Integrative and Comparative Biology

Integrative and Comparative Biology, volume 56, number 2, pp. 278–289
doi:10.1093/icb/icw056

Society for Integrative and Comparative Biology

SYMPOSIUM

Unravelling the Costs of Flight for Immune Defenses in the Migratory Monarch Butterfly

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From the symposium "Are Migratory Animals Superspreaders of Infection?" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2016 at Portland, Oregon.

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Synopsis Migratory animals undergo extreme physiological changes to prepare for and sustain energetically costly movements; one potential change is reduced investment in immune defenses. However, because some migrants have evolved to minimize the energetic demands of movement (for example, through the temporary atrophy of non-essential organs such as those involved in reproduction), migratory animals could potentially avoid immunosuppression during long-distance journeys. In this study, we used a tethered flight mill to examine immune consequences of experimentally induced powered flight in eastern North American monarch butterflies. These butterflies undergo an annual two-way long-distance migration each year from as far north as Canada to wintering sites in Central Mexico. We quantified immune measures as a function of categorical flight treatment (flown versus control groups) and continuous measures of flight effort (e.g., flight distance, duration, and measures of efficiency). We also examined whether relationships between flight and immune measures depended on reproductive investment by experimentally controlling whether monarchs were reproductive or in state of reproductive diapause (having atrophied reproductive organs) prior to flight. Of the three immune responses we measured, hemocyte concentration (the number of immune cells) was lower in flown monarchs relative to controls but increased with flight distance among flown monarchs; the other two immune measures showed no relationship to monarch flight. We also found that monarchs that were reproductively active were less efficient fliers, as they exerted more power during flight than monarchs in reproductive diapause. However, reproductive status did not modify relationships between flight and immune measures. Results of this study add to a growing body of work suggesting that migratory monarchs—like some other animals that travel vast distances—can complete their journeys with efficient use of resources and minimal costs.

Introduction

Animals that walk, fly, or swim long distances can expend massive amounts of energy (reviewed by Bonte et al. 2012; Matson and VanDijk 2016). In some cases, investment in energetically costly movements can come at the cost of defense against pathogens (Altizer et al. 2011; Buehler et al. 2010). Several observational studies of birds have documented reduced immune defense before or during migratory intervals (Owen and Moore 2006, 2008a, 2008b), or lower immune defenses in migrants versus resident individuals (Eikenaar and Hegemann 2016), although it is important to note that other variables such as temperature, age, and access to food resources can affect immune defense in wild animals. Experimental approaches have been successful in examining the effects of forced movement while controlling for other variables that further affect immunity in the wild (Matson et al. 2012; Nebel et al. 2012). Although migratory birds have been a primary focus of such work, Chapman and colleagues (2015) recently highlighted the potential for tethered flight studies with experimentally tractable migratory

Advanced Access publication June 2, 2016

insects to unravel the link between migratory effort and pathogen defense.

Costs of movement for immune defense might depend on whether or not animals are in reproductive condition during migration (Rankin and Burchsted 1992; Buehler and Piersma 2008;). Migrating animals can vary in the extent to which reproduction and migration are synchronous (Dingle 2006; Ramenofsky and Wingfield 2006, 2007). Some birds, for example, atrophy reproductive organs (and other organ systems) when they enter a pre-migratory state (Bauchinger v 2007; Vézina and Salvante 2010; Vézina et al. 2012). Similarly, migratory or dispersing insects can undergo a phenomenon called the ''oogenesis flight syndrome'', wherein the reproductive system shuts down at the onset of a flight or movement interval (Lorenz 2007, Guerra 2011). Since reproduction is known to be costly to immune defense (Schwenke et al. 2016), being reproductively mature during migration could intensify the costs of movement. In this sense, decoupling reproductive and migratory intervals allows animals to maintain important physiological requirements (such as immunity) during migration (Rankin and Burchsted 1992; Buehler et al. 2010; Vézina and Salvante 2010).

In this study, we examined whether active flight lowers immune defense in a migratory butterfly and further asked whether reproductive development compounds the cost of strenuous activity. We focused on monarch butterflies (*Danaus plexippus*) in eastern North America that undergo an annual long-distance migration traveling up to 3500 km each fall from Canada and the US to wintering sites in Central Mexico. In the fall, monarch adults emerge in a pre-migratory state called reproductive diapause, thought to be a necessary precursor to the long-distance migration (Herman 1973, 1981). This state can be experimentally induced by exposing monarch larvae to cooling temperatures and decreasing day length, mimicking fall conditions (Goehring and Oberhauser 2002). Adult monarchs in reproductive diapause have atrophied reproductive organs and excess stores of lipids needed to survive the overwintering period (Brower et al. 2006). In the early spring, monarchs break reproductive diapause and initiate the reverse migration that re-colonizes the southern part of their breeding range before dying. Subsequent generations of reproductively active monarchs fly substantial distances northward during spring and early summer to complete re-colonization of the breeding range (Malcolm et al. 1993; Miller et al. 2012), and potentially incur simultaneous costs of reproduction and flight on immune defenses.

Monarchs use both soaring and powered flight during migration; in soaring flight, monarchs gain altitude on thermal vents and coast long distances (Gibo 1986; Gibo and Pallett 1979) while in powered flight monarchs must continually flap their wings. Gibo and McCurdy (1993) estimated that a monarch would deplete a 140 mg fat supply in 1060 h of soaring flight versus only 11 h of powered flight. Monarchs would be unlikely to survive the migration by relying on powered flight alone, but sustained flapping is a necessary component of migration under low-wind conditions or when facing adverse weather. Thus, powered flight is crucial to monarch migration and is likely to be energetically costly relative to gliding (Gibo and McCurdy 1993).

To test whether monarchs experience lower immune defense as a result of energetically expensive flight, we induced powered flight by flying monarchs on a tethered flight apparatus over consecutive days and measured subsequent changes in immune defenses. We tested the effects of forced flight on immunity in both reproductive and non-reproductive (diapause) monarchs to explore whether reproductive development compounds the costs of strenuous activity. We predicted that monarchs that were both reproductively active and forced to fly (compared to those in diapause and immobilized controls) would mount the lowest immune responses, and that immune defense would decrease with continuous measures of flight effort, based on a trade-off between flight-related energy expenditure and immune defense.

Methods

Monarch rearing

Five non-inbred monarch genetic lines were used in experiments; these were the grand-progeny of eastern North American migratory monarchs originally collected at St Marks Florida USA (a stopover site during the fall migration) in October 2014 and held to overwinter in the laboratory. Mating and egg laying occurred in a naturally lit room with ambient light from approximately 0630 to 2030 and temperatures from 26 °C (average nighttime low) to 29 °C (average daytime high) during June 2015 in Athens, GA, USA. Eggs were collected on stalks of greenhouse-reared *Aclepias incarnata* (swamp milkweed) and were laid in daily cohorts staggered across 14 days. At the early 2nd instar stage, monarch larvae were transferred from milkweed stalks to individual 0.5 l containers with mesh screen lids and housed in controlled environmental chambers

(Model I-36VL, Percival Scientific, Perry IA) for the remainder of the experiment.

Manipulation of reproductive status

In monarchs, adult reproductive diapause is triggered by environmental conditions, especially temperature, photoperiod and host plant quality experienced at the larval stage (Goehring and Oberhauser 2002). We generated non-reproductive (diapause) monarchs by rearing in "Fall" conditions with cool night temperatures and decreasing day length: temperatures were 17 °C nighttime, 23 °C daytime with a decreasing photoperiod (13:11 l:d reduced by 2 min per 24 h, to a final photoperiod of 11.5:12.5 l:d), similar to conditions used to induce diapause in Goehring and Oberhauser (2002). We generated reproductive monarchs by rearing in "Summer" conditions: 26 °C nighttime, 28 °C daytime with a constant long photoperiod (16:8 l:d). Although the terms "Fall" and "Summer" refer to rearing conditions, we acknowledge that these groups do not represent the distinct summer breeding and fall migratory generations that occur in the wild, but instead represent two groups reared to differ in reproductive status. Monarchs developed at different rates under these temperature regimes (mean days from hatch to adult eclosion: 19.8 days in Summer and 37.1 days in Fall), so all of the Summer monarchs eclosed prior to the Fall monarchs. Upon emergence as adults (eclosion), males were euthanized by freezing; this study used only female monarchs because their reproductive status is easier to assess by dissection, and because reproductive costs of egg production in females are predicted to be greater than costs of sperm production in males (Oberhauser 1988, 1989).

Manipulation of flight activity

On the first day after eclosion, monarchs were assigned to one of three flight treatment groups: Flown, Tethered Control and Unhandled Control. Monarchs in the Flown group ($n = 35$ Summer, $n = 53$ Fall) were forced to fly to exhaustion (or to a maximum of 60 min, whichever came first) on each of four consecutive days between 0900 and 1800 h. In all cases monarchs were flown on days 4–7 post-eclosion to control for effects of age on flight and immune responses. The Tethered Control group ($n = 13$ Summer, $n = 14$ Fall) experienced the same handling procedures as the flown group (application of a wire for tethered flight) but instead of forced flight they were restrained in a glassine envelope for sixty minutes. The Unhandled Control group ($n = 6$ Summer, $n = 6$ Fall) served as a further control for handling stress; they did not have a wire attached and remained unhandled (in glassine envelopes) for the duration of the study, except for approximately 5 min every day when they were manually fed 20% honey water. We acknowledge that confinement in glassine envelopes could be stressful in itself, but this control group did not experience the effects of energy expenditure during forced flights.

We applied wires to monarchs in the flown and tethered control groups on the second day after eclosion. An 8 cm length of lightweight steel wire was affixed to the dorsal side of the monarch's thorax with a small piece of lab tape and rubber cement. Monarchs were weighed immediately before and after wire attachment, and the average mass of the wire and adhesive was 0.20 g (range 0.09–0.27 g, or approximately 35% of adult monarch body weight). Monarchs acclimated to the wire attachment for 48 h in a 0.6 m^2 mesh cage located inside the environmental incubator set to the same environmental conditions experienced as larvae; monarchs remained in these cages for the duration of the experiment except when flight treatments were applied. Flown and Tethered monarchs had *ad libitum* access to 20% honey water in petri dishes in their cages.

Flight trials were conducted in two separate interior rooms (to avoid daily variation in intensity of natural light) with one flight mill apparatus per room. The flight trial rooms and tethered flight mill were configured similarly to Bradley and Altizer (2005) and are described in detail in the Supplementary Material. Briefly, the flight mill consisted of a lightweight carbon rod (120 cm in length and 3 mm in diameter) attached to a stand on a nearly-frictionless steel pivot. At one end of the rod, a tape "flag" passed through a photogate (interrupting an infrared beam and transmitting information to a datalogger) upon each rotation of the monarch affixed to the opposite end of the carbon rod. The datalogger and associated software (Supplementary Material) record the timestamp of each rotation and the instantaneous velocity (m/s) of the flag's passage through the photogate. Given the dimensions of the rod, the circumference of the monarch's circular flight path was 4.23 m.

To initiate a trial, we taped the wire attached to the monarch to the carbon rod of the flight mill and released the monarch. Throughout the trial, if the monarch ceased flight for 10 consecutive seconds, the observer blew lightly on the monarch from behind (in the direction of flight) to stimulate flight. A trial was terminated when the monarch failed to resume flapping after three consecutive

"blows" separated by 10 s of gliding. All trials that did not end by this mechanism were terminated at 60 min (the maximum flight time permitted by logistical constraints). If a monarch's flight was terminated in five or fewer minutes, the monarch was fed 20% honey water and was re-flown one to three hours later. In all cases of re-flight ($n = 27$ total trials across 21 individuals), the second trial was longer in duration than the first and was subsequently used in data analyses. One monarch was excluded from the study after having flown fewer than 5 min on each of the first 2 days of flight trials.

Flight metrics and physical covariates

For each flight trial, we calculated the duration of flight (s), the distance flown (number of rotations * 4.23 m circumference, in m), and the average speed (distance flown/flight duration, in m/s). From these data, we calculated four summary flight effort metrics to assess the cumulative impact of four days of flight on immune measures. First, we summed the total flight duration and total distance flown over four flights. Using monarch weights obtained immediately before and after each flight, we calculated the mass lost per distance flown [(pre-flight mass – post-flight mass)/distance flown], then averaged (and log-transformed) this measure across the four flights to index the monarch's ability to retain mass during flight. Finally, we coarsely estimated mechanical power as an index of energy spent over time in flight (Ellington 1991; Hasselquist et al. 2007; Hedenström et al. 2001). To calculate power, we first estimated energy expended as kinetic energy (Joules) using the formula KE = ½ * mass(kg) * velocity^2 (m/s); we then divided KE by flight duration to obtain power (in Watts or J/s). Given the calculation of power (mass * velocity^2)/(2*time), a high powered flight could result from short duration but high-velocity flights or from a heavy monarch flying at low velocity (large numerator), while a low power could result from a long-duration flight (large denominator). Power was averaged across the four flight trials.

Both flight performance and immune responses in insects can be influenced by physical factors such as body mass, wing size, and relative masses of thorax and abdomen body segments (Berwaerts et al. 2002, 2006; Srygley and Kingsolver 2000), which we measured and controlled for in statistical analyses. On their first day post-eclosion, we measured the length (in mm) of the monarchs' right forewing, and we weighed monarchs to quantify initial mass (in g). Following the conclusion of the study, monarchs were euthanized in a −20 °C freezer for 1 h and the abdomens were removed and dissected to assess reproductive status as the presence or absence of mature oocytes (Goehring and Oberhauser 2002). Dissections were performed in pre-weighed aluminum pans so that abdomen tissue could be retained for drying; the pans and their contents (including eggs, fat, and other tissue) were oven-dried at 60 °C for 72 h and re-weighed (subtracting the original weight of the pan) to obtain the dry mass of the abdomen. We also separated, oven-dried (at 60 °C for 72 h), and weighed the thoraxes to subsequently calculate the abdomen:thorax mass ratio; larger relative thorax mass is associated with higher flight performance in butterflies (Berwaerts et al. 2002, 2006; Saastamoinen et al. 2010).

Monarch immune responses

Approximately 60 min after the conclusion of the fourth and final flight (or restraint in the case of Tethered Controls), we sampled hemolymph (insect blood) by puncturing an intersegmental vein on the dorsal side of the monarch's abdomen. We measured cellular immunity (hemocyte concentration) with fresh hemolymph and two aspects of humoral immunity (phenoloxidase and lysozyme-like activities) on aliquots of hemolymph frozen at −80 °C.

Hemocytes are invertebrate immune cells with functions including phagocytosis, encapsulation, and the production of antimicrobial peptides (Lavine and Strand 2002; Strand 2008). Under phase contrast microscopy at 400×, we counted total hemocytes (and calculated the average number of hemocytes per microliter) and differentially counted each of the four cell types—granulocytes, plasmatocytes, oenocytoids, and spheroid cells—scored as a percentage out of 100 hemocytes. Granulocytes, typically the most abundant, are phagocytic; plasmatocytes aggregate to encapsulate pathogens; oenocytoids produce molecular precursors to the melanization response; spheroid cells have an unknown function in monarchs (Lavine and Strand 2002; Strand 2008).

Melanization is an invertebrate immune response through which the enzyme phenoloxidase (PO) produces melanin, a toxic compound, in response to a bacterial pathogen or elicitor (Söderhäll and Cerenius 1998). Procedural details for this immune assay are provided in Supplementary Material. We define PO activity as the slope of the kinetic curve (absorbance per hour) during the linear phase of the melanization reaction (Hall et al. 1995; Barnes and Siva-Jothy 2000).

Lysozyme-like activity is the capacity of antimicrobial peptides in hemolymph to lyse bacterial cell wall (Adamo 2004). Hemolymph samples were incubated in agar plates containing freeze-dried *M. luteus* bacteria, and we measured the diameter of the clearance zones surrounding sample wells (see Supplementary Material for procedural details). These diameters were calibrated against a standard curve of known concentrations of chicken egg white lysozyme, so here lysozyme-like activity is in units of estimated concentration (μg/ml).

Statistical analyses

Dissections to determine reproductive status showed that 66% of monarchs reared in Fall conditions (intended to induce reproductive diapause) had zero mature oocytes, indicative of diapause (Goehring and Oberhauser 2002). Other studies inducing reproductive diapause experimentally have found that up to 50% of monarchs can emerge reproductive despite rearing conditions intended to produce diapause, and that a notable fraction of wild monarchs sampled during fall migration are also reproductively active (Goehring and Oberhauser 2002; S. Altizer unpublished data). All monarchs reared in summer-like conditions in the present study had mature oocytes. In primary analyses concerning the effect of reproductive status on immunity, flight, or flight–immunity relationships, we restricted the dataset to "Fall Diapause" (fall-reared and absent mature eggs) and "Summer Reproductive" monarchs (summer-reared and present mature eggs).

First, we used three separate one-way ANOVAs to test if reproductive status or rearing conditions affected flight effort metrics (total duration, total distance, average power, and mass lost/distance flown). In this case, mass lost/distance flown was $\log_{10}$ transformed to normalize the error variance. We next asked if flight treatment group and reproductive status affected immune defenses using separate two-way ANOVA models for each of the three immune measures (hemocyte concentration, PO activity, and lysozyme-like activity). We modeled these immune measures as a function of flight treatment category (unhandled control, tethered control, flown), reproductive status (fall diapause and summer reproductive), and the interaction between flight treatment and reproductive status. Initially, the flown flight treatment category included all monarchs that were forced to fly; a second round of analyses restricted this comparison to monarchs that flew a sum total duration of 2 h or longer across four days (approximately 30 min per day, or half of the maximum flight time). Tukey's HSD post hoc tests were used to evaluate differences among treatment groups with more than two levels. The relative percentages of the two most common hemocyte types (granulocytes and plasmatocytes) were modeled with the same predictor variables but using a generalized linear model (glm in base *R*) with a quasi-binomial error structure.

Our final analytical question was to investigate if, among flown monarchs, immune measures were predicted by physical covariates, continuous flight effort metrics, and interactions between flight effort and reproductive status. Initial general linear models (run separately for hemocyte concentration, PO activity, and lysozyme-like activity) included as predictors four flight effort metrics, four physical covariates (initial monarch mass, wing length, mass of the wire attachment, and abdomen:thorax mass ratio), and reproductive status both as main effects and in interaction terms with each flight effort metric. Owing to the large number of predictor variables, we simplified models following (Crawley 2002) until all terms in the model were significant or only the intercept was remaining.

We log-transformed the immune response variables for hemocyte concentration (cells/µl) and lysozyme-like activity (estimated lysozyme concentration in µg/ml) to normalize error variance. All continuous flight metrics and physical covariates were standardized to the consistent unit of standard deviations ($y = (x - \text{mean}(x))/(2*\text{SD}(x))$) prior to inclusion as predictor variables in linear models to facilitate comparisons of estimates and effect sizes. We investigated correlations among continuous flight effort metrics to identify issues with collinearity. For all models of immune response variables, we initially used linear mixed effects models in the R package lme4 (Bates et al. 2015) to test effects of random intercepts for hatch date (cohorts of eggs laid across 14 days) and monarch genetic lineage; however, these random effects explained very low amounts of variance in immune measures so final models contained only fixed effects modeled with general linear models. We used R version 3.1.3 (R Core Team 2015) for all analyses.

Results

General results

Summer Reproductive monarchs ($n = 53$) emerged at lighter weights than fall diapause monarchs ($n = 46$; $F_{1,97} = 10.64$, $P = 0.002$), but these two groups did not differ in wing length ($F_{1,97} = 0.29$, $P = 0.59$). Given the presence of oocytes in the abdomen,

Table 1 Summary metrics of flight parameters across all monarchs as well as separately by fall diapause (non-reproductive) and summer reproductive groups

	Sum flight duration (h)	Sum flight distance (km)	Log_{10} (Average mass lost/distance flown (mg/m)	Average power (Watts * 10^7)
All monarchs combined	2.65 ± 0.96	9.79 ± 4.30	−5.03 ± 0.24	2.41 ± 1.36
Fall diapause	2.95 ± 0.73	10.76 ± 3.47	−5.06 ± 0.24	1.93 ± 1.03
Summer reproductive	2.40 ± 1.07	8.96 ± 4.80	−5.00 ± 0.24	2.85 ± 1.49

Shown are means with standard deviation.

summer reproductive monarchs also had higher ratios of abdomen mass to thorax mass than fall diapause monarchs ($F_{1,97}$=21.58, $P < 0.005$).

On average, monarchs flew for a total of 2.65 h across all 4 days (range: 0.69-4.00 h), for flight distances of 9.79 km on average (range: 1.88–18.47 km; Table 1). Our initial analyses showed relationships among the flight performance metrics. Total flight distance was tightly correlated with total flight duration ($r^2 = 0.86$, $P < 0.005$). Mass lost per distance flown decreased with flight duration ($r^2 = 0.16$, $P < 0.005$), highlighting that mass lost per distance represents flight efficiency needed for long duration flights. Monarchs that had higher flight power flew significantly shorter total flight distances ($r^2 = 0.29$, $P < 0.005$), and also lost significantly more mass per distance flown ($r^2 = 0.09$, $P = 0.01$). Thus, higher flight power corresponds to lower flight efficiency, as more energy is expended per unit time.

Does reproductive status predict flight performance?

Reproductive status did not strongly influence the overall flight distance or duration of monarchs, but did affect measures of flight efficiency. Means (± standard deviation) of each summary flight metric by reproductive status are given in Table 1. Summer reproductive monarchs flew shorter-duration flights than fall diapause monarchs ($F_{1,61} = 5.32$, $P = 0.02$), but the flight distances of these two groups were statistically similar ($F_{1,61} = 2.81$, $P = 0.10$). Summer reproductive monarchs lost similar mass per distance flown as compared to fall diapause monarchs ($F_{1,61} = 0.76$, $P = 0.39$). Summer reproductive monarchs exhibited more power during flight than fall diapause monarchs ($F_{1,59} = 7.76$, $P = 0.01$), even after correcting for mass (power/initial monarch mass: $F_{1,59} = 10.64$, $P = 0.002$); in other words, reproductively active monarchs used more energy per unit time than monarchs in diapause (Fig. 1A). Further, total flight distance (across four days) decreased with average power in summer reproductive monarchs (distance ∼

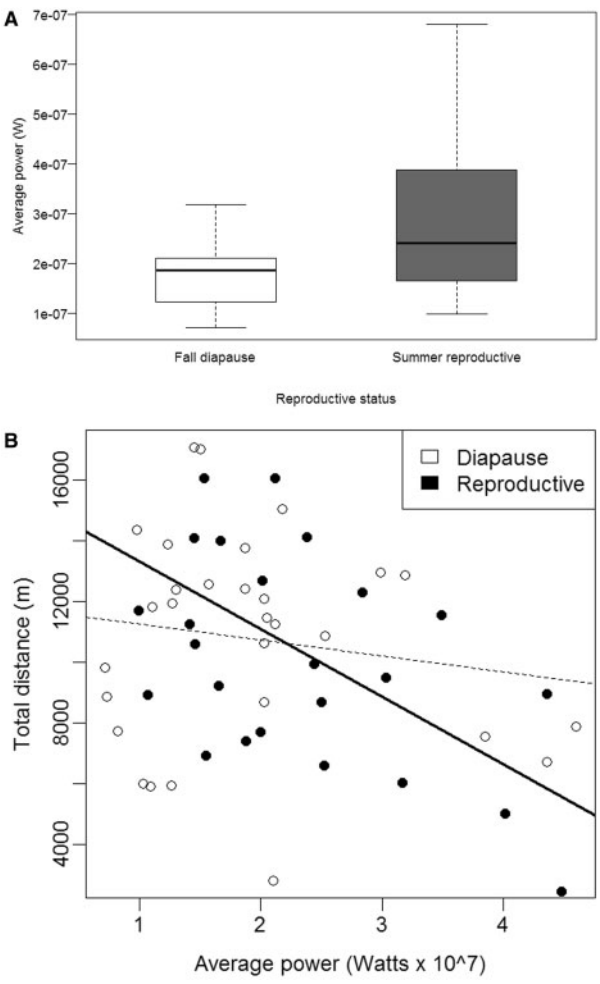

Fig. 1 Fall diapause and summer reproductive monarchs differ in the power exerted during flight. Mechanical power (in Watts) was estimated as *(monarch mass * flight velocity^2)/(2* flight time)*. (**A**) Summer reproductive monarchs had higher average power (used more energy per unit time) than fall diapause monarchs ($F_{1,59}$ =7.76, $P = 0.01$). Boxes designate the interquartile range divided by the median, and whiskers extend to 1.5 times the interquartile range beyond the box. (**B**) In summer reproductive monarchs only, total flight distance (across 4 days) decreased with average power, suggesting a cost of lower flight efficiency in terms of capacity to fly longer distances (ANOVA: Reproductive status * Power; $F_{1,57}$ =4.86, $P = 0.03$).

reproductive status * power; $F_{1,57} = 4.86$, $P = 0.03$; Fig. 1B). FLIGHT distance did not decrease with average power in fall diapause monarchs, suggesting that they do not incur the costs of increased energy expenditure for the capacity to fly longer distances.

Table 2 Results of two-way ANOVA models investigating main and interactive effects of flight treatment category (flown, tethered control, and unhandled control) and reproductive status (fall diapause versus summer reproductive) category on immune defense measures

Response variable Predictors	Mean square	Df	*F*	*P*
Phenoloxidase activity				
Flight treatment	0.75	2	0.38	0.68
Reproductive status	0.71	1	0.36	0.55
Flight treatment * reproductive status	2.03	2	1.04	0.36
Error	1.96	75		
Lysozyme-like activity				
Flight treatment	0.05	2	0.23	0.79
Reproductive status	**2.38**	**1**	**11.10**	**<0.005**
Flight treatment * reproductive status	0.00	2	0.01	0.99
Error	0.21	78		
Hemocyte concentration				
Flight treatment	**0.63**	**2**	**12.86**	**<0.005**
Reproductive status	**0.64**	**1**	**13.20**	**0.005**
Flight treatment * reproductive status	0.10	2	1.96	0.15
Error	0.05	93		

Do forced flight and reproductive status predict immunity?

Our results show limited evidence that flight treatment and reproductive status influenced immunity (Table 2). PO activity did not depend on either flight or reproductive status, but lysozyme-like activity was significantly lower in summer reproductive than fall diapause monarchs (Table 2). Hemocyte concentration was significantly lower in fall diapause than summer reproductive monarchs (Table 2) and post hoc analyses showed that hemocytes were lower in flown monarchs compared to either control group (Fig. 2). Among flown monarchs, hemocytes were lower in fall diapause than summer reproductive individuals ($P = 0.002$). There was no difference, however, in the control groups compared across reproductive status (Fig. 2). These results were qualitatively similar when we restricted this analysis to include only monarchs that flew a total duration of 2 h or greater across the four days of flight (Supplementary Material).

We further investigated whether the reduction in hemocyte concentration in flown monarchs was driven by changes in any particular cell type. The relative percentage of granulocytes (phagocytic cells) was higher in flown monarchs relative to unhandled and tethered controls, regardless of monarch reproductive status (GLM estimate ± standard error for effect of forced flight: 1.07 ± 0.29, $P <$ 0.005). The decline in granulocytes was mirrored by an increase in plasmatocytes (cells involved in the encapsulation response) in flown monarchs

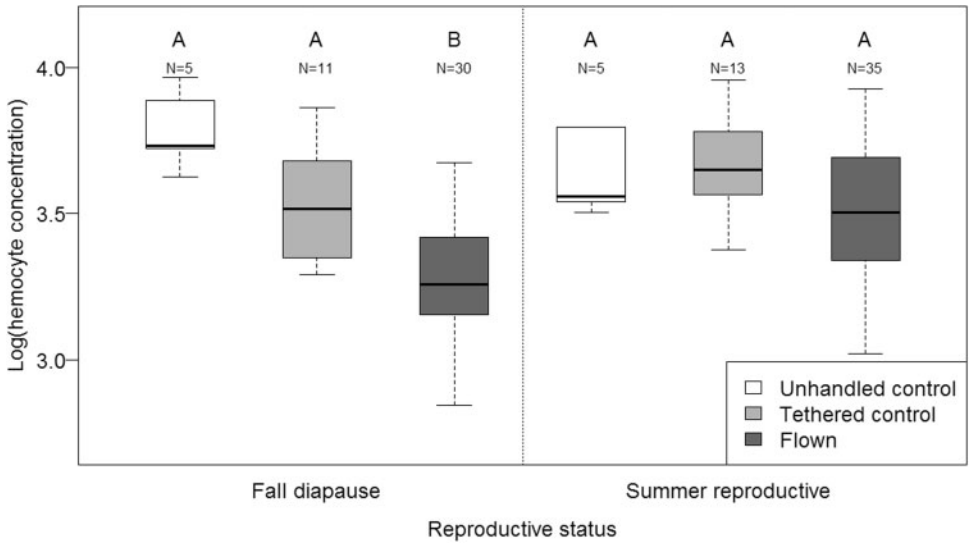

Fig. 2 Effects of flight treatment and reproductive status on hemocyte concentration. Post hoc analyses of the significant main effects (in two-way ANOVA) of flight treatment and reproductive status show that hemocyte concentration is lower in flown monarchs relative to both unhandled and tethered controls and in fall diapause monarchs relative to summer reproductive monarchs. Boxes designate the interquartile range divided by the median, and whiskers extend to 1.5 times the interquartile range beyond the box.

Table 3 Effects of continuous flight measures, physical covariates, and reproductive status on immune measures

Retained model structure and predictor variables	Estimate ± S.E.	*P*
Phenoloxidase activity ~ Wire mass *($P = 0.03$, adjusted $R^2 = 0.05$)*		
Wire mass	0.72 ± 0.33	0.03
Lysosyme-like activity ~ Abdomen:Thorax Ratio *($p = 0.03$, adjusted $R^2 = 0.05$)*		
Abdomen:Thorax ratio	−0.31 ± 0.11	0.005
Hemocyte concentration ~ Distance + Repro. status + Wire mass *($P < 0.005$, adjusted $R^2 = 0.28$)*		
Sum distance	0.12 ± 0.06	0.04
Reproductive status	0.26 ± 0.06	<0.005
Wire mass	−0.15 ± 0.06	0.02

relative to unhandled and tethered controls (GLM estimate ± standard error for effect of forced flight: -1.17 ± 0.32, $P < 0.005$).

Do monarch physical traits and flight effort predict immunity?

There was no evidence that immune measures decreased with measures of flight performance (Table 3). No flight metric or interactions with reproductive status were retained in final models predicting either PO activity or lysozyme-like activity (Table 3). PO activity was only predicted by the mass of the wire attached to the monarch: monarchs showed higher PO activity when they had larger wires attached. Lysozyme-like activity was negatively related to the ratio of abdomen to thorax mass, with larger relative abdomens corresponding to higher lysozyme activity (Table 3). One measure of flight was retained in the hemocyte model. Hemocyte concentration increased with the total distance flown, was higher for monarchs in reproductive status, and also increased with the mass of the wire attachment (Table 3).

Discussion

We tested the hypothesis that reproduction and flight would interact to reduce immune defenses in monarch butterflies. Our results show that reproductively active monarchs were less efficient fliers that monarchs in reproductive diapause during short-term powered flights in the lab environment. While the flights we experimentally induced in the lab cannot represent an entire migratory journey, powered flight is essential to migration and controlled flight effort in captive insects can yield important knowledge of the costs of flight (Chapman et al. 2015). Flight activity was both negatively and positively associated with variation in immune defenses; hemocyte concentrations were lower in flown monarchs compared to unflown controls, but the number of immune cells increased with flight distance in monarchs who flew. Interestingly, there were no interactive effects of reproductive status and flight on immunity suggesting that reproduction does not modulate the costs of short-term powered flight for immunity in monarchs. Overall, this study adds to the body of work suggesting that migration-adapted animals are resilient to the costs of flight despite the large expenditure of energy during long-distance movement and the assumption that this expenditure should come at the cost of immune defenses (Wikelski et al. 2003; Hasselquist et al. 2007).

Differences in flight metrics between summer reproductive and fall diapause monarchs were consistent with the idea that aspects of the migratory condition (induced by environmental cues in autumn) correspond to higher flight efficiency. Reproductive monarchs used more energy per flight time than monarchs in diapause, the reproductive status associated with fall migration, a finding mirrored by differences in flight demand between monarch generations in the wild. Individuals in the summer breeding generations are laden with eggs, have less flight muscle, and should be able to move quickly in short bouts within and among resource patches (i.e., nectar-flower gardens), whereas fall migrating individuals should use less energy during flight to be capable of flying long distances. In some migratory species studied in an experimental context, flight performance metrics measured in the lab have been found to predict actual migratory performance in the wild (Matyjasiak 2012). Importantly,

differences in power (energy expenditure per time) have important ties to migration success. For example, a recent study measuring flight energy expenditure (as overall dynamic body acceleration, ODBA) in wild white storks (*Ciconia ciconia*) found that juvenile birds used more flapping (inefficient flight) rather than soaring flight relative to adults (Rotics et al. 2016). Further, juveniles with higher ODBA during flight were less likely to survive their first migration (Rotics et al. 2016), highlighting the profound consequences of inefficient energy use.

A well-supported pattern in avian migration is that spring migrations are completed faster than fall migrations owing to the pressure to compete for mates and territory on breeding grounds (Karlsson et al. 2012; Nilsson et al. 2013). Accordingly, fall migrants have been found to use energy more efficiently than spring migrants, through behavioral adaptations such as relying on thermal vents for soaring flight and prolonging stopovers in unfavorable conditions (Duerr et al. 2015). Spring migrations in birds are also completed while many species are undergoing reproductive development, whereas fall migrations typically occur when individuals are reproductively refractory (Ramenofsky and Wingfield 2006). Experiencing dual demands of reproduction and movement should intuitively exacerbate the energetic demands of migration, especially given that hormonal pathways involved in both reproduction and the stress response (potentially induced by strenuous movement) can reduce immunity (Ashley and Wingfield 2011). In monarchs and other insects, reproductive status (i.e., diapause) and movement are tightly linked by physiological processes such as the release of adipokinetic hormone and changes to the insulin signaling pathway (Lorenz and Gäde 2009; Nylin 2013), which also influences immune gene regulation (Castillo et al. 2011). In *Drosophila*, for example, reproductive diapause increased the storage (rather than the usage) of lipids and carbohydrates, and also increased the expression of innate immune genes (Kubrak et al. 2014). The authors note that this increase in innate immunity may be adaptive in this species because *Drosophila* are sedentary during reproductive diapause and thus are highly vulnerable to attack by parasitoids (Kubrak et al. 2014). In contrast, monarchs are extremely mobile during the reproductive diapause stage, so it is less clear if a diapause-associated upregulation of immunity during migration would be adaptive.

Our results suggest limited immune costs of short-term powered flight. Other studies with captive animals have not observed immune costs of flight. In red knots (*Calidris canutus*) forced to fly in a wind tunnel, flown and unflown birds mounted similar levels of cell-mediated and humoral immune responses, and birds that failed to fly at all mounted the weakest immune responses, a result which the authors say indicates that poor-condition birds choose not to undertake strenuous journeys (Hasselquist et al. 2007). Similarly, western sandpipers (*Calidris mauri*) experienced immune costs of wind-tunnel flight only when the birds were previously challenged with a non-pathogenic simulated bacterial infection (Nebel et al. 2013). In fact, among healthy birds, bacterial killing ability was positively correlated with flight duration (Nebel et al. 2013), a result comparable to ours showing a positive relationship between hemocyte concentration and flight distance. As an alternative explanation for the lack of differences in immunity between flight treatment groups, we note that monarchs in our study were fed honey water ad libitum, and this unrestricted access to food may have obscured costs of flight for immune measures.

Among insects, there are additional examples of animals maintaining immune defenses during strenuous movement. In the Glanville fritillary (*Melitaea cinxia*), individuals forced to fly for 10 min while shaken in a jar were better able to encapsulate a foreign body compared to unflown controls, indicating that flight (or stress) may actually mobilize immune cells (Saastamoinen and Rantala 2013). During a "fight or flight" response to tethered flight in crickets (*Gryllus texensis*), hemocytes were found to rush into the hemolymph (Adamo 2010). In our study, the only immune measure that responded to forced flight was hemocyte concentration. Hemocyte activity was lower in flown monarchs relative to controls, potentially reflecting that forced flight shifts monarchs' molecular resources (e.g., apolipoporin III, a protein utilized by both stress and immune responses in insects) from immunity towards flight-related functions such as lipid transfer (Adamo and Parsons 2006; Adamo et al. 2008). However, hemocyte concentration is known in other insects to increase with stress, as hemocytes are released from the hemopoietic organ into the hemolymph (Adamo 2010); the positive relationship in our study between hemocyte concentration and flight distance could result from this link between increasing flight stress and mobilization of hemocytes. Alternatively, this result could be interpreted as evidence that monarchs unable to fly long distances (perhaps owing to lower stored lipids) were also constrained in their ability to proliferate hemocytes. It is important to note that we did not restrict

Strenuous activity can also reconfigure versus universally reduce immunity (Nebel et al. 2012; Adamo 2014). Our study shows evidence that different types of immune cells responded in opposing ways to flight stress; phagocytic granulocytes were more abundant and encapsulating plasmatocytes were less abundant in flown monarchs relative to controls. Hemocytes can be mobile (circulating in hemolymph) or sessile (bound to tissues in the body cavity), and this status of proliferation has been known in other insect systems to relate to stress and infection (Perez and Fontanetti 2011; King and Hillyer 2013). There remains very little known about mobilization of hemocytes in adult Lepidoptera, but our finding suggests a nuanced shift in deployed hemocytes that could be related to stress hormones released during flight (Diehl-Jones et al. 1996). Despite the established links between immunity, reproduction, and movement in insects (Rankin and Burchsted 1992; Schwenke et al. 2016) and vertebrates (Ashley and Wingfield 2011), very few studies have aimed to understand how animals re-organize their suite of immune defenses to optimize resources expended for reproduction versus flight (Adamo 2014).

The physiology of the monarch migratory phenotype is just beginning to be understood (Zhan et al. 2011, 2014). Initial work suggests that southwesterly flight orientation—an indicator of fall migration—is attributed to multiple aspects of the migratory phenotype beyond reproductive diapause alone (Zhu et al. 2009). We acknowledge that our use of environmental conditions (at the larval stage) to induce diapause may fail to generate ''migratory condition'' monarchs identical to those that would undergo the fall migration in the wild. For example, we do not know if our fall-reared monarchs would display oriented migratory flight, and we did not observe striking differences in key flight parameters (total distance flown, total flight duration, etc.) between fall diapause and summer reproductive monarchs. However, fall diapause monarchs were heavier than summer reproductive monarchs and had larger proportions of lean mass (thorax tissue) than lipid mass (abdomen tissue), two physical (non-reproductive) traits that are also associated with the fall migratory condition (Brower et al. 2006). Importantly, the physiological characteristics of spring recolonizing monarchs, which are both reproductively active and fly substantial distances, are almost entirely unknown (Herman and Tatar 2001). The results of the present study suggest that spring recolonizers may show reduced flight efficiency (like our summer reproductive monarchs) when compared to fall migrants but not necessarily at the expense of immune defenses. Future work could extend this study by employing the forced flight protocol with monarchs collected from the wild across the summer breeding, fall migratory, and spring recolonizing generations. Testing for immunosuppression in a large sample of actively migrating wild animals across seasons or generations when reproductive status differs will inform predictions about seasonal differences in susceptibility to pathogen infection, as well as increase our understanding of the challenges faced by an imperiled, charismatic migratory species.

Funding

This work was supported by the National Science Foundation Doctoral Dissertation Improvement Grant (1406695 to A.F.M., V.O.E. and S.A); and the Animal Behavior Society small grant to A.F.M. Organization and participation in the symposium was supported by SICB Division of Animal Behavior and Division of Ecoimmunology and Disease Ecology as well as a scientific meeting grant from the Company of Biologists.

Acknowledgments

We thank the UGA Instrumentation Shop for fabricating a second flight mill apparatus, and John Patrick, Ian Yeager, and Michael Holden, and Dara Satterfield for running numerous flight trials. Emilie Morris assisted with post-experiment data processing (dissecting and weighing monarch body segments) and Kyle McKay helped to process and analyze flight metric data. Troy Simon, Paola Barriga, and Ania Majewska provided helpful comments on earlier versions of the article.

Supplementary data

Supplementary data available at *ICB* online.

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