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Adaptive physiological traits of distinct broad-toothed field mouse populations as guidelines for conservation

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Running Head: Adaptive physiological traits of wood mouse
Abstract

Species and Evolutionarily Significant Units (ESU) recognitions have been increasingly based on molecular data, while the identification of ecologically meaningful phenotypic variations that can separate biological entities has been largely neglected. We suggest that in lieu of global environmental changes, adaptive physiological traits, especially those related to arid survival, should be identified and used to better differentiate between species and subspecies. We used the broad-toothed mouse (*Apodemus mystacinus*) as a model species to examine the concept of different biological entities that may be manifest within the same species. We examined the water restriction abilities of two populations of *A. mystacinus*, separated by the Great Rift Valley, under short and long day photoperiod regimes. The two allopatric populations exhibit opposing physiological traits in response to simulated aridity conditions. Under both photoperiod regimes, increased urine production was followed in the high-altitude inhabiting “Hermon” population by a decreased urine osmolarity, whereas in the low-altitude “Carmel” population it was followed by an increased urine osmolarity. In a response to increasing dietary salt loads, the Carmel group increased whilst the Hermon group decreased their urinary sodium and chloride concentrations. Potassium levels of both groups increased during winter, but remained stable during summer, in a response to increasing dietary salt loads. The results show that two populations of woodmouse in the eastern Mediterranean exhibit striking physiological differences related to aridity adaptation, which typify and separate them. We suggest that the case of the woodmouse should set an example to a functional approach to the designation of an ESU. Under fast global changes, it is
expected that protecting the differential physiological traits, exhibited in the two opposing populations, will help the species subsist.

Key words: adaptation, allopatric, ESU, salinity, urine, woodmouse
Introduction

The increasing threats of habitat destruction (Brook, Sodhi & Ng, 2003) and global warming (Thomas et al., 2004) to species’ survival calls for the identification of the patterns of diversity within species (UNEP, 1992). Such recognition will facilitate the protection of biodiversity and the preservation of the evolutionary process. Identifying Evolutionarily Significant Units (ESUs) may be an important step towards this goal, as the importance of ESUs to conservation is widely recognized (Moritz, 1994; Deweerdt, 2002; Ryan, 2006). Adaptive variation in genetic and phenotypic traits has been widely used to define ESUs (Crandall et al., 2000).

However, whilst neutral markers such as molecular determinants may provide insights to the path of evolutionary processes, they do not assist in understanding the adaptive mechanisms of evolutionary change, and thus limit the ecological interpretations underlying the ESU. The identification of species and ESUs has been increasingly based on molecular data (e.g. Holyoake, Waldman & Gemmell, 2001; Wilson et al., 2005) while identifying intra-specific ecologically meaningful variations has been largely neglected. Some authors have suggested that ESUs can be used regardless of adaptive features (see Dimmick et al., 2001; Young, 2001), but adaptation remains an important feature to distinguish between biological entities. Recently Green (2005) has suggested a new term, Designatable Unit (DU) to provide a better definition of which biological entities should receive high priority for protection. This new approach was developed since the extensive definitions for ESU as well as the definitions of species failed to provide practical guidelines for conservation policy. Green’s definition couples previous criteria with conservation status. However, it is not clear how the conservation status is decided upon, as
current conservation status is often determined only after distinguishing one biological entity from the other. Using adaptive physiological traits as tools for distinguishing ESUs or DUs can overcome this obstacle because it is the phenotypes of physiological traits that can affect the performance of species and subspecies during environmental changes (Carey, 2005).

The woodmouse (genus Apodemus) has been the subject of numerous phylogeographic studies, mostly based on molecular markers, resulting in the recognition of new species and subspecies (Filippucci, Macholan & Michaux, 2002; Michaux et al., 2002; Serizawa et al., 2002; Michaux et al., 2003; Bellinvia, 2004; Liu et al., 2004). As a non-commensal murid, this genus provides a good model for studying the inter- and intraspecific radiations of populations and their apparent physiological adaptations. We therefore used the broad-toothed mouse (Apodemus mystacinus, Danford & Alston 1877) as a model species to examine the concept of different biological entities that may be manifest within the same species. We argue that the two studied populations are distinct and that each may have a unique evolutionary course, especially in lieu of the current global changes. A. mystacinus inhabits broad leaves forests (mainly oak) from Asia minor to Iraq, Israel being its southern range limit in the east Mediterranean (Harrison & Bates, 1991).

We identified two populations of A. mystacinus that are separated geographically by the African Great Rift Valley. We hypothesize that one population, originating from the cool, dry and high altitude environment of Mount Hermon would exhibit superior physiological adaptations to conditions of water stress than another population originating from a relatively mild, mesic,
lower-altitude environment of Mount Carmel. We also hypothesized that both populations would show photoperiod-induced differences in their physiological responses.

Materials and Methods

Animals: 16 individuals of broad-toothed mouse (A. mystacinus) were trapped in Mount Hermon (1600 above sea level, 35°46’ E 33°17’ N, a sub-alpine population), and 14 individuals were trapped on Mount Carmel (200 above sea level, 32°43’ E 34°58’ N, a Mediterranean population) during 2001-2002. The mice were brought into the laboratory of the Department of Biology, University of Haifa-Oranim and were housed in individual cages (approx. 35 x 25 x 15 cm) with sawdust as bedding. They were offered rodent chow (21% crude protein, 4% crude fat, 4% cellulose, 13% moisture, 7% ash, 18.7 kJg⁻¹ gross energy; Koffolk, Israel) ad libitum and agar gel (20g of agar gel dissolved in 1000ml of water) as a source of moisture. Mice were kept in the laboratory prior to any experiments to establish whether any of the females were pregnant. Pregnant females were not used. In order to simulate changes in seasonal thermogenic adaptation (Heldmaier et al., 1982), mice were initially acclimated to a short photoperiod (short-day; ‘SD’) (16D: 8L, lights on 08:00-16:00) and then subject to the three different salinity levels (below). They were then acclimated to a long photoperiod (long day; ‘LD’) (8D: 16L, lights on 06:00-22:00) and measurements were repeated. The intensity of the ‘light periods’ was 450 lux, which was provided by white fluorescent lighting. A dim red light was on continuously (< 50 lux), which allowed experimenters to work in the ‘dark’ periods. Ambient temperature was kept constant at 25°C throughout the entire experimental period. Acclimation to each photoperiod regime lasted at least two weeks.
Salinity changes

To simulate changes of osmotic load experienced in the wild, we exposed animals to different levels of water stress by altering the levels of salt (NaCl) dissolved into the agar (that was provided as a water source). This has been found to affect thermoregulatory capabilities of several species of Mediterranean rodent [e.g. *Sekeetamys calurus* (Palgi & Haim, 2003), *Acomys russatus* (Ron & Haim, 2001) and *Acomys cahirinus* (Shanas et al., 2002)]. In the current study we exposed both SD and LD-acclimated mice to salt concentrations of 0.9 % to 1.4 % and then 1.8 %. Several physiological capabilities (below) were then measured for each of the six different treatment conditions (i.e. for the two photoperiods and the three salinities).

We weighed the mice at the end of each acclimation period, prior to the urine collection.

Urine collection

After two weeks of acclimation to a specific salinity, mice were transferred to metabolic cages (19.5 x 11.5 x 9cm) with mesh floors above sheets of Parafilm for 24 hours. They were provided with dried soybeans *ad libitum* and agar gel. Every two to three hours the urine was collected from the Parafilm with a Pasteur pipette and stored in Eppendorf tubes at 4 °C until analysis.

Urine volume, osmolality, electrolytes and urea analysis

Urine volume was measured (±1 l, Pipetman, Gilson) within 24h of collection, and the osmolality was determined by using a vapor pressure osmometer (Wescor 5500). Sodium and potassium concentrations were determined within 2 weeks using a flame photometer (Sherwood...
Chloride concentrations were determined by titration on a chloride analyzer (Corning 925). Urea concentrations were determined using a commercial test kit (Sigma 640) on a spectrophotometer (Milton Roy Spectronic 401).

Statistics

We used a mixed design analysis (Page, Braver & Mackinnon, 2003; Keppel & Wickens, 2004) Groups were set as between-subject effects; season (long and short photoperiod acclimation) and salinity were set as within-subject effects. Because some individuals died during the course of the experiments, mice were grouped such that the analysis used the between-error terms.

Results

Urine volume and body mass did not change as a response to increasing dietary salt content (F2,62=1.030, p>0.05; F2,62=.333, p>0.05 respectively) and there was no interaction between these parameters and the origin of the mice (F1,62=.122, p>0.05; F1,62=.650, p>0.05 respectively) or the photoperiod (F1,62=.601, p>0.05; F1,62=.047, p>0.05 respectively).

Osmolarity: Urine osmolarity did not change with dietary salt concentration (F2,62=.360, p>0.05). Osmolarity did not differ between the two groups or between photoperiods (F1,62=3.938, p>0.05; F1,62=2.054, p>0.05, respectively, Fig. 1).
Fig. 1. The effect of dietary salt concentration on urine osmolarity (average ± SE) of two groups of *A. mystacinus* at different seasons (photoperiod acclimated).

There was a positive correlation between osmolarity and urine volume in the Carmel mice but a negative correlation in the Hermon mice (Carmel winter: \( Y = -3.123 + .002X, r^2=0.781, F_{1,2}=3.571, p=0.3908 \); Carmel summer: \( Y = -1.55 + .001X, r^2=0.998, F_{1,2}=487.991, p=0.0288 \); Hermon winter: \( Y = 3.669 - .001X, r^2=0.954, F_{1,2}=20.731, p=0.1376 \); Hermon summer: \( Y = 3.517 - .001X, r^2=0.74, F_{1,2}=2.852, p=0.3403 \); Fig 2).
Fig. 2. Correlation between urine volume (ml) and osmolarity (mmol/l) in *A. mystacinus* from the Carmel and Hermon populations under SD (winter) and LD (summer) photoperiod acclimation.

Urea: There was a significant effect of salinity on the urea concentration in the urine, with increasing dietary salt resulting in a decrease of urea concentration (Fig. 3; $F_{2,62}=5.754$, $p<0.01$).
However, neither site of origin (F₁,₆₂ = 1.43, p > 0.05) nor photoperiod (F₁,₆₂ = 0, p > 0.05) had a significant effect on urea concentration.

Fig. 3. Effect of dietary salt concentration on urea production (average ± SE) in *A. mystacinus* at different seasons (photoperiod acclimated).

**Sodium:** There was a significant difference in the response of Na⁺ concentration to dietary salt content between the Hermon and Carmel mice (F₁,₆₂ = 11.208, p = 0.001).
Across all mice, increasing dietary salt content produced a significant increase of Na\(^+\) concentration (F\(_{2,62}=6.769\), p<0.01). However, there was no significant effect of photoperiod on the concentration of Na\(^+\) in the excreted urine (F\(_{1,62}=1.178\), p>0.05).

We further found a significant interaction in the response of urinary Na\(^+\) concentration between the dietary salt content and the site of origin of the mice (F\(_{2,62}=3.719\), p<0.05), with Carmel group showing an increasing and Harmon group showing a decreasing (only under SD) of urinary Na\(^+\) concentration (Fig. 4).

Fig. 4. Effect of dietary salt concentration on Na\(^+\) urine concentration (average ± SE) in *A. mystacinus* at different seasons (photoperiod acclimated).
Chloride: There was no significant effect of dietary salt content, photoperiod or origin of the group on the urinary Cl⁻ concentration (F_{2,60}=1.972, p>0.05; F_{1,60}=1.185, p>0.05; F_{1,60}=2.801, p>0.05 respectively). However, there was a significant interaction between site of origin and dietary salt content (F_{2,60}=7.433, p=0.001) with Hermon group showing a decreasing and Carmel group showing an increasing urinary Cl⁻ concentration at 1.4%. There was also an interaction between site of origin and photoperiod (F_{1,60}=5.551, p<0.05), with mice from the Carmel having a greater Cl⁻ concentration under LD. A third interaction was found between dietary salt content and photoperiod (F_{2,60}=5.891, p<0.01), with urinary Cl⁻ decreasing under SD but increasing under LD in response to increasing salt loads (Fig. 5).
Fig. 5. Effect of dietary salt concentration on Cl⁻ urine concentration (average ± SE.) in *A. mystacinus* at different seasons (photoperiod acclimated).

**Potassium:** Dietary salt concentration significantly increased potassium concentration in the urine ($F_{2,61}=4.966$, $p=0.01$). There was no significant effect of the site of origin or photoperiod on the $K^+$ concentration in the urine ($F_{1,61}=3.672$, $p>0.05$, and $F_{1,61}=0.095$, $p>0.01$, respectively). However, there was a significant interaction between dietary salt content and photoperiod ($F_{2,61}=6.269$, $p<0.01$) with an increase of $K^+$ concentration greater under the SD (winter acclimation) than under the LD (summer acclimation) regimes (Fig. 6).

![Graph showing the effect of dietary salt concentration on K⁺ urine concentration in A. mystacinus at different seasons (photoperiod acclimated).](attachment:graph.png)

Fig. 6. Effect of dietary salt concentration on K⁺ urine concentration (average ± SE.) in *A. mystacinus* at different seasons (photoperiod acclimated).
Discussion:

Identifying units of management that can reliably represent evolutionary lineages has been the subject of extensive study and debate (Moritz, 1994; Dimmick et al., 1999; Crandall et al., 2000; Deweerdt, 2002; Green, 2005; King et al., 2006). In the wake of rapid changing environments, it is especially important to identify and study those species and traits that are in risk of extinction. Some traits can be especially important for species to deal with the changing environments (Blondel et al., 2006), particularly those related to physiological adaptations. To this end, the field of ecological physiology can help to predict the performance of species, and maybe even subspecies, during environmental changes (Carey, 2005). Therefore identifying subspecies or ESUs with these same physiological tools should be recognized as a necessary step.

Closely related species may show striking differences in their physiological traits, as presumed adaptation to differences in environmental characteristics (Shkolnik, 1969; Goyal et al., 1988). In some cases, physiological differences may even be apparent within the same species (Haim, 1981; Haim, Rubal & Harari, 1993; Weissenberg, 1994) if its distribution covers diverse habitats. Scantlebury et al. (2003, 2005) and Shanas et al. (2003) demonstrated that populations of the same species within the same locality, but occupying different micro-niches may show significant differences in their physiological traits. These latter experiments were held in two opposing slopes of the same canyon (“Evolutionary Canyon, (Nevo et al., 1998). Can different physiological traits call upon the designation of unique ESUs? The concept of ESU has resulted in many controversial definitions and thus, was occasionally
counterproductive in the efforts to preserve the world biodiversity (Dimmick et al., 1999, 2001; Young, 2001). Since, adaptation stands at the center of this debate, it is surprising that it has not received the necessary attention that would have provided a better definition of what traits may be held as an important physiological difference that would assist in defining a biological entity as an ESU, and what would be considered as a species plasticity manifest within phenotypic variations. 

The results of the present study show contrasting physiological traits in two allopatric populations of broad toothed field mouse. We found that the Hermon population responded, as would be expected in rodents, to increased urine production by a decreased urine osmolarity. Indeed, urine osmolarity is generally inversely related to urine output (Ntshotsho et al., 2004). However, the Carmel mice showed a surprising phenomenon whereby urine osmolarity increased with the increasing urine production (Fig 2). The two populations retained their unique responses in both photoperiod regimes. The two groups inhabit two zoogeographic ecosystems that differ in a wide range of ambient conditions. The Hermon Mountain is relatively high (up to 2814m altitude), and is considered as a part of the Irano-Turanian zone and typified by a subalpine, dry and cold weather. The Carmel Mountain, in contrast, is relatively low (maximum 546m) and is part of the Mediterranean ecosystem, typified by a humid and mild weather. Therefore, the Hermon Mountain imposes high evaporative conditions on its mice populations, which might explain the adaptive negative correlation between urine volume and osmolarity. We suggest that the Carmel group might be more liberal in their water economy, and thus they may increase urine output when osmolarity increases. Previous studies have demonstrated that these two populations
also differ in their thermoregulatory capacities (Haim, Plaut & Zobedat, 1996) and noradrenaline-induced thermogenesis (Spiegel & Haim, 2004). Since the current study shows two opposing responses under the same laboratory conditions for both populations, we suggest they are true adaptive characteristics that typify and physiologically separate the two populations.

Contrary to our expectation (e.g. (Sasidharan et al., 1990), we did not find differences in urine production between photoperiods. Although previous studies have shown that changes in photoperiod regimes exert changes in physiological responses of A. mystacinus (Spiegel & Haim, 2004), it is possible that the purely photoperiod-simulated seasonality in this study was not strong enough to elicit a response in urine production and that photoperiod manipulations may need to be accompanied by changes in ambient temperatures.

In the current study, we found that urea concentrations decreased with the increasing dietary salt concentrations (Fig. 3). Usually, in dry environments, we expect the urea concentration to increase with the increasing dietary salt content, or with increased water deprivation, as the urea production helps to build the osmotic gradient in the kidney (Frean, Downs & Lovegrove, 1998; Peinke & Brown, 1999; Shanas et al., 2003). The decreased urea concentration has possibly resulted from increased water discharge (although no significant change in urine volume was observed), possibly representative of the mesic, less water restrictive northern geographic origin of both populations.
We also found striking differences between the two localities in both the sodium and the chloride urinary concentrations. Under SD, the Carmel group increased while the Hermon group decreased their urinary sodium concentration in response to increasing dietary salt load. Moreover, the Carmel group exhibited no differences in the response to increasing dietary salt concentrations between photoperiods, whereas the Hermon group showed opposing responses (Fig. 4). It appears that the Hermon group acted as might be expected from rodents living in a dry habitat in which the summer is dry and hot and the winter is cooler and wetter. Therefore, the increased LD sodium concentrations are similar to rodents originating or adapted to arid environment (Moro & Bradshaw, 1999; Palgi & Haim, 2003; Shanas et al., 2003). In contrast, the Carmel group exists in a relatively more stable environment with less extreme seasonal differences, reflected in the similar responses at both photoperiods.

The differences in urinary chloride concentration may also be suggestive of physiological differences between the two populations (Fig. 5). We expected an increase in urinary chloride concentration as a result of increasing dietary salt concentrations, especially under LD (Stallone, 1979; Shanas et al., 2003). However, the initial decrease in urinary chloride concentrations in the Hermon group was contrary to our expectation, and it may suggest kidney chloride retention capabilities of this group when aridity increases (Stallone, 1979).

Together with the osmolarity, urea, and sodium concentrations these results are supportive of separating these two groups into two distinct populations.
Overall, we show that two populations of woodmouse in the eastern Mediterranean exhibit physiological differences in the way that their urine is concentrated. This is evidenced by the differing concentrations of solutes present in their urine under varying levels of water stress.

Since the two populations inhabit different ecosystems, separated by the Great Rift Valley, it is very likely that they do not interbreed. We therefore suggest that the two populations should be regarded as distinct ESUs. Interestingly, it has also been demonstrated that the two populations do indeed diverge based on genetic markers, and it has been suggested that the Great Rift Valley may present a geographical and therefore a genetic barrier for this species (Nir, 2006).

Global climate change may result in mass extinction. It has been speculated that 37% of the global species diversity may be compelled to extinction within the next 50 years (Thomas et al., 2004). Therefore, identifying populations of species that show unique ecological traits, especially traits that are related to adaptations to warmer and arid environments (e.g. the Hermon population of woodmouse in this study) is of major importance. Under fast global changes, such populations may require special conservation attention. We suggest that the case of the woodmouse should set an example to a functional approach to the designation of an ESU. Physiological data that deal with adaptive traits will considerably enhance conservation efforts, and should therefore be of research priority.

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