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Foreword

Between 2011−2013, *Zoological Research* has released 3 special issues on Primates and Animal Models of Human Diseases. Now, after a year of preparation, we are pleased to release this fourth special issue. In this issue, we compiled 12 review, research articles and letters to the editor, each examining different aspects of scientific and medical research using non-primates as an animal model, with a special emphasis on recent developments in genetic, virological, behavioristic and methodological studies.

One of the key obstacles preventing wider applications of non-human primates (including their closest relative, tree shrew) in biomedical research is a lack of fundamental biological information on these models. To that end, Fan et al carried out a genome-wide study on positive gene selection to interpret the genetic bases of locomotor adaptation in the Chinese tree shrew (*Tupaia belangeri chinensis*), and Zhang et al measured serum immunoglobulin IgG, IgM, IgA, complement C3, C4 and CRP levels in 3−11 year old captive northern pig-tailed macaques (*Macaca leonina*) located at the Kunming Primate Research Center of Chinese Academy of Sciences, which currently maintains the largest captive population of *Macaca leonina* in China.

Another focus of this special issue is certain human diseases that are difficult to study using traditional rodent animal models, such as *Mycobacterium tuberculosis* (*M. tb*) and human immunodeficiency virus (HIV) co-infection, which is growing global public health problem. More effective research on such diseases could be done if there was a more effective animal model. In this issue, Guo et al accordingly reviewed recent developments in animal models for *M. tb*/HIV co-infection, with a focus on the non-human primate models. Similarly, Lei et al showed that *Macaca leonina* has the potential to be a promising animal model for human HIV/AIDS studies. Mo et al compared the different parameter settings of white matter diffusion tensor imaging (DTI) in rhesus macaques (*Macaca mulatta*) and screened out the optimal setting.

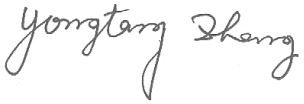
While finding more effective models is an important step in furthering the use of non-human primates in biomedical research, there is also the issue of the methodologies used to create such models. Tian & Ma pointed out that a common restriction in conventional animal models is that most of the human diseases-like abnormalities are induced via artificial interventions, such as drug administration. However, more elegant and subtle animal models should accurately exhibit the pathological symptoms of human mental diseases and not simply imitate certain syndromes. Consequently, effective animal models are supposed to be developed through psychological methods consistently associated with known theories of mental disorders. In their large-sized study on mental illness and cynomolgus monkeys (*Maca fascicularis*), Tian & Ma highlighted the need to more deeply consider both genetic factors and environmental factors in animal models, rather than simply relying on drugs that can induce mimicked symptoms. Wang addressed the advantages of using non-human primates and tree shrews in the studies of drug addictions.

As a corollary to much of the research into non-human primates we highlighted in this issue, animal behaviors are among the most direct indexes needed to demonstrate the effects of medications and the outcomes of diseases, but they also serve as a foundation in wild species conservation. Chu et al reported a huddling-based paradigm that represents

the postpartrum depression (PPD) in primates and provides a great translational efficiency and research platform for systematically investigating the etiology, treatment, prevention of PPD. Self-directed behavior (SDB) is characterized as an indicator of anxiety, frustration and stress in nonhuman primates. Zhang et al collected SDB data from one group of free-ranging Tibetan macaques (*Macaca thibetana*) at Mt. Huangshan, China, and their observations suggest that SDB is not only an index of anxiety in Tibetan macaques, but also can provide a new insight into evaluation of social relationships between individuals. Cui et al likewise clarified the hierarchy and social relationships in a one-male unit of captive *Rhinopithecus bieti* observed between August 1998 and March 1999, and found that in adult male Tibetan (*Macaca thibetana*), Barbary (*M. sylvanus*), and stump-tailed macaques (*M. arctoides*), bridging is a ritualized infant-handling behavior. Collecting further data from a group of habituated, provisioned Tibetan macaques, Bauer et al supported the agonistic buffering hypothesis of the underlying mechanisms of bridging. Such behaviors observed in these studies can also be profoundly influenced by human social industrialization, which may have implications for human-animal interaction in developing or using non-human primate models for scientific research. To that end, Yuan et al evaluated the influences of tsaoko plantation on the habitats of eastern hoolock gibbon (*Hoolock leuconedys*) and provided useful suggestions for future species conservation.

Ultimately, we hope that this new collection of papers on “Primates and Animal Models of Human Diseases” will continue to raise the profile of primate research, and provide some valuable insights into the implications for the future use of non-human primate models for biomedical research. We warmly welcome your contributions and thoughts on these issues for inclusion in our next special issue on this topic.


Editor-in-Chief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Animal models to study *Mycobacterium tuberculosis* and HIV co-infection

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Abstract: *Mycobacterium tuberculosis* (*M.tb*) and human immunodeficiency virus (HIV) co-infection has become a public health issue worldwide. Up to now, there have been many unresolved issues either in the clinical diagnosis and treatment of *M.tb*/HIV co-infection or in the basic understanding of the mechanisms for the impairments to the immune system by interactions of these two pathogens. One important reason for these unsolved issues is the lack of appropriate animal models for the study of *M.tb*/HIV co-infection. This paper reviews the recent development of research on the animal models of *M.tb*/HIV co-infection, with a focus on the non-human primate models.

Keywords: HIV; *Mycobacterium tuberculosis*; Co-infection; Animal model

Tuberculosis (TB) and HIV/AIDS remain two major global health problems. An estimated 12 (11−13) million people worldwide suffered from active TB, and 35.3 (32.2−38.8) million people were living with HIV in 2012 (UNAIDS, 2013; WHO, 2013). In 2012, 2.3 (1.9−2.7) million people were newly infected with HIV, and 1.6 (1.4−1.9) million people died from AIDS-related causes globally (UNAIDS, 2013). In 2012, there were an estimated 8.6 million new cases of TB (13% co-infected with HIV), and 1.3 million people died from TB, including 320 000 among people who were HIV-positive (UNAIDS, 2013; WHO, 2013).

Starting in the mid-1980s, the HIV epidemic led to a major upsurge in TB cases (Ye & Lu, 2004). TB is the most common opportunistic infection affecting 20%−50% of HIV patients. About one-quarter of deaths among people with HIV are due to TB (WHO, 2013). HIV is the most powerful known risk factor for reactivation of latent TB infection, which is a leading killer of people living with HIV. Co-infection with TB and HIV leads to challenges in both the diagnosis and treatment of TB. The mechanisms of *M.tb*/HIV co-infection/interaction and the breakdown of the immune defense of the co-infected individual are not well known. The research in the biology of concurrent *M.tb* and HIV infection is urgently needed, as it will help to reduce the risk of morbidity and mortality and the socioeconomic burden associated with these two diseases.

One of the most important challenges in studies of *M.tb*/HIV co-infection is to establish an appropriate animal model. Although small animals such as mice can be infected by *M.tb*, they are not hosts of HIV and are not suitable for the *M.tb*/HIV co-infection modelling. But some complementary mouse models, such as humanized mouse, can be used to reproduce the relevant features of *M.tb* and HIV infections. By contrast, some nonhuman primates, such as *Macaca* (macaques: rhesus, cynomolgus), can be infected by simian immunodeficiency virus (SIV), a retrovirus similar to HIV causing immunodeficiency in macaques (Lei et al, 2013; Xia et al, 2010; Zhou et al, 2013). These SIV macaque models have been widely used in HIV/AIDS research (Zhang et al, 2007a). Macaques can also be infected by *M.tb* and develop TB that is closely resemble humans. Addition-

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ally, similar to humans, macaques can maintain TB latency for years and only a small proportion progress to active disease (Gormus et al, 2004). Therefore, macaques may be ideal for establishing future *M.tb*/HIV co-infection animal models.

M.tb/HIV co-infection mouse model

To circumvent the limitation that the mice cannot be infected by HIV, researchers developed two complementary mouse models. The first model is called the humanized mouse model. In this paradigm, the human immune system is reconstituted in immunodeficient mice by transplanting human hematopoietic progenitor cells ($CD34^+$) from human cord blood (Traggiai et al, 2004). The second model is the BLT (bone marrow, liver, thymic) mice model. NOD/SCID mice are co-transplated with live human fetal thymus tissues along with autologous $CD34^+$ hematopoietic stem cells (Gorantla et al, 2010). These BLT mice gain human immunity as a functional human thymus can produce more proper humanized T cells. Therefore, these mice could be infected by HIV, causing $CD4^+$ T cells depletion and prolonged viremia (Baenziger et al, 2006; Denton et al, 2010; Gorantla et al, 2010; Sun et al, 2007; Zhang et al, 2007b). In addition, as the transplanted human cells can be maintained in the mucosal surface, the mice can be infected with HIV by the intravaginal and intrarectal routes (Joseph et al, 2010). This model has been used extensively to study the prevention or treatment of HIV infection with human-neutralizing antibodies, antiretroviral drugs and T cell-specific siRNA (Denton et al, 2010; Joseph et al, 2010; Kumar et al, 2008). Recently, the humanized mouse model has also been used in the TB infection research. Heuts et al (2013) reported that humanized mice were infected with either Bacillus Calmette-Guerin (BCG) by intravenous injection or *M.tb* by aerosol. In contrast to the nonhumanized BCG-infected control, the core of the granulomas in humanized mice contained giant cells, human $CD68^+$ macrophages, and high bacilli numbers surrounded by a layer of human $CD3^+$ T cells and a fibrotic response encapsulating the lesion in the liver and lungs (Heuts et al, 2013). Paradoxically, humanized mice contained higher mycobacterial numbers in organs than nonhumanized controls (Heuts et al, 2013). The higher mycobacterial loads was mediated by the human $CD4^+$ T cells, as BCG loads in the lungs or liver of anti-human CD4-treated humanized mice were reduced compared with nontreated humanized mice (Heuts et al, 2013). These humanized mice can be a good model in pathogenic infection and in the study of the formation and maintenance of human granulomas in TB.

Calderon et al (2013) recently developed an improved humanized mouse model using NOD-SCID mice, which were engrafted with human fetal liver and thymus tissue, and supplemented with $CD34^+$ fetal liver cells. The peripheral blood in well-reconstituted mice expressed high levels of human CD45 pan-leukocyte marker. Human T cells (CD3, CD4 and CD8), natural killer and monocyte/macrophages were observed within the human leukocyte population 12 weeks after engraftment. Human T cells in humanized mice were functionally competent as determined by proliferative capacity and effector molecule expression in response to positive stimuli. Once intranasally infected with *M.tb*, these humanized mice had progressive *M.tb* infection in the lung, which disseminated to the spleens and livers 2−8 weeks post-infection (Calderon et al, 2013). In addition to the organized granulomatous lesions, caseous necrosis, bronchial obstruction and crystallization of cholesterol deposits could be found in the sites of infected lungs. Importantly, human T cells were found in the lungs, liver and spleen at the site of inflammation and *M.tb* growth (Calderon et al, 2013). These findings clearly demonstrated the feasibility of using this mouse model for *M.tb* infection. However, there has been no report about using this model for *M.tb*/HIV co-infection studies.

HIV transgenic mice incorporating the entire vial genome have also been used in *M.tb* infection (Scanga et al, 2007). *M.tb*-infected mice showed an increase in HIV expression at the site of bacterial replication and associated with the areas of inflammation. *M.tb* induces HIV transgene expression by both TNF-dependent and independent mechanisms, the former playing a more substantial role at a later stage of infection. Antibiotic treatment of *M.tb* can markedly reduce HIV transgene expression. These data suggest that this animal model is useful in testing therapeutic regimens for reducing the disease burden in patients with HIV-associated TB (Scanga et al, 2007).

MAC/SIV co-infection macaque model

Macaques provide a valuable model for SIV and *Mycobacterium avium* complex (MAC) co-infection.

MAC is known to cause a severe opportunistic infection for HIV-infected people. Although the murine model of MAC infection is well established, this model is not suitable for investigating the interaction among the host, MAC and HIV. MAC in SIV-infected macaques is a frequent opportunistic infection that shares many features with humans infected with AIDS.

Through a retrospective analysis, Mansfield et al (1995) found that 17% (23/135) of SIV-induced simian AIDS macaques which received neither antiretroviral nor antimicrobial therapy were infected with MAC, and 31.3% (21/67) of the SIVmac251-infected macaques were MAC-positive. MAC positivity specifically for SIVmac29 and SIVmac239/316EM was detected in 1.9% (1/53) and 6.7% (1/15), respectively. In addition, compared to the SIV mono-infected macaques, animals with MAC had a longer mean survival after SIV primary infection and lower CD4 cell counts at death. However, the mechanism of the longer survival time in co-infected macaques remains unclear. Mansfield et al (2001) also developed an experimental system to co-inoculate rhesus macaques with SIV and a clinical MAC strain. By using this animal model, they found that the development of disseminated MAC is dependent on the SIV strain. The rhesus monkeys co-infected with SIVmac251 and MAC developed progressive disease, whereas the control animals infected with only MAC and animals co-infected with SIVmac239 or SIVmac239MER and MAC developed self-limiting infection. The ability of self-limiting infection which can eliminate mycobacterial disease was independent of the CD4 T cell counts and the viral load but associated with the size and composition of micro-granulomas.

Whether infection with MAC among HIV patients is a result of recent exposure to virulent strains or reactivation of latent MAC infection is unclear at present. To address this question, Maslow et al (2003) cultured the tissue samples from SIV-infected as well as uninfected rhesus macaques, showing that 68.1% (32/47) SIV-infected and 22.2% (14/63) SIV-uninfected macaques were MAC-positive. Twenty-five SIV-infected animals and one uninfected animal were infected with MAC strain K128A, which is highly virulent for rhesus macaques (Newman et al, 1999). These data demonstrate that disseminated MAC disease appears to be from reactivation of latent infection, as well as from recent infection with virulent MAC strains.

BCG/SIV co-infection macaque model

The safety of BCG use in people infected with HIV has been controversial (Nuttall & Eley, 2011). To examine the impact of BCG inoculation on SIV replication, Cheynier et al (1998) repeatedly inoculated BCG to three SIVmac251-infected macaques by intravenous injection and showed that the recruitment of BCG-specific T cells facilitated SIV replication and dissemination. This finding was supported by another study (Zhou et al, 1999), showing that BCG-mediated T cell activation correlated with a marked increase in viral loads in SIV-infected macaques. Moreover, the prolonged T cell activation coincided with the enhanced depletion of CD4 T cells and the accelerated progression to clinical AIDS in the co-infected monkeys. Within 2 to 7 months after BCG co-infection, all chronically SIV infected monkeys died from AIDS, including TB-like disease. These results suggested that BCG-driven T cell activation may be an important mechanism that enhances the pathogenicity of HIV (Cheynier et al, 1998; Zhou et al, 1999). Surprisingly, in this study (Zhou et al, 1999), 2 weeks after simultaneous SIV and BCG inoculation, naïve monkeys manifested a T cell activation-related toxic shock syndrome and a profound depletion of $CD4^+$ T cells. These co-infected naive monkeys all died of AIDS within 2 months after co-infection. In contrast, the control SIV mono-infected naïve monkeys showed only a natural course of chronic SIV infection, and the BCG mono-infected naïve monkeys survived BCG infection. Thus, this SIV/BCG co-infection model supports the hypothesis that HIV and *M.tb* co-infection can remarkably impact AIDS virus-induced disease.

In another study (Croix et al, 2000), rhesus macaques were first infected with SIV/DeltaB670 and then inoculated with BCG. All animals had a dramatic transient increase in plasma viral loads and CCR5 expression on T lymphocytes after BCG inoculation. Two of the four SIV-infected animals had strong proliferative responses to PPD; the other two with poor responses developed disseminated BCG during the course of the experiment. The interaction of BCG with SIV was also examined in SIV-infected long-term non-progressor (LTNP) monkeys (Croix et al, 2000). Similar to the acutely infected monkeys, two of three LTNPs had an increase in plasma viral loads and CCR5 expression, but none had accelerated progression to AIDS (Croix et al, 2000).

Newly acquired *M.tb* infection in HIV-infected patients can spread readily and progress to active TB disease (Daley et al, 1992). Following BCG co-infection, the SIV-infected macaques with high viral loads developed a SIV-related TB-like disease (Shen et al, 2004b; Shen et al, 2002a). The clinical symptoms included diarrhea, anorexia, weight loss and altered levels of consciousness, and pathological studies revealed the presence of disseminated granulomas. In contrast, co-infected macaques with low viral loads either showed no evidence of BCG-induced disease or developed focal granulomatous lesions (Shen et al, 2002a). The interaction between SIV and BCG may play a critical role in triggering the development of SIV-related TB-like disease. BCG infection can enhance the destruction of $CD4^+$ T cells in macaques with high SIV loads. The progression of SIV disease led to marked suppression of BCG-specific T cell responses, causing the persistence of BCG infection and development of SIV-related TB-like disease. The naïve macaques simultaneously infected with SIV and BCG also developed the SIV-related TB-like disease (Shen et al, 2002a).

In order to examine antiretroviral therapy-induced restoration of memory anti-mycobacterial immunity, macaques were inoculated intravenously with BCG, then SIVmac251 and finally BCG again in 2-month intervals (Shen et al, 2001). The co-infected macaques received antiretroviral treatment, which controlled the SIV replication and the SIV-related BCG-induced disease. The resolution of this disease coincided with the restoration of the PPD-specific T cell response. By contrast, the co-infected macaques which did not receive antiretroviral treatment had a depressed PPD-specific response and they all died from TB-like disease (Shen et al, 2001). The results of this study suggested that antiretroviral agents can improve the outcome of AIDS virus-related TB-like disease by restoring the *M.tb* specific immune response. $V\gamma2V\delta2^+$ T cells may contribute to adaptive immunity to mycobacterial infection (Shen et al, 2002b). Zhou et al (2003) investigated the $V\gamma2V\delta2^+$ T cells in SIV and BCG co-infected macaques. The primary and recall expansions of phosphoantigen-specific $V\gamma2V\delta2^+$ T cells can be found after BCG infection and BCG reinfection in control SIVmac negative macaques (Shen et al, 2002b; Zhou et al, 2003). Conversely, SIV infected macaques showed only subtle expansions of $V\gamma2V\delta2^+$ T cells in both peripheral and bronchoalveolar lavage fluid (Zhou et al, 2003). But this adaptive $V\gamma2V\delta2^+$ T cell responses during SIV/*M.tb* co-infection can be generated by effective antiretroviral treatment (Shen et al, 2004a).

M.tb/SIV co-infection macaque model

Safi et al (2003) was among the first to characterize the manifestations of *M.tb* and SIV co-infection, using Indian rhesus monkeys. In this study, 15 animals were vaginally infected with SIV/Delta B670; three animals died within 6 months after infection (Safi et al, 2003). The surviving 12 animals were divided into two groups at about 400 days post SIV infection. Six animals were infected intrabronchially with *M.tb* H37Rv (200 cfu/animal). Two of three co-infected animals with high levels of plasma viral loads had significant body weight loss, and died within 15 weeks after *M.tb* infection. All three animals with high levels of plasma viremia yielded *M.tb* from multiple organs with extensive inflammation and caseous necrosis. The other three animals with moderate levels of plasma viremia survived for 6 months after the *M.tb* challenge and showed no loss in body weight. Necropsy showed pulmonary granulomata and acid-fast organisms. Four of the six SIV mono-infected monkeys were alive without weight loss at the end of the study; the other two died of pneumonia and *M. avium* complex enteritis. In this study, the clinical, immunologic and pathologic findings in survived macaques were similar compared to humans with latent tuberculosis infection (LTBI). These observations suggested that an animal model of LTBI in SIV-infected macaques can be developed. Such a model can be used in investigating the immunologic and microbial factors in HIV and *M.tb* co-infection.

The cellular and molecular mechanisms of TB reactivation by HIV infection are unclear. HIV-infected patients with latent *M.tb* infection have a significantly greater risk of TB reactivation than HIV-negative individuals with latent TB, even if the CD4 T cells are well-preserved (Hanson et al, 1995; Mukadi et al, 1993; Post et al, 1995). To study the reactivation of TB, the latent *M.tb*-infected macaque models are needed. It appears that cynomolgus macaques are more suitable for latent *M.tb* infection than rhesus macaques. Diedrich et al (2010) showed that out of 10 cynomolgus macaques infected with *M.tb* Erdman strain, six were classified as latently infected, as these animals only showed TST positive but no signs of other clinical manifestation. However, when these animals were infected with

SIVmac251, they developed activated TB. Reactivation was independent of viral load but with early depletion of peripheral T cells during acute SIV infection (Diedrich et al, 2010). Comparing with SIV-mono-infected animals, co-infected animals had fewer CD4 T cells in involved lungs. The granulomas from the co-infected animals had histopathologic characteristics that are consistent with a chronically active disease process. These results suggested that initial T cell depletion may contribute reactivation of TB in HIV-*M.tb* co-infection. They also found the T cell cytokines responses in SIV/*M.tb* co-infected macaques are associated with timing of reactivation (Mattila et al, 2011). The *M.tb*-specific Th1 T cells responses were increased 3−5 weeks post SIV infection; more multi-functional $CD4^+$ T cells 3−5 weeks post SIV infection and more Th2-polarized and fewer Th0, Th1-polarized $CD8^+$ T cells 1-10 weeks post SIV infection were founded in animals reactivating <17 weeks post SIV infection than animals reactivating >26 weeks post SIV infection (Mattila et al, 2011). The distortions in pro-inflammatory and anti-inflammatory T cell responses have significant effects on the reactivation of latent TB.

Besides the TB reactivation cynomolgus model, Mehra et al (2011) also developed a similar model by using rhesus macaques at the same time. First, twelve Indian adult rhesus macaques were infected with 500 cfu *M.tb* CDC1551 via a head-only aerosol method. Eight weeks later, six animals with latent tuberculosis and four *M.tb*-negative animals were infected with SIVmac239 via intravenous injection (200 TCID50/animal). The co-infected group had significantly higher body temperature, CRP levels and body weight loss than the *M.tb* mono-infected group (Mehra et al, 2011). SIV not only reactive TB but also increase *M.tb* dissemination in latently infected monkeys as the co-infected group had shorter survival time and higher *M.tb* loads in multi-organs (Mehra et al, 2011). By using confocal microscopy, they also found *M.tb* and SIV can be in the same cells; numerous SIV-positive cells were located in the vicinity of *M.tb* infected cells (Mehra et al, 2011). This study suggests that rhesus macaques serve as an Excellent model to study the phenomena of *M.tb* latency and reactivation.

Although HIV patients are highly susceptible to *M.tb,* even with well-preserved CD4 T cell levels, the risk of *M.tb* infection increases as CD4 T cell levels decrease (Lawn et al, 2006; Mtei et al, 2005). CD4 T cells are still important in the protection against *M.tb*. CD4-depleted monkeys significantly had more severe gross pathology, bacterial burden, and dissemination of bacteria than the control animals (Lin et al, 2012). Out of six latently infected macaques treated with neutralizing antibody to CD4, three had clinical signs of reactivation, although *M.tb*-specific production of IFN-γ was similar to the latently infected control between CD4-depleted reactivators and nonreactivators (Lin et al, 2012). In contrast, SIV infection of latently-infected macaques led to reactivation in all the co-infected animals (Diedrich et al, 2010). CD4 T cells are important for the control of *M.tb* infection, but it is not the only immune factor impaired by HIV. HIV still has other ways to contribute to enhanced susceptibility of the host to *M.tb* infection.

Conclusion

The global HIV epidemic is driving the re-emergence of *M.tb* infection, resulting in an increasingly prevalent TB epidemic across the globe. The significant impact of both HIV and TB on socioeconomic and public health underscore the need for the development of new preventives and therapeutics, which require appropriate animal models for the evaluation. Fortunately, macaques have been recognized as a suitable animal model for both SIV and *M.tb* infection. The data generated from the literature clearly indicate that macaques are appropriate for the study of *M.tb*/HIV co-infection. However, more investigations on testing newly-developed TB or HIV vaccines in co-infected macaque model are very much needed. Such future studies should further prove that macaque model is a valuable animal model for *M.tb* and/or HIV research.

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Involve both genetic and environmental factors to build monkey models of mental disorders

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Dear Editor,

Mental diseases, such as schizophrenia, are typically the result of multiple abnormalities, including neurobiological, psychological and sociological processes, particularly the environmental and genetic disorders (Bailey, 2000). Consequently, in psychic medicine, animal models should be developed via methods consistent with their associated theories of mental disorders. However, many conventional models are constructed via genetic manipulations or surgical operations to the nervous system (Bezard, 2006, van der Staay, 2006, 2009), e.g. administrate animals with agonists or antagonists of various neurotransmitters or drugs to reproduce human mental disorders. The usage of these methods, which should be considered as simple neuro-pharmacological interventions (Duan et al, 2013, van der Staay, 2009; Zugno et al, 2014,), only a limited number of clinical symptoms can be simulated. Moreover, several critical environmental and genetic factors can be easily overlooked. A famous study on a set of identical female quadruplets who all developed schizophrenia between the ages of 20−24 demonstrated the critical roles that genetic backgrounds play in this disease (Mirsky et al, 2000). However, heredity is not able to explain all the cases of schizophrenia. Some 60% of schizophrenia patients have no close relatives with the illness, suggesting that a variety of other factors aside from genetic background play important roles in schizophrenia, especially individuals with an existing genetic predisposition.

Given the complex poly-genic nature of schizophrenia and the multitude of other risk-factors, an ideal animal model of the disease requires screening individuals with similar genetic predisposition under similar environmental conditions that humans experience. To do so requires large populations that provide adequate samples. In southern China, there are numerous monkey breeding centers with populations ranging from the thousands to tens of thousands. Recently, we conducted a screening at the primate center of Jin Gang Biotech International in Haikou, Hainan Province, China. This center breeds more than 20 000 cynomolgus monkeys (*Maca fascicularis*) in different colonies (Table 1).

Table 1 Monkey colonies at the primate center of Jin Gang Biotech International, Haikou

Colony (each colony has approximately 20 monkeys)	Numbers of colonies
Breeding	335
All-male (adult)	100
All-male (immature)	200
All-female (adult)	95
All-female (immature)	195

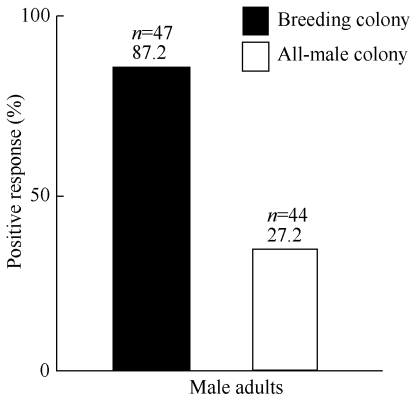

Figure 1 Male monkeys from breeding colonies exhibit more positive responses to the external stimuli than those from the all-male colonies (*n*: numbers of colonies observed)

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To aid in the development of future depression and schizophrenia models, we collected the samples from more than 500 cynomolgus monkeys, and in collaboration with the Huaxi Hospital of Sichuan University, began behavioral observations. To date, we found a significant difference in the social behaviors between the male monkeys from the breeding colonies and the all-male colonies. Males from the all-male colonies exhibit more indifferent responses to external stimuli (Figure 1) and individuals with lower social rank may prone to be depressed than males from the breeding colonies. Moving forward, we need to next determine if there are some correlations between genetic background, social rank, and phenotypes of the disorders in these cynomolgus monkeys. We expect that monkeys from all-male or all-female colonies may prove more suitable for monkey models of depression or schizophrenia than those from the breeding colonies, though only further testing will bear out this hypothesis.

Ultimately, what we would like to address in this letter is that building better animal models of mental disorders is not simply about developing more effective drugs or pharmacological interventions, but should instead carefully consider both genetic and environmental factors.

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Non-human primate models in drug addiction deserve more attention

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Dear Editor,

The process of relapse involves firm or aberrant memories of environmental cues associated with drug craving or addiction. To date, it is not known where these memories are stored in the brain, what kinds of regulatory biological factors or molecules are involved, nor why it is so difficult to stop addiction psychologically. Currently, rodent animal models, such as the self-administration and conditioning place preference / aversion paradigm are still widely used in the studies of drug withdrawal syndromes or drug-associate memories. However, the differences between humans and rodents—particularly in terms of genetics, and pathology and pharmacology—have significantly limited the application of further studies on this topic. Essentially, rodents lack the long-term or life-time memories humans possess and lose their drug-associated memory only after a few weeks of withdrawal.

Compared to rodents, non-human primates have numerous intrinsic advantages that make them an irreplaceable animal model for studying drug addiction, especial relapse. Non-human primates are not only closely related to humans in terms of taxonomic status, but also possess a sophisticated developed prefrontal cortex (PFC) and experience patterns of addictions similar to humans. For example, similar to humans, rhesus monkeys (*Macaca mulatta*) are able to remember morphine-associated cues for at least 36.3±1.3 months after six injections of morphine (Wang et al, 2012; Wu et al, 2012). Some laboratories have also applied monkeys in drug addictive self-administration paradigm (Foltin & Evans 2001) and have combined it with brain imaging techniques (MRI and PET) to explore the changes in the white matter, gray matter, and other brain regions, especially the PFC (Nader& Banks，2014; Smith et al, 2014). Non-human primates have also been used in screening genes of addiction vulnerability, molecules and pathways in addiction memory (e.g., CREB, ΔFosB, PKMzeta, ERK pathway, etc.) (Nestler, 2013;Shema et al, 2011), as well as epigenetic alterations to drug addiction (DNA methylation, histone acetylation/methylation, non-coding RNA, etc.; Nestler, 2014).

Addiction is a complicated process involving both brain malfunction and homeostatic dysfunction (Naqvi, 2014; Paulus et al, 2013). Previous studies indicated that insula and viscerosensory responses play active roles in addiction (Contreras et al, 2007; Naqvi et al, 2007). Alongside PFC, which is one of the most highly focused areas in addiction research (Chen, 2013), other brain areas such as the orbito-frotal cortex (OFC), limbic system and striatum, are increasingly becoming research targets in non-human primate studies. Similarly, other key aspects of addiction including interoceptive reflexes, emotional and environmental contexts, and social status, are being examined using non-human primates.

In addition, as another close relative to human, tree shrews (*Tupaia belangeri chinensis*) are quickly becoming a common animal model in biomedical research (Xu et al, 2013). Wiens et al (2008) reported that the pentailed tree shrew (*Ptilocercus lowii*) evolves a specific metabolic system to avoid alcohol intoxication, while Sun et al (2012) found that tree shrews can develop morphine addiction. Therefore, tree shrews may be considered as a viable animal model in addiction studies.

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A natural model of behavioral depression in postpartum adult female cynomolgus monkeys (*Macaca fascicularis*)

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Abstract: Postpartum depression (PPD) is a modified form of major depressive disorders (MDD) that can exert profound negative effects on both mothers and infants than MDD. Within the postpartum period, both mothers and infants are susceptible; but because PPD typically occurs for short durations and has moderate symptoms, there exists challenges in exploring and addressing the underlying cause of the depression. This fact highlights the need for relevant animal models. In the present study, postpartum adult female cynomolgus monkeys (*Macaca fascicularis*) living in breeding groups were observed for typical depressive behavior. The huddle posture behavior was utilized as an indicator of behavioral depression postpartum (BDP) as it has been established as the core depressive-like behavior in primates. Monkeys were divided into two groups: A BDP group (n=6), which were found to spend more time huddling over the first two weeks postpartum than other individuals that formed a non-depression control group (n=4). The two groups were then further analyzed for locomotive activity, stressful events, hair cortisol levels and for maternal interactive behaviors. No differences were found between the BDP and control groups in locomotive activity, in the frequencies of stressful events experienced and in hair cortisol levels. These findings suggested that the postpartum depression witnessed in the monkeys was not related to external factors other than puerperium period. Interestingly, the BDP monkeys displayed an abnormal maternal relationship consisting of increased infant grooming. Taken together, these findings suggest that the adult female cynomolgus monkeys provide a natural model of behavioral postpartum depression that holds a number of advantages over commonly used rodent systems in PPD modeling. The cynomolgus monkeys have a highly-organized social hierarchy and reproductive characteristics without seasonal restriction—similar to humans—as well as much greater homology to humans than rodents. As such, this model may provide a greater translational efficiency and research platform for systematically investigating the etiology, treatment, prevention of PPD.

Keywords: Postpartum depression; Cynomolgus monkeys; Huddle behavior; Locomotion activity; Stressful events; Hair cortisol; Maternal relationship

Postpartum depression (PPD) is commonly identified as a subtype of major depressive disorder (MDD) with the specification of depression onset within two months after delivery (Friedman & Resnick, 2009). It is estimated that 15% of mothers overall suffer from PPD, with symptoms that last at least a few weeks to months (England, 1994; Flores & Hendrick, 2002; Ghubash & Abou-Saleh, 1997; O'Hara, 1987; Sit & Wisner, 2009). In general, PPD primarily exerts profound adverse effects on mothers and their infants through disturbing the maternal relationship, which may be a disabling or life-threatening disruption (Epperson & Ballew, 2004). However, the cause of PPD is not understood and the exact mechanisms remain unclear (O'Hara & McCabe, 2013).

Still, a further confounding factor to understanding

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PPD development is the crucial and susceptible period, including the time over pregnancy and puerperium, which affects the mental health of mothers and the maturation of infants. This limited window is a difficult period to perform scientific investigation and ultimately slows down the pace of PPD research. The fact highlights the importance of animal models in studying and developing treatments for PPD. However, most models of PPD have been adopted in rodents, and have focused on exploring only a few biological factors involved in PPD, such as ovarian hormones withdrawal, stress and corticosterone exposures, etc. (Brummelte & Galea, 2010). No PPD rodent model fully contains the disorders full spectrum, as there are large differences in rodent and human homology (Willner, 1991), and there are, thus, translational limitations to using rodent models to investigate PPD. Conversely, non-human primates possess physiological, behavioral, and genetic characteristics similar to humans (Sibley & Ahlquist, 1987; Kalin & Shelton, 2003), which make them potential tools in the development of natural models of PPD.

In the present study, adult female cynomolgus monkeys in puerperium were selected as subjects to investigate their use as a natural model of PPD because they have very similar reproductive behaviors, without seasonal restrictions, as humans and a well-organized hierarchical society similar to humans (Sapolsky, 2005; Van Esch et al, 2008) in addition to the biologically similar characteristics between monkeys and humans mentioned above. Furthermore, depression-associated behaviors in non-human primates have long been established by studying maternal separation (Bowlby, 1962; Spitz & Wolf, 1946), and are now widely used in the definition of primate models of mood-related disorders (Shively et al, 2002, 2005, 2006; Strome et al, 2002). As such, researchers have observed that primates demonstrate, along with depressive behaviors, a series of behavioral and neurophysiological abnormalities which are analogous to relevant changes in patients with MDD and PPD, including an increased responsiveness in the sympathetic nervous system (Pryce et al, 2004), dysfunction of the HPA axis (Shively, 1998; Shively et al, 1997), monoamine transmitter deficits (Shively, 1998; Willard & Shively, 2012), and anhedonia (Pryce et al, 2004). These characteristics make the adult female cynomolgus monkey a potentially useful system to explore the etiology and clinical interventions of PPD. And the huddle posture behavior, generally considered to be the core depressive-associated behaviors in non-human primates (Chilton et al, 2011; Shively et al, 2005), was accordingly used as the behavioral indicator to differentiate between monkeys displaying depression postpartum from monkeys who were not in the study.

Numerous stressors, including stressful life events, have been identified as the predictors of PPD (Liu & Tronick, 2013; O'Hara & Swain, 1996). Therefore, the monkeys displaying PPD-like behaviors in this study were then evaluated for stress related indicators. In primates, most sources of stress originate from two main types of conflict events: the receipt of aggression and the display of submission. These stressful events are opposed by two other conflict events or non-stressful events: the receipt of submission and the display of aggression (Koolhaas et al, 1999). Stressful events are often regarded as alternative strategies of coping with stress and non-stressful events are ways of alleviating stress (Folkman et al, 1986; Koolhaas et al, 1999). Therefore, the numbers of times receipt of aggression and submission displays by the monkeys were used to reflect the intensity of stress the monkeys experienced. Previous studies have shown that conflict events have a link with hyperactivity of the HPA axis in both animals and humans, but that non-stressful events did not (Koolhaas et al, 1999). The continuous hypersecretions of the glucocorticoids, such as cortisol, by the HPA axis provides a crucial link between chronic stress exposure and mental disorders in humans (Carroll et al, 1976; McClure, 1966; Parker et al, 2003; Schüle, 2007) and has been commonly utilized in assessing stress conditions experienced in other species (Abbott et al, 2003; Burke et al, 2005; Cattet et al, 2003; Constable et al, 2006; Keay et al, 2006; Millspaugh et al, 2002; Touma & Palme, 2005; Whitten et al, 1998). However, the relevant relationship of stress in PPD patients is still an unclear issue (Gard et al, 1986; Groer & Morgan, 2007; Harris et al, 1989, 1996; Jolley et al, 2007; Nierop et al, 2006; Okano & Nomura, 1992). Therefore, this study further evaluated hair cortisol levels in the subjects from hair samples taken on the first day of the third week postpartum, and were used as an approximate reflection of the accumulated stress experienced by the monkeys over last trimester before delivery until sampling (Russell et al, 2012). A substantial body of evidence indicates that cortisol in hair provides a functional instrument in qualifying the degree of long-term stress in both humans and primates (Davenport et al, 2006; Feng et al, 2011; Stalder et al, 2012) and that hair cortisol is a stable biomarker to assess the state of stress over time, whereas cortisol levels evaluated in plasma, saliva, urine, or feces only reflect cortisol levels at a single point in time (Wennig, 2000; Russell et al, 2012).

Monkeys displaying PPD-like behaviors in this study were then evaluated for the quality of the mother-infant relationships formed after birth. A poor mother-infant relationship is the prominent outcome in PPD patients (Field, 2010). As such, the monkeys displaying PPD-like behaviors in a breeding group provided a unique and controlled environment to assess the nature of the maternal-child relationship as the mother-infant bond forms the fundamental foundation of well-

organized social groups. In breeding groups of cynomolgus monkeys, there are two predominantly observable forms of mother-infant interactions: (1) mothers holding infants and (2) mothers grooming infants (Nakamichi et al, 1990). Conversely, poor maternal relationships have been demonstrated where the maternal monkeys abuse and neglect their babies, which resulted in decreased times in holding and grooming their infants (Maestripieri & Carroll, 2000; Nakamichi et al, 1990). Moreover, grooming interactions have been correlated with levels of stress. While, rodent studies have shown that grooming positively correlated with the state of stress (Kalueff & Tuohimaa, 2005), grooming by maternal primates paralleled with reduced levels of stress (Nakamichi, 2003; Shutt et al, 2007). Thus, by observing behavioral depression postpartum in a breeding group of adult female cynomolgus monkeys, new knowledge can be gained into abnormal parental behaviors and physiological stress states that will provide vital clues for investigating PPD in the future.

METHODS

Subjects

Ten healthy adult female cynomolgus monkeys (5.80±0.79 years, mean±*SE*) were chosen which selected randomly from 62 healthy adult female cynomolgus monkeys we observed in the preliminary study, and were distributing in 8 breeding groups (population numbers ranked from 22 to 29 in each group, where two males were included). Each of the subjects was multiparous with only one infant being reared with their colonies. The colonies were housed in a cage with a roof, which was divided equally into three connected quarters (each of the quarters measured 3.0 m×3.3 m×2.9 m). All the subjects were provided commercial biscuits twice a day and fruit & vegetables once daily. The animals had tap water available *ad libitum*. All animal procedures were carried out in accordance with the Kunming Institute of Zoology Animal Care and Use Committee and with the National Institute of Health's (USA) Guide for the Care and Use of Laboratory Animals.

Group classification and experimental design

All animals were identified for their exhibition of huddle behavior. The huddle behavior is a typical depressive-like behavior that is commonly used as an indicator of behavioral depression (Chilton et al, 2011). Behavior recordings of all subjects were performed immediately on the first day after giving birth and then for 14 consecutive days of postpartum observation in order to calculate the score of the huddling behavior and other behaviors, e.g., locomotive activity, stressful events, and parental behaviors. As a result, subjects were classified into two groups: (1) a behavioral depression postpartum (BDP) group (n=6) and (2) a non-behavioral depression postpartum control (control) group (n=4). On To evaluate cortisol levels, on the 15th postpartum day, hair samples were obtained.

Behavior sampling

Behavior data were collected using a high-resolution portable digital video camera fixed on a tripod. For habituating the animals to the recording activity, the observer entered the monkey farm at least two weeks before recording began; and while videotaping, the observation site was set up on a platform as far away as possible from the cage to prevent disturbing the monkeys. Two 25-minute recordings were collected on each day of observation during two separate time windows: 0900h-1230h and 1400h-1730h, respectively. All data recordings were stored on a hard disk before being analyzed blindly by two trained technicians; the two viewers reached a consensus to the behavioral classification, and all their analyses were done on the computer.

Behavioral categories and definitions

In the present study, four behavioral categories were measured in the subjects: huddling, locomotive activity, stressful events, and parental behavior. First, both depressed macaques and MDD patients have been reported to exhibit an increased huddle time (Canales et al, 2010; Harlow and Suomi, 1974) and the huddle behavior, specifically, predicted behavioral depression in cynomolgus monkeys. The huddle behavior is characteristic of head flexion and thoracic kyphosis (Shively et al, 2005). Second, locomotive activity was appointed to evaluate general physiological functions of the subjects after child birth. Lastly, stressful events and parental behavior were analyzed in both BDP group and control group as incremental stressful life events and disrupted maternal relationships have been associated with the onset of PPD (Field, 2010; Hammen, 2005). In general, stressful events were classified as either aggressive behaviors in the cynomolgus monkeys, which included stare, threat, open-mouth threat, chase, displace, bite, and slap/grab; or submissive behaviors, which included lip smack, grimace, submissive present, move away, scream, scream threat, crouch, flee (Shively et al, 2005). The maternal parental relationship was monitored by counting mother to infant grooming and holding times.

Hair sampling and cortisol RIA assay

Hair sampling was conducted on the first day of the third postpartum week, following the completion of the behavioral recording. The animals were captured by an experienced technician with a net and removed from the colonies. Then, hair samples were taken from their backs of the subjects using a pair of scissors under manual

restraint. The hair samples were placed into a small punch of aluminum foil for protection and storage (Davenport et al, 2006; Wennig, 2000). As mentioned before, the cortisol levels in the hair samples would be used to evaluate approximate amounts of accumulated stress experienced by the monkeys in the study over last three months prior to delivery (Russell et al, 2012).

Before being assayed, cortisol was extracted from the hair sample as detailed previously (Davenport et al, 2006; Feng et al, 2011). In brief, approximately 500 mg of hair was washed twice in 10 mL isopropanol for 3 minutes to remove surface contaminants, dried at 37 ºC for 8 hours, and then pulverized using a Retsch ball mill (Retsch M400) at 26 Hz for 2.5 minutes. Afterwards, 400 mg of the powderized hair was weighed precisely and incubated in 8 mL of methanol at room temperature for 24 h with a slow rotation to extract the cortisol. The samples were then centrifuged at 8000 rpm for 5 minutes and 4 mL of the supernatant was pipetted into a centrifuge tube and dried under a stream of nitrogen gas. The precipitate was reconstituted in 0.25 mL of PBS and stored at −20 ℃.

All measurements of cortisol were performed with a commercially available radioimmunoassay kit (Cortisol RIA DSL-2000, America) at the Radioimmunolaboratory of Yunnan Second People's Hospital. The cortisol extraction and RIA assay were performed under a double-blind procedure, and each hair sample was tested three times with the mean of the three hair cortisol values used to diminish measurement error.

Data analysis

Huddle behavior, locomotive activity, stressful events, and parental behaviors were all quantified for duration (in seconds) and/or frequency of occurrence. The data was analyzed with the Mann-Whitney U (Wilcoxon rank-sum) test to compare differences between the BDP and control group for each behavioral measure. For all analyses, significance was set at $P<0.05$ and determined via two-tailed tests. All data analysis was done using SPSS 16.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Depressive-like behavior

The animals were grouped into a BDP group (n=6) and control group (n=4) based on their time spent in the huddle posture during the first two weeks postpartum. As shown in Figure 1A, the values of huddle time of the BDP monkeys were higher than the control monkeys over the first 6 days, but persistently dropped down over time. Despite this drop, the BDP monkeys still had higher huddle times than those of the control monkeys at each time point; until the last two time points (day13−14 postpartum) where the behaviors of both groups started to match and had relatively low huddle times (Figure 1A). On average, the BDP monkeys had significantly higher huddle times in comparison with the control monkeys (Figure 1B; 108.40±19.14 *vs*. 11.44±4.57, $P<0.001$).

Locomotive activity

No significant differences were discovered for both locomotion duration (Figure 2A; 140.31±7.52 *vs*. 146.85±9.077, $P=0.23$) and frequency (Figure 2B; 39.86±2.16 *vs*. 45.54±3.26, $P=0.49$) between BDP and control monkeys, suggesting that monkeys in the BDP group behaved as normally as their counterparts in the control group.

Stressful events and stress hormone levels

There were no statistically significant differences found between the BDP group and the control group for both stressful events experienced (Figure 3A; 14.71±1.40 vs. 15.56±1.75, $P=0.58$) and hair cortisol levels (Figure 3B; 28.62±4.66 *vs*. 32.01±4.57, $P=0.52$). This suggests that the social stress suffered by both the BDP monkeys and the control monkeys were at similar levels following child birth and during pregnancy.

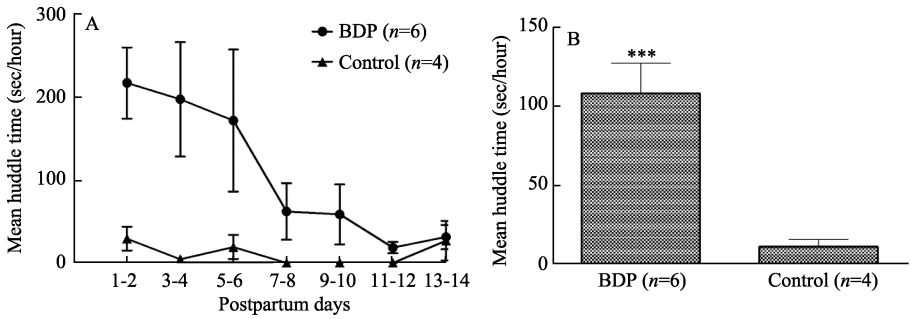

Figure 1 Time being spent in the huddle posture over the first two weeks postpartum in BDP monkeys (n=6) and control monkeys (n=4)

A: Time profiles; B: Average durations of huddling in the BDP group and control group; ***: $P<0.001$.

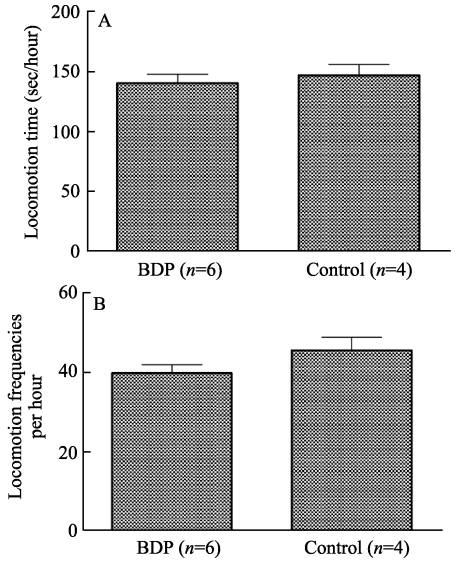

Figure 2 Comparison of locomotive activity between the BDP group (n=6) and the control group (n=4)

A: Duration of locomotive activity; B: The frequency of locomotive activity in the monkeys.

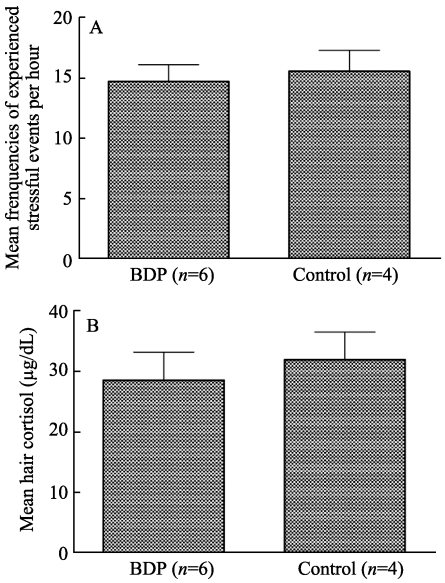

Figure 3 Measures of stressful events experienced (A) and hair cortisol levels (stress hormone) (B) in the BDP group (n=6) and control group (n=4) monkeys

Maternal relationship

Two kinds of recognizable behaviors were used to evaluate the maternal relationship: grooming infants and holding infants. Observations revealed that there were no differences in the "holding" between the BPD monkeys and the control monkeys (Figure 4A; 59.49±0.30 *vs*. 59.85±0.09, P=0.50). However, the BPD monkeys demonstrated a significantly higher amount of time performing the "grooming" behavior compared with control monkeys (Figure 4B; 91.81±12.17 *vs*. 65.64±15.56, P<0.001). Specifically, as shown in Figure 4C, the values of groom time of both groups stayed even in total before the third time point (day 5−6 postpartum), and were both slightly declining over this period. Afterwards, the BDP monkeys started to spend more time grooming than those of the control monkeys over the last 8 days, in spite of almost having the same values of groom time with the control monkeys at the fifth time point (day 9−10 postpartum) (Figure 4C). Overall, this suggested there was an abnormal mother-infant relationship in the BPD monkeys.

DISCUSSION

To the best our knowledge, this report is the first on a natural model of behavioral postpartum depression in non-human primates which is similar to PPD in humans. The BDP-like patterns in adult female cynomolgus monkeys reflected a miniature version of PPD (Epperson & Ballew, 2006) with onset occurring on the first day of delivery and lasting a two-week duration. This study used the huddle behavior as the core indicator of behavioral depression in primates. While this behavior is not the only candidate depression measure available, due to the recognition and effectiveness of the huddle posture measurement provides adequate utilization for measuring behavioral depression in the development of primate depression models (Chilton et al, 2011; Shively et al, 2002, 2005, 2006; Strome et al, 2002). Furthermore, the BDP monkeys demonstrated no differences in locomotive activity in comparison with the control monkeys. The locomotive activity of animals has been found to be affected when parturition is accompanied by loss of blood. The fact that BDP monkeys and control monkeys had similar locomotive behaviors suggested that blood

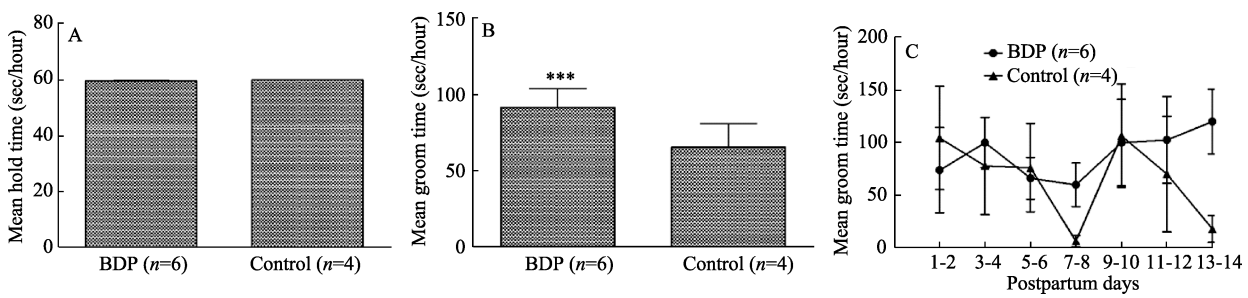

Figure 4 Analyses of the maternal relationship in the BDP monkeys (n=6) and control monkeys (n=4)

A：Holding behavior; B: Grooming behavior; C: time profiles of the grooming behavior over the first two weeks postpartum in the BDP group and control group; ***: P<0.001.

hemorrhaging during delivery was not a confounding factor in the occurrence of the behavioral depression in the postpartum adult female cynomolgus monkeys observed here. All told, these findings represent the first demonstration of behavioral postpartum depression in primates.

In a stable society of most macaques, the behavioral and physiological stress levels of individuals in groups can be predicted by their social status, meaning that subordinate animals experience more stressors such as stressful events and stress hormone responsiveness (Michopoulos et al, 2012; Sapolsky, 2005). In the present study, no associations were found in the BDP group with stressful events and hair cortisol levels, suggesting that stress played little role in the behavioral postpartum depression we observed. By extension, if the stressful events were not an external biological factor in the development of the BDP-like patterns in the adult female cynomolgus monkeys, they may instead be an indicator of a balanced social status in both the BDP and control groups, which corresponded to no differences in stress hormone levels as well (Michopoulos et al, 2012; Shively et al, 2005). This finding contradicts several previous studies in which the presumptive manifestation of behavioral depression in the adult female cynomolgus monkeys positively correlated with stressful events and stress hormone responsiveness (Shively et al, 2005). Therefore, it is plausible that the underlying mechanisms of the behavioral depression phenomena related to either postpartum or stress experiences are significantly different, with wide implications on the elucidation of the basis of PPD and MDD, respectively (Douma et al, 2005; Hammen, 2005).

In addition, the BDP monkeys in this study were found to spend more time grooming their infants than the control monkeys, despite no significant differences being found in the time spent holding infants in both groups. These findings may suggest that "grooming" behavior was more vulnerable to the occurrence of behavioral depression in postpartum adult female cynomolgus monkeys, while the "holding" behavior might reflect a manner of infant abuse and neglect that is separate from the BDP. In fact, infant abuse and neglect by the adult female cynomolgus monkeys is considered a severe form of maltreatment, where as the "grooming" infant behavior represents a mild form of maternal behaviors (Carroll & Maestripieri, 1998; Tsuchida et al, 2008). In a common sense, "grooming" has been understood as a socially affiliative behavior, and has been considered in correlation with lower physiological stress levels (Nakamichi, 2003; Shutt et al, 2007). However, the increased "grooming" behavior in the BDP monkeys does not explicitly correspond to the null differences in stressful events and stress hormone levels in the monkeys as referred to above. The "infant grooming" observed here may be different than the normalized "social grooming" observed in monkey social hierarchies, where the "infant grooming" may have reflected a state of compensation for a certain kind of psychopathological condition associated with the behavioral postpartum depression. In any case, the abnormal parenting relationship in the BDP monkey may be an important behavioral dysfunction in this natural model that may be related to PPD in humans. Cumulative evidence has shown that most PPD patients have poor maternal relationships with their children, which can lead to adverse effects on the behavioral, cognitive, emotional development of their infants (Field, 2010; Goodman et al, 2011), such that this model may be furthered to investigate both physiological and behavioral changes to infant monkeys in response to BDP in maternal monkeys.

Furthermore, this natural model to in the adult female cynomolgus monkeys can be expanded to examine other precipitating factors contributing to behavioral postpartum depression in the monkeys. Previous studies in PPD patients and in rodent systems have provided many clues to what underlying physiological factors are associated with BDP symptoms, such as ovarian hormone withdrawal (Brummelte & Galea, 2010; Douma et al, 2005; Osterlund, 2010). Human clinical trials have found that the ovarian hormone withdrawal can trigger depressive symptoms in susceptible women with a history of PPD (Bloch et al, 2000). Meanwhile, in rodents a hormone-stimulated pregnancy/hormone withdrawal protocol was developed to investigate this issue. The research found that behavioral depression was predicted in the withdrawal phase (Galea et al, 2001; Green et al, 2009; Stoffel and Craft, 2004; Suda et al, 2008). Similar postpartum hormonal changes have been found in cynomolgus macaques. Goodman & Hodgen (1978) reported that progesterone levels dropped over the first two weeks postpartum and then leveled off following this period. Likewise, estradiol levels followed a similar pattern to progesterone, except the lower levels lasted less than one week postpartum. It then stands to reason that the BDP-like patterns witnessed during this study on adult female cynomolgus monkeys may be associated pathologically with ovarian hormones withdrawal. Going forward, this natural PPD model will provide an adequate system to investigate the correlation between ovarian hormone withdrawal in depressed and non-depressed monkeys, as well as investigate the uses of hormone replacement therapy and/or novel drug therapeutics in the treatment of postpartum depression.

In summary, this novel natural model is attempted to define PPD in non-human primates. The BDP-like patterns occurring spontaneously in the postpartum adult female cynomolgus macaque show a much closer relationship to PPD than any other existing model (Brummelte & Galea, 2010). This suggests that it will be a very useful tool to systematically explore the etiology, treatment, and prevention of PPD in future.

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Parameter comparison of white matter diffusion tensor imaging (DTI) in rhesus macaques (*Macaca mulatta*)

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Abstract: In this study, we analyzed diffusion tensor imaging (DTI) results of brain white matter in rhesus macaques (*Macaca mulatta*) with four different parameter settings and found that the sequence A (b=1 000 s/mm^2, spatial resolution=1.25 mm×1.25 mm× 1.25 mm, numbers of direction=33, NSA=3) and B (b=800 s/mm^2, spatial resolution=1.25 mm×1.25 mm×1.25 mm, numbers of direction=33, NSA=3) could accurately track coarse fibers. The fractional anisotropy (FA) derived from sequence C (b=1 000s/mm^2, spatial resolution=0.55 mm×0.55 mm×2.5 mm, direction number=33, NSA=3) was too fuzzy to be used in tracking white matter fibers. By comparison, the high resolution and the FA with high contrast of gray matter and white matter derived from sequence D (b=800 s/mm^2, spatial resolution=1.0 mm×1.0 mm ×1.0 mm, numbers of direction=33, NSA=3) qualified in its application in tracking both thick and thin fibers, making it an optimal DTI setting for rhesus macaques.

Keywords: DTI; Whiter matter; Rhesus macaque

Neuropsychiatric disorders such as Parkinson's syndrome and senile dementia remain global health problems worldwide, and are only expected to grow. In conducting research to gain a clearer picture of these disorders and developing novel therapeutics, animal models have proved critical. Due to their phylogenetic closeness to humans and to circumvent ethical issues, nonhuman primates in particular are widely used in the pathological and etiological researches of these diseases.

In recent years, magnetic resonance imaging (MRI) has become an important tool in brain diseases study because it is non-invasive and can be used to observe the chronic changes in brain function and morphology. Among the various forms of MRI, diffusion MRI, also referred to as diffusion tensor imaging (DTI) has been extraordinarily successful, especially in the study of brain anatomical connectivity. Molecular diffusion in tissues reflects interactions with many obstacles. Therefore, water molecule diffusion patterns can reveal microscopic details about tissue architecture. The neural axons of the white matter in the brain have an internal fibrous structure analogous to the anisotropy of certain crystals. Water will then diffuse more rapidly in the direction aligned with the internal structure. This means that the measured rate of diffusion will differ depending on the observing direction. The neural tract directional information derived from DTI, are sufficient to compute the diffusion tensor, and then from the diffusion tensor, diffusion anisotropy measures such as the fractional

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anisotropy (FA), can be computed (Kubicki et al, 2002). Thus the principal direction of the diffusion tensor can be used to infer the white-matter connectivity of the brain (Behrens et al, 2003; Schmahmann et al, 2007).

The brain volume of humans is 1,400 mL, but is only 100 mL in monkeys. Because the brain volume is important to image quality, scanning parameters applied to humans cannot be used directly on monkeys. Yet the lack of normative descriptions has limited the application of DTI on monkeys. Currently, only a few research institutions have dedicated MRI scanners for animals and the clinical MRI is still widely in use. In this study, four different parameter settings were compared by performing the PHILIPS Achieve 3.0T MRI on rhesus macaques (*Macaca mulatta*) to confirm the optimal settings.

MATERIALS AND METHODS

Experimental animals

Rhesus macaques used in this study were 1.5–8 years old and weighed 3.2–6.8 kg (n=30, 24 males, 6 females). All monkeys were obtained from the Animal Center of Kunming Institute of Zoology, CAS. The animals were habituated for at least four weeks in a temperature-controlled (21 ℃) colony room with food and water available *ad libitum* using a 12h:12h (light : dark) schedule (white lights on: 8:00–20:00). Experimental protocols were consistent with the Society for Neuroscience and National Institute of Health guidelines for the humane use and care of animals.

Experiment procedure

Anesthesia

Subjects were deprived of water 12 hours before the experiment. Prior to the scanning, atropine (i.m, 0.2−0.5 mg/kg, Batch Number: 120502, Shanghai Harvest Pharmaceutical Co., LTD.) was injected to avoid vomiting or oral secretions blocking airways. Following with the onset of preanesthetic ketamine (i.m, 10 mg/kg, Batch Number: KH121011, Jiangsu Hengrui Medicine Co., LTD), subjects were maintained at deep anesthesia with amyl sodium pentobarbital (i.m, 35 mg/kg, Batch Number: WS20110112, Shanghai Westang Bio-Tech Co., LTD).

DTI image acquisition

All subjects were scanned on a PHILIPS 3.0T (Achieve) superconducting MRI scanner by referring the knee coil as the scan coil. The head of subject was fixed with a sponge to reduce noise and prevent head shaking. Each monkey received at least two types of the acquisition sequences. Single-shot echo planar imaging (EPI) combined with parallel acquisition techniques were used in the axial DTI data acquisition. Each monkey was scanned by four different scanning parameter settings, and all scans were conducted by a same MRI technician.

Sequence A: b=1000 s/mm^2, resolution=1.25 mm× 1.25 mm×1.25 mm, numbers of direction=33, numbers of signal acquisition (NSA)=3;

Sequence B: b=800 s/mm^2, resolution=1.25 mm× 1.25 mm×1.25 mm, numbers of direction=33, NSA=3;

Sequence C: b=1000 s/mm^2, resolution= 0.55 mm× 0.55 mm×2.5 mm, numbers of direction=33, NSA=3;

Sequence D: b=800 s/mm^2, resolution=1.0 mm× 1.0 mm×1.0 mm, numbers of direction=33, NSA=3.

Other parameters: frequency direction (FOV)=100, time echo (TE)=shortest, time repetition (TR)=shortest, scanning layers=48−60, flip angle=90℃, scanning time= 12−22 min.

Statistical analysis

All values were presented as mean±SD, with P<0.05 was taken as statistically significant. Statistical analyses were performed by one-way ANOVA. When statistical differences were revealed by ANOVA, the statistical post-hoc analysis of LSD was applied.

RESULTS

Effects of different parameters on the image quality

By comparing the image quality and tracking data, we found that the sequence A performed well in the accurate tracking of thick fibers, e.g. white matter fibers (Figure 3). No significant difference was found between the results obtained from sequence A and B. Relative to the sequence A, sequence D was high in noise level, because this sequence has a relatively high resolution, but the contrast of FA in 1 mm is higher than that in 1.25 mm (FA of gray matter to FA of white matter). This sequence can track both the thick and the fine fibers (Figure 2), so the white matter can be more abundantly displayed. In sequence C which was high in resolution, but significantly low in the signal to noise ratio (SNR), although the b value was reduced to 700, the display of FA values was still completely fuzzy (Figure 1) and cannot be used to track white matter fiber. Thus, our results indicate that the sequence D is the optimal setting to acquire DTI data in rhesus macaques.

Effects of different parameters on the scan time

Table 1 Effects of different parameters on scanning time

b value (s/mm^2)	Resolution (mm^3)	NSA and the corresponding scanning time (min)		
		NSA=2	NSA=3	NSA=4
1000	1.25×1.25×1.25	7.59	12.59	17.41
800	1.0×1.0×1.0	14	21.32	28.37
800	1.25×1.25×1.25	7.21	12.16	16.43
1000	1.0×1.0×1.0	14.23	22.17	29.39

White matter growth in baby monkeys

The DTI tractography of white matter growth in four baby monkeys (from 6 to 16 months after birth) indicates that the myelination of the corpus callosum is faster in 16-month-old subject than that in 6-month and 8-month-old subjects, whereas, the myelination of the prefrontal cortex is relatively slow (Figure 4).

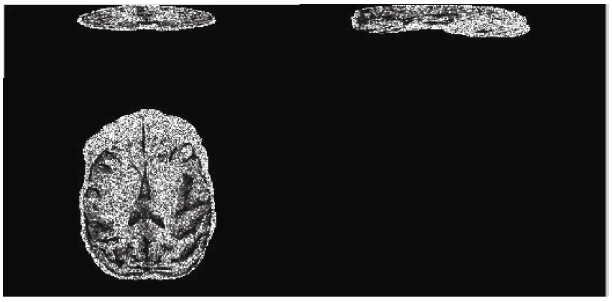
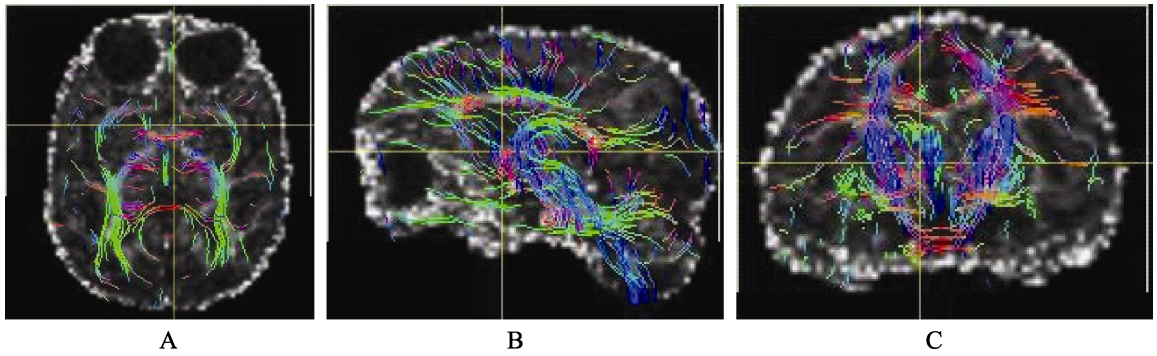
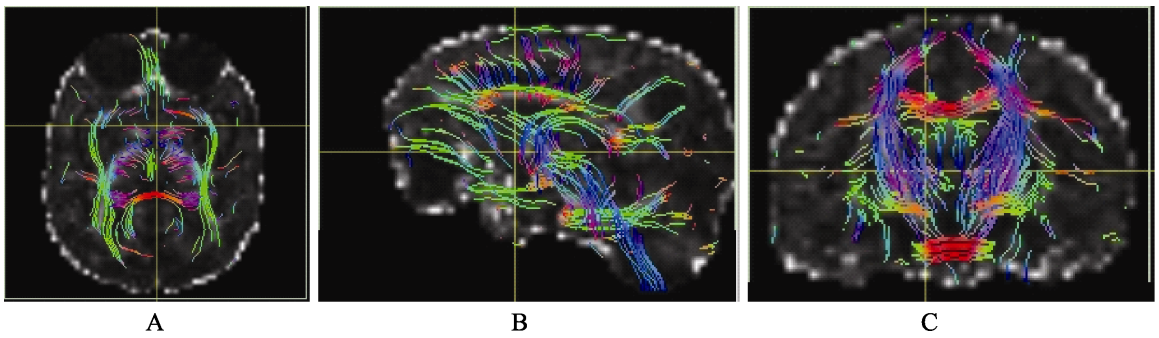


Figure 1 Original DTI images based on sequence C



A B C

Figure 2 Transverse (A), sagittal (B) and coronal DTI tractography based on sequence D



A B C

Figure 3 Transverse (A), sagittal (B) and coronal DTI tractography based on sequence A

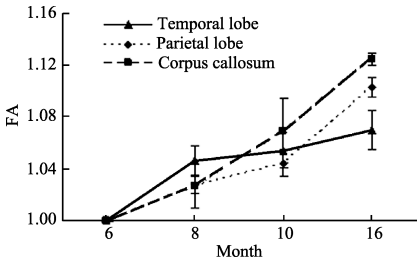


Figure 4 Myelination in baby monkeys

DISCUSSION

Spatial resolution, *b* value and motivate frequency are critical for DTI image quality and fiber tracking (Kim et al, 2006; Liu et al, 2009; Oouchi et al, 2007; Roebroeck et al, 2008). The significant difference in brain volume of monkeys and humans determines that high spatial resolution is necessary to image sophisticated brain structures in monkeys.

The resolution level of 2 mm×2 mm×2 mm or even higher commonly used in humans may induce a signify-cant partial volume effect in monkeys (Alexander et al, 2001), and thereafter, increase errors in FA and MD (average diffusion coefficient) values (Kim et al, 2006; Papanikolaou et al, 2006). Thus, with a certain SNR, high spatial resolution should be applied to reduce the

partial volume effect. Moreover, the higher the resolution is, the better the white matter fibers can be displayed.

In this study, with the resolution of 1.25 mm× 1.25 mm×1.25 mm, the SNR and contrast were good, the scan time was acceptable, but only the thick white matter fibers were well shown, whereas, with the resolution of 1.0 mm×1.0 mm×1.0 mm more white matter fibers were shown. However, due to the EPI sequence we used in DTI, the high resolution had remarkably reduced the SNR of the obtained image, therefore, it is necessary to keep the spatial resolution at certain level. With the resolution of 0.55 mm×0.55 mm×2.5 mm (Yundi et al, Shi et al, 2013), neither the image contrast or SNR were too low to infer FA, nor the white matter fiber direction could be tracked.

In general, the higher the *b* value, the more sensitive the DTI is in the differences in proton diffusion rate but meanwhile, the signal is quite reduced and less informative with lower SNR. To obtain a relatively good image quality and tracking, b=1 000 s/mm^2 is the most widely used setting in DTI. Naganawa et al (2004) reported that good tracking can also be realized at b= 700 s/mm^2, In our study, no significant differences in SNR and contrast were found between sequence A (b=1 000 s/mm^2) and B (b=800 s/mm^2).

The diffusion gradient directions and image sensitivity are not always positively correlated in a linear way. Jones et al (2004) showed that qualified FA and MD values could be obtained at a direction of 20−30. In this study, within an acceptable time range, a high diffusion gradient direction (33) was used to improve SNR.

Keeping subjects under anaesthetic during the whole procedure requires brief scanning time. Among all the parameters, spatial resolution is the most critical one affects scanning time, e.g. in this study the scanning time at the resolution of 1.0 mm×1.0 mm×1.0 mm almost doubled as that at a resolution of 1.25 mm×1.25 mm×1.25 mm (Table 1). Because the increasing of NSA significantly prolongs scanning time, so in this study, to get high SNR while also keeping the scanning time within an acceptable range, we set NSA=3. In this study, all the 30 monkeys were under anaesthesia during the scanning, indicating that the scanning time we used meets the requirement of anesthesia.

In summary, our results indicate that resolution= 1.0 mm×1.0 mm×1.0 mm, b=800 and NSA=3 are the optimal setting to obtain satisfactory SNR and contrast, as well as the thereafter fiber tracking and FA value calculation.

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Replication potentials of HIV-1/HSIV in PBMCs from northern pig-tailed macaque (*Macaca leonina*)

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Abstract: The northern pig-tailed macaque (*Macaca leonina*) has been identified as an independent species of Old World monkey, and we previously found that PBMCs from *M. leonina* were susceptible to human immunodeficiency virus type 1 (HIV-1), which may be due to the absence of a TRIM5 protein restricting HIV-1 replication. Here we investigated the infection potentials of six laboratory adapted HIV-1 strains and three primary HIV-1 isolates in PBMCs from *M. leonina*. The results indicate that these strains are characterized by various but low replication levels, and among which, HIV-1_{NL4-3} shows the highest replication ability. Based on the abundant evidence of species-specific interactions between restriction factors APOBEC3 and HIV/SIV-derived Vif protein, we subsequently examined the replication potentials of *vif*-substituted HIV-1 (HSIV) in *M. leonina* PBMCs. Notably, HSIV-vif_{mac} and st$HIV\text{-}1_{SV}$ chimeras, two HIV-1_{NL4-3}-derived viruses encoding the viral infectivity factor (Vif) protein from SIV_{mac239}, replicated robustly in cells from *M. leonina*, which suggests that HSIV could effectively antagonize the antiviral activity of APOBEC3 proteins expressed in cells of *M. leonina*. Therefore, our data demonstrate that *M. leonina* has the potential to be developed into a promising animal model for human AIDS.

Keywords: HIV-1; HSIV; Replication; PBMC; Northern pig-tailed macaque (*Macaca leonina*)

The lack of animal models that can be efficiently infected by HIV-1 has been a major impediment to the study of AIDS, anti-HIV-1 drugs and vaccines (Hatziioannou & Evans, 2012; Zhang et al, 2007). Presently, the most widely used non-human primate models for HIV/AIDS research are rhesus (*Macaca mulatta*), pig-tailed and cynomolgus macaques (*M. fascicularis*) infected with simian immunodeficiency viruses (SIVs) or SIV/HIV chimeric viruses (SHIVs) encompassing the HIV-1 *env*, *tat*, *rev* and *vpu* genes or reverse transcriptase gene (Baroncelli et al, 2008; Hatziioannou & Evans, 2012). Although these models have offered us with abundant information on immunopathogenesis and antiretroviral strategies (Evans & Silvestri, 2013; Lackner & Veazey, 2007), they have limitations largely due to the significant genetic differences between HIV-1 and SIV (Ambrose et al, 2007). SHIVs contain certain HIV-1 genes, but the absence of other HIV-1 genes has restricted their functional evaluation in viral pathogenesis or as targets for antiretroviral therapies *in vivo*.

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Among Old World monkeys, the pig-tailed macaque is the only primate that can be infected by HIV-1, though the infection is transient and limited (Agy et al, 1992; Bosch et al, 2000; Hu, 2005; Kent et al, 1995). Of note, some HIV-2 strains have been adapted in pig-tailed macaques and can result in persistent infection, $CD4^+$ T cell depletion, and AIDS (Kuller et al, 2001; Otten et al, 1999). Additionally, in contrast to rhesus macaques, the course of SIV infection in pig-tailed macaques more closely resemble that of HIV-1 infection in humans (Batten et al, 2006). Consequently, there has been increasing interest in the use of pig-tailed macaques in HIV/AIDS research (Lei et al, 2013). Based on morphological characteristics and phylogeographic studies, pig-tailed macaques have been classified into three species: Sunda pig-tailed macaques or Southern pig-tailed macaques (*M. nemestrina*), locating in Borneo, Bangka, Sumatra and the Malay peninsula; Northern pig-tailed macaques (*M. leonina*), mainly occurring in China (southwestern Yunnan), eastern Bangladesh, Cambodia, Laos, Myanmar, Thailand, southern Vietnam and India; and Mentawai macaques (*M. pagensis*), living in the Mentawai islands (Groves, 2001; Gippoliti, 2001; Kuang et al, 2009; Rosenblum et al, 1997). So far, the majority of pig-tailed macaques used in SIV/SHIV infection is *M. nemestrina*.

In recent years, it has been reported that the restriction factors, TRIM5α and APOBEC3, are the major barriers for HIV-1 to infect non-human primate cells (Huthoff & Towers, 2008; Liu et al, 2005; Thippeshappa et al, 2012). The TRIM5α protein mediates a post-entry block to retroviral infection by binding to incoming viral capsids through its C-terminal domain (Stremlau et al, 2004, 2006). The cytidine deaminases APOBEC3, especially APOBEC3G/3F, can be packaged into progeny virions, which can then inhibit viral replication largely by causing lethal hypermutations in viral genomes during reverse transcription. However, this restriction can be counteracted by HIV/ SIV-derived Vif protein in a species-specific manner (Mariani et al, 2003; Sheehy et al, 2002; Stopak et al, 2003; Thippeshappa et al, 2012; Zennou & Bieniasz, 2006). Interestingly, our laboratory previously found that *M. leonina* lacks a TRIM5α, and its novel TRIM5-Cyclophilin A (TRIM5-CypA) fusion protein is dysfunctional in blocking HIV-1 infection, which may explain why pig-tailed macaques are susceptible to HIV-1 (Kuang et al, 2009; Liao et al, 2007). These findings are consistent with previous studies in *M. nemestrina* (Brennan et al, 2007, 2008; Newman et al, 2008; Virgen et al, 2008). To overcome barriers imposed by APOBEC3, functional substitution of the *vif* gene with that from pathogenic SIV enables persistent infection of HIV-1 in *M. nemestrina* both *in vitro* and *in vivo* (Hatziioannou et al, 2009; Thippeshappa et al, 2011). Therefore, pig-tailed macaques appear to be a promising animal model for HIV-1 infection.

Here, to identify an isolate that can replicate efficiently in *M. leonina* cells, we investigated the replication potentials of six laboratory-adapted HIV-1 strains and three primary HIV-1 isolates in *M. leonina* peripheral blood mononuclear cells (PBMCs). The results showed that the replications in these HIV-1 strains are various and transient, whereas, constructed HSIV strains based on HIV-1_{NL4-3} with a substitutional *vif* gene from SIV_{mac239} replicate robustly. These results suggest that HSIV strains are resistant to APOBEC3G/3F proteins in *M. leonina* cells and can be applied to infect *M. leonina*, *in vivo*.

MATERIALS AND METHODS

HIV-1 strains and HSIV proviral plasmids

A panel of six lab-adapted subtype B HIV-1 strains, including HIV-1_{IIIB}, HIV-1_{RF}, HIV-1_{MN}, HIV-1_{SF2}, HIV-1_{NL4-3}, and HIV-1_{SF162}, were obtained from the NIH AIDS Research and Reference Reagent Program (USA) or MRC AIDS Research Project (UK). Primary isolates HIV-1_{KM018}, HIV-1_{TC2} and HIV-1_{WAN} were isolated from HIV-1 infected individual in Yunnan Province, China (Huang et al, 2013). All the above-mentioned HIV-1 strains are X4-tropic except that HIV-1_{SF162} and HIV-1_{KM018} are R5 tropism. Viral stocks were stored at −80 °C.

The infectious molecular clone of SIV_{mac239} (Shibata et al, 1991) has been previously described (Li et al, 2007). Proviral plasmids of HIV-1_{NL4-3} (Adachi et al, 1986), stHIV-1_{SV} (Hatziioannou et al, 2009) and HSIV-vif_{mac} were kindly contributed by Prof. Guang-Xia GAO of Institute of Biophysics from Chinese Academy of sciences (Figure 1). stHIV-1_{SV}, a simian-tropic(st) HIV-1, containing a macaque-adapted HIV-1 *env* gene (from $SHIV_{KB9}$) and its *vif* gene from SIV_{mac239} has been described in detail previously (Hatziioannou et al, 2009). HSIV-vif_{mac} chimera differs from HIV-1_{NL4-3} only in the *vif* gene.

Cell culture

H9 (human lymphoblastoma) and CEM×174 suspension cells were cultured in RPMI-1640 medium

(Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. The adherent cell lines TZM-bl and 293T were maintained in complete DMEM containing 10% FBS.

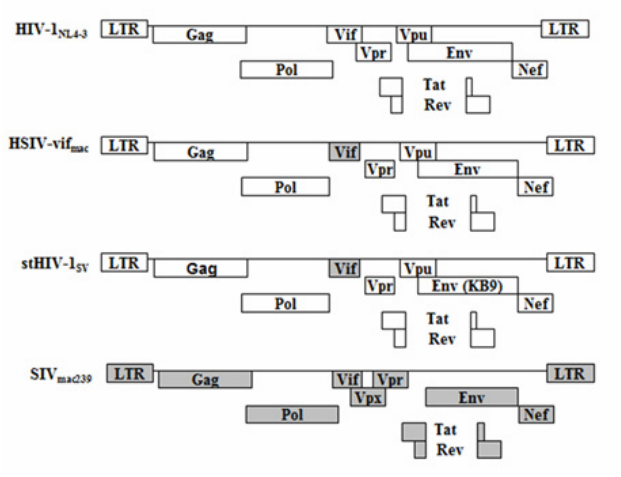

Figure 1 Schematic representation of viral clones used in this study

PBMCs were isolated from adult healthy *M. leonina*, Chinese rhesus macaques, or human peripheral blood by Ficoll-Paque gradient centrifugation as described previously (Dai et al, 2013). All PBMCs were cultured in RPMI-1640 medium with 10% FBS and 50 U/mL IL-2 (Sigma) before infection and thereafter. Human PBMCs were activated with 5 μg/mL phytohemagglutinin (PHA; Sigma) for 72 h. PBMCs from *M. leonina* and Chinese rhesus were activated for 72 h by 10 μg/mL and 5 μg/mL Concanavalin A (ConA; Sigma), respectively.

Transfection and replication assays

To obtain HIV-1/HSIV stocks, 293T cells in a 6-well plate were transfected with infectious molecular clones by using Lipofectamine 2000 (Invitrogen). After 48 h, culture supernatants were harvested and stored at −80 °C until use. Infectious virus titers were determined by serial 5-fold dilutions of the virus stock using TZM-bl reporter cells in a 96-well plate. After 48 h, cells were lysed and treated with Bright-Glo™ Reagent to read relative luminescence units (RLU) in the luminometer (Molecular Devices). $TCID_{50}$ of SIV_{mac239} was determined by infecting CEM×174 cells with serial dilutions of the stock as described previously (Aldovini & Walker, 1990).

For replication assays, 1×10^7 activated *M. leonina* PBMCs were infected with each of the nine HIV-1 strains (40 pg p24) mentioned above in duplicates for 3 h at 37 °C. Then the cells were rinsed three times with PBS to remove cell-free virus and resuspended in fresh medium. To monitor viral replication, supernatants were harvested and replaced every three days for p24 antigen quantification using an enzyme-linked immunosorbent assay kit (ELISA; ZeptoMetrix, Bufflaflo, NY). To compare $HIV\text{-}1_{NL4\text{-}3}$ and HSIV replication in H9 and stimulated PBMCs, viral stocks were mixed with 5×10^5 H9 cells or 1×10^6 activated PBMCs at a multiplicity of infection (MOI) of 0.01. Cells were rinsed three times after incubation, and supernatants were collected with the half of the medium being replaced at 3−4 day intervals postinfection for p24 analysis.

qRT-PCR assay

PBS-rinsed H9 and PBMCs (from human, *M. leonina* and Chinese rhesus macaque) were prepared for RNA isolation. Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocols. cDNA was synthesized using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China). To examine TRIM5 and APOBEC3G/3F mRNA expression, qRT-PCR was performed in triplicate with SYBR® Premix Ex Taq™ II (Tli RNase H Plus) kit as described by the manufacturer (Takara, Dalian, China) in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Human and macaque target gene expression were normalized to the endogenous GAPDH mRNA level and ribosomal protein L13A (RPL13A) mRNA level, respectively (Ahn et al, 2008). The mRNA expression levels of target genes in different cells are calculated using the $2^{-\triangle Ct}\times100\%$ ($\triangle Ct=Ct_{(Target\ gene)}-Ct_{(Internal\ reference\ gene)}$) method. Chinese rhesus macaque TRIM5α primers (Arhel et al, 2008) and other gene-specific primers used in our study are presented in Table 1.

RESULTS

Replication of HIV-1 strains in *M. leonina* PBMCs

Our laboratory has previously reported that in contrast to Chinese rhesus macaque cells, *M. leonina* cells are susceptible to HIV-1, which may be due to the dysfunctional TRIM5-CypA in the TRIM5 locus (Kuang et al, 2009; Liao et al, 2007). To examine the replication potentials of different HIV-1 strains in *M. leonina* PBMCs, six lab-adapted HIV-1 strains and three primary HIV-1 isolates were used to infect *M. leonina* cells *in vitro*. Lab-adapted subtype B $HIV\text{-}1_{NL4\text{-}3}$ and $HIV\text{-}1_{IIIB}$ were chosen because of their ability in infecting T cells

Table 1 Primers used for qRT-PCR

Gene	Primer[a]	Sequence
Human-A3G	F	5' -CACGTGAGCCTGTGCATCTTC-3'
	R	5'-AAAGGTGTCCCAGCAGTGCTTA-3'
Human-A3F	F	5' -GTCCTGAAACCTGGAGCCT-3'
	R	5'-AGACGGTATTCCGACGAGA-3'
Human-TRIM5α	F	5' -ATGTCCGACGCTACTGGGTTGATGT-3'
	R	5' -TGTCTGGTATCTTGTCCCTCGTGCC-3'
Human-GAPDH	F	5'-GAAATCCCATCACCATCTTCCAGG-3'
	R	5' -GAGCCCCAGCCTTCTCCATG-3'
NPM[b]-A3G	F	5'-TACCACCCAGAGATGAGATT-3'
	R	5' -GTTTCCAGAAGTAGTAGAGG-3'
NPM-TRIM5-CypA	F	5' -CAAAGTCTGAAACGAAGATGGT-3'
	R	5'-GCGGCAGCGTCTCTAAACA-3'
Macaque-A3F	F	5'-CTTTAATAACAGACCCATCCTT-3'
	R	5' -GTTGCCACAGAACCGAGA-3'
ChRM[c]-A3G	F	5'-AACCTTGGGTCAGTGGACAGC-3'
	R	5'-TGGAGCCTGGTTGCGTAGA-3'
ChRM-A3F	F	5'-CTTTAATAACAGACCCATCCTT-3'
	R	5'-GTTGCCACAGAACCGAGA-3'
ChRM-TRIM5α	F	5'-TTGGATCCTGGGGGTATGTGCTGG-3'
	R	5'-TGATATTGAAGAATGAGACAGTGCAAG-3'
Macaque-RPL13A	F	5'-AAGGTGTTTGACGGCATCCC-3'
	R	5'-CTTCTCCTCCAAGGTGGCTGT-3'

[a]: The primers are presented as forward (F) and reverse (R); [b]: denotes northern pig-tailed macaque; [c]: denotes Chinese rhesus macaques.

of pig-tailed macaques in previous studies (Agy et al, 1992, 1997; Gartner et al, 1994). HIV-1_{MN}, HIV-1_{RF}, HIV-1_{SF162} and HIV-1_{SF2} were used due to their close genetic similarity with HIV-1_{NL4-3} and HIV-1_{IIIB}. The primary isolates HIV-1_{KM018} (Wang et al, 2011), HIV-1_{TC2} (Zhang et al, 2010) and HIV-1_{WAN}, which were often used in studies of anti-HIV-1 drugs in our lab, were also chosen to assess their replication potentials in *M. leonina* cells.

As shown in Figure 2, all the HIV-1 strains replicated transiently with different susceptibility in *M. leonina* PBMCs from four different donors and there was no significant increasing trend after day 3 post-infection. In lab-adapted HIV-1 strains, HIV-1_{NL4-3}, and to a lesser extent, HIV-1_{IIIB}, HIV-1_{MN} and HIV-1_{SF162} were all able to replicate productively in *M. leonina* cells. Nevertheless, the replication levels of HIV-1_{RF} and HIV-1_{SF2} were low, which suggested that they were not adapted well in *M. leonina* cells. Meanwhile, primary isolates HIV-1_{WAN} and HIV-1_{KM018} were unable to replicate productively in *M. leonina* PBMCs. In contrast, the replication level of clinical isolated HIV-1_{TC2} in *M. leonina* PBMCs was much higher than that of HIV-1_{WAN} and HIV-1_{KM018}, though was little lower than that of HIV-1_{NL4-3}. Taken together, our results indicate that although lab-adapted HIV-1 strains and primary HIV-1 isolates replicate differently in *M. leonina* cells, their replication levels are low.

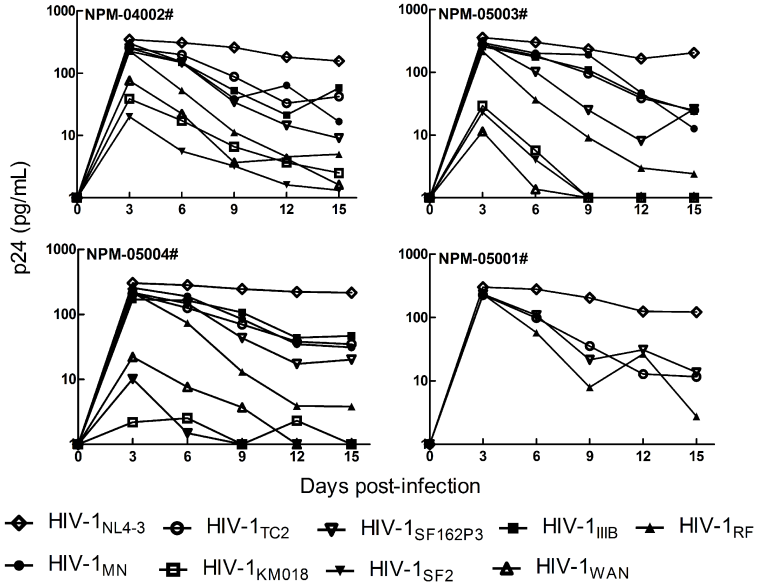

Figure 2 Replication of HIV-1 strains in *M. leonina* PBMCs

Infections of four different macaque donors are shown; Experiments were carried out with equal amounts of viruses (40 pg p24); Replication was monitored by determining the amount of p24 in culture supernatants every three days post-infection. NPM: northern pig-tailed macaque (*M. leonina*)

HIV-1 with *vif* substitution replicates robustly in *M. leonina* cells

The fact that HIV-1 replicates transiently in *M. leonina* cells despite the absence of a post-entry block to viral infection prompted us to consider other factors restricting HIV-1 replication. Several studies have demonstrated that APOBEC3 proteins in cells from rhesus macaque and African green macaque, which are

resistant to HIV-1 Vif protein, can effectively inhibit HIV-1 infection (Mariani et al, 2003; Virgen & Hatziioannou, 2007). Therefore, we determined the mRNA expressions of APOBEC3G/3F, the two potent antiviral proteins among APOBEC3 family members (Albin & Harris, 2010), in *M. leonina* PBMCs, human and Chinese rhesus macaque cells (Figure 3). As expected, TRIM5α mRNA was expressed in H9, human PBMCs and Chinese rhesus macaque PBMCs, whereas, *M. leonina* cells expressed TRIM5-CypA mRNA rather than TRIM5 α mRNA (Fiugre 3). Accordingly, we hypothesized that the reason why HIV-1 failed to infect M. leonina cells may be due to the potent anti-HIV-1 activity imposed by APOBEC3 proteins.

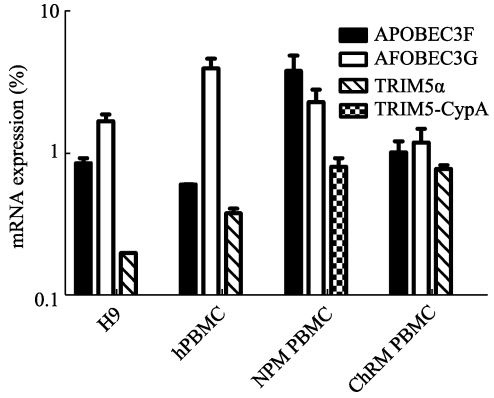

Figure 3 TRIM5 and APOBEC3G/3F mRNA expression in human and macaque cells

mRNA expression levels of human and macaque cells normalized to GAPDH and RPL13A mRNA using the $2^{-\Delta Ct}\times$ 100% method, respectively; Mean values of PBMCs isolations from three healthy donors are shown, and error bars represent standard errors of the means. hPBMC: human PBMCs; NPM: *M.leonina*; ChRM: Chinese rhesus macaque.

To overcome the antiretroviral activity of APOBEC3 in *M. leonina* cells, we subsequently investigated the replication potentials of two HIV-1-derived infectious clones in *M. leonina* PBMCs, stHIV-1_{SV} and HSIV-vif_{mac} chimeras containing the *vif* gene from SIV_{mac239} (Figure 1). Of note, stHIV-1_{SV}, encompassing a macaque-adapted HIV-1 *env* gene from $SHIV_{KB9}$, replicates efficiently in cells of *M. nemestrina* as described previously (Hatziioannou et al, 2009). Additionally, it has been previously reported that SIV_{mac}-derived Vif proteins could potently counteract the antiviral activity of APOBEC3 proteins in rhesus and pig-tailed macaques (Hatziioannou et al, 2009; Kamada et al, 2006). In our experiment, we used HIV-1_{NL4-3} and SIV_{mac239} to infect *M. leonina* PBMCs, meanwhile, took assays of HIV-1/HSIV infection in human T cell line H9, as well as human and Chinese rhesus macaque PBMCs as controls. We found that the replication levels of wild-type HIV-1_{NL4-3} and HSIV-vif_{mac} in H9 cell were higher than those of stHIV-1_{SV} (Figure 4A), suggesting that the inclusion of the *env* gene from $SHIV_{KB9}$ might, at least to some extent, affect HIV-1 infection. However, stHIV-1_{SV} and HSIV-vif_{mac} replicated as efficiently as HIV-1_{NL4-3} in human PBMCs from different donors (Figure 4A), demonstrating that the *vif* substitution could not influence HIV-1 infection. Notably, we observed that the replication levels of stHIV-1_{SV} and HSIV-vif_{mac} in *M. leonina* PBMCs were almost as high as those of SIV_{mac239} (Figure 4B), a pathogenic virus that can result in AIDS in pig-tailed macaques (Klatt et al, 2012). However, the replication level of HIV-1_{NL4-3} was much lower than that of HSIV, indicating that the *vif* replacement was sufficient for HIV-1 to robustly infect *M. leonina* cells *in vitro*. Meanwhile, stHIV-1_{SV} replicated better than HSIV-vif_{mac} in *M. leonina* cells, suggesting the incorporation of macaque-adapted HIV-1 envelope proteins might be conducive to *vif*-substituted HIV-1 replication in *M. leonina* cells. More importantly, based on the well-studied interactions between Vif protein and APOBEC3 proteins, HSIV-derived Vif protein might effectively antagonize the antiretroviral activity of APOBEC3G/3F proteins in *M. leonina* cells.

We next examined the replication potentials of HIV-1/HSIV in Chinese rhesus macaque PBMCs, which were resistent to HIV-1 infection as observed previously (Agy et al, 1992). As we expected, the replication level of HIV-1_{NL4-3} in Chinese rhesus macaque PBMCs was rather low compared with that of the pathogenic SIV_{mac239} (Figure 4C). *M. leonina* cells were more susceptible than Chinese rhesus macaque cells to HIV-1 (Figure 4B). Surprisingly, we found no difference between HIV-1_{NL4-3} and HSIV in their ability to infect Chinese rhesus macaque PBMCs, indicating that the *vif* substitution was insufficient for HIV-1 to replicate robustly in Chinese rhesus macaque PBMCs. Collectively, our results reveal that APOBEC3 proteins in *M. leonina* cells may function as an important barrier for HIV-1 infection and by replacing HIV-1 *vif* gene with that from pathogenic SIV can overcome this potent block.

DISCUSSION

According to primate taxonomy, *M. leonina* and *M. nemestrina* are two independent species in Old World monkeys (Groves, 2001; Malaivijitnond et al, 2012). Our

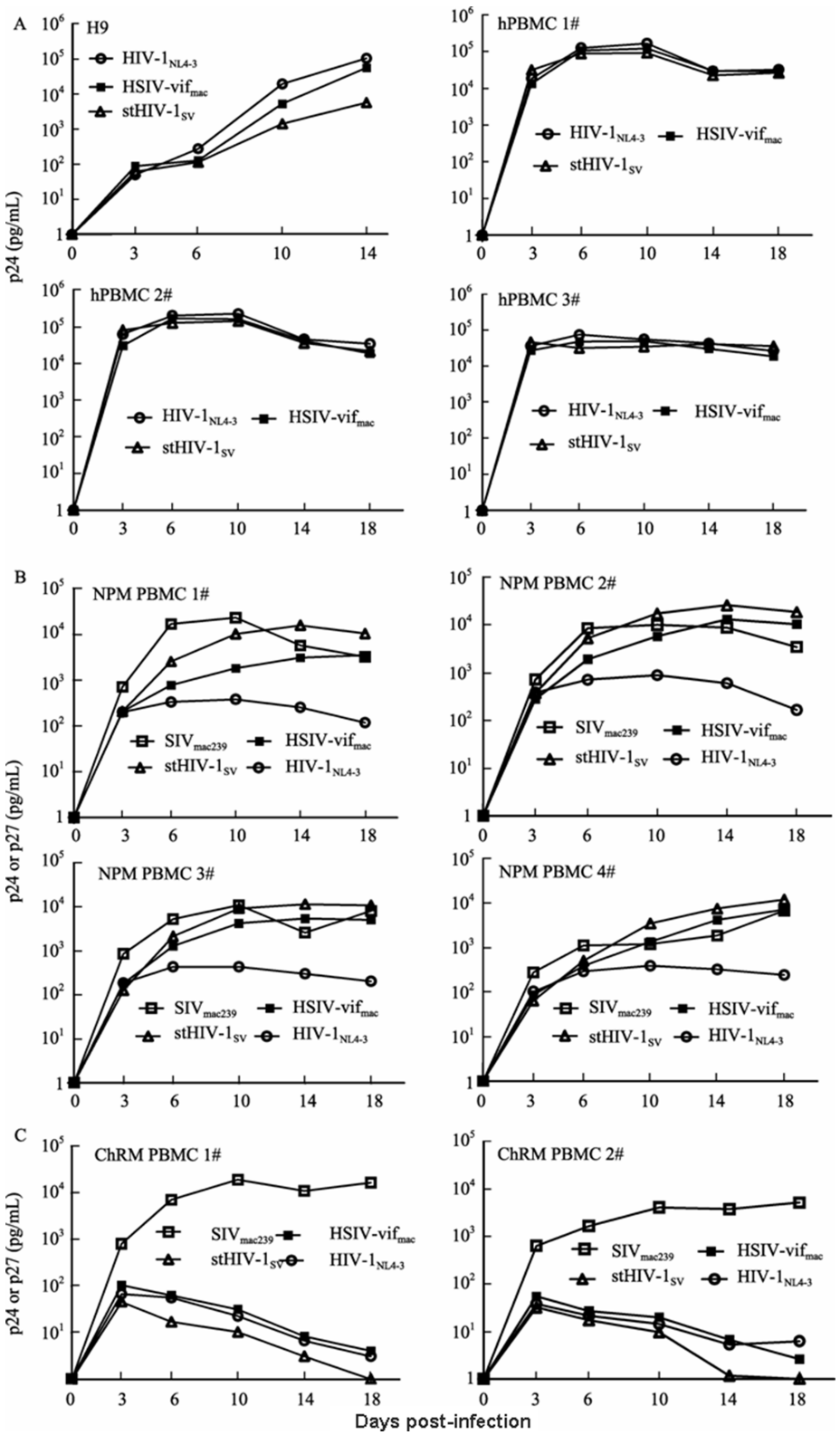

Figure 4 Replication of HIV and HSIV chimeras *in vitro*

A, B and C are replication of HIV and HSIV in H9 and human PBMCs (hPBMCs), *M. leonina* (NPM) PBMCs, and Chinese rhesus macaque (ChRM) PBMCs, respectively; Infections were carried out at an MOI of 0.01; Replication was monitored by determining the amount of p24 (for HIV/HSIV) or p27 (for SIV_{mac239}) per mL at 3- to 4-day intervals post-infection; Cells and donors are indicated at the top of each panel.

lab has previously reported that *M. leonina* cells are prone to HIV-1 infection, which may be due to the absence of a post-entry restriction imposed by a TRIM5 protein (Kuang et al, 2009; Liao et al, 2007). In this study, we showed that the replication levels of six lab-adapted HIV-1 strains and three primary HIV-1 isolates were various but low in *M. leonina* cells. Among the HIV-1 strains, HIV-1_{NL4-3} replicated best in *M. leonina* cells, which was consistent with the previous studies (Agy et al, 1997). However, HIV-1_{RF} and HIV-1_{SF2}, the two HIV-1 strains possessing similar biological properties with HIV-1_{NL4-3} (X4-tropic and lab-adapted subtype B virus), replicated poorly in *M. leonina* cells. Primary isolate HIV-1_{TC2}, rather than HIV-1_{KM018} and HIV-$1_{WAN,}$ could replicate in *M. leonina* cells. A similar phenomenon has also been reported regarding HIV-1 infection in cells of pig-tailed macaques, which suggested that the limited permissivity of macaque cells for HIV-1 may account for certain HIV-1 strains' failing in infecting pig-tailed macaque cells productively (Gartner et al, 1994). Moreover, a recent study suggested that the adaptation of HIV-1-derived envelope protein, which is responsible for viral entry, was necessary for the virus to infect cells from pig-tailed macaques (Humes & Overbaugh, 2011). However, whether the inability of certain HIV-1 isolates to infect *M. leonina* cells is related with viral entry or some other factors remains to be elucidated.

It is well known that APOBEC3 proteins in macaque cells is a major impediment in HIV-1 replication in Old World monkeys and their antiviral activity can be abrogated by some SIV Vif proteins (Huthoff & Towers, 2008; Thippeshappa et al, 2012). Recently, some studies showed that SIV *vif* substitution is sufficient for HIV-1 to persistently infect *M.nemestrina* cells both *in vitro* and *in vivo* (Thippeshappa et al, 2012). Thus, we subsequently examined the replication potentials of stHIV-1_{SV} and HSIV-vif_{mac} (two chimeras encoding Vif protein from SIV_{mac239}) in PBMCs from human and *M. leonina*. Consistent with previous studies, we found that these two chimeras could replicate productively in human PBMCs, implying that SIV_{mac} Vif protein could inactivate human APOBEC3 proteins (Gaddis et al, 2004; Thippeshappa et al, 2011).

Importantly, we showed that stHIV-1_{SV} and HSIV-vif_{mac} are able to replicate robustly in *M. leonina* cells *in vitro*, suggesting that APOBEC3 proteins expressed by *M. leonina* cells are a major barrier to HIV-1 infection in this primate species. We also showed that the replication level of stHIV-1_{SV} expressing the $SHIV_{KB9}$-derived envelope protein is higher than that of HSIV-vif_{mac} in *M. leonina* PBMCs, implying that the modified *env* gene may be conducive for HSIV to infect *M. leonina* cells, which is consistent with previous reports that stHIV-1 can replicate efficiently in cells from *M. nemestrina* both *in vitro* and *in vivo* (Hatziioannou et al, 2009). A more recent study also showed that HSIV-vif, in which the HIV-1_{NL4-3} *vif* gene was functionally substituted by the *vif* gene from SIV_{mne027}, can replicate as efficiently as SIV_{mne027} in cells from *M. Nemestrina* (Thippeshappa et al, 2012). By contrast, HIV-$1_{NL-DT5R}$, a virus containing a part of the Gag CA sequence (corresponding to the HIV-1 CypA-binding loop) and a *vif* gene from SIV_{mac239}, is unable to achieve the replication level of SIV_{mac239} in pig-tailed macaque cells *in vitro* (Kamada et al, 2006), which may result in its transient infection in pig-tailed macaques (Igarashi et al, 2007). Although further modification or passaging *in vitro* of HIV-$1_{NL-DT5R}$ to some extent had enhanced its replication in cells from cynomolgus macaques, a macaque-tropic virus that can replicate as efficiently as SIV_{mac239} in macaque cells could not be obtained (Kamada et al, 2009; Kuroishi et al, 2009; Nomaguchi et al, 2013a, 2013b; Saito et al, 2011). In comparison, the HSIV-vif_{mac} we used seemed to replicate better than HIV-$1_{NL-DT5R}$ with SIV_{mac239} serving as a control, which may be either due to HIV-$1_{NL-DT5R}$ expressing modified Gag protein that was unnecessary for high- level of HIV-1 infection in cells of pig-tailed macaques or because the cells we used in this study was from *M. leonina* instead of *M. nemestrina*. However, whether there are significant differences between *M. nemestrina* and *M. leonina* after HSIV infection needs to be determined.

We also observed that HIV-1 and HSIV replication in Chinese rhesus macaque PBMCs were potently inhibited, which was consistent with a previous study (Hatziioannou et al, 2006). However, it has also been demonstrated that stHIV-1 containing both Gag CA (expressing viral capsid) sequence and a *vif* gene from SIV_{mac}, after serial passage *in vitro*, could replicate robustly in rhesus macaque lymphocytes, whereas, the replication of HIV(SCA) carrying only SIV_{mac} CA was low and transient (Hatziioannou et al, 2006). Additionally, HIV-$1_{NL-DT5R}$ as described above could also replicate in CD8-depleted PBMCs from a rhesus

macaque (Kamada et al, 2006). It is known that TRIM5α protein mediates the early block to HIV-1 infection in Old World monkey cells (Blanco-Melo et al, 2012). Thus, we conclude that TRIM5α, a potent retrovirus inhibitor, may function as a major impediment in Chinese rhesus macaque cells to HIV-1/HSIV replication. Comparatively, the absence of a TRIM5 blocking HIV-1 replication in *M. leonina* cells may partly explain why *M. leonina* cells are more sensitive than Chinese rhesus macaque cells to HIV-1 infection.

In summary, our results indicate that the abilities of HIV-1 strains to persistently infect *M. leonina* cells *in vitro* are various and limited. Notably, HSIV chimeras based on HIV-1_{NL4-3} encoding the SIV_{mac239} Vif protein can achieve the SIV_{mac239} replication level in *M. leonina* cells. Thus, HSIV infection in *M. leonina* may be developed into a promising animal model for human AIDS.

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Analysis of immunoglobulin, complements and CRP levels in serum of captive northern pig-tailed macaques (*Macaca leonina*)

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Abstract: The northern pig-tailed macaque (NPM，*Macaca leonina*) has become a widely used animal model in biomedical research. In this study, we measured serum immunoglobulin IgG, IgM, IgA, complement C3, C4 and CRP levels in 3−11 year old captive northern pig-tailed macaques using HITACHI 7600-20 automated chemistry analyzer in order to determine the influences of age and gender on these items. The results showed that serum IgA, IgM, C3 and C4 levels were not correlated with age (P>0.05), while serum IgG levels increased progressively with age (r=0.202； P=0.045). Serum IgG, IgA, IgM and C3 levels were higher in females than in males (P<0.05). Moreover, serum C3 concentration was both positively and strongly correlated with that of C4 (r=0.700; P<0.0001). This study provides basic serum immunoglobulin and complement data of captive northern pig-tailed macaques, which may prove useful for future breeding efforts and biomedical research.

Keywords: Northern pig-tailed macaque (*Macaca leonina*); Immunoglobulin; Complement; C-reactive protein

Macaque species, including pig-tailed macaques (*Macaca nemestrina* group), rhesus macaques (*M. Mulatta*) and cynomolgus macaques (*M. fascicularis*), have been widely used in biomedical research (Yoshino et al, 2000) because of their phylogenetic proximity to humans, such as in HIV infection (Kuang et al, 2009; Lei et al, 2013; Zhu et al, 2010), chlamydial infection (Patton et al, 2001), *Campylobacter* infection (Flores et al, 1990), immunogenetics (Knapp et al, 1996), immune cell function (Li et al, 2012; Shaulov & Murali-Krishna, 2008), xenotransplantation (Watts et al, 2012), neurophysiology (Rausell et al, 1998) and cognitive behavioural studies (Macellini et al, 2010; Sussman & Ha, 2011). The taxonomic status of Macaque species have been re-evaluated, and three subspecies have been corrected into three independent species: northern pig-tailed macaques (NPMs, *M. leonina*), southern pig-tailed macaques (*M. nemestrina*) and Mentawai macaques (*M. pagensis*) (Groves, 2001; Kuang et al, 2009).

While these studies provide critical information, to date that physiological and biochemical characteristics of captive NPMs have not been as well described as those of the other commonly-used macaques. We have previously reported the reference values of blood chemistry and hematology of NPMs (Pang et al, 2013), but little is known about the normal levels of its basic immunological parameters, such as serum immunoglobulins, complements and CRP levels, which may closely correlate with age,

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gender, breeding environment and sampling methods (Curry, 2001).

In this study, we analyzed the levels of serum IgG, IgM, IgA, C3, C4 and CRP of healthy captive NPMs with different age and gender, which will likely benefit further applications towards infectious diseases, immuneology and other biomedical researches.

MATERIALS AND METHODS

Animals

One hundred NPMs used in this study were housed in Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ, CAS). Animals were free of any known virus, bacteria and parasites and visually showed with no trauma, no pregnancy and no estrus. Age, gender and body weight information were presented in Table 1. These NPMs were maintained in social groups in enclosures at room temperature and provided with a diet of fruits, monkey biscuits, and vegetables at 0800h, 1300h and 1600h, respectively. Animal care was performed according to the regulations and recommendations of the Animal Care Committee of KIZ, CAS.

Serum samples

Blood samples (1−2 mL) from each subject were collected sterilely from the saphenous vein without anesthesia, and allowed to clot at room temperature. Serum was further separated by centrifuging at 2,500 r/min, 4 °C for 20 minutes 1-hour later after bleeding and stored at −80 °C until use.

Table 1 Age, gender and body weight of NPMs

Groups	Female			Male		
	Number (*n*)	Age (years)	Body weight (kg)	Number (*n*)	Age (years)	Body weight (kg)
3−4 years old (Juveniles)	11	3.4±0.5	2.37±0.33	24	3.5±0.5	2.51±0.29
5−11 years old (Adults)	39	6.5±1.5	3.83±0.86	26	6.6±1.7	3.87±1.38
Total	50	5.8±1.9	3.51±0.98	50	5.1±2.0	3.21±1.22

Assays for serum immunoglobulins, complements and CRP

Serum specimens were delivered to Department of Clinical Laboratory, the Second Hospital of Yunnan Province, China, and then the turbidimetric immuneoassay determining levels of serum IgG, IgM, IgA, C3, C4 and CRP was performed on a HITACHI 7600-020 automated chemistry analyzer using rabbit anti-human polyclonal antibodies, protein standards and reagents from Orion Corporation according to users' manuals. IgA, IgG, IgM, C3, C4 and CRP standards were diluted into known concentrations. Next, protein standards dilutions and serum specimens were measured simultaneously. Upon adding a serum sample, target antigen and polyclonal antibodies would form an immune complex that would precipitate and then increase the turbidity of the reaction solution. When light was shone on the sample, some were scattered, some were absorbed, and the rest passed through. The automated chemistry analyzer can measure the sample's light absorbance, which is positively correlated with the protein concentrations in it. Accordingly, target serum protein level could be calculated by referring to this protein's known diluted concentration series. Detailed protocols are available from the manufacturer.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5.0. The normal distributions were tested by the Kolmogorov-Smirnov test. Spearman partial correlation coefficients were calculated by taking gender as control and taking each serum immunological parameters as dependent variables against age. When dependent variables were significantly correlated with age, then gender effects were analyzed under covariance analysis taking gender as a factor and age as a covariate. Other times, an unpaired *t*-test was used to compare the means between male and female groups. Pearson's *r*-test was used to analyze the significance in correlations between each serum immunological variables. All analyses were two-tailed, with results expressed as mean±*SD*, with P=0.05 being statistically significant.

RESULTS

Levels of serum immunoglobulins, complements and CRP

The levels of serum immunoglobulins, complements and CRP in captive NPMs from different gender and age groups are shown in Table 2. Among the total serum samples, 34 samples were showed with no detectable CRP signals, and another 34 samples displayed CRP levels less than 0.1 mg/L (data not shown).

Effects of age on the levels of serum immunoglobulins and complements

While the development, maturation and functional decline of immune system are age-related biological processes, we assessed the effects of age on the levels of serum immunoglobulins and complements in the 3−11 years old subjects. We found that the serum IgG levels were significantly increased with age (r=0.202; P=0.045), whereas, the serum IgA (r=0.146; P=0.149), IgM (r=–0.073; P=0.474), C3 (r=–0.055; P= 0.591) and C4 (r=–0.164; P=0.105) levels showed no significant correlations with age (Figure 1).

Gendered levels of serum IgG, IgA, IgM, C3 and C4

Given the differences between female and male immune systems, we tested whether levels of serum IgA, IgG, IgM, C3 and C4 of females were significantly different with those of males. As shown in Figure 2, significant gender-related differences were observed in the levels of serum IgG (P<0.001), IgA (P=0.001), IgM (P<0.001) and C3 (P=0.031). All of the female serum IgA, IgG, IgM and C3 levels, especially IgM levels, were higher than those of the male. However, there was no significant gender-related difference in the serum C4 levels (P=0.777) between male and female groups.

Correlation analysis of serum immunoglobulins and complements

The complement system is closely correlated with immunoglobulins. Certain immunoglobulins can activate the complement system, and the activated complement system can therefore enhance the biological activities of immunoglobulins. We analyzed the correlations among serum IgA, IgG, IgM, C3 and C4 levels in NPMs and the statistical analysis showed significantly positive correlations only between the following pairs of components: IgA and IgM (r=0.457; P<0.0001), C3 and IgM (r=0.246; P=0.014), C3 and IgA (r=0.295; P=0.003), C3 and IgG (r=0.289; P=0.004), C3 and C4 (r=0.700; P<0.0001) (Figure 3).

Table 2 Levels of serum immunoglobulins and complements of NPMs

Grouping		3−4 years old (Juveniles)	5−11 years old (Adults)	Total
IgG (g/L)	Female	17.236±2.900	19.808±3.47	19.242±3.491
	Male	16.954±2.509	17.570±2.875	17.274±2.696
	Total	17.043±2.598	18.912±3.403	18.258±3.257
	Range	12.400 −22.900	10.400 −30.000	10.400 −30.000
IgA (g/L)	Female	0.924±0.377	1.041±0.060	1.015±0.375
	Male	0.739±0.327	0.815±0.359	0.779±0.343
	Total	0.797±0.349	0.951±0.383	0.897±0.377
	Range	0.260 −1.550	0.290 −2.290	0.260 −2.290
IgM (g/L)	Female	2.025±0.652	1.864±0.693	1.886±0.682
	Male	1.488±0.503	1.406±0.485	1.445±0.490
	Total	1.656±0.600	1.670±0.651	1.665±0.631
	Range	0.820 −3.590	0.580 −4.040	0.580 −4.030
C3 (g/L)	Female	1.851±0.419	1.892±0.316	1.883±0.337
	Male	1.813±0.240	1.688±0.297	1.748±0.276
	Total	1.825±0.301	1.810±0.322	1.816±0.314
	Range	1.310 −2.650	1.030 −2.560	1.030 −2.650
C4 (g/L)	Female	0.315±0.097	0.298±0.087	0.302±0.089
	Male	0.321±0.095	0.274±0.091	0.297±0.095
	Total	0.319±0.094	0.228±0.089	0.299±0.091
	Range	0.100 −0.510	0.100 −0.480	0.100 −0.510

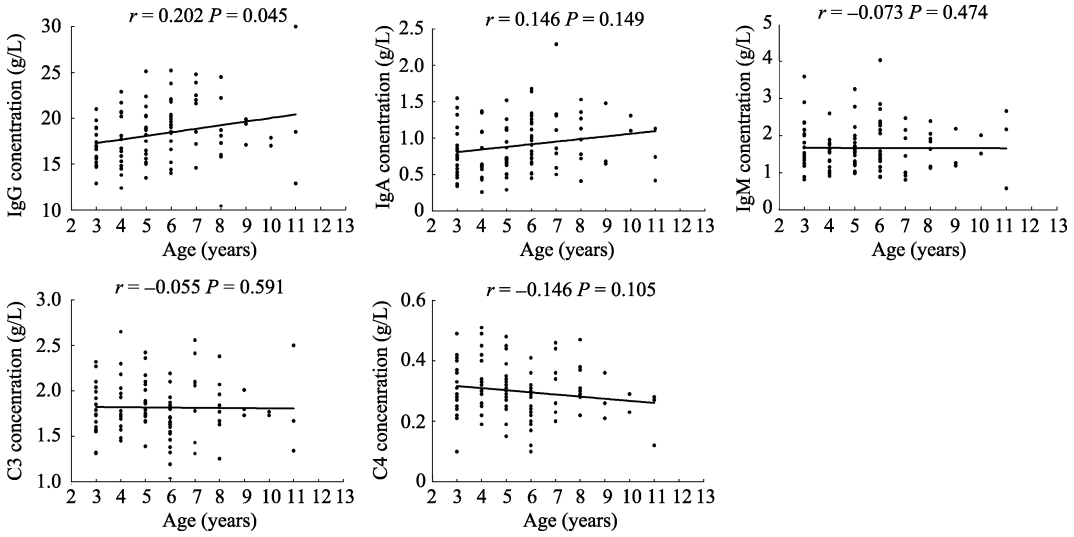

Figure 1 Effects of age on the levels of serum IgG, IgA, IgM, C3 and C4 in NPMs

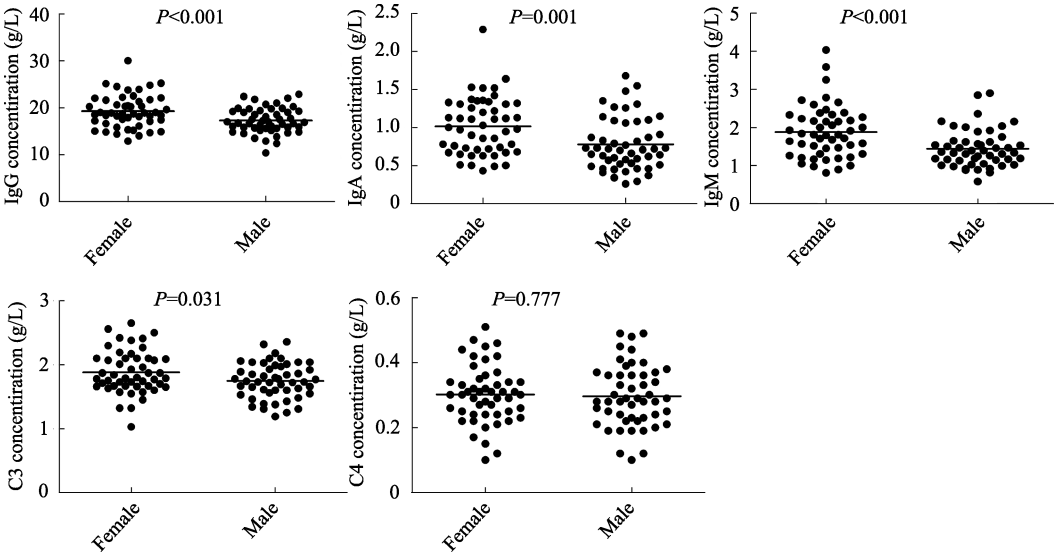

Figure 2 Effects of gender on the levels of serum IgG, IgA, IgM, C3 and C4 in NPMs

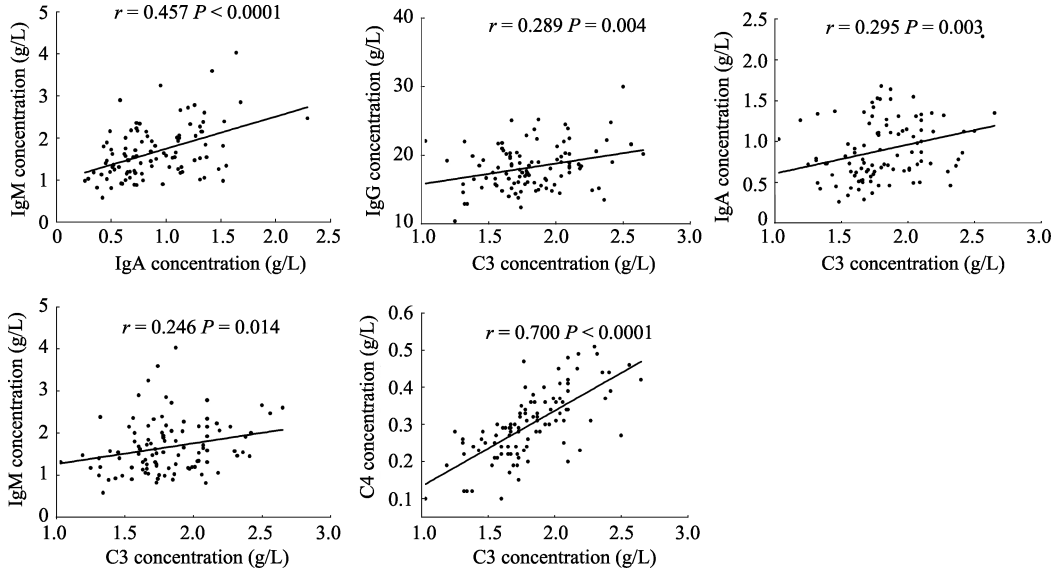

Figure 3 Correlations of serum immunoglobulins and complements in NPMs

DISCUSSION

Due to the high phylogenetic proximity between humans and non-human primates, proteins—such as immunoglobulins, complements and CRP—share a high degree of homology. Earlier studies evaluated the cross-reactivities between anti-human antibodies and non-human primate antigens and demonstrated that the homology could reach to 80%−100% (Jinbo et al, 1998; Shuster et al, 1969). Therefore, lots of monkey proteins have been measured with homologous antibodies of human (Axberg et al, 1991; Chen et al, 2006; Cheng et al, 2003; Cole & Bowen, 1976; Fujimoto et al, 1982; Mayer et al, 1993). Moreover, in many previous studies, anti-human polyclonal antibodies have been successfully used in determining serum immunoglobulin, complement and CRP levels in rhesus macaques and/or cynomolgus macaques (Jinbo et al, 1998; Socha et al, 1993; Yoshino et al, 2000).

Immunoglobulins play a critical role in the immediate defense against infection and its quantitative internal concentration determination is helpful in disease diagnosis, monitoring and medication. In our study, we found no effects of age (3−11 years old) on the serum IgA and IgM levels of NPMs, which was consistent with the findings in rhesus macaques and cynomolgus macaques. However, serum IgG levels increased progressively with age, which was inconsistent with previous studies on rhesus monkeys and cynomolgus macaques (Cheng et al, 2003; Cole & Bowen, 1976). Such contradictory results are probably due to the interspecies differences in growth and development. The serum IgA, IgG and IgM levels of female NPMs were higher than those of males, which was in accordance with studies on other species. It has been documented that in general, females have better humoral immunity than their male counterparts (Ahmed et al, 1985; Ahmed & Talal, 1990; Grossman, 1984), by showing higher immunoglobulin levels in serum, particularly IgM (Allansmith et al, 1968). This gender-specific differences in serum IgA, IgG and IgM levels could mainly due to the effects of sex hormones (Ahmed et al, 1985; Ahmed & Talal, 1990). NPMs were characterized with similar levels of serum IgG and IgM, but lower level of serum IgA compared with rhesus macaques and cynomolgus macaques. However, the serum IgA levels between rhesus macaques and cynomolgus macaques was similar (Cole & Bowen, 1976; Miller et al, 1992; Patterson et al, 1976). This phenomenon could be explained by the perspectives of species evolution. Pig-tailed macaques were estimated to have evolutionarily differentiated from rhesus macaques and cynomolgus macaques approximately 5 million years ago (mya). However, the divergence between rhesus macaques and cynomolgus macaques occurred approximately 2.4 mya (Morales & Melnick, 1998). Additionally, blood chemistry and hematology parameters of captive populations of NPMs also have indicated that the differences between NPMs and rhesus macaques or cynomolgus macaques were greater than those between rhesus macaques and cynomolgus macaques (Pang et al, 2013). As the most important and richest immunoglobulin in the mucosal immune system, the low level of IgA in the mucosal immune system may result in reduced mucosal resistance to microbial infection. In the future, it may be worth exploring and then exploiting whether or not the lower serum IgA level is related with the lower IgA of the mucosal immune system. Consequently, understanding the differences of serum and mucosal IgA levels between NPMs and other experimental non-human primates will likely benefit from its application to infectious disease studies, vaccine evaluation, as well as other fields of viral immunology.

Compared with antibodies, the complement system had an earlier origin. Complements not only act as cofactors and enhancers of antibody activities, but also have other biological functions independent of antibodies. The complement system plays an important role in innate defense against common pathogens, the modulation of inflammatory response and coagulation. For example, C3 is critical in activating the whole complement system, and C4 is the major protein of classical pathways (Kasperska-Zajac et al, 2013). As such, determining the serum levels of C3 and C4 in healthy population is helpful in disease diagnosis. The levels of complements increased after delivery, so that by 3−6 months of age the mean levels of serum C3 and C4 in the infant populations are already similar to those in the normal adults (Fireman et al, 1969). Our results showed that serum C3 and C4 levels did not fluctuate with age (3−11 years old), which was consistent with Cheng et al's (2003) study on rhesus macaques. While serum C3 levels of female NPMs were higher than those of males, no significant gender-specific differences were found in serum C4 levels. This phenomenon could be explained by sex hormone differences, since it has long been documented that sex hormones can markedly affect the immune system (Ahmed et al, 1985; Verthelyi &

Klinman, 2000), and also influence pro-inflammatory cytokines IL-6 and IL-1 production (Angstwurm et al, 1997; Cannon & Dinarello, 1985). Moreover, evidence suggested that IL-1 and IL-6 have an enhancing effect on the production of C3, whereas neither IL-1 nor IL-6 affect the biosynthesis of C4 (Falus et al, 1990). Serum C3 levels of NPMs were close to those of rhesus macaques and cynomolgus macaques, whereas, serum C4 levels of NPMs were higher than those of rhesus macaques but lower than those of cynomolgus macaques (Cheng et al, 2003; Poskitt et al, 1974), which had indicated that non-human primates displayed marked interspecies variations regarding complements (Ellingsworth et al, 1983). Additionally, the changes in complement pathway activities were involved in the acute rejection after xenotransplantation and the pathogenesis of systemic autoimmune diseases (Chen et al, 2010; Saadi et al, 2004; Yu & Whitacre, 2004). Ultimately then, when non-human primate animal models are applied to studying human complement-related diseases, it is necessary to consider and understand the species-specific characteristics of the complement systems.

CRP is a classical acute-phase serum protein, which interacts with complements, neutrophils, monocytes, etc, and functions not only as an inflammation regulator, but also as a host defender against infection. CRP is primarily synthesized in livers and is simultaneously released into the bloodstream during acute phase response. CRP testing has been applied in differential diagnosis of infectious diseases (Du Clos & Mold, 2004), assessing cardiovascular risks (Albert et al, 2002) and monitoring autoimmune diseases progression. Previous studies reported that both the detection methods and many other factors may influence the quantifications of serum CRP levels (Zhang et al, 2011; Khera et al, 2005)). In our study, no detectable signals of CRP were found In 34 serum samples. We assume this is due to traces of serum CRP in NPMs had outreached the instrumental detection limit (0.01 mg/L), which is special designed for human beings.

We also analyzed correlations among serum IgA, IgG, IgM, C3 and C4 levels of NPMs. As we known, complement systems, immunoglobulins, and the related components are closely interacted with each other. Serum levels of certain complement components are highly correlated with those of certain immunoglobulins (Plebani et al, 1984), and are functioned as bridges between innate and adaptive immune responses. The classic pathway is activated by immune complexes of IgG, IgM and complements, meanwhile, IgG and IgA immune complexes are acting as activators of the alternative pathways (Roach et al, 1981; Wagner & Frank, 2009). In addition, pro-inflammatory cytokines, for example IL-6, are modulators of the levels of lgG, IgM, IgA and some complements (Maes et al, 1997; Ritchie et al, 2004). Roach et al (1981) found that serum components of certain classical pathways and their alternative pathways were significantly correlated (r>0.537). Yilmazer et al (2003) showed that serum C3 and C4 levels were highly correlated (r>0.6, P<0.001). In some diseases, the correlations between serum complement components and immunoglobulins levels was markedly altered (Gewurz et al, 1968; Kohler & Muller-Eberhard, 1969). In our study, we found significant positive correlations between the following pairs: IgA and IgM, C3 and IgM, C3 and IgA, C3 and IgG, C3 and C4, all of which are consistent with the findings in other species.

In conclusion, this study shows that serum IgA, IgM, C3 and C4 levels in NPMs were irrelevant with age, whereas, IgG levels increased progressively with age. Serum IgG, IgA, IgM and C3 levels were higher in females than in males, meanwhile, C3 concentrations were positively and closely correlated with those of C4. These basic data of captive NPMs may, in the future, promote its future and more detailed application to infectious diseases, immunology and other fields of biomedical research.

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Dominance hierarchy and social relationships in a group of Captive black-and-white snub-nosed monkeys (*Rhinopithecus bieti*)

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Abstract: Different types of dominance hierarchies reflect different social relationships in primates. In this study, we clarified the hierarchy and social relationships in a one-male unit of captive *Rhinopithecus bieti* observed between August 1998 and March 1999. Mean frequency of agonistic behaviour among adult females was 0.13 interactions per hour. Adult females exhibited a linear hierarchy with a reversal of 10.9%, indicating an unstable relationship; therefore, *R. bieti* appears to be a relaxed/tolerant species. The lack of a relationship between the agonistic ratio of the adult male towards adult females and their ranks indicated that males did not show increased aggression towards low-ranking females. Differentiated female affiliative relationships were loosely formed in terms of the male, and to some extent influenced by female estrus, implying that relationships between the male and females is influenced by estrus and not rank alone. A positive correlation between the agonistic ratio of adult females and their ranks showed that the degree to which one female negatively impacted others decreased with reduction in rank. Similarly, a positive correlation between the agonistic ratio of females and differences in rank suggests that a female had fewer negative effects on closely ranked individuals than distantly ranked ones. These data indicate that rank may influence relationships between females. A steeper slope of regression between the agonistic ratio and inter-female rank differences indicated that the extent of the power difference in high-ranking females exerting negative effects on low-ranking ones was larger during the mating season than the birth season, suggesting that rank may influence the mating success of females.

Keywords: Dominance style; Hierarchy; Linearity; *Rhinopithecus bieti*; Social relationship

Social dominance is considered important in studies of animal behaviour, is generally defined in terms of the consistent direction of agonistic behaviour between individuals (Walters & Seyfarth, 1987), and may be measured by asymmetry in repeated interactions (de Waal & Luttrell, 1989). Dominance style, which refers to the way dominants treat subordinates and vice versa (de Waal, 1996), is classified on a continuum with despotic at one end and tolerant/relaxed at the other (Thierry, 2000). In tolerant/relaxed species, high-ranking animals display weak, symmetrical patterns of aggression, more tolerance around resources and reconcile frequently; despotic species show opposite tendencies (Berman et al, 2004). A dichotomy of hierarchies explains dominance relationships in nonhuman primates. A strong dominance hierarchy is characterized by common agonistic interactions, and less than 5% of reversals indicating stable dominance relationships. By contrast, a weak hierarchy is typified by rare agonistic interactions, and by as much as 15% reversal, suggesting an unstable relationship (Isbell & Young, 2002).

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Socio-ecological theory predicts that food resources and predation risk shape the competitive regime and therefore the relationship and dominance structure among group-living female diurnal primates (Sterck et al, 1997; van Schaik, 1989). Scramble competition has been associated with egalitarian dominance relationships, in which hierarchies are unclear and non-linear. Contest competition is linked to despotic dominance relationships, in which hierarchies are clearly established and linear. Such females often formalize dominance relationships, which are expressed in ritualized signals (de Waal, 1989). Contest competition occurs when the distribution of food can accommodate only some individuals and exclude others. Contest competition will increase with the ability to monopolize resources and the number of competitors. A linear hierarchy is adaptive, and reduces the intensity of competition through clear hierarchies when contests are strong (Wittig & Boesch, 2003).

Dominance hierarchies are either poorly defined or not apparent in some colobine species (reviewed by Struhsaker & Leland, 1987). However, in other studies, adult females exhibit a linear hierarchy (*Presbytis entellus*: Borries et al, 1991; *Semnopithecus entellus*: Koenig, 2000; *Trachypithecus phayrei*: Koenig et al, 2004) stable over short periods but fluctuating year to year, and inversely related to female age (Borries et al, 1991). The number of adult females in a group is thought to influence hierarchy linearity indices and reversals as well as dominance relationships. Dominance ranks change more frequently in larger groups than in small and medium-sized groups (Koenig, 2000). Dominance hierarchy is related to size (and therefore age) as well as genealogy in many species of nonhuman primates (see Borries et al, 1991). Moreover, linear dominance hierarchies have been reported amongst one male units (OMUs) in *R. roxellana*, with ambiguous and reversal interactions at 17.7% (Zhang et al, 2008a). In *R. roxellana* OMUs, the dominance ranks of females are determined by displacement, and the hierarchy may regulate the strategy of female mating competition since high-ranking females mate more than low-ranging ones (He et al, 2013).

Black-and-white snub-nosed monkeys (*Rhinopithecus bieti*) travel together as a large and cohesive cohort, predominately as one-male multi-female units (OMUs), but also an all-male unit (AMU) (Kirkpatrick et al, 1998). Adult female *R. bieti* migrate among OMUs, OMUs sometime split, and merge with individuals from different OMUs (Cui, unpublished data). Relationships among wild colobine females are infrequent and hard to record in comparison to cercopithecidae females (Newton & Dunbar, 1994). The habitat of wild *R. bieti* is characterized by steep slopes and deep valleys and also because they are shy, it is difficult to habituate them to allow for individual identification. Until now, it has been impossible to obtain systematic data on agonistic interactions between individuals within OMUs for *R. bieti* in the wild.

Captive *R. bieti* display a low agonistic interactions rate (0.3 per hour) and reconcile frequently (54.5%). The adult male intervenes frequently and peacefully in conflicts among adult females (Grüter, 2004). According to predictions from socio-ecological models (van Schaik, 1989), a weak or nonlinear hierarchy would be expected in this species. In terms of the dichotomous hierarchy system (Isbell & Young, 2002), the dominance hierarchy of this species would be characterized by frequent reversals, indicating an unstable dominance relationship. Moreover, in OMUs of *R. roxellana*, adult females can be arranged by dominance rank (He et al, 2013), but they have no tendency to build a strong relationship with each other (Wang et al, 2013). Both male and female immature *R. bieti* emigrate from natal groups in the wild (Cui, unpublished data), but there is a lack of information on natal dispersal. The overall objective of this study is then to better understand the social structure in snub-nosed langurs by analysing dominance hierarchy and affiliative patterns. Specifically, this study aimed to (1) examine dominance style and social relationships in an OMU of *R. bieti*; (2) explore the role of dominance rank in adult female social relationships; and (3) better understand natal dispersal mechanisms in this species, for example, do females disperse from the natal group and under what conditions? And what is the role played by adult individuals during the dispersal course of their offspring?

MATERIALS AND METHODS

Subjects

Study animals comprised one adult male and six adult females and their seven offspring housed at the Kunming Institute of Zoology (KIZ) (Kunming, Yunnan, China). Two of the adult females (Fa and Fc) were transferred from Kunming Zoo (KZ) for impregnation on October 12, 1998, and returned after this research. All adults were wild-captured from a band in Weixi county

(N27°27′, E 99°11′), northwest Yunnan. The adult male and juveniles≥3 years old were separately housed since the male is often agonistic toward them. This captive group has a similar composition to wild OMUs (Cui et al, 2008). Age-sex composition and the matrilines of these individuals appear in Cui & Xiao (2004).

Enclosure environment

Study subjects were kept in an enclosure that contained an indoor room (5.2 m long, 2.0 m wide and 2.7 m high) and an outdoor octahedronal cage (66 $m^2 \times 4$ m). The enclosure is evenly divided into two sections. A cement wall separated the indoor room, and the wire mesh fence divided the outdoor pen, which was surrounded by 8 cm-interval vertical metal bars. There was a basin full of water in each cage. Animals on each side could see each other through the metallic mesh, and the observer could also see the animals clearly through the metal fence. A keeper fed the monkeys plants (e.g., privet, cherry, willow leaves), some fruit (apple, banana, pear, tomato) and nutritionally balanced artificial food at 0900h, 1200h and 1600h each day. The plants and fruit were dispersed at two sites in each part of the cage to avoid food competition among animals; artificial food was allocated based on body-size for each animal.

Data collection

Linearity relies on the number of established binary dominance relationships and on the degree of transitivity in these relationships (Appleby, 1983; de Vries, 1995). It should be adaptive for a linear hierarchy when contest competition is strong and where the strength of aggression needs to be reduced by clear dominance relationships among competitors (de Vries et al, 2006). Data on affiliative and agonistic behaviours of all individuals in two cages were collected from August 20, 1998 to March 10, 1999. All animals were observed for 7−8 hours per day, and 2−3 days per week. Total observations were 364 hours, with 243 hours in the mating season and 121 hours in the birth season. Affiliative interactions, recorded by focal-animal scan sampling (every 5 minutes) (Altmann, 1974), are comprised of proximity (being within one meter, but not in contact) and contact (all body contact except agonism and playing). All dyadic interactions with the actor and receiver were recorded using all occurrences sampling (Altmann, 1974) during the period between scanning points. Dyadic interactions include dominant and submissive behaviours. Dominant behaviour is defined as one of the following performed by the initiator: threat, lunge, slap and chase. Submissive behaviour includes responses by the recipient: cower, displace, flee and scream. Animals were names as the following rules: Mk represents the male adult; female adults are named as F*x* (*x* represents different individuals); immatures are named as age and mother' s identity, e.g., Ib is the infant of Fb. This research complied with protocols approved by the Animal Care Committee of Yunnan Province and adhered to Chinese National Laws on Protection of Wild Animals.

Data analysis

It has been reported that wild adult *R. rexallana* females in OMUs change dominance ranks between the mating and birth seasons (Li et al, 2006), thus dominance relationships were analysed in two periods in this study: from August 20 to December 31, 1998, and from January 1 to March 10, 1999. The first period took place primarily in the mating season (MS), and the second period was within the birth season (BS). A combined dominance matrix was constructed for the whole study period due to the absence of agonistic behaviour between some adults and juveniles either in MS or BS. We analysed the frequency of agonistic interactions (*n* per individual observation hour).

Dominance ranking of individuals was measured using David's score (Gammell et al, 2003). Six or more individuals were required to test the linearity of dominance hierarchy, thus the linearity of hierarchy among seven adults in this study was measured using Kendall's coefficient *K* (Appleby, 1983). To describe the extent to which agonistic interactions were asymmetric within dyads, the directional inconsistency index (DII) was calculated as the percentage of all agonism directed to the less frequent direction within dyads (de Waal, 1977). The term "reversal" refers to those episodes below the diagonal of a matrix, and is usually expressed as the percentage of the total number of interactions (e.g., Isbell & Pruetz, 1998). The procedure for calculating the rank of individuals on an interval scale followed Singh et al (2003).

The agonistic ratio for each individual was calculated in terms of the equation: [*n* (agonistic interactions won+1)]/[*n* (agonistic interactions lost)+1]](Newton-Fisher, 2004). To test for differences across seasons, we compared slopes of two linear regressions between agonistic ratios of adult females and their ordinal dominance ranking, and between agonistic ratios of females

and their rank difference (Zar, 1999). Spearman rank correlation tests (R_s) were used to examine the relationship between dominance ranks of adult females and age. Multiple linear regression tests were used to check the relationship between immature rank and age, between the agonistic ratio of the adult male to adult females and their ranks, and between the agonistic ratio of females and rank distance. The Kolmogorov-Smirnov test was used to examine differences in the agonistic ratio of the adult male to adult females between the two seasons. The Wilcoxon Matched Pairs Test was used to investigate differences in agonistic frequencies of adult females between both seasons. The relationship between parents and their offspring was tested using One-way ANOVA and *t*-tests. The index of similarity can be calculated using the method of clustering (Lehner, 1979). A single-link cluster analysis dendrogram was constructed for affiliative similarities. All tests were two-tailed and significance was set at $P<0.05$.

RESULTS

Dominance hierarchy

The dominance matrix was constructed from 717 agonistic interactions and revealed a linear hierarchy among adults in the mating season (Kendall's coefficient: $K=1$, $P=0.002$) (Table 1) and a reversal of 10.7% indicating an unstable relationship. Similarly, there was also a linear hierarchy among adults in the birth season ($K=1$, $P<0.01$), and the reversal of 9.3% suggested an unstable relationship (Table 2). David's score indicated that the adult male was dominant to adult females, and adult females to immature animals. Interval scales of female ranks changed with season (Figure 1). Two pairs of adult females (Fd *vs*. Fq, and Fa *vs*. Fc) changed rank across seasons, but others consistently occupied higher positions in both seasons (Table 1, Table 2). The ranks of adult females were not correlated with age in either season ($R_s<0.09$, $n=6$, $P>0.05$ for both).

There was a significant linear hierarchy of adults in the whole period ($K=1$, $P<0.01$), with a reversal of 10.3%, thus implying an unstable relationship (Table 3). The interval scale of ranks for all monkeys is displayed in Figure 2. Immature ranks were correlated with age ($R^2=0.96$, $F_{1,5}=123.2$, $P<0.001$).

Relationships between the adult male and adult females

The overall mean frequency of agonistic behaviour between the male and females was 0.12 interactions per hour (MS: 0.13 *vs*. BS: 0.12). The agonistic ratio of the male towards females did not differ between seasons (MS: 12.2 *vs*. BS: 13.3, K-S test: $Z=1.16$, $P=0.14$). No relationship was found between the agonistic ratio and female ordinal ranking for both seasons (MS: $R^2=0.63$, $F_{1,4}=6.81$, $P=0.059$; BS: $R^2=0.39$, $F_{1,4}=2.57$, $P=0.18$).

Table 1 Frequencies of agonistic interactions among adults during the mating period based on dominance matrix

Actor	Recipient							∑	DS
	Mk	Fb	Fi	Fd	Fq	Fc	Fa		
Mk	—	20	9	25	19	70	25	168	15.6
Fb	5	—	2	1	18	46	14	86	13.9
Fi[a]	3		—	3	21	85	47	159	4.9
Fd[a]	5		1	—	9	67	93	175	−0.6
Fq[a]	3	2	7	5	—	20	16	53	−5.2
Fc[a]	1			8	7	—	30	46	−13.9
Fa			2	8	3	17	—	30	−16.6
∑	17	22	21	50	77	305	225	717	

[a]Individuals were estrous in the mating season, estimated by mating activities, and further corroborated by newborns in 1999. The descending order for onset of female estrus is as follows: Fi>Fq>Fd>Fc, in which Fc was in estrus before being transferred from Kunming Zoo. DS: David's score; Mk: Male adult; F*x*: Female adults (*x* represents different individuals).

Table 2 Frequencies of agonistic interactions for adults during the birth period

Actor	Recipient							∑	DS
	Mk	Fb	Fi	Fq	Fd	Fa	Fc		
Mk	—	7	5	9	9	5	44	79	16.3
Fb		—	1	9	1	8	22	41	12.6
Fi	1		—	27	5	30	38	101	4.9
Fq	4	2	8	—	2	6	10	32	−0.6
Fd	2		1	1	—	2	20	26	−2.3
Fa			2	5		—	12	19	−11.5
Fc						2	—	2	−20.1
∑	7	9	17	51	17	53	146	300	

DS: David's score; Mk: Male adult; F*x*: Female adults (*x* represents different individuals).

Relationships among adult females

There was a significant linear hierarchy among females in each season ($K=1$, $P=0.022$ for both) with a reversal of 9.2% in MS and 8.1% in BS. In the social unit, females were tolerant of each other. The overall mean frequency of agonistic behavior among females was 0.13 interactions per hour over the whole study period. No difference was found in inter-female agonistic rates

between seasons (MS: 0.15 *vs*. BS: 0.12 interactions per hour, Wilcoxon matched pairs test: Z=0.45, P=0.65). There was a positive correlation between the agonistic ratio of each female and its ordinal ranking in both seasons (R^2>0.73, $F_{1,\ 4}$>11.62, P<0.05 for both), and no difference was found in slopes from linear regressions between two seasons (t=1.47, $t_{0.05\ (1),\ 8}$=1.86, P>0.05). A positive correlation was found between the agonistic ratio of females and ordinal rank differences in both seasons (R^2>0.85, $F_{1,\ 3}$>18.5, P<0.05 for both), and the slope of the linear regression was significantly larger in MS than in BS (t=7.70, $t_{0.001\ (2),\ 6}$=5.96, P<0.001).

Bi-directionality of aggression

The DII suggested that aggression was primarily unidirectional. A total of 105 interactions were directed in the less common direction within dyads (DII=10.3%), in which DII was 10.7% in MS and 9.3% in BS. DIIs (8.1%−11.3%) were consistent in each period and across each partner (Table 4).

Relationships between parents and offspring

The agonistic frequency for father-to-offspring correlated positively with offspring age (R^2=0.84, $F_{1,3}$=15.32, P=0.03), but not for immature animals less than 2 years old (LSD: P>0.05 for both). In contrast, the agonistic frequency for mother-to-offspring did not correlate with offspring age (R^2=0.17, $F_{1,3}$=0.68, P=0.48) and shifted with age ($F_{1,341}$=35.98, P<0.001): mothers displayed aggression towards 2-year-old offspring more frequently than 1-year-old animals (LSD: P<0.01), and 3-year-old offspring more than 4-year-old animals (LSD: P<0.05).

The father did not direct more agonisms towards his infants than mothers did (0.01 *vs*. 0.03 times/h, t_{253}=1.70, P=0.09). Mothers directed more agonisms towards their 2-year-old offspring than the father did (0.20 *vs*. 0.11 times/h, t_{243}=2.30, P=0.02), but the father directed more agonisms towards≥3-year-old offspring than mothers did (0.94 *vs*. 0. 27 times/hour, t_{187}=4.40, P<0.001 for 3-year-old; 2.33 *vs*. 0.06 times/hour, t_{85}=2.04, P=0.045 for 4-year-old). Moreover, the father directed more agonisms towards his offspring during MS than BS (0.90 *vs* 0.32 times/ h, t_{428}=2.71, P=0.007), particularly towards≥3-year-old offspring (3-year-old: t_{144}=3.24, P=0.0015; 4-year-old: t_{71}=2.27, P=0.026); this was not true for mothers (0.11 *vs* 0.17 times/hour, t_{346}=1.66, P=0.10).

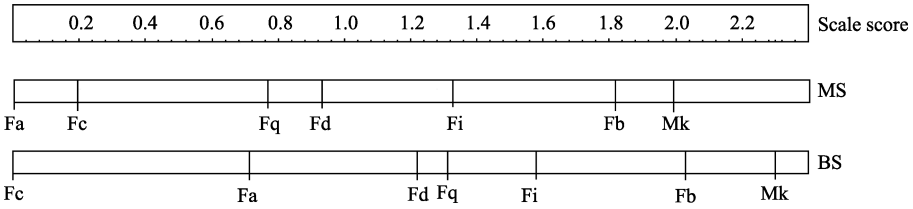

Figure 1 Dominance rank of adult individuals on an interval scale in the mating season (MS) and birth season (BS)

Calculation of individuals' ranks on an interval scale refers to the method presented by Singh et al (2003).

Table 3 Frequencies of agonistic interactions for all individuals over the whole study period

Actor	Recipient														∑	DS
	Mk	Fb	Fi	Fd	Fq	Fc	Fa	4i	3b	3q	2i	2q	1d	Ib		
Mk	—	27	14	34	28	114	30	864	444	249	36	22	3	0	1865	80.4
Fb	5	—	3	2	27	68	22	8	14	21	65	47	7	52	341	73.5
Fi	4		—	8	48	123	77	6	70	49	21	90	40	8	544	57.3
Fd	7		2	—	10	87	95	10	24	40	198	72	57	7	609	41.2
Fq	7	4	15	7	—	30	22	96	200	62	245	140	89	21	938	38.3
Fc	1			8	7	—	32	173	330	402	162	96	63	7	1281	17.0
Fa			4	8	8	29	—	104	273	243	164	118	8	1	960	16.8
4i		1		1	12	2	8	—	162	252	33	49	12	13	545	−12.5
3b			5	5	35	7	13	65	—	163	22	20	2	4	341	−16.4
3q		4	4	4		4	12	102	75	—	36	45	6	5	297	−23.4
2i					6	1				3	—	10	24	6	50	−55.3
2q						1		3	2	3	5	—	15	12	41	−55.4
1y						2		2			2		—	6	12	−73.6
Ib											1			—	1	−101.3
∑	24	36	47	77	181	468	311	1433	1594	1487	990	709	326	142	7825	

DS: David's score; Mk: Male adult; F*x*: Female adults (*x* represents different individuals); Immatures are named as ages and mother's identity (e.g., Ib: the infant of Fb).

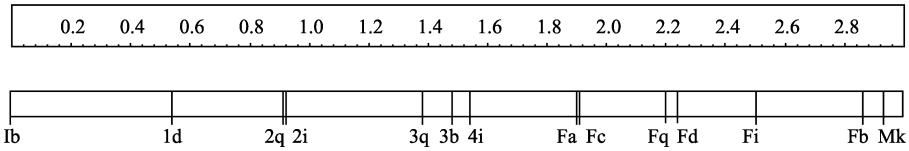

Figure 2 Rank of all individuals on an interval scale during the whole study period

Calculation of individuals' ranks on an interval scale refers to the method presented by Singh et al (2003).

Table 4 Directional inconsistency index of agonistic interactions among adult monkeys during the study period

	20/08/98−31/12/98	01/01/99−10/03/99	20/08/98−10/03/99
All partner combination	77/717 (10.7%)	28/300 (9.3%)	105/1017 (10.3%)
Male-female dyads	17/185 (9.2%)	7/86 (8.1%)	24/271 (8.9%)
Female-female dyads	60/532 (11.3%)	21/214 (9.8%)	81/746 (10.9%)

Affiliative interactions

There were three clusters in the OMU (Figure 3). Three nursing mothers, the adult male and two adult females from KZ formed a loose association in cluster A. Cluster B was comprised of one adult female and her offspring. Three juveniles formed cluster C. The strongest social bond appeared in one mother-infant pair at the 268 level of affiliative similarity, and the next were two mother-2-year-old-juvenile and one mother-yearling pairs at the 255 level of similarity. Affiliative bonds among females were clearly differentiated. Adult females (Fa and Fc) with the lowest ranks in the adult class tended to stay together, and associate with the adult male through the adult female Fi. Because mothers followed their yearlings around, offspring less than two years of age were seldom left alone. They were also often found near their tolerant father. An adult female (Fd) with a crippled left leg was unable to follow her yearlings, and this may have resulted in a smaller level of similarity. The three>2--year-old juveniles were more isolated than the≤2-year-old ones because their father directed more agonism towards them.

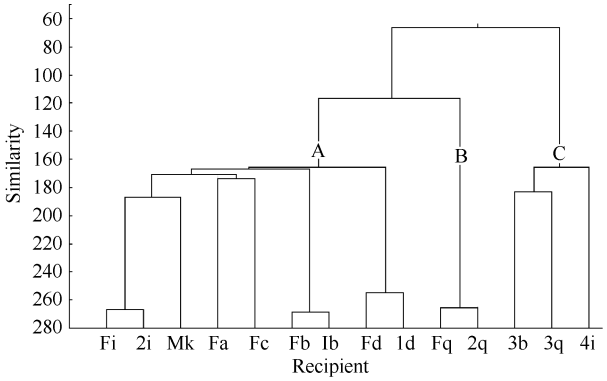

Figure 3 Dendrogram based on affiliation among members of a captive *Rhinopithecus bieti* OMU

Calculation of similarity index refers to the method reported by Lehner (1979).

DISCUSSION

Dominance hierarchy

Adult female *R. bieti* displayed an average of 0.13 interactions per hour and this rate is at the low end when compared with other colobines (1.20 for *P. thomasi*: Sterck & Steenbeek, 1997; 0.6 for *C. polykomos*, 0.19 for *Procolobus badius*; Korstjens et al, 2002; 0.25 for *T. phayrei*: Koenig et al, 2004; and 0.22 displacement rate per hour for *R. roxellana*: He et al, 2013). A previous study of captive *R. bieti*, consisting of three adults, one sub-adult and five immatures, reported an overall mean frequency of agonistic interactions per hour of 0.30 for individuals over one year of age (Grüter, 2004). This inconsistency in agonistic rate may be the result of two possibilities. First, we did not collect data on agonisms during feeding. Second, it might relate to differences in the age-sex composition of subjects between the two studies. In our study, 87% of agonisms were made by immature animals, thus the higher rate might result from the higher proportion of immatures (67%) in the previous study. *R. bieti* feed mainly on evenly distributed lichens in its northern range (Kirkpatrick et al, 1998), but their primary foods, such as deciduous broadleaves (Ding & Zhao, 2004) and bamboo leaves (Yang & Zhao, 2001), are patchily distributed in its southern range. If food competition plays an important role in inter-female interactions, the agonistic rate may be higher in its northern range than southern range; however, further research is needed to clarify this.

Agonistic behaviour might be more common in captive than wild animals, but appears to follow a linear hierarchy in guenons (*Cercopithecus diana*, *C. solatus*, *C. mitis*) and patas monkeys (*Erythrocebus patas*) in both captivity and the wild (see Lemasson et al, 2006). We found a linear dominance hierarchy among adult females

in our *R. bieti*, as has been reported in other colobines (Borries et al, 1991; Koenig, 2000; Koenig et al, 2004) and in patas monkeys (Isbell & Pruetz, 1998). Consequently, our study does not support the idea that lower agonistic rates coincide with an indiscernible hierarchy (Isbell & Young, 2002). The consensus is that the dominance hierarchy between females in group-living primates relates to factors such as resource competition and kinship. The linear hierarchy of female *R. bieti* outside of feeding contexts may reflect scramble competition among females in the wild. Here, the adult male was dominant to adult females and adult females to immatures, consistent with reports from wild *R. roxellana* (Li et al, 2006). Dominance ranks of adult female *R. bieti* are not based on age, in contrast to other colobines (Borries et al, 1991; Koenig et al, 2004). Colobine daughters do not acquire ranks similar to those of their mothers, unlike in most cercopithecines (Melnick & Pearl, 1987). Juvenile hanuman langur females rise in rank above older and even larger females (Borries et al, 1991). During this study, however, all juvenile females may have been too young (3−4 years) to enter higher rank, and were subordinate to adult females. These young females may begin to rise in rank at an age of five or even six years, if they rise in rank as in hanuman langurs (Borries et al, 1991; Koenig, 2000).

Hierarchy style

A previous study of *M. thibetana* provided quantitative indices to identify dominance style of a species (Berman et al, 2004). The DII of a typically despotic species ranges from 0.7% to 4.1%, and one value of 9.0% is available for a relaxed species. The conciliatory tendency ranges from 5% to 15% for despotic species, and from 35% to 48% for relaxed species. Notably, comparable values are for all adults were combined together (Berman et al, 2004). In our study, the DII of 10.3% is larger than 9.0%, thus within the range of DIIs for relaxed species. The dominance style hypothesis predicts that a more relaxed style is correlated with high levels of post-conflict reconciliation (de Waal & Luttrell, 1989). The conciliatory tendency is 54.5% for captive *R. bieti* monkeys over 1-year-old age (Grüter, 2004), 51.3% for all captive adult spectacled leaf monkeys (Arnold & Barton, 2001), and 40% for captive *R. roxellana* (Ren et al, 1991). Moreover, bidirectional aggression of captive *R. bieti* is common (28% of all conflicts) (Grüter, 2004), and many wild *R. bieti* usually forage in one tree with rare agonisms (Kirkpatrick et al, 1998). Therefore, *R. bieti* is likely a relaxed or tolerant species.

Relationships between the male and adult females

The similar agonistic ratio of the adult male towards adult female *R. bieti* showed that the male directed unbiased agonisms towards females in MS and BS. Moreover, the lack of correlation between agonistic ratios of the male towards females and rank suggests that the male did not direct more agonism towards low-ranking females than high-ranking ones. Therefore, dominance rank appears to not play a role in maintaining the relationship between the adult male and adult females in OMUs, that is, the adult male does not sustain relationships with adult females in terms of their dominance rank.

Inter-female relationships in OMUs

Dominance gradient, rather than linearity, is considered the key element of a hierarchy because it dictates the degree to which one female can exert a negative effect on another (Henzi & Barrett, 1999). A steep gradient increases the extent of power differential between high- and low-ranking females, allowing the former to exert a strong negative influence on the latter (Barrett et al, 2002). The positive correlation between the agonistic ratio of adult females and their rank indicates that the degree to which one female influences others declined with a decrease in their rank. A similar positive correlation between the agonistic ratio of adult females and their rank differential indicates that they directed less negative behaviour to closely ranked females than distantly ranked one. These patterns mean that dominance rank should play an important role in relationships among adult females in the OMU of *R. bieti*. A steeper gradient in MS implied a strong power of high-ranking females to exert negative effects on low-ranking ones as compared with that in BS, which implies that dominance rank may affect the mating success of females, as in *R. roxellana* (He et al, 2013).

Relationships between parents and offspring

Extra-group males are common in many colobines, which typically show male-biased dispersal (Newton & Dunbar, 1994), but extra-group females do occur in some species (*P. senex*: Rudran, 1973; *R. roxellana*: Zhang et al, 2008b) and at times in *N. larvatus*, *T. auratus*, *T.*

johnii and *P. thomasi* (Yeager & Kool, 2000). Young males in some primate species encounter increased rates of aggression from resident adult males, which has been assumed to be the cause of their eventual emigration (Struhsaker & Leland, 1987). However, male lowe's guenons living in one-male groups emigrate voluntarily if their father is still present in the group when they mature (Bourlière et al, 1970). Nulliparous females may disperse for inbreeding avoidance when they mature and their father still has a breeding status (Clutton-Brock, 1989). In our study, continual agonisms of a father towards older offspring of both sexes suggest they must emigrate from their natal group and their father. The intensity of agonism of the father towards his offspring escalated with age, but this was not true for mothers, suggesting that natal dispersal of immature animals is caused by their father rather than their mother, at least when the father still resides in the group. If an adult male enters a bisexual group and drives out the previous breeding male, individuals are presumably expelled by males that are not their fathers (Pusey & Packer, 1987).

Female *R. bieti* become mature at 4.5 years of age, and males at about 6.5−7.0 years of age (Zou, 2002). The father directed agonisms towards his≥3-year-old offspring more frequently than<3-year-old ones, suggesting that individuals of both sexes begin emigration at puberty (Pusey & Packer, 1987). In addition, the father evicted his≥3-year-old of offspring more frequently in MS than BS, and accordingly we predict that natal dispersal occurs before or during MS.

Mothers directed agonisms towards 2-year-old offspring more frequently than 1-year-old ones because mothers began weaning their offspring at 1.5 years of age. In wild-provisioned and captive populations of hanuman langurs, infants are weaned at an age of 12.8 months. However, for wild langurs at Ramnagar, infants were on average weaned at an age of two years. This difference in weaning age is related to nutritional conditions between two different living environments (Koenig & Borries, 2001).

Affiliative relations in the OMU

The breeding male has the strongest social association with the first estrus female (Fi), then unfamiliar females (Fa and Fc), and then other females of decreasing rank. This suggests that social bonds between adults of both sexes are first affected by female estrus, and then by their rank. Conversely, *R. roxellana* females have no strong tendency to build a relationship with the adult male in OMUs in the wild (Wang et al, 2013). The female affiliative relationship is differentiated, and weakly formed on the basis of the breeding male, and also influenced by their rank. Future research is needed to confirm the pattern among adults of OMUs in the wild. On the other hand, the strongest social association occurs in mother-infant pairs in the OMU of *R. bieti*. Affiliation between the mother and offspring is weaker as they grow, implying that immature animals gradually socialize and become independent. In most polygynous species, males provide little paternal care (Pusey & Packer, 1987). In this study, the observed adult male *R. bieti* provided little direct paternal care to infants, but was tolerant of their staying and playing in/around him, and occasionally sniffed them.

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Influence of dominance rank and affiliation relationships on self-directed behavior in female Tibetan macaques (*Macaca thibetana*)

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Abstract: Self-directed behavior (SDB) is characterized as an indicator of anxiety, frustration and stress in nonhuman primates. In this study, we collected self-directed behavior data from one group of free-ranging Tibetan macaques (*Macaca thibetana*) at Mt. Huangshan, China (September 2012–May 2013) using a combination of behavioral sampling methods including focal animal sampling, behavioral sampling, continuous sampling and instantaneous sampling. Our results showed that females engaged in significantly higher rates of self-directed behavior when they were in proximity to dominant individuals compared to subordinate ones. Conflict losers significantly increased their SDB rates after agonistic episodes, indicating that SDB might also serve as an index of anxiety in *M. thibetana*. We further found that females significantly increased their SDB rates when focal individual was proximity to weakly affiliation relationship higher rank members than to strongly affiliation relationship higher rank members. If conflicts were not reconciled, the postconflict SDB rates of losers were higher when they stayed with strongly affiliation opponents; if conflicts were reconciled, victims of strongly affiliation relationships opponents engaged in more SDB rates before reconciliation than after reconciliation, while victims of moderately affiliation relationships opponents did not engaged in more SDB rates before reconciliation than after reconciliation. We conclude that both of dominance rank and affiliation relationships might both influence the SDB rates of female Tibetan macaques significantly, suggesting that SDB is not only an index of anxiety in Tibetan macaques, but also can provide a new insight into evaluation of social relationships between individuals.

Keywords: Tibetan macaques (*Macaca thibetana*); Female; Self-directed behavior (SDB); Dominance Rank; Affiliation relationship

Animals, including humans, can exhibit some activities unconsciously when facing with stress or anxiety (Lantz, 1979; Maestripieri et al, 1992). For example, passerine birds clean their bills or feathers in some sexual or agonistic contexts; non-human primates show self-scratch behavior after being attacked by conspecific members (Maestripieri et al, 1992; Radford, 2012). Thus, a behavior pattern which is "apparently irrelevant" to an animal's ongoing activities called self-directed behavior (SDB) (Maestripieri et al, 1992). It exists in a wide range of animals such as arthropods, fish, birds and mammals (Duncan & Wood-Gush, 1972; Hansen & Drake Af Hagelsrum, 1984; Roper, 1984; Rowell, 1961). Self-directed behavior, such as self-scratching, self-grooming, self-touching, yawning and body shaking (Schino et al, 1988), have been widely used as an indicator of anxiety in non-human primates. Pharmacological and behavioral evidence showed that there was a high correlation between SDB and anxiety (Maestripieri et al, 1992). For example, anxiolytic and anxiogenic drugs could reduce or increase the individual SDB rates in wild gregarious non-human primates (Crawley et al, 1985; Maestripieri et al, 1992; Ninan et al, 1982; Redmond Jr & Huang, 1979; Schino et

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al, 1996; Schino et al, 1991). With purely observational method, researchers attempted to link SDB and anxiety by observing the individual SDB rates in different contexts. In the study of *Macaca fascicularis*, Pavani et al (1991) found that the individual engaged in higher scratching rates when stayed with a dominant neighbor rather than with a subordinate neighbor. Similar results were received in Olive Baboon (*Papio anubis*) (Castles et al, 1999). Although Captive vervets (*Cercopithecus aethiops*) didn't engage in more SDB rates when near to a dominant neighbor, it showed higher SDB rates when in postconflict situation (Daniel et al, 2008). Analogous studies about *M. fuscata fuscata*, *M. Sylvanus*, *M. fascicularis* suggested that individual's SDB rates tended to increase in postconflict situation (Aureli, 1997; Aureli & Schaik, 1991; Kutsukake & Castles, 2001).

Earlier studies about SDB in non-human primates mainly concentrated in two uncertain circumstances: (1) close proximity to dominant individuals. Subjects would engaged in anxiety emotion since the dominant neighbor increased the possibility being attacked (Daniel et al, 2008). (2) postconflict. After a conflict, individuals became stressful as a result of 2 ambivalent motivations : 1) withdrawing for fear of renewed attacks and 2) approach to reconcile (Aureli, 1997; Aureli & Schaik, 1991). Thus, the individual's SDB rates after conflict increased significantly. These two uncertain circumstances are both related to dominance rank, that is to say, subordinates will engage in higher SDB rates under threaten or potential threaten from dominants. However, individual's SDB levels are not only influenced by the dominance rank, but also social relationships (Castles et al, 1999). For example, Castles et al (1999) found that the Olive Baboons' SDB rates were higher when proximate to dominants, but had no correlation with the rank distance. Based on this, if the SDB rates are influenced by the dyadic relationships between the partners? Moreover, if the SDB rates are influenced by the quality of the relationship between former opponents after conflict? All these questions remain to be answered.

Tibetan macaques(*M. thibetana*) are highly gregarious and display female philopatry, male dispersal (Li, 1999; Xia et al, 2013). Male-male relationship are mostly competitive, while female-male relationships are affected by different factors such as mating season, non-mating season, temporary spouse relationship and population sex ratio(Li, 1999; Wang et al, 2009). Female Tibetan macaques are reported to form linear dominance hierarchies, establish strong and well-differentiated relationships with other adult females in their group, and female-female relationships play a very important role in maintenance of the group's stability (Xia et al, 2012). Therefore, female Tibetan macaques are ideal model to study influence of dominance rank and dyadic affiliation on SDB. Proximity to dominant individuals and postconflict contexts were set in this study, to examine: (1) the correlation between SDB and anxiety; (2) the effect of dyadic affiliation relationships on SDB.

MATERIALS AND METHODS

Study site and subjects

We conducted this study at the Valley of Wild Monkeys, Mt. Huangshan, Anhui Province, China (E118°10′, N30°29′), a well-known tourist destination and research site, environment about this area refer to Li (1999). There are two groups at the site: Yulingkeng A1 (YA1) and Yulingkeng A2 (YA2), for this project we collected behavioral samples in YA1. The nine female adult individuals were selected for study which can be distinguished by facial/body characteristics, and the abbreviations rules of the subjects' name referred to Li (1999). Observations of YA1 continued for 27 years since 1986, recording dynamic data of immigration, emigration, death, birth and so on. During the study period, the YA1 group consisted of 4 adult males, 9 adult females, 5 subadults and 18 juveniles/infants. Table 1 shows the study individuals and duration for sampling during study.

Behavioral definitions

In this study, self-directed behavior definitions see Table 2.

Data collection

This study was conducted from September 2012 to May 2013, using behavioral sampling, focal animal sampling, continuous recording, instantaneous recording to collect behavioral data (Altmann, 1974).

Behavioral sampling was used to record the displacements, aggressions and avoidances among females, these data were used to construct the win/lose matrix in order to confirm dominance hierarchy, 288 samples were recorded.

Focal sample duration (using a digital voice record)

Table 1 Subjects' duration for sampling and SDB rates

Subjects	Focal duration (min)	SS (bouts/min)	SG (bouts/min)	ST (bouts/min)	SHAKE (bouts/min)	YAWN (bouts/min)	Total SDB (bouts/min)
YH	1200	0.243	0.049	0.007	0.003	0.002	0.303
Hhui	1200	0.213	0.073	0.011	0.008	0004	0.309
YM	1197	0.203	0.046	0.018	0.005	0.008	0.279
TH	1200	0.168	0.088	0.013	0.011	0.001	0.282
TXX	1193	0.244	0.049	0.013	0.010	0.010	0.326
HH	1196	0.219	0.078	0.024	0.016	0.006	0.343
TR	1200	0.193	0.094	0.023	0.010	0.003	0.323
TT	1200	0.157	0.051	0.017	0.004	0.003	0.231
YZ	1200	0.198	0.068	0.011	0.003	0.003	0.283

Dominance rank of 9 focal female descended from top to bottom.

Table 2 Behavioral definitions

Catalog	Definition
Self-scratching (SS)	(usually repeated) Movement of the hand or foot during which the fingertips are drawn across fur or skin.
Self-grooming (SG)	Picking through and/or slowly brushing aside fur with one or both hands.
Self-touching (ST)	Other forms of body touching with the hand including wiping eyes, inspecting feet and placing hand to mouth.
Shaking	Shaking movement of entire body (similar to that of a wet dog).
Yawning	Brief gaping movement of the mouth. Not recorded as an SDB if accompanied by aggressive signals such as eye flash or canine whetting.
Postconflict reconciliation behavior	
Teeth-chatter	Clicking sounds are made with the teeth by rapidly moving the jaw up and down. Eyelids are lowered, the chin is raised, and the tongue may move rapidly across the teeth.
Embrace	One individual approaches another and lipsmack or both individuals hold each other and may lightly bite one another.
Touch	One individual lightly touches another usually on the head, shoulders, or back.
Present	One individual displays her bottom to another.
Genital inspection	One individual touches, licks, or sniffs the genitals of another.
Groom	One individual orally or manually manipulates the fur of another
Bridge	A complex sequence of behavior in which an individual approaches another, alternating glances at the receiver and an infant that is carried by either the approacher or the approached. The pair holds the infant between them and simultaneously licks the infant's genitals or body while teeth-chattering vigorously.
Hold bottom	One individual approaches another and holds or embraces her bottom for a few seconds.

Self-directed behavior definitions were modified by Schino et al (1988), aggressive behavior definitions were modified by Berman et al (2006) and Li (1999).

was set at 20 min to score the activity of focal individual. To balance samples, we sampled each focal individual randomly, with no subject observed twice before all others were watched once. Focal sampling and continuous recording were used to score grooming, proximity and all that affiliation behaviors to evaluate the dyadic affiliation relationships. During the focal sampling, we also recorded the SDB and set breaks in SDB lasting >2 s or switches to another class of SDB as separate bouts. Whenever we recorded an SDB bout, we also recorded the identity of the nearest individual within 2 m. Instantaneous recording was proceeded with focal sampling to record the identity of the nearest individual at 1-min intervals. Samples were discarded if the focal individuals disappeared and samples were less than 15min (Castles et al, 1999; Manson & Perry, 2000).

We initiated focal observations on the victim of aggression once a conflict happen and record: victim and aggressor (Li, 1999). Then we collected 5 min postconflict (PC) focal sample, including SDB and affiliative interactions. We also recorded the time both when a SDB and postconflict affiliative interactions (see table 2) occurred. If conflict reoccurred between former opponents during the PC period, we restarted the PC observation after the new encounters. The next day at the same time, we conducted matched control (MC) focal observations of the victim, the contents and methods recorded in MC according to PC. If the subjects were out of vision or involved in a conflict 5min before MC, we postponed the MC until the all

conditions were met. If MC observation could not be conducted within one week of the PC, the PC was discarded.

Data analysis

We assessed individual dominance ranks by calculating David's Score(DS). We also calculated linearity for the obtained dominance hierarchy (De Vries, 1995; Gammell et al, 2003). Rank distance is the number of individuals ranking between the focal animal and a given partner, plus 1 (Castles et al, 1999).

Individual ranks calculated with DS: P_{ij} (The proportion of wins by individual i in his interactions with another individual j)=α_{ij} / n_{ij}, P_{ji}=1−P_{ij}; α_{ij} is the number of times that i defeats j, n_{ij} is the total number of interactions between i and j. If there was no aggression and avoidances between individual i and j, then P_{ij}=P_{ji}=0. For each member i, DS=w+w_2−l−l_2, w represents the sum of i's P_{ij}; w_2 represents the summed w values (weighted by the appropriate P_{ij} values) of those individuals with which i interacted; l represents the sum of j's P_{ji}; l_2 represents the summed l values (weighted by the appropriate P_{ji} values) of those individuals with which j interacted. Individual dominance rank is determined by DS, the higher the DS value, the higher the rank, and vice versa. For more details, see Gammel et al (2003).

To determine the number of proximity point samples, we followed Castles et al (1999), wherein s is the number of SDB bouts shown by focal individual, when individual X was nearest neighbor within 2 m; P equals the number of proximity point sample in which individual X was nearest neighbor within 2 m. For individual X, the focal individual's neighbor SDB rates is s/q. According to the way of calculation above, the unit of SDB rates is bouts/min.

We measured the relationship between females by using the Dyadic Association Index (DAI):

$$\mathrm{DAI}_{\mathrm{AB}} = \frac{\sum(A+B)}{\sum A+\sum B-\sum(A+B)}$$

wherein A is the time individual A was seen, B is the time individual B was seen and A+B is the time A and B were seen together (Nishida, 1968). Then the dyadic scores were ranked individually. In this study, each one of the 9 focal animals corresponds to 8 dyadic relationships, wherein the top quartile were labeled as strongly affiliation relationship and the lowest quartile were labeled as weakly affiliation relationship, and the mid 50% was labeled as moderately affiliation relationship (Arnold & Whiten, 2001; Cords & Aureli, 2000; Preuschoft et al, 2002).

We analyzed only overall rates (all SDBs summed) because most of the individual SDBs, besides scratching, had very low occurrences. We used mean values (±*SE*) as quantitative criteria. A paired *t*-test was used to analyze differences of SDB rates influenced by different dominance rank and different affiliation relationship. Spearman Rank Correlation Test was used to test the correlation between dominance rank and SDB rates, also, the rank distance and SDB rates. All analyses were two tailed and carried out using the SPSS 16.0 software, with the significance level set at 0.05.

RESULTS

Dominance hierarchy

Dominance hierarchy for adult females showed a linear dominance hierarchy (h'=0.991, n=9, P<0.001; see Table 3).

Table 3 Dominance hierarchy for females

	YH	HHUI	YM	TH	TXX	HH	TR	TT	YZ	w	w_2	DS
YH		8(1.0)	13(1.0)	10(1.0)	12(1.0)	5(1.0)	8(1.0)	6(1.0)	5(1.0)	8	28	**36**
HHUI			9(1.0)	12(1.0)	11(1.0)	3(1.0)	1(1.0)	7(1.0)	3(1.0)	7	21	**27**
YM				14(1.0)	12(1.0)	1(1.0)	14(1.0)	7(1.0)	2(1.0)	6	15	**18**
TH					6(1.0)	11(0.85)	15(1.0)	9(1.0)	7(1.0)	4.85	9.68	**7.65**
TXX						1(1.0)	12(1.0)	5(1.0)	4(1.0)	4	6.15	**0**
HH				2(0.15)			11(1.0)	7(1.0)	5(1.0)	3.15	3.73	**−7.65**
TR								13(1.0)	11(1.0)	2	1	**−18**
TT									6(1.0)	1	0	**−27**
YZ										0	0	**−36**
l	0	1	2	3.15	4	4.85	6	7	8			
l_2	0	0	1	3.73	6.15	9.68	15	21	28			

The individual on the vertical axis scored as winner.

Correlation between SDB and anxiety in female Tibetan macaques

In the study group, the mean±*SE* SDB rates among 9 subjects was 0.297±0.011 bouts/min (see Table 1). There is no correlation between dominance rank and any SDB rates (overall: *Rs*=–0.050, *n*=9, *P*=0.898; SS: *Rs*=−0.500, *n*=9, *P*=0.170; SG: *Rs*= 0.283, *n*=9, *P*=0.460; ST: *Rs*= 0.393, *n*=9, *P*=0.295; SHAKE: *Rs*=−0.017, *n*=9, *P*=0.966; YAWN: *Rs*= 0.000, *n*=9, *P*=1.000).

All subjects had ⩾1 other females within 2 m on 34.9% (±4.92%) of the proximity point samples. Focal subjects engaged in significantly more SDB rates when their nearest neighbor was a dominant than the neighbor was a subordinate (*t*=4.629, *n*=7, *P*=0.004) or there was no neighbor(*t*=2.622, *n*=7, *P*=0.039) within 2 m. In addition, they engaged in more SDB rates when there was no neighbor than their nearest neighbor was a subordinate within 2m (*t*=2.898, *n*=7, *P*=0.027) (Figure 1) (There was no dominants near YH and there was no subordinates near YZ, so we did not analysis the neighbors of YH and YZ in this part).

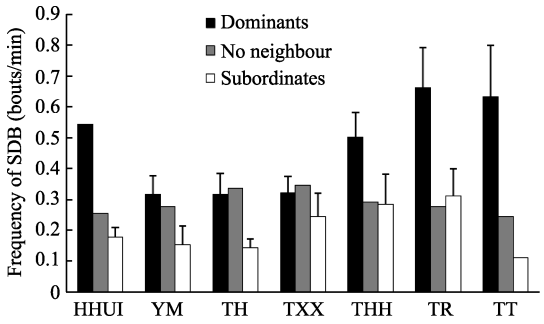

Figure 1 Self-directed behavior rates when a dominant or subordinate conspecific was nearest neighbor within 2 m and when there was no nearest neighbor within 2 m

The SDB rate was not correlated with rank distance (dominant point samples near YH, Hhui, YM and subordinate point samples near YZ, TT, TR were all less than 3, we didn't analysis in this part). Spearman rank correlation coefficients ranged between –0.600 and 0.500 for dominant near neighbors (TH: subordinate partners: *Rs*=0.500, *n*=3, *P*=0.667; TXX: subordinate partners: *Rs*=0.000, *n*=4, *P*=1.000; HH: subordinate partners: *Rs*=0.100, *n*=5, *P*=0.873; TR: subordinate partners: *Rs*=–0.600, *n*=6, *P*=0.208; TT: subordinate partners: *Rs*=–0.179, *n*=7, *P*=0.702; YZ: subordinate partners: *Rs*=–0.214, *n*=7, *P*=0.645. we didn't analysis YZ, TT and TR for the number of subordinates partner of YZ, TT and TR were less than 3) ; between-0.976 and 0.800 for subordinate near neighbors (YH: dominant partners: *Rs*=–0.976, *n*=8, *P*=0.000; Hhui: dominant partners: *Rs*=–0.536, *n*=7, *P*=0.215; YM: dominant partners: *Rs*=0.800, *n*=4, *P*=0.200; TH: dominant partners: *Rs*=–0.300, *n*=5, *P*=0.624; TXX: dominant partners: *Rs*=–0.400, *n*=4, *P*=0.600; HH: dominant partners: *Rs*=0.500, *n*=3, *P*=0.667. we didn't analysis YH, Hhui and YM for the number of subordinates partner of YH, Hhui and YM did not reach 3). Only 1 of 12 coefficients were significant (YH: subordinate partners: *Rs*=−0.976, *n*=8, *P*=0.000).

In this study, we had 60 valid PC-MC observations. Postconflict SDB rates without reconciliation were significantly more than MC (*t*=6.317, *n*=8, *P*=0.000) and after reconciliation (*t*=5.142, *n*=7, *P*=0.002). The SDB rates before reconciliation were significantly more than MC (*t*=3.675, *n*=7, *P*=0.010) and after reconciliation (*t*=3.654, *n*=7, *P*=0.008). It was close to significant that the postconflict SDB rates with no reconciliation were more than before reconciliation (*t*=–2.550, *n*=7, *P*=0.043), while there was no significant differences between after reconciliation and MC (*t*=1.058, *n*=7, *P*=0.331) (Figure 2).

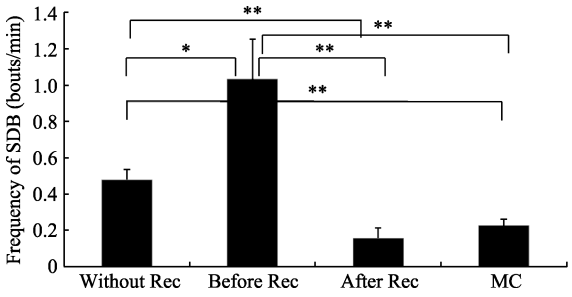

Figure 2 Victims' self-directed behavior rates with reconciliation (Rec) or without reconciliation between opponents and MC

**: *P*<0.01; *:*P*<0.05.

There was no correlation between postconflict SDB rates and rank distance (the data of YH, Hhui, YM was not used in this analysis because there were not enough individuals nearby for spearman correlation analysis). Spearman rank correlation coefficients ranged between -0.522 and 0.718(TH: *Rs*=−0.866, *n*=3, *P*=0.333; TXX: *Rs*=–0.500, *n*=3, *P*=0.667; HH: *Rs*=–0.112, *n*= 5, *P*=0.858; TR: *Rs*=–0.800, *n*=4, *P*=0.200; TT: *Rs*= 0.500, *n*=3, *P*=0.667; YZ: *Rs*=–0.316, *n*=4, *P*=0.684).

Female affiliation relationship and proximity SDB

Comparisons of individuals' mean SDB rates with

dominant partners of different affiliation relationships, we found that subjects engaged in more SDB rates with a neighbor of strong affiliation relationship than of weakly affiliation relationship(t=−2.818, n=5, P=0.048). There was no significant differences between with a neighbor of strong affiliation relationship and moderately affiliation relationship (t=−0.568, n=6, P=0.595), also, moderately affiliation relationship and weakly affiliation relationship (t=−0.377, n=5, P=0.726).

Comparisons of individual mean SDB rates with subordinate partners of different affiliation relationships, we found no differences among them (strong affiliation relationship and moderately affiliation relationship: t=2.641, n=3, P=0.118; strong affiliation relationship and weakly affiliation relationship: t=0.417, n=4, P=0.705; moderately affiliation relationship and weakly affiliation relationship: t=0.676, n=6, P=0.529).

Female affiliation relationship and postconflict SDB

Postconflict with no reconciliation, the subjects engaged in significantly more SDB rates aggressed by individuals with strong affiliation relationship than weakly affiliation relationship (t=0.900, n=4, P=0.003). But there were no differences between attacked by strong affiliation relationship and moderately affiliation relationship (t=0.987, n=5, P=0.379), moderately affiliation relationship and weakly affiliation relationship (t=1.353, n=5, P=0.247).

Postconflict with reconciliation, subjects engaged in significantly more SDB rates attacked by strongly affiliation relationships before reconciliation than after reconciliation (t=3.008, n=8, P=0.020) and MC (t=3.006, n=8, P=0.020). There were no differences between attacked by moderately affiliation relationships before reconciliation and after reconciliation (t=2.955, n=4, P=0.060) or MC (t=2.039, n=4, P=0.134) (Figure 3).

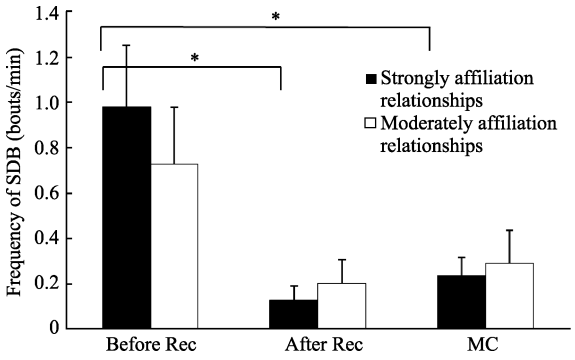

Figure 3 The effects of opponents with different affiliation relationships on self-directed behavior frequency before reconciliation and after reconciliation

*: P<0.05.

DISCUSSION

The correlation of SDB and anxiety in female Tibetan macaques

Female Tibetan macaques showed significantly higher SDB rates while proximity to dominants than subordinates, which may be resulted from the strict dominance hierarchy. Aggression among Tibetan macaques is directed predominantly down the hierarchy (Li, 1999), thus a dominant partner will present more danger of receiving aggression than a subordinate partner, and increased anxiety should be expected. The SDB rates is significantly higher when there was no neighbor within 2 m than in proximity to a subordinate, suggesting that detachment from near neighbors increased the potential surrounding risk and separation from allies. Moreover, the presence of subordinates reduced the subjects' SDB rates significantly, this is because both the two potential risks presented above were removed, that is, the dominants scarcely received threaten from subordinates, and the potential separation from allies was eliminated, thus the individual was in a state of emotional stability.

Similar with the results reported for long-tailed macaques (*M. fascicularis*) (Aureli, 1992;1997; Aureli & Schaik, 1991; Aureli et al, 1989; Das et al, 1998), Barbary macaques (*M. sylvanus*) (Aureli, 1997), Olive baboons (Castles & Whiten, 1998), Chimpanzees (*Pan troglodytes*) (Fraser et al, 2010), after conflicts, female Tibetan macaques showed higher SDB rates without reconciliation or before reconciliation, while the opponents reconciled, the SDB rates decreased. It can be explained that the victim was fear of renewed attacks without reconciliation or attempt to reconcile before reconciliation (SDB rates was higher before reconciliation than without reconciliation confirmed the second assumption, see Figure 2), thus the presence of anxiety emotion is expected. If this emotion can not be disposed immediately and cumulated, which would increase the likelihood of worse outcome such as growing development, disease resistance and fertility (Henry, 1982; Nederhof & Schmidt, 2012), and even the damage of brain (Uno et al, 1989). When the opponents reconciled, the emotion of anxiety and stress got released, which decreased the bad consequence greatly, suggesting the importance of postconflict reconciliation to individual survival and even the stability of population.

Both of the two uncertain situation were established on dominance rank difference in social interaction, which showed that there was some correlation between SDB and anxiety in Tibetan macaques.

The influence of affiliation relationship on SDB

In this study, SDB rates of focal subjects had no correlation with rank distance of the proximate partners and the aggressors, suggesting that SDB varies not only according to the dominance rank, Castles et al (1999) supposed that SDB rates also varies according to other aspects of its relationship with the partner in close proximity.

We divided dyadic affiliation relationship into three levels: strongly affiliation relationship, moderately affiliation relationship and weakly affiliation relationship, and found that focal subjects engaged in more SDB rates in proximity of dominants with strongly affiliation relationship than weakly affiliation relationship, which is consistent with the speculation of Castles et al (1999): the comparison of SDB rate across proximity partners could, therefore, provide insight into relationship security. In secure relationship, whether or not the tolerance, low aggressive rates, gaining support would be common remained to be verified in further study.

In the study of genus *Macaca* (*M. fascicularis*), Aureli (1997) found that the victim showed higher SDB rates aggressed by more valuable group members. A similar result was received in female Tibetan macaques that the victim showed more SDB rates attacked by strongly affiliation relationship partners than weakly affiliation relationship partners. Aggressed by strongly affiliation relationship partners with reconciliation, the victim showed higher SDB rates before reconciliation than after reconciliation, while the SDB rates did not show difference before reconciliation and after reconciliation aggressed by moderately affiliation relationship partners. It may be because that the Tibetan macaques are highly gregarious dominated by males, the status of females mainly depended on the supports of male and females alliance particularly (Li, 1999) and female alliance mainly depended on partners with strongly affiliation relationship. If the bond between strongly affiliation relationship partners was break, it would be greatly harmful to females' status and value in the group. Focal subjects were anxious to repair the relationship after conflicts, while, the break of strongly affiliation relationship had worst influence, thus the higher SDB rates performed after conflicts between partners with strongly affiliation relationship is expected.

In conclusion, SDB had a positive correlation with anxiety in Tibetan macaques, and the influence of dyadic affiliation relationship on SDB was reflected in our study.

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Male Tibetan macaques' (*Macaca thibetana*) choice of infant bridging partners

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Abstract: Adult male Tibetan (*Macaca thibetana*), Barbary (*M. sylvanus*), and stump-tailed macaques (*M. arctoides*) engage in bridging, a ritualized infant-handling behavior. Previous researchers found a bias toward the use of male infants for this behavior, but its function is debated. Explanations include three hypotheses: paternal care, mating effort, and agonistic buffering. We studied a group of habituated, provisioned Tibetan macaques to test whether adult males' affiliative relationships with females predicted their use of an infant for bridging. We also examined biases for sex, age, and individual in males' choice of bridging infant. We collected data via all occurrences, focal animal, and scan methods, from August to September 2011 at the Valley of the Wild Monkeys, China. We found that male infants were significantly preferred over females for bridging, but of three male infants in the group, only one was used by all males, while one male infant was used less often than expected. Adult males had females they were significantly more likely to be proximate to and/or to groom, but these corresponded to the mother of the bridging infant for only one male. Our results are most consistent with the agonistic buffering hypothesis: lower-ranked males used the alpha male's preferred bridging infant in an attempt to regulate their interactions with the alpha.

Keywords: Agonistic buffering; Affiliated infant; Paternal care

Tibetan macaques (*Macaca thibetana*) live in stable, multi-male/multi-female groups and are distributed across east and central China (Ogawa, 1995a; Zhao, 1996). The species is female-philopatric and forms linear dominance hierarchies (Thierry & Aureli, 2006). Adult male-infant interactions are generally rare in multi-male/multi-female social groups such as those found in macaques and baboons (Estrada & Sandoval, 1984; Kurland & Gaulin, 1984; Packer, 1980; Ransom & Ransom, 1971; Smith & Whitten, 1988; Smuts, 1985); however, Tibetan, Barbary (*M. sylvanus*), and stump-tailed (*M. arctoides*) macaques exhibit a type of triadic affiliation called bridging (Deag & Crook, 1971; Estrada & Sandoval, 1984; Ogawa, 1995a). Bridging is defined as two individuals simultaneously lifting an infant accompanied by affiliative behaviors such as teeth chattering (Berman et al, 2004; Ogawa, 1995a). While adult females and juveniles participate in bridging, primatologists have focused on adult males' use of infants (Deag, 1980; Ogawa, 1995a).

Three hypotheses have been proposed to explain bridging and other male-infant interactions: 1) paternal investment/enforced babysitting (Kümmerli & Martin,

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2008; Taub, 1980); 2) mating effort (Smuts, 1985; Taub, 1980; Thierry & Aureli, 2006); and 3) agonistic buffering (Deag & Crook, 1971; Ogawa, 1995a; Paul et al, 1996; Kümmerli & Martin, 2008; Zhao, 1996). The paternal investment and enforced babysitting hypotheses make similar predictions: that males preferentially interact in ways that enhance survival of infants related to them (Paul et al, 1996). The enforced babysitting hypothesis proposes that matrilineally-related males use a related infant to bridge and form a caretaking relationship with the infant (Ogawa, 1995a). Neither of these hypotheses have strong support from field observations. Paul et al (1996) found that while males did prefer specific infants for triadic interactions, these were not infants who were related to them. Ogawa (1995a) found that males did not prefer to use infants of their own matriline, and newly immigrated males who lacked biological relatives in the group still bridged.

The mating effort hypothesis suggests that males use interactions with infants to influence female mate choice (Ménard et al, 1992; Paul et al, 1996). In olive baboons (*Papio anubis*), Smuts (1985) found affiliations between particular males and females, which in turn mediated males' interactions with infants. Similar adult male-female relationships have been observed in macaques, but Paul et al (1996) found no support for the mating effort hypothesis in their study of Barbary macaques (*M. sylvanus*).

Deag & Crook (1971) proposed that adult male primates use infants in order to regulate relationships with other males and called such interactions agonistic buffering. This hypothesis predicts that subordinate males use infants to reduce or avoid aggression from more dominant males (Deag, 1980; Thierry & Aureli, 2006). There is support for this hypothesis in Tibetan (Ogawa, 1995a) and Barbary (Paul et al, 1996) macaques. Subordinate male macaques in both species were more likely to approach a dominant male than the reverse and often accompanied the approach with affiliative behaviors; in Tibetan macaques, bridging is followed by proximity and grooming between the adult male bridging partners. Male Barbary and Tibetan macaques prefer the same infant (the male's affiliated or primary infant) for bridging and for male-infant dyadic interactions. The affiliated infant hypothesis predicts that a male is more likely to accept a bridge from a male carrying his affiliated infant. Affiliated infant choice may be based on kinship, infant sex, social rank of the adult male, infant's birth order, and consortships or friendships with the infant's mother (Deag, 1980; Ogawa, 1995a, b; Taub, 1980; Zhao, 1996). Some baboon species (*Theropithecus gelada*: Dunbar, 1984; *P. cynocephalus*: Smith & Whitten, 1988; *P. anubis*: Smuts, 1985) display triadic male-infant interactions in which a male may look for support from, and develop a social relationship with, the infant's mother. In *P. anubis*, males are more likely to carry the infants of their female friends (Smuts, 1985; Stein, 1984). Deag (1980) proposed that male Barbary macaques choose infants for triadic interactions based on the existence of a friendship with the infant's mother, and Ogawa (1995a, b) and Zhao (1996) both noted that adult male Tibetan macaques showed a preference for the infants of consort partners. Alternatively, Paul et al (1996) found that in Barbary macaques adult male-female friendships did not typically extend to the females' infants, and for Tibetan macaques, the most consistent observation with respect to bridging is that adult males prefer to bridge with male offspring <1 year old (Ogawa, 1995a, b, c; Wang et al, 2008).

We hypothesized that adult male Tibetan macaques would bridge using particular infants. To test the mating effort hypothesis for bridging, we predicted that males would bridge more often using the infants of their preferred adult female grooming and proximity partners.

MATERIALS AND METHODS

Study Site and Species

We conducted this study from 04 August to 28 September 2011 at Mt. Huangshan, Anhui Province, China. Mt. Huangshan is a popular tourist area and UNESCO World Heritage site. The study site is south of the main park and is called the Valley of the Wild Monkeys. The research site is described in further detail by Berman & Li (2002).

The study group, Yulingkeng A1 (YA1), has been observed and monitored by researchers since 1986, resulting in known individual identities and maternal lineages (Berman et al, 2004). During the study period the group consisted of 27 individuals (Table 1), including 4 adult males, 8 adult females, 11 juveniles (1−4 year), and 4 infants. Tibetan macaques mate from July to December, with most births occurring between January and April (Li et al, 2005). Our data collection occurred during part of the mating season, and this likely impacted on adult male-female affiliation.

Observation platforms were built in 1994 (Berman et al, 2007) for tourists and researchers to easily view the monkeys. We collected data from these platforms. Park staff provisioned the monkeys with corn approximately 4 times per day. Data were collected between 0830h to 1700h during provisioning and non-provisioning times.

Data Collection

We collected bridging data and data on adult male-female grooming and proximity. All researchers achieved an inter-observer reliability of >90% on location, individual identification, and target behaviors. We used sections of ethograms published in Ogawa (1995a: bridging and sequence of bridging initiation) and Berman et al (2004: affiliative and aggressive interactions) to collect bridging and other behaviors.

Zhu et al (2013) conducted a study of the dominance hierarchy for this population that overlapped with our study period, so we used his rank results and a separate hierarchy for each sex (Table 1).

Table 1 YA1 Group composition (Jiang Ting and Zhu Lei, personal communication)

Age/Sex class[a]	Name	ID (Rank[b])	Mother	Birth or immigration year
Adult ♂	TouGui	TG (1)	TouTai	b. 2003 (natal male)
	ZiLong	ZL (2)	??	i. 2006
	GaoShan[c]	GS (3)	??	b. 1984 (natal male)
	BaiTou	BT (4)	??	i. 2011
Adult ♀	YeMai	YM (6)	Ye[d]	1990
	TouTai	TT (3)	Tou[d]	1991
	YeZhen	YZ (2)	Ye[d]	1992
	TouHong	TH (7)	TouGou[d]	2003
	YeHong	YH (5)	YeMai	2003
	HuaHong	HH (8)	Hua[d]	2003
	TouRui	TR (1)	TouTai	2004
	HuaHui	HHU (4)	Hua[d]	2005
♂ 3 year	TouRongBing	TRB	TouTai	2008
	YeRongBing	YRB	YeZhen	2008
♀ 3 year	TouXiaXue	TXX	TouHong	2008
♀ 2 year[e]	YeChunYu	YCY	YeMai	2009
	TouRongYu	TRY	TouTai	2009
	TouHuaYu	THY	TouRui	2009
♂ 1 year	HuaXiaMing	HXM	HuaHong	2010
	TouRongGang	TRG	TouTai	2010
	YeRongQiang	YRQ	YeZhen	2010
	YeChungLong	YCL	YeMai	2010
♀ 1 year	YeXiaXue	YXX	YeHong	2010
♂ Infant	Dumbo	DM	HuaHong	04 May 2011
	Scar Face	SF	TouRui	11 June 2011
	Wee-Wee	WE	HuaHui	23 June 2011
♀ Infant	Sissy	SS	TouHong	21 Feb 2011

[a]: In 2006 and 2007, only two male offspring survived to adulthood. Both had emigrated by the time of our study in 2011; [b]: See Zhu et al, 2013 for calculation of YA1 dominance rankings in 2011; [c]: GS was likely born in YA1 (Ogawa, 2006) but has emigrated several times. He returned to YA1 in 2005 and remained there continuously through the time of our study in 2011; [d]: Denotes dead individuals; [e]: In 2009 only females were born.

We conducted 5 minute focal samples, with instantaneous recording at 30 second intervals, on all adult males in a predetermined, random order (Martin & Bateson, 2007). During focal samples, we recorded grooming bouts between adult males and females. Following each focal sample, we conducted location scans, during which we recorded the identity and location of all adults and infants. To facilitate data collection on the

location of individuals, Matheson et al (2006) divided the provisioning area into approximately equal zones based on natural breaks, and we used these designations to indicate monkey location. We considered adult males and females to be proximate if they were touching or within arm's length as recorded during both focal samples and location scans (Sheeran et al, 2010). During location scans, we also noted whether or not mothers were carrying their infants.

We recorded data on adult male bridging behaviors through all occurrences sampling (Martin & Bateson, 2007). When we noted bridging or suspected that it would occur (e.g., an adult male approached or picked up an infant), we suspended focal samples and location scans, and the bridging individuals became the focus of our attention until the bridging behavior ended. We recorded the duration, sequence of initiation, and participants for each bridge. Following Ogawa (1995b), we defined the bridge initiator as the male who held the infant as he approached another male. We determined the observed frequencies of bridges for each male by counting the times a male successfully initiated a bridge with another male. We also recorded failed bridges, defined as cases when one male failed to accept the infant from a male initiator (Ogawa, 1995b).

Data analysis

We used *chi*-square tests to determine if each adult male was proximate to and/or groomed an adult female more often than expected, with the proximity frequency equal to the number of instantaneous records collected during scan samples. We converted the number of instantaneous records of adult male-female grooming into a rate for each focal sample, and then averaged the rates for each dyad. We used *chi*-square tests to determine whether adult males: 1) preferred to use particular infant (s) to initiate bridges; 2) had an affiliated infant and, if so; 3) preferentially received affiliated infants during bridging. We report standardized residuals $\geq |2|$ as indicative of individuals used significantly more or less often than expected. All analyses were conducted in Vassar Stats Website for Statistical Computations (©Richard Lowry 1998−2013), with alpha set to 0.05.

RESULTS

Dominance hierarchy and bridging behavior

During the study period, the bridges an adult male initiated with an infant versus the bridges he received followed his rank: alpha male TG had the lowest ratio (5 initiated:29 received=0.17), followed by beta ZL (12:17=0.82), gamma GS (11:5=2.20), and recent immigrant BT (29:6=4.80).

Bridging partner preference: Infants versus juveniles

For both successful and failed bridges males used infants (n=80) more often than they used juveniles (n=5). The majority of bridges with infants (57/80 or 71%) and with juveniles (4/5 or 80%) were accepted.

Infant preference: males versus females

One female and 3 male infants (<1 year) were born in in the group prior to our study. In chi square analyses, we weighted expected values based on the 2011 infant sex ratio. Male offspring were used in 75/80 successful and failed bridges compared to 5/80 for the female infant (Table 2). The female infant was used less than expected for all adult males' successful (n=57, χ^2=7.16, df=1, P=0.0075, standardized residual [SR] ♀=−2.45) and failed (n=23, χ^2=6.39, df=1, P=0.0115, SR ♀=−2.40) bridges. Sufficient data existed to test for significant differences in BT's successful (n=29) and total (n=44) bridges. BT showed no significant preference for infants by sex (df=1; successful bridges: χ^2=0.56, P=0.4543; total bridges: χ^2=3.67, P=0.0554). In 2011, there was 1 female and 4 male juveniles (b. 2010) in the study group. Two male juveniles were exclusively used in the 5 juvenile bridges we observed.

Thus, for successful and failed bridges, 3 adult males used male infants more often than expected by chance. Juveniles were used infrequently for bridging, but all juvenile bridges were with males.

Infant preference: individual

Males' preferences for infant bridging partners were not evenly distributed across the 4 infants for successful (n=57) or failed (n=23) bridges (df=3; successful bridges: χ^2=49.74, P<0.0001, SR ♂DM=+5.76, SR ♂ WE=−3.25, SR ♀SS=−2.45; failed bridges: χ^2=13.35, P=0.0039, SR ♀SS=−2.40). Out of BT's successful (n=29) and total (n=44) bridges, he used ♂WE less than expected in his successful bridges (χ^2=11.14, df=3, P=0.011, SR ♂WE=−2.32). He selected ♂SF more often and ♂WE less often than expected in his total bridges (χ^2=23.09, df=3, P<0.0001, SR ♂SF=+3.32, SR ♂WE=−2.71).

We further examined male use of infants by analyzing cases when >1 infant was proximate to a male before

Table 2 Distribution of infants used in bridges by each adult male; male ranks in parentheses

		Infant			Juveniles[a]		
		♂DM	♂SF	♂WE	♀SS	♂Yerongang	♂Tourongang
BT (4)	Successful	11	12	1	5	0	0
	Failed	4	10	1	0	0	0
GS (3)	Successful	10	0	1	0	0	0
	Failed	4	0	1	0	0	0
ZL (2)	Successful	10	2	0	0	0	2
	Failed	2	0	1	0	0	0
TG (1)	Successful	5	0	0	0	1	1
	Failed	0	0	0	0	1	0
All males	Successful	36	14	2	5	1	3
	Failed	10	10	3	0	1	0
	Total	46	24	5	5	2	3

[a]: No bridges were observed using other juvenile males or the female born in 2010.

he initiated a bridge. Such situations were rare and never involved all 4 infants, but there were limited occurrences of >1 infant being proximate to a male when the male picked up an infant for bridging. ♂DM was chosen over ♂SF 7 times, and over ♂WE 4 times. ♂SF was chosen over ♂DM 5 times and over ♂WE 4 times. ♂WE was chosen over ♂SF once. These data indicate that adult males tended to prefer 2 male infants (DM and SF) over ♂WE when choices of >1 infant were available.

An infant's availability for bridging might be related to its age and/or how often it was carried by its mother. We examined how often mothers carried each infant using data from location scans during which the infant in question was visible. All of the infants spent the majority of their time off of their mothers' bodies, though there was variability in the percentage of time each was being carried, which was related to infant age: oldest infant ♀SS was carried in 6.5% (n=23/356 scans) of location scans compared to 17.5% (n=114/651) for ♂DM, 37.8% (n=141/373) for ♂SF, and 45.0% (n=163/362) for the youngest infant ♂WE. ♂DM was the infant most often used in bridges and he was off of his mother's body about 80% of the time. ♀SS was the infant most often off of her mother (93% of the time), but she was the infant least frequently used for bridging.

The affiliated infant hypothesis stipulates that adult males will receive bridges more often from an initiator carrying the recipient's affiliated infant. In our dataset, the alpha and beta males did not have an infant with whom they interacted dyadically, but gamma male GS and lowest-ranked male BT did (♂DM for GS, χ^2=27.09, df=3, P<0.0001; ♂SF for BT, χ^2=15.06, df=3, P<0.01). GS was offered ♂DM in 6 bridges, and ♂SF and ♂WE were each offered to him once. GS accepted bridges only with ♂DM (n=5), his affiliated infant. BT received all bridges offered to him (n=6): 4 using ♂DM and 2 using ♂SF, his affiliated infant.

Adult Female Proximity and Grooming Partners

We observed 305 scans in which adults were proximate to one another (Table 3). Three of the 4 adult males (TG, GS, BT) were significantly proximate to, or out of proximity with, 1 or more adult females. BT was the only male who was proximate to the mother of 1 of the 2 infants he used for bridging. TG was proximate to the mother of his bridging infant less often than expected. We recorded a total of 220 focal samples from adult males (TG 53, ZL 59, GS 56, BT 52), and these were evenly distributed among males (χ^2=0.55, df=3, P=0.9078). To avoid pseudo-replication in our grooming analysis, we calculated the rate of grooming for each focal sample and then averaged the rates of grooming for each dyad (Table 4). For each of the 4 males, associations with female grooming partners were not evenly distributed (Table 4). TG and ZL groomed the mother of the males' preferred bridging infant *less* often than expected, while GS and BT groomed with her *more* often.

TG and BT each associated with the same female for both proximity and grooming. GS had different partners for proximity and grooming. ZL had no female proximity partner preference, but he groomed 3 females significantly more often than other females. In a comparison

Table 3 Instantaneous records of male-female proximity per dyad; infants in brackets; significant (≥|2|) standardized residuals in parentheses

	Females							
Males	TH [♀SS]	YZ	TT	YM	TR [♂SF]	YH	HHU [♂WE]	HH [♂DM]
TG[a]	7	13	5 (−2.27)	35 (+5.98)	9	24 (+2.95)	9	4 (−2.54)
ZL[b]	5	9	5	8	4	4	5	1
GS[c]	13	0 (−3.14)	5	24 (+4.49)	8	13	5	11
BT[d]	14	1 (−2.83)	5	11	5	4	0 (−3.14)	39 (+9.26)

[a]:χ^2=61.70, *df*=7, *P*<0.0001; [b]:χ^2=8.37, *df*=7, *P*=0.2011; [c]:χ^2=37.35, *df*=7, *P*<0.0001; [d]:χ^2=113.91, *df*=7, *P*<0.0001.

Table 4 Average rates of male-female grooming per dyad per focal sample; infants in brackets; significant (⩾|2|) standardized residuals in parentheses

	Females							
Males	TH [♀SS]	YZ	TT	YM	TR [♂SF]	YH	HHU [♂WE]	HH [♂DM]
TG[a]	0 (−6.81)	0.6 (+2.00)	0 (−6.81)	0.8 (+4.64)	1.0 (+7.87)	0.6 (+2.00)	0.6 (+2.00)	0.1 (−4.90)
ZL[b]	0 (−6.20)	0.9 (+8.17)	0.7 (+5.59)	0.5	1.0 (+9.95)	0 (−6.12)	0 (−6.12)	0 (−6.12)
GS[c]	0.1 (−3.32)	0 (−5.23)	0 (−5.23)	0 (−5.23)	0.5 (+3.56)	0.2 (−2.37)	0.6 (+6.43)	0.9 (+11.39)
BT[d]	0 (−3.02)	0 (−3.02)	0 (−3.02)	0 (−3.02)	0 (−3.02)	0 (−3.02)	0 (−3.02)	0.7 (+21.14)

[a]:χ^2=212.35, *df*=7, *P*<0.0001; [b]:χ^2=351.63, *df*=7, *P*<0.0001; [c]:χ^2=282.59, *df*=7, *P*<0.0001; [d]:χ^2=511, *df*=7, *P*<0.0001.

of infant and female affiliation partners, 2 males demonstrated a relationship. One of GS's grooming partners was the mother of his bridging infant. BT was proximate to and frequently groomed the mother of 1 of his bridging infants.

Bridges involving juveniles can be used in a preliminary fashion to explore whether adult males used the juvenile offspring of their female affiliates. The 5 bridges involving juveniles were initiated by alpha TG and beta ZL. They used 2 of the 5 1-year-olds, the male offspring of TT and YZ. TG and ZL both groomed YZ more often than expected, and ZL groomed TT more often than expected.

These results in aggregate suggest that YA1 male Tibetan macaques have particular associations with females for grooming and proximity, but these preferences may be unrelated or coincidental to the identity of the infants used by males for bridging, at least for long term group residents (TG, ZL, GS). Males' competition for reproductive females likely also influenced their affiliation with females.

DISCUSSION

Dominance hierarchy and bridging behavior during the study period

Zhao (*M. thibetana*, 1996) and Ménard et al (*M. sylvanus*, 2001) found that lower-ranked males received fewer bridges but initiated more compared to higher-ranked males. This was true of our data, too: alpha male TG initiated the fewest bridges and received the most (0.17), followed by beta ZL (0.82), gamma GS (2.20), and finally BT (4.80). BT joined the study group approximately 6 months before the study began. He initiated more than half (44/80) of all failed and successful bridges we observed involving infants. His comparatively high bridging rate may have influenced his choice of infants, causing him to be less selective of the infants used than was true of adult males with lower rates of initiating bridges.

Bridging partner preference: infants over juveniles

In their study of YA1 Tibetan macaques, Wang et al (2008) noted that males used infants rather than juveniles for bridging (see also Zhao, 1996). We found a similar result: in successful and failed bridges, adult males used infants 80 times, compared to 5 bridges using juveniles. The bridges using juveniles were rare compared to those using infants, but the majority of bridges using either type of immature were successful. Only the alpha and beta males initiated bridges using juveniles, and alpha male TG's only failed bridge was initiated with a juvenile.

Infant preference: males versus females

Wang et al (2008) found that YA1 Tibetan macaque

males preferred to use male infants in bridging. Zhao (1996) reported that in a Tibetan macaque group with 2 male and 2 female infants, males were used in 78 bridges compared to only 26 for females. Ogawa (1995a) showed that YA1 adult male Tibetan macaques held male infants more often than they held female infants, and that male infants were used in bridging 0.43 times/hour compared to 0.04 times/hour for female infants. We found a similar sex-preference pattern for adult males in aggregate, with male infants preferentially used in both successful and failed bridges. The top 3-ranked adult males never used the female infant when initiating their bridges. Adult male BT, however, did not significantly prefer male infants over the female infant and was the only male to use her in his bridges, perhaps because of his high overall bridging rate. BT's bridges with the female infant were successfully completed, indicating that other adult males would receive her as the bridging medium even though they did not use her themselves in bridges that they initiated.

There were 5 young juveniles (b. 2010) in the group (4 male, 1 female). All 5 bridges we observed using juveniles occurred with 2 of the male juveniles. Since there were more male juveniles present, it is not surprising that they were more often used in bridging; however, our data are consistent with previous researchers' observations that males are used for bridging more often than are females.

Infant Preference: Individual

Previous studies found two preferences in adult males' choice of bridging partners: infants chosen over juveniles, and males chosen over females. However, past researchers did not explore whether a bias existed for a particular male infant (see Zhao, 1996). Uniquely compared to other bridging studies in this species, we individually identified infants in the 80 bridges observed. We found that, while male infants were used more often in bridging than was the female infant in our study group, one of the three male infants was used less often than expected, while one male infant was used more often. In aggregate, all of the adult males used lowest-ranked female HH's infant (♂DM) more often than expected in their bridges. In addition to often using ♂DM in his bridges, low-ranked BT also used ♂SF more often than expected. The general preference for ♂DM persisted in those relatively few cases in which males appeared to have a choice of infants before the bridge was initiated. ♂DM, and to a lesser extent, ♂SF were both preferred over ♂WE when > 1 infant was in close proximity to the male before bridging was initiated. Thus, particular male infants were preferred for bridging, rather than males choosing infants based on proximity. We considered infant availability as another potential factor in a male's choice of bridging partner. ♂WE, the infant least often used for bridging, was also being carried by his mother during 45% of location scans, perhaps making him unlikely to be used in adventitious bridges because he would have to be taken from his mother for that purpose. ♀SS, however, was often available (she was being carried during 6.5% of her location scans), but she was used for bridging less often than expected, while ♂SF was infrequently available (he was being carried during 37.8% of his location scans) but was used for bridging by BT more often than expected. Zhao (1996) found that male Tibetan macaques at Mt. Emei would use for bridging an infant being carried by its mother. Therefore, whether the infant is spending a lot of time off of her or his mother does not seem to influence adult male choice of bridging partner in either population.

Previous researchers studying Tibetan (Ogawa, 1995a, b) and Barbary (Taub, 1980) macaques hypothesized that a male's choice of bridging infant may match his preferred infant for other male-infant dyadic interactions (the male's affiliated or primary infant). They predicted that adult males were more likely to accept a bridge from a male carrying his affiliated infant. In our data set, 2 adult males had affiliated male infants: ♂DM for gamma GS, and ♂SF for lowest-ranked BT. GS only accepted bridges using his affiliated infant (N = 5). BT received his affiliated infant 2 times and another infant 4 times. Our data are not, therefore, conclusive with respect to the affiliated infant hypothesis, although it may be that recent immigrants and/or low-ranked individuals such as BT receive any bridges offered to them, regardless of infant being used.

In summary, with respect to males' preferences for particular infants, our data indicate that there was a strong preference for 1 male infant, even though 2 other male infants were in the population. The preferred infant was the son of the lowest-ranked adult female. This preference cannot be explained by the infant's availability in terms of being off of his mother's body or in terms of his affiliation with a particular adult male. Barbary macaques at Gibraltar appeared anecdotally to prefer the youngest male infant in the group and would

approach the infant's mother and take the infant for this purpose (Bauer personal observation). In our study population, the preferred infant was the second born in the 2011 birth cohort, so males were not choosing the youngest male infant in the group. This infant was exclusively used by alpha TG in the 5 bridges he initiated. Other males may have followed suit using his favored infant, even if he was not TG's affiliated infant.

Adult female proximity and grooming partners

Ogawa (1995a, b) reported that YA1 Tibetan macaque males preferred as bridging partners the infants of their female consort partners, which suggested that short-term, adult, male-female relationships might influence infant and juvenile bridging partners. We hypothesized that adult males have regular, preferred female partners for proximity and grooming, and that those patterns might predispose males to use those females' infants in bridging.

In our study population, 3 of the 4 males were proximate to a specific adult female more often than expected. Alpha male TG was proximate to females YM and YH more often than expected. Gamma male GS shared with TG a proximity preference for YM. The lowest-ranked male in the group, BT, was more often in proximity with the lowest ranked female, HH. HH's infant ♂DM was significantly preferred by males for bridging, but only BT was frequently in proximity with her, and he did not exclusively use her infant for his bridges (Table 2). Thus, male-female proximity does not explain the pattern we observed in males' choices of infant or juvenile bridging partners and may be more reflective of mating competition. TG and GS were more often proximate to females who did not have dependent offspring during the study period and were likely fertile.

We predicted that a male's grooming partner would be the mother of the infant he used for bridging. All 4 males groomed at least 1 adult female more often than expected. In the case of low-ranked BT, he was proximate to and more often groomed the same female, HH, and she was the mother of 1 of his 2 bridging infants. However, he neither groomed nor remained proximate to the mother (TR) of his other bridging infant. Gamma male GS had 3 female grooming partners, all of whom were different from his proximity females. Both of these females had infants<1 year, but GS groomed more with the mother of the infant he used for bridging less often than expected. Only in our small juvenile bridge data set (n=5) did the expected pattern emerge: alpha TG and beta ZL both groomed with the mothers of the juveniles they used in bridging. In summary, our results indicate that adult males do not necessarily spend time grooming or affiliating with the mother of the infant most often used for bridging, and this is consistent with the findings of Paul et al (1996), who also failed to find support for the mating effort hypothesis in Barbary macaques.

CONCLUSIONS

Three hypotheses have been proposed to explain male-infant interactions: mating effort, paternal investment, and agonistic buffering. Our data showed that males are biased in the choice of individuals used for bridging: infants were used for bridging more than juveniles were, and males were used more than females. Only 1 male was strongly favored by all 4 males, but he was not the youngest member of his birth cohort. Adult males in our study did not necessarily have a grooming or proximity preference for this infant's mother; in fact, 2 males groomed with her significantly less than expected, and 1 male was proximate to her significantly less than expected. The mother of this infant was the lowest-ranked female in the group, so the males were not biased in favor of dominant females' offspring. In this population, it appears that having a strong male-female affiliation is not a necessary prerequisite for using the female's infant in bridges, at least for long-term male residents. Thus, our data do not support the mating effort hypothesis. We could not fully test the paternal investment hypothesis as we lack paternity data for this population, but the three males who could have fathered any of the infants were biased in favor of a particular infant. Our preliminary results are most consistent with an agonistic buffering framework, in which infant choice of bridging partner may aid in regulating male-male relationships through the use of the infant preferred by the alpha male, a pattern which pertained exclusively to the beta and gamma males.

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Effects of tsaoko (*Fructus tsaoko*) cultivating on tree diversity and canopy structure in the habitats of eastern hoolock gibbon (*Hoolock leuconedys*)

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Abstract: In this study, the quadrat method was used to study the effects of tsaoko (*Fructus tsaoko*) plantation on tree diversity and canopy structure of two natural habitats of eastern hoolock gibbon (*Hoolock leuconedys*): Nankang (characterized by extensive tsaoko plantation) and Banchang (relatively well reserved and without tsaoko plantation). Totally, 102 tree species from 25 families and 16 woody liana species from 10 families were recorded in Nankang, whereas 108 tree species from 30 families and 17 woody liana species from 12 families were recorded in Banchang. Although the tree species between two habitats is different, both habitats are characterized by enriched food resources for eastern hoolock gibbons, sharing similar dominant plant families. Due to tsaoko plantation, tree density proportion and diversity of forest layer Ⅰ (>20 m) in Nankang were both significantly decreased, but the tree density of layer Ⅱ (10−20 m) increased. Likewise, in conjunction with these behavioral observations, we also address potential impacts of tsaoko plantation on the behavior of eastern hoolock gibbon.

Keywords: Eastern hoolock gibbon (*Hoolock leuconedys*); Habitat; Tree diversity; Canopy structure; *Fructus tsaoko* plantation; Mt. Gaoligong

Habitats are essential to animal survival. They provide shelter for protection and food resources for sustenance and reproduction. Currently, deforestation and fragmentation are two of the primary threats to species conservation, which then often result in changes to plant composition and distribution (Saunders et al, 1991; Benítez-Malvido & Martínez-Ramos, 2003; Laurance et al, 2006), reducing food resources (Das et al, 2009; Boyle & Smith, 2010; Wang et al, 2000), and influencing animal behavior and species diversity (Feeraz et al, 2003).

Different species, such as arboreal primates, have developed various strategies to respond to environmental alterations. For example, the bearded saki monkey (*Chiropotes satanas chiropotes*) moves less and rests more (Boyle & Smith, 2010), while the western hoolock gibbon (*Hoolock hoolock*) spends more time resting and less time feeding, while increasing its alternative food consumption (Das, 2002; Das et al, 2009). The howler monkey (*Alouatta palliate*) has also been seen to broaden its diet choices (Cristóbal-Azkarate & Arroyo-Rodríguez, 2007). The black crested gibbon (*Nomascus concolor*) in Mt. Wuliang meanwhile avoids areas with high human disturbance, while also increasing feeding and movement and decreasing rest (Fan & Jiang, 2010). The François's

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languor (*Trachypithecus francoisi*), in small populations in Fusui, Guangxi Province, appear to choose mountain tops with relatively low human disturbance or deforestation to feed (Zhou et al, 2010).

Habitat degradation also affects the quality and quantity of sleeping sites, and subsequently impacts the animal's choice of sleeping sites (Wang et al, 2011). Francois's languors living in places with high disturbance and food scarcity, for example, use proximity to food instead of predator avoidance as the determining factor in choosing a sleeping site as compared to others living in better preserved habitats (Wang et al, 2011).

Habitat degradation has increased deforested areas which many monkeys are loath to cross (Das et al, 2009; Fan et al, 2011). Hoolock gibbons must walk on the ground or jump over big gaps in India's severely deforested Borajan area, thereby increasing their chance of dropping their young (Das et al, 2009). However, some reports claimed that habitat degradation benefited certain primate populations. Plumptre & Reynolds (1994) found that population densities of blue monkey (also names diademed monkey) (*Cercopithecus mitis*), red-tailed monkey (*C. ascanius*), and mantled guereza (*Colobus guereza*) in selectively logged Budongo forest reserves, Uganda, were even higher than the ones in well preserved habitats.

The eastern hoolock gibbon (*H. leuconedys*) is a primate from the Hylobatidae (gibbon) family, *Hoolock* genus. The species is found in extreme eastern corner of Assam, India, Myanmar east of the Chindwin River, and in southwest portion Yunnan in China (Das et al, 2006). Updated surveys of hoolock gibbon in China showed that they were only distributed in 17 forest patches around the towns of Baoshan, Tengchong and Yingjiang, with less than 200 individuals in total (Fan et al, 2011). Commercial logging, illegal hunting and farmland expansion are the main factors in its declining population in China (Fan et al, 2011). Due to the small population size and narrow distribution area, eastern hoolock gibbon has been listed as "EN" or endangered in IUCN and is a Grade I National Protected Species in China. Moreover, because eastern hoolock gibbons mainly inhabit Myanmar, little is actually known about this species due to a lack of observational studies in the area and the remoteness of some of these regions. Lan et al (1999) and Zhang et al (2011) analyzed their vocalizations. Bai et al (2007, 2008, 2011), Zhang et al (2008a, b) and Wu et al (2009, 2010) reported their habitat utilization, diet and food choice, time budget and daily food consumption based on short time research. Fan et al (2013) and Zhang et al (2014) studied the seasonal variation in diet and time budget, and the ranging pattern of an eastern hoolock gibbon population in Nankang based on a 14-month observation period.

The eastern hoolock gibbon is a typical diurnal and arboreal primate that lives together in monogamous pairs that stake out territory. Like other gibbons, they brachiate through the trees with their long arms, as it is the most efficient form of movement (Feeroz & Islam, 1992; Islam & Feeroz, 1992; Fan et al, 2013). Similarly, because of their unique moving pattern, eastern hoolock gibbons spend most of their lives in the crown canopy. They obtain approximately 49.1% of their diet from fruit and the rest from leaves and flowers (Fan et al, 2013). In Mt. Gaoligong, which has a high altitude and high latitude, one group's home range size was 93 hm^2 (Zhang et al, 2014).

Fructus tsaoko (*Amomum tsaoko*), also named tsaoko, is a perennial shade tolerance herb of Zingiberaceae family, *Amomum* genus, and grows in high altitude areas that are cool in summer and warm in winter, in enriched soil and under forest shading ranging from 50% to 60% (Dai et al, 2004; Qin et al, 2008). To reach the moisture, ventilation and shading requirements of tsaoko cultures, farmers remove trees, shrubs and weed (Dai et al, 2004). Since the 1980s, as an economically important cash crop, tsaoko has been planted in a large-scale area and has become the primary household income of local residents in the Mt. Gaoligong Reserve. As a result, the impact of tsaoko cultivating on habitats of eastern hoolock gibbons below 2300 m a.s.l. has become increasingly obvious. In Nankang and Houqiao, Mt. Gaoligong, the habitat degradation caused by tsaoko plantations was responsible for more than 50% of the decrease of eastern hoolock gibbon populations. For example, in one population in Nankang, 3 infants were born during the past 8 years, with the first 2 resulting in death (Fan et al, 2011).

In this study, to investigate the impact of tsaoko plantations on the eastern hoolock gibbon and offer a theoretical basis for species conservation, we analyzed the differences in tree species diversity and canopy structure of their two habitats, Nankang and Banchang (with and without tsaoko plantations, respectively). We further discussed the potential influence of tsaoko plantations on the behaviors of the eastern hoolock gibbon.

MATERIALS AND METHODS

Study site

We collected data at two areas of Mt. Gaoligong National Nature Reserve: Banchang (precinct of Baihualing management station, N25°12′, E98°46′), and Nankang (precinct of Nankang management station, N25°49′, E98°46′). Three eastern hoolock gibbon groups in Banchang and two groups (one family group and one solitary female) in Nankang were recorded. The vegetation type of Banchang and Nankang are both mid-montane evergreen broad-leaved forests, and the latter is characterized by large-scale tsaoko plantation.

Quadrats setting and data collection

In the habitats of eastern hoolock gibbons in Banchang and Nankang, quadrats (20 m×20 m) were set along the contour lines 100 m apart using systematic sampling methods and taking 100 m contour lines as transect lines. Due to topographic factors in Banchang, 50 quadrats were set along 6 lines with altitude ranging from 2,000 m to2,400 m, and length ranging from 200 m to 1,000 m. In Nankang, 33 quadrats were set along 4 lines with altitude ranging from 2,000 m to 2,300 m, with lengths ranging from 200 m to 1,100 m. According to the height of trees inside the quadrats, the forest was categorized as 3 sub-layers, layer Ⅰ (height>20 m), layer Ⅱ (10<height≤20 m) and layer Ⅲ (height≤10 m).

An altitude logger and GPS were used to record quadrat locations. Based on our field observations, eastern hoolock gibbons rarely use trees with diameter-at-breast-height (dbh) smaller than 10 cm, so we only measured trees with dbh≥10 cm, and these trees with dbh≥10 cm were identified at the species level. Circumference-at-breast-height (dbh=circumference-at-breast-height/3.14) was measured with ring ruler and height was determined by hand-hold laser altimeter. In Banchang, among the 1 286 labeled trees, 10 had a dbh of <10 cm, having with broken or fallen treetops or were dead due to natural reasons, and 6 were lacking data. So, data was collected for 1 270 trees. In Nankang, 1 010 trees were labeled. Meanwhile, name and abundance of edible woody liana species were recorded by the aid of keys provided by Wu et al (2009) and Fan et al (2013) in Nankang, and the behavioral data of eastern hoolock gibbons in Banchang was recorded from June, 2012 to June, 2013. Unidentified specimens were brought back to laboratory for further analysis.

Data processing

Basal coverage and abundance of edible woody liana (Sun et al, 2007)

$$\text{Basal coverage}=\sum_{i=1}^{n} Ai \quad (1)$$

n: number of trees in quadrat, *Ai*: cross sectional area at breast height of the *i*th labled tree in quadrat.

Average abundance of edible woody liana= abundance of edible woody liana/ the total number of quadrats.

α diversity, Jaccard index and importance value

α diversity, Jaccard index and importance value were calculated according to the protocols of Survey Plan for Plant Species Diversity of China's Mountains (PKU-PSD) (Fang et al, 2004).

Shannon-Wiener index: $H=-\sum_{i=1}^{s} Pi \ln Pi$

Pi=total area-at-breast-height of the *i*th tree species/total area-at-breast-height of all the tree species in one quadrant.

Pielou index: $E=H/\ln S$

S (abundance index): total tree species in quadrant.

Jaccard index (similarity index of species diversity): $cj=c/(a+b-c)$, in which *a* and *b*: number of tree species in habitat a and b, respectively; *c*: common tree species of habitat a and b, respectively.

Importance value of a family or species (*V*)=(relative abundance of a family or species + relative frequency of a family or species+relative dominance of area-at-breast-height of a family or species)/3.

Relative abundance (%)=100%×total number of one tree species in quadrat/total numbers of all tree species in quadrat.

Relative frequency (%)=100%×occurrence frequency of one tree species in quadrats/sum of occurrence frequency of all species;

Relative dominance of area-at-breast-height (%)= 100%×total area-at-breast-height of one tree species/ total area-at-breast-height of all tree species.

We used *t*-tests to assess the height, dbh, crown diameter, abundance and basal coverage of the trees in two habitats. 2×2 *Chi*-square test was used to evaluate the density differences of trees and edible woody liana between two habitats. Mann-Whitney test was used to determine the diversity among the forest layers. All statistics were completed using Excel and SPSS 16.0.

RESULTS

Characteristics and diversities of trees in habitats

The height (t=–4.438, df=2 278, P<0.001), dbh (t=–6.432, df=2 278, P<0.001) and basal coverage (45.36 m^2/hm^2, t=4.494, df=81, P<0.001) of trees in Banchang were significantly higher than those in Nankang. However, no difference was found in crown diameters (t=–0.447, df=2278, P=0.655) (Table 1).

Forest layer Ⅱ (10–20 m) was the most important component of habitat vegetation (45.0% and 59.8% in Banchang and Nankang, respectively), and the percentage of layer Ⅱ in Nankang was significantly higher than that in Banchang (Mann-Whitney Test: Z=–4.078, $n_{Banchang}$= 50, $n_{Nankang}$ =33, P<0.001) (Figure 1). Layer Ⅲ (<10 m) was the second major component (41.9% and 37.3% in Banchang and Nankang, respectively), and no difference was found between two habitats (Mann-Whitney Test: Z=–0.656, $n_{Banchang}$=50, $n_{Nankang}$=33, P=0.512) (Figure 1). Although layer Ⅰ (>20 m) was only a small part of habitat forest, its percentage in Banchang was significantly higher than in Nankang (Mann-Whitney Test: Z=−5.620, $n_{Banchang}$=50, $n_{Nankang}$=33, P<0.001) (13.1% and 2.9% in Banchang and Nankang, respectively) (Figure 1)

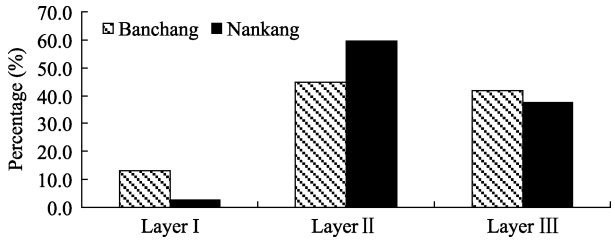

Figure 1 Percentage of forest layers in the habitats of eastern hoolock gibbon at two sites. Sites included Nankang (with tsaoko plantation) and Banchang (without tsaoko plantation)

There was no difference between tree density of forest layer Ⅲ between the two habitats (266 and 286 individual/hm^2 in Banchang and Nankang, respectively) (*Chi*-square test: χ^2=0.362, df=1, P=0.547). Tree density of layer Ⅰ in Banchang was significantly higher than in Nankang (84 and 22 individual/ha in Banchang and Nankang, respectively) (*Chi*-square test: χ^2=19.828, df=1, P<0.001), whereas, the tree density of layer Ⅱ in Nankang was significantly higher than in Banchang (285 and 457 individual/ha in Banchang and Nankang, respectively) (*Chi*-square test: χ^2=20.207, df=1, P<0.001). t-test showed that the average heights of both layer Ⅰ (25.1 and 22.5 m in Banchang and Nankang, respectively)(t-test: t=8.120, df=908, P<0.001) and layer Ⅱ (13.9 and 13.3 m in Banchang and Nankang, respectively)(t-test: t=–4.735, df=1171, P<0.001) in Banchang were significantly higher than in Nankang, whereas, the average height of layer Ⅲ in Banchang (7.3 m) was shorter than in Nankang (8.2 m, t-test: t=–2.969, df=195, P=0.003).

The Jaccard index of family, genus and species of trees in the two habitats was 0.719, 0.514 and 0.346, respectively. The abundance index of trees in Banchang (H=3.631) was slightly higher than in Nankang (H=3.550), but without significant difference (t-test: t=–0.104, df=208, P=0.914). Evenness index showed no significant difference between the two habitats either (E=0.775 and 0.768 in Banchang and Nankang, respectively) (t-test: t=–1.951, df=80, P=0.055). Among different forest layers, both the highest diversity index and evenness index were found in layer Ⅱ in Banchang, whereas, in Nankang, the highest diversity and highest evenness were found in layer Ⅲ and layer I, respectively (Table 2).

Table 1 Characteristics of tree composition in the habitats of eastern hoolock gibbons at two sites
Nankang (NK, with tsaoko plantation) and Banchang (BC, without tsaoko plantation)

	Maximum value		Minimum value		Mean		*SD*	
	BC	NK	BC	NK	BC	NK	BC	NK
Number of trees in quardat (idi.)	56	56	5	15	25	31	12	9
Height (m)	39.8	28.3	1.5	1.4	12.6	11.6	6.4	3.7
Crown diameter (m)	20.0	11.0	1.0	1.0	4.6	4.9	2.8	1.8
DBH (cm)	88.4	53.2	4.8	4.8	14.3	11.7	11.3	7.2

Table 2 Diversity and Evenness index of different forest layers in the habitats of eastern hoolock gibbons at two sites.
Nankang (NK, with tsaoko plantation) and Banchang (BC, without tsaoko plantation)

	Species number (*S*)		Diversity index (*H*)		Evenness index (*E*)	
	BC	NK	BC	NK	BC	NK
Layer Ⅰ	44	16	2.879	2.500	0.761	0.902
Layer Ⅱ	87	77	3.678	3.356	0.821	0.770
Layer Ⅲ	85	79	3.643	3.632	0.820	0.831

Although the lowest diversity of both habitats occurred in layer I, layer I in Banchang was still much more diversified than in Nankang (*t*-test, t=–4.210, df=58, P<0.001). In layer I of Banchang, 44 tree species were recorded, but in Nankang, only 16 were recorded (Table 2). The diversity of layer Ⅱ and layer Ⅲ between two habitats showed no significant difference (Table 2).

Difference in dominant species in two habitats

Tree species belonging to 23 families were found in both habitats, with Tiliaceae, Flacourtiaceae, Meliaceae, Celastraceae, Tetracentraceae, Oleaceae, and Caprifoliaceae in Banchang only, whereas, Daphniphyllaceae, and Rutaceae in Nankang only (Table 3). Except Rosaceae, Juglandaceae in Banchang and Moraceae, Aceraceae in Nankang, the rests of the top 10 dominant families (shadowed areas in Table 3) in the two habitats were the same. Among the identified tree species, 54 were distributed in both habitats, 55 were only found in Banchang and 48 were only found in Nankang. There were 32 and 33 species could provide food to eastern hoolock gibbons in Banchang and Nankang, respectively. Among the top 20 dominant species in the quadrats of the two habitats, 9 were common (shadowed areas in Table 4).

Difference in edible woody lianas in two habitats

A total of 13 families of edible woody lianas were found in the quadrats of both habitats. Among them, 12 families and 17 genus were distributed in Banchang and 10 families and 16 genus in Nankang. 11 species were found in both habitats (Table 5). Neither the density (202 and 192 individuals/ha in Banchang and Nankang, respectively) (*Chi*-square test: χ^2=0.103, df=1, P=0.748) nor average abundance (8.08 and 7.67 in Banchang and Nankang, respectively) (*t*-test: t=–0.112, df=32, P=0.904) of edible woody lianas between two habitats was of any significant difference.

DISCUSSION

Effect of tsaoko plantations on forest layer structure and diversity

This study was conducted within Mt. Gaoligong National Nature Reserve, and the vegetation types of both habitats are mid-montane evergreen broad-leaved forests. So, the diversity and evenness index of the tree species in quadrats were similar, and because of the closed flora of seed plants (Chen, 2008; Li et al, 2008), the similarity index of both families (0.719) and genus (0.514) in two habitats were high. However, due to the differences in local precipitation, thermal energy reallocation and human disturbances, the similarity index of tree species (0.346) in the two habitats was relatively low (Chen, 2008; Li et al, 2008).

Tsaoko cultivation had impacts on vegetation diversities (Dai et al, 2004; Guo et al, 2010), in specific, it caused degradation of fine vertical partitioning of forest strata and the decease or loss of tree and shrub species on

Table 3 Importance value of tree families in the habitats of eastern hoolock gibbons, Nankang (NK, with tsaoko plantation) and Banchang (BC, without tsaoko plantation)

Families	Importance values in BC (v1)	Importance values in NK (v2)
Fagaceae	17.56	23.27
Lauraceae	15.87	13.77
Magnoliaceae	11.59	15.07
Theaceae	10.93	8.9
Ericaceae	5.44	8.76
Rosaceae	4.51	1.58
Araliaceae	4.2	3.82
Elaeocarpaceae	3.81	7.82
Aquifoliaceae	3.26	2.69
Juglandaceae	2.62	0.11
Moraceae	2.48	3.47
Euphorbiaceae	2.26	0.98
Hamamelidaceae	1.98	0.11
Betulaceae	1.96	0.16
Proteaceae	1.55	1.31
Staphyleaceae	1.38	0.61
Symplocaceae	1.16	0.99
Myrsinaceae	1.02	1.13
Aceraceae	0.89	2.35
Tiliaceae	0.88	−
Rubiaceae	0.58	0.34
Nyssaceae	0.37	0.97
Flacourtiaceae	0.36	−
Meliaceae	0.36	0.08
Celastraceae	0.24	−
Styracaceae	0.22	0.14
Santalaceae	0.21	0.11
Oleaceae	0.11	−
Caprifoliaceae	0.11	−
Tetracentraceae	0.11	−
Rutaceae	−	0.8
Daphniphyllaceae	−	0.15

Families in shadow areas were top 10 dominant families in each habitat; −: absent in the quardats.

Table 4 Top 20 dominant tree species in the habitats of eastern hoolock gibbons, Nankang (NK, with tsaoko plantation) and Banchang (BC, without tsaoko plantation)

Species in BC	Importance values in BC (v1)	Species in NK	Importance values in NK (v1)
Phoebe yunnanensi	6.48	*Castanopsis hystrix*	11.33
Castanopsis hystrix	6.41	*Alcimandra cathcartii*	6.61
Michelia floribunda	6.40	*Elaeocarpus boreali-yunnanensis*	5.41
Lithocarpus fenestratus	5.24	*Eurya pseudocerasifera*	4.42
Laurocerasus undulata	4.04	*Rhododendron delavayi* var.	4.36
Schima wallichii	3.42	*Lithocarpus hancei*	4.12
Lithocarpus hypoglaucus	3.30	*Michelia doltsopa*	4.02
Eurya pseudocerasifera	2.92	*Schefflera minutistellata*	3.36
Vaccinium duclouxii var.*pubipes*	2.67	*Ficus neriifolia*	3.35
Elaeocarpus boreali-yunnanensis	2.63	*Manglietia insignis*	2.91
Manglietia insignis	2.34	*Neolitsea lunglingensis*	2.90
Exbucklandia populnea	2.27	*Lithocarpus fenestratus*	2.71
Ficus neriifolia	2.05	*Vaccinium duclouxii* var. *pubipes*	2.51
Lithocarpus variolosus	1.93	*Beilschmiedia yunnanensis*	2.41
Juglans cathayensis var. cathayensis	1.87	*Elaeocarpus duclouxii*	2.06
Camellia caudata	1.83	*Lyonia ovalifolia*	0.60
Schefflera hoi	1.76	*Cinnamomum pauciflorum*	1.75
Ilex delavayi	1.47	*Lithocarpus variolosus*	1.58
Elaeocarpus duclouxii	1.54	*Lindera foveolata*	1.39
Alnus nepalensis	1.38	*Acer pubipetiolatum*	1.35

Species in shadow areas were common top dominant species in two habitats.

middle level or ground level. As a result of this degradation, alongside reduced community stability and species diversity, the entire ecosystem had become quite fragile (Dai et al, 2004). In this investigation, the degraded forest vertical layer structures were primarily seen in Nankang. To open closed canopies, trees in layer I in the tsaoko plantation areas have been largely logged. Therefore, the density, proportion, average height, diversity and species abundance in layer I in Nankang were all lower than in Banchang. On the other hand, because of the massive clearance of trees in layer I, better illumination conditions provided for the growth and regeneration of trees in layer Ⅱ and layer Ⅲ. Therefore, in Nankang, the trees in layer Ⅱ were shorter than in Banchang, but their density was higher, whereas due to the increased shadow of layer Ⅱ, the regeneration of trees in layer Ⅲ in Nankang was hindered and showed no difference either in proportion or density with the trees in Banchang.

Implications of tsaoko plantation on the behaviors of eastern hoolock gibbons

Providing enough food to animals is the most basic and important function of habitats. Gibbons feed on leaves, flowers, and especially fruit (51%−89%) (Bartlett, 2007). Habitats degradation could result in the migration and loss of gibbon populations (Eudey, 1990; Marshall, 1990). Deforestation and fragmentation are the main threats to eastern hoolock gibbons' survival and reproduction. Fan et al (2013) reported that tree species, such as *Castanopsis hystrix*, *Lindera foveolata*, *Elaeocarpus duclouxii*, *Nyssa javanica*, *Ficus neriifolia*, *Eurya pseudocerasifera* and *Schefflera minutistellata*, and woody lianas, such as *Embelia floribunda*, *Rhaphidophora decursiva*, *Embelia procumbens* and *Cayratia japonica* were the main food species of eastern hoolock gibbons in Nankang. These species were distributed in both Banchang and Nankang. According to the importance values of tree species, some of the 9 common dominant species (the species with the top 20 importance values) in both habitats were also the important components of the diet of eastern hoolock gibbons (e.g. *Ficus neriifolia*).

Moreover, the average abundance of woody lianas in the two habitats was comparable (Table 5). Some reports claimed that herbivorous primates in disturbed habitats could fully explore the woody lianas resources to meet their food requirements (*Alouatta fusca*: Chiarello, 1994; *Trachypithecus francoisi*: Li et al, 2009).

Table 5 Species composition and average abundance of edible woody lianas in the habitats of eastern hoolock gibbons, Nankang (NK, with tsaoko plantation) and Banchang (BC, without tsaoko plantation)

Species	Families	Average abundance of edible woody lianas	
		BC	NK
Smilax mairei	Liliaceae	0.02	−
Smilax bockii	Liliaceae	0.04	0.09
Smilax lunglingensis	Liliaceae	−	0.27
Pterolobium punctatum	Leguminosae	0.44	−
Cyclea polypetala	Menispermaceae	0.06	0.42
Stephania japonica	Menispermaceae	0.02	0.15
Melodinus khasianus	Apocynaceae	0.08	0.51
Actinidia venosa	Actinidiaceae	0.10	−
Schisandra micrantha	Magnoliaceae	−	0.12
Schisandra propinqua	Magnoliaceae	−	0.06
Sargentodoxa cuneata	Lardizabalaceae	0.26	−
Cayratia japonica	Vitaceae	0.90	2.39
Tetrastigma sp.	Vitaceae	−	0.03
Tetrastigma planicaule	Vitaceae	1.2	−
Tetrastigma delavayi	Vitaceae	0.30	−
Rubus xanthoneurus	Rosaceae	0.12	0.39
Ficus sarmentosa	Moraceae	0.14	0.03
Rhaphidophora decursiva	Araceae	3.08	1.91
Toddalia asiatica	Rutaceae	0.02	0.09
Embelia procumbens	Myrsinaceae	0.74	1.00
Embelia floribunda	Myrsinaceae	0.50	0.18

−: Absent in quardats.

Based on those observations the Nankang habitat might still be capable of providing food to eastern hoolock gibbons and supporting populations despite the influence of tsaoko plantation.

Like other gibbons (Tenaza & Tilson, 1985; Reichard, 1998; Fan & Jiang, 2010; Phoonjampa et al, 2010; Fei et al, 2012), eastern hoolock gibbons prefer to sleep on large trees in forest layer Ⅰ to avoid predation (unpublished data). Due to tsaoko plantations, large trees in layer Ⅰ had been isolated because of the large clearance in Nankang. This could result in the decreased quantity of suitable trees for gibbons to sleep in, and increase their chance of being exposed to predators.

Moreover, like the other gibbons (Feeroz & Islam, 1992; Islam & Feeroz, 1992; Fan et al, 2009), most of the activities of eastern hoolock gibbons happen in canopies (unpublished data). But in Nankang, because of deforestation, the top canopies were patched and fragmented, which made it difficult and energy consuming for eastern hoolock gibbons to move in the top layer of trees. Meanwhile, the chance of infants falling increased significantly. During our field survey in Nankang, we had witnessed a 3-year-old gibbon fall from the canopy while moving; fortunately, it was not wounded. In Nankang, although layer Ⅰ has been destroyed, layer Ⅱ and layer Ⅲ were still relatively intact and the canopy was continuous, allowing it to support the movement and feeding behaviors of gibbons.

Habitats vegetation protection

Although Nankang Station has bolstered its management practices since 2007, there was still a lack of control of the commercial-driven tsaoko plantations. Indeed, the plantation area is expanding every year, increasing both the frequency and density of human disturbance. Based on this study, tsaoko plantations have had a significant impact on the forest structure of the Nankang habitat and therefore, a potential negative effect on the movement and sleep patterns of gibbons. Acco-

rdingly, to conserve both eastern hoolock gibbons and other arboreal species, we have suggested several new initiatives, including controlling tsaoko plantation expansion or even decreasing plantation area, and restricting cultivation activities.

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Positively selected genes of the Chinese tree shrew (*Tupaia belangeri chinensis*) locomotion system

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Abstract: While the recent release of the Chinese tree shrew (*Tupaia belangeri chinensis*) genome has made the tree shrew an increasingly viable experimental animal model for biomedical research, further study of the genome may facilitate new insights into the applicability of this model. For example, though the tree shrew has a rapid rate of speed and strong jumping ability, there are limited studies on its locomotion ability. In this study we used the available Chinese tree shrew genome information and compared the evolutionary pattern of 407 locomotion system related orthologs among five mammals (human, rhesus monkey, mouse, rat and dog) and the Chinese tree shrew. Our analyses identified 29 genes with significantly high ω (Ka/Ks ratio) values and 48 amino acid sites in 14 genes showed significant evidence of positive selection in the Chinese tree shrew. Some of these positively selected genes, e.g. *HOXA6* (homeobox A6) and *AVP* (arginine vasopressin), play important roles in muscle contraction or skeletal morphogenesis. These results provide important clues in understanding the genetic bases of locomotor adaptation in the Chinese tree shrew.

Keywords: Chinese tree shrew; Locomotion system; Positively selected genes

The Chinese tree shrew (*Tupaia belangeri chinensis*), currently placed in the order Scandentia, has attracted wide attention as a viable alternative animal model for biomedical research due to a variety of unique characteristics, e.g., small adult body size, high brain-to-body mass ratio, short reproductive cycle and life span, low cost of maintenance, and most importantly, a closer affinity to primates, particularly humans. These physical factors make the tree shrew an attractive alternative experimental animal to primates, while the closer affinity to humans make them preferable to rats or mice (Cao et al, 2003; Fuchs & Corbach-Söhle, 2010; Peng et al, 1991; Xu et al, 2013). The Chinese tree shrew is widely distributed in the tropical forests of South Asia, Southeast Asia and Southwest China (Peng et al, 1991). As an arboreal mammal living in the wild, tree shrew needs to cope with unfavorable circumstances (e.g., insufficient food and the attack of predators), and accordingly has developed an important adaption ability, that is, the ability of quick climbing and jumping movement (Fuchs & Corbach-Söhle, 2010; Peng et al, 1991). To adapt to the inevitably dangerous circumstances, the ancestor of tree shrew gradually developed typical arboreal adaptation patterns of the muscular system, including well-developed deltoideus and acromion of the scapula, existence of post-scapular fossa as in many arboreal mammals, the triceps brachii and the teres major with large bundles in favor of forelimb flexion, and strong and massive structure of the biceps femoris. These features may play a role in knee flexion during the locomotion on the branches of trees (Endo et al, 1999). Therefore, tree shrew has a significant advantage in locomotion ability of climbing and jumping, and in strong explosive power.

The evolution of these typically arboreal traits poses several interesting questions, the most pertinent being

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whether or not there any selective signals in the tree shrew's locomotion system. During every evolutionary process, a series of sequence changes occurs on multiple genes to induce phenotypic changes in morphology and physiology, allowing species to adapt to their novel environments (Hoekstra & Coyne, 2007). Because positive selection could leave an imprint on genes, the identification of positively selected genes via genome-wide scanning may help explain the genetic bases underlying adaptive evolution in mammals (e.g. Zeng et al, 2013). The currently available high-quality genome sequence of Chinese tree shrew (Fan et al, 2013) provides an opportunity to more fully characterize the potential molecular underpinnings of the tree shrew's locomotion ability, as well as give potentially significant clues to how the tree shrew evolved and differentiated itself from other related mammals, many of which are also used as animal models in biomedical research.

In this paper, we searched for locomotion system related genes against the Gene Ontology (GO) database (http://www.geneontology.org/) and then reconstructed gene sets of six mammalian species, including the Chinese tree shrew, human, rhesus monkey, mouse, rat and dog, to identify genes under positive selection along the Chinese tree shrew lineage. We also investigated rapidly evolving genes of locomotion system related GO category in Chinese tree shrew. Collectively, the results of these analyses provide some important clues to understanding the genetic bases of locomotion ability and adaptation of Chinese tree shrew.

METHODS

Source of gene dataset and identification of orthologs

A total of 848 human locomotion system related genes were obtained from the Gene Ontology database (GO:0007626 locomotory behavior, GO:0040017 positive regulation of locomotion, GO:0003012 muscle system process, GO:0001501 skeletal system development) for comparative study of the Chinese tree shrew's locomotive system. To determine orthologous relationships between Chinese tree shrew and human, we downloaded human protein sequence data from Ensembl (release 64; http://www.ensembl.org/index.html) for comparison. The longest transcript was chosen to represent each gene with alternative splicing variants. We aligned the orthologous human protein sequences of these 848 genes onto the Chinese tree shrew genomes using tblastn (Mcginnis & Madden, 2004), and then the best hit regions of each gene with a 5kb flanking sequence were cut down and re-aligned using GeneWise (Birney et al, 2004) in order to define the detailed exon-intron structure of each gene. The identified orthologous sequences that may have potential errors such as frame-shift and premature termination were further eliminated, and ortholog sequences from the other surveyed species were identified using a two-step method, as follows. First, using human genes as a reference, we obtained orthologous sequences of other species from Ensembl one2one orthology gene list, and downloaded the corresponding coding region nucleotide sequences and amino acid sequences according to IDs from Ensembl (release 64). Next, we identified the sequences of these genes not included in Ensembl one2one orthology gene list using computational gene prediction, as described in the Chinese tree shrew genome project (Fan et al, 2013).

Sequence alignment and filtering

All coding sequences (CDS) from the six surveyed species were aligned via MUSCLE 3.7 (Edgar, 2004) with the guidance of aligned protein sequences. To reduce the occurrence of false positive prediction, we carried out a series of filtering processes. First, we deleted all gaps and "N" from the alignments. Second, the aligned regions with more than 4 non-synonymous mutations in 7 continuous amino acids were filtered. Finally, the entire alignment was discarded if the remaining sequences after alignment were shorter than 100 bp.

Detection of positively selected genes

To detect potential candidate genes under positive selection along the Chinese tree shrew lineage, we applied CODEML from the PAML4 package, which is based on the Maximum-Likelihood method of molecular evolution (Yang, 2007), to the human, rhesus monkey, Chinese tree shrew, mouse, rat and dog ortholog gene sets. The same guide tree present in our previous study (Fan et al, 2013) was used to detect the positive selection genes using the following models: (a) branch model, which allows different ω values (ω is the ratio of non-synonymous to synonymous substitution rates, i.e., the Ka/Ks ratio) between the foreground branch (ω_2) and background branch (ω_1) while its corresponding null model assumes all branches have an identical ω_0 value; and (b) branch-site model with fixed foreground branch ω_2=1 and non-fixed foreground branch ω_2, which is used to test whether a gene has undergone positive selection on a foreground branch. Finally, likelihood ratio test (LRT) was performed on following model pairs: (a) Method 1, to test whether the foreground branches ω ratio were significantly different from that of background branches; and (b) Method 2, to test whether a proportion of sites in the sequence provided statistically significant support for ω>1 on foreground branches.

Phylogenetic analysis

Maximum Likelihood (ML) trees were reconstructed based on amino acid sequence using MEGA5.0

(Tamura et al, 2011). For the phylogenetic analysis, dog sequence obtained served as an outgroup to root the phylogenetic tree. Accuracy of phylogenetic tree were measured with 1 000 bootstrap replicates.

Analysis of rapidly evolving function categories

Based on GO annotation of human, we sought to detect rapidly evolving function categories of genes between Chinese tree shrews and humans, or between humans and rats. If a given GO category contained 20 or more genes from our ortholog dataset, it was selected for further analysis. The values of *K*a and *K*s and the *K*a/*K*s ratio (ω) were estimated for each gene using the KaKs_Calculator program (Zhang et al, 2006). The average values of *K*a and *K*s for all genes annotated to a given GO were calculated using the following equations (Cho et al, 2013).

$$k_a = \frac{\sum_{ieT} a_i}{\sum_{ieT} A_i}, \quad k_s = \frac{\sum_{ieT} s_i}{\sum_{ieT} S_i}$$

Where a_i is the number of non-synonymous substitutions and A_i is the number of non-synonymous sites in gene i; s_i is the number of synonymous substitutions and S_i is the number of synonymous sites in gene i; T is the number of genes annotated by GO. The expected proportion of non-synonymous substitutions to all substitutions P_A in a GO category was then estimated as previously described in Cho et al (2013):

$$P_A = \frac{k_a \sum_{ieC} A_i}{k_a \sum_{ieC} A_i + k_s \sum_{ieC} S_i}$$

Finally, for a given GO category, we used binomial distributions (Chimpanzee Sequencing and Analysis Consortium, 2005) to estimate the divergence of the proportion of non-synonymous substitutions and synonymous sites between the observed and expected values. The pbinom package in R (http://www.r-project.org/) was used to calculate the *P*-value. Rapidly evolving function categories were defined as $P<0.05$, with the parameter of lower.tail=FALSE.

RESULTS

Filtered ortholog genes

From the gene list of the GO database, we identified 604 locomotion system related orthologs between the genomes of the Chinese tree shrew, mouse, rat, dog, rhesus monkey and human. After alignment and filtering, 197 genes were eliminated (see Methods) leaving 407 genes for further positive selection tests.

Genes under positive selection

To detect the difference in selective pressure between Chinese tree shrew and other species, each aligned gene was evaluated in terms of its ratio of non-synonymous to synonymous substitution rates (ω value) using CODEML in the PAML package (Yang, 2007) under the guide tree previously described by Fan et al (Fan et al, 2013). Testing under the branch model (Method 1) showed 29 genes (7.1 % of the total number of genes) had a significantly higher ω value ($P<0.05$) in the Chinese tree shrew (Table 1) than in the other five surveyed species. Next, we used the branch-site test of positive selection (Method 2) to detect signals of positive selection on each alignment, identifying 48 amino acid sites in 14 genes (3.4 % of the total number of genes) that exhibited significant evidence of positive selection ($P<0.05$) in the Chinese tree shrew lineage (Table 2). While the final gene lists retrieved from these two methods were largely different, two genes, *HOXA6* (homeobox A6) and *AVP* (arginine vasopressin) that showed positive selection signals were identified by both of these methods (Figure 1).

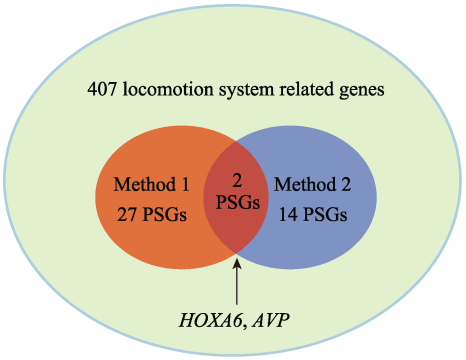

Figure 1 Summary of positively selected genes identified by two different detection methods

Method 1: likelihood ratio test under branch model; Method 2: likelihood ratio test under branch-site model (positive selection). PSGs: positively selected genes.

Phylogenetic analysis

To gain a clearer picture of these two potential locomotive related genes showing positive selection, two maximum likelihood (ML) trees were reconstructed based on the amino acid sequences of HOXA6 and AVP, respectively. Both gene trees showed a clustering pattern inconsistent with the recognized species tree (Fan et al, 2013; Lindblad-Toh et al, 2011; Murphy et al, 2004) and had a long branch for the Chinese tree shrew (Figure 2). Several other gene trees were constructed for the other positively selected genes, and only 9 trees showed a clustering pattern that was consistent with the species tree (data not shown). The observed discrepancy between the gene tree and species tree was in agreement with results of the above prediction of selection signal.

Table 1 29 positively selected genes detected by method 1 in Chinese tree shrew

Gene	GO category	*P*-value
ACTC1	GO:0030049 muscle filament sliding; GO:0060048 cardiac muscle contraction; GO:0030049 muscle filament sliding; GO:0003012 muscle system process	0.031449
ACTN2	GO:0030049 muscle filament sliding; GO: 0006936 muscle contraction	0.013104
ARRB2	GO:0007628 adult walking behavior	0.016337
AVP	GO:0007626 locomotory behavior	0.005769
AXIN2	GO:0003413 chondrocyte differentiation involved in endochondral bone morphogenesis	0.00714
BSX	GO:0007626 locomotory behavior	0.026649
CALM1	GO:0006936 muscle contraction; GO: 0010881 regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion; GO: 0055117 regulation of cardiac muscle contraction	5.26E-05
FGF1	GO:0030335 positive regulation of cell migration; GO:0040017 positive regulation of locomotion	0.001138
GJC1	GO:0006936 muscle contraction	0.037772
GNB2L1	GO:0030335 positive regulation of cell migration	0.005448
HOXA6	GO:0048704 embryonic skeletal morphogenesis	0.009921
HSBP1	GO:0006936 muscle contraction; GO:0003012 muscle system process	0.025508
KCNJ10	GO:0007628 adult walking behavior; GO:0007626 locomotory behavior	0.049375
LAMB1	GO:0030335 positive regulation of cell migration	0.039006
OPRD1	GO:0008344 adult locomotory behavior	2.92E-05
PDGFB	GO:0014911 positive regulation of smooth muscle cell migration; GO: 0030335 positive regulation of cell migration; GO: 2000591 positive regulation of metanephric mesenchymal cell migration	0.002046
PDGFRB	GO:0014911 positive regulation of smooth muscle cell migration; GO: 0030335 positive regulation of cell migration; GO: 0048705 skeletal morphogenesis	0.012328
PLN	GO:0003012 muscle system process; GO: 0010881 regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion	0.016657
PRKRA	GO:0048705 skeletal morphogenesis	0.047973
PRRX1	GO:0048701 embryonic cranial skeleton morphogenesis; GO: 0048705 skeletal morphogenesis	0.000916
RRAS2	GO:0030335 positive regulation of cell migration	0.008652
SATB2	GO:0048704 embryonic skeletal morphogenesis	0.043399
SRSF1	GO:0060048 cardiac muscle contraction	0.048949
TBX20	GO:0006936 muscle contraction	0.012294
TGFB3	GO:0048702 embryonic neurocranium morphogenesis; GO: 0060364 frontal suture morphogenesis	0.040708
TGFBR1	GO:0048701 embryonic cranial skeleton morphogenesis; GO: 0048705 skeletal morphogenesis	0.018154
TNNC2	GO:0003009 skeletal muscle contraction; GO: 0006937 regulation of muscle contraction; GO: 0030049 muscle filament sliding	0.017249
VIPR1	GO:0006936 muscle contraction	0.009035
WNT11	GO:0030335 positive regulation of cell migration	0.024982

Method 1 is likelihood ratio test under branch model.

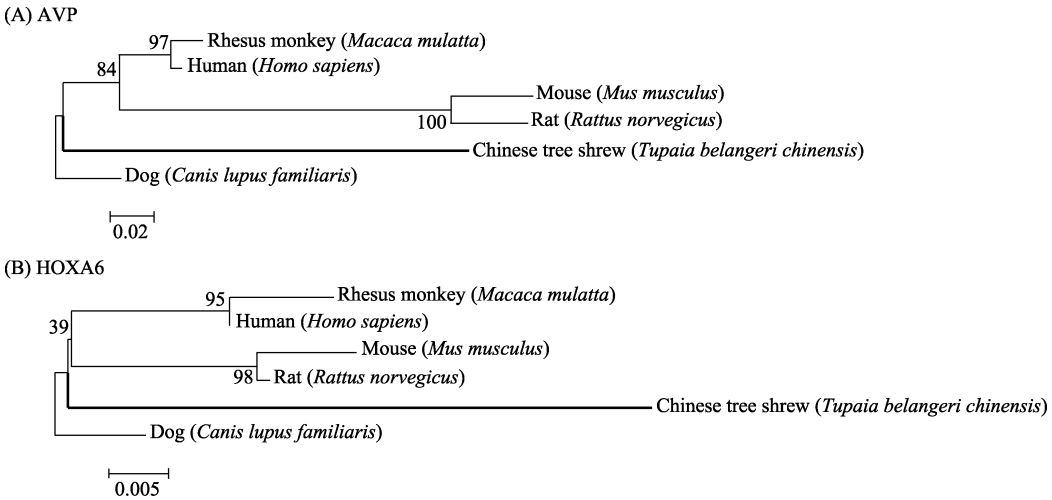

Figure 2 Maximum likelihood (ML) trees based on amino acid sequences of AVP (A) and HOXA6 (B)

Numbers on branches denote ML bootstrap values.

Table 2 14 Positively selected genes detected by method 2 in Chinese tree shrew

Gene	GO category	*P*-value
APLP2	GO:0007626 locomotory behavior	0.006183
AVP	GO:0007626 locomotory behavior	0.024482
C1QBP	GO:0040017 positive regulation of locomotion	0.000929
CACNA1E	GO:0007626 locomotory behavior	0.005731
CAV1	GO:0003012 muscle system process	8.85E-05
CBS	GO:0001958; GO:0048705 skeletal morphogenesis	0.000192
CCR7	GO:0040017 positive regulation of locomotion	0.014505
CHRNA1	GO:0003009 skeletal muscle contraction; GO:0003012 muscle system process	0.010052
FOXC2	GO:0040017 positive regulation of locomotion	0.017599
HOXA6	GO:0048704 embryonic skeletal morphogenesis	6.50E-10
HOXC8	GO:0048705 skeletal morphogenesis	0.032523
MMP14	GO:0030335 positive regulation of cell migration; GO:0040017 positive regulation of locomotion	0.002603
MYH4	GO:0030049 muscle filament sliding; GO:0006936 muscle contraction; GO:0003012 muscle system process	0.004462
MYOT	GO:0006936 muscle contraction; GO:0003012 muscle system process	0.001562

Method 2 is likelihood ratio test under branch-site model.

Rapidly evolving function categories

To further determine the rapidly evolution of locomotion system related GO category in Chinese tree shrew, we calculated the average *K*a and *K*s values for all GO category containing 20 genes or less. When compared to humans, Chinese tree shrews had 23 GO categories showing rapid evolvement ($P<0.05$), of which 7 categories were closely related to locomotion (GO: 0040012 regulation of locomotion; GO:0040017 positive regulation of locomotion; GO:0006937 regulation of muscle contraction; GO:0007626 locomotory behavior; GO:0003012 muscle system process and GO:0030534 adult behavior) (Table 3). Compared to rats, the Chinese tree shrew had 26 GO categories showing a rapid evolvement ($P<0.05$). Among these GO categories, 10 were closely related to locomotion (GO:0060537 muscle tissue development; GO:0014706 striated muscle tissue development; GO:0040012 regulation of locomotion; GO:0042692 muscle cell differentiation; GO:0040017 positive regulation of locomotion; GO:0006941 striated muscle contraction; GO:0003012 muscle system process; GO:0007517 muscle organ development; GO:0006936 muscle contraction; GO:0006937 regulation of muscle contraction) (Table 4). Comparisons between GO categories revealed four rapidly evolving categories relevant to locomotion (GO:0040012 regulation of locomotion, GO:0040017 positive regulation of locomotion, GO: 0003012 muscle system process, GO:0006937 regulation of muscle contraction), which were commonly observed in the Chinese tree shrew, human, and rat.

DISCUSSION

As a small mammal with a close affinity to primates, tree shrews are increasingly considered a viable alternative to primates in biomedical research (Cao et al, 2003; Fuchs & Corbach-Söhle, 2010; Peng et al, 1991). While the recently published genome of the Chinese tree shrew greatly extends the necessary knowledge needed to make it an effective animal model, there are still many aspects of Chinese tree shrew evolution and genetics that we still do not entirely understand. Take for example locomotion: previous studies have shown that to adapt to dangerous living environments in the wild, the ancestor of tree shrew gradually developed a faster rate of speed and stronger jumping ability that partially differentiated it from other mammals (Fuchs & Corbach-Söhle, 2010; Peng et al, 1991). While these changes are well known and fit well with both the extant empirical observations and theoretical models, relatively little is known about the underlying genetic mechanisms that partly distinguish the tree shrew from other related mammalian species—in particular those mechanisms that may serve to make the Chinese tree shrew a more valuable animal model.

In the present study, we used the recently published Chinese tree shrew genome as a foundation to begin understanding the development of the tree shrew's locomotive system by examining related orthologs in six mammals. We identified several genes under positive selection in Chinese tree shrew that may help explain its significant advantage in locomotion ability of explosive power and jumping (Fuchs & Corbach-Söhle, 2010; Peng et al, 1991), but *HOXA6* and *AVP* were the most attractive targets, being detected commonly by two distinct detection methods. *HOXA6* was previously reported to be involved in skeletal morphogenesis (Wellik, 2007), but little is known about the function of *AVP*, though future research on the function of *AVP* may uncover putatively unknown function of *AVP* related to locomotion.

Among the other 39 genes under positive selection, 13 genes have been reported to be involved in muscle contraction, especially with fast-twitch fibers contraction. Fast-twitch fibers have several unique characteristics, including shorter contraction time, more powerful and more advantage in explosive power, all of which would likely prove useful to the tree shrew in its natural environment (Eberstein & Goodgold, 1968). *TNNC2* and *MYH4* were detected to be under positive selection by both Method 1 and Method 2. Previously, both genes were shown to play important roles in fast-twitch fibers contraction (Davoli et al, 2003; Farah & Reinach, 1995). As a subunit of Troponin C, *TNNC2* is expressed exclusively in fast-twitch skeletal muscle in human and plays a critical role in regulating fast-twitch skeletal muscle contraction (Farah & Reinach, 1995). *MYH4* (myosin, heavy chain 4, skeletal muscle), an isoform of myosin, is highly expressed in adult fast-twitch fibers in human (Davoli et al, 2003). Myosins, which are composed of a family of ATP-dependent motor proteins, are best known for their role in muscle contraction and are responsible for actin-based motility (Sellers, 2000). The positive selection of this gene may potentially contribute to the improvement in explosive power of Chinese tree shrew, which is consistent with a previous observation (Schmidt & Schilling, 2007) that tree shrews possess a high content of fast-twitch fibers in infraspinatus muscle. Together, the rapid evolution of *TNNC2* and *MYH4* in Chinese tree shrew may have increased fast-twitch muscle content and strength, thus improving explosive power and jumping ability.

Another identified gene, *ACTN2* (actinin, alpha 2), which encodes alpha-actinin protein (a member of family of the actin-binding proteins), was previously reported to be closely related to explosive power (Norman et al, 2009). ACTN2 is the major structural components of the sarcomeric Z-line involved in anchoring together the actin-containing thin filaments (Tiso et al, 1999). ACTN2 also takes part in the anaerobic muscle metabolism and compensates for the function of ACTN3, an actin protein associated with explosive power (Macarthur & North, 2004). The detected rapid evolution of *ACTN2* in Chinese tree shrew supports the view that this species has evolved an advantage in explosive power. In addition, another positively selected gene in Chinese tree shrew, *MYOT* gene that encodes myotilin, is a skeletal muscle protein found within the Z-disc of sarcomeres, and it was previously found that myotilin could induce the formation of actin bundles (Salmikangas et al, 2003).

While we found several interesting candidate genes that may explain the locomotive system of the tree shrew, previous studies have effectively argued that some complex function is determined not only by one gene, but also by a series of genes or even by evolution of the whole pathway or network (Huynen et al, 2005). In both our key comparisons of the Chinese tree shrew to humans and rats, we observed many rapidly evolving GO categories related to the locomotion system, especially regarding muscle contraction. These rapidly evolving functions may suggest that Chinese tree shrew has evolved an arboreal locomotion ability to adapt to its living circumstance at a broader level.

While our results may greatly expand the knowledge of the tree shrews locomotive system, the current study has several limitations worth noting. First, some key genes may not have been detected due to the limited power of the statistical methods (Kosakovsky Pond et al, 2011). Second, the current study utilizes the list of locomotor genes in human as reference genes, and this may lead to a partial coverage of all possible locomotion related genes in Chinese tree shrew, meaning there may still be more as of yet unidentified genes at work contributing to the tree shrew locomotive system. Third, no functional assay was performed to validate the findings. Future study taking these limitations into account will likely facilitate greater in-depth understandings of the Chinese tree shrew's locomotion ability.

In summary, our screening for putative locomotor genes under positive selection provided some important clues for understanding the locomotion adaptation in Chinese tree shrew. Further study is indispensable to characterize the exact function of these positively selected locomotor genes.

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Table 3 GO categories showing a rapid evolution in Chinese tree shrew as compared with human

GO ID	Gene number	GO name	*Ka*/*Ks*	Amino Acid divergence	*P*-value
GO:0040012	48	Regulation of locomotion	0.083451	0.049934	2.25E-23
GO:0040017	38	Positive regulation of locomotion	0.078734	0.049505	9.93E-18
GO:0030335	35	Positive regulation of cell migration	0.075789	0.048236	2.46E-14
GO:0043085	57	Positive regulation of catalytic activity	0.072898	0.044996	6.25E-14
GO:0051270	46	Regulation of cell motion	0.075461	0.046457	3.75E-13
GO:0030334	41	Regulation of cell migration	0.07441	0.04616	4.62E-12
GO:0051272	38	Positive regulation of cell motion	0.073068	0.046683	8.31E-12
GO:0007610	88	Behavior	0.067098	0.042765	8.81E-09
GO:0001558	24	Regulation of cell growth	0.07434	0.049782	2.07E-08
GO:0008283	44	Cell proliferation	0.06937	0.044489	1.96E-07
GO:0006937	30	Regulation of muscle contraction	0.065469	0.046684	2.48E-06
GO:0007626	73	Locomotory behavior	0.065127	0.042458	4.95E-06
GO:0006928	63	Cell motion	0.06289	0.039346	0.00096
GO:0030097	23	Hemopoiesis	0.065893	0.040171	0.001051
GO:0016477	42	Cell migration	0.065758	0.038147	0.001705
GO:0048870	43	Cell motility	0.063748	0.038383	0.004006
GO:0048534	28	Hemopoietic or lymphoid organ development	0.064639	0.038657	0.004228
GO:0016044	27	Membrane organization	0.068598	0.041368	0.004382
GO:0014070	21	Response to organic cyclic substance	0.061588	4.17E-02	0.00834
GO:0003012	74	Muscle system process	0.057302	0.040496	0.008362
GO:0030534	29	Adult behavior	0.063637	0.04071	0.009514
GO:0007010	33	Cytoskeleton organization	0.061117	0.043273	0.013121
GO:0030155	21	Regulation of cell adhesion	0.068809	0.036182	0.025276

Table 4 GO categories showing a rapid evolution in Chinese tree shrew as compared with rat

GO ID	Gene number	GO name	*Ka*/*Ks*	Amino Acid divergence	*P*-value
GO:0030155	21	Regulation of cell adhesion	0.097633	0.119016	3.55E-49
GO:0048514	44	Blood vessel morphogenesis	0.082959	0.108724	1.92E-46
GO:0043085	57	Positive regulation of catalytic activity	0.084787	0.107279	4.26E-43
GO:0001568	48	Blood vessel development	0.079933	0.106575	1.32E-42
GO:0001944	49	Vasculature development	0.080965	0.105684	2.00E-42
GO:0060537	31	Muscle tissue development	0.077758	0.117899	3.24E-37
GO:0014706	29	Striated muscle tissue development	0.073017	0.116237	1.87E-31
GO:0040012	48	Regulation of locomotion	0.08633	0.10316	1.10E-27
GO:0030334	41	Regulation of cell migration	0.083986	0.101519	2.35E-23
GO:0042692	22	Muscle cell differentiation	0.065632	0.112218	1.37E-18
GO:0051270	46	Regulation of cell motion	0.080463	0.096135	1.32E-15
GO:0040017	38	Positive regulation of locomotion	0.0823	0.098041	1.48E-15
GO:0030335	35	Positive regulation of cell migration	0.080413	0.096529	1.47E-13
GO:0008015	39	Blood circulation	0.07289	0.097362	4.87E-13
GO:0006941	21	Striated muscle contraction	0.055351	0.103401	1.45E-10
GO:0051272	38	Positive regulation of cell motion	0.078057	0.092413	3.92E-10
GO:0001558	24	Regulation of cell growth	0.079741	0.100792	7.15E-09
GO:0001666	23	Response to hypoxia	0.072469	0.088672	1.44E-08
GO:0016044	27	Membrane organization	0.078575	0.094073	5.07E-07
GO:0003012	74	Muscle system process	0.06129	0.086659	9.54E-06
GO:0007517	46	Muscle organ development	0.057935	0.088743	1.23E-05
GO:0006936	68	Muscle contraction	0.057845	0.086065	0.000125
GO:0006937	30	Regulation of muscle contraction	0.069204	0.084317	0.00053
GO:0048870	43	Cell motility	0.075263	0.081279	0.000704
GO:0016477	42	Cell migration	0.07596	0.081453	0.00111
GO:0051094	48	Positive regulation of developmental process	0.06674	0.076614	0.004085

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基因通过代谢控制性状

现在国内过于关注基因克隆和表达，相对忽视了代谢的研究。其实，生命科学研究的最终目的是了解某种性状是如何产生和改变的，从而希望人为干预性状变化，以实现预期目的。基因要实现对性状的控制，就必须通过影响代谢才能达到。此外，不仅基因表达变化通过代谢来影响性状，而且代谢本身也是基因表达变化调控的基础。如果关注一些高影响因子的 SCI 论文，其研究必然牵涉到代谢变化的研究，即贯穿基因、代谢和性状。提请大家注意增加代谢研究的分量。

酶基因克隆和表达的研究中必须测定酶活性

测定到某种酶活性发生了显著改变，表明该酶在您研究的生物学现象中可能具有重要作用，值得进一步克隆并且分析该基因的表达。WB 测定的只是某种蛋白质含量的变化，不能直接代表酶活性大小。酶活性的调控可以发生在基因表达水平上，也可以发生在翻译后修饰、变构调节和反馈作用等水平上。如果 WB 测定表明酶蛋白质数量未发生变化，而酶活性发生了显著变化，说明该酶活性的调控可能发生翻译后水平上。因此，酶基因表达的研究中必须测定酶活性。

酶通过底物和产物改变性状

代谢研究中通常更注重酶活性的测定，而忽视酶催化底物和产物水平的测定。其实，酶本身不能直接改变某种性状，而是通过改变其底物或者产物的含量，进一步引起相关性状发生改变。因此，在代谢测定中，酶活性变化只是用来分析底物或者产物含量变化的原因，一定要注意底物和产物含量的测定。

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