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The role of alpha-melanocyte stimulating hormone in environmentally induced color change

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THE ROLE OF ALPHA-MELANOCYTE STIMULATING HORMONE IN ENVIRONMENTALLY INDUCED COLOR CHANGE

by

Philip Joseph Fernandez, Jr.

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Philip J. Fernandez, Jr.

entitled THE ROLE OF ALPHA-MELANOCYTE STIMULATING HORMONE IN ENVIRONMENTALLY INDUCED COLOR CHANGE

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Joseph T. Bagnara	7/18/90 Date

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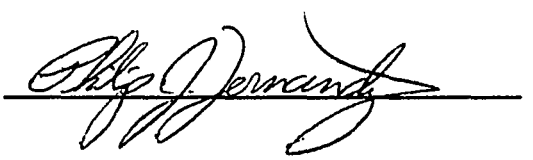
SIGNED: 

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TABLE OF CONTENTS

TABLE OF CONTENTS-continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS-continued

ABSTRACT

It is generally accepted that the endocrine basis of integumental color change in vertebrates is due to pituitary secretion of alpha-melanocyte stimulating hormone (α-MSH). However, physiological levels of α-MSH had not been reported for several species that have historically been the models of choice for investigating changes in pigmentation. The goal of this research was to quantitatively measure levels of circulating α-MSH in two important genera: leopard frogs (Rana pipiens and R. chiricahuensis) and the green anole (Anolis carolinensis) under a variety of physical conditions. Histology of the ventral skin of R. chiricahuensis was also examined due to its unusual melanization and ability to change color. Effects of background color and low temperature on α-MSH levels were examined by radioimmunoassay. In all three species examined, black background color induced dark skin color and high levels of α-MSH. Rana chiricahuensis exhibited much greater plasma α-MSH than the other species. Anolis and R. chiricahuensis darkened in response to low temperature, but R. pipiens did not. Cold induced darkening was associated with increased plasma α-MSH, but not to the extent observed during background adaptation. Ventral skin of R. chiricahuensis, in vivo, darkened at low temperature, and the skin histology revealed numerous large dermal melanophores

dispersed among iridophores. These ventral dermal melanophores are active components of R. chiricahuensis physiological color change.

INTRODUCTION

The interaction of α-melanocyte stimulating hormone (α-MSH) and specialized pigment cells (chromatophores) is a valuable system for the study of many basic cellular mechanisms. Chromatophores are large (20-200μm diameter) and easily observed at low magnification. They are naturally pigmented so they require no special staining for gross inspection *in vivo* or *in vitro*. Cytosolic location and quantity of pigment conveniently indicate important aspects of the cell's physiological state. Degree of pigment dispersion can be objectively quantified and correlated with physiological states (Hogben and Slome, 1931; Hadley and Castrucci, 1988). All chromatophores arise from the neural crest and subsequently differentiate into morphologically and physiologically distinct cell types (Bagnara et al., 1979). Therefore, some similar characteristics among types of chromatophores can be attributed to their common origin. Interestingly, among the most obvious differences among pigment cells are their endocrine sensitivity and pigment products. These black (melanophores), yellow (xanthophores), red (erythrophores), and white (iridophores) cells each produce unique pigments that embellish animal integuments with intricate color patterns in a variety of hues (Bagnara and Hadley, 1973).

The principle element affecting pigment expression is α-MSH. Differential effects of α-MSH on chromatophores during development and differentiation profoundly affect pigment pattern. The precisely controlled mechanism of α-MSH release imparts great flexibility of color and pattern in many species due to effects on mature chromatophores. The focus of this dissertation was to investigate how two environmental stimuli, background color and temperature, influence secretion of α-MSH in three poikilothermic vertebrates.

Poikilotherms synthesize α-MSH in the pars intermedia of the pituitary. Pituitary secretory cells, including melanotrophs, corticotrophs, and neurons of the midbrain and medial basal hypothalamus synthesize a large pro-hormone, pro-opiomelanocortin (POMC), which is the parent molecule of α-MSH. In addition, POMC contains the amino acid sequences for the peptide hormones ß-MSH, gamma-MSH, corticotropin-like intermediate lobe protein (CLIP), adrenocorticotropic hormone (ACTH), ß-lipotropic hormone (ß-LPH), and ß-endorphin (ß-EP) (O'Donohue and Dorsa, 1982).

Appropriate tissue specific expression of POMC derived peptides is due to differential expression of enzymes that post-translationally process the pro-hormone. Four distinct enzymes have been implicated in post-translational modification of POMC (O'Donohue and Dorsa, 1982; Farah, et.

al., 1986). Pro-opiomelanocortin converting enzyme (PCE) cleaves between pairs of basic amino acids. There are soluble and membrane-associated forms of PCE and each has different enzymatic specificity. The soluble form found in secretory granules preferentially cleaves between ACTH and a 16 Kd fragment. The form associated with secretory vesicle membranes cleaves between ACTH and ß-LPH. Another enzyme, carboxypeptide B-like enzyme (CPB) cleaves dibasic amino acid residues from peptide fragments produced from PCE action. Final peptide products are amidated and acetylated in secretory granules by the enzymes peptidyl-glycine α-amidating monooxygenase (PAM) and opiomelanocortin acetyltransferase (OMAT). Amidation and acetylation enhance biological activity of POMC derived peptides. It is thought that acetyl and amide groups increase the longevity of the peptides by impeding their enzymatic degradation (O'Donohue and Dorsa, 1982).

Secretion of α-MSH from pars intermedia melanotrophs is thought to be primarily controlled by neurons whose cell bodies reside in nuclei of the hypothalamus (Davis and Hadley, 1979; Jacobowitz, 1988). Several neurotransmitters have been implicated in this control. Thyrotropin-releasing hormone (TRH), corticotropin-releasing factor (CRF) and serotonin (5-HT) stimulate secretion of α-MSH, and neurons impinging on the

intermediate lobe contain these factors (Lamacz, et al., 1987; Saland, et al., 1988). Both calcium and potassium ions are necessary for α-MSH release in vitro (Hadley, et al., 1973). Epinephrine, norepinephrine, dopamine (DA), and gamma-aminobutyric acid (GABA) are potent inhibitors of α-MSH secretion (Bower et al., 1974; Davis and Hadley, 1979; Adjeroud, et al., 1986).

Evidence for inhibitory control of α-MSH secretion comes from several sources. If intermediate lobe tissue is transplanted to an ectopic site in a frog (e.g. the anterior chamber of the eye), the animal's skin becomes dark as long as the transplant remains viable, regardless of illumination or background color (Iturriza, 1967; Perryman, 1974). Hypothalamic lesion of larval or adult frogs has the same effect (Pehlemann, 1967; Kastin and Ross, 1965). At the level of the gene, it has been shown that conditions that increase spontaneous depolarizations of intermediate lobe melanotrophs increase expression of mRNA that codes for POMC and enzymes that post-translationally modify it (Farah, et al., 1986). Other studies have confirmed the effects of catecholamines and releasing factors on melanotroph secretion in vitro (Adjeroud, et al., 1986; Lamacz, et al., 1986).

The phenomenon of background adaptation is ultimately the result of fine control over intermediate lobe secretion of α-

MSH. Most animals that change the color of their skin do so in response to the relative reflectance of their environment, or its albedo. Animals are dark-colored on a dark-colored background, and lighter-colored on a light-colored background. This response is mediated through the animal's eyes. Elegant experiments by Butcher (1938) in which he rotated the eyes 180°, or placed colored patches over half the eyes of fish (Fundulus), revealed that the dorsal and ventral hemispheres of the retina are functionally distinct. The dorsal half is important for perceiving the albedo of its substrate. Numerous studies have documented how surface albedo influences skin color change during background adaptation (Bagnara and Hadley, 1973). The precise mechanism of how this information is processed into control of intermediate lobe secretion is not entirely known.

As the hormone's name implies, α-MSH causes an acute dispersion of pigment granules (melanosomes) in melanophores. Iridophores respond to α-MSH in an opposite manner by reducing amounts of crystalline purine pigments (Bagnara, 1958; Butman, et al., 1979). Xanthophores of fish and some amphibians disperse pigment in the presence of α-MSH, but the effect is not consistent among all species examined (Bagnara and Hadley, 1969).

Receptors for α-MSH are located in the cell membrane.

Experiments in which large molecules or virus have been conjugated to α-MSH indicate that the hormone does not need to penetrate the plasmalemma for activity (Sawyer, et al., 1988). In fact, microinjection of α-MSH into frog melanocytes does not cause pigment dispersion (Horowitz, et al., 1980). Receptor density of murine and human melanoma cells in culture has been estimated from radioligand receptor assays to be approximately 10^4 receptors per murine cell (Sawyer, et al., 1988) and 10^3 receptors per human cell (Ghanem, et al., 1988).

Signal transduction of the α-MSH-receptor complex is mediated through adenylate cyclase, and activated pigment cells exhibit elevated levels of cyclic-adenosinemonophosphate (cAMP) (Bitensky and Burstein, 1965). Protein kinase C (PKC) is important for activation of lizard (Anolis) melanocytes, probably by phosphorylating the catalytic subunit of adenylate cyclase (Lucas, at al., 1987). A comprehensive model for α-MSH mechanism of action has been proposed that involves calcium channels and metabolism of membrane phospholipids (Sawyer, et al., 1988), but several aspects of these pathways remain to be demonstrated.

In addition to α-MSH receptors, melanophores of some species also have α-adrenergic and ß-adrenergic receptors that respond to catecholamines (epinephrine and norepinephrine).

Early studies established that catecholamines from the adrenal medulla affect lizard coloration (Kleinholz, 1938). Stimulation of Anolis melanophore α-adrenergic receptors results in melanin granule aggregation, whereas stimulation of ß-adrenergic receptors causes melanin granule dispersion (Goldman and Hadley, 1969a). Pigment cells can have both α-adrenergic and ß-adrenergic receptors on the same cell. The α-adrenergic receptors are dominant and mask the dispersing effect of ß-adrenergic receptors when they are simultaneously activated. The α-adrenergic receptors must be blocked with specific pharmacologic agents for the effect of catecholamines to be expressed by ß-adrenergic receptors. The response of Rana to catecholamines depends on the particular species in question. Some races have predominantly α-adrenergic receptors, while others have only ß-adrenergic receptors (Goldman and Hadley, 1970). Melanocytes of the spadefoot toad, Scaphiopus couchi, have only ß-adrenergic receptors, and will only disperse pigment when stimulated by catecholamines (Goldman and Hadley, 1969b).

Once melanocytes are activated, integument color can be modified by either physiological or morphological color change (Parker, 1948). Physiological color change is achieved by moving melanosomes from the central area around the nucleus to the peripheral processes of the cell. The cytoskeleton has

been implicated as the motive force of pigment translocation. Schliwa and Eutereuer (1983) reviewed many studies of intracellular transport of pigment. Most analyses reveal that polymerization of tubulin and adenosine triphosphate (ATP) hydrolysis are associated with pigment dispersion. Cyclic-AMP and protein phosphorylation are also involved in dispersion (Rozdzial and Haimo, 1986). Pigment aggregation may be an energy-requiring process, but the data are conflicting (Iga and Bagnara, 1976; Saidel, 1977; Negishi, et al., 1985).

A second type of color change, morphological color change, results from de novo synthesis of melanin pigment (Parker, 1948). Melanogenesis is controlled by the enzyme tyrosinase which regulates two steps in conversion of the amino acid tyrosine into a polymeric indole (Prota, 1988). In living cells, melanogenesis occurs only within melanosomes where tyrosinase is localized on a protein matrix. Chronic exposure to α-MSH results in increased melanocyte mitosis as well as melanogenesis (Hadley and Quevedo, 1966; Pehlmann, 1967).

Melanocytes of the dermis and epidermis contribute to adaptive color changes in amphibians (Hadley and Quevedo, 1966). Dermal melanocytes, in combination with xanthophores and iridophores, act as a chromatophore unit (Bagnara, et.

al., 1968). In the dermis, iridophores and xanthophores overlie melanocytes and reflect bright colors when melanocytes are punctate. But in the presence of α-MSH, skin color darkens because melanosomes move into cellular processes that overlie the other pigment cells.

Epidermal melanocytes disperse and aggregate pigment like dermal cells, but they are unique in that they inject melanin into adjacent epithelial cells during morphological color change (Hadley and Quevedo, 1966). The melanin in epithelial cells of morphologically adapted animals does not disperse or aggregate during color change, but it persists until the cells are shed.

Color pattern of many vertebrates is dark colored dorsally and light colored ventrally. This pattern is typical of most amphibia. Dorsal-ventral pigment pattern is thought to arise from differentiation of pigment cells according to influence of trophic and inhibitory factors in the local tissue environment (Fukuzawa and Ide, 1988; Fukuzawa and Bagnara, 1989). Models for embryonic determination of integument pigment patterns have been proposed that also include the influence of such factors as gestation period and diffusion coefficients of substances in tissues (Bard, 1977; Murray, 1981; Murray and Maini, 1986). One element, a melanization inhibiting factor, has been isolated from white

ventral frog (Xenopus and R. pipiens) skin, and implicated as a cause for establishing and maintaining dorsal-ventral pigment pattern (Fukuzawa and Ide, 1988; Fukuzawa and Bagnara, 1989).

The intricate integumental pigment patterns seen in various species are ultimately the result of the complex process of natural selection. Survival of many species relies on subtle pigmentary differences that are critical for proper social signalling and species recognition. A distinct pigment pattern can also be important for crypsis or warning (Hadley, 1972). Coloration of many species is flexible, and the ability to change color could potentially increase the activity time and habitat range of such adaptable animals.

Animals change the color of their skin in response to diverse stimuli including humidity (Rowlands, 1952), diurnal rhythms (Redfield, 1918), psychic stimuli such as excitement or fear (Ketterer and Remilton, 1954; Sumpter, et al., 1985), seasonal changes (Stebbins, 1985), age (Fernandez and Collins, 1988), light (Butcher, 1938; Sumner, 1944; Fernandez, 1988), background color (Bagnara and Hadley, 1973), and temperature (Parker, 1938; Edgren, 1954; Kats and VanDragt, 1986). The pituitary melanotropin α-MSH is considered to be the immediate cause of color change in most vertebrates. Many studies quantify color change in terms of the Melanophore Index

(Hogben and Slome, 1931) which describes the degree of melanosome dispersion on a scale from 1-5. Sensitive *in vitro* bioassays for α-MSH rely on this scale either through visual assessment of melanocytes or change in light reflected from skin samples as determined by a reflectometer (Shizume, et al., 1954; Hadley and Castrucci, 1988). However, many questions about how environmental stimuli affect α-MSH secretion have been unaddressed.

Coloration of some amphibians changes with temperature (Parker, 1930; Edgren, 1954; Schmuck, et al., 1988). The Chiricahua leopard frog, *Rana chiricahuensis*, is phenotypically similar to Northern leopard frogs, but it has several unique chromatic adaptations, including cold induced darkening, that make it an interesting model for study of environmental influence on endocrine control. It has been reported that *R. chiricahuensis* is much darker in winter than summer, and that the dark pigmentation includes its ventral surface (Bagnara, 1982). It is not known whether this darkening is due to increased secretion of α-MSH, some other humoral factor, or if temperature has a direct effect on chromatophores in the skin.

Development of a sensitive and specific radioimmunoassay (RIA) for α-MSH has provided a precise means of directly measuring the effects of specific environmental stimuli such

as background color and temperature on α-MSH release during color change. It is unknown how this hormone's concentration varies over time, or how levels vary within or between species. Thus far, α-MSH has been confirmed quantitatively (by RIA) during integument color change in light-mediated responses for one species of frog (Wilson and Morgan, 1979), two species of trout (Rodrigues and Sumpter, 1984; Baker, et al., 1984), an eel (Bowley, et al., 1982), and a carp (Follenius, et al., 1986).

Sensitivity of frog and lizard skin to α-MSH has been established *in vitro* (Hadley and Castrucci, 1988) and it seems clear that α-MSH regulates integumentary pigmentation of amphibians and reptiles (Bagnara and Hadley, 1973). However, in light of the fact that pigment cells have non-α-MSH endocrine receptors that control pigment dispersion, α-MSH may not be the sole modulator of color change under all circumstances. Furthermore, the actual physiological levels of the hormone *in vivo* have not yet been reported for leopard frogs (*Rana pipiens*) or the American chameleon (*Anolis carolinensis*). This information would be extremely valuable for future research into α-MSH controlled pigmentation. The localization and effects of α-MSH have been studied extensively in mammalian systems (O'Donohue and Dorsa, 1982), but α-MSH seems to have a nominal effect on pigmentation in

mammals, and its action may be most important as a neurotransmitter in the central nervous system (Kastin, et al., 1986). Therefore, amphibians and reptiles remain valuable species for investigating the important aspects of hormonal control of color change.

The research presented in the following chapters is comparative at the organismal level in an effort to reveal variations and commonalities in endocrine control and action under a variety of environmental conditions. Such evidence is useful in determining evolutionary and phylogenetic relationships among species and peptides. Plasma α-MSH levels will also be correlated with the unusual structure of the ventral pigmentation of R. chiricahuensis ventral skin. In addition, the effect of low temperature on amounts of α-MSH released by the pituitary may reveal novel mechanisms of secretory homeostasis.

CHAPTER 1

EFFECT OF BACKGROUND COLOR AND LOW TEMPERATURE ON SKIN COLOR AND CIRCULATING α-MELANOCYTE STIMULATING HORMONE IN TWO SPECIES OF LEOPARD FROG

ABSTRACT

Circulating levels of α-MSH in two species of leopard frog, Rana pipiens and R. chiricahuensis, were measured by radioimmunoassay to reveal the correlation between skin color change induced by background color and low temperature. High levels of α-MSH were found in both species of frog on a black background, but R. chiricahuensis had eight times higher levels than R. pipiens. R. chiricahuensis also exhibited the ability to darken its ventral surface, whereas the ventral surface of R. pipiens remains white. Dorsal and ventral skin of R. chiricahuensis darken in a dose-dependent manner to α-MSH in vitro. Neither skin color nor plasma α-MSH of R. pipiens was affected by cold. Low temperature did, however, darken dorsal and ventral skin of R. chiricahuensis, which corresponded to increased levels of plasma α-MSH. Effects of low temperature on skin pigmentation in vitro indicate that temperature alone does not darken skin of R. chiricahuensis.

INTRODUCTION

The principal element thought to affect pigmentation of many animals is α-melanocyte-stimulating hormone (α-MSH), a peptide secreted from the intermediate lobe of the pituitary gland. The differential effects of α-MSH on dermal pigment cells (chromatophores) impart great flexibility of color and pattern in many species of fish, amphibians, and reptiles (Bagnara and Hadley, 1973). These black (melanophores), yellow-red (xanthophores), and silvery (iridophores) chromatophores produce unique pigments that embellish animal integuments with intricate color patterns in a variety of hues (Vevers, 1982). Of the different chromatophores, dermal melanophores are usually most active during color change (Bagnara, et al., 1968). As the hormone's name implies, α-MSH causes an acute dispersion of pigment granules (melanosomes) in melanophores resulting in a darkening of the skin.

The role of α-MSH in regulating pigmentation, specifically the ability to quickly change the color of skin, has been the focus of numerous studies. Rapid physiological color change in animals such as frogs often results in a less conspicuous (cryptic) appearance that makes it less visible to predators. The phenomenon of background adaptation is ultimately thought to result from fine control over

intermediate lobe secretion of α-MSH. Most animals that change the color of their skin do so in response to the relative reflectance of their environment, or its albedo. Animals are dark-colored on a dark-colored background, and lighter-colored on a light-colored background. This response is mediated through the animal's eyes (Butcher, 1938; Sumner, 1944). Many studies have relied, by necessity, on indirect evidence for the role of α-MSH in color change because it was not directly measured in the blood. Relatively few studies have reported this valuable information about the correlation between skin color and circulating levels of α-MSH. Thus far, α-MSH has been confirmed quantitatively (by RIA) during integument color change in light-mediated responses for the African clawed toad (Wilson and Morgan, 1979, Van Zoest, et al., 1989), two species of trout (Rodrigues and Sumpter, 1984; Baker, et al., 1984), an eel (Bowley, et al., 1982), and a carp (Follenius, et al., 1986). Notably lacking is a report of α-MSH levels in background-adapted leopard frogs.

Another aspect of animal coloration that has been discussed in some detail, but without benefit of much empirical data, is the phenomenon of cold-induced darkening. It is reported that many poikilothermic animals are dark-colored when cold and lighten when warmed (Norris, 1967; Sherbrooke, 1988). The most common explanation for this

response is that the dark pigmentation facilitates absorption of solar radiation, and thereby facilitates achieving a more optimal body temperature for activity (Norris, 1967).

The focus of the research presented here was to investigate how two important environmental stimuli, background color and temperature, influence circulating levels of α-MSH in two species of leopard frog, Rana pipiens and R. chiricahuensis. Although these two species are closely related, their pigmentation differs in several respects. Alpha-MSH was measured directly, and correlated with the animal's skin color during background and temperature adaptation. The ability of R. pipiens to background adapt is well documented and they have been examined in numerous studies of endocrine control of color change (Hadley and Quevedo, 1966; Hadley and Bagnara, 1969; Hadley, et al., 1973; Brown, 1973; Perryman, 1974). R. chiricahuensis has received relatively little attention, despite its unusual ventral melanization (Platz and Mecham, 1979) and reported cold-induced darkening (Bagnara, 1982).

MATERIALS AND METHODS

Animals

Northern leopard frogs, Rana pipiens, were obtained from Charles D. Sullivan Company (Nashville, TN) and William A. Lemberger Company (Oshkosh, WI). Chiricahua leopard frogs, Rana chiricahuensis, were collected in northern Arizona under permission of the Arizona Game and Fish Department (Management Research permit number 104). Frogs were maintained in containers that provided them access to a dry surface or aged tap water. Frogs were fed as many crickets, Acheta domestica, (Fluker's Cricket Farm, Baton Rouge, LA) as they could eat 2-3 times a week. Frogs were housed under lamps that contained both fluorescent and incandescent wide-spectrum bulbs. Photoperiod was controlled by an electronic timer adjusted to the natural photoperiod of the season. Air temperature in the room where the frogs were housed ranged from 20-25°C.

Blood Collection

Hemolysis free plasma was obtained by clipping lingual blood vessels and collecting blood into heparinized 80 µl capillary tubes (VWR Scientific Supply, Phoenix, AZ). Tubes were sealed and centrifuged (4 minutes at 13,000 x g) then broken at the cell/plasma interface. Plasma was expelled into a collection tube and 30 µg/ml of the protease inhibitor

Aprotinin (Sigma Chemical Co., St. Louis, MO) and 1.44 mg/ml of the anticoagulant ethylene dinitrilo tetraacetic acid were added. Plasma samples were stored frozen in liquid nitrogen (-196°C) until assay.

Radioimmunoassay (RIA)

The RIA used for α-MSH is a disequilibrium double antibody assay (INCSTAR Corporation, Stillwater, MN). The procedure, in brief, is as follows. Rabbit anti-α-MSH antibody (diluted 1:35,000) is added to all standard and plasma samples (50 μl aliquots in duplicate) and incubated for 20 hours at 5°C. Then I^{125}-labeled α-MSH (chloramine T iodination technique, 79-145 μCi/μg specific activity) is added to all assay tubes and incubated for 20 hours at 5°C. Goat anti-rabbit precipitating complex is then added and incubated for 20 minutes at room temperature. Samples are then centrifuged 20 minutes at 760 x g to pellet the precipitate. Supernatant is discarded and radioactivity of the precipitate in each tube are then measured for 60 seconds in an LKB-Wallac CliniGamma 1272-004 gamma counter.

Radioactivity of samples were analyzed with a National Institutes of Health-RIA data processing program (Rodbard and Munson, 1980, adapted to the IBM-PC by C.A. Leadem and J. Marshall). Each standard point is expressed as a percentage

of B/Bo, where B is the fraction of labeled α-MSH bound to antibody in the presence of unlabeled peptide, and Bo is the fraction of labeled α-MSH bound to antibody in the absence of unlabeled peptide. Standard curves are linearized via a logit transformation, and logit(B/Bo%) is plotted against log(α-MSH mass). The value for logit(B/Bo%) is derived from $\log_e$[B/Bo%/(100-B/Bo%)]. A line is then fitted to the linearized data using a weighted least-squares regression method. Weighting of the curve is biased towards the middle where variance is smallest.

Quality control was monitored within assays by comparing coefficients of variation at 50% B/Bo. Variation among assays was monitored by comparing the standard deviation of counts associated with samples taken from a pool of plasma that was assayed repeatedly.

Radioimmunoassay Validation

Specificity of this radioimmunoassay for α-MSH of R. pipiens and R. chiricahuensis had not been previously established. Specificity of the antibody for Rana α-MSH was determined by making serial dilutions of plasma high in native α-MSH with assay buffer (1% bovine serum albumin-borate, pH 7.4). The regression lines of the plasma dilutions were compared with the standard dilutions using analysis of covariance (Sokal and Rohlf, 1981).

To test for components in the plasma that might interfere with the anti-MSH antibody, serial dilutions of synthetic α-MSH (INCSTAR Corporation, Stillwater, MN) were made in plasma low in native α-MSH, and plasma diluted 1:2 with distilled water. The regression lines of the α-MSH dilutions were compared with the regression of the standard curve using analysis of covariance (Sokal and Rohlf, 1981).

Analysis of covariance (ANCOVA) was employed to determine how the data fit the regression lines, and whether there were differences in slope or intercept of regression lines.

Background Adaptation

Frogs were placed in plastic containers (47x25x20 cm) painted on the outside with flat black (low reflectance), grey (intermediate reflectance), and gloss white (high reflectance). Illumination impinging on the containers was equilibrated with an electronic lux meter (Lutron LX-101). A combination of cool-white and gro-lux 15 watt fluorescent tubes provided a broad spectrum of visible wavelengths. To objectively monitor changes in skin color, frogs were initially held in white or grey containers until all individuals were similarly colored, then they were randomly divided into groups in white, grey, or black containers. Change in skin color was monitored by a Photovolt 670

reflection meter (Photovolt Corporation, New York). The reflection meter was standardized against a white enamel reflectance standard plate with a green tristimulus filter in the light path. A clear plastic dish was placed over the photocell of the reflectance meter, then frogs were held against the plastic over the photocell. The filter was left in place for measurement of ventral skin, but was removed for measurement of dorsal skin. Average skin reflectance of groups (n = 4 frogs per group) was compared initially to insure equivalence among groups at the onset of each experiment. Blood samples were collected after 8 days of background adaptation.

Temperature Adaptation

Effects of temperature on dorsal and ventral skin color were examined in both frog species. Frogs were adapted at room temperature (22°C) to a neutral grey background to reduce the influence of strong visual stimuli. Then they were transferred to chambers maintained at either 25°C or 5°C (n = 5 frogs at each temperature). The grey containers had enough water to completely cover the frogs, thereby reducing the potential for increased cooling by evaporation. Fluorescent lights were adjusted for equal in nsity between chambers with the lux meter. Dorsal and ventral skin reflectance was

recorded for the frogs over time courses ranging from 8-24 hours. Blood samples were collected after 8-10 hours. During blood collection frogs were held in a water bath at the same temperature as their treatment to maintain the temperature stimuli.

Air and water temperature were measured with an electronic microcomputer thermometer (Omega model HH-72T) equipped with type-T thermocouples. Air temperature was measured with glass-braid insulated thermocouples, and water temperature was measured with a 30 x 0.2 cm stainless steel thermocouple.

Effect of α-MSH and Low Temperature on Skin Pigmentation In Vitro

The procedures of Shizume, et al. (1954) for in vitro bioassay of α-MSH were used to examine the effects of α-MSH and low temperature on skin color of R. chiricahuensis. Frogs were sacrificed by decapitation and dorsal and ventral skin were removed and placed on a 25 mm diameter circular frame consisting of an inner aluminum ring and an outer overlapping plastic ring. Frog skin stretched across the aluminum ring was held in place by fitting the plastic ring over it. The frame was then immersed in a 50 ml glass beaker containing 20

ml of frog Ringer's solution (NaCl 6.50 g/l, KCl 0.14 g/l, $CaCl_2$ 0.12 g/l, $NaHCO_3$ 0.20 g/l). Five skin samples were used in each treatment. Controls were incubated in the Ringer's solution at room temperature (22-23°C). Other skin samples were treated with α-MSH (Sigma Chemical Co., St. Louis, MO) at molar concentrations of $5x10^{-9}$, $1x10^{-9}$, $4x10^{-10}$. Other skin samples were held at low temperature (5°C) for 9 hours to determine the effects of temperature alone on skin pigmentation. The low temperature chamber had fluorescent illumination adjusted with a lux meter to the same intensity that the room temperature skin samples were exposed to. Skin reflectance was recorded by placing beakers on the photocell of the reflection meter, and centering them so that the light source struck the frog skin.

General Statistics

Differences between mean values of skin reflectance or plasma α-MSH were determined by Student's t test (Sokal and Rohlf, 1981). Equality of variances was determined via an F test to determine appropriate degrees of freedom for t tests (Sokal and Rohlf, 1981). Confidence limits are standard error of the mean, unless otherwise indicated.

RESULTS

Radioimmunoassay

The RIA of R. chiricahuensis and R. pipiens plasma (from white-background adapted frogs), to which synthetic α-MSH had been added in the same concentration as the standards, is presented in Figure 1.1. The ANCOVA revealed that the regression of each dilution explains the variation in the data ($p < 0.001$), and that the separate regressions of B/Bo% on α-MSH mass are not significantly different in slope. Further analysis showed that a single regression line and Y-intercept explain the variation of the α-MSH dilutions. This information indicates that components of the plasma of these species do not significantly interfere with binding of the rabbit-anti-α-MSH antibody and synthetic α-MSH.

To test the affinity of the rabbit-anti-α-MSH antibody for frog α-MSH, serial dilutions of plasma high in native α-MSH (i.e., plasma from black-background adapted frogs) were made with distilled water and assayed (Figure 1.2). The agreement of the standard and plasma dilutions from the two species of frog indicate that the rabbit-anti-α-MSH antibody binds to frog and synthetic α-MSH with similar affinity (ANCOVA, $p < 0.001$).

The intra-assay coefficient of variation in eight assays at 50% B/Bo was 7.6% ± 2.9 (s.d.). The mean interassay

coefficient of variation of four separate assays was 2.02% ± 0.94 (s.d.).

Background Adaptation

Frogs of both species held in white, grey, and black containers at room temperature for eight days became pale, intermediate, and dark-colored, respectively. Although an intermediate grey background was provided, there was sufficient variation in the mean dorsal skin reflectance that white and grey background adapted frogs were not consistently different (Figures 1.3). However, for both species, dorsal skin of black background adapted frogs was much darker than those on white or grey backgrounds. Rana chiricahuensis became very dark-colored on black backgrounds, and were darker than R. pipiens under similar conditions (Figures 1.3).

Ventral skin of R. pipiens remained white regardless of background color (Figure 1.4). Ventral skin of R. chiricahuensis was also light-colored on white or grey backgrounds, but became much darker on a black background (Figure 1.4).

Plasma α-MSH (as determined by RIA) from the frogs held on white and black backgrounds corresponded well with their coloration (Figure 1.5). On a white, grey, or black background, R. pipiens had 49.6 ± 2.4, 55.8 ± 1.3, and 163.2 ± 27.3 pg α-MSH/ml, respectively. The corresponding levels

of α-MSH for R. chiricahuensis were 81.3 ± 15.8, 67.4 ± 9.9, and 1378.5 ± 338.3. There was no difference between α-MSH of R. pipiens on white or grey backgrounds, but α-MSH was approximately three times greater ($p < 0.05$) in frogs on a black background. Rana chiricahuensis were similar in this respect, except the amount of α-MSH on a black background was almost 20 times greater ($p < 0.05$) than on white or grey. Some plasma samples had amounts of α-MSH that were beyond the range of the assay, so they were subsequently diluted and assayed again for more accurate quantification. On black backgrounds, the amount of α-MSH secreted by R. chiricahuensis was eight times greater than that secreted by R. pipiens ($p < .05$).

Temperature Adaptation

Under conditions where light and background color were held constant, neither dorsal nor ventral reflectance of R. pipiens was affected by low temperature (Figure 1.6). Frogs held 12-48 hours at 5°C were indistinguishable from those held at 25°C. However, under those same conditions R. chiricahuensis were significantly darker at 5°C (Figure 1.7). Both dorsal and ventral skin were consistently darker at low temperature, sometimes to the extent that it resembled skin of a black background adapted frog.

Skin color of the same frogs whose reflectance is illustrated in Figures 1.6 and 1.7 corresponded with plasma α-MSH levels as determined by RIA. There were no differences in plasma α-MSH levels in R. pipiens. In contrast, even though they were on the same colored background and under identical illumination, cold-adapted R. chiricahuensis had a greater amount ($p < 0.05$) of α-MSH (135.3 ± 24.1 pg/ml α-MSH), than frogs at room temperature (64.5 ± 5.2 pg/ml α-MSH) (Figure 1.8).

Effect of α-MSH and Low Temperature on Skin Pigmentation In Vitro

Dorsal and ventral skin samples of R. chiricahuensis darkened within 30 minutes after application of α-MSH and remained dark for several hours unless washed off with fresh Ringer's solution (Figure 1.9). Skin darkened in a dose dependent manner over a range of α-MSH concentrations from $4x10^{-10}$ M to $5x10^{-9}$ M (Figure 1.10). ANCOVA revealed that the dose response curves of dorsal and ventral skin have different slope, indicating that sensitivity of dorsal and ventral skin to α-MSH are different.

Temperature alone had no effect on pigmentation of dorsal or ventral R. chiricahuensis skin in vitro over eight hours (Figure 1.11). Viability of melanophores in cold and warm

skin after eight hours at 5°C was confirmed by its rapid darkening after adding α-MSH to a concentration of $5x10^{-9}$ M in the Ringer's solution (Figure 1.11).

DISCUSSION

The results of this study reveal new information about the dynamic process of animal color change. Normal physiological levels of α-MSH in two species of Ranid frogs are reported under variable conditions of albedo and temperature. Dorsal skin of both species darkens when surface reflectance is low (low albedo). Ventral skin of R. pipiens is white regardless of visual or thermal stimuli. The ventral integument of R. chiricahuensis was found to be an active component of the animal's ability to change color. Ventral skin of R. chiricahuensis darkens profoundly under conditions of low albedo and low temperature. The skin of R. chiricahuensis in vitro responds to α-MSH but not to low temperature, indicating the response is endocrine mediated.

In light of the many previous studies of frog color change, it was expected that skin color of background adapted frogs would correspond well with plasma α-MSH levels. For example, Wilson and Morgan (1979) found the expected correlation between albedo and circulating α-MSH in Xenopus laevis. However, the magnitude of the difference in α-MSH

between black background adapted Xenopus, R. pipiens, and R. chiricahuensis was much greater than expected. The α-MSH of R. chiricahuensis on a black background was three times greater than Xenopus, and over eight times greater than R. pipiens.

There are several interesting implications of the high α-MSH titers in R. chiricahuensis. For example, the comparatively high levels of α-MSH may be related to the degree of melanization in this species. Even on a white background, R. chiricahuensis are 26% darker than R. pipiens (Figure 1.3). It has been shown that α-MSH has a trophic effect on developing pigmentary systems (Chen, et al., 1974; Fukuzawa and Bagnara, 1989) and prolonged exposure to high amounts of α-MSH can lead to proliferation of melanophores in the integument of fish (Chevins and Dodd, 1970) and amphibian larvae (Pehlemann, 1967). Chronically high levels of α-MSH from the period of metamorphosis to adulthood may increase the number of melanophores in the integument, and may even override the effect of the melanization inhibiting factor in ventral skin (Fukuzawa and Ide, 1988) responsible for the typical dorsal-ventral pigment pattern (i.e., dark dorsum and white ventrum) seen in R. pipiens and other species. The trophic effect of α-MSH and other factors on undifferentiated "chromatoblasts" (Bagnara, et al., 1979) in the ventral skin

may lead to the observed abundance of melanophores. Plasma α-MSH levels of larval frogs have not been reported. Chromatoblast determination may be affected by high α-MSH levels in very early larval stages in some species. The ontogeny of ventral skin pigmentation of both R. pipiens and R. chiricahuensis is histologically described in Chapter Two.

The ultimate value of control of pigmentation is undoubtedly multifaceted, and the result of many powerful but opposing selective pressures (Hadley, 1972). Differences in pigmentation and the endocrine control of pigmentation are related to the life history and habitat of species. Inconspicuous, or cryptic coloration is an adaptive strategy employed by many species to avoid predation. R. pipiens are commonly called grass frogs because of their occurrence in damp meadows and around the grassy perimeter of ponds. Hence, light brown and green dorsal skin improve this species' crypsis in its natural habitat. The white ventrum may also be considered cryptic coloration. For example, to a predatory fish near the bottom of a pond, the silvery-white ventrum of a frog floating at the surface may appear similar to the reflective surface of the water.

In keeping with this strategy, the melanistic skin of R. chiricahuensis seems appropriate to its native habitat. Typical terrestrial habitat of this frog includes rocky banks

of clear streams. Rocks in the regions where these frogs occur are volcanic and are typically black in color. In fact, the region from which the R. chiricahuensis examined in this study were collected is named the Black River because of the appearance of the black rocks in the stream bed. There are well-described examples of regionally melanistic animals that correspond to regions of black volcanic substrata (Norris, 1967). These occurrences are presumed to be due to increased survival of cryptic individuals.

Predators of R. chiricahuensis include garter snakes (Thamnophis elegans) and trout (Oncorhynchus apache) which both hunt using visual stimuli. Dark-colored dorsal and ventral skin probably facilitates crypsis of these amphibians that live among black rocks on land and in the water. The dark-colored dorsal skin blends in well with damp soil and black rocks along the banks. Dark-colored ventral skin may be advantageous because of the swiftly-moving, clear, and shallow water of the stream. In most places the streams are too shallow and swift for frogs to float at the surface and be viewed from below. Frogs that leap into the water are carried by the current for some distance before they come to rest on the bottom. While in the current they can be turned over, thereby exposing the ventral skin. If that skin were white, it could potentially attract the attention of

predaceous trout, like the bright flash of a fishing lure. Dark-colored, non-reflective skin is more cryptic in clear water (Fernandez and Collins, 1988), and that could be crucial to these frogs during this vulnerable time.

Another interesting adaptation of R. chiricahuensis is cold-induced darkening. It had been reported that R. chiricahuensis is darker-colored in winter than summer, and that the dark pigmentation included its ventral surface (Bagnara, 1982). Cold-induced darkening and ventral melanization of this species are quantified for the first time in this report. The ability of R. chiricahuensis to darken its ventral skin and darkening in response to low temperature are unusual traits, and distinguish them from the common leopard frog, R. pipiens. Despite their low body temperature, the time course for cold-induced darkening indicates an endocrine-mediated event. However, plasma α-MSH of cold-darkened frogs was not as high as room temperature black background adapted frogs. This is probably related to the depressed metabolic state of induced by the low temperature. Some aspect of α-MSH secretion must be enhanced by low temperature.

In addition, this study has shown that cold does not directly alter skin pigmentation of R. chiricahuensis, but melanophores continue to respond to α-MSH at low temperature.

This is in agreement with the findings of Wright (1955) who found that skin of R. pipiens and R. clamitans did not darken at low temperature, and the response to α-MSH was reduced. However, melanophores of some amphibians do appear to disperse at low temperature. Moriya and Miyashita (1989) report that, in vitro, melanophores of a frog (Rana chensinensis) and a salamander (Hynobius retardatus) dispersed in response to cold, while melanophores of Xenopus laevis did not. The source of this interspecific variation remains unclear, but it is probably related to the consequences of balancing selective pressures in each habitat.

Coloration of other amphibians have also been observed to change with temperature (Parker, 1930; Edgren, 1954; Kats and Van Dragt, 1986; Schmuck, et al., 1988). It has been suggested that this may be a thermoregulatory phenomenon that affects potential absorption of solar radiation in order to facilitate maintaining a favorable body temperature for a greater period during the day.

Some poikilotherms control their body temperature in ways that could be potentially affected by integument color. Behavioral thermoregulation has been shown to significantly increase body temperature above ambient temperature in butterflies (Watt, 1968), toads (Sherman, 1980), salamanders (Feder, 1982), lizards (Pearson and Bradford, 1976), and birds

(Ohmart and Lasiewski, 1971). Semiaquatic amphibians and terrestrial reptiles increase body temperature by basking in the sun and cool off by seeking shade or water (Lillywhite, 1970; Muth, 1977). A relatively higher body temperature may improve efficiency of metabolic processes such as digestion and muscle contraction (Brill, 1979). In addition, using solar radiation reduces the metabolic cost of maintaining a greater temperature than the environment (Ohmart and Lasiewski, 1971). In terms of survival and fitness, it would seem advantageous to adjust the absorptive characteristics (color) of the integument to control body temperature and maximize the amount of time available for foraging and reproduction.

Under certain conditions, a dark-colored integument absorbs more heat more quickly than light-colored skin. The extra heat absorbed by a dark integument could reduce the time necessary to reach an optimal body temperature on a cool but sunny morning. If ambient temperature exceeds the optimal body temperate later in the day, the same animal could benefit from a more reflective light colored integument that would reduce its heat load and potentially extend time for activity.

However, the fact that amphibian skin is moist may negate any thermal benefit gained from its pigmentation. Studies of Lillywhite (1975) reveal that evaporative water loss from skin

of frogs increases with temperature. Hydration of the skin is determined by centrally controlled blood flow to the skin. Since evaporation effectively cools surfaces, heat gained from dark pigment is probably quickly lost.

Ultimately, the secretion of α-MSH at low temperature is probably related to crypsis. Activity of amphibia is greatly reduced at low temperatures. The two main means by which amphibia avoid predation are cryptic coloration and rapid escape (i.e., jumping). Decreased metabolism at low temperature reduces the effectiveness of rapid escape because of reduced muscular response. Therefore, at low temperature, crypsis becomes the more important option. Dark coloration and reduced movement is probably the best strategy for survival of this species when it is cold. Other poikilothermic species have been described as "stone mimics" due to their appearance and inactivity (Sherbrooke, 1988), and R. chiricahuensis appears have a similar cryptic strategy.

This research establishes precedent for looking at the sources of environmental control of α-MSH secretion. An organ or dispersed cell perfusion system may be an appropriate method for this type of investigation. Neurointermediate lobes could be maintained in vitro at 5°C and 25°C to determine the effect of temperature directly on pituitary melanotrophs. Frog pituitaries have been successfully maintained in vitro

(Adjerod, et al., 1986), and measurable amounts of α-MSH have been recovered from incubation media in as little as 15 minutes (Bower, et. al., 1974). Perfusion systems have also been successfully employed to measure secretion of α-MSH from frog pituitaries in response to various pharmacological agents (Tonon, et al., 1986).

The concentration of α-MSH in the incubation or perfusion media as measured by RIA, could determine if secretion increases as temperature decreases. The possible dependence of a factor from the hypothalamus could be investigated by perfusing neurointermediate lobes with hypothalamic neurotransmitters (e.g. dopamine, norepinephrine, thyrotropin releasing hormone). It has been shown in vitro that dopamine is inhibitory to α-MSH secretion (Bower, et al., 1974), and that the hypothalamus normally exerts a tonic inhibition on melanotrophic cells of the pituitary. If low temperature increases α-MSH secretion, it may be due to decreased hypothalamic inhibition. Decreased melanotroph sensitivity to dopamine could also be investigated by treating pituitary cells with dopaminergic agonists and antagonists. Decreased melanotroph sensitivity to an antagonist such as haloperidol at low temperatures might indicate that dopaminergic receptors are less responsive due to conformational changes or decreased turnover of active receptors. Response to the agonist

thyrotropin releasing hormone could be tested as well to see if melanotroph sensitivity to a putative releasing factor is increased.

The ultimate value of ventral darkening and cold-induced darkening should also be investigated to more clearly identify the benefits of these adaptations. The thermoregulatory and cryptic effects of this pigmentation may reveal significant value to the survival of this intriguing species.

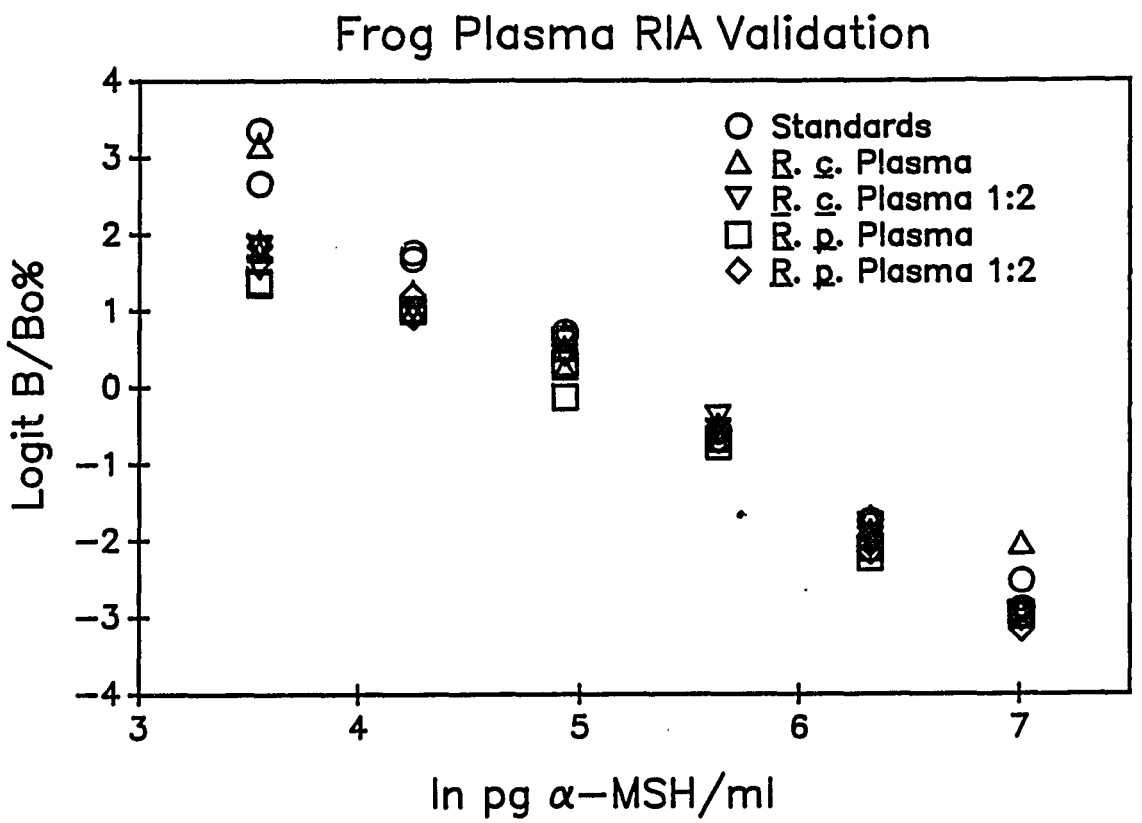


Figure 1.1 Frog plasma RIA validation. Each symbol represents assay replicates of synthetic α-MSH diluted in assay buffer (Standards), in R. chiricahuensis (R.c.) or R. pipiens (R.p.) plasma, or in plasma diluted in half with water (Plasma 1:2).

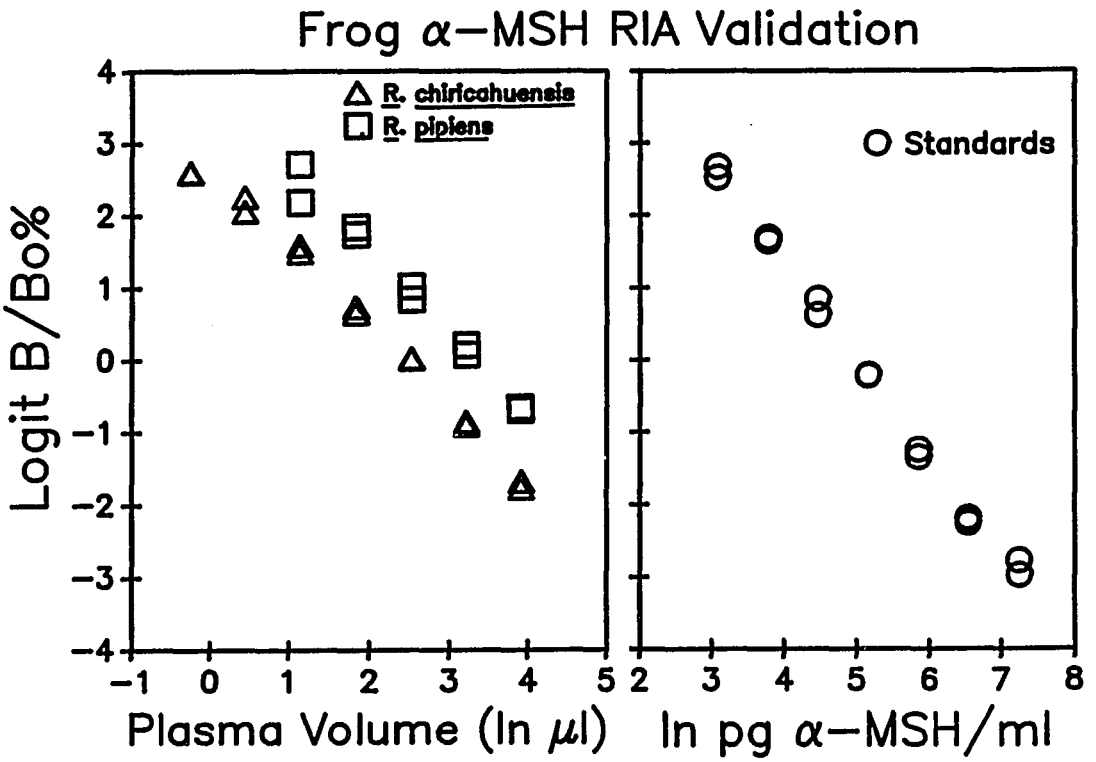


Figure 1.2 Frog α-MSH RIA validation. Each symbol represents assay replicates of serial dilutions of α-MSH standards, and plasma from black background adapted R. chiricahuensis and R. pipiens.

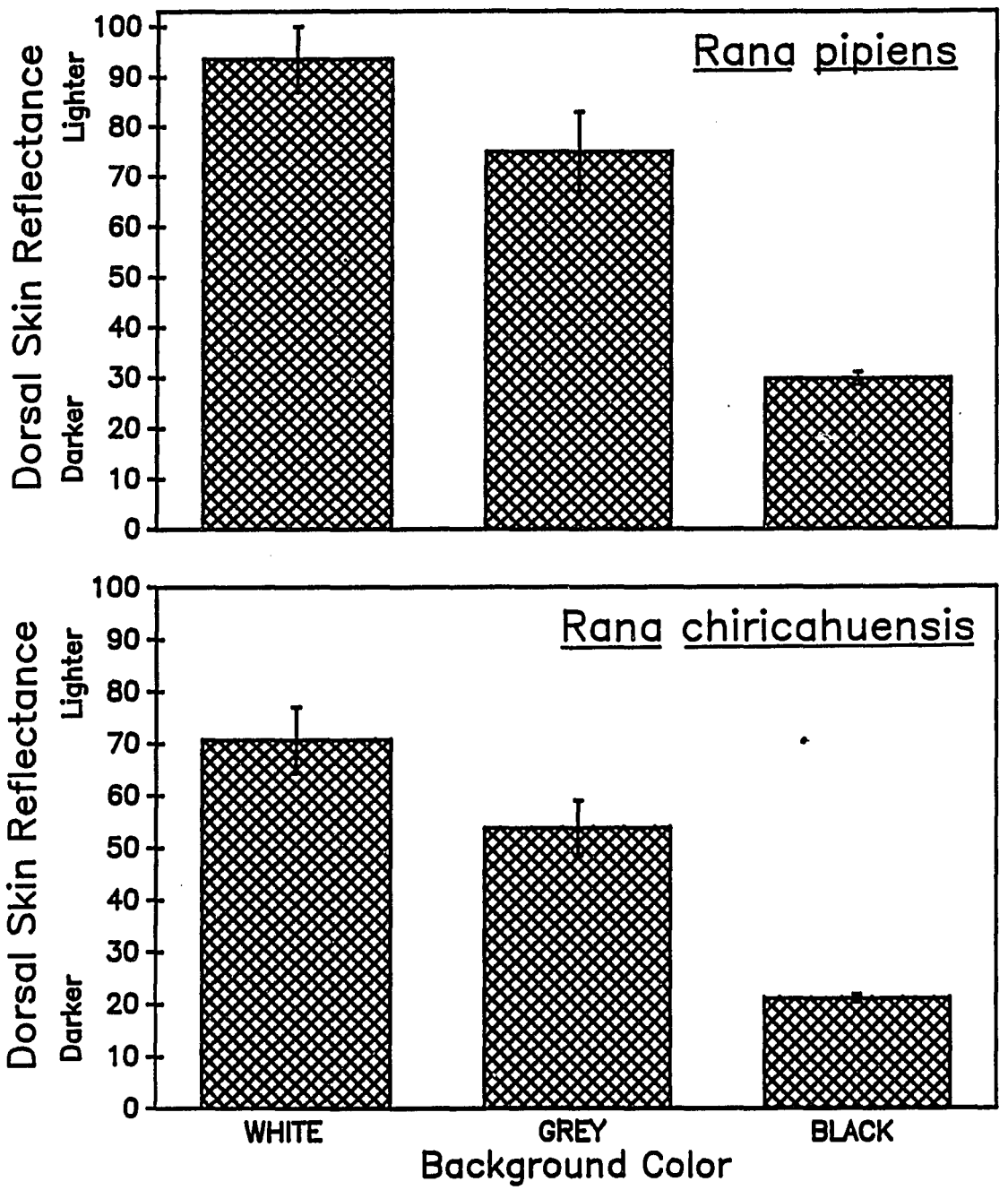


Figure 1.3 Effect of white, grey and black backgrounds on dorsal skin reflectance of R. pipiens and R. chiricahuensis. Bars represent the mean dorsal reflectance (± SE) of 4 frogs. Skin reflectance of R. pipiens on white is greater than black ($p < 0.01$), and grey is greater than black ($p < 0.02$). Skin reflectance of R. chiricahuensis on white is greater than black ($p < 0.05$), and grey is greater than black ($p < 0.01$).

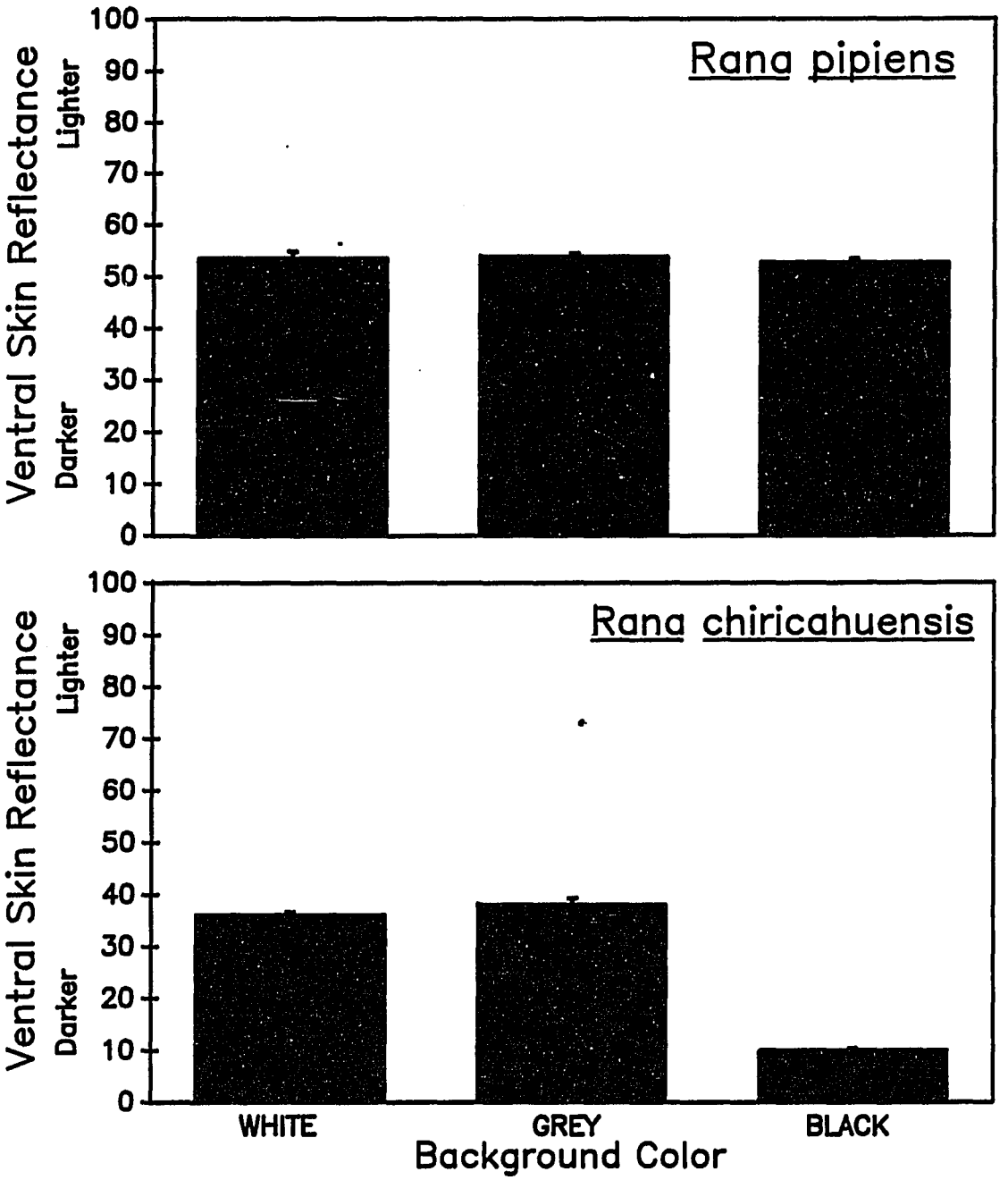


Figure 1.4 Effect of white, grey, and black backgrounds on ventral skin reflectance of R. pipiens and R. chiricahuensis. Bars represent the mean (± SE) of 4 frogs. There are no significant differences among mean reflectance of R. pipiens. Skin reflectance of R. chiricahuensis on white and grey is greater than black ($p < 0.001$).

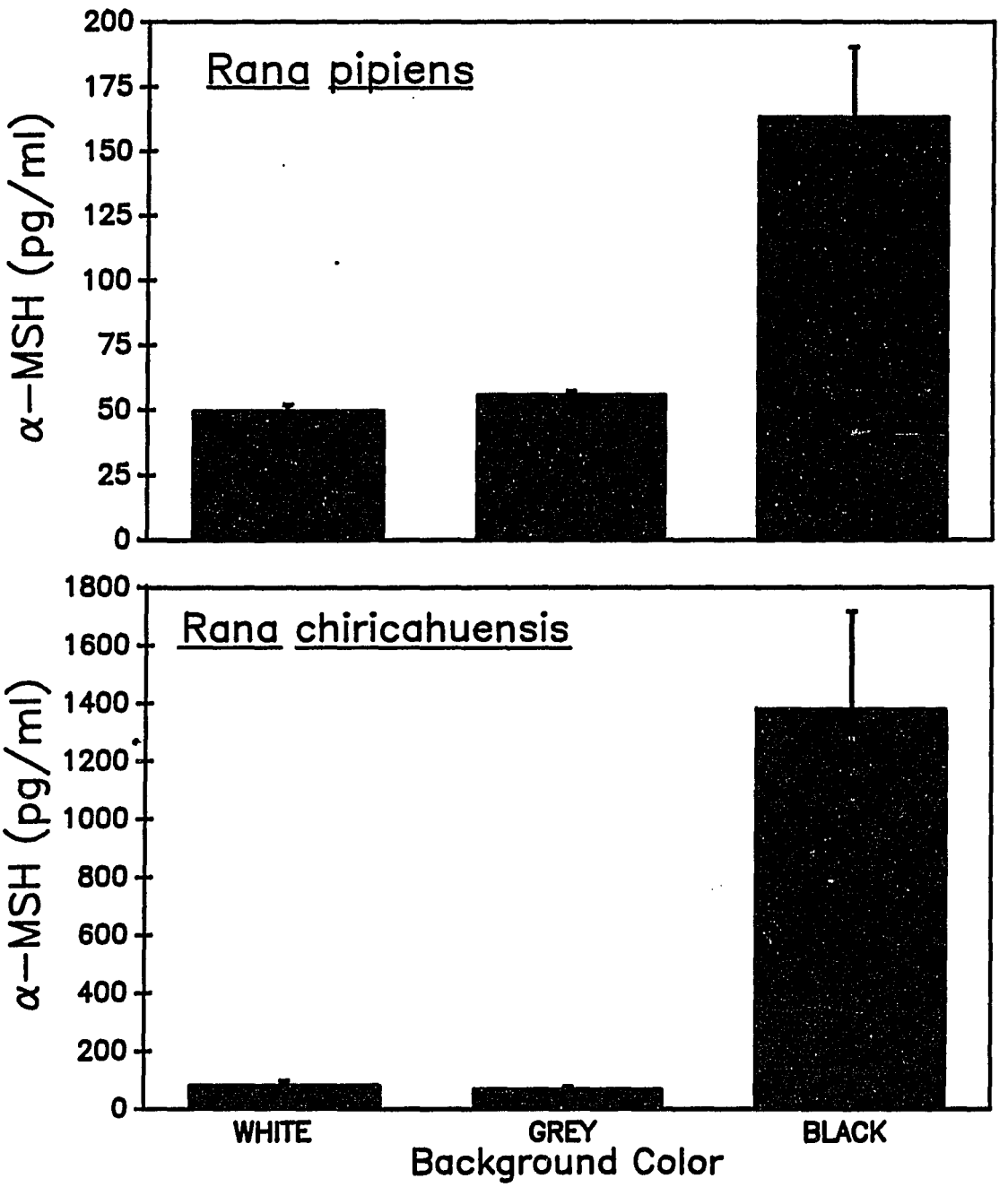


Figure 1.5 Plasma α-MSH of R. pipiens and R. chiricahuensis on white, grey, and black backgrounds. Bars represent the mean (± SE) of the same frogs represented in Figures 3 and 4. Plasma α-MSH of frogs on a black background is greater than those on white or grey ($p < 0.05$).

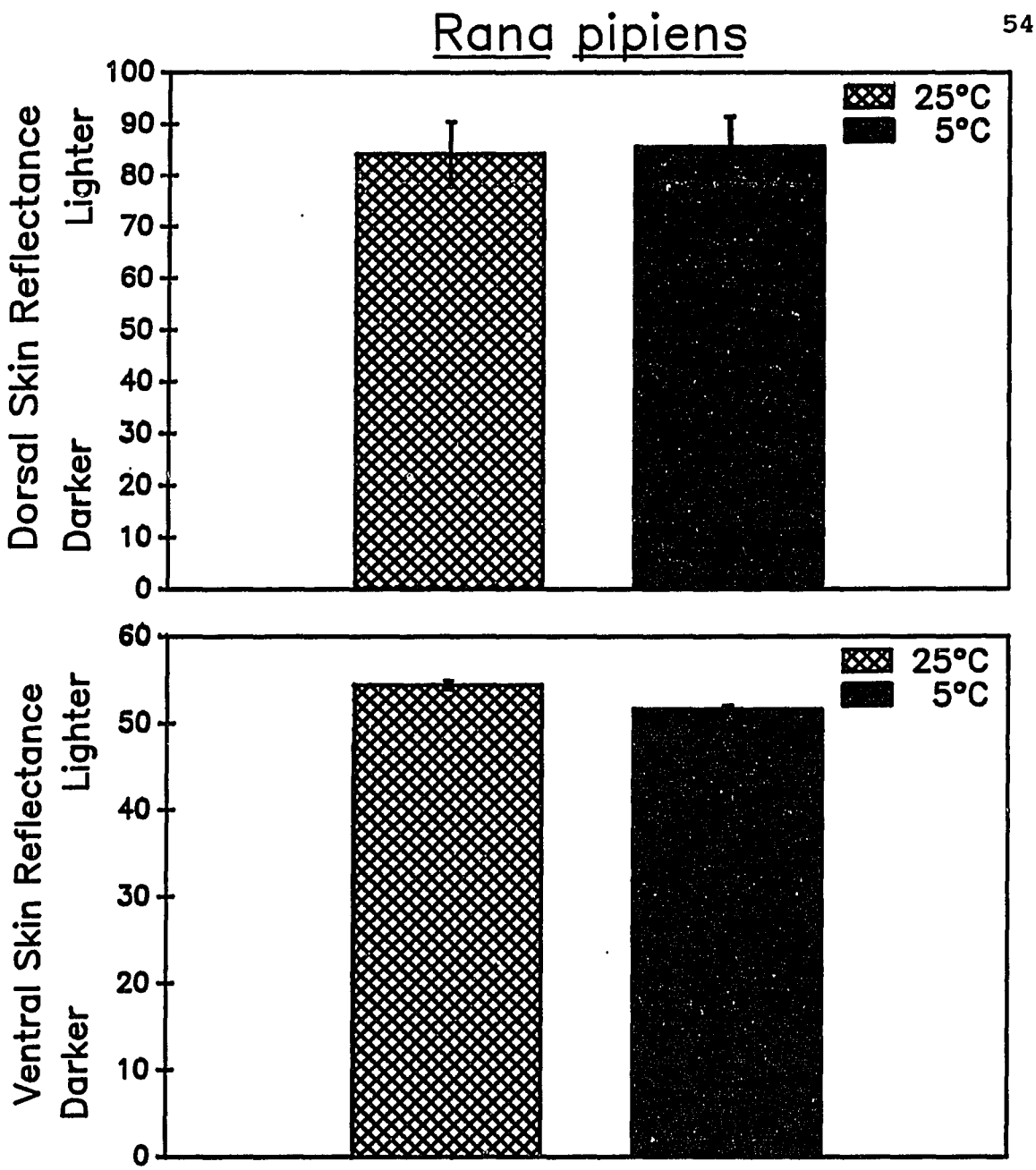


Figure 1.6 Effect of low temperature on dorsal and ventral skin reflectance of R. pipiens held on grey backgrounds at 25° C and 5° C. Bars represent the mean (± SE) of 5 frogs. Temperature had no effect on skin reflectance.

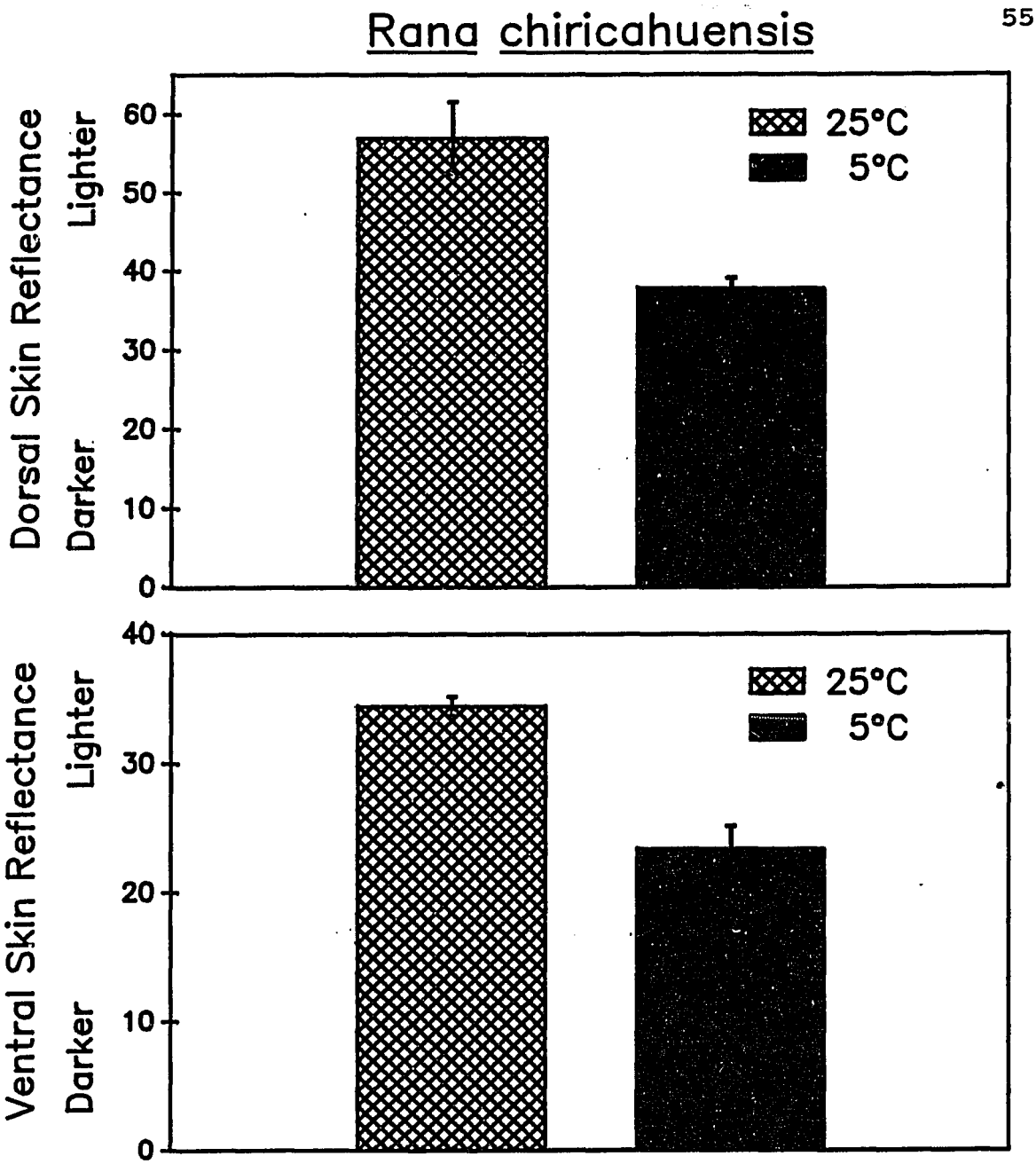


Figure 1.7 Effect of low temperature on dorsal and ventral skin reflectance of R. chiricahuensis held on grey backgrounds at 25° C and 5° C. Bars represent the mean (± SE) of 5 frogs. Frogs at 5° C are significantly darker than those at 25° C ($p < 0.001$).

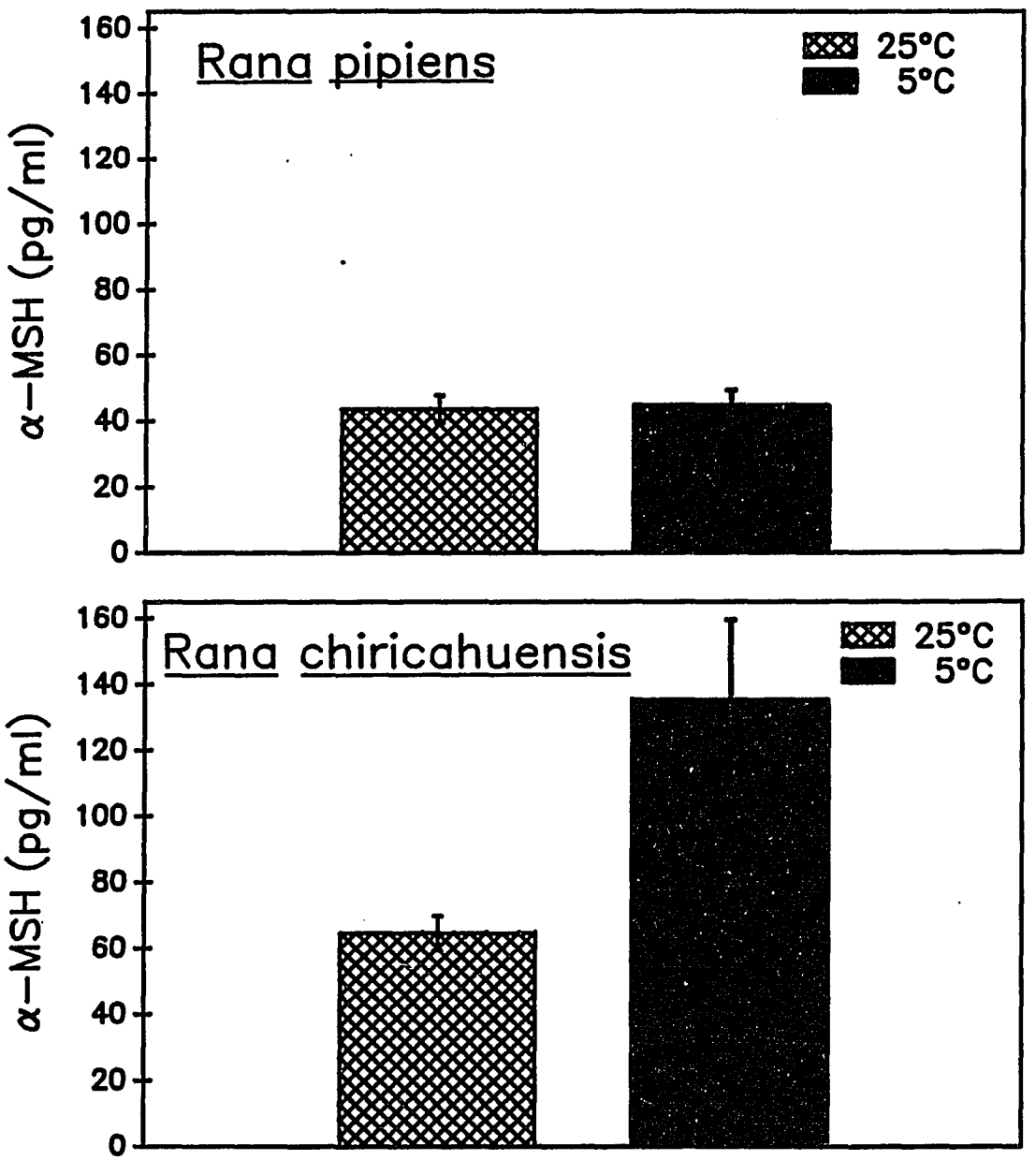


Figure 1.8 Plasma α-MSH of R. pipiens and R. chiricahuensis held at 25° C and 5° C. Bars represent the mean (± SE) α-MSH levels of the same 5 frogs in Figures 6 and 7. There is no difference among R. pipiens, but R. chiricahuensis at 5° C have higher levels of α-MSH than those at 25° C ($p < 0.05$).

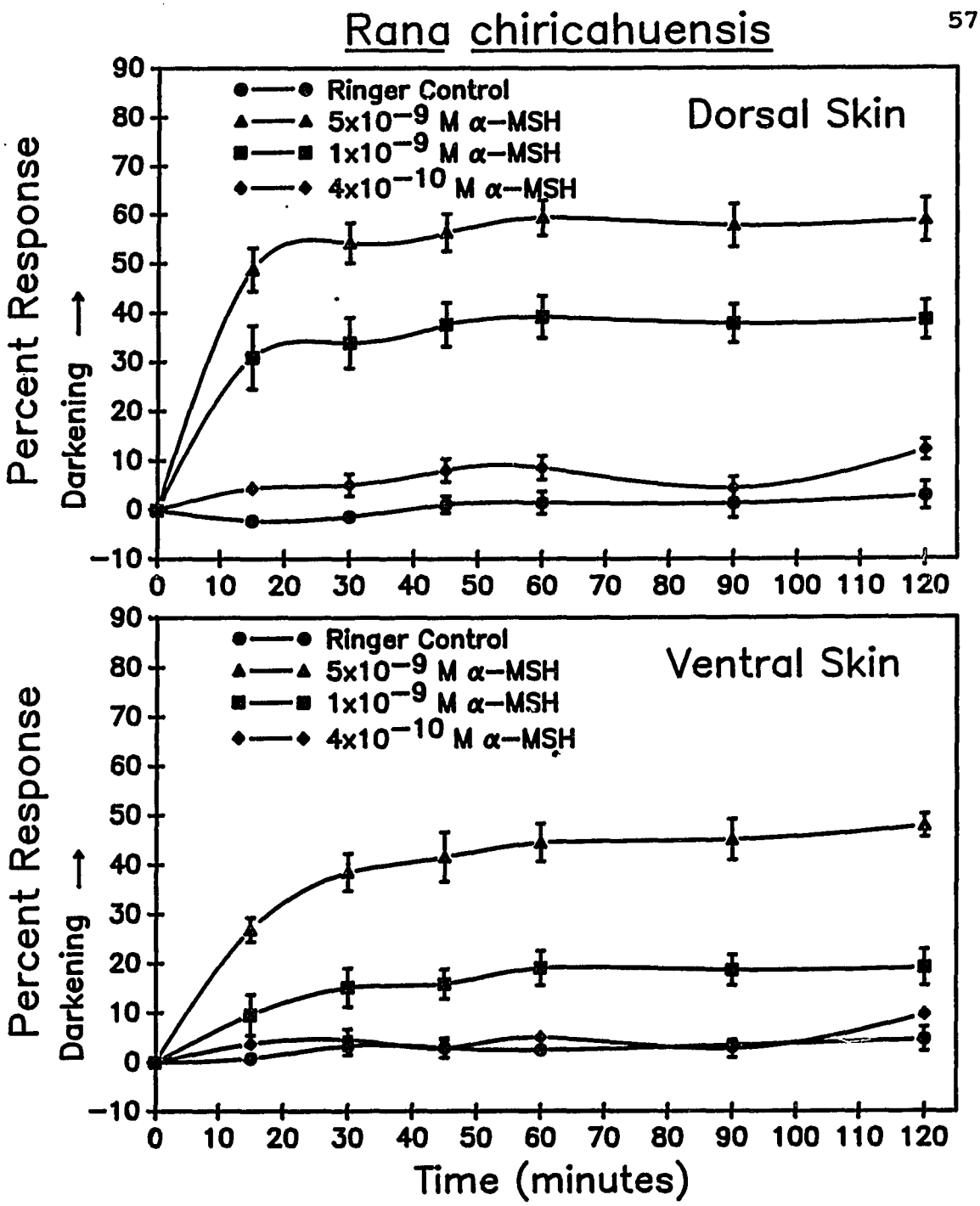


Figure 1.9 Response of dorsal and ventral R. chiricahuensis skin to α-MSH in vitro over a two hour period. Symbols represent the mean (± SE) reflectance of 5 skin samples.

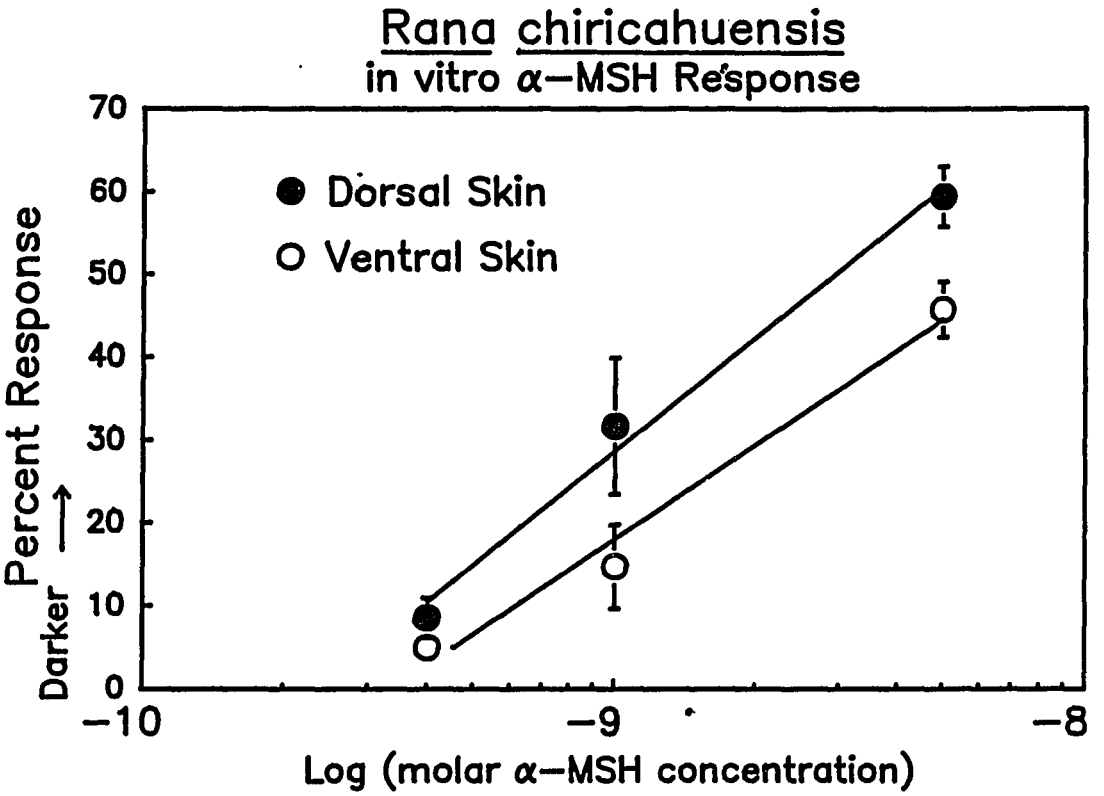


Figure 1.10 Dose response of dorsal and ventral R. chiricahuensis skin in vitro after 60 minutes exposure to α-MSH. Symbols represent the mean (± SE) reflectance of 5 skin samples.

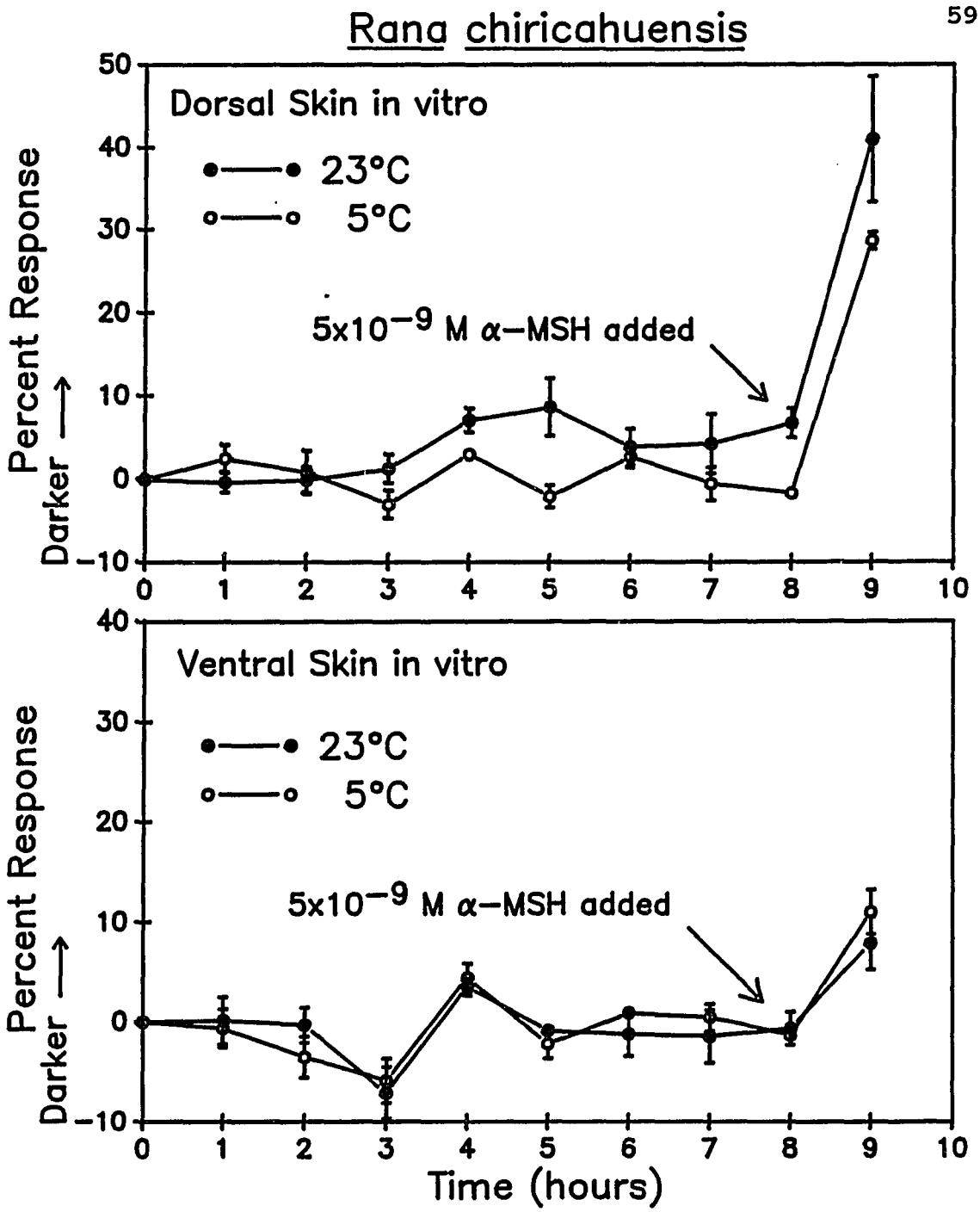


Figure 1.11 Response of dorsal and ventral R. chiricahuensis skin to low temperature in vitro. α-MSH was added to the Ringer's solution bathing skin samples after 8 hours to confirm melanophore viability. Symbols represent the mean (± SE) reflectance of 5 skin samples.

CHAPTER 2

OBSERVATIONS ON DEVELOPMENT OF UNUSUAL MELANIZATION OF LEOPARD FROG VENTRAL SKIN

ABSTRACT

The ontogeny of ventral pigmentation of two species of leopard frog, Rana pipiens and R. chiricahuensis, was examined using light microscopy and transmission electron microscopy to reveal how the unusual melanistic ventral pigmentation of R. chiricahuensis is achieved at the cellular level. Ventral skin of R. pipiens is always white. Ventral skin of adult R. chiricahuensis is white when frogs are background adapted to a white substrate, but ventral skin becomes nearly as dark-colored as the dorsal skin when frogs darken in response to a black background. Skin samples from both species of tadpole, newly metamorphosed frog, and adult frog were analyzed for chromatophore composition and distribution. Ventral skin of R. pipiens is white due to abundant iridophores. Ventral skin of R. chiricahuensis contained iridophores and melanophores. Melanophores appeared in the integument in significant numbers after metamorphosis, and continued to increased in abundance as frogs aged. Pigment in ventral melanophores migrated during physiological color change.

INTRODUCTION

Many vertebrate animals exhibit complex skin pigment patterns achieved by differential distribution of three pigment cell (chromatophore) types: melanophores, xanthophores, and iridophores. All chromatophores are embryologically derived from the neural crest, and ultimately, the migration and destination of these undifferentiated, pluripotential cells determines pigment pattern (Bagnara, 1987).

The bright colors of many anuran species' dorsal integument is attained by layering different types of chromatophores. Development of dorsal pigmentation has been extensively investigated and development of the leopard frog, Rana pipiens, dorsal pigment pattern has been especially well described (Volpe, 1964; Smith-Gill, 1974; Bagnara, 1982a). In addition, it has been determined that the either brown or green dorsal color of newly metamorphosed R. pipiens is a genetic trait (Fogleman, et al., 1980).

Development of amphibian ventral pigmentation has also received some attention (Fukuzawa and Ide, 1988). Typically, ventral skin of the African clawed toad, Xenopus laevis and R. pipiens, is white due to an abundance of only iridophores. However, white ventral skin of Xenopus contains dendritic, dopa-positive cells that lack melanin, implying the presence

of a tissue environment unfavorable for melanophore differentiation. The dopa-positive reaction indicates the presence of tyrosinase and this is consistent with observations of Chavin (1969). It would appear that an agent is present that inhibits melanophore differentiation. A melanization inhibiting factor (MIF) has been isolated, characterized, and implicated as a determining component of pigment pattern development (Fukuzawa and Ide, 1988; Fukuzawa and Bagnara, 1989). It appears that the putative MIF's role in ventral pigmentation is, at least, two-fold since it apparently has a stimulatory effect on iridophores (Bagnara, et al., 1989).

An interesting exception to the typical dorsal-ventral pigment pattern of R. pipiens is the closely related species, the Chiricahua leopard frog, Rana chiricahuensis (Platz and Mecham, 1979). This leopard frog has the unusual ability to rapidly change the color of its ventral skin from white to nearly as dark as its dorsal skin (Bagnara, 1982b; Fernandez, 1990). This unusual ability is interesting in several respects. The coexistence of dermal melanophores and iridophores in the ventral skin is intriguing in light of the presence of the putative MIF in ventral skin of two similar species, R. pipiens and R. forreri (Fukuzawa, et al., 1989).

Rana chiricahuensis have been found to have extremely

high plasma α-MSH under conditions of low temperature or black background color (Fernandez, 1990). It has been suggested that the chronicly high α-MSH may have a trophic affect on undifferentiated pigment cells increasing the proportion of melanophores as compared to other pigment cell types in the skin (Fukuzawa and Bagnara, 1989). This may partially explain R. chiricahuensis ventral melanization.

In this report, development of ventral pigmentation in two closely related leopard frogs, R. pipiens and R. chiricahuensis, were described. Rana pipiens served as a model of typical ventral pigmentation, since its ventrum is white at all stages of its development. Ultrastructural comparison was made directly with R. chiricahuensis at identical stages of development from larva to adult. Aspects of this species' ventral pigmentation are identified that allow it to use the ventrum as an active component of its ability to rapidly change color in response to the environment.

MATERIALS AND METHODS

Animals

Northern leopard frogs, R. pipiens, were obtained from Charles D. Sullivan Company (Nashville, TN) and William A. Lemberger Company (Oshkosh, WI). Chiricahua leopard frogs,

R. chiricahuensis, were collected in northern Arizona under permission of the Arizona Game and Fish Department (Management Research permit number 104). Frogs were maintained in containers that provided them access to either a dry surface or aged tap water. These containers were positioned under lamps that contained both fluorescent and incandescent wide-spectrum bulbs. Photoperiod was controlled by an electronic timer adjusted to the natural photoperiod of the season. Air temperature ranged from 20-25°C. Frogs were fed as many crickets, Acheta domestica, (Fluker's Cricket Farm, Baton Rouge, LA) as they could eat 2-3 times a week.

Frogs were background adapted in white or black containers (47x25x20 cm) under the described lighting conditions until physiological color change was observed before obtaining skin biopsies.

Whole Mounted Tissue

The technique described by Bagnara (1958) was employed for low magnification examination of chromatophores. Full thickness skin samples were taken from the center of the back and belly. Fresh biopsies of frog skin were trimmed into approximately 5 mm square pieces and laid flat on a microscope slide. A concentrated sugar solution (Karo syrup) was applied evenly over the tissue and then a cover slip was placed on it.

Specimens prepared in this manner last indefinitely (at least 20 years) in an extremely well-preserved state. Skin samples were examined with reflected light objectives to facilitate identification of iridophores, as well as melanophores and xanthophores.

Light and Electron Microscopy

Part of the tissue collected for whole mounts was simultaneously prepared for light and electron microscopy. Techniques described by Bagnara, et al. (1968) were followed. In brief, tissue was fixed 2 hours in 6% glutaraldehyde in 0.1 M-collidine buffer, pH 7.4, then post-fixed one hour in 2% osmium tetroxide in 0.1 M collidine. After dehydration in a graded ethanol series, the tissue was embedded in epoxy resin (Epox 812, Ernest F. Fullam) and cured 48 hours at 60°C. Sections were cut with a diamond knife on a Reichert-Jung Ultracut E ultramicrotome. Sections for light microscopy were stained with toluidine blue and viewed under an oil immersion objective.

Sections with silver to gold interference colors were selected for electron microscopy. Sections were collected on Formvar-coated, carbon-stabilized grids (Electron Microscopy Specialists), stained with uranyl acetate and lead citrate, and examined in a Jeol JEM 1000 CXII transmission electron

microscope operating at 60 kV.

RESULTS

Larvae

Larval R. pipiens and R. chiricahuensis are phenotypically similar. In larvae, ventral skin over the viscera was pigmented by iridophores (Figure 2.1 A,E). Ventral skin of both species was white regardless of background color or light intensity.

Newly Metamorphosed Frogs

Ventral skin of newly metamorphosed R. pipiens had only iridophores, and they could be seen around openings of skin glands (Figure 2.1 B and 2 A). Ventral skin of newly metamorphosed R. chiricahuensis exhibited both iridophores and melanophores (Figure 2.1 F). Iridophores were the more abundant chromatophore. Although not shown in this report, a small number of xanthophores were found in R. chiricahuensis ventral skin laterally in the region adjacent to where dorsal pigmentation begins.

Density of melanophores in the sample of newly metamorphosed R. chiricahuensis ventral skin in Figure 2.1 F was 60/mm^2. Melanophore processes do not usually overlap. Ventral skin of newly metamorphosed R. chiricahuensis was

white when frogs were adapted to a white background, but the skin turned grey with black flecks when the frogs were adapted to a black background. The degree of ventral darkening in response to a black background increases as the frogs mature. Development of skin glands is evident at metamorphosis. Gland openings are ringed by chromatophores (Figures 2.1 and 2.2).

Adult Frogs

Ventral skin of adult R. pipiens was pigmented solely by iridophores, and the skin did not change color when frogs were on white or black backgrounds (Figure 2.1 C,D). Iridophores contain reflecting platelets that are similar in size and shape to those of R. chiricahuensis (Figure 2.3). Melanophores in skin of adult R. chiricahuensis (Figure 2.1 G,H) are more heavily pigmented than newly metamorphosed frog skin (Figure 2.1 F). Melanophore densities of the samples illustrated in Figures 2.1 G and H were 71/mm^2 and 220/mm^2, respectively.

Ventral melanophores actively dispersed and aggregated melanin pigment according to background reflectance. On a black background pigment dispersed into dendritic processes (Figure 2.1 G) and aggregated into the cell body on a white background (Figure 2.1 H). There was extensive overlap of melanophore dendritic processes when pigment was dispersed

(Figure 2.1 G). Ventral melanophores are large cells (Figure 2.2 D) and contain abundant, well formed melanosomes (Figure 2.3 A,B). Skin glands were larger in adult frogs than newly metamorphosed frogs, and iridophores were aggregated around the periphery of gland openings (Figure 1.1 C,D,G,H).

DISCUSSION

Results of this study reveal that ventral skin of the two species of leopard frog differ in dermal pigmentation. Rana pipiens have only iridophores while R. chiricahuensis become progressively more melanistic due to increase in number of melanophores mixed among iridophores. This difference in pigmentation is significant in several respects.

First of all, it is interesting that two similar species of frog should have such different ventral pigmentation. Rana chiricahuensis exhibits considerable geographic range overlap with other Ranid species that have a typical white ventrum (Platz and Mecham, 1979). Therefore, there are probably similar selective pressures on these species. This melanistic pigmentation may in some way enhance the frog's crypsis, making it less conspicuous in a unique niche of the general habitat it shares with the other species.

The ventral pigmentation of R. chiricahuensis could be explained by the process of morphological color change.

Morphological color change, pigmentation due to an increased number and synthetic activity of pigment cells, is due to prolonged hypersecretion of α-MSH (Parker, 1948; Bagnara and Hadley, 1973). In species that normally have a white ventrum, constantly high α-MSH secretion, accomplished by surgical manipulation, induced ventral melanization (Pehlemann, 1967; Chevins and Dodd, 1970). Presence of ventral melanophores in R. chiricahuensis may be due to differentiation of latent chromatoblasts in ventral skin caused by exposure to the extremely high levels of α-MSH secreted by this species. A type of morphological color change involving the ventrum may be maintained by natural environmental conditions that promote α-MSH secretion. Low environmental albedo significantly darkens pigment pattern in tiger salamanders and decreases amounts of bright-colored pigments in the skin (Fernandez and Collins, 1988). Two environmental stimuli, dark-colored substrata and low temperature, have been demonstrated to elevate plasma α-MSH in R. chiricahuensis.

Ventral chromatophores of R. chiricahuensis are arranged like dorsal dermal chromatophore units (Bagnara et al. 1968). Large dendritic melanophores underlie a layer of iridophores directly beneath the epidermal basal lamina. Xanthophores, however, are not found in adult ventral skin. Presence of dynamic melanophores in the ventral dermis may have much to

do with pigment cell response to trophic and inhibitory factors in the chromatophore tissue environment. Since melanophores and xanthophores are both stimulated by α-MSH (Bagnara and Hadley, 1969), and only melanophores are present, the two cell types may respond differently to the influence of high levels of α-MSH in the presence of a melanization inhibiting factor (Fukuzawa and Bagnara, 1989). Latent melanophores of R. chiricahuensis may also be less responsive to the melanization inhibiting factor than other Ranid species.

Ventral skin of R. chiricahuensis has not yet been tested for presence of a melanization inhibiting factor. This information, and data on its potency as compared to other Ranids, would be useful in determining a more complete scenario for ventral melanization in R. chiricahuensis.

Rana chiricahuensis is a unique and valuable model for studying development of pigment pattern and pigment cell differentiation. Further comparison with species like R. pipiens could reveal much about the role of trophic and inhibitory factors that determine chromatophore expression.

Figure 2.1 Whole mounts of R. pipiens (A,B,C,D) and R. chiricahuensis (E,F,G,H) ventral skin photographed at 20x magnification with reflected-light optics. Skin samples are from tadpoles (A,E), newly metamorphosed frogs (B,F,), adult frogs from a black background (C,G), adult frogs from a white background (D,H). Scale bar: 100μm.

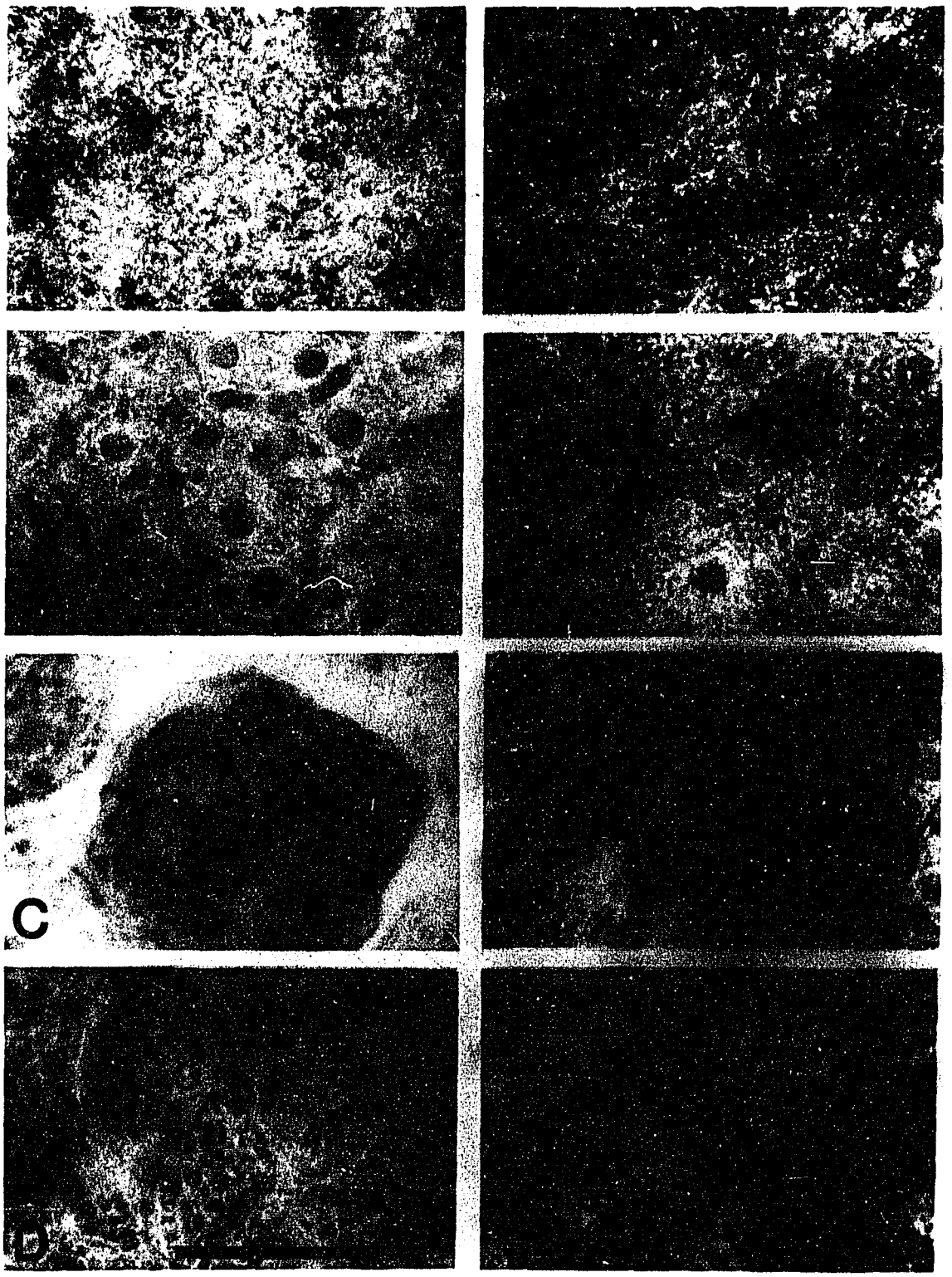

C
D

Figure 2.2 Sections of R. pipiens (A,B) and R. chiricahuensis (C,D,) ventral skin photographed at 63x magnification. Skin samples are from newly metamorphosed frogs (A,C) and adult frogs (B,D). The chromatophores indicated by arrows are iridophores (I) and melanophores (M). Scale bar: 20μm.

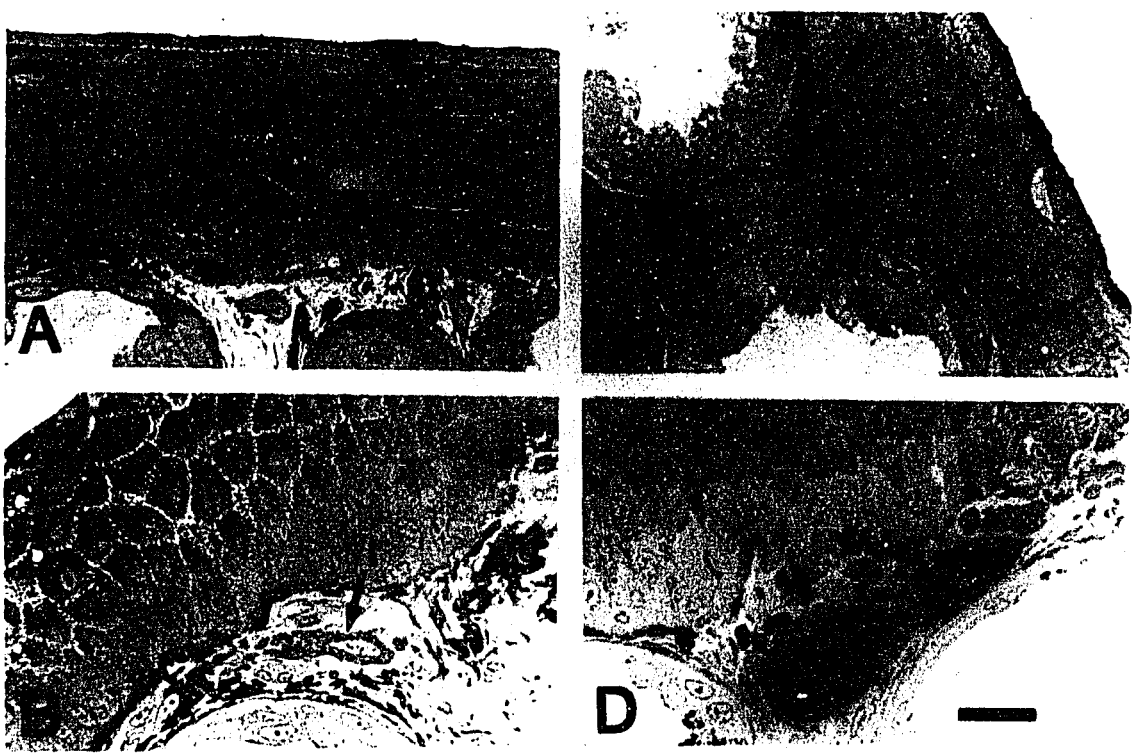
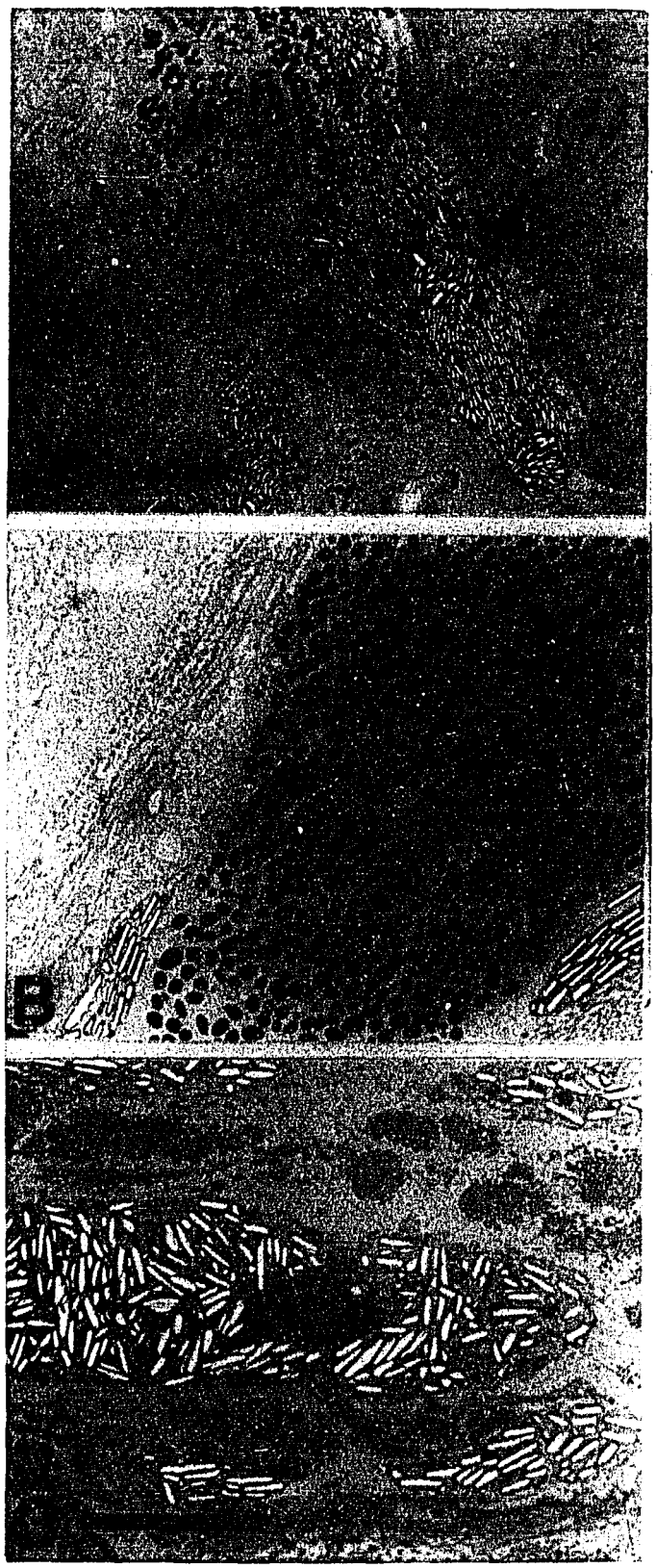

A
D

Figure 2.3 Transmission electron micrographs of R. chiricahuensis (A,B) and R. pipiens (C) ventral skin. Dermal iridophores (I) and melanophores (M) are immediately beneath the epidermis (E). Scale bars: A: 10μm; B and C: 5μm.


B

CHAPTER 3

EFFECT OF BACKGROUND COLOR AND LOW TEMPERATURE ON SKIN COLOR AND CIRCULATING α-MELANOCYTE STIMULATING HORMONE IN THE LIZARD, ANOLIS CAROLINENSIS

ABSTRACT

Plasma α-melanocyte stimulating hormone (α-MSH) was measured in Anolis carolinensis after adaptation to black and white backgrounds and to low temperature. Plasma α-MSH levels of black background adapted lizards, and lizards held at low temperature were significantly higher than plasma α-MSH of lizards at room temperature adapted to white backgrounds. Black background adapted lizards were darker-colored than cold adapted lizards. Black background adapted lizards had higher levels of α-MSH than cold adapted lizards. These results reveal that α-MSH plays a significant role in darkening skin of Anolis during background and temperature adaptation.

INTRODUCTION

The anatomy and physiology of skin color change in the green anole, Anolis carolinensis, may be the most completely described of any vertebrate species. However, the principal hormone thought to control this process, α-melanocyte stimulating hormone (α-MSH), has not been measured directly during different skin color phases.

Earlier work of Parker and Starratt (1904) and Kleinholz (1936) established many facts that support the role of α-MSH and other humoral agents in control of skin color during various physiological and environmental conditions. These results were summarized by Hadley and Goldman (1969). Sensitivity of A. carolinensis skin in vitro to α-MSH and other hormones has been thoroughly investigated (Hadley and Castrucci (1988).

Cold-induced darkening, a phenomenon observed in several poikiloltherms (Parker, 1938; Atsatt, 1939; Norris, 1967; Sherbrooke and Frost, 1989), has also been described in A. carolinensis. At 10°C these lizards are brown regardless of illumination (Parker and Starratt, 1904). Experiments by Smith (1929) demonstrated that Anolis skin in vitro became brown below 8°C and turned green above 43°C, indicating that cold directly caused melanophores to disperse pigment, and thus darken the integument. Further evidence that temperature

has a direct affect on melanophore dispersion, rather than pituitary secretion, was that melanophores were less responsive to α-MSH after incubation at 4°C (Hadley and Goldman, 1969). However, these data do not exclude a possible additional contribution of α-MSH in cold induced darkening.

Investigations of the endocrinology of Anolis color change have to this point indirectly quantified the role of α-MSH in vivo. The research reported here employed radioimmunoassay to quantify physiological levels of α-MSH in the circulation of Anolis carolinensis during adaptation to black and white backgrounds and to low temperature.

MATERIALS AND METHODS

Animals

Green anoles, Anolis carolinensis, were purchased commercially (Dan Vicknair Snake Farm, La Place, LA) and held in a terrarium where they were provided water and crickets (Acheta domestica, Fluker's Cricket Farm, Baton Rouge, LA).

Blood Collection

Blood samples were collected from the lizards' trunks after decapitation. Hemolysis free plasma was obtained by collecting blood into heparinized 80 μl capillary tubes (VWR Scientific Supply, Phoenix, AZ). Extractable blood volume of

each Anolis was about 150 μl, so plasma samples from two or three lizards were pooled to obtain a suitable plasma volume for assay replicates. Capillary tubes were sealed and centrifuged (4 minutes at 13,000 x g) then broken at the cell-plasma interface. Plasma was expelled into a collection tube and 30 μg/ml Aprotinin (Sigma Chemical Co., St. Louis, MO) and 1.44 mg/ml ethylene dinitrilo tetraacetic acid were added. Plasma samples were stored frozen in liquid nitrogen (-196°C) until assay.

Radioimmunoassay (RIA)

The radioimmunoassay (RIA) used for α-MSH is a disequilibrium double antibody assay (INCSTAR Corporation, Stillwater, MN). The procedure, in brief, is as follows. Rabbit anti-α-MSH antibody (diluted 1:35,000) is added to all standard and plasma samples (50 μl aliquots in duplicate) and incubated for 20 hours at 5°C. Then I^{125}-labeled α-MSH (chloramine T iodination technique, 79-145 μCi/μg specific activity) is added to all assay tubes and incubated for 20 hours at 5°C. Goat anti-rabbit precipitating complex is then added and incubated for 20 minutes at room temperature. Samples are then centrifuged 20 minutes at 760 x g to pellet the precipitate. Supernatant is discarded and gamma counts of the precipitate in each tube are then measured for 60

seconds in an LKB-Wallac CliniGamma 1272-004 gamma counter.

Radioactivity of samples was analyzed with a National Institutes of Health-RIA data processing program (Rodbard and Munson, 1980, adapted to the IBM PC by C.A. Leadem and J. Marshall). Each standard point is expressed as a percentage of B/Bo, where B is the fraction of labeled α-MSH bound to antibody in the presence of unlabeled peptide, and Bo is the fraction of labeled α-MSH bound to antibody in the absence of unlabeled peptide. Standard curves were linearized via logit transformation, and logit(B/Bo%) was plotted against log(α-MSH mass). The value for logit(B/Bo%) is derived from $\log_e$[B/Bo%/(100-B/Bo%)]. A line was fitted to the linearized data using a weighted least-squares regression method. Weighting of the curve is biased towards the middle where variance is smallest.

Quality control was monitored within assays by comparing coefficients of variation at 50% B/Bo. Variation among assays was monitored by comparing the standard deviation of counts associated with samples taken from a pool of plasma that was assayed repeatedly.

Radioimmunoassay Validation

Specificity of the antibody for Anolis α-MSH was determined by making serial dilutions of plasma high in native

α-MSH with assay buffer (1% bovine serum albumin-borate, pH 7.4). The regression lines of the plasma dilutions were compared with the standard dilutions using analysis of covariance (Sokal and Rohlf, 1981).

To test for components in the plasma that might interfere with the anti-MSH antibody, serial dilutions of synthetic α-MSH (INCSTAR Corporation, Stillwater, MN) were made in plasma low in native α-MSH, and plasma diluted 1:2 with distilled water. The regression lines of the α-MSH dilutions were compared with the regression of the standard curve using analysis of covariance (Sokal and Rohlf, 1981).

Analysis of covariance (ANCOVA) was employed to determine how the data fit the regression lines, and whether there were differences in slope or intercept of regression lines.

Skin Reflectance Measurement

Change in skin color was monitored with a Photovolt 670 reflection meter (Photovolt Corporation, New York). The reflection meter was standardized against a white enamel reflectance standard plate with a green tristimulus filter in the light path. A clear plastic dish was placed over the photocell of the reflectance meter, then lizards were held against the plastic over the photocell.

Background Adaptation

Lizards were placed in plastic containers painted with flat black (low reflectance) and gloss white (high reflectance) paint. Illumination impinging on the containers was equilibrated with an electronic lux meter (Lutron LX-101). Broad spectrum lamps provided a range of wavelengths. Ten experiments were performed using a total of 168 Anolis. Plasma from five of the ten experiments was assayed for α-MSH. Data from a typical experiment are presented here in detail.

Temperature Adaptation

Anolis were tested for their response to low temperature on a grey background to avoid influence of strong visual stimuli. Lizards were placed for 17 hours in grey containers that were held in the laboratory at 27°C or held in an illuminated low temperature incubator at 10°C. Air temperature in lizard containers was measured with an electronic microcomputer thermometer (Omega model HH-72T) equipped with a type-T thermocouple. Five experiments were performed using a total of 105 Anolis. Plasma from three of the five experiments was assayed for α-MSH. Data from a typical experiment are presented here in detail.

RESULTS

Radioimmunoassay Validation

Affinity of rabbit-anti-α-MSH antibody for *Anolis* α-MSH was determined by making serial dilutions of plasma high in native α-MSH (i.e., plasma from black-background adapted lizards) with distilled water (Figure 3.1). Agreement of the standard and plasma dilutions indicate that rabbit-anti-α-MSH antibody binds to *Anolis* and synthetic α-MSH with similar affinity (ANCOVA, $p < 0.001$).

The RIA of *Anolis* plasma (from white-background adapted lizards), to which synthetic α-MSH had been added in the same concentration as the standards, is presented in Figure 3.2. The ANCOVA revealed that the regression of each dilution explains the variation in the data ($p < 0.001$), and the separate regressions of B/Bo% on α-MSH mass are not significantly different in slope. Further analysis showed that a single regression line and Y-intercept explain the variation of α-MSH dilutions. This information indicates that components of the plasma of *Anolis* do not interfere with binding of rabbit-anti-α-MSH antibody and synthetic α-MSH.

The intra-assay coefficient of variation in eight assays at 50% B/Bo was 7.6% ± 2.9 (s.d.). The mean interassay coefficient of variation of four separate assays was 2.02% ± 0.94 (s.d.).

Background Adaptation

Skin reflectance of lizards held on a black background was dark brown and significantly darker ($P < 0.001$) than lizards on white backgrounds whose skin was bright green (Figure 3.3). Low skin reflectance correlated with high α-MSH. Lizards on white backgrounds averaged 40.4 (± 6.2 SE) pg/ml α-MSH and lizards on black backgrounds had 660.2 (± 43.95 SE) pg/ml α-MSH (Figure 3.3).

Temperature Adaptation

Skin reflectance of lizards on a grey backgrounds was darker at 10°C than 27°C (Figure 3.4). Plasma α-MSH was almost 3 times higher in the cold (Figure 3.4). Lizards at 10°C averaged 268.3 (± 56.8) pg/ml α-MSH while lizards at 27°C had 85.5 (± 14.7) pg/ml α-MSH. Coloration of cold-darkened lizards was different than brown lizards at room temperature in that cold lizards had an uneven, mottled brown coloration with prominent darkly pigmented postorbital patches.

DISCUSSION

The range of plasma α-MSH levels found by RIA in this report was in good agreement with the range of concentrations A. carolinensis skin responds to in vitro. Green lizards from white backgrounds had approximately 2×10^{-11} M plasma α-MSH

which is similar to the minimal effective dose of 8 x 10^{-11} M reported by Hadley and Castrucci (1988). Maximum darkening of skin samples *in vitro* occurs at an α-MSH concentration in the 10^{-9} M range (Hadley and Castrucci, 1988), while plasma α-MSH of black background adapted lizards average 4 x 10^{-10} M.

Occasionally a lizard would be exceptionally dark colored. Plasma from these rare individuals had α-MSH concentrations in the 10^{-9} M range. This response was not observed often and usually occurred during what appeared to be territorial behavior when two male lizards were confined together in small containers. Greenberg and Chen (1987) found that social stress alters skin color and α-MSH levels. Lizards ranked as subordinates had significantly elevated melanotropin levels. A more complete analysis of the role of α-MSH in coloration elicited by social interactions has yet to be made.

On a grey background at room temperature, skin color and plasma α-MSH concentration were similar to white background adapted lizards. However, cold-adapted lizards on grey backgrounds had higher plasma α-MSH than those at room temperature (Figure 3.4). This result was not completely expected since the coloration of cold lizards was different than brown lizards at room temperature. Cold lizards had a mottled brown coloration with prominent black post-orbital

patches similar to that elicited by electrical stimulation or injection with adrenaline (Kleinholz, 1938).

Anolis melanophores have α-adrenergic and ß-adrenergic receptors that effectively modulate pigment aggregation and dispersion, respectively (Hadley and Goldman, 1969). Certain stress events cause sequential stimulation of α-adrenergic and ß-adrenergic receptors resulting in an initial excitement pallor followed by darkening. Therefore, melanin dispersion can be accomplished by both α-MSH and catecholamines. It would be expected then, based on lizard coloration at low temperature, and Anolis melanophore physiology, that catecholamine secretion would be the primary effector of pigmentation at low temperature. However, since α-MSH is elevated at low temperature, it may contribute to cold induced darkening.

Another factor contributing to cold induced darkening may be the direct effect of cold on melanophores. Skin of A. carolinensis darkens in vitro at low temperatures (Smith, 1929; Hadley and Goldman, 1969). However, response to α-MSH is impaired at low temperature (Hadley and Goldman, 1969). It remains unclear how low temperature and increased α-MSH secretion at low temperature achieve pigment dispersion in melanophores. It may be that low levels of α-MSH enhance the direct effects of cold on Anolis melanophores.

Dark coloration at low temperature is conceivably advantageous to a poikilotherm such as Anolis in terms of crypsis and thermoregulation. Brown colors are abundant in most habitats during periods of cold weather. Dark skin coloration would help lizards be less conspicuous under these conditions. Dark pigmentation could also potentially increase absorption of solar radiation when basking on cool mornings. Since the overall metabolic rate of cold lizards is depressed, a dual endocrine signal (catecholamines and α-MSH) combined with the direct effect of cold on melanophores, may be an efficient means of achieving dark coloration at low temperature.

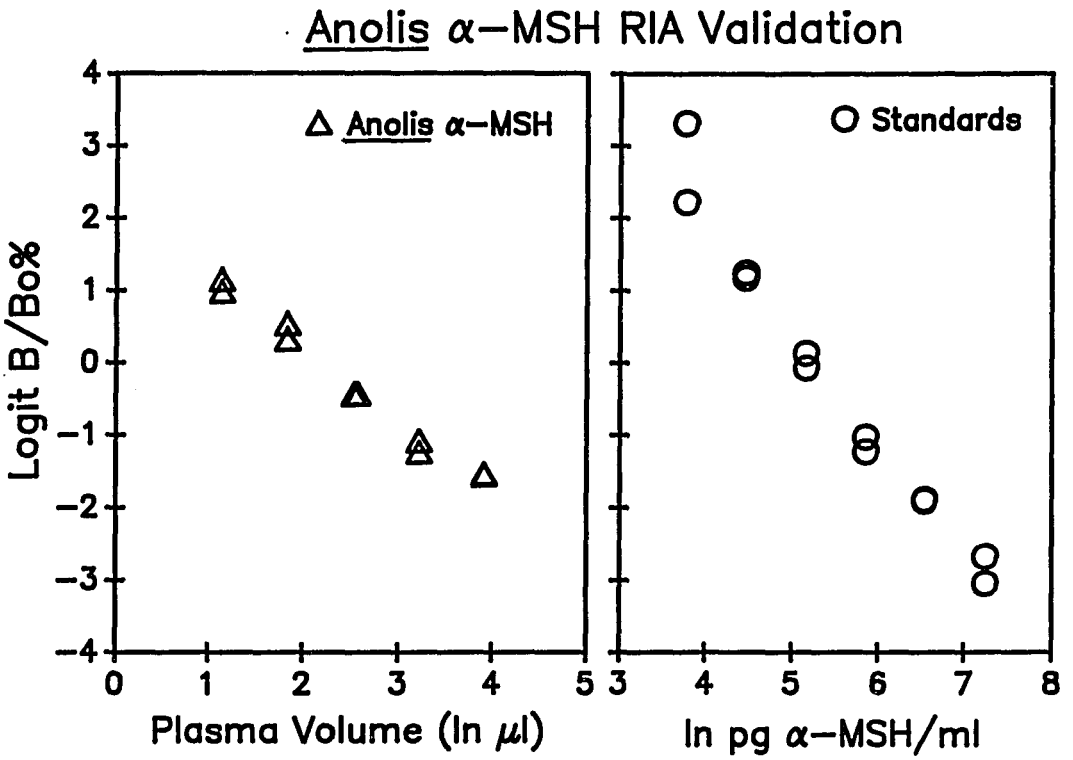


Figure 3.1 Anolis α-MSH RIA validation. Each symbol represents assay replicates of serial dilutions of either synthetic α-MSH (standards) or plasma from black background adapted lizards (Anolis).

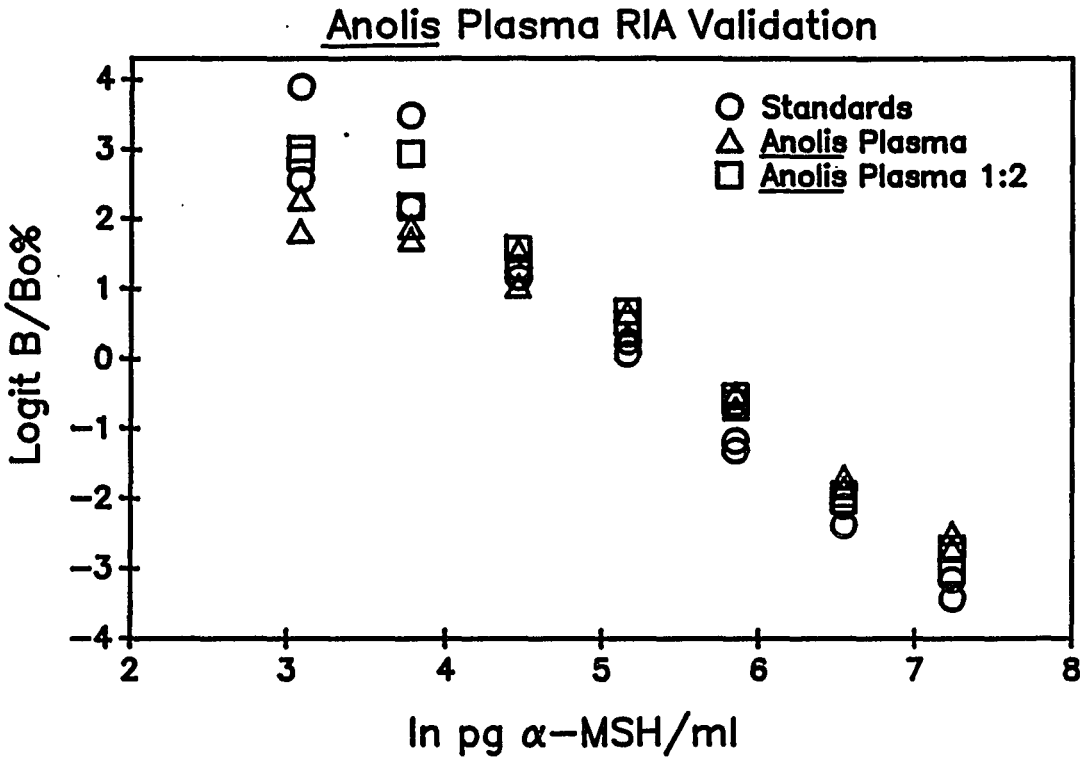


Figure 3.2 Anolis plasma RIA validation. Each symbol represents assay replicates of synthetic α-MSH diluted in assay buffer, or in Anolis plasma, or in Anolis plasma diluted in half with water.

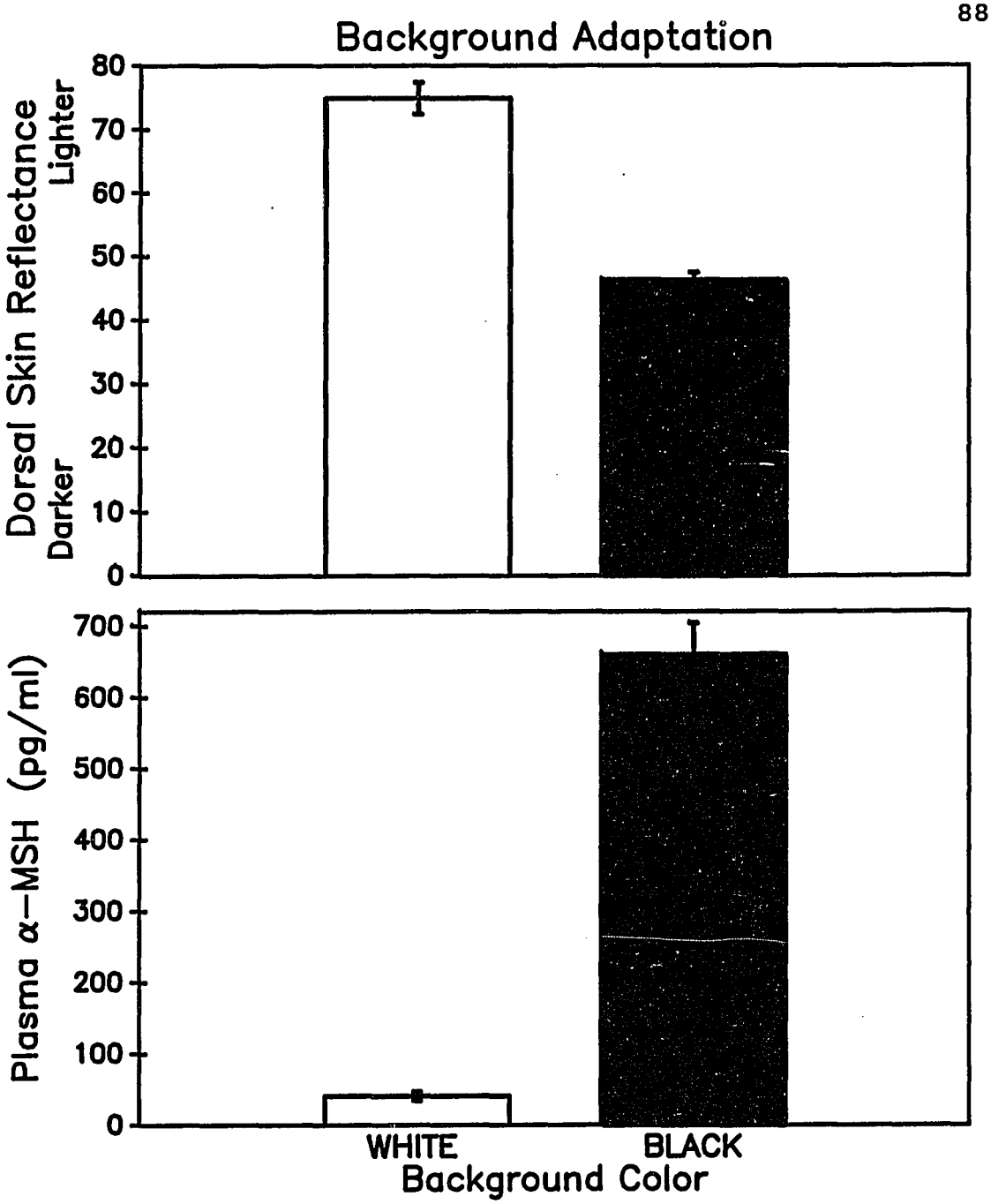


Figure 3.3 Effect of white and black backgrounds on Anolis dorsal skin reflectance and plasma α-MSH. Black background adapted lizards (n = 5) are darker ($p < 0.001$) and have greater α-MSH than white background adapted lizards (n = 8) ($p < .001$). Bars represent mean (± SE) values.

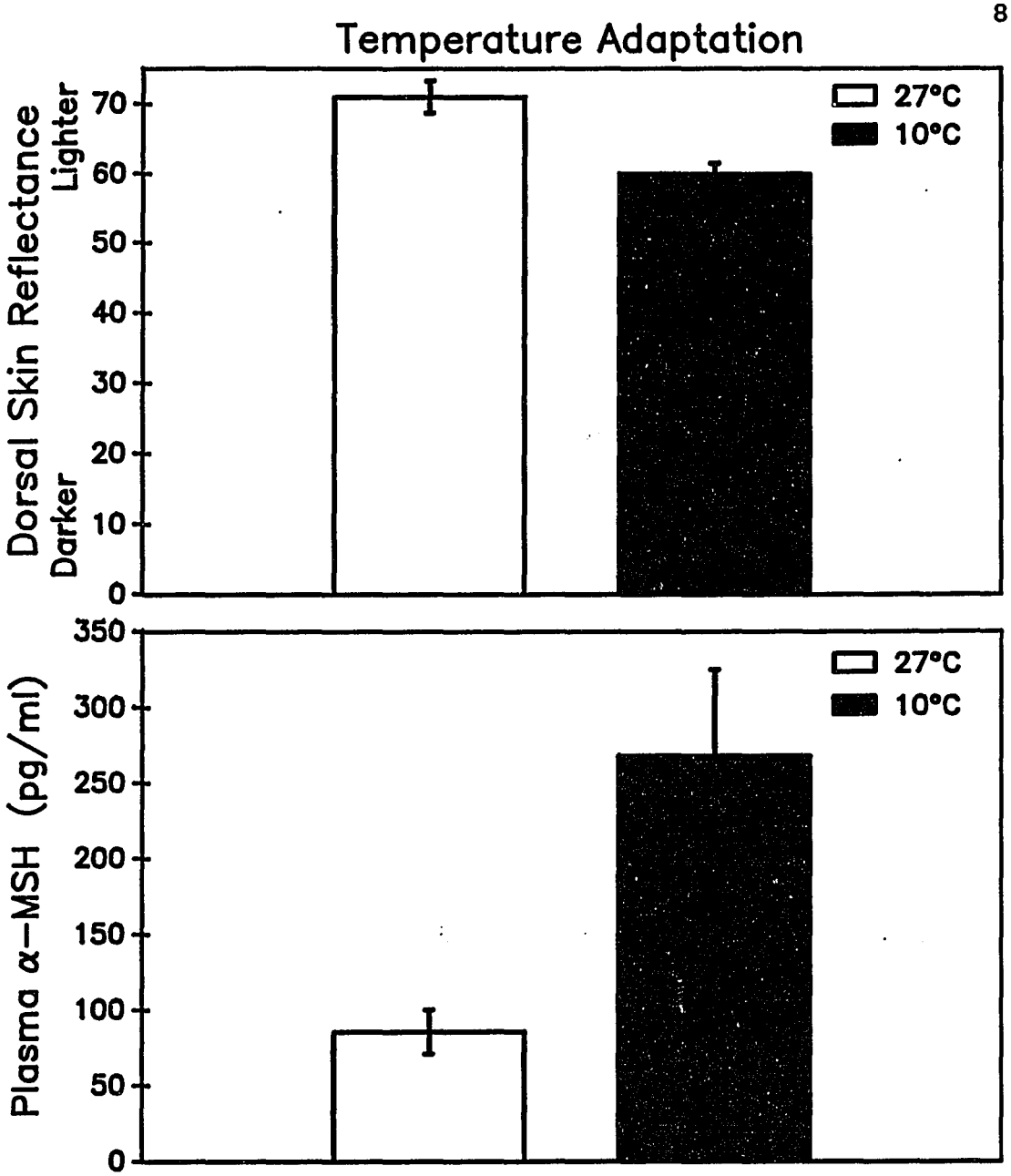


Figure 3.4 Effect of low temperature on Anolis dorsal skin reflectance and plasma α-MSH. Lizards were maintained at 27°C and 10°C on grey backgrounds (n = 6 at each temperature). Lizards at 10°C were significantly darker ($p < 0.01$) and had greater α-MSH ($p < 0.05$) than those at 27°C. Bars represent mean (± SE) values.

SUMMARY

Animal coloration is an intriguing phenomenon. The importance of skin color to an amphibian or reptile is multifaceted, and ultimately results from the influence of natural selection on the evolution of species' phenotypes (Hadley, 1972). Aspects of the process of color change ranging from molecular events that stimulate or inhibit secretion of melanotropic hormones, to the influence of skin color on animal behavior have been investigated (Bagnara and Hadley, 1973; Hadley, 1972; Greenberg and Chen, 1987). This research has focused on two environmental factors, light and temperature, that dramatically affect pigmentation. Rapid color change, in a natural setting facilitates crypsis and potentially affects thermoregulation. Animals that change skin color are better adapted to a variety of suitable habitats, and therefore are potentially more successful in many environments.

The results of the research presented here quantifies plasma α-MSH for the first time during change in skin color of three important species. Because of their ability to change color in response to visual and thermal stimuli, leopard frogs and the green anole are valuable models for study of the anatomy, physiology, and ecology of the color change process.

Dark skin color and high plasma α-MSH were observed in Rana pipiens, R. chiricahuensis, and Anolis carolinensis during adaptation to dark-colored backgrounds. Plasma α-MSH fell to nearly undetectable levels when these animals were on light-colored, reflective backgrounds. The history of investigations of background adaptation has clearly demonstrated the importance of animals' eyes and pituitary gland (Butcher, 1938; Hogben and Slome, 1931; Kleinholz, 1938a and 1938b; Parker, 1948; Sumner, 1944). The degree of substrate reflection, or albedo, perceived by the eyes is integrated by the central nervous system and results in regulation of α-MSH secretion by the pars intermedia (Jenks, et al., 1988). Evidence for this scenario was largely based on indirect measurement of α-MSH. This report provides the first direct measurements of α-MSH in plasma of leopard frogs and Anolis during background adaptation. Levels of α-MSH found in plasma were similar to concentrations of α-MSH that frog and lizard skin respond to in vitro. These results provide further evidence that α-MSH is the principal hormone involved in darkening skin during background adaptation in these species.

While all three species had elevated plasma α-MSH during adaptation to a black background, plasma α-MSH of R. chiricahuensis was many times higher than that of R. pipiens

or A. carolinensis. Some aspect of R. chiricahuensis pituitary secretion is extremely active. It remains to be seen which part of the α-MSH secretory pathway is responsible for production of such large amounts of the melanotropin. Ultrastructure of the pituitary of R. chiricahuensis has not yet been described. It would be interesting to determine the relative abundance of the two melanotroph cell types found in pituitary intermediate lobes in both R. pipiens and R. chiricahuensis. It may be the case that there are proportionately more of the synthetically active dark cells (Chronwall, et al., 1988) in R. chiricahuensis. This could partially account for the abundance of plasma α-MSH.

Knowledge of the unusually high plasma α-MSH of R. chiricahuensis provides some insight as to why the ventral skin of this frog is unusually melanistic. Dermal melanophores are dispersed among iridophores of the ventral integument. Pigment of these dermal melanophores actively migrates like dorsal pigment cells. It may be the case that secretion of large amounts of α-MSH during maturation of larvae and juvenile frogs leads to differentiation of latent melanophores in the typically white frog ventrum. Rana chiricahuensis is a potentially interesting model for study of the putative melanization inhibiting factor that induces white pigmentation of other frogs' ventral skin (Fukuzawa and

Ide, 1988). The trophic effect of α-MSH, and other factors in skin, on undifferentiated ventral pigment cells could moderate the inhibitor's influence. Quantitative characterization of the actions of the melanization inhibiting factor in R. pipiens and R. chiricahuensis would reveal whether its actions are dose dependent or if its actions are overridden by α-MSH or other skin factors.

Influence of the habitat of R. chiricahuensis must also be considered. Environmental albedo has a significant effect on development of color pattern in amphibians. Development and maturation in habitats of low albedo leads to significantly darker individuals than others that grow in high albedo environments (Fernandez and Collins, 1988). Much of the habitat of R. chiricahuensis is of low albedo. The soil and rocks are black, and the water is clear. These conditions of low reflectance also dictate dark skin color for animals whose main defense from predation is through crypsis.

Since both dorsal and ventral skin of R. chiricahuensis respond during background adaptation, it seems reasonable that the dark ventrum facilitates crypsis. In some way, this unusual coloration may render R. chiricahuensis less conspicuous to predators in its natural environment. Predators of R. chiricahuensis usually hunt in the water or at the water's edge. Dark skin color facilitates crypsis in

both these regions of low albedo.

Temperature was found to influence coloration of R. chiricahuensis and A. carolinensis, but not R. pipiens. The species that darkened at low temperature also exhibited increased plasma α-MSH. This indicates that in addition to its important role in background adaptation, α-MSH is also involved in the process of cold-induced darkening. However, melanotropin levels were not as high in cold-darkened individuals as in black-background adapted individuals. This probably reflects the overall depressed metabolic state of these poikilothermic animals at low temperature. The color pattern of cold-adapted Anolis also indicates that catecholamines are involved in the darkening response. Therefore, cold-induced darkening may result from a combination of endocrine secretions, including α-MSH, mediated through different pigment cell receptors.

Ability to darken at low temperature indicates that there is some benefit to dark coloration when it is cold. When considering the colors of the physical environment of the natural habitat of R. chiricahuensis and A. carolinensis during periods of cold, dark pigmentation could enhance crypsis of these animals during a period when they are potentially vulnerable. Seasons of prolonged cold cause vegetation to turn brown and die back revealing dark-colored

soil and rock. Again, dark skin is cryptic in this low albedo habitat.

The primary means of predator avoidance for both R. chiricahuensis and A. carolinensis is cryptic coloration, and secondarily, rapid escape. If discovered by a predator, the primary means (crypsis) has failed. At low temperatures the secondary means (rapid escape) is impaired. Cold-induced darkening may significantly enhance crypsis at low temperatures and compensate for the animals' reduced capacity to flee.

Dark pigmentation facilitates thermoregulation in some species (Norris, 1967; Walsberg, et al., 1978; Walsberg, 1983). Since dark pigments absorb radiation more quickly than more reflective colors, dark-colored Anolis may be able to increase body temperature by basking in the sun on cool, sunny mornings, speeding their warming rate and thereby increasing time for feeding and other behaviors. Green Anolis would gain less benefit than brown lizards because green skin reflects more radiation than brown skin. Since α-MSH is elevated at low temperature, it appears that the melanotropin plays a role in thermoregulation of this species.

It is less likely that an amphibian such as R. chiricahuensis could thermally benefit from a dark integument. Cooling due to evaporative water loss from moist, permeable

skin would probably negate any gains from increased absorption of radiation by pigment dispersion (Lillywhite, 1970). Since frogs frequently move between land and water, energy gained by basking would be quickly lost through conduction to water.

The three species examined, R. chiricahuensis, R. pipiens, and A. carolinensis, exhibit diverse morphology, behavior, and ecology. Yet all three share a common means of controlling skin color by secretion of α-MSH. It is impressive that the color change process of these animals share so much in common at the cellular and molecular level, yet each expresses a variety of skin colors under diverse environmental conditions. Differences in pigmentation reflect a "general chromatic compromise" (Norris and Lowe, 1964) resulting from natural selection acting at many levels. Each species interprets an assortment of cues from a milieu, then uses the same hormone, α-MSH, to change skin color in ways that undoubtedly increase their survival.

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