

Research Article

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Effects of the parasite, *Ophryocystis elektroscirrha*, on wing characteristics important for migration in the monarch butterfly

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Abstract: There is mounting evidence that the long-term declines of overwintering monarchs in Mexico are exacerbated by losses during the fall migratory journey. Infection with the protozoan, *Ophryocystis elektroscirrha* (OE), is known to negatively impact migration success. Here we examine how infections affect specific wing traits of monarchs that are important for migratory success. We used a collection of infected and uninfected monarchs reared under identical conditions, and from the (deceased) specimens, measured wing area (larger monarchs are known to have greater migratory success), wing color (the shade of orange pigmentation in monarchs is a known predictor of migration and flight ability), and the physical density of wings (a measure of wing mass per unit area). We also measured the tear-resistance of wings, using an apparatus that measured the force needed to cause a tear in the wing. Results showed no effect of OE on overall wing size, nor on the shade of orange pigmentation, but a clear effect on measures of physical density and tensile strength. Wings of infected monarchs weighed less per unit area (by 6%), and there was a 20% reduction in tear-resistance of wings. All results were qualitatively similar in a follow-up investigation using freshly-killed specimens. Collectively, this indicates infected monarchs are more prone to wing damage, which would be costly during long-distance migration. As such, this would be one more way in which OE infections reduce migratory success. Given the toll of OE to the monarch population, especially during migration, it would be prudent to focus conservation efforts on mitigating human activities that spread this disease.

Keywords: monarch butterfly, migration, *Ophryocystis elektroscirrha* (OE), wing morphology, tear-resistance

1 Introduction

For animals that migrate long distances each year, attempting to migrate while being sick can be costly. Migrating animals are often infected with one or more internal or external parasites or pathogens (1). New comparative research compiling information across a wide variety of animals that migrate indicates the costs of these parasites to their host are often small, but they can include lower body stores, reduced movement capacity, delayed migration phenology and lower rates of survival during migration (2). Given that one of the hallmarks of infection is reductions in energy and locomotion, it is not surprising that a majority of research has focused on how infections affect animal movement or behavior during migration (3) or energy storage ability (4-6). Less appreciated is the fact that infections could also impact the physical development of animals, if the disease is contracted during early life, leading to poorly-developed locomotor structures that could in turn reduce migratory performance.

Migrating while infected is an issue that is faced by the world's most famous insect migrant, the monarch butterfly, *Danaus plexippus* in eastern North America. Monarchs around the world are prone to a naturally-occurring, protozoan parasite, *Ophryocystis elektroscirrha* (OE) (Fig. 1), which is transmitted when larvae consume parasite spores (Fig. 1, inset) on their hostplants (milkweeds). The parasite develops within the larvae, replicates during host metamorphosis, and the adult butterfly emerges covered with new spores. This parasite, in heavy infections, can have a range of deleterious effects on the host, including reductions in larval survival, adult fecundity and adult lifespan (7-9), and reductions in flying efficiency (10). Importantly, within the eastern N.

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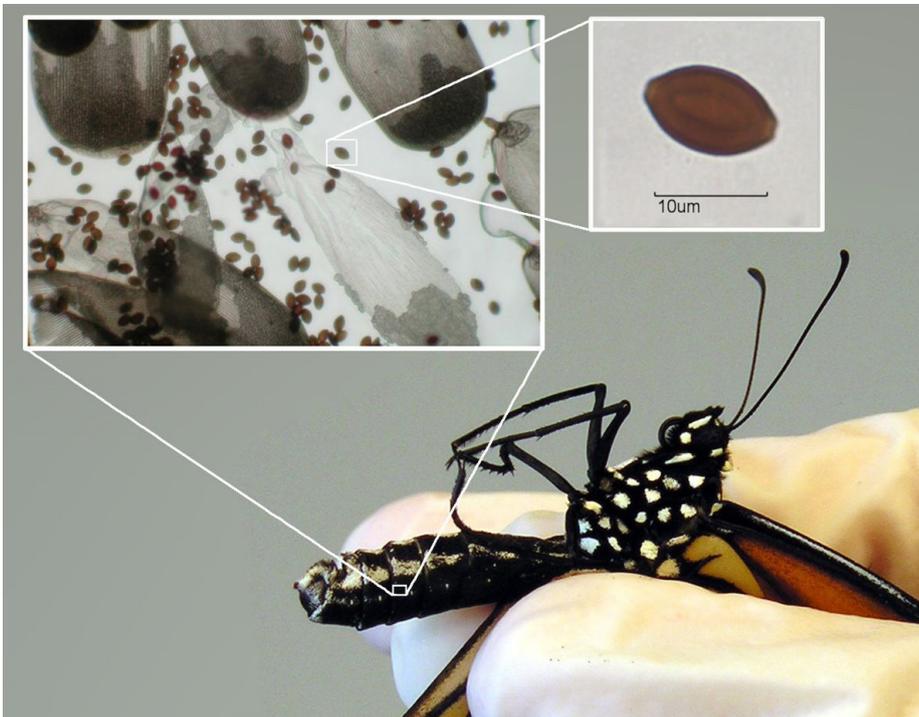


Figure 1. Depiction of the protozoan parasite, *Ophryocystis elektroscirrha* (OE), which affects monarch butterflies. The parasite replicates during the monarch's larval and pupal stages, then forms microscopic spores on the adult butterfly that can be seen between the scales on the abdomen.

American population, the parasite tends to build up over time during the summer (11, 12), leading to the highest prevalence (10-25%) in the migratory generation produced at the end of the summer. Since the most recent estimates of the number of (overwintering) monarchs is ~70 million, (13), it is entirely possible then that ~7-17 million infected monarchs begin the migration. However, during the migratory journey, there is also evidence that infected individuals drop out, leading to reductions in prevalence southward along the flyway (11, 14), and a final prevalence at the overwintering sites that is usually less than 5% (15). Collectively then, all evidence to date suggests that OE infections lead to significant losses of monarchs during migration.

What are the mechanisms by which OE causes migratory monarchs to fail to reach their destination? As indicated above, simply having a reduced lifespan (9) is one possibility, as is reductions in flight capacity (10). However, one mechanism that has never been explored is how the infection alters physical development of the very structures needed to migrate – the wings. The physical characteristics of monarch wings have been shaped (literally) by natural selection over thousands of years of migration; around the world, populations of monarchs that migrate tend to have larger wings than those that do not migrate (16-18). Moreover, for reasons that are

not yet clear, even the shade of orange pigmentation on monarch wings seems to be linked with migratory ability (19, 20). While the pigmentation itself likely does not convey any aerodynamic or flying benefits, it may simply be a correlate of overall condition, which does correlate with migratory ability (21). And finally, while there is only limited research on this to date (22), the physical strength of monarch wings must also be important for migrants, to resist against damage.

In this study, we evaluated the effects of OE infection on the physical characteristics of monarch wings, using an archived collection of monarch specimens from an experiment conducted a number of years earlier in which infected and uninfected monarchs had been reared under controlled (laboratory) conditions. We specifically examined how infections impact the size (surface area), color (orange pigmentation) and density (mass per unit area) of monarch forewings, using a combination of computer-assisted image analysis techniques as well as a novel device we designed for measuring tear-resistance of butterfly wings. A follow-up test was performed using fresh specimens to verify results from the archived specimens. Results of this effort will be useful to more fully understand the impact of this disease on monarch migration, which in turn will aid in conservation efforts of the North American monarch.

2 Methods

2.1 Rearing monarchs and parasite inoculation

Monarch specimens for the current project came from an earlier experiment conducted in 2009, which investigated the effects of milkweed species on monarch butterfly resistance and tolerance to OE (23). In this experiment, monarch larvae were reared on different species of milkweed under controlled laboratory conditions, and experimentally inoculated with 10 spores of an OE strain derived from California. Monarch larvae were the outbred grand-progeny of monarchs originally collected from Pismo Beach, California. Pismo Beach is one of the main areas where monarchs west of the Rocky Mountains overwinter, as opposed to Mexico, where most eastern North American monarchs migrate. Note that while the monarchs used originated from the western North American population, which has a shorter migration than that of the eastern population, the overall results concerning infection are of importance here. Also, a follow-up test was conducted using monarchs from the eastern population (see “tests of fresh wings” below).

From the project above, we selected a total of 147 monarchs for inclusion in the current study (66 uninfected, 81 infected). They had been reared on seven different milkweed species, *Asclepias curassavica*, *A. fascicularis*, *A. incarnata*, *A. physocarpa*, *A. speciosa*, *A. sullivantii*, and *A. syriaca*. Note that in all statistical analyses of wing traits, milkweed species was a predictor (see below). Also note that there were more infected monarchs than this

in the rearing experiment, thus we randomly selected a representative sample of infected monarchs from each hostplant group.

2.2 Wing measurements

Morphological features of monarch forewings were measured using computer-assisted procedures that have been employed in multiple prior studies of monarchs (16, 21, 22, 24, 25). A single forewing was removed from the monarch specimen and scanned using a standard flatbed scanner connected to a desktop computer, resulting in a high-resolution digital version of the forewing (Fig. 2). We scanned the monarch’s left forewing, unless it was damaged, and if so, used the right.

Using image-analysis software (FoveaPro 4.0, www.reindeergraphics.com), we measured the surface area of the wing (mm^2), as a measure of overall wing size (Fig. 2A). Next we digitally selected the central orange cell of the forewing (Fig. 2B), and obtained the average pixel hue score of the entire selection. Computer images are made up of thousands of pixels, and each one is labelled with a hue, saturation and brightness value. From prior work on monarchs, the hue score appears to convey the most biological meaning (19, 25), and is also the simplest to interpret; hue is generally thought of as the difference between orange, brown and blue, etc. Saturation can be thought of as the difference between pink and red. Hue is measured in degrees (0-360), and the orange hue score on monarchs wings tends to vary between 15 and 45, with lower values representing more red, and higher values being more yellow (26), although these scores can vary between scanners (Davis, *pers. obs.*). This image-analysis

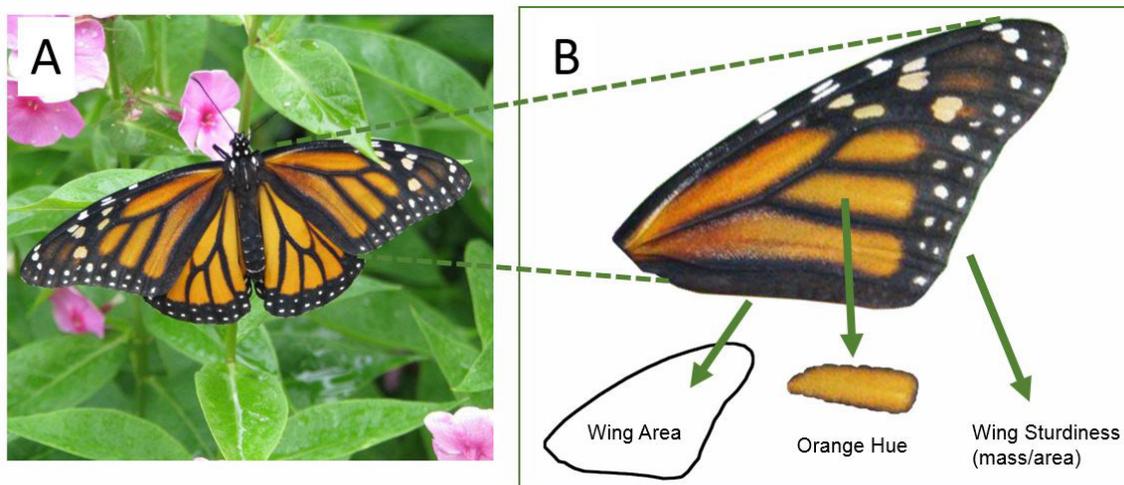


Figure 2. (A) Female monarch butterfly, photographed by Pat Davis. (B) Scanned forewing, showing the morphological measurements obtained in this study, including forewing surface area, hue of orange pigmentation, and forewing density (wing mass divided by wing area).

approach to measuring wing color differs from other Lepidopteran work where spectrophotometers are used to assess pigmentation (27-29), however, prior work has shown consistencies between approaches for measuring the range of frequencies that span the orange and red colors (19).

Lastly, we weighed each forewing using a precision electronic balance (in mg). Note that since these specimens had been in storage for nine years, this value is likely lower than the mass of a live wing, and may even be closer to the 'dry mass' of the wing (i.e. if the specimen had been dried in an oven). Even so, our intention here was not to estimate or even simulate dry mass, but merely to compare *relative* differences among individuals in this collection (which had all been handled and stored similarly). Also note that we subsequently tested a separate collection of living monarchs (see below) to verify results from the archived specimens. We then used the wing mass and wing area values for each individual to compute an index of wing thickness, or density (mass/area), which has been used before in studies of monarchs (22) and other butterflies (30).

2.3 Tear-resistance measurement

Based on preliminary results gathered from the wing morphology analyses, we also tested the tear-resistance

of a subset of monarch wings in this study. For this, we constructed a unique benchtop apparatus (Fig. 3) that allowed us to measure the force required to tear a wing. A single forewing was secured (by its base) in a tabletop vice (Fig. 3A). The distal tip of the wing was held by a clamp, which was attached to a force-gauge. The gauge was attached to a vertical post but with an adjustable height-dial that allowed it to move straight upward. To measure the tear-resistance of the wing we slowly moved the gauge and clamp upward until the wing tore (Fig. 3B). The gauge recorded the maximum force (i.e. Newtons, N) required to tear the wing. We performed this measurement on half of the monarch specimens, with similar numbers of infected (n=38) and uninfected (n=39) monarchs.

2.4 Tests of fresh wings

Since the results garnered during this project hinged on the use of archived (frozen and stored) monarch specimens, we conducted a follow-up investigation using fresh specimens, to understand how storage itself affects wing properties, and to verify the results from the archived specimens. For this we used a set of 18 infected and 21 uninfected monarchs that had been reared on *Asclepias incarnata* in our lab, under similar conditions as those from the original experiment. Infected monarchs had been inoculated with OE spores as larvae, and all larvae

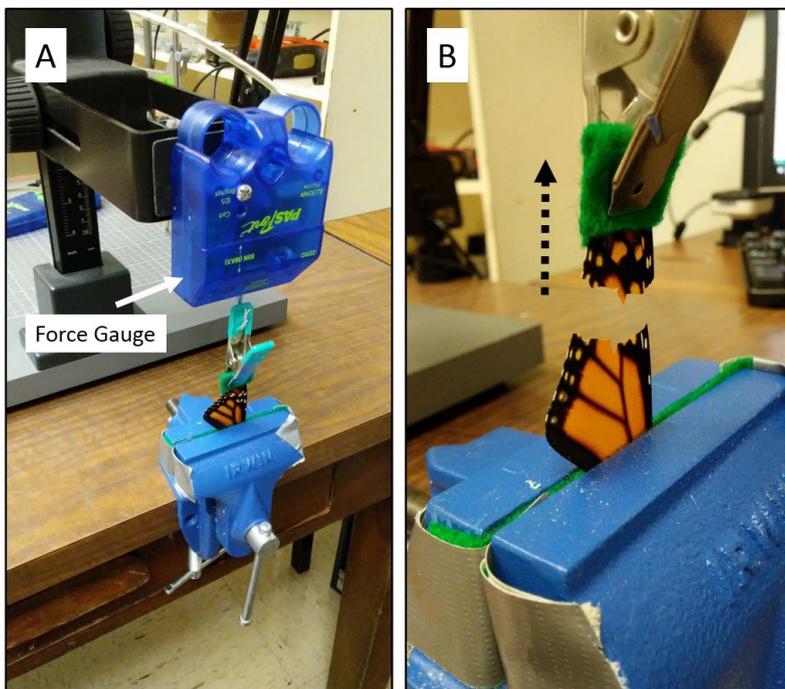


Figure 3. Apparatus used to measure tear-resistance of monarch forewings. Wings were held in place using a tabletop vice (A), while a clamp held the distal end of the wing. The clamp connected to a force gauge, which could move upward (B), and it measured the force needed (in Newtons) to tear the forewing.

were reared until eclosion. The adults were stored in glassine envelopes for 4 days at 13°C. On the day of testing, the adults were killed and their left forewing removed. The wings were immediately scanned and weighed (i.e. within minutes of killing). This allowed us to calculate their “living” wing density (mass/area). Immediately after scanning, we used the force meter setup as before, and measured the breakforce needed to cause a tear in each wing. We note also that the specimens for this test originated from the eastern North American population of monarchs.

2.5 Data analyses

The dataset for the analyses of wing traits (of the archived specimens) included measurements of 147 monarchs, with data on the larval hostplant species (7 species of milkweeds), the sex of the adult, OE infection status (yes, no), and the three wing traits of interest: forewing area, wing hue score, and wing density. All continuous variables were normally-distributed. We evaluated the effects of OE infection on the three wing traits using factorial ANOVA models (one model for each wing trait), with the hostplant, sex and infection as predictors. Two-way

interactions involving infection were also included. In our follow-up experiment on tear-resistance, we had data on 77 monarchs. To examine the effect of infection on tear-resistance we used ANCOVA, with sex, infection and hostplant (4 plant species) as predictors, and wing density, as a covariate (to determine if density predicts tear-resistance). Finally, in the follow-up tests of freshly-killed specimens, we compared infected and uninfected monarch wings using t-tests, and specifically tested wing density, and tear-resistance, as these two variables would (in theory) be most affected by long-term storage. All data for this project were analyzed using the Statistica 13.3 software package (Tibco Software, Inc.).

3 Results

3.1 Wing Characteristics

Forewing areas of all 147 monarchs in this study ranged from 744mm² to 1104mm², with an overall average of 962mm² (± 63 mm² SD). In the ANOVA model that examined predictors of wing area there was no significant variation due to OE infection status ($p=0.2138$, Table 1), although

Table 1. Results of ANOVA models that examined effects of OE infection on monarch wing traits, wing area, orange hue score and wing density (mass/area). Significant predictors are indicated in bold.

<i>Response</i>	<i>Predictor</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p</i>
<i>Forewing Area</i>	Hostplant	6	11534	3.29	0.0048
	Sex	1	2025	0.58	0.4487
	Infection	1	5472	1.56	0.2138
	Hostplant*Infection	6	6680	1.91	0.0846
	Sex*Infection	1	202	0.06	0.8108
	Error	131	3507		
<i>Orange Hue</i>	Hostplant	6	5.67	2.27	0.0403
	Sex	1	38.28	15.35	0.0001
	Infection	1	3.30	1.32	0.2520
	Hostplant*Infection	6	0.80	0.32	0.9254
	Sex*Infection	1	1.01	0.40	0.5265
	Error	131	2.49		
<i>Wing Density</i>	Hostplant	6	0.000001	0.69	0.6541
	Sex	1	0.000011	5.48	0.0208
	Infection	1	0.000016	8.07	0.0052
	Hostplant*Infection	6	0.000002	1.20	0.3129
	Sex*Infection	1	0.000001	0.64	0.4259
	Error	131	0.000002		

there was a slight trend for infected monarchs to have larger wings (Fig. 4A). There was also no effect of sex on wing area ($p=0.4487$), but there was a significant effect of hostplant ($p=0.0048$, Table 1). This hostplant effect appeared to be driven by a single plant species that produced larger monarchs than the others; monarchs reared on *A. curassavica* had an average wing area of 1003mm^2 , compared to the others, which ranged from $930\text{--}978\text{mm}^2$.

Orange hue scores also did not differ between infected and uninfected monarchs; the effect of infection in our ANOVA model of hue was not significant ($p=0.2520$, Table 1, Fig. 4B). There was an expected difference in hue between males and females in this model ($p<0.0001$), though this pattern is known already in monarchs (21, 25, 31). There was again a significant hostplant effect ($p=0.0403$), which again, appeared to be driven by a single plant species, *A. curassavica*. Monarchs reared on this plant tended to have higher hue scores (more yellow) than those reared on other plants.

Infection with OE did affect the density of monarch forewings; in this ANOVA model, the infection predictor was significant ($p=0.0052$, Table 1). This effect is also visualized in Fig. 4C, where the mean of all infected monarchs ($0.0120\text{mg}/\text{mm}^2$) was less than that of uninfected monarchs ($0.0127\text{mg}/\text{mm}^2$). The magnitude of this difference is approximately 6%. In other words, infected monarchs tended to have wings that were lower in mass, given their size (i.e. less sturdy). There was also a difference between males and females with this trait ($p=0.0208$), with females having higher density values than males, consistent with prior research (22).

3.2 Tear-resistance

The results of the wing morphology analyses above (specifically, the density results) compelled us to conduct further tests on the effect of infection on tear-resistance of forewings. Here we discovered a clear effect of OE infection on the tear-resistance of monarch wings; infected monarch wings were more easily torn. The mean level of force required to tear wings of uninfected monarchs ($n=39$) was 2.18N , compared to an average of 1.77N needed to tear wings of infected individuals ($n=38$) (Fig. 5), or a difference of 20%. And, in the ANCOVA model that examined tear-resistance, the effect of infection was significant ($F_{1,69}=7.58$, $p=0.0075$). In the same model there was no effect of sex ($F_{1,69}=0.13$, $p=0.7163$), or plant species ($F_{3,69}=1.82$, $p=0.1522$). Interestingly, this model also showed there was no significant relationship between wing density and tear-resistance among all individuals ($F_{1,69}=0.05$, $p=0.8144$).

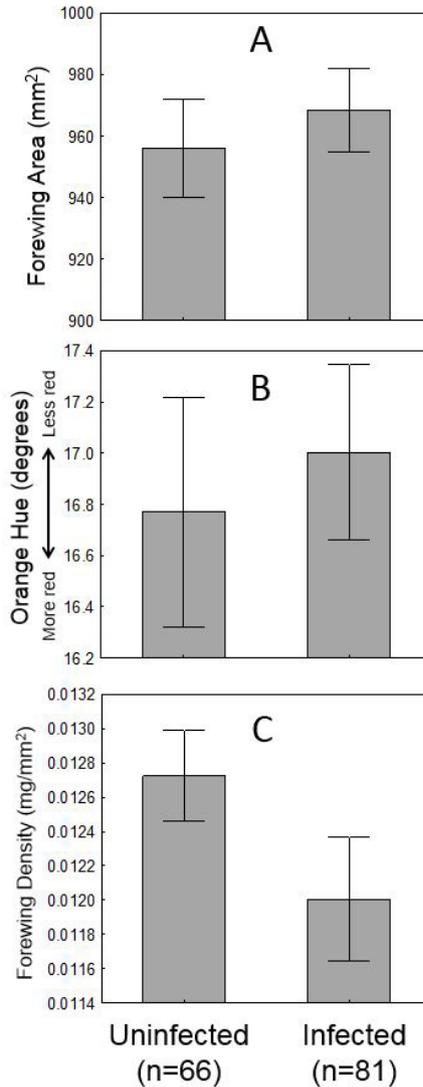


Figure 4. Comparison of migration-related wing characteristics between uninfected and infected monarchs, including mean surface area (A), orange hue score (B) and wing density (C). Of these, only wing density was significantly different between healthy and infected individuals. Whiskers represent 95% confidence intervals.

3.3 Tests of fresh wings

Comparison of physical properties in recently-killed specimens showed qualitatively-similar results as the analyses of archived specimens. The average forewing density of infected monarchs ($\bar{x}=0.017\text{mg}/\text{mm}^2$) was significantly lower than that of uninfected monarchs ($\bar{x}=0.018\text{mg}/\text{mm}^2$) based on a Student's t-test ($t=2.36$, $df=37$, $p=0.0234$), or a difference of ~8%. The force needed to tear wings of infected monarchs ($\bar{x}=2.27\text{N}$) was also significantly lower ($t=2.83$, $df=37$, $p=0.0074$) than that of uninfected monarchs ($\bar{x}=2.84$), or a difference of ~20% (Fig. 5B). We noted also that the breakforce of freshly-

killed (uninfected) specimens was approximately 23% higher than that of archived specimens (compare Fig 5A vs. 5B).

4 Discussion

Identifying causes of migration success or failure for the monarch butterfly population in eastern North America is an issue of immense importance, because of the mounting evidence pointing to migration failure as one of the major causes for the long-term declines of overwintering monarchs in Mexico (32-35). Infection with the protozoan parasite, *Ophryocystis elektroscirrha* (OE), is one of the few known factors that can reduce migratory success (11, 14), though in the past it was always thought to do so by reducing flight capability (10). Here, we uncovered new evidence that points to an additional mechanism by which OE could reduce migratory ability: reductions in wing strength. Monarchs with OE infections appear to have lower wing mass (6% lower) than uninfected monarchs, and their wings tear more easily (20% reduction in tear-resistance). These results were similar whether we used archived or fresh monarch specimens, and in both western and eastern North American monarchs. This evidence indicates the OE parasite infection makes monarch wings weaker and more prone to damage, and this would be problematic during migration.

At least some wing damage is bound to happen to monarchs during their fall migration, given the two-month long, treacherous and risky journey (36, 37). In support of this, collections of fall migrants in Texas tended to have more wing damage than migrants in Minnesota (38). However, monarchs that incur too much wing damage during migration probably have a poor outlook for finishing the journey, and the evidence for this comes from several sources. First, researchers studying the overwintering sites in Mexico have long noted how the majority of monarchs that arrive there appear to have virtually undamaged wings (L. Brower, pers. comm.), suggesting those with damaged wings did not make it. Also, from a multi-year study of migratory monarchs on the Atlantic coast, monarchs with damaged wings were found to require more frequent, and longer stopovers (39), which over time could result in a slower overall pace of migration, and eventual failure. From a mechanical standpoint, it is not difficult to visualize how wing damage would affect monarch flight capability. In fact, experimental work with other flying insects has directly demonstrated how wing damage reduces flight performance (40). Thus, wing damage incurred during migration would force monarchs

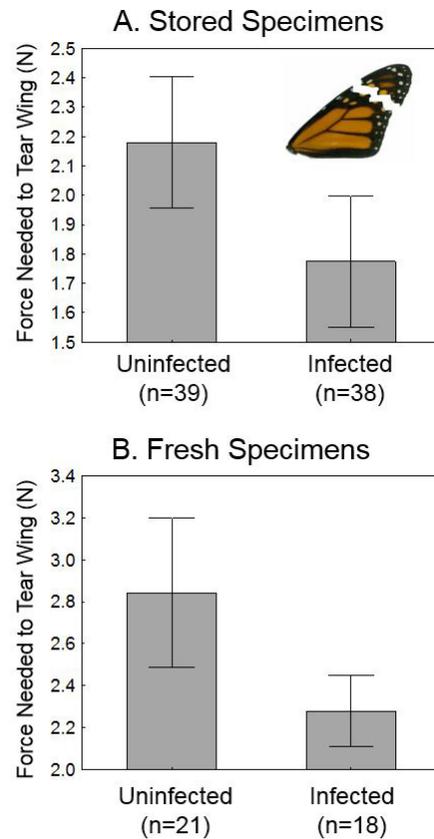


Figure 5. Effects of *Ophryocystis elektroscirrha* on the tear-resistance of monarch wings, using stored specimens (A) and freshly-killed specimens (B). In both collections, the mean level of force needed to tear wings was higher in uninfected monarchs than in infected monarchs. Whiskers represent 95% confidence intervals.

to expend more energy flying, causing them to require more frequent stopovers to refuel.

Our results imply that OE infections cause monarch wings to be more prone to damage, and that wing damage could negatively affect migration success. However, there may be some threshold level of damage that must occur for this to happen; not only do at least some infected monarchs succeed in reaching the Mexican overwintering sites each year, examinations of such monarchs nearly 2 decades ago showed that these infected monarchs tended to have greater amounts of “wing tatter” than uninfected butterflies (41). On the one hand, this observation confirms the association we found between infection and reduced damage-resistance, but on the other, it signifies that not all damage is fatal during migration. It is also possible that there is a distance threshold for this mechanism to work; recent work using stable isotopes to infer natal origins of overwintering monarchs indicated that infected monarchs did not travel as far to reach the site as healthy monarchs did (14). Thus in theory, an infected monarch

with wing damage could arrive at the overwintering site if it originated from a nearby region (i.e. Texas, or northern Mexico), and did not have to migrate far.

The reductions in wing form and strength found here suggest that OE infections can impair development of adult body structures during metamorphosis (when wings are physically formed). Indeed, the life cycle of OE is such that it undergoes most replication during host metamorphosis, and therefore should have the largest impact on the butterfly during this stage, i.e. during tissue development. In support of this idea, detailed examinations of infected and uninfected monarchs collected during fall migration showed both groups were similar in size (wing area), but dry mass was significantly lower in the infected monarchs (42). This implies that infected butterflies have reduced muscle mass (or other internal tissue), consistent with the wing mass results found here.

Similar to the findings of Satterfield and colleagues (42), who examined migrating monarchs in Georgia, USA, we found no reduction in wing area as a result of OE infection (our monarchs were descended from adults collected in California). A similar pattern was found (no difference in wing size) in collections of eastern migrants in Kansas and Texas (43). Interestingly, other work has shown clear negative effects of OE on monarch wing size (length) in wild-caught collections from western North America (41). Such variation among studies may indicate the impact of the parasite on monarch wing development can vary across studies. It also argues that blanket statements suggesting OE infections cause reductions in wing size are not accurate.

An ancillary finding here deserves comment. While not the focus of this study, we discovered that milkweed hostplant species can affect the resulting wing characteristics of adult monarchs. We stress however, that our sample sizes for these results are small (20 monarchs per plant type). Interestingly, we found that the hostplant effect was driven by one plant species, *A. curassavica*, which produced monarchs with larger forewings, but that were lighter orange. On the one hand, large wings would be beneficial for long-distance migration, but on the other, lighter shades of orange in monarchs are associated with poorer flight performance and migration success (19, 20). Thus, these results concerning monarchs reared on *A. curassavica* seem paradoxical. We note that other research in this journal issue has more thoroughly investigated this topic (Freedman and Dingle).

Finally, the long-term declines in overwintering colony size in Mexico (44) have spurred numerous efforts to conserve the monarch migration, though most of these appear to be focused on breeding habitat enhancement

(e.g. 45, 46). However, conservation efforts should also take into account the many known and unknown factors responsible for migration failure, of which OE infection is one (10, 11, 14). Recall that by our estimate, as many as 7-17 million monarchs are infected within the migratory generation, and most of these will not succeed in reaching Mexico. As such, it would behoove all who have a stake in the management or conservation of monarchs, to be aware of the ramifications of OE infections to the monarch population, and know that the largest effect of OE is likely to be felt during the migratory journey.

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