ZONGULDAK BÜLENT ECEVİT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ALTITUDINAL EFFECTS ON INNATE IMMUNE RESPONSE AND LOCAL POPULATION GENETIC STRUCTURE OF *NANNOSPALAX XANTHODON* (ANATOLIAN BLIND MOLE RAT)

DEPARTMENT OF BIOLOGY

MASTER OF SCIENCE THESIS

HALİL MERT SOLAK

DECEMBER 2019

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"With this thesis, it is declared that all the information in this thesis is obtained and presented according to academic rules and ethical principles. Also as required by academic rules and ethical principles all works that are not result of this study are cited properly."

Halil Mert SOLAK

ABSTRACT

Master of Science Thesis

ALTITUDINAL EFFECTS ON INNATE IMMUNE RESPONSE AND LOCAL POPULATION GENETIC STRUCTURE OF *NANNOSPALAX XANTHODON* (ANATOLIAN BLIND MOLE RAT)

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Immune defense is costly to maintain and deploy, and the optimal investment into immune defense should depend on the risk of infection. Altitude is a natural environmental factor predicted to affect parasite abundance, with lower parasite abundance expected at higher altitudes because of stronger environmental stressors that reduce parasite transmission. Therefore, investment in immune response predicted to depend on the strength of selection imposed by parasites and the availability of resources. The possible differences in immune response may also be increased by neutral divergence between the populations, or they can be decreased by gene flow between geographically close populations.

Using high, middle and low altitude populations of the Anatolian blind mole rat (ABMR), *Nannospalax xanthodon,* we tested for effects of altitude on constitutive innate immune defense, and the amount of neutral genetic divergence between these populations.

ABSTRACT (continued)

Field studies were performed with 48 wild animals in 2017, 2018 and 2019 from two high, one middle and three low altitude populations in Niğde and Konya region, at respective altitudes of 1010 m, 1115 m, 1350 m, 1700 m, 2580 m and 2900 m above sea level. We first compared standing innate immune defense among these populations as measured by bacteria killing ability of blood plasma. We then measured corticosterone stress hormone levels because stressful conditions may affect immune response. Furthermore, we compared prevalence and intensity of gastrointestinal parasites of field-captured ABMR. Finally, we tested the neutral genetic divergence between altitude populations by eight microsatellite markers.

We found that bacteria killing ability of plasma is greater in the mole-rat samples from high altitude populations. There was no significant difference in stress (corticosterone) levels between altitude categories. Parasite prevalence was significantly higher in 2017 samples but there was no significant difference in abundance or intensity between altitudes nor between sexes. The amount of neutral genetic differences between altitudes were likely too small to be detected with the given markers. Small sample sizes may have reduced our power to detect true differences, nevertheless, this study provides preliminary support that greater standing innate immunity in high altitude animals may reflect greater investment into constitutive defense. These functional differences could be caused by strong natural selection for adaptation to the mountain environment.

Keywords: Ecoimmunology, Altitude, Stress, Gastro-intestinal parasites, Immune response, *Nannospalax,* Microsatellite markers, Population genetic structure.

Science Code: 401.04.00.

ÖZET

Yüksek Lisans Tezi

YÜKSEKLİĞİN ANADOLU KÖRFARESİ *NANNOSPALAX XANTHODON*'UN DOĞUŞTAN GELEN BAĞIŞIKLIK SİSTEMİNE ETKİSİ VE LOKAL POPÜLASYONLARIN GENETİK YAPILARININ TESPİTİ

Halil Mert SOLAK

Zonguldak Bülent Ecevit Üniversitesi
Fen Bilimleri Enstitüsü
Biyoloji Anabilim Dalı

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Bağışıklık yanıtını sürekli olarak aktif tutmak ve bunu sürdürmek canlılar için çok fazla enerjiye mal olmaktadır ve bağışıklık yanıtına yapılacak olan ideal enerji yatırımı enfeksiyon riskine bağlıdır. Yükseklik ise parazit bolluğunu etkileyen doğal çevresel bir faktördür, yüksek rakımda zorlu çevre koşullarının parazit transmisyonunun azaltmasına bağlı olarak parazit bolluğunu düşürdüğü tahmin edilmektedir. Bu yüksekliğe bağlı muhtemel fenotipik farklılık, popülasyonlar arasındaki nötral ayrılma ile artacağı gibi, coğrafi olarak yakın popülasyonlar arasındaki gen akışı ile de azalabilir.

Bu çalışmada Anadolu Körfaresi'nin yüksek, orta ve alçak rakımdaki popülasyonları kullanılarak, yüksekliğin doğuştan gelen özgül olmayan bağışıklık sistemine olan etkileri ve farklı yükseklikteki popülasyonların nötral genetik ayrılma miktarları test edildi.

ÖZET (devam ediyor)

Devam eden üç yıl bolunca (2017, 2018 ve 2019) Niğde, Konya ve çevresindeki altı farklı popülasyonda (iki yüksek rakım, bir ortalama rakım ve üç alçak rakım) 48 örnek toplandı. Popülasyonların rakımları sırasıyla denizden 1010 m, 1115 m, 1350 m, 1700 m, 2580 m ve 2900 m yükseklikte dir. İlk olarak farklı yükseklikteki popülasyonlardan alınan kan plazmasının bakteri öldürme yeteneği ölçülerek doğuştan gelen özgül olmayan bağışıklık sistemi karşılaştırıldı. Sonrasında yükseltiye bağlı olarak stresli koşullar bağışıklık sistemini etkileyebileceği için, kortikosteron (stres) hormonunun seviyeleri karşılaştırıldı. Daha sonra mide-bağırsak parazitlerinin yaygınlık ve yoğunluğu karşılaştırıldı. Son olarak sekiz floresan mikrosatelit beliteç kullanılarak popülasyonlar arasındaki nötral genetik ayrılma miktarları test edildi.

Yüksek rakımdan alınan örneklere ait plazmaların bakteri öldürme yeteneklerinin alçaktakilere oranla daha yüksek olduğu gözlenirken, korikosteron seviyelerinde yükseltiye bağlı anlamlı bir fark görülmedi. Mide-bağırsak parazitlerinin yoğunluğu ve bolluğunun yükseltiye ya da cinsiyete bağlı olarak değişimezken, yaygınlığının 2017 yılında 2018 yılına göre daha yüksek olduğu gözlendi. Farklı yüksekliklerdeki popülasyonların nötral genetik ayrılmalarının muhtemelen elimizdeki mikrosatelit markırlar ile tespit edilemeyecek kadar az olduğu gözlendi. Düşük örnek sayısı gerçek farklılıkların ortaya çıkartılmasını engellemiş olabilir fakat yine de bu çalışma yüksek rakımda yaşayan hayvanların doğuştan gelen özgül olmayan bağışıklık sistemine daha fazla yatırım yaptığına dair öncül sonuçlar göstermektedir ve bu fonksiyönel farklılıklar ise muhtemelen güçlü bir doğal seçilimin etkisinde, dağlık ortama adaptasyon amacı ile gelişmiştir.

Anahtar Kelimeler: Ekoimmünoloji, rakım, stres, mide-bağırsak parazitleri, bağışıklık yanıtı, *Nannospalax*, mikrosatelit markırlar, popülasyon genetik yapısı.

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SYMBOLS

µl	: Microliter
°C	: Centigrade Degree

ABBREVIATIONS

ABMR	**:** Anatolian Blind Mole-rat
BEÜ	**:** Bülent Ecevit Üniversitesi
BKA	**:** Bacteria Killing Assay
CFU	**:** Colony Forming Units
CytB	**:** Cytochrome oxidase b gene
HWE	**:** Hardy-Weinberg Equilibrium
M	**:** Mole
mL	**:** Mililiter
mm	**:** Milimeter
PBS	**:** Phosphate Buffered Saline
RPM	**:** Round Per Minute
TSB	**:** Tryptic Soy Broth

CHAPTER 1

INTRODUCTION

Parasites are ubiquitous in wild populations and can affect host birth and death rates (Hudson et al. 2002), and shape population demographics (Hudson et al. 1998, Pedersen and Greives 2008, Forbes et al. 2014). Thus, it is no surprise that parasites are powerful agents of selection that promote rapid evolution of host resistance (Altizer et al. 2003). Yet immunity is not without its own costs (Lochmiller and Deerenberg 2000). These costs can take the forms of energy consumption and immunopathology during immune system activation (Råberg et al. 2009). For an individual, investment in immune response should depend on the strength of selection imposed by parasites and the availability of resources that can be allocated to different physiological functions (Sheldon and Verhulst 1996, Durso and French 2017).

In light of this balance, investment in immune response may be particularly costly at high altitudes, which are characterized by environments with extremely limited resources. These limited resources include less land area and thus lower food supply, lower atmospheric pressure and thus oxygen availability, and lower temperatures at high altitude compared to low altitude environments (Körner 2007). However, mammals have been shown to adapt to some of these stressors. For instance, to cope with the stressor of low oxygen availability at high-altitude, many mammals possess hemoglobin variants with stronger oxygen affinity (reviewed in Storz 2007), and high-altitude Andean mouse (*Andinomys edax*) species brought to sea-level still show increased carbohydrate use rather than lipids, it is an oxygen-saving metabolic strategy to conserve energy (Schippers et al. 2012). Thus, environmental pressures at high-altitudes may promote immunologic adaptations in animals living under those harsher conditions.

In addition to affecting host adaptation, extreme conditions at high altitude can also affect parasite abundance. High altitude conditions can limit parasite abundance by reducing speed of development (LaPointe et al. 2010), by increasing mortality from low temperature and solar radiation during free-living stages (Zanet et al. 2017), and by reducing the availability of

vectors for transmission (van Riper et al. 1986, Michel et al. 2014). While these previous studies point to reduced parasite abundance at higher altitudes, others have found increased parasite burden for individual hosts (Tadin et al. 2014, Tollenaere et al. 2010, Tollenaere et al. 2008, Tollenaere et al. 2011). This finding is possibly explained by greater nutritional stress leading to decreased physiological condition and increased susceptibility to infection among high-altitude animals. For example, higher helminth egg output rates in montane compared to lowland baboons were linked to lower resource availability and increased malnutrition in montane areas (Appleton and Henzi 1993). In addition, host species that are concentrated on less space at higher altitudes may experience increased parasite burden because higher host density increases the transmission of many parasites (McCallum et al. 2001) and density promotes greater parasite abundance (Arneberg et al. 1998). Thus, it is unclear if parasite prevalence and intensity are directionally correlated with altitude, and more work is needed to address these questions.

In summary, individuals are expected to maximize fitness by minimizing costs from both parasites and immune defenses. Therefore, in the absence of parasites, animals should reallocate energy resources away from unnecessary and energetically expensive immune defense mechanisms to biological functions associated with greater fitness (Blossey and Notzold 1995, Sheldon and Verhulst 1996, Zuk and Stoehr 2002, Viney et al. 2005, Lee 2006). Accordingly, if parasite pressure is lower at higher altitude, adaptations should lead to different optimal immune defense among high and low altitude populations.

The possible difference in immune defense between populations of Anatolian blind mole-rat may also be caused by neutral divergence between populations or it can be affected by gene flow between geographically close populations. Therefore, it is important to know the amount of genetic divergence or gene flow between the populations. Microsatellite nuclear genetic markers are cost- and time-effective, and versatile to estimate genetic differences between populations (Guichoux et al. 2011). Previous authors used two panels of microsatellite markers on *Nannospalax* genus, 1) a set of 15 markers developed for *Nannospalax ehrenbergi* by Karanth (Karanth et al. 2004) and used in Malik et al. 2011 and Matur et al. 2019 and 2) a set of eight markers developed for *Nannospalax leucodon* by Popa et al. 2014. We chose the set 2, because the number of alleles and the levels of polymorphisim of these loci are expected to better reflect the differences between populations. Also, we expected the set 2 to amplify better in *Nannospalax xanthodon* since *N. leucodon* and *N. xanthodon* are closer to each other than both of them to N. ehrenbergi (Matur et al. 2019).

The Anatolian blind mole-rat (ABMR), *Nannospalax xanthodon* (Satunin 1898), is a subterranean and solitary rodent found from the sea level up to 3000 m asl (above sea level) in Anatolia. Each individual constructs its own system of underground tunnels, and rarely leaves it unless forced to do so. Population density of *Nannospalax* genus is up to 6 animals per 1000 m^2 in Turkey (Sözen 2005). To our knowledge, there is no study on the dispersal range of the ABMR, however, a closely related species, the Palestinian mole-rat (*Nannospalax ehrenbergi*) is known to occasionally make tunnels in a long straight line up to 300 m during the mating season (Rado et al, 1992). The ABMR has the highest diversity of chromosomal forms (cytotypes) among the Palearctic mammals, with each cytotype occupying its own distinct geographical area (Arslan et al. 2016). It is very likely that most if not all of these chromosomal forms are reproductively isolated (Arslan et al. 2016). Both low dispersal ability and high degree of cryptic speciation create high potential for local adaptation between populations (e.g. Bultin et al. 2014).

In light of these ideas, we compared the innate standing immune defense, stress levels, and gastrointestinal parasite prevalence, abundance, intensity and genetic difference among three populations of ABMR from low and high altitude (1010 m asl to 2900 m asl) to address three interrelated questions: (i) Are ABMR innate standing immune response and stress levels influenced by altitude? (ii) Do parasite richness, prevalence, and intensity differ among ABMR populations by altitude? (iii) What is the amount of neutral divergence between populations of ABMR?

CHAPTER 2

MATERIALS AND METHODS

2.1. SITES AND FIELD SAMPLING

ABMRs were captured for three consecutive years (2017, 2018, 2019, Table 2.1) at three sites (six populations) in Turkey (Figure 2.1): low altitude (Ereğli1 1010 m asl, Ereğli2 1115 m asl. and Ulukışla 1350 m asl), 'middle altitude' (Madenköy 2000 m asl) and 'high altitude' (Medetsiztepe / Bolkar Mountains 2900 m asl).

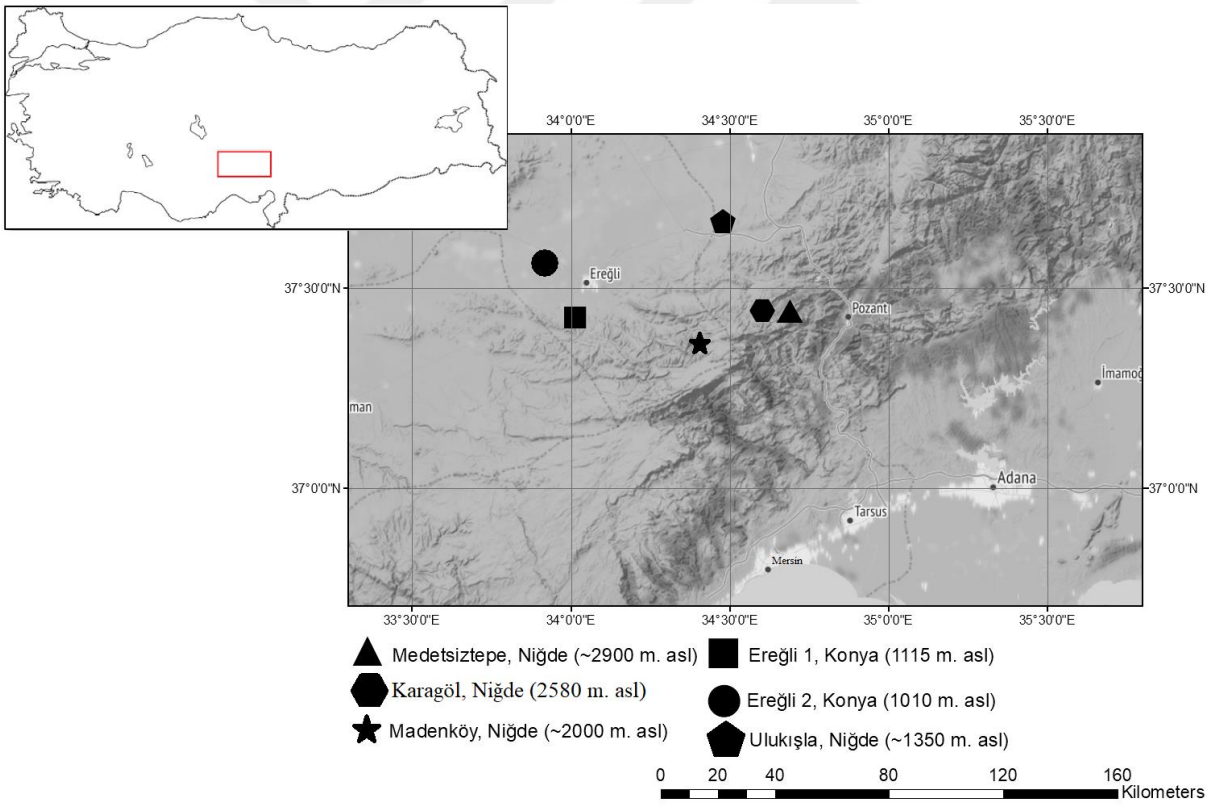

Figure 2.1. Map of sampling locations. Previous surveys in the region revealed the two areas of Eregli and Medetsiztepe as the geographically closest sites with the greatest elevation difference (Sözen and Kıvanç 1998, Arslan et al. 2016).

ABMRs are known for the exceptional diversity of chromosomal races. All populations used in this study were previously described as a part of the same chromosomal race (*cilicicus*), diploid chromosome number 2n=58 (Sözen and Kıvanç 1998, Arslan et al. 2016). Annual mean temperature and mean precipitation at the high altitude is 0.16 °C and 744.6 mm, respectively; and 11 °C and 344.7 mm at the low altitude, respectively. Partial snow cover is present until June at high altitude and may limit digging activities and access to water for these animals. All altitude sites were characterized by grassland (Leguminosae, Asteraceae and Poaceae families). Bulbous plants (such as *Ornithogalum* sp., *Gagea* sp.) are the preferred food source for TMBR (Sözen 2005) and were present at high altitude. Food availability is one of the major factors affecting population density in a closely related species *Nannospalax ehrenbergi* (Lövy et al. 2015), as it is more generally for omnivorous, granivorous and herbivorous small mammals (Prevedello et al. 2013).

We caught 20 animals from high altitude, 8 from middle altitude and 20 animals from low altitude. Sample size for each assay by population and altitude is shown in Table 2.2. Animals were live captured by opening the burrow passageways at specific sections and blocking retreat when the animal is mending the tunnel (Wertheim and Nevo 1971).

Table 2.1. Sample size for each altitude.

Altitude category	**2017**	**2018**	**2019**
High	3	5	12
Middle	0	0	10
Low	3	10	5

Table 2.2 Sample size for each study by population and altitude.

Altitude category	Population (N=48)	Elevation (meters asl)	N for BKA	N for stress assay	N for parasite assay	N for genetic study
High	Medetsiztepe (N=9)	2900 m	9	9	8	20
	Karagöl (N=12)	2000 m	0	0		10
Middle	Madenköy (N=10)	1700 m	0	0	0	8
Low	Ereğli 1 (N=2)	1010 m	2	2	3	3
	Ereğli 2 (N=8)	1115 m	8	8	8	12
	Ulukışla (N=5)	1350 m	0	0	0	5
	Total		19	19	19	48

On capture, we weighed animals and determined their sex and sexual maturity status. We collected fecal samples. We disected animals and collected liver samples for genetic studies. Liver and fecal samples stored in 95% ethanol. For blood collection we could not use the saphenous vein because it was impossible to locate under field conditions, so we collected approximately 100 µl blood by heart puncture with a 0.30 mm (30G) x 8 mm gauge needle injector (BD micro-Fine™ Plus, U-100 Insulin) and placed the whole blood in an (2 mL) Eppendorf tube. The time spent between the first capture until blood collection was recorded as "time held (minutes)". After collecting blood, the animals were monitored for two hours and then released back into their own nests. Blood was allowed to coagulate and then put on ice and stored for 5 – 6 hours after which the serum was separated and frozen until further processing.

After collection, blood samples were centrifuged to separate serum and red blood cells via portable centrifuge (Alfagen Mini Centrifuge, Mini-7K) at 7000 rpm for 15 minutes. Serum and whole red blood cells were stored separately in (1.5 mL) Eppendorf tubes. Samples were stored at +4°C in the field and then moved to -80°C once at the lab. For the bacteria killing assay (BKA) and corticosterone assay we only used the 2018 samples because prolonged freezing and frost-defrost conditions experienced by 2017 samples may affect concentrations of biochemical components and hormones in serum (Bielohuby et al. 2012). Thus, running

frozen and fresh samples in same assay is not recommended (but see Hegemann et al. 2017). Moreover, it is suggested to use fresh samples whenever possible (Jacobs and Fair 2015).

Animal handling and usage were approved by the Animal Ethics committee of Bülent Ecevit University (#91330202).

2.2. ECOIMMUNOLOGY

2.2.1. Bacteria Killing Assay

The majority of immune assays developed for domesticated or model organisms which are species-specific. However, bacteria killing assay is not species-specific and can be applied for various vertebrates with optimization. The assay quantifies the antimicrobial capacity of blood or plasma in vitro which measures the standing innate immune response. This technique can provide comprehensive measures of immune function with minimal sample size and equipment (Liebl and Martin 2009, French and Neuman-Lee 2012). So, it is an ideal method for non-model wild organisms like ABMR.

2.2.1.1. Bacteria Preparation

Prior to the assay, we autoclaved TSB (Tryptic Soy Broth, Sigma; 15 g broth/500 ml nanopure water) and stored it overnight at 4°C. Additionally, we reconstituted Escherichia coli in lyophilized 1 pellet (E-Power, Escherichia coli ATCC® 8739TM) into 10 ml pre-warmed 1X PBS (Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium, cat. No: 02-023-1A). Using flame-sterilized forceps, we transferred the pellet to the warm PBS, incubated the solution in a shaker for 30 min (37°C) to ensure hydration. Finally, we vortexed the stock solution until the pellet was completely dissolved and stored the solution for no more than 24 h at 4°C. We used this stock solution to make a working solution (105 colony-forming unit; CFU) by adding 1 ml of bacteria stock to 9 ml of pre-warmed PBS.

2.2.1.2. Optimization of Incubation Time and Serum Concentration

Before testing the bacteria killing ability of our samples, we optimized the incubation time of bateria (interval to log phase growth) and bacterial concentration. The concentration of E. coli used was 105 CFU/ml incubated at 37°C.

We added 6 µl of bacteria working solution, 18 µl PBS and 125 µl TSB into two wells. We then incubated the plate in a plate shaker adjusted to 150 rpm for 30 min at 37°C, and measured the absorbance at 300 nm, 340 nm, 405 nm, 490 nm, and 592 nm sampled at 0, 4, 8, 12, 14, 20, and 24 hours to determine log-phase growth of *E. coli*.

The procedure is the same for the bacteria killing assay (below) except for this optimization we used two pooled serum samples of 4 individuals (2 from the high altitude population and 2 from low altitude populations) to ensure variation of bacteria killing ability between individuals/populations. We used serial dilutions to find the best dilution (from 1:1 to 1:128). To do this we added 18 µl (9 µl from each of two individuals) of serum to 18 µl of PBS, mixed these by pipetting, and then took 18 µl of the serum-PBS mixture to the next well. 18 µl of PBS was added, mixed together, and then 18 µl was moved to the next row and the process was repeated until the dilution reached 1:128 (8 times). We then measured absorbance at the optimized time and wavelength (10 hrs. and 340 nm.).

We observed log-phase growth between 5 to 12 hours of incubation at 37°C, and we selected 10 hours as our optimized incubation period for subsequent sample assays (Fig. 3.1). Additionally, we selected the absorbance filter 340 nm because it allowed for better resolution of our data. Plasma dilution was determined to be 1:2 as this was the concentration at which 50% of bacteria were killed (Fig. 3.2).

2.2.1.3. Bacteria Killing Assay Procedures

Optimization of incubation time and serum dilution was carried out prior to the bacteria killing assay. Samples were thawed (15 min at room temperature) and then run in duplicate to provide greater accuracy. We modified the method of French and Neuman-Lee (2012) to prepare the plates. First, we plated positive controls and negative controls by adding 18 µl of PBS and 24 µl of PBS, respectively (TPP 96 well cell culture round bottom microplates). To

the duplicate sample wells, we then added 6 µl of serum and 12 µl sterile PBS (1M 10x PBS; 1:2, or 3x dilution) to each and added 6 µl of the bacteria working solution to all wells except negative controls. Plates were incubated in the plate shaker for 30 min at 37°C, at 150 rpm and then we added 125 µl TSB to each well. We then incubated plates in the plate shaker for a further 10 hours at 37°C, at 150 rpm. Next we read the plate at both 300 nm and 340 nm to measure the absorbance at time 0. We also read two blank wells to determine the background absorbance.

To calculate bacterial killing ability, we followed previous authors (Liebl et al. 2009, French and Neuman-Lee 2012) and first subtracted the background absorbance readings (blanks) from the sample absorbance readings to control for the initial coloration in the wells. Microbicidal capacity was calculated as one minus the mean absorbance for each sample (samples were run in duplicates), divided by the mean absorbance for the positive controls (wells containing only bacteria and TSB), and multiplied by 100 (i.e., % bacteria killed relative to the positive control). We ensured that the negative control absorbance values did not vary between the pre and post-incubation read.

2.2.2. Stress (Corticosterone) Assay

We proceeded with the stress assay shortly after (10-15 min) initiating the bacterial killing assays to avoid re-thawing the samples (as this may affect the quality of samples). The level of corticosterone, which is the main stress hormone in *Spalax* syn. *Nannospalax* and other subterranean rodents (Ganem and Nevo 1996, Moshkin et al. 2002, Vera at al. 2012), was measured in serum using a commercial enzyme immunoassay kit, according to the instructions provided (DetectX® Corticosterone Enzyme Immunoassay Kit). Samples were run in duplicate and we calculated the coefficient of variation to determine the repeatability of our corticosterone measurements. The intra-assay (average percent) coefficient of variation for corticosterone concentration for duplicates (n = 19) was 7.8% (range = 0.0 to 25.7%). Four individuals had CV greater than 15% (range 15.9 to 25.7%), but we were unable to repeat the assay because of limited serum. The mean (and SD) corticosterone concentration across all individuals was 11.04 µg/dL (SD=6.38) and did not significantly change by removing these four individuals (mean = 12.61 µg/dL (SD=6.20), two sample t-test: $t = -0.720$, $df = 30.58$, $p=0.477$). Thus, we retained all individuals for statistical analyses. Mean corticosterone concentrations were calculated from duplicates, referred to as MCC hereafter. Absorbance

was read at 450 nm by plate reader (Thermo Scientific, Multiskan™ GO Microplate Spectrophotometer). The standard curve corticosterone levels was calculated using the online software (www.myassays.com/arbor-assays-corticosterone-enzyme-immunoassay-kit.assay).

2.2.3. Gastrointestinal Parasite Assay

Spalacinae are commonly infected by numerous parasite species, including gastrointestinal helminths and coccidian (Wertheim and Nevo 1971, Sayin et al. 1997, Sayin 1980, Nalbantoglu et al. 2010). Oocysts of coccidia are shed in the host feces and transmitted via a fecal–oral route. Oocysts are only infective after the sporulation stage which occurs in the environment and so are subject to environmental conditions. The most important environmental factors are temperature, moisture, oxygen-level, and UV (Fayer 1980), all strongly associated with environmental gradients. Similarly, helminth parasites common to Spalax are either soil-borne nematodes, or insect-transmitted nematodes and cestodes (Wertheim and Nevo 1971) and thus are also subject to environmental factors that become increasingly severe with altitude.

To identify the presence and intensity of gastrointestinal parasites of *N. xanthodon* we collected fecal samples from animals in the field. Noninvasive fecal egg counts are commonly used as an approximation of worm burden and have been shown to correlate with the ability of the host's immune system to regulate worm burden and fecundity (Stear et al. 1995). However, due to our method of capture, we were unable to consistently collect mole-rat fecal samples during the same hours of the day, and this may increase noise in any true patterns of sampling time. (Lopez et al. 2007). Using samples collected from 19 individuals (8 high and 11 low altitude; 8 from 2017 and 11 from 2018), we used salt flotation with sodium nitrate solution (specific gravity 1.2–1.5) to isolate the oocytes and eggs of gastrointestinal parasites (Dryden et al. 2005, Winternitz et al. 2012). We scanned the cover slips in five replicate zigzag transects at both 100x and 400x, and then quantified the parasite oocytes and eggs per gram of feces. To obtain the fecal mass (g) we weighed collection tubes containing the fecal samples stored in 95% EtOH and subtracted the mass of collection tubes with the same volume of EtOH.

2.3. DATA ANALYSIS

2.3.1. Bacteria Killing and Corticosterone Assay

We analyzed the serum samples from 19 individuals (15 Female, 4 Male) for the bacteria killing assay (BKA) and for the corticosterone assay. For these assays we only used 2018 samples as our serum samples from 2017 were not consistently frozen and this may affect the accuracy of results. We had unequal representation of the sexes, with almost 4 times as many females as males, so we ran models for males and females combined and for females separately.

We performed general linear model selection to strike a balance between overfitting and underfitting due to our small sample size. Global models were fit for the response variables BKA and mean corticosterone concentration (pg/mL) using the R package *glmulti* (Calcagno and de Mazancourt 2010). We used "altitude", "sex" and "time held" as explanatory varşables. The best model had the lowest AICc value and was selected from the combination of variables thought to be important for predicting the BKA and the mean corticosterone concentration. Models within 2 ΔAICc units of the top model are considered to have substantial support (Symonds and Moussalli 2011) and were included in our confidence set of models. The following predictor variables were used for the BKA and mean corticosterone concentration (MCC) model selection: altitude (high or low), sex, mass (g) of animal, and time held. Handling time was included as a covariate in the BKA models, as acute stress from handling could also affect innate immunity (e.g., Matson et al. 2006). Time held was included as a covariate for MCC models because it has also previously been shown to associate with increased corticosterone levels in wild captured birds (Romero and Reed 2005), although longer time in traps did not appear to increase stress levels in wild rodents (Fletcher and Boonstra 2006). We also ran separate BKA model selection to test for an explicit effect of stress on bacteria killing ability by adding MCC as a covariate to the global model, excluding time held, as the two variables are expected to be correlated. We confirmed that all numeric predictor variables (mass, time held, and MCC) all showed correlation coefficients below 0.3.

We used the "maxsize" parameter of *glmulti* to set the maximum number of terms to be included in any model to two, to avoid overfitting. The distribution of our response variables were not normally distributed based on formal tests, however, multiple regression models

estimated using ordinary least squares (Gaussian) only require an assumption of normally distributed errors, not normally distributed response variables (Williams et al. 2013). In addition, Gaussian models are fairly robust to non-normality (Knief and Forstmeier, 2018). We verified that residuals are normally distributed and that simulated data from the model matched distributions of observed data.

2.3.2. Gastrointestinal Parasite Assay

We analyzed 29 fecal samples from 19 animals (10 Female, 3 Male and, 6 unidentified sex; 2017, N=8; 2018, N=11). In cases where the same individual (from the same capture) had more than one fecal sample we used the mean value.

We calculated prevalence, abundance, and intensity for the years (2017 and 2018) and for the altitudes (low and high). Prevalence was calculated as the percentage of infected from sampled individuals; abundance was calculated as the number of eggs/oocysts per gram of feces for each individual, including zeros; intensity was calculated as the number of eggs/oocysts per gram of feces for infected individuals (not including zeros). We then calculated the confidence interval for the parasite prevalence (traditional Clopper-Pearson CI), confidence interval for the mean intensity (bias-corrected and accelerated (BCa) Bootstrap with 5000 replications) and confidence interval for the mean abundance (Bootstrap BCa with 5000 replications). Fisher's exact test was used to analyze the parasite prevalence data. Since a large number of parasites can be concentrated within very few hosts, bootstrap tests are recommended as they do not assume a specific distribution of infection among hosts (Rózsa et al. 2000). We used a bootstrap t-test with 5000 replications to compare intensity and abundance by the population, sex and year.

All parasite statistical analyses were conducted using the Quantitative Parasitology Website (http://www2.univet.hu/qpweb/qp10/index.php). All other statistics were conducted with R 3.5.1 (https://www.r-project.org/).

2.4. POPULATION GENETIC STRUCTURE

We used 48 individuals comprised of the following: 20 high altitude (from two population), 20 low altitude (from three populations), 8 middle altitude (from one population) individuals, plus two individuals from Gerede, Turkey and eight individuals from Javakheti, Georgia (Matur, Yanchukov, Şahin, Kandaurov: unpublished data). We used eight fluorescent microsatellite markers, which were described for one of the closely related species of ABMR, *Nannospalax leucodon* in Popa et al. 2014. We used a Multiplex PCR technique which allows amplification of multiple targets in a single reaction. In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture. Our primers were labeled with two different kinds of fluorescent labels, FAM (blue) and HEX (yellow), so we could run multiple samples in one PCR reaction. Then we could distinguish fragments according to their sizes as well as their fluorescent labels.

First, we extracted DNA from liver tissues with QIAamp DNA Mini Kit (QIAGEN, Cat. No: 51304) and followed the instructions provided. DNA samples were stored in -80°C freezer. For the PCR reactions, we used QIAGEN Multiplex PCR Kit (QIAGEN, Cat. No: 206143). Instead of provided instructions, we ran PCR reactions in PCR plates with total volume of 7 µl (containing 3 µl Multiplex PCR Buffer, 0.6 µl Q-solution, 1.5 µl DNA, 0.5 to 1 µl primer and distilled water to make 7 µl of total volume). In our study, HEX labeled microsatellite markers needed higher concentrations (10 picomole) than FAM markers (5 picomole) to work well. We used Techne® PrimeG gradient thermal cycler (Techne, Cat. No: 5PRIMEG / 02) for amplification. The temperature profile of the PCR reaction consisted of an initial denaturation step at 95°C for 15 min followed by 10 cycles of denaturation at 93°C for 40 s, annealing 60°C for 40 s (decreasing 0.4°C per each cycle), and extension at 72°C for 80 s; 20 cycle of denaturation at 93°C for 30 s, annealing at 56°C for 40s, extension at 72°C for 80 s, followed by a final extension step at 60°C for 30 min.

For the fragment analysis, we used GeneScan™ 500 ROX™ dye Size Standard (Applied Biosystems™, Cat. No: 401734). First we diluted the size standard with Hi-Di™ Formamide (Cat. no. 4311320), 40 size standard to 100 formamide. Then we put 10 µl of diluted standard to each well and added 2-3 µl of PCR product. After that, we put the plate on the thermal cycler at 95°C for 3 min and then we immediately chilled the plate in -20 freezer for a few

minutes. Then we used 3130 Genetic Analyzer (Applied Biosystems™, Cat. No: 313001R) for the fragment analysis. Alleles were identified using Gene Mapper Software (version: 5.0).

Population genetic analyses were conducted using R version 3.5.1. Using the "*df2genind"* command (R package "*adegenet*"; Jombart 2008, Jombart and Ahmed 2011) we converted the data frame of genotypes to genind object. Then we tested the Hardy-Weingberg Equilibrium per populations by "*hw.test*" command (R package "*pegas*"; Paradise 2010). Then, we calculate the mean proportions of shared alleles by using the *propShared* function in *adegenet*, averaged per respective populations. We also calculated Nei's pairwise Fst (Nei 1983) between all pairs of populations by "pairwise.fst" command (R package "*hierfstat*", Goudet J and Jombart T 2015). Finally, we run Principle Component Analysis (PCA) by "*dudi.pca*" command (R package "*ade4*"; Chessel D et al. 2004, Dray and Dufour 2007, Bougeard and Dray 2018) and created heat map of the Bruvo distances (Bruvo et al. 2004) between individual microsatellite genotypes by "heatmap" command (R package "*stats*"). Bruvo genetic distance is a common way to calculate microsatellite genotype distances which consider mutation processes and allows comparison of individuals with different ploidy levels. While *d* is the total distance between two individuals and $|x|$ is the number of repeat units between the two alleles, calculation of the genetic distance between two alleles, *d*, is shown below.

$$d = 1 - 2^{-|x|} \qquad (2.1)$$

CHAPTER 3

RESULTS

3.1. ECOIMMUNOLOGY

3.1.1. Bacteria Killing Assay

Bacteria killing assay is not a species-specific method which measures the standing innate immune response by quantifying the antimicrobial capacity of serum. Incubation time for bacteria and serum dilutions optimized for this study. We observed log-phase growth at 10 hours of incubation at 37°C and used this for sample assays (see Fig. 3.1). Additionally, we selected 340 nm reading because it allowed for better resolution of our data. Serum dilution was determined to be 1:2 as this was the concentration at which 50% of bacteria were killed (Fig. 3.2).

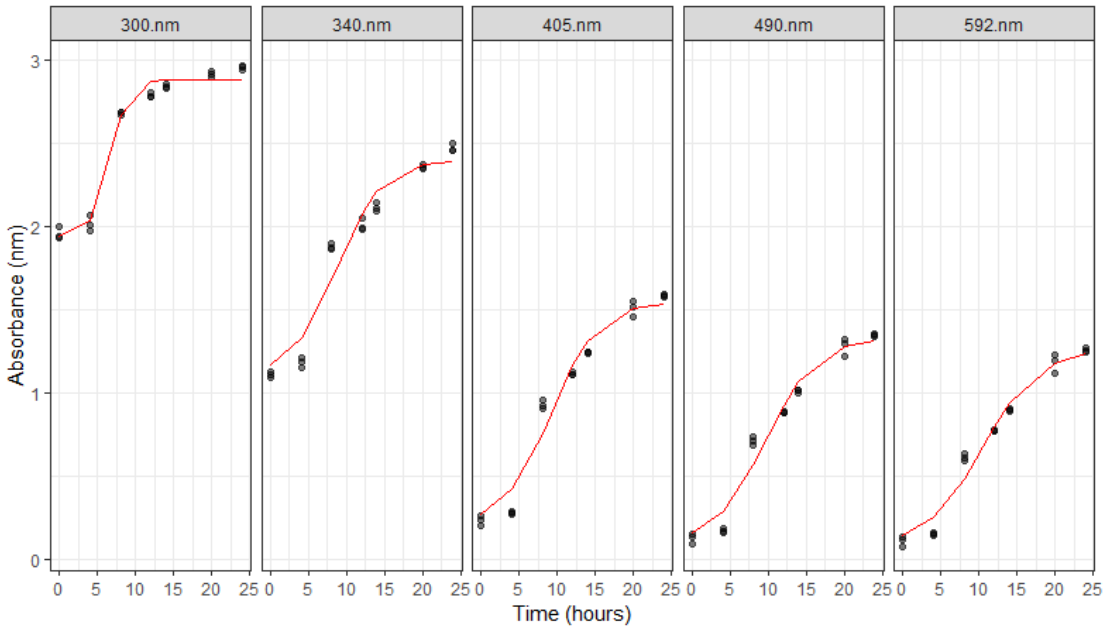

Figure 3.1 Bacterial growth for all absorbance filters by time (hours). The 300 nm and 340 nm absorbance filters were most effective for measuring microbial growth (i.e., a clear lag-phase, growth, and saturation curve were identifiable). The log phase (exponential) growth can be seen between 5 to 12 hours of incubation, and we selected 10 hours as our optimized incubation period. Red lines indicate predicted log-growth curves.

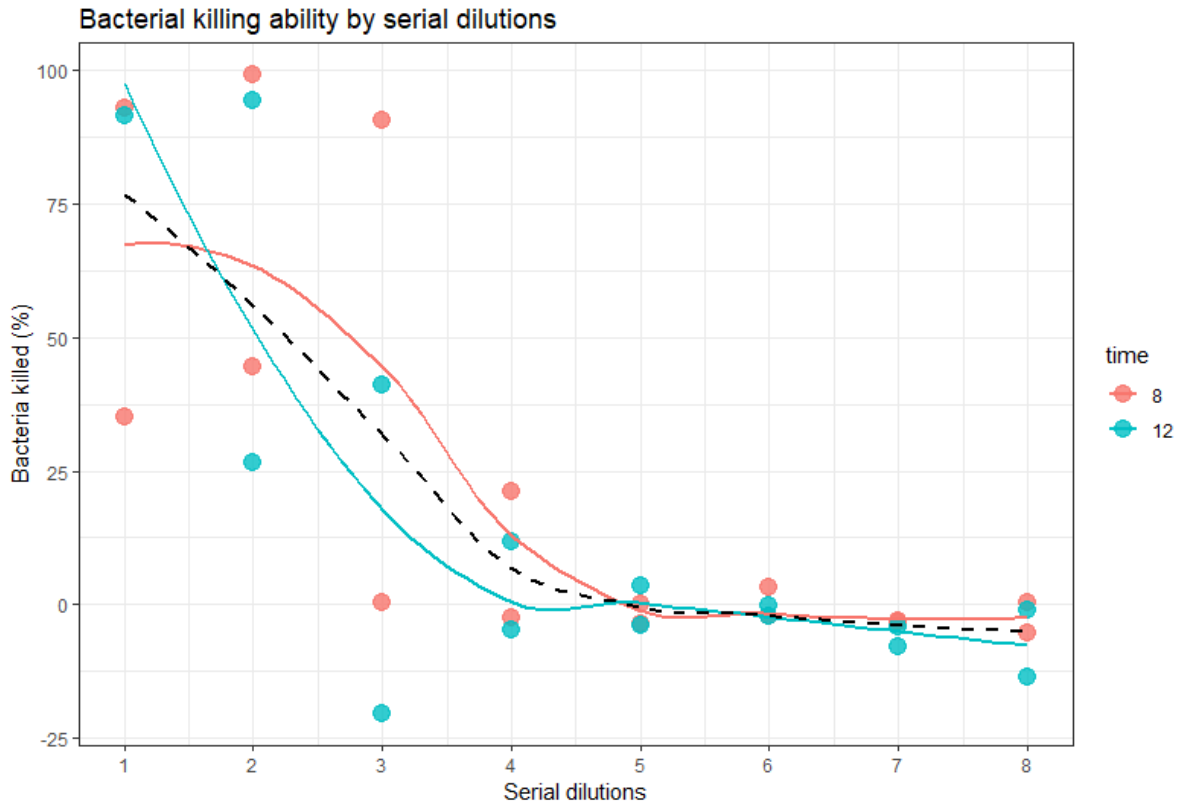

Figure 3.2 Bacteria killing ability by serum dilutions for 340nm (8 hours and 12 hours incubation). Serum dilution with 50% bacteria killing ability is optimal. Therefore, we choose the second dilution, which is closest to 50% and represents 1:2 dilution of serum. (1 = 1:1 dilution, or 2x; 2 = 1:2 dilution, or 3x, and so on). This would be a 3x dilution. (Curves are polynomial fit curves of certain growth ours).

After applying the model selection to the full dataset (with both sexes, 15 Female, 4 Male), we found that three models were within $\Delta AICc < 2$ (Table A1). The best model was BKA = intercept + altitude with a weight of 0.585 (Table 3.1). The other two models in the confidence set included the predictor time held (weight = 0.226) and altitude + time held (weight = 0.121).

Table 3.1 Best-fit general linear model coefficients for predictors of bacteria killing ability (BKA) of Anatolian blind mole rats, including both sexes (N=19, F=15, M=4).

Variable	**Estimate (SE)**	**t**	**P**
Intercept	25.33 (6.72)	3.77	0.002
Altitude (Low)	-27.50 (9.27)	-2.97	0.009
Adjusted R^2 = 0.303, F = (1,17) = 8.81, p = 0.009			

Bacteria killing ability was significantly greater at higher altitude than at lower altitude (GLM, low altitude estimate = -27.50 (SE=9.27), t= -2.97, p=0.009, Table 3.1, Fig. 3.3A). Other predictor variables were not significantly associated with BKA.

Model selection for female samples only (N=15) identified only one model within ΔAICc < 2 of the best model (Table A2). The best model for the entire dataset including males was qualitatively the same as for female-only models (BKA = intercept + altitude, weight = 0.579). This model also showed that bacteria killing ability was significantly greater at higher than lower altitude (GLM, low altitude estimate = -35.30 (SE=10.63), t= -3.32, p=0.006, Fig. 3.3B).

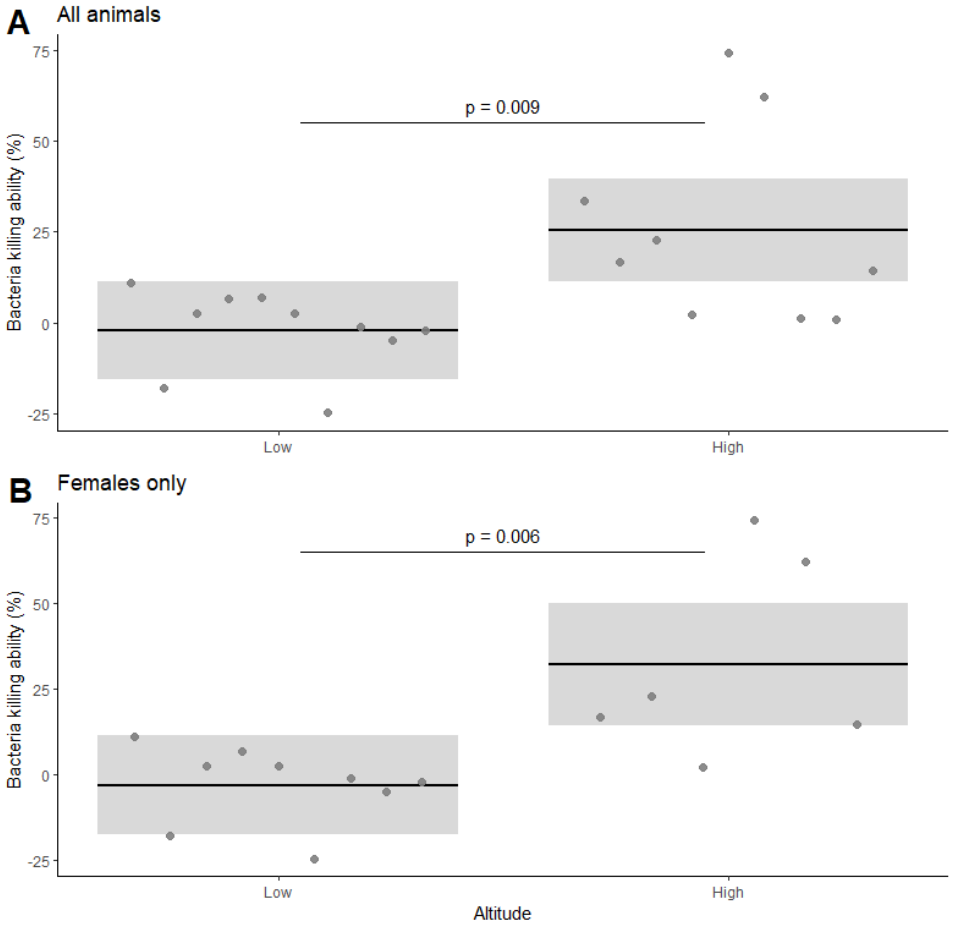

Figure 3.3 Partial regression plots of the effect of altitude on bacteria killing ability. (A) Bacteria killing ability by altitude for full dataset (N=19) and (B) for only females (N=15), from the lowest AICc model, BKA ~ Intercept + altitude. The negative BKA values stem from a known phenomenon that occurs when the bacteria grow faster in the samples than the controls due to extra nutrients provided by the serum (Tieleman et al, 2005; reviewed in Brooks and Mateo, 2013). Points represent the bacteria killing ability (%), the black line indicates predicted values from the model, and the grey area indicates 95% CI of the predicted values.

To explicitly test for an effect of stress on bacteria killing ability we added MCC as a covariate to the global model (excluding time held) predicting BKA and ran model selection for the full dataset and for females separately. Model selection for the full dataset (with both sexes, 15 Female, 4 Male) identified one model within ΔAICc < 2 of the best model (Table A.3), and the best model was again BKA ~ 1 + altitude with a weight = 0.512. Model selection for the just female dataset also identified only one model within ΔAICc < 2 of the best model (Table A.4), and the best model was again BKA ~ 1 + altitude with a weight = 0.634.

3.1.2. Stress (Corticosterone) Assay

Model selection for the full dataset (with both sexes, 15 Female, 4 Male) identified four models within ΔAICc < 2 of the best model and the best model was Mean Corticosterone Concentration (MCC) = Intercept, i.e. it did not include any predictor variables (Table A.5). Univariate models for altitude, sex, and time held were in the confidence set of models (ΔAICc < 2) in that order (weights = 0.191, 0.124, 0.105, respectively, Table A.5). However, none of these models were significantly better than the intercept-only model based on overall F-tests (all p>0.05). The regression plot for MCC by altitude is shown in Figure 3.4.

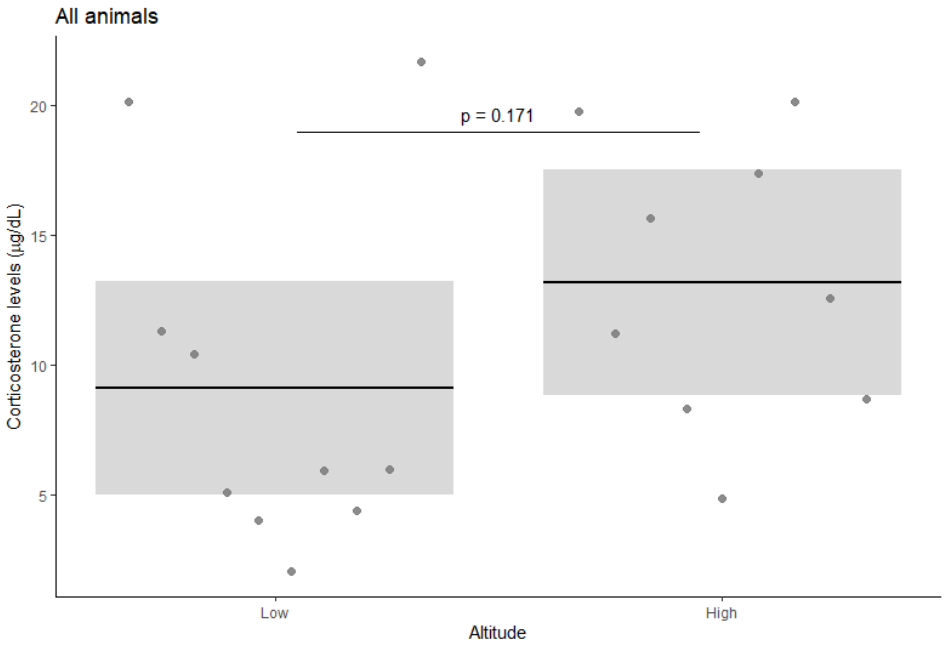

Figure 3.4. Partial regression plots of the effect of altitude on the mean corticosterone concentration (µg/dL) for both sexes (N=19), from the second-best model (MCC ~ Intercept + altitude). The effect of altitude on MCC is non-significant (p=0.171). Points represent the partial residuals, the black line indicates predicted values from the model, and the grey area indicates 95% CI of the predicted values.

Model selection applied to female samples only (N=15) identified only one model within ΔAICc < 2 of the best model and this model was also the intercept-only model with a weight = 0.514 (Table A.6).

3.1.3. Gastrointestinal Parasite Assay

Parasites are powerful agents of selection that can affect host birth and death rates. We assume that with increasing altitude parasite abundance and intensity should decrease. To test this hypothesis we measured the parasite load of individuals by populations. 14 of 19 individuals were found to be infected by gastrointestinal parasites. Morphological measurements were used to identify two major taxonomic groups of gastrointestinal parasites (Table 4): coccidian of genus *Eimeria* (Protozoa) and a parasitic roundworm most likely belonging to the genus *Strongyloides* (Nematoda). Coccidia dominated the infections, with a mean intensity score of 56.4 oocytes (Bootstrap BC_a CI 25.6 to 116) (Table 3.2). Since only one individual was found with nematode eggs, we focus on results from coccidia.

Table 3.2 Mean parasite prevalence, species richness (by taxonomic group), and parasite intensity for all Anatolian blind mole-rats (*Nannospalax xanthodon*) captured in 2017 and 2018 (N = 19). (Bootstrap BCa with 5000 replicates).

Parasite taxa	N	Prevalence (Clopper-Pearson CI)	Mean intensity (bootstrap Bca CI)	Mean abundance (bootstrap Bca CI)
Coccidian	14/19	73.7% (48.8 to 90.9)	56.4 (25.4 to 117)	41.6 (17.6 to 91.6)
Nematode	1/19	5.3% (0.1 to 26.0)	0.59	0.03 (0 to 0.09)

The prevalence of coccidia was high, at least 50% for each year and altitude category, and was not significantly greater at high than at low altitude (87.5% and 63%, respectively; low, N=11; high, N=8; Fisher's exact test 2-sided p= 0.338). The prevalence in 2017 samples was greater than in 2018 samples (100% and 54%, respectively; 2017, N=8; 2018, N=11; Fisher's exact test 2-sided p= 0.045, Fig. 6A). We found no significant difference in prevalence by sex (70 % and 33.3%, respectively; F, N=10; M, N=3; Fisher's exact test 2-sided p= 0.511). See Table 3.3 for parasite prevelance, mean intensity and mean abundance by year and altitude.

Table 3.3 Parasite (coccidian and nematode) parameters measured for each sampling site and year. (Bootstrap BCa with 5000 replicates). For definitions of parasitism indices, see Table 3.2.

Altitude	Year	N	Prevalence (Clopper-Pearson CI)	Mean intensity (bootstrap Bca CI)	Mean abundance (bootstrap Bca CI)
Low	2017	3	100% (29 to 100)	65.60 (33.70 to 96.10)	65.60 (33.70 to 125)
	2018	8	50% (16 to 84)	8.19 (3.39 to 14.70)	4.10 (1.21 to 9.51)
	Total	11	63% (30 to 89)	32.80 (13.20 to 82.80)	20.90 (7.10 to 58.50)
High	2017	5	100% (47 to 100)	110 (28.50 to 222)	110 (28.10 to 223)
	2018	3	66% (9 to 99)	14.60 (10.90 to 14.60)	9.75 (0 to 15.90)
	Total	8	87% (47 to 99)	83.10 (26.30 to 180)	72.70 (21.90 to 161)
Total		19	73% (49 to 91)	58 (28 to 119)	42.70 (19.40 to 91.50)

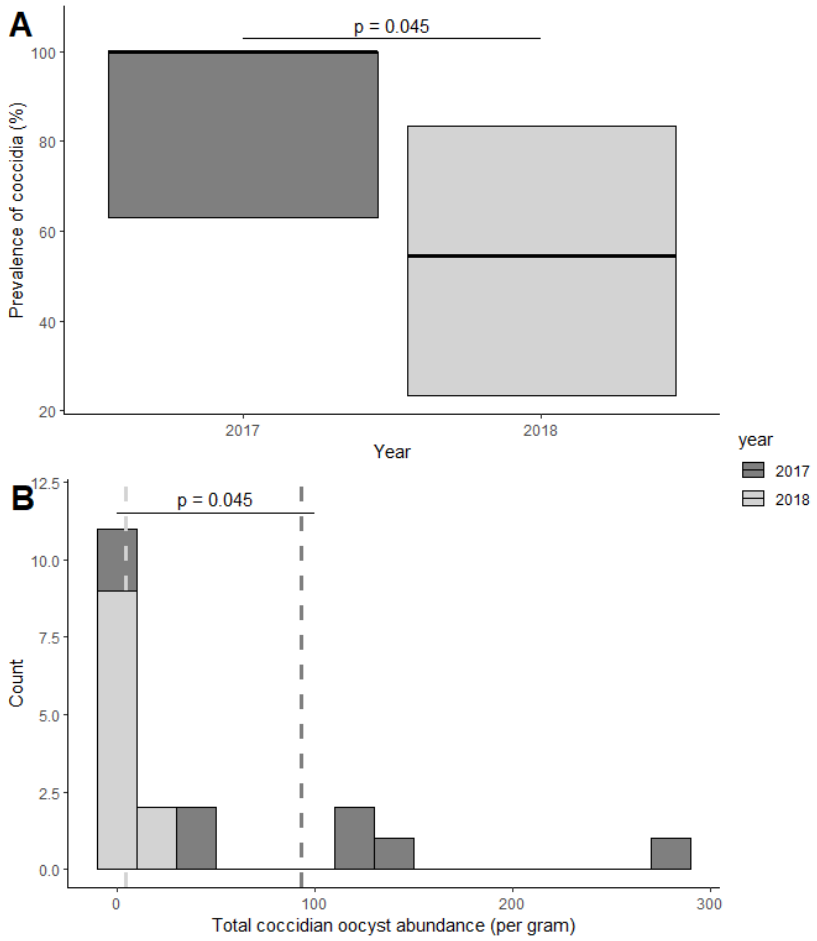

Figure 3.5 Coccidian parasitism measures in Anatolian blind mole-rats by year. **(A)** The prevalence of coccidian is significantly higher in October 2017 than June 2018 (Fisher's exact test 2-sided p= 0.045). Boxes represent the 95% Clopper-Pearson CI and black lines indicate the observed prevalence. **(B)** Coccidian abundance is also significantly greater in October 2017 than June 2018 (bootstrap t-test with 5000 replications p = 0.045). Dotted vertical lines indicate the mean coccidian abundance per year. 2017 and 2018 year samples sizes are N=8 and N=11, respectively.

Coccidian intensity was not significantly different between years and altitudes (Table A.7 and A.8 for results from statistical tests). However, coccidian abundance was significantly greater in 2017 than 2018 (2017, N=8, mean (SD) = 93.22(95.65); 2018, N=11, mean (SD) = 3.99(6.08); bootstrap t-test with 5000 replications p = 0.045, Fig. 3.5B). We could not compare the intensity between the sexes because when we excluded animals with missing values, there was only one male infected. Coccidian abundance was also not significantly different between the sexes (F, N=10, mean (SD) = 48.33 (91.57); M, N=3, mean (SD) = 41.74 (72.29); bootstrap t-test with 5000 replications p= 0.901).

3.2. POPULATION GENETIC STRUCTURE

We found greater immune response in high altitude populations. Functional differences between populations may also be caused by the long time spent in isolation without gene flow. To estimate, the amount of neutral divergence and/or connectivity between populations, we genotyped our samples at eight nuclear microsatellite genetic markers.

A total of 48 ABMR samples were genotyped at 8 loci previously reported by Popa et al. 2014, with 26.6 % of missing data. We also included the distant populations from Georgia (eight individuals) and Gerede (two individuals) to compare the polymorphisms at two different geographic scales. Except for SL49, all of the loci worked well in our study, and SL49 was excluded from all analyses. The sizes of loci were similar to those previously recorded, the number of alleles per locus was slightly different from Popa et al. 2014 (values calculated including Georgian and Gerede samples, see Table 3.4). The number of alleles per locus ranged from 4 (SL 96) to 12 (SL 88).

Table 3.4 Eight fluorescent microsatellite markers with sequences and fluorescent modifications (described in Popa et. al. 2014). NA: No. Alleles.

Locus	Direction	Primer sequence (5' - 3')	modification on 5` end	Size bp (Popa et al. 2010 / current study)	No. Alleles (Popa et al. 2010 /current study)
SL18	F	tgttcatggctatagaaagcaaa	6FAM	165/189	8/10
	R	ggtcaaccgttgggatttta			
SL49	F	gtacctcgcattctggaaca	HEX	242/NA	12/NA
	R	tgacatcatgagctccttgg			
SL53	F	tggcctctaatctaccctca	6FAM	248/249	9/11
	R	ttccaggatctttggaactg			
SL98	F	caacgggttctctgaggttc	HEX	148/154	9/5
	R	tttgtcgtgtctttccattcc			
SL48	F	ccagtaaggaaacatgaccca	HEX	193/185	7/6
	R	cctgtgtttattgcatgagagtg			
SL51	F	tcaatccctcacaaccatcc	6FAM	205/202	11/11
	R	ctcccttgtttcccatgact			
SL88	F	ccctgtgtctgaggggagat	6FAM	146/141	10/12
	R	ggcatgataagcattgtgga			
SL96	F	ttggtctgcaacaccacata	HEX	127/108	8/4
	R	agcctgcttatgttcctcca			

The observed heterozygosity ranged from 0.00 to 1.00 while the expected heterozygosity ranged from 0.00 to 0.86. Hardy-Weinberg test was not significant in almost all populations by every locus, means these populations not in Hardy-Weinberg equilibrium (HWE) or it is not possible to detect deviation from HWE due to small sample sizes, but Eregli 2 had significant p value for all loci (except SL18) plus Medetsiz had significant p value for two loci (SL18 and SL96) and Karagöl had significant p value for one locus (SL18). Hardy-Weinberg test, observed and expected heterozygosity were not calculated for Gerede and Georgian 2 populations due to low sample sizes (Table 3.5). Observed heterozygosity was usually smaller than expected heterozygosity.

Table 3.5. Observed, expected heterozygosity and test for Hardy-Weinberg Equilibrium by population. N: Sample Size; H_o: Observed Heterozygosity; H_e: Expected Heterozygosity; H_p: p value for Hardy–Weinberg equilibrium test; *: $p < .05$.

Population	Altitude	N		SL18	SL51	SL88	SL96	SL98	SL53	SL48
Eregli1	Low	3	Ho	0.50	1.00	0.50	0.00	0.67	0.00	0.00
			He	0.62	0.72	0.44	0.50	0.61	0.50	0.00
			Hp	0.334	0.197	NA	0.333	1.000	0.326	1.000
Eregli2	Low	12	Ho	0.42	0.55	0.33	0.00	0.89	0.38	0.00
			He	0.61	0.80	0.50	0.48	0.62	0.68	0.57
			Hp	NA	0.000*	0.455	0.002*	0.006*	0.001*	0.000*
Ulukisla	Low	5	Ho	0.80	0.40	0.00	0.00	1.00	0.20	0.20
			He	0.74	0.58	0.00	0.00	0.70	0.54	0.18
			Hp	0.466	0.364	1.000	1.000	0.664	0.056	1.000
Madenkoy	Middle	8	Ho	0.71	0.60	0.17	0.00	0.00	0.50	0.50
			He	0.67	0.80	0.68	0.41	0.64	0.59	0.62
			Hp	0.487	0.157	0.003*	0.026*	0.003*	1.000	0.324
Karagöl	High	12	Ho	0.50	0.50	0.88	0.00	0.56	0.25	0.17
			He	0.77	0.79	0.80	0.00	0.64	0.73	0.15
			Hp	0.015*	0.171	NA	1.000	0.630	0.520	0.820
Medetsiz	High	8	Ho	0.14	0.57	0.67	0.00	0.40	0.00	0.17
			He	0.54	0.80	0.83	0.44	0.56	0.00	0.17
			Hp	0.020*	NA	0.090	0.014*	0.135	1.000	NA
Gerede	Gerede	2					NA			
Georgian1	Georgian1	6	Ho	0.17	1.00	1.00	0.00	0.25	0.50	0.00
			He	0.49	0.00	0.50	0.00	0.22	0.65	0.00
			Hp	0.150	NA	0.093	1.000	1.000	0.025*	1.000
Georgian2	Georgian2	2	Ho				NA			

F_{st} is one of the fundamental model-based statistics and measures differences between populations and M_{spa} is a basic statistic that computes the proportion of shared alleles in a set of genotypes. F_{st} values ranged from -0.371 (Georgian1 and Georgian2; Georgian2 population has just two samples) to 0.340 (Georgian1 and Madenköy) and indicates stronger divergence between Georgian1 and Madenköy populations. M_{spa} values ranged from 0.00 to 0.76 (Georgian1 and Georgian2) indicates that there is a high shared proportion of alleles between Georgian populations compared to other pairs (see Table 3.6 and 3.7). The current data show that populations from different altitudes do not differ genetically, according to F_{st} and M_{spa} values, however, there is a clear distinction between Anatolian and Georgian samples, as

expected due to geographical and phylogenetic distance between them. The principle component analysis confirms the same pattern as well as the heatmap (Fig. 3.6 and Fig. 3.7).

Table 3.6 A: Pairwise F_{st} and **B:** mean of the shared proportions of alleles between populations. Negative F_{st} values (effectively seen as zero) indicate that there is no detectable genetic difference between the populations considered.

A	Eregli1	Eregli2	Georgian1	Georgian2	Gerede	Karagol	Madenkoy	Medetsiz	Ulukisla
Eregli1	0.000								
Eregli2	0.079	0.000							
Georgian1	0.427	0.346	0.000						
Georgian2	-0.026	0.062	-0.371	0.000					
Gerede	-0.179	-0.046	-0.030	-0.366	0.000				
Karagol	0.080	0.106	0.372	0.050	-0.066	0.000			
Madenkoy	0.099	0.061	0.330	0.050	-0.036	0.075	0.000		
Medetsiz	0.199	0.155	0.467	-0.011	-0.115	0.134	0.138	0.000	
Ulukisla	0.249	0.119	0.519	0.109	-0.240	0.128	0.170	0.266	0.000

B	Eregli1	Eregli2	Georgian1	Georgian2	Gerede	Karagol	Madenkoy	Medetsiz	Ulukisla
Eregli1	0.000								
Eregli2	0.372	0.000							
Georgian1	0.000	0.000	0.000						
Georgian2	0.000	0.000	0.763	0.000					
Gerede	0.222	0.444	0.111	0.000	0.000				
Karagol	0.257	0.316	0.003	0.000	0.371	0.000			
Madenkoy	0.272	0.333	0.008	0.000	0.276	0.328	0.000		
Medetsiz	0.157	0.355	0.000	0.000	0.312	0.280	0.227	0.000	
Ulukisla	0.210	0.374	0.010	0.000	0.316	0.372	0.335	0.311	0.000

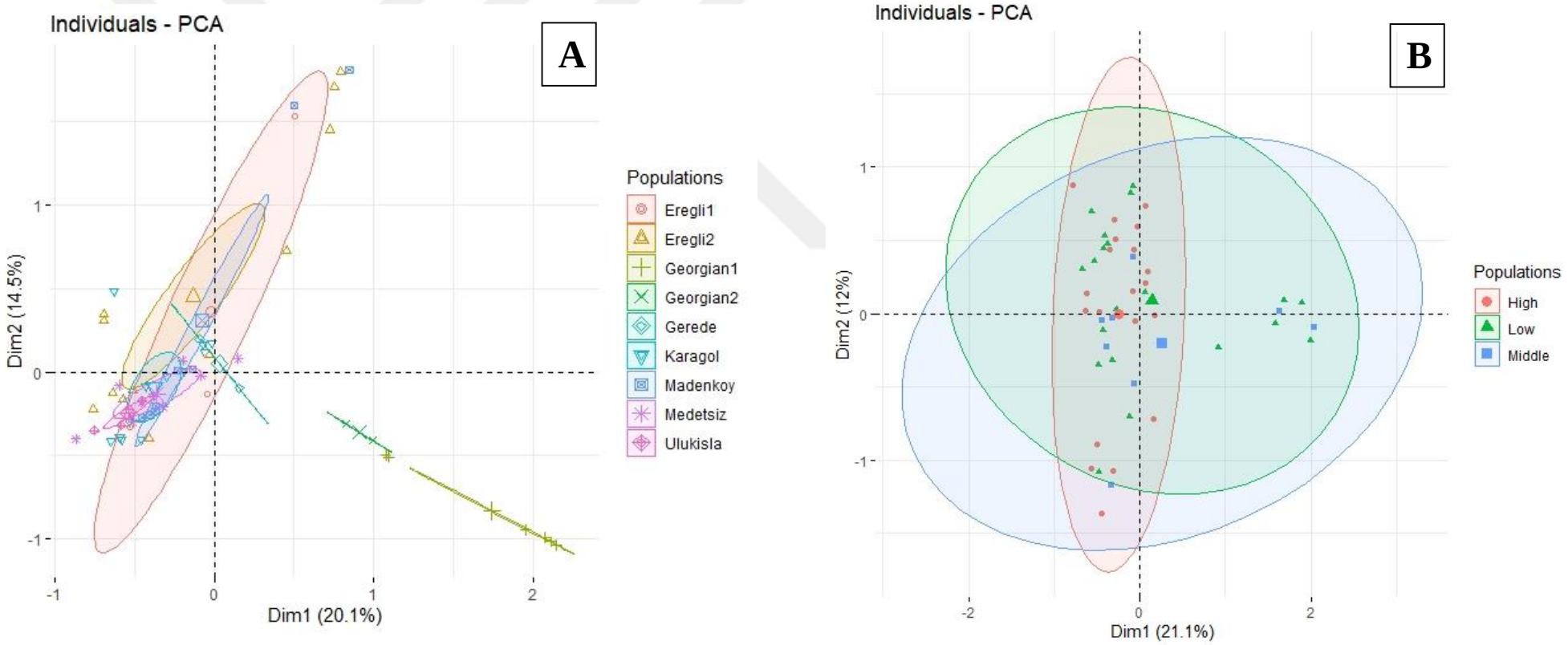

Figure 3.6 Principal Component Analysis (PCA) performed on individual microsatellite genotypes **A:** for the complete dataset (including Gerede and Georgia) and **B:** just altitudinal populations. Ellipses are drawn according to confidence ellipses around group mean points. The proportion of variance explained by the first two PC axes is given in brackets.

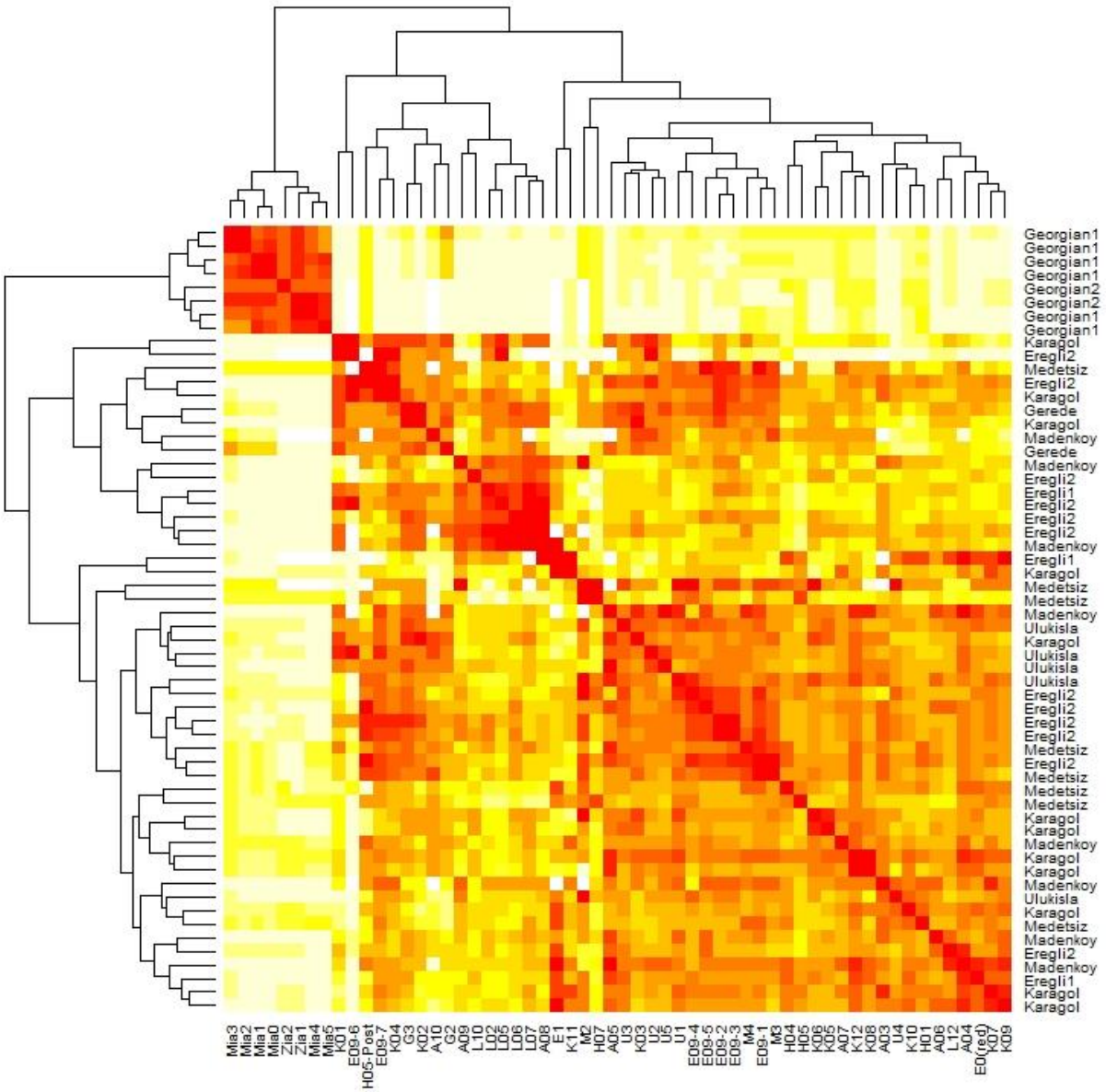

Figure 3.7 The heat map of Bruvo distances between individual genotypes shows clear difference between Georgian and Anatolian samples. However, there is no clear pattern between populations by altitude.

Table 3.7 A: Pairwise F_{st} and **B:** mean of the shared proportions of alleles between the altitudinal populations.

A	High	Low	Middle
High	0		
Low	0.069	0	
Middle	0.052	0.054	0

B	High	Low	Middle
High	0		
Low	0.312	0	
Middle	0.313	0.323	0

CHAPTER 4

DISCUSSION

In this study, we compared the effect of altitude on standing innate immune defense and stress levels, as well as on prevalence, abundance, and intensity of gastrointestinal parasites and neutral genetic diversity between populations of ABMR. We found that (i) standing innate immunity is greater in the high altitude mole-rat population than in the low altitude populations, (ii) neither stress (as measured by corticosterone concentration) nor (iii) gastro-intestinal parasite measurements differed by altitude and (iii) there is no significant genetic difference between altitudinal populations of ABMR.

4.1. INNATE IMMUNE RESPONSE VS ALTITUDE

Having a greater innate immune response at the high altitude can be explained by the high cost of infection on survival at high altitude. Simple infections may cause more serious damage in harsher mountain conditions, leading to stronger selection pressure for pathogen resistance. Thus, high altitude populations may need to make a greater investment into innate standing immune defense. Altitude effects on immunity have been of interest for over 60 years (reviewed in Richard and Meehan 1987, Eisen et al. 2013). However, previous studies have almost exclusively focused on humans and lab animals and on changes in the immune function of subjects *exposed* to high altitude, not *adapted* to high altitude. Our current study makes an important contribution to the fields of alpine ecology and ecoimmunology by investigating potential immune adaptations to altitude in wild populations. Being able to predict environmental correlates of immune response will be important to mitigate effects of ongoing climate-mediated shifts in pathogen ranges (Altizer et al. 2013).

Immune responses can differ by sex (Klein and Flanagan 2016, Jaillon et al. 2017, but see Kelley et al. 2018 for a recent meta-analysis). In our dataset, the sexes were unequally represented (F=15, M=4). This is simply due to the fact that collecting live female TMBRs is

much easier in the field than collecting males (see Sözen, 2005 for an example of high female to male ratio in a large sample). This prevented us from testing for an effect of sex independent of altitude on the innate immune response in Anatolian blind mole-rats. However, we could show that bacteria killing ability is significantly higher at higher altitude both for the entire dataset (Fig. 3.3A) and when only females are considered (Fig. 3.3B). Therefore, our main result appears robust despite the possibility of an effect of sex on the immune response. While we acknowledge that our sample size is small, the use of approximately eight replicates per category is common for immunological studies using non-model organisms (Gardner et al. 2018, cane toad, N=9 per group, total 27; Gao and Deviche 2018, house sparrow, N=10 per group, total 20; Georgiev et al. 2015, rhesus macaques, N=10-15 per immunological test; Matson et al. 2012, homing pigeons, N=8 per group, total 16; Moeller et al. 2017, Gila monster, N=3 females, 5 males, 8 total; Neuman-Lee et al. 2019, common gartersnake, N=22).

4.2. STRESS

Stress has previously been shown to augment immune activity (reviewed by Martin 2009), so we wanted to control for its potential confounding effect in our study. According to the assay protocol we used, all animals were in the natural range of corticosterone concentration (1.68 – 21.95 μg/dL, Fig. 5). We found no significant effect of altitude, mass, sex, or time held in captivity on mean corticosterone concentration. MCC was positively correlated with time held, though not significantly so (Spearman's rho = 0.208, p=0.393, N=19). It is thus not surprising that including MCC as a potential covariate in BKA model selection did not influence the confidence set of models. One explanation for the lack of relationship between corticosterone concentration and BKA and time held is that mole-rats may simply produce low levels of glucocorticoid after acute stress. There is evidence that fossorial rodents, living in relatively stable microclimatic conditions, are adapted to an environment with fewer extreme external stressors and are thus less reactive to stress (Moshkin et al. 2002).

4.3. GASTROINTESTINAL PARASITES

Increasing altitude is generally known to decrease abundance, mortality and transmission of pathogens (LaPointe et al. 2010, Zanet et al. 2017, van Riper et al. 1986, Michel et al. 2014). While some studies have found positive correlations between pathogen (plague and viruses)

prevalence in rodent hosts and altitude (Tadin et al. 2014, Tollenaere et al. 2010, Tollenaere et al. 2008, Tollenaere et al. 2011), other studies have reported no correlation between altitude and ectoparasite (flea) abundance but found positive correlations between the diversity of ectoparasites and altitude (Moore et al. 2015). In our study, we also found no relationship between gastrointestinal parasite prevalence, abundance and intensity, and altitude. This may be partly due to the fact that fecal examinations have significantly lower detection ability of gastrointestinal parasites compared to direct intestine examination (Jorge et al 2013), or to PCR based screening methods (Morgan et al. 1998). In addition, the localization of parasites in host organs and the randomness of the presence of parasites in feces are important factors that we could not address (Jorge et al 2013).

4.3. POPULATION GENETIC STRUCTURE

In our microsatellite results, fragment sizes and the number of alleles per locus were similar to Popa et al. 2010. This indicates that we amplified the same microsatellite loci in Anatolian and in Georgian TMBR samples. We found large genetic differences between the Georgian and the Anatolian samples. However, we could not see any significant genetic neutral difference between the ABMR populations by altitude.

Observed heterozygosity was almost always lower than expected heterozygosity (see Table 3.5). This could be caused by the Wahlund Effect which is a case of reduction of heterozygosity in a metapopulation caused by geographical structure. However, we calculated the values for each local population separately. On the other hand, lower heterozygosity can also be caused by inbreeding. This option can be more realistic since ABMR has very low dispersal ability (Nevo et al. 1999 and 2000). Though high proportion of missing alleles and non-significant HWE tests suggest that null alleles might be present. Null microsatellite alleles are an allele at a microsatellite locus that does not amplify to detectable levels in a polymerase chain reaction test. Therefore, a null alele apears as a missing data point causing the bias in the population genetic analysis. As mentioned before, these primers designed for *Nannospalax leucodon* and these primer binding regions maybe different in *Nannospalax xanthodon*.

Nannospalax genus is known for numerous variations in the number of chromosomes, which is mostly caused by the Robertsonian translocations. In our study, we see that there is a large difference between Georgian (2N=50) chromosomal race *nehringi*) and Anatolian (2N=58, from Ereğli-Niğde area, see Figure 2.1) chromosomal race *cilicicus* according to Arslan et al. 2016. Hadid et al. 2012 reported that 2N=50 from Eastern Turkey (same chromosomal race found in Georgia) and 2N=60 (which is separated from 2N=58, used in our study, by one Robertsonian fusion, see Arslan et al. 2016) belong to phylogenetically distant clades. Our results are in line with this finding, indicated by large genetic differences between Georgian (2N=50) and Anatolian (2N=58) samples.

Conclusion: Our initial hypothesis was based on the assumptions that parasite abundance is higher in lower altitude environments, and that investment into immune defense correlates positively with the risk of infection. Thus, we expected to see greater standing innate immunity in the low altitude populations. However, the results of the bacteria killing assay showed that contrary to our expectations, high altitude animals showed greater standing innate immunity, presumably because there is higher cost of repeated or sustained infection at high altitude. This could be mediated by relatively greater energy costs from repeated or prolonged immune activation at high altitudes, as basal metabolic rate and energy expenditure can increase between 17-27% at high altitude (4300m, Butterfield et al. 1992). In addition to increasing metabolic rate, high altitude environments are generally colder than low altitude environments and have lower available oxygen, which contributes to greater thermoregulatory demands and lower capacity for sustained thermogenesis. Just as these factors can select for increased thermogenetic capacity at high altitude (~3800 m) in deer mice (Hayes and O'Connor 1999), they could also select for increased immune system efficacy. One point to consider is that adaptations to tolerate hypoxic stress underground are shared among subterranean rodents (Lewis et al. 2016). Therefore, differences in oxygen availability between the altitudes we sampled may be physiologically equivalent to mole-rats adapted to hypoxic conditions. In other words, reduced oxygen, a key altitudinal stressor, may not be much of a stressor in our study system.

The immune defense component model (Schmid-Hempel and Ebert 2003) separates the immune system by two intersecting axes: one a continuum of specific to non-specific defense, the other a continuum of constantly maintained (constitutive) defenses to (induced) defenses deployed in response to activation. In this study, we only focused on constitutive innate

immunity. However, because of the complexity of the immune system, it is suggested that multiple immune indices are measured to more fully understand which components are contributing most to an organism's immune response (Sheldon and Verhulst 1996, Schmid-Hempel and Ebert 2003, Lee 2006; Martin et al. 2008, Demas et al. 2011). To better understand the complex relationship between altitude and the immune system, we provide some general recommendations for future research. Sampling across altitudinal gradients rather than binary low and high altitude categories, and sampling multiple species will provide more power for testing immune system adaptations to altitude (e.g. Sun et al. 2018, for identifying genetic adaptations to high altitude). In addition, recording climatic conditions (e.g. temperature, humidity) may be helpful for teasing apart mechanisms. Surveying multiple types of parasites, such as ectoparasites, blood protozoa, and viruses could reveal stronger links between parasitism and altitude. Common garden experiments, where animals adapted to higher and lower altitude environments are brought to the laboratory for concurrent study, will be important to confirm that differences in immune response traits are genetically-based instead of environmentally-dependent. In addition, we stress the need to measure multiple measures of immune function, or even whole organism traits such as host competence, tolerance, or resistance (e.g. Martin et al. 2016, Burgan et al. 2019), realistically accomplished in the field by measuring fitness indices before and after parasite removal and immune challenge experiments. Finally, it is important to validate assays for non-model study systems being investigated.

In this study, we found that the amount of neutral genetic differentiation between the altitudes as well as between local populations is too small to be detected by the given microsatellite markers and our sample sizes. The evolutionary time since the split of these local populations could be too short to result in substantial neutral divergence, or our sample sizes were insufficent to detect weak population structure. At the same time we found significant functional differences in immune response between the altitudes. These results could suggest that functional differences have been caused by strong natural selection for adaptation to the mountain environment.

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APPENDICES

Appendix A: Supplementary Tables

Table A.1 Top set of general linear models for bacteria killing ability (BKA). Models represent the full dataset including both sexes (N=19, F=15, M=4). All models within Δ2 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	BKA_340nm ~ 1 + altitude	0.00	0.314	17	0.303
2	BKA_340nm ~ 1 + time_held	0.66	0.226	17	0.278
3	BKA_340nm ~ 1 + altitude + time_held	1.91	0.121	16	0.309

Table A.2 Top set of general linear models for bacteria killing ability (BKA). Models represent the dataset including only female samples (N=15). All models within Δ2 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	BKA ~ 1 + altitude	0.00	0.579	13	0.417

Table A.3. Top set of general linear models for bacteria killing ability (BKA) with MCC variable as a covariate. Models represent the full dataset including both sexes (N=19, F=15, M=4). All models within Δ2 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	BKA ~ 1 + altitude	0.00	0.512	17	0.303

Table A.4 Top set of general linear models for bacteria killing ability (BKA) with MCC variable. Models represent the full dataset including only female samples (N=15). All models within Δ4 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	BKA ~ 1 + altitude	0.00	0.634	13	0.417

Table A.5 Top set of general linear models for mean corticosterone concentrations (MCC). Models represent the full dataset including both sexes (N=19, F=15, M=4). All models within Δ2 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	MCC ~ 1	0.00	0.270	18	NA
2	MCC ~ 1 + altitude	0.69	0.191	17	0.055
3	MCC ~ 1 + sex	1.55	0.124	17	0.011
4	MCC ~ 1 + time_held	1.88	0.105	17	-0.006

Table A.6 Top set of general linear models for mean corticosterone concentrations (MCC). Models represent the dataset including only female samples (N=15). All models within Δ2 AICc of the top model are shown.

#	Models	ΔAICc Values	Weights	Df	R^2_{adj}
1	MCC ~ 1	0.00	0.514	14	NA

Table A. 7 Comparisons of coccidian parasitism measures in Anatolian blind mole-rats by year. Statistical results are provided for each test.

Group	Infecte	Total	Prevalence	Mean intensity (SD)	Mean abundance (SD)
2017	8	8	100.0%	93.22 (95.65)	93.22 (95.65)
2018	6	11	54.5%	7.33 (6.68)	3.99 (6.08)

Statistical tests for year differences:	Two-sided with p = 0.045	Bootstrap t-test with 5000 replications, p = 0.055	Bootstrap t-test 5000 replications, p = 0.045

Table A.8 Comparisons of coccidian parasitism measures in Anatolian blind mole-rats by altitude. Statistical results are provided for each test.

Group	Infected	Total	Prevalence	Mean intensity (SD)	Mean abundance (SD)
Low altitude	7	11	63.6%	30.01 (44.60)	19.09 (37.22)
High altitude	7	8	87.5%	82.81 (106.40)	72.46 (102.70)

Statistical tests for year differences:	Two-sided p = 0.338	Bootstrap t-test with 5000 replications, p = 0.260	Bootstrap t-test with 5000 replications, p = 0.218

Appendix B: Field Trip Photos

Figure A.1 Field Team left to right (Ferhat Matur, Alexey Yanchukov, Jamie Winternitz, Faruk Çolak, Halil Mert Solak).

Figure A.2 *Nannospalax xanthodon* from Medetsiztepe.

Figure A.3 One of the high altitude population, Medetsiztepe, around 3000 meters above sea level.

Figure A.4 One of the low altitude population, Ereğli 2, around 1200 meters above sea level (Halil Mert Solak on the right).

CURRICULUM VITAE

Halil Mert SOLAK was born in 1994 in İzmit, Kocaeli, Turkey. He graduated from primary school in Gölcük, Kocaeli. After he graduated from Atatürk Anatolia High School in 2012, he started BSc education at Biology Department of Bolu Abant İzzet Baysal University. After he completed BSc in 2017, he started MSc education at Biology Department of Graduate School of Science, Engineering, and Technology of Bülent Ecevit University.

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