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Cover image: *Phcnonotus sinensis*, Photo by Hao-Tian BAI

Editorial

The impact of special issues

With support from many authors from many different scientific fields, *Zoological Research* (*ZR*) successfully released eight special issues from 2011 to 2014 on various topics, including *Animal Ecology and Resources*, *Animal Genetic Diversity*, *Development and Evolution*, *Fish Diversity* and *Primates and Animal Models of Human Diseases*. These special issues strongly align with our aims and scope, which have been fostered since our inception some 35 years ago.

Our special issues have promoted the visibility and influence of *ZR*, particularly evident in regards to the increased click rates from PubMed and high citation rates from Web of Science. For example, the click rate for the *Special Issue on Primates and Animal Models of Human Diseases* (2011, Feb. 32(1)) was more than 500 times higher than that of the previous issue, while the citation rates for the *Special Issue on Animal Ecology and Resources* (2011, Apr 32(2)) and the *Special Issue on Primates and Animal Models of Human Diseases* (2011, Feb. 32(1)) were reasonably high.

The articles and reviews published in our special issues have occasioned considerable attention. As of the end of 2014, the original research article *A behavioral ecology approach to traffic accidents: interspecific variation in causes of traffic casualties among birds* (2011, Apr. 32(2): 115-127) has been cited over 10 times and downloaded over 1000 times, while the *Checklist of fishes of Yunnan* (2013, Aug. 34(4): 281-343) has been reported in several newspapers, websites and other mainstream media.

The research and development on primate and tree shrew models of human diseases is an area of particular interest for *ZR*. Over the last four years, five special issues have been published with great success. The leading research on tree shrews and depression models, *Tree shrew models: a chronic social defeat model of depression and a one-trial captive conditioning model of learning and memory* (2011, Feb. 32(1): 24-30) has been downloaded almost 2000 times as of the end of 2014. Two review papers regarding Chinese tree shrews (*Tree shrews under the spot light: emerging model of human diseases* (2013, Apr. 34(2):59-69) and *Molecular evidence on the phylogenetic position of tree shrews* (2013, Apr. 34(2):70-76)) hit the top 10 cited list from 2011-2014, and both have been selected in the “Project of Frontrunner 5000” (F5000) due to their roles in promoting scientific communication. [1]

We are privileged to provide a platform in which exciting and groundbreaking research can be published. Should you, or your colleagues, be interested in contributing papers or developing a special issue on specific topics, we would be delighted to invite you as single-issue Guest Editors. We guarantee the timely publication of research, and we believe with your continued support, *ZR* will maintain its contribution to and influence on the field of science.

Again, we greatly appreciate the help provided by our authors and readers during the preparation of our special issues, and we look forward to further support in our upcoming publications.

Sincerely yours,

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*All the statistical data were from Web of Science and PubMed, from January 01 2011 to December 31 2014.

ZOOLOGICAL RESEARCH

Emerging directions in the study of the ecology and evolution of plant-animal mutualistic networks: a review

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ABSTRACT

The study of mutualistic plant and animal networks is an emerging field of ecological research. We reviewed progress in this field over the past 30 years. While earlier studies mostly focused on network structure, stability, and biodiversity maintenance, recent studies have investigated the conservation implications of mutualistic networks, specifically the influence of invasive species and how networks respond to habitat loss. Current research has also focused on evolutionary questions including phylogenetic signal in networks, impact of networks on the coevolution of interacting partners, and network influences on the evolution of interacting species. We outline some directions for future research, particularly the evolution of specialization in mutualistic networks, and provide concrete recommendations for environmental managers.

Keywords: Mutualistic networks; Coevolution; Speciation; Phylogenetic signal; Specialization

INTRODUCTION

Plant-animal mutualistic interactions, such as pollination and seed dispersal, have been regarded as one of the principle examples of coevolution and have long drawn the sustained attention of researchers (Fleming & Kress, 2013). The study of mutualistic interactions is part of the larger field of ecological networks, which also includes 'antagonistic' interactions between different species at varying trophic levels in the food chain (Pimm, 1982; Thompson, 2009). However, since the evolutionary forces that shape antagonistic networks may differ from those that shape mutualistic ones, we focused on mutualistic plant-animal interactions, a subject with now sufficient studies to stand on its own (Thompson, 2005; Thébault & Fontaine, 2010). Indeed, the published literature is so large and complex that is it useful to categorize the different kinds of questions that have been asked. Our review had the following objectives: (1) outline the major ecological and evolutionary questions about mutualistic networks that have become prominent in the last 30 years, and (2) point out lines of research where further development would be particularly fruitful. [1]

BRIEF HISTORY OF RESEARCH ON PLANT-ANIMAL MUTUALISTIC NETWORKS

The idea of plant-animal interaction networks was first proposed 150 years ago with the publication of Darwin's *On the Origin of Species*, who wrote "I am tempted to give one more instance showing how plants and animals, most remote in the scale of nature, are bound together by a web of complex relations" (Darwin, 1859, p74). A large number of studies have looked at specific mutualistic interactions between plants and animals, such as yucca moths and yuccas (Pellmyr, 2003), ants and Acacia (Janzen, 1966) and fig wasps and figs (Cook & Rasplus, 2003). However, the interactions among organisms are far more complex than pair-wise relationships, as many different organisms interact together in networks that include many species (Thompson, 2005). With more biological data available and the advance of algorithms for complex network analysis derived from physics and computer science, ecologists and evolutionary biologists have started to explore plant-animal mutualistic interactions from the network perspective (Guimarães et al, 2011; Olesen et al, 2007; Rezende et al, 2007a, b).

To study the growth in this literature, we searched the topics 'mutualistic network', 'plant animal interaction' or 'mutualistic interaction' with the timespan from 1970 to 2013 on Web of Science, and then further refined the results using the Web of Science ecology category. A total of 1 945 publications were closely related to the topics, with rapid growth year-to-year (Figure 1). In terms of the total number of publications in a year, most years had more publications than the year before. This trend is expected to keep on increasing annually in the future.

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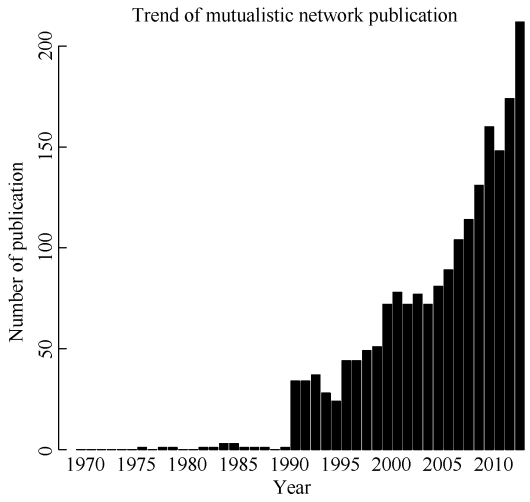

Figure 1 Literature found on Web of Science by searching 'mutualistic network', 'plant animal interaction' or 'mutualistic interaction' with the time span 1970 to 2013, plotted against the date of publication

Each bar represents the number of publications published in a single year. This search was conducted in June 2014.

Tracing early literature, plant-animal mutualistic studies began to appear in the late 1970s, with an escalation in publications observed in the early 1990s. Most early studies were field experiments, which laid the foundation for later, more theoretical research. Jordano (1987) detailed the first well-recognized study on mutualistic networks from the theoretical perspective in *American Naturalist*. A rapid increase in publications on networks was observed from 2005 (Figure 1). Bascompte et al (2003) introduced an important technique for network analysis to measure the nestedness of the interaction network, opening new avenues in the field. Beyond studies on network architecture, increasing research has focused on the implications of network theory for ecology and evolution, and we address these two major kinds of studies separately below.

MUTUALISTIC NETWORKS IN ECOLOGICAL TIME

Mutualistic network properties

Plant-animal mutualistic networks can be described by interaction matrices, with plant species in the columns and animal species in the rows; we refer to an interaction between a plant and animal species as a "link". According to the kind of values in the matrix cells, network matrices can be categorized into "weighted" networks ("quantitative" networks) and "unweighted" networks ("binary" networks or "qualitative" networks, as they only indicate whether a pair of species interact, not the intensity). Several network properties have been generated to describe the network structure, such as connectance and interaction strength (Table 1).

Table 1 Glossary of main network terms and key references

Metric	Definition
Asymmetry	Measures the imbalance in the interaction strength of two interacting species (Bascompte et al, 2006). It is defined as $AS_{ij}=(b_{ij}-b_{ji})/(b_{ij}+b_{ji})$, where b_{ji} is the reciprocal dependence of species j on species i (b_{ij} see interaction strength, Bascompte et al, 2006; Blüthgen et al, 2007).
Binary network	In binary network matrix, the value is 0 or 1, if the interaction occurs, the value is 1, otherwise 0 (Jordano, 1987).
Connectance	Proportion of the realized interactions in all possible interactions (Yodzism 1980). In mutualistic networks, connectance (C) is: $C=L/(IJ)$. L describes the number of realized links; I and J are the number of species of each bipartite network (Blüthgen et al, 2008).
Degree	Number of interactions a species has (Jordano et al, 2003).
Interaction strength	Interaction strength of species j on species i (b_{ij}) can be defined by the proportion of interactions between i and j (a_{ij}) of the total interactions recorded for i; thus $b_{ij}=a_{ij}\Big/\sum_{j=1}^{J}a_{ij}$. For mutualistic network, b_{ij} measures the dependence of species i on its partner j (Jordano, 1987; Blüthgen et al, 2007).
Modularity	Measures the degree to which the network is organized into clearly delimited modules (Olesen et al, 2007). Modularity (M): $M=\sum_{s=1}^{N_M}(\frac{I_s}{I}-(\frac{k_s}{2I})^2)$, where N_M is number of modules in the network, I_s is the number of links in the network, and k_s is the sum of degrees of all species in s. M values belong to the interval [0; -1/N_M].
Module	A set of weakly interlinked subsets of species that consist of strongly connected species (Olesen et al, 2007).
Nestedness	A nonrandom pattern of the network structure, which entails the tendency of specialized species to interact with a subset of the interaction partners of more generalized species. The nestedness temperature (T) measures the departure from a perfectly nested interaction matrix, ranging from 0 to 100, which indicates the degree of disorder. T=0 is defined for maximum nestedness: when rows and columns are ordered by decreasing number of links, links of each row and column exactly represent a subset of the previous ones. Nestedness can be defined as N=(100°-T)/100° (Bascompte et al, 2003).
Weighted network	Networks that include information on the intensity or weight of the interactions among nodes (Bascompte & Jordano, 2007).

The topology of ecological interaction webs (such as mutualistic networks) holds important information for biodiversity, ecosystem stability and theories of coevolution (Bascompte et al, 2006; Jordano, 1987; Montoya et al, 2006). On the whole, mutualistic networks are neither a collection of pair-wise, highly specific interactions nor diffuse, random assemblages (Bascompte & Jordano, 2007). Rather, mutualistic networks have common, well-defined network architecture regardless of the type of mutualism, species composition, and geographic region (Bascompte et al, 2003). Several topological features are characteristic, including skewed distribution of links per species (i.e., a few species with many more interactions than expected by chance, and many species with a few interactions; Vázquez & Aizen, 2003; Jordano et al, 2003), the nested organization of the interaction matrix (Bascompte et al, 2003) and the frequent occurrence of asymmetric interactions (i.e., a plant species depending strongly on an animal species, the animal depending weakly on the plant; Bascompte et al, 2006). In addition to these properties, some networks are also modular (especially pollination networks; Olesen et al, 2007), whereby clusters of species interact more closely with each other than with species in other clusters or species outside the clusters. In comparison to antagonistic networks, mutualistic networks demonstrate high asymmetry and connectance, whereas antagonistic networks tend to be weakly connected, with many groups of species ("compartments") interacting only within their group (Cagnolo et al, 2011; Krause et al, 2003; Prado & Lewinsohn, 2004; Thébault & Fontaine, 2010).

Stability and diversity of mutualistic networks

The maintenance of stability in complex communities has been a long-standing debate since the classic work of May (1974). Most network studies have found that the properties of mutualistic networks contribute to their diversity and stability; for example, the asymmetric nature (i.e., asymmetric interactions and asymmetry in interaction strength) of mutualistic networks promotes community coexistence, which favors the persistence of biodiversity (Bascompte et al, 2006). Nestedness has been shown to reduce interspecific competition, and hence promote diversity and nested networks stability (Bastolla et al, 2009; Thébault & Fontaine, 2010). Other network properties, including community size, species degree, species strength, and symmetry of the interaction, may also positively contribute to stability (Okuyama & Holland, 2008), while modularity decreases stability (Thébault & Fontaine, 2010). Recent developments have questioned the idea that nestedness itself affects stability, suggesting that more simple features, such as species degree, are more important drivers (Feng & Takemoto, 2014; James et al, 2012; Jonhson et al, 2013; Staniczenko et al, 2013). A further complication is that other interaction types, such as antagonistic networks, may interact with mutualistic networks to affect overall community stability (Mougi & Kondoh, 2012).

Spatial-temporal variation of the mutualistic network

Studies have indicated that both spatial and temporal dimensions impose constraints on plant-animal mutualistic interactions and influence network patterns (Burkle & Alarcón, 2011). Nevertheless, some general network properties remain constant over time and space. For example, Plein et al (2013) found that none of the characteristics of the seed-dispersal networks (e.g., interaction diversity, interaction evenness, and network specialization) used in their study changed with landscape type (farmland, orchard, forest edge) or season. Other studies have shown that although seasonal species turnover exists in the network, general network patterns (such as nestedness, connectance and modularity) remain relatively constant (Dupont et al, 2009; Dupont & Olesen, 2012; Olesen et al, 2008, 2011; Petanidou et al, 2008; Plein et al, 2013). However, these studies were conducted over a relatively short time period (often two to four years), and further studies with longer time spans are needed to test these conclusions. Additional attention needs to be paid when comparing different networks or when pooling data from different networks for meta-analysis. Networks are only comparable in this manner if they are at the same time scales or over the same phenological periods; for example, mixing networks from different seasons may prove confusing (Burkle & Alarcón, 2011).

Conservation implications of mutualistic networks

One potential problem for networks in our changing world is that alien mutualists, including both plants and pollinators, can integrate into native pollination networks, and sometimes end up acting as super-generalist species (a few species that interact with an extremely large number of species) of the network (Aizen et al, 2008; Bartomeus et al, 2008; Olesen et al, 2002). Highly invaded networks exhibit weaker mutualism than less invaded networks, and the connectivity among native mutualists declines, although overall network connectivity may not change (Aizen et al, 2008) and other aspects of network structure such as nestedness are relatively robust to the introduction of invasive species (Vilà et al, 2009). However, the removal of invasive alien species from the network can change the network structure, imposing a pronounced effect on degree distribution and modularity of the network, leading to higher species loss, which could affect the evolution of the interaction network architecture (Valdovinos et al, 2009).

Habitat loss and fragmentation also have large, consistently negative effects on biodiversity (Fahrig, 2003). The extinction of species and loss of mutualistic partners may impose a cascading effect on mutualistic networks, which can consequently lead to greater biodiversity loss (Anderson et al, 2011). Several theoretical studies have looked at how mutualistic networks respond to such disturbances. For example, Fortuna & Bascompte (2006) used a modeling method to understand how mutualistic networks respond to habitat loss, and found that real communities started to decay sooner than random communities, although they persisted better at high levels of destruction. Pollination network field research also demonstrated that habitat loss not only leads to species loss, but also indirectly causes the reorganization of interspecific interactions in the local community (Spiesman & Inouye, 2013). The reduction in suitable habitats is associated with species loss, which is correlated with reduced nestedness

and increased modularity (Tilman et al, 1994).

Given the work on how mutualistic networks respond to invasive species and anthropogenic disturbance, it is clear that network theory has important implications for conservation. For example, by looking at the network structure, we can identify the network's susceptibility to alien species invasion (Olesen et al, 2002; Morales & Aizen, 2006). Network theory can also clarify the role of different species (i.e., species with different specialization degree) and their impacts on the whole network architecture and stability. For example, super-generalist species can greatly affect the overall topology of a network (Aizen et al, 2008; Hansen & Galetti, 2009), which has a direct consequence on the protection of the endangered system (Kiers et al, 2010) as these super-generalists can be targeted in conservation plans. Other issues that lead to species loss in the network, such as the effect of habitat loss on networks, are also important to conservation practice. Additionally, it has been suggested that the extinction of phylogenetically related species can lead to cascading coextinction events (Rezende et al, 2007a). Understanding this potential effect with the assistance of network theory could help conservationists make decisions on species priority.

Unfortunately, despite the theoretical advances in networks and their potential use in conservation, their actual implementation in conservation management is rare. There appears to be a communication failure among scientists, practitioners, and government officials that requires the assistance of all parties to resolve (Heleno et al, 2014).

MUTUALISTIC NETWORK STUDIES FROM AN EVOLUTIONARY PERSPECTIVE

As discussed above, studies on network structural properties and their implication for ecology have been extensively investigated in recent years. More attention has been spent on investigating mutualistic networks from the ecological perspective than the evolutionary one. However, species are not independent entities but rather related to each other through common evolutionary histories. Phylogenetic constraints may influence mutualistic interactions, imprinting a phylogenetic signal (i.e., the tendency of phylogenetically similar species to have similar phenotypic attributes; Bascompte & Jordano, 2007) on network structure (Ives & Godfray, 2006; Rezende et al, 2007a, b). To understand plant-animal mutualistic networks, we must examine the phylogenetic signal of species' positions (i.e., species centrality and their placement relative to modules; Olesen et al, 2007) in the network and observe which species form the network.

In addition, studies that have attempted to link network literature to explore the evolution of networks (e.g., Guimarães et al, 2011) remain scarce. The synthesis of network studies and phylogeny may change our understanding of the coevolutionary process. For example, recent work that combined phylogeny and pollination data found coevolution to be an important driver of species diversification (Van der Niet & Johnson, 2012). Despite some limitations of using phylogeny to explain the processes that influence speciation, work of this kind may nevertheless suggest novel directions towards understanding speciation, diversification, and biodiversity. Below, we discuss three topics in the evolution of mutualistic networks in which there has been recent research activity.

Phylogenetic signal in mutualistic networks

A revolutionary article on network evolution used phylogenetic methods for the first time to study how the interaction pattern was associated with phylogeny in mutualistic networks (Rezende et al, 2007a). By incorporating a large dataset of 36 plant-pollinator and 23 plant-frugivore mutualistic networks, they found that the phylogenetic signal in species degree (number of other species with which a species interacts) could be detected in more than one third of the networks. Meanwhile, the actual identity of interaction partners had a phylogenetic component in about half the interaction networks. Simulated extinction events triggered cascading coextinction, wherein phylogenetically related species went extinct together.

Having realized the importance of evolutionary history on network structure, more researchers now include phylogenetic studies in their work. For example, a recent study by Schleuning et al (2014) has further improved our understanding on network modularity. These researchers associated both weighted and binary network data with species traits and phylogenetic information to study how modularity is related with these ecological and evolutionary factors. The study followed the methodology of Olesen et al (2007), which identifies species connectedness within (*z*-score) and between (*c*-score) modules. The results showed that for both weighted and binary networks, no phylogenetic signal was detected in within-module degree (z), but significant phylogenetic signal was found in the c value: the tendency of a species to interact with species in other modules. Results also showed significant phylogenetic signal in species degree, concordant with the results of Rezende et al (2007a).

How do mutualistic networks affect coevolution of their species?

Guimarães et al (2011) combined a model for trait evolution with data from 20 plant-animal mutualistic networks to explore coevolution. They found that both evolution and coevolution contributed to the increase in convergence (e.g., trait similarities emerge in response to similar selective pressures) and complementarity (e.g., degree of trait matching between interactive partners) among traits within the network, and that coevolution significantly sped up the rate of trait evolution within networks (Guimarães et al, 2011). They also found that super-generalists facilitated trait evolution in mutualistic networks by greatly increasing complementarity (match of traits between partners) and convergence. Because super-generalists serve as connections between different modules, evolutionary and coevolutionary forces that affect them multiply throughout the whole network (Guimarães et al, 2011).

Thompson (2009) proposed a new hypothesis from an evolutionary perspective, in which plant-animal interaction networks act as vortexes, absorbing increasing numbers of species into the interaction network. As the web grows bigger, it

has more capacity to hold diverse species. In this way, plant-animal interaction networks may promote the evolution of biodiversity. However, more evidence is needed to support or reject this hypothesis.

How could mutualistic networks affect the rate of evolution of participating species?

The above studies either combined data on mutualistic networks with the phylogeny of the interacting species to look at how evolutionary history influenced network properties or simulated how coevolution shaped the patterns of complementarity and convergence between species in networks. However, the question of the rate at which species with different specialization levels evolve and diverge (i.e., speciation) in mutualistic networks remains largely unexplored. As has been well studied, mutualistic networks have a wide range of species that differ in their connectedness to other species, with many species interacting with only a few species ("specialists"), and other species interacting with many other species ("generalists"). In the field of classical ecological evolution, many studies have compared the speciation rate between specialists and generalists (e.g., Colles et al, 2009; Fernández & Vrba, 2005); however, do different kinds of species in networks differ in their speed of evolutionary divergence?

We recently conducted a study along the lines of this question, asking if specialist and generalist frugivorous birds have different speeds of evolutionary divergence (Gu et al, 2015). A recent time-calibrated phylogeny of birds that included all extant species (Jetz et al, 2012) allowed such a study, as every species had an estimated time of divergence from its sister species. Using 16 seed dispersal networks in the published literature, we found that specialists (defined as per Olesen et al, 2007) had significantly shorter divergence times than did generalists. This result is somewhat surprising as most birds are thought to be rather unspecialized and not dependent on specific fruiting trees, in contrast to the very strong and sometimes obligate relationships found between pollinators and plant species (Blüthgen et al, 2007). We therefore treat the result quite tentatively, noting some confounding variables, such as specialists being generally rare, which could be driving the result. Nevertheless, we think the question is important, and believe future research should investigate this in other taxa such as insects and plants.

FUTURE DIRECTIONS AND CHALLENGES

A general challenge for studies of plant-animal mutualistic networks is the paucity of data sampling. Field observations are time-consuming, and certain habitats (e.g., canopies) are difficult to access. Fundamentally, there is a lack of properly trained personnel to make the observations. General problems for flora and insect taxonomy lie in the shortage of funding, the low Impact Factor index of taxonomic journals, low scientific regard of taxonomic knowledge, and a lack of investment and training programs for a new generation of taxonomists (Dangles et al, 2009; Ma, 2014; Rafael et al, 2009). These problems greatly hamper the development of plant-animal interaction network studies, leading to misidentification of species.

There are potential alternative methods of network construction, including DNA barcoding (Carcía-Robledo et al, 2013; Heber et al, 2004). For example, researchers recently found that DNA barcoding gave comparable results to direct observations in a plant-herbivore system (Carcía-Robledo et al, 2013). However, the authors only studied a small group of well-known taxa (with plants only from order Zingiberales and rolled-leaf beetles from only two genera, *Cephaloleia* and *Chelobasis*). They emphasized that DNA information is unavailable in public databases for many organisms that participate in plant-animal interaction networks. This is especially true for mutualistic networks, in which the organisms come from a wide range of taxa.

Additionally, the size of the network from different studies varies greatly, with total species ranging from a few dozen to several hundreds. This range in network size could reflect biological reality or could be due to differences in sampling effort. If the differences are due to sampling effort, these can be accounted for during analysis, as recently shown by Schleuning et al (2014). To answer large ecological or evolutionary questions, data from many different networks needs to be gathered together and a general protocol needs to be established (Heleno et al, 2014).

Another challenge of network studies is to find an effective way to quantify the network, which should reveal the real ecological processes behind the interactions. For example, for most existing pollination networks and seed dispersal networks, flower visitation and frugivory are recorded as proxies without evaluating the effectiveness of the ecological services, that is, how these services affect plant reproduction (Heleno et al, 2014). By reconsidering how networks are quantified, we can better understand the real ecological and evolutionary mechanisms. In addition, as weighted data are more informative than binary data (Barrat et al, 2004; Schleuning et al, 2014), we should encourage future studies to use weighted network data.

Further development of plant-animal mutualistic network theory requires more complete and informative datasets, which allow the evaluation of multiple mechanisms simultaneously (Vázquez et al, 2009). According to the geographic mosaic theory (Thompson, 2009), coevolution varies in time and space. Thus, network data with explicit spatio-temporal information are needed to provide more reliable and explanatory interpretations on both the ecological and evolutionary study of mutualistic networks. To answer questions of how networks affect the evolution of participating species, both comprehensive ecological information and phylogenetic information of participating species are required. We believe that field studies, particularly those that use the same systematic technique to look at networks in multiple study sites, and hence better understand consistency and variation in the qualities of the networks, are just as vital to the development of the field as theoretical studies.

New insights will undoubtedly arise as our knowledge about phylogeny increases. In pollination networks, for example, there

are extremely large numbers of diverse pollinators from Diptera, Hymenoptera, Lepidoptera and Coleoptera. Yet only a small proportion of these species have been well studied taxonomically and phylogenetically. Time calibrated phylogenies for species in these groups are extremely limited (to some species from genus *Bombus*, family Apidae), and few of these species have DNA sequences accessible in GenBank. New developments in obtaining further phylogenetic information and better techniques will surely open new doors to analyses, just as Jetz et al (2012) did for our study (Gu et al, 2015).

Despite all the challenges in mutualistic network studies, there are many opportunities in this field. It is difficult to predict the directions of future research to come, after all who could have foreseen the network literature blossoming as it has over the last 30 years, but certainly phylogenetic and bar-coding techniques may ignite new possibilities. From our evolutionary perspective, it is clear that there are many questions yet unanswered about how the network phenomenon has influenced the evolution of participating species. We also believe that conservation is a priority. However, despite the rapidly accumulating research in this field, there is little consensus on how environmental managers should incorporate networks into their planning (Heleno et al, 2014). For example, should networks be used to evaluate habitat quality rather than individual species counts (Valiente-Banuet et al, 2014)? Are networks where invasive species are super-generalists irreparable or do remedial actions exist (Aizen et al, 2008; Valdovinos et al, 2009)? Further progress on these issues will demonstrate that our growing knowledge about ecological networks can be applied to solve environmental problems.

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Species identification refined by molecular scatology in a community of sympatric carnivores in Xinjiang, China

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ABSTRACT

Many ecological studies and conservation management plans employ noninvasive scat sampling based on the assumption that species' scats can be correctly identified in the field. However, in habitats with sympatric similarly sized carnivores, misidentification of scats is frequent and can lead to bias in research results. To address the scat identification dilemma, molecular scatology techniques have been developed to extract DNA from the donor cells present on the outer lining of the scat samples. A total of 100 samples were collected in the winter of 2009 and 2011 in Taxkorgan region of Xinjiang, China. DNA was extracted successfully from 88% of samples and genetic species identification showed that more than half the scats identified in the field as snow leopard (*Panthera uncia*) actually belonged to fox (*Vulpes vulpes*). Correlation between scat characteristics and species were investigated, showing that diameter and dry weight of the scat were significantly different between the species. However it was not possible to define a precise range of values for each species because of extensive overlap between the morphological values. This preliminary study confirms that identification of snow leopard feces in the field is misleading. Research that relies upon scat samples to assess distribution or diet of the snow leopard should therefore employ molecular scatology techniques. These methods are financially accessible and employ relatively simple laboratory procedures that can give an indisputable response to species identification from scats.

Keywords: DNA analysis; Snow leopard; Scats; Noninvasive genetics; Carnivore

INTRODUCTION

Information gathered from scats, such as diet, distribution, abundance and community dynamics, is widely used in many ecological studies and conservation management plans and requires reliable identification of scats (Gibbs, 2000; Long et al, 2008). However the identification of species from field signs alone is not always accurate and misidentification of scats is frequent, in particular in habitats with sympatric, similarly sized carnivore species (Farrell et al, 2000). Traditional methods of identifying scats in the field include size (length and/or diameter), shape (segmented, pointed ends, etc.), color (black, brown, white, etc.), pH or smell, in addition to the co-occurrence of other signs at the site such as pugmarks, scratches or hair (Danner & Dodd, 1982; Green & Flinders, 1981; Jackson & Hunter, 1996). However, these field methods have proven unreliable for the following reasons: body size can vary greatly within species which affects scat dimensions (e.g. between juvenile and adult, and male and female), and other species may investigate the scat sites of the target species, confusing identification by leaving their signs. [1]

To address the scat identification dilemma, molecular scatology techniques have been developed to extract DNA from the donor cells present on the outer lining of the scat samples (Foran et al, 1997; Höss et al, 1992). Target DNA, that enables species identification, is then amplified using polymerase chain reaction (PCR) based methods. These techniques have been employed for scat recognition in numerous studies and for a variety of species, for example from identification of European brown bears (*Ursus arctos*) (Kohn et al, 1995), to sympatric confamilial groups (Mills et al, 2001), and discerning species in an entire community of carnivores (Fernandes et al, 2008) or even telling apart hybrids (Adams et al, 2003).The use of molecular scatology has also revealed and confirmed that species identification of scats in the field is often inaccurate (Davison et al, 2002; Farrell et al, 2000; Harrington et al, 2009; Prugh & Ritland, 2005; Zuercher et al, 2003).

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Snow leopards are very elusive, extremely rare and inhabit harsh environments (Jackson & Ahlborn, 1988; Sunquist & Sunquist, 2002) making research through direct observation almost impossible. Research on this species therefore relies on gathering information in the absence of direct contact, primarily through non-invasive techniques (Ma et al, 2011; Wei et al, 2001; Xu et al, 2005). One of the main survey techniques used is scat collection with the following objectives: to determine prey preference (Bagchi & Mishra, 2006; Liu et al, 2003; Oli, 1993; Shehzad et al, 2012; Wang et al, 2014), to estimate population size (Ale et al, 2007; Fox et al, 1991; Hussain, 2003; Karmacharya et al, 2011; McCarthy et al, 2008), to analyse community structure (Lovari et al, 2013), etc. In most of their ranges, snow leopards co-occur with other similarly sized carnivores, such as wolves, foxes and jackals (*Canis aureus*), potentially creating uncertainty in the field identification of scat. It is not surprising that evidence of extensive scat misidentification confirmed by molecular techniques has been reported for snow leopards in previous publications. Janečka et al (2008) first observed a high level of scat misidentification, ranging from 35% in Ladakh, India to 54% in South Gobi, Mongolia. Of 71 samples collected in the study by Karmacharya et al (2011) in Nepal, 42% were misidentified as snow leopard in the field, but in fact belonged to other carnivores. Anwar et al (2011) in Pakistan found 52% of scats to be correctly identified as snow leopard while the rest were from other sympatric species. Likewise Shehzad et al (2012) in Mongolia correctly identified in the field 43% of putative snow leopard scats as confirmed by DNA analysis, while 57% were excluded from the study because they actually belonged to other species.

Despite misidentification being a source of significant bias, scat sampling still remains a fundamental tool in the study and conservation of snow leopards, so it is important to address and reduce the described inaccuracies.

This study aims to: (1) assess the extent of misidentification in the field for snow leopard scat; (2) determine if variables associated with the scat, such as morphological characteristics of the feces, are correlated with species identification; and (3) understand if accurate field identification of snow leopard scats is possible, if so establish more specific field collection protocols that include morphological features statistically relevant for successful species identification of scats.

MATERIALS AND METHODS

Study area and sample collection

Scats were collected from February 22, to March 12, 2009, and March 12 to April 12 in 2011 in Taxkorgan Nature Reserve (TNR; E74°30'-77°00', N36°38'-37°30'), located in the east plateau of the Pamir Mountains, Xinjiang Uygur Autonomous Region, China. TNR has a mean elevation of approximately 4000 m and it is characterized by a cold desert climate, with long very cold winters. The average temperature during the survey months is 1.03 °C, with 3.27 mm average precipitation per month. The carnivore guild includes snow leopards, wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and lynx (*Lynx lynx*). Pallas' cat (*Otocolobus manul*) may also be present, but extended surveys using camera traps have never recorded in our study sites.

Data for this study were collected in two sites, Mariang and Mazar; both sites had minimum altitudes above 3 000 m. The study sites straddled the southeastern and eastern boundaries of the TNR respectively, with some transects extending as far as 40 km outside the reserve (Figure 1). In

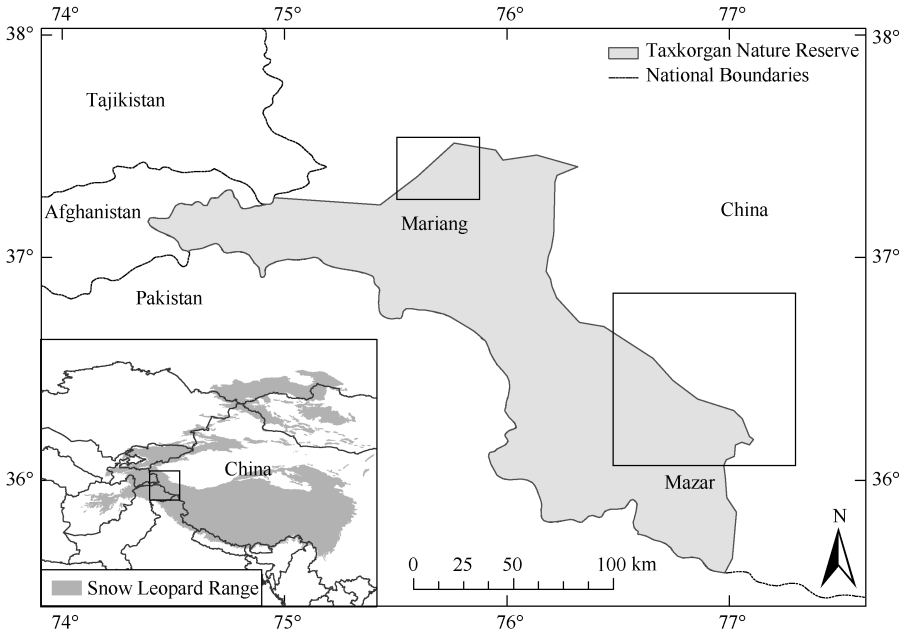

Figure 1 Location of Taxkorgan Nature Reserve including the two study sites (Mariang and Mazar)

2009, 24 transects with a total length of 242.7 km were divided between the two study areas, with 13 transects covering 204.1 km in Mazar, 11 transects totalling 38.6 km in Mariang. In 2011, 40 transects with a total length of 131.6 km were conducted in Mariang. Each transect was located within a distinct valley or along a ridgeline. In the field, scat samples were attributed to a certain species according to morphological characteristics (Table 1), the presence of other discerning signs (e.g. pugmarks, scratches, urine etc.) and the scat's location (e.g. under a cliff, open area, on trail etc.). These parameters were used in combination as guidance to identify the correct donor species. This was left to some extent to the collectors' discretion and decisions could be based on experience and individual opinion. The field team was composed of academic professionals specialized in carnivore studies and local Nature Reserve staff.

Table 1 Scat morphological parameters used in the field as guidance to identify the species.

Species	Color	Shape	Number of segments (*n*)	Length (cm)	Diameter (cm)
Snow Leopard	Dark	Long pointed tail	≤3	5-6	>2
Lynx	Dark	Long pointed tail	≤3	4-5	~2
Wolf/dog	White or grey	Round end, no tail	≤3	5	>2
Red Fox	Dark, grey old samples	Round end, short pointed tail	2-5	2-4	<2

Each sample at time of collection was placed individually in a labeled zip lock plastic bag to avoid contamination. To prevent degradation the samples were air-dried in the field by placing the open bags in a ventilated, cool and dry environment away from direct sunlight. In the laboratory, samples were then transferred to a freezer at −4 °C and finally to −80 °C for long-term storage. The morphological scat data recorded included number of segments (S), mean segment length (ML, cm), total length of scat (TL, cm), mean diameter (MD, cm) and weight after drying (DW, g). Original weight at collection was also noted but not used in these analyses, as it is greatly dependent on the age of the scat (fresh scats are heavier due to a higher water content, while old scats are lighter) and location of the scat (exposure to ice and snow may increase water content of old scats). Weight after drying was preferred as it does not present this constraint and was considered more appropriate.

Scat samples (number of samples in brackets next to the species Latin name in bold) were assigned to reference species based on nucleotide diversity <0.03 and node bootstrap value >90%.

DNA extraction and species identification

Laboratory analysis was performed at the Key Laboratory of Animal Ecology and Conservation Biology (Institute of Zoology, Chinese Academy of Sciences, Beijing). The DNA extraction was performed using the QIAamp DNA stool minikit (Qiagen). A 146 bp region of the mitochondrial cytochrome b gene was amplified by PCR using carnivore specific primers from Farrell et al (2000) (5′-AAACTGCAGCCCCTCAGAATG ATATTTGTCCTCA-3′ and 5′-TATTCTTTATCTGCCTATACAT RC ACG-3′). It is not possible to distinguish between wolf and domestic dog using this primer due to their close phylogenetic relationship, so they will be referred to as "wolf/dog" in the rest of this paper. Amplifications were conducted following the protocol developed by Janecka et al (2008), although changes to the volume of reagents (total 50 μL instead of 10 μL) and thermo cycling conditions were made as follows: 5× PrimeSTAR buffer 10 μL, 4 μL of dNTP mixture (2.5 mmol/L), forward primer 1 μL (10 μmol/L), reverse primer 1 μL (10 μmol/L), PrimeSTAR HS DNA polymerase 0.5 μL (2.5 U/μL), DNA extract 4 μL and DNA-free water 29.5 μL. The PCR conditions included an initial denaturing step of 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 30 sec, and a final extension step of 72 °C for 2 min. PCR products (3 μL amplified DNA plus 1.5 μL of loading dye) were fractionated on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Primers were provided by Invitrogen (Beijing, China) and all reagents by TaKaRa Biotechnology Co. (Dalian, China). PCR products were sent for sequencing to SinoGenoMax Company Ltd. (Beijing, China). Sequences obtained from one strand were examined in 4PEAKS version 1.7.2 (©2006 Mek& Tosj.com) and submitted to a BLAST search (Madden, 2002) to be compared with entries in GenBank sequences in order to identify the species for each sample (Zhang et al, 2000). Sequence matches with an E-value equal to 0.0 and maximum identity value ≥90%, were considered positive species identifications (DeMatteo et al, 2014; Keehner, 2009; Naidu et al, 2011; Rozhnov et al, 2011). In addition, a phylogenetic tree was constructed with reference species (downloaded from GenBank with accession numbers: KJ637144, JQ003577, AB303951, AB194817, EF689046, AY928671, KF990330, EF551002, AF053050, JF357970, JF357968, EF551004, KF661088) to double-check species identification, using the neighbor-joining algorithm based on the Kimura 2-parameter model (Kimura, 1980) with MEGA v6.06 (Tamura et al. 2013). Node support was evaluated using 1 000 bootstrap replicates.

Variables associated with species identification

The genetic species identification results were used to calculate field identification error for snow leopard feces (i.e. the percentage of scats that were misidentified as snow leopard in the field). A confusion matrix (or contingency table) was built to summarize the results of the misidentification for all collected samples belonging to the carnivore guild.

A one-way ANOVA test was used to look for correlation between the presumed snow leopard samples (categorical independent variables) and the morphological variables

(continuous dependent variables) associated with the scat as previously done by Anwar et al (2011). For those variables that were found to be significant, a post-hoc test of least significant difference (*LSD*) to explore all possible pair-wise comparisons of means comprising a factor using the equivalent of multiple *t*-tests.

To analyze the relationship between the variables associated with the presumed snow leopard scat and the actual species, principal component analysis (PCA) was used. This method is a true eigenvector-based multivariate analysis, which reduces the effective dimensionality of a multivariate data set by producing linear combinations of the original variables that summarize the predominant patterns in the data (Peres-Neto et al, 2003).

All analyses were performed using software R 2.11.1 (R Development Core Team, 2013). The total number of samples included in the analysis varied as some samples had missing values (i.e. in 2009 total length, mean segment length and number of segments were not recorded) and could not be used in certain statistical tests.

RESULTS

Identification of species from scat

During the survey 100 scats were collected and were attributed to a species in the field: 51 presumed snow leopard scats, 40 presumed fox scats and 9 could not be identified (Figure 2). No wolf/dog samples or other carnivores were categorized in the field. DNA was successfully extracted, amplified and sequenced for species identification from 88% of scats and all species were categorized with maximum identity value≥90% (Appendix 1, available online). Nucleotide diversity (number of nucleotide base substitutions per site averaged over all sequence pairs within each species) was 0.005 for snow leopard, 0.009 for fox and 0.024 for wolf/dog. Samples were assigned to reference species based on pairwise distance <0.03 and node bootstrap value >90% (Figure 3; Appendix 2, available online). Unidentified scats (12 samples) could either be from species for which the primers were not appropriate

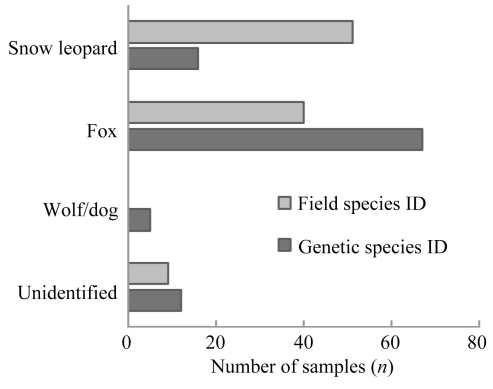

Figure 2 Species identification of scat samples ("Field species ID") in the field compared to results of genetic identification ("Genetic species ID")

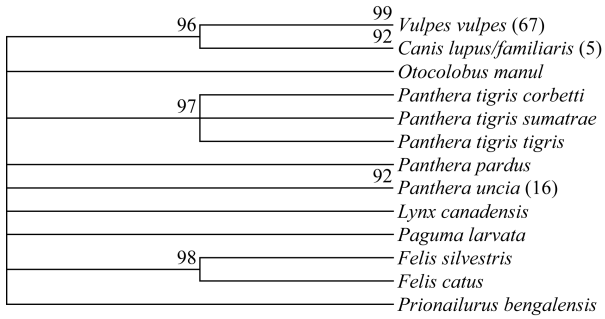

Figure 3 Condensed neighbor-joining tree with cut-off value of 90% built using Kimura 2-parameter model

(non-carnivore species) or represent samples with low DNA quality not suitable for genetic analysis. The discrepancy between species identification in the field and identification by genetic analysis is shown in Figure 2.

Among the samples identified in the field as snow leopard scats (51 samples), the majority was genetically identified as actually belonging to fox (28 samples, 55%), 4 were wolf/dog (8%), while 16 were snow leopard (31%). The remaining 3 were unidentified samples (6%). Therefore snow leopard scat identification error, calculated based on genetically verified scats for snow leopard scats in our study is 67%.

Genetic analysis showed that scats categorized as fox in the field were correctly identified in all cases, except when DNA analysis was unsuccessful (Table 2, total 40 samples, 32 confirmed fox and 8 were unidentified). A confusion matrix was built (Table 3), showing that while Type 1 errors were made in 32 occasions (scats identified as snow leopard in the field were not snow leopard scats), Type 2 errors didn't occur (snow leopard scats were not present amongst scats identified in the field as other species).

Table 2 Morphological characteristics (diameter and dry weight) for snow leopard, fox and wolf/dog.

Species	Total samples	Diameter (cm)	Dry weight (g)
Snow leopard	16	2.5±0.5	16.2±15.1
Fox	67	1.8±0.4	6.8±7.0
Wolf/dog	5	2.4±0.6	14.8±8.3

Table 3 Confusion matrix summarizing the results of 2009 and 2011 field seasons

		Predicted class				
		Snow leopard	Fox	Wolf/ dog	Unidentified	Total
Actual class	Snow leopard	16	0	0	0	16
	Fox	28	32	0	7	67
	Wolf/dog	4	0	0	1	5
	Unidentified	3	8	0	1	12
	Total	51	40	0	9	100

Type 1 errors were made (*n*=32) but no Type 2 errors were made when trying to identify snow leopard scats in the field.

Table 4 Results of snow leopard scat identification studies

Author	Country	Total samples	Snow leopard	Fox	Corsac fox	Lynx	Wolf/ dog	Unknown	ID error
Janecka	India	32	53%	19%	NP	0	6%	22%	35%
Janecka	Mongolia	27	41%	48%	NP	0	0	11%	54%
Anwar	Pakistan	95	52%	21%	3%	NP	11%	13%	40%
Karmacharya	Nepal	71	27%	42%				31%	61%
Shahzad	Mongolia	203	43%	Not analyzed					57%
Present study	China	51	31%	55%	NP	0	8%	6%	67%

Describe percentage of samples for each species and misidentification; ID error: percentage of feces that were incorrectly identified as snow leopard in the field; NP: not present in study site.

Scat morphological variables of feces and their association with species

The length of snow leopard scat samples was on average 11.0±4.3 cm, ranging from shortest 3.6 cm to longest 18.1 cm. The average diameter was 2.1±0.5 cm and the weight after drying 16.2±15.1 g. The number of segments was between 1 and 2 (2.2±1.2) with each segment having a mean length of 5.9±3.1 cm. However the values for wolf/dog scats should only be used as reference value as the low sample size does not allow for statistical accurate results.

One-way ANOVA tests showed that the diameter of scat was significantly different between snow leopard, fox and wolf/dog ($F_{(2,85)}$=18.13 and P<0.01), with significant difference between snow leopard and fox scats (*LSD* test, P<0.01) and wolf and fox (*LSD* test, P<0.01), but snow leopard and wolf/dog scats were not significantly different. In particular fox scats had a considerably smaller diameter (1.8±0.4 cm), while snow leopard and wolf/dog scats were larger and similar in size (2.5±0.5 cm and 2.4±0.6 cm for snow leopard and wolf/dog respectively) (Table 2).

Dry weight also differed significantly between species ($F_{(2,85)}$=7.99 and P<0.01). Snow leopard and fox dry weights were significantly different (*LSD* test, P<0.01) but highly variable (16.2±15.1 g and 6.8±7.0 g for snow leopard and fox respectively), while wolf/dog scats were more consistent in weight (14.8±8.3 g) (Table 2). None of the other morphological variables tested were significantly different. The first two dimensions from the principal component analysis (PC1 and PC2) represented a cumulative proportion of variance of 79% (43% and 36% respectively). Snow leopard scat samples were distributed throughout the component space and no discriminating patterns could be identified.

DISCUSSION

The high misidentification rate of snow leopard scats indicates that field identification is problematic, so erroneous conclusions on the overlapping diet of these two species and incorrect prey preference were likely to be made if our study had proceeded without first employing molecular analysis. However, it is reassuring to know that amongst the feces field-identified as fox, no snow leopard samples were found, hence valuable samples were not lost. Other researchers studying snow leopards have encountered similar issues with scat identification and their results are compared in Table 4. Overall, only a few of the total samples collected in these studies actually belong to the target species (Anwar et al, 2011; Janecka et al; 2008, Janecka et al, 2011; Karmacharya et al, 2011; Shehzad et al, 2012), raising concern for research conclusions inferred from studies that did not employ molecular analysis.

Identification error in our study decreased significantly between 2009 and 2011 (Pearson's *Chi*-squared test with Yates' continuity correction, χ^2=0.08, do=1, P=0.77), from 71% to 65% respectively, suggesting that the collector's experience may have a role in reducing error. This can be verified in the future subsequent to several sampling seasons being carried out.

Very few samples were unsuccessfully genetically analyzed with only 6% of presumed snow leopard scats unidentified (and 12% of total scats). This is likely due to the dry climate and the constant freezing temperatures of the study site that helped to preserve high quality DNA. For this study simple air-drying was sufficient for mtDNA analysis and species identification. Other studies presented in Table 4 show higher percentages of unidentified samples. This is likely due to DNA degradation that could result from differences in collection method such as different season, sun exposure, storage method etc. (Stenglein et al, 2010). Temperature, humidity and microorganisms may affect final DNA quality and have proven to be detrimental for molecular analysis of scats, so it is advisable to seek the best protocol available to prevent degradation and contamination. Studies have highlighted that silica and ethanol are two effective ways of preserving samples (Conradi, 2006; Santini et al, 2007).

Amongst the morphological variables tested, none could confidently be used as guidance to correctly discriminate snow leopard from fox or wolf scats. In common with previous results by Anwar et al (2011), statistical significance was found in scat diameter and dry weight. It was not possible, however, to define precise ranges for each species because of extensive overlap between the scat morphological values. Other variables related to the surrounding environment of the scat, such as presence of other signs, location and substrate type, may also play a role in correct species identification, and should be recorded and verified in future studies (Ma et al, 2005).

Davison et al (2002) have emphasized that scat morphological identification methods need to be more rigorous when used in surveys and suggest using multi-evidence approaches involving a variety of methods to correctly identify species presence. This is also the case for snow leopard surveys as scat misidentification can lead to confusion about the species' presence and risk of overestimating populations. It is important that research not rely solely on the identification of scats in the field, but scat identification be improved by including other techniques such as molecular analysis, scat detection dogs and camera trapping (Janecka et al, 2011; Long et al, 2007a; Long et al, 2007b;). An extensive sampling survey required to collect sufficient snow leopard scat samples can incur high financial and time costs, so an additional cost of 70¥ (about US$12) per sample for molecular analysis is a reasonable price to make these surveys more accurate. These costs will likely decrease in the future and more cost-effective analyses can be used such as real-time PCR or PCR-RFLP (Cossíos & Angers, 2006; Harrington et al, 2009; Mukherjee et al, 2010; Rodgers & Janečka, 2012).

This preliminary study confirms that there is a high rate of misidentification of snow leopard scats in the field and that morphological characteristics of scats can't be used to reliably differentiate between sympatric carnivore species. Therefore any research project that requires species identification (diet studies, sign surveys etc.) is advised to employ noninvasive DNA testing of scats in order to avoid serious bias in results. Genetic methods involve rather straightforward laboratory procedures, are relatively inexpensive and provide indisputable species identification of scats.

It is also important to note that only a small portion of samples collected actually belonged to the target species, and since small sample size is already considered to be a problem when studying elusive animals such as the snow leopard, it is important to remind researchers that they must take this into further account and plan accordingly by increasing the study area, extending duration of surveys or using multiple non-invasive sampling methods.

Finally, molecular species identification is not only a verification tool to be used in the laboratory. The results, in combination with other information on scat morphology, can also provide valuable feedback to field workers to improve collection guidelines and ultimately create effective conservation action plans.

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Effect of food restriction on the energy metabolism of the Chinese bulbul (*Pycnonotus sinensis*)

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ABSTRACT

Food resources play an important role in the regulation of animals' physiology and behavior. We investigated the effect of short-term food restriction on metabolic thermogenesis of Chinese bulbuls (*Pycnonotus sinensis*) by measuring changes in body mass, body fat, basic metabolic rate (BMR), and organ mass of wild-caught Chinese bulbuls from Wenzhou, China. Short-term food restriction induced a significant decrease in body mass and body fat but body mass returned to normal levels soon after food was no longer restricted. Food restriction caused a significant reduction in BMR after 7 days (P<0.05), which returned to normal levels after food restriction ceased. Log total BMR was positively correlated with log body mass (r^2=0.126, P<0.05). The dry masses of livers and the digestive tract were higher in birds that had been subject to temporary food restriction than in control birds and those subject to continual food restriction (P<0.001 and P<0.05, respectively). There was also significant differences in the dry mass of the lungs (P<0.05), heart (P<0.01), and spleen (P<0.05) in birds subject to short-term food restriction compared to control birds and those subject to continual food restriction. BMR was positively correlated with body and organ (heart, kidney and stomach) mass. These results suggest that the Chinese bulbul adjusts to restricted food availability by utilizing its energy reserves, lowering its BMR and changing the weight of various internal organs so as to balance total energy requirements. These may all be survival strategies that allow birds to cope with unpredictable variation in food abundance.

Keywords: Basic metabolic rate; Energy metabolism; Food restriction; *Pycnonotus sinensis*

INTRODUCTION

Migrating birds alternate between periods of short-term food restriction during flight and periods of increased food intake at stopover sites (Bairlein, 1987; Pierce & McWilliams, 2004. The capacity to sustain a period of food restriction can be affected by external factors such as food intake and ambient temperature (Kendeigh, 1945; Ni et al, 2011), or internal factors such as body mass, body composition (Blem, 2000; Liknes & Swanson, 2011a) and the amount of stored fat (Burns, 2013; Liknes et al, 2014; Sartori et al, 1995). [1]

Shallow nocturnal hypothermia, hypometabolism during periods of restricted food abundance, or fasting that induces adaptive changes in metabolism, are wide-spread facultative mechanisms for reducing energy requirements in birds (McKechnie & Lovegrove, 2002; Reinertsen, 1996), e.g., young Japanese quails (*Coturnix japonica*) (Schew & Ricklefs, 1998; Wall & Cockrem, 2009) respond by either actively decreasing their body temperature by 2 ˚C or reducing heat production by approximately 40% during fasting in thermoneutrality. Adaptive and active decreases in energy expenditure in response to food shortage can be pronounced, e.g., some galliformes can reduce their metabolic rate by 30-40% in thermoneutral conditions eventually entering torpor (Prinzinger & Siedle, 1988; Schew & Ricklefs, 1998). Such changes to heat production include changes in both physiology and metabolism (Marjoniemi, 2000). Theoretically, basal metabolic rate (BMR) is the minimum metabolic rate required for maintenance in endotherms (Swanson, 2001, 2010). It often serves as a baseline for comparisons of the metabolic costs of activities within species, and for comparisons of the "cost of living" among species or species groups (Kersten & Piersma, 1987; McNab, 1988; McKechnie & Wolf, 2004; Wiersma et al, 2007). BMR has become an important parameter for interspecific and intraspecific comparisons of energy metabolism that can indicate the relative energy consumption of different individuals

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and species, and thereby infer animals' degree of adaptation to various environment models (Liknes et al, 1996; McNab, 1997).

For homeotherm, the ability to regulate BMR, energy reserves, morphology, and organ function can be an effective way of making short-term adaptations to unpredictable, or reduced, food resources (Gebhardt-Henrich & Richner, 1998; Liknes & Swanson, 2011b; Vézina & Williams, 2003). Recent studies have shown that some bird species migrate with relatively small digestive organs and a lower digestive capacity (Bauchinger, 2002; Chediack et al, 2012; McCue, 2010; Pierce & McWilliams, 2004). One hypothetical explanation is that atrophy of these organs reduces BMR and wing loading thereby increasing flight capacity (Karasov et al, 2004). The 'digestive adaptation paradigm' (Karasov et al, 2004) suggests that, either decreasing digestive capacity, and/or down-regulating digestive tract activity to save energy, compensates for food shortage during migration by more efficient energy processing.

The Chinese bulbul (*Pycnonotus sinensis*) is a small passerine that breeds throughout central, southern and eastern China (MacKinnon & Phillipps, 2000; Zheng & Zhang, 2002). Within its natural range, the Chinese bulbul preferentially inhabits scrubland, open woodland, secondary forest, parks, gardens and villages on plains and hills up to 1 000 m a. s. l. (Zheng & Zhang, 2002). Chinese bulbuls are omnivorous, feeding primarily on arthropods (insects and spiders) and gastropods (snails and slugs) in the breeding season, but also eating fruits and seeds in autumn and winter (Peng et al, 2008; Zheng & Zhang, 2002). Chinese bulbul have been found to have higher temperature, lower BMR and a relatively wide thermal neutral zone (TNZ) (Zhang et al, 2006), as well as an obvious circadian rhythm and seasonal variations in the metabolic heat production (Zheng et al, 2008a; Zhou et al, 2010). We here test the following hypotheses: (1) during the migration, Chinese bulbul response to food shortage by the change of plasticity; and (2) the response to food deprivation is flexibility of body composition. We predict that Chinese bulbuls will show an increase in digestive efficiency and/or decreases in body mass, fat reserves and BMR during food restriction; their digestive tract mass will be adjusted in response to food availability; and the birds will regain lost condition after the period of food restriction ends.

MATERIALS AND METHODS

Animals

Chinese bulbuls were captured using mist nets in Wenzhou city, Zhejiang Province, China in May 2011. The climate is warm-temperate, with an average annual rainfall of 1 300 mm spread across all months with slightly more precipitation during spring and summer. Mean daily temperature is 18 ˚C. Body mass (to the nearest 0.1 g) was determined immediately upon capture with a Sartorius balance (model BT25S). Birds were transported to the laboratory on the day of capture, housed individually in metal cages (length×width×height, 50 cm×30 cm×20 cm) with a perch and containers for water and food and maintained at 21±1 ˚C with a 12L:12D cycle (lights on at 0600h). Food (20% crude protein, 6% crude fat, 4% crude fiber, 1% Calcium, 0.5% Lys, 0.5% Met+Cys, Jiangsu Xie Tong Biological Engineering Company Ltd.) and water were supplied *ad libitum*. Birds were acclimated to these conditions for at least 2 weeks before experiments began. All animal procedures were licensed under the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Experiment 1

In order to examine the effects of food limitation on the body mass and digestive efficiency of Chinese bulbuls, we randomly assigned 16 bulbuls to either a control (n=8) or a treatment group (n=8). Birds in the control group continued to be provided with food *ad libitum* whereas those in the treatment group were provided with 33.3% of the usual quantity of food for 7 days after which food was once again provided *ad libitum* for 8 days (Karasov et al, 2004).

Body mass and food intake were measured throughout the experiment on a daily basis; body mass was measured daily between 2000h and 2200h. Energy intake and digestibility were measured by collecting, manually separating and oven-drying (60 ˚C) food residues and feces to obtain their dry mass, after which their caloric content was determined with a C200 oxygen bomb calorimeter (IKA Instrument, Germany). Gross energy intake (GEI) (Q_{food}=14 374 kJ/g), feces energy (FE), digestible energy intake (DEI), and digestibility of energy were calculated according to Grodziński & Wunder (1975) and Ni et al (2011):

$$\text{GEI (kJ/day)}=\text{dry food intake (g/day)}\times\text{caloric value of dry food (kJ/g)} \quad (1)$$

$$\text{FE (kJ/day)}=\text{dry mass of feces (g/day)}\times\text{caloric value of dry feces (kJ/g)} \quad (2)$$

$$\text{DEI (kJ/day)}=\text{GEI (kJ/day)}-\text{FE (kJ/day)} \quad (3)$$

$$\text{Digestibility (\%)}=\text{DEI (kJ/day)}/\text{GEI (kJ/day)}\times 100\% \quad (4)$$

Experiment 2

To examine the effects of different durations of food limitation on BMR, organ mass and body fat over time, 48 bulbuls were randomly assigned to six groups (n=8 in each group). These were then assigned to the following experimental groups: (1) a control group in which birds were supplied with food and water *ad libitum*; (2) four continual food-restriction (CFR) groups in which birds were provided with 33.3% of the usual amount of food for different periods of time (i.e. 1 day, 3, 5, and 7 days); (3) a temporary food-restriction (TFR) group in which birds were provided with 33.3% of the usual amount of food for 7 days after which they were fed *ad libitum* for 8 days.

Metabolic trials

Metabolic rates of bulbuls were measured with an open-circuit respirometry system (AEI Technologies Model S-3A/I, USA). For these measurements, birds were first acclimated in darkness for 1 h inside 3.5 L plastic metabolic chambers housed inside a temperature-controlled cabinet capable of regulating temperature within ±0.5 ˚C of a defined setpoint (Artificial climatic engine BIC-300, China). Air was scrubbed of water and CO_2 before entering, and after exiting the metabolic

chamber with Drierite and Ascarite. Dry CO_2-free air was pumped through the chamber at 300 mL/min using a flow control system (AEI technologies R-1, USA) calibrated with a general purpose thermal mass flowmeter (TSI 4100 Series, USA) (McNab, 2006). The fractional concentration of O_2 in inlet chamber air was determined using an oxygen sensor (AEI Technologies N-22M, USA). Oxygen consumption rates were measured at 30±0.5 ˚C, which is within the thermal neutral zone of Chinese bulbuls (Zheng et al, 2008a). Oxygen content of excurrent air was recorded at 10 s intervals for 1 h, after the 1 h equilibration period. BMR was calculated for each individual as the average of the 30 lowest consecutive oxygen consumption recordings (about 5 min). Food was removed 4 h before each test to provide post absorptive conditions. Metabolic rates of bulbuls were calculated from equation 2 of Hill (1972), and expressed as O_2 (mL)/g/h corrected to standard temperature and pressure, dry (STPD) conditions (Schmidt-Nielsen 1997). All measurements were made daily between 2000h and 2400h.

Measurement of organ mass

Birds were euthanized at the end of the experiment and their brain, heart, lungs, liver, kidneys, gizzard, small intestine, rectum and pectoral muscle were removed and weighed to the nearest 0.1 mg. Internal organs were dried to constant mass over 2 d at 60 ˚C and weighed to 0.1 mg (Liu & Li 2006; Williams & Tieleman, 2000; Zheng et al, 2008a).

Carcasses were dried to constant mass in an oven at 60 °C, and then weighed (to 1 mg) to determine their dry mass. Total fat was extracted from the dried carcasses by ether extraction in a Soxtec 2050 Soxhlet apparatus (FOSS Instrument, Germany). Body fat content was calculated according to Dawson et al (1983) and Zhao et al (2010):

$$\text{Body fat content (\%)} = \frac{\text{total fat of carcass}}{\text{wet carcass mass (mg)}} \times 100\% \quad (5)$$

Statistical analyses

We used SPSS (version 12.0 for Windows) for all statistical analyses and considered $P<0.05$ as significant for all statistical tests. All results are expressed as means±*SE*. Distributions of all variables were tested for normality using the Kolmogorov-Smirnov test. Non-normally distributed data were log-transformed. Repeated-measures analysis of variance (RM-ANOVA) was used to determine the significance of changes in body mass. Differences among groups were determined by one-way ANOVA. The significance of differences in BMR and organ mass were determined using ANCOVA with M_b as the covariate. Fisher's least significant difference (*LSD*) *post hoc* test was used to detect which groups differed significantly. We used least-squares linear regression to test for allometric correlations between log dry organ mass and log BMR with log M_b. If allometric correlations for organ masses were significant, we calculated residuals from the allometric equations and regressed the residuals of log dry organ mass against those of log BMR to determine if the mass of specific organs was significantly correlated with BMR. If allometric correlations were not significant, we regressed raw values for log dry organ mass against log BMR to test for BMR-organ mass correlations (Zheng et al, 2013).

RESULTS

Variation in body mass

There was no significant difference in the initial body mass of the Chinese bulbuls assigned to the control, the four CFR, or the TFR, experimental groups (ANOVA, $F_{(5,30)}=0.137$, $P=0.982$, Figure 1, Figure 3A) but after acclimation a significant difference in body mass between treatment and control groups was apparent (RM ANOVA, $F_{(1,3)}=532.8$, $P<0.01$; *LSD* test, 10 day, $P>0.05$; other groups, $P<0.01$, Figure 1). There was a significant, negative, linear relationship between body mass and the duration of food restriction in the TFR group ($r^2=-0.925$, $P<0.01$, Figure 1) but the body mass of the TFR group increased to become essentially the same as that of the control 2 days after the end of food restriction ($P>0.05$, Figure 1).

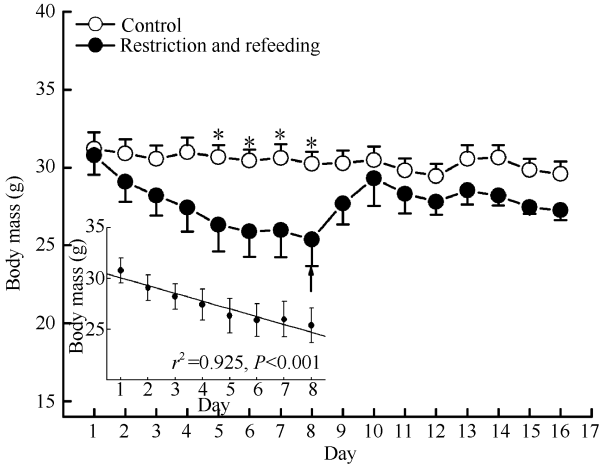

Figure 1 Relationship between body mass and food availability in the Chinese Bulbul (*Pycnonotus sinensis*)

Arrow indicates end of a 7 day period of temporary food restriction (TFR); *: $P<0.05$.

Digestibility and body fat

Digestibility of acclimatized control birds was 35.4%, 6.6%, 17.7% and 22.4% lower than that of the 1 day, 3 day, 5 day and 7 day acclimatized CFR groups, respectively. Digestibility of the 1 day and 7 day CFR groups was significantly higher than that of both the control and TFR groups (*LSD* test, $P<0.01$, Figure 2). Digestibility of the TFR group was essentially the same as that of the control group ($P>0.05$) but was significantly different to that of the 1 day, 5 day and 7 day CFR groups ($P<0.01$). In addition, comparison of digestibility of the 3 day, 5 day, 7 day CFR groups suggests that this increases with increased duration of food limitation (Figure 2).

The mean body fat content of the control group was significantly higher than those of the 3 day, 5 day and 7 day CFR groups (*LSD* test, $P<0.01$, Figure 3B) but markedly lower

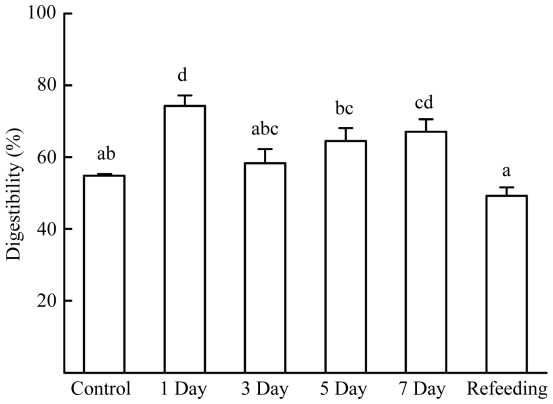

Figure 2 Differences in digestive efficiency in Chinese Bulbuls (*Pycnonotus sinensis*) subject to continuous food limitation for 1, 3, 5 or 7 days and those subject to temporary food limitation for 7 days (TFR)

Different letters labeled above each bars indicate significant differences.

than that of the TFR group (P<0.05), which was also significantly higher than that of the 3 day, 5 day and 7 day CFR groups (P<0.01, Figure 3B). Body fat content of control birds was 53.7%, 57.4% and 61.7% higher than that of the 3 day, 5 day and 7 day CFR groups, respectively, but 57.4% lower than that of the TFR group. As expected, comparison of the body fat content of the 1 day, 3 day, 5 day and 7 day CFR groups indicates a decline in body fat with increased duration of food restriction (Figure 3B).

BMR

Feeding regime and the energy intake levels significantly affected BMR. The 7 day CFR group had significantly lower unit BMR than both the control (*LSD* test, t=2.463, P<0.05) and TFR groups (P<0.05, Figure 3C), whereas no significant differences in unit BMR was found between the control and the 1 day, 3 day, 5 day, CFR groups (*LSD* test, P>0.05 in both comparisons). The unit BMR of the 1 day, 3 day, 5 day and 7 day CFR groups were 2.9%, 22.9%, 22.9% and 28.6% lower than that of the control group, respectively.

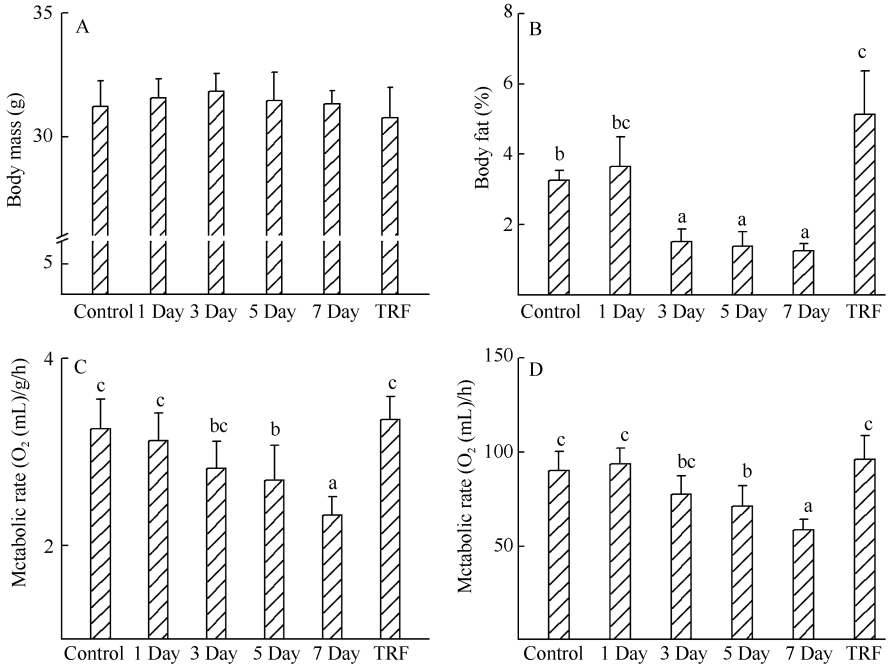

Figure 3 Differences in (A) initial body weight (P=0.982), (B) body fat, (C) total BMR and (D) unit BMR between Chinese Bulbuls (*Pycnonotus sinensis*) subject to either continual food restriction for 1, 3, 5 or 7 days and those subject to temporary food limitation for 7 days (TFR)

Different letters labeled above each bars indicate significant differences.

The total BMR of the 7 day CFR group was significantly lower than that of the control (*LSD* test, t=2.717, P<0.05) and the TFR groups (P<0.05, Figure 3D), but there were no significant differences between the BMR of the control and those of the 1 day, 3 day, and 5 day CFR groups (*LSD* test, P>0.05 in both comparisons). The total BMR of the 1 day CFR group was 12.6% higher than that of the control, but those of the 3 day, 5 day and 7 day CFR groups were 22.2%, 20.7% and 30.0% lower than the control, respectively.

As expected, comparison of total and unit BMR values of the control, 1 day, 3 day, 5 day, and 7 day CFR groups shows a decline in BMR with increasing duration of food limitation (Figure 3C, D). Total and unit BMR of the TFR group was, however, essentially the same level as that of the control group.

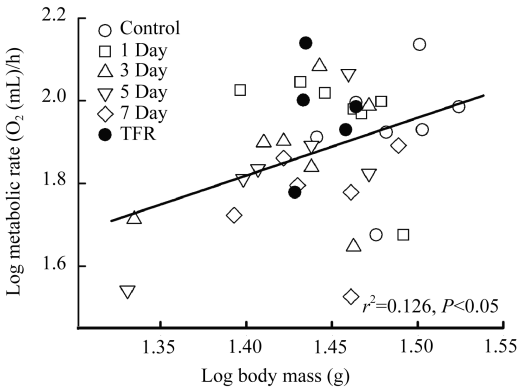

Figure 4 Relationship between log BMR and log body mass in Chinese Bulbuls (*Pycnonotus sinensis*) subject to either continual food restriction for 1, 3, 5 or 7 days or temporary food limitation for 7 days (TFR)

Log total BMR was positively correlated with log body mass (r^2=0.126, P<0.05, Figure 4).

Effects of temporary food restriction on body composition and digestive tract morphology Mass of internal organs

ANCOVA with mass as a covariate revealed marked differences in the dry mass of the lung, heart and spleen (lung, $F_{(5,33)}$=2.560, P<0.05; heart, $F_{(5,33)}$=8.019, P<0.01; spleen, $F_{(5,33)}$=2.651, P<0.05, Table 1) of different experimental groups. The control, 1 day CFR and TFR groups differed significantly in heart and lung dry mass compared to the 5 day (P<0.01) and 7 day (P<0.01) CFR groups (Table 1). Similar results were obtained for the brain, spleen, lung, and kidney but these differences were not always statistically significant. However, a comparison of the group means shows a tendency towards reduced organ mass with increasing duration of food restriction for all internal organs except the spleen (Table 1).

Table 1 Dry mass of various internal organs of Chinese bulbuls (*Pycnonotus sinensis*) subjected to either continual food restriction for 1, 3, 5 and 7 days, temporary food limitation for 7 days (TFR), or provided with food *ad libitum* (control)

Dry mass (mg)	Control	1 day	3 day	5 day	7 day	TFR	$F_{(5,32)}$	P
Brain	200.5±6.8	215.3±6.1	214.0±6.2	203.4±6.8	210.0±6.1	192.9±7.1	1.726	0.157
Heart	84.9±6.2^{a}	108.9±5.5^{b}	83.7±5.6^{a}	77.9±6.1^{a}	68.3±5.5^{a}	103.3±6.4^{b}	7.263	<0.001
Lung	48.2±3.4^{ab}	56.4±3.0^{b}	52.0±3.0^{ab}	49.0±3.4^{ab}	44.2±3.0^{a}	56.6±3.5^{b}	2.607	<0.05
Liver	333.8±20.5^{ad}	281.1±18.2^{ac}	285.4±18.6^{ac}	274.6±20.5^{ac}	268.8±18.2^{c}	394.2±21.4^{bd}	5.471	<0.001
Kidney	102.9±5.9	97.7±5.3	94.2±5.4	93.2±5.9	91.3±5.3	103.0±6.2	0.661	0.655
Spleen	8.5±2.3^{b}	10.5±2.0^{ab}	7.0±2.1^{b}	16.2±2.3^{a}	10.7±2.0^{ab}	15.5±2.4^{a}	2.829	<0.05
Stomach	138.9±9.6	142.5±8.6	134.5±8.7	123.8±9.6	126.3±8.6	157.3±10.1	1.554	0.201
Digestive tract	332.0±24.7^{a}	329.1±21.9^{a}	355.1±22.4^{a}	333.3±24.6^{a}	328.6±21.9^{a}	445.3±25.8^{b}	3.330	<0.05

Different superscripts in the same row indicate significant differences between groups.

Mass of digestive tract morphology

The stomach dry mass of the CFR groups were not significantly different to those of the control group (ANOVA, $F_{(4,29)}$=2.465, P>0.05, Table 1) but the digestive tract dry mass of the TFR group was significantly different to that of both the control and CFR groups (*LSD* test, P<0.05, Table 1). The digestive tract dry masses of the TFR group were 24.3%, 25.8%, 26.5%, 25.8% and 24.2% greater than those of the control and CFR. No other significant differences in digestive tract weight were detected. The stomach mass of birds in the 5 day and 7 day CFR groups was also significantly lighter than that of birds in the control and TFR groups but these differences ceased to be significant after stomach mass had been adjusted for differences in body mass (ANCOVA, Table 1).

Birds in the CFR groups had significantly lighter liver than those in the TFR and control groups (ANCOVA, P<0.01, Table 1). The liver dry mass of 1 day, 3 day, 5 day and 7 day CFR birds were 17.9%, 15.9%, 12.9% and 22.9% less than those of the control, whereas that of the TFR group was 12.4%, 28.2%, 26.4%, 32.2% and 32.5% greater than that of the control, 1 day, 3 day, 5 day and 7 day CFR groups respectively. Indeed, comparison of the group mean values suggested that liver mass declined with increasing duration of food restriction.

Allometric relationships between log dry organ mass and log Mb (minus organ wet mass) were positive except for the brain, but only the dry masses of the liver, heart, and lungs were significantly correlated with body mass (Table 2). No other residual organ masses were significantly correlated with BMR residuals, but there were significantly positive correlations between BMR residuals and heart and lung dry mass residuals (Table 2).

DISCUSSION

Our results indicated that food restriction significantly affected body mass, fat content and BMR, all of which decreased significantly over a 7 day period. We did not find the effect of short-term food restriction following a hyperphagic period in the current study.

Effects of food restriction on body mass and fat reserves

Seasonal changes in body mass, especially in small passerine

Table 2 Linear regression statistics for allometric and residual correlations of log dry organ mass versus log body mass (minus wet mass of the organ), and dry organ mass versus metabolic rate (BMR) in Chinese bulbuls (*Pycnonotus sinensis*) from Wenzhou City, Zhejiang Province, China

	Liver	Heart	Brain	Lung	Kidney	Spleen	Stomach	Digestive tract
Allometric correlations:								
R^2	.169	.421	.001	.273	.105	.066	.011	.014
P	<.001	<.001	.944	<.001	<.05	.116	.534	.467
Slope	.497	.948	–.006	.551	.271	.793	.101	.136
Residual correlations:								
R^2	.050	.125	.007	.145	.088	.029	.138	.003
P	.171	<.05	.603	<.05	.066	.298	<.05	.732
Slope	.129	.242	–.023	.199	.124	.270	.185	.033

birds, are considered to be an essential adaptive strategy for survival (Cooper, 2000; Pendergast & Boag, 1973). More recent studies of migrating birds have shown that appreciable amounts of protein and fat may be stored to be used during migration (Bordel & Haase, 2000; Karasov & Pinshow, 1998; Liknes et al, 2014; Piersma & Lindström, 1997). Ecological field studies have revealed that migrants arriving at stopover sites recover body condition slowly for one to two days after which there is a much more rapid increase in body mass (Gannes, 1999; Hume & Biebach, 1996; Langslow, 1976; Lindström, 1995; Pierce & McWilliams, 2004; Rappole & Warner, 1976; Yong & Moore, 1997).

We found that bulbuls subject to restricted food intake for 7 day were 16.2% lighter than control birds. Body weight increased rapidly after food restriction was lifted but was still 6.4% lower than that of controls after 6 days. There was a significant linear relationship between body mass and the duration of food restriction and the body fat content of control birds was 53.7%, 57.4% and 61.7% higher than that of 3 day, 5 day and 7 day CFR birds, respectively.

Many studies have documented similar changes in fat levels in birds. For example, Karasov & Pinshow (1998) found that every gram of body mass lost by fasting blackcaps (*Sylvia atricapilla*) was mainly accounted for by fat loss. Pierce & Williams (2004) found that there was a significant difference in the body mass of *Zonotrichia albicollis* on a restricted diet compared to controls. This suggests that birds must rebuild fat reserves during migration stopovers. In any case, there is considerable evidence to suggest that body mass regulation is a very important adaptation to unpredictable food availability (Ekman & Hake, 1990).

Effect of food restriction on BMR

The regulation of energy metabolism is the main means by which birds maintain energy balance (Marsh & Dawson, 1989). The most prudent and tentative conclusion is that, in order to maintain energy balance, many resident and migratory birds lower their BMR in response to a decrease in ambient food. Such variation in BMR may reflect species, and environment specific, evolutionary survival strategies (McKechnie & Wolf, 2004). As in many previous studies, our results showed that the BMR of birds subject to food restriction was about 20% less than that of control group. There was also a trend toward declining BMR with increasing duration of food restriction. The BMR of TFR birds was essentially the same as that of the control group after food restriction ceased. Lindström (1995) also found that BMR decreased during food restriction and returned to the level of the control in a species of migratory passerine. Prinzinger & Siedle (1988) reported that house martin (*Delichon urbica*) nestlings' metabolic rate decreased in response to food shortage. We also found that bulbuls subject to food restriction decreased their energy expenditure in order to maintain body mass. Within 8 days from the beginning of food restriction, a clear change in behavior was evident; bulbuls reduced movement to decrease heat loss. Schew & Ricklefs (1998) argue that there is an adaptive and active decrease in energy expenditure in response to food restriction. In addition, most bulbuls probably do face major changes in food abundance each year. Our results support the view that, 'despite the potential costs involved in exhibiting physiological flexibility (DeWitt et al, 1998), metabolic flexibility is a basic trait of a bird, not a result of evolutionary adaptation in populations in cold areas' (Klaassen et al, 2004).

Effects of food restriction on digestibility and digestive organs

In birds, the size of the digestive tract is likely to be limited by constraints associated with migration and/or flying. Change in digestive organs in response to temporary food restriction has been observed in many birds (Karasov & Pinshow, 2000) and may help maintain digestive efficiency and the body's energy reserves (Karasov & Pinshow, 2000; Starck & Rahmaan, 2003). Because a large digestive system requires increased energy to function, short-term food deprivation and migration may cause atrophy of the digestive tract (McWilliams & Karaso, 2001). The ability to reduce the size of the digestive tract is one of the ways by which birds successfully meet the conflicting physiological challenges of migration.

We found that bulbuls in the CFR groups had higher digestive efficiency than those in the control and TFR groups

(maximum 35.4%，minimum 6.6%). Digestibility of bulbuls in the 1 day and 7 day CFR groups was significantly higher than that of those in the control and TFR groups. However, there was no significant difference in digestibility of the control and TFR groups, probably because that of the TFR group returned to normal levels after food limitation ceased. There was no significant difference in the digestive tract and stomach dry mass of the CFR and control groups. Although, ANOVA indicated significant differences in the digestive tract mass of different experimental groups these disappeared when adjusted for differences in body mass. We conclude that bulbuls may enhance their digestive efficiency to compensate for reduced digestive organ mass during migration (Pierce & McWilliams, 2004) and when food is limited. This phenotypic plasticity and flexibility of the digestive tract is of vital significance in the life history of birds.

Effects of food restriction on body composition

Chinese bulbuls that were subject to food restriction had lighter livers than control birds, and liver, kidney, heart, lung mass generally declined with increased duration of food restriction. Similar changes in digestive organs associated with food restriction and migration have been observed in other birds like yellow-rumped warblers (*Setophaga coronate*), Semipalmated Sandpiper (*Calidris pusilla*), yellow-legged gulls (*Larus michahellis*), Songbird (*Zonotrichia albicollis*) (Lee et al, 2002; McWilliams & Karasov, 2005; Piersma & Gill, 1998; Pierce & McWilliams, 2004). This suggests that food deprivation has a profound effect on the body composition and digestive organs of birds. The liver is the largest and most important organ involved in metabolic activity in homeothermic animals (Villarin et al, 2003). Our results suggest that bulbuls slowly reduce the weight of the liver as the duration of food limitation increases. This may be an adaptive strategy for migration conferring the benefits of reduced metabolic rate and/or energy consumption. Interestingly, the size of the liver, kidneys, heart, spleen, stomach, digestive tract and lungs of the TFR group was greater than those of both the control and CFR groups, suggesting that organs necessary for flight may increase in size before departing a stopover site.

In conclusion, our results support the conclusions of previous research showing that body mass declines in response to food deprivation. When food is limited, bulbuls consume energy reserves, reducing body weight and body fat in order to maintain essential metabolic functions. In addition, they reduce energy consumption by reducing their basal metabolic rate and enhance digestive efficiency to compensate for reduced digestive organ mass. When adequate food resources once again become available all these parameters quickly return to normal. All these changes are of obvious adaptive benefit to a species that experiences marked variation in food availability. Along with the global climate warming, chinese bulbuls have showed a general adaptability to the shortage of food with a tendency to spread to the north.

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Diversity and habitat association of small mammals in Aridtsy forest, Awi Zone, Ethiopia

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ABSTRACT

Here, we conducted a survey to examine the diversity, distribution and habitat association of small mammals from August 2011 to February 2012 incorporating both wet and dry seasons in Aridtsy forest, Awi Zone, Ethiopia. Using Sherman live traps and snap traps in four randomly selected trapping grids, namely, natural forest, bushland, grassland and farmland, a total of 468 individuals comprising eight species of small mammals (live traps) and 89 rodents of six species (snap traps) were trapped in 2352 and 1200 trap nights, respectively. The trapped small mammals included seven rodents and one insectivore: *Lophuromys flavopuntatus* (30.6%), *Arvicanthis dembeensis* (25.8%), *Stenocephalemys albipes* (20%), *Mastomys natalensis* (11.6%), *Pelomys harringtoni* (6.4%), *Acomys cahirinus* (4.3%), *Lemniscomys zebra* (0.2%) and the greater red musk shrew (*Crocidura flavescens*, 1.1%). Analysis showed statistically significant variations in the abundance and habitat preferences of small mammals between habitats during wet and dry seasons.

Keywords: Abundance; Aridtsy forest; Awi Zone; Diversity; Distribution; Small mammals

INTRODUCTION

Varied climatic conditions helped create a diversity of ecosystems in terms of climate, topography and vegetation, which are home to a large number of endemic species. On a global scale, Wilson & Reeder (2005) estimated some 5 416 mammalian species, of which a large number were small mammals. To date, rodentia remains the largest order of mammals in the world comprising 2 277 species 41% of which the small mammal species, 481 Genera and 33 Families (Wolff & Sherman, 2007). Insectivore fauna are also diverse, having 429 species worldwide, of which 312 are shrews, 140 of which are found in East Africa (Kingdon, 1997). Small mammals such as rodents and insectivores are highly mobile animals whose distribution is influenced by the altitude and vegetation types (Mulungu et al, 2008; Prakash & Sing, 2001) as well as human disturbance (Liu et al, 2008) and the presence of large mammals (Hoffmann & Zeller, 2005), whose intensive grazing degrades the land and makes it uninhabitable for rodents because of loss of cover and food (Baker et al, 2003; Vieira, 2003; Liu et al, 2008). Rainfall also plays a significant role in the occurrence of high population of rodents during the wet season (Linzey & Kesner, 1997; Prakash & Sing, 2001; Tadesse & Afework, 2008). [1]

Previous reports of high faunal biodiversity in Ethiopia highlight the existence of a large number of species of mammals and other higher vertebrates (Jacobs & Schloeder, 2001; Melaku, 2011; Yalden & Largen, 1992). In line with global trends, of the 284 mammal species of Ethiopia, a large share (84, 29.6%) of species are rodents (Afework & Leirs, 1997). Out of the total rodent species of the country, the endemic rodent species comprise 21% (Afework, 1996), which make up nearly half of the endemic mammals of Ethiopia (Afework & Corti, 1997). Endemicity itself is associated with particular faunal regions or habitat types (Yalden & Largen, 1992), but despite numerous reports on the small mammals of Ethiopia (Afework & Corti, 1997; Afework & Leirs, 1997; Yalden et al, 1976; Yalden & Largen, 1992), no attempt was made to investigate the population status and habitat association of small mammals in Aridtsy forest in Awi Zone. Accordingly, in the present study we report on a survey undertaken aimed at collecting data on the diversity, distribution and habitat association of rodents and shrews in this largely unexplored habitat.

MATERIALS AND METHODS

Study area

This study was conducted in Aridtsy Forest in Awi Zone,

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northwestern Ethiopia, Amhara Regional State. Aridtsy Forest is located in Ankesha Guagusa Woreda, about 30 km from the main administrative Zone in Injibara). The area coverage of the forest is around 127 ha, and it is a natural forest. The study area of our survey was located between N10°43'40"—10°44'20" and E36°46'40"—36° 48'0" (Figure 1). The maximum and minimum temperature and the mean annual rainfall near the study site at Ayehu agricultural development station are collected by the Ethiopian National Meteorology Agency. The climatic condition of the study area is in the warm agro-climatic zone. This area has one long rainy season, mainly from early May to mid November, and a dry season from January to April. This particular seasonal difference explains the large variations in average rainfall, with 6.47 mm in February and 240.84 mm in August. In aggregate, annual rainfall of the study area is 2 890.1 mm. Mean monthly temperature also vary, ranging form 9.7 to 32.0 ℃. Suitable agro-climatic conditions allow for the production of different commercial and food crops in the area.

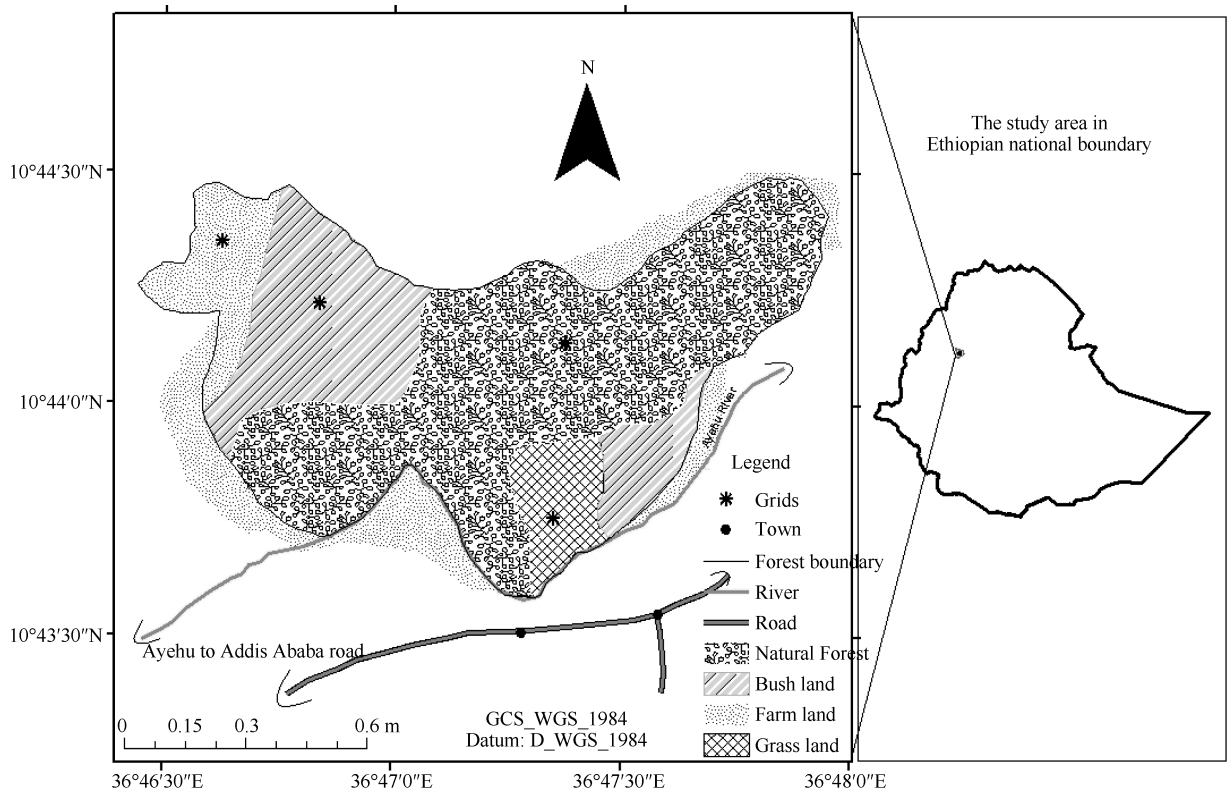

Figure 1 Map of the study area

Methods

Following the preliminary survey, continuous field work on ecological study of small mammals in the study area was carried out during August 8, 2011 to February 3, 2012, covering both wet and dry seasons. Based on the topography and habitat type, four sampling plots were selected, one for each habitat type. The inclusions of the four randomly selected habitats was based on different criteria, including altitudinal differences and vegetation cover were natural forest, bushland, grassland and farmland. In each sampling at each locaiton, permanent trapping stations at 10 m intervals square grid were placed at randomly selected plots and 25 snap traps at an interval of 20 m square grid were used at least 200 m away from the live-trapping sites. Both Sherman live-traps and snap-traps were utilized during the study period, in order to better sample small mammals specifically able to be captured by Sherman live-traps and snap-traps (Lee, 1997).

In each habitat type, a 3 600 m^2 (0.36 ha) live-trapping grid was established. In total, 49 live traps were set over an area of 60 mx60 m positioned at 10 m intervals for each grid. Trapping stations were marked with red plastic tap approximately one meter above the traps. The traps were baited by peanut butter and checked twice daily, early in the morning hours (0600h—0800h) and late in the afternoon hours (1700h—1800h) and rebaited as necessary. For each individual trapped, the grid and trap station number, body mass and sexual conditions were recorded and a toe-clipping was performed. Furthermore, trapped specimens were also distinguished as adults, subadults and juveniles on the basis of weight, pelage colour (which is usually very grey in juveniles). Reproductive condition of males was assessed via examination of scrotal and abdominal testes, while for females conditions were recorded including being

pregnant or with suckling nipples, perforate or imperforate vagina. All live trapped individuals were released with the same site as they were trapped.

Species identification was carried out based on the taxonomic characteristics listed in Nowak (1999), Yalden et al (1976) & Kingdon (1997). Additionally, when species identification was difficult in the field; skin and skull measurements were prepared and compared with the specimens deposited in the Zoological Natural History Museum of Addis Ababa University. Dissection was carried out on the snap trapped specimens for stomach content analysis and in pregnant females for embryo count. Stomach contents were removed and preserved in 5% formalin for diet analysis. The stomach contents were spread onto a petridish and mixed thoroughly, after which the contents were sieved through 0.25 mm sieve, washed by distilled water to remove finely digested food and fine particles for proper identification of the remaining parts and dried in open air for a day. For each sample, four slides were prepared and the contents were put on a glass slide to observe the type and proportion of food items under a compound microscope. The food items were grouped into plant matter (leaves, roots, and seed), animal matter (earthworms and arthropods) and unrecognizable items. From the entire slide, the particles were counted, then summed up and converted to the mean percentage for each sample.

Data analysis

Abundance of small mammals in each habitat was assessed by the percentage of trap success between the seasons. The Shannon-Weiner diversity index was used to estimate the diversity for the small mammals trapped in the study area. In addition, a *Chi*-square test was used to interpret variations of small mammal species in different trapping seasons and grids. Habitat association of small mammal species (rodents and insectivores) was also analyzed using *Chi*-square test. All statistical data were analyzed using SPSS 15.0 (SPSS inc., Chicago, IL, USA).

RESULTS

Results of this study revealed the presence of eight species of small mammals in the study area, of which seven species were the following rodents: *Lophuromys flavopuntatus*, *Arvicanthis dembeensis, Stenocephalemys albipes*, *Mastomys natalensis*, *Pelomys harringtoni*, *Acomys cahirinus* and *Lemniscomys zebra*. The eighth species was an insectivore, the greater red musk shrew (*Crocidura flavescens*). A total of 2 352 trap nights yielded 468 live-trapped individuals. Among 468 live-trapped individuals, 372 individuals were new captures and 96 individuals were recaptures (Table 1). Porcupine (*Hystrix cristata*), bush squirrel (*Parexerus flavovittis*) and hyraxes (*Procavia capensis*) were observed throughout the study period, but not trapped. In addition, indirect evidences of mound of *Tachyoryctes splendens* was observed in study site, especially in the grassland habitat.

Arvicanthis dembeensis, A. cahirinus and *C. flavescens* were not captured from the natural forest both during wet and dry seasons. *Lemniscomys zebra* was exclusively captured from the maize farm during the second dry season. *Lophuromys flavopunctatus*, *S. albipes* and *M. natalensis* were recorded from all habitat types during both seasons (Table 2). *Lophuromys flavopunctatus* was the most abundant species in the bushland habitat, followed by *S. albipes*. *Arvicanthis dembeensis* being the most abundant rodent in the grassland habitat.

There was a significant difference in the number of *L. flavopunctatus* trapped between habitat types (χ^2=42.49, *df*=3, *P*<0.05), *A. dembensis* (χ^2=69.76, *df*=3, *P*<0.05), *S. albipes* (χ^2=40.7, *df*=3, *P*<0.05), *M. natalensis* (χ^2=22.95, *df*=3, *P*<0.05) and *P. harringtoni* (χ^2=12.34, *df*=3, *P*<0.05), but the captures of *A. cahirinus* (χ^2=6.5, *df*=3, *P*>0.05), *L. zebra* (χ^2=3, *df*=3, *P*>0.05) and *C. flavescens* (χ^2=2, *df*=3, *P*>0.05) were

Table 1 Species composition and relative abundance (%) of live-trapped small mammals in wet and dry seasons in the study area (figures in parenthesis are recaptures)

Family	Species	Wet	Dry	Total catch	Relative abundance (%)
Muridae	*Lophuromys flavopuntatus*	69	45	114 (29)	30.6
	Arvicanthis dembeensis	42	54	96 (25)	25.8
	Stenocephalemys albipes	54	20	74 (18)	20
	Mastomys natalensis	37	6	43 (11)	11.6
	Pelomys harringtoni	19	5	24 (7)	6.4
	Acomys cahirinus	-	16	16 (6)	4.3
	Lemniscomys zebra	-	1	1	0.2
Soricidae	*Crocidura flavescens*	3	1		1.1
Hystricidae	*Hystrix cristata*			*	*
Sciuridae	*Parexerus flavovittis*			*	*
Procaviidae	*Procavia capensis*			*	*
Rhizomyidae	*Tachyoryctes splendens*			*	*
Total				372 (96)	100

*: Observed species.

Table 2 Seasonal Species composition, distribution and abundance of live- trapped small mammals from different habitats with both wet and dry seasons

Species	Individuals trapped from different habitats during wet and dry seasons								
	NF		BL		GL		FL		
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Total
Lophuromys flavopunctatus	24	15	31	20	9	8	5	2	114
Arvicanthis dembeensis	–	–	4	5	21	30	17	19	96
Stenocephalemys albipes	13	5	31	10	4	2	6	3	74
Mastomys natalensis	3	1	6	3	4	2	24	–	43
Pelomys harringtoni	6	1	9	3	4	1	–	–	24
Acomys cahirinus	–	–	–	7	–	4	–	5	16
Lemniscomys zebra	–	–	–	–	–	–	–	1	1
Crocidura flavescens	–	–	1	–	1	–	1	1	4
Total	46	22	82	48	43	47	53	31	372
Total/season	68		130		90		84		

–: Absence of trapped individuals; NF: Natural forest; BL: Bushland; GL: Grassland; FL: Farmland.

not statistically significant. The natural forest had the least number of species, while Bushland, grassland and farmland habitats shared similar abundance, all with seven species. There was no significant variation in species abundance among habitats (χ^2=1.08, *df*=3, *P*>0.05). As expected, the number of individuals trapped during the wet season was significantly higher than those trapped during the dry season (χ^2=15.52, *df*=1, *P*<0.05).

The distribution of species varied between the wet and dry seasons in all habitat types. The difference between habitat preference of small mammals during the wet and the dry seasons (Table 2) was statistically significant (χ^2 =20.04, *df*=3, *P*<0.05 and χ^2=13.02, *df*=3, *P*<0.05, respectively), with variation in species diversity between habitat types. Species diversity indices of small mammals were relatively high in farmland and low in natural forest. The result of Shannon-Weiner Index (H') for the species diversity from highest to least was 2.34, 1.55, 1.37 and 1.07 for farmland, bushland, grassland and natural forest, respectively. The variation in species diversity across the different habitat types not statistically significant (χ^2=0.56, *df*=3, *P*>0.05).

Results also showed a marked variation in richness index of small mammal species along with habitat types (Table 3). The highest species richness index was registered by *L. flavopunctatus* with RI value of (0.82) from forest habitat and the lowest by *C. flavescens* (8.7) from the farmland habitat. Among the four habitat types, farmland comprised the lowest species richness index, while both bushland and grassland had the highest value of species richness index; for example, the highest species richness of *A. dembeensis* was obtained from farmland (1.7) and the least was from the bushland (2.41).

During the wet season, more pregnant rodents were trapped (65.8%). One *L. flavopuntatus* gave birth to four youngs inside the trap during the second wet season. A greater number of (25 individuals) pregnant females were caught during the wet season than during the dry season (13 individuals), explaining the significant variation between seasons (χ^2=3.78, *df*=1, *P*<0.01). Males contributed for higher number from the total catch for all species than females, and each species had a higher proportion of males from all seasons and habitats, except for *L. zebra* (no captured male).

Table 3 Species richness index (RI) and Simpson's similarity index (SI) of small mammal species in the study area among the habitat types (cumulative for wet and dry seasons)

Species	Species richness index of small mammals from different habitat types				
	NF	BL	GL	FL	SI
Lophuromys	0.82	1.53	2.12	3.1	
Arvicanthis	–	2.41	1.53	1.7	
Stenocephalemys	1.04	1.62	3.35	2.7	
Mastomys natalensis	2.2	2.73	3.35	1.9	
Pelomys harringtoni	1.54	2.41	3.73	–	
Acomys cahirinus	–	3.08	4.33	3.7	
Lemniscomys zebra	–	–	–	0	
Crocidura flavescens	–	0	0	8.7	
Species richness (S)	4	7	7	7	0.48

NF: Natural forest; BL: Bushland; GL: Grassland; FL: Farmland.

Dietary information was obtained from six snap-trapped rodent species, *Lophuromys flavopunctatus* with seventeen specimens, *Arvicanthis dembeensis* with twelve specimens, *Mastomys natalensis* with seven specimens, *Stenocephalemys albipes* with eleven specimens, *Pelomys harringtoni* with twelve specimens and *Acomys cahirinus* with seven specimens, which is presented in Table 4. In the present study, plant matter was seen in the diets of all species. Animal matter in the stomach contents of rodents averaged (7.8±9.7) and plant matter

averaged (12.9±8.2) in all observed species of rodents. The remaining unidentified food items were averaged (5.9±3.9).

Overall, the highest percentage of animal matter was found in the diet of *L. flavopunctatus* during the wet season. However, plant matter, particularly grasses were the major type of food item in the diet of *A. dembeensis. Mastomys natalensis* preferred plant matter the major type of food item in the entire study. Stomach contents of *S. albipes* consisted with highest percentage of grasses, monocot leaves and dicot seeds, which form a major part of their diet during both wet and dry season. A higher percentages of animal matter was found in the diet of *A. cahirinus* during the wet season. Among the species, there was significant variation in types and relative amounts of food items in the stomach sample (χ^2=1262.74, *df*=2, *P*<0.05). Statistically, there was also significant variation in the proportion of food items consumed by the rodents between seasons (χ^2=3.9, *df*=1, *P*<0.05). Statistical test illustrate a significant difference of food items between each species, *L. flavopunctatus* (χ2=83.94, *df*=5, *P*<0.05), *A dembeensis* (χ^2=338.79, *df*=5, *P*<0.05), *M. natalensis* (χ^2=217.6, *df*=5, *P*<0.05), *S. albipes* (χ^2=207.5, df=5, *P*<0.05), *P.harringtoni*(χ^2=273.6, *df*=5, *P*<0.05),*A. Cahirinus* (χ^2=141.3, *df*=5, *P*<0.05).

Table 4 Percentage of food items of six snap-trapped rodent species in the stomach contents collected during wet and dry seasons

Species	Food items (%)									
	Season	Ml	Ms	Dl	Ds	Ro	Gr	E	A	U	H
Lophuromys flavopunctuats	Wet	5	2.5	10.6	5.9	2.9	11.5	30.2	29.8	1.9	*
	Dry	6.5	11.8	5.4	10.8	7.5	9.7	8.6	35.5	4.3	–
Arvicanthis dembeensis	Wet	11.5	10.7	6.1	22.1	3.1	42.7	1.5	–	2.3	–
	Dry	16.3	8.8	8.2	18.4	5.4	36.1	2	0.7	4.1	–
Mastomys natalensis	Wet	16.3	12.5	7.7	28	3.8	10.6	7.7	4.8	8.7	*
	Dry	16	10.6	19.1	25.5	6.4	8.5	4.3	2.1	7.4	–
Stenocephalemys albipes	Wet	9.2	18.3	7.1	12.2	4.1	28.6	5.1	7.1	8.2	*
	Dry	5.3	20	4.2	21.1	6.3	26.3	4.2	3.2	9.5	
Pelomys harringtoni	Wet	17.3	16.7	13.3	23.7	10.3	10.7	–	4	4	*
	Dry	13.8	20	6.2	24.6	3.1	16.9	–	1.5	13.8	–
Acomys cahirinus	Wet	–	–	–	–	–	–	–	–	–	–
	Dry	20.2	16.7	14.3	21.4	6	10.7	–	3.6	7.1	–

Ml: Monocot leaf; Ms: Monocot seed; Dl: Dicot leaf; Ds: Dicot seed; Ro: Root; Gr: Grass; E: Earthworm; A: Arthropods; U: Unrecognized food items; H: Hairs; *: Observed hairs; –: Absence.

DISCUSSION

One interesting result of the present survey was that although the total capture included eight species of small mammals, only 1.1% of all were shrews. The type of traps used (Sherman)—as opposed to pitfall traps—may be one of the key reasons for the capture of low number of shrews. Similarly, Fichet-Calvet et al (2010) only collected a few species of shrews from Upper Guinea. Some other variations are also worth noting. For example, our results show a largely male biased sex ratio, likely attributable to the males traveling over greater distances. Such assumptions are consistent with those made by Hansson (1978), making them more likely to be trapped, as well as results from both Smith et al (1975) & Tilahun et al (2012) who have recorded higher capture frequency of males. Another variation worth noting is that *Lophuromys flavopunctatus* is one of the most common rodents in the moist areas of East Africa (Clausnitzer & Kityo, 2001) and in the Magamba Forest Reserve in Tanzania (Makundi et al, 2007). Here, *Lophuromys flavopunctatus* was commonly captured in all grids, but was predominate in bushland habitat. This distribution may be due to the species' diverse feeding habits, a highly adaptable feature that allows it to inhabit a wide range of habitats.

Seasonal variations were also seen in terms of overall capture numbers. For example, *A. cahirinus* and *L. zebra* were not captured during the wet season, potentially due to the availability of food, or changes in seasonal diet that may include arthropods, seeds and snail in their natural habitat during the wet season, which may limit their capture. This result is not unexpected; studies have shown seasonal variation and availability of food results less trap success (Kronfeld-Schor & Dayan, 1999). This is also consistent with our survey, wherein the rarest rodent species captured area was *L. zebra,* as well as earlier reports (Tadesse & Afework, 2008) in which only few individuals were trapped out of a total captures from Alatish National Park, Ethiopia. Meanwhile, across all habitats, *M. natalensis* was trapped, but was most abundant in the farmland and less abundant in forest, potentially due to the preference for crop fields. Similarly Massawe et al (2005) revealed that agricultural activities increase the abundance of *M. natalensis.*

Overall, more individuals were caught in bushland habitat as compared to the other habitats, likely as a result of the habitat's composition of plants such as, *Pterolobium stellatum*, *Capparis tomentosa* and *Urtica simensis*, which are thorny, and relatively prevent movement of humans and livestock, thereby resulting in more shelter for small mammals. Similarly, food and

adequate cover influence diversity of rodents (Tadesse & Afework, 2008).

The Forest habitat contributed for the least number of small mammals compared to other habitats, likely because the habitat is quite steep as compared to other habitats. This topography directly results in flooding during the wet season, thereby reducing ground cover. Bennett (1990) stated that, clearing and fragmentation of the natural forest vegetation have had a marked impact on the small mammal fauna. The lowest species richness in the natural forest habitat is mostly due to the absence of small mammals typical of other habitats, including *A. dembeensis*, *A. cahirinus*, *L. zebra* and *C. flavescens*. The high species richness and diversity of small mammals in the farmland contradicts with the findings of Aplin & Singleton (2002), who noted that agricultural landscapes are structurally simple and contain relatively low biotic diversity.

The observations made in the current study suggest that breeding of most of the small mammal species in the study area was during the wet season, which is consistent with several earlier investigations (Prakash & Sing, 2001; Tadesse & Afework, 2008) reported that that reproduction is often linked with the rainy season and with the availability of sufficient resources for rearing the young. Moreover, several studies suggest that seasonal variations in weather, particularly rainfall, influences the nutritional aspects, which affects the life strategies of rodents (Makundi et al, 2007). Indeed, Linzey & Kesner (1997) reported that rainfall could indirectly govern reproductive success by affecting the supply of insects.

Stomach content analysis showed a variety of food items consumed by rodents, illustrating both omnivorous and granivorous feeding habits. *Lophuromys flavopunctatus* had more animal matters, consistent with previous reports by Barnett et al (2000). This situation is related to the type of habitat they live in and abundance of prey species in the vicinity. For example, consumption of animal matter was highest during the wet season, potentially due to the formation of a suitably moist environment allowing for an abundance of arthropods and worms, which consistently increase the relative abundance of small mammals during the wet season. Significant differences in consumption of food items were previously observed among rodent species and seasons, such as Ellis et al (1998) in Argentina. Conversely, plant matters were consumed by all species throughout the entire study periods. *Arvicanthis dembeensis* highly preferred grass as a major food item. Workneh et al (2006) from Maynugus irrigation field, northern Ethiopia also revealed similar findings. *Mastomys natalensis* showed a relatively a high percentage of dicot seed in their stomach contents during entire the study period, but it consumed all analyzed food items. According to Massawe et al (2005), opportunistic behavior enables *M. natalensis* to take advantage of changes in habitats, particularly in relation to food resources. During the present study, out of the total species captured, *S. albipes* and *P. harringtoni* were endemics of Ethiopia.

Overall, the present results of our survey indicate that variations in small mammal abundance occurred not only in terms of habitat, but also in terms of season. These results suggest that all analyzed rodent species seem to be near generalists in relation to diet composition, containing both animal and plant materials.

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ZOOLOGICAL RESEARCH

Energy intake, oxidative stress and antioxidant in mice during lactation

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ABSTRACT

Reproduction is the highest energy demand period for small mammals, during which both energy intake and expenditure are increased to cope with elevated energy requirements of offspring growth and somatic protection. Oxidative stress life history theory proposed that reactive oxygen species (ROS) were produced in direct proportion to metabolic rate, resulting in oxidative stress and damage to macromolecules. In the present study, several markers of oxidative stress and antioxidants activities were examined in brain, liver, kidneys, skeletal muscle and small intestine in non-lactating (Non-Lac) and lactating (Lac) KM mice. Uncoupling protein (ucp_s) gene expression was examined in brain, liver and muscle. During peak lactation, gross energy intake was 254% higher in Lac mice than in Non-Lac mice. Levels of H_2O_2 of Lac mice were 17.7% higher in brain (P<0.05), but 21.1% (P<0.01) and 14.5% (P<0.05) lower in liver and small intestine than that of Non-Lac mice. Malonadialdehyde (MDA) levels of Lac mice were significantly higher in brain, but lower in liver, kidneys, muscle and small intestine than that of Non-Lac mice. Activity of glutathione peroxidase (GSH-PX) was significantly decreased in brain and liver in the Lac group compared with that in the Non-Lac group. Total antioxidant capacity (T-AOC) activity of Lac mice was significantly higher in muscle, but lower in kidneys than Non-Lac mice. Ucp_4 and ucp_5 gene expression of brain was 394% and 577% higher in Lac mice than in Non-Lac mice. These findings suggest that KM mice show tissue-dependent changes in both oxidative stress and antioxidants. Activities of antioxidants may be regulated physiologically in response to the elevated ROS production in several tissues during peak lactation. Regulations of brain ucp_4 and ucp_5 gene expression may be involved in the prevention of oxidative damage to the tissue.

Keywords: Antioxidant; Energy intake; Lactation; Metabolic rate; Oxidative stress; Uncoupling protein

INTRODUCTION

Reproduction is the highest energy demand period for almost all mammals, during which sustained energy intake and expenditure are suggested to reach a ceiling, indicating that limited energy resources could be allocated between competing demands for reproduction and somatic protection (Speakman, 2008; Speakman & Garratt, 2014; Speakman & Król, 2005; 2011). Oxidative stress is suggested as a possible physiological cost of somatic protection that may limit investment on reproduction (Dowling & Simmons, 2009; Salmon et al, 2001; Selman et al, 2000; Xu et al, 2014). Oxidative stress life history theory proposed that free radicals, more specifically reactive oxygen species (ROS), were produced in direct proportion to metabolic rate as a consequence of the molecular functioning of mitochondria and the electron transport chain (Monaghan et al, 2009; Speakman & Garratt, 2014). An increase in metabolic rate in parallel with reproduction therefore leads to an elevation of ROS production (Dowling & Simmons, 2009; Selman et al, 2012; Speakman & Selman, 2011). If this is the case, there may be a trade-off in energy allocation between the investment in offspring growth and in physiological antioxidant against the elevated ROS levels. [1]

However, data from several studies performed recently were less consistent and somewhat paradoxical, which might not be evident in the oxidative stress life history. The links between ROS production and rates of metabolism were not very strong in lactating females compared with that in their non-lactating counterparts (Fletcher et al, 2013; Garratt et al, 2012; Nussey et al, 2009; Speakman & Garratt, 2014; Xu et al, 2014). For example, Speakman & Garratt (2014) compared several markers indicative of oxidative stress between reproductive and

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non-reproductive animals in both laboratory and field animals, but observed that oxidative stress was unchanged, or was lower, in reproductive individuals in comparison with those that did not reproduce. These studies showed a weak positive correlation between oxidative damage and reproduction (Bergeron et al, 2011; Fletcher et al, 2013; Speakman & Garratt, 2014; Xu et al, 2014).

Mitochondria are the major producers of cellular ROS (Brand, 2000; Toime & Brand, 2010). There is a strong positive relationship between inner mitochondria membrane potential and ROS production, by which just a small increase in the membrane potential induces a large stimulation of ROS, and a small decrease is suggested to reduce ROS production (Boveris et al, 2006; Miwa & Brand, 2003). It has been suggested that UCPs (uncoupling proteins) partially uncouple mitochondria, and activate the proton conductance, leading to lowered proton motive force and decreased ROS production, consequently protecting against excessive superoxide production (Brand et al, 2004). Five mitochondrial UCPs exist: UCP_2, expressed ubiquitously; UCP_1, exclusively in brown adipose tissue; UCP_3, predominantly in muscle; UCP_4 and UCP_5, in brain (Ježek, 2002). It is well established that lactating animals usually increase the rate of metabolism, but decrease *ucp*s gene expression and/or protein content (Li & Wang, 2005; Martin et al, 1989; Pedraza et al, 2000; Xiao et al, 2004). However, it remains uncertain if changes of *ucp*s expression are involved in ROS production and oxidative damage in lactating animals.

Kunming (KM) mice, a domestic laboratory rodent, are well known as a widely used mice model in the study of many field, including physiology and pharmacology. However, the oxidative stress and antioxidant, as well as UCPs expression, in several tissues related to digestion in KM mice during the peak lactation remain uncertain. The aim of this study was to examine the relationship between oxidative stress and reproduction in mice, using serial markers of both oxidative stress and antioxidants, including H_2O_2 and malonadialdehyde (MDA), as well as SOD, total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-PX). We manipulated female energy demand during lactation by altering female litter size at birth to 12 pups. This allowed us to test the relationship after correcting for the effect of litter size. In addition to oxidative stress and antioxidant, *ucp*s gene expression in brain, liver and skeletal muscle was measured.

MATERIALS AND METHODS

Animals

Virgin female KM mice, aged 9-10 weeks, were obtained from the colony maintained in the animal house at Wenzhou University. Animals were housed individually in plastic cages (29 cm×18 cm×16 cm) with sawdust bedding, and maintained on a 12: 12 h light–dark cycle (lights on at 0800h) at a constant temperature of 21±1 °C. Animals were fed standard rodent chow (produced by Beijing KeAo FeedCo., Beijing, China) and water *ad libitum*. All experimental procedures were in compliance with the Animal Care and Use Committee of Wenzhou University.

Twenty-four female mice were assigned into two groups: 15 females were mated with males for 11 days, then males were moved out. 14 females became pregnant and gave birth (lactation, Lac, *n*=14) and the rest of females were not paired with males (non-lactation, Non-Lac, *n*=10). To correct for the effect of litter size, females were artificially regulated to raise 12 pups on parturition day. Body mass, food intake, energy intake, as well as litter size and litter mass were measured during the peak lactation (day 13-14 of lactation). Litters were weaned on day 17 of lactation.

Gross energy intake (GEI), digestive energy intake (DEI) and digestibility

GEI, DEI and digestibility were measured from day 13 to 14 of lactation, as described previously (Grodziński & Wunder, 1975; Liu et al, 2003). In detail, food was provided on the start of day 13, any uneaten food or food mixed within the bedding were collected along with any feces from each animal on the end of day 14 of lactation (over two days). They were separated manually after they were dried at 60 °C to constant mass. Gross energy contents of the food and feces were determined using a Parr 1281 oxygen bomb calorimeter (Parr Instrument, USA). GEI, DEI and digestibility were calculated using the following the equations (Grodziński & Wunder, 1975; Liu et al, 2003; Zhao et al, 2013, 2014a):

GEI (kJ/d)=[food provided (g/d)×dry matter content of food (%)–dry spillage of food and uneaten food]× gross energy content of food (kJ/g) (1)

DEI (kJ/d)=GEI–[dry feces mass (g/d)×gross energy content of feces (kJ/g)] (2)

Digestibility (%)=DEI/GEI×100% (3)

H_2O_2 and MDA

The female mice were euthanised by decapitation. Brain, leg skeletal muscle, liver and kidneys were separated and weighed (to 1 mg). The small intestine was also separated quickly and weighed (to 1 mg) after the contents were removed. All tissues were put into liquid nitrogen immediately after the weighing. Tissues were homogenized using ice-cold 0.9% NaCl solution. The homogenates were centrifuged at 3 000 g for 15 min and the supernatant was taken for later assay of the markers of oxidative stress and antioxidant enzymes. Levels of hydrogen peroxide (H_2O_2) were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute) in accordance with the manufacturer's instructions and guidelines. A pre-experiment performed on these tissues demonstrated that these assay kits were effective for KM mice. Level of H_2O_2 (405 nm) was expressed as mmol/g protein (Chen et al, 2014). The lipid peroxidation level was measured using the levels of malonadialdehyde (MDA), which is the end product of lipid peroxidation and reacts with thibabituric acid (TBA) as a thiobarbituric reactive species (TBARS) to produce a pink colored complex that has peak absorbance at 532 nm (Manivannan et al, 2013). Level of tissue MDA was expressed as nmol/mg protein (Chen et al, 2014).

Activity of SOD and T-AOC

Antioxidant enzymes activities, including SOD, and total

antioxidant capacity (T-AOC) were determined using commercial kits (produced by Nanjing Jiancheng Bioengineering Institute), strictly according to the instructions. These kits were also sensitive for mouse tissues. 1 unit of SOD activity was defined as the amount of proteins causing 50% inhibition of the rate of nitroblue tetrazolium (NBT) reduction, and 1 unit of T-AOC activity was defined as the increment of 0.01 of absorbance OD value per min (Chen et al, 2014).

Activity of glutathione peroxidase (GSH-PX)

Activity of GSH-PX was also measured using a commercial kit (produced by Nanjing Jiancheng Bioengineering Institute) according to the instructions. The assay is based on the reaction of reduced glutathione (GSH) with H_2O_2, that produced oxidized glutathione (GSSG) and H_2O. GSH reacted with disulfide generation sulfydryl reagent 5, 5'-dithio-bis (2 nitrobenzoic acid, DTNB), producing a 2-nitro-5-thiobenzoic acid a yellow colored compound, that was read at 412 nm. GSH-PX activity was calculated as the reduction rate of levels of GSH with the presence of GSH-PX relative to absence of GSH-PX. 1 unit of GSH-PX activity was defined as 1μmol of GSH consumption/min/mg protein at 37 °C. The protein concentration was determined by the method of Lowry (Lowry et al, 1951), using bovine serum albumin (BSA) as standard (Chen et al, 2014).

Real-time RT-qPCR analysis

Total RNA was prepared from brain, liver and skeletal muscle using TRIzol agent (TAKARA, Dalian, China). Real-time RT-qPCR analysis was carried out as described previously (Zhao et al, 2014b). Briefly, RNA concentration was determined spectrophotometrically, and 2 μg of total RNA was taken to synthesize cDNAs in a final reaction volume of 50 μLwith AMV Reverse Transcriptase (TAKARA) using random primer oligo $(dT)_{18}$. The cDNA samples (2 μL) were used as a template for the subsequent PCR reaction using gene-specific primers, listed as follows: *ucp*$_2$, forward, 5'-CCCAATGTTGCCCGTAAT-3' and reverse, 5'-CCCAAGCGGAGAAAGGAA-3'; *ucp*$_3$, forward, 5'-GTTTACTGACAACTTCCCCT-3' and reverse, 5'-CTCCTGAGCCACCATCT-3'; *ucp*$_4$, forward, 5'-GCCGAATAAT GAACCA AC-3' and reverse, 5'-ACCAAGGGGTCATTCTCA-3'; *ucp*$_5$, forward, 5'-CGTTACTAAGACAGGCATCA-3' and reverse, 5'-ACACCCCTCCACAGACCC-3'. The final reaction volume of 20 μL contained 10 μL of 2× SYBR Premix EX Tag TM (TAKARA), 0.4 μL of forward prime and reverse primer (final concentration 0.2 μmol/L per primer) and 2 μL cDNA template. qPCR was performed using Roche LightCycler480 real-time qPCR system (Forrentrasse CH-6343 Rotkreuz, Switzerland). After an initial polymerase activation step at 95 °C for 60 s, amplification followed, by 40 cycles of (95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s) and the reaction finished by the built-in melt curve. All samples were quantified for relative quantity of gene expression by using actin expression as an internal standard, actin, forwards, 5'-CGTAAAGACCTCTATGCCAA-3' and reverse, 5'-GCGCAAGTTAGGTTTTGTC-3' (Zhao et al, 2014b).

Statistics

Data were expressed as mean±*SE*. and analyzed using SPSS 13.0 statistic software. Differences in body mass, DMI, GEI, DEI and digestibility, levels of H_2O_2 and MDA, activities of GSH-PX, SOD and T-AOC, as well as *ucp*$_s$ gene expression between Non-Lac and Lac groups were examined using independent sample *t* tests. Correlations of H_2O_2, MDA, GSH-PX, SOD and T-AOC among different tissues were examined using Pearson's correlation analysis. The level of significance was set at $P<0.05$.

RESULTS

Body mass, energy intake, litter size and litter mass

Body mass was significantly different between the two groups, and it was higher by 31.3% in Lac females than in their Non-Lac counterparts (Table 1). Lac females consumed significantly more food and produced more feces than Non-Lac females (Table 1). During peak lactation, GEI and DEI were 254% and 253% higher in the Lac group, respectively, than that in the Non-Lac group (Table 1). No difference was observed in digestibility between the two groups (Table 1). Litter size averaged 12 throughout lactation and mean litter mass was 97.5±2.6 g during peak lactation (Table 1).

Table 1 Energy intake, digestibility and litter size and mass in non-lactating and lactating mice during peak lactation

	Non-Lac	Lac	*t*	*P*
Body mass (g)	41.8±1.0	54.9±0.9	9.48	**
DMI (kJ/d)	7.0±0.3	25.0±0.8	16.75	**
GEI (kJ/d)	124.0±5.4	438.9±14.5	16.75	**
GE of feces (kJ/d)	28.5±1.1	102.2±3.5	16.44	**
DEI (kJ/d)	95.5±4.5	336.7±11.8	15.78	**
Digestibility (%)	76.9±0.5	76.7±0.5	0.30	ns
Litter size	-	12.0±0.7	-	-
Litter mass (g)	-	97.5±2.6	-	-

DMI: Dry matter intake; GEI: Gross energy intake; GE: Gross energy; DEI: Digestive energy intake; **: $P<0.01$; ns: Non-significance ($P>0.05$).

H_2O_2 and MDA

Brain showed 17.7% higher H_2O_2 levels in Lac than Non-Lac group ($t_{22}=2.34$, $P<0.05$). Levels of H_2O_2 in liver and small intestine were significantly lower in Lac group than that in Non-Lac group (liver, $t_{22}=4.57$, $P<0.01$; small intestine, $t_{22}=2.20$, $P<0.05$). No statistical differences were observed in H_2O_2 levels between the two groups in kidneys ($t_{22}=1.14$, $P>0.05$) and skeletal muscle ($t_{22}=2.20$, $P>0.05$) (Figure 1A). There were significantly negative correlations of H_2O_2 between liver and brain and small intestine, and the relationships among other tissues were not significant (Table 2).

Levels of MDA in brain were significantly higher in Lac than in Non-Lac group ($t_{22}=4.69$, $P<0.01$). However, MDA levels of Lac females were 32.7%, 36.7%, 42.3% and 39.4% lower in liver ($t_{22}=4.09$, $P<0.01$), kidneys ($t_{22}=4.98$, $P<0.01$), skeletal muscle ($t_{22}=2.14$, $P<0.05$) and small intestine ($t_{22}=2.80$, $P<0.01$) than

Table 2 Correlations of H_2O_2, MDA, GSH-PX, SOD and T-AOC among different tissues in lactating mice

		Brain	Liver	Kidneys	SM	SI
H_2O_2	Brain	1	–0.50*	–0.11	–0.14	0.27
	Liver		1	–0.25	0.36	–0.42*
	Kidneys			1	–0.31	–0.12
	SM				1	–0.03
	SI					1
MDA	Brain	1	–0.43*	0.29	–0.08	–0.44*
	Liver		1	0.07	0.26	0.82**
	Kidneys			1	0.19	0.05
	SM				1	–0.02
	SI					1
GSH-PX	Brain	1	–0.19	–0.09	0.08	0.15
	Liver		1	–0.17	0.22	0.32
	Kidneys			1	–0.01	0.47*
	SM				1	–0.15
	SI					1
SOD	Brain	1	–0.16	–0.46*	0.04	0.01
	Liver		1	0.08	0.09	0.09
	Kidneys			1	0.06	0.04
	SM				1	–0.06
	SI					1
T-AOC	Brain	1	–0.13	0.11	0.29	0.30
	Liver		1	–0.13	0.21	–0.16
	Kidneys			1	–0.04	–0.12
	SM				1	0.26
	SI					1

SM: Skeletal muscle; SI: Small intestine; *: P<0.05; **: P<0.01.

that of Non-Lac females (Figure 1B). There were significant correlations of MDA levels between small intestine and brain and liver, as well as brain and liver (Table 2). No correlations were observed between H_2O_2 and MDA and litter mass in any tissues (Table 3).

Table 3 Correlations of litter mass with H_2O_2, MDA, GSH-PX, SOD and T-AOC of different tissues in lactating mice

	Brain	Liver	Kidneys	SM	SI
H_2O_2	0.13	–0.29	0.03	–0.30	0.36
MDA	0.23	–0.34	–0.33	0.07	–0.37
GSH-PX	–0.19	–0.30	0.33	0.25	–0.04
SOD	–3.7	–0.17	0.29	–0.38	0.23
T-AOC	0.05	–0.13	0.16	0.24	–0.03

SM: Skeletal muscle; SI: Small intestine.

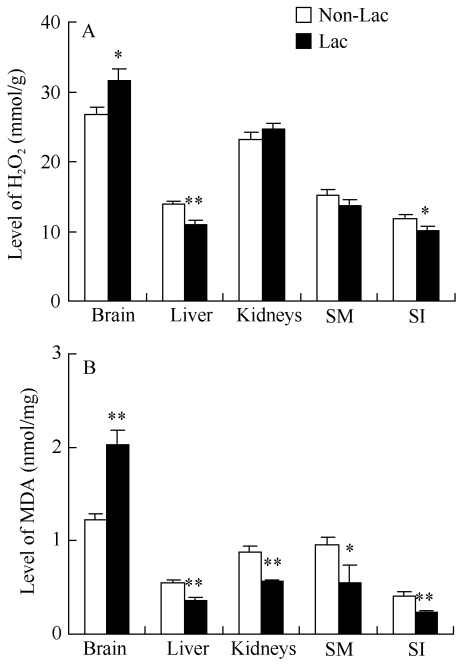

Figure 1 Levels of H_2O_2 (A) and MDA (B) in different tissues in mice during peak lactation

SM: Skeletal muscle; SI: Small intestine; *: P<0.05; **: P<0.01.

GSH-PX, SOD and T-AOC

Activity of GSH-PX in the Lac females was significantly lower in the brain (t_{22}=2.73, P<0.05) and liver (t_{22}=5.65, P<0.01) than in Non-Lac females, while it did not differ between the two groups in kidneys (t_{22}=0.08, P>0.05), skeletal muscle (t_{22}=1.01, P>0.05) and small intestine (t_{22}=0.49, P>0.05, Figure 2A). A significant correlation of GSH-PX was observed between kidneys and small intestine only (Table 2). There was no difference in levels of SOD between the two groups in any tissues (Figure 2B). No correlations were found in SOD among the several tissues except for significant relationship between brain and kidneys (Table 2). Activity of T-AOC did not differ between the two groups in brain (t_{22}=1.52, P>0.05), liver (t_{22}=0.43, P>0.05) and small intestine (t_{22}=0.83, P>0.05), whereas it did in kidneys (t_{22}=2.18, P<0.05) and skeletal muscle (t_{22}=5.27, P<0.01, Figure 2C). No correlations were found in T-AOC activity among any tissues (Table 2). There were no correlations between litter mass and levels of GSH-PX, SOD and T-AOC in any tissues (Table 3).

Ucp_s gene expression

No significant difference was observed in brain ucp_2 gene expression between Non-Lac and Lac groups (t_{26}=0.97, P>0.05, Figure 3A). Ucp_4 gene expression of Lac females was 394% higher than that in the Non-Lac control group (t_{26}=1.73, P=0.096, Figure 3A). There was a significant difference in ucp_5 gene expression between the two groups, and Lac females showed 577% higher ucp_5 gene expression than the Non-Lac females (t_{26}=2.06, P<0.05, Figure 3A). Ucp_2 in liver did not differ

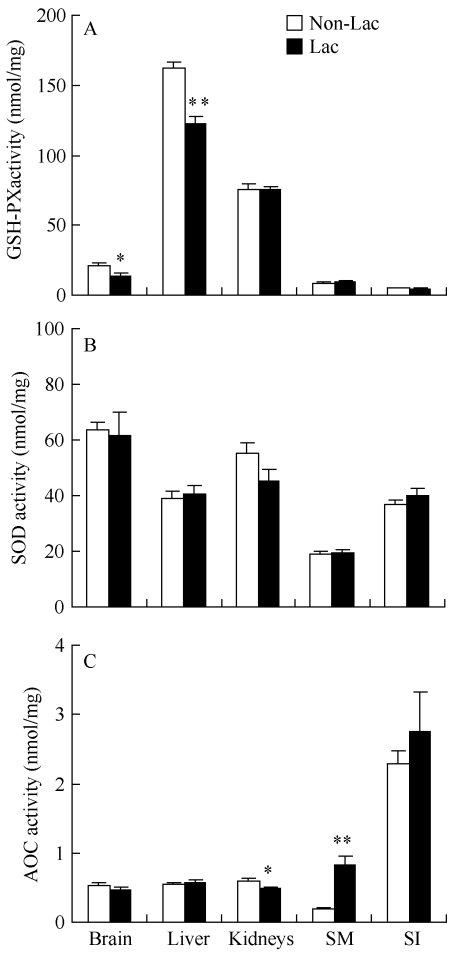

Figure 2 Activities of GSH-PX (A), SOD (B) and T-AOC (C) in different tissues in mice during peak lactation

SM: Skeletal muscle; SI: Small intestine; *: $P<0.05$; *: $P<0.01$.

between the two groups (t_{26}=0.97, $P>0.05$, Figure 3B). Lac mice showed 41.4% lower *ucp*$_3$ expression in skeletal muscle than the Non-Lac females (t_{26}=1.94, P=0.06, Figure 3B).

DISCUSSION

Lactation is the most energetically demanding period encountered by small mammals, during which a female must allocate a large part of their energy to support their offspring, and also have to increase the energy utilised for maintenance (Speakman & Król, 2005). In the present study, female mice consumed 2.5-fold higher energy during peak lactation than non-reproductive females. This was consistent with results observed in the same strain of mouse (Zhao & Cao, 2009). Similarly, food intake during peak lactation was 3.4-fold higher in MF1 mice (Johnson et al, 2001), 1.2-fold in Brandt's voles (*Lasiopodomys brandtii*) (Zhang and Wang, 2007), around 3-fold in Siberian hamsters (*Phodopus sungorus*) (Paul et al, 2010) and 2.1-fold in striped hamster (*Cricetulus barabensis*)

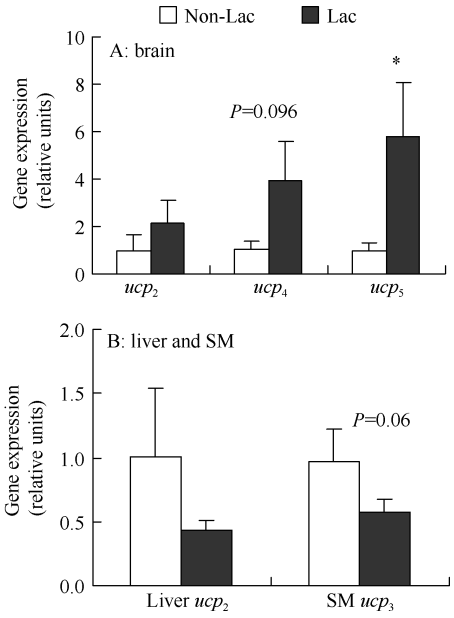

Figure 3 Gene expressions of *ucp*$_2$, *ucp*$_4$ and *ucp*$_5$ in brain (A) and *ucp*$_2$ in liver and *ucp*$_3$ in skeletal muscle (B) in mice raising litters of 12

SM: Skeletal muscle; *: $P<0.05$.

(Zhao, 2011). Although, energy intake is notably increased in a majority of mammals during reproduction, it actually reaches a ceiling at peak lactation, called limitation on sustained energy intake (Hammond & Diamond, 1997; Speakman & Król, 2005, 2011). Life-history theory assumed that there was a trade-off between the allocation of limited resources to the competing demands of reproduction and somatic maintenance (Speakman & Garratt, 2014; Speakman & Król, 2005, 2011).

As mentioned above, the main energy expenditure during reproduction in lactating females is the energy utilised for milk production, the only energy resources that offspring could used for growth and activity (Speakman, 2008). In the current study, the weaned offspring were 97.5 g in KM mice. This was consistent with the average of litter mass in this strain of mouse (Zhao & Cao, 2009). That means lactating mice must export as much as 103kJ/d, on average, to support their offspring, accounting for 32.3% of daily gross energy intake (Zhao et al, 2010). Similarly, milk energy output accounts for 45.1% for MF1 mice (Johnson et al, 2001; Król & Speakman, 2003). It was assumed that the reproductive value would be higher if females invest more in their offspring (Speakman & Król, 2005, 2011). However, based on the life-history theory, animals do not reproduce maximally because this would reduce the probability of their own survival (Speakman & Król, 2005). One such possibility is oxidative stress, which has been suggested as a possible physiological cost of reproduction (Xu et al, 2014). The possible explanation is that the females usually increase their rate of metabolism during lactation, resulting in elevated generation of ROS that have potential to damage macromolecules unless quenched by antioxidants (Garratt et al, 2011).

In the present study, we expected that females during peak lactation would elevate oxidative stress and antioxidant activity. In contrast to our expectation, several markers of oxidative stress did not increase in lactating mice compared with that in non-reproductive counterparts. MDA levels were significantly lower in liver, kidneys, skeletal muscle and small intestine in lactating females than non-reproductive controls, indicating that no evidence of increased oxidative stress was found in reproductive mice. Consistently, significant reduction of MDA and/or protein carbonyls in liver were observed in lactating Brandt's voles (Xu et al, 2014) and Mongolian gerbils (*Meriones unguiculatus*) (Yang et al, 2013), and house mice (*Mus musculus domesticus*) (Garratt et al, 2011, 2013) compared with non-reproductive controls. In addition, Garratt and colleague found that reproductive animals did not have higher lipid peroxidation, which was even lower in inner tissues, like liver, indicating that no oxidative damage occurred to the females (Garratt et al, 2011). As a large proportion of energy was metabolized in lactating females directly to offspring in milk, showing significant increases in rate of energy metabolism, it was unlikely that the absence of oxidative damage was owing to an insufficient increase in metabolism (Garratt et al, 2011).

It has been suggested that oxidative stress occurs only when ROS production exceeds the capacities of protection, resulting in oxidative damage to macromolecules (Beckman & Ames, 1998; Selman et al, 2002). In the present study, activities of GSH-PX, SOD and T-AOC, the markers of antioxidants, in lactating mice were almost the same as that observed in non-reproductive mice. Organisms have a variety of defensive mechanisms that can protect against oxidative stress (Garratt et al, 2011). Physiological regulation of antioxidant systems are effective mechanisms to maintain the oxidant-antioxidant balance, and consequently may play important roles in preventing oxidative damage to macromolecules (Garratt et al, 2011). Actually, the potential associations between the both sides have been observed in the studies previously performed in house mice (Aloise et al, 2013; Garratt et al, 2011, 2013), Brandt's voles (Xu et al, 2014), Mongolian gerbils (Yang et al, 2013), Wistar rats (*Rattus norvegicus*) (Davidović et al, 1999; Venditti et al, 2004) and short-tailed field voles (*Microtus agrestis*) (Selman et al, 2000). Among the different tissues in the current study, the brain was only one showing a significant increase in oxidative stress, indicated by levels of H_2O_2 and MDA, which might be partly caused by significant reduction of antioxidant of GSH-PX activity. The decreased GSH-PX activity might impair the oxidant-antioxidant balance. The data from the current study may also demonstrate in reproductive mice that antioxidants were regulated physiologically in response to ROS, by which oxidant-antioxidant balance occurs during peak lactation. In addition, in the present study tissue-dependent changes in both oxidative stress and antioxidants were observed in lactating KM mice. This might be due to the difference in the rate of metabolism among the different tissues. Unfortunately, the tissue-specific rate of metabolism was not measured in these tissues.

UCP_s are suggested to uncouple mitochondria and lower proton motive force, and play roles in decreasing ROS production (Brand et al, 2004). It has been suggested that lactating animals usually decrease ucp_s gene expression in peripheral tissues, including brown adipose tissue, liver and skeletal muscle (Li & Wang, 2005; Martin et al, 1989; Pedraza et al, 2000; Xiao et al, 2004). In the present study, we observed that in lactating mice ucp_5 gene expression of brain were 577% higher in lactating mice than non-reproductive mice (P<0.05), indicating potential roles of ucp_5 in prohibition of ROS production. UCP_5 passes protons through the inner mitochondrial membrane to the matrix, and thus performs the essential function of an uncoupler of oxidative phosphorylation (Ramsden et al, 2012). This process is accompanied by a reduction in oxidative stress, and consequentially exerts a protective influence on cells (Ramsden et al, 2012). In the present study, UCP_5 expression was also up-regulated in the brain in lactating mice compared with that in non-lactating mice. This may reflect an adaptive response to the increased oxidative stress, indicated by the elevations of H_2O_2 and MDA, during peak lactation. However, the data from the present study were only associated with ucp_s gene expression in brain, liver and skeletal muscle, which might not draw a generally strong conclusion about the relationship between ROS production and UCP_s expression in animals during peak lactation.

KM mice showed significantly higher energy intake and exported more energy in milk production during peak lactation than their non-reproductive counterparts. However, several markers of both oxidative stress and antioxidant activities were not significantly higher in liver, kidneys, skeletal muscle and small intestine in reproductive mice compared with that in controls. This was inconsistent with the prediction of the oxidative stress life history theory. Activities of antioxidants might be regulated physiologically in response to elevated ROS production in several tissues of lactating mice, by which oxidant-antioxidant balance might prevent oxidative damage to the tissues. Regulations of brain ucp_4 and ucp_5 gene expression might be involved in the prevention of oxidative damage to the tissue.

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ZOOLOGICAL RESEARCH

Sex differences in morphine-induced behavioral sensitization and social behaviors in ICR mice

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ABSTRACT

Gender and genetic strain are two prominent variants that influence drug abuse. Although certain sex-related behavioral responses have been previously characterized in ICR mice, little is known about the effects of sex on morphine-induced behavioral responses in this outbred strain. Therefore, in this study, we investigated the sex differences of morphine-induced locomotion, anxiety-like and social behaviors in ICR mice. After morphine or saline exposure for four consecutive days (twice daily), increased locomotion, more time spent in the central area, as well as attenuated rearing and self-grooming behaviors were found in morphine-treated females in an open field; no differences were found in locomotion and the time spent in the central area between male and female controls. When interacting with the same-sex individuals, female controls were engaged in more social investigation, following, body contacting and self-grooming behaviors than controls; morphine exposure reduced contacting and self-grooming behaviors in females; in contrast, these effects were not found in males. These results indicate that female ICR mice are more prosocial and are more susceptible to morphine exposure than males.

Keywords: Morphine; ICR mice; Locomotion; Social behavior

INTRODUCTION

Gender and genetic strain are two prominent variants that influence drug abuse (Belzung & Barreau, 2000; Orsini et al, 2005; Phillips et al, 2008). It has been reported that females are more sensitive to the addictive properties of drugs compared with males (Anker, et al, 2011; Becker & Hu, 2008; Carroll & Anker, 2010). Besides, differences in behavioral responses to abused drugs may reflect differences in genetic vulnerability to drug abuse (Grabus et al, 2004). Due to the high genetic homogeneity, many inbred strains have been used in drug-related behavioral tests (Eisener-Dorman et al, 2011; Kennedy et al, 2012). For example, C57BL/6 and BALB/c mice, the two of the most commonly used inbred mouse strains (or sometimes their sublines) have been widely implicated in drug abuse research. In fact, outbred mice with genetically variable compositions, vigorous physiques and economical prices have also been considered for neuroethological studies (Dell'omo et al, 1993; Ge et al, 2013). For example, in addition to applications in immunology, pathology and pharmacology, the ICR mouse is also employed in behavioral neuroscience (Ge et al, 2013; Li et al, 2014; Liang et al, 2014) including drug-related studies (Kitanaka et al, 2014; Mao et al, 2011). Recent research has revealed that the ICR males display distinct paternity, including father-pup social interaction, and shed light on parental behaviors (Liang et al, 2014). They spent less time to get used to a new environment and have better environment memory capacity as compared to inbred C57BL/6 and BALB/c mice (Shi et al, 2008). The female ICR mice showed superior learning and memory abilities than males, but there were no sex differences in locomotion (Ge et al, 2013). Although some sex-related responses have been previously characterized in this strain (Aoki et al, 2010; Ge et al, 2013; Yamaura et al, 2013), to our knowledge, little is known about the sex differences of ICR mice in drug-related behaviors. [1]

Behavioral sensitization is a phenomenon in which neural changes are elicited by exposure to repeated intermittent administration of psychostimulants or opioids (Stewart & Badiani, 1993; Wang et al, 2010). These neuroadaptations are responsible for drug-induced increases in locomotor activity, reinforcing and rewarding effects (Francès et al, 2000; Wang et al, 2010). The endogenous opioid systems are related to changes in social emotional responsivity and social behavior (Kennedy et al, 2011; Nocjar & Panksepp, 2007). The effects of

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morphine on rewarding effects, locomotion or social play behaviors have been explored in some other mice strains (Cunningham et al, 1992; Campbell et al, 2000; Cole et al, 2013; Vanderschuren et al, 1995). Although morphine-induced conditioned place preference (CPP) has been conducted in male ICR mice (Mao et al, 2011), some studies have proposed the possibility of a disassociation between rewarding effects and behavioral sensitization (Miner, 1997; Wang et al, 2012; Zhang et al, 2002). In this study, we explore the sex differences in morphine-induced behavioral sensitization, anxiety and sociability in ICR mice.

MATERIALS AND METHODS

Animal subjects

Male and female ICR mice (18-22 g) were purchased from Ningxia Medical University Laboratory Animal Center (Yinchuan, China). The animals were housed in groups of four in standard transparent Makrolon cages (32 cm×21.5 cm×17 cm). The colony room was illuminated on a 12:12 light-dark cycle (lights on 2000h) and the temperature was maintained at 23±2 ℃. Food and water were available *ad libitum*. Mice were allowed to adapt to housing conditions for one week and handled daily by the same experimenter for three days prior to testing. All experiment procedures were performed strictly in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Drug administration

Morphine-hydrochloride (Northwest Pharmaceutical Co., Ltd. Sinopharm, Xi'an, China) was diluted in saline. Mice received subcutaneously a daily binge injection of morphine or saline for four consecutive days. The daily binge pattern was consisted of two injections of morphine (10 mg/kg) or euqal vulume of saline at 0800h and 1400h, respectively. The dose of morphine applied in this study was according to the previous studies on behavioral sensitization, social motivated behaviors and CPP performance in mice (Mao et al, 2011;Nocjar & Panksepp, 2007; Xu et al, 2007).

Open-field test

Animal subjects were randomly assigned to one of the four treatment groups: male mice receiving physiological saline (MS, n=8) and morphine administration (MC, n=8), and female mice receiving physiological saline (FS, n=8) and morphine administration (FC, n=8). Twenty minutes following the last injection, motor activity and anxiety-like behavior were assessed in an open-field chamber. The chamber is a brightly and evenly illuminated square arena (50 cm×50 cm×25 cm) made of white glacial polyvinyl chloride and illuminated with four 60 W lamps mounted 1.5 m above the arena. The area was divided into 16 quadrants (four central and 12 peripheral) (Fiore & Ratti, 2007). Mice were placed individually in the center of the open-field and left to explore for 5 min and videotaped under white illumination. After the test trial was completed, the open-field was thoroughly cleaned with 70% ethanol solution. To assess anxiety-like behavior, the time spent in the center of the open-field was measured. The number of crossings between quadrants was used to assess locomotion. Additionally, rearing (raising on the hind legs and sniffing into the air or the wall of the box) and self grooming (licking own fur, sometimes using forepaws, passing them over the nose with a series of brief, horizontal movements) were recorded. All focal animals were videotaped during experiments using a Sony camera. The frequency and total duration of these behaviors were later scored by a researcher blind to experimental treatment using Jwatcher 1.0.

Same-sex social interaction test

After the open-field test, social interaction test was conducted. The interaction test was performed using only same-sex dyads to eliminate the possibility that even a low frequency of sexually motivated behavior might confound the results of tests. Testing was conducted in a neutral plastic cage (46 cm×31.5 cm×20 cm), with approximately 2 cm of wood shavings covering the floor and a removable opaque divider in the middle. One mouse was placed on each side of the arena with the divider still in place and given 5 min to adapt to the arena. Once the divider was removed a video-recorder mounted 70 cm above the apparatus monitored the arena for 15 min.

The behavior of the individual was classified as social investigation (sniffing the face, body or anogenital area of an individual), following (moving in the direction of or pursuing the test partner who moves away), contact behavior (contact with another individual including staying together or amicable grooming), aggressive behavior (pouncing (jumps or lunges), fighting (tumbling and biting) and chasing), self-grooming (cephalocaudal progression that begins with rhythmic movements of the paws around the mouth and face, ears, descending to the ventrum, flank, anogenital area and tail). Since the aggressive behaviors were rarely observed during social interaction, the related data were not shown.

Statistical analysis

Statistical analyses were carried out using SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). Data were checked for normality using the one-sample Kolmogorov–Smirnov test. Data from the open-field test and social interaction test were compared using two-way ANOVA with sex and morphine treatment as factors. Group differences were compared using post-hoc tests. All data are presented here as mean±*SE*. Statistical significance was taken at $P \leq 0.05$.

RESULTS

Behaviors in open-field

Two-way ANOVA revealed the effect of sex on transition ($F_{(3,28)}$=4.479, P=0.043) and rearing (duration: $F_{(3,28)}$=5.272, P=0.029; frequency: $F_{(3,28)}$=9.810, P=0.004). Significant effects of morphine were found on the time spent in the central area ($F_{(3,28)}$=6.039, P=0.020), rearing (duration: $F_{(3,28)}$=5.307, P=0.029; frequency: $F_{(3,28)}$=0.016, P=0.692) and self-grooming (duration: $F_{(3,28)}$=4.748, P=0.038; frequency: $F_{(3,28)}$=7.136, P=0.012). The effect of interactions between sex and morphine was found in self-grooming frequency ($F_{(3,28)}$= 9.017, P=0.006).

Specifically, no differences were found in total transition

(*mean difference*=12.71, *P*=0.195) or time spent in the central area *(mean difference*=1.75, *P*=0.667) between female and male controls. Morphine-treated females showed more transitions (*mean difference*=19.663, *P*=0.049) and spent more time in the central area than the saline-treated females (*mean difference*=9.789, *P*=0.021) (Figure 1 A, B).

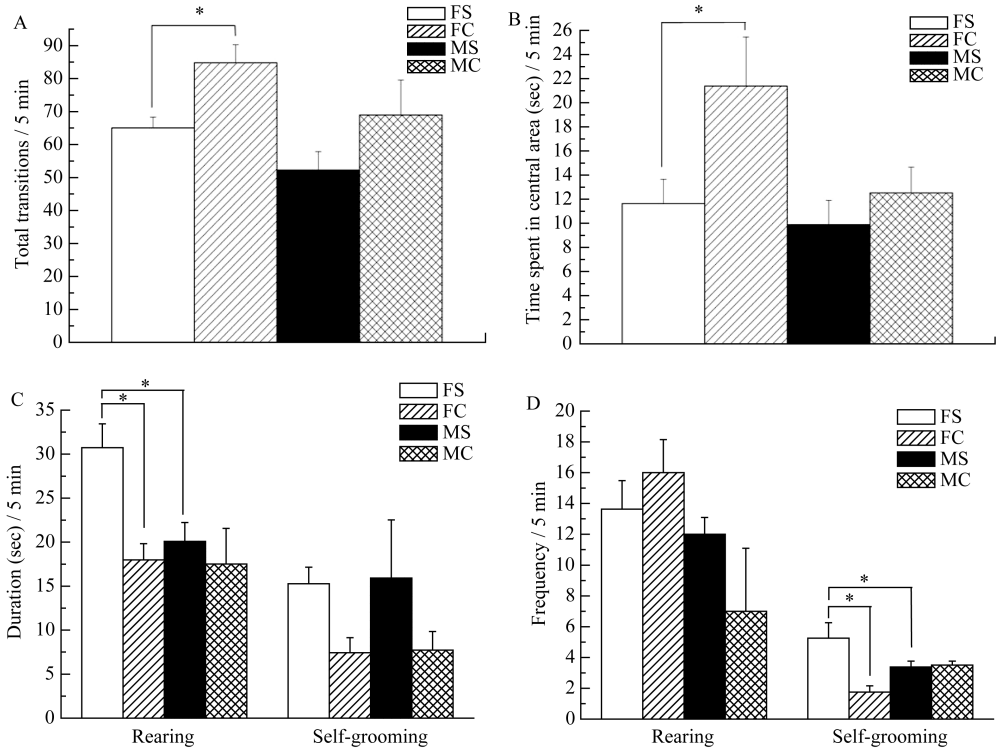

Figure 1 Behaviours of ICR mice during an open-field test

A: Total transitions; B: Total time spent in the central area; C: Duration of rearing and self-grooming behaviors; D: Frequency of rearing and self-grooming behaviors; *: *P*≤0.05.

Compared with male controls, female controls have higher levels of rearing (duration: *mean difference*=10.633, *P*=0.012; frequency: *mean difference=1.625, P=0.530*) and self-grooming behaviors (duration: *mean difference*=–0.655, *P*=0.901; frequency*: mean difference*=1.875, *P*=0.032)). Moreover, morphine-treated females showed less rearing (duration: *mean difference*=–12.743, *P*=0.003; frequency: *mean difference*=2.375, *P*=0.361) and self-grooming behaviors (duration: *mean difference*=–7.84, *P*=0.146; frequency: *mean difference*=–3.500, *P*<0.001) than the saline-treated females (Figure1 C, D). No morphine-induced differences were found in the transition numbers, the time spent in the central area, the rearing and self-grooming behaviors in males (*P*>0.05, data not shown) (Figure 1).

Social interaction

The effects of sex and morphine on the frequency of social investigation (sex: $F_{(3,28)}$=22.846, *P*<0.001; morphine: $F_{(3,28)}$=4.892, *P*=0.035) and self-grooming (sex: $F_{(3,28)}$=23.086, *P*<0.001; morphine: $F_{(3,28)}$=10.024, *P*=0.004) were revealed by two-way ANOVA test. The significant effects of sex on both the frequency of following ($F_{(3,28)}$=4.904, *P*=0.035) and contact behavior ($F_{(3,28)}$=5.931, *P*=0.022) as well the self-grooming duration ($F_{(3,28)}$=4.753, *P*=0.038) were also found. The frequency of contact behavior ($F_{(3,28)}$=7.026, *P*=0.013) and duration of self-grooming behavior ($F_{(3,28)}$=4.281, *P*=0.048) were significantly affected by the interactions between sex and morphine.

Specifically, in saline groups, compared with male controls, female controls showed higher frequencies of social investigation (*mean difference*=6.375, *P*=0.004), following (*mean difference*=3.125, *P*=0.041), contact behaviors (*mean difference*= 3.625, *P*=0.004) and self-grooming behaviors (duration: *mean difference*=52.94, *P*=0.005; frequency: *mean difference*=5.500, *P*<0.001) (Figure 2). Compared with saline controls, morphine-treated female mice exhibited lower frequencies of contact behaviors (*mean difference*=–3.750, *P*=0.003) and self-grooming behaviors (duration: *mean difference*=-48.61, *P*=0.009; frequency: *mean difference*=–4.750, *P*=0.001) (Figure 2C, D). However, no influences of morphine were found in the social investigation, following, contact and self-grooming behaviors in male mice (*P*>0.05, data not shown) (Figure 2).

DISCUSSION

Morphine-induced locomotion and anxiety-like behaviors

In the present study, no sex differences were observed in locomotion or anxiety levels under saline treatment, which are

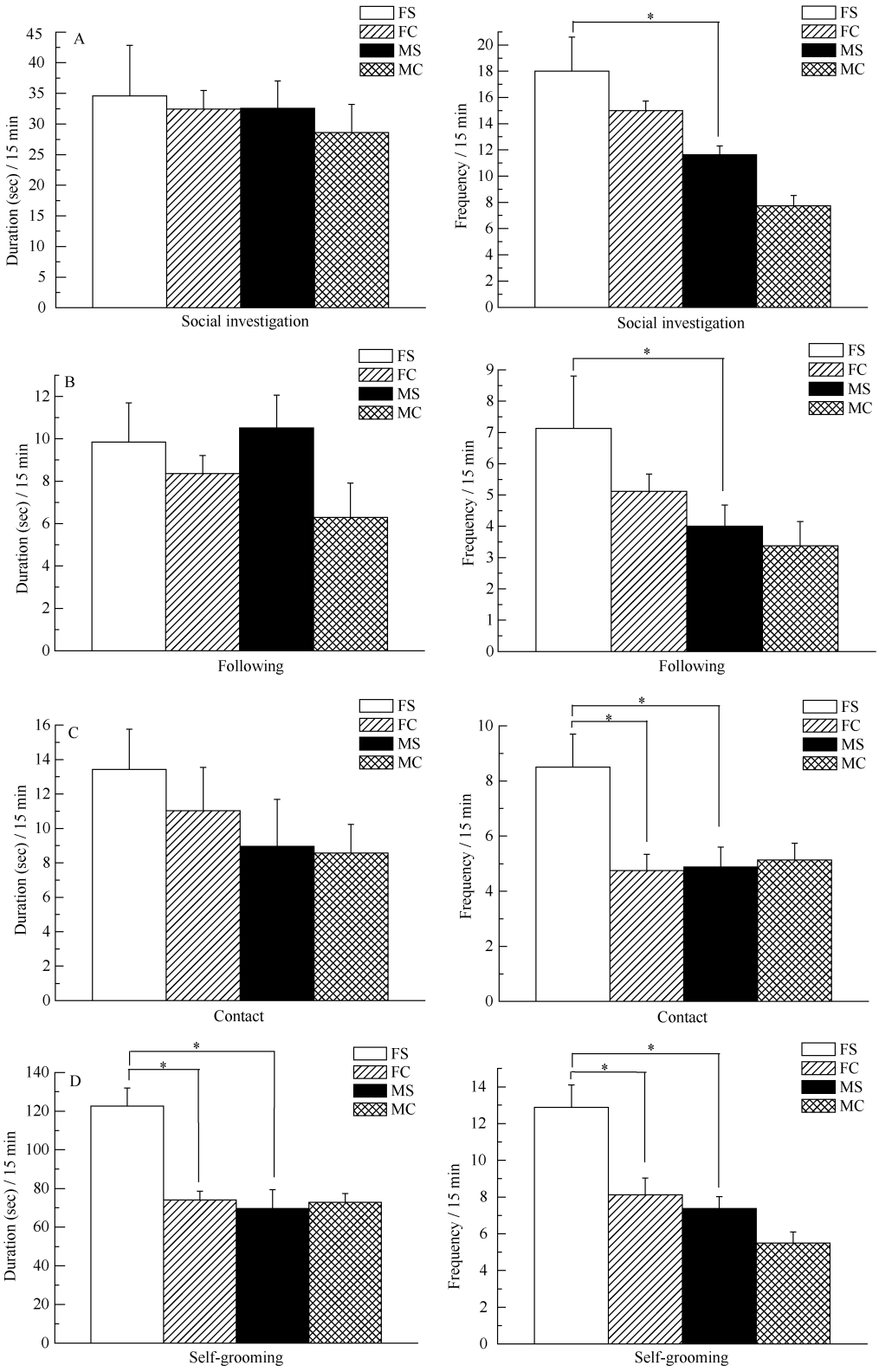

Figure 2 Behaviors of ICR mice in the same-sex social interaction test

A: Social investigation; B: Following behavior; C: Contact behavior; D: Self-grooming; *: $P \leq 0.05$.

consistent with previous findings in ICR mice (Ge et al, 2013). According to An et al (2011), similar phenomena in C57BL/6J and BALB/cJ mice were found in the open field and elevated plus maze tests as well. Here, female ICR mice exhibited more rearing and self-grooming behaviors than males. Since rearing behaviors of mice, to a certain degree, reflected its exploratory and cognitive ability (Edsbagge et al, 2004; Shi et al, 2008), our results indicate that the female ICR mice have higher levels of exploratory abilities than males. In addition to the time spent in the central zone, the changes in rearing and self-grooming are generally considered as indices of emotional behavioral processes (Carey et al, 2005; To & Bagdy, 1999; To & Bagdy, 1999). Thus, the differences between males and females in rearing and self-grooming behaviors suggest their different emotional changes in an open-field.

Several studies have shown that mice exhibit a heightened sensitivity to the effects of morphine (Niu et al, 2013; Xu et al, 2009). In this study, morphine increased the locomotion whereas attenuated the levels of anxiety, rearing and self-grooming behaviors in females; the time spent in the central area of an open field implies the morphine-induced anxiolytic effects in females. Similarly, cocaine treatments also decrease levels of rearing and grooming behaviors (Carey et al, 2005). These findings illustrate the importance of rearing and self-grooming phenotypes in drug-related behavioral researches. Additionally, our results showed that the locomotion, the time spent in the central zone or the rearing and self-grooming behaviors in males were not altered by morphine, indicating that female and male ICR mice respond differently to repeated morphine exposure.

Morphine-induced social behaviors

When interacting with the same-sex individuals, we found that under saline treatment, females were engaged in more social investigation, following, body contact and self-grooming behaviors than males. And these differences between males and females were inconsistent with their patterns in locomotion and anxiety-like behaviors. Interestingly, similar phenomena of high levels of sociability in females were also found in C57BL/6J and BALB/cJ mice (An et al, 2011; Wang et al, 2014). In addition, the decreased contact and self-grooming behaviors in morphine-treated females showed that morphine changed the social behaviors in females, whereas, morphine did not alter any social behaviors in males, combining our findings that morphine increased locomotor activity in females, indicating that female ICR mice are more susceptible to morphine than males.

Addition to ovarian hormones, sensitivity to the effects of drugs has been proposed to be associated with the sociability, emotion and memory (Curtis & Wang, 2007; Niigaki et al, 2010; Perrine et al, 2008). Compare with males, female ICR mice exhibit more sociability and better memory ability (Ge et al, 2013). These traits may influence behavioral responses to morphine in females. Besides, it has been reported that female ICR mice exhibit higher responses to stress and higher levels of serum corticosterone under both basal and stressed conditions as compared with males (Aoki et al, 2010; Yamaura et al, 2013). Sex differences in stress may contribute to sexually dimorphic patterns in morphine-induced behavioral sensitization (Holly et al, 2012; Wang et al, 2010).

In conclusion, this study compared the morphine-induced behavioral sensitization and social behaviors in female and male ICR mice. Their different behavioral responses to repeated morphine exposure indicate that female ICR mice are more susceptible to morphine than males.

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The validity of *Sarcocystis sinensis*

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DEAR EDITOR:

Recently, in a publication of Dubey et al (2014) was suggested that *Sarcocystis sinensis* was a *nomen nudum* based on what they considered insufficient description of the pararasite and lack of publicly available voucher material. They singled out that Zuo et al (1990) was not an appropriate venue for publishing a new species description, but we believe they did not adequately acknowledge two other reports in 1992 and 1995 when reaching their conclusion. Dubey et al (2014) correctly state that "the species of *Sarcocystis* from the water buffalo in China was presented under the name '*S. sinensis*' by Zuo (Zuo et al, 1990) at a national meeting (Fifth Symposium of the Chinese Society of Protozoology, Chongqing), and an abstract without figures was released."(Dubey et al, 2014). That presentation would not serve as a valid basis for naming the new species.

However, *S. sinensis* was first formally published by Zuo et al (1995). There, in the book "*the Proceedings of the Tenth Anniversary of the Founding of the China Parasitological Society*" (*ISBN 7-5046-2012-2, Chinese Science and Technology Express, Beijing, China, 1995*), and a book "*Coccidians in Livestock and Birds and Human Coccidiosis*" (*ISBN 7-5308-1195-9, Science and Technology Publishing Company of Tianjin, Tianjin, China, 1992*), the cyst's clear and detailed morphological structure was described and the life cycle was studied by Zuo (Zuo, 1992; Zuo et al, 1995). We present here the translation of the original descriptions as well as the detailed morphology of muscular cysts of *Sarcocystis sinensis* and infection experiments.

In the book (Zuo et al, 1995), the new species was described from cysts in muscles of water buffalo (*Bubalus bubalis*) from Kunming, Yunnan, China. Cysts were long and thin and/or fusiform in shape, 1 250 μm×100 μm (480-3 570 μm×45-152 μm) in average size. Leaning, finger-like protrusions (Figure 1, 10), 4.2-10.1 μm in size, averaged 5.8 μm, contained microtubules and few electron-dense granules (Figure 1, 11). There were invaginations on the surface of the protrusions' mostly in the middle and base parts. Ground substrate was 0.4-1.7 μm in thickness, extending inside of the cyst formed the septa-like structures or segmentation inside the cyst, in which a lot of concentrated banana-shaped bradyzoites, 13.0 μm×3.6 μm (10.9-16.1 μm×2.7-4.7 μm) in size could be seen. Under the scanning electron microscopy (SEM), the tip surface of the protrusions was arranged as regular square-shaped structures with space measured 0.4×0.8 μm, between reach other (Figure 1, 12). Natural prevalence was estimated at 58.0%. Location of parasite was skeletal muscle. Light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were presented. The cysts structure under LM, TEM and SEM were presented from both books were copied as Figure 1. [1]

In order to identify its definitive host, experimental infections were conducted initially in carnivorous animals. The first experiment was performed from April to May, 1988. Two dogs, two cats and two rhesus monkeys (*Macaca mulatta*) were infected with fresh *S. sinensis* cysts isolated from water buffalo. Each animal was fed with 60 cysts. Stools of the infected animals were examined for the presence of sporocysts and oocysts from day 5 to day 40 post feeding. Finally, animal were killed and the scrape from the intestinal mucosa was examined for *Sarcocystis* sporocysts and oocysts. No sporocysts and oocysts were found. Second experiment was performed from December 1989 to June1990, and three cats, three dogs, one eurasian sparrow hawk (*Accipiter nisus*), a boreal owl (*Aegolius funereus*), an eurasian tawny owl (*Strix aluco*), and a little-banded goshawk (*A. badius*), two vultures (*A. monachus*) were infected with *S. sinensis* cysts. Each animal were fed with 200 cysts. Stools of the infected animals were examined for the presence of sporocysts and oocysts from day 5 to day 40 post feeding. Except of goshawk and vultures, all animals were checked as first experiment in 1988, killed and intestinal mucosa were examined. No sporocysts and oocysts were found. The third infection experiment was performed in 1995. Two volunteers (one 55 years old woman and one 29 years old man) swallowed 1 600 and 2 000 cysts respectively, but no sporocysts and oocysts were found later.

These attempts demonstrated that none of these hosts serves as the definitive host for this parasite (Zuo et al, 1995). The definitive host of *S. sinensis* remains unknown to this day.

The identification of *S. sinensis* is as following:

Intermediate host: water buffalo

Definitive host: unknown

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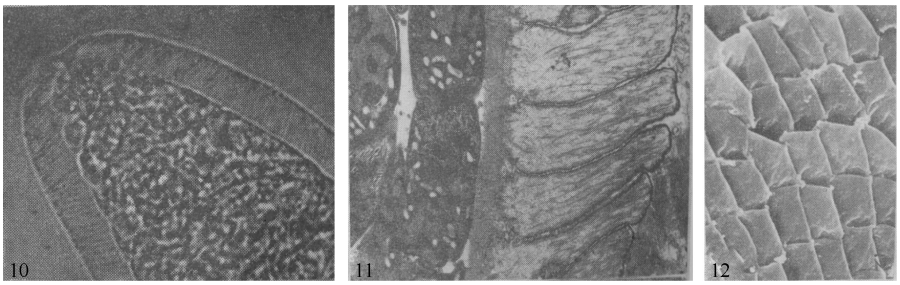

Figure 1 Cysts structure under LM, TEM and SEM presented from the book of Zuo et al (1995)

10: *S. sinensis* cyst from naturally infected water buffalo (1 400×, LM); 11: Cyst wall section of *S. sinensis*, showing the protrusions, microtubules few electron dense granules (7 000×, TEM); 12: Cyst surface protrusions of *S. sinensis* (10 000×, SEM).

Found place: Kunming, China

S. sinensis samples were stored at the museum of Department of Biology, Yunnan University, Kunming, China. The samples were cysts fixed in glutaraldehyde.

We consider that naming the new species as *S. sinensis* completely met the requirements of *The International Code of Zoological Nomenclature* (ICZN). The book (a collection) was published formally in 1995 (ISBN 7-5046-2012-2). It wasn't the 1985-1995 proceedings mentioned by Dubey et al (2014), but rather was an open publication to commemorate the China Society for Parasitology, founded a decade earlier. It was a peer-reviewed book. Its preface specified that only 93 studies were selected from more than 200 manuscripts submitted for publication. Eight new species from *Metahaliotrema*, *Protogyrodactylus*, *Bicotyle*, *Diplectanum*, *Diplectanum*, *Lamellodiscus*, *Pseudocryptotropa*, as well as *Sarcocystis* respectively were published. Although it is true that the ICZN stipulates that "materials issued primarily to participants at meetings (e.g. symposia, colloquia, congresses, or workshops) including abstracts and texts of presentations or posters" do not constitute a published work, we believe that Zuo (Zuo et al, 1995) fully meets the criteria required for describing a new species. We emphasize that Zuo's work was not published in collections of meeting proceedings from 1985-1995 mentioned by Dubey et al (2014) (although such an unpublished collection, in Chinese, does exist). Rather, this was a formally published in a book with an ISBN designation. The ICZN code does not stipulate that publishing a new species must take place in a journal. Moreover, article 8 of the ICZN code defines a published work as an edited material, which since origin is available and which constitutes a high number of identical copies of long term material. Two formally published books have been issued 2100 copies since published, and all of them were commercialized.

Some samples of *S. sinensis* were subsequently identified by investigators according to the initial morphological description. Photos were showed by several investigators who found *S. sinensis* (Chen et al, 2011; Jehle et al, 2009; Li et al, 2002; Moré et al, 2014; Yang et al, 2001, 2002). Additionally, Chen et al (2011) made a specific introduction of *S. sinensis* in English as well as an attempt to differentiate it from *S. hominis*. Several of the mentioned studies have also provided molecular evidence of differentiating *S. sinensis* from other *Sarcocystis* species.

The fourth *Sarcocystis* species mentioned by Dubey et al (2014) in cattle, *Sarcocystis sp.* was named as *S. sinensis* in the original publication. Suppression of the name *S. sinensis*, would, in our view, lead to further confusion in scientific literature. For these reasons, we consider that *S. sinensis,* as described in 1995 by Zuo et al, remains as a valid name. Probably, ICZN should provide a statement about this topic.

Huong & Uggla named a new species as *S. dubeyi* n. sp. (PROTOZOA: SARCOCYSTIDAE) in water buffalo (*Bubalus bubalis*) in 1999. Samples from esophagus, tongue, heart, masseter, cervical, scapular, diaphragm, psoas, thigh, and abdominal muscles were collected from 60 carcasses of adult beef water buffaloes from Vietnam. They reported 13% prevalence rate of *S. dubeyi* from examined animals and presented the morphological structures under LM and TEM. They did not report any attempts at experimental infection. Their abstract states "The definitive host of *S. dubeyi* was not determined, but it could possibly be humans or other primates". On what basis was this possibility suggested? Should experiments not have attempted to use cats and dogs as definitive hosts, given their frequent role as definitive hosts for species of *Sarcocystis* using domesticated animals as intermediate hosts? Later, Dubey et al (2014) stated the "*S. dubeyi* (definitive host unknown but not cat or dog)" but without citing published evidence for this parenthetical statement. According to the morphological structure under LM and TEM (Huong et al, 1999), *S. dubeyi* is similar to *S. sinensis.* Moreover, there are no reports of gene sequences of *S. dubeyi* reported on the GenBank. Based on the fact that *S. sinensis* was reported earlier, and based on the fact that more experimental infection attempts have been made in searching for its definitive host, we hold that *S. sinensis* deserves priority. If the two taxa are to be synonymized, we recommend that *S. dubeyi* be relegated as a junior synonym in accordance to the priority statement of the ICZN.

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Absence of mutation in *miR-34a* gene in a Chinese longevity population

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DEAR EDITOR:

Centenarians are a typical longevity model characterized by delayed onset of morbidity in age-related diseases such as cancer, cardiovascular disease, dementia, and stroke (Andersen et al, 2012). Though there may be a number of underlying mechanisms behind this longevity, curiously it seems that the survival advantage persists in their offspring (Terry et al, 2003), suggesting a potentially important role for genetic factors. Previous studies suggested that the heritability of human longevity may be ~25% (Herskind et al, 1996; Mcgue et al, 1993), whicih is consistent with other studies on model organisms that identified several longevity-related genes, such as *age-1, daf-2, daf-16, and sir-2* (Friedman & Johnson, 1988; Kenyon et al, 1993; Lin et al, 1997; Tissenbaum & Guarente, 2001). Likewise, several studies have reported the existence of many mutations related to human longevity (Holstege et al, 2014; Sebastiani et al, 2012).

Alongside gene mutations, microRNAs have been shown to influence gene function in general, and more particularly have been implicated in various age-related diseases (Boehm & Slack, 2005; Bonauer et al, 2010; de Lencastre et al, 2010; Esquela-Kerscher & Slack, 2006; Eacker et al, 2009; Jordan et al, 2011; Provost, 2010; Somel et al, 2010; Schraml & Grillari, 2012). Typically, the expression of most miRNAs are downregulated with human age (Noren Hooten et al, 2010), but are upregulated in centenarians as compared with the octogenarians, being somewhat similar to those of younger people (Serna et al, 2012). Consequently, it is not surprising that miRNAs have been suggested to play crucial roles in longevity (Ibáñez-Ventoso et al, 2006; Pincus et al, 2011). Of these miRNAs, *miR-34a* was reported to determine life span of *C. elegans* and modulate aging in *Drosophlia* (de Lencastre et al, 2010; Liu et al, 2012; Yang et al, 2013). Mutations located in the region of pri-*miR-34a* were shown to affect the function of miR-34a (Gong et al, 2012; Locke et al, 2014). *MiR-34a* itself plays an important role in development and various diseases (Rokavec et al, 2014); previously, it was reported to determine life-span and modulate aging in model organisms (de Lencastre et al, 2010; Liu et al, 2012; Yang et al, 2013). The inhibition of *miR-34a* regulates cardiac aging through silencing or genetic deletion in mice (Boon et al, 2013). Likewise, *miR-34a* was recognized as a tumor suppressor gene in multiple kinds of cancers (Chim et al, 2010; Cole et al, 2008; Li et al, 2009; Welch et al, 2007; Wiggins et al, 2010). [1]

Based on these evidences, we speculated that *miR-34a* gene may be associated with human longevity. Mutations in the *miR-34a* gene were revealed to affect its function (Gong et al, 2012; Locke et al, 2014). Data from 1000G Database showed 5 rare mutations (rs201359809, rs72631823, rs35301225, rs369892834, and rs372904298) located in the *miR-34a* gene (http://www.1000genomes.org/), two of which have been shown to associate with the *miR-34a* function (Gong et al, 2012; Locke et al, 2014). The minor allele A of rs72631823 in pre-*miR-34a* resulted in higher level of mature *miR-34a* expression, which increased apoptosis in pancreatic beta-cell (Locke et al, 2014). The rs35301225 residing in the miR-34a mature sequence shows an effect on its target binding (Gong et al, 2012). Despite these suggestive lines of evidence, to our knowledge, there has not been any data available on the association of *miR-34a* SNPs with longevity.

Here, we aim to locate SNPs potentially associated with longevity by sequencing the *miR-34a* gene. In total, 439 genetically unrelated subjects of Han nationality (203 centenarians and 236 normal control subjects) were recruited

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from Hainan, China. Both centenarians and controls were in good healthy states without severe diseases (He et al, 2014a, b). The age of centenarians ranged from 100 to 109 years with an average of 102.7±2.3 years, and the average age of controls was 48.1±11.3 years. Total genomic DNA was extracted from the whole blood using the standard phenol-chloroform method (Sambrook & Russell, 2006). The purity and concentration of the extracted DNA were determined by Synergy H1 hybrid multimode microplate reader (BioTek, USA).

The *miR-34a* coding gene is located in the region of Chromosome 1p36.23 with a length of 110 bp. The *miR-34a* gene was amplified using primers as follows: the forward primer, 5'- ACTTCTCCCAGCCAAAAGCC-3'; reverse primer, 5'-TTATCAACAGGTGCTGGGGA-3'. The polymerase chain reaction (PCR) conditions were as follows: 3 min at 94 °C for one circle; followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; final extension was completed at 72 °C for 7 min. The PCR products were sequenced on an ABI3730xl Genetic Analyzer (ABI, USA).

After sequencing the *miR-34a* gene among the 439 Chinese subjects, comprised of 203 centenarians and 236 young controls, we did not find any SNPs in the *miR-34a* gene (Table 1), though this may be attributable to the sample size. This failure to observe variations in *miR-34a* gene suggests that other mechanisms affecting the function of *miR-34a* may exist, such as epigenetic silencing, aberrant miRNA processing or other molecular ways (Garzon et al, 2009; Liang et al, 2009). Further targeted studies to explore this possibility would be invaluable in elucidating the potential association of *miR-34a* functionality and longevity.

Table1 Sequencing results of *miR-34a* gene in Hainan population by using DNA direct sequencing

Sample	Number (*n*)	Mean age (years)	SNPs				
			rs201359809	rs72631823	rs35301225	rs369892834	rs372904298
Controls	236	48.1	0	0	0	0	0
Centenarians	203	102.7	0	0	0	0	0
1000G	-	-	0	0	-	0	0
NCBI	-	-	0.02%	0.01%	-	-	-

SNPs: Single nucleotide polymorphisms; 1000G: 1000 Genomes Project (http://www.1000genomes.org/); NCBI: National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/); Dash indicates that the data was unavailable.

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Three Australian leg-flagged Great Knots (*Calidris tenuirostris*) found on the islet coast of Jinmen (Quimoy) in Fujian, China

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DEAR EDITOR:

On April 7, 2013, approximately 50 Great Knots (*Calidris tenuirostris*), Red Knots (*C. canutus*) and Bar-tailed Godwits (*Limosa lapponica*) were seen on the coast of Jinmen (Quimoy) (N24°26'; E118°18') in Fujian, China. Among these birds, three Great Knots were banded with Australian yellow leg-flags (ENE, LCC and USP in Figures 1, 2 and 3, respectively), which are new to Jinmen.

Great Knots are long distance migrants on the trans-pacific route. During the spring northbound flight to Siberia, Great Knots fly nonstop for 5 420 km from Australia to Chongming Dongtan in the Yangtze River estuary of China (Barter & Wang, 1990; Battley et al, 2001; Choi et al, 2009; Ma et al, 2011; Pennycuick & Battley, 2003; Zhang et al, 2011), followed by a stopover at the Yalujiang estuary of northern China (Choi et al, 2009; Ma et al, 2011) before reaching their breeding grounds in Siberia. Jinmen is a small island located on the southern coast of Fujian, China. During the spring northbound migration, many shorebirds such as Great Knots, Red Knots, Dunlins (*C. alpine*), Red-necked Stints (*C. ruficollis*), Lesser Sand Plovers (*Charadrius mongolus*) and Bar-tailed Godwits stopover in Jinmen and gain small fuel stores for further migration flight (Yen & Shu, 2002). Our observations are the first to report on Great Knot stopover at Jinmen.

Figure 1 Leg-flag ENE of Great Knot (*Calidris tenuirostris*)

Figure 2 Leg-flag LCC of Great Knot (*Calidris tenuirostris*)

Figure 3 Leg-flag USP of Great Knot (*Calidris tenuirostris*)

According to Australian records, Great Knot ENE was banded as a one-year-old on November 27, 2007, at Roebuck Bay in Broome (S18°00'; E122°37'), Australia. From 2008 to 2012, it was resighted at Roebuck Bay every year, and it was recaptured

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on August 28, 2011, when it was five years old. Bird LCC was banded on November 10, 2008, at Roebuck Bay, at two years of age. It was resighted in Roebuck Bay every year. Bird USP was banded on October 15, 2011, at Roebuck Bay. It was recaptured at the same bay on March 6, 2012, at two years of age.

The migration strategies of shorebirds are categorized into hop, skip and jump patterns (Boere & Stroud, 2006; Colwell, 2010). Some shorebirds hop short distances between stopover areas and gain small fuel stores. Other species skip among stopover sites and accumulate moderate fuel loads, while some species jump long distances after accumulating large lipid reserves. The migration flight of Great Knots in autumn exhibits a jump pattern, whereby they move long distances nonstop. In spring, however, they skip between stopover sites to accumulate moderate fuel loads as they move towards their breeding grounds. The three leg-flagged Great Knots observed in this research provide evidence that some individuals stopover at Jinmen in Fujian, China, during their northbound migration flight, which was previously unknown.

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Journal Correction

The description of the cover image of the volume 35, issue 6, released on 18 November 2014, contained error.

The scientific name of the cover image was incorrect and should have been *Macaca leonine*. The online version has been corrected. *Zoological Research* apologizes to the authors and readers for the mistake.

Zoological Research Editorial Board

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