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Lepidopteran species differ in susceptibility to winter warming

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ABSTRACT: Predicting assemblage-level impacts of climate change requires knowledge of among-species variation in susceptibility to warming. Increased winter temperatures intuitively reduce cold stress for overwintering ectotherms, but can decrease fitness by increasing consumption of energy reserves. We kept overwintering stages of the butterflies *Papilio glaucus*, *P. troilus* (Papilionidae), and *Erynnis propertius* (Hesperiidae) under current temperatures and conditions simulating a 4°C increase in mean temperature, and compared mass, water, energy reserves (lipid, protein and carbohydrate content), and, in *E. propertius* and *P. troilus*, post-winter development time, adult size, and mortality. All 3 species lost mass over winter, more so during warm winters. *Erynnis propertius* lost more mass than either *Papilio*. Warm winters increased energy reserve depletion in *E. propertius*, but had no effect on energy reserves of either *Papilio*. Warming reduced development time, but did not affect mortality or adult size of *P. troilus*. Mortality in *E. propertius* was uniformly high preventing assessment of treatment effects on survival, development, or adult size. The observed overwinter mass loss by *E. propertius* and *P. troilus* was predominantly due to water loss; thus we conclude that mass loss is not an appropriate measurement of energy drain in insects, rather body composition should be measured directly. Thus, *P. glaucus* and *P. troilus* have low vulnerability to winter warming, while *E. propertius* shows some vulnerability. Measuring the impact of winter warming on many species will enable the identification of species traits that predict vulnerability, and facilitate identification of clades particularly susceptible to winter warming.

KEY WORDS: Climate change · Ectotherm · Dormancy · Overwintering · Energy reserves · Development time

1. INTRODUCTION

Anthropogenic climate change is altering mean temperatures and the relationships among environmental variables such as growing season and winter temperatures (IPCC 2007a, Jackson et al. 2009); leading to shifts in phenology and geographic range of many species (Thackeray et al. 2010, Chen et al. 2011). Inter-specific differences in sensitivity to climate are exacerbating phenological asynchronies within ecosystems (Singer and Parmesan 2010, Walther 2010), while differential mobility causes disassembly of communities and novel biotic interactions as species move to track their preferred climates at different rates (Thuiller 2004, Devictor et al. 2012). Increasing habitat fragmentation and anthropogenic barriers to dispersal signify that population persistence will increasingly rely on species' ability to tolerate changing thermal environments, but in most cases these physiological tolerances are unknown. To identify species with high vulnerability to various aspects of climate change (e.g. increases in mean,

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maximum or minimum temperatures), we need a comprehensive framework that can use life-history and demographic traits to predict the physiological consequences of warming on focal species and to eventually allow prediction at the assemblage level (Chown & Gaston 2008).

In North America, regional climate change projections include a relatively greater warming in winter than during the growing season (IPCC 2007b, Bonsal & Kochtubjada 2009). Most temperate insects spend the winter in a state of dormancy (diapause or quiescence; Koštál 2006), in which metabolic rate is suppressed (but still temperature-dependent), and activity and feeding cease (Hahn & Denlinger 2011). The intuitive outcome of winter warming is a release from cold-induced mortality, and this is important in some species, for example *Atalopedes campestris* (Lepidoptera: Hesperiidae; Crozier 2004) and 2 species of geometrid moth (Jepsen et al. 2008). However, increased winter temperatures can also decrease fitness of dormant ectotherms. Increased pre- or over-wintering temperatures lead to higher rates of resource consumption and a concurrent depletion of stored reserves, which negatively impact survival and fecundity as reduced resources are available for reproduction in the spring (Bosch & Kemp 2003, 2004, Irwin & Lee 2003, Williams et al. 2003, Sorvari et al. 2011, Mercader & Scriber 2008, Koštál et al. 2011).

Not all species are equally susceptible to energetic drain as a result of winter warming. Species from diverse taxonomic groups show no energetic drain as a result of winter warming (e.g. Jean et al. 1990, Beekman et al. 1998), and even within genera the vulnerability to warming may differ. For example, pupae of the cold-adapted *Papilio canadensis* (Lepidoptera: Papilionidae) suffered increased mortality and increased mass loss at a constant 10°C compared to 4°C over winter; while the closely-related, more widely-distributed *P. glaucus* did not show any negative effects of warming on survival or mass loss (Mercader & Scriber 2008). Similarly, a warm-adapted gall-forming wasp (*Diplolepis variabilis*; Hymenoptera: Cynipidae) was not affected by warming, whereas survival and fecundity were reduced in response to winter warming in a congener with cool-climate distribution (Williams et al. 2003). Thus, it is possible that inter-specific vulnerability to warming can be predicted by life history traits such as the degree of cold-adaptation, the climatic diversity of a species' geographic distribution, the amount of pre-feeding development remaining post-winter, or larval diet breadth. To date, there are few studies comparing multiple species' responses to ecologically-relevant levels of winter warming (but see Williams et al. 2003, Mercader & Scriber 2008), thus insufficient data exist to test these preliminary hypotheses. A trait-based approach has been successfully utilized in a climate change context to predict susceptibility to phenological shifts in response to warming (Diamond et al. 2011) and the vulnerability of terrestrial organisms to warming (Deutsch et al. 2008).

We examined the physiological vulnerability to winter warming of 3 butterfly species with contrasting life-history characteristics. *Papilio troilus* (Spicebush swallowtail) and *P. glaucus* (Eastern Tiger swallowtail; Lepidoptera: Papilionidae) are sympatric species that occur from southern Ontario and Michigan to Florida (Wagner 2005), although the distribution of *P. glaucus* extends further north and west than that of *P. troilus* (Brock & Kaufman 2003) over a wider range of climatic zones (Peel et al. 2007). *Papilio glaucus* larvae are polyphagous, while *P. troilus* larvae are oligophagous (Wagner 2005, Scriber et al. 2008); both species overwinter as pupae. *Erynnis propertius* (Lepidoptera: Hesperiidae) is a skipper that inhabits warm-temperate coastal oak ecosystems on the West Coast of North America from Vancouver Island in the north to Baja California in the south (Peel et al. 2007, Pelini et al. 2009). Larvae are monophagous and overwinter in a final instar (Prior et al. 2009).

Papilio glaucus is relatively insensitive to increases in overwintering temperature, although energy reserves have not been assessed directly (Mercader & Scriber 2008). We predicted greater susceptibility to warming in *P. troilus*, based on the lower climatic diversity of its geographical distribution, and higher resource specialisation. We predicted the highest susceptibility to warming in *Erynnis propertius,* which is sensitive to changes in overwintering temperature (Pelini et al. 2009), has a distribution confined to a narrow climatic range, and a narrow larval diet breadth. We measured both energy reserve depletion and the impact of depletion on subsequent life-history parameters that may be associated with fitness. Our results suggest that *E. propertius* will be susceptible to energetic drain in warming winters but that the *Papilio* species we studied will not. Although our sample size is too low to perform phylogenetically-independent analyses, we aim to quantify the sensitivity of these 3 species to winter warming, to contribute to a larger body of knowledge that will permit such analyses.

2. MATERIALS AND METHODS

2.1. Collection and rearing

Eggs were obtained from females wild-caught in Norfolk County, Ontario, Canada (*Papilio glaucus* and *P. troilus*) or lower Vancouver Island, British Columbia, Canada (*Erynnis propertius,* as described in Pelini et al. 2009). Larvae were reared on potted host plant (*P. glaucus*: *Liriodendron tulipifera*, tulip tree; *P. troilus*: *Lindera benzoin*, spicebush; *E. propertius*: *Quercus garryana*, Garry oak) in incubators (EGC-TC2, Environmental Growth Chambers) in the Biotron Centre for Experimental Climate Change, at the University of Western Ontario, on a 12:12 h light: dark (L:D) photoperiod, at diurnally-cycling temperatures (19:10°C day:night). When larvae pupated (*P. glaucus* [N = 37] and *P. troilus* [N = 85]) or when 3 d passed without evidence of feeding (*E. propertius* [N = 59]), they were considered dormant and transferred to MIR-153 incubators (Sanyo Scientific) in 40 ml plastic containers on moist vermiculite to prevent desiccation. The containers were sealed with tight-fitting lids that had 5 small perforations for air flow. The vermiculite was misted lightly with tap water every third day, which kept the substrate damp (and humidity high) throughout the experiment. Insects were kept on an 11:13 h L:D photoperiod, with temperatures fluctuating between 14:10°C day:night for 2 wk; then 10:14 h L:D, 10:6°C day:night for 2 wks; then a constant 6°C (8:16 h L:D) until 20 November 2008 (when all individuals had entered dormancy). This stepwise decrease in temperature and photoperiod was chosen to approximate natural seasonal changes and allow larvae and pupae to acclimate to winter conditions. On 20 November 2008, animals were divided between warm and cool winter treatments, and placed in incubators cycling between 8: 2°C day:night (warm treatment); or between 4:–2°C day:night (cool treatment) on a 12:12 h thermoperiod, in constant darkness. The difference between the treatments (4°C) reflects increases in global mean temperature over the next century predicted by the SRES A2 scenario (IPCC 2007a). The cool treatment reflects mean daily maximum and minimum microclimate temperatures in London (Ontario) during the month of January (C. M. Williams & B. J. Sinclair pers. obs.), which is close to where the *Papilio* species were collected and thus approximates their current average overwintering microclimate temperatures. Microclimate temperatures in Vancouver Island, origin of *E. propertius*, are somewhat warmer (winter average 5.1°C; Pelini et al. 2009), and thus the 'cool' treatment represents an unusually cool (but not unprecedented) winter for this species. For all species, the 'warm' treatment imposes warming compared to their current native microclimate conditions. We examined body size, phenology and energy reserves over the winter as described below, chosen to represent key parameters for overwintering fitness.

2.2. Life-history measurements

We measured survival (in *Erynnis propertius* and *Papilio troilus* only, due to insufficient sample sizes of *P. glaucus*) and overwinter mass loss in pupae or larvae exposed to these warm or cold thermal regimes. Larvae and pupae were weighed (Mettler-Toledo MX5; d = 0.1 µg) at the beginning, middle and end of winter (20 November 2008, 20 January and 20 March 2009). The length of *Papilio* spp. pupae from cremaster to tip was measured using digital calipers (±0.5 mm; Mastercraft). Mass at the end of winter was subtracted from mass at the beginning of winter to obtain overwinter mass loss. Mortality can be detected in larval *E. propertius* as larvae desiccate and do not respond to stimulus; thus individuals that died over the winter were excluded from further analyses. On 20 March 2009, all remaining *E. propertius* larvae and *P. troilus* pupae were placed into an incubator cycling between 25:15°C day:night, on a 16:8 h L:D photoperiod, and checked daily for emergence. When adults emerged, they were killed at −20°C, weighed, and the right wing was measured from the proximal attachment point to apex. This measurement is not influenced by water content, and has fitness implications due to the requirements of flight for mate finding, and oviposition in females (e.g. Shirai 1995).

2.3. Energy reserve assays

We measured total protein, storage lipids, and carbohydrates of individuals from the warm and cool treatment groups at the beginning and end of winter (*Papilio troilus* and *Erynnis propertius*; N = 7–10 for each species, treatment and time point combination); or, due to insufficient sample sizes, at the end of winter only in *P. glaucus* (N = 9 for each treatment). *P. glaucus* and *P. troilus* pupae were sexed by whether the genital opening intersected the first abdominal sternite (females) or was closer to the cremaster (males; cf. Posada et al. 2011). We weighed the pupae and larvae to determine fresh mass (FM),

then punctured them 5 times with a dissecting pin and transferred them frozen to be lyophilized (48 h, ~13 Pa; Lyocentre lyophilizer, Virtis). We then re-weighed them (dry mass; DM) and calculated water content as: FM – DM.

Lyophilized pupae and larvae were homogenised using a mortar and pestle, and aliquots of tissue were weighed for determination of concentrations of lipid (~1 mg tissue), protein (~1 mg tissue), and carbohydrate (~22 mg tissue *Papilio glaucus* and *P. troilus*; ~16 mg tissue *Erynnis propertius*). The aliquots of tissue were placed directly into 2.5 ml chloroform: methanol (2:1 v/v; lipid assay), 1 ml Tween-20 0.05% (protein assay), or 1.2 ml 30% KOH (carbohydrate assay).

We quantified protein using the bicinchoninic acid (BCA) assay, as described previously (Gefen et al. 2006). Triplicates of 10 µl of each sample were loaded on microplates and 200 µl BCA assay reagent (50 parts BCA:1 part 4% $CuSO_4$) were added to each well. Plates were incubated overnight, and absorbance at 562 nm measured the following day. Concentrations were determined by comparison to standards (0.2–1 mg ml^{-1} bovine albumin serum, Sigma-Aldrich). We validated the assay using 3 bovine serum albumin standards, of which 103 ± 8% (mean ± SD) were recovered.

We quantified triacylglycerides (TAGs) by thin layer chromatography coupled to a flame-ionisation detector (Iatroscan MK-6, Shell-USA), as described in Williams et al. (2011). Briefly, we extracted neutral lipids using chloroform: methanol 2:1 v/v (Folch et al. 1957), then spotted triplicates of 1.5 µl of sample or a standard (containing a cholesterol ester, a mono-, di-, and tri-acylglyceride, a free fatty acid, and cholesterol) onto silica rods (Shell-USA), developed the rods in benzene:chloroform:formic acid (70:30:0.5 v/v/v) for 35 min and quantified the lipids using a flame ionisation detector. Initial analyses did not detect monoacylglyceride in samples, so we used a monoacylglyceride (1-stearoyl-*rac*-glycerol; Sigma Aldrich) as an internal standard, to correct for losses during extraction. We identified compounds by comparison to known standards, normalized values to internal standard peaks, and quantified TAGs using previously determined reference curves (Williams et al. 2011). This assay returns 99% of TAG (Williams et al. 2011).

We quantified total carbohydrates using the anthrone method (Caroll et al. 1955). Briefly, the homogenised samples received 1.2 ml 30% KOH and were incubated (100°C, 20 min) to extract the glycogen from the tissue, then centrifuged (2000 × *g*, 10 min). A 1 ml aliquot of the supernatant was combined with 2 ml 95% ethanol, then the glycogen precipitated by the addition of 50 µl saturated $NaSO_4$ aqueous solution. Samples were centrifuged (2000 × *g*, 10 min) and the pellet dried at 100°C and reconstituted in 500 µl distilled water. An aliquot of 2.5 ml of cold anthrone reagent (0.05% anthrone [w/v], 1% thiourea [w/v], 28% H_2O [v/v], 72% H_2SO_4 [v/v]) was added to samples and standards (0.1–0.5 mg ml^{-1} glycogen; Sigma Aldrich) and cooled on ice. Once ice-cold, samples and standards were incubated (100°C, 15 min), before being returned to ice. Triplicate 200 µl aliquots were transferred to an acid-proof polypropylene 96-well plate (Brandtech Scientific) and absorbance measured at 620 nm. The recovery of triplicate glycogen standards was 99 ± 2% (mean ± SD). The anthrone method measures total carbohydrates including free glucose and glycogen, but does not measure polyols such as glycerol and sorbitol (Graham 1963).

We expressed TAG, carbohydrate and protein concentrations in µg mg DM^{-1}, and estimated whole-animal values by multiplying by total DM. We subtracted whole-animal TAG and carbohydrate from DM to give lipid- (and carbohydrate-) free DM. We calculated total energy content in J per pupa assuming 39.3 J mg TAG^{-1} and 17.6 J mg $carbohydrate^{-1}$ (Djawdan et al. 1997).

2.4. Data analysis

All statistical analyses were performed in R 2.13.0 (R Core Development Team 2009), and preliminary data exploration was performed according to Zuur et al. 2010. Our general modeling approach was to start with maximal models and sequentially drop terms using Akaike's information criterion (AIC) to judge the improved fit of the simplified model (models with $\Delta AIC > 2$ were retained; Crawley 2007). We excluded individuals that did not eclose as adults from the analysis of life-history variables (11 *Papilio troilus* pupae). All mass loss measurements and life-history variables were normally distributed, apart from masses of *P. glaucus* and *P. troilus*, which were bimodal in both November and March, with small modes at very low values. For *P. troilus* (the only species for which we have robust survival data), mass loss over winter was the only significant predictor of survival, with high mass loss being associated with failure to emerge as an adult (survived: 6.2 ± 2.7% initial mass, range 3.4–15.5%; died: 29.5 ± 25.8% initial mass, range 4–79%). We thus excluded *Papilio* spp. pupae with water loss of >16% of their initial

mass, resulting in the exclusion of 3 *P. glaucus* pupae from the warm treatment.

We compared pupal length, mass (October, December, and January), overwinter mass loss, and adult mass, wing length and development time, where applicable, separately within each species using analysis of variance (ANOVA) with treatment (and sex, in the case of *Papilio troilus*) as a factor. Development time in *P. troilus* was $\log_{10}$-transformed to meet assumptions of normality. We compared rates of mass loss over time among the 3 species using a mixed general linear model with treatment, species and time (beginning, middle and end of winter) as fixed factors, mass at the beginning of winter as a covariate, and individual as an error term. We compared survival rates of *P. troilus* between treatments using a generalised linear model with a binomial error structure with treatment as a factor, and mass in November and overwinter mass loss as covariates.

For *Papilio troilus*, the only species for which we had robust adult size measurements, we assessed the multivariate structure of interactions among life-history variables (date of pupation, mass at the beginning, middle and end of winter, overwinter mass loss, development time, adult mass, and wing length) using a principal components analysis (PCA), using the 'prcomp' function. We centered and scaled the data to standardise variances then rotated the loading matrix to obtain orthogonal factors. Principal components (PCs) that were above the inflection point of a scree plot and had eigenvalues of >1 were retained (Tabachnik & Fidell 2007), and the PCs were defined based on variables that loaded strongly (factor loadings > 0.3). Scores on each PC were compared among treatments using ANOVA.

We used total protein as a proxy for metabolising tissue. Total carbohydrate (*Papilio glaucus*) was $\log_{10}$-transformed to meet assumptions of normality. Total protein, water content, and TAG and carbohydrate concentration were compared between treatments using ANOVAs with treatment (warm or cool) and sex (for *P. troilus* and *P. glaucus* only) as factors. Date of dormancy was used as a covariate in analyses of total protein in *Erynnis propertius*; total protein was used as a covariate to TAG and carbohydrate content in all species, and dry mass was included as a covariate in comparisons of water content in all species.

3. RESULTS

3.1. Life-history traits

The temperature treatments did not affect pupal length (*Papilio glaucus* and *P. troilus*); mass at any given time-point (*P. glaucus, P. troilus* and *Erynnis propertius*); or adult wing length, adult mass or development time (*P. troilus*; Tables 1 & 2). However, mass declined significantly during the winter in all 3 species ($F_{2,138}$ = 597.1, p < 0.001), and mass declined significantly faster in the warm treatment than in the cool treatment (treatment × time interaction: $F_{2,138}$ = 25.0, p < 0.001; Table 2). *Erynnis propertius* lost mass more quickly than for either of the *Papilio* species (species × time interaction: $F_{4,138}$ = 32.8, p < 0.001), but there were no inter-specific differences in the effects of overwintering temperature on the rate of mass loss (species × treatment interaction: $F_{2,68}$ = 1.8, p = 0.167; Fig. 1)

Table 1. *Papilio glaucus, P. troilus* and *Erynnis propertius*. Life-history and experimental design parameters for 3 butterfly species overwintered in warm or cool thermal conditions. Mass loss = $Mass_{Nov} - Mass_{Mar}$. Cool–warm pairs in **bold** indicate a significant difference. Development: days to eclose as adult post-transfer to 25°C. Data are mean ± SD, or values for each individual (*E. propertius*: adult mass, wing length, and development). $N_{overwintering}$ includes those used for body composition analysis in March, while % survival is calculated excluding those individuals (i.e. using only individuals transferred to 25°C to monitor emergence)

	P. glaucus		*P. troilus*		*E. propertius*	
Date of diapause	28 September 2008 ± 29 d		17 September 2008 ± 20 d		2 October 2008 ± 12 d	
Treatment	Cool	Warm	Cool	Warm	Cool	Warm
$N_{overwintering}$	19	18	43	42	28	31
Pupal length (mm)	27.0 ± 1.8	28.0 ± 2.7	29.0 ± 1.6	28.7 ± 2.0		
$Mass_{Nov}$ (mg)	678.2 ± 138.7	736.2 ± 210.4	800.7 ± 141.4	792.4 ± 142.1	273.3 ± 55.3	296.1 ± 48.3
Mass loss (mg)	**31.6 ± 30.4**	**49.5 ± 14.8**	**36.4 ± 9.1**	**54.9 ± 17.7**	**72.7 ± 12.4**	**99.4 ± 30.6**
N_{adults}			13	12	2	1
% survival			68.4	70.5	25.0	9.1
Adult mass (mg)			212.4 ± 82.1	252.5 ± 74.9	18.1, 75.6	80.1
Wing length (mm)			41.6 ± 3.3	43.1 ± 3.4	8.7, 16.4	15.7
Development			21.4 ± 11.1	16.5 ± 4.4	28,18	16

Table 2. *Papilio glaucus, P. troilus* and *Erynnis propertius*. Between-treatment comparison of life-history variables of *P. glaucus, P. troilus,* and *E. propertius* kept in warm or cool conditions over winter. **Bold**: significant effects of treatment. Mass loss = Pupal $Mass_{Nov}$ – Pupal $Mass_{Mar}$. Development: days to eclose as adult post-transfer to 25°C

Variable	*P. glaucus*			*P. troilus*			*E. propertius*		
	F	df	p	*F*	df	p	*F*	df	p
Pupal length	1.73	1,32	0.197	0.25	1,51	0.616			
Pupal $Mass_{Nov}$	0.94	1,32	0.341	0.05	1,51	0.832	2.74	1,55	0.103
Pupal $Mass_{Jan}$	0.72	1,32	0.402	0.23	1,51	0.635	0.09	1,35	0.766
Pupal $Mass_{Mar}$	0.62	1,32	0.436	0.06	1,21	0.814	0.48	1,15	0.497
Mass loss	**5.44**	**1,33**	**0.026**	**9.67**	**1,21**	**0.005**	**4.70**	**1,15**	**0.047**
Adult mass				0.91	1,22	0.349			
Wing length				1.24	1,22	0.276			
Development				1.05	1,22	0.317			

There was no difference in survival between warm and cool winters in *Papilio troilus* ($F_{1,34}$ = 0.3, p = 0.598; Table 1). Mass in November did not influence survival ($F_{1,34}$ = 0.1, p = 0.773), but overwinter mass loss was a significant predictor of survival ($F_{1,34}$ = 8.0, p = 0.009). We did not have sufficient sample size to perform survival assays in *P. glaucus,* and only 3 *Erynnis propertius* individuals emerged as adults (Table 1).

A PCA of life-history variables in *Papilio troilus* provided 3 PCs that cumulatively explained 89% of variation in the data set (Table 3). The first PC (PC1) accounted for the effects of size, with all pupal and adult size measurements loading positively; PC2 represented mass loss and development time, with mass loss loading positively while development time loaded negatively; and PC3 was defined by pupal length and date of diapause, whereby later diapause was associated with increased pupal length (Table 3). There was no significant effect of treatment on PC1 ($F_{1,21}$ = 0.61, p = 0.443) or PC3 ($F_{1,21}$ = 0.23, p = 0.635), but individuals in the warm treatment had significantly higher factor scores on PC2 ($F_{1,21}$ = 6.41, p = 0.019), confirming that they had higher mass loss and shorter development times than individuals in the cool treatment (Fig. 2).

3.2. Energy reserves

There were strong correlations between total protein and lipid- and carbohydrate-free dry mass in all 3 species (*Papilio troilus*: r_{32} = 0.63, p < 0.001; *P. glaucus*: r_{16} = 0.89, p < 0.001; *Erynnis propertius*: r_{35} = 0.57, p < 0.001). In *E. propertius* date of dormancy was negatively correlated with total protein (r_{35} = –0.35, p = 0.033) and lipid- and carbohydrate-free dry mass (r_{35} = –0.64, p < 0.001), but not with TAG or carbohydrate concentration (p > 0.05, data not shown). Date of dormancy did not affect total protein, TAG concentration, or carbohydrate concentration for *P. troilus* or *P. glaucus* (p > 0.05, data not shown).

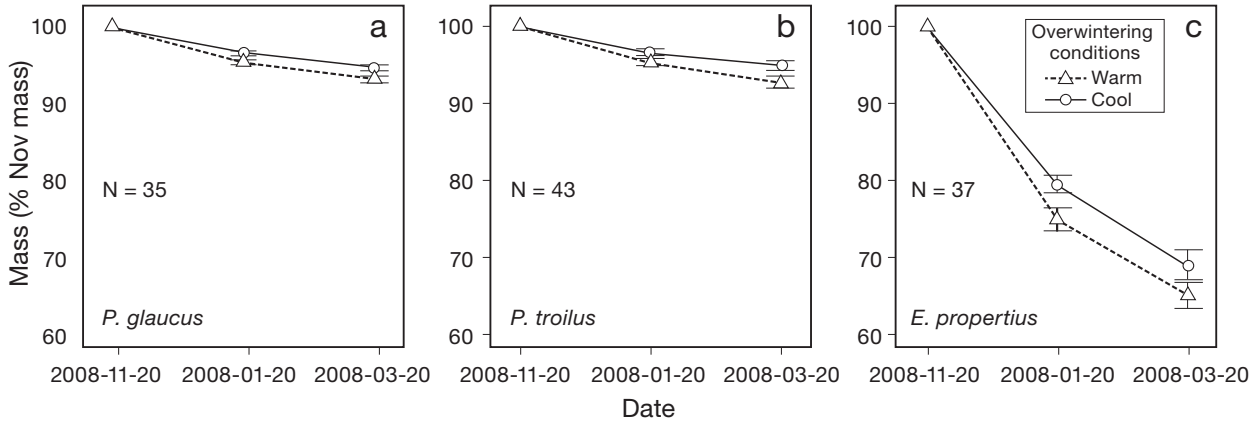

Fig. 1. *Papilio glaucus, P. troilus* and *Erynnis propertius.* Overwinter mass change of 3 butterfly species exposed to cool or warm overwintering conditions. Mass of (a) *P. glaucus*, (b) *P. troilus*, and (c) *Erynnis propertius* in November, January, and March under warm and cool conditions expressed as % of individual's mass in November, to standardize for inter- and intraspecific size differences (although analyses were performed on raw mass data with mass in November as a covariate, see text for details). Data are mean ± SE; if not visible, error bars are obscured by symbol. Mass declines over time for all species; rates are significantly faster in warm than in cool conditions, and *E. propertius* exhibits significantly greater rates of mass loss than do *P. glaucus* and *P. troilus*) (see Section 3.1 for details)

Table 3. *Papilio troilus*. Principal components (PCs) for life-history variables of *P. troilus* subjected to warm or cool winters. Values are factor loadings for the first 3 PCs (total variation in parentheses) of a PCA. NS: non-significant loading (<0.3). Individuals in the warm treatment had significantly higher scores on PC2 (see Section 3.1 for details)

Name of PC	PC1 loading (61.2%) Size	PC2 loading (15.7%) Large mass loss, short development time	PC3 loading (12.1%) Late diapause, long pupae
Pupal $mass_{Nov}$	0.43	NS	NS
Pupal $mass_{Jan}$	0.42	NS	NS
Pupal $mass_{Mar}$	0.42	NS	NS
Pupal length	0.33	NS	0.46
Overwinter mass loss	NS	0.71	NS
Adult mass	0.37	NS	NS
Wing length	0.39	NS	NS
Development time	NS	−0.56	NS
Diapause date	NS	NS	0.81

Total protein did not differ between overwintering treatments for *Papilio glaucus, P. troilus* or *Erynnis propertius* (Tables 4 & 5). As suggested by the correlation between date of dormancy and total protein in *E. propertius*, date of entering dormancy significantly affected total protein (Table 5), with late entry into dormancy associated with lower total protein content. Water content was positively related to total protein (i.e. size) in all 3 species, and declined significantly over time in *P. troilus* and *E. propertius* (Tables 4 & 5). TAG content was positively related to total protein in *P. glaucus* and *P. troilus*, with no effect of overwintering temperature on TAG content (Tables 4 & 5). In *E. propertius*, there was a 3-way interaction of overwintering temperature, total protein, and sampling time, such that TAG per unit total protein declined between the beginning and end of winter in the warm treatment but not in the cool treatment (Tables 4 & 5). Total energy content was strongly correlated with TAG (r = 0.99 for all 3 species), and in all cases the conclusions are identical for energy content and TAG content. Mass-specific energy content was not affected by overwintering temperature in *P. glaucus* (not shown) or *P. troilus*, nor in *E. propertius* in the cool overwintering treatment (Fig. 3). However, mass-specific energy content of *E. propertius* declined over the course of the winter in the warm overwintering treatment (Fig. 3).

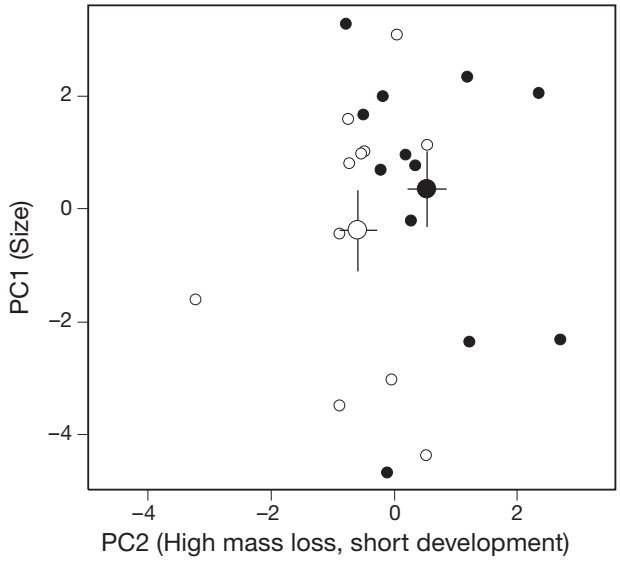

Fig. 2. *Papilio troilus*. The first 2 principal components of an analysis (PC1, PC2) of life-history characteristics of *P. troilus* (N = 24) exposed to warm (black) or cool (white) overwintering conditions. Small symbols: individual data points; large symbols: mean ± SE. Only individuals that survived the winter are included. See Table 3 for details of components. Individuals overwintering at warm temperatures have significantly higher mass loss and shorter development times than those overwintering at cool temperatures

4. DISCUSSION

We examined the responses of the overwintering stages of 3 butterfly species to the degree of winter warming that is predicted over the next century (IPCC 2007a). We found that winter warming increased rates of overwinter mass loss in all 3 species, but that this mass loss was largely due to water loss rather than increased consumption of energy reserves. This indicates that whole animal mass loss is not an appropriate measure of energy depletion in animals such as insects that can tolerate large fluctuations in water content, but our results contrast with those of Scriber et al. (2012) who found that water content was tightly regulated in *Papilio glaucus and P. canadensis* (Scriber et al. 2012). Although all species showed a trend towards energy decline over winter (Fig. 3), this was only significant for *Erynnis propertius* at warm temperatures. Thus our hypotheses were partially supported: *E. propertius* was sensitive to winter warming and *P. glaucus* was insensitive, but there was no evidence for any sensitivity to winter warming in *P. troilus*. The parameters measured in this study (i.e. multiple size measurements

Table 4. *Papilio glaucus, P. troilus* and *Erynnis propertius*. Physiological variables of *P. glaucus, P. troilus* and *E. propertius* overwintered in warm or cool thermal conditions. Due to sample size, *P. glaucus* was measured only at the beginning of winter. Significant factors or covariates are derived from models in Table 5. TAG: triacylglyceride; Protein: total protein; Carb: total carbohydrate; Time: sampling time (beginning or end of winter). NS: not significant

	N	Protein (mg)	Water (µl)	Water (% fresh mass)	TAG (mg mg protein^{-1})	Carb (mg mg protein^{-1})
P. glaucus						
End of winter						
Cool	9	41.2 ± 8.4	488.4 ± 82.5	78.3 ± 1.8	0.380 ± 0.166	0.038 ± 0.031
Warm	9	41.0 ± 13.1	517.2 ± 142.7	76.8 ± 4.0	0.406 ± 0.241	0.043 ± 0.035
Significant factors or covariates		NS	Dry mass		Protein	NS
P. troilus						
Beginning of winter						
Cool	7	41.2 ± 8.2	665.9 ± 51.0	83.0 ± 3.0	0.248 ± 0.070	0.032 ± 0.041
Warm	9	36.7 ± 10.5	655.9 ± 107.3	84.2 ± 2.2	0.248 ± 0.074	0.071 ± 0.036
End of winter						
Cool	9	61.6 ± 19.3	612.2 ± 64.5	79.5 ± 1.9	0.236 ± 0.146	0.049 ± 0.025
Warm	9	47.9 ± 14.3	597.6 ± 114.5	80.7 ± 2.1	0.214 ± 0.112	0.050 ± 0.030
Significant factors or covariates		NS	Dry mass, Time		Protein	NS
P. propertius						
Beginning of winter						
Cool	9	19.9 ± 5.3	216.8 ± 46.9	81.5 ± 2.1	0.344 ± 0.120	0.059 ± 0.051
Warm	10	22.0 ± 7.5	236.0 ± 42.5	80.3 ± 2.2	0.356 ± 0.118	0.054 ± 0.045
End of winter						
Cool	9	23.8 ± 7.5	145.8 ± 45.0	70.9 ± 4.8	0.287 ± 0.096	0.047 ± 0.031
Warm	10	28.0 ± 10.2	147.9 ± 36.8	70.7 ± 4.5	0.274 ± 0.097	0.027 ± 0.027
Significant factors or covariates		Date of dormancy	Dry mass, Time		Treatment × Protein × Time	Protein

Table 5. *Papilio glaucus, P. troilus* and *Erynnis propertius*. ANOVA or ANCOVA statistics describing the effect of overwintering treatment (warm or cool), sex, and sampling time (beginning or end of winter, *P. troilus* and *E. propertius* only) on energy reserves of *P. glaucus* and *P. troilus* pupae, and *E. propertius* larvae. Dormancy: date of dormancy; DM: dry mass; TAG: triacylglyceride; NA: not applicable

Parameter (y)	Initial model	Minimal adequate model	Term	F	df	p
P. glaucus						
Protein	y = Treatment × Sex	Null model	NA			
Water	y = Treatment × DM × Sex	y = DM	Dry mass	43.24	1,16	<0.001
TAG	y = Treatment × Protein	y = Protein	Protein	8.33	1,16	0.011
Carbohydrate	y = Treatment × Protein	Null model	NA			
P. troilus						
Protein	y = Treatment × Sex × Time	y = Time	Time	10.22	1,31	0.003
Water	y = Treatment × DM × Time × Sex	y = DM + Time	Dry mass	24.15	1,31	<0.001
			Time	16.11	1,31	<0.001
TAG	y = Treatment × Protein × Time	y = Protein	Protein	4.50	1,32	0.042
Carbohydrate	log(y) = Treatment × Protein × Time	Null model	NA			
E. propertius						
Protein	y = Treatment × Dormancy × Time	y = Dormancy	Dormancy	4.88	1,35	0.034
Water	y = Treatment × DM × Time	y = DM + Time	Dry mass	70.27	1,34	<0.001
			Time	126.12	1,34	<0.001
TAG	y = Treatment × Protein × time	y = Treatment × Protein × Time	Treatment × Protein × Time	4.35	1,29	0.047
Carbohydrate	log(y) = Treatment × Protein × Time	log(y) = Protein	Protein	7.54	1,35	0.009

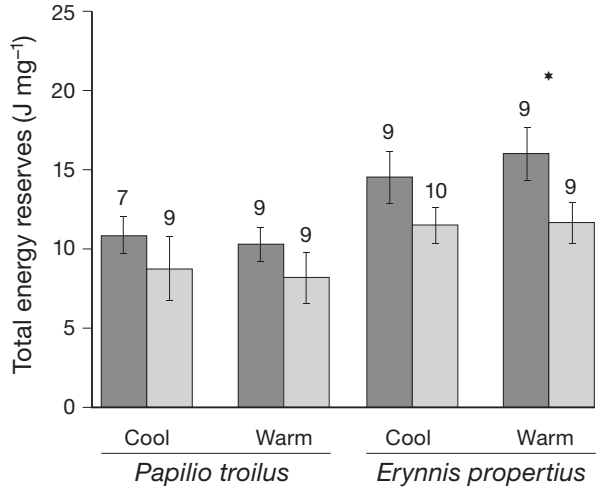

Fig. 3. *Papilio troilus* and *Erynnis propertius*. Total energy reserves of *P. troilus* and *E. propertius* at the beginning (gray) or end (light gray bars) of the overwintering period, for individuals exposed to warm or cool overwintering temperatures. *Significant energy depletion ($p < 0.05$) from the beginning to the end of winter; sample sizes above bars

over winter, body composition pre- and post- winter, length of dormancy, and ideally mortality rates and adult size measurements) are key to overwintering fitness and are necessary to robustly quantify the sensitivity of insects to winter warming.

The current study has thus provided a basis for classifying the physiological vulnerability of these 3 species of Lepidoptera to winter warming. *Papilio glaucus* and *P. troilus* have low vulnerability to energetic drain resulting from winter warming, as there were no discernable lethal or sub-lethal effects of the levels of warming that are predicted over the coming century. This is concordant with a previous study that also found a low vulnerability of *P. glaucus* to winter warming, although a sister species *P. canadensis* is vulnerable to winter warming (Mercader & Scriber 2008). *Papilio glaucus* also exhibits low vulnerability to acute thermal stress, in contrast to *P. canadensis* (Scriber et al. 2012). *Erynnis propertius* has at least moderate vulnerability to energetic drain due to winter warming: sub-lethal effects of warming were detected, although it was not possible to assess lethal effects of warming as even the control group had almost complete mortality, possibly due to desiccation. These classifications are broadly in line with our hypotheses based on species traits: the species with the highest degree of resource specialisation, greatest amount of development remaining post-winter, and most restricted range showed the highest sensitivity to warming. Once data on many more species are collected, it will be possible to conduct meta-analyses to assess species traits that are predictors of vulnerability to winter warming in a phylogenetically-informed context, guiding conservation efforts and identifying species most at risk from winter warming. A further avenue of interest will be to examine intraspecific variation in vulnerability to winter warming, as insect species can differ in their thermal physiology across their geographic range (Scriber 2011; Sinclair et al. 2012). As poleward populations of insects sometimes show raised metabolic costs relative to lower-latitude populations (e.g. Kukal et al. 1991), this could put northern populations at relatively greater risk of energetic drain resulting from winter warming.

The decreased sensitivity to warming in the 2 *Papilio* spp. compared to *Erynnis propertius* may be caused by a deeper degree of overwinter metabolic suppression in *P. glaucus* and *P. troilus*, making them less sensitive to temperature. Mass-specific metabolic rates of *E. propertius* pupae are about twice those of *P. zelicaon* pupae under identical conditions (Pelini et al. 2009); a similar degree of metabolic suppression in *P. glaucus* and *P. troilus* would explain their decreased sensitivity to temperature. A deep diapause, with a high degree of metabolic suppression, has been proposed to explain the low vulnerability of *P. glaucus* to stressful winter conditions (Scriber et al. 2012). The degree of metabolic depression experienced by insects during diapause corresponds roughly to the energetic demands of the overwintering stage (Hahn & Denlinger 2011); thus we would expect a more shallow diapause in insects that retain the capacity to move during dormancy (e.g. *E. propertius* and other larval or adult diapausers) compared to stationary stages such as eggs or pupae. This suggests that overwintering stage may be a good predictor of vulnerability to warming.

Water content declined over time in *Erynnis propertius* and *Papilio troilus* and was sufficient to fully account for the observed mass loss in both species. If we assume *P. glaucus* began the winter with similar water content to *P. troilus,* then it appears likely that *P. glaucus* also experienced significant desiccation over the course of the winter, which could fully explain the observed mass loss. This is supported by the observation that overwinter mass loss of *P. troilus* and *P. glaucus* were similar. If however we assume that water content of *P. glaucus* was regulated tightly throughout the winter at close to the finishing value of ~73%, as has been found by Scriber et al. (2012), then the observed mass losses over the winter may have been due to losses in energy reserves or protein. The high overwintering water loss by *E. propertius*

larvae (~40% of their initial water content) may be a cause of the high mortality observed in this species and may result from increased permeability of the larval compared to pupal integument, or interspecific differences in cuticular hydrocarbons (Chown et al. 2011). It is possible that the mortality in winter-warmed *P. canadensis* pupae (which also lose a lot of mass, Mercader & Scriber 2008) may be mediated by desiccation stress rather than energetic drain.

Winter warming thus brings with it the potential for a disruption of water balance, in addition to energetic drain. Warmer temperatures increase rates of water loss in insects with a Q_{10} of ~2 (Hadley 1994); thus warmer winters will increase rates of water loss. We found support for this in that rates of mass loss were faster in warm than cool conditions in all 3 species (Fig. 1), and mass loss appears to predominantly consist of water loss in at least *Erynnis propertius* and *Papilio troilus*. It thus seems likely that winter warming will increase desiccation stress in these species, and likely other insects that overwinter in dry environments, or in the presence of ice (see also Danks 2000). We suggest that understanding temperature-water loss rate relationships may be an important component of future work considering susceptibility to changing winters.

Warm winter conditions also impact post-winter development: *Papilio troilus* had decreased emergence time following a warm compared to a cool winter. Emergence time also decreases with increased overwintering temperature in *Osmia cornuta* and *O. lignaria* (Hymenoptera: Megachilidae; Bosch & Kemp 2003, 2004), and may be a result of higher respiration rates at warmer winter temperatures (Bosch & Kemp 2004), or some development occurring during the winter under warmer temperatures. A shortening of development time after warm winters will counteract the negative impacts of winter warming by truncating the period of dormancy with concomitant energetic savings. However, some species have a chilling requirement without which development times are extended (Gray et al. 2001), and these chilling requirements are species-specific and correlate negatively with habitat temperatures (e.g. Neven et al. 2000, Irwin et al. 2001, Forrest & Thomson 2011, Papanastasiou et al. 2011). For such species, increases in overwintering temperatures may slow development and extend the length of dormancy. This means that a positive feedback loop could amplify the negative effects of warming on cold-adapted species, while a negative feedback loop could mitigate the effects of winter warming on warm-adapted species.

The negative relationship between date of dormancy and total protein content (a proxy for metabolizing tissue and thus body size) of *Erynnis propertius* pupae may represent a general relationship between rates of growth and development and overall quality of individuals, such that higher quality individuals grow and develop faster (and thus reach the overwintering stage earlier), and also attain greater mass. Alternatively, later-diapausing individuals may be smaller due to a decline in host plant quality caused by seasonal fluctuations in phytochemistry (Bracho-Nunez et al. 2011) or inducible plant defenses (Rieske & Dillaway 2008). As climate changes, warmer growing seasons will allow faster rates of growth and development and may also extend the availability of host plant in the autumn, potentially mitigating the size penalties of delayed phenology and perhaps allowing the development of an additional generation (Altermatt 2010).

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