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

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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 contrast, 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 sympathy, 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 wing spots, possibly attributable to faster immune defence against parasites caused by higher body temperatures (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
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

| 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) |
| 2011        | 29.7 (0.6) | 15.5 (1.5)         | 52.2 (5.1)      | 205.0 (96.8) |
| 2012        | 29.9 (0.8) | 13.8 (1.7)         | 46.2 (6.0)      | 290.5 (99.1) |
| Tarvasjoki  |           |                    |                 |   |
| 2010        | 29.3 (1.1) | 14.1 (1.4)         | 48.1 (4.7)      | 146.9 (55.0) |
| 2011        | 29.1 (1.2) | 15.8 (1.4)         | 54.3 (5.9)      | 197.7 (59.3) |
| 2012        | 29.7 (0.9) | 14.6 (1.7)         | 49.1 (5.5)      | 279.1 (80.0) |
| Total       | 29.6 (0.9) | 14.6 (1.7)         | 49.4 (6.0)      | 233.6 (104.9) |
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; 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:

\[
\text{RWP (\%)} = 100 \times \frac{\text{wing spot length}}{\text{wing length}}.
\]

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 \( \Delta \text{AIC} = (\text{AIC}_i - \text{AIC}_{\text{min}}) \) 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, 51.4–465.9 s; \( N = 108 \)).

There were no differences in wing length between years (GLMs, \( F_{2,106} = 3.71, P = 0.212 \)) or between populations (\( F_{2,106} = 0.46, P = 0.658 \)). Moreover, there was no interaction between populations and study years (\( F_{2,106} = 1.37, P = 0.259 \)). There were no differences in wing spot size between years (GLMs, \( F_{2,106} = 1.41, P = 0.415 \)) or between populations (\( F_{2,106} = 0.02, P = 0.904 \)). However, wing spot size was affected by the interaction between population and study year (\( F_{2,106} = 4.47, P = 0.014 \)). 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 (\( F_{1,106} = 11.41, P = 0.001 \; \text{Fig. 1} \)). 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

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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.
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 *Calopteryx* in southern Sweden (Svensson & Waller, 2013) and two sympatric *C*alopteryx 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 sunlight more efficiently. 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 laneti*) 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 intraspecific 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...
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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

| Population | Spot | Intercept | Slope | f | p-value | f | p-value |
|------------|------|-----------|-------|---|---------|---|---------|
| Mietoinen  | 2010 | 339.9     | −12.0 | 0.021 | 0.31 | 0.021 | 0.31 |
|            | 2011 | 446.6     | −15.6 | 0.062 | 0.005 | 0.005 | 0.005 |
|            | 2012 | 347.9     | −4.2  | 1.12  | 0.005 | 1.12  | 0.005 |
| Turvasjoki | 2010 | 369.5     | −15.8 | 0.056 | 2.04  | 2.04  | 2.04  |
|            | 2011 | −107.3    | −17.6 | 0.062 | 0.005 | 0.005 | 0.005 |
|            | 2012 | 535.0     | −17.6 | 0.062 | 0.005 | 0.005 | 0.005 |

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

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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. Larger 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 calopterygids 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 & Muehler, 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 competitively, at least for a while, against males with larger 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.

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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.