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Effects of Diet and Temperature Stressors on Fluctuating Asymmetry of Wing Traits, Mortality and Dry Mass in a Lepidopteran (*Vanessa cardui* Linnaeus)

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DEDICATION

To friends I met at the Rocky Mountain Biological Laboratory, where I was invigorated to work with insects and biologists. I will be back!

ABSTRACT OF THE THESIS

Effects of Diet and Temperature Stressors on Fluctuating Asymmetry of Wing Traits, Mortality and Dry Mass in a Lepidopteran (*Vanessa cardui* Linnaeus)

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Biologists have long been interested in using population parameters not only to assess species' growth trajectories, but also to make inferences about the processes underlying animal behavior. One parameter of considerable contemporary interest, fluctuating asymmetry, is characteristic of all bilaterally symmetrical animals. In the first chapter of this thesis, fluctuating asymmetry is defined in empirical terms and by how it relates to organism stress. Its importance to biologists is briefly reviewed, and some potential limitations to its utility are outlined. The second chapter is a report of a laboratory experiment designed to detect fluctuating asymmetry in wing traits of the model butterfly *Vanessa cardui*, and to test whether fluctuating asymmetry varied with the degree of environmental stress to which larvae and pupae were exposed. Finally, the third chapter critiques the author's experiment and evaluates the likely usefulness of fluctuating asymmetry as an indicator of changing environments in nature. Recommendations are made for those interested in studying fluctuating asymmetry further.

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Chapter 1

Questions and Answers about Fluctuating Asymmetry

There are numerous types of symmetry in nature (Graham *et al.* 2010), many of which are appreciated by the human eye (Enquist & Arak 1994; Chen *et al.* 2011). But the eye is usually not critical of, or precise enough to capture, the small imperfections in symmetry that are ubiquitous in nature. These asymmetries must be quantified by instruments such as micrometers or imaging software. This thesis is about one such type of imperfection – fluctuating asymmetry – that occurs in bilaterally symmetrical animals, which comprise about 99% of all extant metazoans (Freeman *et al.* 2014). In this chapter I pose some basic questions about fluctuating asymmetry and provide answers based on my reading of the literature. In Chapter 2, results of an experiment conducted on fluctuating asymmetry in a lepidopteran are reported, and in Chapter 3, a summary of the implications of results from chapter 2 for future work are discussed.

What is fluctuating asymmetry?

Fluctuating asymmetry can be visualized as the unsigned difference between two sides of a trait of a bilaterally symmetrical organism. For a trait to be an indicator of fluctuating asymmetry, its asymmetries must be small in magnitude and random in their direction, such that when measured in a large sample of a population, the distribution of asymmetries is normal around a mean of zero. The amount of fluctuating asymmetry that an individual displays is thought to reflect that individual's ability (relative to others in the population) to prevent environmental perturbations from negatively affecting developmental processes that maintain symmetry. This idea traces to the 1930's (Ludwig 1932, cited in Van Valen 1962). The rationale for expecting fluctuating asymmetry to reflect organism developmental processes is briefly outlined below.

Developmental canalization (Waddington 1942, 1957) refers to the ability of organisms to produce a consistent, species-typical phenotype despite the fact that individuals experience different environmental conditions during development, and despite the fact that (for sexually reproducing species) each individual has a unique genetic background. Implied in this concept is recognition that environments regularly present challenges to organisms in maintaining a trajectory toward optimal growth and development. When a facet of the environment taxes an organism's ability to maintain homeostasis, it is referred to as a "stressor" (Odum 1985). Organism capacity to prevent environmental perturbations from adversely affecting growth processes is finite (*i.e.,* canalization is imperfect), and some individuals have less ability to maintain homeostatic control of morphological development than others. Since both sides of bilaterally symmetric physical traits possess the same genetic background and experience similar environments during development, the amount of fluctuating asymmetry that an individual displays is thought to represent the physical manifestation of organismal limits on control over development (Graham *et al.* 1998).

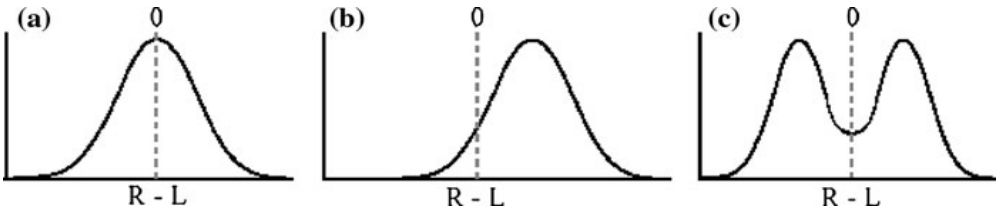
In addition to environmental stress, other factors may also contribute to fluctuating asymmetry. These include developmental stochasticity (measureable in clonal animals maintained under constant conditions; Babbitt 2008) and a range of genetic disturbances (including, but not limited to, increased homozygosity such as that occurring in small populations and the effects of rare major genes/mutations; Leary & Allendorf 1989; Parsons 1992; Habel *et al.* 2012). Researchers interested in effects of environmental stress on fluctuating asymmetry ignore the effects of developmental stochasticity (assuming its effects random with respect to questions under study). The rarely encountered individuals that exhibit asymmetries much larger than others of their population are typically excluded from studies of environmental effects, under the

assumption that such asymmetries are the results of genetic disturbances (Palmer & Strobeck 1986).

The reasoning developed above suggests that fluctuating asymmetry is not adaptive and that natural selection should act to reduce its occurrence, typically resulting in small deviations from perfect symmetry. Also, heritability of fluctuating asymmetry should be low as a result of consistent selection against it (Van Valen 1962). Empirical evidence is largely consistent with these expectations (*e.g.,* Van Valen 1962; Leamy & Klingenberg 2005; Polak 2008; Tsujino & Takahashi 2014). This suite of characteristics differentiates fluctuating asymmetry from two other types of asymmetry that are prevalent in bilaterally symmetric organisms, namely directional asymmetry (one side of a trait is consistently larger than the other) and antisymmetry (bimodality of trait distribution) (Figure 1). While bilateral symmetry had a single origin deep in the evolution of metazoans, antisymmetry and directional asymmetry have subsequently evolved numerous times in various lineages of bilaterians (Palmer 1996). In contrast to fluctuating asymmetry, the other prevalent types of asymmetry are often known or suspected to be functional in that they allow for role specialization of affected organs or appendages. For example, male crabs of the genus *Uca* show pronounced asymmetry in the size of their claws: one large ("major") claw is specialized for display, while the other ("minor") claw is small and used for food gathering (Levinton *et al.* 1995). The brains of humans show lateralization indicative of specialization: notably, the left forebrain tends to be larger and side difference is greater in right-handed individuals (Gotts *et al.* 2013). While early studies suggested that both directional asymmetry and antisymmetry have significant heritabilities (*e.g.,* Mather 1953; Van Valen 1962), recent research indicates a more complicated story (Palmer 1996) that is largely irrelevant to the topic of fluctuating asymmetry. The important point here is that these forms of asymmetry are thought to have evolved due to advantages of lateral specialization; therefore, their presence is not evidence

of failure of homeostatic control over development. However, where these forms of asymmetry do occur, their presence makes it more difficult to quantify fluctuating asymmetry, as fluctuating asymmetry typically manifests as minor side differences compared to these other types of asymmetry (Palmer 1994).

Figure 1. Distributions of right side minus left side (R-L) means in bilaterally symmetrical organisms: (a) fluctuating asymmetry, (b) directional asymmetry, (c) antisymmetry. From Allenbach (2011), who adapted from Palmer (1994).



Theoretically, fluctuating asymmetry will be present in all bilateral traits, yet some traits may be more suitable than others for detecting it. Since the relationship between environmental stressors and development of specific traits is often unknown, selection of traits is predicated by practical and statistical considerations (see Chapter 2). Nevertheless, for reasons unknown, selected traits will often differ in their magnitude of fluctuating asymmetry. Since the utility of measures of fluctuating asymmetry is that they provide estimates of individual and population developmental stability, multiple traits should therefore be measured, because fluctuating asymmetry of one trait does not accurately represent the overall developmental stability of the individual, due to several reasons, including sampling error and the small magnitude of fluctuating asymmetry (Whitlock 1996; Leung *et al.* 2000; Van Dongen 2006). Nevertheless, a fairly recent review (Lens & Eggermont 2008) found that only a small proportion of studies included more than one trait as a measure of an individual's fluctuating asymmetry. In addition, some reviewers of fluctuating asymmetry have advocated for the use of trait composites (integrated fluctuating asymmetry score across multiple traits; *e.g.*, Leung *et al.* 2000, Gangestad

et al. 2001, Palmer & Strobeck 2003); when this approach is taken, the signed asymmetries of traits included in such a composite should not be correlated, as this would suggest they are also developmentally correlated, and thus not independent measures (Palmer & Strobeck 2003). A composite index is favored under the premise that it provides a more sensitive barometer of individual developmental stability than do multiple individual traits. An underlying assumption here is that there exists a mechanism that buffers development throughout the body, but does not exert local control (Mitton 1993; Polak *et al.* 2003).

Why do ecologists and evolutionists study fluctuating asymmetry?

While researchers in a number of disciplines, including medicine (Aw & Levin 2009; Thomas *et al.* 2012) and (captive) animal welfare (Knierim *et al.* 2007) have begun to use fluctuating asymmetry as a tool, most work has been concentrated in the fields of sexual selection (as studied by behavioral ecologists) and conservation biology. The rationale for the interest in fluctuating asymmetry by researchers in these two fields is briefly described below.

Sexual selection

Sexual selection favors the evolution of costly secondary sexual traits, such as those that are large or especially colorful (Andersson 1994). Theory predicts that these traits are expected to evolve condition-dependent expression, such that only (in most systems) males of high quality are able to develop and maintain extreme trait expression that is attractive to potential mating partners and/or intimidating to competitors (Zahavi 1975; Iwasa *et al.* 1991). On the expectation that most traits are subject to stabilizing selection while sexually selected traits are under directional selection, Møller & Pomiankowski (1993) predicted that fluctuating asymmetry would be greater in sexually selected traits than in other traits and that degree of expression of a sexually selected trait would be inversely proportional to its fluctuating asymmetry. Thus, they suggested, fluctuating asymmetry of such traits could be used an as indicator of male quality and should

negatively correlate with male fitness (see also Leung & Forbes 1996). They also predicted that fluctuating asymmetry of secondary sexual traits is heritable (heritability being maintained by the unrelenting directional selection imposed on the trait), so that by choosing males that display large or otherwise extreme trait expression and low asymmetry, females increase average genetic quality of their offspring.

Given that sexual selection is one of the most researched topics in behavioral ecology (Owens 2006), it is not surprising that there have been numerous tests of these predictions over the more than 20 years since these ideas were developed. The most recent general review of this literature (Polak 2008) indicates that symmetrical traits are often preferred in mate choice; however, authors of the review found little support for the idea that fluctuating asymmetry is a quality indicator. Specifically, fluctuating asymmetry of sexual traits generally has low heritability; there is no evidence of a consistent association between trait size and trait asymmetry; and there is no evidence that trait asymmetry is a condition-dependent indicator. Nevertheless, there is much heterogeneity of results across studies, indicating that fluctuating asymmetry of secondary sexual traits may be an indicator of male quality in some systems. However, there appears to be no theoretical development or set of predictions about the circumstances under which fluctuating asymmetry should be informative of male quality.

Despite these criticisms, it is important to acknowledge that there have been recent advances in elucidating the genetic bases of fluctuating asymmetry (Leamy & Klingenberg 2005). Also, given that measures of trait fluctuating asymmetry often have heritabilities and genetic architecture similar to traits with large fitness implications, such as litter size in mammals (Leamy & Klingenberg 2005), it is premature to conclude that fluctuating asymmetry is not useful as an indicator of individual quality. Thus far, however, there appears to be no theoretical development

or set of predictions about the circumstances under which fluctuating asymmetry should be a quality indicator.

Conservation

Contemporary biologists are becoming increasingly invested in efforts associated with conservation, especially as the issue of widespread negative anthropogenic impact on the world's biomes has risen to eminence. Worldwide, it is expected that nearly 1000 species will go extinct every year (De Vos *et al.* 2014). Anthropogenic factors, which have both large direct and indirect effects (including genetic factors such as heterozygosity and hybridization; Leary & Allendorf 1989) on local environments and global climate, are in large part responsible for the extinction epidemic. Increasing temperatures worldwide have begun to have cascading effects on local ecosystems. Fluctuating asymmetry has been advanced as a potentially powerful tool for measuring the effect of such environmental stressors (Lens & Eggermont 2008; Beasley *et al.* 2013), as well as a potential tool for helping conservationists prevent further extinctions (Leary & Allendorf 1989).

In conservation studies, animal populations are assessed for indications of growth or decline. One way investigators do this is to construct life tables to examine how lifetime fitness varies in populations over time. Traditional measures of fitness include fecundity and survival estimates, but these are time- and labor-intensive to collect and are sometimes simply unobtainable (Clarke 1995; Lens & Eggermont 2008; Schmeller *et al.* 2011). Traditional fecundity measures have included sacrificing organisms to measure ova or allocation to gonadal tissue, which is not ethically feasible in vulnerable populations (Clarke 1995; Lens & Eggermont 2008). Accordingly, ecologists have increasingly sought for surrogate measures of these parameters by emphasizing measures that reflect changes in life history patterns, such as body size and age at maturity (Odum 1985). More recently, some researchers have embraced

fluctuating asymmetry as a superior surrogate to these measures, on the grounds that by serving as a barometer of stress organisms experience during development, measures of fluctuating asymmetry will permit tracking of population health with minimal invasiveness (*e.g.*, short handling time; Lens & Eggermont 2008; Beasley *et al.* 2013) and without major investments of resources (Beasley *et al*. 2013). Proponents of using fluctuating asymmetry as a measure of response to stress point out that no other morphological or physiological trait has been found to reflect stress reliably (Van Dongen 2008; Beasley *et al.* 2013). Some studies have demonstrated that historical (*e.g.* museum) specimens can be utilized to track changes of fluctuating asymmetry – and thereby track changes in population stress – over time (see Schmeller *et al.* 2011)

Fluctuating asymmetry is thought to be particularly useful for studying organisms that exist at the edges of their distribution, where it may capture a signal indicating convergence of multiple sources of stress that include genetic drift, abiotic extremes, and stochastic processes (Kark *et al.* 2004; Ashton *et al.* 2009; Schmeller *et al.* 2011). Studies of birds and mammals have indeed demonstrated that populations experiencing habitat fragmentation (Anciaes & Marini 2000) and those found at the limits of their range (Møller 1995; Auffray *et al*. 1999) display high fluctuating asymmetry in comparison with other populations. Studies on lepidopterans have not been so successful in this regard, however. After failing to find a pattern similar to those cited above for birds and mammals in two species of Pierid butterflies, Kark *et al.* (2004) offered this as explanation: perhaps the butterflies in their study did not experience abiotic stress, but were rather simply limited in further expansion by host plant distribution. If this interpretation is correct, then fluctuating asymmetry remains useful as a means to detect stressful circumstances.

Fluctuating asymmetry is thought to offer several practical advantages. In addition to those mentioned above (low invasiveness, low equipment needs), the technique allows for easy comparison of spatially and temporally separated individuals (Hogg *et al.* 2001). This idea is

highlighted by a recent study of the near-threatened butterfly *Parnassius apollo* (Schmeller *et al.* 2011). Authors measured fluctuating asymmetry of wing traits in both historical museum specimens and live specimens in hopes of finding indication of population recovery after the International Union for Conservation of Nature listed the butterfly as Vulnerable; they were able to demonstrate changing levels of fluctuating asymmetry over a span of more than a century.

What are major barriers and issues faced when applying fluctuating asymmetry?

Historically, fluctuating asymmetry studies have suffered from poor statistical design. That is no longer a barrier as Palmer & Strobeck (2003) have provided clear guidelines (their approach is emulated in Chapter 2). But several challenges and issues are ongoing, the most important of which are discussed below.

Difficulty of measuring fluctuating asymmetry

Fluctuating asymmetry manifests as very small deviations from symmetry. These deviations are often two orders of magnitude smaller than the traits they appear in (Lens *et al.* 2002). Depending on the size of an animal, measuring fluctuating asymmetry may require the use of fine calipers, high-powered ocular equipment, and/or digital equipment. If physical traits are to be measured digitally, organisms should be photographed on the same physical plane. Together, these requirements make the prospect of accurately measuring fluctuating asymmetry difficult, especially if done in field conditions.

Measurement error is a large concern for fluctuating asymmetry studies (Leung & Forbes 1995; Lens *et al.* 2002; Palmer & Strobeck 2003) because the effect size of fluctuating asymmetry is often small relative to trait size, and similar in magnitude compared to measurement error (Lens *et al.* 2002). To describe fluctuating asymmetry, one must demonstrate that measurement error in a sample is much smaller than the between sides variation in trait size. For larger animals (which have larger traits), measurement error relative to fluctuating asymmetry

is likely to be smaller. However, researchers are sometimes limited in the sample sizes they can gather, especially when focal animals are large or have relatively low fecundity (*e.g.* fish, birds, mammals) (Beasley *et al.* 2013). Where sample sizes are small, the effect of measurement error may be compounded. Also, when fluctuating asymmetry occurs alongside directional asymmetry or antisymmetry, it may be especially hard to detect (Lens *et al.* 2002).

Organisms with different life histories may show different levels of fluctuating asymmetry

Some organisms are more suited for the detection of fluctuating asymmetry because of their ecological and or developmental characteristics. Fluctuations in temperature can cause serious problems for some holometabolous insects, the degree to which may depend on habitat or intervening ecological variables. For example, investigators working on *Parnassius apollo* have found that unusually warm spells in winter cause "false spring" events, in which larvae hatch early and then later starve when temperatures fall and they are unable to feed. In addition, early snow melt can cause drought conditions to occur early in the season, with the result that host plants become unsuitable (Descimon *et al.* 2005, cited in Ashton *et al.* 2009) and drive high mortality of pre-adults. In a study of immature stoneflies, however, investigators found that local increases in temperature had no effect on fluctuating asymmetry levels (Hogg *et al.* 2001). The authors concluded that the small temperature shifts (like those that can be driven by global warming) observed in their study are likely not driving a level of stress required to induce detectable fluctuating asymmetry. Mobile aquatic insects, however, may have more opportunities to escape local temperature maximums or to forage in areas with more optimal conditions than terrestrial insects that may have limited dispersal ability, like the Apollo butterfly. Indeed, as Lens & Eggermont (2008) have pointed out, roving animals are likely less susceptible to local environmental stresses than sedentary animals.

Depending on taxon, fluctuating asymmetry may only signal some types of stress and then only in some traits (Breuker & Brakefield 2003), and in certain developmental stages (Van Dongen 2006; Windig & Nylin 2002; but see Talloen *et al.* 2004). Some investigators posit that invertebrates have accelerated complex developmental programs that make them more susceptible to environmental stresses than vertebrates (Beasley *et al.* 2013) and which will make fluctuating asymmetry more apparent. Yet, several other hypotheses contradict this idea. For example, the Adaptive Decoupling hypothesis (Moran 1994) posits that the evolution of complex life cycles is predicated on the decoupling of traits over multiple life stages (see also Developmental Selection hypothesis, Møller 1997). Campero *et al.* (2008) have since proposed the Stressful Metamorphosis hypothesis, which states that early stressors (*e.g.*, those occurring in the larval stage) have less impact on adult phenotype than later stressors (*e.g.*, those occurring during the pupal stage), which takes from the idea that the advent of metamorphosis reduces asymmetries accrued during development. Furthermore, there is accumulating evidence that in insects, life stages are not developmentally independent (Pechenik 2006). This suggests that punctuated anatomical reorganizations experienced by organisms with complex life cycles may allow stressed individuals opportunities to "fix" asymmetries accumulated during early life stages, with the result that fluctuating asymmetry could be reduced in adults that have undergone metamorphosis. In their review of Odonate life cycles, Stoks & Cordoba-Aguilar (2012) suggested that conclusions about the effects of environmental inputs on an organism's life history may not be accurate or illuminating unless inputs and life events that occurred before and after metamorphosis are both considered.

Lack of clear predictions about effects of stressors

As explained above, many studies of fluctuating asymmetry use this metric as an index of organismal response to environmental stress. But what is known about the nature of stressors that predictably influence fluctuating asymmetry? In this section, I briefly address this question.

One review paper listed the following as examples of types of environmental stress whose effects on fluctuating asymmetry have been studied: "temperature, nutrition, radiation, chemicals, population density, noise, parasites, light conditions, predation risk, and habitat structure" (Leamy & Klingenberg 2005). This varied list would appear to suffice to identify stressors that predictably influence fluctuating asymmetry. However, several reviews of the literature have concluded that putative environmental "stressors" do not consistently cause increased trait fluctuating asymmetry in populations (Leung & Forbes 1996; Hogg *et al.* 2001; Lens *et al.* 2002; Van Dongen 2008). Some of the proposed explanations for the observed inconsistencies in results among studies follow. (1) Stressors may impact certain traits (*e.g.*, sexually selected ones – see above) more than others, so selection of traits for study must be made carefully. (2) Particular types of environmental stimuli may be more likely to impact fluctuating asymmetry than others (*e.g.*, Polak *et al.* 2004). However, highly variable results have been found in response to some presumed stressors, such as heavy metal exposure. Monna and colleagues (2011) reported, for example, that levels of fluctuating asymmetry of brown trout (*Salmo trutta fario*) varied directly with cadmium and lead concentrations found at several locations in France; on the other hand, Polak and colleagues (2004) found that lead exposure did not increase fluctuating asymmetry in laboratory strains of fruit flies (*Drosophila melanogaster*). (3) Results like those found for lead exposure cited in the previous sentence could easily result from different taxa being sensitive to different stressors (Palmer, 1994). However, continuing to use heavy metal exposure as an example, it is easy to find contradictory results regarding its impact on a single species (*e.g.*, bristle number on sternopleural plates of *Drosophila melanogaster*: Graham *et al.*

1993; Polak *et al*. 2004; tarsus length of the great tit, *Parus major:* Eeva *et al.* 2000; Dauwe *et al*. 2006). (4) Various combinations of stressors may have unpredictable interaction effects, including one stressor dampening the effect on fluctuating asymmetry of another (Polak *et al.* 2004). (5) Failure to demonstrate that a presumed stressor impacts development, maintenance, and/or reproduction of an organism may contribute to erroneous interpretation, especially when negative results are found (Leung & Forbes 1996). (6) Particularly for early studies, failure to consider measurement error may have led to many spurious results (Leung & Forbes, 1996); inclusion of these studies in meta-analyses and reviews complicates interpretation.

In sum, the general utility of fluctuating asymmetry as an indicator of organism resistance to environmental stress suffers from that fact that there is no group of stressors that has been identified to reliably impact fluctuating asymmetry. Equally if not more important are the problems that there is no body of theory to predict which sort of stressors should be most impactful and no conceptual perspective to allow prediction regarding how ecological conditions may impact the relationships among stress, fluctuating asymmetry, and organism fitness (Lens & Eggermont 2008). Without progress in these areas, it is unlikely that the scientific community will reach a consensus that fluctuating asymmetry is an important tool for conservation research.

Further Reading

Fluctuating asymmetry is a topic that has been studied for over 50 years in the context of measuring developmental stability of organisms. Given the range of practical problems and conceptual issues surrounding this area of research, it is not surprising that authors are deeply divided about the usefulness of fluctuating asymmetry as either a measure of individual quality or environmental stress experienced by populations. For favorable reviews, readers are pointed to Van Dongen (2006) and Beasley *et al.* (2013). For less favorable ones, see Lens *et al.* (2002) and Palmer & Strobeck (2003).

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Chapter 2

Does FA of wing traits capture relative environmental stress in a lepidopteran?

Abstract

Numerous investigators have hypothesized that fluctuating asymmetry may be a useful predictor of population canalization, especially for organisms at risk from environmental change. However, identification of traits that meet statistical criteria as measures of functional asymmetry remains a challenge. This study was undertaken to specifically address this problem in the context of an experiment performed under controlled laboratory conditions. Poor quality diet and elevated temperature were used as stressors on adult wing phenotype of *Vanessa cardui*, a cosmopolitan butterfly. Variation in larval diet stress was introduced by manipulating the macronutrient ratio of protein to carbohydrate. Thermal stress was varied by housing larvae/pupae in growth chambers maintained at optimal (25°C) or elevated (32°C) temperatures. Individuals subjected to stressful conditions (especially a low protein diet and high temperature throughout development) were predicted to show elevated fluctuating asymmetry of three wing traits. While fluctuating asymmetry proved measureable for all three traits, it did not vary among treatment levels. Instead, the combined percentage of individuals that died prior to completing eclosion and of those that showed significant malformation of wing characters (together, "inviability") increased in the treatment levels that were predicted to increase fluctuating asymmetry. In addition, treatment differences in adult dry mass were observed that reflected predicted stress levels. These results suggest that potentially measureable variation in fluctuating asymmetry was underrepresented in the study population because a substantial proportion of individuals predicted to display increased fluctuating asymmetry either died or were developmentally aberrant. This experiment illustrates

important constraints on the investigation of fluctuating asymmetry, including choice of appropriate traits and prevention of elevated mortality through identification of ideal levels of stress. The last concern brings into question the utility of fluctuating asymmetry as an indicator of stress in vulnerable, natural populations, where level of stress is rarely controlled, and mortality (which, where elevated by stress, may mask fluctuating asymmetry) and effects on fitness can often not be quantified.

Introduction

In bilaterally symmetrical organisms, fluctuating asymmetry of a trait is defined as deviation from perfect symmetry that is random in its direction (left or right side biased), but normally distributed around a mean of zero (Palmer & Strobeck 2003). Under favorable conditions, homeostatic mechanisms operating during development buffer effects of random perturbations, a phenomenon that has been termed "developmental stability" (Gibbs & Breuker 2006; Ludoski *et al.* 2014; Habel *et al.* 2012); effective mechanisms result in low fluctuating asymmetry. Homeostatic mechanisms can be overwhelmed by a range of environmental conditions, including poor diet quality (Windig & Nylin 2002), food shortages (Stoks 2000), high parasite density (Møller 2005), and interference competition for limited resources (Clark & McKenzie 1992), as well as abiotic stresses such as extreme temperature (Parsons 1992; Sisodia & Singh 2009); such biotic and abiotic factors, often called "stressors", lessen an organism's ability to allocate resources to development and reproduction (Bilsma & Loeschcke 2005; Beasley *et al.* 2013). When stressors cause developing individuals to shift allocation away from maintaining developmental homeostasis to more pressing needs, such as insuring survival (Møller 2005), fluctuating asymmetry is expected to increase. This increase in fluctuating asymmetry is considered evidence of poor developmental stability.

The purpose of the current experiment is to investigate whether environmental stressors influence fluctuating asymmetry in an insect species, the painted lady butterfly (*Vanessa*

cardui Linnaeus), under laboratory conditions that allow good control over stressors and make possible determination of fates of all subjects. Temperature and diet manipulations were selected as stressors. Temperature was selected for two reasons. First, small changes in rearing temperature of lepidopteran larvae and other insects can result in large changes in the rate of development (Wagner *et al.* 1984; Khadioli *et al.* 2014). These changes may reflect increased demands on the developmental program of the organism. Thus, temperature is a logical candidate for a stressor that could increase fluctuating asymmetry (*e.g.*, Parsons 1992; Polak *et al.* 2004; Beasley *et al.* 2013). Second, due to global climate change, temperature is of great contemporary interest to investigators of fluctuating asymmetry. One reason for this is that diet quality may often be ecologically tied to temperature (Descimon *et al.* 2005; Ashton *et al.* 2009). Accordingly, in this experiment, the macronutrient composition of the larval diet was manipulated in order to test an ecologically realistic combination of stressors.

The painted lady is hardy, easy to culture in the lab, and thrives on general lepidopteran diets (*e.g.*, Ahmad *et al.* 1989). This sexually monomorphic species is a widespread generalist herbivore (Janz 2005; VanOverbeke 2011) that is a model organism in studies of migration. Adults possess conspicuous wing eyespots that are characteristic of other lepidopterans (*Pararge aegeria*, *Parnassius apollo,* & *Bicyclus anynana*) for which fluctuating asymmetry of wing traits has been investigated (Talloen *et al.* 2004; Gibbs & Breuker 2006; Habel *et al.* 2012).

The following predictions were made at the outset of the study. First, diet and temperature treatments would impact fluctuating asymmetry. Specifically, elevated temperature throughout development should result in greater fluctuating asymmetry than would a rearing temperature near the species optimum. Immatures experiencing elevated temperature for only part of development should display intermediate levels of fluctuating asymmetry. Regarding diet treatments, all diets produced in the lab (chemically defined artificial diets) were expected to result in greater fluctuating asymmetry than the commercial diet on which the study population

had been reared for a number of generations. Caterpillars reared on lab diets were expected to show higher mortality and fluctuating asymmetry. Likewise, based on previous work on this species (VanOverbeke 2011), it was expected that diets with unequal macronutrient ratios – especially carbohydrate-biased diets – would effect greater mortality and fluctuating asymmetry. Dry mass data were expected to follow patterns predicted for fluctuating asymmetry, such that more stressful conditions should result in individuals of lower mass. Also, since differential mortality among treatments reflects relative stress, mortality patterns should show similar patterns to those predicted for fluctuating asymmetry. An important caveat here is that, where stress causes high mortality, those individuals most likely to display high fluctuating asymmetry are also those most likely to die before completing development (Polak *et al.* 2004). Where high mortality occurs, differential effects of stress may be reflected in dry weight and mortality patterns, but patterns of fluctuating asymmetry may not show predicted effects, because fluctuating asymmetry can only be measured in adults that survive eclosion without damage.

Methods

This experiment incorporates several criteria for the selection of candidate traits established by Palmer & Strobeck (2003) for the study of fluctuating asymmetry: (1) multiple traits should be included and should display low phenotypic correlation in the direction and size of their left-right side differences. (2) Selected traits must display left-right side variances significantly greater than that which is caused by measurement error, and these traits should display similar magnitudes of measurement error. (3) Candidate traits must be screened for the presence of other types of asymmetry; those that exhibit anti-symmetry (bimodality) obscure fluctuating asymmetry and are not suitable. Palmer & Strobeck (2003) also provided a framework for the statistical evaluation of fluctuating asymmetry, and the analysis here follows the steps they outlined.

Butterfly culture and experiment initiation

The butterflies in this study were obtained as five separate shipments of 100 eggs from a commercial supplier (Carolina Biological Supply: Burlington, N.C., U.S.A.) that also supplied a proprietary medium (Carolina Biological Painted lady culture medium; referred to as "Carolina" below) for rearing this species. The commercial stock had been maintained on this diet under standard conditions for numerous generations, with periodic additions of wild-caught butterflies. Purchased eggs were reared to adulthood on Carolina medium. Individuals were allowed to breed for two generations before the experiment was initiated.

The rearing protocol involved several steps. Adults were housed in laboratory flight cages (24" wide x 36" tall cylinders) that held 30 individuals and were supplied with food (sugar-honey water) and oviposition sites (flower cuttings of *Achillea millefolium*, which were refreshed every other day). Eggs were collected every two days, sterilized in a 5% bleach solution for two minutes, and placed in group-rearing chambers containing Carolina medium. These chambers were inspected daily for pupae. Pupae were transferred to an eclosion box within two days of pupation. Within 24 hours of eclosion, butterflies were transferred to flight cages. Between generations, butterflies were randomly shuffled among flight cages to ensure outcrossing.

The F2 generation of eggs was produced in the lab in December 2015, at which time eggs were collected over a 48 hr period from 10 flight cages of 30 butterflies each. Upon collection, eggs were sterilized and transferred to rearing chambers as above, where they developed on the Carolina diet for the first two instars. When a sufficient number of larvae simultaneously reached third instar, individuals were randomly assigned, one at a time, to one of 10 experimental treatments described below. A total of 600 F2 larvae were individually transferred with a paint brush into 1oz (Solo®) soufflé cups. These cups were then placed into their respective growth chambers, using a randomization design to assign individual larvae to shelves. Experimental conditions commenced immediately thereafter.

Stressors

To investigate the impact of stressors on adult wing trait fluctuating asymmetry, larvae were reared in growth chambers under varying thermal and dietary regimes. Growth chambers (Percival incubators – model 136LL) were maintained on a L16: D8 photoperiod for all treatments. Temperature was maintained at 25 +/- 1°C in Chamber 1 and at 32 +/- 1°C in Chamber 2. Chambers were maintained at 60 +/- 10% humidity. Each chamber possessed two shelves.

The experiment was designed to be replicated through the use of two sets of two growth chambers, the maximum number of chambers that could be obtained for use in this experiment at the time. However, development did not proceed as smoothly in the chambers used for the second replicate. Pre-eclosion mortality was approximately double that which occurred in the first replicate chambers, with disproportionate mortality occurring in one of them. Accordingly, here I report results only for the first replicate.

Temperature levels were selected based on several considerations found in the work of Poston *et al.* (1977) as well as those gleaned from preliminary investigations, which involved rearing butterflies under a variety of conditions before the start of the experiment. An upper-limit temperature was chosen as a stress inducer rather than a lower-limit temperature for practical considerations: mean expected rearing time from egg to eclosion at a mid-range temperature (36 days at 24°C) is much closer to that for a high temperature (22 days at 32°C) than a low temperature (72 days at 18°C) (Poston *et al.* 1977). Various commercial suppliers that maintain populations of *V. cardui* year-round suggest that optimal rearing conditions include a "room-temperature" environment that does not exceed 26-27°C and that permits completion of development (egg to adult) over the course of three to four weeks. During the preliminary investigation of candidate traits for this study, nearly complete larval mortality (especially in the first two instars) was observed at a constant rearing temperature of 35°C, while at 32°C larval

mortality was not much greater (10-15 %) than that observed at 25°C. Accordingly, 25°C was chosen as a temperature close to optimal for development (temperature level 1), and 32°C was chosen as the stressful temperature, as it appeared nearly neutral for mortality but close to the temperature at which high mortality is observed (Poston *et al.* 1977). To investigate the prediction that immatures subjected to stressful temperatures throughout development would show higher fluctuating asymmetry than those stressed for only part of development, temperature treatment levels included rearing individuals at elevated temperature (32°C) during both the larval and pupal stages (temperature level 3) and rearing subjects at elevated temperature only during the larval stage (temperature level 2; Table 2).

As described above, the stock of *V. cardui* used in this experiment had been maintained on Carolina medium for a number of generations. The Carolina diet was included in the experiment (diet level 1) as a baseline condition; it was expected that larvae would thrive on this diet, to which they had become adapted. For all other diet levels, a chemically defined artificial diet was used (see Table 1 for full diet recipes) in order to test the effect of macronutrient ratio on induction of fluctuating asymmetry. The chemically defined diet was adapted for rearing *Vanessa* by a past lab member (VanOverbeke 2011) from a formulation originally created for use in *Manduca sexta* (Ahmad *et al.* 1989). During preliminary trials, normal pupation was observed for larvae reared on this diet, but many pupae failed to initiate eclosion; of those that did initiate eclosion, many failed to complete the process. As a result, the recipe was modified to include linseed oil, a source of fatty acids (linoleic and linolenic acids) necessary for ecdysis in some insects and particularly lepidopterans (Nation 2015). Diet treatments included equal amounts of protein and carbohydrate (1:1; diet level 2), as well as high protein (3:1; diet level 3) and low protein (1:3; diet level 4) ratios (Table 2). Based on VanOverbeke's (2011) finding that *V. cardui* suffered most on a carbohydrate-biased artificial diet, it was predicted that adult butterflies would show the greatest fluctuating asymmetry when supplied with a 1:3 protein:carbohydrate diet.

In sum, the experiment included a total of 10 treatments, generated from two factors: diet and temperature. There were three temperature levels and four diet levels (Table 2). Three treatments involved transfer of newly formed pupae from a high temperature (32°C) to a lower temperature (25° C) condition (T5, T7 and T9 in Table 2). Transfer was performed when pupae were one day old. Larvae in one diet regime (Carolina diet) were subjected to only one temperature regime (25° throughout development) as a baseline or control condition.

Eclosion and Processing of Specimens

Starting three days after the first pupae developed, chambers were monitored every 12 hours for signs of imminent eclosion. Soufflé cups housing pupae were cleaned out (food and frass removed) or replaced to allow enclosing butterflies room to dry wings. Butterflies were allowed to dry wings fully in the cups housing them, and were thereafter quickly removed to preserve the integrity of wing characteristics. Adults were placed into a growth chamber maintained at 18°C and held for 24 hr to allow them to evacuate meconium. Butterflies were subsequently placed individually, wings folded behind the ventral edge of the body and on either side of the abdomen, into glassine envelopes and were then euthanized in a freezer.

Frozen specimens were sorted into two categories: (1) those with wing deformities and those that failed to fully escape their puparia versus (2) those that appeared to have fully developed wings. Butterflies in the first category were classified as "inviable", as they would be unlikely to survive and reproduce; their non-directional wing asymmetries would not be measureable. Butterflies in the second category were deemed "viable" and were included as subject in the investigation of fluctuating asymmetry (Table 2).

Specimens were subsequently rehydrated for up to an hour to make wings pliant for removal from the abdomen. Wings were cut at abdomen base in the same order (right forewing/left forewing/left hindwing/right hindwing). Wings of viable specimens were then

sealed in left-right pairs in clear packaging tape for imaging. Scans of these wings were taken on an HP printer (LaserJet M1522n) at 1080dp.

After dissection, the head, thorax, and abdomen of each specimen (both viable and inviable) were placed together in a glassine envelope and allowed to desiccate on the lab bench for two weeks. Total dry mass of these body parts (legs were not included because of breakage problems) was obtained using a NewClassic MF balance (Mettler Toledo model MS205DU).

Trait Selection and Measurement Procedures

During preliminary investigations, seven candidate wing traits were identified (forewing area, forewing vein, forewing spot, hindspot 1, hindspot 2, hindvein, hindwing area). These traits were then measured (and later re-measured) for 50 randomly selected individuals that were used only for measurement practice and trait selection (*i.e.*, they were not part of the experiment). Using the data from these 50 individuals, the seven traits were analyzed for relative measurement error and for correlations among the left-right side differences in size (*i.e.*, trait asymmetry). For four traits (forewing vein, forewing spot, hindspot 2, hindwing area), differences in signed asymmetry were strongly inter-correlated. This non-independence of phenotypic expression implies developmental interdependence (Palmer & Strobeck 2003), and so these traits were dropped from further consideration. Three remaining traits – forewing area, hindwing spot area (spot at the anal angle of the wing), and length of a hindwing vein (second branch of cubitum vein) – were not strongly inter-correlated and did not differ in measurement error. These three are the traits used in analyses included here.

Measurement of forewing area (100% image magnification) and hindwing spot area (400% image magnification) was accomplished using the "polygon" tool in ImageJ (Schneider *et al.* 2012) to manually outline the border of these traits (see Figures 2 and 3 for examples of polygons for these traits). Hindwing vein length (100% image magnification) was measured,

using the "segmented line" tool, as the shortest length from a starting point near the abdomen to the border of the flange at the outer edge of the wing (Figure 3).

Indices of Fluctuating Asymmetry

Fluctuating asymmetry of individual traits was evaluated using the mean of the absolute value of the left side minus the right side measurement of the trait (mean|left- right| size for twice-measured individuals and |left- right| size for individuals measured once), after establishing that this value was greater than measurement error and met various statistical criteria (named "FA1" in Palmer 1994; Palmer & Strobeck 2003). Hindvein length was measured in millimeters and forewing and hindspot area in millimeters^2.

An individual butterfly's composite fluctuating asymmetry ("FA11" in Palmer 1994) was calculated by summing observed FA1 scores for individual traits. Only traits that had similar measurement errors in the full data set (forewing area and hindvein length) were included in the composite index. Since traits differ in their magnitude of fluctuating asymmetry, values were ln-transformed prior to adding them together so that traits contributed approximately equally to the composite fluctuating asymmetry index (Palmer & Strobeck 2003). For example, if the composite includes two traits, Trait 1 and Trait 2, then, for individual *x*,

$FA11_x = |(\ln(\text{left Trait}1_x) - \ln(\text{right Trait}1_x))| + |(\ln(\text{left Trait } 2_x) - \ln(\text{right Trait } 2_x))|$.

Analyses

A total of 310 individuals were included in the study. The first 101 individuals to be measured were re-measured after approximately two-thirds of all subjects had been measured once. Due to time constraints, the remaining individuals were measured only one time. Individuals that were measured twice were selected using a stratified random design to ensure that approximately 10 individuals from each treatment were included.

Palmer & Strobeck's (2003) protocol for analysis of trait fluctuating asymmetry was used as a reference for completing the analysis procedure described below. Data for the individuals

measured twice were analyzed first and were used to quantify measurement error. Under the assumption that measurement error was the same for individuals measured once as for those measured twice, the remaining data analysis was conducted on the full data set derived from 310 individuals.

Screening for outliers – The first two series of analyses screened the data set for problems that inflate the estimate of fluctuating asymmetry of traits. These analyses were initially performed for each diet and temperature level (seven separate analyses for each of three traits). The rationale for performing analysis on each factor level is that if outliers are disproportionately represented in one or two levels, they would be difficult to detect in the aggregate data set, yet potentially bias outcome of analyses of fluctuating asymmetry of the full data set (Palmer & Strobeck 2003).

The first set of analyses was performed to identify possible cases of "bad" raw measurements. This category includes instances in which larger-than-usual error was made in measuring a specimen, as well as errors unrelated to measurement precision *per se* (such as data entry errors). This analysis could only be performed for the replicated data set (sample sizes ranged from 10 [diet level 1] to 46 [temperature level 1]). Scatterplots were created to display the difference between replicate measurements of one trait (x-axis) against the difference between replicate measurements of another trait (y-axis) for a group of individuals in a given diet or treatment level.

The second set of analyses was performed for both the replicated data set and the full data set (where sample size ranged from 41 to 140). Here, scatterplots were made to identify possibly "aberrant" individuals, *i.e.*, those that are outliers for asymmetry of one or more traits. This set of inspections involved separate plots of size of the left side (x-axis) versus the size of right side (y-axis) of each individual trait, as well as plots of the difference between left and right sides of one

trait (x-axis) versus the difference between left and right sides of another trait (y-axis) for each of the seven treatment levels.

Once possible outliers for bad raw measurements or aberrant individuals were visually identified, either Dixon's test (when $N \leq 25$) or Grubb's test (when $N > 25$) was performed to calculate the distance of each measurement from the sample mean. Because data inspection involved multiple groupings of the data (four diet levels; three temperature levels), sequential Bonferroni corrections were made to the p-values obtained from these analyses.

When a data point was found to be statistically significant as a "bad measurement", two new measurements were taken on separate days, and these replaced the original measurements. When a specimen was deemed to be significantly "aberrant" for a single trait (forewing, hindspot, or hindvein), its values for that trait were dropped from the data set. Such outliers are likely caused by aberrant developmental programming, and are not representations of fluctuating asymmetry (Palmer 1994). When more than one trait of a given individual was found to be an outlier for asymmetry, all measurements for that individual were dropped from the data set. This procedure was implemented after discovery that about half the individuals found to be outliers for one trait were also outliers for at least one other trait.

After completion of data inspection for each of the seven levels, the data set (replicated or full) was aggregated and the steps outlined above repeated, as per recommendations of Palmer and Strobeck (2003a).

Departures from "ideal" fluctuating asymmetry – Following elimination of outliers, a series of two-way, mixed-model ANOVAs was performed on the replicated data set to test whether small non-directional asymmetries (asymmetries random in their direction, but centered around zero) were greater than measurement error for each trait. This step is important because non-directional asymmetries are valid as indices of fluctuating asymmetry only for traits in which the asymmetry is greater than measurement error. These tests also examined whether traits

showed directional asymmetry (one side consistently larger than the other). When directional asymmetry is present, estimates of fluctuating asymmetry are artificially inflated. Therefore, such asymmetry was corrected by adding half the value of the mean difference between sides to the smaller side and subtracting the same amount from the larger side in order to obtain a better measure of fluctuating asymmetry (Palmer 1994).

In these ANOVAs, the dependent variable was size of one trait (*e.g.*, forewing area), and the independent variables were body "side" (left or right; a fixed factor), and "individual" (a random factor). These analyses were initially performed on each diet and temperature level (seven separate analyses on sample sizes that varied between nine and 44) and later repeated on the aggregate data set. The reason for doing analyses on each treatment level, in addition to the data set as a whole, is to establish that non-directional asymmetry is greater than measurement error at all treatment levels. This is an important precaution here because of possible treatment influences on the occurrence of damaged wings: if subtle deformities were more frequent in certain treatment levels, this might impact relative measurement error (Palmer & Strobeck 2003).

Trait differences in measurement error – To determine whether the three traits differed in measurement error, correction for differences in trait size were carried out by ln-transformation of raw measurements. Then, Levene's test of homogeneity of variances (Brown & Forsythe 1974) was performed on the aggregate replicated data set (N = 101) to compare the absolute values of the second versus first set of ln-transformed measurements for each trait; these tests were performed using the STATA® "robvar" command. Similarity of measurement error is necessary in order to combine indices of fluctuating asymmetry of individual traits into a composite index. *Remaining analyses were performed on the complete data set (N = 310).*

Relationship between magnitude of trait asymmetry and trait size – Spearman tests were used to assess whether amount of asymmetry (absolute value of left minus right side) of a given

trait was correlated with size of that trait. This step is important because the existence of a positive correlation is problematic for interpretation of significance of asymmetry (Palmer 1994).

Inspection of trait distributions – For traits to be used as estimates of fluctuating asymmetry, they should exhibit a normal distribution, with a mean centered near zero (Palmer & Strobeck 2003). Tests for departures from these criteria included examination for antisymmetry (bimodality), skewness and kurtosis. Skewness and kurtosis were statistically evaluated using the skewness-kurtosis test in STATA®. One-sample t-tests were used to ascertain departure from a mean asymmetry value of zero. Since each trait was subjected to three statistical tests for departure from normality, sequential Bonferroni tests were applied in the evaluation of whether trait asymmetry distributions deviated from normality (Palmer & Strobeck 2003).

Treatment effects on Fluctuating Asymmetry – Finally, if trait asymmetries were found to be normally distributed and the magnitude of asymmetry was not correlated with trait size, such asymmetries were deemed measures of fluctuating asymmetry. At this point, linear mixed models were used to ask whether fluctuating asymmetry of individual traits ("FA1") or the trait composite ("FA11"; see above for formula) differed among treatments. In these tests, a measure of fluctuating asymmetry was the dependent variable, diet and temperature were included as fixed factors, while rearing shelf (top or bottom) was a block. An equivalent linear mixed model was performed to assess influence of treatments on dry mass. Throughout, non-significant interaction terms were dropped. When parametric analysis generated significant residuals, the validity of the linear mixed model was called into question. Also, because the inclusion of subjects reared on the Carolina diet (diet level 1) at only one temperature resulted in an unbalanced design, the statistical routine could not produce an overall error term for the linear mixed models. For these reasons, non-parametric analysis of variance (Kruskal-Wallis tests) were also performed to provide comparison.

Additional tests – For the 600 subjects that entered the experiment, Pearson's X^2 tests were used to determine whether diet or temperature levels had differential effects on viability. In these analyses, subjects that died prior to and during eclosion and those that damaged wings were considered together as inviable, and analyses determined whether the proportion of inviable specimens varied as a function of diet or treatment level.

All analyses were two-tailed and were performed in STATA 14® (StataCorp LP, College Station, TX).

Results

An overview of final sample sizes in each treatment is provided in Table 3. Overall, 53.5% of the experimental population survived eclosion with undamaged wings and were measured for trait asymmetry. A substantial fraction of the population failed to survive through eclosion (27.2%), and an additional fraction had wing deformities inconsistent with survival in nature (19.3%).

Data set with replicate measurements

Screening for outliers – Three individuals were found to be statistically significant outliers for [L-R] differences in two or more traits and were dropped from the study. For three additional individuals, a single trait showed anomalous asymmetry; in these cases, values for outlier trait were dropped, but the individuals remained in the data set. In addition, human measurement error was detected, and corrected, in three (of 318) measurements.

Departures from ideal fluctuating asymmetry – Two-way ANOVAs to evaluate relative magnitude of asymmetries of each trait were performed for each diet and temperature level of the replicated data set. These analyses showed that non-directional asymmetry was significantly greater than measurement error in all cases (Table 4). Comparable tests for directional asymmetry were significant for five of seven comparisons involving hindvein, but not significant in analyses of forewing and hindspot (Table 5). After correction for the average difference between right and

left hindvein lengths (0.1017 mm), the ANOVA procedure was repeated on the pooled data set. At this point, all three traits exhibited highly significant non-directional asymmetry (Table 6), and directional asymmetry was no longer significant for any trait (Table 7).

Trait differences in measurement error – Measurement error significantly differed among traits: specifically, the average absolute difference between the second set of measurements versus the first was about twice as great for hindspot (mean ± S.E.: 0064 ± .0049 mm^2) than either forewing (.0032 ± .0024 mm^2) or hindvein (.0034 ± .0027 mm) (Levene's test, W = 12.468, df = 2, 299, P <.0001). No differences in measurement error were found as function of diet level (W = 0.183, df = 3,298, P = .91) or temperature level (W = 0.619, df = 2, 299, P = .54).

Full data set

Screening for outliers – Altogether eight specimens were found to be significant outliers for two or more traits and seven specimens were outliers for a single trait (including those in the replicated portion of the data set).

Departures from ideal fluctuating asymmetry – The average difference in left-right hindvein was 0.061 mm; this number differed significantly from zero (t = -4.00, N = 308, P = 0.001), so a correction for directional asymmetry was again applied.

Relationship between magnitude of trait asymmetry and trait size – Neither forewing (Spearman rho = 0.05, N = 303, P = .39) nor hindvein (rho = -0.006, N = 308, P = 0.92) displayed a correlation between the absolute size of trait asymmetry and trait size. A weak positive correlation was found for hindspot (rho = 0.12, N = 295), but this was not significant (P = 0.12) after Bonferroni correction.

Inspection of trait distributions – Forewing and hindvein were found to be normally distributed (Figure 4), while the distribution of hindspot showed significant kurtosis (P = 0.014 after correction for multiple comparisons). Accordingly, subsequent analyses were performed only for forewing and hindvein.

Treatment effects on fluctuating asymmetry – None of three linear mixed models to determine if diet and temperature levels predicted fluctuating asymmetry of forewing or hindvein (FA1) or composite trait (forewing + hindvein) fluctuating asymmetry (FA11) was significant. No interaction between factors was significant, so the interaction term was removed before reporting the model. Shelf effects were also non-significant (Table 8). However, the parametric models all generated highly significant residuals, so these results were supplemented by non-parametric analyses. None of six Kruskal-Wallis tests showed a significant influence of either diet or temperature levels on any fluctuating asymmetry measure (Table 9). Means and standard errors of fluctuating asymmetry for all levels of both factors are reported in Table 10.

Additional tests – *Viability*. Frequency of inviability (specimens that failed to survive eclosion and those with substantial wing defects: see Table 3) differed on the basis of diet level (X^2 = 14.764, 3 df, P < .005) and temperature level (X^2= 10.952, 2 df, P < .01 after correction for multiple comparisons). Post-hoc analyses indicated that viability was highest on the Carolina diet (P< .025), and was lowest among specimens reared on a low-protein diet (diet level 4: P < 0.025); Figure 5) and when subjects experienced elevated temperature throughout development (temperature level 3: P < .01, Figure 5).

Dry mass. The linear mixed model predicting dry mass of viable specimens was highly significant (Wald X^2= 355.51, 297 observations, 2 blocks, P < .0001). Both diet (X^2 = 238.56, 3 df, P <.0001) and temperature (X^2= 24.70, 2 df, P <.0001) made significant contributions to the model. Dry mass differed significantly among all diet levels: butterflies reared on the Carolina diet had the greatest dry mass, and those on the low protein diet had the lowest (Figure 6). Dry mass differed significantly between butterflies reared at 25°C throughout development (temperature level 1) and those exposed to higher temperatures as larvae. However, no mass difference was observed between butterflies maintained at 32°C throughout development

(temperature level 3) and those that experienced a 25°C environment as pupae (temperature level 2; Figure 6).

The linear mixed model exploring factor effects on dry mass of specimens classified as inviable showed the same pattern as found for viable specimens (Wald X^2= 120.73, 83 observations, 2 blocks, P < .0001), with both significant diet (X^2= 82.65, 3 df, P < .0001) and temperature (X^2= 6.49, 2 df, P = 0.039) effects; in addition, specimens with residual meconium weighed on average 13.4% more (marginal mean ± S.E.: 0.0694 ± .002g) than those that had expelled their meconium before death (marginal mean ± S.E.: 0.0612 ± .002g; X^2= 8.32, 1 df, P = 0.004).

Next, a linear mixed model to determine whether inviable individuals tended to be lighter than viable ones; this model included diet and temperature as fixed factors, as well as viability status. Since the above analysis indicates that meconium retention significantly inflates estimates of dry mass of inviable samples, this model included only specimens with no evident retained meconium. In the resulting model (Wald X^2= 316.74, 338 observations, 2 blocks, P < .0001), inviable specimens (marginal mean ± S.E.: 0.0674 ± .0034 g) were found to weigh an average of 7% less (marginal mean ± S.E.: 0.0674 ± .0034 g) than the viable specimens (marginal mean ± S.E.: 0.0725 ± .0008 g; X^2 = 3.99, 1 df, P = 0.046); effects of diet (X^2= 188.43, 3 df, P <.0001) and temperature (X^2= 26.86, df = 2, P < .0001) on dry mass remained the same as previously observed.

Discussion

The primary objective of this experiment was to determine whether temperature and diet stressors during butterfly development would influence fluctuating asymmetry of adult wing traits. The methods used proved sufficient to detect fluctuating asymmetry. However, statistical tests did not reveal any differences in fluctuating asymmetry among diet or temperature treatment

levels. On the other hand, viability patterns indicated that diet and temperature levels were differentially stressful. Specifically, greater inviability occurred among treatments in which subjects experiencing higher temperature throughout development, as well as among those in which individuals were reared on the high carbohydrate diet. The greater inviability found in the high temperature treatment level had not been observed when larvae/pupae were exposed to this regime during project development. I suggest that this result was caused by the interaction between high temperature stress and the low protein diet. Unfortunately, this hypothesis cannot be directly examined with the data, due to the unbalanced treatment design (specifically, that individuals fed the Carolina diet were reared only on temperature level 1 (25°C throughout development).

One interpretation of these results is that the most stressful conditions induced higher mortality among lower-quality individuals. By this reasoning, the individuals that survived such conditions were those most able to buffer against environmental perturbations and therefore exhibited relatively low fluctuating asymmetry. Thus, the effect on fluctuating asymmetry of extreme stressors may have been masked by the differential survival of high- versus low-quality individuals.

Support is provided for the idea that inviability resulting from increased developmental stress masked treatment effects on fluctuating asymmetry by the finding that dry mass of specimens was lower in both diet and temperature treatments predicted to be more stressful. This suggests there may be a critical minimum size that larvae must generally obtain prior to pupation in order to metamorphose successfully, and that when environmental stress is higher, larvae of lower intrinsic have more difficulty reaching the target size needed for development to proceed. In any case, the finding that the dry mass patterns cohere with mortality patterns is strong evidence that the temperature and diet levels selected for this experiment were differentially stressful as predicted.

There are two major, interrelated implications of these results. First, results indicate that lack of evidence for stressors causing increased fluctuating asymmetry may be hard to interpret in field settings in which rates of death (or "inviability") cannot be readily determined. In turn, this consideration suggests that variation in fluctuating asymmetry of traits may only be measureable within a prescribed range of environmental conditions, i.e., where "stress" does not cause inviability. This range would have to be determined on a population-by-population basis (Bijlsma & Loeschcke 2005). If these implications are valid and have generality within and across taxa, they suggest that the investigation of fluctuating asymmetry has limited utility to detect the presence of impactful environmental perturbations in natural populations.

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Chapter 3

Reflections on experiment and suggestions for future study

Abstract

In this chapter, limitations of the study detailed in Chapter 2 are discussed. Attention is given to design elements that perhaps could have been anticipated and which made statistical exploration of factor effects difficult. Also, several design aspects of peculiar import to studies of fluctuating asymmetry are briefly discussed, including (1) the importance of stringently screening traits for similarities in distributions and measurement error, (2) implementation of stressors during specific life-stages may strain experiment design, and (3) the need to temper the impact of stressors so that mortality is not inflated amongst the most stressed individuals.

Study limitations

In the design of the experiment, Carolina diet was included as a baseline for performance, under the expectation that performance would be highest on this diet. Due to concern over post-mortality sample sizes of the various treatment groups, this diet was not included in factor groups exposed to elevated temperature. This resulted in an unbalanced experimental design that did not allow for complete measurement of factor interaction effects.

A second problem was the limited availability of growth chambers for the study. After discounting the second replicate, all subjects were reared in one of two growth chambers. As a result, it is not possible to cleanly separate effects of chambers from those of temperature factors. However, the absence of shelf effects in the data analyses and the observed mortality patterns suggest that the factors imposed resulted in observed effects.

There is another flaw in the design that, going forward, would need to be rectified. One objective of the experiment was to test the idea that later stresses are more impactful on adult phenotype than earlier ones in organisms that exhibit a complex life cycle and undergo

metamorphosis (see Chapter 1). However, the experimental design did not separate the effect of overall time exposed to a high temperature environment from the stage during which the stress was imposed. Specifically, factor groups 6, 8 and 10 received more time at high temperature than groups 5, 7 and 9. Consideration of this problem indicates that testing the stressful metamorphosis hypothesis while balancing absolute amount of time exposed to a stressor (as well as controlling for the physical movement between temperature environments) would present complicated design issues and the need for several growth chambers.

Another limitation of this study is the inability to sex the organisms. The design of the study precluded sexing 600 individuals at the larval stage (in fact, including those in the second replicate, there were nearly 1100 larvae). Prior to the start of the experiment, the literature was scoured for evidence of sexual dimorphism of adult traits. No information was found implicating adult traits that would objectively indicate sex. Nevertheless, butterfly morphology was closely examined – especially the 7 candidate traits – and included statistical exploration of trait distributions (*i.e.,* bimodal distribution of trait size or size ratios such as forewing/hindwing area). The lack of information on sex could complicate interpretation of the results if the sexes differ in response to experimental conditions or have intrinsic differences in fluctuating asymmetry of traits measured here.

Implications for further study and lessons learned

This study illustrates two major problems for the investigation of fluctuating asymmetry. The first is the difficulty of minimizing mortality effects while imposing stressors: this is a critical issue for reasons exemplified by this study. In concept, fluctuating asymmetry could be a useful tool as a descriptor of relative vulnerability of populations to decline due to the presence of one or more environmental stressors. However, the effect of the stressors must be measureable among some traits of the surviving individuals. If a large proportion of a population dies, the

absence of factor-imposed differences in fluctuating asymmetry does not inform us about the relative quality and potential fluctuating asymmetry expression by the individuals that perished.

The second issue concerns the identification of suitable traits for inclusion in measures of individual (composite) fluctuating asymmetry. As mentioned in Chapter 1, these traits should possess several properties: (1) their expression must show developmental independence. (2) Despite differences in shape and size and relative location, they must display similar, low levels of measurement error. (3) Traits need to exhibit variation in response to applied stressors. (4) Ideally, such traits should be easy to measure in order to expedite the collection of significant samples needed to detect the small effect size that fluctuating asymmetry is likely to display. These criteria were considered at the outset of this study and much effort was accordingly devoted to evaluating candidate traits. Despite these efforts, the three selected traits did not, in the end, have similar measurement error. Hindsight has illuminated the inherent limitations of achieving low relative measurement error in one of the selected traits (hindspot, the trait excluded from the composite fluctuating asymmetry score). In the absence of copious preliminary data, it remains unclear how to identify suitable traits with a reasonable amount of effort. Overcoming both of the above obstacles presents a barrier to future investigation of fluctuating asymmetry.

Were another similar experiment on this topic warranted, the investigator could endeavor to streamline the design by eliminating a number of factor groups, notably those involved in investigation of effects of different developmental stages on fluctuating asymmetry. Any such design should, however, endeavor to retain at least two stressor types to increase the probability of detection of effects of factor levels on fluctuating asymmetry. The intensity of stressors should be toned-down in an effort to reduce mortality effects, ideally even amongst organisms exposed to multiple stressors, as masking of induced stress is implicated in fluctuating asymmetry effect size-reduction (Møller 1997; Polak *et al.* 2002). If temperature were to be included as a stressor, it would be advisable to have available at least six growth chambers. This number of chambers

would permit a balanced design and hedge against catastrophic population failure that could result from disease or growth chamber failure.

Author's recommendation for evaluating the impact of a potential study

After extensively reviewing the fluctuating asymmetry literature, several themes have become apparent. Some reviewers express a lack of confidence in the imminent development of a framework that elucidates the relative impact of mechanisms of developmental stability (Fuller and Houle 2003; Møller 2005; Leamy and Klingenberg 2005). This is unsurprising, given that the heritability of fluctuating asymmetry appears to be low. While strong advances are currently being made in our understanding of ontogenetics, it may be that there is waning interest (and/or motivation) in the factors that lead to organism-wide failures of development, as opposed to specific pathways that can be co-opted or engineered with genetic tools to tackle specific diseases. This is perhaps a problem of the state of scientific funding. Regardless, it does not bode well for those interested in the potential use of fluctuating asymmetry as a general tool for conservation. More investigation of the how and why some types of stressors effect fluctuating asymmetry in some types of traits is needed for interested scientists to determine whether fluctuating asymmetry will be useful in their system of interest.

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Appendices

Tables

Table 1. Lab diet recipes used to feed 3rd through 5th instar larvae in this experiment^.

Ingredients	Diet factor levels (FL)		
	1:1 P:C (FL2)	3:1 P:C (FL3)	1:3 P:C (FL4)
Water (ml)	850	850	850
Casein (g)	60	90	30
Sucrose (g)	60	30	90
Agar (g)	20	20	20
Wesson's salt (g)	14	14	14
Vitamin mixture (g)	10	10	10
Ascorbic acid (g)	5	5	5
Antibiotic (g)	4	4	4
Linseed oil (ml)	4	4	4
Cholesterol (g)	4	4	4
Kanamycin sulfate (g)	3	3	3
Methylparabenzoate (g)	2	2	2
Sorbic acid (g)	2	2	2
Choline chloride (g)	1	1	1

^ Differs from painted lady chemically defined lab diet used by Vanoverbeke (2011) in that no formaldehyde was included here and linseed oil and kanamycin sulfate were not components of Vanoverbeke's diet. Linseed oil contains steroids important for eclosion.

Table 2. Description of treatments (T) and diet and temperature levels (L).

T	Diet		Temperature	
	L	description	L	description
F1	1	Carolina painted lady diet	1	25° C from 3rd instar larva → eclosion
F2	2	lab diet - 1:1 protein: carbohydrate	1	25° C from 3rd instar larva → eclosion
F3	3	lab diet - 3:1 protein: carbohydrate	1	25° C from 3rd instar larva → eclosion
F4	4	lab diet - 1:3 protein: carbohydrate	1	25° C from 3rd instar larva → eclosion
F5^	2	lab diet - 1:1 protein: carbohydrate	2	25° C from 3rd instar larva → pupation; thereafter, 32° C → eclosion
F6	2	lab diet - 1:1 protein: carbohydrate	3	32° C from 3rd instar larva → eclosion
F7^	3	lab diet - 3:1 protein: carbohydrate	2	25° C from 3rd instar larva → pupation; thereafter, 32° C → eclosion
F8	3	lab diet - 3:1 protein: carbohydrate	3	32° C from 3rd instar larva → eclosion
F9^	4	lab diet - 1:3 protein: carbohydrate	2	25° C from 3rd instar larva → pupation; thereafter, 32° C → eclosion
F10	4	lab diet - 1:3 protein: carbohydrate	3	32° C from 3rd instar larva → eclosion

^Treatments moved from chamber 2 to chamber 1 one day after pupation in order to accommodate the change in environmental temperature.

Table 3. Summary of fates of experimental subjects, and final sample sizes for analyses.

Treat-ment	Diet level	Temp level	N Assigned	N Died	N Damaged Wings*	N Inviable°	N Viable/ Measured	N Excluded^	N Included#
1	1	1	60	7	10	17	43	3	40
2	2	1	60	11	8	19	41	0	41
3	3	1	60	19	12	31	29	0	29
4	4	1	60	21	8	29	31	1	30
5	2	2	60	11	10	21	39	0	39
6	2	3	60	17	13	30	30	1	29
7	3	2	60	9	14	23	37	3	34
8	3	3	60	18	16	34	26	1	25
9	4	2	60	22	8	30	30	1	29
10	4	3	60	28	17	35	15	1	14
Total subjects			600	163	116	279	321	11	310

*Damaged individuals had wings that failed to dry properly (both forewings and hindwings damaged).

°Sum of $N_{died} + N_{damaged\ wings}$

^Individuals excluded from fluctuating asymmetry results because 2 or more traits met statistical criteria for exclusion and/or had handling or other damage that precluded measurement of traits.

#Final sample size: number of individuals included in fluctuating asymmetry results (had at least 2 traits that were not excluded).

Table 4. Results of tests* for non-directional asymmetry, by factor level, for the replicated data set.

Dependent variable	Factor	Level	F-ratio	Degrees of freedom	P^
Forewing area	Diet	1	10.914	7/272	<.001
		2	18.790	27/3192	<.001
		3	38.553	25/2756	<.001
		4	42.676	33/4692	<.001
	Temperature	1	26.423	42/7482	<.001
		2	27.878	27/3192	<.001
		3	30.385	25/2970	<.001
Hindvein length	Diet	1	3.224	7/272	<.005
		2	15.252	27/3192	<.001
		3	10.826	26/2970	<.001
		4	20.710	33/4692	<.001
	Temperature	1	10.733	42/7482	<.001
		2	14.125	27/3192	<.001
		3	14.765	26/2970	<.001
Hindspot area	Diet	1	22.604	7/272	<.001
		2	58.045	26/2970	<.001
		3	39.630	26/2970	<.001
		4	54.909	32/4422	<.001
	Temperature	1	50.556	42/7482	<.001
		2	26.044	26/2970	<.001
		3	46.167	25/2756	<.001

*Two-way, mixed model ANOVAs with side as a fixed effect, and individual as a random effect.

^P values from Rohlf & Sokal (2012), Table F.

Table 5. Results of tests* for directional asymmetry, by factor level, for the replicated data set.

Dependent variable	Factor	Level	F-ratio	Degrees of freedom	P^
Forewing area	Diet	1	0.004	1/7	>.75
		2	0.854	1/27	>.25
		3	0.232	1/25	>.50
		4	0.509	1/33	>.25
	Temperature	1	0.161	1/42	>.50
		2	1.081	1/27	>.25
		3	0.650	1/25	>.25
Hindvein length	Diet	1	0.631	1/7	>.25
		2	11.256	1/27	<.005
		3	0.232	1/26	>.75
		4	5.760	1/33	<.025
	Temperature	1	7.875	1/42	<.01
		2	4.662	1/27	<.05
		3	7.167	1/26	<.025
Hindspot area	Diet	1	0.024	1/7	>.75
		2	0.225	1/26	>.50
		3	0.773	1/26	>.25
		4	0.121	1/32	>.50
	Temperature	1	0.437	1/42	>.50
		2	0.383	1/25	>.50
		3	0.390	1/25	>.50

*Two-way, mixed model ANOVAs with side as a fixed effect, and individual as a random effect.

^ P values from Rohlf & Sokal (2012), Table F.

Table 6. Results of tests* for non-directional asymmetry in the replicated data set (data pooled across all factors).

Dependent Variable	F-ratio	Degrees of freedom	P^
Forewing area (FW)	29.134	98/39,402	<.001
Hindvein length (HV)	12.304	99/40,200	<.001
Hindspot area (HS)	49.545	97/38,612	<.001

*Two-way, mixed model ANOVAs with side as a fixed effect, and individual as a random effect.

^ P values from Rohlf & Sokal (2012), Table F.

Table 7. Results of tests* for directional asymmetry in the replicated data set (data pooled across all factors) following correction for side differences in hindvein length.

Dependent Variable	F-ratio	Degrees of freedom	P^
Forewing area (FW)	0.156	1/98	>.50
Hindvein length (HV)	0.014	1/99	>.75
Hindspot area (HS)	0.006	1/97	>.75

*Two-way, mixed model ANOVAs with side as a fixed effect, and individual as a random effect.

^ P values from Rohlf & Sokal (2012), Table F.

Table 8. Influence of diet and temperature factors on FA1 and FA11 in the full data set (linear mixed models, after interaction term removed, ^with shelf as a random effect; no corrections for multiple comparisons).

Dependent variable	N	Wald X^2	Model P	Diet effect P	Temperature effect P
Forewing FA1*	303	6.46	0.26	0.23	0.72
Hindvein FA1*	308	5.10	0.40	0.21	0.63
Composite FA11*	301	4.29	0.51	0.25	0.75

^Variance contribution of shelf effect approached zero in all models.

*Model residuals are highly significant.

Table 9. Non-parametric models for effects of diet and temperature on FA1 and FA11 (Kruskal-Wallis tests) for the full data set (no corrections for multiple comparisons)

Dependent variable	N	Diet effect			Temperature effect		
		X^2	df	P	X^2	df	P
Forewing FA1	303	3.473	3	0.32	1.829	2	0.40
Hindvein FA1	308	1.810	3	0.61	1.966	2	0.37
Composite FA11	301	3.557	3	0.31	1.902	2	0.39

Table 10. Descriptive statistics of FA scores by factor level for full data set (raw means).

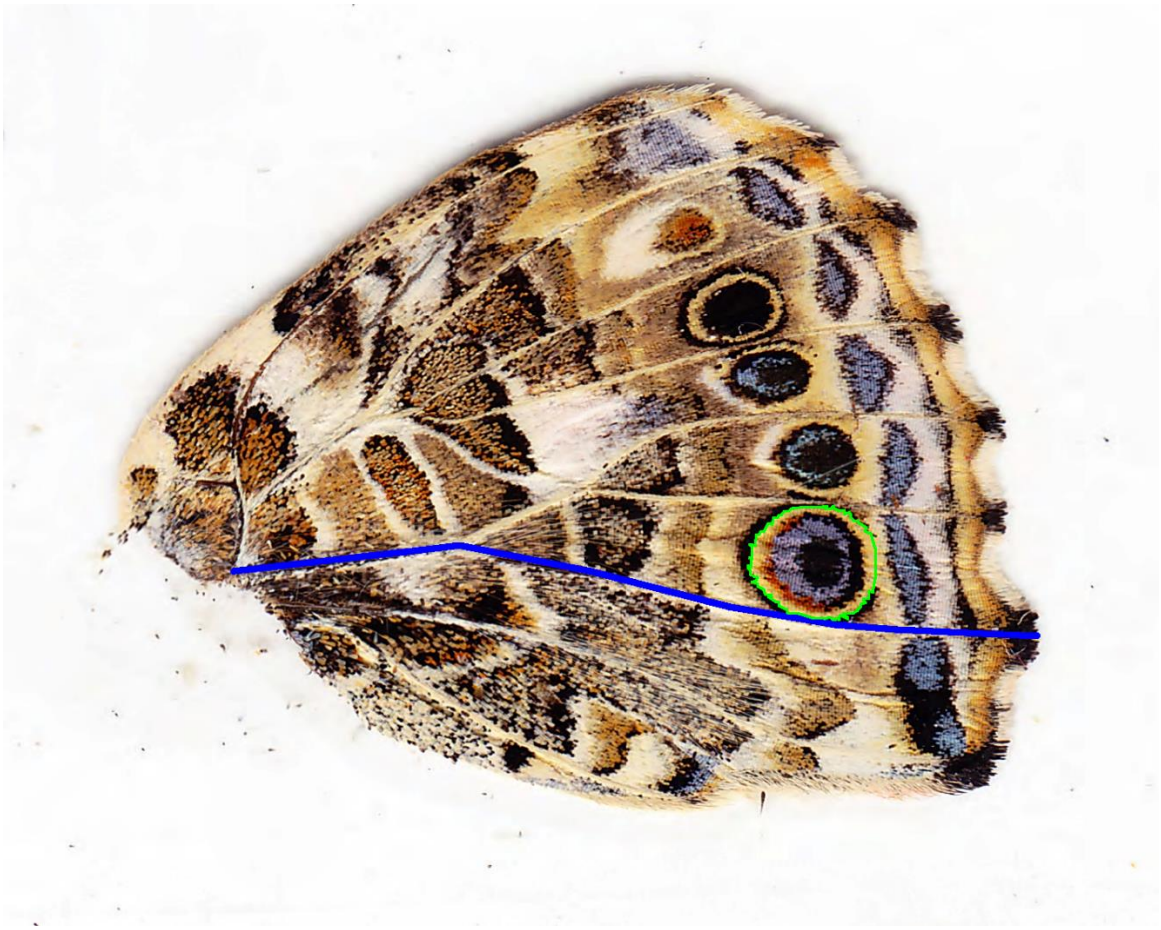
Factor	Level	Variable	Mean ± S.E.	N
Diet	1	FW FA1	5.66±0.72	40
	1	HV FA1	0.25±0.04	39
	1	FA11	0.027±0.002	39
	2	FW FA1	4.09±0.31	106
	2	HV FA1	0.21±0.01	108
	2	FA11	0.021±0.001	105
	3	FW FA1	4.59±0.44	86
	3	HV FA1	0.21±0.02	88
	3	FA11	0.024±0.002	86
	4	FW FA1	4.17±0.40	71
	4	HV FA1	0.19±0.02	73
	4	FA11	0.023±0.001	71
Temperature	1	FW FA1	4.76±0.33	138
	1	HV FA1	0.21±0.01	138
	1	FA11	0.023±0.001	136
	2	FW FA1	4.04±0.34	100
	2	HV FA1	0.19±.02	102
	2	FA11	0.023±0.002	100
	3	FW FA1	4.45±0.48	65
	3	HV FA1	0.022±0.02	68
	3	FA11	0.024±0.002	65

Figures

Figure 2. Illustration of trait forewing area.

Ventral view of a left forewing. Area under blue outline is an example of the variable forewing area. Note that the flange surrounding the perimeter of the wing was excluded from measurement. There is also a small negative space inside the polygon that was created erroneously when cutting the wing.

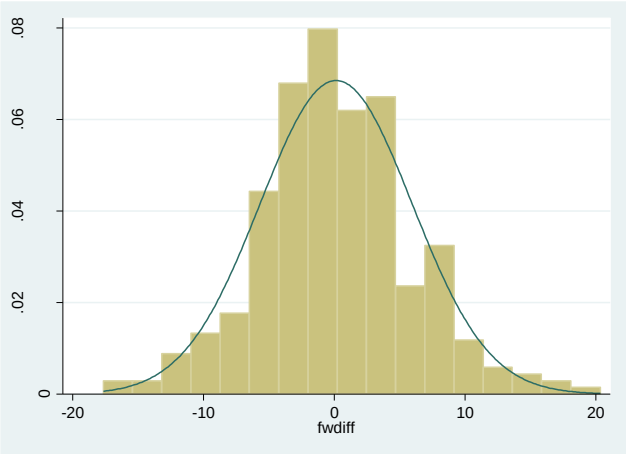
Figure 3. Illustration of traits hindvein length and hindspot area.



Ventral view of a left hindwing. The blue line is an example of the variable hindvein length. The green polygon is an example of the variable hindspot area.

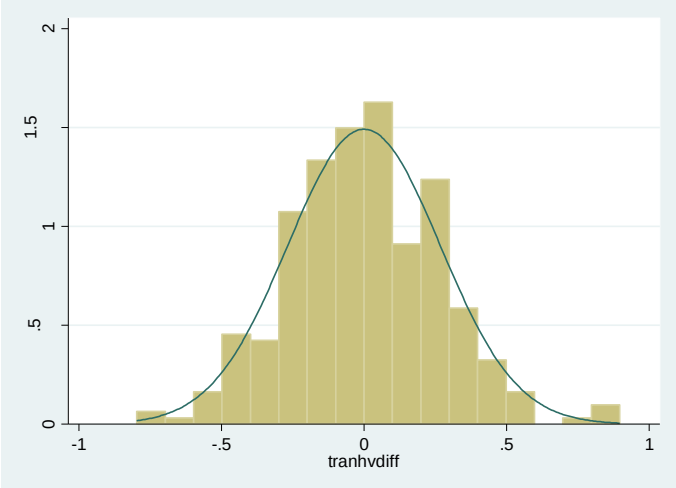
Figure 4. Distribution of left-right asymmetries of forewing area, hindvein length, and hindspot area in the full data set.

A. Forewing^



^Distribution of mean (left-right) forewing area differences (N =303, skew P = 0.17, kurtosis P = 0.07 after Bonferroni correction, t-test for mean differs from zero P = 0.68).

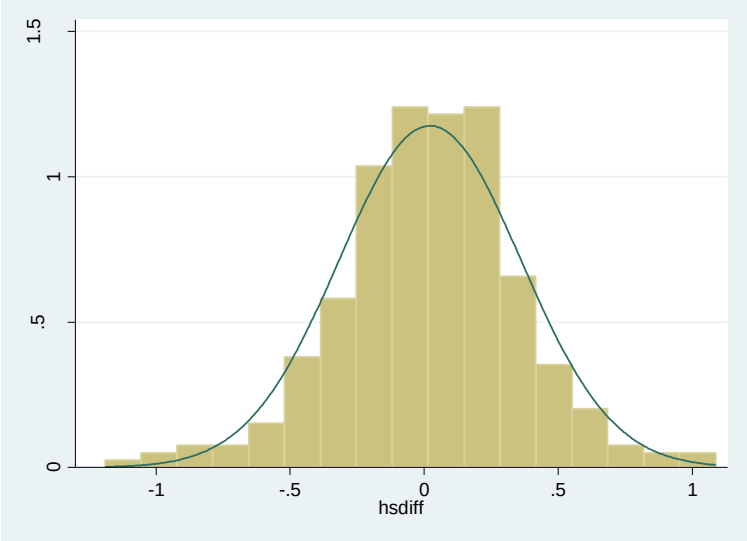
B. Hindvein*



*Distribution of mean (left-right) hindvein length differences after correction for directional asymmetry (N = 303, skew P = 0.26, kurtosis P = 0.17, t-test for mean differs from zero P = 0.99).

(Figure 4 continued)

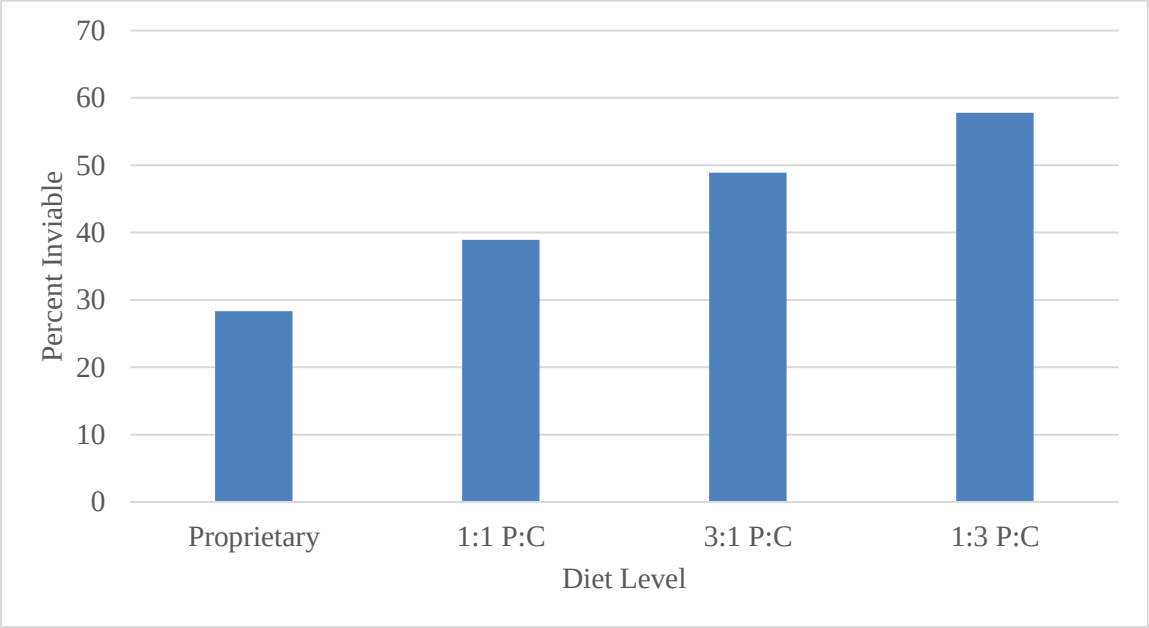
C. Hindspot[#]



[#]Distribution of mean (left-right) hindspot area differences (N = 295, skew P = 0.16, kurtosis P = 0.014 after Bonferroni correction, t-test for mean differs from zero P = 0.27).

Figure 5. Inviability as a function of diet (A) and temperature (B).

A. Inviability by Diet Level



B. Inviability by Temperature Level

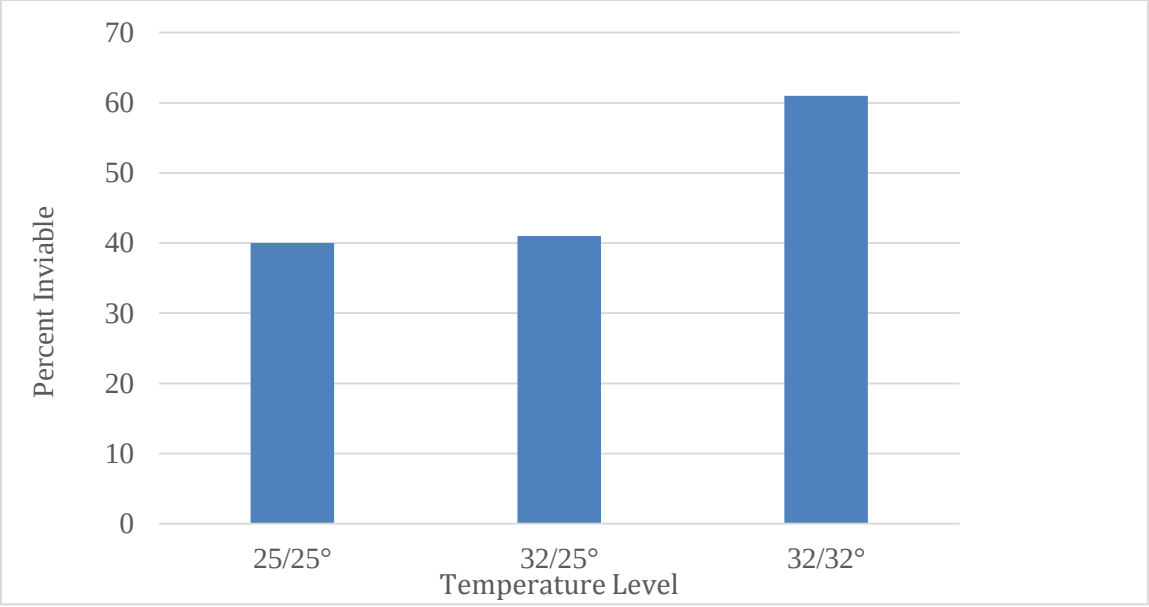
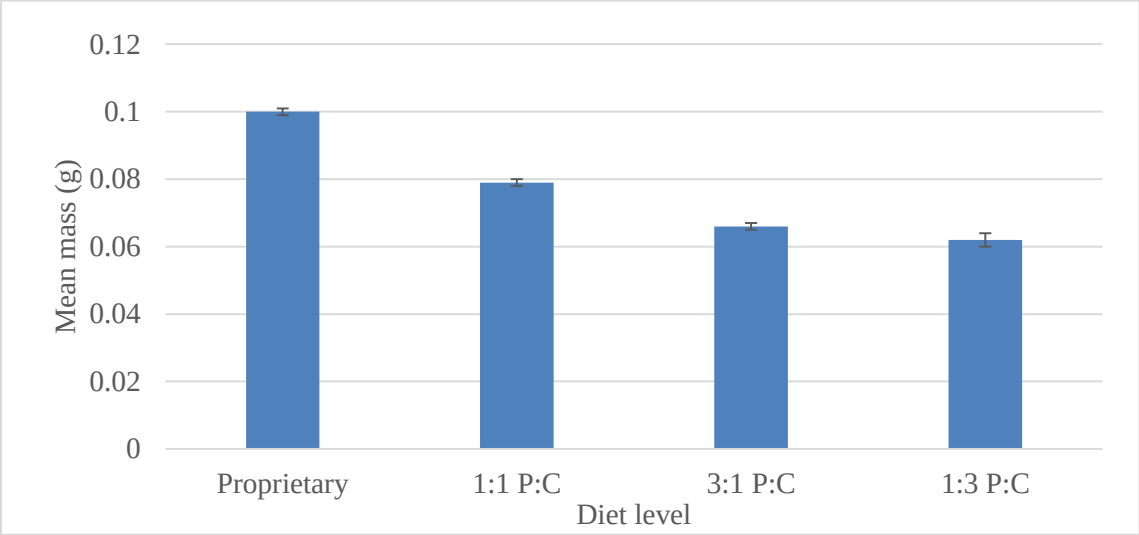


Figure 6. Dry mass (mean ± S.E.) as a function of diet (A) and temperature (B).

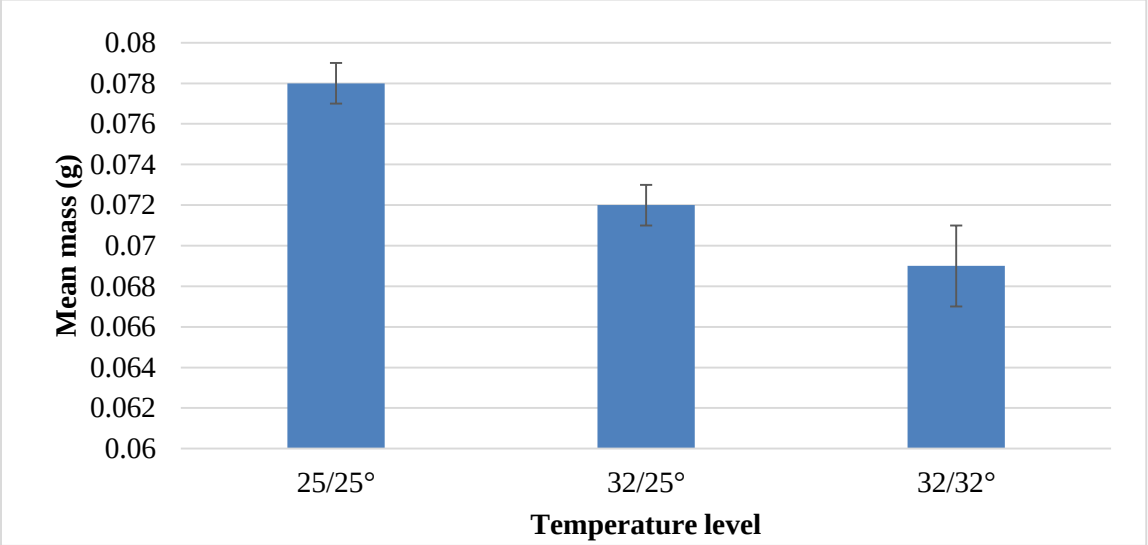
A. Dry Mass by Diet Level



All *a posteriori* comparisons of the dry mass means of proprietary diet, the 1:1 protein: carbohydrate diet, and the 3:1 protein: carbohydrate diet were highly significant (P's < 0.0001); the comparison between the 3:1 and 1:3 diet ratios was also significant (z = - 2.32, P = 0.021).

(Figure 6 continued)

B. Mean Mass by Temperature Level



Mean dry mass differed between butterflies reared at 25°C throughout development and other factor levels (P's < 0.0001), but not between the higher temperature factor levels (z = -1.47, P = 0.14).