Biological Journal of the Linnean Society, 2021, **134**, 685–696. With 2 figures.

Phenotypic variation in male *Calopteryx splendens* damselflies: the role of wing pigmentation and body size in thermoregulation

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Received 7 April 2021; revised 24 June 2021; accepted for publication 28 June 2021

For ectothermic insects, their colour and size are important determinants of body temperature: larger bodies require more heat to reach a certain temperature, and dark colours absorb heat more efficiently. These dark colours are expressed using melanin, which has been intimately linked with the thermoregulatory capabilities of insects. Melanin is also linked with immune defence and is often used as a secondary sexual character in insects. There is a potential trade-off situation between thermoregulatory capabilities, immune defence and secondary sexual characters, all of which use melanin. Some *Calopteryx* damselflies, such as *Calopteryx splendens*, have melanin-based wing pigmentation that is sexually selected and drives intra- and interspecific territorial aggression. Our goal was to study experimentally how the wing pigmentation and body size of *C. splendens* males affect their thermoregulation and, especially, their ability to become active (hereafter, 'activate') after being cooled down. Our results were in line with our hypotheses, showing that individuals with larger wing spots had significantly faster activation times than those with smaller wing spots, and that individuals with larger body size had significantly slower activation times than those with smaller body size. Both variables showed an interaction and are therefore important in damselfly warm-up and activation. We discuss the role of wing pigmentation and thermoregulation in the behavioural patterns observed in *Calopteryx* species.

ADDITIONAL KEYWORDS: Calopterygidae – invertebrate – melanism – Odonata.

INTRODUCTION

In thermodynamics, colour and size are important determinants of body temperature: dark colours absorb heat more efficiently, and larger bodies require more heat to reach a certain temperature (Guggenheim, 1985). Insects are primarily ectothermic, meaning that they heat their bodies using external energy sources, mainly solar radiation (May, 1979). Phenotypic variation of body pigmentation is a phenomenon in the animal kingdom whereby some individuals have darker coloration and are more pigmented than others (Kettlewell, 1973; de Jong *et al.*, 1996; Van Dyck *et al.*, 1998; True, 2003; Clusella-Trullas *et al.*, 2007; Angilletta, 2009; Heidrich *et al.*, 2018). Individuals with darker pigmentation absorb solar radiation more efficiently than lighter ones (Brakefield, 1984; Clusella-Trullas *et al.*, 2007); therefore, darker insects heat up faster than lighter-pigmented ones (Watt, 1968; de Jong *et al.*, 1996; Forsman *et al.*, 2002; Hegna *et al.*, 2013). In conrast, overheating might be a serious problem for darker insect individuals, especially at lower latitudes (Sunday *et al.*, 2014; Guillermo-Ferreira & Gorb, 2021), where average temperatures are higher and solar radiation is stronger. Therefore, it is not surprising that insects with darker pigmentation are more prevalent in colder environments, and correspondingly, lighter insects are more prevalent in warmer habitats (Watt, 1968; Clusella-Trullas *et al.*, 2007; Hegna *et al.*, 2013; Svensson & Waller, 2013; Hassall, 2014; Zeuss *et al.*, 2014; Bishop *et al.*, 2016; Pinkert *et al.*, 2016; Van Dievel *et al.*, 2019). In addition, the minimum body temperatures required for insect flight have been demonstrated to be higher in tropical species compared with temperate species (May, 1976).

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Dragonflies and damselflies have been the focus of several studies of thermoregulation (e.g. May, 1976; Stoks *et al.*, 1996; Clusella-Trullas *et al.*, 2007; Bots *et al.*, 2008; Corbet & May, 2008; Tsubaki *et al.*, 2010; Outomuro & Ocharan, 2011; Svensson & Waller, 2013; Rivas *et al.*, 2016; Schreiner *et al.*, 2020; Guillermo-Ferreira & Gorb, 2021). They show a wide range of variation in body and wing pigmentation, both within species (Bots *et al.*, 2008; Tynkkynen *et al.*, 2004; Hassall, 2014) and between species (Outomuro & Ocharan, 2011; Svensson & Waller, 2013; Zeuss *et al.*, 2014). In certain *Calopteryx* species, males have pigmented wing spots (Svensson & Waller, 2013) that serve as secondary sexual characters (see the review by Suhonen *et al.*, 2008). It has been suggested that males with these large and dark sexual characters have faster physiological activity, such as faster encapsulation rate against parasites (Rantala *et al.*, 2000; Siva-Jothy, 2000; Moore & Martin, 2016). In addition, there is indirect evidence that patterns of pigmentation are important in odonate thermoregulation: in populations of single odonate species and in communities with many species, it has been observed that darker individuals often occur at higher latitudes (Svensson & Waller, 2013; Hassall, 2014; Zeuss *et al.*, 2014). However, it is inconclusive how strongly pigmentation affects odonate thermoregulation. For example, Svensson & Waller (2013) and Schreiner *et al.* (2020) found that in species of the genus *Calopteryx*, wing pigmentation can affect thermoregulation, whereas Outomuro & Ocharan (2011) found no evidence of this interaction. Moreover, Rivas *et al.* (2016) studied *Hetaerina* damselflies and found no relationship between wing pigmentation and thermoregulation. Given that odonate species display different thermal tolerances between southern and northern latitudes (Svensson & Waller, 2013; Hassall, 2014; Zeuss *et al.*, 2014), it seems possible that ambient temperatures explain, at least in part, why wing pigmentation affects thermoregulation of certain species but not others. Ambient temperatures are generally higher at latitudes closer to the equator (*Hetaerina* species; Rivas *et al.*, 2016), meaning that the effect of direct solar radiation is lower regarding the interplay between wing pigmentation and thermoregulation. In addition, methodological differences might explain some of the differences between these studies.

In ectothermic insects, body size has been shown to affect thermoregulation (Digby, 1955; May, 1976; Willmer & Unwin, 1981; Heinrich, 1996). Body size also seems to be linked with odonate thermoregulation (Samejima & Tsubaki, 2010) and with the minimum body temperature required for flying (May, 1976); also, owing to their greater mass, larger individuals and species take longer to heat up. However, this association between body size and thermoregulation has not always been found; for example, in the damselfly species *Enallagma cyathigerum* (Bots *et al.*, 2008).

The aim of this study was to investigate the relationship between thermoregulation, wing pigmentation and body size in damselflies. We used the damselfly *Calopteryx splendens*, because it is an ideal study object. This species has large phenotypic variation in their body size and wing pigmentation (wing spot) attributable, in part, to interspecific interactions with *Calopteryx virgo* (Tynkkynen *et al.*, 2004). Both species often live in sympatry, have similar ecology and habitat requirements and often engage in territorial battles that arise from similar male appearances. In these fights, *C. virgo* is often victorious owing to its larger body size (Tynkkynen *et al.*, 2004). The interspecific aggression is more likely when the *C. splendens* male has larger wing spots (Tynkkynen *et al.*, 2004), which causes negative directional selection pressure against wing spot size (Tynkkynen *et al.*, 2005). Although interspecific aggression decreases wing spot size, large wing spots still exist in the *C. splendens* populations (Tynkkynen *et al.*, 2004, 2005, 2006). This implies a positive directional selection towards larger wing spots, possibly attributable to faster immune defence against parasites caused by higher body temperatures (see also Rantala *et al.*, 2000; Siva-Jothy, 2000).

We examined the effect of wing pigmentation and body size on the time taken for solar radiation to increase the body temperature of a cooled damselfly to a temperature high enough to allow activity. In this experiment, we used *C. splendens* males, because they have dark melanin-based spots in the middle of their translucent wings (Askew, 2004). Also, their body size and wing pigmentation show a high degree of variation between individuals and populations (Tynkkynen *et al.*, 2004).

We made two predictions. First, because more pigmented males occur at higher latitudes and elevations (Svensson & Waller, 2013; Hassall, 2014; Zeuss *et al.*, 2014), we predicted that *C. splendens* males that have more pigmented wings would activate faster than males with less wing pigmentation. Second, we predicted that smaller *C. splendens* males would warm up faster than larger ones, because a larger body requires more energy to warm up (Guggenheim, 1985).

MATERIAL AND METHODS

FIELD STUDIES

We chose two populations and three study years, because we wanted to have more phenotypic variability between males in wing length and wing spot size (Table 1). We captured mature *C. splendens* males from two different populations in southwestern

Table 1. Mean (and SD) of wing length (in millimetres), spot size (in millimetres), relative spot size (as a percentage) and activation time (in seconds) in two populations, Mietoinen and Tarvasjoki, during the three study years (2010, 2011 and 2012); sample size is indicated as *N*

	Wing length		Spot size		Relative spot size		Activation time		*N*
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Mietoinen									
2010	29.0	0.9	15.4	1.2	53.3	4.8	154.7	98.0	16
2011	29.7	0.6	15.5	1.5	52.2	5.1	205.0	96.8	14
2012	29.9	0.8	13.8	1.7	46.2	6.0	290.5	99.1	35
Tarvasjoki									
2010	29.3	1.1	14.1	1.4	48.1	4.7	146.9	55.0	13
2011	29.1	1.2	15.8	1.4	54.3	5.9	197.7	59.3	7
2012	29.7	0.9	14.6	1.7	49.1	5.5	279.1	80.0	23
Total	29.6	0.9	14.6	1.7	49.4	6.0	233.6	104.9	108

Finland: Mietoinen (60°37′N, 21°55′E) and Tarvasjoki (60°34′N, 22°43′E). Both study sites were open small creeks (~2 m wide), with an abundant aquatic macrophyte vegetation and a gravel/sand bed. Study sites were densely populated with *C. splendens* and a few *C. virgo*. Both study sites were relatively isolated, because the rivers immediately up- and downstream provided no suitable habitats for *C. splendens*. The operational sex ratio was highly male biased in both creeks (see also Suhonen *et al.*, 2008).

In total, we collected 108 *C. splendens* males over three summers (2010–2012) between early June and early August (Table 1; Supporting Information, Table S1). Individuals were captured using an entomological net from a population during warm and sunny days. Within a few hours, they were transported to a laboratory, experimented on, marked and released back to their original population no later than the following day. To reduce individual differences between their natural body temperatures, each damselfly was transported in an 0.5 L plastic jar with a wet paper tissue that was placed in a polystyrene cooler with ice packs. In the laboratory, the plastic jars were put in a cooling room (10 °C), where they were kept for 1 h to keep the body temperatures low before the experiment, to calm the individuals down and to mitigate environmental causes of differences in body temperatures.

LABORATORY WORK

Measurements were made in a temperature-controlled (15 °C) room (6.0 m long, 5.0 m wide and 2.5 m high) without any windows. We used a white Styrofoam box (0.22 m long, 0.35 m wide and 0.32 m high) as an experimental chamber (Supporting Information, Fig. S1). We chose a white Styrofoam box as the chamber, because we wanted to direct most of the radiation to the damselfly instead of the walls. One side of the chamber was a window (0.25 m wide and 0.18 m high), through which we observed the behaviour of the study specimen (see also Bots *et al.*, 2008). The chamber was not covered. The chamber was placed under a light source (a lamp) in a dark, temperature-controlled room. We used a sodium lamp (IDMAN, 681 20 SON-T-400, 1 × SON-T 400 W, 230 W, IP 23) as a light source radiating a spectrum that corresponds to the spectrum of natural sunlight. The lamp was 45 cm above each study specimen.

Specimens were placed in the experimental chamber with four ice bricks to cool the inside temperature of the chamber to 3–4 °C. We started our heating experiment by making a hole in a plastic test tube and placing the tip of an insect thermal probe (a copper-constant thermocouple Type MT-29/1B with time constant 0.015 s mounted in a 29-gauge hypothermic needle; Physitemp Instruments, Clifton, NJ, USA) through the hole. A damselfly was then placed on the test tube so that the tip of the thermal probe was touching the surface of the thorax of the insect on the ventral side to prevent direct exposure to the light (Supporting Information, Fig. S2). This location was also appropriate because insect flight muscles are in the thorax (Dudley, 2000; but see Bots *et al.*, 2008). The damselflies were bound to the test tube by their abdomen and their prothorax using a sewing thread. The body temperature of the damselfly was then allowed to drop to 3–4 °C. Next, the ice bricks were removed from the box and the body temperature of the damselfly was allowed to rise to 5 °C. While continually measuring the body temperature, we started to measure the time to the point when the damselfly started to flap its wings [hereafter, activation time (in seconds)]. Each individual was experimented on only once. Hindwing length was used as a proxy for individual size because it is a reliable measure of fresh body mass in damselflies (Koskimäki *et al.*, 2009; Aromaa *et al.*, 2019). Wing spot size (in millimetres) was used as a proxy for wing pigmentation because it is a reliable measure for relative darkness in *Calopteryx* species (J. Suhonen, unpublished data), it is a measure often used in studies (Tynkkynen *et al.*, 2004, 2005, 2006) and it has been shown to influence both intra- and interspecies behaviour (Tynkkynen *et al.*, 2004, 2005, 2006; Rantala *et al.*, 2010, 2011). The lengths of both the wing and the wing spot were measured with Mitutoyo digital callipers to the nearest ±0.01 mm. After wing measurements, we marked damselflies with a silver marker (Edding 780) to prevent measuring of the same individual twice. Then, we placed each male back into the 0.5 L plastic jar with a wet paper tissue and freed them back to their original population.

STATISTICAL ANALYSES

We used Pearson's correlation coefficient to test for an association between wing length and wing spot size (see also Hardersen, 2010). We used ANOVA to test differences in wing length and wing spot size between years (fixed factor) and between populations (fixed factor) in addition to their interaction to observe any potential yearly and/or population effects that might affect our results. Relative wing pigmentation (as a percentage; hereafter, RWP) was calculated using the following formula:

$$\mathrm{RWP}\ (\%) = 100 \times \text{wing spot length}/\text{wing length}.$$

We tested how RWP affects the activation time in *C. splendens* males using regression analyses. Activation time was the dependent variable, and RWP was the independent variable.

Effects of body size and wing spot size on the activation time of a damselfly individual were analysed using general linear models (GLMs). The dependent variable was the time taken for the damselfly to start flapping its wings, i.e. for it to become active. The explanatory factors were population and year, and continuous variables were wing length and wing spot size. All two-way interactions were also added to the model. We added an interaction term in the statistical models between wing length and wing spot size to determine whether there was an antagonistic or a synergistic interaction between variables. The activation time did not differ from a Gaussian (normal) distribution (one-sample Kolmogorov–Smirnov normal test, $z = 0.076$, $P = 0.147$); therefore, the assumption of a GLM was filled.

We used linear regression analyses to calculate the slope and intercept for each population and each study year. The dependent variable was the activation time (in seconds) from commencement of the heating experiment.

The Akaike information criterion (AIC) was used to compare the four possible models, where the one with the smallest AIC is the best model, based on the Kullback–Leibler distance (Burnham & Anderson, 2000). We used GLMs to estimate the AIC values in IBM SPSS STATISTICS v.26. This approach works well for detecting differences between models when values for $\Delta\mathrm{AIC}_i = (\mathrm{AIC}_i - \mathrm{AIC}_{min})$ are higher than seven (Anderson *et al.*, 2000; Burnham & Anderson, 2000; Burnham *et al.*, 2011). Statistical tests were calculated using the function lm in the 'stats' package of RSTUDIO v.1.2.5042 and IBM SPSS STATISTICS v.26.

RESULTS

In total, we analysed and used 108 *C. splendens* males in our experiments (Supporting Information, Fig. S1 and S2). The average wing length was 29.6 mm (SD = 0.9 mm; range, 26.8–31.4 mm; $N = 108$) and wing spot size was 14.6 mm (SD = 1.7 mm; range, 10.0–18.1 mm; $N = 108$). The RWP was 49.4% on average (range, 32.9–62.1%). Wing spot size was not correlated with wing length ($r = -0.093$, $N = 108$, $P = 0.341$). On average, *C. splendens* males became active in 234 s (SD = 105 s; range, 5.1–465.9 s; $N = 108$).

There were no differences in wing length between years (GLMs, $F_{2,2} = 3.71$, $P = 0.212$) or between populations ($F_{1,2.28} = 0.46$, $P = 0.558$). Moreover, there was no interaction between populations and study years ($F_{2,102} = 1.37$, $P = 0.259$). There were no differences in wing spot size between years (GLMs, $F_{2,2} = 1.41$, $P = 0.415$) or between populations ($F_{2,2.08} = 0.02$, $P = 0.904$). However, wing spot size was affected by the interaction between population and study year ($F_{2,102} = 4.47$, $P = 0.014$). There was phenotypic variation in wing spot size between populations and years (Table 1).

There was a negative association between high RWP and the activation time in *C. splendens* males ($F_{1,106} = 11.41$, $P = 0.001$; Fig. 1). The wing spot size, the wing length and their interaction influenced the activation time of *C. splendens* males (Table 2; Fig. 2). Population, year or their interaction did not affect the activation time (Table 2). Males with large wing spots activated faster than males with small wing spots (Table 2; Fig. 2A). Males with short wings (i.e. smaller individuals) activated faster than males with long wings (Table 2; Fig. 2B).

The best model (the smallest AIC) included wing spot size, wing length and their interaction as variables (Table 3). The interaction term between wing length and wing spot size was positive (Table 3), indicating a synergistic effect between variables. In

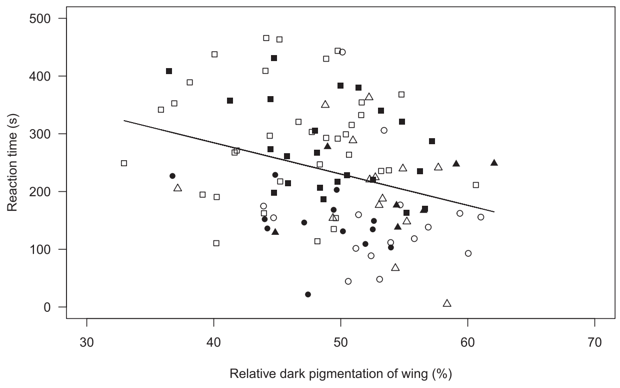

Figure 1. Effects of relative wing pigmentation (RWP) on activation time in *Calopteryx splendens* males in the laboratory experiment. Activation time (in seconds) = 501.3 (SE = 79.3) – 5.4 (1.6) × RWP (%), r^2 = 0.097. Open symbols indicate the Mietoinen population; filled symbols indicate the Tarvasjoki population. Study years are indicated as circles (2010), triangles (2011) and squares (2012).

Table 2. Thermoregulation of *Calopteryx splendens* males

Source	Wald	d.f.	*P*-value
Intercept	8.09	1	0.004
Population	0.75	1	0.387
Year	0.79	2	0.674
Wing	7.05	1	0.006
Spot	8.28	1	0.004
Population × year	1.56	2	0.458
Population × wing	0.83	1	0.361
Population × spot	0.03	1	0.870
Year × wing	0.11	2	0.947
Year × spot	2.02	2	0.364
Wing × spot	8.05	1	0.005

The dependent variable is the activation time of a male (in seconds). Explanatory factors are population (Mietoinen and Tarvasjoki) and year (2010, 2011 and 2012). The continuous explanatory variables are the wing length (in millimetres), the wing spot size (in millimetres) and the interaction between these two. The statistical model includes all main explanatory variables and their two-way interactions. The data were analysed in a generalized linear model with identity link. Statistically significant variables are in bold.

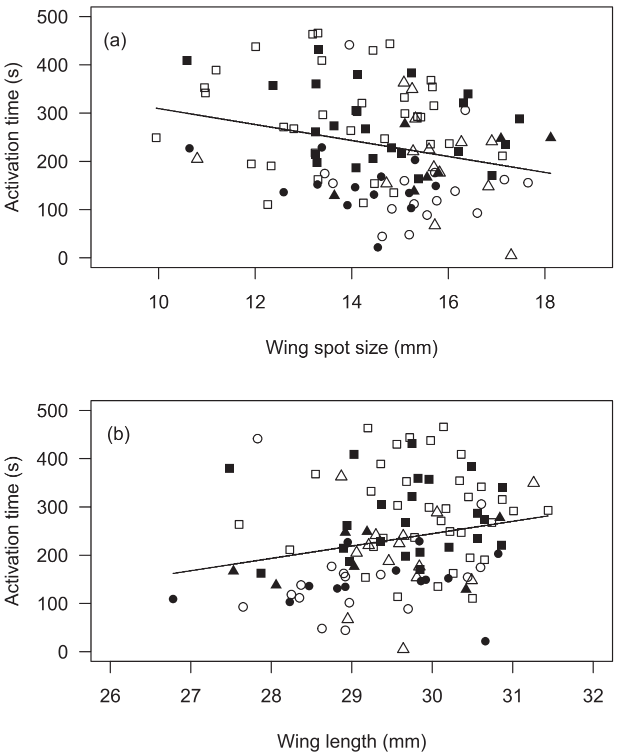

Figure 2. A, the effect of wing spot size (in millimetres) on observed activation time (in seconds) of *Calopteryx splendens* males. Activation time (in seconds) = 474.7 (SE = 85.9) – 16.5 (5.8) × wing spot size (in millimetres), r^2 = 0.070. B, the effect of wing length (in millimetres) on observed activation time (in seconds) of *C. splendens* males. Activation time (in seconds) = –523.4 (SE = 320.0) + 25.6 (10.8) × wing length (in millimetres), r^2 = 0.050. Open symbols indicate the Mietoinen population; filled symbols indicate the Tarvasjoki population. Study years are indicated as circles (2010), triangles (2011) and squares (2012).

this model, all estimated parameters were statistically non-significant (Table 3). The second model, without the interaction term, fitted almost as well, and the estimated parameters were statistically significant (Table 1). Two other models (wing spot size and wing length) fitted less well (Table 3).

When regression analysis between the activation time and wing spot size was calculated separately in different populations and different years, we found that in five out of six cases, wing spot size affected the activation time of the damselflies (Table 4). Likewise, in five out of six cases, smaller males warmed up and activated faster than larger ones (Table 4).

DISCUSSION

In this study, we examined the relationships of relative wing pigmentation, wing spot size and wing length with the activation time of male damselflies, and the results can be summarized in five main findings. First, males with relatively more pigmented wings activated faster than less pigmented ones. Second, individuals with larger wing spots activated faster than males with smaller wing spots. Third, male activation time increased with increasing wing length. Fourth, the best-fitting model according to AIC values included the wing spot size, wing length and their interaction.

Table 3. Thermoregulation of *Calopteryx splendens* males

Model	Variable									
	Intercept	Wing	Spot	Wing × spot	Statistical test				AIC	
	Estimate	Estimate	Estimate	Estimate	d.f.$_1$	d.f.$_2$	*F*	*P*-value	AIC	ΔAIC
Wing + spot + wing × spot	5913.3	−184.1	−429.8	14.0	3	104	5.61	0.001	1304.4	0.0
Wing + spot	−222.2	**23.0**	**−15.4**	–	2	105	6.51	0.002	1306.0	1.6
Spot	**474.7**	–	**−16.5**	–	1	106	8.00	0.006	1308.7	4.3
Wing	−523.4	**25.6**	–	–	1	106	5.60	0.020	1311.0	6.6

The dependent variable is the activation time of a male (in seconds). Explanatory variables are wing length (in millimetres), wing spot size (in millimetres) and the interaction between wing length and wing spot size. Data were analysed with a general linear model. Statistically significant variables are in bold. Abbreviation: AIC, Akaike information criterion.

Fifth, wing spot size did not increase with increasing wing length.

According to our results, damselflies with relatively darker wings had significantly faster activation times than those with relatively less pigmented wings. This was confirmed by our regression analyses that used different populations and years. This is in accordance with a previously published study of *C. splendens* in southern Sweden (Svensson & Waller, 2013) and two sympatric *Calopteryx* species from North America (Schreiner *et al.*, 2020), in which a link was found between wing pigmentation and thermoregulation. This also seems a logical finding, given that larger dark patches on the wing absorb more sunlight. Similar results have been found using a ladybird species (*Adalia bipunctata*; de Jong *et al.*, 1996) and wood tiger moths (*Parasemia plantaginis*; Hegna *et al.*, 2013). Surprisingly, however, Svensson & Waller (2013) found that the *C. virgo* with more wing melanization became cooler as ambient temperature increased. Also, Outomuro & Ocharan (2011) found, when using two *Calopteryx* species, no connection between the wing spot size of individuals and heat gain. Rivas *et al.* (2016) manipulated wing spot sizes, but the size of these spots did not affect the internal temperature of individuals of the two *Hetaerina* species examined. Bots *et al.* (2008), in contrast, examined the effect of body mass and body coloration on heating rate and activation time, but no significant effect was found. Moreover, it was recently found (Guillermo-Ferreira & Gorb, 2021) that at least one odonate species (*Zenithoptera lanei*) can use the dorsal side of the wings as a cooling system and the ventral side as a heating system. Our results provide more clues for understanding how wing pigmentation affects thermoregulation of *Calopteryx* species, but highlight that a universally applicable way of testing insect temperature changes is still lacking. We continue to improve our methods and encourage other researchers to do the same.

Alternatively, large wing spot size might be linked to a better body condition of the *C. splendens* males, which might explain why males with larger wing spots activate faster. It has been shown that males with larger wing spots have a higher immunocompetence than small-spotted males, indicating better physiological condition (Rantala *et al.*, 2000; Siva-Jothy, 2000). Therefore, faster activation might be the result of better fitness rather than increased solar absorption owing to larger wing spots. However, better condition does not explain the geographical variation in wing spot size within *Calopteryx* species (Hassall, 2014) or between species (Svensson & Waller, 2013). Interestingly, these are not mutually exclusive explanations, begging for more research to gain a full understanding of this immunocompetence/thermoregulation situation.

The positive effect of an increasing wing spot size on the activation of an ectothermic insect seems logical and easy to understand: a dark wing spot absorbs sunlight more efficiently than a translucent part of the wing; hence, a larger wing spot increases the heating ability of an individual more efficiently than a smaller wing spot. In *Calopteryx* species, these dark wing spots also serve as secondary sexual characters, and they play a particularly important role in intra- and interspecific interactions. *Calopteryx* species are territorial, such that males acquire high-quality breeding areas and fight over these areas against other males in order to increase their chances of copulation (Plaistow & Siva-Jothy, 1996; Serrano-Meneses *et al.*, 2007). High-quality males are the ones that can obtain the best territories and are more likely to reproduce, and wing spot size is a likely determinant of these high-quality males (Rantala *et al.*, 2010). It seems that individuals with larger wing spots activated earlier in the field (Fält, 2011), which can lead to longer foraging times and, subsequently, larger fat reserves in males. It has been observed that males with larger wing ornaments have more fat reserves (Anderson *et al.*, 2007; Contreras-Garduño *et al.*, 2008; Córdoba-Aguilar, 2009), which increases their ability to obtain territories (Marden & Waage, 1990; Marden & Rollins, 1994; Koskimäki *et al.*, 2004; Contreras-Garduño *et al.*, 2006). Faster activation in the morning also means that these males are the first ones to arrive in the reproductive areas and obtain territories, improving their chances of winning territorial fights (Waage, 1988). Therefore, it is not surprising that calopterygid males with larger wing spots are able to obtain higher-quality territories (Grether, 1996; Rantala *et al.*, 2010), win more fights (Contreras-Garduño *et al.*, 2008), are favoured by females (Siva-Jothy, 1999) and acquire more copulations (Grether, 1996; Córdoba-Aguilar, 2002, 2009). Faster activation owing to larger wing spots might also help insect individuals physiologically; for example, through increased metabolism. Studies have reported that larger wing spots are associated with increased immune responses (Rantala *et al.*, 2000; Contreras-Garduño *et al.*, 2006; Anderson *et al.*, 2007; Córdoba-Aguilar *et al.*, 2009) and lower rates of parasitism (Siva-Jothy, 2000; Anderson *et al.*, 2007), but the results on the effect of wing spot size on immune defense are conflicting (González-Santoyo *et al.*, 2010; González-Tokman *et al.*, 2010).

Larger wing spots might also help individuals to maintain higher body temperatures. This might benefit them in evasion of avian predators (Svensson & Friberg, 2007; Rantala *et al.*, 2010, 2011) or enable them to capture prey more efficiently and build up larger fat reserves. Given the apparent benefits of having larger wing spots, it would seem likely that wing spot size increases generation after generation, as predicted by the sexual selection theory. However, there might be significant costs in having larger wing spots that might stabilize the evolution of wing spot size. Interspecific aggression often occurs in sympatric populations, because large-spotted *C. splendens* males resemble *C. virgo* males; therefore, *C. virgo* males attack them (Tynkkynen *et al.*, 2004). This causes agonistic character displacement (Grether *et al.*, 2009), as often observed in calopterygids (Anderson & Grether, 2010), and in *C. splendens* this causes negative selection on wing spot size (Tynkkynen *et al.*, 2004, 2005, 2006; Honkavaara *et al.*, 2011; but see Kuitunen *et al.*, 2011). In addition, larger wing spots might increase the visibility of individuals, thus increasing avian predation (Svensson & Friberg, 2007; Rantala *et al.*, 2010, 2011) and reducing prey capture rates (Grether & Grey, 1996) and subsequent fat accumulation.

It seems that *C. splendens* males with larger wings require more time to activate compared with males having smaller wings. This is in accordance with our previous findings, where smaller individuals activated earlier in the field than larger individuals (Fält, 2011). However, this relationship was not found in

Table 4. Results from linear regression analyses from each population and each study year

Population	Spot						Wing					
	Intercept	Slope	r^2	*F*	d.f.$_1$,d.f.$_2$	*P*-value	Intercept	Slope	r^2	*F*	d.f.$_1$,d.f.$_2$	*P*-value
Mietoinen												
2010	339.9	−12.0	0.021	0.31	1,14	0.588	−47.7	7.0	0.004	0.06	1,14	0.810
2011	446.6	−15.6	0.062	0.79	1,12	0.392	−702.6	30.6	0.041	0.51	1,12	0.488
2012	347.9	−4.2	0.005	0.16	1,33	0.688	451.3	−5.4	0.002	0.06	1,33	0.807
Tarvasjoki												
2010	369.5	−15.8	0.156	2,04	1,11	0.182	−63.2	7.2	0.021	0.23	1,11	0.638
2011	−197.3	25.0	0.371	2.94	1,5	0.147	−345.6	18.6	0.137	0.80	1,5	0.413
2012	535.0	−17.6	0.134	3,26	1,21	0.085	146.0	4.5	0.02	0.05	1,21	0.825

The dependent variable was activation time and the independent variable was wing spot size or wing length.

another damselfly species, *Enallagma cyathigerum* (Bots *et al.*, 2008). Given that wing length is a good indicator of body mass in odonates (Koskimäki *et al.*, 2009; Aromaa *et al.*, 2019), our results mean that it takes a longer time for larger odonates to heat up to the point of activation. Large body mass is a likely explanation for the reliance of the largest odonates on 'wing-whirring' to elevate their body temperatures endothermically (May, 1976; Stoks *et al.*, 1996; Corbet & May, 2008; Worthen, 2016). It would take these large odonate species too long to heat up, for example after a cool morning, using only sunlight radiation. Larger body size, especially in females, is often considered advantageous in reproduction ('fecundity advantage hypothesis', Darwin, 1896; Shine, 1989; Cox *et al.*, 2003), and it is positively associated with territory size in odonates (Aromaa *et al.*, 2019). However, a slower activation time probably functions as a stabilizing factor to limit the body size. It has also been linked to a higher risk of extinction (Suárez-Tovar *et al.*, 2019; Rocha-Ortega *et al.*, 2020), further decreasing gradual size increase over time.

We found that smaller individuals, in contrast, activate faster. Although the males in calopterygid species have been shown to benefit from a larger body size in territorial contests (Serrano-Meneses *et al.*, 2007; Suhonen *et al.*, 2008; Córdoba-Aguilar, 2009; Córdoba-Aguilar *et al.*, 2009; Koskimäki *et al.*, 2009), there are studies that indicate equal opportunities regardless of male body size (Córdoba-Aguilar, 1995; Lefevre & Muehter, 2004; Ramírez-Delgado *et al.*, 2015). These findings also mean that small males with pigmented wings are the first ones to activate, increasing their competitiveness, at least for a while, against males with larger bodies. The smaller body size and earlier activation might allow these individuals to operate successfully using alternative reproductive tactics, such as the sneaker or wanderer tactics (Córdoba-Aguilar, 1995; Plaistow, 1997; Candolin & Voigt, 2003; Serrano-Meneses *et al.*, 2007; Suhonen *et al.*, 2008; Koskimäki *et al.*, 2009), therefore maintaining body size variation within populations.

According to our data, wing spot size was not related to wing length. This means that there are individuals with large wing spots but short wings and individuals with small wing spots but long wings. This is in contrast to previous studies on calopterygids (Córdoba-Aguilar *et al.*, 2009; Rodríguez-Escobar *et al.*, 2019), where wing spot size was positively related to wing length.

The fact that larger wing spots might speed up damselfly activation offers further insight into the distribution of insect species, their dispersal ability and the impact of rising global temperatures on this animal group. It seems that in *Calopteryx* species, more pigmented species and individuals within a species increase towards northern latitudes (Outomuro & Ocharan, 2011; Svensson & Waller, 2013; Hassall, 2014), a pigmentation pattern that is also observed in damselfly larvae (Van Dievel *et al.*, 2019), in dragonflies in general (Zeuss *et al.*, 2014; Pinkert *et al.*, 2016; Rocha-Ortega *et al.*, 2020) and in other insect groups (Hegna *et al.*, 2013; Zeuss *et al.*, 2014; Bishop *et al.*, 2016; Heidrich *et al.*, 2018). Based on our results and the general pattern described above, it appears that relatively darker pigmentation of individuals is beneficial in colder and higher latitudes and higher-elevation environments, owing to the heat-absorbing effect. On the contrary, it might cause overheating at lower latitudes (Sunday *et al.*, 2014), further enhancing the geographical shift of more pigmented individuals towards northern latitudes. Insects have evolved to adapt to temperature fluctuations and peak temperatures, and their pigmentation is tightly linked to thermal tolerance (Sunday *et al.*, 2014; Gunderson & Stillman, 2015; MacLean *et al.*, 2016; Van Dievel *et al.*, 2019). Rising global temperatures might affect the wing pigmentation pattern of *C. splendens* and, subsequently, the distribution of the species and the relationship with the often sympatric *C. virgo* species. A recent study in North America found that greater extinction risk is linked to narrow thermal limits and larger body size in odonates (Rocha-Ortega *et al.*, 2020). Moreover, they found that southern damselfly and dragonfly species were more climate tolerant than northern species. They concluded that subboreal species need to be protected, because they are not able to tolerate climate change as well as those in warmer areas (Rocha-Ortega *et al.*, 2020).

Previous studies used a proven thermal probe (Bots *et al.* 2008), thermal cameras (Tsubaki *et al.*, 2010; Svensson & Waller, 2013) and measured temperatures from removed wings (Outomuro & Ocharan, 2011). However, comparing these results with each other is difficult owing to their methodological differences. Furthermore, it has been suggested that damselfly wings are too thin to store heat and that their veins might be too narrow to enable efficient circulation and heat transfer to the thorax (Tsubaki *et al.*, 2010; but see Guillermo-Ferreira & Gorb, 2021). It seems clear that studying insect thermoregulation in the future requires more understanding of insect wing physiology and whether wings can be used to heat the thorax. In addition, we suggest that future studies: (1) use thermal imaging simultaneously in the field and in a controlled environment; (2) photograph entire specimens (wings, thoraxes and heads) of study individuals in order that further analysis can determine, for example, their melanin levels and distributions; and (3) measure thermoregulation of different species and individuals.

To conclude, our results emphasize the role of wing pigmentation and body size in insect thermoregulation and how quickly individuals can become active after cooling down. However, the negative effect of larger size on activation suggests a trade-off situation between size and activation speed. Our results also highlight the interesting cost-and-benefit situation in male *Calopteryx* damselflies and the effects that dark and large wing spot size might have on their reproduction, territorial contests, species coexistence, avian predation and their ability to cope with increasing global temperatures.

ACKNOWLEDGEMENTS

We would like to thank Suomen Hyönteistieteellinen Seura (L.K.L.), Suomen Biologian Seura Vanamo (L.K.L.), TOP-säätiö (L.K.L.) and the Academy of Finland (J.S.) for funding our research. We would also like to thank Talvikki Suhonen for helping us with the laboratory experiment, Juha Ojanperä for reading our text and checking the spelling and grammar, and the anonymous reviewers who helped us to make this article better.

DATA AVAILABILITY

The data underlying this research work are available in the Supporting Information (Table S1) and in Dryad data repository (https://doi.org/10.5061/dryad.4mw6m909v; Laakso *et al.*, 2021). The data are also available directly from the authors on reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Activation times and wing data for the 108 specimens of *Calopteryx splendens*.
Figure S1. Laboratory equipment.
Figure S2. *Calopteryx virgo* male cooling down in the chamber.