverbank was 64% on average. It is reasonable to expect search efficacy to be lower than this in a pool where the entire area cannot be covered, as opposed to a linear search. The previous *L. dubia* reintroduction at Foulshaw Moss in Cumbria found that 43% - 68% of the exuviae from the translocated mature larvae were recovered (Clarke, 2014). Although higher than the numbers retrieved from Doolittle Moss, the receiving pools searched at Foulshaw Moss were much smaller and therefore easier to fully search, similar to the pools at Chartley Moss and FWBM.

In 2016 a section of low garden fence was set up around the receptor plots in an attempt to increase the proportion of exuviae recovered by providing a secure support for emerging dragonflies and, although 12 exuviae were found on the fence, most were found on vegetation within the 2 x 2 metre plots. *L. dubia* are known to be very mobile within Sphagnum and so separating emergence by translocated larvae and that from larvae that have spent at least a year in the pool is difficult.

The observation of adults was also not easy at Doolittle Moss as the best vantage point over the vegetation was from the bank, which was about 30 metres away from the centre of the pool. Attempts to observe adult *L. dubia* from within the water were obscured by tall vegetation, in particular Soft Rush. As all translocated material was released into the same pool, separating on-site breeding from translocated larvae and eggs was impossible from 2015 onwards given the two year life cycle.

The weekly exuviae searches at the donor sites gave a very good picture of the number of *L. dubia* emerging from the pools, as the large number of volunteers and the methodical searching around the pool edges meant that the detection rate of exuviae is likely to be close to 95% (DuBois, 2015). *Leucorrhinia dubia* has previously been noted as showing high variability in population size between years (Cham *et al.*, 2014). The results of the present study showed that, over the time that stock was taken from the donor sites, the population numbers at most pools fluctuated substantially. If this fluctuation was due to the removal of *L. dubia* larvae and eggs it would be expected that the number of exuviae recorded at all pools would change in the same direction but this was not the case. Hence changes in population size between years is more likely a consequence of weather conditions and changes in vegetation, for example Birch saplings maturing and shading pool margins to a greater extent. Due to the two year life cycle of *L. dubia* it is worth bearing in mind that the impact of taking eggs and young larvae in 2016 will not be seen until 2018. There are no previous data with which to compare numbers of *L. dubia* at FWBM, and the only similar data are those for Shooters pool at Chartley Moss (Beynon, 1997). Using estimates of emergence numbers at Shooters pool at Chartley Moss for 1994 to 1996 (Beynon, 1997), *L. dubia* have 'moved' their primary breeding location from Shooters pool to Europa pool, only 10 metres away. This could be due to the removal of aquatic Sphagnum from Shooters pool prior to the present project starting. This was carried out to slow succession and keep the pool open for *L. dubia* to breed. As the Sphagnum in Shooters pool expands once more, increasing *L. dubia* larval habitat, the *L. dubia* population should also increase.

The emergence times of male and female *L. dubia* are of particular interest in relation to the reintroduction attempt. Despite finding no significant difference between emergence times of male and female *L. dubia* in these data, the results suggest that there may be a tendency for female *L. dubia* to emerge later in the season than males do, the opposite of the more territorial *Sympetrum sanguineum* (Falck & Johansson, 2000). As all larvae for the translocation were collected before emergence began, to avoid disturbing fragile emergent dragonflies, there was a chance that more male *L. dubia* may have been moved than females. Of the exuviae recovered and sexed from Doolittle 44% were

female, although only a small number of the total translocated larvae were recovered. Sexing a larger number of *L. dubia* throughout an emergence season should provide a clearer picture as to whether females do emerge later. If so, this could make the collection of final instar larvae early in the emergence period likely to be male biased. Sexual size dimorphism is well documented in odonates (Johansson *et al.*, 2005) and therefore it was not surprising to find that male and female exuviae differed in mean size in *L. dubia*. Species that show larger male body size are usually territorial, the larger size conferring additional fitness with which to fight off competitors. The fact male *L. dubia* may emerge earlier and grow larger than females in the larval stage suggests a higher larval mortality of males, associated with riskier foraging strategies (Falck & Johansson, 2000). This leads to a female biased sex ratio, which despite not finding the result significant, was found here.

Leucorrhinia dubia do seem to show a preference for emerging from within the larval pools, using emergent vegetation. However, where there is little vegetation within the pool they will emerge on the bank, in particular at FWBM where the old peat cuttings have steep sided banks. Few exuviae were found on the bank over 30 cm from the water's edge, making the vegetation in the marginal zone especially important for emergence. The vast majority of *L. dubia* exuviae were found on Common Cotton-grass within the pools at both donor sites. However, it is unclear whether this is in response to a preference for the quality of the species as an emergence support or because it is the most common species at most *L. dubia* breeding sites. At both donor sites *L. dubia* appear to emerge most frequently on the most common species of emergent vegetation, the second most common emergence support being the second most common species of vegetation (Fig. 5). Pool 15.1 at FWBM illustrates this nicely as there is little Common Cotton-grass there, despite over 400 exuviae being recorded from the pool in 2015 and 2016. Here the adults lay eggs in abundance and larvae use Purple Moor Grass as their primary emergence support. At the receiving site, Doolittle Moss, many exuviae were found on Soft Rush, despite there being Common Cotton-grass within a few centimetres.

It seems that, when emerging on emergent vegetation, *L. dubia* are able to judge the strength of the support and show a tendency to climb higher when the support is sturdier or offers more protection from wind and rain. There were some cases where *L. dubia* larvae climbed high up Common Cotton-grass but most were found within 5 cm of the water surface, requiring the adults to move up the vegetation as their wings expanded. This response to emergence support structure could increase emergence success by decreasing the risk of their fragile wings getting wet or wind blowing the emergence support and emergent dragonfly into the water.

When data on the size of *L. dubia* exuviae were broken down into individual

years, there was often a difference between donor sites, with both sites appearing to have smaller *L. dubia* in different years. However, when all data were analysed together there was no significant difference in size between donor sites. It is hoped that, in due course, the genetics work at Manchester Metropolitan University will provide information of the most successful donor site of the reintroduced population.

The location of *L. dubia* exuviae was recorded from both donor sites within a 2 metre grid square in the hope of finding why the exuviae are often found in groups. Despite there being quite obvious grouping in certain grid squares, those grid squares showing a high density of *L. dubia* exuviae were scattered across the pools and changed from year to year. Vegetation surveys and assessments of the density of Sphagnum moss were carried out but analysis of the results did not shed any light on what caused the grouping behaviour. It is possible that it is a behavioural response to reduce the individual likelihood of predation by emerging 'en masse' in groups, so that even the most voracious predator is unable to take all of the *L. dubia* present.

Conclusion

This reintroduction has not been without its difficulties, although it is hoped that the data presented here may help others considering *Leucorrhinia dubia* translocations to fully appreciate some of the obstacles that they may be presented with. Despite the difficulties, this reintroduction attempt has allowed an in-depth study of the emergence preferences of this endangered dragonfly, which will add to the collective knowledge of *L. dubia* in Britain and problems that may face other conservation reintroductions, whether of odonates or other groups. Although it is too early to speculate on the success of the reintroduction there are many positives. We know that female *L. dubia* have oviposited in Doolittle, several larval stages were found in Doolittle following emergence in 2016, there is great support from the local community to bring the species back to Delamere and, following restoration of mossland habitats in Delamere, there are more pools suitable for *L. dubia* to spread into. 2019 will be the first year that proof of breeding in Doolittle Moss can be obtained since, upto 2018, any emergences could be from introduced larvae. After four years of translocations the focus will now shift to monitoring the reintroduced population over the next few years to see how the *L. dubia* population in Delamere Forest develops.

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