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### Evolution of Molt Phenology in Dwarf Hamsters

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EVOLUTION OF MOLT PHENOLOGY IN DWARF HAMSTERS

By

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Thesis

Presented in partial fulfillment of the requirements  
for the degree of

Master of Science  
in Organismal Biology, Ecology, and Evolution

The University of Montana  
Missoula, MT

May 2017

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## **Abstract**

Clare-Salzler, Zachary J., M.S. May 2017

Organismal Biology, Ecology, and Evolution

### EVOLUTION OF MOLT PHENOLOGY IN DWARF HAMSTERS

Chairperson: Jeffrey Good

Environmental seasonality presents many challenges for survival. In response, organisms from across the tree of life exhibit adaptive phenology, which can facilitate survival from season to season. In particular, many species are white in the winter and brown in the summer where winter snow cover alters the visual environment. There is an extensive body of literature describing the physiological and cellular regulation of this trait in the Siberian dwarf hamster (*Phodopus sungorus*), but there are major shortcomings in our understanding concerning the evolution and molecular regulation of mammalian skin phenology. Notably, little is known about the genetic basis or the transcriptional regulation of seasonal coat color change and previous quantification of coat color during the molt to the winter pelage has substantial qualitative bias, rendering comparative frameworks futile. Resolving these gaps in knowledge will illuminate how adaptive phenology evolves and how seasonal coat color change is regulated in the skin. I used two species of dwarf hamster to address these issues: *P. sungorus*, a species that expresses a white winter coat, and the sister species *P. campbelli*, which expresses phenology similar to *P. sungorus*, but does not molt to a white winter coat. I conducted a series of crosses between *P. campbelli* and *P. sungorus* to dissect the genetic basis of pelage phenology. I also quantified pelage reflectance on an objective, continuous scale in both species, and their F1 and backcross hybrids. Measuring coat color on this scale allows for unbiased, direct comparisons between genotypes. Finally, I used RNA-seq to further understand the regulation of molt phenology in the skin of both species, and in hybrid individuals. In our backcross experiment, F1 hybrids expressed a winter pelage phenotype more similar to *P. campbelli* than the white phenotype in *P. sungorus*, indicating that molting to a white winter coat is generally recessive. There was also considerable variation in terminal coat color in backcross individuals, suggesting that mutations in relatively few genes underlie the evolution of seasonal coat color change. *Phodopus sungorus* exhibited more robust patterns of phenology in the skin than what was found in *P. campbelli* or F1 hybrids, which brings into question the validity of direct phenology comparisons made in the past between the two parental species. Our crossing experiment demonstrates that evolution of a complex adaptive trait, like seasonal coat color change, may only require changes in relatively few loci. Despite the fact that these two species share many seasonal changes, gene expression changes during seasonal molts appear fairly divergent. *Phodopus sungorus* shows weak temporal patterning in gene expression changes while *P. campbelli* and F1 hybrids do not. The hybrids, in particular, showed dampened expression changes, potentially reflecting hybrid incompatibilities that disrupt seasonal phenology.

## ***Acknowledgments***

First, I would like to acknowledge my advisor Jeffrey Good for his support and guidance during my time as his graduate student at the University of Montana. He was the source of incredible insight that went into this thesis and making sense of an unexpectedly complicated and convoluted story. Also, I would not have had the opportunity to come to the incredible state of Montana, which has shaped my life in so many ways, if it was not for his trust in me to be a contributing member of his lab. Living here and building professional and personal relationships has taught me more than I could have imagined. I would also like to thank my lab mates, friends, and family who helped me along the way. Tom Brekke provided an incredible amount of help throughout my thesis work especially with animal husbandry and bioinformatics, even from across the Atlantic Ocean. Erica Larson generously guided me through differential expression analyses and helped edit parts of this thesis. Mafalda Ferreira and Brice Sarver offered bioinformatic assistance as well as reference genome and transcriptome assembly expertise. Tiago Antao was an unparalleled resource for all server and bioinformatics needs. Nick Hinricher, an undergraduate student, assisted with RNA sampling and colony maintenance. Colin Callahan was always able to answer questions related to wet lab work and acted as a constant source of Wu Tang and The Wire references. Matt Jones knows the literature better than most in our field and was always willing to guide me toward the best sources of information. Nathanael Herrera is about as solid of an office mate as they come and he was always able to give me invaluable advice, encouragement, or a whole-hearted laugh. Eric Bittman from the University of Connecticut and Robert Johnston from Cornell University were an integral part of supplying the animals that were ultimately the model for this Master's thesis. Lastly, all supplies, sequencing costs, and bioinformatic resources were supported with funds from University of Montana Small Research Grant Program, University of Montana start-up funds to Dr. Good, and an instrumentation grant from the Murdock Charitable Trust.

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## CHAPTER 1

### *Introduction*

Seasonal environmental variation presents various adaptive challenges for many living organisms. Changes in temperature and precipitation can vastly alter landscapes and decrease food availability, especially in cold climates. Survival in regions with dramatic seasonal changes in the environment requires seasonally plastic traits to coordinate needs for survival with variable environmental stressors. Seasonality in climate is a predictable, circannual pattern and in response many organisms exhibit phenological phenotypes that co-vary with seasons (Piersma and Drent 2003). In order to synchronize phenological traits with the seasonal environment, seasonally adapted species often co-opt environmental cues as signals for induction of a given seasonal phenotype (Stuart-Fox and Moussalli 2009, West-Eberhard 1989). These adaptive traits can compensate for ecological challenges due to inadequate food availability, extremely low temperatures, and differences in the visual environment due to snow cover. Many seasonally adapted traits are regulated by the environment via changes in daily photoperiod or temperature (Lincoln et al. 2003, Lincoln et al. 2006).

Camouflage is an effective and taxonomically common strategy for predator avoidance and as a result, animal pigmentation is often highly correlated with the background environment (Caro 2005). In particular, there is a rich literature describing the evolution and maintenance of locally adapted coat color polymorphisms in rodent populations where pelage color matches the predominant substrate color in the local environment (Nachman 2003, Hoekstra et al. 2005, Hoekstra 2006, Steiner et al. 2007, Linnen et al. 2009, Manceau et al. 2011, Linnen et al. 2013). In addition, there are similar examples of adaptive color

polymorphisms that match local environmental substrate color in a variety of squamates (Rosenblum et al. 2004) and insects (Farkas et al. 2013).

Seasonally variable environments present additional challenges to camouflage since environmental substrate color varies with and without snowfall. Numerous boreal and arctic species exhibit phenology in coat color that is synchronized with the presence or absence of snow. Molting to a white winter coat has evolved multiple times in temperate and boreal mammalian species, including in *Phodopus sungorus* (Stoner et al. 2003, Mills et al. 2013, Zimova et al. 2014). This dwarf hamster species is endemic to the steppes of the Altai and Tien Shan mountains in northern central Asia (Ross 1998) where the climate is highly seasonal and winters are extremely cold and snowy. A suite of other seasonally adaptive traits also varies in concert with seasonal coat color in *P. sungorus*, including thermoregulatory and metabolic acclimatization (Stullken and Hiestand 1953, Heldmaier and Steinlechner 1981, Bockler and Heldmaier 1983, Russel and Tumlinson 1996). Previous studies have characterized the physiological regulation of seasonal coat color change in *P. sungorus* (Bartness and Wade 1985, Heldmaier et al. 1981, Teubner et al. 2008, Duncan and Goldman 1984); however, the evolution and genetic basis of this trait remains poorly understood (Lynch et al. 1989).

Characterizing the genetic basis of adaptive phenotypes is crucial for understanding patterns of adaptive evolution and the origins of biodiversity. Evolution of traits with little regulatory complexity require changes to only one or a few genes, but traits with complex regulatory pathways, like seasonal coat color change, may evolve by changes in many genes or just a few (Flint et al. 1995). Drawing connections between genotype and phenotype allows evolutionary biologists to address many longstanding questions. For example, how do



adaptations function at the molecular level (Long et al. 2015) and do adaptations generally involve a few genes of large effect or myriads of genes of nearly infinitesimal effect (Orr 2005, Mackay et al. 2009)? For adaptive phenotypes, coat color is a particularly tractable trait to dissect because it is a non-cryptic phenotype and there is a rich history of exploration into animal pigmentation (Caro 2005, Caro 2009, Hofreiter and Schoneberg 2010, Hubbard et al. 2010, Poelstra et al. 2013). While the genetic basis of adaptive coat color continues to receive considerable attention (Nachman et al. 2003, Rosenblum et al. 2004, Hoekstra et al. 2005, Steiner et al. 2007, Linnenn et al. 2009, Manceau et al. 2011, Linnen et al. 2013), little is known about the genetic basis of seasonal coat color although this form of adaptive coat color variation has been shown to be crucial to in some species (Zimova et al. 2016).

The genus *Phodopus* provides a tractable and appropriate model to understand the evolution of seasonal coat color. Preceding winter months when natural photoperiod decreases relative to summer months, *P. sungorus* molts to a white pelage. *P. campbelli*, sister species to *P. sungorus*, is native to the cold, arid steppes and semi-deserts of central Asia (Ross 1995) and exhibits photoperiod-regulated winter adaptations mainly associated with thermoregulation and energetics (Wynne-Edwards 1998, Wynne-Edwards et al. 1999) However, unlike *P. sungorus*, the pelage of *P. campbelli* remains mostly brown throughout the year (Heldmaier and Steinlechner 1981, Timonin et al. 2006), presumably reflecting adaptation to drier and less snow covered winter environments in this species (Wynne-Edwards 1998). Since winter phenotypes are induced via reduction in photoperiod, the winter molt can be experimentally induced by artificially reducing daily photoperiod for captive hamsters (Duncan and Goldman 1984). Additionally, *P. sungorus* and *P. campbelli* can produce viable first-generation hybrid

Table 1. Summary of phenologies in *P. campbelli* and *P. sungorus* and associated references for each set of phenotypes. \*only Bilbo et al. 2003 supports this.

	Body mass	Testes mass	Ovary mass	Testosterone	Leptin	Prolactin	Estradiol	Coat Color
<i>P. campbelli</i>	↓ (25%)	↓ (90%)	↓ (30%)	No change	No change	No change	No change	Lighten to white*
<i>P. sungorus</i>	↓ (25%)	↓ (90%)	↓ (30%)	↓ (95%)	↓ (70%)	↓ (85%)	↓ (65%)	Lighten to white
<b>References</b>	Bilbo et al. 2003, Hoffman 1973, Heldmaier et al. 1982	Bilbo et al. 2003, Timonin et al. 2006, Hoffman 1973	Timonin et al. 2006	Timonin et al. 2006	Timonin et al. 2006	Timonin et al. 2006, Dodge et al. 2002	Bilbo et al. 2003	Bilbo et al. 2003, Heldmaier et al. 1981, Hoffman 1978

offspring and second-generation backcrosses (Brekke and Good 2014, Safronova and Vasil'eva 1996), which allows us to map genetic variation by exploiting F1 gamete recombination entering backcross individuals and begin to dissect the genetic basis of this seasonal and adaptive trait. Despite alternative winter coat colors, *P. sungorus* and *P. campbelli* express many similar winter-adapted phenologies (table 1). In addition, one paper has reported that *P. campbelli* also lightens coat during the winter molt (Bilbo et al. 2003). The apparent phenotypic similarities between *P. campbelli* and *P. sungorus* phenologies may afford these two species as a useful system for studying adaptive coat color evolution using both quantitative genetic and comparative frameworks.

This study used laboratory crosses to first examine the genetic basis of seasonal coat color change and further test whether this is an appropriate system for dissection of this trait through direct comparisons between species. First, I will generate a comparative timeline of the seasonal molts and quantify overall phenotypic differences between *P. campbelli* and *P. sungorus* on an objective, continuous scale. There has been considerable work done on the

phenology of *P. sungorus* and, to a lesser extent, *P. campbelli*. However, an objective, high-resolution measure of coat color change along the molt timeline is still lacking in the literature. Measuring coat color on an absolute, continuous scale not only provides a more complete metric for assessing coat color (Nachman et al. 2003, Hoekstra et al. 2005, Steiner et al. 2007, Linnen et al. 2009, Linnen et al. 2013), but also provides the means to make more direct comparisons between the molt timelines in two divergent species. Second, I will use crosses between the species to understand the genetic architecture of this adaptive seasonal phenotype. Elucidating the basic genetic underpinnings of seasonal coat color change will provide useful insight into how this form of adaptive coat color evolves. Color polymorphisms among populations of mice appear to have a somewhat simple genetic basis with a few loci of large effect (Steiner et al. 2007), but the evolution of seasonal coat color differences between species is potentially a much more complex trait and therefore may have a much more complex genetic basis. Thus, dissection of the genetic basis of seasonal coat color change between two hamsters species will be useful for evaluating the generality of other genetic studies on coat color evolution, while providing much needed insight into the evolution of an important phenological trait.

## **Methods**

### **Animals**

Six breeding pairs of *Phodopus sungorus* and six breeding pairs of *Phodopus campbelli* hamsters were used to establish our colony at the University of Montana. *Phodopus sungorus* were provided by Eric Bittman from the University of Massachusetts. This colony originated

from a colony established by Klaus Hoffman that was later managed by Bruce Goldman at the University of Connecticut. *Phodopus campbelli* individuals were provided by Robert Johnston from Cornell University. *Phodopus campbelli* breeding families originated from wild populations that were sampled in 1981 by Katherine Wynn-Edwards and then supplemented in 1990 with additional wild hamsters (Scribner and Wynn-Edwards 1994). The colonies at the University of Montana were managed under a crossing scheme to minimize inbreeding (Wright 1921). All breeding individuals were housed in 14-hr light/10-hr dark lighting regimen in accordance with the University of Montana's Institutional Animal Care and Use Committee regulations.

Two commonly implemented approaches to understand the genetic architecture of novel traits include genome wide association studies, which can be utilized in wild populations, and controlled line crosses between captive populations that differ in a trait of interest (i.e., line crosses). With sufficiently large sample sizes, line crosses can be used in tandem with genotyping to map quantitative trait loci (QTL). Here I used a line crosses to begin to characterize the genetic architecture of seasonal coat color change in captive, breeding *Phodopus* populations. Although previous studies have investigated the physiological basis and regulation of seasonal coat color change in *P. sungorus*, the genetic basis of this novel trait remains a mystery across boreal mammals with seasonal variation in coat color. Quantitative genetic analysis of seasonal coat color change will provide the initial insights for the genetic basis of this novel trait.

Two principal aims motivated this study. First, to generate a timeline for phenological change in coat color during the winter molt in *P. campbelli* and *P. sungorus*. Only two previous studies aimed to make direct comparisons between *P. campbelli* and *P. sungorus* phenology

(Bilbo et al. 2003, Timonin et al. 2006). However, Bilbo et al. (and all previous studies measuring pelage color change in *P. sungorus*) quantified pelage color using a highly subjective, categorical scale, which measures relative change from the darker summer pelage and was developed for use in *P. sungorus* (Duncan and Goldman 1984). Here I quantified changes in coat color using an objective, continuous measure by quantifying reflectance of coat along the molt timeline using a spectrophotometer. This allowed for a direct comparison of the coat color phenotypes between the two species and their F1 hybrids, quantified at a level of detail previously unattained. Second, these more robust phenotypic measures were then used to begin to dissect the genetic basis of this trait by examining patterns of phenotypic variation observed in a backcross experiment. Although not enough backcross hybrids could be generated to successfully map QTL associated with this trait (see below), observed phenological responses in the F1 and backcross hybrids did provide novel insights into the quantitative genetic basis of this trait.

### **Photoperiod and Sampling Schema**

Twenty adult female *P. campbelli*, 20 adult female *P. sungorus*, and 20 adult female hybrid hamsters were housed individually under the same 8-hr light/16-hr dark lighting regimen (Hoffman 1973) for 14 weeks. Previous work demonstrated that high levels of testosterone in *P. sungorus* dampened phenological responses to reduced photoperiod conditions (Paul et al. 2007) so only females were used in this study. Immediately following weaning, backcross litters were moved to a separate room and housed with littermates of the same sex under an 8-hr light/16-hr dark lighting regimen to emulate winter photoperiod conditions and to induce the

winter molt (Hoffman 1978). Littermates were identified using an ear notch identification system. All 32 backcross animals were kept on this lighting regimen for 15 weeks.

### **Quantifying Coat Color Phenology**

Previous studies quantified coat color change using a standardized, categorical 4-point scale to identify stages of pelage color from maximal summer morph (1, darkest) to maximal winter morph (4, white) (Duncan and Goldman 1984). While this method is useful for quantifying relative coat color throughout standardized transitions, this scale was developed specifically for quantifying coat color change in *P. sungorus* and therefore may not be sufficient for making quantitative comparisons between two species or among hybrid individuals. Furthermore, this method lacks resolution for quantifying different aspects of coat color and does not encapsulate variation at each color category during coat color change. Alternatively, spectrophotometer reflectance has been used to generate accurate, continuous, and high-resolution measurements of adult (non-changing) pelage color in other rodent systems (Hoekstra et al. 2005, Steiner et al. 2007). Spectrophotometer reflectance therefore may provide a higher resolution means of quantifying seasonal coat color change in *Phodopus* and this study will be the first to utilize this method to quantify changes in seasonal coat color in a mammal. Although reflectance measurements were used for most analyses, I also recorded hamster pelage coloration on the 4-point categorical scale (Duncan and Goldman 1984) to make direct comparisons with previous studies that implemented a categorical quantification approach.

I used a USB4000 Miniature Fiber Optic Spectrophotometer (Ocean Optics) with a PX-2

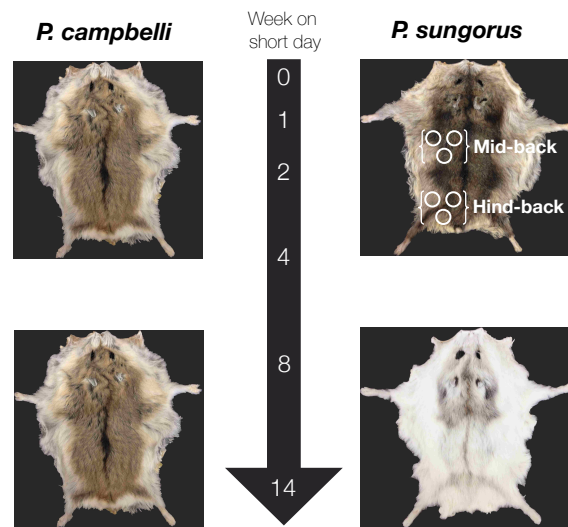


Figure 1. General sampling design as exemplified for coat color change between *P. campbelli* and *P. sungorus*. Circles on top right panel indicate locations where reflectance was measured and skin was sampled. Sampling was conducted at 0, 1, 4, 8, and 14 weeks on SD for *P. campbelli*, *P. sungorus*, and F1 hybrids. Backcrosses were sampled at 0, 3, 6, 9, 12, 15 weeks on SD and skin was removed only on week 15.

pulsed xenon light source and SpectraSuite software (Ocean Optics, version 2.0.162). For both species, I measured reflectance on animals (n=20 each, n=60 total) under anesthesia with isoflurane in samples of four at 0, 1, 4, 8, and 14-week time points under the simulated winter photoperiod conditions (figure 1). Three measurements were taken at a 30-degree angle directly on the specimen at the mid-back adjacent to the dorsal mid-line and at the hind-back adjacent to the dorsal mid-line. After reflectance was measured across all time points, these individuals were euthanized by asphyxiation with CO<sub>2</sub>. Liver samples were then taken for genetic material, which would contribute to the mapping panel and for genotyping. Skin samples were taken for gene expression analysis, which is covered in chapter 2 of this thesis.

Output text files from SpectraSuite were processed and seven color metrics that non-

independently measure components of reflectance were extracted using CLR: Colour Analysis Programs v1.05 (Montgomerie 2008) and then re-formatted using a custom Python script. The seven color metrics measured mean brightness (B2), intensity (B3), chroma (S3 and S5c), contrast (S6), and hue (H3 and H4c). Linear mixed-effect models for each of the seven color metrics were fit to the time series data with genotype and week treated as covariates. Reflectance measurements were used in concert with the relative scale developed by Duncan and Goldman (1984) and digital images were taken of each animal prior to measurement. In addition to coat color metrics, animals were also weighed at each data collection time point.

### **Using Hybrid Genomes to Examine the Genetic Basis of a Complex Phenotype**

A QTL experiment necessitates recombining genetic information from two distinct breeding lines or species that differ in a certain quantitative trait or set of traits. All following crossing schema are listed with female first and male second. New genetic combinations are generated by crossing hybrid F1 progeny (F1 x F1) or through a backcross (F1 x species 1 or species 2, figure 2). Crossing female *P. campbelli* and male *P. sungorus* will produce viable female offspring, but males are sterile. The reciprocal cross is non-viable due to extensive placental and embryonic overgrowth (Brekke and Good 2014, Safronova and Vasil'eva 1996). Therefore, this experiment utilized a backcross breeding design using F1 females.

Sampling and pelage reflectance was quantified in F1 hamsters (n=20) using the same methods as the in the parental strains (figure 1). Coat color reflectance was measured on all backcross animals under anesthesia with isoflurane every 3 weeks for 15 weeks including at time of wean. Again, three measurements were taken at a 30-degree angle directly on the



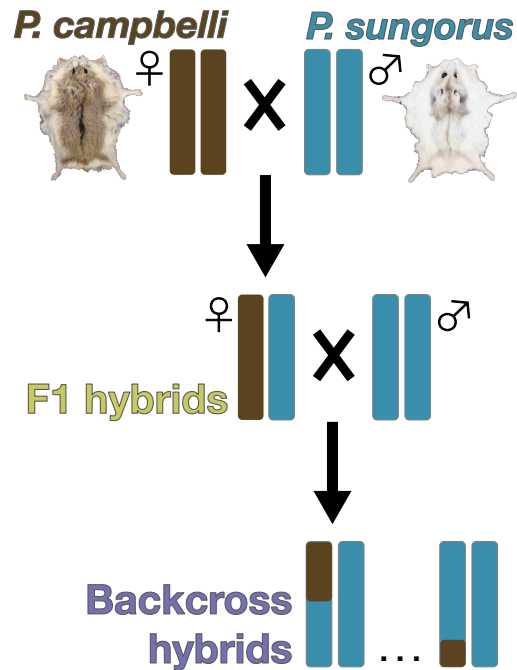


Figure 2. Breeding design for producing experimental animals. *P. sungorus*, *P. campbelli*, F1 hybrids, and backcross hybrids.

specimen at the mid-back adjacent to the dorsal mid-line and at the hind-back adjacent to the dorsal mid-line. After 15 weeks, backcross animals were euthanized by asphyxiation with CO<sub>2</sub> followed by cervical dislocation, skins were removed and catalogued, and liver harvested for genetic material. All F1 skin samples were taken for gene expression analysis, which is covered in chapter 2 of this thesis. Additionally, reflectance data for F1 and backcross individuals were processed and analyzed using the same pipeline as discussed with the parental strains in the previous section.

Although I was not able to generate enough backcross hybrids to have reasonable power to map QTL (table 2) associated with seasonal coat color change, the data generated using the 32 backcross hybrids were applied to the Castle-Write Estimator to generate a rough

Table 2. QTL parameters calculated with R/qtldesign (Sen et al. 2007)

Sample Size	Power	Effect Size	% Variance Explained
50	50%	1.09	22.82
50	75%	1.28	28.99
50	90%	1.45	34.44
100	50%	0.77	12.87
100	75%	0.90	16.96
100	90%	1.03	20.81
150	50%	0.63	8.97
150	75%	0.74	11.98
150	90%	0.84	14.90
200	50%	0.54	6.88
200	75%	0.64	9.26
200	90%	0.72	11.61

approximation of the number of genes associated with this trait. The Castle-Write Estimator is the simplest method to quantify the number of factors underlying a quantitative trait and takes into account the difference between the phenotypic means and variances between the parental strains as a proportion of the difference in variances between backcrosses and parental lines (Castle 1921).

$$n_e = \frac{(\bar{x}_{P1} - \bar{x}_{P2})^2 - (\sigma_{P1}^2 + \sigma_{P2}^2)}{8(\sigma_B^2 - \frac{(\sigma_{P1}^2 + \sigma_{P2}^2)}{2})}$$

This approach makes important assumptions, which limit the efficacy of the estimation and introduce bias. First, all alleles that increase the value are fixed in one parental strain and all alleles that decrease the value are fixed in the other parental strain. Second, allelic differences are equal at all loci and all loci are not linked. Finally, there is no dominance or epistasis among

alleles associated with the trait. Additionally, this approach is more accurate with large sample sizes (>200) and when the two parental means do not overlap in their distribution—10 or more standard deviations apart (Serebrovsky 1928, Zeng 1992). Although some assumptions of the Castle-Wright approach are violated, these estimates provide a sound, preliminary analysis for our data.

## **Results**

### **P. sungorus Exhibits Inducible Coat Color Change Compared to P. campbelli**

All *P. sungorus* individuals molted to a predominantly white pelage during exposure to short photoperiod conditions (8-hr light/16-hr dark), while no *P. campbelli* individuals exhibited detectable coat color change (figure 3). While this result is contradictory to some previous research (Bilbo et al. 2003), both *P. campbelli* and *P. sungorus* showed decreased body mass over time on reduced photoperiod conditions (figure 4), which confirms that all animals were photoresponsive and is consistent with previous studies (Hoffman 1973, Hoffman 1978, Heldmaier and Steinlechner 1981, Duncan and Goldman 1984, Kuhlmann et al. 2003).

Reflectance data were then processed into seven individual components of color with CLR and each component was plotted over time on short photoperiod conditions for each genotype category (*P. campbelli*, *P. sungorus*, F1 hybrids, and backcrosses). The linear mixed-effect models for each color component with genotype and week on short photoperiod as explanatory variables yielded significant effect of genotype and week only for *P. sungorus* and multiple components of color (figure 5; B2: brightness; B3: intensity; S3: chroma). Whiteness

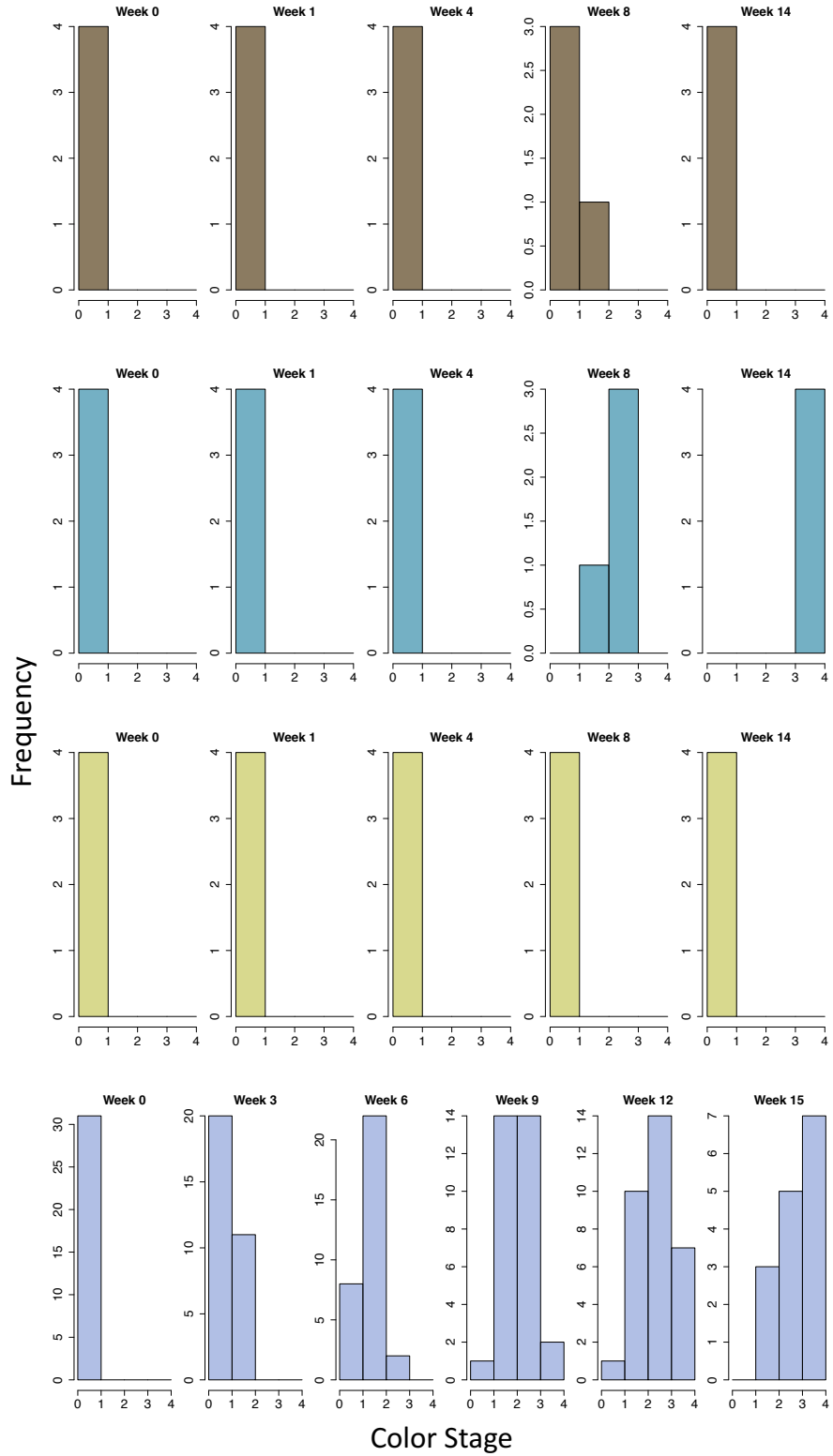


Figure 3. Categorical color value over the timeline of the molt for all individuals of all genotypes. *P. campbelli* (brown), *P. sungorus* (blue), F1 hybrids (green), backcross hybrids (purple).

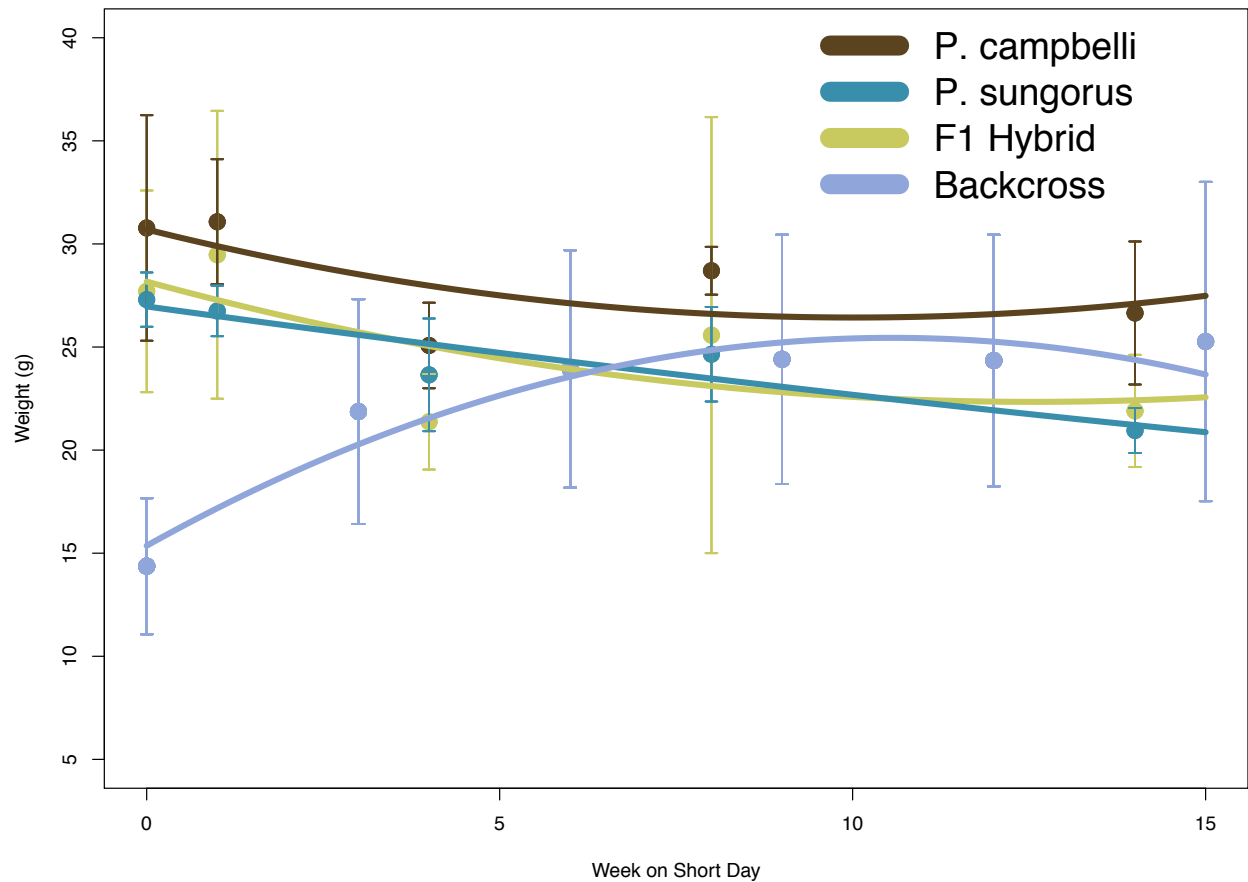


Figure 4. Average weight of each genotype over time with standard deviations. *P. campbelli* (brown), *P. sungorus* (blue), F1 hybrids (green), backcross hybrids (purple). Backcross hybrids increased in weight while all other genotypes decreased. Backcross hybrids were not fully grown at the time of the experiments since they were placed on short day conditions following wean while all others were full-grown adults on the first day of the short day treatment.

should reflect an increase in brightness and intensity of reflected light, but also be associated with a decrease in chroma, or intensity of reflected light within the visible color spectrum.

There was a significant increase in brightness ( $p < 0.01$ ) and intensity ( $p = 0.05$ ), and a significant decrease in chroma ( $p = 0.00$ ). These three color components that show significant effects of genotype and week corroborate the expectations of component association and direction with

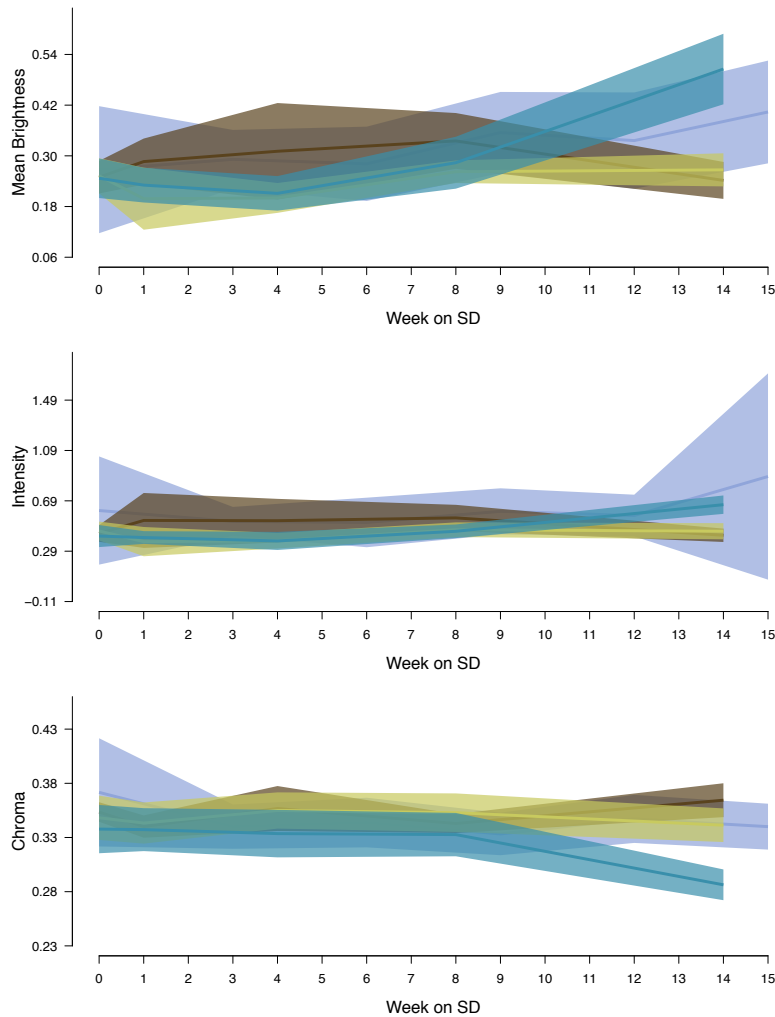


Figure 5. Reflectance measures showing significant differences over week and between genotypes for *P. campbelli* (brown), *P. sungorus* (blue), F1 hybrids (green), and backcross hybrids (purple).

pelage whiteness. The linear mixed-effect models for the four other color components (S3: Chroma; S5c: Chroma; S6: Contrast; H3: Hue; H4c: Hue—as a function of chroma) did not exhibit significant patterns of differentiation between the four genotypes over time and thus were not considered further in the analysis.

## The Genetic Basis of Seasonal Coat Color Change

F1 hybrid individuals (n = 20) exhibited a darker winter coat color similar to *P. campbelli* (figure 3, figure 5). As all F1 genomes are an equal autosomal pairing the *P. campbelli* and *P. sungorus* genomes, this experiment elegantly displays that the alleles for seasonal coat color change in *P. sungorus* are generally recessive. Given this general pattern of trait dominance, all F1 females were backcrossed to the recessive line, *P. sungorus*, to generate phenotypic variation in the backcross. Only 32 backcross offspring were generated from this cross (see below), however, there was sufficient variation across individuals in terminal pelage color to provide some basic insights into the genetic underpinnings of this trait (figure 5). Furthermore, average coefficient of variance for the three significant components among backcrosses throughout the molt was consistently greater than the coefficient of variance in all other genotypes (figure 6). Due to recombination in the F1 gametes entering the backcross, some backcross individuals will carry genotypes that more closely reflect *P. campbelli* or *P. sungorus* at causative loci. Of the 32 backcross offspring, nine (28%) exhibited terminal coat color phenotypes that were similar to the recessive phenotype in *P. sungorus*. While this is likely too few to quantitatively map a trait, the noticeable variation in terminal coat color among backcross offspring suggests that seasonal coat color change may have a relatively simple genetic basis involving relatively few linked loci.

Since a QTL study was not feasible, I applied the Castle-Write Estimator equation to the terminal coat color reflectance and 4-point categorical data collected from the parental lines and backcross offspring. For the categorical scale, this equation estimated that 1.33 factors (linked loci) were associated with seasonal coat color change. However, when applying

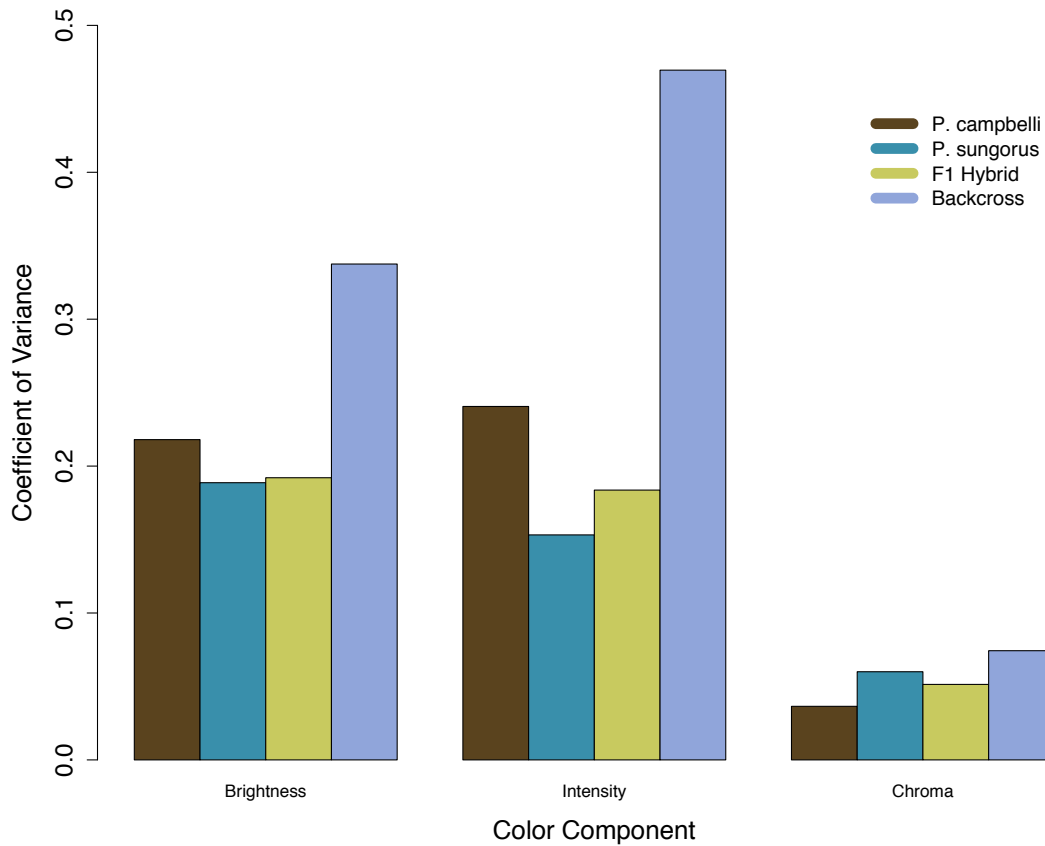


Figure 6. Average coefficient of variance for all genotypes for each of the significant reflectance measures.

reflectance data to this equation, reflectance measures returned very different estimates for genetic complexity. Measures for mean brightness, intensity, and chroma estimated there to be 0.84, 0.01, and 3.38 factors associated with the given measure, respectively. While these results should be considered as preliminary, they do suggest that there are likely a few loci of relative major effect that are associated with terminal coat color. These results to come with caveats given that many of the assumptions of the Castle-Wright estimator are likely not met, and that the sample size is too small (Zeng 1992).



### Low Fecundity in Backcross Females

We conducted a total of 13 crosses between the two species, *P. campbelli* (“gold” strain) x *P. sungorus*, to generate 20 female hybrid hamsters. Using all of these hybrid females, we conducted 20 backcrosses (F1 x *P. sungorus*) to generate 32 backcross individuals (15 females, 17 males) from 11 F1 females that had successful pregnancies. Compared to other crosses over similar lengths of time in our colony (table 3), this result indicates that F1 females had unexpected and prohibitively low fertility for the feasibility of a quantitative genetic mapping experiment.

Table 3. Summary of breeding success for different crosses in hamster colony.

	Avg litter size	Avg number of days crossed	Avg number of offspring per days crossed	
F1 x P. sun. “gold”	1.52	102	0.31	
F1 x P. sun.	1.78	95	0.34	
F1 x P. cam.	4.00	61	2.36	
P. cam. x P. cam.	4.55	102	0.89	
P. sun. “gold” x P. sun. “gold”	3.55	107	2.12	
P. sun. x P. sun.	5.63	66	0.68	
	Total crossed	Total females with a pregnancy	Total litters	Total offspring
F1 x P. sun. “gold”	20	11	21	32
F1 x P. sun.	7	7	18	32
F1 x P. cam.	28	28	36	144
P. cam. x P. cam.	5	5	20	91
P. sun. “gold” x P. sun. “gold”	26	26	64	227
P. sun. x P. sun.	4	4	8	45

## **Discussion**

Seasonally variable environments present additional challenges to survival because of dynamic and circannual fluctuations in the environment. *Phodopus* has emerged as a model system used to understand the regulation of seasonal crypsis in mammals (Bartness and Wade 1985, Heldmaier et al. 1981, Lynch et al. 1989, Teubner et al. 2008, Duncan and Goldman 1984). However, previous work does not provide a comprehensive quantification of seasonal coat color change nor does it provide a good understanding for the genetic basis of this unique, adaptive phenotype. This experiment aimed to improve our understanding of seasonal coat color change by implementing an objective and continuous measure of pelage color through the molt to the white winter coat in *P. sungorus*. Also, using an unbiased, absolute measure of pelage color allows for direct comparison of coat color between species and hybrids. Secondly, this chapter aimed to quantify the genetic basis of seasonal coat color change. While the evolution and genetic basis of adaptive coat color polymorphisms between populations of mice, for example, is well studied (Nachman 2003, Hoekstra et al. 2005, Steiner et al. 2007, Linnen et al. 2009, Manceau et al. 2011, Linnen et al. 2013) the genetic basis of seasonal coat color change is largely unexplored. Characterizing the genetic basis of seasonal coat color change in *Phodopus* will illuminate how adaptive phenologies evolve.

### **Comparative Molt Timelines in Two Seasonally Changing Species**

The first goal of this study was to develop timelines of high-resolution coat color measures over the course of the winter molt for *P. campbelli*, *P. sungorus*, F1 hybrids, and backcross hybrids. I was able to successfully track change in coat color during the molt to winter pelage in *P.*

*sungorus* using reflectance measured with a spectrophotometer (figure 5). Previous work using a categorical scale of color change showed that *P. sungorus* does gradually change color over the course of the molt to a white winter coat (Helmaier and Steinlechner 1981, Duncan and Goldman 1984, Kuhlman et al. 2003, Bilbo et al. 2006). Our results extend these general patterns by using a more advanced measure of coat color and thus offers additional insights into how coat color changes over the course of the molt. First, our approach allowed us to isolate separate components of color to draw a much more complete picture of how coat color changes over time. Our results suggest that brightness, reflectance intensity, and chroma are the main components of color that change over time in *P. sungorus*. Brightness and intensity of reflectance increased and chroma (purity or intensity of color) decreased in *P. sungorus* over time (figure 5). Although these results are not surprising given change in overall pelage color during the winter molt, there were four other reflectance measures that were found to not be significant. These included a second measure for chroma, contrast, and two measures of hue. Considering that the molt goes from a dark coat to a predominantly white coat, it is also unsurprising that these metrics did not show significance. Chroma and hue are related to color, which is absent in white hairs, and since most hairs are the same color at the dimensions of the sampling locale, contrast should not be an appreciable measure, especially later in the molt for *P. sungorus*.

Second, our continuous and absolute quantification of pelage color provides the means to make direct, unbiased phenotype comparisons with other species and hybrids, whether they express white winter phenotypes or not. In contrast to *P. sungorus*, our quantification approach showed that *P. campbelli* and F1 hybrids do not change color to nearly the same degree as *P.*

*sungorus*. While *P. sungorus* changed, no components of color significantly changed over time in *P. campbelli* or the F1 hybrids. The species-specific results confirm reports made by Kuhlmann et al (2003), but directly refute other reports that *P. sungorus* and *P. campbelli* lighten to a similar degree (Bilbo et al. 2003). Furthermore, backcross hybrids showed increased variation over the course of the molt and individuals exhibited terminal coat colors more similar to *P. campbelli* or *P. sungorus*. This result is exciting and resolves some dispute in the literature about whether *P. campbelli* pelage lightens as much as *P. sungorus* during the molt to the winter coat (Bilbo et al. 2003, Kuhlmann et al. 2003, Timonin et al. 2006). Collectively, these results demonstrate that using a high-resolution spectrophotometer is a more objective and comprehensive way to quantify coat color than previous methods and should be incorporated into future studies on coat color change in these and other seasonally changing species.

*P. sungorus* and *P. campbelli* display striking differences in coat color phenology, yet both species exhibit other shared phenologies, such as change in body weight and circulating concentration of various hormones (Bilbo et al. 2003, Timonin et al. 2006, Figure 4). Seasonal changes are generally induced by changes in circulating melatonin concentrations (Lincoln et al. 2003, Lincoln et al. 2006). Since two closely related species both exhibit phenology for some traits, but not other traits suggests that although phenology is triggered by master regulating hormones, there is flexibility in downstream mechanisms across species. Overall, seasonal molt phenology may therefore evolve in a modular fashion and changes in those modules can occur relatively rapidly between closely related species.

## Ecological Context

One of the most obvious and common changes in circannual variable environments is the presence or absence of snow cover on the ground. For higher latitude environments in the winter, the ground is often covered with snow for many months, which significantly alters the visual environment and many organisms in these environments may need to sync phenotypes to cope with additional survival challenges related to this change (Lincoln et al. 2003, Mills et al. 2013, Zimova et al. 2016). Camouflage is an effective strategy for predator avoidance, but for organisms that are active all year in environments with winter snow cover, darker coloration for camouflage in the summer will conspicuously mismatch on the snowy white background in the winter leading to higher predation risk (Zimova et al. 2016). Thus, many boreal species exhibit seasonal coat color adaptations that fluctuate with the seasons to match the circannual changes in environmental substrate color (Mills et al. 2013).

*Phodopus* has been used as a model system to study seasonal coat color change, but what natural selective pressures have driven the evolution of the white winter coat in *P. sungorus* has not been investigated. However, other work suggests that selection may drive reproductive and behavioral differences between *P. campbelli* and *P. sungorus* (Wynne-Edwards 1998, Wynne-Edwards et al. 1999). This work showed that *P. campbelli* has special energetically efficient behaviors during reproduction, which may be due to greater thermogenic challenges and resource limitation in the species' range. For example, there is only biparental care in *P. campbelli*, pups grow slower, and adults are less heat tolerant than *P. sungorus*. On the other hand, the environment for *P. sungorus* is less harsh and as a result displays behavior and reproductive strategies that are less energetically efficient (Scribner and Wynne-Edwards

1993, Wynne-Edwards 1998, Timonin et al. 2006).

The striking difference between *P. sungorus* and *P. campbelli* winter coat colors supports that white winter pelage in *P. sungorus* is also likely to be locally adaptive, similar to some behaviors exemplified by Wynne-Edwards and colleagues. The *P. sungorus* range is characterized by circannual summer and winter environmental substrate color due to winter snow cover. However, *P. campbelli* inhabits more arid regions of Mongolia and northeastern China where the visual environment is less perturbed by snow cover in the winter (figure 7). The general association between winter snow cover and molting to a white winter coat and vice versa is consistent with the hypothesis that winter color in each species reflects natural

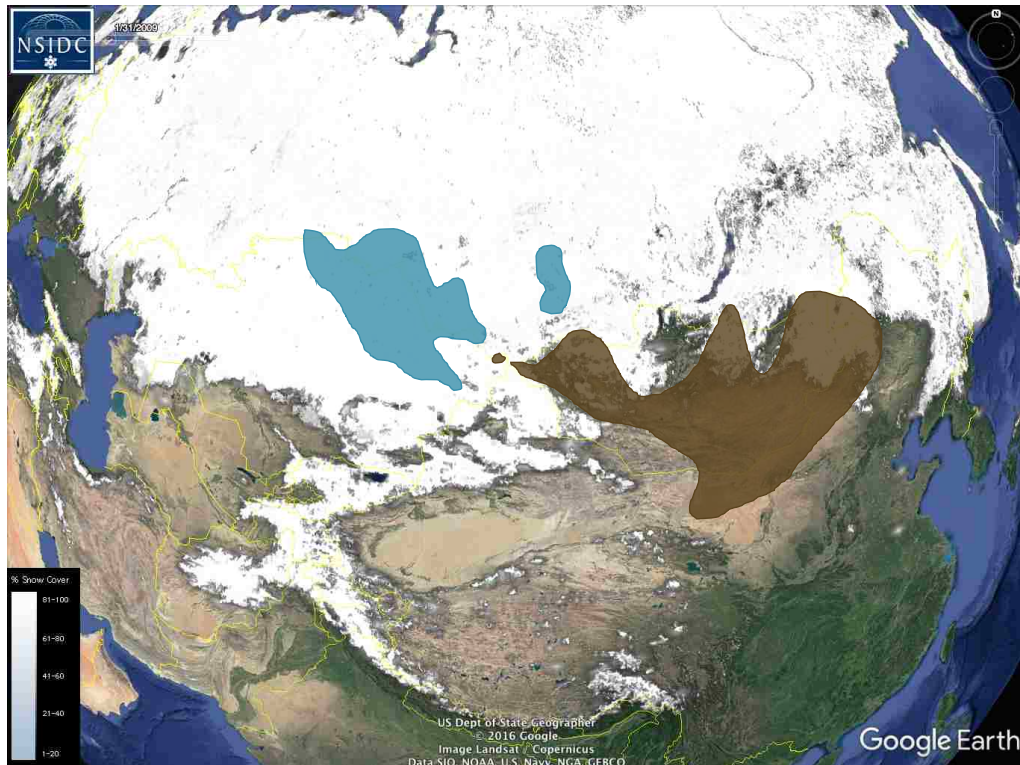


Figure 7. Ranges of *P. campbelli* (brown) and *P. sungorus* (blue) in the winter (Jan 31, 2009). The *P. campbelli* range is characterized by almost entirely no snow cover while the *P. sungorus* range is completely covered by snow. Satellite image acquired from Google Earth.

selection driven by seasonal differences in their native ranges.

### **Insights into the Genetic Basis of Seasonal Coat Color Change**

At this point, it is not well understood whether differences in adaptive traits between species are explained by changes in a few genes of large effect or whether adaptive traits are highly polygenic in nature (Orr 2005, Mackay et al. 2009). Although well-reasoned *a priori* predictions for the genetic bases of adaptive traits are difficult to formulate, we do know that *P. campbelli* and *P. sungorus* are relatively closely related and share many aspects of seasonal phenology, seasonal coat color change notwithstanding. Additionally, these two species are able to produce viable, fertile offspring, further indicating that large portions of these two genomes are still highly compatible when combined in the same individual. The seemingly low level of genetic divergence between these two species is encouraging and suggests that there may be few genes contributing to the evolution of divergent winter coat colors in *P. campbelli* and *P. sungorus*.

This is the first study to examine coat color phenology in hybrid animals and offers initial insights into the genetic basis of phenology and coat color. Seasonal coat color change in *P. sungorus* acts in a recessive fashion in F1 hybrids. Therefore, by crossing F1 females with *P. sungorus* males, we were able to recover variation in winter pelage color among backcross hybrids. The simple observation that approximately one third (28%) of backcross offspring developed a white winter coat is consistent with a relatively simple genetic basis. Extending this idea, we also used an early quantitative genetic methodology called the Castle-Wright Estimator (Castle 1921) to analyze parental and backcross reflectance and categorical color

data. The Castle-Write approach estimates the number of “factors” or loci associated with a quantitative trait by taking the variation around the phenotypic means in each parental species as a proportion of the variation measured in backcrosses relative to the average of parental variation. This approach is convenient for approximating the number of loci associated with the genetic basis of a trait because all that it requires is measures of a quantitative trait from individuals in a traditional genetic test cross, but it makes some critical assumptions that are not always met (Zeng 1992). First, the Castle-Wright Estimator requires parental lines that differ in phenotypic mean by many phenotypic standard deviations. Second, a large sample size is needed so that phenotypic variances are not inflated. Finally, this model also assumes no linkage disequilibrium between corresponding loci. While *P. sungorus* and *P. campbelli* show large differences in terminal coat color, these means may not be different enough for this approach. Using two parental lines that are not different enough in phenotypic mean could ultimately underestimate the number of associated loci. Furthermore, the sample sizes for the data applied to this approach are quite small. Only four parental and 32 backcross individuals were used for each phenotype mean and variance. Zeng et al. (1992) recommends that phenotype data from at least 200 individuals be used with this approach. Applying inflated standard deviations due to low sample size to this equation (especially among the backcrosses, which should show considerably more variation than either parental line) will underestimate the total number of corresponding genes. Considering the final main assumption, it is difficult to justify that all loci underlying the genetic basis of seasonal coat color change are not linked. However, given the proportion of individuals that expressed recessive phenotypes and exhibited pelage colors as white as *P. sungorus*, our estimate that only a few genes are involved



is probably not too far off from what would be measured with a QTL approach and is almost certainly at least within the same magnitude.

Given the phenotypes recovered in the backcross hybrids, it is unfortunate that not enough recombinant offspring could be produced to quantitatively map seasonal coat color change. However, reduced fecundity in the F1 females used for this backcross is an interesting result. F1 males from this cross are sterile and F1 females have reduced fertility, which is evidence for skewed hybrid incompatibilities between the sexes. While we were not able to dissect the genetic basis of seasonal coat color change at the resolution at which we hoped, this crossing experiment did offer insight into potential hybrid incompatibilities (Dobzhansky 1937) in F1 females and evolved barriers of reproduction between *P. campbelli* and *P. sungorus* for both sexes of offspring, not just males (Safronova and Vasil'eva 1996). Our difficulty to generate the necessary number of offspring in the F1 and backcross hybrids was somewhat unexpected since previous work by Brekke and colleagues (2016) used the same two hamster species for a separate backcross mapping experiment and did not experience these same breeding difficulties. However, one caveat is that Brekke et al. used a different strain of *P. sungorus* that generally breeds better than the strain used in this study (referred to as the “gold” strain), but exhibited a damped phenological response to short photoperiod conditions in preliminary experiments (not shown). On the other hand, the “gold” strain consistently molts to a white coat under short photoperiod conditions, but shows poorer breeding performance. Future quantitative genetic experiments in this system will likely need to rely on strains that are both reliably photoresponsive and fully fertile.

Limitations of the study system aside, the general conclusion that the genetic basis of

seasonal coat color change is relatively simple is exciting and allows us to make connections between these results and previous work investigating the genetic basis of adaptive coat color. In particular, work in the pocket mice showed that evolution of melanic polymorphism involved up to four mutations at a single locus, *Mc1r* (Nachman et al. 2003). Later work done in this same system showed that evolution of melanic pocket mice was strongly selected for and evolved rapidly (Hoekstra et al. 2005). Additionally, coat color polymorphisms between populations of deer mice evolved through selection for a few changes at a single gene, *Agouti*, and an increase in *Agouti* expression in lighter variants is caused by *cis*-regulatory changes (Linnen et al. 2009, Linnen et al. 2013). It is plausible that the evolution of seasonal coat color change also primarily reflects selection on genetic variation at these or other related coat color genes.

## **Conclusions**

This is the first study to use reflectance to measure seasonal coat color over a comparative timeline between two closely related species. This experiment implements an improved approach to measure changes in absolute coat color in an unbiased manner on a continuous scale. Here, I show that using this method for quantifying coat color should be the preferred method over traditional, less objective and less accurate approaches (Duncan and Goldman 1984). Previous work investigating the regulation of seasonal coat color change focused on the physiological and cellular mechanisms underlying this trait. For example, the *Phodopus* system provided the ground work for examining the effects of various steroid hormones on the expression of phenotypes in boreal mammals (Hoffman 1973, Heldmaier et al.

1981, Duncan and Goldman 1984, Niklowitz and Hoffman 1988, Steward et al. 2003, Butler et al. 2008) as well as the effects of reduced photoperiod, the focal environmental cue, which increases circulating melatonin concentrations and ultimately triggers a cascade of physiological changes (Heldmaier and Steinlechner 1981a, Heldmaier and Steinlechner 1981b, Lincoln et al. 2003, Lincoln et al. 2006). However, the genetic basis and insight into the evolution of this trait was previously uninvestigated. Although this thesis failed in providing hard, quantitative evidence on the genetic basis of seasonal coat color change, the crossing experiments did illuminate more general patterns regarding the genetic basis of the phenotype. These experiments have begun to disentangle how we understand the evolution of adaptive phenology. If seasonal coat color is indeed a derived trait in the genus *Phodopus*, it may not come as a surprise that it demonstrates a recessive nature. However, the variation detected in only relatively few recombinant offspring suggests that seasonal coat color change requires evolution in only a few genes and is an exciting and somewhat unexpected result given the complex physiological regulation of this trait.

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## CHAPTER 2

### *Introduction*

Organisms in highly seasonal environments are subject to circannual variation in food availability, energetic requirements for survival, and the ability to camouflage from predators. Because of the repeated and predictable nature of seasonality, many organisms adjust physiology, morphology, or behavior to maximize survival or reproduction from season to season (Paul et al. 2007). The quality of having reversible and plastic traits is referred to as phenotypic flexibility (Piersma et al. 2003). There is extensive work published on the physiological and cellular regulation of the phenology of winter adaptations associated with thermoregulation (Heldmaier et al. 1982), energetics (Helmaier and Steinlechner 1981), and crypsis (Abdel-Malek et al. 2001, Manceau et al. 2010). However, less is known regarding the regulation of phenotypic flexibility at the molecular level.

RNA-seq is a powerful tool that uses high throughput sequencing technology and allows for accurate quantification of transcripts and isoforms. This approach is well suited for understanding the molecular regulation of a given trait and for identifying changes in gene expression over a developmental timeline (Wang et al. 2009, Wang et al. 2010). Using this approach, we can quantify relative transcripts abundance genome-wide for a given tissue (i.e., transcriptomes) and can have the versatility to discover associations between gene expression and phenotypes without the need for *a priori* expectations. Furthermore, RNA-seq can be used to identify transcripts or to find general changes in expression that are associated with phenotypic plasticity and phenotypic flexibility (Gui et al. 2013, Henning et al. 2013, Marra et al. 2014).

While analyses of RNA-seq transcriptomes have limited power to directly link genetic and phenotypic variation, a comparative framework allows for a more complete understanding of how the expression of a phenotype is regulated on the molecular level (Wang et al. 2009, Todd et al. 2016). This methodology categorizes how genes are differentially expressed under certain treatments or between genotypes without requiring a complete reference genome or other genomic resources. For example, differential gene expression can be assessed with a *de novo* transcriptome along a developmental timeline (Wang et al. 2010), to understand differences in pigmentation (Henning et al. 2013), and to characterize evolution of important cellular function resulting from adaptation to new environments (Gui et al. 2013, Marra et al. 2014). Investigating the regulatory divergence of phenotypes between species and hybrids is informative for understanding the genetic basis of a given trait and how adaptive, complex phenotypes may evolve (Todd et al. 2016).

*P. sungorus* is a model system for understanding the physiological and environmental regulation of one notable flexible phenotype, seasonal coat color change. Although the physiological and environmental regulation of seasonal coat color change is well understood (Heldmaier et al. 1981, Duncan and Goldman 1984, Bartness and Wade 1985, Teubner et al. 2008), the molecular regulation of this complex trait is at this point largely unexplored. *P. campbelli* and sister species, *P. sungorus*, share multiple phenological changes in traits associated with winter survival but differ in that *P. sungorus* molts to a predominantly white coat in the winter while *P. campbelli* remains mostly brown (Chapter 1, Heldmaier and Steinlechner 1981, Timonin et al. 2006). Because both species appear to molt and respond to short day conditions, but only one species turns white, we reasoned that a comparative

functional approach could help inform us about the evolution of species specific phenology. Furthermore, since *P. campbelli* and *P. sungorus* can be crossed to produce viable hybrid offspring (Brekke and Good 2014, Brekke et al. 2016), expression can be measured in F1 hybrids to further understand how seasonal regulation responds to the combination of divergent haplotypes in the same individual. This allows for testing of the regulation of two different genomes in the same individual or “environment”; similar to the design of traditional common garden experiments. Therefore, expectations about dominance of a phenotype can be tested in heterozygous, hybrid individuals.

Here I combine detailed phenotypic characterization of seasonal molts (Chapter 1) in two species of dwarf hamsters with a genome-wide analysis of gene expression. This experiment integrated thorough quantification of phenotype with a developmental timeline of gene expression to provide the first functional genomic assessment of seasonal molt between two species. There are three main goals of this study: (1) Characterize differential expression along a seasonal molt timeline for *P. campbelli* and *P. sungorus* using RNAseq. Is there coordinated expression associated with molt (i.e., hair growth), but differences in pigment production between the two timelines? RNA-seq is more flexible than other functional genomic approaches and allows for gene expression analysis for the entire transcriptome without *a priori* predictions. This study will also (2) assess conservation of the molt regulation between these two species. In other words, is genetic regulation of the molt to the winter coat conserved except for differences in coat color, or are the underlying developmental processes sufficiently diverged to obscure insights into color change? Finally, we will (3) measure differential expression in F1s and assess how divergent expression profiles in the two species

behave in the same individual. Do fixed differences between the two divergent parental haplotypes reflect dominance patterns observed in the F1 hybrid phenotype or does expression appear to be disrupted, consistent with hybrid incompatibilities (Dobzhansky 1937, Orr 1996, Tang and Presgraves 2009) between these two species in the context of phenology? This is the first study that seeks to understand the genetic and regulatory basis of seasonal molts using a comparative functional genomic framework. Evaluating genetic regulation of phenology between two divergent species will inform how seasonally flexible phenotypes evolve as well as contribute a well-needed deeper understanding of how seasonal coat color change functions in *Phodopus*.

## **Methods**

### **Animals**

All animals used for this study were bred in our animal facility at the University of Montana (Chapter 1). All breeding individuals were housed in 14-hr light/10-hr dark lighting regimen in accordance with the University of Montana's Institutional Animal Care and Use Committee regulations.

### **Experimental Crosses**

Twenty adult female F1 hybrid hamsters from crosses described in Chapter 1, twenty adult female *P. campbelli*, and twenty adult female *P. sungorus* hamsters were used for this experiment. Experimental animals were moved to a separate room and housed separately under an 8-hr light/16-hr dark lighting regimen to emulate winter photoperiod conditions and

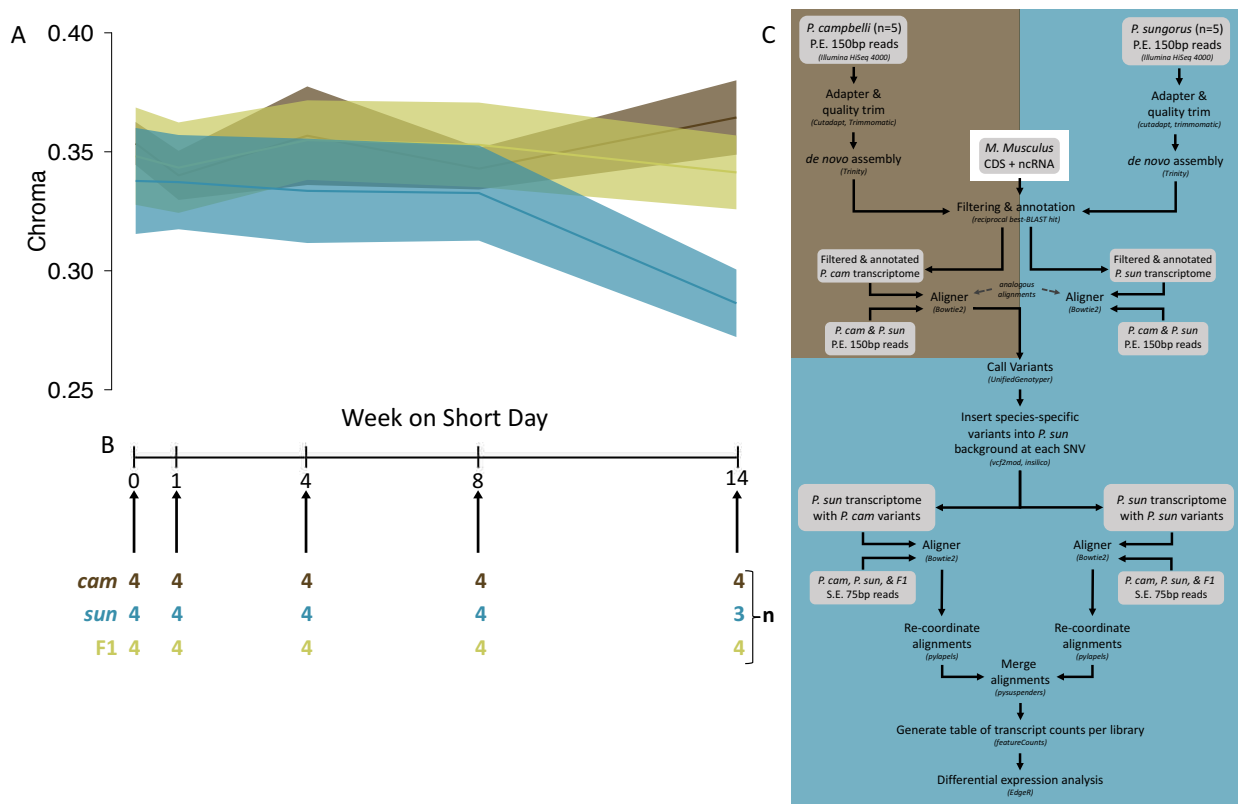


Figure 1. Overall experimental design showing change in phenotype over time (A), skin sampling schema for RNA extractions (B), and the bioinformatics pipeline for transcriptome assembly and expression counting (C). Brown denotes *P. campbelli*, blue denotes *P. sungorus*, and green denotes F1 hybrids. Colors in the bioinformatics pipeline (C) indicate which species background was used for those steps.

to induce the winter molt. Hamsters were kept on this lighting regimen for up to 14 weeks. At 0, 1, 4, 8, and 14 weeks into the experimental lighting regimen, 4 hamsters of each genotype were removed from experimental conditions, euthanized using CO<sub>2</sub> followed by cervical dislocation, and skin and liver were sampled for RNA extraction (figure 1).

### Tissue Sample and Library Preparation

For RNA extraction, the entire back skin was immediately removed and snap frozen on a

block of dry ice. During snap freezing, liver was sampled and placed in RNA-later. Once skin was frozen, hair was shaved with a razor and two pairs of 4mm biopsy skin samples from the mid-back and hind-back were placed in RNA-later. Tissue samples were incubated overnight in RNA-later and then frozen at -80 Celsius. These skin samples were used for RNA extraction and expression analysis. Basic structure of this protocol was adapted from Silva et al. (2014), but I developed and iteratively optimized the sampling and extraction protocol for the purposes of this experiment and based on the constraints of this system prior to sampling experimental animals.

To control for confounding batch effects with actual experimental factors (time point and genotype) during RNA extraction and cDNA library preparation, samples were prepared in batches of 15, which consisted of five individuals of each genotype and 1 from each of the sampling time points (0, 1, 4, 8, and 14 weeks). If there was a batch effect, this would not bias the effect of week or genotype in our experiment. One batch at a time, RNA-later saturated, frozen skin tissue samples were thawed at room temperature and homogenized to a powder in liquid nitrogen using a frozen and sterilized mortar and pestle. Homogenized tissue was immediately placed in 2-mercaptoethanol and further homogenized further with a 18G needle and syringe. I extracted RNA using an RNeasy kit (Quiagen), treated with 80  $\mu$ l of DNase (Quiagen). RNA extractions were quantified using an Agilent 2200 TapeStation. Dual-index barcoded RNA-seq libraries were prepared per individual using an Illumina TruSeq stranded mRNA library preparation kit. Libraries were quality checked on an Agilent 2200 TapeStation and then quantified with qPCR on an Agilent Mx3000 qPCR machine using a KAPA SYBR FAST ROX low qPCR kit (KAPA Biosystems). Libraries were diluted to 30nM in EBT buffer and pooled



for sequencing following the Illumina library pooling guidelines.

A reference genome has not yet been established for *Phodopus*, and exploratory bioinformatic analyses of transcriptome and whole exome data indicate that existing references from other hamster species (*Cricetulus griseus*, CriGri\_1.0; Xu et al. 2011) are too divergent to be used for reference assemblies (analyses not shown). Briefly, I first attempted to generate a reference genome using an iterative mapping and variant-calling approach to generate a pseudo-genome using *pseudo-it* (Sarver et al. 2017) and the Chinese hamster genome; a whole genome shotgun assembly from a Chinese hamster ovary cell line (Xu et al. 2011). We inferred that this approach was not feasible for this system given the fragmented structure of the Chinese hamster genome. I also attempted the same pseudo-genome approach using the more complete *Mus musculus* genome (mm10 build), but this attempt did not improve our ability to map single-end short reads to the pseudo-reference, likely due to the fact that mouse and hamster may be too divergent for this method. After multiple systematic iterations to generate a pseudo-genome using the above approach, I used paired-end sequence reads to generate *de novo* transcriptome assemblies with *Trinity*.

For *de novo* transcriptome assembly, two different Illumina sequencing experiments were performed on *P. campbelli* and *P. sungorus* libraries. To establish species-specific references, one individual from each species was selected from each time-point (i.e., n=5 per species, n=10 total) and paired-end sequenced twice on 30% of one lane at 150 bp on an Illumina HiSeq 4000 platform. We manually selected the individuals so that we had even representation from each time point within each species (compensate for variable coverage over the course of the molt) and for libraries that shared P7 barcode sequences within each

species. This design was implemented so that demultiplexing would result in two distinct informatic libraries (one for each species) with all individuals within each species as a single library. These longer, paired-end reads were used to generate de novo reference assemblies for *Phodopus* skin transcriptomes. To quantify transcript abundances, all libraries prepared from *P. campbelli*, *P. sungorus*, and F1 hybrid skin (i.e., n=20 per species, n=60 total) were single-end sequenced at 75 bp on an Illumina NextSeq platform. These short read, single-end data were aligned to de novo transcriptomes for differential gene expression analysis.

### **Transcriptome Assembly**

Raw 150 bp paired-end reads were filtered by removing Illumina universal and TrueSeq adapters with *cutadapt* v1.12 with parameters `-O 5` and `-e 0.1` (Martin 2011). Reads were then quality trimmed with *trimmomatic* v0.36 with parameters set to `LEADING:5 TRAILING:5 SLIDINGWINDOW:4:15 MINLEN:36` (Bolger et al. 2014). Library read qualities were quantified at each step using *FastQC* v0.11.5. Adapter and quality trimmed paired-end reads for each species were used to create two species-specific de novo transcriptomes using *Trinity* v2.1.1 with parameters `--seqType fq, --SS_lib_type FR, --full_cleanup, and --max_memory 50G` (Grabherr et al. 2011). These parameters were selected based on previous *Trinity* assemblies for other placenta-specific *de novo* transcriptomes (Brekke et al. 2016). The *de novo* Trinity transcriptome assemblies yielded >190,000 genes and >220,000 isoform variants (see below). Given that there are ~25,000 known protein-coding genes in most mammalian genomes, the number of genes assembled by *Trinity* is likely inflated due to spurious and/or incomplete assembly of splice variants. While this redundancy is expected and at least partially mitigated

within standard analytical pipelines of differential expression (*EdgeR*, etc), these issues present additional challenges when comparing expression levels between species. To narrow down the *de novo* assemblies in *Phodopus* to a more realistic number of genes, I filtered out spurious assembled genes using a three-way *reciprocal-best-BLAST-hit* (RBBH) approach (Brekke et al. 2016). I first conducted a reciprocal BLAST between the two species-specific *de novo* transcriptomes and then a reciprocal BLAST between each hamster species and the mouse GRCm38 CDS and ncRNA assembly. Only genes that returned a high-quality hit (e-value less than  $1e-20$ ) and only the top hit were kept in the final filtered assemblies (14,823 genes, see below), which were the longest isoform variant per gene. Additionally, three splice variants were manually identified for the known coat color gene *Agouti* and all three isoforms were also included in the final transcriptomes.

In addition to direct comparisons between species, this experiment utilizes F1 hybrid hamsters to understand patterns of expression. It is common practice to align sequences to a single reference genome; however, this can lead to a mapping bias or uneven mapping success rate depending on genetic distance of the different species to the reference (Sarver et al. 2017). In RNA-seq experiments, expression level is calculated as a function of coverage, or number of reads mapped, for a given gene. Because of the potential for underlying mapping bias in RNA-seq studies, it is imperative to remove mapping bias between the two transcriptomes when aligning short reads during differential expression analysis (Huang et al. 2014, Sarver et al. 2017). To do this, I followed the *modtools* pipeline (Huang et al. 2014, Brekke et al. 2016) to generate two pseudotranscriptomes with a *P. sungorus* background and variants from either species at all variable sites. Since both filtered and annotated *P. campbelli* and *P. sungorus de*

*novo* transcriptomes had equivalent number of transcripts and paired-end data from each species mapped at equivalent rates to each transcriptome, it was not necessary to continue while iterating downstream steps in both species (figure 1). However, the unfiltered *P. sungorus* transcriptome had three assembled isoforms for an important candidate gene, *Agouti*, instead of the two assembled isoforms discovered in *P. campbelli*. Therefore, I chose to continue with downstream analyses using the *P. sungorus* background. Paired-end reads from both species were mapped to the *de novo* transcriptome using *bwa mem* and single-nucleotide variants (SNVs) were called between the two species using *UnifiedGenotyper* from *GATK* v3.4-46 with the parameter `-stand_call_conf 20.0` (McKenna et al. 2010, DePristo et al. 2011). The SNVs were filtered using *vcftools* v0.1.14 with the parameters `--minDP 0 --min-meanDP 0 --minQ 0 --minQ 500 --min-alleles 2 --max-alleles 2`. Sites with fixed differences between the two species were kept if all individuals for a species were homozygous for the same allele at that site, which yielded 169,264 SNVs in 12,137 genes out of the total 14,823 genes in the transcriptome.

Pseudotranscriptomes and mod files were then generated using *vcf2mod* and *insilico* from the *modtools* package v1.0.5 (Huang et al. 2014). Single-end reads from all individuals were reciprocally mapped to both pseudotranscriptomes with *Bowtie2* v2.3.0. Transcripts from each individual were then re-coordinated to a reference coordinate system using *pylapels* v0.2.0 in *Lapels* v1.1.1 and then all re-coordinated alignments were merged and reads assigned to a parent of origin based on mapping quality in the alignment using *pysuspenders* v0.2.5 in *Suspenders* v0.2.5 (figure 1).

## Differential Expression Analysis

Merged alignments from *Suspenders* were then read into *featureCounts* v1.5.0 with parameters to count the number of fragments (-f) that align to each transcript (Liao et al. 2014). Tables of counts were then used as input into *EdgeR* v3.16.5 to examine differential expression across all transcripts with a generalized linear model approach (Robinson et al. 2010). In *EdgeR*, mitochondrial genes were removed from the count and annotation tables to account for increased maternal (*P. campbelli*) expression in hybrids before filtering out those genes (table 2). *EdgeR* uses a Generalized Linear Model (GLM) to test for differential expression in transcripts across all pairwise contrasts in the experimental design. We filtered out transcripts that were expressed below one fragment per kilobase of exon per million reads mapped (FPKM) in at least 3 of the samples across the timeline (Angelini et al. 2014). Expression level was ultimately calculated as FPKM using log<sub>2</sub> transformed and mean-centered normalized read counts (Angelini et al. 2014). The counts were then normalized using the “TMM” method (Robinson and Oshlack 2014). All filtered count data and counts per species were separately plotted using a multidimensional scaling (MDS) plot to identify any preliminary patterns across the experimental design and potential outliers that needed to be removed from the dataset. Distances in this matrix were calculated as the average root-mean-square (Euclidean distance) of the largest absolute log<sub>2</sub>-fold change between each sample’s count. Additionally, the common negative binomial dispersion was calculated for all counts and counts per species using a Cox-Reid profile-adjusted likelihood and the biological coefficient of variance (BCV) was calculated as the square-root of the dispersion.

I calculated differential gene expression for all pairwise contrasts. These pairwise

contrasts fell into two categories, temporal and genotype differential expression. Temporal differential expression was calculated for each genotype (*P. campbelli*, *P. sungoru*, and F1 hybrids) by finding differences in expression per transcript between all possible comparisons along the molt timeline. Temporal differential expression tests will identify genes differentially expressed across the molt timeline. These patterns underlay biological phenomena within a species and an assessment of overall phenological patterns between species can be done with temporally differentially expressed gene. Genotype differential expression was calculated by finding differences in expression per transcript at each time point between *P. campbelli* and *P. sungorus*, *P. campbelli* and F1 hybrids, and *P. sungorus* and F1 hybrids. Genotype differential expression tests for divergence along molt timeline while controlling for time point between the two species and assesses how expression of those genomes behave in hybrids. The following analyses were calculated for all pairwise contrasts described above. A genewise negative binomial GLM was fit to the data for each contrast and the likelihood ratio for one or more of the coefficients in the linear model was calculated (McCarthy et al. 2012). The top differentially expressed transcripts with a false discovery rate (FDR) that was less than 0.05 based on the Benjamin-Hochberg multiple test correction and had a Log2 fold-change greater than 2 (McCarthy et al. 2012) were classified as either temporal or genotype differentially expressed depending on contrast. This cutoff was used because using a Log2 fold-change cutoff greater than 1 introduced additional variation and resulted in lower temporal signal (data not shown).

Transcripts that showed temporal differential expression were used to identify blocks of correlated expression across the molt and between the two species. To do this, a hierarchical

clustering analysis across all time points in both species and across all time points within each species was conducted using the *heatmap.2* function in the R package *gplots* 3.0.1 with the “complete” linkage option for the hierarchical clustering method.

Next, the *venn* function in *gplots* was used to assess overlap in temporal differentially expressed transcripts between the two species. Temporal differentially expressed transcripts in *P. campbelli* were compared to differentially expressed transcripts in *P. sungorus* and we determined the intersection of those two sets of genes with a Venn diagram approach. Additionally, comparisons were also made between the two species for temporal differentially expressed transcripts in contrasts between consecutive time points. For example, the transcripts that were differentially expressed between week 1 and week 4 were compared to transcripts in the other species that were differentially expressed between week 1 and week 4. These comparisons were made for each pair of consecutive time point contrasts.

Genotype differentially expressed genes were used to identify divergence between the two species. The numbers of genotype differentially expressed genes were calculated for pairwise contrasts between the two parental species and the F1 hybrids. Additionally, spearman rho correlations were calculated for all genes for each genotype pairwise contrast and with 97.5% confidence intervals estimated with 1000 bootstraps.

### **Gene Ontology Enrichment Analysis**

We tested for gene ontology (GO) enrichment using the DAVID Bioinformatics Database (Dennis et al. 2003) for each species to further understand the functional role of transcripts that were differentially expressed across the molt timeline. GO analyses used the *Mus musculus*

background and differentially expressed transcript Ensembl gene IDs were used as the gene list. Functional annotation clustering was run for the gene ontology term categories “molecular function”, “biological process”, and “cellular component”.

## Results

### Seasonal coat color change is inducible under reduced photoperiod conditions

Detailed discussion of the change in coat color phenotypes over the course of the molt for *P. campbelli*, *P. sungorus*, and F1 hybrids are presented in Chapter 1. Briefly, *P. campbelli*

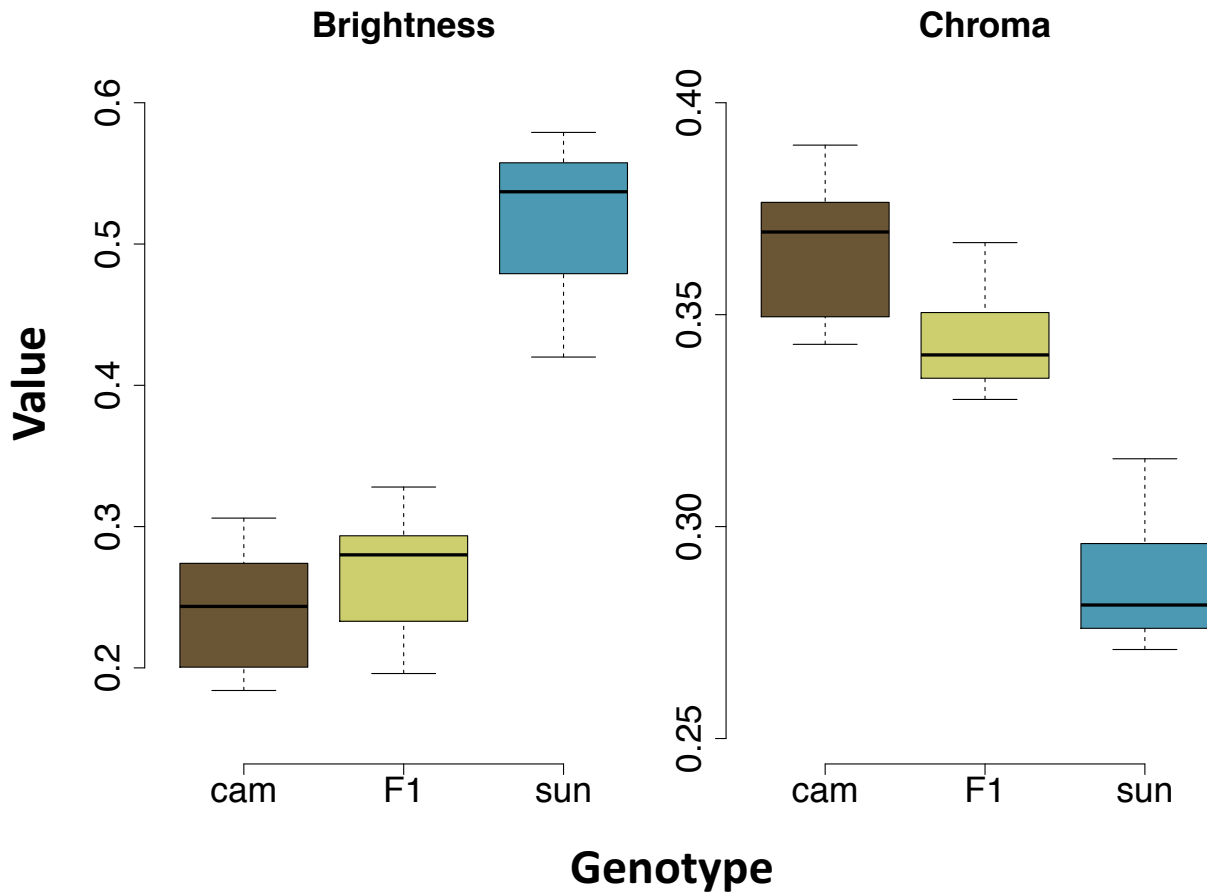


Figure 2. Terminal (week 14) values for coat color brightness and chroma in each genotype. *P. sungorus* white coat color is associated with higher brightness and lower chroma.



and F1 hybrids equally showed no effect of time point for any measure of reflectance over the molt timeline while *P. sungorus* exhibited temporal change at five reflectance measures (mean brightness, intensity, hue, chroma, and contrast) over the course of the molt. Additionally, it is important to note that since F1 hybrids exhibited a phenotype similar to *P. cambelli*, the ability to molt to a white winter coat seems to be mostly recessive and can inform expression patterns found in the F1 hybrids (figure 2).

### Raw data and quality control

Sixty strand-specific cDNA libraries were prepped from mRNA extracted from skin tissue samples. These library fragments ranged in length between 276 and 335bp (including adapters) and were single-end sequenced over two high-output runs of an Illumina NextSeq to generate 837,695,387 75bp single-end reads. After trimming adapters and filtering out low quality raw

Table 1. Summary of de novo transcriptome parameters before and after RBBH filtering and annotation. Parameters are reported for longest isoform per gene in assembly.

	Raw Assembly		Filtered Assembly	
	<i>P. cambelli</i>	<i>P. sungorus</i>	<i>P. cambelli</i>	<i>P. sungorus</i>
<b>Components/Genes</b>	189406	199333	13985	14040
<b>Transcripts</b>	220045	230668	14823	14823
<b>N50</b>	886	927	3319	3260
<b>Median Contig Length</b>	336	332	2178	2152
<b>Mean Contig Length</b>	618.79	621.85	2574.72	2537.38
<b>Total Assembled Bases</b>	117203280	123955879	36007416	35624801

reads, the number of total reads was 828,996,807. Out of the 60 strand-specific cDNA libraries mentioned above, 5 *P. campbelli* libraries and 5 *P. sungorus* libraries were selected across time points and were paired-end sequenced on 60% of one lane on an Illumina HiSeq 4000 at 150bp. This generated a total of 89,423,719 raw paired-end reads. After trimming adapters and filtering out low quality raw reads, the total number of paired-end reads was 87,569,213 paired-end reads. These reads were used as input for the de novo Trinity transcriptome assembly.

### **Assembly and Transcript Abundance Estimation**

The *de novo* Trinity assemblies for the two parental species were similar in quality. *P. campbelli* yielded 134,614 genes and 159,875 isoforms and *P. sungorus* yielded 142,434 genes and 168,705 isoform variants (table 1). Since our experiment makes direct comparisons between these two species, we could not retain the full complexity of the two *de novo* assemblies. *De novo* assemblies often generate erroneous, chimeric contigs from two or more real transcripts. Areas of the genome with repetitive elements or sequences with low sequence coverage are particularly prone to this type of assembly error (Graberr et al. 2011, Cahais et al. 2012, Singhal et al. 2013). The assembly of erroneous transcripts is not just an issue with *Trinity*, but is rather a complication associated with most *de novo* transcriptome assemblers (Vijay et al. 2013). Our assemblies appeared to include erroneous contigs as well and it is impossible to make unbiased comparisons between two species with full complexity of each *de novo* reference transcriptome. Thus, an annotation and filtering approach is needed to filter down transcriptomes to a core set of orthologous transcripts that include only the longest isoform

detected. A three-way RBBH approach that kept only the longest isoform filtered the de novo transcriptomes down to 14,823 annotated genes for *P. sungorus* and *P. campbelli* (table 1). However, only the *P. sungorus* transcriptome was used as the background reference for all downstream analyses since it is unnecessary to reciprocally create modified transcriptomes for each parental background (Huang et al. 2014).

*P. campbelli* paired-end reads were mapped to the *P. sungorus* transcriptome and 169,264 SNVs were discovered in 12,137 genes out of the total 14,823 genes and *P. campbelli* variants were inserted into the *P. sungorus* background. *P. sungorus* paired-end reads were also mapped to the *P. campbelli* transcriptome, but both reciprocal alignments resulted in equivalent mapping success rates and only the *P. sungorus* background was used for downstream analyses. Species specific haplotypes were called from these paired-end alignments and I generated two modified reference transcriptomes, one that was *P. sungorus* and one that consisted of *P. campbelli* SNVs inserted into the *P. sungorus* background. Single-end reads from *P. campbelli*, *P. sungorus*, and F1 hybrids were mapped to each to each modified reference transcriptome mentioned above. The separate alignments for each modified reference transcriptome were merged and parent-of-origin was determined for each read alignment across all individuals. Note that one *P. sungorus* individual was removed from downstream analyses because the parent-of-origin ratio did not reflect the expectation for *P. sungorus* and instead had a 1:1 parent-of-origin ratio, which is the expectation for hybrid individuals (table 2).

The table of counts generated with *featureCounts* v1.5.0 was read into *EdgeR* and transcripts were removed if more than 3 individuals for all species across the timeline

Table 2. Parent-of-origin (PO) identity in F1 hybrids for merged alignments to modified transcriptomes. Proportions of reads aligning to *P. campbelli* (cam PO) or *P. sungorus* (sun PO) are calculated with and without mitochondrial (cam MT) genes.

cam PO	cam MT	cam PO (minus cam MT)	sun PO	Proportion cam PO (before MT removed)	Proportion cam PO (after MT removed)
2310683	521026	1789657	1729812	0.57	0.51
1661627	523068	1138559	1086750	0.60	0.51
2064486	458223	1606263	1549040	0.57	0.51
1441499	324335	1117164	1153672	0.56	0.49
2630617	669256	1961361	2044214	0.56	0.49
1687161	588909	1098252	1087864	0.61	0.50
1610865	392407	1218458	1235270	0.57	0.50
322364	74166	248198	246518	0.57	0.50
742402	203733	538669	522102	0.59	0.51
1352312	289692	1062620	1074756	0.56	0.50
1786584	420199	1366385	1349129	0.57	0.50
1921605	777222	1144383	1148365	0.63	0.50
1941251	659502	1281749	1282684	0.60	0.50
3488701	1036889	2451812	2513184	0.58	0.49
2003208	574298	1428910	1403962	0.59	0.50
1318117	279101	1039016	1031963	0.56	0.50
3474149	870951	2603198	2569706	0.57	0.50
2842842	813673	2029169	2079578	0.58	0.49
1634728	333398	1301330	1310304	0.56	0.50
1439913	375008	1064905	1017664	0.59	0.51

expressed that transcript at less than 1 FPKM. Additionally, the analysis of parent-of-origin ratios demonstrated that alignments were biased toward *P. campbelli* at mitochondrial genes in the F1 hybrids, which is expected since F1 hybrids have *P. campbelli* mothers. Therefore, I removed counts for mitochondrial genes from the dataset and did not include these genes in downstream analyses. This resulted in a total of 13,998 transcripts that were used for differential expression analysis.

## Differential expression analysis

### Overall transcriptomic structure

To examine overall patterns of expression within and between species, expression levels (read counts) for all individuals were plotted using an MDS plot to identify temporal or genotype clustering based on expression (figure 3). There was clear grouping of points within each species that was mainly distributed across the dimension 2 of the MDS plot. Although

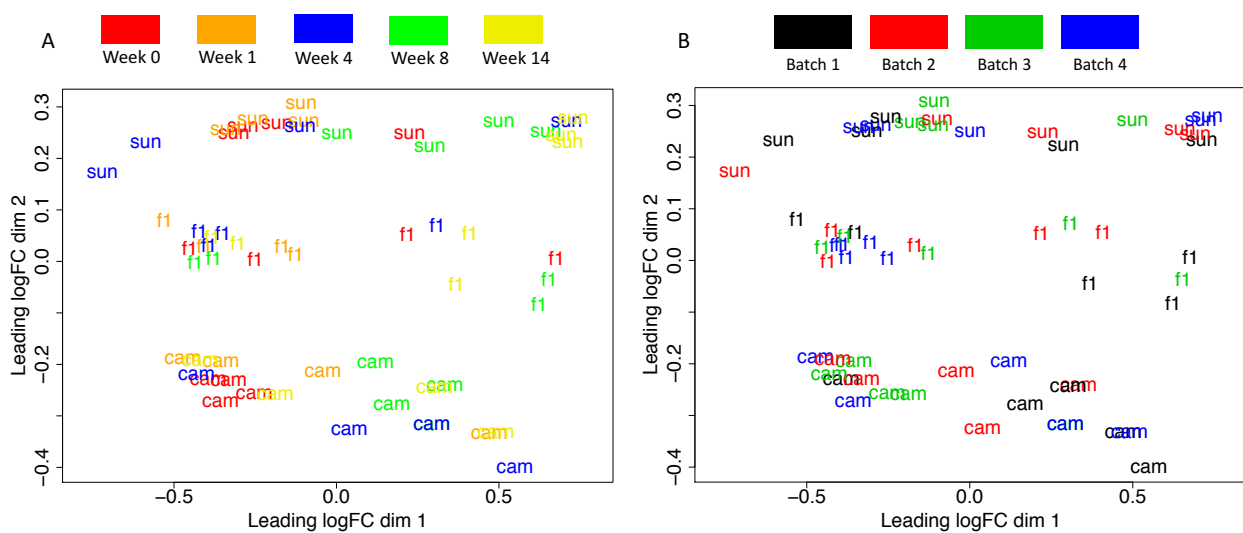


Figure 3. MDS plot for all individuals. A) points colored by week in molt timeline. B) points colored by extraction and library prep batch.

points for each species were distributed across dimension 1 of the MDS plot, there was little signal of temporal structure. MDS plots were then generated for each species separately to reduce noise due to comparisons between species and recover distribution of points based on the time point. However, this approach also did not result in a strong temporal signal for any single genotype (figure 4). However, a lack of strong temporal signal may be unsurprising considering this analysis uses expression of all genes and only temporal differential expressed genes should show a strong temporal signal.

Sample extraction and library preparation for each genotype and each time point were stratified across four batches as to not confound batch effects with differences in temporal or genotype differential expression. However, structuring RNA extractions and library preparations in this fashion may lead to loss of temporal signal and instead a signal associated

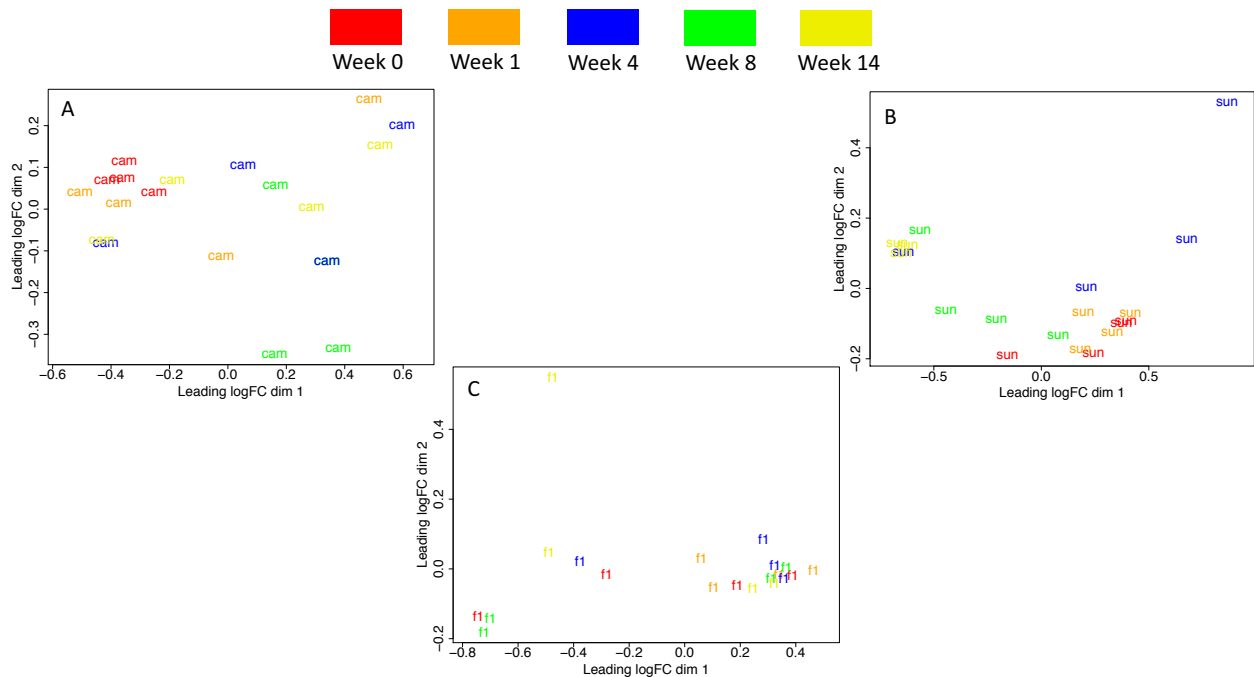


Figure 4. MDS plot for all *P. cambelli* (A), *P. sungorus* (B), and F1 hybrid (C) individuals with points colored by week.

with batch effect. To test for potential batch effects during RNA extraction and library prep, sample points were colored based on batch and plotted on the same MDS plots (figure 3). Points in these plots did not cluster on batch, suggesting that batch did not affect the lack of temporal signal.

To evaluate the amount of variation between replicate RNA samples found in our dataset, I calculated the biological coefficient of variance (BCV). This metric represents the coefficient of variance that one would find between biological replicates if sequencing depth was unrestricted. BCV is agnostic of the size of counts and is the most appropriate way to assess uncertainty for highly expressed transcripts and is essential for identifying potential bias in differential expression experiments (Chen et al. 2016). Common dispersion was 0.08104 and the BCV was 0.2847 for the entire dataset with all individuals. Common dispersion and BCV were also calculated for each species. For *P. campbelli*, the common dispersion was 0.07552 and the BCV was 0.2748. *P. sungorus* had a common dispersion of 0.0714 and a BCV of 0.2672. F1 hybrids had greater variation with a common dispersion of 0.09507 and a BCV of 0.3083 (table 3). The manual for *EdgeR* recommends that BCV is 0.1 for genetically identical model organisms and 0.4 for human data (Chen et al. 2016). Our values fall within this range and are relatively close to the value for genetically identical model organisms, which indicates that there should be little bias in our dataset based on variation between replicates.

Table 3. Common dispersion and biological coefficient of variance.

	<i>P. campbelli</i>	<i>P. sungorus</i>	F1 Hybrid
<b>Dispersion</b>	0.07552	0.0714	0.09507
<b>BCV</b>	0.2748	0.2672	0.3083

### *Transcriptome Phenology*

Temporal differential expression was calculated for all pairwise contrasts between time points within each species and the F1 hybrids for all 13,998 transcripts that remained after filtering (see above). Temporal differential expression tests for a signal associated with phenology along the molt time line for each genotype. These patterns in gene expression are the basis for biological phenomena in the skin within a species. Furthermore, an overall comparison of phenological patterns between genotypes can be done with temporal differentially expressed gene. 147 genes were differentially expressed between any two time points in *P. campbelli* and 494 for *P. sungorus*. F1s exhibited much lower differential expression with a total of 7 genes differentially expressed between any two points along the timeline (table 4). Furthermore, the low number of differentially expressed genes in the F1s is likely related to the increased BCV value relative to the parental species; that is the F1s likely show reduced differential expression because a greater amount of variation in the expression of all transcripts across the timeline.

Temporal differentially expressed transcripts were clustered based on Euclidean distance for all individuals within each species using the *heatmap.2* function in the R package *gplots* v3.0.1 (figure 5). However, clustering of individuals did not reproduce timeline structure, which was somewhat expected based on the results from the overall transcriptomic patterns tested in the MDS plots. Additionally, there were no obvious blocks of clustered transcripts with coordinated patterns of expression in this clustering analysis. To further test for timeline structure within each species, a matrix of pairwise spearman rho correlations was calculated for





Table 4. Total number of differentially expressed (LogFC > 2) genes.

P. cam vs P. sun	P. campbelli	P. sungorus	F1 Hybrid	P. cam vs F1	P. sun vs F1
781	147	494	7	224	342

differentially expressed genes among all individuals for each species (figure 6). There was a weak signal of timeline structure in *P. sungorus*, but more so than in *P. campbelli* or the F1 hybrids. These results suggest that there little change in expression of genes across time points, there is considerable variation between individuals at a given time point, and that there are relatively few genes out of all differentially expressed genes that are potentially involved in the regulation of seasonal coat color change and other traits that change along the timeline. This suggests that there is discordance between the two molt timelines in terms of which genes are differentially expressed and when differentially expressed genes are induced. Divergence between molt timelines in *P. campbelli* and *P. sungorus* is investigated further in genotype differential expression analyses.

Based on these results, Venn diagrams were used to further investigated whether *P. campbelli* and *P. sungorus* induce different sets of genes during the molt and whether the same genes were differentially expressed between consecutive time points between the two species (figure 7). Out of the 494 differentially expressed transcripts across the in *P. sungorus* timeline and the 147 differentially expressed transcripts across the *P. campbelli* timeline, only 41 of those transcripts were differentially expressed in both (figure 7). When looking at only genes that are differentially expressed between consecutive time points within each species, *P. campbelli* and *P. sungorus* do not share any of the same genes for any contrast. These results

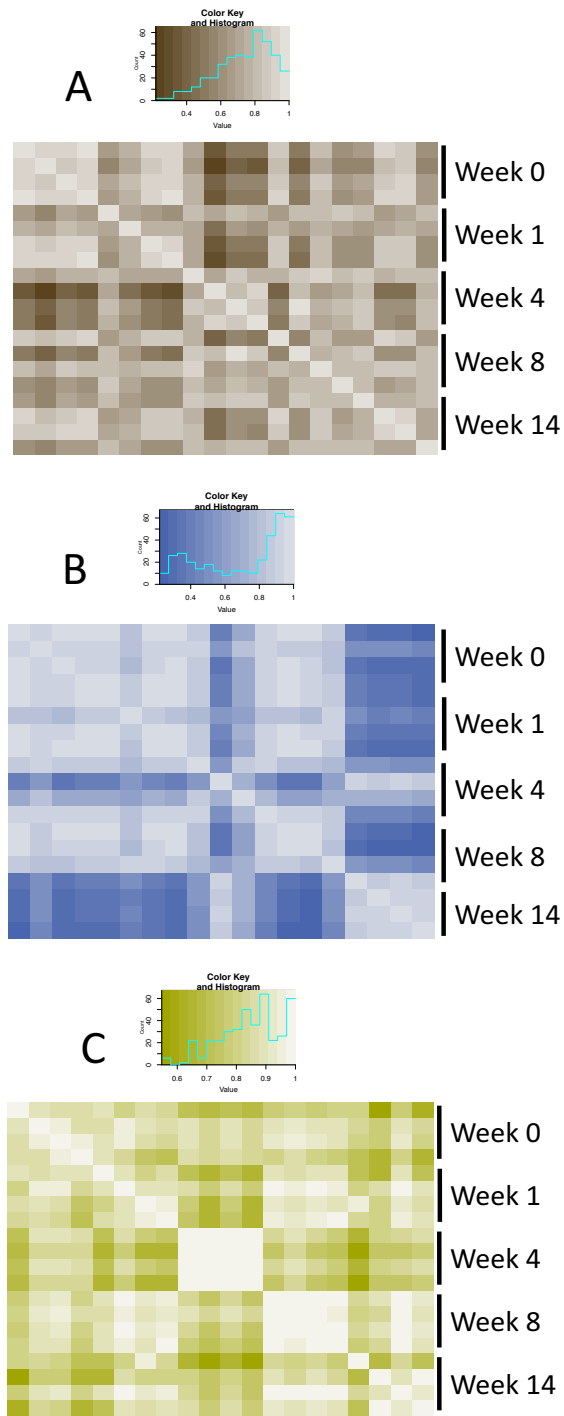


Figure 6. Heatmap showing pairwise spearman correlations between individuals by time point for each species: (A) *P. campbelli*, (B) *P. sungorus*, and (C) F1 hybrids. Lighter colors denote higher spearman rho values and darker colors denote lower spearman rho values.

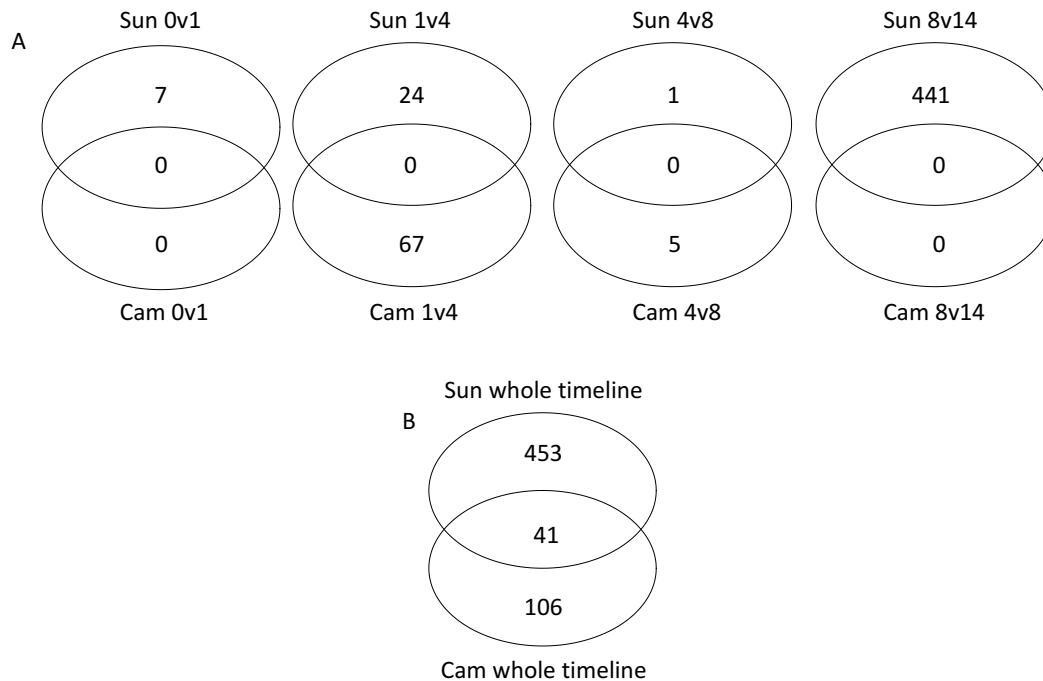


Figure 7. Venn diagrams comparing differentially expressed genes in (A) consecutive time points and (B) any differentially expressed gene along the timeline between *P. campbelli* and *P. sungorus*. Panels A and B do not add up since B includes all pairwise contrasts between all time points.

show that not only are many genes that are differentially expressed within a species different than those genes in the other, but there is no overlap between the species for temporal differentially expressed genes between consecutive time points.

Finally, I performed a GO enrichment analysis for transcripts that were differentially expressed between any two time points for *P. campbelli* and *P. sungorus*. The top enrichment group for *P. campbelli* had an enrichment score of 3.849 and was generally associated with metabolic functional pathways. The top three enrichment groups for *P. sungorus* had enrichment scores of 8.236, 4.538, and 4.345, respectively. These three groups were

respectively associated with cell cycle, melanogenesis, and melanogenesis/metabolism functional pathways (table 5).

These results all suggest that there is little similarity between the species in which genes are differentially expressed during the molt to the winter coat and when they are up- or down-regulated. However, this all come as somewhat of a surprise considering these two dwarf hamster species are relatively closely related and were previously used as a comparative model system for understanding the regulation of seasonal coat color change (Bilbo et al. 2003). Additionally, both species show evidence of seasonal change in body weight on short days, for example (Chapter 1).

#### *Expression Divergence*

Based on evidence for divergence of gene regulation during the molt timeline between the species in the temporal differential expression analysis, I further investigated differences between *P. campbelli* and *P. sungorus* by identifying genotype differential expression, while controlling for time point in the molt. Genotype differential expression was calculated for contrasts between *P. sungorus* and *P. campbelli* and between each parental species and F1 hybrids at each time point. 781 genes showed genotype differential expression across the timeline between *P. campbelli* and *P. sungorus*. 224 and 342 genes showed genotype differential expression between F1 hybrids, and *P. campbelli* and *P. sungorus*, respectively (table 4).

Next, to further understand how genotype differential expression varied between each genotype contrast (i.e., *P. campbelli* vs *P. sungorus*, *P. campbelli* vs F1, and *P. sungorus* vs F1) I

Table 5. DAVID enrichment for differentially expressed genes in *P. campbelli* and *P. sungorus*.

<i>P. campbelli</i>			
Enrichment group	Enrichment score	Summary term	Enrichment terms
1	3.849	Metabolism	Retinol binding Retinol metabolic process
<i>P. sungorus</i>			
Enrichment group	Enrichment score	Summary term	Enrichment terms
1	8.236	Cell cycle	Cell division Mitotic nuclear division Chromosome segregation Kinetichore
2	4.538	Melanogenesis	Melanosome membrane Melanin biosynthetic process Developmental pigmentation Pigmentation
3	4.345	Melanogenesis/Cell cycle	Melanosome membrane Melanosome Melanosome organization

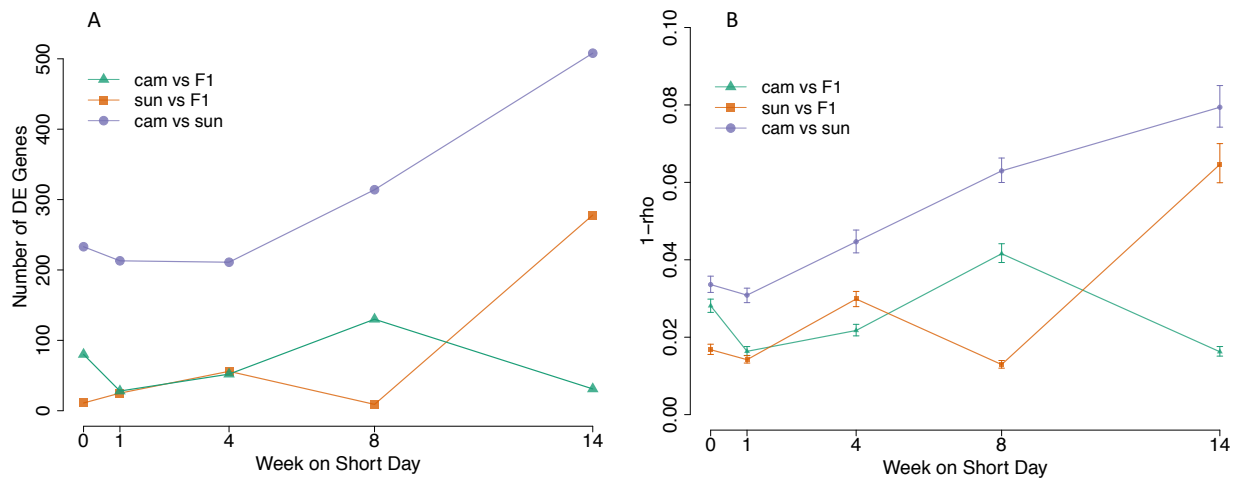


Figure 8. (A) Number of differentially expressed genes and (B) spearman correlations in expression for all genes at each time point between *P. campbelli* and F1 hybrids (triangles), *P. sungorus* and F1 hybrids (squares), and *P. campbelli* and *P. sungorus* (circles).

calculated the number of genotype differential expressed genes at each time point for each genotype contrast. Spearman rho correlations were also calculated for the same genotype differential expressed genes and confidence intervals were bootstrap estimated (figure 8).

These analyses indicate that molt timelines in *P. campbelli* and *P. sungorus* are divergent and the number of differentially expressed genes increases further into the molt timeline. This trend is confirmed in the correlation patterns—later time points show lower correlation in expression with increasing differential expression between *P. campbelli* and *P. sungorus*. There is less genotype differential expression in contrasts contain F1 hybrids and the number of differentially expressed genes in F1 contrasts are somewhat similar, but show greater divergence in later time points, especially at the last time point. Furthermore, there are fewer differentially expressed genes between *P. campbelli* and F1 hybrids than between *P. sungorus*

and F1 hybrids. This is an interesting result because it confirms phenotypic similarities between *P. campbelli* and F1 hybrids that was discussed in Chapter 1. Spearman rho correlations for genotype differential expressed genes in contrasts between parental species and F1 hybrids show similar patterns as the number of differentially expressed genes. In addition, rho values at week 14 between parental genotype differential expression and *P. sungorus* vs F1 genotype differential expression are much more similar than the value for *P. campbelli* vs F1 genotype differential expression.

### **Discussion**

*P. campbelli* and *P. sungorus* both exhibit phenology that is induced by the reduction in daily photoperiod (Bilbo et al. 2003, Timonin et al. 2006). At this point, there is extensive work detailing the physiological and cellular regulation of seasonal coat color change in *P. sungorus*, but there is a poor understanding of the genetic basis for or molecular regulation of this novel and likely adaptive trait (Mills et al. 2013, Zimova et al. 2016). In fact, this is the first study to attempt to understand the regulatory basis of seasonal coat color change in an interspecific comparative framework. There were three main goals of this chapter: First, characterize differential expression along the molt timeline for the two species using RNA-seq. Second, also using transcriptomic data, assess whether regulatory differences between the species and whether large regulatory divergence precludes this system from being used in a comparative framework. to characterize differential gene expression along the molt timeline for *P. campbelli*, *P. sungorus*, and F1 hybrids. Differential expression in these contrasts are defined as temporal differential expression. Temporal differential expression will offer insight into how



phenology is regulated in each species. Finally, we also sought to measure differential expression in F1s and assess how divergent expression profiles in the two species behave in the same individual. Differential expression in these contrasts are defined as genotype differential expression. Genotype differential expression for contrasts involving the F1 hybrids will suggest how the two parental species haplotypes behave in the same individual, further elucidating the regulatory nature of seasonal coat color change and phenology. Below, I discuss insights from the three aims listed above and how our results inform the broader understanding of the evolution of seasonally flexible phenotypes.

### **Overall Transcriptomic Patterns**

To generate reference transcriptomes and measure expression I used a rigorous approach designed to remove biases and promote comparisons between the two parental dwarf hamster species. I first examined overall patterns of expression in the three transcriptomes (*P. campbelli*, *P. sungorus*, and F1 hybrids). Using MDS plots, I attempted to elucidate patterns associated with phenology within each transcriptome and make distinctions between the three molt timelines. These plots showed clear distinct clustering for the three genotypes on dimension two, but the distribution of samples along dimension one did not appear to be associated with time point or reflect a simple signal of phenology. Since there was no overall phenology signal with all samples, I attempted to systematically recover a pattern associated with time point by generating MDS plots for each genotype separately. However, this also did not produce a distribution of samples by time point on either dimension one or dimension two. During RNA extraction and cDNA library preparation, I stratified samples across

four batches so that there was no close association between batch and time point or genotype. While this approach ensures against type 1 error and that that batch will not drive patterns that appear to be associated with time or genotype, distributing samples in this way can interrupt signals of treatment in an experiment. Effects due to extraction and library preparation batches may have disrupted the signal of a developmental timeline in our dataset, so to test this the same MDS plots were generated with sample points colored according to batch number. Sample points did not cluster based on batch number, thus this approach did not indicate that batch effect disrupted clear patterns of phenology in the MDS plots.

### **Molt Phenology**

Neither the MDS plots nor clustering samples based on transcript expression by Euclidean distance using heat maps indicated a strong signal of phenology for *P. campbelli*, *P. sungorus*, or F1 hybrids. In other words, these results indicated that there is not a strong signal of induction of large groups of genes during the molt, regardless of whether it is a species that has a white winter coat or not. This was surprising considering that we found clear changes in coat color in *P. sungorus* and extensive work shows evidence of phenology throughout the organism in both *P. campbelli* and *P. sungorus* (Stullken and Hiestand 1953, Heldmaier and Steinlechner 1981, Bockler and Heldmaier 1983, Russel and Tumlinson 1996, Bilbo et al. 2003, Timonin et al. 2006).

BCV for biological replicates within each genotype was slightly higher than the expectation for genetically identical replicates. However, biological replicates were sourced from our outbred colony and individuals are closely related, but not genetically identical so the

elevated level of variation between biological replicates in our experiment this is not a concern. This variation between biological replicates may introduce too much noise between replicates and make it difficult to identify time points of differential gene expression that reflect induction of phenology in the phenotype. Furthermore, it was shown in *P. sungorus* that seasonal molting is a continuous and mosaic process (Kuhlmann et al. 2003), which may have introduced variance into our measure of gene expression. Paul et al. (2007) corroborated that *P. sungorus* continuously molts, but added that reduced photoperiod results in increased rate and density of fur regrowth compared to that in long day conditions. This work suggests that although *P. sungorus* exhibits a continuous molt, rate of hair regrowth and hair density is a seasonal component of the molting process. Furthermore, Paul et al. (2007) also argued in line with Kuhlmann et al. (2003) that there may be a limited photorefractory synchrony between individuals during photoperiod changes. Both of these studies indicate that there may be appreciable variation in molting rate and photoresponsiveness between individuals. We implemented a highly-controlled and replicated sampling schema to exclude any additional variation into our measures of expression. However, given the continuous and mosaic nature of the *Phodopus* molt, even though I sampled biological replicates at the same location and at the same time point I may have not captured skin cells at the same point in anagen among replicates. This character of the molt would likely introduce more variation in gene expression counts than in our phenotype measurements because hair follicles across the organism are incredible dense variability in hair follicle activity would be dampened at the hair phenotype level.

The continuous molt in *P. sungorus* may be a bit unique among molting organisms (Ling

1970). Previous work demonstrates that molt is seasonally coordinated based on environmental or physiological cues in snowshoe hares (Mills et al. 2013, Ferreira et al. *in review*), birds (Gonzalez-Gurriaran et al. 1995, Dawson et al. 2001), arthropods (Gates et al. 1993), and sheep (Lincoln et al. 2006) rather than continuous throughout the year. While, continuous versus seasonal molting cycles has not been tested across most biodiversity, there appears to be strong indication that many species and most mammals molt seasonally instead of continuously.

*P. campbelli* shows less temporal differential expression than *P. sungorus* and in *P. sungorus* the greatest amount of temporal differential expression is between week 8 and week 14. Since we see a clear induction of the winter coat color phenotype in *P. sungorus* around week 4 (see Chapter 1), the expectation would be to see temporal differential expression before or during that phenotype induction period. Instead, our data indicate that the largest change in expression is between week 8 and week 14, which is probably associated with a transition from most hairs in the anagen phase, or hair growth phase, to telogen, the more transcriptionally quiescent hair follicle phase (Botchkarev and Kishimoto 2003, Schlake et al. 2003, Lin et al. 2004, Geyfman et al. 2015). However, that justification still does not explain consistently low temporal differential expression between time points in *P. campbelli*. Contrasting overall temporally differentially expressed genes between the two species reveal that only 41 genes are both temporally differentially expressed in both species. Considering the apparent shared phenologies between these two species (Bilbo et al. 2003, Timonin et al. 2006), more genes may be expected to fall into this category than what was found in this experiment. Although my quantitative genetic data in Chapter 1 suggests that changes in only a

few genes allows for evolution of seasonal coat color change, our differential expression analysis detected many more genes that are associated with general molt and skin reorganization processes and not just pigment production.

Gene ontology (GO) enrichment analysis for temporal differential expression in each parental species did confirm some expectations based on the expression patterns. There was generally poor enrichment for ontology terms in *P. campbelli*, which may be related to the lack of temporal differential expression. GO terms were all related to retinol binding and retinol metabolism. Temporal differentially expressed genes in *P. sungorus* showed greater enrichment for various pathways and/or biological processes. The most significant enrichment group was associated with cell cycle processes and specifically cell division, mitotic nuclear division, chromosome segregation, and kinetochore development. The other two most significant enrichment groups were associated with melanogenesis. We expected the general patterns of enrichment discovered in the GO analysis. Although *P. sungorus* molt is not entirely seasonal, this species does become less active during the winter months (Puchalski and Lynch 1986, Elliot et al. 1987), which may include reduced hair regrowth. Geyfman et al. (2015) argues that the telogen stage in hair growth is most energy-efficient state of the hair growth cycle and based on winter reduction in other energetic expenditures, most hair follicles are probably in telogen at the end of the molt to the winter pelage in *P. sungorus*. GO enrichment in *P. sungorus* is unsurprising considering the largest change in temporal differential expression was at the end of the molt cycle when transcriptional activity decreases as hair follicles enter telogen phase.

## Divergence in Transcriptome Phenology Between Species

Comparisons between the overall timelines between *P. campbelli* and *P. sungorus* indicate that there may be divergence in the fall molt of these two species. Specifically, there was no overlap of temporal differentially expressed genes at consecutive time points between *P. campbelli* and *P. sungorus*. Additionally, only 41 genes were found to be temporal differentially expressed across the entire molt between the two parental species. To further investigate the possibility of divergent phenologies we identified genotype differentially expressed genes. To do this, I measured differential expression between the species or between each species and F1 hybrids along the molt at each time point. Compared to genotype contrasts including F1 hybrids, genotype differential expression between the species was much greater. Also, there was lower correlation in overall expression between the species than there was with the F1 and either species.

Based on the literature, *P. campbelli* and *P. sungorus* are closely related (Brekke et al. 2016) and exhibit similar phenology (Bilbo et al. 2003, Timonin et al. 2006). Therefore, our *a priori* expectation would be that these two species exhibit similar temporal differential expression profiles. Furthermore, based on this line of reasoning we would expect there to be little differential expression between the two species at any given time point. A large network of genes control hair growth (Botchkarev and Kishimoto 2003, Schlake et al. 2004, Kawano et al. 2005) and as far as we are aware, both species molt during the transition to winter phenotypes (Bilbo et al. 2003, Kuhlmann et al. 2003, Paul et al. 2007). All of these results indicate that the molecular regulation of molt phenologies of *P. campbelli* and *P. sungorus* are highly divergent.

Furthermore, taking the differences in ecology and life history of these species may shed

light on why there is seemingly little overlap in the regulatory nature of winter phenology between these two species. *P. sungorus* inhabits an environment that is seasonally highly variable and is characterized cold, snowy winters. Non-winter months in the range are typically less harsh (Ross 1998). On the other hand, *P. campbelli* inhabits large regions of the Gobi desert and the surrounding region, which is much more harsh than the environment experienced by *P. sungorus*. Although winters are colder than the rest of the year in this area, the environment is extremely arid and less variable as there is less consistent snow cover during the winter and temperatures during non-winter months can still be very cold (Ross 1995).

Considering what we found in temporal and genotype differential expression, there is the possibility that molt phenology is driven by upstream regulation that signals to the skin and that cascading effects of this signal in the skin is are dampened. Alternatively, very subtle expression changes in a few genes in the skin may ultimately regulate molt phenology. Both of these alternative hypotheses could explain the surprising lack of expression differences associated with phenology in the skin.

Temporal differential expression in F1 hybrids appeared further dampened with only seven genes were differentially expressed across the timeline. This amount of temporal differential expression is surprisingly low compared to what was found in the parental species. Estimates for BCV show that F1 hybrid biological replicates had slightly higher variability than what was found in the species. Additionally, F1 hybrids had fewer genotype differentially expressed genes compared to the parental species than there were between the parental species. Also, there was greater correlation (Spearman) of total expression between F1 hybrids and parentals than between the parentals. These results indicate that the F1 hybrids may be

expressing both parental haplotypes equally or nearly equally. If there is in fact equal expression of both parental haplotypes in the hybrids, this would suggest that *trans*-regulatory evolution is driving divergence in phenology between *P. campbelli* and *P. sungorus*. Intriguingly, most work throughout the regulatory evolution literature suggests that adaptive evolution of gene regulation is primarily driven by *cis*-regulatory changes (Wittkopp 2005, Wray 2007, Wittkopp 2012, Coolon 2014). Given the contradictions of these initial observations with what is typically found throughout the literature, a more comprehensive allele-specific expression analysis should be conducted to definitively say whether this is the case or not.

### **Conclusions and Future Directions**

This is the first study to understand the genetic regulation of seasonal coat color change in an interspecific comparative framework. While there were no clear patterns of phenology by examining temporal differential expression within either species, we were able to draw some qualitative conclusions regarding regulation of skin phenology and divergence in phenology between *P. campbelli* and *P. sungorus*. In particular, our data suggest that skin phenology is regulated by subtle changes over time in a few genes in both species. Likewise, skin phenology may be more generally regulated by upstream changes in gene expression outside of the skin. Perhaps once this signal reaches skin cells, the induction of gene expression associated with skin phenology is dampened and large, coordinated changes in expression are difficult to detect. Alternatively, the amount of noise between biological replicates or our inability to detect organized temporal differential expression may be implicated by the biology of the molting process in *Phodopus*. Kuhlmann et al. showed that the molting process in *P. sungorus* is



a continuous, mosaic process across the organism. Even though I controlled for location and timing of sampling in this experiment, the fact that the sampling location may or may not have included hairs currently in anagen or if samples were at different points in hair growth could contribute to increased variation between biological replicates.

Although these two species have been used to study phenology in a comparative framework in the past, this study suggests that there is a great deal of divergence in which genes are differentially expressed at a given time point in the molt. Furthermore, based on greater differential expression and greater correlation in expression between the F1 hybrids and either parental species than between the two parental species, there appears to be equal expression of maternal and paternal haplotypes in the hybrid hamsters. This pattern suggests that *trans*-regulatory evolution may be an influential factor for the regulatory divergence between these two species. It would be worthwhile to further investigate transcript-wise allele-specific expression in F1 hybrids and distinguish between *cis*- and *trans*-regulatory changes that promote divergent phenology. Understanding the role of *cis*- vs *trans*-regulatory changes is the first step in detailing regulatory evolution and may provide *a priori* predictions for specific nucleotide changes that lead to expression differences and foster adaptation.

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