

Coloration Selection in *Ensatina* at Fort Ord UC Reserve

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Abstract: The ring complex of *Ensatina* salamanders represent a classic example of locally adapted subspecies that are parapatrically distributed. The Monterey *Ensatina* (*E. e. eschscholtzii*) is a cryptic subspecies typically found to with a brown dorsal and white to orange tinted underbelly. However, at Fort Ord Natural Reserve in Monterey County, CA, dark brown morph salamanders are uncommon while both leucistic morph (pink) and low melanin (light orange) appear to be more common. The presence of these morphs, which are uncharacteristic for *E. e. eschscholtzii* present the possibility that this population may be experiencing different selection pressures than other populations of *E. e. eschscholtzii*. This maritime chaparral habitat lies on light-colored wind-blown sand, and we hypothesize that this population has evolved the lighter coloration to background match the substrate to reduce predation. We tested this hypothesis by tracking predator attack marks on clay models of *E. e. eschscholtzii* consisting of three colorations: 1) pinkish colored (or leucistic) morphs, b) orange morphs, and c) dark brown dorsal morphs. These three morphs were placed on both light-colored sand and darker colored leaf-litter/dirt to measure relative attack rates of each morph on light and dark colored backgrounds. Brown morph models have equal attack rates (0.0164) on both light and dark backgrounds. Orange and Leucistic morph models had lower attack rates on light substrate (0.0104 and 0.0145, respectively) and much higher attack rates on dark substrate (0.0397 and 0.0326). These results suggest that the lighter coloration of Fort Ord *Ensatina* population may have adapted to background match the light colored sand to reduce predation.

Keywords: local adaptation, speciation, natural selection, color polymorphism, clay models, predation, ring species, hybrid zones

Introduction:

The study of morphological and behavioral effects of predator-prey interactions are fundamental to understanding interspecies interactions in ecology (Bateman 2017). Prey organisms may experience strong selection through crypsis to avoid predator detection (Skelhorn *et al.* 2010). This can result in phenotypic variation across diverse geographical regions leading to potential local adaptations (Gifford 2007). Biodiversity hotspots such as California contain many habitat types that have led to intraspecific local adaptation and ultimately speciation (Rissler *et al.* 2006). However, not all species complete allopatric speciation and instead unite at secondary contact zones allowing for gene flow between divergent lineages (Sinervo 2007).

One such taxa that has locally adapted to various environmental conditions throughout its' range is the *Ensatina eschscholtzii* ring species which displays a number of locally adapted subspecies that occur from British Columbia to northern Baja California (Wake 1997). In California, *Ensatina* occur in mountainous areas such as the Sierra Nevada and the Coast Ranges forming a ring around the Central Valley (Wake 1997; *Figure 1*). All but one of the seven subspecies of *Ensatina eschscholtzii* use crypsis to avoid predation, possibly leading to early stages of species formation through locally adapted coloration (Wake 1997). The morphological diversification among the seven subspecies throughout the ring complex have commonly resulted from biogeographic barriers, such as the Central Valley and the Monterey Bay (Kuchta *et. al* 2009).

The Monterey Bay is a historical geological formation that has impacted the isolation, phylogeographic, and morphological evolution of many taxa in the central California coast (Kuchta *et. al* 2009). The subspecies *E. e. xanthoptica* and *E. e. eschscholtzii* adjoin in the

Monterey Bay region near the Pajaro Gap, along the east-to-west oriented Pajaro River (Pereira and Wake 2009). This secondary contact zone has been disrupted by agriculture, development, and habitat restrictions (Kuchta *et. al* 2009).

Ensatina eschscholtzii eschscholtzii are typically found to have a uniform dark brown dorsal coloration, and the absence of ventral melanophores can create a whitish-orange tinted underbelly, limbs, and tail (Stebbins 1949). However, within Fort Ord Natural Reserve (FONR), both leucistic (no melanin) and low melanin orange morphed *Ensatina* have been found. Leucistic amphibians maintain normal dark eye pigmentation but lack integumentary pigment (Mitchell 2002). Genetically determined pigmentation abnormalities naturally occur but are very rare in vertebrate populations (Mitchell 2002). The cause of inherited color defects such as albinism and leucism are attributed to lack of activity in the enzyme tyrosinase which stimulates the formation of melanin (Bechtel 1991). Melanin is a dark brown to black pigment that occurs in the skin of people and animals, and when sparse or absent in *Ensatina* results in a light orange or pinkish coloration (Rawles 1960). Generally, leucistic animals stand out on darker soils and presumably result in high predation frequencies due to the high contrast of a light animal on a dark background, providing an easily recognizable search-image for predators. The high frequency of lighter colored morphs at Ft. Ord may be driven by natural selection and suggests that *Ensatina* with lighter coloration may better match the light-colored sands of the reserve.

Geologically the parabolic coastal dunes at Fort Ord are young dunes, developed along the coastline approximately 11,000-18,000 years ago when sea level dropped in the Monterey region (Dupre 1990, Thornton 2006). During the Pleistocene these dunes originated from onshore winds which deposited Salinas river sediments inland (Thornton *et. al* 2006). Given this timescale, adaptation to background match light substrates in this population would have occurred over a relatively short evolutionary timescale. Evolutionary speciation and ecological

dynamics that lead to species divergence are influenced strongly by habitat alteration and isolation (Whittaker *et al.* 2009, Irschick & Reznick 2009). Due to the dry, exposed nature of the maritime chaparral habitat, and the population's position at the edge of the subspecies range, *Ensatina* at Fort Ord may have low connectivity to other nearby populations. This low connectivity may reduce the influx of brown-morph *Ensatina* genes and could potentially allow locally adapted traits to persist. Additionally, brown-morph individuals that migrate into the light sand habitat may incur higher predation frequencies.

Observing and gathering quantifiable data of natural predation events is difficult, however, some success has been found through the use of replacement methods with prey mimicking clay models (Irschick & Reznick 2009, Bateman 2017). In order to measure relative predation rates among brown, orange, and leucistic *Ensatina* morphs we deployed soft clay models of each morph and recorded attack events by documenting predation imprints in the clay (Kuchta 2005). We deployed these models only at night (when these nocturnal salamanders are active at the surface) and recorded if they were deployed on a light-sand or dark-dirt /leaf litter background (the two distinct substrate types at the reserve). We hypothesize that 1) brown-morph salamander models will have higher predation rates on light versus dark backgrounds, 2) both orange and leucistic morph salamander models will have higher predation rates on dark versus light backgrounds, and 3) that orange and leucistic morph salamander models will have lower overall predation rates than brown morph models.

Methods and Materials:

2.1 Model Construction:

Models were created with plastic green salamander toys from the Tennessee Aquarium made in 1995, coated in black non-toxic modeling clay. The models were 55mm from snout to vent and 105 mm in total length, a size and proportion that is typical of adult *Ensatina*. These

models consisted of three color morphs of *E. e. eschscholtzii* a) light pinkish colored morphs resembling leucistic salamanders, b) an orange morphed variation and c) normal dark brown-backed *Ensatina* resembling those of the San Lucia Mountains (*Figure 2*). The leucistic morph was spray painted with a mixture of tan camouflage Rust-Oleum and gloss coral Ultra Cover paint+primer Rust-Oleum spray paint, in order to achieve a pinkish coloration. The orange morph was sprayed with a mixture of tan camouflage Rust-Oleum and orange Gloss Protective Enamel Rust-Oleum spray paint. The typical *E. e. eschscholtzii* morph was given a brown dorsum with brown camouflage non-reflective finish Rust-Oleum spray paint and an orange ventral painted with the same orange Rust-Oleum spray paint as orange morph; with additional orange pigment added to upper leg segments with pure pumpkin orange DecoArt all-purpose paint. The models received a final coat of Rust-Oleum clear enamel gloss finish to appear shiny and wet similar to amphibians (Kuchta 2005; *Figure 3*).

Twenty-four ounce single compartment Nutriboxes were used to cover models during the day to avoid marks from diurnal predators that would not typically come in contact with *Ensatina*, which take shelter during the day. Nutriboxes were colored with gloss meadow green Ultra Cover paint+primer Rust-Oleum spray paint to block model visibility from diurnal predators.

2.2 Model Placement-

Models were deployed on UC Santa Cruz FONR, which has restricted access to the public (*Figure 1*). These models were placed into 80 clusters of three models (240 models total), each cluster containing one of each morph. These eighty points were placed randomly throughout the reserve and cover the four most common habitat types on the reserve: oak forest, chaparral, grassland, and open shrub. Each of the three models were tied with Shur Strike fishing line one meter away from an Alazco steel tent peg and placed in a triangle formation. Each

model was set approximately two meters apart and placed on either light-colored sandy area or a darker colored dirt or leaf litter area. Models were deployed in the field on November 28th and collected on January 11th, 14 nights per point was collected, totaling 3281 model nights. One model set out for one night will be referred to as a model night. Models were uncovered each night between 15:30-17:30 and recovered and checked for predation each morning between 5:30-8:30.

2.3 Predation scores:

When predators such as rodents or birds attack the models, they will leave indentations that will be scored. Multiple marks on a model from one night are considered a single “attack event”. If there are significantly fewer marks on the leucistic models compared to the other two darker models this will support the hypothesis that natural selection is favoring the lighter coloration of the leucistic salamanders by having lower predation rates when compared to the darker *E. e. eschscholtzii*.

2.4 Statistical Analysis

Predation events were scored by marks on models in a single night. Models were categorized as either attack or not. Multiple predator strikes on the same model in the same night were counted as a single predation event in order to avoid misrepresenting multiple attack marks by a single predator. The proportion of total predation events out of the total model nights were compared across each morph on dark versus light substrate.

All statistical analyses were conducted in JMP statistical software v14. To compare how morph coloration affects predation frequency on dark versus light substrate background, contingency tests were used to determine statistical significance, with $P \leq 0.05$ established as significant (Kuchta 2005).

Results:

All data regarding model nights, attack, and predation rates for each morph can be found in Table 1. The total number of model nights for each morph were within 1% variance of each other, with brown morphs deployed for 1,098 model nights, orange morphs for 1,085 model nights, and leucistic morphs for 1,097 model nights. Eighteen separate predation events for brown morphs were recorded for a predation rate of 1.64% (18 attacks/1,098 model nights), with no variation between light and dark substrates (1.64% attacked; light substrate=8 attacks/487 model nights, dark substrate=10 attacks/611 model nights). Orange morphs had 29 separate predation events recorded for a predation rate of 2.67% (29 attacks/1,085 model nights), with 5 attacks on light substrate (1.04% attacked; 5 attacks/481 model nights) and 24 attacks on dark substrates (3.97% attacked; 24 attacks/604 model nights). Leucistic morphs had 29 separate predation events recorded for a predation rate of 2.46% (27 attacks/1,097 model nights), with 7 attacks on light substrate (1.45% attacked; 7 attacks/484 model nights) and 20 attacks on dark substrate (3.26% attacked; 20 attacks/613 model nights).

Brown morphs did not display more predation on light versus dark substrate ($N_{\text{strikes on light}}=8/479$, $N_{\text{strikes on dark}}=10/601$, $\chi^2=0$, $P=0.99$; Figure 5). However, orange morphs were displayed more predation on light versus dark substrate ($N_{\text{strikes on light}}=5/476$, $N_{\text{strikes on dark}}=24/580$, $\chi^2=0.03$, $P=0.0017$; Figure 5). Similarly, leucistic morphs displayed more predation on light versus dark substrate ($N_{\text{strikes on light}}=7/477$, $N_{\text{strikes on dark}}=20/592$, $\chi^2=0.01$, $P=0.047$; Figure 5). Brown morphs were 18.5% of all strikes on dark substrate; orange morphs (44.7%) and leucistic morphs (36.8%) made up the remaining strikes (Figure 6). Brown morphs were 39.8% of all strikes on light substrate; orange morphs (25.2%) and leucistic morphs (35%) made up the remaining strikes on light substrate (Figure 6).

Low predation frequencies were observed on all model morphs; 1.64% of brown morphs, 2.67% orange morphs, and 2.46% leucistic morphs experienced predation. Total model predation frequency was 2.22% and averaged 2.25%.

Discussion:

The results of this experiment suggest there could be different predation rates among normal brown morph, light colored orange morph, and even lighter colored leucistic morph *Ensatina* in the maritime chaparral habitat of Fort Ord Natural Reserve. Brown morph *Ensatina* showed a lower overall predation rate than the two lighter colored morphs and have comparable predation rates on both light and dark substrates. This finding does not support two of our hypotheses which predicted 1) lower predation rates on dark backgrounds than on light backgrounds due to crypsis, and 2) higher predation rates overall. These results imply that brown morph *Ensatina* may in fact have lower predation rates, and with enough immigration of brown morphs into the Fort Ord population, there could be an increase of brown morphs in the future.

Low melanin *Ensatina* models experienced a higher overall predation rate than brown morphs. However, the lowest predation rates of any morph on any background were those of the orange and leucistic morphs on a light background. This may explain why there is a high proportion of light morphs in the Fort Ord region. Leucistic animals tend to be rare in nature and are typically highly unsuccessful comparative to normally pigmented individuals (Mitchell 2002, Bruni 2017). Despite this most *Ensatina* encountered in Fort Ord have either an orange or leucistic coloration, which suggests that either in the past they have had strong selection pressures to match a light background and/or are currently experiencing selection for crypsis. Currently the majority of Fort Ord's substrate is dark, but historically Fort Ord had extensive dunes throughout the region containing light sands (Thornton *et al.* 2006). Therefore, these

morphs may support previous evidence that massive reduction in melanin loading in the skin allows for greater crypsis on the white sand substrate (Rosenblum Harmon 2010).

All model types exhibited low predation frequencies however, variation between models was observed. These small differences over a long timescale can amount to large differences in survival and may have huge impacts on selection pressure over time. Therefore, the nocturnal predation events observed in these models support natural selection as a large driving force behind their selection on coloration.

Color variation is a key trait in the advancement in ecological divergence for predator avoidance coinciding with substrate color and environmental conditions (Rosenblum *et al.* 2004). Fort Ord's dry, sandy environment is not characteristic of the moist forest habitats typically inhabited by *Ensatina* therefore, creating unique selection pressure on this population. *Ensatina* typically have low dispersal ability, which may influence the morphology and behavior in regional populations; this allows different regions across its' range to become adapted to local conditions (Whittaker 2009, Staub *et al.* 1995).

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Figures:

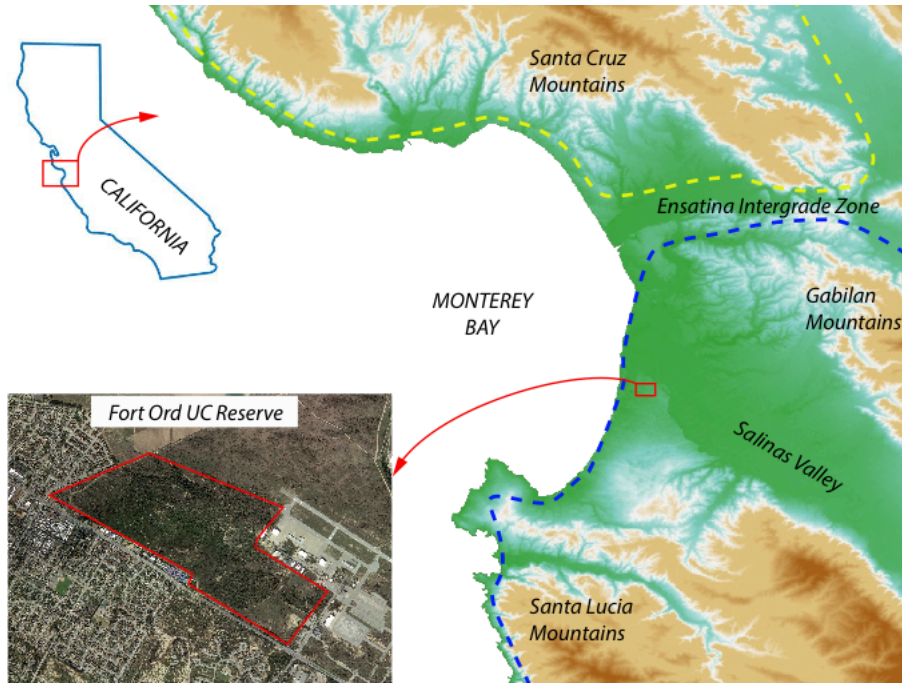


Figure 1. Location Map. Ring Species Ranges. Fort Ord Specific. Our study site is outline in red throughout this diagram. On the right side the Ensatina Intergrade Zone is expressed by dotted lines. The yellow line represents the zone of *E. e. xanthoptica* distribution and the blue represents the zone of *E. e. eschscholtzii* distribution.



Figure 2. San Lucia Ensatina. (*E. e. eschscholtzii*) Pictures show the dark pigmentation typically seen of *Ensatina* in the mountain ranges near Fort Ord Reserve. (Photos accessed from CaliforniaHerps.com)

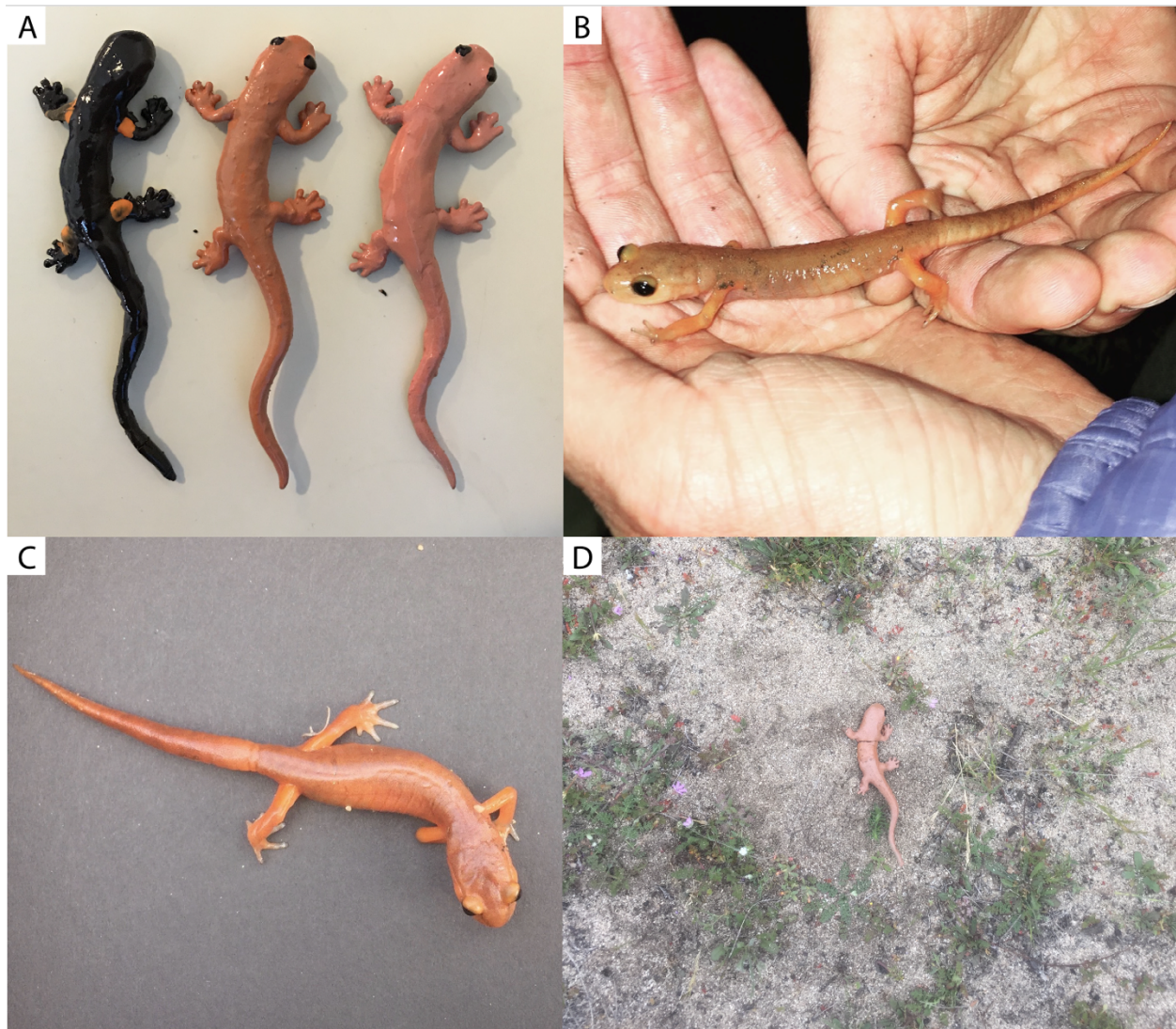


Figure 3. Model and Morphs. Photo A shows the three types of clay models deployed in this experiment. B and C display live orange morphed salamanders that have been found at Fort Ord Reserve. Photo D is a photo graph of an orange model placed against light substrate in the study site.



Figure 4. Predated Models. Images of models post-predation events. Indentations in the clay demonstrate predation strikes.

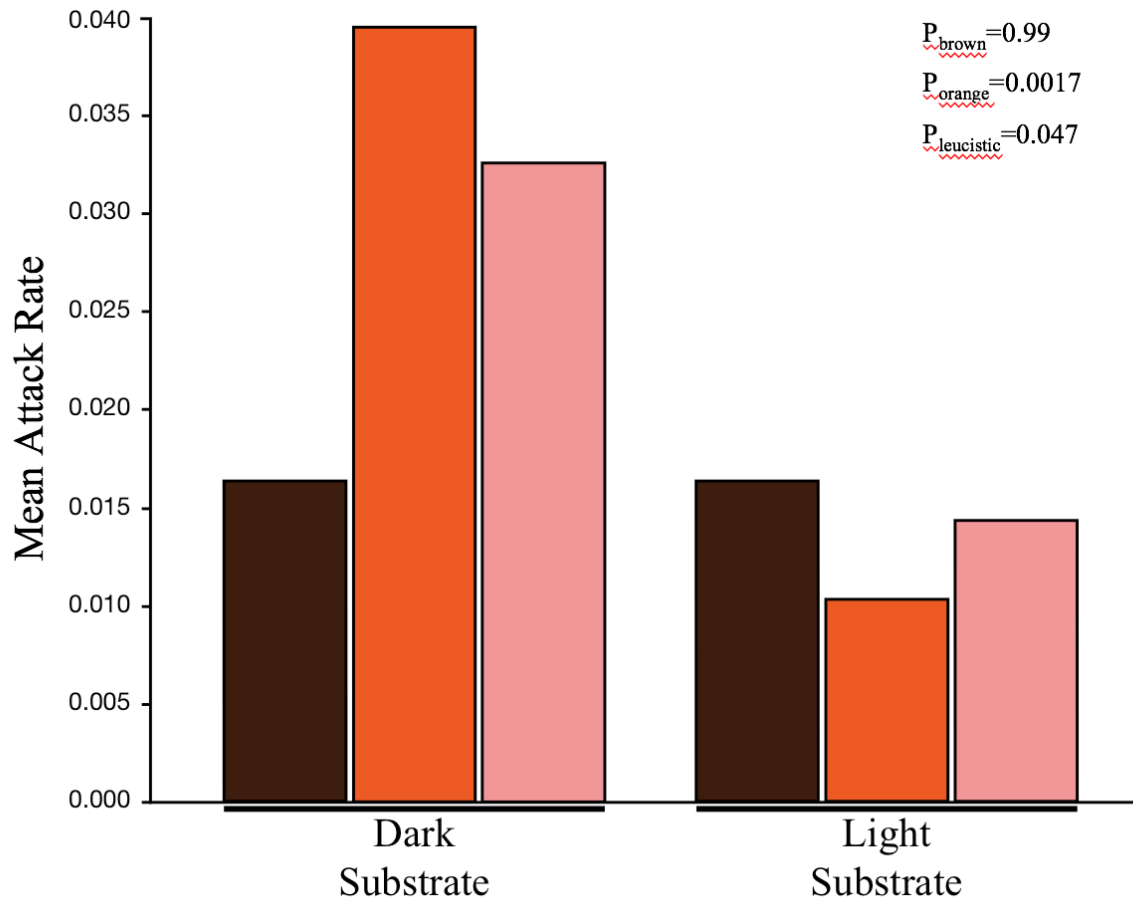


Figure 5. Substrate Effect on Morphs. This graph shows how morph color on a specific substrate affects the mean predation rates. Brown morphs (dark brown bars) did not vary on different substrates, however orange (orange bars) and leucistic (pink bars) morphs show large amounts of variation between dark and light substrate.

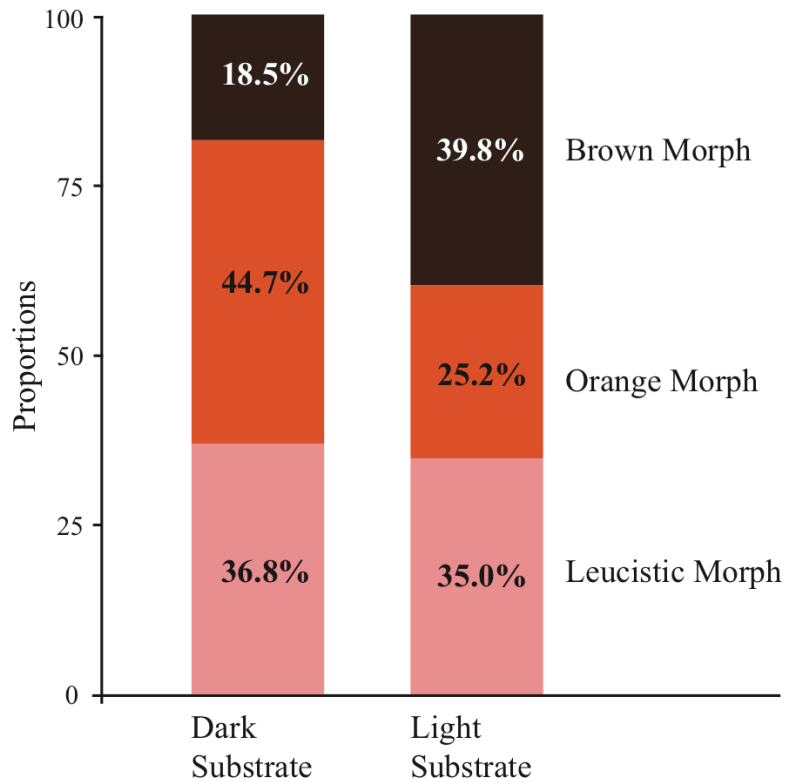


Figure 6. Substrate Effect on Morphs. This graph shows the percentage that each morph was struck out of the total amount of strikes that occurred on dark versus light substrate type.

Tables:

Ensatina Morph	Model Nights	Total Attacks	Predation Rate
<i>Brown</i>	1098	18	0.0164
-light sand	487	8	0.0164
-dark sand/leaf litter	611	10	0.0164
<i>Orange</i>	1085	29	0.0267
-light sand	481	5	0.0104
-dark sand/leaf litter	604	24	0.0397
<i>Leucistic</i>	1097	27	0.0246
-light sand	484	7	0.0145
-dark sand/leaf litter	613	20	0.0326

Table 1. Raw Data Table. Taken and struck uncovered models were included in total attacked but not in total strike count.