TITLE PAGE

Title:

Flight behaviour diverges more between seasonal forms than between species in *Pieris* butterflies

Running title: seasonal divergence in flight behaviour

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Abstract

In flying animals, wing morphology jointly evolves with flight behaviours. Whether seasonal polymorphism in butterfly wing morphology is linked to divergent flight behaviour is however not known. Here we compared the flight behaviour and the morphology between the spring and the summer forms of two closely related, temperate species, *Pieris napi* and *P. rapae*. We first quantify three-dimensional flight behaviour by reconstructing individual flight trajectories using stereoscopic high-speed videography in an experimental cage. We then measure wing size and shape characteristics assumed to influence flight behaviours in butterflies. We show that seasonal, but not interspecific, differences in flight behaviour might be associated with divergent forewing shapes, either elongated or rounded wings. In *Pieris* spring forms, individuals are small and have elongated forewings, which are significantly correlated with low speed and acceleration, but with high flight curvature. Large *Pieris* summer individuals exhibit rounded forewings, which are correlated with high flight speed, turning acceleration, and advance ratio. We interpret such results as potentially adaptive flight behaviours in *Pieris*. Curvy flight trajectories in the spring forms may be a specialised foraging behaviour evolving in response to newly emerging feeding and oviposition resources. In contrast, the summer forms' high flight speed and flight efficiency may be advantageous for dispersal to new habitats. Further, the recorded higher acceleration capacities of the summer form could have evolved in response to higher predation pressure. Our study provides quantitative evidence of divergent flight behaviours between seasonal forms of *Pieris* butterflies correlated with differences in wing morphology. Seasonal flight behaviour could be adaptive, possibly driven by the interaction of various seasonal selection pressures, including resource distribution (nectar and larval food plants), predator and parasitoid pressure, and thermoregulation.

Introduction

Seasonal changes in the environment induce in some species the development of polymorphic forms exhibiting traits adapted to specific ecological conditions (Xue & Leibler, 2018). Such seasonal phenotypes are typically influenced by shifts in temperature (Nijhout, 2003), species interactions, such as the availability of feeding and breeding resources (Brakefield & Reitsma, 1991) or predators' abundance (Brakefield & Reitsma, 1991; Lyytinen et al., 2004). These adaptations are most common in regions with strong seasonality, where animals need to adjust their foraging, breeding, or dispersal strategies for a few months in the year.

For flying insects living in temperate environments, wing shape and colouration are key polyphenic traits. Polyphenism of insect wings has been shown to play roles in thermoregulation (Kingsolver, 1985), inter-specific signalling (Lyytinen et al., 2004) and powered flight (Harrison, 1980). While flight is a primary function of insect wings, little is known about its relation with wing morphology in the context of seasonal polyphenism. Adaptive changes in flight behaviour can be driven by various ecological including microhabitat use (Dell'Aglio et al., 2022; DeVries et al., 2010), mate-locating strategies (Berwaerts et al., 2002) or locomotor mimicry (Srygley, 1997, 1999). To our knowledge, however, possible divergence in flight behaviour between seasonal forms has never been investigated. Here we examine whether seasonal variation in wing morphology (size or shape) have jointly evolved with divergent flight behaviour.

Differences in wing morphology are often associated with contrasted flight behaviours. For instance, wing loading being positively associated with flight speed (Le Roy et al., 2019). However, our understanding of which specific wing traits influence distinct flight behaviours, including agility and manoeuvrability, remains unclear. Seasonal flight behaviours may be adaptive as, for example, increased manoeuvrability seems to be advantageous when foraging for newly emerging feeding resources (Evans et al., 2020). Furthermore, increasing wing area might enhance dispersal ability, as quantified in migrating generations vs. sedentary populations of *Danaus* butterflies (Tenger-Trolander et al., 2023). Behavioural variation can also influence flight patterns in seasonal butterflies, as the preference in flight direction of migrating populations of *Pieris brassicae* seems to depend on the season of emergence (Spieth & Cordes, 2012). Thus, in the case of polyvoltine seasonal butterflies, the factors modifying intra-specific flight behaviours

likely involve both morphological and behavioural adaptations (Spieth & Cordes, 2012; Tenger-Trolander et al., 2023).

To assess whether seasonal changes in the environment affect the evolution of morphology and flight behaviour, we study two polyvoltine butterfly species, *Pieris napi* (Linnaeus, 1758) and *P. rapae* (Linnaeus, 1758) (Pieridae: Pierini). These two species are closely-related (Okamura et al., 2019), co-occur in Europe and are ecologically very similar (Tenger-Trolander et al., 2023). They are known to differ in wing size and shape in spring and summer (Fric et al., 2006; Shkurikhin & Oslina, 2016). It is thought that the spring forms of both species have a more manoeuvrable flight due to their elongated and pointed forewings (Shkurikhin & Oslina, 2016), whereas the summer forms might have improved dispersal capacity due to their larger wings and lower wing loading (Fric et al., 2006; Shkurikhin & Oslina, 2016). Furthermore, variation in habitat use is expected among both species and seasonal forms (Friberg & Wiklund, 2019). While *Pieris napi* usually flies in habitats such as forest edges and feeds on biennial and perennial plants, *P. rapae* explores temporary and degraded habitats with ephemeral plants (Ohsaki & Sato, 1999), including agricultural landscapes (Ryan et al., 2019). Seasonal differences in population dynamics driven by climate have also been reported (Okamura et al., 2019; von Schmalensee et al., 2023), with *Pieris napi* having a higher overwintering survival than *Pieris rapae*, resulting in higher abundances during the spring but lower population growth during the summer (von Schmalensee et al., 2023).

Here we quantified the flight behaviour of spring and summer forms of both *Pieris napi* and *P. rapae*, using a stereoscopic high-speed videography system in a controlled environment. To test if seasonality drives divergent flight behaviour in *Pieris* butterflies, we compared flight characteristics (i) between *Pieris napi* and *P. rapae* and (ii) between seasonal forms in spring and summer. A stronger difference between seasonal forms than between species would point at an effect of contrasted environmental conditions on the evolution of flight behaviour. To the contrary, more divergent flight behaviour between species could suggest flight adaptation related to known habitat preferences between the two *Pieris* species.

Methods

Butterfly flight trajectories recording

The recordings took place from the end of May to mid-August 2020. We caught *Pieris napi* and *P. rapae* during the morning (~8:00 – 11:00) in the surroundings of České Budějovice, Czechia (48°59'36"N 14°26'10"E). *Pieris* individuals captured between May and mid-June represented the spring form, whereas the ones captured during July and August represented the summer form (von Schmalensee et al., 2023). Flight trajectories were filmed between 10:00 and 16:00 in a tunnel-like cage (6 m × 2.5 m × 2.5 m, length × width × height). Films were recorded using two GoPro Hero8 Black cameras (San Mateo, USA) mounted on tripods at a height of 80 cm, and positioned at perpendicular angle to each other, following a similar setup to that of Le Roy et al., (2021). The cameras recorded at 120 frames per second using the wide lens option with a resolution of 2560×1440 pixels. To uncover within-individual variation, we filmed up to 6 flight trajectories per individual, depending on the fatigue of the individual. After the experiments, the butterflies were killed, stored at -20°C, and later spread and mounted for further morphological measurements.

Quantifying flight parameters

To quantify the butterfly flight trajectories in three dimensions (3D), we used the software Argus v2.1 (Jackson et al., 2016) implemented in Python v. 3 (Van Rossum & Drake Jr., 2009). Because of the wide filming option, the coordinate systems from the camera videos were warped. We thus undistorted all videos using the Argus package DWarp. The camera setup was calibrated using the direct linear transformation (DLT) technique (Theriault et al. 2014). For this, we used a 24-cm ruler with Styrofoam balls fixed to both ends, which was moved across the cage. We then tracked the centre of the Styrofoam balls using the Argus package *Clicker*. The butterfly flight trajectories were similarly tracked by digitizing the positions of the butterfly thorax. But given its small size, we were not able to record the positions in certain video frames. We reconstructed the 3D coordinates per frame by merging the two synchronized 2D point trajectories and calibration coefficients using the Argus package *Wand*. The butterfly positions throughout the trajectory were then post-processed using a linear Kalman filter in MATLAB (Muijres et al., 2014) resulting in

smoothed positional coordinates as well as estimated missing positions. Additionally, the total number of wingbeats per butterfly trajectory were counted during the video processing.

The flight behaviour was described using **11** parameters for every butterfly individual:

- As general flight characteristics, we calculated **(1)** wingbeat frequency (average number of wing beats per second, in Hz), **(2)** covered distance (m), which was calculated as the consecutive distance between all tracked 3D positions, **(3)** average flight height (m), **(4)** mean velocity (m/s) and **(5)** mean acceleration (m/s2), calculated as the first and second temporal derivative of butterfly positions through time, respectively.
- As a measure of flight efficiency, we calculated **(6)** mean advance ratio, which describes the flapping efficiency and was calculated as the ratio of mean velocity to the wingbeat frequency (Ellington, 1984).
- To describe individual turning abilities, which encompass manoeuvrability and agility, we calculated **(7)** turning acceleration, which is a component of acceleration strictly attributable to changes in flight direction, and **(8)** turning rate, which describes how quickly the butterfly turns, which was calculated as the angular change in the direction of subsequent velocity vectors in the 3D space.
- To describe the flight trajectory shape, we calculated **(9)** sinuosity, which is the ratio of the straight distance between the starting and ending positions against the covered distance, **(10)** curvature, which is a measure of how sharply the butterfly turns (Jantzen & Eisner, 2008), and **(11)** ascent angle (degrees), calculated as the angle between the velocity vector and the horizontal.

All the calculations were made using a custom-written R script from Le Roy et al., (2021)

Wing morphometrics

All butterflies were mounted and photographed with a millimetre scale, using a Canon EOS250D camera with an 18-55mm EF-S lens (Tokyo, Japan). We measured conventional wing morphometrics (Figure 1) using ImageJ v. 1.53 (Schneider et al., 2012) and, for wing area calculations, we used GIMP (GIMP Development Team, 2019). We measured the left wings for

all calculations; if the left wings were damaged, the right wings were used. For all specimens, we calculated: **(1)** forewing length (cm), which is the distance between the wing base (landmark point 1; Figure 1) and the outer edge of vein R4 (landmark point 13; Figure 1), **(2)** forewing width (cm), which is the highest width perpendicular to the measured forewing length, **(3)** aspect ratio, which characterises the relative elongation and narrowness of the forewings and is calculated by the ratio between forewing length and width, **(4)** thoracic volume (cm^3), which served as proxy to body mass, and was calculated as the cylindrical volume inferred from the thorax length and width, **(5)** total wing area (cm^2), measured as twice the forewing area (dorsal view) plus twice the hindwing area (ventral view), using the histogram function of GIMP, and **(6)** wing loading, being the ratio of thoracic volume (as proxy for mass) and the total wing area.

In addition, we calculated the **(7)** curvature of the forewing outer edge, which is known to differ between the spring and the summer forms (Shkurikhin & Oslina, 2016). It was calculated as the sum of distances around the terminal veins at the outer edge (i.e., R4, R5+M1, M2, M3, Cu1, Cu2 and A2) (landmark points 13, 14, 15, 16, 17, 18 and 19, respectively; Figures 1) divided by the straight distance between veins R4 and A2 (landmark points 13 to 19, respectively; Figure 1). A curvature of the forewing outer edge close to 1 represents the rounded forewings of summer *Pieris*, while an index higher than 1 depicts the elongated forewings of spring *Pieris* (Shkurikhin & Oslina, 2016). We also calculated the **(8)** relative length of the marginal region, measured as the distance between landmark 1 to 9 divided by the straight distance between landmarks 1 to 13; Figure 1). The shape characteristics of one *P. napi* (specimen b220) were not possible to calculate as its wing margins were damaged during handling after the video recordings; the specimen was excluded from the analyses of the effect of wing shape on flight behaviour.

Interspecific and seasonal differences in flight behaviour

We compared differences in flight behaviour between the species (*P. napi* and *P. rapae*), and between their seasonal forms, spring (May to mid-June) and summer (July and August). First, the measured flight characteristics were reduced in dimensionality and visualized using Principal Component Analysis (PCA) in the R v. 4.2.1 (R Core Team, 2022). The *missMDA*'s function '*imputePCA*'of the package *missMDA* (Josse & Husson, 2016) was used to estimate missing values of average wingbeat frequency and advance ratio in four 3D trajectories where wingbeats were

difficult to score. To visualize possible interspecific and seasonal differentiation in flight behaviour, we used the function *fviz_pca* of the package *devtools* (Wickham et al., 2022).

Second, the differences in flight behaviour among species and their seasonal forms were estimated using linear models with mixed effects (LMMs), as implemented in the R package *lme4* (Bates et. al. 2015) via the function '*lmer*'. The response variables were the flight characteristics (log-transformed), except covered distance, fitted in separate models with species identity or seasonal form as predictor variables. As we recorded multiple trajectories per individual, specimen identity was included as a random factor and the covered distance (log-transformed) per trajectory as a fixed factor. Then, post hoc, pairwise comparisons were performed for flight characteristics found to differ significantly among species and seasonal forms in the LMM analyses. We used Tukey post hoc tests implemented in the R package *emmeans* (Lenth, 2022) to calculate mean values of the flight characteristics for each species and seasonal form and estimated marginal (*emmeans*) values of the LMMs.

Associations of wing morphologies with flight behaviour

To test whether wing morphological traits were associated with specific flight characteristics, we used LMMs with flight characteristics as response variables in separate models. As explanatory variables we used wing morphology and shape (wing area, forewing length, aspect ratio, wing loading, curvature of the forewing outer edge and the relative length of the marginal region). All morphological measures were log-transformed for statistical analyses, and, as in the previous LMM analyses, specimen identity was treated as a random factor whereas species identity and covered distance (log-transformed) were treated as fixed effects.

Results

Interspecific and seasonal differences in morphology

We quantified 106 flight trajectories of 31 butterfly individuals: 14 *Pieris rapae* and 17 *P. napi* (Table 1). The differences in wing morphology between the two *Pieris* species were lower than between their seasonal forms. The two *Pieris* species differed only in the relative length of

marginal region ($F_{1,28}$ = 9.307, p =0.005), as *P. napi* had a longer marginal region relative to the forewing length than *P. rapae* (Figure 1, Table 1). *Pieris napi* and *P. rapae* did not significantly differ in their wing areas ($F_{1,29}$=0.344, p = 0.562), aspect ratios ($F_{1,29}$=0.006, p = 0.941), wing lengths ($F_{1,29}$ = 0.724, p = 0.402), wing loadings ($F_{1,29}$ = 0.069, p = 0.795) and curvature of the forewing outer edge ($F_{1,28}$ **=** 0.853**,** p = 0.364).

The spring individuals were overall smaller than the individuals of the summer forms in both species. The spring *Pieris* had significantly shorter forewing length ($F_{1,29}$**=**5.343**,** p = 0.028) and lower wing area ($F_{1,29}$**=** 5.911**,** p = 0.022) than summer *Pieris*. Although only marginally significant, wing loading ($F_{1,29}$ **=** 3.757**,** p = 0.0624) and aspect ratio ($F_{1,29}$ **=** 3.668**,** p = 0.065) tended to be higher in the spring forms (Table 1). Higher values of aspect ratio described long and slender wings. The curvature of the forewing outer edge was significantly higher in spring *Pieris* ($F_{1,28}$ = 9.535, p = 0.005), meaning the spring generation had more elongated forewings with rounded edges. The curvature of the forewing outer edge was closer to 1 in summer *Pieris* meaning that the summer form had more rounded forewings (Figure 1). The relative length of the fore wing marginal region did not differ between spring and summer forms ($F_{1,28}$ **=** 0.244**,** p = 0.625).

Interspecific and seasonal differences in flight

The first axis of the PCA carrying most of the variance (32.8%) in flight parameters depicted changes in mean velocity, mean acceleration, mean advance ratio, and turning acceleration, as opposed to average flight height. The second PCA axis carried variation (25.1% explained variance) in turning rate, sinuosity, and curvature, as opposed to ascent angle. The PCA space did not reveal substantial differences between individuals of *P. napi* and *P. rapae*, which had a large variation in the measured flight parameters (Figure 2). Conversely, the flight behaviours of the spring and summer forms clustered separately, although higher variability in flight characteristics was present within summer individuals. Spring individuals had lower advance ratio, mean velocity, mean and turning acceleration, but higher flight curvature and height compared to the summer form.

Based on the results of the LMMs, the two *Pieris* species did not significantly differ in any of the measured flight characteristics (Table 2). In contrast, the seasonal forms differed significantly, in agreement with the clustering pattern highlighted by the PCA, as the spring

individuals had lower advance ratio ($p < 0.01$), mean velocity ($p < 0.01$), mean acceleration ($p = 0.03$) and turning acceleration ($p < 0.01$), but higher flight curvature ($p < 0.01$) than the summer individuals (Table 2).

Associations of wing morphologies with flight behaviour

The shape of the forewing was the most significant morphological variable correlated with different flight behaviour of the two seasonal forms (Table 3). The higher values of the curvature of the forewing outer edge (i.e., the elongated forewings found in spring *Pieris*) were associated with higher flight curvature ($p = 0.01$), whereas the lower values of the curvature of the forewing outer edge (i.e., the rounded forewings in summer *Pieris*) were associated with higher velocity ($p = 0.02$) and higher advance ratio ($p = 0.04$). The larger wing area, found in summer *Pieris*, was correlated with higher advance ratio ($p = 0.05$) and tended to also correlate with higher acceleration ($p = 0.06$) (Table 3). Although we have detected a significantly higher turning acceleration (measure of turning ability; $p < 0.01$) in summer *Pieris*, we did not find any measured wing morphology trait connected to such a difference. Contrary to previous assumptions, we have not detected any significant effect of wing loading and forewing length on the measured flight parameters between seasonal forms in *Pieris napi* and *P. rapae*.

Discussion

In this study, we used stereoscopic high-speed videography to quantify and compare the flight behaviours of two closely related *Pieris* species and their seasonal forms. The hypothesised divergence in flight behaviours between species due to habitat preference (*Pieris napi* associated with forest edges and *P. rapae* with agricultural open landscapes) was not supported by our findings (Table 2), suggesting that any flight adaptation driven by slight differences in habitat use has not evolved in either species. Interestingly, our results suggest that seasonal variation in wing size and shape is associated with contrasting flight characteristics (Table 3). On one hand, the elongated forewings of spring *Pieris* are associated with lower velocity, advance ratio, and higher flight curvature, indicating manoeuvrable flight with tortuous trajectories. On the other hand, the rounded forewings of summer *Pieris* are associated with higher velocity, and advance ratio. The divergent flight behaviours observed between seasonal forms

could possibly be adaptive. The higher curvature of flight in spring *Pieris* might for example reflect seasonal habitat use. Also, higher acceleration and turning acceleration (agile flight with quick changes of direction) in summer *Pieris* (Table 2), which cannot be explained by wing morphology variation alone, may represent a behavioural response to higher predator pressure during that season (Sang & Teder, 2011; Zvereva & Kozlov, 2021).

Despite the artificial conditions of the experimental cage, our approach efficiently detected the effect of wing morphology on flight. Using wild specimens for our study, moreover, likely resulted in uncontrolled variables such as variation in age, mating or feeding status, possibly impacting flight behaviour (Almbro & Kullberg, 2008, 2012). Nevertheless, our study revealed significant differences in flight behaviour between seasonal forms of *Pieris*. Similarly, we were not able to test the effect of the sex (Van Dyck & Wiklund, 2002), as only 5 females were collected (Table 1). Finally, although wing morphology and behavioural adaptations may explain the divergent flight behaviour between seasonal forms, we cannot rule out an effect of plasticity conditioned by maternal or developmental conditions (Nylin, 1994). This could be addressed in future studies using cage experiments and the quantification of flight patterns following our approach.

Seasonal but not interspecific morphological differences affect flight behaviour

Differences in environmental conditions between spring and summer may have driven divergent evolution of flight behaviours in *Pieris* butterflies. While the two closely related species, *P. napi* and *P. rapae* have variable, but overall undistinguishable flight and morphology (Table 2), their respective seasonal forms appear to diverge in morphology (Table 1) and flight behaviour (Table 2). The observed morphological differences between seasonal forms are consistent with previous studies. Fric et al. (2006) and Shkurikhin & Oslina (2016) reported that spring individuals were smaller with more elongated wings compared to larger summer individuals with more rounded wings. It has been hypothesised that the spring individuals of both *Pieris* species are adapted to explore the scattered feeding and oviposition resources on their natal patch, while the summer forms might be better adapted for dispersal to novel localities (Fric et al., 2006; Shkurikhin & Oslina, 2016). Dispersal may indeed be favoured because it permits access to more newly

emerged larval feeding plants and possibly reduced parasitoid pressure (Kerr et al., 2020; Ohsaki & Sato, 1999).

Based on the morphological differences, it has been expected that spring individuals fly faster and with greater manoeuvrability than the summer individuals (Fric et al., 2006; Shkurikhin & Oslina, 2016). Contrary to such expectations, the flight of spring *Pieris* was significantly slower than that of the summer individuals (Table 2). Although flight speed usually increases with wing loading (Le Roy et al., 2019), this is not the case for *Pieris*, as the spring forms have higher wing loading (Table 1; Fric et al., 2006) yet significantly lower flight velocity than summer *Pieris*. We argue that the lower flight speed of spring *Pieris* may be conditioned by the lower ambient temperature, which decreases body temperatures and affects flight performance (Tsuji et al., 1986).

We provide quantitative evidence for higher manoeuvrability in spring individuals, suggested by a higher flight curvature (Table 2), which was significantly associated with the forewing shape. The small and elongated forewings typical for the spring individuals were linked to flight trajectories with higher curvature. Such flight characteristics might increase exploration efficiency in individuals living during spring (Root & Kareiva, 1984), which may be beneficial for locating emerging nectar sources and larval feeding plants (Danks, 2007). Although the benefits of adopting a curvy flight pattern for foraging in a dispersed resource environment remain hypothetical, the higher flight curvature of spring *Pieris* may have evolved jointly with a specialised foraging strategy. Altogether, the divergent flight behaviour of spring *Pieris* might be adaptive and linked to movements across the landscape, which might result in more effective exploitation of newly emerging feeding and oviposition resources (Cant et al., 2005).

The larger wing area and, consequently, the lower wing loading typical of summer *Pieris* (Table 1) were predicted to enhance dispersal abilities across landscapes (Fric et al., 2006; Shkurikhin & Oslina, 2016), including fragmented habitats (Merckx & Van Dyck, 2006; Skórka et al., 2013; Thomas et al., 1998). One explanation was that more rounded wings, for example in *P. rapae*, might be adaptive for using ascending thermal air currents for dispersal (Fric et al., 2006). Indeed, it was the rounded forewing shape of the summer form that was significantly associated with high advance ratio, thus, might contribute to effective dispersal (Table 3). Next, we found that larger wing area, but not wing loading, seems to be positively correlated with advance ratio and acceleration, though with marginal statistical significance (Table 3). Furthermore, we did not

find evidence supporting that the larger wing size of summer *Pieris* decreases acceleration predicted by Shkurikhin & Oslina, 2016. On the contrary, we found higher acceleration and turning acceleration in the summer form of *Pieris*. Such a quick flight may reflect fast linear flight used during dispersal (Cant et al., 2005), but it is also recognised that fast flight is affected by higher summer temperatures (Cormont et al., 2011). The higher acceleration and turning acceleration of the summer form might also represent seasonal behavioural response to increased predator pressure on adult butterflies during summer for example by naïve juvenile birds (Zvereva & Kozlov, 2021) or dragonflies (Sang & Teder, 2011). However, we take such a claim with a caution because it has also been predicted that the predation pressure on insects is likely higher during spring than during summer in some temperate regions (Remmel et al., 2009).

Our results highlight the high variability in butterfly flight behaviours, which challenge the constancy of simple biomechanics assumptions based on wing morphology alone (for a review, see Le Roy et al., 2019). The divergent flight behaviours of two seasonal *Pieris* species are not explained by forewing aspect ratio, wing loading nor by total wing area. The main morphological change that might be involved in the flight divergence observed between spring and summer *Pieris* is the shape of the forewings (Figure 1, Table 3). Wing shape variation in temperate butterflies might be either plastic or have a genetic background, as revealed in *Pararge aegeria* (Nymphalidae) (Van Dyck & Wiklund, 2002, Berwaerts & Van Dyck, 2004). Similarly, subtle differences in wing shape are linked with distinct dispersal capacity in females of *Melitaea cinxia* (Nymphalidae) (Breuker et al., 2007). We also cannot rule out that the seasonal wing morphologies may be related to differences in thermoregulation properties, in addition to divergent flight characteristics, thus, acting as a multi-role trait. For instance, the larger wing size of summer forms might provide higher thermal buffering ability (Bladon et al., 2020; Kleckova et al., 2014). Thus, the morphological divergence between seasonal forms may be conditioned by multiple selective pressures. Our results add to mounting evidence that the predictors of flight behaviour such as manoeuvrability and dispersal capacity are complex and are likely the result of both morphological adaptation and behavioural variation.

Conclusions

Our study sheds light on the divergence in flight behaviour between seasonal forms of two *Pieris* species. We provide quantitative evidence relating wing morphology, in particular the shape of forewings, to difference in flight behaviour (Le Roy et al., 20190. Different life strategies (e.g., higher flight curvature of spring *Pieris* in relation to resource exploration) probably contributes to the adaptive seasonal divergence of *Pieris* flight, which has rarely been documented in butterflies (e.g., Dell'Aglio et al., 2022; Mena et al., 2020; Spieth & Cordes, 2012). In the future, a combination of experiments with laboratory bred individuals (Almbro & Kullberg, 2008) together with recording flight behaviour of wild butterfly individuals in both, controlled and natural settings (e.g., Cant et al., 2005; Maggiora et al., 2019) will improve our understanding of the mechanisms driving seasonal differences in flight behaviour.

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Authors contributions

I.K. - Conceptualization, Investigation, methodology, data curation and analysis, writing original draft, review and editing. **D.L.** - data analysis, writing original draft, review and editing. **F.M.R.**- Conceptualization, Investigation, and editing, **L.R. –** data curation and investigation, review and editing, **C.L.R.** - data analysis, review and editing, **P.M.M.** - Funding acquisition, investigation, methodology, conceptualization, review and editing.

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Competing Interests

We declare no competing interests.

Data Accessibility

All data and scripts are archived in the Figshare repository, (DOI: https://doi.org/10.6084/m9.figshare.24631410.v1).

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Figures and Tables

Figure 1. Wing morphology of *Pieris* white butterflies and their seasonal forms**.** Forewing length and landmarks used to describe relative with of marginal region (measured as the distance between landmarks 1 to 9 divided by the distance between landmarks 1 to 13**)** and outer edge of forewing (the sum of distances around the outer edge from landmarks 13 to 19 divided by straight distance between landmarks 13 and 19).

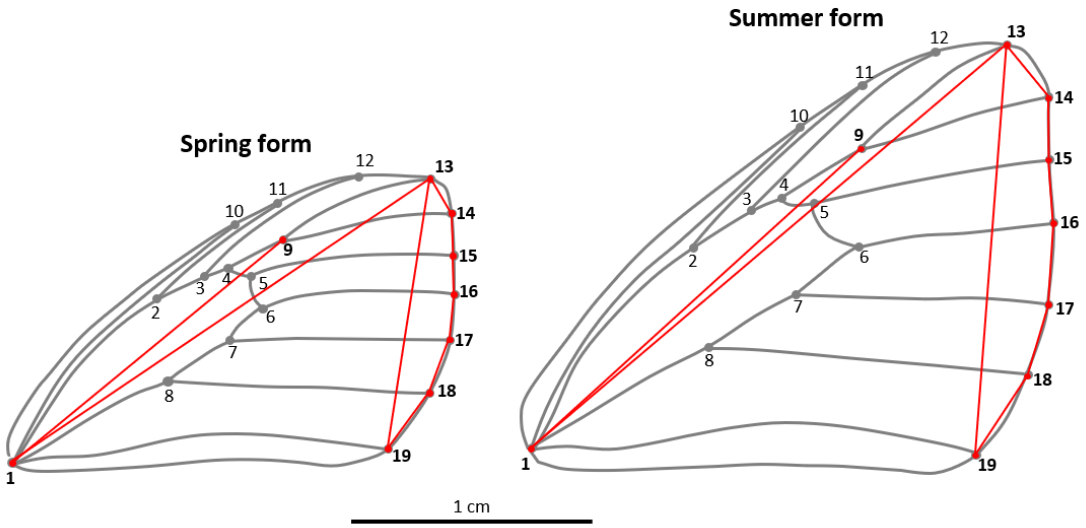
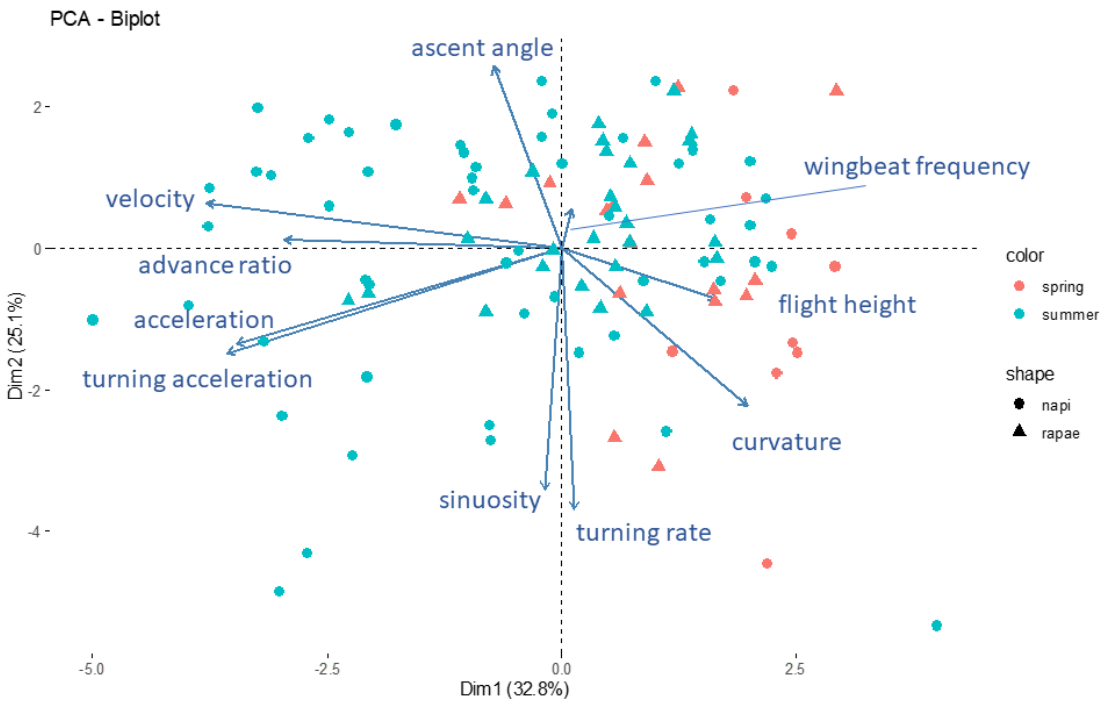


Figure 2. Seasonal forms but not species of *Pieris* butterflies differed in their flight characteristics. The first and second axes of PCA analyses of flight characteristics of the two closely related sympatric *Pieris napi* and *P. rapae* (Pieridae) and their seasonal, spring and summer, forms. Each point represents a recorded trajectory.



				Size				Forewing shape	
	N (M/F)	Spring /Summer form	Nu of tracks	Wing area (mm^2)	Aspect ratio	Fore wing length (mm)	Wing loading (mm^3/mm^2)	Outer edge	Marginal region
Pieris napi	17 (14/3)	4/13	63	933 (33.4)	1.75 (0.014)	24.6 (0.44)	0.02127 (0.00162)	1.09 (0.007)	0.72 (0.007)
Pieris rapae	14 (12/2)	5/9	43	957 (32.8)	1.75 (0.02)	25.1 (0.38)	0.02063 (0.0018)	1.08 (0.008)	0.69 (0.007)
Spring	9 (8/1)		26	864 (42.6)	1.78 (0.024)	23.9 (0.51)	0.02442 (0.00219)	1.11 (0.011)	0.70 (0.010)
Summer	22 (18/4)		80	977 (25.1)	1.73 (0.011)	25.2 (0.32)	0.01957 (0.00133)	1.08 (0.005)	0.70 (0.007)

Table 1. Overview table summarising the number of individuals (males and females) of the two *Pieris* species and their seasonal forms, number of tracks recorded, and mean values (±SE) of wing area, aspect ratio, forewing length and wing-loading and shapes of the curvature of the forewing outer edge and of relative length of marginal region (see Methods for more details). *Pieris* species differed only in relative length of marginal region, however, their seasonal forms differed in multiple morphological variables (see Result section for more details). Generally, the spring form is smaller with elongated forewings and more rounded shape of the forewing edge. The summer form was larger with more straight edge of forewings.

Flight characteristics	Species effect			Seasonal form effect			emmean (SE)	
	F	**Dendf**	**P**	**F**	**Dendf**	**P**	**spring (N=26)**	**summer (N=80)**
Wingbeat frequency	0.55	29.246	0.46	0.73	32.2	0.40		
Flight height	0.02	29.589	0.90	0.97	31.82	0.33		
Velocity	0.33	27.599	0.57	**12.089**	**30.48**	**<0.01**	**1.13 (0.10)**	**1.62 (0.09)**
Advance ratio	1.25	27.54	0.27	**10.86**	**31.7**	**<0.01**	**0.10 (0.01)**	**0.15 (0.01)**
Acceleration	0.28	27.57	0.60	**5.76**	**27.821**	**0.02**	**2.30 (0.34)**	**3.5 (0.30)**
Turning acceleration	0.71	28.968	0.40	**12.46**	**27.96**	**<0.01**	**1.29 (0.20)**	**2.4 (0.21)**
Turning rate	0.03	28.421	0.86	0.20	30.87	0.67		
Curvature	0.77	26.853	0.36	**14.69**	**34.06**	**<0.01**	**4.74 (1.05)**	**1.83 (0.23)**
Ascent angle	0.96	26.846	0.34	1.40	28.51	0.25		
Sinuosity	0.087	73.337	0.36	0.395	35.173	0.53		

Table 2. *Pieris napi* and *P. rapae* species did not differ in flight behaviour, but their seasonal forms do. Comparison of interspecific differences in flight characteristics in two closely related species, *Pieris napi* (17 individuals, N=63 trajectories) and *P. rapae* (14 individuals, N=43 trajectories), and between their seasonal forms, spring (9 individuals, 26 trajectories) and summer (22 individuals, 80 trajectories). For each *Pieris* individual, flight characteristics were inferred for several trajectories of differing length, thus linear mixed models (LMMs) include individual identity as a random factor and covered distance as fixed factor. For flight characteristics, which significantly differed among seasonal forms were estimated marginal means of LMM (emmean) and transformed to real values of the flight characteristics. Significant relationships are highlighted **in bold**.

Flight characteristic	Trait	Slope	SE	F	Df	P
Wingbeat frequency	Wing area					n.s.
	Aspect ratio					n.s.
	Forewing edge					n.s.
Flight height	Wing area					n.s.
	Aspect ratio					n.s.
	Forewing edge					n.s.
Velocity	Wing area					n.s.
	Aspect ratio					n.s.
	Forewing edge	**-4.8**	**2.01**	**5.70**	**29.252**	**0.02**
Advance ratio	Wing area	**0.9**	**0.45**	**4.25**	**28.75**	**0.05**
	Aspect ratio	-3.5	1.80	3.84	26.423	0.06
	Forewing edge	**-5.2**	**2.37**	**4.74**	**28.126**	**0.04**
Acceleration	Wing area	1.1	0.57	3.78	27.43	0.06
	Aspect ratio					n.s.
	Forewing edge					n.s.
Turning acceleration	Wing area					n.s.
	Aspect ratio					n.s.
	Forewing edge					n.s.
Turning rate	Wing area					n.s.
	Aspect ratio					n.s.
	Fore wing edge					n.s.
Curvature	Wing area					n.s.
	Aspect ratio					n.s.
	Fore wing edge	**13.3**	**4.73**	**7.97**	**29.63**	**0.01**
Ascent angle	Wing area					n.s.
	Aspect ratio					n.s.
	Fore wing edge					n.s.
Sinuosity	Wing area					n.s.
	Aspect ratio					n.s.
	Fore wing edge					n.s.

Table 3. Flight performance of *Pieris napi* and *P. rapae* (Pieridae) butterflies was determined by individual morphological traits. The effect of individual morphological traits on flight characteristics was tested by linear mixed models (LMMs). For each individual, flight characteristics were inferred for several trajectories of differing length, thus, an individual identity was included in the LMMs as a random factor and covered distance (log transformed) as fixed

factor. Further, species identity was included in the models as a fixed factor. Significant effects of morphology traits are highlighted in bold, effects close to being significant are underlined. We have not detected significant effects of wing loading, forewing length and the relative size of the relative length of the marginal region on flight characteristics.