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Effects Of The Brown Locus On Melanin Accumulation In The Skins Of Neonatal Mice

by

Kim Stanford

A THESIS

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ABSTRACT

Experiments were conducted to determine the effects of the brown locus on the accumulation of melanin in the skins of neonatal mice. For C57/BL/10J (black, BB) and crossbred (black x brown, Bb) neonatal mice, melanin accumulation in the skin increases curvilinearly until 10 to 12 days post parturition, after which it decreases until 20 days of age. C57/BR/cdJ (brown, bb) mice showed a linear increase in skin melanin concentration until 15 days of age, with melanin decreasing thereafter until 20 days of age. Black mice reached a significantly higher maximum ($P < 0.05$) skin melanin content than crossbred mice at 10-12 days of age, with crossbreds having approximately 27% less melanin at maximum. From birth to 15 days, black and crossbred mice had significantly ($P < 0.05$) higher skin melanin content than brown mice, the maximum skin melanin attained by brown animals was 50% that of black mice. From 20 to 25 days of age there was no significant difference in skin melanin content among all lines of mice, as melanin content approached zero in all lines. These results indicate that the b allele reduces skin melanin content in homozygote mice. In heterozygotes, the b allele limits the maximum level of skin melanin attained, but otherwise has no significant effects on the melanin content of the skin.

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CHAPTER 1. INTRODUCTION

1.1. MELANINS, THEIR STRUCTURE AND FUNCTION

1.1.1. Types of Melanin

Mammalian pigmentation is dependant on a group of compounds known as melanins. Melanins can be divided into two main groups: the black-brown eumelanins which are derived from the enzymatic oxidation of tyrosine or related metabolites, and the sulfur containing pheomelanins which range from yellow to red (Riley 1977). Pheomelanins arise by the addition of the amino acid cysteine or other sulfhydryl compounds such as glutathione into the eumelanin pathway (Prota 1980). The pathway for the production of eumelanins and pheomelanins is illustrated in Figure 1.1. The exact structures of these melanins are not known as the molecules are huge heteropolymers, or random polymers of various melanin precursors, which in the case of eumelanin include 5,6-dihydroxyindole and indole-5,6-quinone (Jimbow et al. 1976).

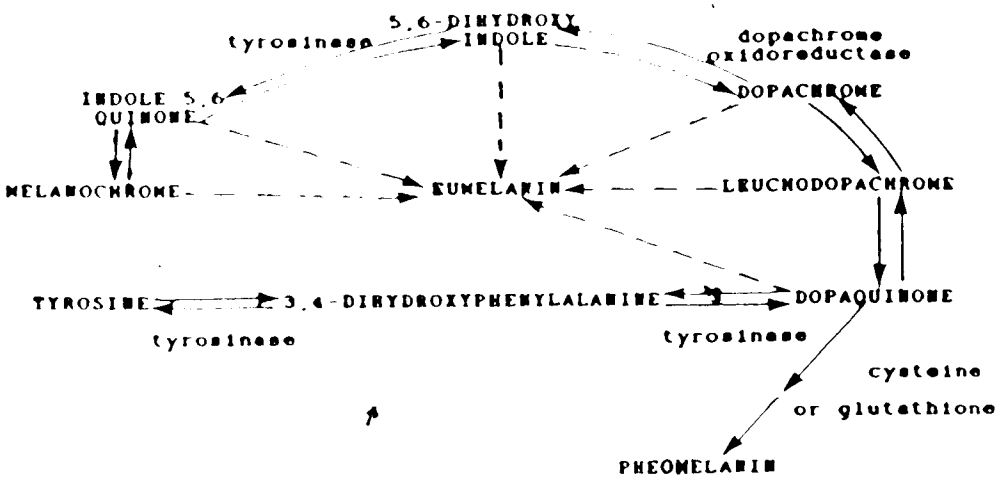
1.1.2. The Function of Melanin

Melanin functions in mammals by providing some protection from ultraviolet light, as a part of camouflage protection, for temperature

Figure 1.1 The Eumelanin Pathway. Eumelanin is a heteropolymer made of all intermediates in the pathway after and including dopaquinone. Broken arrows indicate condensation of the intermediates to form the heteropolymer. Solid arrows indicate enzymatic or spontaneous conversion between intermediates.

FIGURE 1-1

THE EUMELANIN PATHWAY



control and possibly for detoxification of toxic metabolites.

1.1.2.1 Protection from Ultraviolet Light

The primary function of melanins is to protect the cells of the skin and retina from the mutagenic effect of ultraviolet (UV) radiation (Hill and Setlow 1982). The skin of mammals responds to UV radiation by thickening the epidermis and increasing production of melanin (Prota 1980). Eumelanins have been shown to be significantly more effective than pheomelanins in this protective role (Limbow and Takeuchi 1979). In man, the protective role of melanins is demonstrated by the less frequent occurrence of skin tumors in dark than in lightly pigmented individuals exposed to solar irradiation at the same latitude (Harrison 1961).

In other mammals, body hair is effective in protecting the underlying tissues from UV light. In mice, Kodama et al. (1984) found that hair protected the skin from 94% of potential DNA damage by UV light. Even in mammals with thick protective hair coats, skin melanin provides important protection in more exposed, hairless areas.

In cattle, increasing eyelid pigmentation reduces the incidence of ocular squamous carcinoma ('cancer-eye') (Troutt and Schurig 1985) and also decreases the incidence and severity of bovine infectious keratoconjunctivitis ('pink-eye') (Ward and Neilson 1979). It is significant that maximal outbreaks of these diseases occur primarily

in early to mid summer, during the period of peak UV radiation (Trout and Sobrio, 1985).

In mice and other animals which are born without hair, epidermal melanin remains an important defence against UV radiation until the growth of the hair coat. In mice, hair growth is initiated on or around the second day after birth and is only complete between days 16 and 21 after birth (Fraser 1951).

1.1.2.2 Protective Coloration

Besides serving as protection from UV light, the melanins also provide protective coloration or camouflage for many animals (Prota 1980). Both pheomelanin and eumelanin play a major role in pattern formation in the hair and feathers of animals (Morison 1985).

1.1.2.3 Temperature Control

The melanins are also active in body temperature control, functioning as a heat absorber in animals exposed to cold temperatures (Morison 1985). Conversely, a lack of melanin can reduce heat stress. For example, the hair of tropical cattle is most often light colored. Light colored hair reflects solar radiation and is thought to aid in keeping the animal from being overheated (Brody 1956).

1.1.2.4. Detoxification

Other views of the function of melanins also exist. Pawelek and Lerner (1978) believe that the process of pigmentation can be seen as a detoxification mechanism by which highly reactive quinones are rapidly converted through a series of spontaneous reactions into an insoluble, relatively inert, nondiffusible polymer. In their view, detoxification is the primary function of melanin, other functions being purely incidental.

1.2 THE EPIDERMAL MELANIN UNIT

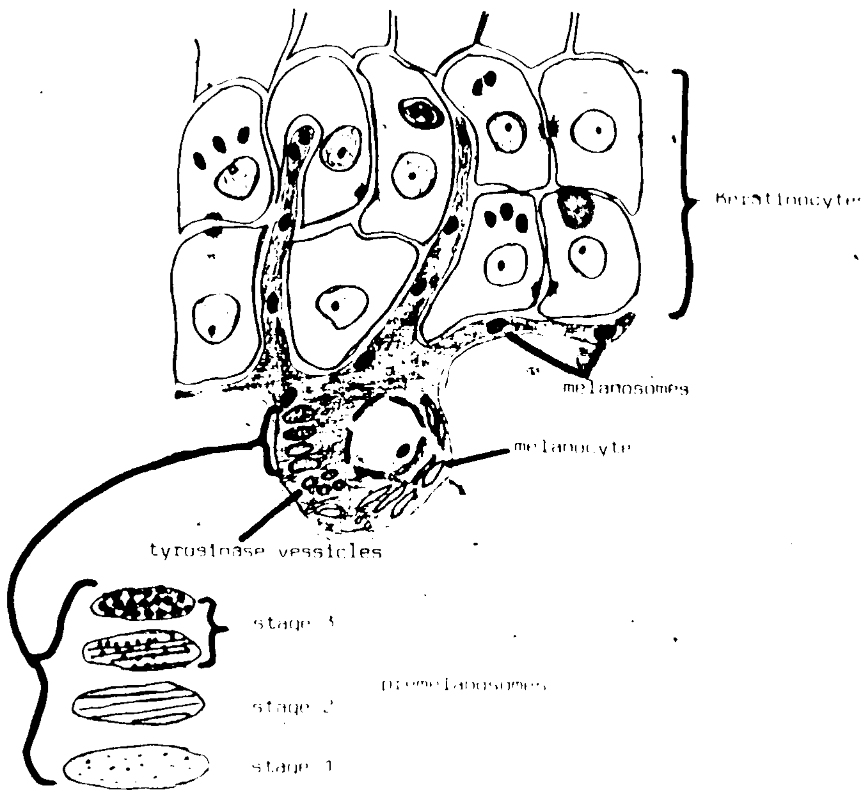
The epidermal melanin unit is illustrated in Figure 1.2.

1.2.1. Melanocytes

Melanin producing cells are commonly referred to as melanocytes. Melanoblasts, which are undifferentiated melanocytes, migrate from the neural crest through the dermis into the epidermis in early embryonic life (Rawles 1948). In mice, the number of active, melanin producing, epidermal melanocytes peaks around 5 days after birth. Thereafter, the number of melanocytes shows a steep decline until virtually none are found in the epidermis 30 days after birth, the melanocytes having either died or become inactive (Hirobe and Takeuchi 1977). Exposure to UV light reactivates the small population of melanocytes remaining

Figure 1.2 The Epidermal Melanin Unit. Melanosomal development is divided into 4 stages. Stages 1-3 are premelanosomes. Stage 4 is the mature melanosome.

AN EPIDERMAL MELANIN UNIT



From Prota and Thomson 1976

in the epidermis (Quevedo and Smith 1963). UV light also stimulates the mitotic proliferation of these newly active melanocytes, causing up to a seven fold increase in melanocyte numbers (Jimbow and Uesugi 1982).

Some melanoblasts also migrate from the epidermis into the hair bulbs and differentiate into melanocytes (Silvers 1956). Once within the hairbulb, the melanocytes are protected from the pre programmed death that occurs in the epidermis. Instead, they remain functional during periods of active hair growth, producing the melanin found in hair (Fraser 1951).

1.2.2. Melanosomes

Melanin is deposited within the melanocyte in organelles known as melanosomes (Seiji et al. 1963). Although controversy exists as to the exact sequence of events in melanosome formation (Quevedo and Fleischmann 1980), the general structure of melanosomes is well characterized. Melanosomes consist of three basic components: (1) limiting membranes, (2) structural or matrix proteins, (3) the enzyme tyrosinase (Nakagawa et al. 1984). In addition to tyrosinase, melanosomes exhibit activity for other enzymes such as acid phosphatase, ATPase and the recently characterized dopachrome oxidoreductase (Barber et al. 1984). Toda and Fitzpatrick (1971) outlined four stages in the development of eumelanosomes (eumelanin containing melanosomes). In stage 1, the melanosomes consist of a

spherical membrane-limited vesicle containing no melanin. In stage 2, the organelles become oval and the inner lammellae which consist of longitudinal fibers which are cross linked, form. The enzymes necessary for melanogenesis bind at regular intervals along the matrix. In stage 3, melanization begins, with melanin deposition at first localized around the enzymatic complexes. In stage 4, melanization is complete, with the melanosomal matrix being completely obscured by melanin. The development of pheomelanosomes (pheomelanin containing melanosomes) is identical to that of eumelanosomes until stage 2 (Jimbow and Takeuchi 1979). Pheomelanosomes are spherical and develop without an organized protein matrix. Instead, the pheomelanin is deposited on randomly distributed areas on a tangled mat of very fine protein fibers with no organized aggregation or cross-linking (Moyer 1966).

Once fully melaninized, melanosomes migrate into the dendrites of the melanocytes and are then transferred to surrounding keratinocytes, thereby distributing melanin throughout the epidermis (Cohen and Szabo 1968). The exact means of melanosome transfer is still in dispute, but several theories exist (Wolff 1973, Garcia et al. 1979). Once spread throughout the epidermis, the protein matrix of the melanosome can be degraded by lysosomal enzymes (Ohtaki and Seiji 1971), but the melanin itself is highly resistant to degradation (Quevedo 1973).

1.2.3. Enzymes Involved in Eumelanogenesis

1.2.3.1. Tyrosinase

Tyrosinase catalyses two and possibly three reactions in the conversion of tyrosine to eumelanin (Korner and Pawelek 1982) as illustrated in Figure 1.1. As the deposition of melanin on the melanosomes (melanization) progresses, tyrosinase activity decreases. This decrease in tyrosinase activity was once thought to be due to the newly synthesized melanin blocking the active sites of the enzyme (Seiji and Fitzpatrick 1961). More recently Tomita et al. (1980) have demonstrated that the inactivation of tyrosinase is due to as yet unknown changes in the enzyme itself.

1.1.3.2. Other Enzymes

The role of enzymes other than tyrosinase in the production of eumelanins remains in dispute, as dopa quinone and other intermediates can spontaneously form melanin through self-oxidation (Murray et al. 1983). However, Barber et al. (1984) have demonstrated the existence of an enzyme they term dopachrome oxidoreductase which catalyses the conversion of dopachrome to 5,6-dihydroxyindole (Figure 1.1), greatly speeding melanin formation.

1.2.4. Hormones Involved in Melanogenesis

The pituitary hormone, α-melanocyte-stimulating hormone (MSH) plays an important role in the development of pigmentation. Hirobe and Takeuchi (1977) showed that the injection of MSH stimulates the mitosis and differentiation of epidermal melanoblasts into melanocytes in newborn mice. According to Lerner (1980), the binding of MSH to its receptors on the melanocytes causes the activation of adenylate cyclase. In turn, adenylate cyclase catalyses the conversion of ATP to cyclic-AMP. Cyclic-AMP, through the activation of an as yet unknown protein kinase, causes the activation of tyrosinase. MSH also appears to stimulate the formation and translocation of melanosomes and the formation of dendrites in the melanocyte (Hirobe and Takeuchi 1977).

Melatonin, a hormone produced in the pineal gland, is also important in melanogenesis. Weatherhead and Logan (1981) have shown that melatonin blocks the stimulatory effect of MSH and cyclic AMP on melanin production. Tyrosinase activity is unaffected by melatonin, so this hormone must block some post-tyrosinase step in the melanin biosynthetic pathway (Ibid.).

1.3. THE DEVELOPMENT OF THE SKIN AND HAIR FROM BIRTH TO 15 DAYS IN MICE

An early histological study, Gibbs (1941), described in detail four stages in the post-natal development of the skin and hair of

mice. In stage 1 (1-4 days) the epidermis increases in thickness and primary hair fibers pierce the surface. From day 1 to day 3 the dermis increases in thickness. In stage 2 (4-13 days), the epidermis decreases in thickness, while the dermis increases rapidly in thickness. Hair proliferation is maximal from day 7 to 12. The subcutaneous adipose layer reaches greatest thickness 9 days after birth, decreasing in thickness thereafter until day 28 (Slee 1962). In stage 3 (13-15 days) the decrease in thickness of the epidermis ceases. Follicle formation and growth ceases. From day 11 to day 15 the dermis decreases in thickness. In stage 4 (15-21 days), the epidermis reaches maturity, assuming the characteristics of adult skin. Hair growth ceases until the mice reach 28 days of age.

The continuously recurring events of hair growth, quiescence, and loss are referred to as the hair cycle. In mice the complete cycle lasts 28 days (Fraser 1951).

1.4. GENOTYPIC DIFFERENCES BETWEEN BLACK AND BROWN MICE

C57BL/10J (black) and C57BR/cdJ (brown) mice are thought to differ at only one locus known to affect coat color in mice, this being the brown locus (Hirobe 1984). Both C57BL/10J and C57BR/cdJ are non-agouti strains, with non-agouti referring to the abscence of pheomelanin (Ito et al. 1984). C57Br mice arose as a naturally occurring mutation from C57Bl stocks in the 1920's (Silvers 1979). Presently, both strains are maintained by Jackson Laboratories of Bar

Harbor, Maine.

1.5. PHENOTYPIC DIFFERENCES BETWEEN BLACK AND BROWN MICE

A summary of the phenotypic differences between black and brown mice is presented in Table 1.1.

1.5.1. Structural

1.5.1.1. Melanocytes

Hirobe (1984) found that the epidermis of brown mice had significantly more melanocytes than that of black mice from day 2 to day 10 after birth. Hirobe also found the total population of melanocytes and melanoblasts to be identical in these two lines of mice, but after 3 days of age all the melanoblasts had differentiated into melanocytes in brown mice, whereas numerous melanoblasts continued to be present in black epidermis at all developmental ages. Therefore, if all other aspects of melanogenesis were identical in black and brown mice, neo-natal brown mice would be capable of having a higher skin melanin content as they have many more active, melanin-producing melanocytes than do black mice.

TABLE 1.1

PHENOTYPIC DIFFERENCES BETWEEN BLACK AND BROWN MICE

	BLACK	BROWN
1. Melanocytes	1. fewer melanocytes	1. more active melanocytes day 2 to 10 after birth
	2. undifferentiated melanoblasts at all ages	2. all melanoblasts differ-entiate into melanocytes
2. Melanosomes	1. oval shaped	1. round
	2. larger	2. smaller, more uniform size
	3. matrix organized more regular framework	3. matrix less organized, more tangled
	4. melanosomal matrix obscured by melanin	4. melanosomal matrix visible even in mature melanosomes
3. Melanin		
A. In hair	1. 2 to 3 as times much melanin as in brown	1. 1/3 to 1/2 as much melanin as in black
	2. melanin finely granular	2. melanin coarsely granular
B. In skin	1. natural melanin accumulations higher?	1. natural melanin accumulations lower?
	2. incubation with precursors less produced	2. incubation with precursors more melanin produced
4. Tyrosinase	1. more T4 (active *in vivo*)	1. less T4?
	2. less T1 (active *in vitro*)	2. more T1?

1.5 1.2. Melanosomes

Melanosome structure also differs in black and brown mice. Brown melanosomes are smaller than black ones and show less variation in size (Russell 1949). Black melanosomes are usually oval in shape, whereas brown melanosomes are usually round (Russell 1948). Differences also exist in the form of the melanosomal matrix, with black mice having a more regular framework of membranes and brown mice having a more tangled, less organized matrix (Rittenhouse 1968). Due to these structural differences the melanosomes of brown mice share certain similarities with pheomelanosomes which are also spherical in shape, but are slightly smaller and completely lack an organized matrix (Jimbow et al. 1981)

1.5.1.3. Melanin

Brown mice have only 1/3 to 1/2 the hair melanin content of their black counterparts (Ibid.). Foster (1963) reported that the skin of homozygous black mice had more melanin than the skin of crossbred (black x brown), with crossbred mice having a higher melanin content than homozygous brown mice. Foster did not specifically state the age of these mice, merely referring to them as 'young' mice. When viewed under the electron microscope by Moyer (1966), melanin in brown melanosomes was coarsely granular and flocculent, with the underlying

matrix often visible even in mature melanosomes. The melanin of black melanosomes was very finely granular and almost appeared homogeneous, with the underlying matrix often obscured within three days after birth.

It has been assumed that the observed difference in color between black and brown mice was due solely to changes in the amount of melanin and melanosome size and shape (Silvers 1979). More recently, Ito et al. 1984a) concluded that due to the lack of exhaustive tests, the possibility remains that black melanin is not structurally identical to brown melanin.

1.5.2 Enzymes and Hormones

1.5.2.1 Enzymes

Coleman (1962) found that peak tyrosinase activity occurred four to six days after birth in both black and brown mice. However, brown mice showed more than twice the tyrosinase activity of black mice when skin samples were incubated with tyrosine. Foster (1963) also found that brown mice had a significantly higher tyrosinase activity than black mice under conditions of substrate saturation, with crossbred mice having an intermediate activity. Paradoxically, crossbred mice were found to have a significantly higher tyrosinase activity than brown mice when skin samples were incubated with dilute concentrations of tyrosine and 3,4 dihydroxyphenylalanine (dopa). A problem with

these studies was in all cases tyrosinase activity was equated with melanin production. The existence of other enzymes influencing melanogenesis was not considered.

Hearing et al. (1987) demonstrated the existence of four isozymes of the enzyme tyrosinase. Of the four isozymes only isozyme 4 is associated with the melanosomes and is actively involved in melanin synthesis. The other isozymes are thought to be precursors of isozyme 4. Isozymes 1 and 3 do not appear to promote melanogenesis in vivo as they are not associated with the melanosomes, but are active in melanogenesis in vitro. An early study, Holstein et al. (1967) reported only three isozymes. In brown mice, two of these were found to be significantly less reactive to dopa as compared to the same isozymes in black mice. It is unknown how the isozymes of Holstein and co workers relate to those of Hearing et al. However, it is possible that brown mice have a relative shortage of isozyme 4 which would greatly limit their rate of melanin synthesis in comparision to that of black mice. It is also possible that brown mice have an abundance of the tyrosinase isozymes which are inactive in vivo, but which are active under in vitro conditions. If true, this would explain some of the apparent contradictions found in in vivo and in vitro studies. Other than differences in tyrosinase, non-agouti black and brown mice have not been demonstrated to differ in any other enzymes known to affect melanogenesis such as dopachrome oxidoreductase (Murray et al. 1983). However, the possibility of differences in enzymes as yet uncharacterized cannot be discounted.

1.5.2.2 Hormones

Although Geschwind et al. (1972) found that the injection of MSH had no effect on the hair pigmentation of black or brown mice, Hirobe (1984) speculated that brown mice may have an increased amount of MSH or MSH receptors in melanoblasts, due to the higher frequency of differentiation of melanoblasts into melanocytes in brown mice. Of course, it remains entirely possible that the increased differention of melanoblasts in brown mice could instead be due to an inhibition in the effects of melatonin.

1.6. THE BROWN LOCUS, THEORIES EXPLAINING ITS ACTION

Although it has been determined through traditional breeding experiments that the brown locus is on the fourth chromosome in mice (Hirobe 1984), its gene products and their exact mode of action have not been determined. The most commonly proposed theory is that the brown locus specifies the synthesis of a melanosomal matrix protein (Foster 1963, Moyer 1966, Holstein et al. 1967, Rittenhouse 1968). Foster (1963) proposes three possible functions for this matrix protein. The first possible function is binding tyrosinase to other matrix proteins. This could secondarily alter the activity or stability of tyrosinase, which would in turn influence melanin deposition. A second possible function lies in the provision of

conjugation sights for melanin polymers, which would also influence the amount of melanin bound to the melanosomal matrix. The third proposed function is the binding of matrix proteins to each other, which would influence the structure of the melanosome. This postulated matrix protein could perform one or a combination of these functions. Brumbaugh et al (1979) speculate that the brown locus in mice codes for a matrix protein quantity regulator, with brown homozygotes producing too much matrix protein. Presently, these theories remain unsupported by experimental evidence.

In opposition to these matrix protein theories, Murray et al (1983) found that melanosome-free homogenates of brown skin synthesized brown melanin and black skin synthesized black melanin. They speculate that the skin contains enzymes which regulate the formation of black and brown melanin. This enzyme difference may be in the conversion of dopa to dopaquinone, as Ito et al (1984) found the levels of free dopa to be significantly higher in non agouti brown mice as compared to black mice.

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CHAPTER 2. EFFECTS OF THE BROWN LOCUS ON MELANIN DEPOSITION IN NEONATAL MICE

2.1. INTRODUCTION

A specific phenotypic expression is generally a result of the action and interaction of many genes. It is therefore usually difficult to determine the role of individual genes in the manifestation of the phenotype. However, in some instances a single gene can have a major influence on a phenotype. The genes affecting pigmentation in mice offer some extremely simple systems for study where alleles of a single gene have readily apparent influences on the phenotype. Through traditional breeding experiments, many different loci and alleles of these loci affecting pigmentation in mice have been identified. Of these loci, the brown locus was selected for study for two reasons: (1) it involves a single gene (Hirobe 1984), (2) its effects on the phenotype are limited to differences in eumelanin without the added complication of pheomelanin differences (Ito et al. 1984).

In this study, two alleles of the brown locus, black (B) and brown (b) were chosen. The effect of these alleles on the pigmentation of the mouse coat has been well documented, hairs of black mice contain two to three times more melanin than those of brown mice (Ito et al. 1984). Differences are also present in the shape and structure of the melanosomes (pigment granules), with melanosomes of black mice being

larger, less uniform in size (Russell 1948) and having a more organized matrix (Rittenhouse 1968) than those of brown animals. However, the effect of interaction of these two alleles in crossbred mice has not been investigated. It has been generally assumed that the B allele masks the effects of the b allele in the heterozygous state (Silvers 1979), but quantitative assays for the melanin content of crossbred hair have never been reported.

The relationship between hair and skin pigmentation in black and brown mice has been even less clearly defined. The melanocyte populations of the hair follicles and skin are identical in origin, both migrating from the neural crest in early embryonic life (Rawles 1948). Foster (1963) reported that the skins of young black mice contained more melanin than the skins of crossbred mice of the same age, while brown skins contained significantly less melanin than did those of crossbred mice. A seeming contradiction also reported by Foster (1963) is that when skin samples were incubated in vitro with melanin precursors, brown skin produced more melanin than did crossbred, with black skin producing the least amount of melanin.

One difference between skin and hair is that the melanin content of hair has not been reported to fluctuate over time except in the case of old, greying mice and mice carrying mutations such as 'Silver'(Silvers 1979). In contrast, the melanin content of mouse skin

is thought to show a large variation with age. Mice are born with very few active melanin producing melanocytes in their skin and therefore with very little melanin (Hirobe and Takeuchi 1977). Melanoblasts rapidly differentiate into melanocytes and melanin is then produced. After the mice begin to develop hair, the melanocytes in the skin gradually cease melanin production, with the majority of melanocytes dying. A small proportion of melanocytes remains alive, but ceases melanin production so that by 15 days after birth essentialy no active melanocytes remain in the skin (Ibid).

Although brown mice have significantly more melanocytes in their skin than black mice from 2 to 10 days of age (Hirobe 1984), Foster (1963) reported that young brown mice produce less melanin than black mice. However, Foster (1963) did not report the age of his mice. As the melanin content of the skin changes with age, the possibility exists that the relationship between the amounts of melanin in the skins of black and brown mice might also vary with age. The development of melanin in the skins of crossbred mice would also be of interest as it would reveal the interaction between these two alleles of the brown locus.

This study had three major objectives:

(1) to compare the melanin content from birth to 25 days of age in skins of black, brown and crossbred mice in order to determine the age-related effects of the brown locus on skin pigmentation;

(2) to compare the effects of the brown locus on skin with

its known effects on hair pigmentation; and

(3) to compare values for skin melanin content with the melanocyte counting data of Hirobe and Takeuchi (1977) and Hirobe (1984) for the same strains of mice.

From this data, it was hoped that the effects of the B and b alleles on skin pigmentation could be better understood. It was also hoped that, with this information, a theory could be postulated as to the nature of the gene products of alleles that produce the differing phenotypes.

2.1. MATERIALS AND METHODS

2.1.1. Animals

C57BL/10J black (genotype BB) mice (mus musculus), C57BR/cdJ brown (genotype bb) and crossbred mice (genotype Bb) served as subjects. The only difference in loci known to affect the pigmentation of these mice is the allelic difference at the brown locus. All strains of mice were non-agouti (lacking pheomelanin, genotype aa), non-albino (genotype CC) and lacked the dilution gene (genotype DD) (Hirobe 1984). Brown mice were obtained from Jackson Laboratories of Bar Harbor, Maine. Black mice were obtained from the MSB Animal Unit of the University of Alberta from a population derived from Jackson Laboratory stock. All mice received commercial mouse

chow and water ad libitum. Mice were maintained at 23 ± 1 C with 35 % relative humidity, with 12 hours of fluorescent light provided daily. Noticeably pregnant mice were placed under close supervision and checked every 2 hours for the presence of pups.

2.1.2. Sample Collection.

For the first experiment, skin samples were collected from mice at 17 ages after birth ranging from 2 hours to 15 days (Table 2.1). In the second experiment, samples were collected at 15, 20 and 25 days after birth (Table 2.2). The error in age estimation was ± 2.5% of age in all cases except for mice under 2 days of age. For these mice, error was equal to ± 1 hour.

2.1.3. Skin Homogenates

Mice were sacrificed by cervical dislocation and hair was then removed from the skins by the application of a commercial depilatory (Neet, Whitehall Laboratories). Neet was applied to the skins of all mice, regardless of age. Neet was tested and found not to influence assay of melanin (Appendix 2). The skin of the torso was removed and scraped free of underlying connective tissue and subcutaneous fat. Skins were then stored at -70 C until assayed for melanin content. For assay, the skin was finely minced with scissors into pieces approximately 2 mm^2. A 0.25 g sample of minced skin was

TABLE 2.1

EXPERIMENT 1 MEAN MELANIN CONTENT OF THE SKINS OF BLACK, BROWN AND CROSSBRED MICE FROM BIRTH TO 15 DAYS OF AGE, NUMBER OF OBSERVATIONS PER MEAN AND STANDARD ERRORS OF THE MEAN

Each sample collected from a separate litter

	BLACK			BROWN			CROSSBRED		
Age (days)	Melanin (mean)	SEM	n	Melanin (mean)	SEM	n	Melanin (mean)	SEM	n
0.1	.47	.13	3	.62	.23	3	.10	.12	3
0.3	.71	.08	3	.43	.14	3	.89	.46	3
0.5	.37	.26	3	.54	.00	1	.54	.01	3
1.0	.34	.19	3	.03	.23	2	.06	.20	3
1.5	.31	.07	3	.18	.38	3	.16	.79	2
2.0	.72	.24	3	.13	.00	1	1.40	.55	3
2.5	1.10	.26	3	.62	.00	1	1.49	.44	3
3.0	1.76	.45	3	.21	.00	1	1.52	.31	3
3.5	1.63	.38	3	.76	.00	1	3.60	1.30	3
4.0	2.33	.11	3	1.15	.12	2	2.98	.33	3
5.0	4.70	.42	3	1.85	.16	3	4.35	1.39	3
6.0	7.15	.68	3	2.45	.54	3	6.29	.55	2
7.0	8.60	.39	4	3.30	.17	3	8.25	.01	3
8.0	9.98	.98	3	2.96	.08	2	8.00	.05	2
10.0	11.31	.50	3	4.11	.21	3	9.44	.57	4
12.0	13.00	.72	4	4.24	.79	3	9.01	.02	3
15.0	8.58	.45	3	6.01	.10	2	7.88	.74	3

SEM--standard error of the mean

Mean melanin expressed as ug melanin/mg protein

TABLE 2.2

EXPERIMENT 2 MEAN MELANIN CONTENT OF THE SKIN OF BLACK, BROWN AND CROSSBRED MICE FROM 15 TO 25 DAYS OF AGE. STANDARD ERRORS OF THE MEAN NUMBER OF OBSERVATIONS PER MEAN

	BLACK			BROWN			CROSSBRED		
Age (days)	Melanin (mean)	SEM	n	Melanin (mean)	SEM	n	Melanin (mean)	SEM	n
15	8.81	.05	6	5.00	.10	6	7.95	.06	19
20	1.22	.24	10	1.23	.15	6	1.99	.22	16
25	1.02	.17	11	.72	.26	6	1.47	.13	10

In all cases ≥ 2 samples were collected per litter

SEM Standard error of the mean

Mean melanin expressed as ug melanin/mg protein

removed and homogenized in a glass to glass homogenizer with 4 ml of a 0.1 M phosphate buffer (pH 6.8).

2.1.4. Melanin Determination

Melanin content of the skin homogenate was determined through a modification of the method for hair follicles reported by Logan and Weatherhead (1978). Triplicate 0.25 ml aliquots of skin homogenate were incubated for 3 hours at 37 C with 2.5 ml pronase (200 ug/ml, Sigma) in 0.01 M Na_2CO_3, adjusted to pH 7.8 with 2 N HCl. Following incubation, 3.5 ml of 0.06 M Na_2CO_3 and 15 ul 30% H_2O_2 were added. The tubes were then boiled for 30 minutes and allowed to cool. Three ml of a chloroform:methanol (2:1) mixture were added to remove the lipid from the homogenate and the tubes were vortexed. The tubes were then centrifuged at 1000g for 10 minutes. The fluorescence of the aqueous phase was measured with a Perkin-Elmer spectrophotofluorimeter at 410 nm excitation and 500 nm emission. All sample readings were corrected for nonspecific skin fluorescence values determined for the skin of 1-day-old ICR (albino) mice. The melanin content of the skin homogenates was then determined by comparison to a standard curve of dilutions of a synthetic melanin (Sigma) which was treated in the same manner as the skin homogenates. Protein content of the homogenate was measured by the method of Lowry et al. (1951) and results were expressed on a ug melanin/mg protein basis.

STATISTICAL ANALYSIS

Melanin content of the skin homogenates was calculated as follows. $F_{obs} = F_{melanin} + F_{nonspecific} + \Delta$. F_{obs} being observed fluorescence, $F_{melanin}$ being fluorescence due to melanin, $F_{nonspecific}$ being fluorescence due to skin contituents other than melanin, and Δ being variation between standards on different days of assay.

The fluorescence observed on analysis of skins of 1 day old albino mice (n = 11 in experiment 1, n = 7 in experiment 2) was taken to represent the nonspecific fluorescence and was divided into two components: (1) the fluorescence due to skin protein and (2) the fluorescence due to unidentified skin constituents. Fluorescence of albino homogenate samples was found to have a correlation of approximately .99 with skin protein content in both experiments. Ignoring the minor effect of unidentified skin constituents, $F_{nonspecific}$ was calculated by means of a simple regression of fluorescence of albino homogenate samples on their protein contents. The regression equations were $F_{nonspecific} = 10.4 \times$ protein for experiment 1, with $F_{nonspecific} = 6.75 \times$ protein in experiment 2. $F_{nonspecific}$ was then subtracted from F_{obs}. Δ, which represents variation in fluorescence between days of assay was calculated as follows. In each assay, four melanin standards were run in triplicate. The fluorescence values obtained for standards of the same concentration were averaged in order to determine the standard

curve for each experiment. The standard curves from 16 and 7 assays were averaged to make up the mean standard curves for experiments 1 and 2, respectively. For individual assays, the values obtained for the melanin standards were then subtracted from the overall average values. These deviations from average were totaled and divided by 4 (the number of standards) in order to determine the mean deviation of each standard on a particular day of assay from average values, this being Δ. Δ was then subtracted from F_{obs}. Melanin content of the samples was then determined by a simple regression of average fluorescence values of standards of the same concentration on melanin concentration. For experiment 1, melanin = $0.80064 \times F_{melanin}$. In experiment 2, melanin = $1.371 \times F_{melanin}$.

For each strain of mice, samples were collected at 20 ages. Up to four samples were collected per age, with each sample taken from a separate litter in experiment 1 (Table 2.1). In experiment 2, up to 2 samples were collected per litter (Table 2.2). In both experiments, problems were encountered during sample collection due to maternal misbehaviors leading to death of the pups. These maternal misbehaviors and other behavioral observations are outlined in Appendix 1.

Once sample collection was completed, average melanin content and standard error were then calculated. Once average melanin contents of the skin homogenates were determined, melanin curves were fitted to the data points. The three separate curves for black, brown, and crossbred animals were then compared at six age intervals in separate

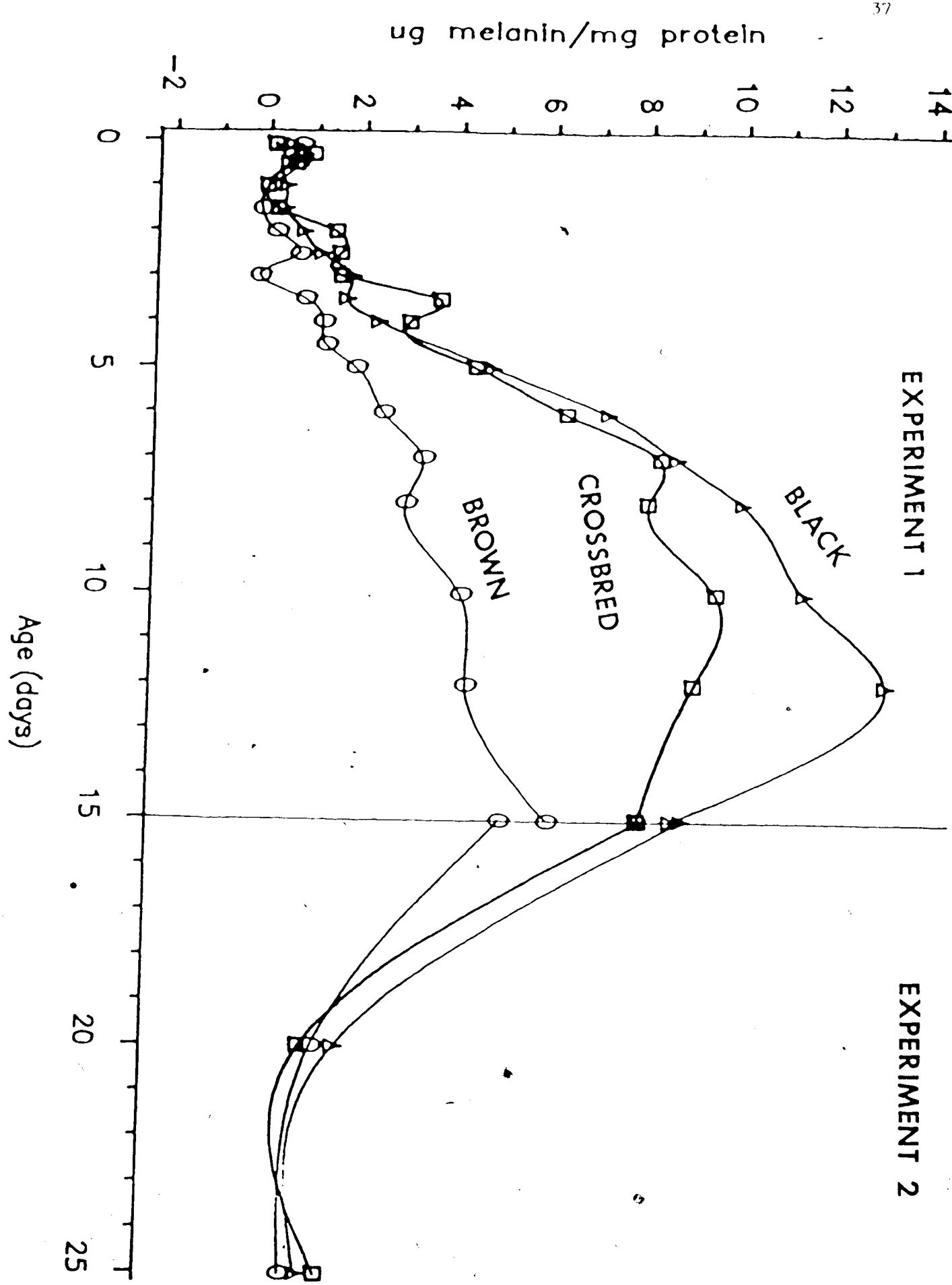
analyses using Student Newman-Keuls (SNK) multiple comparison of means (Steele and Tory 1980).

2.4. RESULTS

2.4.1. EXPERIMENT 1 (newborn to 15 days of age)

Mean melanin contents of skin of black, brown and crossbred mice from an age of 2 hours to 25 days are plotted in Figure 2.1 and presented in Tables 2.1 and 2.2. Brown mice showed a linear trend for development of melanin up to 15 days of age, with the equation for this line being melanin (m)= .4058 age (a) - .1369, R^2= .86. For crossbred and black strains, the development of melanin with age most closely followed the curve of a third degree polynomial. The equation for the curve in black mice was m = -.0032 a^3 + 1.54 a - 1.5507, R^2= .90 and for crossbred mice was m = -.0031 a^3 + 1.303 a - 1.312, R^2= .91 as determined by stepwise multiple regression. Initial accumulation of melanin began at 1 to 1.5 days of age in all lines of mice. The most rapid rate of melanin accumulation in black and crossbred mice occurred approximately 5 to 6 days after birth, with peak skin melanin occurring at approximately 10 to 12 days after birth. These three age ranges (1 to 1.5, 5 to 6 and 10 to 12 days) were then chosen as a basis of comparison between the three lines of mice. Using SNK comparison of means, black mice were found to have significantly higher ($P < 0.05$) melanin contents than brown mice over

Figure 2.1. The accumulation of melanin in the skins of neonatal mice. Mean values for ug melanin/mg protein. Numbers of observations per mean and SNK comparisons of means are presented in Tables 2.1 and 2.2.


ug melanin/mg protein
-2
0
2
4
6
8
10
12
14
0
5
10
15
20
25
Age (days)
EXPERIMENT 1
EXPERIMENT 2
BLACK
CROSSBRED
BROWN

the entire range of ages. Crossbred animals were found to have higher melanin contents ($P < 0.05$) than brown animals over the entire range of ages. Black mice were found to have significantly higher melanin contents ($P < 0.05$) than crossbreds only for the 10 to 12 days age group. Brown mice had a slower rate of accumulation of skin melanin than either black or crossbred mice, but they also did not peak at 10-12 days as did the black mice. After 12 days of age, both black and crossbred mice showed marked decreases in skin melanin content.

2.4.2. EXPERIMENT 2 (15 to 25 days of age)

Results for melanin content of skins of each line at 15 days of age were similar in experiment 1 and 2. At 15 days of age, brown mice had a significantly lower skin melanin content ($P < .05$) than did all other strains of mice. There was no significant difference in melanin content between the other strains of mice (black and crossbred) at 15 days of age. The melanin content of brown skin began to decrease after 15 days of age and by 20 days of age no significant difference existed between the melanin content of all lines. From 20 to 25 days of age, very little melanin was present in the skin of all lines of mice.

As skin samples were taken from the same litter at 15, 20 and 25 days in experiment 2, it was possible to measure variation between litters in melanin content. Variation between litters of the same breeding was not significant except in the case of black mice.

However, interlitter variation was not measurable in brown mice due to the low number of litters obtained. Intra-litter variation appeared reduced in black mice as compared to that of crossbred mice as reflected by reduced residual values, but this difference was not significant.

2.5. DISCUSSION

2.5.1. EFFECTS OF THE BROWN LOCUS OVER TIME

The alleles of the black/brown locus (B) and (b) have been assumed to be a textbook example of Mendelian dominance, where the effects of the b allele are masked by the presence of the B allele (Silvers 1979). In homozygous brown (bb), the b allele was found to greatly reduce the accumulation of skin melanin over time as compared to homozygous black (BB) mice. Peak skin melanin content in brown mice occurred at 15 days of age and was approximately 50% of the maximum skin melanin atta ned in black mice. Contrary to classical dominance beliefs, in heterozygotes, the b allele also reduced skin melanin accumulation. Apparently heterozygotes were unable to maintain maximal rates of melanin accumulation for the same length of time as homozygous black mice. Peak melanin content of crossbred skin was 27% less than the maximum melanin of black skin. Other than this difference in maximal melanin (from age 10-12 days), black and crossbred mice followed similar patterns of skin melanin accumulation

from birth to 25 days of age. After 15 days of age, as melanin levels approached zero in all lines of mice, substitution of alleles at the brown locus had no significant effect on skin pigmentation.

2.5.2. COMPARISON OF EFFECTS OF BROWN LOCUS IN SKIN AND HAIR

The action of the brown locus appears to be similar in both hair and skin. In hair, Ito and co-workers (1984) determined that black mice have 2 to 3 times more melanin than do brown mice. In skin, when comparing maximum levels of melanin attained, black mice have approximately 2 times more melanin than do brown mice. If crossbreds follow the same pattern as black and brown mice, it is possible that crossbred hair contains less melanin than black hair. Further experimentation would be necessary to confirm this hypothesis.

2.5.3. MELANOCTYE COUNTING VS. MELANIN ASSAY

Hirobe (1984) found that the number of active melanocytes in the skin reached a maximum in C57Br/cdj (brown) mice at approximately three days after birth, while in C57Bl/6j (black) mice the number of active melanocytes peaked at four days of age. After this time, Hirobe and Takeuchi (1977) found that the number of active melanocytes decreased sharply until essentially none were present in the epidermis 15 days after birth. Comparing this melanocyte counting data to the results of the present study, 2 observations can be made: (1) melanin

content of the skin continues to increase after 3 to 4 days of age even though melanocyte numbers are decreasing by this time, (2) a lag exists between the times of maximum melanocyte numbers and maximum melanin content. To explain these observations, the factors influencing the measurement of melanin content of the skin must be considered. The melanin level present in the skin is the result of two opposing functions, melanin production and melanin loss. Melanin is produced by active melanocytes and is lost irreversibly with the continuous sloughing of the outer layer of the epidermis (Wood 1985). The melanin polymer is highly resistant to degradation. However, the protein matrix of the melanosome is degraded, resulting in melanin fragments (Quevedo 1973). In this study, prior to assay of melanin, skin samples were incubated with a non-specific protease (Pronase, Sigma). As all melanin would therefore be in the form of melanin fragments, any naturally occurring degradation of the protein matrix of the melanosome would not result in melanin loss within the bounds of this assay. Therefore, the only means of melanin loss would be through the sloughing of the uppermost layer of the epidermis.

A typical cell of the human epidermis takes 2 to 3 weeks to migrate upwards through the different epidermal layers before being shed (Wood 1985). Values for the time of epidermal turnover in neonatal mice have not been reported. Melanin content of the skin would therefore continue to increase as long as less melanin was lost from sloughing the top layer of the epidermis than was produced by the melanocytes in the basal layer of the epidermis. The time lag between

maximum melanocyte number and maximum melanin content may reflect the approximate time needed for an epidermal cell to migrate upward through the layers of the epidermis before being shed. This time lag was 8 and 12 days in black and brown mice, respectively. It is therefore possible that the epidermis of black mice turns over more rapidly than the epidermis of brown mice. Further experimentation to measure the rate of epidermal turnover directly in these mice would be necessary to test this hypothesis.

Viewing the results of these studies it is apparent that the b allele limits melanin accumulation. The question still remains, how does the b allele cause a reduction in melanin accumulation? Theories have been proposed explaining the action of the brown locus. The most common theory is that the brown locus specifies the synthesis of a melanosomal matrix protein (Foster 1963, Moyer 1966, Holstein et al. 1967, Rittenhouse 1968). This theory could explain the structural differences between the melanosomes of black and brown mice. Brown mice have also been shown to be almost completely lacking in one of the isozymes of tyrosinase (Holstein et al. 1967). As only one isozyme of tyrosinase is known to promote melanogenesis <u>in vivo</u> although the other isozymes are active <u>in vitro</u> (Hearing et al. 1982), it is possible that brown mice are in short supply of the tyrosinase that is active <u>in vivo</u>. Hearing and co-workers (1982) theorized that tyrosinase becomes active <u>in vivo</u> with the addition of a polypeptide that links the enzyme to the melanosomal matrix. The brown locus may

code for this polypeptide. Additional research will be required to test this hypothesis.

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CHAPTER 3 GENERAL DISCUSSION

In this study, curves for melanin deposition over time in black and crossbred mice were best described as third degree polynomials. The curves of the two lines followed similar trends but maximum skin melanin attained from 10 to 12 days of age in black mice exceeded that of crossbreds. After 12 days of age, skin melanin decreased so that by 20 days after birth melanin content approached zero in both lines of mice. Brown mice have a different curve that increased linearly until 15 days of age. After 15 days of age, the melanin content of the skins of brown mice, also began to decrease. By 20 days after birth, as melanin content of the skin approached zero, brown mice also followed the same pattern as both blacks and crossbreds. It is therefore obvious that the presence of the homozygous b allele in brown mice influences melanin accumulation in the skin until 15 days of age, while the influence of the b allele in heterozygotes is only apparent in the reduced maximum from 10 to 12 days of age. In order to speculate on the mechanism by which the b allele influences melanin deposition in both homozygous brown and crossbred mice, other factors known to influence the melanin deposition curves in all lines of mice must first be discussed.

Mice are born with very few active, melanin producing melanocytes (Hirobe and Takeuchi 1977). As the melanoblasts that are present in the skins of newborn pups differentiate into melanocytes, they begin to produce melanin. Hirobe (1984) found that by 3 and 4 days after birth in brown and black mice, respectively, the maximum number of active melanocytes are present in the skin.

For the initial days after birth until significant numbers of melanoblasts differentiate, the melanin concentration of the skin would be very low. In this study, melanin concentration did not reach appreciable levels until approximately 2 days after birth. Therefore, the initial stages of the melanin deposition curve in all lines of mice could be governed by the rate of differentiation of melanoblasts into melanocytes. As increasingly more melanoblasts differentiated into melanocytes, melanin content of the skin would continue to increase.

Once mice begin to develop hair, the number of melanocytes present in the skin begins to decline as the melanocytes either die or become inactive (Hirobe and Takeuchi 1977). In the present study, an eventual decline in skin melanin content was observed in all lines of mice. Slee (1962) reported that mice grow a complete hair coat by 15 days of age. In the present study, melanin content of the skin approached zero by 20 days of age in all lines of mice. Hair growth influences skin melanin content because hair shields the skin from

exposure to ultraviolet (UV) light. Kodama et al (1984) found that in mice, hair protected the skin from 94% of the DNA damage due to UV light. Exposure to UV radiation stimulates the production of melanin (Prota 1980). If the hair was removed from the mice, exposure to UV light would reactivate the small population of melanocytes remaining in the epidermis (Quevedo and Smith 1963). The final stage of the melanin deposition curve in mice could therefore be governed by the rate of death or inactivation of the melanocytes indirectly due to the growth of the hair coat. As more melanocytes died or became inactive, skin melanin content would continue to decrease until it approached zero as Hirobe and Takeuchi (1977) found virtually no melanocytes in the epidermis of mice by 30 days after birth.

Hirobe (1984) found that brown mice had significantly more melanocytes in their skin than black mice from 2 to 10 days after birth. If all other factors were equal, it would therefore be expected that brown mice would have more melanin in their skins than black mice over this time period. However, the results of the present study and others indicate that this is not the case.

Factors that may contribute to brown mice producing less melanin than black mice include: (1) changes to the structure of the melanosomal matrix in brown mice, resulting in a less organized, more tangled matrix (Rittenhouse 1968); and (2) less fully melanized melanosomes in brown mice (Moyer 1966). In vitro studies have revealed that brown mice have twice as much tyrosinase present in

their skin and can produce approximately twice as much melanin as black mice when skin samples are incubated with melanin precursors such as tyrosine (Coleman 1962).

Many theories exist as to the cause of these differences between black and brown mice, most of which speculate that the brown locus specifies the synthesis of a melanosomal matrix protein (Foster 1963, Moyer 1966, Holstein et. al 1967, Rittenhouse 1968). More recently, Hearing et. al (1982) demonstrated the existence of 4 isozymes of the enzyme tyrosinase. Only one of these isozymes is thought to be active in promoting melanogenesis *in vivo*; while 2 other isozymes appear to promote melanogenesis *in vitro*. The isozyme that is active *in vivo* is linked to a polypeptide which may be needed to join the tyrosinase to the matrix of the melanosome before melanogenesis can begin. It is possible that the brown locus may code for this linker polypeptide.

The melanin accumulation curve in brown mice could be controlled by the availability of an effective tyrosinase-linker polypeptide. The melanin deposition curve in brown mice would be linear if there was a constant, limited amount of this factor available. As the existence of tyrosinase-linker polypeptide remains as yet an unproven theory, it is possible that the melanin deposition curve is linear due to the shortage of some other factor necessary for melanogenesis. However, until disproven, the tyrosinase-linker polypeptide theory offers the most complete explanation for the observed phenotypic differences between black and brown mice.

The melanin accumulation curve in crossbred mice differs from that

of black mice only at the time of maximum melanin deposition which occurs from 10 to 12 days of age. It is possible that crossbred mice can produce sufficient quantities of the polypeptide specified by the brown locus to support melanogenesis at rates comparable to those of black mice until the rate of melanin deposition reaches its maximum. At this time, crossbred mice may not be able to produce enough of the necessary polypeptide to support maximal rates of melanin deposition. This would result in crossbred mice having lower maximum melanin concentrations in their skins than black mice.

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CHAPTER 4 APPENDICES

APPENDIX 1 SOME OBSERVATIONS ON THE MATERNAL BEHAVIOR OF C57/BL/10J (BLACK) AND C57/BR/cdJ (BROWN) MICE

4.1.1. INTRODUCTION

For studies of neonatal development of mice, it is often necessary to have accurate estimates of the time the pups were born. However, little previous work has examined the factors affecting the distribution of births throughout the day in mice. Some very early work by Merton (1938) reported that 2/3 of 164 births in albino mice occurred in the dark, between the hours of 4 p.m. and 4 a.m., with parturition lasting from 1/2 to 3 hours depending on the degree of difficulty of the birth. Other than darkness, no other factors have been reported to affect the time of mouse parturition.

Another crucial factor in studies of neonatal development of mice is pup survival. Female mice exhibit a variety of maternal activities which serve to maintain their pups, including nest-building, feeding, retrieval, cleaning and defence of young (Gandelman and Simon 1977). Less commonly, female mice engage in behavior which leads to the death of the young. This behavior can be of two types: (1) neglect of the young such as lack of feeding and (2) active killing and eating of offspring (Fraser and Barnett 1975). The causes of this abberant

maternal behavior have not been fully determined. Gandelman and Simon (1977) found that the relatively undernourished young of large litters (11-16 pups) were killed preferentially. They postulated that these pups were killed because they either do not emit cues that initiate or maintain maternal behavior, or they emit cues which specifically elicit killing. Pup killing behavior has also been elicted through removal of the dam's olfactory bulbs (Gandelman et al. 1971) and by administration of testosterone (Gandelman and Davis 1972). Pup killing has also been found to decrease with parity (Fraser and Barnett 1975). However, as litter size also decreases with parity (Ibid), it is unknown whether the mice become better mothers with parity or if less pups are killed only because fewer are born.

Observations of maternal behavior reported here were made as a consequence of a related study involving neonatal mice. Three variables affecting maternal behavior were noted:

(1) the effect of darkness on parturition in black and brown mice;

(2) the effect of relative level of noise on parturition in black and brown mice; and

(3) The effect of light intensity on pup survival.

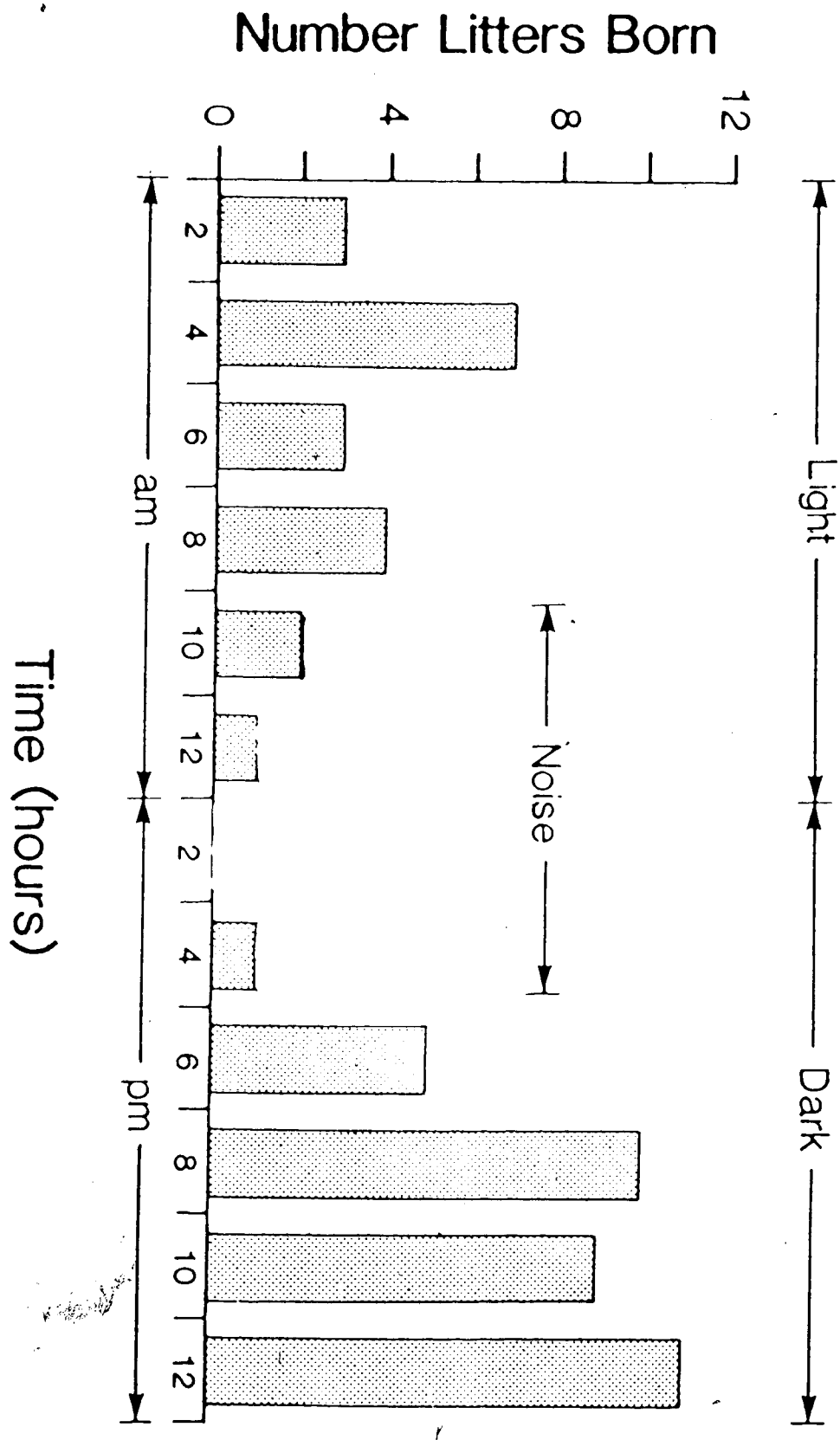
4.1.2. MATERIALS AND METHODS

Adult female mice of the C57/BL/10J (black) and C57/BR/cdJ (brown) strains were provided commercial diets and water ad libitum. Twelve hours of fluorescent light were provided daily, with the period of light from 12 a.m. to 12 p.m. The mice were acclimatized to this altered light-dark cycle for one month prior to parturition. When noticeably pregnant, females were removed from the breeding cages, placed in individual cages and supplied with a cotton nestlet. Pregnant mice were checked every 2 hours for the presence of pups. During the dark period, a flashlight was used to check for pups. The approximate time of birth ± 1 hour, and number of pups born/litter were recorded. The percentage of litters in which no pups lived 3 days post-partum served as a measure of pup survival. Pregnant females were originally housed under 30 candles/ft^2 (footcandles). Light was increased to 48 footcandles for 3 weeks and later decreased to its original intensity. Pup survival was recorded for litters born under these different intensities of light, as measured by a Spectra Combi-500 light meter.

4.1.3. RESULTS

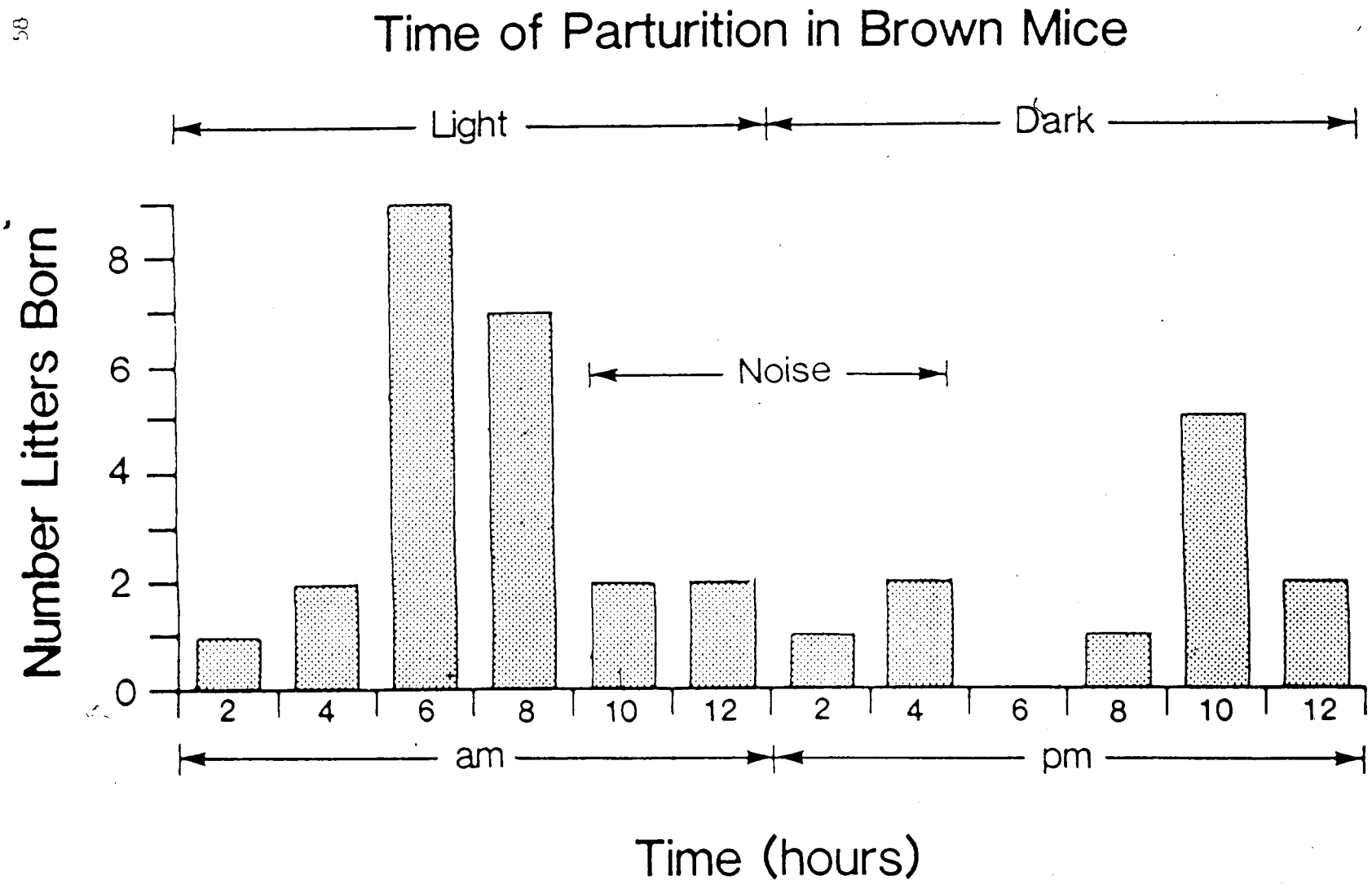
Frequency distributions for number of litters born at 2 hour intervals in black and brown mice are illustrated in Figures A1.1 and A1.2. Using Fisher's exact test, no significant differences were

Figure A1.1 Time of Parturition in Black Mice. All litters born on the weekends were grouped for analysis with those born in the quiet.



Time of Parturition in Black Mice

Figure A1.2 Time of Parturition in Brown Mice. All litters born on weekends were grouped for analysis with litters born in the quiet.


Time of Parturition in Brown Mice
Light
Dark
Noise
Number Litters Born
0
2
4
6
8
2
4
6
8
10
12
2
4
6
8
10
12
am
pm
Time (hours)

found between black and brown mice in the number of litters that were born in the light vs dark or in the noise vs quiet (Tables A1.1 and A1.2). Using a two way analysis of variance, light was found to have no significant effect on time of parturition in black and brown mice. Relative noise level was also found to have no significant effect ($P > .05$) on time of birth in these strains of mice, but a strong trend existed towards more mice being born in the quiet.

When comparing the effects of light intensity on survival of pups (Table A1.3), the percentage survival under the initial light intensity (30 footcandles) and the survival once light intensity was changed back to 30 footcandles were pooled. Using Fisher's exact test, increased light intensity was found to significantly decrease survival in black and brown pups ($P < .05$) and ($P < .001$), respectively. Under both light intensities, black pups were found to have a signicantly higher ($P < .001$) survival rate as compared to brown pups.

4.1.4. DISCUSSION

4.1.4.1. Effect of darkness and noise level on time of parturition

Merton (1938) reported that two thirds of albino mice were born in the dark between the hours of 4:00 p.m. and 4:00 a.m. In the present study, approximately two thirds of the black mice were also born over this time interval. However, this interval did not correspond exactly

Table A1.1 Litters born in light and dark in C57/Bl/10j and C57/Br/cdj mice

	C57/Bl/10j (Black)	C57/Br/cdj (Brown)
Light	35	29
Dark	39	14
Total	74*	43*

*An additional 8 litters in each color type could not be classified as light or dark.

Table A1.2 Litters born in quiet and noise in C5/Bl/10j and C5/Br/cdj mice

	C5/Bl/10j (Black)	C5/Br/cdj (Brown)
Quiet[1]	78	44
Noise[2]	4	7
Total	82	51

[1]Quiet hours included weekends, holidays and weekdays from 6:00 p.m. to 8:00 a.m.

[2]Noise hours from 8:00 a.m. to 6:00 p.m. weekdays

Table A1.4. Litter Mortality in C57Bl/10J (Black) and C57Bl/6J (Brown) Mice Under 2 Light Intensities

	LITTERS	LIGHTING 1 30 FOOTCANDLES	LIGHTING 2 88 FOOTCANDLES	LATER 2 90 FOOTCANDLES
BLACK	SURVIVED	50	13	90
	DIED	10	12	7
BROWN	SURVIVED	33	5	15
	DIED	42	27	10

with the dark period. When the entire light and dark periods were considered, approximately equal percentages of black mice were born in the light and dark. For the brown strain, less than 1/3 of the litters were born between the hours of 4:00 p.m. and 4:00 a.m. and only 1/3 of all litters were born in the dark. No significant effect of darkness on time of parturition was found in either strain of mice. Perhaps strain differences exist, although none were found between black and brown mice. It is therefore possible that darkness has no effect on the time of parturition in mice, but additional research would be necessary to test this hypothesis.

Noise has been recognized as a stressor in rodents causing increases in pulse, frequency of respiration, blood pressure and activity levels (Anthony 1962). In the present study, noise level was not measured directly. The time that the maximum number of people were present either in or around the animal rooms was considered the time of maximum noise. No significant effects of relative noise level on the time of mouse parturition was found. This was due in part to having only one error degree of freedom in a two-way analysis of variance. However, a trend exists toward increased numbers of litters being born in the relative quiet. This trend is strong enough ($P < .22$) to warrant studies directly investigating the effect of noise on parturition.

4.1.4.2. Effect of Light Intensity on Pup Survival

The light intensity under which the mice were maintained was not changed deliberately. Maintenance personnel replaced the bulbs in a previously nonfunctional light fixture. A sudden increase in pup mortality was then noted over a 3 week period. When the light was changed back to its original intensity, pup mortality declined sharply. A 60% increase in light intensity caused a significant increase in pup mortality in both black and brown mice, with brown mice showing a significantly larger increase than black mice.

Thompson (1956) reported striking differences in the activity level of 5 inbred mouse strains. C57/BR (brown) mice, used in the present study were the most hyperactive strain, followed by C57/BL (black). In an earlier study, Thompson (1953) reported that C57/BR mice were more nervous as compared to C57BL. Nervousness was measured by the percentage of mice defecating over ten 15 minute intervals. An increase in light intensity could cause an increase in mouse activity. As a result, the female mice may not remain long enough in the nest to provide the pups with adequate opportunities for nursing and warming. The pups would eventually die over the course of several days. The increased light intensity may have had a more severe effect on pup mortality in brown mice due to their increased nervousness and activity level as compared to black mice.

Both pup neglect leading to the eventual death of the pups and active pup killing were observed in black and brown mice. The number

of litters in which a proportion of the pups survived is unknown. Partial survival of litters would be very difficult to measure as females have been known to consume several of their offspring when eating the placenta (Gandelman and Simon 1977). Continuous surveillance of mice would therefore be necessary in order to measure the proportion of pups surviving in all litters.

These behavioral observations indicate the need for studies directly measuring the effect of light intensity on pup survival and the effects of noise and darkness on time of parturition. These observations might prove valuable as pup survival might be increased and better estimations of the time of parturition might be made without the necessity of 24 hour surveillance.

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APPENDIX 2 EFFECTS OF THE DEPILATORY NEET ON THE ASSAY OF SKIN MELANIN

4.2.1 INTRODUCTION

The objective of this study was to test if the depilatory Neet (Whitehall Laboratories) had any effect on the assay of skin melanin by the method outlined in Chapter 2.

4.2.2 MATERIALS AND METHODS

A litter of 12 C57BL/10J mice aged approximately 15 days was sacrificed by cervical dislocation. Neet was applied to the hair of 6 mice. After 5 min the mice were rinsed with warm water and all traces of hair and Neet were removed. The remaining 6 mice had their hair removed by means of shaving. The skins of all mice were removed and assayed for melanin content by the method outlined in Chapter 2.

4.2.3 RESULTS AND DISCUSSION

Raw fluorescence values for the skins of mice shaved and treated with Neet are shown in Table A2.1 By observation, no differences between treatments were noted. It can therefore be concluded that use

of the depilatory Neet (Whitehall Laboratories) has no effect on the assay of melanin in the skins of mice.

TABLE A2.1 Effects of the Depilatory Neet on the Assay of Melanin in the Skins of Mice

FLUORESCENCE VALUES

	SHAVED		NEET	
	42		44	
	43		41	
	41		43	
	42		44	
	41		43	
	42		45	
Means	41.8	S.D. 1.8	43.3	S.D. 3.15

S.D. Standard deviation