Costs and Benefits of Melanism in the *Malacosoma disstria* Moth: Investigating the Maintenance of a Stable Polymorphism

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ABSTRACT

Costs and Benefits of Melanism in the *Malacosoma disstria* Moth: Investigating the Maintenance of a Stable Polymorphism

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The objective of this work is to investigate potential mechanisms maintaining a melanic polymorphism in the *Malacosoma disstria* moth. Laboratory experiments determined that the male-limited melanic phenotype is measurably darker than the simple phenotype. The melanic allele follows inheritance patterns expected for a Mendelian autosomal dominant. Melanization varies within phenotypes and is strongly dependent on dietary nitrogen availability. In female moths, melanization shows condition dependence. Melanic males tend to be smaller than simple males, allocate higher proportions of resources to melanin synthesis, and may be more susceptible to suboptimal conditions. These costs to the melanic phenotype may be balanced by thermoregulatory advantages and decreased predation pressure. Selection against the melanic phenotype may be strongest during outbreaks, but sex-limitation of the phenotype can protect the melanic allele from negative selection. The frequency of the melanic allele varies geographically;

this may be related to temperature, population dynamics, or both. Multiple mechanisms appear to be balancing selection on the two colour phenotypes in *M. disstria* moths, resulting in a stable polymorphism that is present throughout the species' wide distribution.

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Contribution of Authors

I was the principle investigator for all of the research work undertaken in this thesis, under the supervision of Dr. Despland. Chapter 2 resulted in a manuscript which has been has been published in an international peer-reviewed journal, and for which I was the main author. The experiments for Chapter 3 were conducted by Michael Gasse under my supervision; in the event that the manuscript is accepted for future publication, the authors will be credited in the following order: Ethier J, Gasse M, Despland E.

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Chapter 1 - General introduction

Polymorphic traits, found in diverse morphological, behavioural, and physiological traits of most living organisms, have long fascinated the scientific community. Whereas many traits show continuous variation across a range of potential trait expression, and others are the product of strong stabilizing selection to a single trait optimum, polymorphic traits are characterized by discontinuous variation and multiple trait optima. In an attempt to explain discontinuous trait variation and patterns of heritability, early scientists such as Gregor Mendel and William Bateson helped pioneer genetic theory as we know it today (Huxley 1955). The term polymorphism, as defined by Ford (1945) and further refined by Huxley (1955), refers to a trait consisting of multiple, discrete, and genetically-based morphs (phenotypes) that co-occur within a single population.

1.1 Maintenance of polymorphisms

In order for a stable polymorphism to be maintained, phenotypes must be under balanced selection; otherwise, selection will favour one phenotype and the population will become monomorphic. Balanced selection may result from several different mechanisms, including disruptive selection caused by temporal or geographical variation in selection pressures, negative frequency-dependent (apostatic) selection, and the heterozygote advantage (also termed overdominance or heterosis) (Hedrick et al. 1976; Roulin 2004; Gray and McKinnon 2007). In general, balanced selection changes relative phenotypic fitness over space or time so that no phenotype is consistently the most advantageous. The exception to this is the heterozygote advantage; the heterozygote phenotype has

greater fitness than either homozygote phenotype and thus both alleles are maintained (e.g. human sickle-cell (Allison 1954)).

Spatial variation in selection pressures plays an important role in the maintenance of polymorphisms (reviewed in Hedrick 2006). Phenotypic frequencies of populations often correspond to variation in a particular biotic or abiotic environmental factor, seen as simple geographic patterns or as clines that correspond to an environmental gradient (Brakefield 1984a; Sandoval 1994; Oxford and Gillespie 1998; Hoffman and Blouin 2000; Cook 2003; Galeotti et al. 2003; Hoekstra et al. 2004; Rajpurohit et al. 2008; Robertson and Robertson 2008). Sufficient gene flow between populations can prevent populations from becoming monomorphic due to local adaptation even under strong local selection (Cook 1998; Merilaita 2001). Environmental heterogeneity on a smaller scale can also maintain polymorphisms when phenotypes within populations exploit a different ecological niche (sometimes, but not necessarily, in conjunction with habitat selection), resulting in habitat- or microhabitat-dependent fitness (Roulin 2004; Forsman et al. 2008; Gray et al. 2008).

Temporal variation in selection pressures may be of particular importance in polymorphic species with cyclical population dynamics where density-dependent selection is a factor (e.g. Soay sheep (Moorcroft et al. 1996); side-blotched lizards (Sinervo and Lively 1996; Sinervo et al. 2000b); leaf beetles (Zvereva et al. 2002)). Recurrent fluctuations in density-independent environmental conditions will also result in temporal variation in selection pressures which may promote polymorphism (e.g. Madsen and Stille 1988; Karlsson et al. 2008). Indeed, polymorphisms may be a common adaptation to environments where fluctuations occur on a time scale greater than the lifespan of the

individual organisms in the population (Stehr 1964). However, temporal variation alone is generally not enough to maintain stable polymorphisms (Hedrick et al. 1976; Roulin 2004; Hedrick 2006).

Under apostatic selection, rare phenotypes are favoured until they become common, at which point they are selected against. This mechanism can arise from several different selective forces, including predators forming search images for the common prey type and then switching once the rare type becomes common (Cook 1998; Bond and Kamil 2002; Roulin 2004; Bond 2007; but see Punzalan et al. 2005); sexual selection where a rare or novel type of mate is preferred over common types or types from previous matings (Singh and Chatterjee 1989; Eakley and Houde 2004; Hampton et al. 2009); or by intra-sexual competition for access to mates through competitive interactions or territorial defence (Gross 1985; Sinervo and Lively 1996; Dijkstra et al. 2009).

Selection can also act indirectly on polymorphisms through correlated traits. Morphological, behavioural, and physiological traits can be linked as a result of pleiotropy, linkage disequilibrium, or shared resource pools (Zera and Harshman 2001; Roulin 2004; McKinnon and Pierotti 2010). Negative relationships between correlated traits can balance selection on polymorphisms, but sometimes results in apparent maladaptation (e.g. reduced parental care by red morph Gouldian finches under nutritional stress (Pryke et al. 2012)). However, the presence of beneficial phenotypic combinations maintained through pleiotropy or gene linkage can result in distinct life history strategies composed of multiple complementary traits (e.g. Brodie 1989; Shuster and Wade 1991; Sinervo and Lively 1996). Although this may promote maintenance of polymorphisms, it could also contribute to eventual reproductive isolation. Thus,

polymorphisms can have a profound effect on population dynamics, and may be involved in patterns of speciation and evolution (Gray and McKinnon 2007; Forsman et al. 2008; McKinnon and Pierotti 2010).

Despite the genetic basis of polymorphisms, extrinsic factors may affect the expression of polymorphic traits to a certain extent (e.g. reductions in orange spot expression in guppies (*Poecilia reticulata*) due to parasitic infection (Houde and Torio 1992); throat colour transformation and increased testosterone in yellow-morph side-blotched lizards (*Uta stansburiana*) due to disappearance of orange-morph neighbours (Sinervo et al. 2000a)). However, the genetic polymorphisms discussed here should not be confused with non-genetic polyphenisms where phenotypes are inducible. Although sometimes referred to in the literature as polymorphisms, polyphenisms have no genetic basis; they result from alternate developmental pathways triggered by environmental cues and other factors, such as temperature, photoperiod, hormones, nutrition and growth rate, or the presence of predators or competitors (e.g. Dempster 1963; Brakefield 1987; Harvell 1994; Applebaum and Heifetz 1999; Nijhout 2003).

1.2 Colour polymorphisms

Polymorphisms can be found in diverse morphological, behavioural, and physiological traits, but polymorphisms in colour and pattern are of particular interest to researchers. The easily discernible differences in colour and pattern facilitate visual phenotypic identification, and also hold a certain aesthetic appeal. Colour polymorphisms are widespread and occur in diverse taxa (e.g. mollusks (Whiteley et al. 1997; Cook 1998);

crustaceans (Merilaita 2001); arachnids (Oxford and Gillespie 1998); insects (True 2003; Van Gossum et al. 2008); amphibians (Hoffman and Blouin 2000); reptiles (Forsman 1995; Sinervo et al. 2001); fish (Basolo 2006; Gray et al. 2008; Dijkstra et al. 2009); birds (Galeotti et al. 2003; Roulin 2004); and mammals (Moorcroft et al. 1996; Hoekstra et al. 2004); also see Gray and McKinnon (2007) for a general review). Colour polymorphisms also occur in many plant species; however, this discussion will be restricted to animals.

Animal colouration can be chemically- or structurally-based. Structural colour is caused by refraction and reflection of light off or through layers of tissue and generally has an iridescent quality (e.g. Nijhout 1985; McGraw et al. 2002; Basolo 2006; Stoehr 2006). Chemical colouration results from the deposition of pigments in tissues; two common and well-studied groups of pigments are the carotenoids and the melanins. Carotenoids are a group of hydrocarbon-chain-based pigments that account for many of the bright reds, oranges, and yellows seen in animals. Carotenoid pigments must be obtained in the diet; animals cannot synthesize carotenoids *de novo*, although in some cases pigment structure is altered before being expressed in the target tissue (e.g Goodwin 1986; Hill 2000). Melanin pigments, which are responsible for most black and brown colouration (but also red, orange, yellow, or green in certain cases), are synthesized from amino acid precursors (e.g. Jawor and Breitwisch 2003). Carotenoid-based colour expression is highly dependent on the environment; thus, carotenoid expression often shows condition dependence and is a reliable indicator of individual quality. Conversely, melanin-based colour traits are traditionally thought to be neither affected by environmental factors nor

related to quality, although this view has recently been questioned (e.g. Griffith et al. 2006; see Chapter 3 Introduction).

Colour has numerous functions in animals; as a result, a variety of selection pressures can act directly on colour polymorphisms. Colour traits can have non-visual functions in animals, such as thermoregulation, immune function, and resistance to UV rays, desiccation, or physical abrasion (Bonser 1995; Oxford and Gillespie 1998; Wilson et al. 2001; True 2003; Rees 2004; Rajpurohit et al. 2008). Colour and pattern are often under visual selection by predators or mates. Some animals use cryptic colouration to hide from predation, either by blending into their natural background or by resembling a specific object such as a twig, leaf, or rock (Bond 2007). Other animals use conspicuous aposematic colouration to warn predators that they are distasteful or dangerous, or to mimic other animals that are unpalatable (Mallet and Joron 1999). Colouration can also provide information to potential mates. Positive and negative assortative mating occur when individuals preferentially mate with phenotypically like (Findlay et al. 1985; Pryke et al. 2012) or unlike mates (Sheppard 1952; Lowther 1961; Smith 1973), respectively. Both types of assortative mating occur simultaneously when the same type of mate in one sex is preferred by both phenotypes in the other sex (Roulin 2004 and references therein).

Differences in selection pressures acting on males and females can result in sex-limited colour polymorphisms, as reviewed by Van Gossum et al. (2008) and Svensson et al. (2009). Female polymorphisms may involve a gynochrome (the typical female phenotype) and an androchrome (a phenotype which resembles that of the males). Androchromes may be more likely to avoid unwanted harassment from males seeking mating opportunities. Female-limited phenotypes can also be Batesian mimics of an

unpalatable model species, or be linked to other life history traits such as behaviour, size, or reproductive output. Male-limited colour polymorphisms frequently reflect alternative mating strategies, generally consisting of an aggressive territorial male and a 'sneaky' non-territorial male. Territorial males will fight to defend a territory, which gives them access to females. The sneaky male phenotype often resembles the female phenotype, giving these males access to true females within a fighter male's territory. Sex-limited polymorphisms may be sex-linked or simply sex-limited (i.e. autosomal but not expressed in one sex).

Aside from selection pressures acting directly on colour polymorphisms, selection may also act indirectly on colour phenotypes through correlated traits (e.g. McKinnon and Pierotti 2010). Thus, multiple selection pressures, both direct and indirect, can act simultaneously on colour polymorphisms. Although each form of selection by itself may or may not promote the maintenance of a stable colour polymorphism, the polymorphism will be maintained if the net effect is balanced selection.

1.3 Insects and melanism

The details of the invertebrate melanin synthesis pathway have not yet been fully described, but the pathway is at least superficially similar to that of vertebrates (reviewed in Sugumaran 2002). For example, both vertebrates and insects begin melanin synthesis with the oxidation of tyrosine into dopa (dihydroxyphenylalanine), but each uses a different, and unrelated, enzyme (tyrosinase in vertebrates; phenoloxidase in insects). Despite biochemical differences in the synthesis pathways, and potentially in final

pigment structure, melanins in both vertebrates and invertebrates are nitrogen-rich phenolic polymers that are synthesized from amino acid precursors. Production of these pigments may not be costly in animals that are not nitrogen-limited, but many herbivores (particularly folivores), are nitrogen-limited due to the low levels of available nitrogen present in plants (Mattson 1980). Furthermore, insects (many of which are folivores) have additional nitrogen requirements that vertebrate animals do not.

The insect cuticle is not only rich in nitrogen, but is discarded and replaced multiple times during development through moulting (Andersen 1979). Many insects also construct webs, mats, trails, tents, and cocoons out of nitrogen-rich silk (Berenbaum et al. 1993). Finally, melanin in insects is not only used as a colour pigment, it is also a vital component of the insect immune system. Melanin is involved in the encapsulation and isolation of foreign bodies that have penetrated the cuticle, promotes rapid wound healing, and has cytotoxic effects (Sugumaran 2002). The production and use of melanin for colouration is therefore likely to be costly in insects as it diverts nitrogenous resources away from body construction and other important functions. Indeed, several studies have found that poor nutrition can reduce insect melanization (Allegret 1964; Talloen et al. 2004; Lee et al. 2008; Punzalan et al. 2008a). Melanic insects may suffer increased mortality under adverse conditions (Zvereva et al. 2002); melanism may also negatively affect life history traits such as fecundity, body size, and development time (Cook and Jones 1996; True 2003).

Despite the potential costs to melanin synthesis, melanin is a common colour pigment among insects; polymorphic melanism has been documented in many insect species of various orders (reviewed in True 2003). The Lepidoptera (moths and butterflies) are

particularly good candidates for studying melanism due to their wing structure. The wings consist of a thin membrane with a top and bottom layer of scales one cell thick. These scales are individually pigmented; colouration results from the two-dimensional patterns of pigment synthesis and deposition (Nijhout 1985). Individuals which have more melanin pigments deposited in their wings are melanic (Kettlewell 1973). Thus, melanic phenotypes are not necessarily black; they may simply be darker brown, or have more or larger dark elements (i.e. spots, bars) on the wing, compared to the non-melanic phenotype. Similarly, non-melanic (or typical) phenotypes are not necessarily white; they may be pale brown or have fewer or smaller dark elements on the wing compared to the melanic phenotype.

An interesting model species to investigate polymorphic melanism is *Malacosoma disstria*, the forest tent caterpillar. *Malacosoma disstria* is a holometabolous insect; that is, the life cycle (described in Stehr and Cook 1968; Fitzgerald 1995) is divided into distinct stages (egg, larva, pupa, and adult), with complete metamorphosis occurring during the pupal stage. The larvae hatch from their eggs in early spring and immediately begin feeding on foliage. *Malacosoma disstria* larvae pass through a minimum of five instars, each separated by a moult. They also construct silk mats and trails throughout the majority of their development. At the end of the final instar, the larvae spin silk cocoons in which to pupate. During the pupal stage, the resources that were acquired and stored by the larva are irreversibly allocated to the body, reproductive structures, and energy reserves of the adult moth. The non-feeding adults emerge fully formed and capable of immediate reproduction. After mating, the female moth lays her entire complement of eggs in a single cluster. All larvae hatching from a single egg mass can be assumed to be

full siblings (Fitzgerald 1995 and references therein), making this an ideal system to study inheritance patterns. Lorimer (1979) reported the presence of a melanic polymorphism in the adult *M. disstria* moth, with the melanic phenotype being expressed only in males. As is the case for many other moths with polymorphic melanism (see Chapter 2), she proposed simple Mendelian inheritance where the melanic allele is autosomal and dominant. However, Lorimer did not measure melanization in her study, and melanism in this moth has not been investigated since.

Both the distribution and population dynamics of *M. disstria* make the species well-suited to studying the presence and maintenance of a colour polymorphism. *Malacosoma disstria* shows strong cyclical population dynamics; during the peak of the population cycles, populations reach outbreak densities and the larvae cause severe defoliation of their host trees. During outbreaks, the larvae suffer high levels of starvation, parasitism, predation, and disease. However, *M. disstria* populations remain at very low densities between outbreaks, and the levels of defoliation, starvation, parasitism, predation, and disease are much reduced (Fitzgerald 1995). Thus, selection pressures acting on these insects change drastically over time. *Malacosoma disstria* also has an extremely broad distribution, spanning most of the United States and southern Canada from coast to coast (Fitzgerald 1995); *M. disstria* populations must experience very different selection pressures due to geographical differences in environmental conditions. For example, latitudinal differences have been demonstrated in key life history traits, including reproductive output (Parry et al. 2001) and performance (i.e. survival and development) on different host tree species (Parry and Goyer 2004). The severity and frequency of outbreaks also tends be highest in southern regions of the species' distribution (i.e.

southern United States compared to northern U.S. and Canada (Fitzgerald 1995)). Population dynamics and outbreak patterns vary geographically on a smaller scale as well (e.g. within Quebec), with outbreak incidence particularly reduced near the northern limits of the species' distribution (Cooke and Lorenzetti 2006).

1.4 Purpose

The purpose of my research is to characterize polymorphic melanism in the *M. disstria* moth, and to investigate how selection may be acting on and maintaining the polymorphism in this species. In Chapter 2, I will characterize melanism in *M. disstria* by (a) testing Lorimer's (1979) inheritance hypothesis using insects collected from a temporally and geographically distinct population; (b) determining if measurable differences in melanization exist between phenotypes; and (c) investigating the effects of phenotype on life history traits and trait responses to larval diet quality. In Chapter 3, I will investigate the effects of nitrogen limitation on melanization and determine whether melanization shows condition dependence in this species. To determine what selection pressures may be involved in the maintenance of the polymorphism, Chapter 4 will investigate (a) selection pressures which act directly on the colour trait; and (b) indirect selection pressures and secondary effects of melanism. This work will further our understanding of the mechanisms and selective forces maintaining stable polymorphisms, as well as the conditions which promote the very presence of polymorphic traits as opposed to monomorphic or continuously varying traits.

Chapter 2 – Effects of polymorphic melanism and larval diet on life history traits of *Malacosoma disstria* moths

The following chapter is based on the published manuscript: Ethier J, Despland E (2012) Effects of polymorphic melanism and larval diet on life history traits of *Malacosoma disstria* moths. Journal of Insect Physiology 58: 67–74

2.1 Abstract

In this study we investigated the presence and possible genetic basis of polymorphic melanism in the forest tent caterpillar (*Malacosoma disstria*) moth. Adult moths were classified into pattern-based phenotypes and wing darkness was measured to quantify the degree of melanization. We found that two distinct phenotypes, melanic and simple, are present in these moths. Although the full melanic phenotype is sex-limited to males, it is partially expressed in females. We also provide support for the hypothesis that the melanic allele is autosomal and dominant. The effects of larval diet quality on the survival, development and wing melanization of each phenotype were studied by rearing larvae on the foliage of either a primary or secondary host. Diet quality did not differentially affect the two phenotypes; however, melanic males were found to be smaller than simple males regardless of larval diet. Such inherent developmental differences between the two phenotypes could have important consequences for the frequencies of the two morphs.

2.2 Introduction

Many Lepidoptera have wing colour polymorphisms where two or more distinct phenotypes derive from multiple alleles at a single gene locus (or a few tightly linked gene loci) (Beldade and Brakefield 2002). In moths, melanins are the most common colour pigments, giving rise to various shades of colour from white or pale brown to dark brown or black. Individuals that have synthesized and deposited more melanin in their cuticle have darker phenotypes and are termed melanic (Kettlewell 1973; Majerus 1998). Basic crossing experiments have indicated that adult moth melanic polymorphisms are often controlled by a single gene locus, with melanic alleles being dominant (e.g. Lees 1974; West 1977; Lorimer 1979; also see Kettlewell 1973; Beldade and Brakefield 2002; Cook et al. 2002). More rarely, polymorphic melanism is inherited as a Mendelian recessive in either the adult or the larvae, or over multiple stages of development (Kettlewell 1973; Majerus 1981; Futahashi et al. 2008; Bear et al. 2010).

A well-known case of an adult dominant melanic polymorphism is that of the peppered moth, *Biston betularia*, because of drastic increases in the frequency of melanic phenotypes in certain areas of Great Britain as a result of the industrial revolution (e.g. Kettlewell 1973; Berry 1990; Majerus 1998; Grant 1999; Cook 2003). However, melanic phenotypes are also present at low frequencies in rural populations of *B. betularia* unaffected by industrial emissions (Cook et al. 2002). Melanic phenotypes have also been documented in populations of several other moth species in Great Britain (Lees 1971; Cook et al. 2002) and North America (Owen 1962; West 1977; Lorimer 1979; Sargent 1985). Unfortunately, the ecological and physiological impacts of melanic phenotypes

have received little attention outside the context of industrial melanism, and the selection pressures maintaining polymorphisms in these populations are unknown.

Melanin pigments are not ingested; rather, they are synthesized from amino acid precursors and are rich in nitrogen (Nijhout 1985; Blarzino et al. 1999; Stoehr 2006; McKinnon and Pierotti 2010). Nitrogen is already an important, and often limiting, nutrient for herbivorous insects (e.g. White 1984; Schowalter 1986); and indeed, high levels of melanization have been shown to be costly in some insects. In the leaf beetle, *Chrysomela lapponica*, dark morph beetles had much higher hibernation mortality than pale morph beetles after the beetles were fed on a low quality diet (Zvereva et al. 2002). Talloen et al. (2004) found that darker-winged *Pararge aegeria* butterflies had longer larval stages and more asymmetrical wings, independent of diet quality. However, the cost of melanin production may be offset by a decrease in melanin synthesis when conditions are poor. Wing melanization of *Pararge aegeria* butterflies was reduced when the larvae were reared on a low quality diet (Talloen et al. 2004). Similarly, Allegret (1964) reported that protein deprivation in the late larval stages of *Galleria mellonella* resulted in moths with reduced pigmentation.

An interesting model species with which to investigate polymorphic melanism and the effects of larval diet quality on the development, survival, and melanization of individuals of different phenotypes is the forest tent caterpillar, *Malacosoma disstria*. *Malacosoma disstria* is a North American pest insect with outbreak population dynamics and a large distribution that covers the United States and southern Canada from coast to coast (Fitzgerald 1995). High levels of intra-population variation in wing colour are present in *M. disstria* moths, particularly in males (Stehr and Cook 1968). Male wings

not only range in colour from very dark brown to pale tan, but also exhibit differences in wing patterns, whereas female wing colour is more homogenous in both colour and pattern (Lorimer 1979).

Lorimer proposed that melanism in *M. disstria* is polymorphic but sex-limited to males, with autosomal dominant inheritance. She tested possible mechanisms for inheritance of the melanic allele by comparing the observed frequency of family types (i.e. all males melanic, all males non-melanic, or a mix of both) to those expected if the melanic allele is autosomal dominant, autosomal recessive, sex-linked dominant, or sex-linked recessive, and found that the autosomal dominant mode of inheritance best fit the data. Furthermore, she found that the observed phenotypic ratios of males within mixed families most closely resembled those expected if the melanic allele is autosomal and dominant. Finally, Lorimer found no evidence of the melanic allele being lethal in females; this suggests that females carry the melanic allele but simply do not express the phenotype.

Malacosoma disstria moths are non-feeding; all the energy and nutrients required for an individual to successfully metamorphose into an adult and survive to fulfill its reproductive functions are accumulated during the larval stage (Fitzgerald 1995). Environmental conditions experienced by the larvae can therefore be expected to have a direct effect on the physical traits of the adult moth through irreversible allocation of available resources during the pupal stage. Furthermore, the high insect population densities that occur during outbreaks result in a decrease in both the quantity and quality of food available to individual larvae; hence, they suffer periodic starvation and are often forced to feed on secondary host trees (Fitzgerald 1995). Due to the cost of melanin

production, melanic and non-melanic phenotypes may be expected to show differences in life history traits, such as development time and body size. Melanic males may also suffer increased mortality, particularly under suboptimal conditions such as those experienced during outbreaks. Alternatively, the negative effects of increased melanization could be counteracted by an induced reduction in melanization under poor conditions.

In this study we test several hypotheses. The insects used in Lorimer's (1979) experiment were collected from Michigan, Minnesota, Indiana, and Alabama (USA). However, as mentioned previously, this species' distribution spans a large part of North America. Lorimer's hypothesis of a sex-limited, autosomal dominant melanic allele is therefore further tested here on a sample collected from a population in northern Alberta (Canada), much further north and west than any of Lorimer's sample populations. Comparing phenotypic ratios within and between families to those expected by autosomal dominance of the melanic allele (as per Lorimer 1979) will determine whether the polymorphism, and its proposed genetic basis, is widespread in *M. disstria*. Furthermore, Lorimer's (1979) melanic phenotype, which she termed 'Dark', was based on a purely visual assessment of wing darkness and patterns. Here, moths will similarly be classified into phenotypes, but on the basis of wing patterns only. Wing darkness will then be quantified as an indicator of melanization and compared between pattern-based phenotypes to test the hypothesis that one phenotype is measurably darker than the other, and therefore melanic. Finally, the effects of larval diet quality on the development, survival, and melanization of moths will be investigated to determine whether differences exist between phenotypes.

2.3 Materials and Methods

2.3.1 Larval rearing

Unhatched egg masses were collected in the early spring of 2008 from a high density (outbreak) population near Wabasca in northern Alberta, Canada (56°17.5N, 113°93.9W). They were stored at 4°C and 80%R.H. until local budbreak of trembling aspen (*Populus tremuloides*), the primary host of northern populations of *M. disstria* (e.g. Stehr and Cook 1968; Parry and Goyer 2004). Egg masses were sterilized in a 5% bleach solution before hatching (Grisdale 1985). All larvae hatching from a single egg mass can be assumed to be full siblings (Fitzgerald 1995 and references therein) and are henceforth referred to as a family. Families (n = 28) were reared in separate plastic containers at 21±1°C and 70%R.H., under a 16hr light: 8hr dark photoperiod.

Larvae were fed fresh trembling aspen leaves *ad libitum* from time of hatch to the middle of the fourth larval instar. Once all larvae within a family finished moulting to the fourth instar, the family was haphazardly divided in two and each half was moved to a new container. One half was fed trembling aspen leaves and the other half was fed white birch (*Betula papyrifera*) leaves until completion of the larval stage. White birch is a less-preferred secondary host in northern *M. disstria* populations (Hodson 1941), and represents the low-quality food that larvae may be forced to feed on during outbreaks.

In seven families, infection and mortality from the highly epizootic nuclear polyhedrosis virus was so prolific in the fourth or fifth instar that the colonies were lost and could not be included in this study. Pupae were weighed and placed in small individual containers; immediately after eclosion, the moths were stored at -16°C until processing of the wings.

Development time was recorded for each individual as the number of days from hatching of the egg mass to eclosion of the moth. Individuals that failed to construct a silk cocoon and pupated on the bottom of the container were recorded as having no cocoon.

2.3.2 Characterizing adult wing patterns and melanization

Each moth was sexed and visually classified as having a patterned or simple phenotype based on the presence or absence of specific pattern elements on the forewing (Fig. 2.1). Patterned moths have two pale bars on their forewings adjacent to the dark proximal and distal bars (as did Lorimer's (1979) 'Dark' phenotype), as well as greater variation in shading along the top (anterior) edge of the forewing (pers. obs.). These extra pattern elements were only observed in males, so females were all classified as simple. Thus, moths were divided into three 'adult types': patterned males, simple males, and (simple) females (Fig. 2.1). In all three adult types, colouration of the wings reflected overall colouration of the body (pers. obs.).

Figure 2.1 Wing samples for (a) patterned male, (b) simple male, and (c) (simple) female *M. disstria*. The proximal area of each wing is on the right and the distal area is on the left; these areas are delineated by the proximal and distal bars, respectively. Unlike simple males and females, patterned males have two pale bars adjacent to the dark proximal and distal bars and greater variation in shading along the top (anterior) edge of the wing.

The following colour scoring procedure is based on a procedure developed by Dr. Maya Evenden of the University of Alberta (pers. comm.) and is similar to procedures described in other studies on animal colouration (e.g. Holloway et al. 1997; Eakley and Houde 2004; Talloen et al. 2004; Davis et al. 2005; Punzalan et al. 2008a; Robertson and Robertson 2008). One forewing was removed from each moth and glued to a sheet of white paper; sheets of wings were then scanned using an HP Scanjet 5590 scanner (along with a paper cm/mm ruler to provide scale). Using the software ImageJ 1.40g (Rasband 1997-2011), each file was converted to an 8-bit greyscale (0 = black, 255 = white) in order to measure darkness as a proxy for melanization (e.g. Talloen et al. 2004).

For each moth, the area in mm^2 and the mean grey value of the forewing were recorded. Boxes measuring 15x15 pixels were used to measure mean grey values of the proximal and distal areas of the forewing (Fig. 2.1). These values were then averaged to find the background darkness of the forewing. Due to the nature of the greyscale, a low grey value indicates darker wings and increased melanization compared to a high grey value. Background darkness was found to be a more reliable index than the mean grey value of the whole forewing, as any areas of damage (such as wrinkles or loss of colour scales) were purposely avoided during placement of the boxes used to score background darkness. Moths with extensively damaged wings were not used. Some moths failed to properly expand their wings after eclosion. Although these unexpanded wings could not be colour scored, many could still be classified by phenotype and were therefore included when calculating phenotypic frequencies.

2.3.3 Statistical analyses

A paired *t*-test was used to determine whether the low-quality birch diet differentially affects survival of male phenotypes by comparing phenotypic frequencies within families across diet treatments. Chi square tests were performed to compare observed frequencies of male phenotypes within and between families to those expected if the patterned allele is autosomal and dominant to the simple allele (as per Lorimer 1979). For this part of the analysis only, alpha was increased to 0.10 instead of the standard 0.05 in order to increase power and reduce the risk of committing a Type II error (failing to reject a false null hypothesis). It is usually more serious to commit a Type I error (rejecting a true null hypothesis) as this will erroneously provide support for the research hypothesis. In this particular case, however, failing to reject the null hypothesis supports the hypothesis being tested. By increasing alpha to 0.10, the risk of erroneously finding support for the proposed hypothesis is reduced. The effects of adult type and diet on wing darkness (melanization), development time, pupal mass, and forewing area were tested using an analysis of variance (ANOVA). The ANOVA controlled for the effect of family (as a random factor) while testing for effects of adult type and diet, and included all 2- and 3-way interactions. Tukey post hoc tests were performed when necessary. All statistical analyses were performed in SPSS v.16.0 (IBM 2007), except for the Chi square tests, which were calculated using Excel (Microsoft Office 2007).

2.4 Results

2.4.1 Genetic basis of phenotypes

Families can be classified into three types based on the types of males present: all males simple, all males patterned, or a mix of both. These families will henceforth be referred to as simple families ($n = 9$), patterned families ($n = 2$), and mixed families ($n = 10$). Simple and patterned families remained so regardless of diet treatment. In mixed families, ratios of patterned to simple males were first calculated separately for family halves (groups raised on either aspen or birch) and compared using a paired-samples *t*-test. It was found that diet treatment had no significant effect on the ratio of patterned to simple males within families (d.f. = 9, $t = 0.673$, $p = 0.518$); thus, diet did not differentially affect survival of the two types of males. The Chi square tests for phenotypic ratios of mixed families were therefore performed on entire families (i.e. both diet treatments combined).

According to Lorimer (1979), polymorphic melanism in *M. disstria* is controlled by two alleles at a single gene locus, the patterned allele being dominant but only expressed in males. From this hypothesis, the expected ratio of patterned to simple males in mixed families is either 3:1 or 1:1, depending on the parental cross. This prediction was tested using Chi square tests ($\alpha = 0.10$), which showed that nine of the ten mixed families were not significantly different from one of the two ratios and the remaining family was not significantly different from either ratio (Table 2.1). These results support the proposed hypothesis that the patterned allele is autosomal and dominant. Furthermore, the expected frequencies of each family type were calculated from the observed numbers of patterned and simple males using Hardy-Weinberg equations (patterned: $p = 0.178$; simple: $q = 0.822$; expected family frequencies: simple = 0.456, patterned = 0.063, mixed = 0.482). The results of the Chi square test showed there was no significant difference between the

observed and expected number of families in each category if the patterned allele is autosomal and dominant (d.f. = 2, $\chi^2 = 0.392$, $p = 0.822$).

Table 2.1 Results of Chi square tests for ratios of male phenotypes (patterned = P; simple = S) in mixed type families. Rows in bold indicate that there is no significant difference between the observed and expected frequencies for a given ratio. Alpha was increased to 0.10 to reduce the risk of committing a Type II error. All d.f. = 1.

Family	P:S	Expected ratio	χ^2	p
1	23:9	**3:1**	**0.167**	**0.683**
		1:1	6.125	0.013
2	20:21	3:1	15.033	0.000
		1:1	**0.024**	**0.876**
3	21:28	3:1	27.000	0.000
		1:1	**1.000**	**0.317**
4	20:13	3:1	3.646	0.056
		1:1	**1.485**	**0.223**
5	11:11	3:1	7.333	0.007
		1:1	**0.000**	**1.000**
6	32:12	**3:1**	**0.121**	**0.728**
		1:1	9.091	0.003
7	11:15	3:1	14.821	0.000
		1:1	**0.615**	**0.433**
8	9:17	3:1	22.615	0.000
		1:1	**2.462**	**0.117**
9	23:15	3:1	4.246	0.039
		1:1	**1.684**	**0.194**
10	16:9	**3:1**	**1.613**	**0.204**
		1:1	**1.960**	**0.162**

2.4.2 Wing melanization and development of adult types

Separate ANOVAs were performed for mixed and simple families, as mixed families include all three adult types (patterned males: $n = 153$; simple males: $n = 133$; females: $n = 326$) while simple families contain only simple males ($n = 297$) and females ($n = 309$). Patterned families were not analyzed due to small sample sizes (patterned males: $n = 54$; females: $n = 38$). All residuals were found to be normally distributed prior to analysis. Standard deviations were reported to accurately represent variability within samples (Curran-Everett 2008).

Wing darkness was significantly affected by adult type in mixed families ($F_{2,19.180} = 130.001$, $p < 0.001$). Tukey post hoc tests showed that each type is significantly different from all other types (all $p < 0.001$) (Fig. 2.2i). Patterned moths (always male) were found to be significantly darker than simple moths (both male and female), and will therefore be referred to as melanic for the remainder of this paper. Although females were found to be significantly darker than simple males in mixed families, there was no significant difference in wing darkness between females and simple males in simple families ($F_{1,8.624} = 0.988$, $p = 0.347$) (Fig. 2.2ii). This suggests that females from mixed families have darker wings than those from simple families. This was tested using a simple nested ANOVA where family (still a random factor) is nested within a fixed term representing the presence or absence of melanic brothers. All three types of families were included in this analysis, but no distinction was made between mixed and melanic families. The results of the nested ANOVA showed that females with melanic brothers ($n = 364$) had significantly darker wings than females without melanic brothers ($n = 309$) ($F_{1,19.330} = 10.833$, $p = 0.004$). However, the presence of melanic brothers did not have a significant

effect on the development time, pupal mass, or forewing area of females. Diet had no significant effect on wing darkness in either mixed or simple families (Fig. 2.2).

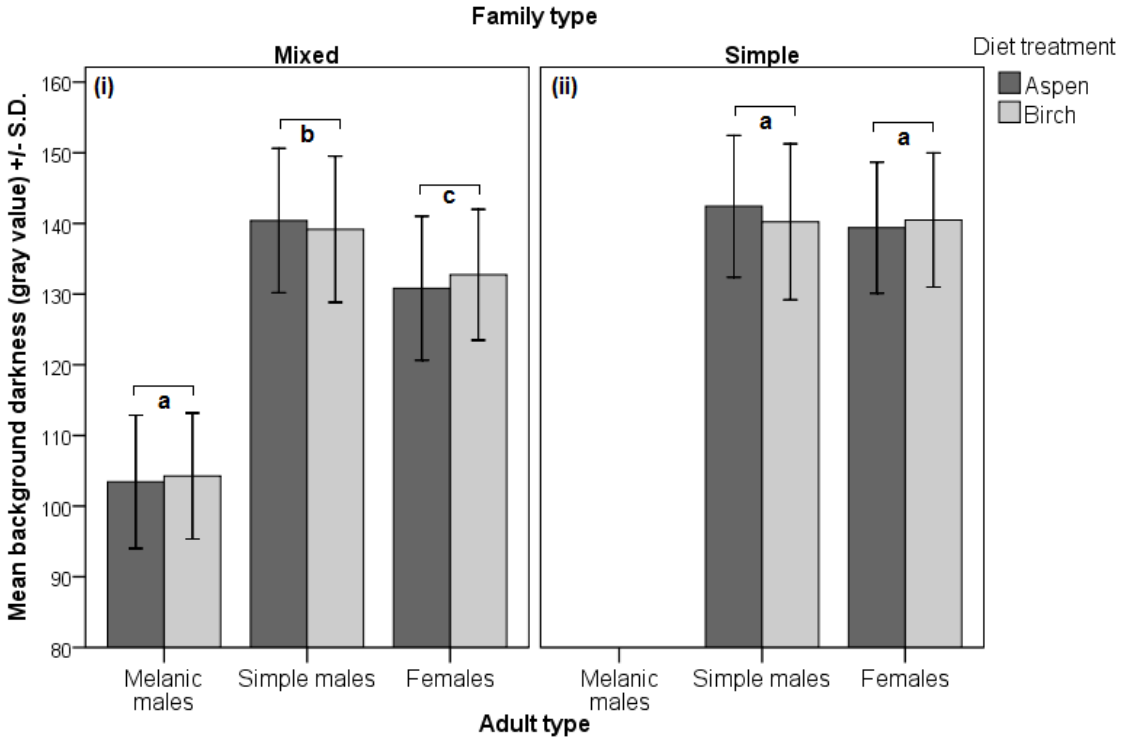

Figure 2.2 Mean (+/- S.D.) wing darkness (as greyscale values: low values are darker, high values are paler) of melanic (patterned) male, simple male, and female *M. disstria* moths on aspen and birch diets from (i) mixed and (ii) simple type families. Letters denote significance between adult types. No significant effect of diet treatment was found.

Development time was significantly affected by adult type in both mixed families ($F_{2,20.789} = 4.775$, $p = 0.020$) and simple families ($F_{1,12.453} = 43.366$, $p < 0.001$) (Fig. 2.3i, ii). In simple families, this simply represented the expected difference between sexes. Similarly, Tukey post hoc tests for mixed families showed that the two types of males did

not differ significantly in their development times ($p = 0.645$), but both had significantly shorter development times than females (both $p < 0.001$). Development time was also significantly affected by diet in both mixed ($F_{1,9.533} = 61.677$, $p < 0.001$) and simple families ($F_{1,8.832} = 76.346$, $p < 0.001$) (Fig. 2.3iii, iv).

Body size was measured at two stages of development as pupal mass and adult forewing area. In mixed families, pupal mass was significantly affected by adult type ($F_{2,19.464} = 391.495$, $p < 0.001$). Tukey post hoc tests revealed that both types of males were significantly smaller than females (both $p < 0.001$) and melanic males weighed significantly less than simple males ($p = 0.013$) (Fig. 2.4i). In simple families, adult type was also found to have a significant effect on pupal mass ($F_{1,10.441} = 968.782$, $p < 0.001$), which simply represented the expected difference between sexes (Fig. 2.4ii). Diet significantly affected pupal mass in simple families ($F_{1,8.903} = 12.417$, $p = 0.007$) but not in mixed families (Fig. 2.4i, ii).

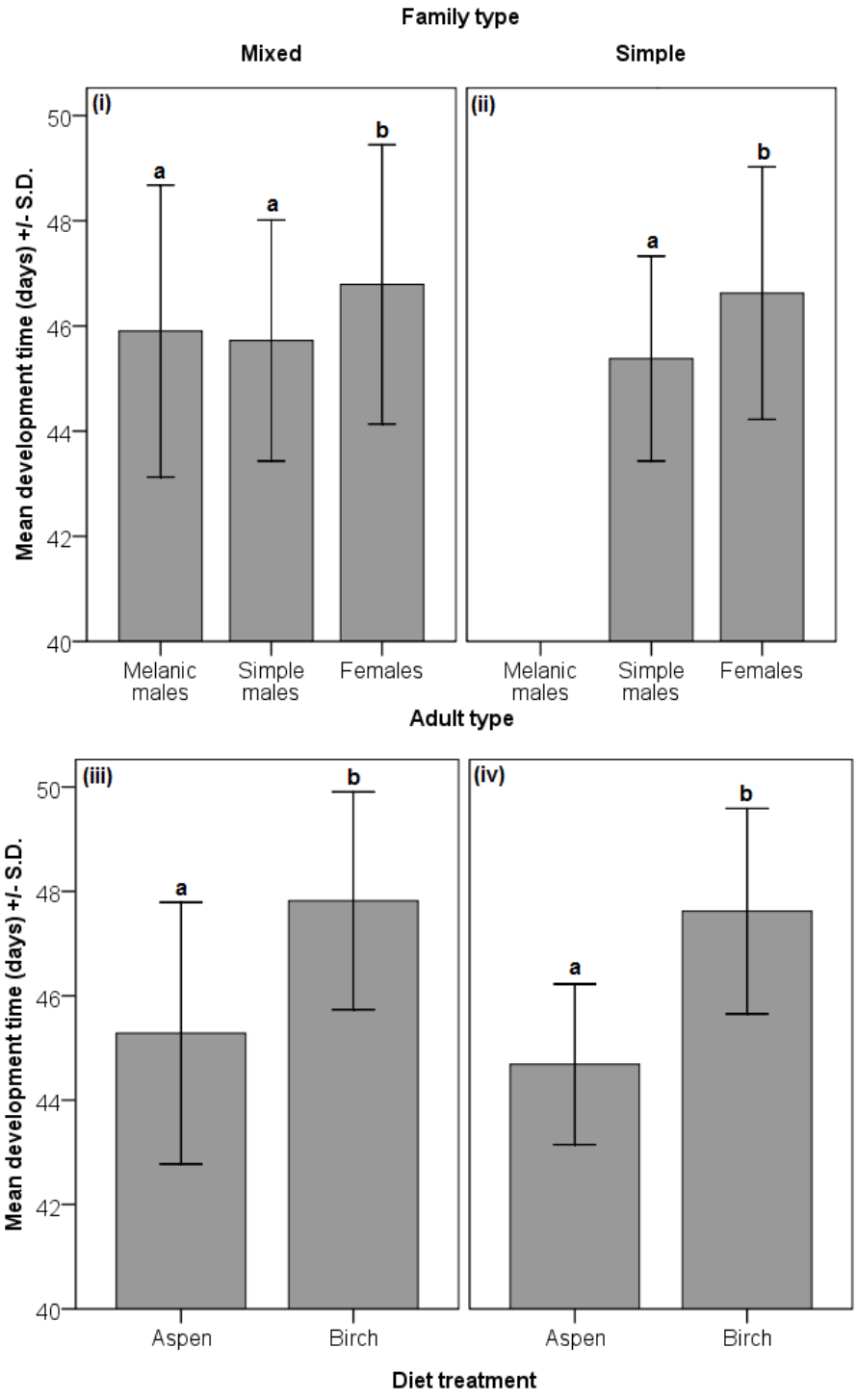

Figure 2.3 Mean (+/- S.D.) development time (in days) of melanic male, simple male, and female *M. disstria* moths in (i) mixed and (ii) simple type families and of moths reared on aspen and birch diets in (iii) mixed and (iv) simple type families. Letters denote significance.

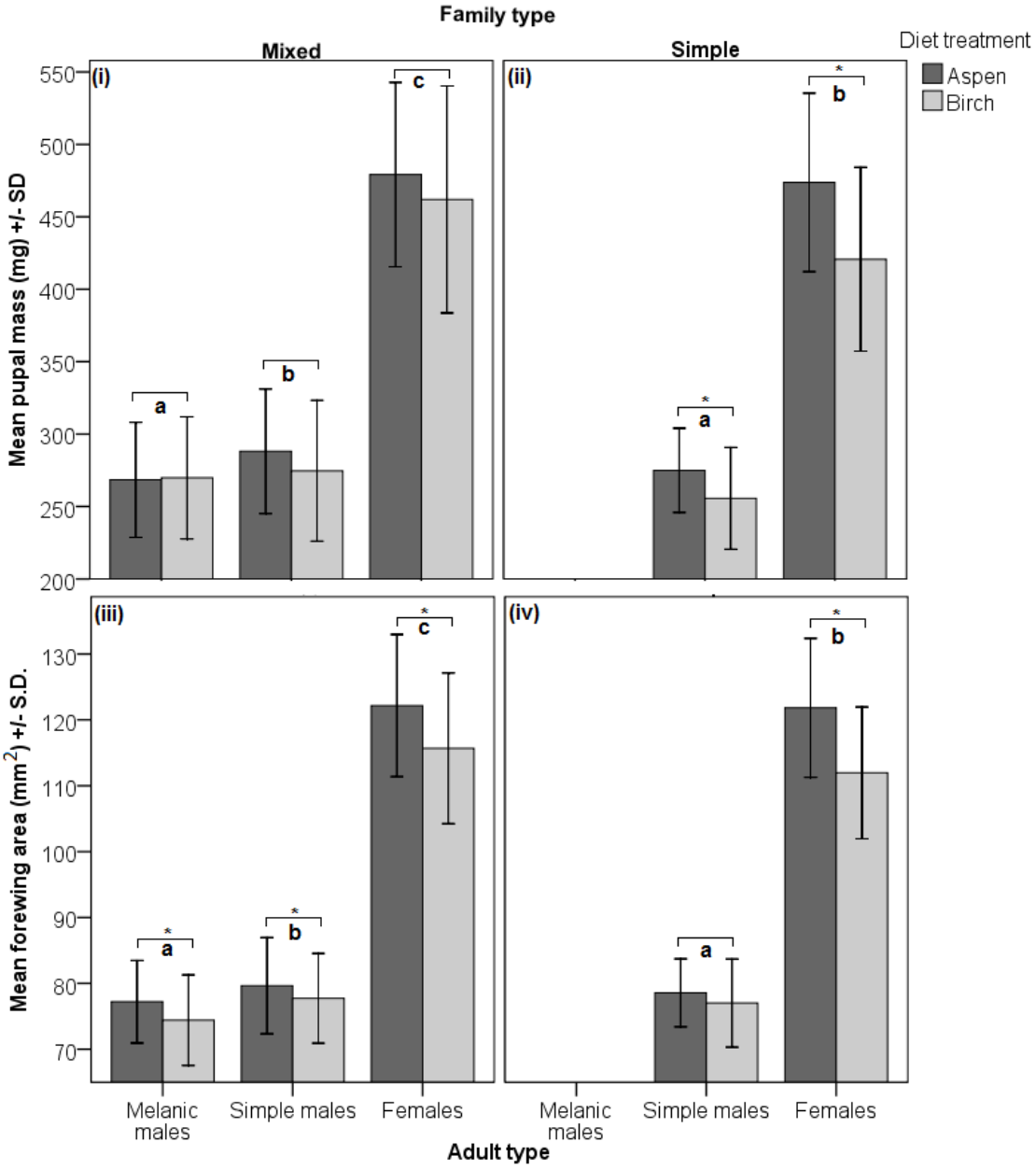

Figure 2.4 Mean (+/- S.D.) pupal mass (in mg) of melanic male, simple male, and female *M. disstria* moths on aspen and birch diets in (i) mixed and (ii) simple type families. Mean (+/- S.D.) forewing area (in mm^2) of melanic male, simple male, and female M. disstria moths on aspen and birch diets in (iii) mixed and (iv) simple type families. Letters denote significance between adult types; stars denote significance between diet treatments.

To test forewing area, a number of moths had to be removed from the analysis due to wing damage that artificially reduced wing area values. This resulted in a decrease in sample sizes in both mixed families (melanic males: n = 147; simple males: n = 127; females: n = 274) and simple families (simple males: n = 283; females: n = 263). In mixed families, forewing area was significantly affected by adult type ($F_{2,21.155} = 845.145$, $p < 0.001$). Tukey post hoc tests indicated that, as was the case with pupal mass, both types of males were significantly smaller than females (both $p < 0.001$) and melanic males were significantly smaller than simple males ($p = 0.011$). Unlike pupal mass, however, forewing area was also significantly affected by diet in mixed families ($F_{1,9.556} = 6.305$, $p = 0.032$) (Fig. 2.4iii). In simple families, forewing area was significantly affected by both adult type ($F_{1,8.644} = 1.030 \times 10^3$, $p < 0.001$) and diet ($F_{1,8.764} = 17.120$, $p = 0.003$). A significant interaction between adult type and diet was also found in simple families ($F_{1,7.714} = 13.439$, $p = 0.007$); Tukey post hoc tests revealed that forewing area was significantly reduced by the birch diet in females ($p = 0.003$) but not in simple males ($p = 0.111$) (Fig. 2.4iv).

During this experiment, a single family (Family 2 from Table 2.1) was observed to have much lower success rates of cocoon formation (52%) than all other families (93-100%). In this mixed family, only 4 of 20 melanic males (20%) successfully formed cocoons before pupating, compared to 14 of 21 simple males (67%) and 26 of 48 females (54%). These success rates were compared using three 2x2 contingency Chi square tests. Melanic males were found to have a significantly lower success rate of cocoon formation than both simple males (d.f. = 1, $\chi^2 = 9.058$, $p = 0.003$) and females (d.f. = 1, $\chi^2 = 6.685$, p

= 0.010), whereas simple males and females did not differ significantly in their success rates (d.f. = 1, $\chi^2 = 0.937$, $p = 0.333$).

2.5 Discussion

2.5.1 Genetic basis of phenotypes

This study showed that two distinct, pattern-based phenotypes are present in *M. disstria* moths, and that one phenotype is measurably darker than the other. Although Lorimer (1979) proposed that the melanic phenotype is sex-limited to males, this study found evidence that females carrying the melanic allele have slightly increased melanization compared to non-carriers. This suggests that melanism in *M. disstria* is only partially sex-limited: only males express the wing patterns and darkness characteristic of the melanic phenotype, but the melanic allele nonetheless affects wing melanization in female carriers. The results of this study also supported the hypothesis that polymorphic melanism in *M. disstria* is controlled by a pair of alleles at a single autosomal gene locus with the melanic allele being dominant (Lorimer 1979). Dominant melanic alleles at an autosomal locus have been shown to be responsible for many other documented cases of polymorphic melanism in moths. Although the sex-limitation is unusual, Kettlewell (1973) reported male-limited melanic phenotypes in three moth species (*Cycnia mendica* (Arctiidae); *Hepialus humuli* (Hepialidae)*;* and *Lasiocampa quercus*, a member of the same family as *M. disstria* (Lasiocampidae)).

The presence of the same phenotypes in Lorimer's (1979) study populations (Michigan, Minnesota, Indiana, and Alabama) and in the Alberta population used in this study

indicate that this melanic polymorphism, and its sex-limitation, is widespread across much of the species' distribution. Furthermore, in this study the frequency of the melanic allele was 18%, while allelic frequencies of 4-19% were calculated from the sample sizes and phenotypic frequencies reported in Lorimer's (1979) study for Michigan and Indiana egg collections in 1976 and 1977. Given the geographical and temporal distances between the populations used in these two studies, allelic frequencies appear to be quite conserved across the species' distribution.

Indeed, the rarity of dominant melanic alleles may be conserved across species of moth as well. In the *Phigalia pedaria* moth, two melanic alleles are present (both dominant to the typical, or non-melanic allele) and occur at combined frequencies of 2-18% in rural areas across the British Isles (Lees 1971). Cook et al. (2002) also report low frequencies (0-39%) of melanic phenotypes of three species of moths in non-industrial areas of Great Britain between 1974 and 1999. The melanic alleles are dominant in all three species, and although the authors did not report the allelic frequencies, the phenotypic frequencies are comparable to those reported by Lorimer (1979) (8-34%) and to that found in the present study (32%), indicating that allelic frequencies are also quite similar.

2.5.2 Melanism and larval development

The low quality larval diet tended to decrease body size and increase development time. Diet quality did not affect wing melanization, however, nor did it differentially affect the survival of melanic and simple (non-melanic) males. Melanic males were found to have both lower pupal mass and smaller wing area than non-melanic males regardless of diet treatment, indicating inherent developmental differences between the two phenotypes.

Lorimer (1979) found no significant difference between mean pupal mass of melanic and non-melanic males; however, the larvae in her experiment were reared on artificial diet rather than on foliage, as was used here.

Trembling aspen is known to promote better larval development than prepared diet, resulting in significantly greater pupal masses (Colasurdo et al. 2009). It is probable that white birch, although a secondary host, also promoted better development than prepared diet. Indeed, mean pupal masses of melanic and simple males in mixed families were much higher in the current study (269mg (n = 153) and 283mg (n = 133), respectively; diet treatments combined) compared to those reported by Lorimer (215mg (n = 108) and 204mg (n = 102), respectively). Furthermore, Lorimer did not report any other information regarding larval rearing conditions in her experiment. Other factors may therefore have contributed to the different results obtained in these two experiments.

Size differences between melanic and non-melanic males could have an important impact on the relative reproductive success of the two types of males due to differences in flight capabilities. Male *M. disstria* must actively search for mates (Fitzgerald 1995); thus, their reproductive success is largely dependent on their ability to fly until they find a receptive female. Flight capabilities are determined by lipid content, thoracic muscle mass, and wing loading (Angelo and Slansky 1984; Marden 2000; Beck and Kitching 2007). However, these traits can be related to pupal mass and forewing area in different ways depending on resource allocation and allometry (Gunn and Gatehouse 1986; Gunn and Gatehouse 1993; Shirai 1993; Muhamad et al. 1994; Boggs and Freeman 2005). Further investigation is needed to determine whether the observed reductions in pupal mass and

adult forewing area are an advantage or a detriment to flight capabilities, and therefore mating success, in melanic male *M. disstria* moths.

This study also found another indication of inherent differences between melanic and non-melanic males. In one family, melanic males were found to have much lower rates of successful cocoon formation compared to non-melanic males and females.The fact that this single family had extremely low levels of cocoon formation in both diet treatments suggests that the larvae were infected with a pathogen that interferes with silk production (e.g. Youssef 1974; Orr et al. 1994). Although normal adults eclosed from naked pupae in the lab, failure to spin a cocoon in nature could result in the larvae falling to the ground and pupating unprotected. These pupae may be more vulnerable to predation than those that successfully formed a cocoon. Melanic males, being more susceptible to this pathogenic infection in the larval stage, might therefore suffer greater pupal mortality than non-melanic males.

Although increased melanism has often been linked to increased immune responses in larvae by virtue of both physical and chemical properties of melanin (e.g. Wilson et al. 2001; Mikkola and Rantala 2010), this is not always the case. Goulson and Cory (1995) reported that at constant larval density, melanic *Mamestra brassicae* larvae were more susceptible to viral infection in the fifth instar than non-melanic larvae. Furthermore, no melanic polymorphisms were observed in the *M. disstria* larvae reared for this experiment. Without a link between adult and larval melanism, there is no reason to expect adult melanism to increase immune function in the larval stage.

The melanic phenotype must provide some advantage for male moths; otherwise, it would not persist in the species due to the costs of melanin production. In cooler weather, melanic males may have a thermoregulatory advantage over non-melanic males, increasing their chances of mating success (e.g. Punzalan et al. 2008b). It is also more difficult for visual predators such as birds to spot darker insects when they are flying in low light conditions (Majerus 1998), which could result in lower predation pressures on melanic males. In females, however, such advantages of the melanic phenotype may not be enough to overcome the costs of melanin synthesis. Although sex-limitation of melanic phenotypes is rare, it has been reported before (Majerus 1998) and may be favoured when males and females are under different sets of selection pressures (e.g. Ohsaki 1995). Flight is less important for the reproductive fitness of female *M. disstria* moths than for the males; rather, body mass is of utmost importance as it is tightly linked with fecundity (e.g. Parry et al. 2001). Indeed, although females carrying the melanic allele have increased melanization, it is not accompanied by the reduction in pupal mass and forewing area seen in males with the full melanic phenotype.

In this study, we have shown that polymorphic melanism is present in *M. disstria* moths. We have also provided support for the hypothesis that the melanic allele is autosomal and dominant (Lorimer 1979). Although the melanic phenotype is only expressed in males, this study showed that the allele nonetheless increases melanization in female carriers. Furthermore, we found inherent developmental differences between the melanic and non-melanic phenotypes, which have important implications for male survival and reproductive success. Further investigation of the possible advantages and disadvantages of both phenotypes would clarify the effects these phenotypic differences may have on

the population dynamics of the species as a whole, and could help explain why the melanic allele is often rare, yet maintained, in *M. disstria* and other polymorphic moth species.

2.6 Appendix

Table 2.2 Melanic allele and phenotype frequencies were calculated for *M. disstria* moths originating from various populations at high (H) or low (L) densities in different years. Some were collected as eggs (host tree indicated when known) and reared in the lab on artificial diet or foliage; others were collected as pupae or moths from the field.

Location	Year	Collected	Total n	Diet	Melanic frequency	
					Allele (%)	Phenotype (%)
Indiana	1976 (H)	Eggs*	275	Artificial diet	4	8
Michigan	1976 (H)	Eggs*	391	Artificial diet	19	34
Alabama	1976 (H)	Pupae*	29	Unknown foliage	9	17
Indiana	1977 (H)	Eggs*	119	Artificial diet	10	19
Michigan	1977 (H)	Eggs*	95	Artificial diet	12	22
Minnesota	1977 (H)	Pupae*	104	Unknown foliage	18	33
Alberta (West of Wabasca)	2005 (L)	Pupae/Moths**	25	Unknown foliage	15.1	28.0
Alberta (South of Wabasca)	2005 (L)	Pupae/Moths**	61	Unknown foliage	19.0	34.4
Alberta (Rocky Mountain House)	2005 (L)	Pupae/Moths**	79	Unknown foliage	16.6	30.4
Alberta (Drayton Valley)	2005 (L)	Pupae/Moths**	50	Unknown foliage	17.5	32.0
Alberta (Wabasca)	2005 (H)	Pupae/Moths**	109	Unknown foliage	14.3	26.6
Alberta (Wabasca) - Chapter 2	2008 (H)	Eggs (aspen)	611	Aspen/birch foliage	18.1	32.9
Quebec (Lac St Jean)	2008 (L)	Eggs (aspen)	170	Aspen/birch foliage	34.5	57.1
Quebec (St Esprit) - Chapter 4	2010 (H)	Eggs (maple)	364	Artificial diet	18.3	33.2
Quebec (St Esprit)	2010 (H)	Pupae	46	Aspen foliage	26.3	45.7
Quebec (St Esprit)	2010 (H)	Pupae	27	Maple foliage	11.8	22.2
Ontario (southern)	2011 (H)	Eggs (maple)	172	Artificial diet	16.5	30.2
Quebec (St Esprit)	2011 (H)	Eggs (aspen)	474	Artificial diet	19.7	35.4
British Columbia (Prince George)	2011 (H)	Eggs (aspen)	338	Artificial diet	43.0	67.5

* Data obtained from Lorimer (1979); **Data obtained from Dr. Maya Evenden (pers. comm.)

Chapter 3 - Condition dependence of a melanin-based colour trait: nitrogen availability and wing melanization in a polymorphic moth

3.1 Abstract

Colour polymorphisms and condition dependence of colour traits are particularly well-studied aspects of animal colouration. Unlike carotenoid-based colour traits, melanin-based colour traits are typically not considered to show condition dependence. Experiments showing no evidence of condition dependence of melanin-based colour traits have been conducted on vertebrate animals that are not nitrogen-limited. These results cannot necessarily be generalized for animals that are greatly affected by dietary nitrogen availability, as melanin synthesis may be more costly in these organisms. In the present study, we make use of a melanic polymorphism in the *Malacosoma disstria* moth to investigate the effects of nitrogen availability on melanization, development, and survival of melanic and non-melanic insects, and to determine whether melanization is condition-dependent in this species. We show that adult melanization is dependent on nitrogen availability during larval development. We also present evidence that melanization is condition-dependent, especially in females, and that melanic males may be more sensitive to reduced nitrogen availability than non-melanic males. This study confirms that patterns of condition dependence should not be over-generalized. Condition dependence of melanin-based colour traits may be most likely to occur in nitrogen-limited animals.

3.2 Introduction

Condition-dependent colour traits are those whose expression depends on the individual's quality; that is, the colour trait is costly to produce or maintain, and only high quality individuals can do so. Many studies have investigated the condition dependence of animal colour by manipulating factors such as food availability, food quality, or parasite load, and measuring the effect on the size or intensity of a colour trait. Carotenoid-based colour traits are generally accepted to be a reliable, or 'honest', indicator of individual quality and condition, reflecting foraging ability and the overall health of individuals (Houde and Torio 1992; Hill and Montgomerie 1994; Hill 2000; McGraw and Hill 2000; but see Cotton et al. 2004). Structural colour traits can also be condition dependent (McGraw et al. 2002; Siefferman and Hill 2005). A number of studies have reported no effect of food or parasite manipulation on the expression of melanin-based colour traits (Gonzalez et al. 1999; McGraw and Hill 2000; McGraw et al. 2002; Siefferman and Hill 2005). Such findings have led to the widespread, but mistaken, assumption that melanin-based colour traits must not be costly to produce and are not condition-dependent (Jawor and Breitwisch 2003; Griffith et al. 2006).

Recent work suggests that expression of melanin-based colour traits may be influenced by factors such as oxidative stress, mineral content of the diet, and hormone levels (McGraw 2007; Galván and Alonso-Alvarez 2008; McGraw 2008; Roulin et al. 2008; Hõrak et al. 2010). Furthermore, the costs of melanin synthesis are not necessarily comparable between taxa (Stoehr 2006), or between feeding guilds. The studies cited above which found no evidence of condition dependence of melanin-based colour traits all used birds as the experimental organism. Three of those studies used birds that feed on

seeds (Gonzalez et al. 1999; McGraw and Hill 2000; McGraw et al. 2002), and one used an insectivorous species (Siefferman and Hill 2005). As these animals are not generally nitrogen-limited, they may be able to divert nitrogen resources to melanin synthesis at relatively low cost. By contrast, folivorous animals that feed exclusively on leaves are often extremely nitrogen-limited. Moreover, insects have additional requirements for nitrogen resources that vertebrates do not. Indeed, there is evidence for condition-dependent melanism in a number of insects, both predatory (Punzalan et al. 2008a) and herbivorous (Allegret 1964; Talloen et al. 2004; Lee et al. 2008; also see Stoehr 2006).

The insect cuticle is rich in nitrogen, being composed mainly of protein and chitin (Hackman 1953a; Hackman 1953b; Hackman 1953c; Wigglesworth 1957; Andersen 1979); hence, nitrogen is lost when the cuticle is shed at each moult. Protein is also a major component of the silk used in structures such as webs and cocoons (Berenbaum et al. 1993). Melanin itself is a vital component in the immune responses of insects (Sugumaran 2002). Melanization of the cuticle functions as both a physical and chemical barrier to infection (Wilson et al. 2001), and melanin pigments are also involved in the encapsulation of foreign bodies such as pathogens or parasites (Nappi and Christensen 2005; Stoehr 2010). For folivorous insects in particular, survival, growth, fecundity, and abundance tend to be nitrogen-limited due to the relatively low levels of nitrogen in leaves (Scriber and Slansky Jr. 1981; White 1984; Awmack and Leather 2002; Fagan et al. 2002; Throop and Lerdau 2004). Melanin-based colour traits are common in insects and have many different functions; furthermore, genetically-based melanic polymorphisms have been documented in numerous insect species (reviewed in True 2003). A well-known example is the peppered moth, *Biston betularia* L. (Lepidoptera:

Geometridae), where the melanic (*carbonaria*) form is black and the non-melanic (*typica*) form is predominantly white (e.g. Cook 2003; also see True 2003).

The vast majority of the Lepidoptera (moths and butterflies) are herbivorous; they are also particularly good candidates for studying melanism due to their wing structure. The wings consist of a thin membrane covered by an upper and a lower layer of scales one cell thick; colour patterns result from simple two-dimensional patterns of melanin deposition (Nijhout 1985). The melanization intensity (darkness) of any given area of the wing depends on the amount of melanin synthesized and deposited in the scales at that location (Kettlewell 1973; Majerus 1998). Thus, darker melanic phenotypes require higher levels of pigment synthesis than non-melanic conspecifics. Melanization of both melanic and non-melanic phenotypes should be dependent on the amount of nitrogen obtained in the diet. Indeed, Stoehr (2006) proposed that melanization should depend on both nitrogen intake and processing efficiency. He predicts that melanization will increase with increasing nitrogen intake up to some maximal level of expression; the slope of the saturating relationship between nitrogen intake and melanization depends on processing efficiency. However, it should be noted that Stoehr's (2006) definition of processing as "everything that happens between resource acquisition and ornament expression" actually encompasses two separate stages of processing: efficiency and allocation. With respect to nitrogen resources, processing efficiency refers to the assimilation rate of ingested nitrogen; allocation refers to the relative amounts of assimilated nitrogen invested into different functions. Phenotypic differences in the slope of the relationship between nitrogen intake and melanization could result from differences in processing efficiency, allocation patterns, or both.

Malacosoma disstria Hübner (Lepidoptera: Lasiocampidae), the forest tent caterpillar, is an ideal species with which to study the relationship between melanization and nitrogen intake in a colour polymorphic folivore. Two distinct melanic phenotypes occur in the adult moth. The melanic phenotype is dark brown and only expressed in males, while the non-melanic "simple" phenotype is light brown or tan (Lorimer 1979; Chapter 2). Although no effect of diet quality (leaves from primary vs. secondary host trees) on melanization was found in Chapter 2, foliar nitrogen levels of the two diet treatments were not measured. As with other folivorous insects, nitrogen is a limiting nutrient for *M. disstria*. Larval development and survival are negatively affected by reduced nitrogen content of natural foliage (Hemming and Lindroth 1995) and of artificial diet (Colasurdo et al. 2009). As single families often include both melanic and simple males (Lorimer 1979; Chapter 2), *M. disstria* provides a unique opportunity to study the relationship between nitrogen intake and melanization in melanic and non-melanic siblings.

Melanic and simple males are expected to differ in their maximal levels of melanization, and we hypothesize that reduced nitrogen intake will decrease melanization. However, the slope of the relationship between nitrogen intake and melanization may or may not differ between the two phenotypes (Fig. 3.1). As discussed above, variation in this slope can result from differences in either processing efficiency or resource allocation. In this species, however, the pupal stage represents the entire resource pool that will be allocated amongst various traits of the non-feeding adult moth (see Materials and Methods). In the relationship between pupal nitrogen content (i.e. net nitrogen intake) and adult melanization, any variation in the slope must be due to differences in the proportion of resources allocated to melanin synthesis. Patterns of resource allocation will therefore

have an impact on the phenotype-specific effects of nitrogen intake. If melanic and simple males show similar patterns of resource allocation (Fig 3.1, cases B and C), then reduced nitrogen intake (Fig 3.1, line X') should affect melanization more strongly in melanic males. Conversely, if melanic males allocate proportionally more resources to melanin synthesis compared to simple males (Fig 3.1, cases A and C), reduced nitrogen intake may have a similar effect on melanization in both types of males, but a stronger negative effect on the survival or development of melanic males.

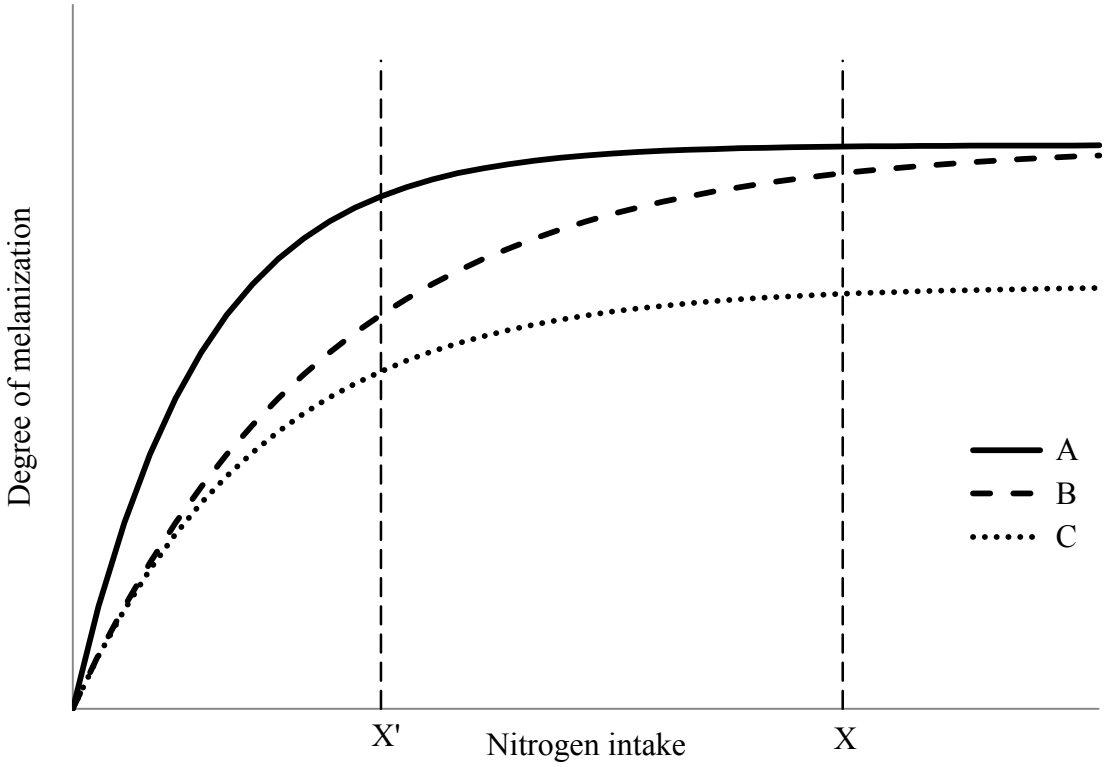

Figure 3.1 Theoretical saturating relationship between nitrogen intake and melanization for three different resource allocation patterns. A and B have the same maximum, but the slope of A is twice that of B; C has a lower maximum but the same slope as B. Line X represents a high level of nitrogen intake; line X' represents a low level of nitrogen intake.

3.3 Materials and Methods

3.3.1 Rearing

Larval nitrogen intake can be easily manipulated by modifying the protein content of artificial agar-based diet. The standard prepared diet used to rear *M. disstria* larvae has a protein:carbohydrate ratio of 20p:22c. It contains equal amounts of casein and dextrose, as well as the protein and carbohydrate in wheat germ (Addy 1969). To artificially increase or reduce nitrogen availability for this experiment, the amounts of casein and dextrose in the diet were manipulated so that the final protein:carbohydrate ratio was either 28p:14c (high-protein), or 14p:28c (low-protein) (see Colasurdo et al. 2009). More extreme manipulations of p:c ratios (i.e. 35:7 and 7:35) are known to have strong negative effects on larval development and survival, and are not representative of the chemical composition of natural foliage (Despland and Noseworthy 2006).

Egg masses were collected before hatching in the spring of 2010 from a high density population near St. Esprit in Québec, Canada (45^{o}55'22.61N, 73^{o}39'53.78W) and stored at 4^{o}C and 80% relative humidity. The egg masses were hatched in individual 1oz. plastic containers where larvae had access to standard diet (20p:22c) for the duration of the first larval stadium. Once all individuals in a family had finished moulting to the second larval stadium, each family was haphazardly split into equal treatment groups which were moved to larger containers and fed *ad libitum* on either the high-protein (28p:14c) or the low-protein (14p:28c) diet for the remainder of the larval stage. Twelve families were used in the experiment for a total of 12 high-protein and 12 low-protein treatment groups.

The 24 groups were reared on their respective diets at 21±1°C and 70% relative humidity, under a 16hr light: 8hr dark photoperiod. Individual cocoons were collected as they formed. Within 48hrs of pupation, each pupa was removed from its cocoon to be weighed and sexed, and placed in a 1oz. container. Development time was recorded as the number of days from hatching of the egg mass to emergence of the moth. Moths were collected immediately after emergence and stored at -16^{o}C until the wings were removed for colour scoring (see Chapter 2, Materials and Methods). One forewing was removed from each moth; total area (in mm^2) and melanization (on a greyscale) of the individual wings were recorded using the software ImageJ v.1.40g (Rasband 1997-2011). Males were also visually categorized as having either the melanic or simple (non-melanic) phenotype; as expected, females all had the simple phenotype (see Lorimer 1979; Chapter 2). Wing patterns in *M. disstria* are very basic (Fig. 2.1), and background wing darkness is representative of overall colouration of the moth (Stehr and Cook 1968; pers. obs.). Individuals with severely damaged wings and males whose phenotype could not be determined with certainty were excluded from all analyses.

Life history traits indicative of individual quality were measured as development time, pupal mass, and adult forewing area (i.e. age and size at maturity). Extended development time can negatively affect survival and fitness due to increased exposure to mortality agents, reduced food quality, a loss of colony synchrony, and fewer mating opportunities for males (Parry et al. 1998; Johansson et al. 2001; Morbey and Ydenberg 2001; Etilé and Despland 2008). Forewing area is an important factor in determining flight capabilities (Berwaerts et al. 2002). Pupal mass is a measure of individual size, and is strongly related to female fecundity (Fitzgerald 1995; Parry et al. 2001; also see Honek

1993). Pupal mass can also be used as a measure of net nitrogen intake, as it represents the entire resource pool an individual has to allocate to structures and traits of the adult moth. Both *M. disstria* and *Spodoptera littoralis* (Boisduval) (Egyptian cotton leaf worm) caterpillars have been shown to regulate the efficiency of post-ingestive protein assimilation when reared on diets with various p:c ratios. As a result, nitrogen concentration of the adult body is constant across high- and low-protein diet treatments (Lee et al. 2002; Colasurdo et al. 2009). Variation in pupal mass therefore represents consistent variation in nitrogen resources available to the adult.

3.3.2 Statistical analyses

To determine whether the survival of melanic and simple males was differentially affected by nitrogen availability, a paired *t*-test was used to compare the proportion of melanic males within families, between diet treatments. Analyses of variance (ANOVAs) were used to test the effects of diet treatment and adult type (melanic male, simple male, and female) on development time, wing melanization, pupal mass, and forewing area in mixed families (n = 9). Individuals with any wing damage (tears, folds, etc.) that artificially changed measured wing area were excluded from analyses that included forewing area. The ANOVAs included family as a random factor, and tested for all 2- and 3-way interactions. Residuals were tested for normality prior to analysis; pupal mass and forewing area were log-transformed, and development time required the Box-Cox transformation. Tukey post hoc tests were performed when necessary. To fit the relationship between melanization and pupal mass to the saturating model proposed by Stoehr (2006), nonlinear regressions were performed for each adult type (diet treatments pooled) using equation 3.1:

$$y = a\left(1 - e^{-x\left(\frac{b}{a}\right)}\right)$$

where y is melanization, x is pupal mass, a is the maximum, and b is the slope. The maximum and slope values obtained from the model were compared between adult types using overlap of the of 95% confidence intervals (95% C.I.) to estimate significance (Cumming and Finch 2005). Individuals from all family types (simple: n = 2; mixed: n = 9; melanic: n = 1) were included in this part of the analysis. The t-test and nonlinear regressions were performed in SPSS v.20.0.0 (IBM 2011); the ANOVAs were performed using JMP v.7.0.1 (SAS Institute Inc. 2007).

3.4 Results

The ratio of simple to melanic males within families was not significantly affected by diet (t = 1.197, p = 0.256, *d.f.* = 11). However, of the 9 mixed families with both simple and melanic males, 8 had either a lower or an equivalent proportion of melanics in the low-protein treatment (data not shown). The total number of simple males was relatively unaffected by diet treatment, and was in fact slightly higher in the low-protein treatment (high-protein n = 48; low-protein n = 54). The total number of melanic males was 27% less in the low-protein treatment compared to the high-protein treatment (high-protein n = 75; low-protein n = 55); similarly, the total number of females was 28% less in the low-protein treatment (high-protein n = 113; low-protein n = 81).

Wing melanization was significantly affected by Adult Type ($F_{2,18.81} = 155.6672$, $p < 0.0001$) and Diet ($F_{1,13.12} = 48.0267$, $p < 0.0001$). The Adult Type*Diet interaction was also significant ($F_{2,26.81} = 8.5450$, $p = 0.0013$). Wing melanization of all three adult types was significantly reduced on the low-protein diet. In both diet treatments, simple and melanic males were both significantly more melanized than females, and melanic males were significantly more melanized than simple males (Fig. 3.2). The significant interaction term may be explained by the fact that the magnitude of the difference in wing melanization between diet treatments differs between adult types. The low-protein diet reduced average wing melanization by 7.1 units in simple males, by 11.2 units in melanic males, and by 15.6 units in females (Table 3.1).

Table 3.1 Mean ± S.D. (*n*) for life history traits of melanic male, simple male, and female *M. disstria* on high- and low-protein diets.

Adult type	Diet (protein content)	Melanization (255-greyscale score)	Pupal mass (mg)	Development time (days)	Forewing area (mm^2)
Melanic male	High	160.3 ± 9.7 (57)	289.0 ± 35.3 (57)	61.8 ± 4.7 (57)	70.3 ± 6.0 (52)
	Low	149.1 ± 13.4 (44)	239.4 ± 27.5 (44)	66.0 ± 4.7 (44)	61.3 ± 5.9 (43)
Simple male	High	132.6 ± 11.7 (39)	283.5 ± 37.5 (39)	61.6 ± 4.9 (39)	70.0 ± 5.6 (39)
	Low	125.5 ± 14.0 (40)	255.1 ± 30.1 (40)	65.5 ± 4.9 (40)	63.4 ± 7.0 (39)
Female	High	121.2 ± 9.7 (91)	473.6 ± 71.1 (91)	63.5 ± 4.8 (91)	109.9 ± 10.5 (83)
	Low	105.6 ± 13.8 (52)	339.4 ± 49.9 (52)	68.5 ± 5.6 (52)	84.1 ± 10.0 (48)

Development time was significantly affected by Adult Type ($F_{2,20.9} = 13.1197$, $p = 0.0002$) and Diet ($F_{1,10} = 21.7155$, $p = 0.0009$). Development times of all adult types were significantly longer on the low-protein diet. Females had significantly longer development times than both simple and melanic males, but melanic and simple males

did not differ in their development times. The low-protein diet increased average development time by 6% (3.9 days) in simple males, by 7% (4.2 days) in melanic males, and by 8% (5.0 days) in females (Table 3.1).

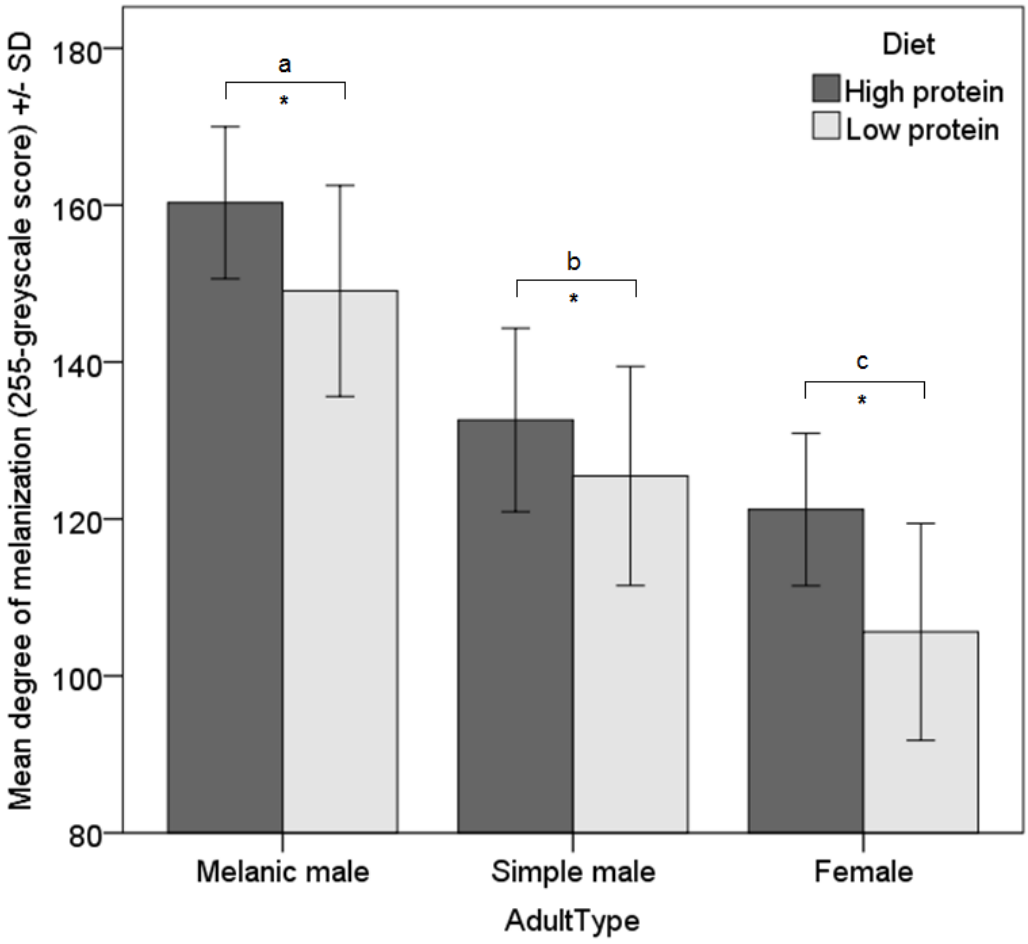

Figure 3.2 Mean (± S.D.) wing melanization (255 - greyscale score) of melanic male, simple male, and female *M. disstria* moths in high- and low-protein diet treatments. Higher melanization scores indicate darker wings. Letters denote significance between adult types; stars denote significance between diet treatments.

Pupal mass was significantly affected by Adult Type ($F_{2,4.56} = 618.9676$, $p < 0.0001$) and Diet ($F_{1,10.28} = 130.9994$, $p < 0.0001$). The Adult Type*Diet interaction was also significant ($F_{2,17.11} = 12.1366$, $p = 0.0005$). Pupal mass of all three adult types was significantly lower on the low-protein diet. In both diet treatments, females weighed significantly more than both simple and melanic males, but there was no difference between the two types of males. The low-protein diet reduced average pupal mass by 10% (28.4 mg) in simple males, by 17% (49.6 mg) in melanic males, and by 28% (134.2 mg) in females (Table 3.1).

Forewing area was significantly affected by Adult Type ($F_{2,8.545} = 492.9308$, $p < 0.0001$) and Diet ($F_{1,9.882} = 145.3937$, $p < 0.0001$). The Adult Type*Diet interaction was again significant ($F_{2,13.03} = 17.6033$, $p = 0.0002$). Forewing area of all three adult types was significantly smaller on the low-protein diet. In both diet treatments, female forewing area ($n = 180$) was significantly greater than forewing area of both types of males, and there was no difference in forewing area of melanic ($n = 123$) and simple males ($n = 99$) (Table 3.1). The low-protein diet reduced average forewing area by 9% (6.6mm^2) in simple males, by 13% (8.9mm^2) in melanic males, and by 24% (25.9mm^2) in females (Table 3.1).

Figure 3.3 shows the relationship between melanization and pupal mass for all three adult types; the results of the nonlinear regressions are summarized in Table 3.2. When comparing 95% C.I. on 2 independent means, an overlap of approximately one quarter of the average interval width corresponds to $p \leq 0.05$; a non-overlap corresponds to $p \leq 0.01$ (Cumming and Finch 2005). This suggests that the maximum for melanic males is

significantly greater than the maxima for simple males and females, but that the maxima for simple males and females do not differ significantly. The slopes are significantly different between all adult types: melanic males have the steepest slope, females the shallowest slope, and simple males are intermediate.

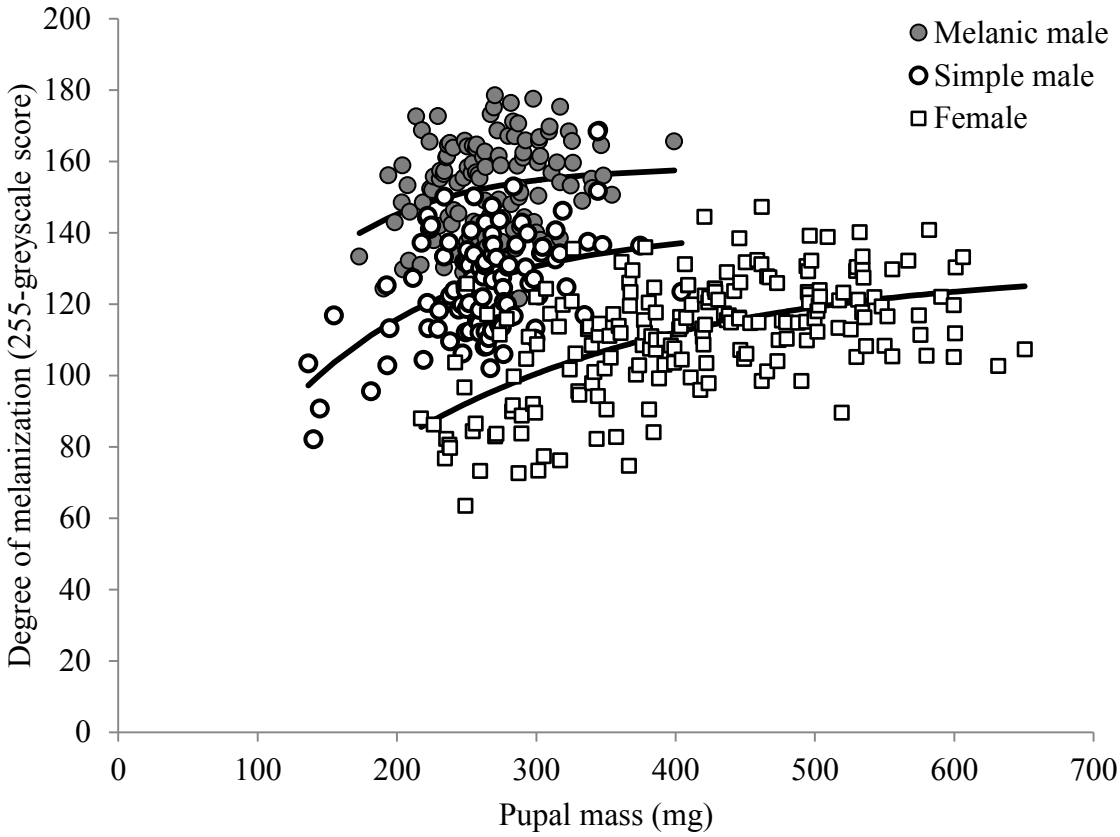

Figure 3.3 Degree of melanization (255 - greyscale score) vs. pupal mass (mg) for melanic male, simple male, and female *M. disstria* moths. Fit lines are the predicted y-values from the nonlinear regression analyses.

Table 3.2 Summary of nonlinear regression results for melanization vs. pupal mass in melanic male, simple male, and female *M. disstria* moths.

Adult type	*n*	Maximum (*a*)	95% C.I.	Slope (*b*)	95% C.I.	r^2
Melanic male	130	158.6	151.1 - 166.1	1.96	1.38 - 2.54	0.050
Simple male	102	141.7	129.8 - 153.5	1.20	0.94 - 1.47	0.236
Female	194	130.3	123.0 - 137.6	0.64	0.56 - 0.72	0.343

3.5 Discussion

Reducing nitrogen availability decreased melanization in all moths, regardless of sex or phenotype. Few studies have investigated the effects of dietary protein on melanization, and to our knowledge, this is the only study which clearly shows that expression of a melanin-based colour trait is directly dependent on broad manipulation of nitrogen availability. Stoehr (2010) investigated possible trade-offs between adult wing melanization, pupal melanization, and the melanization of capsules formed as an immune response in the small cabbage white butterfly (*Pieris rapae* L.) on high- and normal-protein diets. In male butterflies, both wing melanization and capsule melanization of *P. rapae* were increased on the high-protein diet; however, female *P. rapae* actually had reduced wing melanization on the high-protein diet, possibly due to a trade-off with capsule melanization. Lee et al. (2008) found that larval melanization of *S. littoralis* was reduced by a low-quality protein diet deficient in some essential amino acids; similarly, specific dietary deficiencies in tyrosine and phenylalanine (melanin precursors) have been shown to reduce bib melanization in juvenile male house sparrows (*Passer domesticus*) (Poston et al. 2005).

Reduced nitrogen availability also had negative effects on development time, pupal mass, and forewing area in all three adult types. Melanic and simple males did not show significant differences in these traits in either diet treatment. However, the magnitude of the diet effect did depend on adult type. Reducing nitrogen availability increased average development time, and decreased average melanization, pupal mass and forewing area to a greater extent in females than in males (both simple and melanic). Similarly, melanic males showed a slightly greater increase in average development time and a greater decrease in average melanization, pupal mass, and forewing area on the low-protein diet compared to simple males. This suggests that nitrogen limitation affected melanic males more negatively than simple males. Furthermore, although there was no significant difference in the ratios of melanic to simple males across diet treatments, fewer melanic males were obtained on the low-protein diet than on the high-protein diet. This trend was also present in females, but not in simple males, suggesting a difference in survival rates of simple and melanic males. Unfortunately, actual survivorship cannot be determined as it is impossible to identify colour phenotypes at any stage prior to emergence of the adult moth.

The expected saturating relationship between melanization and nitrogen intake is clearly shown in Figure 3.3. Overall, melanic males had the highest maximal level of melanization; simple males and (simple) females shared a similar lower maximum. The slope of the relationship differed for all three adult types, suggesting that females allocated the fewest resources to melanization and melanic males the most. These findings suggest a situation between the two alternate hypotheses outlined in the introduction: melanic males allocate more nitrogen resources to melanization than simple

males, but not so much so that they are able to maintain maximal melanization under dietary nitrogen limitation.

No melanic males were actually obtained in the predicted slope area. A possible explanation is differential mortality of excessively small pupae. A few extremely small simple males with low melanization scores can be seen in Figure 3.3; they may have survived the pupal stage by virtue of low resource allocation to melanin synthesis. Comparably small melanic males are not present; they may not have survived the pupal stage because of higher resource allocation to melanin synthesis. The situation is further complicated by the fact that *M. disstria* larvae must reach a threshold size at the beginning of a larval stadium before they can initiate pupation. The larvae will pass through extra instars until this threshold is reached, thereby extending development time but attaining a higher pupal mass and ingesting more nitrogen (Etilé and Despland 2008). For these reasons, it simply may not be possible to obtain small melanic males that would fall into such a steep slope region.

The results for females provide the strongest support for the predicted relationship between nitrogen intake and melanization, possibly because females have higher nitrogen requirements than males due to their distinctly larger body sizes and the need to produce a maximum number of eggs. From Figure 3.3, it can be seen that only the biggest females were capable of attaining maximal melanization; below this maximum, melanization increased with pupal mass. These results provide strong support for the condition dependence of melanization, as pupal mass is a highly reliable measure of female quality. Female *M. disstria* moths emerge from the cocoon with their lifetime complement of eggs already formed and cannot produce more once these have been laid. Due to this

capital breeding strategy, female fecundity is strongly predicted by pupal mass (Fitzgerald 1995; Parry et al. 2001). Colasurdo et al. (2009) showed that although the relationship between body size and fecundity is unaffected by diet treatment as used in this experiment, nitrogen-limited females change their allocation patterns such that the total mass of the reproductive tissues (eggs) is decreased to a greater extent than somatic (non-reproductive) tissues. This difference is a result of decreased allocation to individual egg size, rather than number of eggs; however, the end result is that a low-protein diet reduces female fitness in two ways. The reduction in pupal mass means absolute fecundity will be lower, and the reduction in the size of individual eggs will have a negative effect on the fitness of progeny (Parry et al. 2001). We suggest that the observed change in relative resource allocation to somatic and reproductive tissues on a low-protein diet is accompanied by a reduction in allocation to melanin synthesis. These changes in resource allocation patterns are indicative of trade-offs that serve to maximize female fitness under nitrogen-limited conditions.

It is unknown what function melanism has in *M. disstria* moths; however, insect melanism can have numerous functions, including, but not limited to, thermoregulation, UV resistance, crypsis (camouflage), and sexual selection (True 2003). Although the vast majority of studies investigating the condition dependence of colour traits focus on those involved in mate choice, condition dependence of colour traits should be expected under any strong selection pressure that opposes the costs of colour production. For example, colouration in the predatory ambush bug (*Phymata americana* Melin) has been shown to be condition-dependent (Punzalan et al. 2008a), and darker males tend to have higher mating success as a result of increased thermoregulatory capabilities (Punzalan et al.

2008b). Colour polymorphisms in animals can also be correlated to other important traits, and therefore be under indirect selection (McKinnon and Pierotti 2010). Such is the case in the side blotched lizard, *Uta stansburiana*, where a colour polymorphism for throat colour is associated with differences in life history traits and behavioural patterns in both male and female lizards (Sinervo et al. 2001). Further investigation is needed to determine exactly what selection pressures, both direct and indirect, may be acting on melanism in male and female *M. disstria* moths.

In conclusion, this study shows that expression of a melanin-based colour trait is dependent on dietary nitrogen availability. These results support the hypothesis that melanin synthesis is costly in nitrogen-limited animals. The negative effects of protein limitation were more pronounced in melanic males compared to simple males, and distinctly more pronounced in females. Based on Stoehr's (2006) model, resource allocation patterns differed between melanic males, simple males, and females, where allocation to melanin synthesis was highest in melanic males and lowest in females. These allocation patterns provide strong evidence for the condition dependence of melanism in this species, particularly in females, where only high-quality individuals were capable of maximal melanin expression. This confirms that patterns of condition dependence should not be over-generalized; differences in the food sources and resource requirements of different types of organisms must be considered. Condition dependence of melanin-based colour traits is most likely to occur in animals that tend to be constrained by dietary nitrogen.

Chapter 4 - Potential direct and indirect selection pressures acting on the melanic polymorphism in *Malacosoma disstria* moths

4.1 Abstract

Both direct and indirect selection pressures may be acting on the melanic polymorphism in *Malacosoma disstria* moths. This study investigates the potential roles of male colour phenotype in female mate choice decisions, thermoregulation, crypsis, and mimicry. Indirect selection through correlated traits or secondary effects of melanism is also investigated for both males and females. Mate choice experiments show no evidence of female preference for melanic or simple males. Thermoregulation experiments suggest that melanic males attain higher thoracic temperatures under a radiant light source. Colouration and patterns of both phenotypes are likely to be involved in disruptive cryptic colouration rather than mimicry or aposematism. Melanization was not correlated with pupal mass, development time, pupal mass lost, wing loading, or reproductive traits in females, nor was there any effect of melanic siblings on these female life history traits. Male colour phenotype had no significant effect on life history traits in males. Melanization was correlated with pupal mass lost in simple males, and with wing loading and lipid content in melanic males, but melanization, pupal mass lost, wing loading, and lipid content are all strongly correlated with pupal mass in both types of males. There is no evidence for indirect selection on melanism in male or female *M. disstria* moths. Potential fitness advantages of the melanic phenotype in natural conditions are also discussed.

4.2 Introduction

In *Malacosoma disstria* moths, melanin synthesis may have an energetic or resource cost, resulting in melanic males having a smaller size or being more negatively affected by poor nutrition in the larval stage (Chapters 2 and 3). However, for the apparently widespread melanic phenotype to be maintained in *M. disstria* populations, it must confer some benefit to melanic individuals. Many selection pressures, both direct and indirect, may be acting on colour in *M. disstria* moths to maintain a balanced polymorphism. Selection pressures that may act directly on the melanic polymorphism in male *M. disstria* moths include mate choice (by females), thermoregulation, and predator avoidance through crypsis or mimicry.

Aposematism is highly unlikely to be a function of colour in the *M. disstria* moth. Although *M. disstria* larvae are conspicuously coloured, they also show defensive behavioural responses to invertebrate predators (McClure and Despland 2011) and have long hairs covering their bodies that appear to be an effective physical deterrent to predation by birds (Fitzgerald 1995). The adult moths have no such defenses; they are often fed upon by birds (Fitzgerald 1995), and the inconspicuous brown colouration of both phenotypes and the contrasting bands present on the forewings (Fig. 4.1 a – c) is a typical example of disruptive cryptic colouration. Contrasting patterning elements that break up the otherwise solid shape and outline of stationary prey make it more difficult for visual predators to spot them (Merilaita and Lind 2005).

Increased melanism in insects may be related to other factors such as immune function, protection against UV radiation, and abrasion resistance (True 2003); however, the short

lifespan of the non-feeding *M. disstria* moth makes it unlikely that these factors will have an appreciable impact on the relative fitness of the two melanic phenotypes. Aside from the effects of melanic phenotype on male pupal mass and survival (Chapters 2 and 3), indirect selection may also be acting on other life history traits through pleiotropy or resource allocation trade-offs.

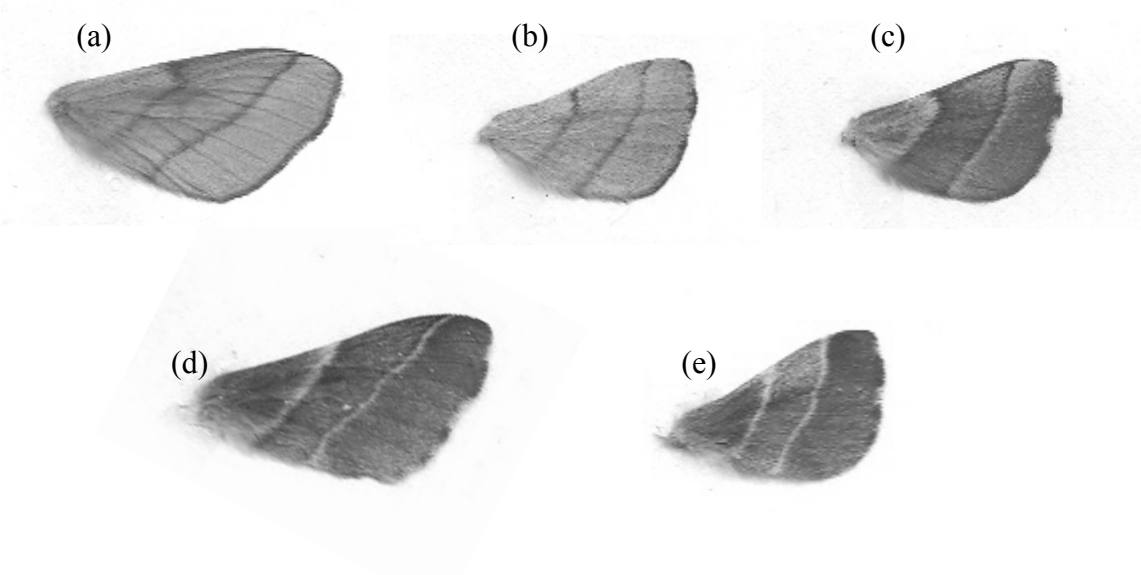

Figure 4.1 Example forewings from (a) female *M. disstria*, (b) simple male *M. disstria*, (c) melanic male *M. disstria*, (d) female *M. americanum*, and (e) male *M. americanum*.

4.2.1 Mate choice

Female Lepidoptera can use cues such as male size, wing damage, colour and pattern, or chemical secretions to make the decision to accept or reject a male's mating attempt (reviewed in Rutowski 1982). Excessive wing damage could indicate an older male who may have already mated and therefore could have sperm that is low in quality or quantity. Chemical secretions and body size could indicate 'good' genes that would be

advantageous for the female's offspring. The males of many moth species transfer nutrient-rich spermatophores to the females during mating, which she uses for nourishment and enhancement of egg production. In these cases, females tend to prefer larger males because they have larger spermatophores. In some Lepidopteran species, females are known to base their choice on male colour patterns. *Pieris occidentalis* (western white butterfly; also known as *Pontia occidentalis*) females preferentially mate with males that have greater melanization in certain areas of the forewing; they may be using melanization to identify conspecific males or to indicate male quality (Wiernasz 1989). Female *Panaxia dominula* (scarlet tiger moths) tend to prefer males with a different colour phenotype from their own, although this may actually be based on chemical cues (Sheppard 1952; Sheppard and Cook 1962).

Mating in *M. disstria* has not been extensively studied, but Bieman and Witter (1983) reported instances of mate rejections by *M. disstria* females in their study on mating behaviour and mate competition. These mate rejections only occurred in high density populations with a sex ratio strongly skewed towards males, and it is unknown on what trait or cue the females based their choice. In *M. disstria*, males either do not transfer spermatophores, or they are so small as to be inconspicuous to human observers (Evenden, pers. comm.). Female *M. disstria* are therefore not likely to choose males based on body size, unless size is used to signal foraging success and 'good' genes. Although *M. disstria* moths tend to communicate information primarily using chemical signals, as is typical for most moths, male *M. disstria* will use visual cues at close range and approach empty cocoons or small brown objects in their search for receptive females (Fitzgerald 1995). In Chapter 3, male melanization was found to be affected by nitrogen

limitation. When population density is high and nitrogen is extremely limiting, melanization could therefore be an indicator of male quality. Furthermore, it is only at high population densities that females are likely to be approached by multiple males in their short lifespan. It is possible, then, that females could use visual cues at close range to assess male darkness as an indicator of quality. In this case, melanic males should be preferred as they are almost always darker than simple males. Experiment 1 will investigate whether female moths show any preference for melanic or simple males.

4.2.2 Thermoregulation

Thermoregulation and heat absorption are generally considered to be important functions of melanism in insects (True 2003). According to thermal melanism theory, under identical conditions, darker ectothermic organisms will warm up faster and reach higher equilibrium temperatures than paler organisms of the same size. Numerous studies have shown geographical clines where insect melanism (both within and between species) tends to increase with increasing latitude or altitude, and others have shown that melanic individuals enjoy fitness benefits (e.g. increased growth rate, activity levels, reproductive success, and survival) at colder temperatures (reviewed in Trullas et al. 2007). Flight activity of adult insects tends to be limited at temperatures between 15-20^{o}C, and the lower threshold below which flight is completely deterred occurs between 10-15^{o}C for various species, including *M. disstria* (e.g. Taylor 1963; Fitzgerald 1995). Although *M. disstria* moths emerge mainly in July in the northern range of the species' distribution (Schmidt and Roland 2003), normal temperatures in many regions can be low enough to limit adult activity (see Table 4.1 for examples).

Malacosoma disstria males tend to fly in search of mates in the afternoon and early evening, but not overnight, and females will recommence secreting sex pheromones in the morning if they fail to attract a mate the previous evening. As with other *Malacosoma* moths, *M. disstria* moths warm up the thoracic muscles before flight by 'shivering' until a threshold muscle temperature is reached and flight can be initiated (Fitzgerald 1995). Casey and Hegel-Little (1987) showed that at low ambient temperatures, pre-flight warm-up is time-consuming and energetically costly for *Malacosoma americanum* (eastern tent caterpillar) moths. If melanic *M. disstria* males are able to warm up more rapidly than simple males on colder days or in the morning because of increased heat absorption, they can begin flying in search of females more quickly, thereby increasing their chances to find mates first. Experiment 2 will test for phenotypic differences in temperature gain under a radiant light source.

Table 4.1 July temperatures (Canadian Climate Normals: 1971-2000) recorded at weather stations near or at locations with known occurrences of *M. disstria*. Temperature data obtained from Environment Canada: National Climate Data and Information Archive.

Location		**July temperatures (^{o}C)**	
Province	Name in archive	Daily average	Daily minimum
QC	Petit Sageunay[1]	17.7	11.9
QC	La Tuque[1]	18.9	12.3
AB	Edmonton Stony Plain[2,3]	16.5	11.2
BC	Prince George A[3]	15.5	8.8

[1] Cooke & Lorenzetti (2006)
[2] Parry et al. (2001)
[3] Schmidt & Roland (2003)

4.2.3 Crypsis and mimicry

Moths generally spend the day sitting motionless on a substrate and often only fly in the evening or at night. There is evidence in some moth species that moths of different phenotypes prefer to rest on substrates on which they are most cryptic (Kettlewell 1973; Majerus 1998); however, no such studies have been conducted on *M. disstria* moths. Bieman and Witter (1983) reported that female *M. disstria* remained on or near their cocoon after emerging, but do not mention how well-concealed these moths were with respect to their resting substrate. Interestingly, although moths tend to be nocturnal, *M. disstria* males were observed by Bieman and Witter (1983) to begin flying in search of females in the afternoon, and to cease flying after dark (possibly due to low overnight temperatures (Fitzgerald 1995)). In low light conditions, such as twilight, darker insects are less visible during flight than are paler insects, a phenomenon known as aerial crypsis (Kettlewell 1961; Majerus 1998). Melanic males may therefore be less conspicuous to predators than simple males when flying in search of mates before dark.

Malacosoma americanum shares the eastern half of *M. disstria*'s distribution (Fitzgerald 1995). The melanic phenotype of *M. disstria* resembles both male and female *M. americanum*, as both sexes of this species have dark brown colouration, with pale distal and proximal bars (see Stehr and Cook 1968; Fig 4.1). It is possible that the melanic phenotype in *M. disstria* is a mimic of *M. americanum*. Experiment 3 will compare colour pattern and melanization of *M. americanum* and *M. disstria* moths.

4.2.4 Correlated traits

Melanism is known to be correlated with other life history traits in numerous insect species, and the melanin synthesis pathway can have important pleiotropic effects in insects (reviewed in True 2003). Melanism may also be expected to be show trade-offs with other life history traits, due to the shared nitrogenous resource pool during metamorphosis. Nitrogen is an essential component of both melanin pigments (Sugumaran 2002) and the adult body and reproductive structures in insects (Colasurdo et al. 2009). Finally, although females do not express the melanic phenotype, Chapter 2 showed that female carriers of the melanic allele show increased melanization compared to non-carriers. The melanic allele could have other indirect effects on female carriers through resource trade-offs or pleiotropy; even though females do not express the melanic phenotype, they may still express correlated traits (Olsson et al. 2012). Finally, linkage disequilibrium could result in correlated traits in both males and females. Experiment 4 will investigate potential secondary effects of the melanic phenotype in males, of the melanic allele in females, and of melanization in both sexes.

4.3 Materials and Methods

Malacosoma disstria egg masses were collected from an outbreaking population north of Kingston, Ontario, Canada (44°33.5N, 76°24.1W) in spring 2009 for use in Experiments 1 and 2, and from an outbreaking population north of Montreal, Québec, Canada (45^{o}55’22.61N, 73^{o}39’53.78W) in spring 2010 for use in Experiment 4. Rearing conditions were the same for both years. Egg masses were stored at 4^{o}C and 80%R.H. until hatching of the larvae. Larvae were reared under a 16hr light: 8hr dark photoperiod

at 21°C and 70% R.H., and given *ad libitum* access to standard prepared diet (20p:22c) for the duration of larval development (Addy 1969). Pupae were collected from rearing containers every 3 days. Within 48hrs of pupation, pupae were removed from their cocoons to be weighed, sexed, and placed in separate containers. Male and female pupae were stored in separate rooms to avoid exposing males to female pheromones.

Experiment 1: mate choice

Mate choice experiments (n = 56) were conducted in the afternoon and early evening. A recently eclosed female moth was placed in a mesh flight cage (39.5cm x 39.5cm x 17.0cm) filled with small-diameter trembling aspen branches at room temperature (22-23°C) and left undisturbed for 30 min to acclimatize to her surroundings. A pair of males, one melanic and one simple, were then added to the cage. To control for possible female choice based on male size or age, only males of approximately the same size (by pupal mass), who had both eclosed within the last 24 hours, were paired. The lights were on for the first 2 hours, and then off for 4 more hours (red lights were used during this time for observations) for a total experimental duration of 6 hours.

At 15 minute intervals, the location and any observed activity of all three moths within each cage was recorded. When a mating pair was observed, the male's phenotype was recorded. When a mating bout ended, any flying moths were allowed to settle, and both males were then removed from the cage to allow the female to oviposit without interference (females are no longer receptive after termination of a mating bout and would normally fly away to oviposit (Fitzgerald 1995)). Chi square tests were performed

in Excel (Microsoft Corporation 2010) to determine whether one type of male had more successful matings than is expected by chance (i.e. 50%).

Experiment 2: thermoregulation

Male moths used in thermoregulation experiments were frozen for 90 min and then allowed to thaw for at least 30 min to return body temperature to ambient. The following procedure for testing thermoregulatory capabilities is based on that of Pivnick and McNeil (1986). A thermocouple probe from a dual-channel thermometer (accuracy= ± 0.3% + 1°C) was inserted into the moth's thorax, keeping the insect 3cm above a square of white Styrofoam. The second probe was also secured to the Styrofoam just beside the moth to record ambient (air) temperature. A 500W photoflood lamp was placed 65cm above the moth as a radiant heat source. The lamp was left off and the setup covered with a brown paper towel while both probes (thoracic and ambient) equalized to room temperature (22-23^{o}C). Once equalized, the initial temperature (temperature at t = 0) was recorded to the nearest 0.1^{o}C, the paper towel removed, and the lamp turned on.

Thoracic and ambient temperatures were recorded at 30 sec intervals for the first 10 min (except for one moth, where temperatures were recorded at 1 min intervals), and at 1 min intervals for the next 10 min; the lamp was turned off at t = 20 min. Temperatures increased rapidly for the first 5 min, and were mostly stable after t = 10 min. Maximum thoracic and ambient temperatures were calculated as the average of the temperatures recorded from time t = 10 to t = 20 because of slight fluctuations in otherwise stable recorded temperatures (≤ 1.4^{o}C in thoracic temperatures; ≤ 1.9^{o}C in ambient temperatures). Temperature gain was calculated as the difference between the maximum

and the initial (t = 0) thoracic temperatures; temperature excess was calculated as the difference between maximum thoracic and ambient temperatures. Due to equipment problems, only 6 replicates were obtained (3 melanic males and 3 simple males); sample sizes were too small to perform statistical analyses.

Experiment 3: mimicry

Malacosoma americanum tents were collected near the end of larval development from a colony in Laval, Quebec, Canada (45°31'58.65"N, 73°49'43.10"W) in summer 2009 and allowed to pupate in the lab; pupae were weighed and sexed as above. Adult *M. americanum* (male n = 37; female n = 10) were frozen upon emergence from the pupal casing and stored at -16^{o}C until the wings could be removed and colour scored using the methods described in Chapter 2. An ANOVA followed by Tukey post hoc tests was used to compare wing melanization of the 5 adult types (female *M. disstria*, simple male *M. disstria*, melanic male *M. disstria*, female *M. americanum*, and male *M. americanum*; *M. disstria* data were obtained from Chapter 2). Residuals were checked for normality prior to analysis. Statistical analysis was performed in JMP v.10.0.0 (SAS Institute Inc. 2012).

Experiment 4: correlated traits

Melanic phenotype (in males) and the melanic allele (in females) could be correlated with other important life history traits. The life history traits investigated here are pupal mass, mass lost during metamorphosis, development time, and wing loading (for both males and females); lipid content (for males only); and fecundity and resource allocation to reproductive and somatic tissues (for females only).

Mass is lost over the course of the pupal stage, which lasts approximately 14 days, due to pupal metabolism. To measure mass lost during metamorphosis, pupae were first weighed within 48hrs of pupation, and then again 11-12 days later (i.e. within 48hrs before eclosion). The difference in mass was calculated as the amount of pupal mass lost (mg) to determine whether melanic males use up more resources during metamorphosis. Development time was recorded as the number of days from hatching of the egg mass to emergence of the moth. One forewing was removed from each moth and used to measure forewing area (mm^2), melanization (255 - greyscale score), and to determine male phenotype (Chapter 2). Females with melanic male siblings were designated as (potential) carriers of the melanic allele; females with only simple male siblings were non-carriers.

Wing loading (mg/mm^2) was calculated for both sexes using adult dry mass and forewing area (e.g. Berwaerts et al. 2002). Fresh (wet) adult mass could not be used; after emergence from the pupal casing, moths expand their wings and expel their meconium (metabolic wastes from metamorphosis). Moths were frozen as soon as possible following eclosion in order to preserve the wings from damage caused by flying and to minimize the loss of lipid reserves to flight. However, not all moths fully expel their meconium immediately upon emerging; thus, fresh moth weight is an unreliable measure of size (Iyengar and Eisner 1999).

To measure male lipid content, male moths were dried at 45^oC for a minimum of 48hrs after removing all legs and wings. Initial dry mass was recorded as adult dry mass; after three 24hr chloroform washes, moths were re-dried as before and final dry mass was recorded. Lipid content in mg was calculated as the difference between initial and final

dry mass (Lee et al. 2002). To measure female fecundity and resource allocation to somatic and reproductive tissues, female moths were dissected and the total number of eggs recorded (females emerge with their lifetime supply of eggs already mature (Fitzgerald 1995)). The soma, ovaries (eggs), and accessory glands were dried at 45^{o}C for a minimum of 48hrs; the body parts were first weighed separately, then together to obtain total adult mass.

The effects of male colour phenotype on pupal mass, melanization, and development time were tested using a random block ANOVA with Family as the random factor. Adult mass was added to the model as a covariate to test lipid content and wing loading; pupal mass was used a covariate to test pupal mass lost. Of the 23 families obtained in this experiment, 9 had only one type of male and were not included in this part of the analysis. The effects of melanic male siblings on female pupal mass, melanization, and development time were tested using a nested ANOVA, where Family is a random factor nested within MelanicSiblings (presence vs. absence; all families included). Pupal mass was added to the model as a covariate to test pupal mass lost; adult mass was used as a covariate to test wing loading, fecundity, soma mass, accessory glands mass, and total mass of the ovaries. Pearson's correlations between melanization and all other life history traits (as listed above) were performed for each adult type. Individuals from all families were included in the analysis.

All residuals were checked for normality prior to analyses; male melanization was square-transformed and male lipid content was square root transformed. ANOVAs were performed using JMP v.10.0.0 (SAS Institute Inc. 2012); correlations were performed in SPSS v.20.0.0 (IBM Corporation 2011).

4.4 Results

4.4.1 Mate choice

Of 56 trials in the mate choice experiments, a total of 35 mating bouts were observed. The 21 null trials were not due to any (observed) mate rejections by females; rather, moths remained mostly stationary and no mating attempts were observed over the 6hr duration of the experiment. Of the 35 successful trials, 15 melanic males and 20 simple males succeeded in mating. These results show no significant effect of wing colour phenotype on mating success (d.f. = 1; χ^2 = 0.714; p = 0.398), nor was there any significant difference in mating success between males that were slightly larger as pupae (n = 16) and those that were slightly smaller (n = 19) than their counterpart (d.f. = 1; χ^2 = 0.257; p = 0.612).

4.4.2 Thermoregulation

The results of the thermoregulation experiment show that melanic males warm up more rapidly than simple males and reach higher thoracic temperatures; temperature gain tends to increase with pupal mass within phenotypes (Table 4.2; Fig 4.2).

4.4.3 Mimicry

Figure 4.1 shows that melanic male *M. disstria* forewings are similar in both colour and pattern to those of male and female *M. americanum*. Adult type had a significant effect on wing melanization ($F_{4,1227}$ = 497.7419, $p < 0.0001$). Post hoc tests show that all adult types differ significantly from all other adult types, except for melanic male *M. disstria* and male *M. americanum* (Fig 4.3).

Table 4.2 Maximum thoracic temperature, temperature gain (above initial), and temperature excess (above ambient) of dead melanic and simple male *M. disstria* moths after 20 min under a 500W photoflood lamp.

Phenotype	Pupal mass (mg)	Maximum thoracic temperature (°C)	Temperature gain (°C)	Temperature excess (°C)
Melanic	188.8	30.9	8.2	6.1
	238.5	31.5	9.2	5.7
	303.3	32.2	9.5	6.5
Average	243.5	31.5	9.0	6.1
Simple	219.7	29.5	7.0	2.7
	230.6	30.4	7.5	5.5
	250.7	30.8	8.3	5.8
Average	233.7	30.2	7.6	4.7

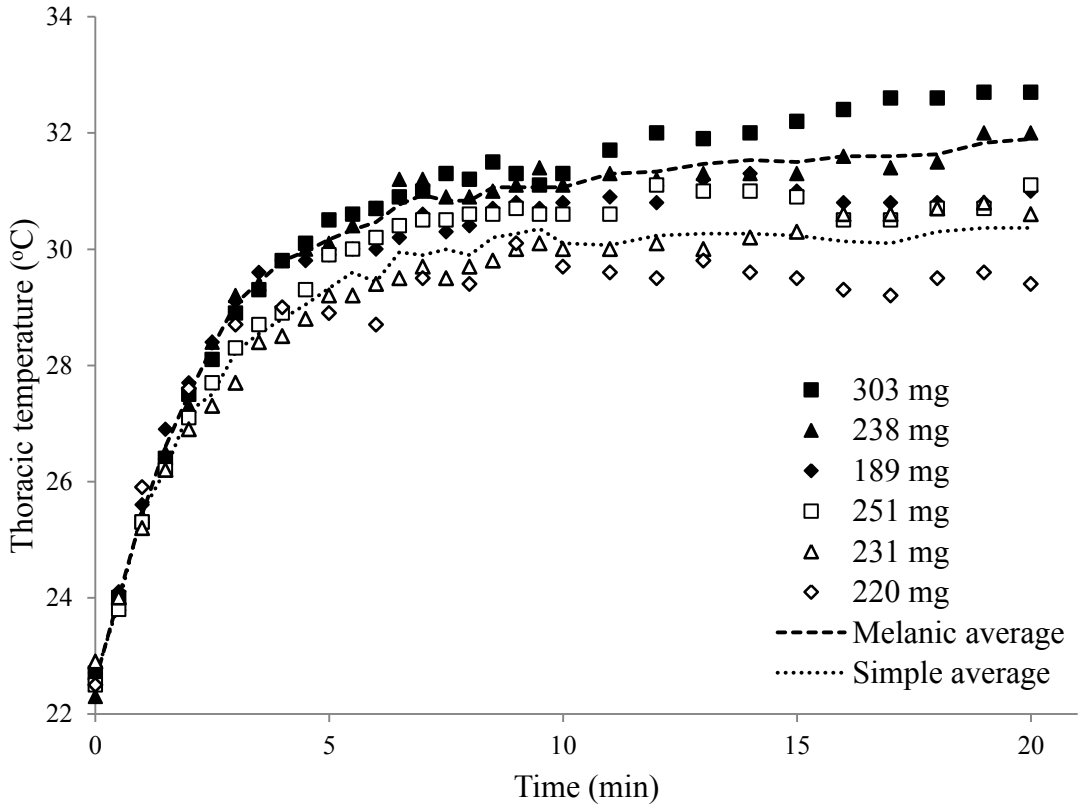

Figure 4.2 Changes in thoracic temperature over time in melanic and simple male *M. disstria* moths under a 500W photoflood lamp. Filled shapes represent melanic males; open shapes represent simple males. Dashed lines show average values for each phenotype.

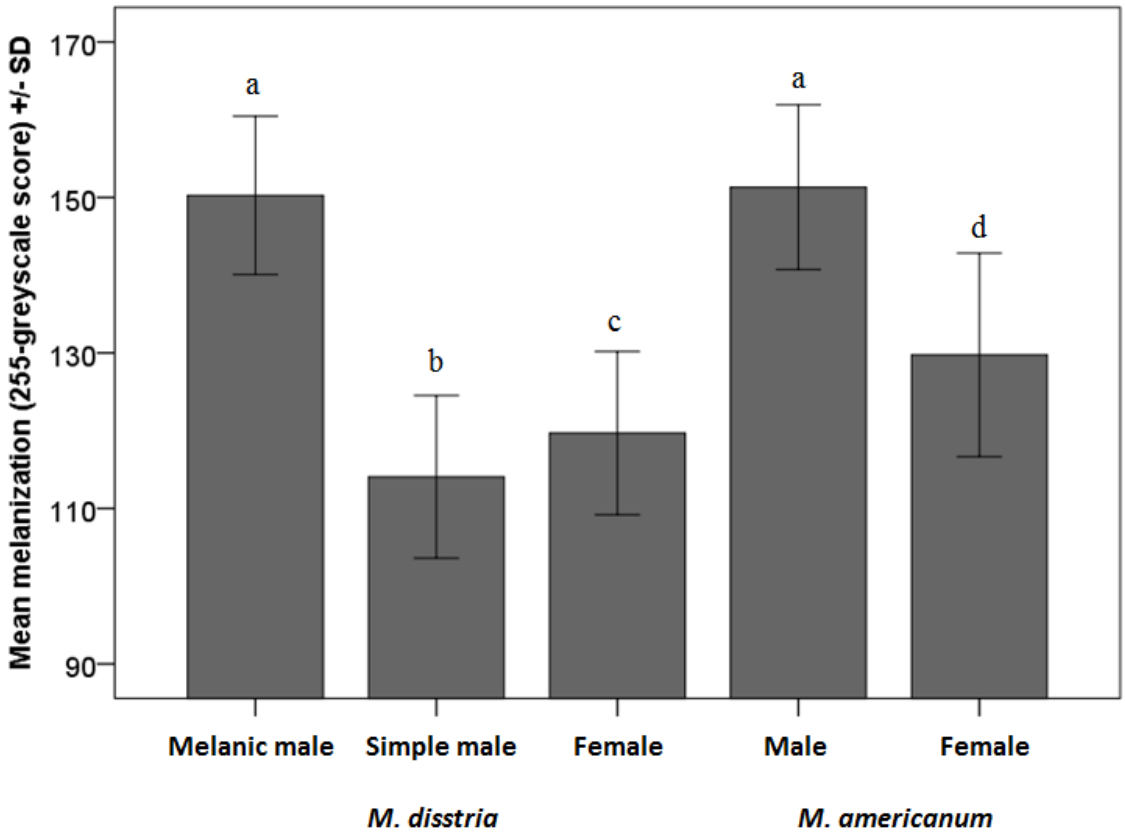

Figure 4.3 Mean melanization (255 - greyscale score) ± S.D. of melanic male *M. disstria* (150.3±10.2; n = 201), simple male *M. disstria* (114.1±10.4; n = 410), female *M. disstria* (119.7±10.5; n = 574), male *M. americanum* (151.3±10.6; n = 37), and female *M. americanum* (129.8±13.1; n = 10) moths. Letters denote significant differences.

4.4.4 Correlated traits

Melanization of melanic males (n = 109) was significantly greater than melanization of simple males (n = 106) ($F_{1,11.62}$ = 89.2149; p < 0.0001). Male pupal mass, development time, lipid content, wing loading, and pupal mass lost were not significantly affected by phenotype (see Table 4.3 for detailed results). Females with melanic siblings (n = 189) had significantly smaller accessory glands than females without melanic siblings (n = 91)

($F_{1,20.72}$ = 4.7315, p = 0.0413). Female pupal mass, melanization, development time, pupal mass lost, wing loading, fecundity, soma mass, and mass of the ovaries were not significantly affected by melanic siblings (see Table 4.4 for detailed results).

Significant correlations between melanization and other life history traits depended on adult type. In females, melanization was not significantly correlated with pupal mass, development time, pupal mass lost, wing loading, fecundity, soma mass, accessory glands mass, or total mass of the ovaries. In simple males, melanization was significantly correlated with pupal mass (r = 0.147, p = 0.022, n = 242) and pupal mass lost (r = 0.198, p = 0.007, n = 188), but not with development time, wing loading, or lipid content. In melanic males, melanization was significantly correlated with pupal mass (r = 0.264, p = 0.003, n = 121), wing loading (r = 0.279, p = 0.003, n = 110), and lipid content (r = 0.245, p = 0.007, n = 121), but not with pupal mass lost or development time.

4.5 Discussion

This study provides no evidence for female mate choice based on male colour phenotype. Indeed, it was observed that the male who succeeded in mating was almost always the first male to begin flying and thus the first to encounter the female. Observed mating behaviours were overall very similar to those reported by Bieman and Witter (1983) and to the mating behaviour of moths in general (Rutowski 1982). Females appeared to have a passive role in mating and showed no observable reaction to the presence of a male. In a single case, a female was observed to avoid a simple male's mating attempt by bending her abdomen away from his, and then by walking away when he persisted in his attempts.

However, moments after the simple male departed, the female expelled her meconium (metabolic wastes from the pupal stage) and was subsequently receptive to a mating attempt by the melanic male 10 minutes later. It is unsurprising that female *M. disstria* do not exhibit strong mate choice behaviour. Although population density reaches high levels during outbreaks, densities are quite low during the intervals between outbreaks. In these conditions, females cannot necessarily count on being approached by more than one potential mate in their short lifespan.

Table 4.3 Results for ANOVAs testing the effects of phenotype on life history traits in male *M. disstria* moths.

Trait	**ANOVA results**		**Melanic males**	**Simple males**
	$F_{(d.f.)}$	*p*	Mean ± S.D. $_{(n)}$	Mean ± S.D. $_{(n)}$
Melanization (255 - greyscale score)	89.2149 (1,11.62)	**< 0.0001**	147.0 ± 9.2 (109)	120.1 ± 13.1 (106)
Pupal mass (mg)	0.0272 (1,7.975)	0.8732	248.0 ± 39.7 (109)	262.8 ± 46.0 (105)
Development time (days)	0.6087 (1,9.983)	0.4534	58.8 ± 6.0 (109)	59.5 ± 5.6 (106)
*Lipid content (mg)	0.0153 (1,200)	0.9016	19.0 ± 4.5 (109)	20.1 ± 4.9 (106)
*Wing load (mg/mm^2)	0.1072 (1,5.514)	0.7554	0.54 ± 0.07 (99)	0.57 ± 0.08 (98)
*Pupal mass lost (mg)	2.5483 (1,9.021)	0.1448	36.9 ± 8.0 (93)	37.5 ± 8.4 (66)

*ANCOVA

Table 4.4 Results for ANOVAs testing the effects of melanic siblings on life history traits in female *M. disstria* moths.

Trait	ANOVA results		Melanic siblings	
			Absent	**Present**
	F (d.f.)	*p*	Mean ± S.D (n)	Mean ± S.D (n)
Melanization (255 - greyscale score)	0.7815 (1,20.55)	0.3869	110.6 ± 12.6 (93)	115.1 ± 12.0 (191)
Pupal mass (mg)	0.4873 (1,20.03)	0.4932	412.1 ± 71.4 (93)	419.5 ± 68.3 (191)
Development time (days)	0.7451 (1,19.52)	0.3985	65.3 ± 7.5 (93)	61.4 ± 6.8 (190)
*Wing load (mg/mm^2)	1.8956 (1,15.38)	0.1883	0.66 ± 0.08 (75)	0.66 ± 0.08 (152)
*Pupal mass lost (mg)	0.4648 (1,16.83)	0.5046	48.8 ± 12.3 (62)	51.4 ±11.1 (122)
*Fecundity (# eggs)	0.3969 (1,19.07)	0.5361	161.5 ± 38.7 (91)	165.5 ± 36.9 (189)
*Soma mass (mg)	0.0071 (1,18.9)	0.9337	21.6 ± 4.4 (91)	21.8 ± 4.5 (189)
*Acessory glands mass (mg)	4.7315 (1,20.72)	**0.0413**	12.7 ± 2.5 (91)	12.5 ± 2.5 (189)
*Ovaries mass (mg)	0.0814 (1,18.41)	0.7786	31.4 ± 9.2 (91)	34.0 ± 8.6 (189)

*ANCOVA

The results of the thermoregulation experiment suggest that melanic males tend to warm up more rapidly and attain higher body temperatures than simple males, which may confer an advantage to melanic males. Punzalan et al. (2008b) found that darker male *Phymata americana* (ambush bugs) had greater mating success than paler males at low ambient temperatures, despite the fact that there was no evidence of mate selection by

females. The authors suggested that the higher thermoregulatory capacity of darker males gives them greater flight capabilities at cooler temperatures, thus allowing them to find females more quickly than paler males do. A similar mechanism may act in favour of melanic phenotypes in the two-spot ladybird (*Adalia bipunctata*) (Brakefield 1984b). Melanic *M. disstria* males may therefore be more likely to find mating opportunities at cooler temperatures or early in the morning, as they can warm up more readily than simple males. However, melanic males may also have a greater risk of overheating on hot summer afternoons. Many other factors also affect ectothermic heat absorption from solar radiation, including light wavelength, wind, and the size, shape, position, and physiology of the organism; it should therefore not be assumed that thermoregulatory differences based on melanization are necessarily enough to affect individual fitness in natural situations (Trullas et al. 2007; Umbers et al. 2012). Investigating phenotypic differences in the body temperature, time to flight initiation, and flight duration of live insects, as well as resting site choice and behaviour at different temperatures and levels of solar radiation would further our understanding of thermoregulatory differences and their potential effects on melanic and simple males.

Although melanic *M. disstria* males have similar wing colouration and patterns as both male and female *M. americanum*, sex-limited mimicry in non-toxic, cryptic prey species involves a distasteful model species and a shared conspicuous (aposematic) colour phenotype (Joron and Mallet 1998). Neither *M. disstria* nor *M. americanum* moths appear to be at all distasteful to predators (Fitzgerald 1995), and the shared colouration cannot be described as conspicuous. It is more likely that dark wings with pale bars is a disruptive cryptic colour pattern, and indeed, the presence of two parallel bars that contrast to some

extent with the background wing darkness is a pattern common to all *Malacosoma* moths (see Stehr and Cook 1968). However, visual predators such as birds may form search images of their most commonly encountered prey items, which results in apostatic selection on colour polymorphic prey (Bond 2007). In *M. disstria*, the simple phenotype should be the most common by far; not only is the melanic phenotype not expressed in females, but it is also usually present at relatively low frequencies in males (see Chapter 2). Melanic males may therefore enjoy disproportionately low rates of predation by avian predators that have formed search images for the simple phenotype during outbreaks.

Male colour phenotype had no significant effect on any of the life history traits measured here, although pupal mass was positively correlated with melanization in both types of males. The amount of pupal mass lost during metamorphosis was positively correlated with melanization in simple males, but not in melanic males. Instead, both wing loading and lipid content of melanic males were positively correlated with melanization. However, the importance of these correlations is not clear due to the fact that pupal mass lost, wing loading and lipid content are all strongly positively correlated with pupal mass ($p < 0.001$) in both melanic and simple males. Thus, correlations between melanization and other life history traits may be an indirect result of shared correlations with pupal mass. The end result is that larger pupae give rise to more heavily melanized male moths. Larger pupae also tend to lose more pupal mass, and to give rise to adults with more lipids and higher wing loading. The overall effect on adult flight capabilities is unknown, given than increases in lipid reserves have a positive effect on flight abilities (Gunn and Gatehouse 1993), while increased wing loading has a negative effect on flight abilities (Angelo and Slansky 1984). Although female carriers (melanic siblings present) had

significantly smaller accessory glands by mass than non-carriers (melanic siblings absent), the biological significance of this result must be questioned. Not only was this difference statistically weak, but the absolute difference between average accessory glands mass of female carriers and non-carriers was 0.2mg. It is unlikely that such a small difference could impact individual fitness to any great extent. The absence of any significant correlations between melanization and other life history traits of female *M. disstria* also does not support the presence of any trade-offs that could account for a difference in resource allocation to accessory glands.

Conclusions

It is probable that numerous selection pressures are acting simultaneously to maintain the melanic polymorphism in *M. disstria* moths. The melanic phenotype may be under positive selection for thermoregulatory abilities, and because of aerial crypsis and apostatic selection by visual predators. Furthermore, the fact that the true melanic phenotype is sex-limited to males may in itself aid in maintaining the polymorphism. The lack of indirect effects of the melanic allele on female life history traits and resource allocation suggests that the allele is effectively hidden from selection in female carriers. It is also possible that a heterozygote advantage exists and the heterozygote melanics have unknown fitness benefits not present in either homozygote; however, there is no obvious way to visually differentiate homozygote and heterozygote melanics. Further study of the behaviour, mating success, and survival of male moths under natural conditions would shed more light on the potential phenotypic differences in male fitness that could be contributing to the maintenance of the melanic polymorphism in this species.

Chapter 5 - General Discussion

The objective of this study was to characterize polymorphic melanism in the *Malacosoma disstria* moth, and to investigate how selection may be acting on and maintaining the polymorphism in this species. In Chapter 2, we confirmed that a melanic polymorphism is present in the male moth, with the melanic phenotype being measurably darker than the simple phenotype. Phenotypes also differ slightly in wing pattern: the paler simple phenotype has two dark bars across the forewing, while two additional pale bars are visible in the darker melanic phenotype. These patterns (i.e. brown colouration broken up by bars of contrasting darkness) are typical of disruptive colouration in cryptic prey species (Chapter 4). The phenotypic ratios within and between families provide support for the hypothesis that the polymorphism is based on a single gene locus with two alleles, the melanic allele being dominant (Lorimer 1979). We also found evidence in Chapter 2 that the melanic phenotype is only partially sex-limited; females do not express the full melanic phenotype, but females potentially carrying the melanic allele (i.e. those with melanic male siblings) show increased melanization compared to non-carriers. Melanic males were smaller than simple males as measured by pupal mass and wing area, but diet quality did not have phenotype-specific effects. Unexpectedly, melanic males also appeared to be more susceptible than simple males to infection by a pathogen that interferes with silk production, although this infection was only present in a single family.

There has been considerable debate in recent literature concerning the potential costs and condition-dependence of melanin-based colour traits. It has been suggested that melanin synthesis should be particularly costly in insects, and dependent on nitrogen intake

(Stoehr 2006). In Chapter 3, we found that melanization of all three types of adults was strongly dependent on nitrogen availability in the larval diet. This shows that even genetically-based melanic colour traits can be influenced by certain environmental conditions. In contrast to the results from Chapter 2, melanic and simple males did not differ in size; however, the negative effects of nitrogen limitation were more pronounced in melanic males. Melanic males also allocated higher proportions of resources to melanin synthesis compared to simple males, and females allocated less to melanin synthesis than either type of male. The saturating relationship between female melanization and pupal mass showed that only the biggest females were capable of maximal melanization. Female fecundity, and therefore reproductive fitness, is strongly correlated with pupal mass; thus, melanization shows condition dependence in female *M. disstria* moths. Although a similar saturating relationship is present in melanic and simple males, the relationship between pupal mass and fitness is not as straightforward for male moths. Male fitness is largely dependent on their ability to find receptive females. Reproductive success should be dependent on flight ability, which is related to thoracic muscle mass, lipid content, and wing loading. However, all three traits are strongly related to pupal mass in different ways, and reductions in pupal mass may therefore have a net positive or negative effect on male flight abilities and reproductive fitness.

Since the effects of pupal mass on male fitness are not clear, further experiments were done in Chapter 4 to investigate the effects of male melanization and phenotype on fitness more directly by measuring lipid content and wing loading. Although melanization was positively correlated with lipid content and wing loading in melanic males but not in simple males, pupal mass was also strongly correlated with

melanization, lipid content, and wing loading in both types of males. All we can conclude here is that in both types of males, smaller pupae tend to have reduced adult melanization and lipid content, but also decreased wing loading. Chapter 4 also tested for phenotypic differences in male life history traits, but no significant effects of phenotype were found. Although melanic males did tend to be smaller as pupae (as was found in Chapter 2), the phenotypic effect was not significant in this experiment. Similarly, Chapter 2 found an effect of the melanic allele on female melanization and Chapter 3 showed a strong relationship between female melanization and pupal mass, but neither of these results was obtained in Chapter 4.

It is unclear why the statistical significance of these results varies between chapters; however, the trends are consistent. Melanic males tended to be smaller than simple males in all cases except on the high-protein diet in Chapter 3 (Table 5.1), while females with melanic male siblings always tended to have increased melanization compared to those without melanic siblings (Table 5.2). Differences in diet, sample size, and source population could all contribute to the differences in statistical significance between chapters. Furthermore, although mean melanization and pupal mass values vary both within and between chapters for all three adult types, the underlying saturating relationship between these two traits remains, as do the resource allocation patterns reported in Chapter 3 (Fig 5.1; Table 5.3). This may help explain why the phenotypic differences in life history traits and survival are present, but not large. Melanic males suffer a cost due to their genetically-dictated high level of melanization and resource allocation to melanin synthesis; however, they are still able to offset some of this cost by virtue of reduced melanization under suboptimal conditions.

Table 5.1 Compiled mean ± S.D. (*n*) melanization (255-greyscale score) and pupal mass (mg) values for melanic and simple male *M. disstria*.

Chapter	Source population	Diet	Melanization (255-greyscale score)		Pupal mass (mg)	
			Melanic	Simple	Melanic	Simple
2	AB 2008 (Wabasca)	Aspen foliage	151.6 ± 9.4 (98)	114.6 ± 10.2 (83)	268.4 ± 39.7 (98)	288.2 ± 43.0 (83)
		Birch foliage	150.7 ± 8.9 (55)	115.8 ± 10.3 (50)	269.8 ± 42.2 (55)	274.6 ± 48.6 (50)
3	QC 2010 (St. Esprit)	High-protein	160.3 ± 9.7 (57)	132.6 ± 11.7 (39)	289.0 ± 35.3 (57)	283.5 ± 37.5 (39)
		Low-protein	149.1 ± 13.4 (44)	125.5 ± 14.0 (40)	239.4 ± 27.5 (44)	255.1 ± 30.1 (40)
4	QC 2010 (St. Esprit)	Artificial diet	147.0 ± 9.2 (109)	120.1 ± 13.1 (106)	248.0 ± 39.7 (109)	262.8 ± 46.0 (105)

Table 5.2 Compiled mean ± S.D. (*n*) melanization (255-greyscale score) and pupal mass (mg) values for female *M. disstria* with melanic male siblings present or absent.

Chapter	Source population	Diet	Melanization (255-greyscale score)		Pupal mass (mg)	
			Present	Absent	Present	Absent
2	AB 2008 (Wabasca)	Aspen foliage	123.9 ± 10.3 (194)	115.6 ± 9.3 (164)	480.4 ± 62.1 (194)	473.8 ± 61.6 (164)
		Birch foliage	122.7 ± 9.5 (170)	114.5 ± 9.5 (145)	464.3 ± 75.6 (170)	420.7 ± 63.5 (145)
3	QC 2010 (St. Esprit)	High-protein	121.2 ± 9.6 (100)	105.9 ± 4.5 (13)	471.9 ± 72.2 (100)	492.6 ± 83.0 (13)
		Low-protein	104.4 ± 13.8 (63)	84.3 ± 11.6 (18)	342.5 ± 55.7 (63)	294.5 ± 58.7 (18)
4	QC 2010 (St. Esprit)	Artificial diet	115.1 ± 12.0 (191)	110.6 ± 12.6 (93)	419.5 ± 68.3 (191)	412.1 ± 71.4 (93)

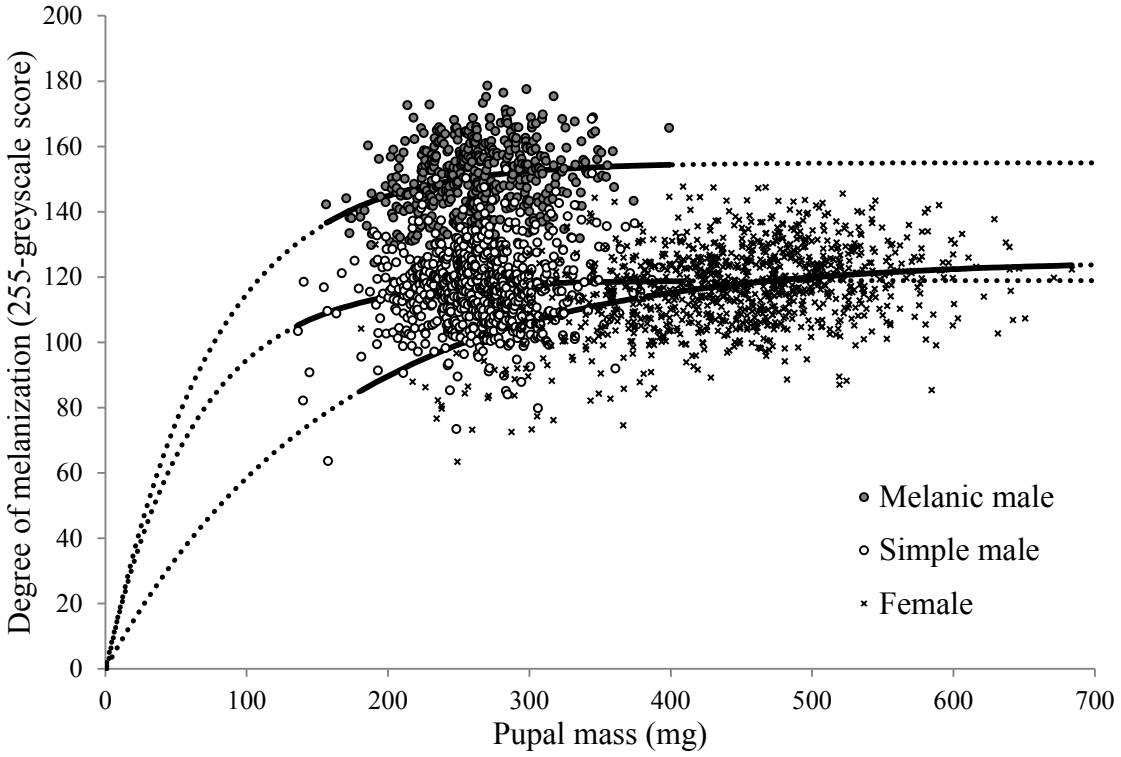

Figure 5.1 Degree of melanization (255 - greyscale score) vs. pupal mass (mg) for melanic male, simple male, and female *M. disstria* moths using combined data from Chapters 2, 3, and 4. Fit lines are the predicted y-values from the nonlinear regression analysis (solid line spans actual data; dashed line shows full theoretical curve).

Table 5.3 Summary of nonlinear regression results for melanization vs. pupal mass in melanic male, simple male, and female *M. disstria* moths using combined data from Chapters 2, 3, and 4.

Adult type	*n*	Maximum (*a*)	95% C.I.	Slope (*b*)	95% C.I.	r^2
Melanic male	452	155.0	152.2 - 157.8	2.11	1.81 - 2.42	0.057
Simple male	695	118.9	117.0 - 120.9	1.89	1.52 - 2.26	0.020
Female	1052	125.3	123.0 - 127.6	0.79	0.73 - 0.85	0.145

The results from this study suggest that increased melanization is costly in M. disstria moths, but that there are mechanisms in place that may reduce the negative effects of melanization on individual fitness. Melanization within *M. disstria* phenotypes is not fixed; a relatively large degree of continuous variation is present with each phenotype. Thus, genetic control of a trait does not preclude condition dependence and strong influences of environmental factors on trait expression. Decreased allocation of resources to melanin synthesis under nitrogen-limited conditions, and the possibility that smaller male pupae give rise to adults with increased flight capabilities (via decreased wing loading) may both reduce the fitness costs of the melanic phenotype. The melanic allele also appears to be effectively hidden from selection in females: despite the effect of the melanic allele on female melanization (Chapter 2), we found no evidence of a cost to the melanic allele in female carriers (Chapter 4). Furthermore, melanic males could potentially enjoy greater mating success than simple males in natural conditions.

Although there was no evidence of female mate choice based on male phenotype (Chapter 4), we found that the first male to locate and approach a receptive female almost always succeeded in mating. In agreement with thermal melanism theory, melanic males tend to warm up faster and attain higher body temperatures than simple males under a radiant light source (Chapter 4). As a result of this thermoregulatory advantage, melanic males may be more likely to reach unmated females first. Melanic males may also be more likely to avoid avian predation during flight due to aerial crypsis and predator search images. Finally, due to the relationship between pupal mass and melanization, melanization may be indicative of quality when population density is high and food and nitrogen are highly limiting. Although melanization of both melanic and simple males

was found to be reduced by nitrogen limitation (Chapter 3), melanic males were still more melanized. Thus, if females show a general preference for darker males at high population densities, melanic males will be favoured despite submaximal melanization.

Selection appears to be acting on the melanic polymorphism in *M. disstria* in several different ways. There are potential costs and benefits to both male phenotypes, supporting the theory that balanced selection is present and maintaining the polymorphism in this species. It is interesting that the phenotypic differences found in this study (i.e. life history traits, development, survival, and the phenotype-specific effects of suboptimal conditions) were all relatively small, suggesting that selection for and against each phenotype may not be particularly strong. A stable polymorphism may be less likely to persist in a system where phenotypes are under very strong selection, even if that selection is balanced overall. Although there may not be one phenotype that is consistently the most advantageous, the strength of the selective forces acting on the polymorphism could result in fixation of one phenotype before variation in selection pressures can act in favour of the other phenotype.

There may also be other mechanisms by which selection could be acting on melanism in *M. disstria* that were not investigated here, such as pleiotropic effects in the larval stage, genetic linkage, a heterozygote advantage, or phenotypic differences in moth behaviour. Apostatic selection cannot be dismissed as a potential mechanism maintaining the melanic polymorphism in *M. disstria*; however, it is unlikely, as the melanic phenotype was the rare phenotype in the majority of the populations sampled (see Table 2.2: Chapter 2 Appendix). The fact that the melanic polymorphism is present at varying frequencies throughout much of the species' already wide distribution indicates that this

is in fact a stable polymorphism, and not a transient polymorphism due to local adaptation. There is a large amount of variation in melanic frequencies in different populations, suggesting that geographical variation in selection may be an important factor. Indeed, the lowest melanic frequencies occur in populations further south (i.e. Indiana and Alabama), while the highest melanic frequencies occur near the northern limits of the species' distribution (i.e. British Columbia and northern Quebec (Lac St Jean)). This supports the theory that melanic males have a fitness advantage at lower temperatures.

The cyclical population dynamics of *M. disstria* adds another dimension to the selection pressures acting on the colour polymorphism, as the melanic phenotype is most likely to be disadvantageous during outbreaks. This is supported by the melanic frequency data collected from populations at different densities (Table 2.2). In the 2005 Alberta populations, melanic frequencies were higher in the low density populations than in the high density population. Similarly, the melanic frequency was quite high in the 2008 Quebec (Lac St Jean) population, which is near the northern limits of the species' range where outbreak densities are rarely reached (Cooke and Lorenzetti 2006) (although this may also be related to temperature as discussed above).

It is interesting to note at this point that *M. americanum* may be monomorphic for a phenotype that strongly resembles the melanic phenotype of *M. disstria*. In Chapter 4, we found that melanization of male *M. americanum* is comparable to that of melanic *M. disstria* males, and female *M. americanum* tend to have greater melanization than female *M. disstria*. Although the *M. americanum* moths used in Chapter 4 were obtained from a single population, sample wings presented by Stehr and Cook (1968) indicate that this

colouration is typical throughout the species' range. Although *M. americanum* populations also exhibit cyclic dynamics, outbreaks are less severe and less frequent that those of *M. disstria* populations (potentially due in a large part to differences in host tree distribution and abundance) (Fitzgerald 1995). The lack of severe outbreaks may therefore remove any strong negative selection on increased melanism that may be present at high population densities.

The phylogenetic relationships between *Malacosoma* species have yet to be clarified (Fitzgerald 1995). It is uncertain what the evolutionary relationship is between *M. americanum* and *M. disstria*, and the ancestral state of colour and pattern in these species is unknown. However, there are three possible evolutionary routes by which the colour polymorphism in *M. disstria* moths may have arisen. The ancestral state may have been continuous variation in melanization, with disruptive selection favouring the presence of two distinct trait optima. Conversely, the ancestral state may have been monomorphic for either the simple or the melanic phenotype, with the second phenotype coming about through mutation and then being favoured enough to stabilize in the species. If the ancestral state was similar to the monomorphic melanism present in *M. americanum*, the simple phenotype could have arisen though a loss-of-function mutation that resulted in a less intensely melanized phenotype. This less costly phenotype could have been selected for by the cyclic population dynamics of this species, and could even contribute to the severity of population cycles. The reverse is also possible; if the ancestral state were the simple phenotype, the melanic phenotype could arise through a gain-of-function mutation that caused increased melanization. Despite the costs to producing the melanic phenotype and the outbreak dynamics of *M. disstria*, the advantages to being melanic and the lack of

expression in females would all contribute to the maintenance of the allele in *M. disstria* populations.

Conclusions

As discussed in Chapter 1, several mechanisms can promote the maintenance of polymorphisms. The cyclical population dynamics of *M. disstria* may cause extensive temporal variation in selection pressures on both phenotypes. The melanic phenotype is likely to be disadvantageous at high population densities due to limited resources, but may have a selective advantage at low densities when resources are not limiting. The sex-limitation of the melanic phenotype may also shield the allele from strong negative selection during outbreaks, and despite the genetic basis of colour phenotypes, plasticity in trait expression under suboptimal conditions may help circumvent the costs of the melanic phenotype. Selection pressures will also vary geographically due to the extremely wide distribution of the species. Thus, stable polymorphisms may be most likely to persist when multiple mechanisms act simultaneously to balance selection on phenotypes.

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