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Heritable Variation in Responsiveness to Photoperiod, Reproduction, and Immune Function in a Population of Peromyscus leucopus

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Heritable Variation in Responsiveness to Photoperiod, Reproduction, and Immune Function in a Population of *Peromyscus leucopus*

Abstract

While neuroendocrine variation is presumed to be important for brain function and natural selection, very little is known about wild-source heritable variation. Within wild populations of rodents living in temperate zones, some individuals respond strongly to changes in environmental conditions, such as photoperiod, while others do not. Changes in photoperiod are assessed by changes in the duration of melatonin secretion. Melatonin affects many physiological processes, such as the stimulation or inhibition of reproduction, changes in body weight and food intake, and changes in immune function. Immune cells can respond to melatonin with circadian and seasonal changes in function.

In this honors thesis, I tested whether differences in neuroendocrine signaling pathways that regulate reproduction are related to differences in immune function in both long-day and short-day photoperiods. For experimental subjects, I used two lines of wild-source *Peromyscus leucopus* that had previously undergone artificial selection for either strong reproductive response to short-day photoperiod or no response. I measured the amount of inflammation in response to a delayed-type hypersensitivity reaction on one of the pinnae of each mouse to measure immune function. In the Run 1, mice in the short-day photoperiod had significantly enhanced delayed-type hypersensitivity responses relative to mice in long-day photoperiod. In the data from Run 1, mice from the non-responsive line in short-day photoperiod had

significantly enhanced delayed-type hypersensitivity responses relative to mice from the responsive line. In the combined data from Runs 1 and 2, the effect of line on inflammation from the delayed-type hypersensitivity test was marginally insignificant. Previous studies indicate that the selection lines vary in seasonal reproductive phenotype and also have differences in neuroendocrine signaling pathways that regulate reproduction. My data indicate that the selection lines may also differ in the strength of immune response to a challenge, suggesting that the wild source population contains biologically significant heritable variation in this aspect of immune function.

Introduction

Neuronal variation in humans and nature

Human populations are genetically diverse (Cheung and Spielman, 2002). This genetic diversity affects brain function and neuronal characteristics. These neuronal characteristics can cause differences in the regulation of neuroendocrine signaling pathways, which can alter fertility. Most populations of humans display at least some seasonal variation in birth rates (Lam and Miron, 1994, Bronson, 1995). This seasonal variation in fertility might be due to environmental factors, such as temperature, food availability, or photoperiod. These environmental factors affect the human reproductive axis, or the hypothalamic-pituitary-gonadal axis. The effects of photoperiod on fertility are well studied in nonhuman mammals, including hamsters

and sheep (Bronson, 1991, Malpaux et al., 1999). For humans living in temperate zones and higher latitudes, the timing of conception is correlated with photoperiod, with more conceptions occurring around the vernal equinox (Roenneberg and Aschof, 1990).

Humans evolved from equatorial African apes, so human reproductive systems have simian origins (Short, 1976, Martin, 2007). Ancestors of modern-day *Homo sapiens* may have lived in regions of Africa that were close to the equator or regions that were more southern (Bronson, 1995). Equatorial habitats would have little variation in photoperiod across seasons. In these habitats, being photoperiodic likely would make little difference in fitness (Bronson, 1995). In more temperate climates in southern Africa, however, photoperiod would vary across seasons. For these populations, it may increase fitness to be able to respond to changes in photoperiod (Bronson, 1995). While it is probable that environmental factors alter the fertility of modern humans, the effects of photoperiod are minor when compared to the effects of cultural factors (Bronson, 1995, Wehr, 1998, Fonken and Nelson, 2011).

Many other animals are photoperiodic (Dawson et al., 2001, Goldman, 2001, Malpaux et al., 2001). Photoperiodism can increase an organism's fitness (Mousseau and Roff, 1987, Prendergast et al., 2001, Emerson et al., 2008). A change in photoperiod can serve as an early signal of future environmental conditions. For example, shortening photoperiod precedes the onset of winter and its associated environmental conditions, e.g. low temperature. Populations of animals also contain much genetic diversity (Reed and Frankham, 2001). Indeed, more genetic diversity

within a population increases the average fitness of its individuals (Reed and

Frankham, 2003). Genetic diversity can affect different parts of the organism,

including brain function and neuronal characteristics (Geschwind, 2000).

Heritable variation in neuroendocrine signaling pathways

Characteristics of neurons, such as their number, location, and connections,

have the potential to alter brain function. If variable neuronal phenotypes increase or

decrease an organism's fitness, these characteristics can be selected for or against by

natural selection. Through natural selection, brain function may become better

adapted to the environment. Over time, natural selection can cause changes in

characteristics of neurons within a population and within a species (Horton and

Rowsemitt, 1992). This neuronal variation can result in variation in an organism's

regulation of physiological processes, such as reproduction, which can affect an

organism's survival. For example, neuroendocrine variation can affect how well

organisms adjust in response to changes in the environment, such as photoperiod.

Further, neuronal variation may be a source of heritable variation in life history

strategies. The success of life history strategies determines which variants are more

likely to occur in the next generation (Heideman, 2004). Responsiveness to

environmental cues, such as changes in photoperiod that indicate the seasons, is an

element of a life history strategy that can increase or decrease fitness, based on

current environmental conditions (Pyter et al., 2005). However, selection may favor

different life history strategies in different environmental conditions, in which case

there is no single optimal life history strategy. This variable selection results in the development and maintenance of heritable variation in life history strategies.

Complicating our understanding of natural populations is the fact that all individuals live in a microgeographically heterogeneous environment that also changes with the seasons. Environmental conditions differ based on time and location. For example, in high latitudes in the Northern Hemisphere, winter is associated with different environmental conditions than summer (Bell, 1997, Mitton, 2000). Because of the changes in environmental conditions, phenotypes and genotypes of individuals are favored or disfavored by selection depending upon what enhances fitness at each time and location. If environmental conditions are highly variable over time and space, phenotypes and genotypes can be highly variable as well (Nelson, 1987, Blank, 1992, Heideman et al., 2005). For example, a population of mice may live near a source of abundant food, such as an oak tree in an acorn mast year (a year of superabundant acorn production) (Gashwiler, 1979, Heideman et al., 2005). In an acorn mast winter, mice could gather food quickly, reducing the risk of predation and conserving energy that would otherwise be expended to forage (Heideman et al., 2005). Mice in this habitat would have increased fitness if the individuals can reproduce all year, including during winter. However, in a year with scarce food, mice might have to forage for longer, which increases the risk of predation and the need for the energy that is used to forage (Heideman et al., 2005). In these alternative conditions, mice might have increased fitness if individuals do not remain fertile during winter. Unpredictable changes in environment maintain variation in phenotypes and genotypes that affect reproductive timing (Heideman et

al., 1999a). Additionally, phenotypic and genotypic variation can arise from mutation and genetic drift, as well as from variation in alleles that impact fitness very little.

Heritable variation is complex, and not much is known about wild-source heritable variation in neuroendocrine signaling pathways (Smale et al., 2005). Traditional animal models, such as laboratory rats and mice, have been useful in discovering how the different parts neuroendocrine signaling pathways function as a system (Phoenix et al., 1959, Ebling, 2005). However, traditional animal models can inaccurately represent human populations or wild populations of other species (Smale et al., 2005). Wild populations can exhibit different behaviors or have variation in neuroendocrine pathways that is not observed in laboratory populations. Wild populations also have pressure from natural selection to eliminate alleles with even small negative effects, unlike laboratory populations.

Many species inhabiting temperate zones conserve energy by limiting the amount of energy devoted to functions, such as reproduction, that are not essential to survival (Heideman and Bronson, 1990, Nelson et al., 1998, Martin et al., 2007, Kaseloo et al., 2012). To survive the winter, endotherms must dedicate more energy into homeostasis in order to maintain body temperature. However, there is less energy available, because available food is often limited. Additionally, obtaining food is more dangerous, because predation is more likely with an increase in the time necessary to forage for food (Bronson, 1991, Bronson and Heideman, 1994). The limited amount of energy and high demand on what energy is available exerts selective pressure on organisms to adapt to their environment. Therefore, it could be advantageous to anticipate the harsh conditions of winter (Moffatt et al., 1993,

Jacobs, 1996). The approach of winter can be predicted by recognizing a shortening photoperiod, a reliable indicator of approaching winter (Bronson and Heideman, 1994, Ebling and Cronin, 2000). Exposure to short-day photoperiod can cause organisms to adjust their physiology to better survive the winter.

Retinopineal pathway and melatonin

Changes in day-length, or photoperiod, are detected by the photoneuroendocrine pathway. The pathway is well described in rodents (Ebling and Cronin, 2000, Prendergast et al., 2002). Variation in this pathway that causes variable reproductive phenotypes in response to winter (short-day) photoperiod occurs in many populations of rodents (Prendergast and Nelson, 2001). This variation in the photoneuroendocrine pathway is used as a model to study heritable variation in the regulation of reproduction and other physiological processes (Ebling and Cronin, 2000, Heideman, 2004).

The photoneuroendocrine pathway begins when light activates photoreceptors in the retina. From the retina, the retinohypothalamic tract transduces the signal to the suprachiasmatic nucleus, which is part of the circadian clock that is essential to timekeeping and interpreting the light signal (Goldman, 2001). In mammals, the signal is carried to the paraventricular nuclei of the hypothalamus, then to the superior cervical ganglion, and then to adrenergic neurons in the sympathetic nervous system (Prendergast et al., 2002). From the sympathetic nervous system, the signal then travels to the pineal gland. The pineal gland releases melatonin only when the retino-pineal pathway is inactive, i.e. when there is no light (Bartness et al., 1993, Goldman,

2001, Prendergast et al., 2002). By limiting the secretion of melatonin to when there is no light, or when it is dark, an organism has a reliable way to detect photoperiod and changes in season.

The duration of melatonin secretion affects an organism's physiology (Silverman, 1988, Bartness et al., 1993, Ebling and Cronin, 2000). Melatonin from the pineal gland binds to receptors on neurons in the hypothalamus, as well as melatonin receptors on many other types of cells, including immune cells. The hormones secreted from the hypothalamus travel through the circulatory system to target cells around the body, including other neuroendocrine organs in the brain. In particular, neurons in the hypothalamus secrete a hormone termed gonadotropin-releasing hormone, which is considered to be the master regulator of reproduction in mammals. From the hypothalamus, gonadotropin-releasing hormone travels through the hypophyseal portal vein to the anterior pituitary gland. The anterior pituitary gland secretes hormones, including the gonadotropins luteinizing hormone and follicle-stimulating hormone, into the bloodstream. Gonadotropins bind to receptors on gonads to signal the gonads to mature and secrete sex steroids, such as estrogen and testosterone. The secretion of gonadotropins is regulated by negative and positive feedback loops. Testosterone inhibits the release of the gonadotropin hormones, partially by inhibiting the release of gonadotropin-releasing hormone from the hypothalamus (Bronson, 1981, Kalra and Kalra, 1983, Smith and Neill, 1987, Meredith et al., 1991, Freeman, 1994).

Factors that affect reproduction can act directly or indirectly on neurons that secrete gonadotropin-releasing hormone (Sisk and Foster, 2004, Kriegsfeld et al.,

2006, Wu et al., 2009, Smith et al., 2010). Glucocorticoid hormones, which are released during stressful situations, help to regulate the release of gonadotropin-releasing hormone and fertility. Glucocorticoids are a product of the hypothalamic-pituitary-adrenal axis, which affects the hypothalamic-pituitary-gonadal axis and thus the regulation of reproduction (Knol, 1991, Rivier and Rivest, 1991). The hypothalamus secretes corticotrophin-releasing hormone, which is transported through the hypophyseal portal vein to the anterior pituitary (Scott and Dinan, 1998). The anterior pituitary secretes adrenocorticotropic hormone, which is transported through the circulatory system to the adrenal gland. The adrenal gland secretes glucocorticoids, which bind to receptors within many types of cells within the body. The hypothalamic-pituitary-adrenal axis can be activated by stress, illness, physical exertion, or cues from the organism's circadian clock (Manteuffel, 2002). The circadian clock is regulated in part by patterns of melatonin secretion.

Melatonin (*N*-acetyl-5-methoxytryptamine) affects many physiological processes in the body, such as circadian entrainment and seasonal reproduction (Reiter, 1991, Morgan et al., 1994, Li and Witt-Enderby, 2000, Masson-Pevet et al., 2000, Witt-Enderby et al., 2003). Melatonin activates at least two known G protein-coupled receptors, known as MT_1 and MT_2 (Dubocovich and Markowska, 2005). These melatonin receptors can be found on cells in the central nervous system and other peripheral target cells. Melatonin receptor activation regulates reproduction through the action of gonadotropin-releasing hormone on the hypothalamus-pituitary-gonadal axis.

Melatonin also directly and indirectly affects the adrenal gland and the daily rhythm of glucocorticoid secretion (Son et al., 2011). In humans, the concentration of plasma glucocorticoids typically reaches a peak during early morning and a nadir around midnight (Friess et al., 1995, Knutsson et al., 1997). Additionally, the concentration of plasma glucocorticoids can vary from season to season (Al-Busaidi et al., 2008). In addition to affecting reproduction, glucocorticoids also affect immune function (Segerstrom and Miller, 2004). With glucocorticoid deficiency, the immune system cannot properly regulate itself (Karalis et al., 1997). When faced with an acute or chronic stressor, the concentration of plasma glucocorticoids increases. Acute stress lasting a few minutes enhances some components of natural immunity while suppressing other components of specific immunity (Segerstrom and Miller, 2004). Chronic stress suppresses both cellular and humoral immunity. Immunity is further affected when glucocorticoids interact with the secretion and efficacy of other hormones, such as melatonin. With the inhibition of the secretion of melatonin by circadian disruption, concentrations of plasma glucocorticoids become elevated (Wright Jr et al., 1997a, Wright Jr et al., 1997b, Spiegel, 1999, Redwine et al., 2000, Shearer et al., 2001, Hu et al., 2010). Because melatonin interacts with the immune system via glucocorticoids and other inputs, it is possible that heritable variation in photoperiod-dependent reproduction is correlated with heritable variation in photoperiod-dependent immune function.

Melatonin is known as an important regulator of the immune system as well as reproduction (Skwarlo-Sonta et al., 2003). Inhibiting melatonin secretion via a surgical or functional pinealectomy is correlated with a decrease in mass of immune

organs, such as the thymus or spleen (Carrillo-Vico et al., 2005). Pinealectomies are also correlated with impaired humoral and cellular immune function. When a subject is given additional melatonin, the number of types of immune cells, such as natural killer cells and monocytes, increases. Melatonin also enhances antigen presentation by macrophages and the expression of major histocompatibility complex class II molecules. Melatonin upregulates the gene expression of different cytokines, including many that stimulate cell proliferation.

Photoperiodism and immune function

Stressors from the environment, such as temperature and the accessibility of food, shelter, and water, can vary from season to season. In temperate zones, winters are usually harsher than summers because of low temperatures and limited food availability (Lack, 1954, Ross et al., 1989, Afoke et al., 1993). Even though access to food is limited, endotherms must use more energy in the winter to keep body temperature high enough to survive. Some individuals in short-day photoperiod (typical of winter), in addition to suppressing reproduction, have a reduced body mass, possibly to require less food to survive (Steinlechner and Heldmaier, 1982, Knopper and Boily, 2000). Energy usually dedicated to non-essential processes, such as reproduction, can be redirected to processes, such as immune function, that are immediately necessary for survival (Heideman and Bronson, 1990, Nelson et al., 1998).

Adjustments of immune function in anticipation of the changing season can be critical for survival. In order to survive the winter, animals must be able to conserve

energy as much as possible without hindering fitness. Because of limited food availability in winter, immune function can be compromised in the wild. However, in laboratory populations of photoperiodic animals, immune function can be enhanced in winter-like short days because food is given *ad libitum* while temperatures in the laboratory are maintained at approximately 23°C (Demas and Nelson, 1996, Demas and Nelson, 1998, Nelson et al., 1998).

Sex steroids, immune function and changes in photoperiod

In a study from a different laboratory, the amount of inflammation resulting from a delayed-type hypersensitivity reaction was not affected in gonadectomized hamsters relative to control hamsters (see Figure 1) (Prendergast et al., 2005). The amount of inflammation resulting from a delayed-type hypersensitivity reaction also was not significantly different in gonadectomized hamsters that were given additional testosterone via Silastic implants relative to gonadectomized hamsters that were not given exogenous testosterone (Prendergast et al., 2005). Additional studies have found that melatonin (Nelson and Drazen, 2000), not sex steroids (Demas and Nelson, 1998), mediates the change in immune response in short-day photoperiod. While sex steroids have not been shown to have a significant effect on the delayed-type hypersensitivity reaction, other parts of the immune system may be enhanced, like antibody production (Bilbo and Nelson, 2001). However, this may be isolated to only certain species, such as the Siberian hamster (Bilbo and Nelson, 2001). Many studies are inconsistent with each other, so the relationship between sex steroids and the immune system remains unclear (Seaman and Gindhart, 1979, Grossman, 1985,

Moshkin et al., 2003, Cutolo et al., 2004, Bouman et al., 2005). Additionally, the

hypothalamic-pituitary-gondal axis and the immune system are both affected by the

hypothalamic-pituitary-adrenal axis and its secretion of glucocorticoids (Da Silva,

1999).
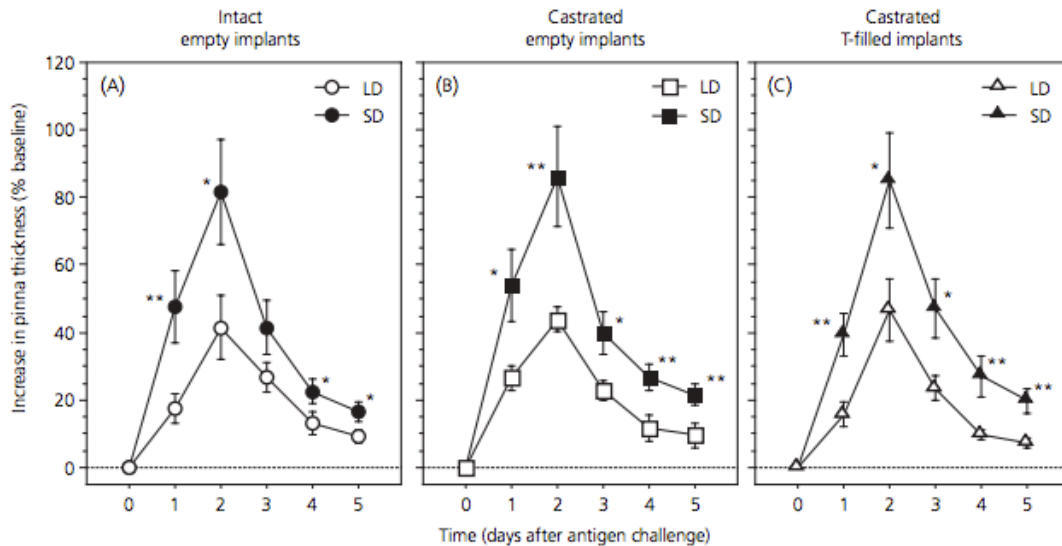
FIGURE 1. Measurements of inflammation induced by delayed-type hypersensitivity
in male Siberian hamsters that (A) had undergone a sham-castration surgery, (B)
were castrated, and (C) were castrated and received exogenous testosterone via a
subcutaneous Silastic implant. Figure from (Prendergast et al., 2005).

Sex did have a significant effect on the amount of inflammation from a

delayed-type hypersensitivity reaction in Siberian hamsters, but the delayed-type

hypersensitivity response was only significantly enhanced in females under stressful

conditions in long-day photoperiod (Bilbo and Nelson, 2003). Interspecies variation

in the effects of sexual dimorphism on the immune system may be more or less

pronounced if the species used was photoperiodic.

Model organism

Populations of *Peromyscus leucopus*, the white-footed mouse, live from southeastern parts of Canada to southern Mexico and the eastern shore of the United States of America to Arizona and the Great Plans states (King, 1968, Jones and Birney, 1988, Kirkland and Layne, 1989, Heideman, 2004). *Peromyscus leucopus* live in a variety of habitats but are usually found in forests in warm and dry climates and brushlands. Home ranges for these mice can be as large as 1 hectare. The average mouse's length ranges from 150 to 205 millimeters and weight varies from 15 to 25 grams (Heideman, 2004). *Peromyscus leucopus* looks similar to closely related species, such as *Peromyscus maniculatus*, with a pale to full reddish brown dorsum and white belly and feet. In higher latitudes, *Peromyscus leucopus* is a seasonal breeder. Both males and females are promiscuous. In the wild, mice are reproductively mature at 2.5 months of age. *Peromyscus leucopus* females near Virginia have approximately 2 litters every year, with and average of 4 pups in each (Kirkland and Layne, 1989). White-footed mice can become reproductively suppressed in winter in response to photoperiodic cues (Heideman et al., 1999a). Mice can also become reproductively suppressed in early summer (Terman, 1998) The mice may be reproductively suppressed because of stress from helminthic infections, as helminthic infections are more prevalent during early summer (Terman, 1998, Vandegrift et al., 2008). The mice have well-developed senses of sight, hearing, smell and touch (especially through their vibrissae, or 'whiskers'). White-footed mice are omnivorous and can eat seeds, nuts, berries, grains, fruit, insects, and

fungi (Heideman, 2004). *Peromyscus leucopus* are nocturnal, which helps them avoid diurnal predators, including many species of snakes and birds of prey.

Individuals of *Peromyscus leucopus* within one population can vary in the degree of responsiveness to photoperiod (Lynch and Gendler, 1980, Heideman and Bronson, 1991, Heideman et al., 1999a). Some individuals become reproductively suppressed in short-day photoperiod by displaying gonadal regression or having significant delays in the development of reproductive organs. Other individuals are non-responsive to changes in photoperiod and remain fertile when exposed to short-day photoperiod. This variation in response to photoperiod has been observed in wild *Peromyscus leucopus* (Terman, 1998) and in the same population in the laboratory (Heideman et al., 1999a). Most individuals within a population have an intermediate response, but a few exhibit either a strong response to photoperiod or no response at all. The range of possible photoperiodic phenotypes indicates that these populations can have heritable variation in the photoneuroendocrine pathway and the regulation of reproduction (Heideman and Bronson, 1991, Heideman et al., 1999a, Prendergast and Nelson, 2001).

Species within the *Peromyscus* genus vary in immune function (see Figures 2 and 3) (Martin et al., 2007). Variation in energy allocation into different immune defenses is a life history strategy (Lochmiller and Deerenberg, 2000, Bonneaud et al., 2003). In one environment, it may be advantageous to invest in non-specific but broadly effective immune defenses, such as the bacterial killing capacity of the innate immune system. In another environment, it may be advantageous to invest in specific but energetically costly immune defenses. According to this study, *Peromyscus*

leucopus exhibit a relatively weak response to delayed-type hypersensitivity (Figures

2 and 3) but have serum with a strong capacity to kill bacteria (measured *in vitro*,

Figure 3). However, the study is only representative of one population of *Peromyscus*

leucopus. While the mice used in Martin et al, 2007 were founded with *Peromyscus*

caught from the wild, the different species of mice were maintained in the

Peromyscus Genetic Stock Center at the University of South Carolina. The original

founder populations were caught many years prior, i.e. in the years between 1948 and

1986 ("Wild Type Stocks") , and may differ in significant ways from populations of

Peromyscus in the wild (Smale et al., 2005).
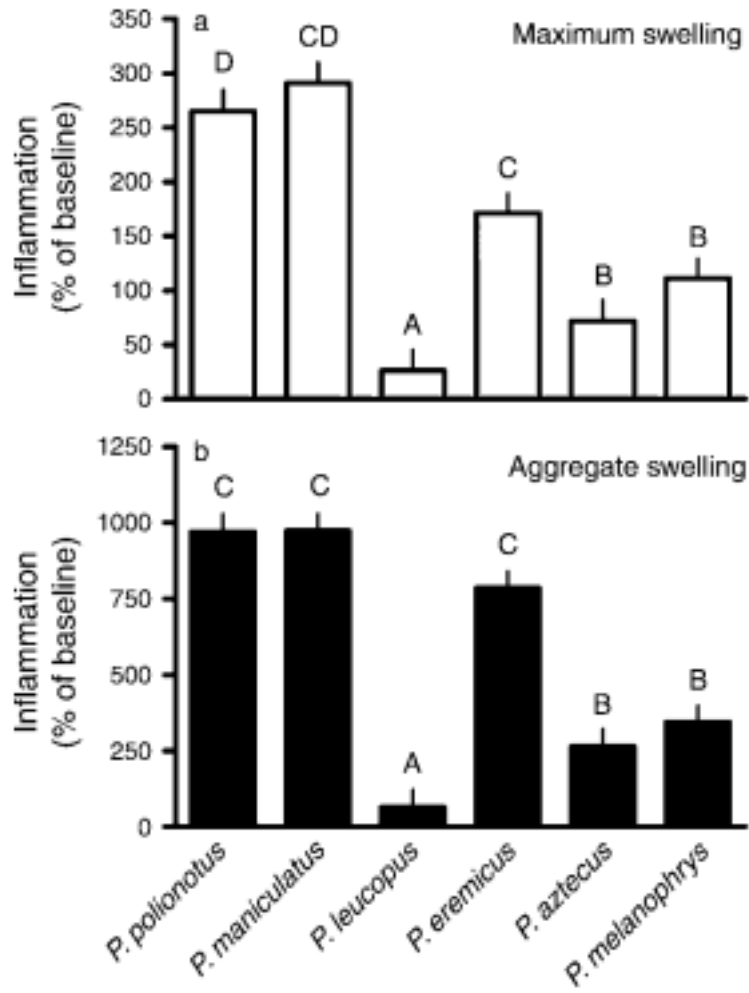
FIGURE 2. The (a) maximum and (b) aggregate inflammation induced by a delayed-type hypersensitivity reaction in Peromyscus species. Figure from (Martin et al., 2007).
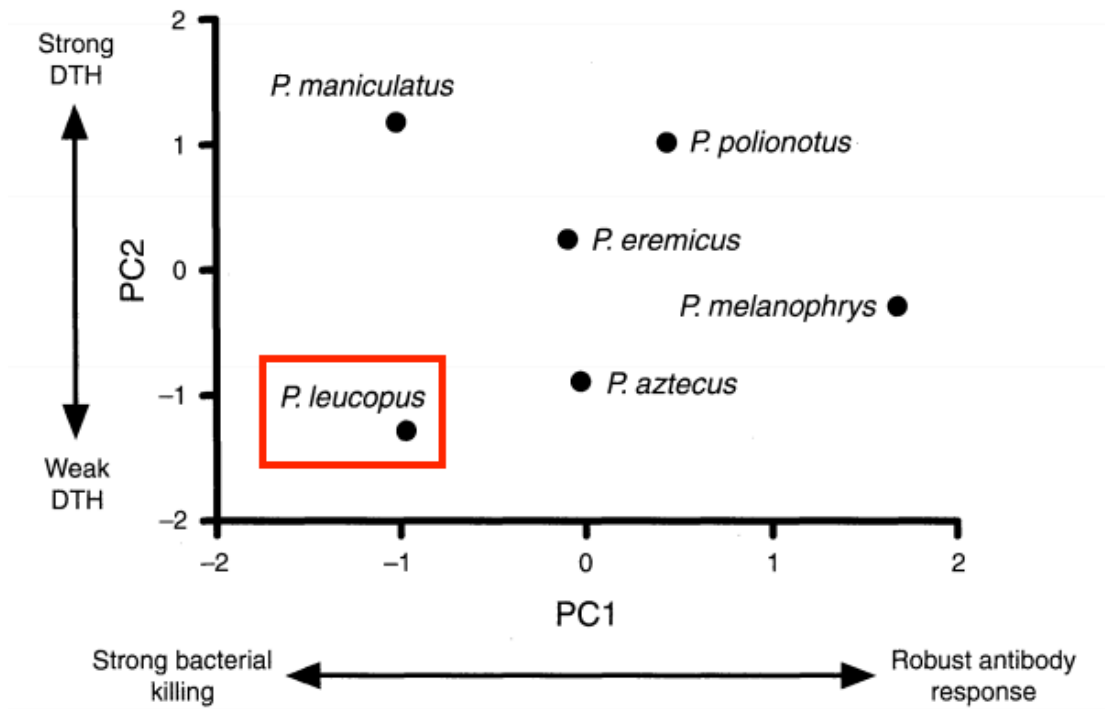
FIGURE 3. *A scatterplot generated with principle components analysis of data measuring immune function in* Peromyscus *species. DTH represents delayed-type hypersensitivity.* Peromyscus leucopus *is highlighted for emphasis. Figure from (Martin et al., 2007).*

Selection lines

Initiated in 1995, Heideman et. al. (1999) used artificial selection to form two lines of wild-source *Peromyscus leucopus*, and these lines have been maintained to the present. One line strongly responds to changes in photoperiod by becoming reproductively suppressed in short-day photoperiod (responsive selection line). The other line is not reproductively suppressed in short-day photoperiod and thus remains fertile regardless of the photoperiod (non-responsive selection line). In a previous study, female mice in the non-responsive line had higher circulating levels of luteinizing hormone than female mice in the responsive line (Heideman et al., 2010). Additionally, mice from the non-responsive line had evidence for significantly more
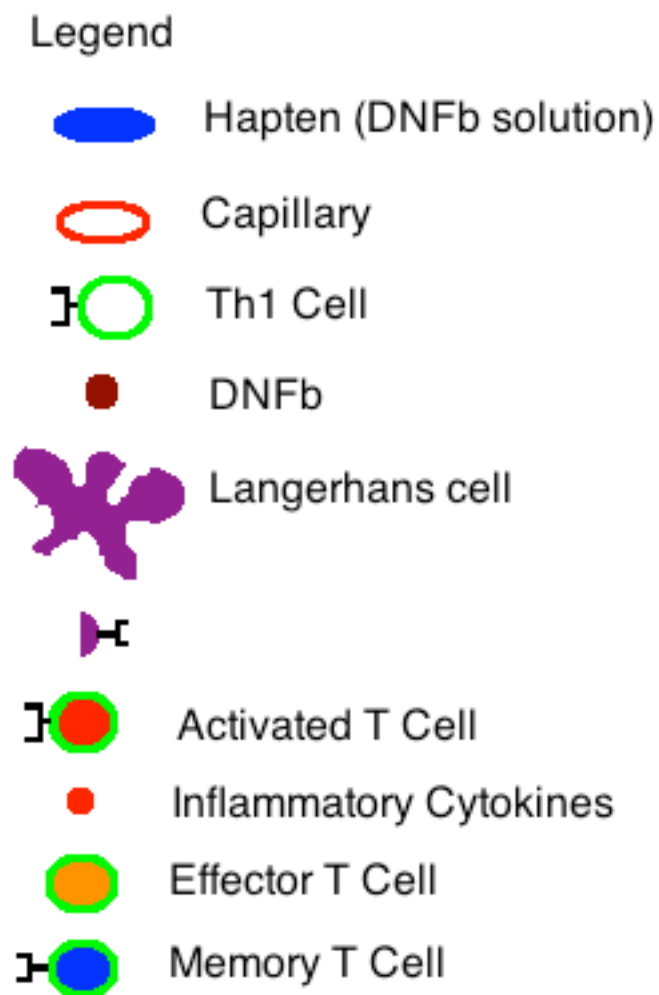
neurons that secrete gonadotropin-releasing hormone and more iodomelatonin

binding in the medial preoptic area and the bed nucleus of the stria terminalis

(Heideman et al., 1999b, Avigdor et al., 2005, Heideman et al., 2007). Mice from the

non-responsive line have been reported to consume more food than mice in the

responsive line (Heideman et al., 2005, Reilly et al., 2006, Heideman and Pittman,

2009), but these findings have not always been replicated in more recent unpublished

studies possibly due to a smaller sample size (Heideman, unpublished data).


Methods

Delayed-type hypersensitivity is a measure of antigen-specific cell-mediated

immunity *in vivo* (Turk, 1980, Dhabhar and McEwen, 1999). The delayed-type

hypersensitivity reaction begins with the first exposure to the antigen, which initiates

the period of sensitization (Figures 4 and 5) (Benjamini and Leskowitz, 1988,

Murphy, 2012). The hapten, 2,4-dinitro-1-fluorobenzene in this experiment, is taken

into Langerhans cells (antigen-presenting dendritic cells) via endocytosis, where it

then alters the gene expression of major histocompatibility complex receptors (Sachs,

1976, Becker et al., 1992). The hapten-presenting Langerhans cells then bind to

inactive T cells. The T cells are activated when bound to both the receptors from the

Langerhans cells and specific cytokines released from cells in the surrounding

environment (Gorbachev and Fairchild, 2001). The activated population of T cells

specific to the antigen grows and begins circulating through the lymphatic system.

After 1 to 2 weeks, the immune system is again exposed to the antigen

(Figures 4 and 6) (Murphy, 2012). The antigen is presented to the antigen-specific T

helper 1 cells by major histocompatibility complex class II dendritic cells. The

cytokine interleukin-1, which activates the T cell, is secreted by other T lymphocytes,

macrophages, or dendritic cells. T helper cells differentiate into T helper 1 cells, T

helper 2 cells, T helper 17 cells, and T regulatory cells. T cells begin to grow in size,

proliferate, and differentiate into various types of T cells, including T helper cells.

Activated T helper cells secrete cytokines. Some cytokines attract other immune cells

to the affected area and facilitate their passage from capillaries into the interstitial

fluid. Other lymphokines activate monocytes and signal the proliferation of T cells.

Activated monocytes mature into macrophages that phagocytize the antigen.

Macrophages can degrade the immunogenic antigen or pathogen, and if it is not easily

degraded, macrophages can form granulomas to isolate and sequester the antigen

from other cells in the body. With the influx of immune cells and fluid into the

affected area, the tissue becomes inflamed. There is a positive correlation between the

amount of inflammation due to an antigenic challenge and the strength of the immune

response, so the delayed-type hypersensitivity reaction can be used to measure

immune function (Phanuphak et al., 1974).

Legend

Hapten (DNFb solution)

Capillary

Th1 Cell

DNFb

Langerhans cell

Activated T Cell

Inflammatory Cytokines

Effector T Cell

Memory T Cell

FIGURE 4. Legend for Figures 2 and 3, depicting the sensitization phase and the elicitation phase of a delayed-type hypersensitivity reaction
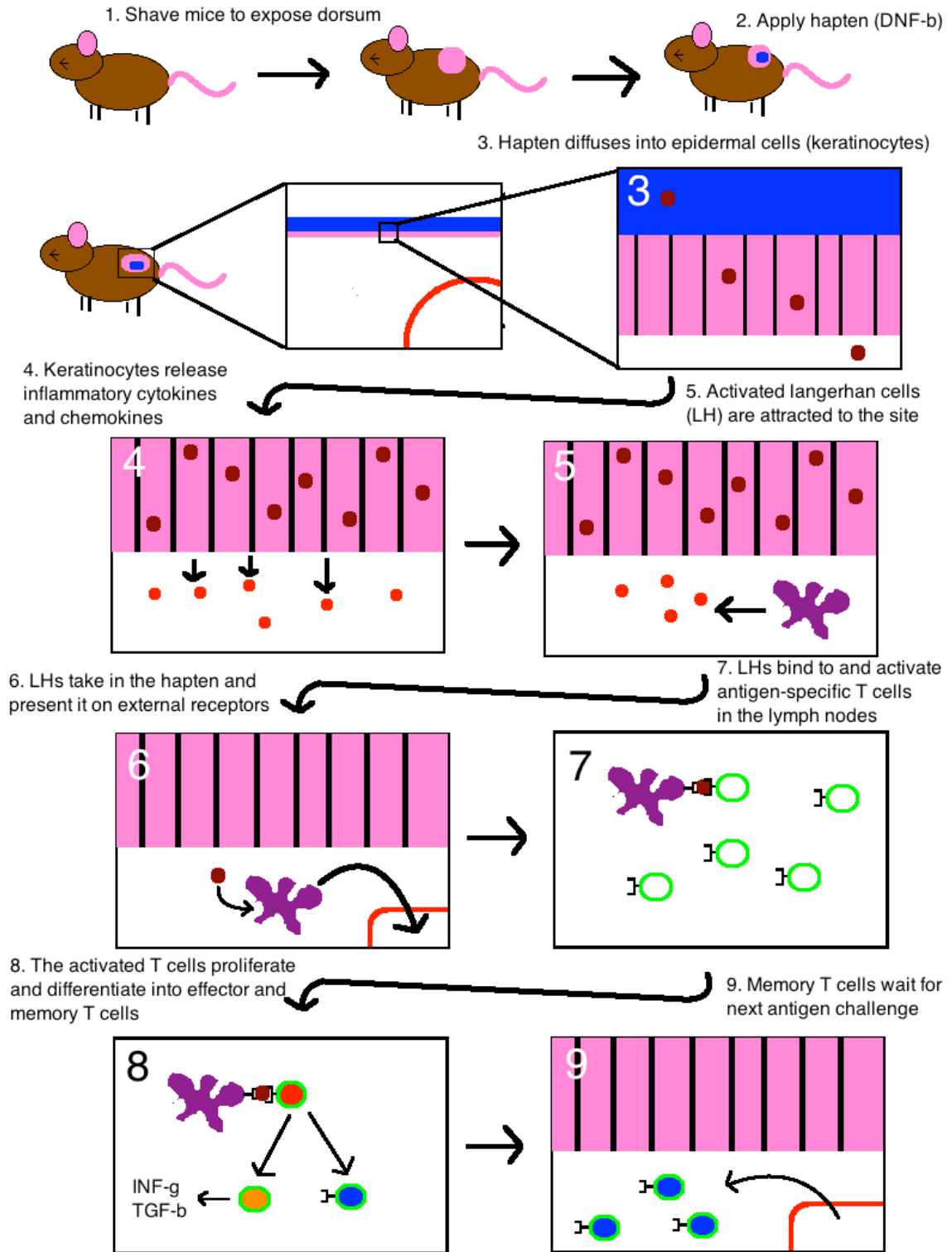
FIGURE 5. The steps occurring the sensitization phase of a delayed-type

hypersensitivity reaction.
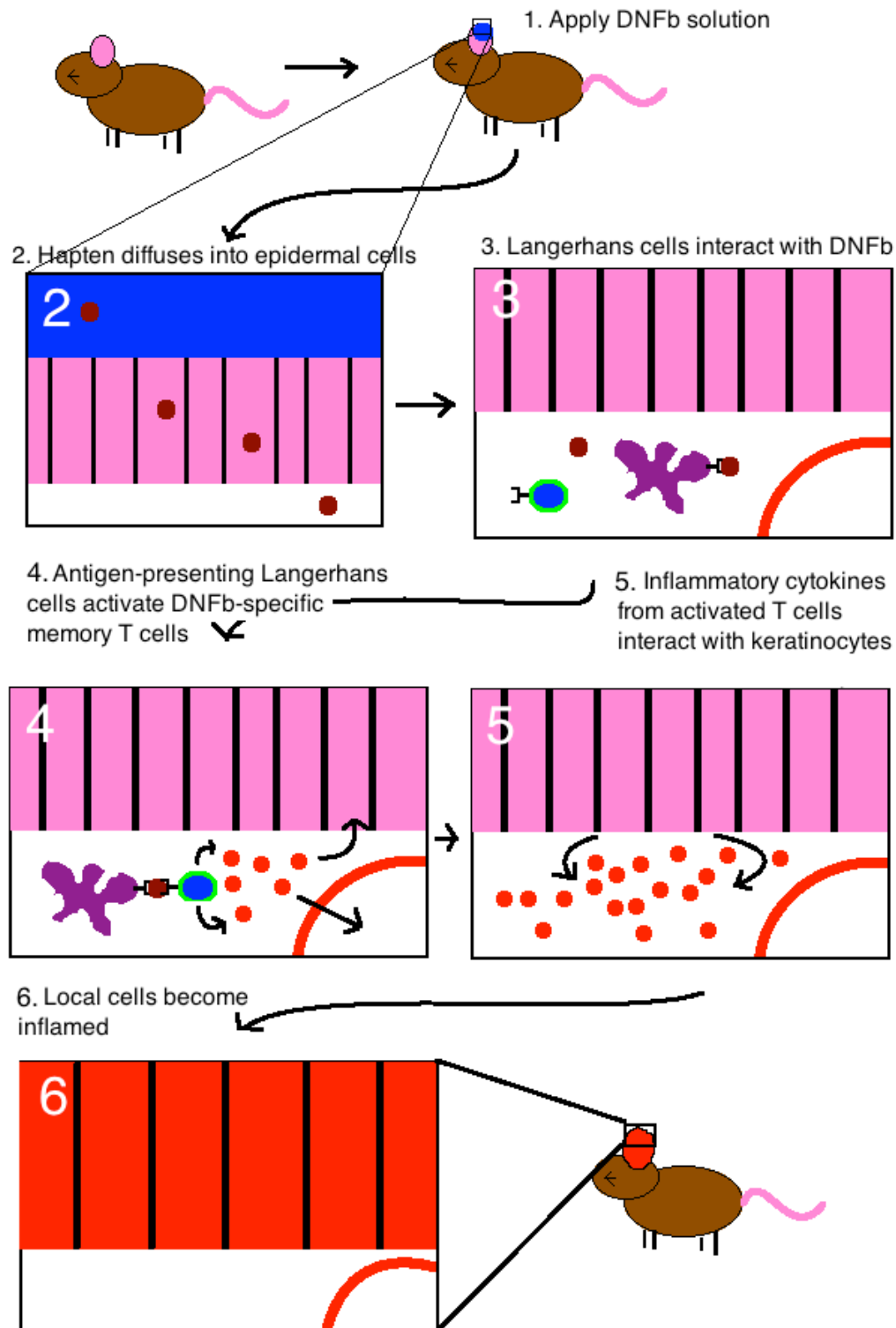
FIGURE 6. The steps occurring during the elicitation reaction of a delayed-type hypersensitivity reaction.

Hypotheses and predictions

To summarize the critical findings from above, natural selection in a changing environment leads to genetic variation in neuroendocrine signaling pathways in natural populations of *Peromyscus leucopus*. In stressful environmental conditions, an organism may need to reallocate energy resources from processes not essential to immediate survival, including reproduction, to processes that are essential to survival, such as homeostasis and immunity. Organisms can anticipate the stressful conditions of the winter season by responding to the shortening of the photoperiod. Our two selection lines of *Peromyscus leucopus* represent two naturally occurring extreme phenotypes: strong reproductive responsiveness to photoperiod and slight reproductive response to photoperiod. Previous studies have indicated that the lines vary genetically in the way that the neuroendocrine pathway that regulates reproduction. Much is still unknown about differences between the lines. Non-reproductive physiological systems, such as the immune system, that share common neuroendocrine regulation with fertility may have heritable variation as well.

In this thesis, I test the hypotheses:

1. The strength of the immune response to a delayed-type hypersensitivity reaction varies in response to changes in photoperiod because mice experience different immunological challenges with the changing seasons and photoperiod is a predictor and indicator of season. The null hypothesis is that immune function does not change in response to photoperiod.

2. There is variation in the strength of the immune response to a delayed-type hypersensitivity reaction that is related to the heritable variation observed in our artificial selection lines of wild-source white-footed mice, *Peromyscus leucopus*. The strength of the immune response would vary because the lines of mice vary in neuroendocrine traits, such as the number of neurons that gonadotropin-releasing hormone, that would alter how the immune system is regulated. The null hypothesis is that there is no difference in immune function between the two selection lines.

Based on these hypotheses, I predicted:

1. Mice in short-day photoperiod would have a stronger response to the delayed-type hypersensitivity test than those in long-day photoperiod because a higher proportion of resources is allocated to immune function in winter.

2. In mice in short-day photoperiod, mice from the responsive line would have a stronger response to the delayed-type hypersensitivity test than mice from the non-responsive line because mice in the non-responsive line in short-day photoperiod allocate a portion of the resources to reproduction, while mice in the responsive line can allocate those resources to the immune system.

Materials and Methods

Generation of selection lines

Mice were taken from a wild-source laboratory colony housed at the

Population and Endocrinology Laboratory at the College of William and Mary. In

1995, forty-eight wild mice were caught as founders for the laboratory population at

latitude 37°16'N near Williamsburg, VA (Heideman et al., 1999a). The mice were

paired in long-day photoperiod (16 hours of light, 8 hours of dark). Offspring were

the parental population used to begin the selection lines. To establish responsive and

non-responsive lines, the parental generation was raised in short-day photoperiod (8

hours of light, 16 hours of dark) from birth. Mice were inspected at 70 ± 3 days of age

to determine reproductive indices (Heideman et al., 1999a). Indices for males were

based on testis size – volume was estimated by multiplying the length by width of the

testis. Males with a testis index less than 24 mm^2 were sorted into the responsive line,

and males with a testis index of greater than 32 mm^2 were sorted into the non-

responsive line. Reproductive indices for females were determined by laproscopically

measuring the size of the ovaries, uterine diameter, and presence or lack of

observable corpora lutea. Females that had ovaries that were less than 2 mm in length,

lacked observable corpora lutea, and had a uterus with a diameter of less than 0.5 mm

were sorted into the responsive line. Females with ovaries that were greater than

approximately 3.5 mm in length, large observable follicles or corpora lutea, and a

uterus with a diameter of greater than 1 mm were sorted into the non-responsive line.

Responsive males and females were paired in long-day photoperiod to begin the

responsive line, and non-responsive mice were paired to begin the non-responsive

line. For multiple generations, selection was imposed on offspring to develop the responsive and non-responsive lines.

Experimental design

For the first run of this experiment, thirty-two mice labeled as male were used from both the responsive and non-responsive lines and long-day and short-day photoperiod. Mice were individually housed after weaning. The four treatment groups were as follows: responsive in long-day photoperiod, non-responsive in long-day photoperiod, responsive in short-day photoperiod, and non-responsive in short-day photoperiod. Mice were between 32 and 160 days old (birth to Day 0), with the mean and standard deviation being 102.4 ± 38.5 days. The mice from the responsive line were significantly younger than the mice from the non-responsive line.

For the second run of the experiment, thirty-two mice were used, sixteen males and sixteen females that had been housed separately after weaning, and separated into four treatment groups as above. Mice were between 69 and 190 days old, with the mean and standard deviation being 120.3 ± 32.4 days. The mice in short-day photoperiod were significantly younger than the mice in long-day photoperiod.

Delayed-type hypersensitivity

On day 0, mice were anesthetized with isoflurane and a small area was shaved on the dorsum (timeline in Figure 7). I applied twenty-five microliters of a 2,4-dinitro-1-fluorobenzene solution (0.5% weight/volume 2,4-dinitro-1-fluorobenzene and 4:1 acetone/olive oil vehicle; Sigma) on days 0 and 1. On day 8, the mice were

anesthetized and the thickness of both pinnae of each mouse was measured with a

thickness gauge to establish a baseline (Mitutoyo, Tokyo; Figure 7). Twenty

microliters of another 2,4-dinitro-1-fluorobenzene solution (0.2% weight/volume 2,4-

dinitro-1-fluorobenzene and 4:1 acetone/olive oil vehicle) was applied to the left

pinna of the mouse. Twenty microliters of a control solution (4:1 acetone/olive oil

vehicle) was applied to the right pinna. The thickness of each pinna was measured

from day 8 to day 13 at 1300 h EST, and all measurement were made in

approximately the same location on the pinna (Figure 7).

Adverse reaction in Run 2

Near the end of Run 2, the non-responsive mice in long-day photoperiod had

an apparent adverse reaction to the 2,4-dinitro-1-fluorobenzene, beginning on Day 3

after the elicitation reaction of the delayed-type hypersensitivity. The left (treated)

pinna of affected individuals became stiff. Superficially, it appeared to be similar to

the effect of loss of blood supply to a thin tissue layer and subsequent desiccation.

This reaction almost exclusively affected the mice from the non-responsive line in

long-day photoperiod; 7 out of 8 mice in the non-responsive, long-day group were

affected, 1 out of the 8 mice in the responsive, long-day group, and none of the mice

in short-day photoperiod were affected. All mice had been treated with the same

solution, and the order of the treatment and handling of mice from the two lines had

been randomized for all treatments and measurements. Members of the laboratory of

Randy Nelson at Ohio State University were consulted, but they had never

encountered this problem before (Tracy Bedrosian, personal communication) and not has it been observed previously in our laboratory.

Body mass, food intake, reproductive organs

      The mice were weighed on day 0 and day 14 (Figure 7). The food in the food hopper of each mouse was weighed on day 0, day 8, and on the final day (day 14 for the first run and day 15 for the second run). On the final day, the mice were euthanized and their reproductive organs were extracted and weighed. Food intake data from mice that ate more than 6 g/day were excluded from analyses, as previous results suggest that higher amounts generally indicated food that is ground by mice but not eaten.
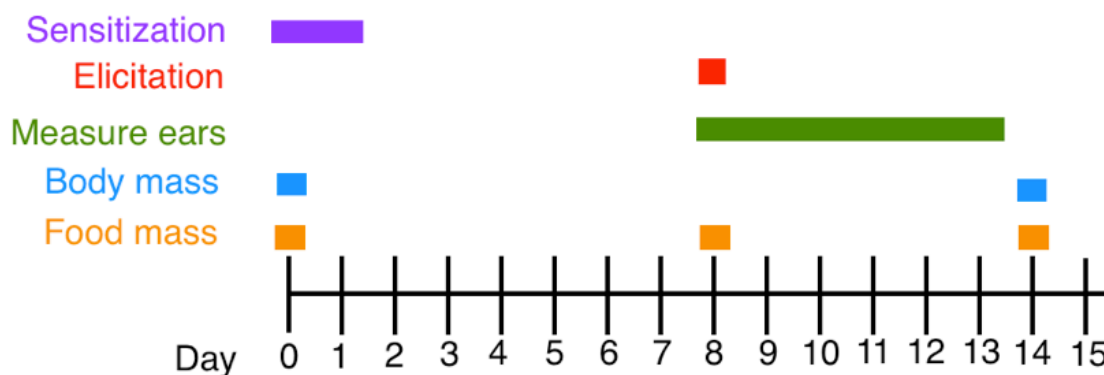
FIGURE 7. A diagram outlining the timeline for all procedures and measurements during Run 1.

Data analysis

Analyses were done using the R statistics software on a Macintosh computer. In Run 1, because the responsive line was significantly younger than the non-responsive line, age was included as a variable in analyses. In Run 2, the mice in short-day photoperiod were significantly younger than the mice in long-day photoperiod, and age was again included in the analyses. All analyses also included photoperiod, line, and the interaction between line and photoperiod.
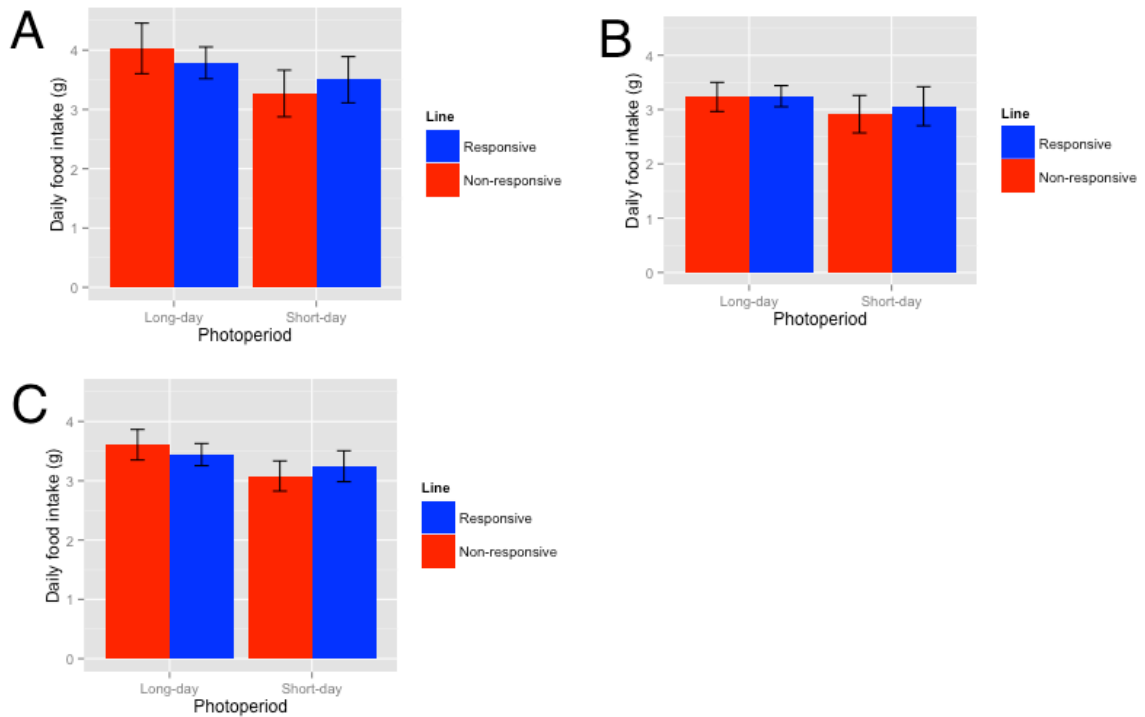
Results

FIGURE 8. Mean (± standard error of the mean) daily food intake from mice in each treatment group in Run 1 (A), Run 2 (B), and the combined data (C); p < 0.05.
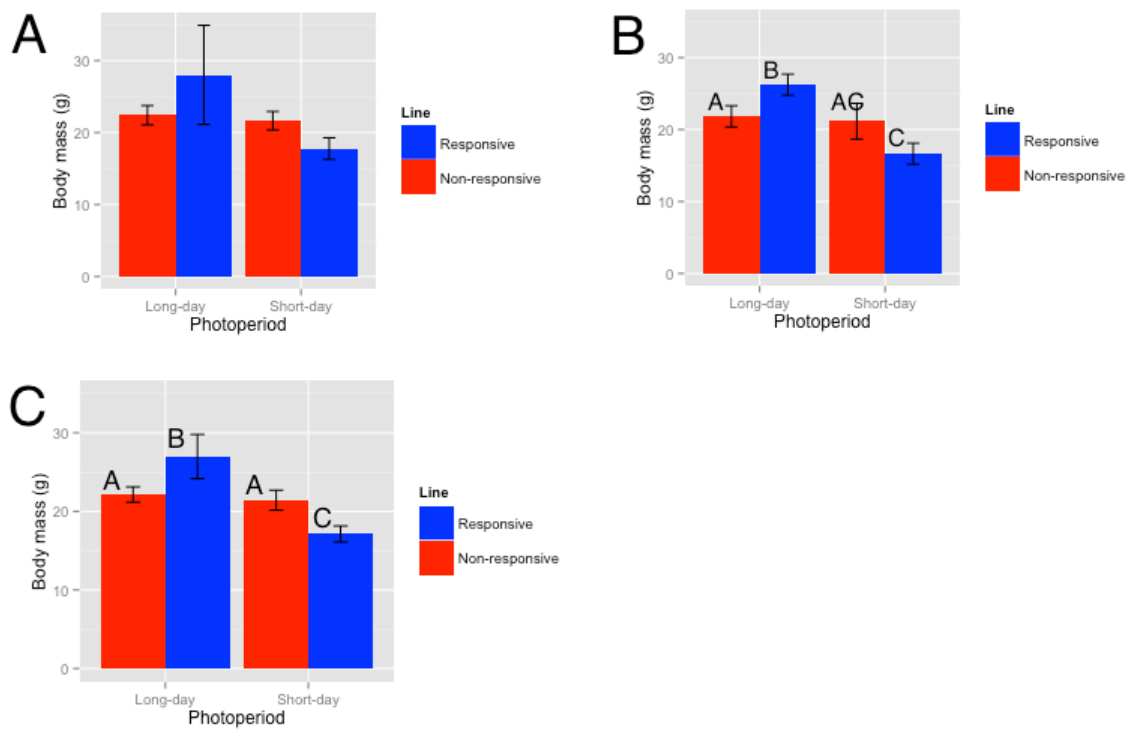
FIGURE 9. Mean (± standard error of the mean) body mass of mice in each treatment group in Run 1 (A), Run 2 (B), and the combined data from Run 1 and Run 2 (C); p < 0.05.
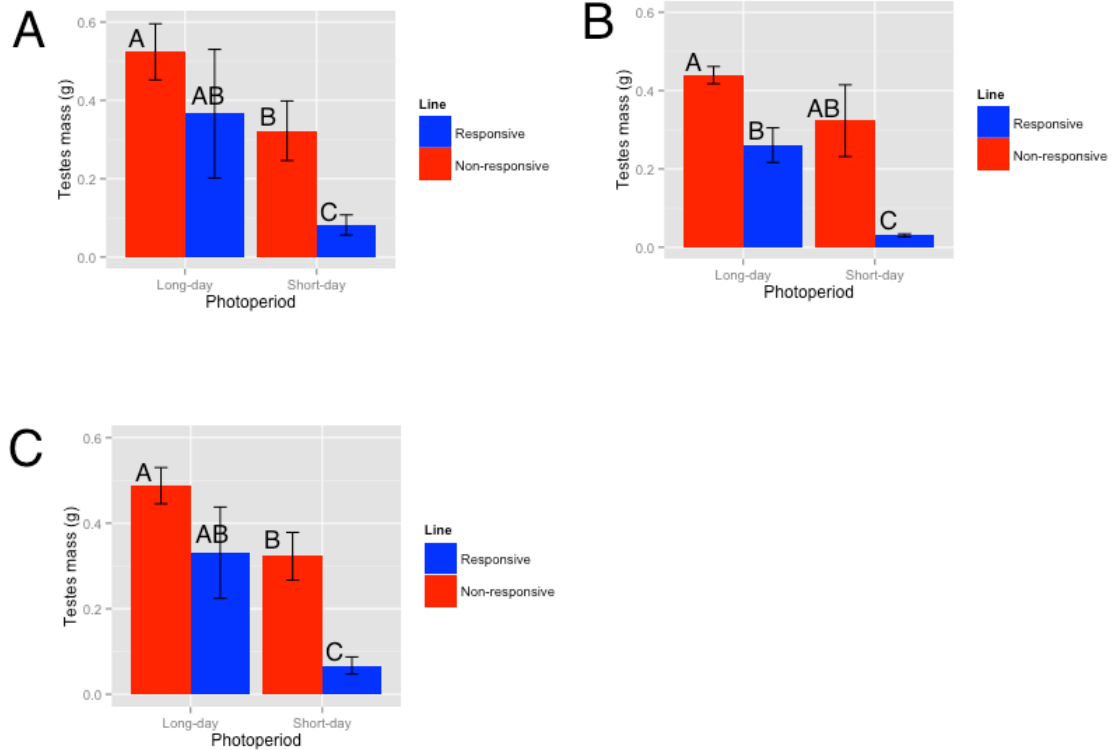
FIGURE 10. Mean (± standard error of the mean) weight of testes mass from male mice in each of the four treatment groups from Run 1(A), Run 2 (B), and the combined data (C); p < 0.05.

Run 1

Delayed-type hypersensitivity

With a two-way within-subjects ANOVA, the effect of photoperiod on inflammation was highly significant (F = 7.05, p < 0.01), whereas the effects of line and the interaction of photoperiod and line were not significant (F = 0.199 and 0.356; p = 0.66 and 0.55, respectively; Figure 11). When analyzing mice only from short-

day photoperiod, the effect of line on inflammation was significant using a one-way within-subjects ANOVA (F = 7.05; p = 0.01). In long-day photoperiod, the effect of line on inflammation was not significant using a one-way within-subjects ANOVA (F = 0.73; p = 0.40).
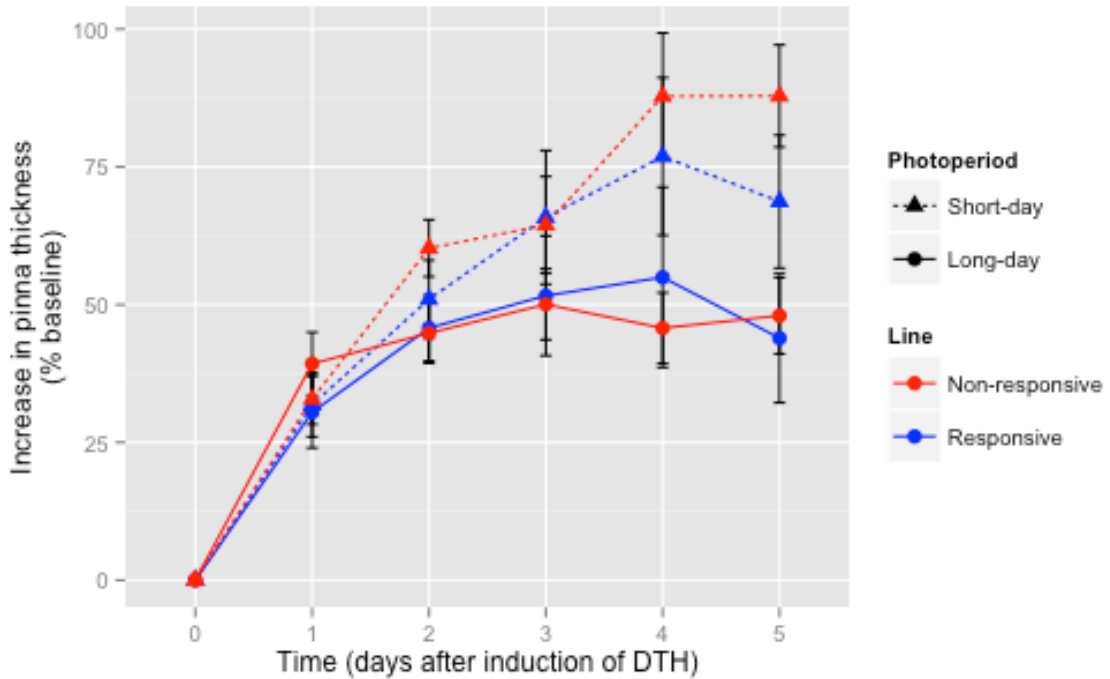

FIGURE 11. Delayed-type hypersensitivity responses in the 4 groups of mice. Data expressed as mean (± standard error of the mean) of the percent difference between the thickness of the treated ear that day and the thickness of the ear on Day 0. Error bars represent the standard error of the mean; p < 0.05.

Line Photoperiod	Age (days)	Body mass (g)	Daily food intake (g)	Testes mass (mg)
Responsive, Long Day	67.8 ± 38.1 [A]	28.0 ± 15.4 [A]	3.79 ± 0.46	366.1 ± 328.4 [A]
Non-responsive, Long Day	113.9 ± 35.8 [B]	22.4 ± 4.5 [A]	4.03 ± 1.20	524.1 ± 202.6 [B]
Responsive, Short Day	81.5 ± 40.2 [A]	17.8 ± 3.7 [B]	3.50 ± 0.87	82.0 ± 57.3 [C]
Non-responsive, Short Day	119.0 ± 26.2 [B]	21.6 ± 4.0 [A]	3.27 ± 1.04	322.5 ± 186.5 [A]

TABLE 1. Mean (± standard deviation) of age (days), body mass (g), daily food intake (g), and testes mass (mg) for the mice from the four treatment groups in Run 1.

Food intake

Food intake was not affected by line (F = 0.02; p = 0.88), photoperiod (F = 1.92; p = 0.18), or their interaction (F = 0.267; p = 0.61; Table 1 and Figure 8).

Age

Age varied with line (F = 10.53; p = 0.003) but not photoperiod (F = 0.49; p = 0.49) or the interaction between line and photoperiod (F = 0.10; p = 0.76; Table 1). The responsive line was significantly younger.

Body mass

Body mass was not affected by the interaction between photoperiod and line (F = 3.32; p = 0.08), line (F = 0.02; p = 0.80), or photoperiod (F = 2.63; p = 0.12) using a two-way ANCOVA (Table 1 and Figure 9). In short-day mice, the effect of line on body mass was marginally insignificant (F = 3.68; p = 0.08).

Reproductive organs

Only mice labeled as male were used in Run 1. Using a two-way ANOVA, testes mass varied with photoperiod (F = 7.37; p = 0.014) and line (F = 6.87; p = 0.017) but not the interaction between line and photoperiod (F = 0.22; p = 0.65; Table 1 and Figure 10).

Run 2

Delayed-type hypersensitivity

One treatment group, non-responsive long-day, had an adverse reaction to the 2,4-dinitro-1-fluorobenzene and the data from several days of ear measurements had to be discarded (Figure 12). Only short-day mice are discussed further in relation to delayed-type hypersensitivity (Figure 13). Inflammation was not affected significantly by line (one-way within-subjects ANOVA; F =1.88; p = 0.17).

Inflammation was not affected by daily food intake (F = 2.77; p = 0.10), body mass

(F = 0.87; p = 0.36), or age (F = 2.34; p = 0.13).

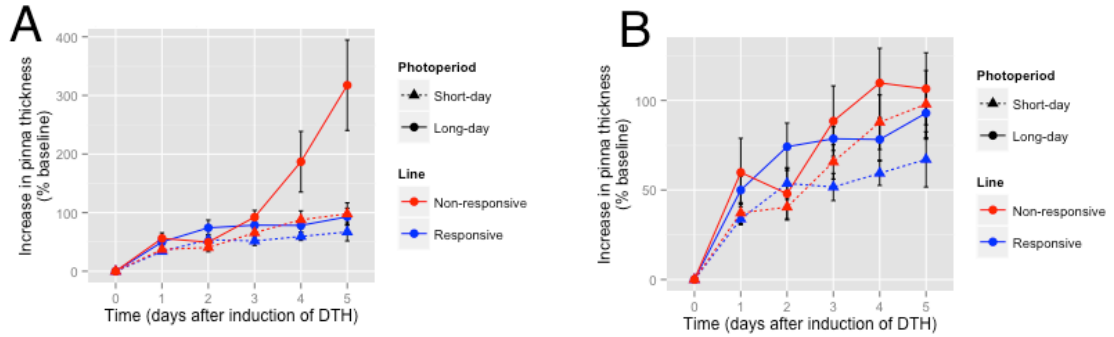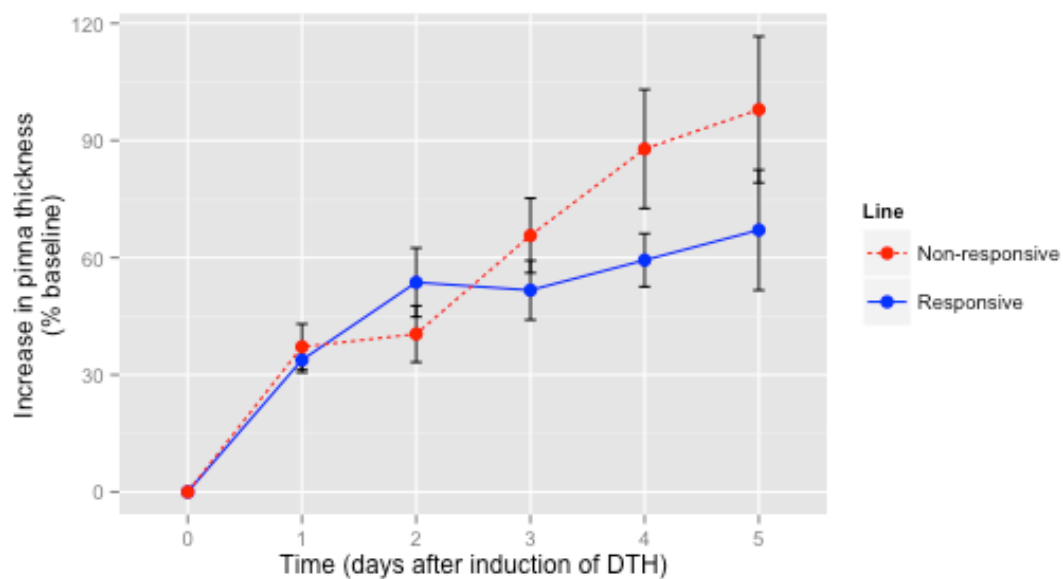*FIGURE 12. Delayed-type hypersensitivity responses in the 4 groups of mice. Data*

expressed as mean (± standard error of the mean) of the percent difference between

the thickness of the treated ear that day and the thickness of the ear on Day 0 in Run

2 for all data points (A) and Run 2 data with elimination of mice with affected ears

(B).

FIGURE 13. Delayed-type hypersensitivity responses in the mice from Run 2 that were housed in short-day photoperiod. Data expressed as mean (± standard error of the mean) of the percent difference between the thickness of the treated ear that day and the thickness of the ear on Day 0.

Line Photoperiod	Age (days)	Body mass (g)	Daily food intake (g)
Responsive, **L**ong Day	141.0 ± 40.3 [A]	26.2 ± 3.9 [A]	3.27 ± 0.56
Non-responsive, **L**ong Day	124.7 ± 24.9 [A]	21.8 ± 4.4 [B]	3.23 ± 0.81
Responsive, **S**hort Day	92.6 ± 23.5 [B]	16.6 ± 4.1 [B]	3.06 ± 0.95
Non-responsive, **S**hort Day	124.8 ± 25.3 [A]	21.2 ± 7.0 [B]	2.92 ± 0.98

TABLE 2. Mean (± standard deviation) of age (days), body mass (g), and daily food intake (g) for the mice from the four treatment groups in Run 2.

Line Photoperiod	Testes (mg)	Uterus (mg)	Ovaries (mg)
Responsive, **L**ong Day	260.8 ± 62.4 [AC]	55.8 ± 17.1 [A]	10.1 ± 2.62
Non-responsive, **L**ong Day	439.5 ± 53.9 [B]	110.6 ± 32.5 [B]	10.9 ± 3.11
Responsive, **S**hort Day	30.6 ± 5.5 [A]	14.1 ± 13.9 [C]	5.2 ± 2.21
Non-responsive, **S**hort Day	323.1 ± 204.8 [B]	39.9 ± 14.5 [AC]	10.3 ± 5.66

TABLE 3. Mean (± standard deviation) of testes mass (mg), uterine mass (mg), and mass of the ovaries (mg) for the mice from the four treatment groups in Run 2.

Food intake

Food intake was not affected by line (F = 0.06; p = 0.82), photoperiod (F = 0.75; p = 0.39), or their interaction (F = 0.03; p = 0.86; Table 2 and Figure 8).

Age

Age varied with photoperiod (F = 4.95; p = 0.03) and the interaction between line and photoperiod (F = 5.66; p = 0.02) but not line (F = 0.88; p = 0.36; Table 2). Mice from the short-day photoperiod (108.7 ± 28.8 days) were significantly younger than those from the long-day photoperiod (131.8 ± 32.4 days).

Body mass

Body mass varied with photoperiod (F = 7.36; p = 0.01) and the interaction between line and photoperiod (F = 6.20; p = 0.02) but not line (F = 0.05; p = 0.82; Table 2 and Figure 9). Mice from the long-day photoperiod (23.8 ± 4.7 g) weighed more than those of the short-day photoperiod (18.9 ± 6.0 g). Body mass also varied with age (F = 5.93; p = 0.02).

Reproductive organs

Using a two-way ANOVA, testes mass varied with line (F = 10.06; p = 0.01) but not the interaction between line and photoperiod (F = 0.56; p = 0.47; Table 3 and Figure 10). The effect of photoperiod was marginally insignificant (F = 4.75; p = 0.052). Uterine mass varied with line (F = 13.37; p = 0.004) and photoperiod (F = 27.29; p = 0.0002) but not the interaction between line and photoperiod (F = 1.95; p =

0.19; Table 3). The mass of the ovaries was not affected by line (F = 2.31; p = 0.16), photoperiod (F = 3.27; p = 0.10), or their interaction (F = 1.42; p = 0.26; Table 3).

Combined data from Run 1 and Run 2

Delayed-type hypersensitivity

Because the delayed-type hypersensitivity results from non-responsive long-day group from Run 2 had to be discarded, only short-day mice were used for the analysis in this section. In a two-way within-subjects ANOVA with line and run as independent variables, the effect of line on the amount of inflammation from the delayed-type hypersensitivity response was marginally insignificant (F = 3.15; p = 0.08; Figures 14 and 15). The effect of run on the amount of inflammation from the delayed-type hypersensitivity response was insignificant (F = 2.42, p = 0.12).
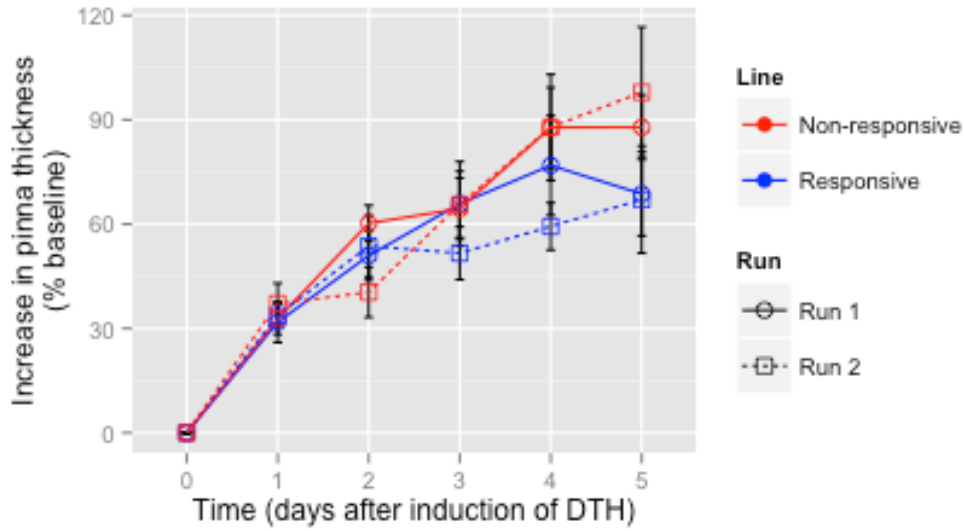
FIGURE 14. Delayed-type hypersensitivity responses in the 4 groups of mice in short-day photoperiod. Data expressed as mean (± standard error of the mean) of the percent difference between the thickness of the treated ear that day and the thickness of the ear on Day 0.
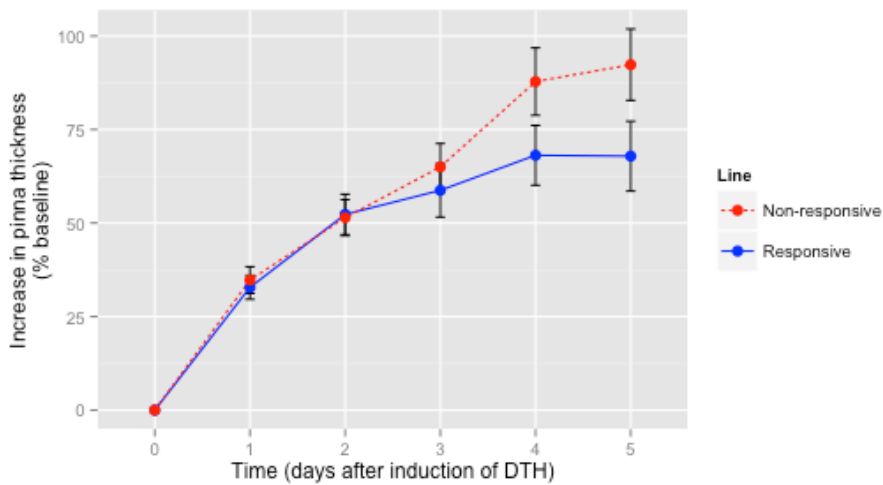

FIGURE 15. Delayed-type hypersensitivity responses in the responsive and non-responsive lines of the combined data in short-day photoperiod. Data expressed as mean (± standard error of the mean) of the percent difference between the thickness of the treated ear that day and the thickness of the ear on Day 0.

Line Photoperiod	Age (days)	Body mass (g)	Daily food intake (g)	Testes mass (mg)
Responsive, Long Day	110.5 ± 53.2 [A]	27.0 ± 9.76 [A]	3.44 ± 0.56	331.0 ± 261.6 [AC]
Non-responsive, Long Day	118.8 ± 31.1 [B]	22.1 ± 4.35 [B]	3.61 ± 1.06	487.8 ± 158.4 [A]
Responsive, Short Day	87.9 ± 30.8 [A]	17.1 ± 3.85 [C]	3.24 ± 0.99	67.3 ± 53.2 [B]
Non-responsive, Short Day	121.9 ± 25.2 [B]	21.4 ± 5.39 [B]	3.08 ± 0.99	322.8 ± 184.9 [C]

TABLE 4. Mean (± standard deviation) of age (days), body mass (g), daily food intake (g), and testes mass (mg) for the mice from the four treatment groups in the combined data from Run 1 and Run 2.

Food intake

Food intake was not affected by line (F = 0.01; p = 0.91) or the interaction between line and photoperiod (F = 0.39; p = 0.54; Table 4 and Figure 8). The effect of photoperiod on daily food intake was insignificant (F = 2.35; p = 0.13; sample sizes = 28 and 31, Cohen's d = 0.429, effect size r = 0.210, power = 0.366).

Age

Age varied with line (F = 6.14; p = 0.02) but not photoperiod (F = 0.70; p = 0.41) the interaction between line and photoperiod (F = 2.12; p = 0.15; Table 4). Mice

from the short-day photoperiod (108.7 ± 28.8 days) were significantly younger than those from the long-day photoperiod (131.8 ± 32.4 days). Mice from the responsive line (98.3 ± 43.3 days) were significantly younger than those from the non-responsive line (120.3 ± 28.1 days).

Body mass

Body mass varied with photoperiod (F = 8.93; p = 0.004) and the interaction between line and photoperiod (F = 9.20; p = 0.004) but not line (F = 0.01; p = 0.93; Table 4 and Figure 9). Mice from the long-day photoperiod (24.0 ± 7.1 g) weighed more than those of the short-day photoperiod (19.5 ± 5.2 g). Body mass also varied with age (F = 4.15; p = 0.05).

Reproductive organs

Only mice labeled as male were used in Run 1, and so measures of testes mass were relevant when combining the data from Run 1 and Run 2. Using a two-way ANOVA, testes mass varied with photoperiod (F = 12.31; p = 0.0013) and line (F = 14.48; p = 0.0006) but not the interaction between line and photoperiod (F = 0.68; p = 0.42; Table 4 and Figure 10).

Discussion

In the introduction, I hypothesized that the strength of the immune response varies in response to changes in photoperiod because mice experience different immunological challenges with the changing seasons and photoperiod is a predictor and indicator of season. I also hypothesized that there is variation in the strength of the immune response to a delayed-type hypersensitivity reaction that is related to the heritable variation observed in our artificial selection lines of wild-source white-footed mice, *Peromyscus leucopus*. The strength of the immune response would vary because the lines of mice vary in neuroendocrine traits, such as the number of neurons that gonadotropin-releasing hormone, which would alter how the immune system is regulated.

Based on these hypotheses, I predicted that mice in short-day photoperiod would have a stronger response to the delayed-type hypersensitivity test than those in long-day photoperiod because a higher proportion of resources is allocated to immune function in winter. I also predicted that in mice in short-day photoperiod, mice from the responsive line would have a stronger response to the delayed-type hypersensitivity test than mice from the non-responsive line, because mice in the non-responsive line in short-day photoperiod allocate a portion of the resources to reproduction, while mice in the responsive line can allocate those resources to the immune system.

The effect of line on the delayed-type hypersensitivity response was significant in Run 1 and marginally insignificant in the combined data (Figures 11 and 15). Only the data from Run 1 can be used to assess the first prediction, because

the analyses including results from the delayed-type hypersensitivity test in Run 2 do

not include mice from long-day photoperiod. The data collected during Run 1

indicates that the first prediction was correct. The second prediction was opposite

from my results; the responsive line had a significantly weaker response to the

delayed-type hypersensitivity test than the non-responsive line. These results suggest

that the selection lines had heritable variation in immune function as well as heritable

variation in seasonal fertility.

Importantly, in this as with previous studies, mice in the non-responsive line

allocated more resources to reproduction, as measured by gonadal mass, than mice in

the responsive line. Mice in the non-responsive line had significantly larger gonads

than mice in the responsive line (Tables 1, 3, and 4 and Figure 10). In addition, mice

in long-day photoperiod had significantly larger gonads than mice in short-day

photoperiod. Consistent with recent unpublished data (Heideman, unpublished data),

the effects of line, photoperiod, and their interaction on daily food intake were not

significant in Run 1, Run 2, or the combined data (Tables 1, 2, and 4 and Figure 8).

For the combined data, the effect of photoperiod on daily food intake was only

marginally insignificant. The lack of significance could be partially due to the small

effect size that photoperiod had on daily food intake ($r = 0.21$).

An important caveat is that the age of subjects was not balanced among

groups. This may or may not have affected the results. Age may not be an important

factor, as the effects of line and photoperiod on testes mass and delayed-type

hypersensitivity responses remained statistically significant when age was included as

a covariate in the analysis. While this suggests that age was not a factor, it would be valuable to repeat this experiment with groups balanced for age.

Another consideration is the fact that *Peromyscus leucopus* may differ from other species in the response to the delayed-type hypersensitivity test. In hamsters, the inflammation in response to delayed-type hypersensitivity peaks during Days 2 and 3 and begins to reduce (Bilbo et al., 2002, Bilbo and Nelson, 2003, 2004, Prendergast et al., 2005, Bedrosian et al., 2011). Other studies involving *Peromyscus leucopus* found that with delayed-type hypersensitivity the pinnae of the mice remained inflamed after Day 6 (Martin et al., 2006), matching results in this study.

The delayed-type hypersensitivity results indicate that there may be a difference in immune function between the lines. By the fourth and fifth days after treatment, the ears of mice from the non-responsive line continued to swell, while the inflammation in the ears from mice in the responsive line seemed to reach a maximum and plateau. I can propose four different hypotheses as to why this might be the case:

1. Mice from the non-responsive line vary in the number of specific types of immune cells, so their reaction to the 2,4-dinitro-1-fluorobenzene can continue to become more inflamed as the days pass. Cell types of interest include T helper cells, cytotoxic T cells, memory T cells, regulatory T cells, B cells, monocytes, dendritic cells, and macrophages.

2. Mice from the responsive line have a larger ratio of T regulatory cells relative to T helper cells and other cells secreting pro-inflammatory cytokines.

3. Mice from the responsive line have a more efficient immune response. Mice from the responsive line are able to clear the 2,4-dinitro-1-fluorobenzene away from the site of application more quickly than mice from the non-responsive line, so the ears of the mice from the non-responsive line continue to become inflamed.

4. Mice from the non-responsive line invest more energy in building an immune response and sustaining an immune response when exposed to an immunogenic molecule than mice from the responsive line.

Maintaining the immune system is not energetically costly (Derting and Compton, 2003). However, mounting an immune response requires more energy (Raberg et al., 2002, Derting and Compton, 2003, Demas, 2004). In the wild, an organism has a limited amount of energy to use (Ricklefs and Wikelski, 2002). This energy has to be used to maintain functions necessary for survival, such as immunity and homeostasis, as well as functions not immediately necessary for survival, such as reproduction. Investment in one system over another represents a trade-off an organism uses to maximize fitness. Mounting an immune response is correlated with an increase in resting metabolic rate (Lochmiller and Deerenberg, 2000, Demas, 2004). The energy used for the immune response may need to be reallocated from reproduction (Bonneaud et al., 2003). A recently published study from the Heideman laboratory found that the basal metabolic rate of mice in the non-responsive line was

significantly higher than that of mice in the responsive line in short-day photoperiod (Kaseloo et al., 2012). The basal metabolic rates of mice in the two selection lines did not differ significantly in long-day photoperiod. Combined with my data, this suggests that the selection lines differ in the amount of energy that is expended.

Organisms have a finite amount of energy to use, and it needs to be allocated well for the organism to survive. The organism must simultaneously regulate many physiological processes, including reproduction and the immune system, and some of these processes regulate others. There are several mechanisms that might cause direct interactions between the reproductive axis and the immune system. Some studies have proposed that sex steroids may act directly on immune cells (Grossman, 1985, Demas and Nelson, 1998). Immune cells have receptors for sex steroids and gonadotropin-releasing hormone (Da Silva, 1999). It is unclear whether sex steroids enhance or depress the immune system (Bilbo and Nelson, 2001). Additionally, other investigators have found significant differences in immune function between males and females of various species, including humans (Paavonen, 1994, Fox, 1995, De Leon-Nava et al., 2009). Both immunity and reproduction are affected by the secretion of glucocorticoids, which is affected by the hypothalamus-pituitary-adrenal axis (Manteuffel, 2002, Tanriverdi et al., 2003). Glucocorticoids can regulate the secretion of sex steroids and vice versa (Brownlee et al., 2005). Heritable variation in the characteristics and functioning of neurons in the hypothalamus or pituitary gland can affect both the secretion of glucocorticoids and sex steroids.

In summary, in the short-day photoperiod, the non-responsive line had a stronger response to the delayed-type hypersensitivity test than mice from the

responsive line. Additionally, the mice from the non-responsive line had significantly

larger gonads than the mice from the responsive line. This suggests that the non-

responsive line allocates energy differently into immunity and reproduction than does

the responsive line. Consistent with this interpretation of my data, Kaseloo et al.

(2012) found that, in these lines, non-responsive mice in short-day photoperiod had a

higher basal metabolic rate than the responsive line, which suggests that non-

responsive mice require more energy to maintain fertility and be able to mount an

immune response.

Future directions

In this first paragraph, I propose a plan for additional runs to produce a dataset

that would have higher statistical power. My foremost suggestion would be to

conduct another trial. The relatively small effect size of line on inflammation from the

delayed-type hypersensitivity reaction suggests testing larger group sizes is necessary

for high statistical power. In addition, future runs should continue for at least three

days longer than Run 1 and Run 2 in this thesis (Figure 16). Ear measurements for

mice should be taken over a total of 9 days, including Day 0. Differences between the

two lines were most pronounced toward the end of the experiment, as has been

reported previously for *Peromyscus leucopus* (Martin et al., 2008a). Ideally, the

inflammation from the delayed-type hypersensitivity response should be declining by

the last day of measurement, as that would show the full time course of the response.

Also, the adverse reaction of the non-responsive, long-day mice in Run 2 would

ideally be avoided or prevented. Unfortunately, the reaction was idiopathic, so the

non-responsive, long-day treatment group can only be watched closely for signs of

pinnae hardening.
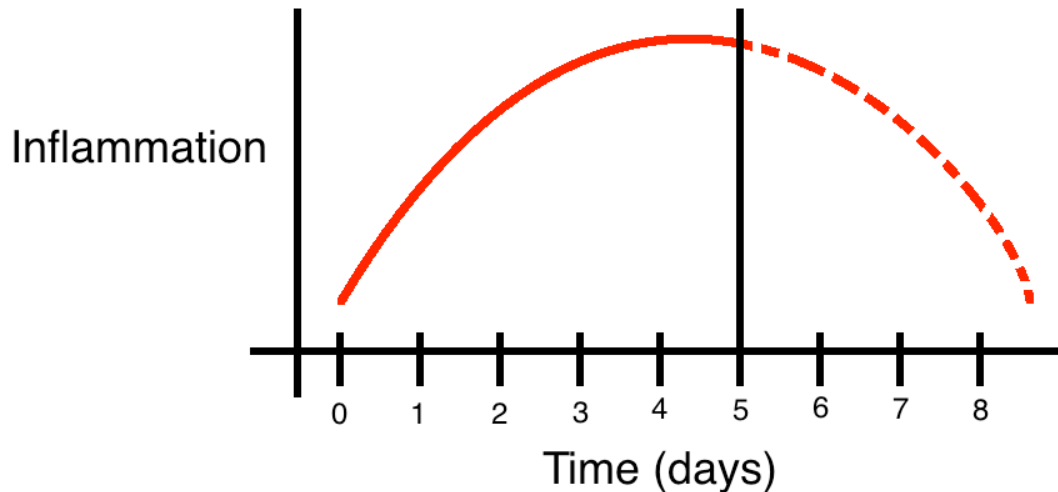
FIGURE 16. Prediction of average measure of inflammation from the delayed-type

hypersensitivity reaction. In this thesis, measurements were continued to Day 5, but if

the number of days that the ears are measured was increased, the inflamed ear may

becoming less inflamed (represented by the dashed line that begins after Day 5).

If additional trials show a significant effect of selection line, more specific

information about how the immune systems of the lines of mice are different could be

investigated [for more possible techniques other than the ones suggested here, see

Figure 16 from (Martin et al., 2008b)]. First, flow cytometry would yield information

about the number of immune cells of certain types, e.g. CD4+ T helper cells,

FOXP3+ T regulatory cells, or CD14+ macrophages. The first two of the four

proposed mechanisms for immune system variation would be able to be refuted by this data. The first hypothesis would be rejected if the effect of selection line on the number of immune cells was not significant. Flow cytometry could be done on various tissues, such as the spleen and blood serum. The second hypothesis would be rejected if the effect of selection line on the ratio of T regulatory cells to T helper cells and other cells secreting pro-inflammatory cytokines was not significant. In a second approach to understand the underlying mechanisms, a lipopolysaccharide challenge would assess the immune response from B cells and macrophages (Anderson et al., 1972, Bucala, 1992). B cells and T cells can sometimes have antagonistic effects on the other (Lampropoulou et al., 2008).

Table 1. Most common assays used to measure immune activity in small mammals and birds.

arm measured	treatment	abbreviation	strong response	indicative of	reference
in vivo					
cell mediated	phytohaemagglutinin[a]	PHA	delayed-type hyper-sensitivity (DTH)	resistance to intracellular infection (e.g. viruses)	Lochmiller et al. (1993), Martin et al. (2006c)
	dinitrofluorobenzene	DNFB			Dhabhar & McEwen (1997)
	keyhole limpet haemocyanin	KLH			Martin et al. (2006b)
humoral	sheep red blood cells	SRBC	generation of antibodies against novel protein(s)	resistance to extra-cellular infection (e.g. bacteria)	Deerenberg et al. (1997)
	keyhole limpet haemocyanin	KLH			Demas & Nelson (1998)
	diphtheria–tetanus virus	DPT			Ilmonen et al. (2000)
innate	lipopolysaccharide	LPS	variable	resistance to multiple novel pathogens	Aubert et al. (1997), Owen-Ashley et al. (2004)
in vitro					
cell mediated	concanavalin A	Con A	extensive prolifer-ation of responsive T-cells	cytokine production, T-cell activity	Fitzgerald et al. (1992)

[a] Unlike KLH and DNFB, PHA is a mitogen that activates a variety of T-cell types, which may lead to different degrees of swelling compared with other antigens (see Martin et al. 2006a,c)

FIGURE 16. Possible techniques to assess the strengths of different arms of the immune system. Figure from (Martin et al., 2008b).

Using the whole organism, while potentially more accurate, does not provide much information about differences between the lines on a cellular level. To may be informative to extract cells to perform *in vitro* experiments. Initially, a peripheral blood smear could be performed from blood extracted from the tip of the tails of the mice. Also, the serum of the blood could be used to measure the antibody titer. *In vitro* call cultures can also be used to evaluated cytokine secretion using an enzyme-linked immuno sorbent assay specific to cytokines of interest, such as tumor necrosis factor-α, interleukin-1, or interleukin-6.

An additional possible experiment can investigate the impact of melatonin on immune function in responsive and non-responsive mice. The mice would be given timed oral doses of melatonin, ramelteon (a melatonin agonist with a high affinity for MT_1 and MT_2 receptors), or luzindole (a melatonin receptor antagonist) (Drazen et al., 2001, Hiebert et al., 2006, Prendergast, 2010).

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