

TEMPERATURE-DEPENDENT PHENOTYPIC PLASTICITY IN WING PATTERN OF *UTETHEISA ORNATRIX BELLA* (EREBIDAE, ARCTIINAE)

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Abstract – *Utetheisa ornatrix* exhibits geographic and intrapopulation variability in wing pattern. Here, evidence is presented that temperature-dependent phenotypic plasticity exists in north Florida populations of the subspecies *U. ornatrix bella*. On six different occasions, experimental groups of late instar larvae and pupae were reared at lower temperatures (15-16°C) and short-day photoperiod, while control groups of sibling larvae were raised through to adult stage at 22°C. Resultant moths from the two groups had different wing pattern phenotypes. Increased melanization is the probable cause of the observed differences, as the cold-affected individuals had more extensive black markings on both surfaces of forewing and hindwing. Cold-induced melanization affected both wings simultaneously, in which it differed from normal genetic variation in melanic markings found in *U. ornatrix*, which inherits separately for fore- and hindwing. Additionally, cold-induced reduction of red/orange pigmentation on the upper forewing surface was sometimes observed. The expression of this phenotypic plasticity varied depending on the brood used in the experiment. Possible adaptive significance of cold-induced melanization in *bella* moth is discussed in the context of our knowledge of similar phenotypic plasticity found in many butterfly species.

Key words: adaptation, reaction norm, polyphenism, polymorphism, Lepidoptera, thermal biology, camouflage, aposematism, *bella* moth, ornate moth, chemical ecology, melanin accumulation, seasonal forms

INTRODUCTION

Seasonal polyphenism is a well-known phenomenon in Lepidoptera. There are numerous examples where it is less than subtle, such as *Araschnia levana* L., 1758 (Nymphalidae), in which the spring form (first generation) is red and black, while the second, summer, generation is black with white stripes. Not only the color, but also the pattern in these two generations of genetically identical butterflies are entirely different, and selective pressures from predators have been proposed as driving this divergence, although this hypothesis has not yet been experimentally confirmed (Ihalainen and Lindstedt 2012). Nijhout (2003: 9), who studied the genetic and physiological controls underlying such polyphenism, stated that: “Phenotypic plasticity can be continuous, in which case it is called a reaction norm, or discontinuous, in which case it is called a polyphenism. Although the morphological discontinuity of some polyphenisms is produced by discrete developmental switches, most polyphenisms are due to discontinuities in the environment that induce only portions of what is in reality a continuous reaction norm.”

Temperature during larval development is frequently a factor that is responsible for the degree of melanization of the wing pattern in Lepidoptera. For example, in the skipper *Copaeodes minima* Edwards, 1870, the dark scales on the anal hindwing margin decreased from 79-82% when reared at 20°C to 27-37% when reared at 30°C (Lewis 1985). Increased melanization of hindwings at lower temperatures suggests an adaptive phenotypic response for thermoregulation.

The trend of seasonal polyphenism is widespread in pierids (e.g., Shapiro 1980). More melanic forms have been linked to shorter day length and lower temperatures, as in the cases, for example, of Indian populations of *Appias libythea* Fab., 1775 and *Cepora nerissa* Fab., 1775 (e.g. Islam et al. 2015). Photoperiod and temperature during development have been experimentally shown to control the phenotype of the butterfly

Catopsilia pomona Fab., 1775, with these environmental cues controlling pattern and color elements to different extents (Rienks 1985). In Florida, in sulphur butterflies of the genus *Eurema* Hübner, 1819, the summer (wet season) forms are paler in color than the winter (dry season) forms. As illustrated by Daniels (1995), these changes are accompanied by variation in other species-specific trends, such as proboscis length, wing size and ovarian activity, and may relate to thermoregulation. Such adaptive advantages have been experimentally demonstrated in pierid butterflies of the genus *Pieris* Schrank, 1801 (Kingsolver 1985, 1995), even though in that system there may be additional selection pressures at play, such as mate recognition (Wiernasz and Kingsolver 1992). Some pierids, such as *Colias eurytheme* Boisduval, 1852 and *Nathalis iole* Boisduval, 1836, apparently evolved an ability to anticipate seasonal changes in thermal conditions by reacting to the shorter photoperiod alone with increased melanin deposition. The resulting darkening of the wings allows them to maintain sexual activity in temporally and geographically cooler environments and has helped in their northward range expansion (Hovanitz 1950, Douglas & Grula 1978). However, the adaptive significance of phenotypic plasticity and melanization are not as clear in every case as in above pierid examples. For instance, in *Pieris rapae* L., 1758, male wing melanization on both wing surfaces shows characteristic seasonal, adaptive plasticity, but only some of the dorsal forewing pattern elements of females conforms to the hypothesis of adaptive melanization, and it has been suggested that sexual dimorphism in wing pattern plasticity may relate to sexual dimorphism in behavior and physiology (Stoehr & Gaux 2008). It is also rather obvious from field observations that while thermoregulation may be driving melanization of winter forms in *Eurema*, the summer and winter forms are more cryptic in their respective environments (e. g., Sourakov 2009), and hence more than one selective pressure may be driving this phenotypic plasticity.

Outside of Pieridae, different mate-locating strategies (perching vs. patrolling) have been shown to correspond to different phenotypes in a satyrine, *Pararge aegeria* L., 1758, with darker males presumably better adapted to more energetic patrolling, supporting the hypothesis that melanization has adaptive thermoregulatory significance (Van Dyck & Matthysen 1998). Similarly, temperature-related genetically controlled variability in larval coloration has been shown to have adaptive significance, with darker forms having faster growing rates during colder periods. This has been shown for both larval cuticle in a swallowtail butterfly (Hazel 2002) and larval hairs in a tiger moth (Fields & McNeil 1988), which are undoubtedly controlled through different pathways and this phenotypic plasticity must therefore have evolved independently.

Adaptive thermoregulatory phenotypic plasticity is not unique to Lepidoptera, but has been shown in other insects such as syrphid flies, where different lineages have also been shown to arrive at similar patterns of seasonal phenotypic plasticity independently, clearly demonstrating their adaptive significance (Holloway et al. 1997).

It has also been suggested (Talloon et al. 2008) that melanin is costly to synthesize and hence that phenotypic plasticity, in addition to its adaptive significance, can reflect environmental conditions (e.g., host quality) under which an animal is reared. Another study, however, demonstrated that in pupae of *Spodoptera exigua* Hübner, 1808, variants with higher melanin, a character with no obvious adaptive significance, also had higher fitness (faster development in all life stages, heavier pupa, longer mating time, and higher fecundity) (Liu et al. 2015). The authors suggested that neither the origin, stage, space or variation in melanization seems to determine whether it will cause fitness gain or loss in an insect species.

Here, a reaction norm is described involving the increase in melanization of the wings in a well-researched species of tiger moth, *Utetheisa ornatrix* (Linnaeus, 1758), which has been the subject of numerous ecological experiments (e.g., Conner 2008). Nevertheless, despite this long experimental history, this reaction norm seems to have been missed by the many researchers who kept colonies of this moth in the lab.

Utetheisa ornatrix has two mainland subspecies, *U. ornatrix ornatrix* that ranges from South Florida/Louisiana and the Caribbean to Argentina, and *U. ornatrix bella* (Linnaeus, 1758) that ranges from the Caribbean and Southern United

States to Nova Scotia. The conspecificity of all *U. ornatrix* populations was proposed by Pease (1968) and later supported by DaCosta's (2010) thorough morphological analysis of the genus. While in Florida (and on some Caribbean islands) the two subspecies meet and interbreed (Pease 1968), the populations in Gainesville, Florida, used in the present study are 100% *U. ornatrix bella*.

The present study therefore only addresses *U. ornatrix bella* and explores the temperature-dependent plasticity of phenotypes associated with this taxon. Around Gainesville, at any given time, every population has a distinct set of morphological characteristics, which produces a unique "fingerprint" if a sample is taken from the population. This situation is typical of this species elsewhere (e.g., Pease 1973). It is almost impossible to find two identical *U. ornatrix* individuals; while the size of individual moths are controlled by both genetics (Iyengar and Eisner 1999) and diet (Sourakov 2015a), the coloration, at first glance, appears to be mostly genetically controlled. In my breeding experiments, F-1 moths tended to resemble their parents, and the same is true of numerous breeding experiments by others such as Charles Remington, whose broods of *U. ornatrix* are preserved at the Peabody Museum (pers. obs.). While greater variability could be found within offspring of wild-caught females, inbreeding usually appears to reduce that variability significantly and Remington's and Pease's work demonstrate that one can selectively breed for even the most unusual phenotypes.

Pease (1968) analyzed natural *U. ornatrix* phenotypic diversity in detail and conducted numerous breeding experiments. He suggested that "five wing characters of pigmentation and black markings comprise both geographic and local variation. These characters are (1) the color of the forewing pigment, (2) the distribution of white pigment on the forewing, (3) the forewing black spotting, (4) the hindwing black markings, and (5) the major color of the hindwing" (Pease, 1968: 719). The intrasubspecific and intrapopulational variability of these characters have been illustrated by him and by others (e.g., Brou 2009). However, the sensitivity of these phenotypes to temperature, as shown in the present study, has been heretofore missed, perhaps due to the overwhelming intrapopulational variability.

Hopefully, the present study will broaden the previous view of this phenotypic variability as expressed by Pease (1968, p. 720): "Variation is genetic rather than environmental

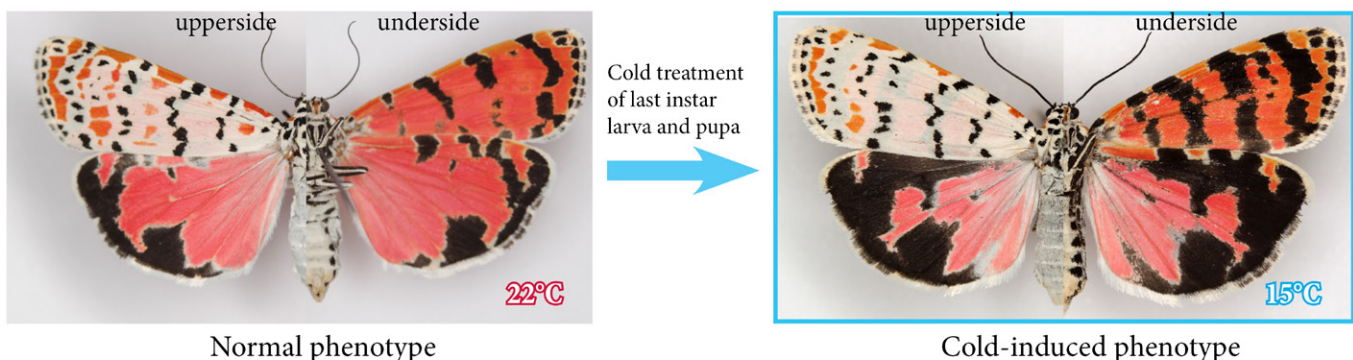
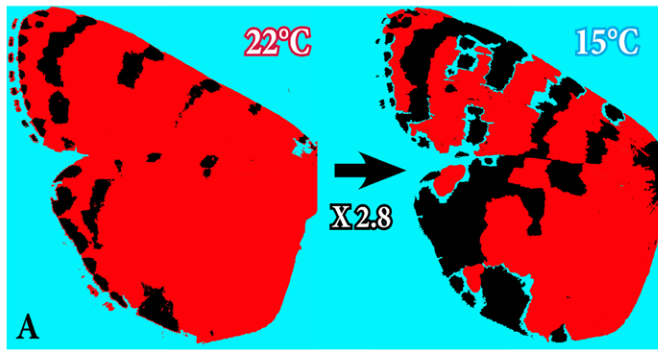
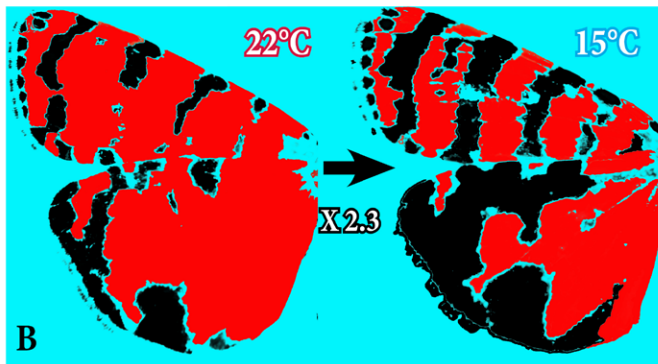


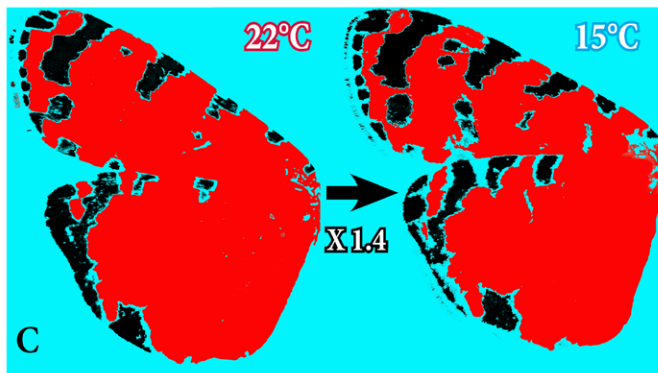
Fig. 1. Example of cold-induced reaction norm (decrease of orange/red on forewing, increase of melanic markings on the hindwings) in *Utetheisa ornatrix bella* (sample female specimens from Trial 2 (see Supplementary materials)).



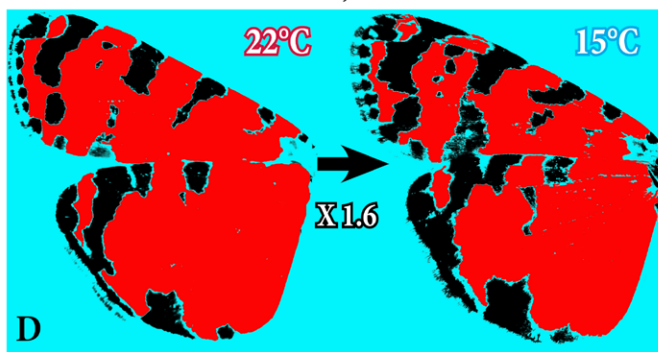
Trial 2, Males



Trial 2-Females



Trial 6, Males



Trial 6, Females

Fig. 2. Schematic illustration of melanic markings on the wing underside of *Utetheisa ornatrix bella* individuals in two temperature reaction norm trials (see text for details). (A) males #1,2 and (B) females #3,4 in Trial 2 (see Supplementary materials), show a 2.3-2.8x increase in black area in cold-induced individuals as compared to controls. (C) males #5,6 and (D) females #7,8 in Trial 6 show a more modest increase of 1.4-1.5x.

Table 1. Examples of increase in melanization of underside wing surface in *Utetheisa ornatrix bella* as a result of larval/pupal development in colder temperatures.

		Wing area (%) colored black		Increase in melanization
		22°C	15°C	
Trial 2	Males #1, #2	14.0%	39.3%	X 2.8
	Females #3, #4	29.0%	66.8%	X 2.3
Trial 6	Males # 5, #6	22.2%	29.9%	X 1.4
	Females #7, #8	32.2%	47.6%	X 1.5

since phenotypically diverse stocks retained their distinctive differences when reared side by side under constant conditions of light and temperature.” In my approach, I have used the opposite method to that of Pease: I reared phenotypically (genetically) similar stocks under different conditions and, when placed side by side, it became obvious that they are (as groups) statistically different from each other in the characters of pigmentation and black markings outlined by Pease’s otherwise excellent revision of the species.

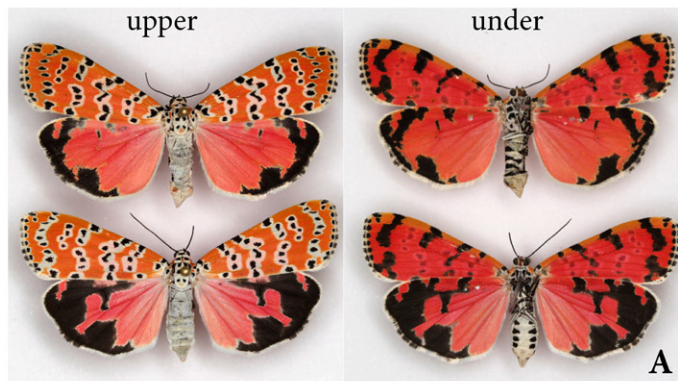
MATERIALS AND METHODS

The study was conducted at the McGuire Center for Lepidoptera and Biodiversity, Gainesville, Florida in 2011-2014. The source populations are located within a 10 mile radius of the University of Florida campus, and collecting localities and dates are provided in the captions to Figs. 1-6. Female moths netted in the wild laid eggs in 2 oz. plastic containers. Larvae were normally raised on *Crotalaria lanceolata* leaves, progressively being divided into smaller and smaller groups as their size increased until they ended up in solitary cups. At the onset of the ultimate instar, the larvae were randomly divided into two groups: one that continued to be raised through to adults in the lab at 22-24°C, where while photoperiod varied, it mostly corresponded to the ambient (window present); the other raised through to adults in a cold room at 15-16°C, where light conditions did not exceed eight hours per day. The diet for the experimental and control groups was consistent within each trial, but depended on food availability. In most trials the larvae were raised on leaves of the widely available, wild-collected *C. lanceolata*, but in one trial the hostplant was the leaves of *Crotalaria pallida* and in another trial it was the seeds of the latter plant. Adult moths were killed by freezing, mounted, and photographed using a Cannon EOS camera with ring-flash. Quantification of patterns with the goal of assessing the degree of melanization was conducted by using Adobe Photoshop CS5.1 to simplify the wing pattern, as in Fig. 2. The resulting bitmap images were then analyzed using custom software, written for this purpose, to calculate the area occupied by black markings.

RESULTS

All six broods obtained under experimental conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C) (e.g. Fig. 1). Photographs

Natural variability in siblings



Cold-induced variability in siblings



Fig. 3. Comparison of black marking variation in *Utetheisa ornatrix bella* found naturally among siblings (A) and induced by temperature (B). Natural variation can be confined to a single wing pair (in this case, hindwing), while induced variation affects simultaneously both wing pairs.

of the broods are provided, in some cases divided into male and female subgroups. One can easily evaluate from these images (Supplementary Materials, Trials 1-6) the wing characteristics that have been affected by temperature: all the cold-reared moths are illustrated in the plates, and one can see that they have more extensive black markings on both upper and underside of the hindwing and on the underside of the forewing than the control moths reared at room temperature; the cold-reared moths also have more extensive white borders surrounding black spots on the upper side of the forewing, hence reducing the orange/red pigmentation. While in some broods (Trial 1, 2) these differences are more pronounced, they are less pronounced in others (Trials 3-6). In one of the broods, in addition to the differences described above, three of the specimens reared under cold conditions exhibited blurred coloration (Trial 4(B)).

In six replications of these experiments the results were somewhat variable, although they all point in the same direction. In Fig. 1 and Table 1, quantification of black markings is provided for two moth pairs selected at random from two trials otherwise figured in Supp. Mat., Trials 1 and 6. In Trial 1, moth individuals showed a greater degree of melanization than in Trial 6. Degree of melanization was similar between males and females within the trial.

While variation in the extent of black hindwing markings can be found frequently in sibling *U. ornatrix* (e.g., Fig. 3A), this mode of variation differs from that induced by cold temperature (Fig. 3B). In naturally-occurring sibling variation, this clearly heritable trait is expressed independently from the forewing coloration – forewings of the siblings in Fig. 3A appear practically identical. In the cold-induced melanism, however (Figs. 3B), the increased melanization of the hindwings is coupled with the increased melanization of the forewing, which is especially apparent on the underside.

DISCUSSION

Due to the tremendous variability of the bella moth, its widespread nature, and perhaps migration (the presence of which has been proposed by Pease (1968)), it is yet to be determined if some form of rudimentary polyphenism already

exists in nature, but it is clear that there is a reaction norm of wing phenotype to temperature that must be naturally occurring, as cold temperatures equivalent to ones used in the present study are frequent during larval/pupal development of *U. ornatrix* within the moth's range. During examination of various samples of *U. ornatrix*, at the National Museum of Natural History, Smithsonian Institution, Washington, DC, I found a sample collected in 1920 in New Jersey. From that sample I illustrate a female collected on 14 October (Fig. 5), which shows a pattern similar to those observed in some of the more melanic individuals obtained in the cold treatment in the present study. Judging by historical weather data, the larva of this later female developed and pupated when conditions were comparable to those used in my experimental treatments (Rutgers University Climate Lab 2015). Also, anecdotally, it seems that moths occasionally flying during the winter and early spring around Gainesville have paler forewings (pers. obs.; e.g., Figs. 4C,F), and perhaps the appearance of these phenotypes was also induced by cold, as seems to be suggested by the present study.

Melanic specimens, such as in Fig. 5, are so rare in the collections probably because it requires a combination of larvae completing their development in colder conditions and a researcher that is sampling this population extensively throughout the year. Penultimate and ultimate instar larvae as well as pupae in the middle of their respective stages were occasionally subjected in my lab to cold conditions for several days; however, none of these individuals resulted in phenotypes that were different from their siblings. From this anecdotal evidence, I conclude that the exposure to cold of developing wing discs is time-sensitive if an increase in melanism is to be induced; cold temperatures should probably be applied during the period between the last day of larval and the first day of pupal stages. This corresponds to the results of others (e.g., Rienks 1985), who showed that weather experienced in the fifth instar and pupal stage by the pierid *Catopsilia pomona* determined the adult phenotype.

According to Suzuki and Nijhout (2008: 64), "Genetic accommodation is a plausible mechanism for the evolution of polyphenisms." What has been shown in the present study



Fig. 4. The coloration of *Utetheisa ornatrix bella* in nature can appear both aposematic (A-B) and cryptic (C-E). (A) freshly emerged adult; (B) same, when wings hardened 2-3 hours later; (C) this moth flying at the end of January appeared more cryptic than aposematic due to highly reduced red/orange coloration of the forewings (Gainesville, Florida); (D) defensive discharge of the moth produced when disturbed; (E) wild moth in mid-December landing on *Crotalaria retusa* - the only hostplant available at the time (Micanopy, Florida); (F) wild moth in early December flying on University of Florida campus, blending with the background of dry grass.

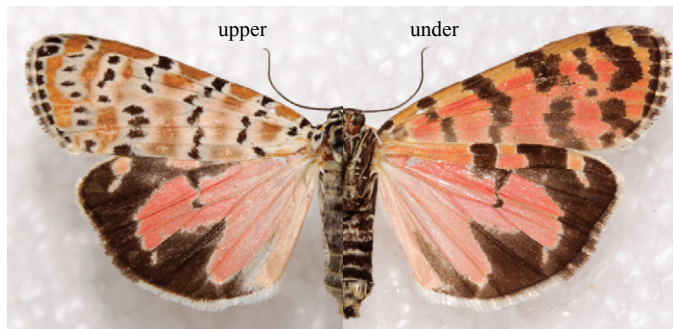


Fig. 5. A female of *Utetheisa ornatrix bella* collected on 14 October 1920 in Plainfield, New Jersey. Its pattern, combined with the lack of such melanism in larger sample collected during warmer part of the year in the same location, suggests that its melanism has been naturally induced by cold.

may or may not be an evolutionarily significant trend for *U. ornatrix*. If the genetic variation in wing pattern stimulated by temperature during larval/pupal development is positively selected, polyphenism may evolve in at least some populations of the bella moth, provided that there is a limited gene exchange with the more tropical populations. Future experiments will be of value to pinpoint the exact timing when melanization is induced, to understand genetic (e.g., polymorphism in genetic accommodation) and physiological mechanisms (e.g., possible hormonal controls) involved in the described phenomenon. For instance, the variation in reaction norm that was found between separate trials in the present study could have potentially been caused by differences in food quality, as has been shown to be the case in the nymphalid *Pararge aegeria* (Talloen et al. 2008). Also, it is interesting to note how the pattern of melanization described here differs from another recently described rare melanic pattern found in the same populations (Sourakov 2015b), where the diffused melanic scales appeared throughout both wings in two aberrant *U. ornatrix* females, which likely represented rare recessive mutations.

The black and red markings on the upper side of the wings add to the memorable pattern when they are exposed to a human (and hence to a potential vertebrate predator) and, no doubt, evolved to instill the corresponding unpleasant experience associated with the moth's defensive discharge (Figs. 4D). The decrease of red/yellow coloration can make the moths more cryptic (e.g., Figs. 4E,F), allowing them to blend in with the background of hostplant flowers and dry grass. The increased markings on the underside may also make the moth more cryptic when it has just emerged and is hanging vulnerably with its wings folded as in Fig. 4A, or may prompt warming up of the moth in cooler temperatures. If increased wing melanization correlates positively with fitness as was found in *Spodoptera* pupae (Liu et al. 2015), it may even prove to be a signal favored by sexual selection. Learning more about predator-prey interactions that involve *U. ornatrix* during different times of the year and about the moth's thermoregulation and mate choice may be key to our understanding of whether these changes in phenotypes indeed have adaptive significance in this species, or whether it is an expression of ancestral genes that have lost their adaptive significance but persist due to neutral selection.

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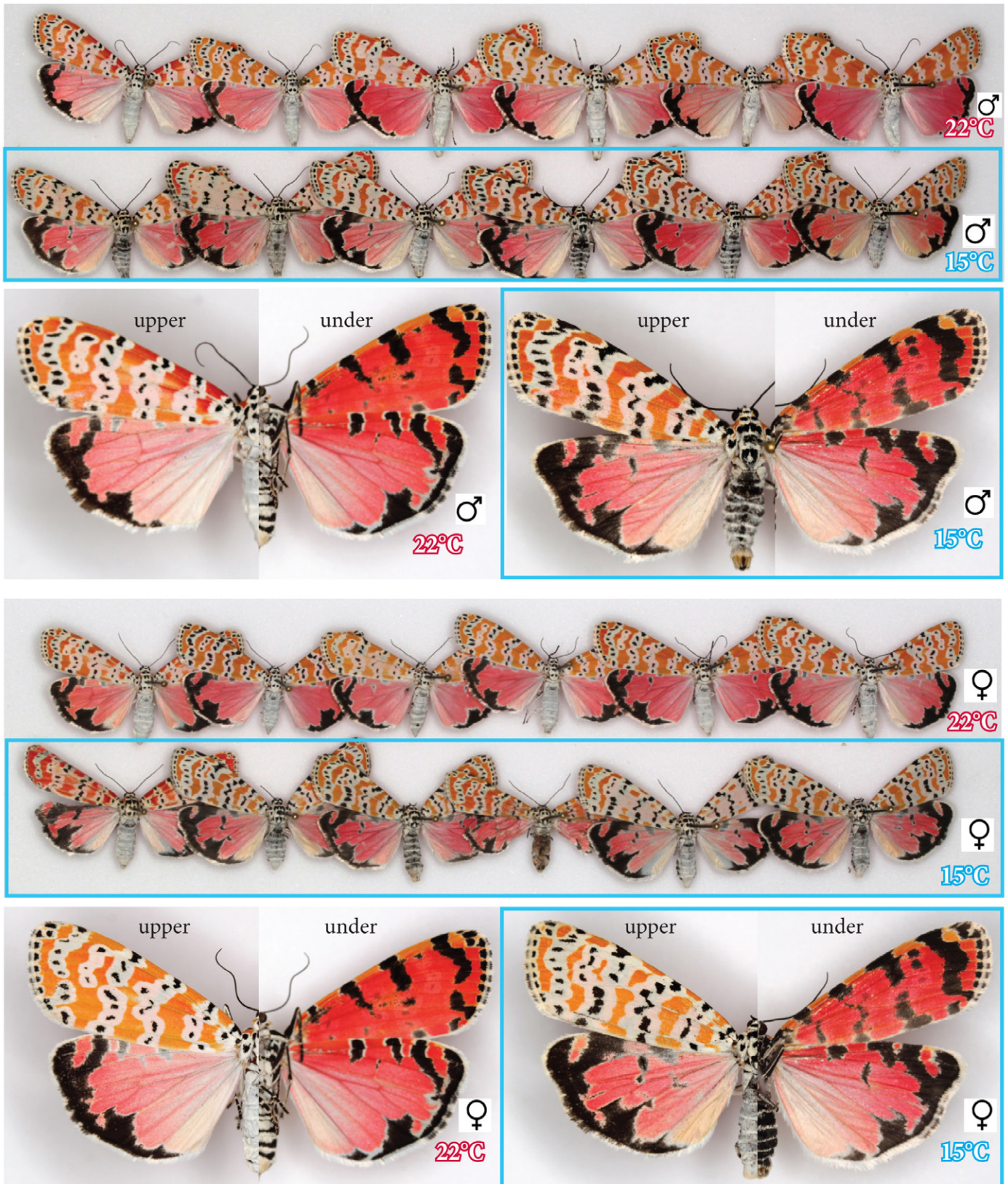
SUPPLEMENTARY MATERIALS:
Photographs of the specimens reared during Trials 1-6.



Trial 1: Individuals of this *Utetheisa ornatrix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded moths that have more extensive black markings on upper and underside of both wings; they also have more extensive white borders surrounding the black spots on the upper side of the forewing, hence reducing the orange/red pigmentation. Brood source: parental female was netted in August 2014, Natural Area Teaching Laboratory, University of Florida.



Trial 2: Individuals of this *Uteheisa ornatix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded moths that have significantly more extensive black markings on upper and underside of hindwing and on the underside of forewing; they also have more extensive white borders surrounding black spots on the upper side of the forewing, and females slightly paler pink coloration of the hindwing. Brood source: parental female was netted in November 2012, Natural Area Teaching Laboratory, University of Florida.



Trial 3: Individuals of this *Utetheisa ornatrix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded moths that have slightly more extensive black markings on upper and underside of both wings; they also have slightly more extensive white borders surrounding the black spots on the upper side of the forewing. Brood source: parental female was netted in December 2013, 4 mi N of the University of Florida campus.



Trial 4: (A) Individuals of this *Utetheisa ornatrix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded three moths with a more blurred pattern. Brood source: parental female was netted in January 2013, 10 mi NW from the University of Florida campus.

(B) The cold-subjected larvae/pupae also yielded three moths with an aberrant faded, blurred pattern. This pattern was not found in other trials.





Trial 5: Individuals of this *Utetheisa ornatrix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded moths that have slightly more extensive black markings on upper and underside of both wings; they also have slightly more extensive white borders surrounding the black spots on the upper side of the forewing, and slightly paler hindwing. Brood source: parental female was netted in January 2013, 10 mi NW of the University of Florida campus.



Trial 6: Individuals of this *Utetheisa ornatrix bella* brood reared under cold conditions (15-16°C) yielded adults that differed from their siblings reared under control conditions (22-24°C). The cold-subjected larvae/pupae yielded moths that have slightly more extensive black markings on upper and underside of both wings; they also have slightly more extensive white borders surrounding the black spots on the upper side of the forewing, and females slightly paler pink coloration of the hindwing and more yellowish forewing color. Brood source: parental female was netted in May 2012, Natural Area Teaching Laboratory, University of Florida.