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Non-migratory monarch butterflies, *Danaus plexippus* (L.), retain developmental plasticity and a navigational mechanism associated with migration.

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Abstract

Monarch butterflies are best known from their migratory North American range, although many resident, year-round breeding populations are established throughout the world. Here, we evaluate two non-exclusive hypotheses for the loss of migration in resident monarch populations: (1) absence of cues that trigger migration and (2) loss of sensory, neural, or physiological systems required for migration. To evaluate the first hypothesis, we exposed resident monarchs from Queensland, Australia to decreasing larval photoperiod and observed reproductive development in resulting females to assess their propensity to show reduced reproductive development, a precursor for long-distance migration. To address the second hypothesis, we measured antennal circadian clock gene expression, a crucial element of the monarch's ability to directionally orient, in a resident and a migratory population. We found that Australian resident monarchs show reduced reproductive development in response to decreasing photoperiod, consistent with the "loss of cues" hypothesis. We found no differences in antennal clock gene expression between migratory and resident populations, inconsistent with the "loss of mechanism" hypothesis. Together, these data indicate that even after hundreds of generations of non-migration, monarchs retain two critical elements of their migratory repertoire: developmental plasticity associated with decreasing photoperiod and antennal circadian rhythms necessary for directional orientation.

Key words: Monarch butterfly, migration, development, reproductive diapause, circadian clock, navigation

Introduction

Long distance migration has evolved across the tree of life as an adaptation to temporal and spatial variation in resource availability (Dingle, 2014). Among insects, perhaps the best-known migration is that of the monarch butterfly in North America (*Danaus plexippus*, Linnaeus, 1758) (Urquhart & Urquhart, 1978; Brower, 1995; Gustafsson *et al.*, 2015). To accomplish their long-distance migration and subsequent overwintering, monarchs exhibit a correlated syndrome of changes in morphology, physiology, and reproductive behavior (Herman, 1981; Masters, Brower & Malcolm, 1988; Brower, Fink & Walford, 2006). Long distance migration distinguishes North American monarch populations from long-established non-migratory populations in Central and South America and the Caribbean, as well more recently established non-migratory populations on many Pacific islands.

Monarch migration is preceded by the onset of a physiological state known as reproductive diapause (Herman, 1973; Brower *et al.*, 1977). In monarchs, reproductive diapause is influenced by juvenile hormone titers (Herman, 1981) and entails decreased investment in reproductive development and greater allocation to lipid reserves required for uninterrupted long-distance flight (Beall, 1948; Brown & Chippendale, 1974). Monarchs from eastern North America exhibit true reproductive diapause, whereby migrating and overwintering adults remain reproductively inactive even after prolonged exposure to summer-like conditions that are conducive to reproduction (Herman, Brower & Calvert, 1989). This is in contrast to other monarch populations, which exhibit less pronounced refractory periods and resume reproductive development after relatively short periods of exposure to favorable conditions (James, 1982; Herman *et al.* 1989). Goehring and Oberhauser (2002) evaluated cues potentially responsible for inducing reproductive diapause in eastern North American monarchs, including factors such as absolute temperature, fluctuations in temperature, photoperiod, decreases in photoperiod, and age of larval host plant material. Among these, they found that decreasing photoperiod, older host plant material, and fluctuating temperatures during larval development—all indicative of the onset of North American autumnal conditions—

were associated with induction of reproductive diapause (Goehring & Oberhauser, 2002).

Migration in monarchs is thought to be highly conserved and dates back to at least the common ancestor of *D. plexippus* and *D. erippus* (Zhan *et al.*, 2014). However, over the past 200 years, monarchs have achieved a nearly global distribution, with at least three independent waves of colonization out of the ancestral North/Central American range (Ackery & Vane Wright, 1984; Zalucki & Clarke, 2004; Pierce *et al.*, 2014; Zhan *et al.*, 2014). Throughout most of their introduced range, monarchs are established as year-round breeding residents, with the exception of southern Australia, where small-scale seasonal migration is known to occur (Smithers, 1965; James, 1979; James, 1993; Dingle, Zalucki & Rochester, 1999). Previous studies have compared resident and migrant populations of monarchs and shown that migrants typically show larger and more elongated forewings, presumably as an adaptation for long-distance flight (Beall & Williams, 1945; Dockx, 2007; Altizer & Davis, 2010; Li, Pierce & de Roode, 2016; Yang *et al.*, 2016). Furthermore, genomic and transcriptomic evidence indicate both fixed differences in haplotype and expression level differences between migratory and resident populations, despite the recency of the monarch's introduction in many of these locations (Zhan *et al.,* 2014).

While it is clear that selection has favored non-migration and associated phenotypes in recently established monarch populations, the proximate causes of the transition to resident status have yet to be fully explored. One possibility is that resident monarchs simply no longer experience the relevant environmental cues that trigger migratory behavior in their North American range, hereby referred to as the "loss of cues" hypothesis. Under this scenario, resident monarchs exposed to conditions akin to those that elicit reproductive diapause in eastern North American monarchs may still respond similarly to their migratory ancestors and exhibit phenotypes conducive to long-distance migration.

Another non-mutually exclusive explanation for the loss of migration in resident populations is that monarchs have lost or suppressed elements of the sensory, neural, or physiological systems that link environmental cues with

103 migratory behavior, hereby referred to as the "loss of mechanism" hypothesis. For
104 example, the sensory system that enables detection of changing photoperiod
105 (suspected to be related to circadian clock genes expressed in the pars lateralis
106 (Sauman *et al.*, 2005; Zhan *et al.*, 2011) may be altered in resident monarchs.
107 Alternatively, non-migratory monarchs may still be capable of sensing and encoding
108 environmental cues relevant for the onset of migration, but simply do not respond
109 to these cues because of selection against individuals that migrate out of areas
110 suitable for year-round breeding. One possible target of selection that could inhibit
111 migration is the set of navigational mechanisms that aid in directional orientation
112 (Merlin, Gegear & Reppert, 2009; Guerra, Gegear & Reppert, 2014).
113 Directional orientation in monarchs involves a time compensated sun
114 compass, which integrates information from visible and polarized wavelengths with
115 an internal clock to track the sun's changing position over the course of the day. The
116 internal clock that communicates with the sun compass is expressed in the
117 monarch's antennae (Froy *et al.*, 2003; Reppert, Zhu & White, 2004; Merlin *et al.*,
118 2009; Guerra *et al.*, 2012). Populations of reproductive summer butterflies in North
119 America express antennal clocks but do not show the directional characteristics of
120 migration (Zhu *et al.*, 2009), although no studies to date have tested antennal clock
121 gene expression in fully resident monarchs. Thus, the shift from migratory to
122 resident status may be related to altered expression of antennal circadian clock
123 genes and disruption of orientation capabilities. Possible patterns of antennal clock
124 gene expression in residents might include (1) loss or alteration of clock gene
125 expression/function due to relaxed selection associated with loss of migration (2)
126 retention of antennal clock gene function due to insufficient time for loss of function
127 (3) retention of clock gene function for use in navigation unrelated to long-distance
128 migration (4) retention of clock gene function for uses unrelated to navigation.
129 In this paper, we evaluate two possible explanations—absence of
130 environmental cues and altered antennal clock gene expression—for the shift to
131 resident status in Pacific populations of monarch butterflies (Figure 1). In one
132 experiment, we evaluated the loss of cues hypothesis by rearing resident monarch
133 butterflies from Queensland, Australia under either constant or decreasing

photoperiod treatments and assessing reproductive development in the adults that emerged. In the second study, we evaluated an element of the loss of mechanism hypothesis by measuring diurnal circadian clock gene expression in resident monarchs from the island of Guam and comparing these to diurnal clock gene expression patterns in a migratory population from California, USA. We found that Australian resident monarchs do indeed show reduced reproductive development in response to decreasing photoperiod, consistent with previous studies in migratory monarchs and consistent with the loss of cues hypothesis. We found no differences in antennal clock gene expression between migratory and resident populations, inconsistent with the loss of mechanism hypothesis and suggesting either retention for use in functions besides migration or insufficient time for loss of function. Together, these data indicate that even after hundreds of generations of resident status, monarchs retain developmental plasticity associated with decreasing photoperiod and a key component of the navigational apparatus necessary for long-distance migration.

Methods

Reproductive diapause experiment

In our first experiment, we sought to determine whether resident butterflies would respond to environmental cues associated with the induction of reproductive diapause and migration in eastern North American monarchs. We collected 11 female butterflies from two populations (Pinjarra Hills: 27°32'26.7"S, 152°54'22.7"E; Mount Crosby: 27°31'45.2"S, 152°47'46.2"E) of resident, winter-breeding monarchs in Queensland, Australia between June 24-28, 2016. Females were all reproductively active upon collection, and all monarch life history stages were present on host plants at the time of collection. This continuous breeding is consistent with previous observations from Queensland (Zalucki & Kitching, 1982a), where temperatures rarely fall below developmental zero for monarchs (Zalucki, 1982). Average temperatures at the sites of collection are typically 21°C : 8°C in late June, with day lengths of approximately 11 hours (Australian Bureau of Meteorology). These 11 females were enclosed in mesh bags on host plants and the

resulting eggs were used for rearing experiments. All female butterflies (and 118/122 overall butterflies collected in Queensland during June and July) used in this experiment were infected with the protozoan parasite *Ophryocystis elektroscirrha* (hereby OE), consistent with high OE infection rates noted in other continuously breeding populations (Satterfield, Maerz & Altizer, 2015; Satterfield *et al.*, 2016). We examined eggs under 40x magnification and removed any visible OE spores with a paintbrush. Eggs from 11 female lines were used for rearing experiments, and eggs from each line were split evenly between experimental treatments.

All larvae used in the experiment hatched within 24 hours of one another and were immediately separated into two Percival growth chambers (GR36-L, Percival Scientific, Inc., Perry, IA) that each included four Phillips F32T8/TL741 32W fluorescent lights averaging 2,470 lumens per light. We chose to focus exclusively on changes in photoperiod as our diapause-inducing cue, since this cue is the most consistent and easily manipulated elicitor of diapause behavior described in the series of experiments carried out by Goehring and Oberhauser (2002). One growth chamber featured a constant 12:12 light/dark (LD) cycle, with a temperature of 28° C during the light period and 18° C during the dark period (hereby constant photoperiod treatment). The other growth chamber featured a LD cycle that started at 14:10, and then decreased by 4 minutes per day until it reached 12:12 30 days later (hereby decreasing photoperiod treatment). Temperatures and rate of decreasing photoperiod were chosen to reflect late August conditions at the northernmost extent of the monarch's North American range. The decreasing photoperiod growth chamber used a temperature ramp that peaked at 28° C during the light to dark transition, and was at its minimum of 18° C during the dark to light transition. The temperature ramp in the decreasing photoperiod treatment ensured that larvae in both growth chambers experienced the same number of developmental degree days in each 24 hour window. Degree day calculations are based on Zalucki (1982), which describes a developmental threshold of approximately 12°C for all larval instars. Both growth chambers were maintained at 70% humidity. Although our treatments conflate the influence of decreasing

photoperiod and total photoperiod—the decreasing photoperiod treatment necessarily featured 43 additional *cumulative* hours of light—Goehring and Oberhauser (2002) manipulated both factors and suggest that absolute photoperiod is unlikely to be the salient feature controlling diapause status.

Larvae were kept in petri dishes (100 x 15 mm) within their respective growth chambers until they reached second instar stage. These larvae were then separated into individual 500 mL clear plastic containers with clear plastic lids and fed using clipped leaf material from the milkweed *Gomphocarpus fruticosis* (Apocynaceae: Asclepiadoideae) collected in the field. All host plant material was washed with a 2% hypochlorite bleach solution and then thoroughly rinsed with tap water to kill OE spores. Containers were cleaned and new leaf material was added every 2-3 days. Individuals pupated in the same containers in which they were reared, and dates of pupation and eclosion were recorded for all individuals.

Upon emergence, any adult butterflies without fully developed wings (n = 12) were discarded, and all other butterflies were placed into glassine envelopes (n = 170 remaining adults). Discarded butterflies came evenly from both larval rearing treatments (n = 7 from decreasing photoperiod treatment, n =5 from constant photoperiod treatment), and so any subtle selection effects associated with OE infection should be minimal. These adults were further split into two temperature treatments to determine whether conditions experienced immediately post-eclosion would influence reproductive development, as per the results of James (1983). Both treatments included 12:12 LD cycles and 70% relative humidity. One treatment (hereby the warm adult treatment) included a 28° C light phase and an 18° C dark phase. The other treatment (hereby the cool adult treatment) had a 21° C light phase and a 15° C dark phase. Adult butterflies in each of these treatments were fed daily with a 20% honey water solution. These adults were raised until they had accumulated 70 degree days above the reported adult reproductive development threshold of 12° C, consistent with the findings that females develop mature oocytes after 6 days of summer conditions (Zalucki, 1981; Oberhauser & Hampton, 1995). This entailed 7 days of development for adults in the warm treatment and 11 days of development for adults in the cool treatment. Developmental zero for adult

227 butterflies is based on the estimate of 12° C provided by Zalucki (1981) that also
228 used Australian monarchs. Adult butterflies were stored in envelopes whose labels
229 did not indicate their larval rearing treatment in order to minimize potential
230 observer bias (Kardish *et al.*, 2015).
231 After accumulating 70 degree days, adult butterflies were dissected and
232 assessed for reproductive development. Female dissections were carried out in the
233 same manner as described in Oberhauser and Hampton (1995). Oocytes were
234 counted and classified as being either yolked (visible yellow coloration) or fully
235 chorionated (ridges along length of oocyte); subsequent analyses use primarily
236 counts of yolked oocytes because of the small number of females that had fully
237 chorionated oocytes (n = 21/81 females). Because vitellogenesis does not
238 commence until eclosion in monarchs (Pan & Wyatt, 1976), we consider yolked
239 oocyte production to be an appropriate measure of adult reproductive development.
240 We also assessed male reproductive development by taking the wet and dry mass of
241 the sac containing the testes; however, because the signature of adult reproductive
242 development for males is more likely to be in the mass of the seminal vesicle, we do
243 not report results for male testes. All butterflies were weighed (at the time of
244 dissection, rather than eclosion), and forewings were scanned and measured using
245 the image processing software ImageJ (Schneider, Rasband & Eliceiri, 2012) to
246 assess the size and shape of the wings in accordance with the methods described in
247 Altizer and Davis (2010). Finally, adults were also assayed for the presence and
248 intensity of OE infection by approximating spore counts on slide mounts and
249 assigning a score based on a relative scale from 0-5 that corresponds to log10
250 transformed spore loads (i.e. a score of 0 indicates no infection, and a score of 5
251 indicates >10,000 spores per individual) (scale adopted from Altizer, Oberhauser &
252 Brower 2000).
253 For the purposes of this study, we refer to the absence of any yolked oocytes
254 as reproductive diapause and treat the number of yolked oocytes produced by
255 females as a continuous measure of reproductive development. Reproductive
256 diapause in monarchs is typified by reduced investment in reproductive structures,
257 reallocation of resources into migration-associated physiology, and a pronounced

refractory period. Although we recognize that other authors distinguish between diapause and temporary reproductive dormancy / oligopause (James, 1982; Pocius, 2014), we consider this distinction to be largely semantic and reflective of different points along a continuum of reproductive responses. Because adult butterflies were exposed to prolonged periods with conditions suitable for reproductive development (7-11 days with temperatures between 15°C - 28°C), we consider the absence of any yolk deposition in these butterflies to indicate a refractory period consistent with diapause.

Data were analyzed using linear and generalized linear models in R version 3.4.1 (R Core Development Team 2017). Briefly, models included the effects of larval treatment (constant vs. decreasing photoperiod) and its interaction with adult treatment (warm vs. cool) and female line, with OE infection status and butterfly sex as covariates. Response variables of interest included whether females were in reproductive diapause (presence/absence of yolked oocytes), number of yolked oocytes, number of mature oocytes, time to eclosion, adult mass, and adult forewing area. Models were initially tested with all possible covariates and interactions between larval treatment, adult treatment, female line, and sex (when appropriate), and then model comparisons based on AIC scores were used to determine whether the inclusion of interaction terms was necessary. For the model that used presence/absence of yolked oocytes as a response variable, we used a binomial GLM with a logit link function. For the model that used mature oocytes as a response variable, we used a zero-inflated Poisson GLM with a logit link function implemented in the pscl package (Zeileis, Kleiber & Jackman, 2008) because of the high proportion of 0s in our count data; for this model, only larval photoperiod and adult temperature were included as predictors to enable model convergence. Summary statistics were generated using Type II analysis of variance implemented in the car package (Fox 2016). For a summary of all models evaluated, see Table 1.

Circadian clock gene expression experiment

To determine whether resident and migratory monarchs possess functional antennal circadian clocks, we measured expression of key clock genes in resident

289 butterflies from Guam and compared expression levels to migratory butterflies from
290 California. Butterflies captured on Guam (n = 12 females) were returned to
291 laboratories in Davis, CA, USA. Their offspring were reared in growth chambers
292 under conditions similar to the July Guam environment (LD 14:10, 28:27.5° C) and
293 within 5-8 days of adult eclosion were processed for detection of diurnal differences
294 in clock gene expression in antennae and the head. Using reverse transcription of
295 total RNA to cDNA followed by quantitative real-time polymerase chain reaction
296 (qPCR), we analyzed *per, tim,* and *cry2* steady state mRNA levels as a function of two
297 circadian time-points (zeitgeber times), ZT5 (day) and ZT14 (night). These times
298 were chosen because in migratory monarchs the circadian expression of these genes
299 was at or near low points 5-6 hours after light onset (ZT5-6) and at or near high
300 points 2-3 hours after the onset of darkness (ZT14-15) (Merlin *et al.*, 2009).
301 Identical analyses were performed on butterflies from known migratory California
302 populations reared under the same conditions

303 At ZT5 and ZT14, butterflies were killed and immediately frozen on dry ice.
304 The antennae and heads were separated from the bodies and stored at -80°C until
305 RNA extraction. The antennae were homogenized as follows: two stainless steel
306 5mm beads (Qiagen, Valencia, California) were placed in a round-bottomed tube
307 containing 3-4 pairs of antennae per pooled sample and 1 ml of TRI-reagent (Sigma,
308 St. Louis, Missouri). The sample was shaken three times at 50 Hz for 45 seconds
309 using a TissueLyser (Qiagen). The heads from the same individuals were frozen in
310 liquid nitrogen and manually ground using a mortar and pestle. 3X TRI-reagent was
311 added to the homogenized head tissue and total RNA extraction was performed as
312 described in Hamby *et al.* (2013). Extracted RNA was treated with Turbo DNA-free
313 kit (Life Technologies, Grand Island, New York).

314 RNA was quantified and its quality assessed using the Experion Bioanalyzer
315 (Bio-Rad, Hercules, California). 1.5 μg of total RNA was used to synthesize cDNA
316 using the Thermoscript RT-PCR System (Life Technologies). Dilutions (1:2) of cDNA
317 samples were used in qPCR. Gene specific primers were designed to amplify
318 monarch *period (per), cryptochrome2 (cry2),* and *timeless (tim)* with amplicon size of
319 around 150 bp and optimized at an annealing temperature of 62°C. Internal control

primers to amplify *rpl32* were optimized at the same annealing temperature for relative quantification. Primer sequences are provided in Table S1. The qPCR reactions were performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) in a CFX 96 Touch Real-Time PCR Detection thermal cycler (Bio-Rad). The cycling parameters were 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and an extension step at 62°C for 30 seconds. The reaction was proceeded with a melt curve analysis ranging from 65° to 95°, with temperature increases of 0.5°C every 5 seconds. Data were analyzed as outlined in Hamby *et al.* (2013) using the ΔΔ-Ct method. At least three biological replicates were performed for each combination of population and ZT, with each biological replicate consisting of three technical replicates for qPCR. We analyzed data in R using analysis of variance that included expression levels nested within technical replicate, with antennae and heads evaluated separately. Here, an effect of ZT time implies differences in expression levels between ZT5 and ZT14, and an interaction between [population*ZT time] implies differential diurnal expression patterns between populations.

Results

Female butterflies reared under the decreasing photoperiod treatment were significantly more likely to exhibit reproductive diapause ($z = 2.41$, $p = 0.016$) and produced significantly fewer yolked oocytes ($F_{1,55} = 7.97$, $p = 0.007$) and marginally fewer mature oocytes ($z = 1.95$, $p = 0.052$) than females reared under a constant photoperiod treatment (Table 1, Figure 2). Of the 16 females that produced no yolked oocytes, 12/40 were from the decreasing photoperiod treatment, compared to 4/40 from the constant photoperiod treatment. Among the 64 females that did show reproductive development, yolked oocyte production was significantly higher in the constant photoperiod treatment (42.4 ± 4.2) compared to the decreasing photoperiod treatment (29.4 ± 3.2) (Figure 2); the same pattern was observed for mature oocytes, with more produced by females from the constant photoperiod treatment (5.3 ± 1.5) compared to the decreasing photoperiod treatment (2.6 ± 1.1)

(Figure 2). The decreasing photoperiod treatment was associated with a significantly longer development period (323.3 ± 11.1 day degrees from egg to eclosion) compared to the constant photoperiod treatment (289.1 ± 13.9 day degrees) ($F_{1,130} = 304.12$, $p < 0.001$) (Table 1). The resulting butterflies from the decreasing photoperiod treatment had significantly higher body masses ($F_{1,124} = 31.02$, $p < 0.001$) (Table 1, Figure 3A) and marginally larger forewings ($F_{1,129} = 2.92$, $p = 0.090$) (Table 1, Figure 3) than those reared under constant photoperiod.

Conditions experienced post-eclosion did not significantly affect the reproductive development of female butterflies, and reproductive development was actually slightly greater in the cool adult treatment for females ($F_{1,55} = 1.46$, $p = 0.233$). Larval treatment and adult treatment interacted significantly to predict female reproductive development ($F_{1,55} = 12.43$, $p < 0.001$), with highest yolked oocyte production in the treatment that combined constant larval photoperiod and cool adult temperature. Post-eclosion conditions significantly affected the body mass of adults, with adults that experienced warm conditions weighing significantly less than those in the cool temperature treatment ($F_{1,124} = 45.02$, $p < 0.001$). Approximately half of the assayed butterflies (86/170) were infected with OE, although OE infection status did not significantly impact reproductive development, development time, body mass, or wing morphology in adult butterflies (Table 1, Figure S1).

We found significant family level effects for female reproductive development ($F_{1,55} = 2.02$, $p = 0.048$), development time ($F_{1,130} = 4.40$, $p < 0.001$), and forewing area ($F_{1,129} = 6.41$, $p = 0.002$) (Table 1). We also found that maternal lines differed in their response to the decreasing photoperiod treatment, as indicated by a significant interaction effect between maternal line * larval treatment ($F_{1,55} = 2.17$, $p = 0.044$) (Figure S2). Maternal line was only a marginal predictor for adult body mass ($F_{1,124} = 1.85$, $p = 0.059$) (Table 1).

Migratory butterflies from California and resident butterflies from Guam displayed identical diurnal patterns of clock gene expression in both heads and antennae (Figure 4). Clock gene expression in heads was significantly greater at ZT14 than ZT5 ($F_{1,96} = 76.28$, $p < 0.001$), but there were no expression differences

between resident vs. migratory populations ($F_{1,96} = 0.31$, $p = 0.58$). Likewise, antennal clock gene expression was significantly greater at ZT14 than ZT5 ($F_{1,96} = 122.76$, $p < 0.001$), but this effect did not differ based on source population ($F_{1,96} = 0.02$, $p = 0.90$).

Discussion

In this paper, we show that monarchs from a resident population in Australia exhibit reduced reproductive development when exposed to environmental conditions known to stimulate migratory behavior in North American monarchs. Furthermore, we demonstrate that larval exposure to decreasing photoperiod is associated with a suite of correlated responses, including a longer development period, greater adult mass, and slightly larger forewings, a pattern that has not been shown in any population of monarch butterfly. These responses varied based on maternal line, suggesting that there may be heritable genetic variation for diapause responses. Finally, we show that resident butterflies from Guam exhibit identical patterns of antennal circadian clock expression to migratory monarchs from California. This suggests that resident butterflies retain a necessary but not sufficient component of their time-compensated sun compass. We discuss possible functions of this sun compass in resident monarch populations.

We found that resident Australian monarchs respond to a decreasing photoperiod treatment during larval development, in accordance with the loss of cues hypothesis for non-migration. The fact that female monarchs reared under a decreasing photoperiod treatment were both more likely to show no reproductive development (i.e. no yolked oocytes), and that females in this treatment produced significantly fewer yolked oocytes, provides strong evidence that monarch butterflies, regardless of source population and migratory status, respond to photoperiod cues during their larval development. Our results from the diapause experiment are consistent with Goehring and Oberhauser (2002) in showing that decreasing photoperiod elicits reduced reproductive development in monarch butterflies. Observing this same result in a non-migratory population suggests that plastic responses to seasonal changes are a common feature of all monarch

412 populations and that the transition to resident status may not be irreversible. These
413 results are also consistent with the deep evolutionary origins of migration within
414 Danaine butterflies. Migration is thought to be the ancestral condition for monarchs
415 and is likely rooted in genetic variation that has been maintained for millions of
416 years (Zhan *et al.*, 2014). Thus, even after hundreds or thousands of generations of
417 non-migration, ancestral variation associated with migration may be maintained
418 and expressed upon exposure to relevant conditions.
419 The finding that Australian females respond to decreasing photoperiod
420 during their larval development is in contrast to the findings of James (1983), who
421 suggested that it is conditions experienced immediately post-eclosion and not
422 during larval development that influence reproductive status in Australian
423 monarchs. However, we note that James (1983) did not formally evaluate the
424 influence of larval rearing conditions and instead made this assertion based on
425 observations of overwintering cluster formation and reproductive development. We
426 also note that the conditions experienced by adult butterflies in our experiment did
427 not significantly affect reproductive development. This may be due to the relatively
428 high temperatures (21:15° C) used in the cool adult treatment, which is warmer
429 than all of the conditions evaluated by James (1983) and consistent with winter
430 temperatures in Queensland, where monarchs breed year round.
431 Monarchs reared under the decreasing photoperiod treatment had
432 significantly higher body mass and somewhat larger forewing area compared to
433 monarchs reared under the constant photoperiod treatment. Although we did not
434 specifically measure lipid content in adult butterflies, higher lean body mass is
435 generally consistent with greater lipid reserves, a characteristic commonly reported
436 for migratory monarchs and monarchs in reproductive diapause (Alonso-Mejia *et*
437 *al.*, 1997; Brower *et al.*, 2006). Previous studies have not shown any link between
438 larval rearing conditions and monarch wing morphology, but larger forewings are
439 thought to be conducive to soaring/gliding and the long distance movements
440 associated with migration (Doccx, 2007; Altizer & Davis, 2010; Yang *et al.*, 2016)..
441 Wing area scaled isometrically with body size and independently of larval
442 photoperiod (Figure S3). Plasticity in monarch wing morphology as a function of

443 larval rearing conditions should be investigated further, as this could help to explain
444 some of the observed morphological differences between migratory and non-
445 migratory monarch populations (e.g. Altizer & Davis, 2010).
446 Given that decreasing/short photoperiod and cool temperatures have been
447 associated with induction of reproductive diapause in monarchs, it is interesting to
448 consider why all of the wild-caught adult females used in this experiment were
449 reproductively competent at the time of capture, despite the short day lengths (LD
450 11:13) and cool temperatures (21°C : 8°C) that they were experiencing. The most
451 likely explanation for the reproductive status of these butterflies is that they are
452 themselves responsive to seasonal changes, but that the year-round availability of
453 their milkweed host plants overrides developmental plasticity associated with
454 seasonality. Previous work has shown that exposure to milkweed can stimulate
455 reproductive development in female monarchs (Goehring & Oberhauser, 2004), and
456 recent research has highlighted that milkweed availability along the monarch's
457 southerly migration route in eastern North America can elicit breeding in adults that
458 were previously in diapause (Batalden & Oberhauser, 2015). Thus, even though
459 monarchs within Queensland may develop and emerge in preparation for adverse
460 conditions, cues associated with the presence of milkweed are likely to supersede
461 other seasonal cues. Another less likely explanation is that there is a threshold level
462 for decreasing photoperiod required to elicit reproductive diapause in monarchs.
463 The latitudes from which we sampled have relatively modest seasonal changes in
464 photoperiod, with maximal daily daylength decreasing by only 1.5 minutes per day,
465 compared to the 4 minutes per day imposed in our experiment treatments.
466 Our data from the photoperiod manipulation experiment show that maternal
467 lines differ in the magnitude of their response to decreasing photoperiod. We found
468 significant family-level effects for female reproductive development, development
469 time, and wing area. Perhaps most interestingly, we also found that there was a
470 significant interaction between maternal line and larval photoperiod treatment for
471 female reproductive development, suggesting heritable variation among family lines
472 in the strength of the response to decreasing photoperiod. Heritable variation for
473 diapause responses has been recorded for numerous species, including milkweed

474 bugs (Dingle, Brown & Hegmann, 1977), ground crickets (Mousseau & Roff, 1989)
475 and pitcher plant mosquitoes (Bradshaw & Holzapfel, 2001). Because we used wild
476 caught females that may have been multiply mated (Oberhauser, 1988), we do not
477 attempt to provide estimates of the narrow-sense heritability of the diapause
478 response, but this is a promising area for future study. We also note that maternal
479 effects could influence diapause responses (Mousseau & Dingle, 1991). However,
480 given that the females used for oviposition in this study were all naturally
481 reproductively active and were experiencing similar environmental conditions at
482 the time of collection, the contribution of maternal effects within this experiment
483 should be similar between female lines.
484 A possible explanation for the observed maintenance of photoperiodic
485 responses in the resident Australian populations described here is ongoing gene
486 flow with putatively migratory populations in southern Australia. While this may
487 indeed be a possibility, currently available population genetic and historical data
488 suggest that Australian monarchs are themselves descended from other non-
489 migratory populations and colonized the Pacific in a stepping-stone manner (Clarke
490 & Zalucki, 2004; Zalucki & Clarke, 2004; Pierce *et al.*, 2014; Zhan *et al.*, 2014). Zhan
491 *et al.* (2014) sampled six Pacific island groups and found that all of them—including
492 Australia—share derived haplotypes with other resident populations from Central
493 and South America, suggesting recurrent selection on ancestral variation associated
494 with resident status. Thus, even if there is gene flow within Australia, this scenario
495 still requires that the genetic variation underlying developmental plasticity and
496 migratory behavior persisted during the monarch's dispersal across the Pacific.
497 While not a primary focus of our study, we were surprised to find that
498 infection by the protozoan parasite OE did not strongly affect the phenotype of adult
499 butterflies in our experiment. Specifically, OE infection load was not significantly
500 associated with adult body mass or wing size, in contrast with numerous studies
501 showing deleterious effects of OE infection in eastern North American monarchs
502 (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; Altizer *et al.*, 2015). We did
503 find modestly stronger impacts of OE infection status for male compared to female
504 monarchs (Figure S2), consistent with the findings of Altizer and Oberhauser

(1999), although the interaction between infection status and sex was not significant. One possible explanation for the apparent lack of association between parasite infection load and adult phenotypes is the evolution of increased tolerance to OE in non-migratory populations. Whereas OE-monarch interactions are thought to be shaped by transmission-virulence tradeoffs in migratory monarch populations (De Roode, Yates & Altizer, 2008), selection may instead favor the evolution of resistance or tolerance mechanisms in non-migratory populations, where monarchs feed recurrently on milkweed patches and OE infection rates are high (Satterfield, Maerz & Altizer, 2015). Such a scenario has been demonstrated in Hawaii: Hawaiian OE is more virulent than OE strains from other monarch populations, yet Hawaiian monarch hosts exhibit only modest reductions in fitness when exposed to OE (Sternberg *et al.*, 2013). Given that we observed extremely high OE infection rates in the wild-caught monarchs that we sampled from Australia (>95%), we tentatively suggest that Australian populations have also evolved mechanisms of tolerance that mitigate the fitness effects of OE infection.

Our second study addressed the loss of mechanisms hypothesis and evaluated whether resident and migratory monarchs exhibit differential expression of clock genes involved in directional orientation and migration. When we examined expression of circadian clock genes in monarch antennae, we found that resident populations from Guam exhibited identical patterns of expression to those seen in migratory California individuals. This indicates that monarchs, even in derived non-migratory populations, retain the antennal clocks necessary for directional orientation in migratory monarchs. We also found identical clock gene expression patterns between residents and migrants in heads. While this experiment only addressed a subset of the loss of mechanism hypothesis, the results of this experiment allow us to rule out the loss of antennal clock gene expression as an explanation for the cessation of migration.

There are a number of possible explanations for retention of antennal circadian clocks in resident populations of monarchs. First, resident monarchs may still utilize antennal clock gene expression for navigational purposes unrelated to long-distance migration. For example, directional orientation could still be adaptive

for locating widely distributed patches of milkweed host plant. Zalucki and Kitching (1982b) showed that monarchs typically fly in straight lines when found not in association with milkweed host patches, and optimal foraging theory dictates that linear movements are adaptive for searching during between-patch movements (Zalucki, 1983; Viswanathan *et al.*, 1999). Second, retention of antennal clock expression may be related to functions entirely unrelated to navigation. For example, antennal clocks in other insects have been shown to coordinate sensitivity of olfactory and gustatory receptors (Rund *et al.*, 2013). It is thus possible that antennal clocks in monarchs may also function similarly and be retained in residents for regulation of receptor sensitivity related to detection of host plants or pheromones. Finally, antennal clocks may no longer serve any useful function in resident populations, but they have not been lost in resident populations due to insufficient time for selection or drift to eliminate their expression. However, given the likely role of monarch antennal clocks in the aforementioned activities, we consider this last possibility unlikely. Again, the deep evolutionary origin of migration within monarchs may help to explain why migration-associated features like antennal clocks have been retained even in populations long-established as residents (Zhan *et al.*, 2014).

The findings that resident monarchs retain their propensity for responding to photoperiodic cues and a critical element of their navigational sun compass raises an interesting question: are non-migratory monarchs capable of resuming long-distance migration? Although resident populations have shorter and rounder wings than migrants (Altizer & Davis, 2010) and fixed and expression level differences in collagen expression related to wing development (Zhan *et al.*, 2014), these differences do not preclude the resumption of migration. One clue to this question comes from the southern parts of the monarch's Australian range. Australian monarchs are themselves derived from non-migratory populations from other Pacific islands (Pierce *et al.*, 2014; Zhan *et al.*, 2014), and strong circumstantial evidence suggests that they may be directly descended from a resident population on New Caledonia (Clarke & Zalucki, 2004). Still, southern Australian monarchs exhibit seasonal migration akin to that seen in western North America, with long-

distance flights of up to 380 km (James, 1983) and overwintering clusters of hundreds to thousands of butterflies in New South Wales and Victoria (Smithers, 1965; James, 1979); similar overwintering colonies have also been reported in New Zealand (Wise, 1980). Further research should attempt to rear permanent resident populations under conditions conducive to diapause and migration and see if these butterflies will attempt to directionally orient in flight simulators (e.g. Mouritsen & Frost, 2002).

It is also interesting to consider why natural selection has not acted more strongly against migration-associated traits in resident monarch populations. One hypothesis is that there has not been enough time for selection to fully erode these traits, and that monarch populations from locations such as Ecuador, where monarchs may have become established as residents longer ago, would show more pronounced loss of migratory capabilities. Another possibility is that in transitions to resident status, monarchs may be exhibiting pre-existing developmental plasticity that is already expressed in the migratory North American population as an adaptation for conditions experienced during periods of summer breeding. Under this scenario, selection in resident populations would act only against genotypes associated with the strongest diapause responses that are capable of being induced by even modest seasonal changes in their introduced range. Other resident monarchs may retain many of the pre-adaptations necessary for migration (e.g. diapause induction responses, directional orientation using antennal clocks, etc.) even after hundreds or thousands of generations of non-migration, either because these genotypes are never expressed and are therefore shielded from selection (Ghalambor *et al.*, 2007) or because these traits have additional functions unrelated to migration. Relaxed selection in the absence of migration versus directional selection for phenotypes well-suited to resident status is a subtle but important distinction that these data do not allow for us to address. However, the monarch's status as an emerging model organism in ecological genomics promises to help answer this question.

In this paper, we show that monarch butterflies that have become established as permanent residents in the Pacific retain two necessary elements of

their migratory repertoire: the ability to respond to diapause inducing cues and the antennal clocks needed for directional orientation. Recent research has begun to highlight the genetic and transcriptional differences between resident and migrant monarch populations and provide hypotheses as to the origins of monarch migration (Zhan *et al.*, 2014; Pfeiler *et al.*, 2017). Especially in light of ongoing population declines in migratory overwintering North American monarchs (Brower *et al.*, 2012), understanding the causes and consequences of shifts to resident status is an important part of understanding monarch butterfly biology.

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Table 1 – Summary of analyses of variance for each of the response variables tested. All predictors with $p < 0.1$ are shown in bold, with asterisks denoting levels of significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$). For response variables #1 and #3, estimates are based on comparison with reference states (constant larval photoperiod and cool adult treatment).

820 **Figure 1** – Map of locations of monarch populations described in this study. Orange
821 locations reflect the locations of monarch populations used for assessing diapause
822 responses in Goehring and Oberhauser (2002) (Minnesota, USA) and this study
823 (Queensland, Australia). Blue locations were used for comparison of antennal
824 circadian clock gene expression between California migrants and Guam residents.
825

826 **Figure 2** – Females reared under decreasing photoperiod conditions (LD 14:10 > LD
827 12:12) produced significantly fewer mature (A) and marginally fewer yolked
828 oocytes (B) when compared with females reared under constant photoperiod
829 conditions (LD 12:12). Error bars represent mean standard error.
830

Figure 3 – Larvae reared under decreasing photoperiod conditions (LD 14:10 > LD 12:12) had significantly higher body mass as adults (A) and marginally larger forewings (B) than larvae reared under constant photoperiod (LD 12:12).

Figure 4—Expression analysis of clock genes in antennae (left panel) and heads (right panel) of migratory (California) and non-migratory (Guam) monarch butterflies. mRNA expression levels of *per*, *tim*, and *Cry2* were assayed in heads and antennae of migratory (top row) and non-migratory (bottom row) butterflies collected at ZT5 (light bars) and ZT14 (dark bars). Steady state mRNA levels were normalized to non-cycling *rpl32* levels, and expressed as a fraction of peak expression level (peak=1). Each biological replicate consists of pooled antennae from 3-4 individuals of the same sex, and at least three biological replicates were performed for combination of population and ZT. Sexes were combined as they did not differ.

Table S1—Primers for gene expression analysis

Figure S1 – OE infection status was not significantly associated with any of the measured response variables, including body mass (A) and forewing area (B). The impacts of OE infection appear to stronger in males than in females, although the interaction between infection status and sex was not significant for either measure. OE infection status reflects $\log_{10}$ spore loads.

Figure S2 – Maternal lines varied significantly in the strength of their response to decreasing photoperiod. Of 11 maternal lines tested, 8 showed greater development under constant larval photoperiod (LD 12:12), 1 showed greater development under decreasing larval photoperiod (LD 14:10 > LD 12:12), and 2 could not be assessed because they were only tested under one condition. Error bars represent mean standard errors.

Figure S3– Wing area and body mass scale isometrically (slope = 0.29 ± 0.05 g/cm^2; isometry = 0.33); the slope of this relationship does not depend on larval photoperiod treatment.