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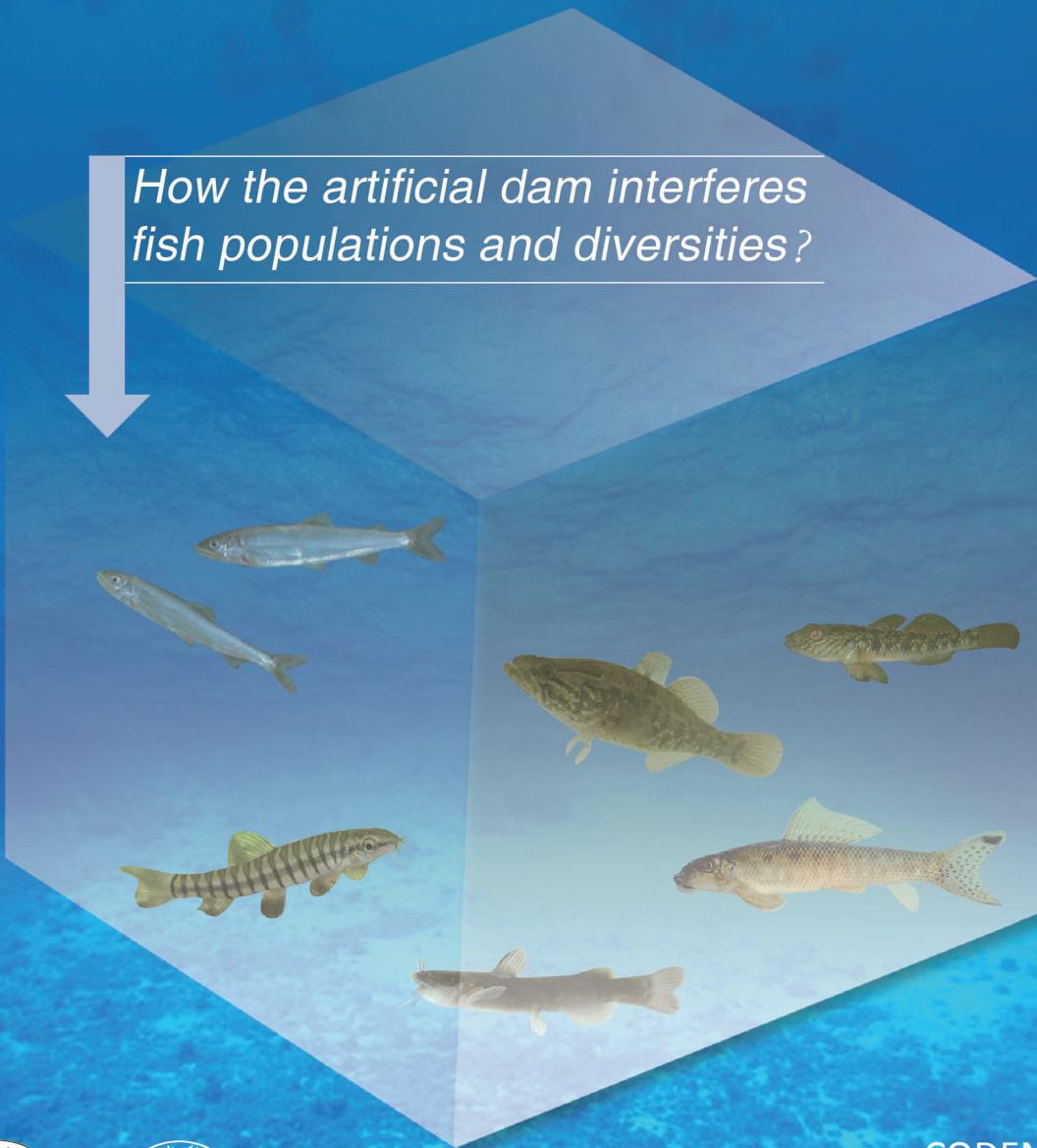
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# ZR

动物研究

# ZOOLOGICAL RESEARCH

*How the artificial dam interferes  
fish populations and diversities?*



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# ZOOLOGICAL RESEARCH

Volume 37, Issue 2 18 March 2016

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**Cover design: Lin LEI**

# Obituary: Professor Ying-Xiang Wang (1938-2016)

Xue-Long JIANG

It is with great sadness that I share the sorrowful news that mammalogy and wildlife science lost a respected scientist, patient teacher, and wonderful mentor on 10 February, 2016, when Professor Ying-Xiang Wang died in Kunming, Yunnan Province, due to heart failure following a lung infection. He is survived by his wife Professor Rui-Qing Liu, daughter Li Wang, son Tao Wang, daughter-in-law Qing Sun and granddaughter Jun-Qi Wang.

Professor Wang was born on 21 July, 1938, in Honghe, Yunnan, where he spent his childhood until the age of seven. He moved to Kunming with his parents following an acute bout of bronchitis, the effects of which he battled all his life.

Professor Wang registered as a zoological student in 1957 and studied at Sichuan University for five years. Following his graduation in 1962, he took a job and began his lifelong career as a mammalogist at the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences. As a young researcher, he participated in numerous fauna surveys in Kunming and the surrounding areas in 1962 and 1963, but undertook his first large field expedition to investigate the mammals and birds of Wuliang Mountain in central Yunnan in 1964. It was during this expedition that he realized the importance of the preparation and education needed to be an outstanding mammalogist, and subsequently trained and worked under Professor Wu-Ping Xia in Inner Mongolia in 1965, with an emphasis on small mammal research.

Many Chinese scientists were impacted with the arrival of the political “Cultural Revolution” in 1966. However, Professor Wang was lucky enough to be able to continue with his mammalian research and related field surveys. Of particular note was his work on the breeding of the threatened *Viverra zibetha* from 1966 to 1973, based upon which he and his colleagues published a book entitled *Breeding of Large Indian Civet and Civet Extraction* (Civet Research Group of Kunming Institute of Zoology & Chinese Academy of Sciences, 1990). Professor Wang also conducted numerous vertebrate surveys and specimen collections in the Gaoligong Mountains, with a focus on small mammals, from 1973-1975. From then, his footprints were left across many mountain ranges in China, such as Baimaxueshan, Biluoxueshan, Xishuangbanna, and Honghe in Yunnan, Shalulishan and Daxueshan in Sichuan, Fanjingshan and Leishan in Guizhou, Wulingshan in Chongqing and Hubei. He also worked as a team leader for a number of vertebrate expeditions, such as in the Honghe Prefecture from 1984 to 1985, and edited a book entitled *Scientific Reports*



**Professor Ying-Xiang WANG, mammalogist, 1938-2016**

of *Biological Resources in Honghe Prefecture, Southern Yunnan: Terrestrial Vertebrates* (Wang, 1987a). With all these impressive achievements, he was promoted to a full Professor of Zoology in KIZ in 1990.

Professor Wang acted as the head of the mammal research group in KIZ, and conducted extensive research on taxonomy, phylogeny, zoogeography, and the conservation of mammals linked to museum specimens and field observations from 1983 until he retired in 2003. However, he worked until his final days as the Editor-in-Chief of *Fauna Sinica Mammalia Vol. 3 Primates, Lagomorpha and Pholidota*, even showing me his work on our last visit together on the evening of 1 February, 2016.

During his career, Professor Wang published 121 scientific papers and key references on mammalian taxonomy and early karyotype studies on birds and mammals (Chen et al., 1992, 1993; Wang et al., 1980, 1982, 1983). He conducted many taxonomic reviews and revisions of different mammal groups, such as shrews (Jiang et al., 2003), bats (Feng et al., 2006, 2007, 2008a,b; Zhou et al., 2009), tree shrews (Wang, 1987b), macaques (Jiang et al., 1991, 1993, 1996), langurs (Wang et al., 1999), gibbons (Groves & Wang, 1990; Ma & Wang, 1986), musk deer (Groves et al., 1995), muntjacs (Ma et al., 1986), squirrels (Li et al., 2005, 2006), voles (Luo et al., 2004; Wang &

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Li, 2000), rats (Wang et al., 1996), and hares (Wang et al., 1985). He and his colleagues described five new mammal species, including *Ochotona gaoligongensis* (Wang et al., 1988), *Muntiacus gongshanensis* (Ma et al., 1990), *Ochotona nigritia* (Gong et al., 2000), *Tylonycteris pygmaeus* (Feng et al., 2008b), and *Rhinolophus xinanzhongguoensis* (Zhou et al., 2009), and many new subspecies, such as *Callosciurus erythraeus wuliangshanensis* (Li & Wang, 1981), *Callosciurus erythraeus gongshanensis*, *Paguma larvata chichingensis*, *Petaurista petaurista nigra*, *Micromys minutus pianmaensis* (Peng & Wang, 1981), *Paradoxurus hermaphroditus hainanus* (Wang & Xu, 1981), *Pipistrellus paterculus yunnanensis*, *Pipistrellus circumdatus drungicus* (Wang, 1982), *Neotetracus sinensis hypolineatus* (Wang & Li, 1982), *Lepus comus pygmaeus*, *Lepus comus peni* (Wang et al., 1985), *Nomascus concolor jingdongensis*, *Nomascus concolor furvogaster*, *Hylobates lar yunnanensis* (Ma & Wang, 1986), *Hadromys humei* (Yang & Wang, 1987), *Tupaia belangeri gongshanensis*, *Tupaia belangeri yaoshanensis* (Wang, 1987b), *Dremomys lokriah nielamuensis* (Li & Wang, 1992), *Typhlomys cinereus guangxiensis*, *Typhlomys cinereus daloushanensis* (Wang et al., 1996), *Macaca thibetana huangshanensis*, *Macaca thibetana guizhouensis* (Jiang et al., 1996), *Rhinopithecus roxellana qinlingensis*, *Rhinopithecus roxellana hubeiensis* (Wang et al., 1998), *Niviventer confucianus yajiangensis*, *Niviventer confucianus deqinensis* (Deng et al., 2000), and *Callosciurus erythraeus zhaotongensis* (Li et al., 2006). He also reported new records for Chinese mammalian fauna, e.g., *Hadromys humei* (Yang et al., 1985), *Niviventer brahma* (Gong et al., 1989), *Manis javanica* (Wu et al., 2005), *Megaerops ecaudatus*, *Megaerops niphanae* (Feng et al., 2006), and *Leopoldamys neilli* (Chen et al., 2014). He achieved a career milestone in publishing *A Complete Checklist of Mammal Species and Subspecies in China: A Taxonomic and Geographic Reference* in 2003, which was the first complete and systematic book on Chinese mammals and is highly cited to this day. In 2007, he and his colleagues published *A Field Guide to the Mammals of China*, in which they provided details on the physical attributes and distributions of 346 mammals in colorful plates, and also updated the checklist of Chinese mammals from 607 to 645 species. He also made great contributions to *Fauna Sinica Mammalia Vol. 8 Carnivora* (Gao, 1987), Vol. 6 Rodentia Part III: Cricetidae (Luo et al., 2000), and many other publications, such as the *Biology of Chinese Tree Shrews* (Peng, 1991), *Evaluation on Animal Resources from Wuling Mountain Areas, Southwestern China* (Song, 1994), *The Natural History of the Doucs and Snub-nosed Monkeys* (Jablonski, 1998), and *Comprehensive Surveys of Xishuangbanna Nature Reserve* (Xu et al., 1987), *Gaoligong Mountain National Nature Reserve* (Xue, 1995), *Nujiang Nature Reserve* (Xu, 1998), *Jingping Fenshuiling Nature Reserve* (Xu, 2002), *Yunnan Luchuan Huanglianshan Nature Reserve* (Xu, 2003), *Baimaxueshan National Nature Reserve* (Li, 2003), *Wuliangshan National Nature Reserve* (Yu, 2004), *Lancangjiang Nature Reserve* (Wang et al., 2010), and *Yunnan Jiaozishan National Nature Reserve* (Peng & Liu, 2015).

Throughout his career, Professor Wang contributed to the

field of mammalian research in many ways. He became a member of the Chinese Society of Mammalogists after it was established in 1980, and served as the vice head and head of the Chinese Primate Research Group from 1990-2002. He and other senior Chinese mammalogists, e.g., Professor Qi-Shan Wang from Anhui University, were significant in pushing forward Chinese primate research and training many young primatologists. He served as the President of the Yunnan Zoological Society from 1992-2003 and as the Associate Editor-in-Chief of *Zoological Research* from 1988-1990 and from 2010 until his death. He was also a member of the *Acta Theriologica Sinica* editorial board from 2001 to 2013 and part of the Scientific Committee for Taxonomy of the Chinese Academy of Sciences from 2006 to 2010. He served as deputy director of the Yunnan Wildlife Conservation Association and Evaluation Committee of Yunnan Nature Reserves from 2000-2008.

Professor Wang was active on the international front as well, and collaborated with many scientists from different countries, including Dr. Jack Fooden from the US on the taxonomic status and distribution of Chinese stump-tailed macaques (Fooden et al., 1985), Dr. Frank E. Poirier from the US on the taxonomy, distribution and behavioral ecology of gibbons and langurs (Jiang et al., 1994a, b; Ma et al., 1988, 1989), and Dr. Colin C. Groves from Australia on the taxonomy and distribution of musk deer and *Nomascus* gibbons (Groves & Wang, 1990; Groves et al., 1995). He also collaborated with Russian and Japanese scientists on the phylogeny and evolution of mammals such as wood mice (Suzuki et al., 2003), talpid moles (Shinohara et al., 2004), flying squirrels (Yu et al., 2004, 2006), weasels (Abramov et al., 2008), and erinaceids (He et al., 2012).

I came to Professor Wang as a graduate student in 1986 and achieved my MS degree in 1989 and PhD in 2000 under his supervision. Professor Wang was an excellent supervisor: fair, supportive, strict and kind. He helped and mentored many students and young scientists, including six MS and four PhD students, and encouraged hard work and self-dependency for their scientific career development. He would often tell us a story of his student days when he was asked by his professor, Hong-Shou Peng, to prepare a specimen of a very dead and decaying house rat found in a rubbish dump – although the preparation was difficult and unpleasant, the end result was work well done. He truly enjoyed working with young researchers and sharing his experience and expertise with his students, especially in regards to mammalian taxonomy and evolution. He worked tirelessly, coming to his office every working day for ten years after he retired at 65, and continuing to work from home even though he was severely ill. He showed this passion until the very last minute. We mourn his passing deeply.

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## In Memory of Professor Ying-Xiang WANG

It is with deep regret and sorrow that *Zoological Research* (ZR) notes the passing away of Professor Ying-Xiang WANG on the 10th February 2016.

Prof. WANG, a former Associate Editor-in-chief of ZR had made a distinguished contribution to the journal. His passing is a great loss for the journal, but all the editorial board and editors of ZR will cherish his contribution.

Prof. WANG was probably one the most internationally renowned Chinese mammalogists of our time. He had made a tremendous contribution to the work of taxonomy and resource conservation of the mammals in China. His pioneer studies paved the way for many younger scholars to carry on their research on wild animals, especially on large beasts and primates. Prof. Wang had a great personality, and it will

be hard to find someone to fill the gap his passing has left in both academic research work and talented thought on the critical ground studies of wild animal resources and their protections.

As a prestigious scientist, Prof. WANG also played active roles in promoting the development of ZR. He joined the editorial board of ZR since 1989 and fulfilled his duty faithfully and diligently. Even during the last few weeks before his death, he was still reviewing the manuscripts and always gave constructive suggestions. His reputation attracted many important national and international submissions, which significantly increased the impact of ZR.

We are greatly saddened by Prof. WANG's passing but his spirit will live on us as a source of inspiration for us.

## Appointment of Dr. Xue-Long JIANG as the Associate Editor-in-Chief of *Zoological Research*



We are very pleased to announce that we have invited Dr. Xue-Long JIANG to serve as the Associate Editor-in-Chief for *Zoological Research* (ZR), effective from 1 March, 2016. Dr. JIANG, Professor and principle investigator from the Laboratory of Mammal Ecology and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, has worked with ZR since 2006 as a member of the editorial board and has played an active and important role in maintaining ZR as a respected academic publishing platform. Currently, he also works as the Associate Editor-in-Chief of *Acta Theriologica Sinica*, *Mammal Research* and as a senior editorial board member of *Biodiversity Science*.

Dr. JIANG's research interests mainly include specimen-based investigations of the biodiversity, inventory, taxonomy and systematics, phylogenetics and phylogeography of small mammals, especially those of the Hengduanshan region in southwest China. He also has particular interest in the ecology and behavior of the black-crested gibbon. The core of his research is to describe the rich but often overlooked biodiversity, and to untangle the formation and radiation of this diversity. These research interests and expertise also extend to applications for the conservation of mammals and resource animals, such as the musk deer. Many of his findings have been published in high-level peer-reviewed journals.

In his new position, with his impressive academic achievements and excellent expertise, Dr. JIANG will be dedicated to promoting the international impact of ZR.

# Influences of local habitat, tributary position, and dam characteristics on fish assemblages within impoundments of low-head dams in the tributaries of the Qingyi River, China

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## ABSTRACT

Low-head dam impoundments modify local habitat and alter fish assemblages; however, to our knowledge, the pattern of how fish assemblages in the impoundments relate to local habitat, tributary position, and dam characteristics is still unclear. We used data collected in 62 impoundments created by low-head dams in headwater streams of the Qingyi River, China, to examine relationships between fish assemblages and local habitat, tributary position, and dam characteristics. We also assessed the relative importance of the three groups of factors in determining fish species richness and composition. Linear regression models showed that fish species richness was related to substrate heterogeneity, confluence link, and dam number upstream. Redundancy analysis showed that fish species compositions were influenced by substrate heterogeneity, confluence link, dam height, dam numbers upstream and downstream. Overall, dam characteristics were more important in affecting fish species richness but less important in determining fish species composition than local habitat (i.e., substrate heterogeneity) and tributary position. Our results suggest that low-head dam may affect fish species richness in impoundments by modifying local habitat and constraining fish movement, and the relative abundances of those fish species may depend more on species habitat presences and stream size than on impoundment size and number.

**Keywords:** Substrate coarseness and heterogeneity; Confluence link; Dam number and area

## INTRODUCTION

Distribution and abundance of stream fishes are influenced jointly by historical processes, abiotic and biotic factors and ecological processes (Dauwalter et al., 2008; Gilliam et al., 1993; Hoeinghaus et al., 2007). At local scale, because of interspecific differences in physiology, behavior, and habitat preference (Jackson et al., 2001), local fish assemblages relate to stream segment habitat features, including flow regime (Yan et al., 2011), water temperature (Wang et al., 2003), dissolved oxygen (Ostrand & Wilde, 2001), and substrate size (Wang et al., 2013). Also, some stream size descriptors, such as water depth (Harvey & Stewart, 1991), stream width (Yan et al., 2010), and discharge (Chu et al., 2015a) are important factors in determining local fish diversity. At a river network scale, the nature of the continuities between mainstems and tributaries, and among tributaries, may result in spatial auto-correlation of abiotic and biotic factors and ecological processes within a watershed (Grant et al., 2007). Local fish assemblages are also determined by the tributary position within the drainage network, which determines fish immigration and extinction rates (Grenouillet et al., 2004; Taylor & Warren, 2001; Yan et al., 2011). Some descriptors of tributary position, such as link magnitude, downstream link, and confluence link, also have been reported to influence local species richness and compositions of stream fishes (Grenouillet et al., 2004; Li et al., 2014; Osborne & Wiley, 1992; Smith & Kraft, 2005; Yan et al., 2011). In addition, such spatial pattern of fish assemblages and their relationship with natural environmental factors are modified by anthropogenic

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activities in the stream channel or in their watersheds, such as land use, dam construction and water pollution (Chu et al., 2015a; Harding et al., 1998; Vila-Gispert et al., 2002).

Dams are widely recognized as one of the primary means by which humans alter or modify fluvial ecosystems (Poff & Hart, 2002; Rosenberg et al., 1997). Numerous investigations have revealed that dams affect stream fish in diverse ways, including blocking fish passage, altering flow and thermal regimes, modifying local habitat condition, and altering the prey base (e.g., Murchie et al., 2008; Nilsson et al., 2005; Poff & Zimmerman, 2010; Rosenberg et al., 2000; Wang et al., 2011). However, most of our knowledge on how dams affect lotic systems and fish assemblages is derived from investigations on large dams, while small low-head dams have been given less attention (Singer & Gangloff, 2011; Thoni et al., 2014; Yan et al., 2013). Although some researchers have found that fish species richness (Tiemann et al., 2004) and assemblage structure (Raborn & Schramm, 2003) in the dammed segments did not differ from free-flowing segments, others observed that low-head dams may substantially reduce local fish species richness (Dodd et al., 2003) and alter fish assemblage structure (Gillette et al., 2005; Poulet, 2007). These differences in results may be associated with dam size and location. Substantial modifications in fish assemblages may occur only in the impoundments immediately upstream, but not downstream of the dams (Yan et al., 2013). Compared with free-flowing stream segments, impoundments created by low-head dams are characterized with slower flows, deeper and wider water bodies, and smaller substrates (Gillette et al., 2005; Tiemann et al., 2004). Such local habitat modifications may alter fish assemblages by decreasing the numbers of lotic species and increasing the numbers of lentic species (Gillette et al., 2005; Tiemann et al., 2004; Yan et al., 2013). Moreover, multiple low-head dams upstream and/or downstream may cumulatively affect local habitat and fish assemblages (Cumming, 2004; Helfrich et al., 1999; Wang et al., 2011). Effects of impoundment are likely to increase with downstream flow past consecutive dams because river transport is largely unidirectional (Santucci et al., 2005).

We hypothesize that the fish assemblages in the impoundments of low-head dams are influenced by size of dams, number of dams upstream and/or downstream, local habitat conditions, and tributary position in the stream network. However, to our knowledge, the combined influences of local habitat, tributary position, and low-head dam characteristics on fish assemblages have not been thoroughly examined. Chu et al. (2015b) collected fishes from 62 impoundments created by low-head dams in headwater streams of the Qingyi River, China. After classifying the 25 fish species collected into 12 indigenous (naturally inhabiting in lotic headwater streams) and 13 native-invading species (naturally preferring lentic or slow-flowing waters of mid to lower reaches of a river network), the authors assessed the influence of abiotic (local habitat) and biotic (native invader) factors on the indigenous fish assemblages. However, they did not examine how the entire fish assemblages, indigenous and native-invasive fishes together, related to abiotic factors. In this study, we used the data of Chu et al.

(2015b) to examine relationships between abiotic factors and fish assemblages in the 62 impoundments. Our aims were: (1) to determine the pure and combined effects of three groups of environmental factors (i.e., local habitat, tributary position, and dam characteristic) on fish species richness; (2) to determine the pure and combined effects of these environmental factors on fish species composition; (3) and to assess the relative importance of the three groups of environmental factors influencing local species richness and species composition.

## MATERIALS AND METHODS

### Study area

The Qingyi River originates in the northern portion of Huangshan Mountain and flows northeast toward its confluence with the lower Yangtze River, China. As a result of a subtropical monsoon climate, this basin is characterized by asymmetric seasonal temperature and precipitation distributions. Monthly mean temperature ranges from -2.1 °C in January to 27.5 °C in July and approximately 79% of the annual rainfall occurs from April to September. Approximately 1 000 low-head dams have been built on the tributaries of this basin for agricultural irrigation, resident water consumption, and recreational fishing (Chu et al., 2015b; Yan et al., 2011, 2013).

### Fish sampling

A total of 62 impoundments created by low-head dams within the first-order (defined from the Anhui Province topographic maps of 1: 300 000 scales using the method of Strahler (1957)) headwater streams were sampled once during October and November 2011. Each sampled impoundment was selected in the field based on criteria that dam height was less than 4 m and impoundment water depth was less than 1 m. Only one site of 50 m long was sampled within each impoundment; however, when impoundments were less than 50 m long, the entire impoundments were sampled. Fish were collected using a backpack electrofishing gear (CWB-2000 P, China; 12 V import and 250 V export) by wading in two passes without blocking nest. Each electrofishing pass was operated with a uniform sampling effort (approximately 30 min sampling time for each 50 m sampling segment) by the same three persons, one operating the gear and the other two capturing fishes. Fish were identified in the field to species, counted, and returned to the sampling sites alive.

### Environmental survey

We characterized local habitat conditions of each sampled impoundment by eight habitat variables, including wetted width (m), water depth (m), water temperature (°C), dissolved oxygen (mg/L), conductivity (mS/s), current velocity (m/s), and substrate coarseness and heterogeneity. Wetted width was measured along five transects equally spacing across the stream channel. Water depth, water temperature, dissolved oxygen, and conductivity were measured at four equal interval points along each transect (JENCO 6350, 9010, USA). Current velocity was taken at 60% of water depth at each point (FP111, USA). Substrate was quantified with a 1 m lead core divided

into 10 cm sections, using the frequency size class method of Bain (1999). Mean and standard deviation of dominant substrate values were regarded as indices of substrate coarseness and heterogeneity, respectively.

We quantified the dam characteristics of each sampled impoundment by two groups of dam variables, including dam size and dam numbers. Dam size consisted of the height (m), length (m) and area ( $m^2$ ) of each low-head dam surveyed. Dam height was estimated as the vertical distance from the natural streambed at the downstream toe of the dam to the lowest point on the dam crest. Dam length was measured as the horizontal distance across channel at the dam crest. Then, dam area was calculated from its height and length, by approximation to a half ellipse. Dam number involved the numbers of upstream and downstream dams for each impoundment surveyed. Because each surveyed impoundment was located at the first-order headwater stream, dam number upstream was counted as the number of all dams (including low-head dams and hydropower stations) upstream of each sampling site along each surveyed headwater stream. Dam number downstream was counted in terms of all dams downstream of each surveyed low-head dam along the mainstem of the Qingyi River before it flows into the Yangtze River.

The impoundments sampled were all located in the first-order streams, suggesting that both stream order (Strahler, 1957) and stream link magnitude (Shreve, 1966) of all tributaries surveyed amounted to one. So, according to Yan et al. (2011), we quantified other two variables (confluence link and downstream link) to describe the tributary position of each impoundment within the Qingyi basin network. Confluence link is the number of confluences downstream from each segment (Fairchild et al., 1998), and downstream link is the linkage number of the stream segment that the sampling stream segment immediately flowing into (Osborne & Wiley, 1992). The two variables were assigned to each segment sampled using Anhui Province topographic maps (1: 300 000 scales).

#### Data analysis

We used stepwise regression to evaluate the effects of environmental variables on fish species richness (Legendre & Legendre, 1998). First, we built three regression models to determine the effects of local habitat alone, tributary position alone, and dam characteristics alone on fish species richness. We entered 10 habitat variables, two tributary variables, and five dam variables into the three regression models, respectively. Second, we entered all the 17 explanatory variables measured into one regression model to identify the combined effects of the three groups of environmental factors on fish species richness. Because only one significant predictor variable was screened out for all the four models, we did not

use Akaike's Information Criterion (AIC) to select the optimal model for explaining the variance in fish species richness any more. Prior to analysis, fish and environment data were log-transformed to meet the assumptions of normality and homogeneity of variances. We used the SPSS 13.0 statistics package to perform statistical analysis, and statistical significance was accepted at  $P<0.05$ .

Using CANOCO 4.5 software package (ter Braak & Verdonschot 1995), we performed a redundancy analysis (RDA) to evaluate the variations in species composition in relation to environmental variables. We used RDA instead of CCA in the relationship analysis because detrended correspondence analyses indicated that our fish data set had a short gradient length (a measure of species turnover) for which the linear model of RDA was more appropriate than CCA (ter Braak & Verdonschot 1995). Similar to our regression analysis, we performed three RDAs to assess the correlations between fish species composition and local habitat alone, tributary position alone, and dam variables alone, respectively; then, we performed one RDA to determine how the three groups of environmental factors affected jointly fish species composition. These analyses included the relative abundances of all fish species except that occurring at two sites or fewer to avoid biased weighting. All the variables entered the analysis after a forward selection procedure, showing their importance in explaining the total variability in species composition. The significance ( $P<0.05$ ) of the RDA gradient was assessed by Monte Carlo permutation tests and their importance measured by the eigenvalues of the first two axes (ter Braak & Verdonschot 1995). All fish and environment data were  $\log_{10}(X+1)$  transformed to meet assumptions of multivariate normality and to moderate the influence of extreme data.

## RESULTS

#### Species richness

When the three groups of environmental variables were considered separately, our results showed that fish species richness was related negatively to substrate heterogeneity (local habitat), and confluence link (tributary position), and positively related to number of upstream dams (dam characteristic) ( $P<0.05$ ). The number of dams upstream explained the most variability (55%) and substrate heterogeneity explained the least variability (30%) in species richness (Table 1). However, when the combined effects of the three groups of factors on fish species richness were considered, only the number of upstream dams explained species richness ( $P<0.05$ ), whereas local habitat and tributary position variables were less important ( $P>0.05$ ) (Table 1).

**Table 1** Linear regression models of fish species richness versus local habitat, tributary position, and dam characteristic

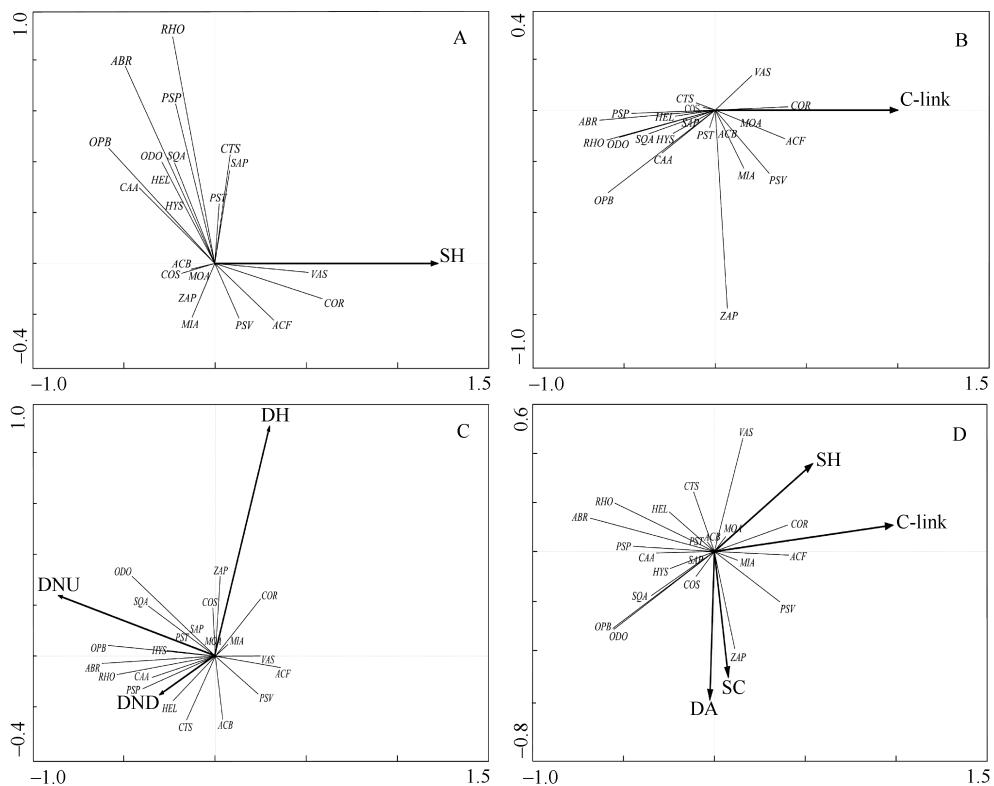
Variables	Independent variables	R	t	P
Local habitat	Substrate heterogeneity	0.30	-2.20	<0.05
Tributary position	Confluence link	0.50	-3.99	<0.01
Dam characteristic	Dam number upstream	0.55	4.64	<0.01
Combined	Dam number upstream	0.55	4.64	<0.01

### Species composition

When the three groups of environmental variables were considered separately, substrate heterogeneity (local habitat) (Figure 1A), confluence link (tributary position) (Figure 1B), and dam height and numbers of dams upstream and downstream (dam characteristic) (Figure 1C) were significantly related to species composition ( $P<0.05$ ). These variables explained 55.6% (substrate heterogeneity), 57.0% (confluence link) and 32.0% (dam characteristics) of the variance of species composition, respectively. When combining local habitat, tributary position, and dam characteristic together, the key factors influencing species composition included substrate coarseness and heterogeneity, confluence link, and dam area ( $P<0.05$ ) (Figure 1D).

Different species responded differently to environmental variables. As substrate heterogeneity increased, abundances of *Vanmanenia stenosoma*, *Cobitis rarus* and *Acrossocheilus*

*fasciatus* increased and *Carassius auratus*, *Opsarrichthys bidens* and *Odontobutis obscura* decreased (Figure 1A). When confluence link increased, *C. rarus* and *A. fasciatus* became more abundant and *Abbottina rivularis*, *Pseudorasbora parva*, *Rhodeus ocellatus*, *Hemiculter leucisculus* and *O. obscura* became less abundant (Figure 1B). As the number of upstream and downstream dams declined, the number of *O. bidens*, *A. rivularis*, *R. ocellatus* and *P. parva* increased whereas *V. stenosoma*, *A. fasciatus* and *Pseudogobio vaillanti* decreased. As dam height increased, *Zacco platypus*, *Cobitis sinensis* and *C. rarus* increased, whereas *P. vaillanti* and *Ctenogobius* spp. decreased (Figure 1C). When the combined effects of the three groups of factors on fish species composition were considered, substrate heterogeneity and confluence link showed positive correlations with the first RDA axis, and substrate coarseness and dam area negatively related to the second RDA axis (Figure 1D).



**Figure 1** Redundancy analysis (RDA) diagrams for fish species composition and local habitat (A), tributary position (B), dam characteristic (C) and their combinations (D) in the impoundments created by low-head dams

Italic codes represent fish species (Appendix I), and bold abbreviations represent environmental variables (Appendix II).

### DISCUSSION

In this study, we found that fish species richness in impoundments behind low-head dams of the Qingyi River was related to substrate heterogeneity, confluence link, and dam number upstream, and fish species composition was influenced by substrate heterogeneity, confluence link, dam height, dam numbers upstream and downstream. Dam characteristics like

number of dam upstream were more important in affecting species richness but less important in determining fish species composition than local habitat like substrate heterogeneity and tributary position like confluence link.

Substrate provides the prerequisite micro-conditions for many stream fishes and can be viewed as an indicator of stream habitat quality (Bain, 1999). Substrate coarseness and heterogeneity, representing substrate size and microhabitat

diversity, may substantially influence stream fish assemblages (Matthews, 1998). The positive relationship between substrate heterogeneity and fish species richness in the free-flowing segments of streams have been observed by many researchers (e.g., Gorman & Karr, 1978; Gratwicke & Spergh, 2005; Li et al., 2014; Wang et al., 2013). However, we found that fish species richness in the impoundments behind low-head dams was negatively related to substrate heterogeneity. This discrepancy may be associated with the difference in environmental conditions and fish species compositions between impoundments and free-flowing segments. Compared with free-flowing segments, impoundments behind low-head dams are characterized by slower flows, deeper water and finer substrate, and by less endemic lotic fishes and more widespread lentic fishes (Gillette et al., 2005; Tiemann et al., 2004; Yan et al., 2013). The total of 25 fish species collected in this study included 12 indigenous specialist species naturally inhabiting upland streams, and 13 invasive generalist species naturally preferring lowland waters (Chu et al., 2015b). Although species richness of indigenous species is positively related to substrate heterogeneity (Chu et al., 2015b), our redundancy analysis showed that the abundances of most invasive species, such as *C. auratus*, *P. parva*, *A. rivularis*, *M. anguillicaudatus*, *R. ocellatus* and *O. obscura*, were negatively related to substrate heterogeneity. Therefore, the habitat-generalist characteristics of invasive fishes in impoundments could lessen the positive correlation between substrate heterogeneity and fish species richness observed elsewhere.

Fluvial systems have interconnected network architectures with complex but definable 'network geometry' (Fausch et al., 2002; Wiens, 2002) or "dendritic ecosystem network" (Grant et al., 2007). At a river network scale, local fish assemblages are determined by tributary position within a watershed network (Grenouillet et al., 2004; Yan et al., 2011), because the rates of fish immigration and emigration influence local fish assemblages in streams and depend on tributary position (Robinson & Rand, 2005; Taylor & Warren, 2001). This may explain why some adventitious streams, defined as streams at least three stream orders smaller than that into which they flow, often hold more diverse fish assemblages than headwater streams with similar size to adventitious streams (Hitt & Angermeier, 2008; Osborne & Wiley, 1992). We found that both species richness and fish assemblages were significantly related to confluence link, suggesting that fish movements may influence fish assemblages within the impoundments by low-head dams. Others have revealed that some variables on tributary position, such as downstream link (Grenouillet et al., 2004; Osborne & Wiley, 1992;) and confluence link (Li et al., 2014; Smith & Kraft, 2005) influence local fish assemblages in free-flowing segments.

We found that both fish species richness and composition in impoundments were related to the number of dams upstream and/or downstream, suggesting of cumulative effects of multiple dams on fish assemblages. These cumulative effects have been also observed by other researchers such as Helfrich et al. (1999), Cumming (2004), and Wang et al. (2011). Because river

transport is largely unidirectional, effects of impoundment often increase with downstream flow past consecutive dams (Santucci et al., 2005). Our redundancy analysis showed that the abundances of most indigenous species were negatively related to the number of dams upstream, but the opposite was observed for invasive species. Similarly, in the same study area, Chu et al. (2015b) found that local species richness of indigenous fishes correlated negatively with the number of dams upstream, while the richness of invasive fishes correlated positively with the number of upstream dams. Therefore, multiple impoundments behind low-head dams may cumulate effects on local fish assemblages, negatively impacting indigenous fishes but benefiting invasive species. In addition, we also found that fish species composition in impoundments was related to dam height and dam area. This is consistent with the opinion that the magnitude of dam effects and the degree to which local habitat conditions and fish assemblages are impacted depend on dam size, because dam size influences the size of their impoundments (March et al., 2003; Poff & Hart, 2002).

The relative importance of different environmental variables in determining fish assemblages may depend on many factors, such as spatial scale at which an investigation is conducted (Jackson et al., 2001; Wang et al., 2006), features of environmental conditions in a particular region (Hughes et al., 2015; Wang et al., 2006), and indicator used to describe fish assemblages (species richness v.s. species composition) (Li et al., 2014; Yan et al., 2011). We demonstrated that dam characteristics (i.e., dam number upstream) were more important in affecting fish species richness in impoundments than local habitat (i.e., substrate heterogeneity) and tributary position (i.e., confluence link). By modifying local habitat features, low-head dams and other co-occurring anthropogenic activities (e.g., land use and water pollution) decrease local species richness, alter the longitudinal pattern of fish species richness along upstream-downstream gradient, and lessen the effects of habitat factors on local species richness (Chu et al., 2015a). In addition, by blocking fish passage, dams also constrain fish movements among stream segments and lower the effects of tributary position on fish species richness (Yan et al., 2011). However, we also demonstrated that dam characteristic was less important in influencing fish species composition than habitat and tributary position. This suggests that the relative abundances of those fish species may depend more on species habitat preferences and stream size than on impoundment size and number (Yan et al., 2011).

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#### Appendix I Species composition, code, and classification (indigenous v.s. invasive) of fishes in 62 impoundments surveyed

Order/Family/Species	Code	Classification
<b>CYPRINIFORMES</b>		
<b>Cyprinidae</b>		
<i>Acrossocheilus fasciatus</i>	ACF	Indigenous
<i>Zacco platypus</i>	ZAP	Indigenous
<i>Pseudogobio vaillanti</i>	PSV	Indigenous
<i>Phoxinus oxycephalus</i>	PHO	Indigenous
<i>Acheilognathus barbatulus</i>	ACB	Indigenous
<i>Opsarrichthys bidens</i>	OPB	Indigenous
<i>Squalidus argentatus</i>	SQA	Invasive
<i>Sarcocheilichthys parvus</i>	SAP	Invasive
<i>Gnathopogon imberbis</i>	GNI	Invasive
<i>Carassius auratus</i>	CAA	Invasive
<i>Pseudorasbora parva</i>	PSP	Invasive
<i>Abbottina rivularis</i>	ABR	Invasive
<i>Rhodeus ocellatus</i>	PHO	Invasive
<i>Hemiculter leucisculus</i>	HEL	Invasive
<b>Cobitidae</b>		
<i>Misgurnus anguillicaudatus</i>	MIA	Invasive
<i>Cobitis sinensis</i>	COS	Indigenous
<i>Cobitis rarus</i>	COR	Indigenous
<b>Homalopteridae</b>		
<i>Vanmanenia stenosoma</i>	VAS	Indigenous

Continued

Order/Family/Species	Code	Classification
<b>PERCIFORMES</b>		
<b>Gobiidae</b>		
<i>Ctenogobius sp</i>	CTS	Indigenous
<b>Mastacembelidae</b>		
<i>Mastacembelus aculeatus</i>	MAA	Invasive
<b>Electridae</b>		
<i>Hypseleotris swinhonis</i>	HYS	Invasive
<i>Odontobutis obscura</i>	ODO	Invasive
<b>SIURIFORMES</b>		
<b>Bagridae</b>		
<i>Pseudobagrus truncatus</i>	PST	Indigenous
<b>Amblycipitidae</b>		
<i>Liobagrus styanii</i>	LIS	Indigenous
<b>SYNBRANCHIFORMES</b>		
<b>Synbranchidae</b>		
<i>Monopterus albus</i>	MOA	Invasive

#### Appendix II Summary statistic for explanatory variables measured for local habitat, tributary position, and dam characteristics

Variables	Abbreviation	Range	Mean
<b>Local habitat</b>			
Wetted width (m)	WW	5.1-48.4	21.1±11.9
Water depth (m)	WD	0.13-0.93	0.47±0.20
Water temperature (°C)	WT	15.9-29.0	19.8±2.5
Dissolved oxygen (mg/l)	DO	5.6-12.7	8.5±1.5
pH	pH		
Conductivity (mS/s)	Con	19.3-156	78.6±34.5
Current velocity (m/s)	CV	0.03-0.63	0.18±0.15
Canopy (%)	Can		
Substrate coarseness	SC	1.3-3.8	2.2±0.9
Substrate heterogeneity	SH	0-1.56	1.02±0.36
<b>Tributary position</b>			
Confluence link	C-link	16-26	20.9±2.6
Downstream link	D-link	2-17	4.28±4.01
<b>Dam characteristics</b>			
Dam height (m)	DH	0.6-3.8	2.5±1.8
Dam length (m)	DL	9.7-70.2	28.4±17.3
Dam area (m <sup>2</sup> )	DA	6.4-500.5	87.8±52.3
Dam number upstream (ind.)	DNU	0-18	3.0±3.4
Dam number downstream (ind.)	DND	3-20	9.0±4.0

# Short photoperiod increases energy intake, metabolic thermogenesis and organ mass in silky starlings *Sturnus sericeus*

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## ABSTRACT

Environmental cues play important roles in the regulation of an animal's physiology and behavior. One such cue, photoperiod, plays an important role in the seasonal acclimatization of birds. It has been demonstrated that an animal's body mass, basal metabolic rate (BMR), and energy intake, are all affected by photoperiod. The present study was designed to examine photoperiod induced changes in the body mass, metabolism and metabolic organs of the silky starling, *Sturnus sericeus*. Captive silky starlings increased their body mass and BMR during four weeks of acclimation to a short photoperiod. Birds acclimated to a short photoperiod also increased the mass of certain organs (liver, gizzard and small intestine), and both gross energy intake (GEI) and digestible energy intake (DEI), relative to those acclimated to a long photoperiod. Furthermore, BMR was positively correlated with body mass, liver mass, GEI and DEI. These results suggest that silky starlings increase metabolic thermogenesis when exposed to a short photoperiod by increasing their body and metabolic organ mass, and their GEI and DEI. These findings support the hypothesis that bird species from temperate climates typically display high phenotypic flexibility in thermogenic capacity.

**Keywords:** Basal metabolic rate (BMR); Body mass; Energy budget; Organ mass; Photoperiod; Silky starling; *Sturnus sericeus*.

## INTRODUCTION

Many organisms experience considerable seasonal changes in environmental conditions, such as fluctuations in temperature, food availability and photoperiod (Swanson, 2010). Physiological demands may also change because of increased energetic

requirements during reproduction or seasonal acclimatization (Starck & Rahmaan, 2003; Williams & Tieleman, 2000; Zheng et al., 2008a; 2014a). Reversible phenotypic flexibility allows individual organisms to adjust their phenotypes to meet different environmental, or ecological, demands (McKechnie, 2008; Piersma & Drent, 2003; Piersma & Gils, 2011). Many resident, small, birds in warm and temperate zones use phenotypic flexibility to cope with seasonal changes in temperature and photoperiod, and to develop morphological, physiological, and behavioural adaptations that assist in coping with various energy demands and enhance reproductive success (Swanson et al., 2014; Zheng et al., 2014a).

Photoperiod acts as an environmental cue for the seasonal acclimatization of thermoregulation in birds (Eyster, 1954; Heldmaier et al., 1989; Swanson et al., 2014). It has been demonstrated that an animal's body mass (Swanson et al., 2014; Wolfson et al., 1952), energy balance (Farner et al., 1961; Johnston, 1962; Ni et al., 2011), and basal metabolic rate (BMR) (Saarela & Heldmaier, 1987) all are affected by photoperiod. BMR is the minimum rate of energy expenditure of a non-growing, non-reproductive homeotherm measured under post-absorptive and thermoneutral conditions during the inactive phase of the circadian cycle (AL-Mansour, 2004; McKechnie & Wolf, 2004). The use of BMR as an index of energy expenditure has been the focus of considerable interest from environmental physiologists and comparative physiologists (McKechnie, 2008; Smit & McKechnie, 2010). The shorter day lengths that precede the onset of winter can induce an increase in the energy expenditure of animals (Heldmaier et al., 1989; Ni et al., 2011; Wolfson et al., 1952). Many birds have a variety of strategies to

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cope with this condition, such as increasing their body mass (Saarela & Heldmaier, 1987) and BMR (Swanson et al., 2014). For example, Chinese bulbuls *Pycnonotus sinensis* acclimatized to a short photoperiod developed significantly higher body mass and BMR than those acclimatized to a long photoperiod (Ni et al., 2011). Similar results have been found in other birds, like the dark-eyed junco *Junco hyemalis* (Swanson et al., 2014) and Japanese quail *Coturnix japonica* (Saarela & Heldmaier, 1987). Furthermore, increased energy intake can compensate for the increased energy expenditure associated with thermogenesis in harsh conditions (Hammond & Diamond, 1997; Williams & Tieleman, 2000). Finally, increasing the mass of metabolically active organs, such as the liver, kidney, heart and gastrointestinal tract, can increase BMR (Liu & Li, 2006; Williams & Tieleman, 2000; Zhang et al., 2006; Zheng et al., 2008b; 2014b).

The silky starling, *Sturnus sericeus*, is resident in most of south and southeast China but also disperses to northern Vietnam and the Philippines in winter (MacKinnon & Phillipps, 2000). This species feeds on fruits and seeds (Zheng & Zhang, 2002), prefers broadleaf and coniferous-broadleaf mixed forest, but is also found in orchards and tillable fields. It has a lower than predicted BMR for its body size (McKechnie & Wolf, 2004; McKechnie & Swanson, 2010), a high body temperature ( $T_b$ ), a high upper critical temperature ( $T_{uc}$ ), high thermal conductance, high evaporative water loss (EWL), and a relatively wide thermal neutral zone (TNZ) (Bao et al., 2014; Zhang et al., 2006). These characteristics suggest that it is adapted to warm climates, where selection for metabolic thermogenesis and water conservation is not strong. However, it is not known if the silky starling can change its body mass and BMR in response to different photoperiods.

In this study, we acclimated wild-caught silky starlings to different two different photoperiods (short vs long day-lengths) and examined the effects of these treatments on body mass, energy budget, metabolic rate and the mass of metabolically active organs. We hypothesized that short photoperiods are a key factor driving metabolic flexibility in silky starlings and consequently predicted that body mass, energy budget, metabolic rate, and organ mass would be higher in starlings acclimated to a short photoperiod than in those acclimated to a long photoperiod.

## MATERIALS AND METHODS

### Animals

Fifteen adult male silky starlings were used in the experiment. All birds were captured in Wenzhou city (N27°29', E120°51'), Zhejiang Province, China. The climate in Wenzhou is warm-temperate with an average annual rainfall of 1 700 mm across all months and slightly more precipitation during winter and spring. Mean daily maximum temperature ranges from 39 °C in July to 8 °C in January. The mean temperature from March to May is 15 °C (Wu et al., 2015; Zheng et al., 2014a). Body mass to the nearest 0.1 g was determined immediately upon capture with a Sartorius balance (model BT25S). After capture, birds were transported to the laboratory at Wenzhou University and

housed in separate plastic cages (50 cm×30 cm×20 cm) at 25 °C with 12L: 12D photoperiod. Food and water were supplied *ad libitum* and replenished daily. After one week of acclimation, starlings were moved into individual cages and then randomly assigned to one of two experimental groups; a short photoperiod (SD, 8L: 16D with lights on at 1000h, n=8) group, and a long photoperiod (LD, 16L: 8D with lights on at 0400h, n=7) group. Each group was acclimated to its respective photoperiod for 4 weeks. Each bird's body mass was monitored weekly during the four week acclimation period (Ni et al., 2011). All experimental procedures were approved by the Wenzhou City Animal Care and Use Committee, Zhejiang Province, China (Wu et al., 2015).

### Measurement of metabolic rate

Birds' metabolic rates were measured with an open-circuit respirometry system (AEI Technologies S-3A/I, USA). To take these measurements, individual birds were placed in 1.5 L plastic metabolic chambers inside a temperature-controlled cabinet at ±0.5 °C (Artificial climatic engine BIC-300, China). No ambient light reached birds within the cabinet so they were effectively in the dark, and therefore more likely to be at inactive, while confined within the apparatus. Dry CO<sub>2</sub>-free air was pumped through the chamber at 300 mL/min using a flow control system (AEI Technologies R-1, USA) (McNab, 2006). The fractional concentration of O<sub>2</sub> in the inlet chamber (dry CO<sub>2</sub>-free air) was determined using an oxygen sensor (AEI Technologies N-22M, USA). Oxygen consumption rates were measured at 30±0.5 °C within the thermal neutral zone and recorded at 20 s intervals (Zhang et al., 2006; Zheng et al., 2013). Each measurement period lasted for 1 hour and began after birds had first acclimated inside the metabolic chamber for about 1 hour. BMR was calculated for each individual as the average of the 30 lowest consecutive oxygen consumption recordings made over about 5 min. Food was removed 4h before each measurement period to minimize the heat increment associated with feeding. Metabolic rates were calculated from equation 2 of Hill (1972), and expressed as O<sub>2</sub>(mL)/h, corrected to STPD conditions (Schmidt-Nielsen, 1997).

### Energy budget

We regarded digestible energy intake as an index of total daily energy expenditure. A set quantity of food was provided during the 28 day experimental period but water was provided *ad libitum*. Food residues and feces were collected during the 2-days before temperature acclimation began (week 0) and weekly (every seventh day) thereafter throughout the 4-week experimental period. These residues were separated manually, then oven-dried at 60 °C until a constant mass was obtained. The caloric content of residual food and feces were determined using a C200 oxygen bomb calorimeter (IKA Instrument, Germany). Gross energy intake (GEI), feces energy (FE), digestible energy intake (DEI), and digestibility of energy were calculated according to Grodzinski & Wunder (1975) and Wu et al. (2014):

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

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

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

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

### Measurements of organ mass

All birds were euthanized by cervical dislocation at the end of the 4 week experimental period, and their heart, liver, spleen, lungs, brain, kidneys, stomach, small intestine and rectum removed and weighed to the nearest 0.1 mg. The gizzard, small intestine and rectum were then rinsed with saline to remove all gut contents before being dried and reweighed. These organs were then dried to a constant mass over 2 d at 75 °C and reweighed to the nearest 0.1 mg (Liu & Li, 2006; Williams & Tieleman, 2000).

### Statistics

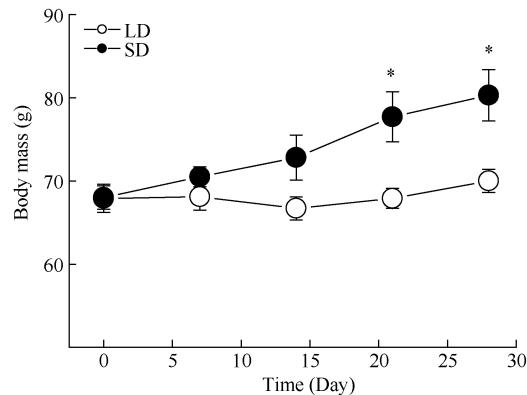
Data were analyzed using SPSS (version 12.0 for Windows). Distributions of all variables were tested for normality using the Kolmogorov-Smirnov test. Non-normally distributed data were normalized by being transformed into their natural logarithm prior to analysis. A repeated-measures analysis of variance (RM-ANOVA) was used to determine the significance of changes in body mass, GEI, FE, DEI and digestibility over time. Direct comparisons of the body mass of starlings acclimatized to LD or SD group were made using independent sample t-tests. With the exception of body mass, differences in the above variables between the LD and SD groups were evaluated using ANOVA or ANCOVA, with body mass as a covariate, where appropriate. Least-squares linear regression was used to test for correlations between log dry organ mass, log BMR and log body mass. For organ mass, body mass minus wet organ mass was used for the organ in question to avoid the statistical problem of part-whole correlations (Christians, 1999). Residuals were calculated from correlations and the residuals of log dry organ mass were regressed against those of log BMR to determine if organ mass was significantly correlated with BMR. Least-squares linear regression was used to evaluate the relationships between log body mass, log GEI and log DEI, and between log BMR, log body mass, log GEI and log DEI. All results are expressed as mean±SE; P<0.05 were considered statistically significant.

## RESULTS

### Body mass and metabolic rate

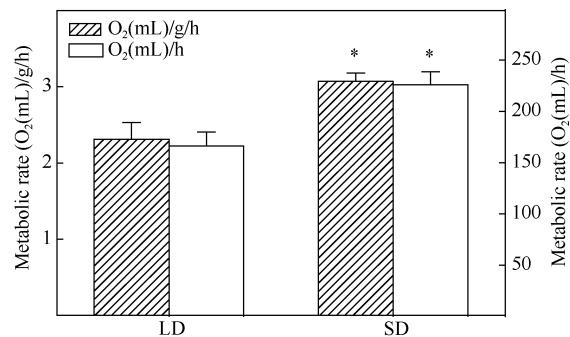
Overall, birds acclimated to the short photoperiod (SD) underwent a significant increase in body mass ( $t_{13}=2.850$ ,  $P<0.05$ ; Figure 1) and weighed on average 15% more than those acclimated to the long photoperiod (LD) by day 28 of the experiment. Significant group by time interactions were also evident for body mass ( $F_{4,28}=20.174$ ,  $P<0.01$ ). No group differences in body mass were apparent prior to photoperiod acclimation ( $t_{13}=0.040$ ,  $P>0.05$ ). However, a significant increase in body mass was apparent in the SD group by day 21 of

acclimation, and this increase was sustained for the 4 week duration of the experiment (Figure 1). An ANCOVA (with body mass as the covariate) indicated that the SD group had undergone a mass-specific ( $O_2(\text{mL})/\text{g/h}$ ) 32% increase in BMR relative to the LD group ( $F_{1,12}=7.814$ ,  $P<0.05$ , Figure 2). Individual birds in the SD group had undergone an average 36% increase in BMR ( $O_2(\text{mL})/\text{h}$ ) by day 28, causing their BMR to be significantly higher than that of LD birds (Figure 2). There was a significant positive correlation between log body mass and log total BMR ( $R^2=0.486$ ,  $P<0.01$ ; Figure 3).



**Figure 1 Trends in the body mass of silky starlings *Sturnus sericeus* acclimated to either a short, or a long, photoperiod for four weeks**

Data are shown as mean±SE, \*:  $P<0.05$ ; SD: short photoperiod; LD: long photoperiod.

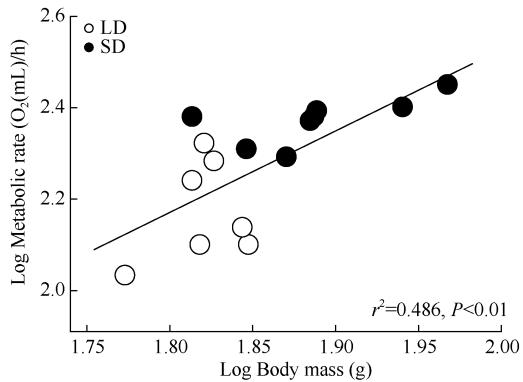


**Figure 2 Basal metabolic rates of silky starlings *Sturnus sericeus* acclimated to either a short, or a long, photoperiod for four weeks**

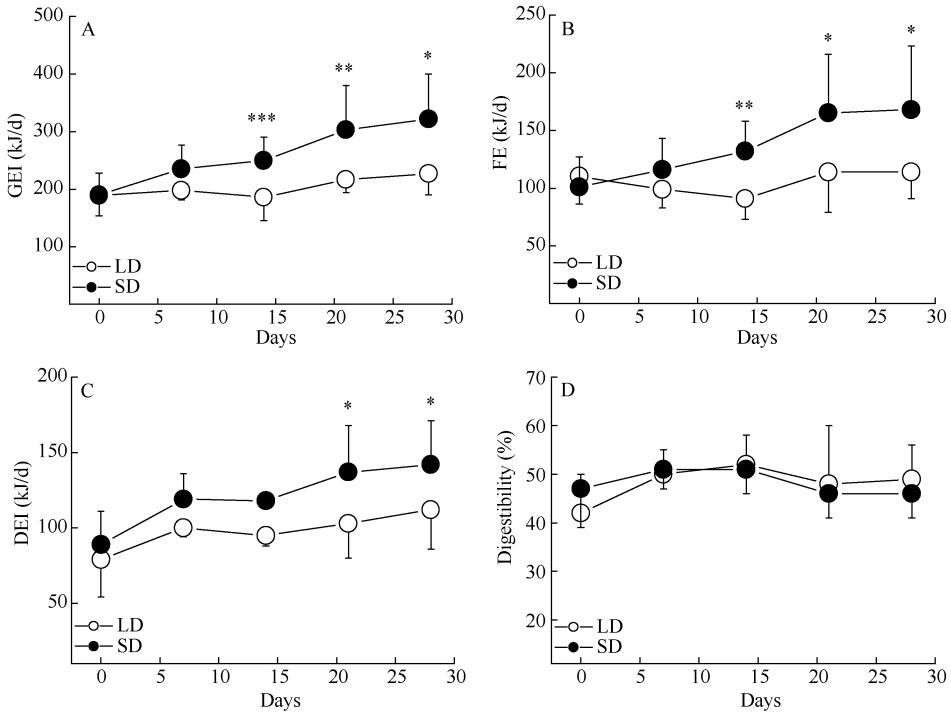
Data are shown as mean±SE, \*:  $P<0.05$ ; SD: short photoperiod; LD: long photoperiod.

### Energy budget

By the end of the 4-week acclimation period the SD group had significantly higher gross energy intake (GEI) ( $F_{1,13}=8.600$ ,  $P<0.05$ ; Figure 4A), feces energy (FE)( $F_{1,13}=5.692$ ,  $P<0.05$ ; Figure 4B), and digestible energy intake (DEI) ( $F_{1,13}=4.026$ ,  $P<0.05$ , Figure 4C), relative to the LD group. There was, however, no significant difference in digestive efficiency



**Figure 3 Correlation between the body mass and basal metabolic rate (BMR) of silky starlings *Sturnus sericeus* acclimated to either a short, or a long, photoperiod for 4 weeks**  
SD: short photoperiod; LD: long photoperiod.



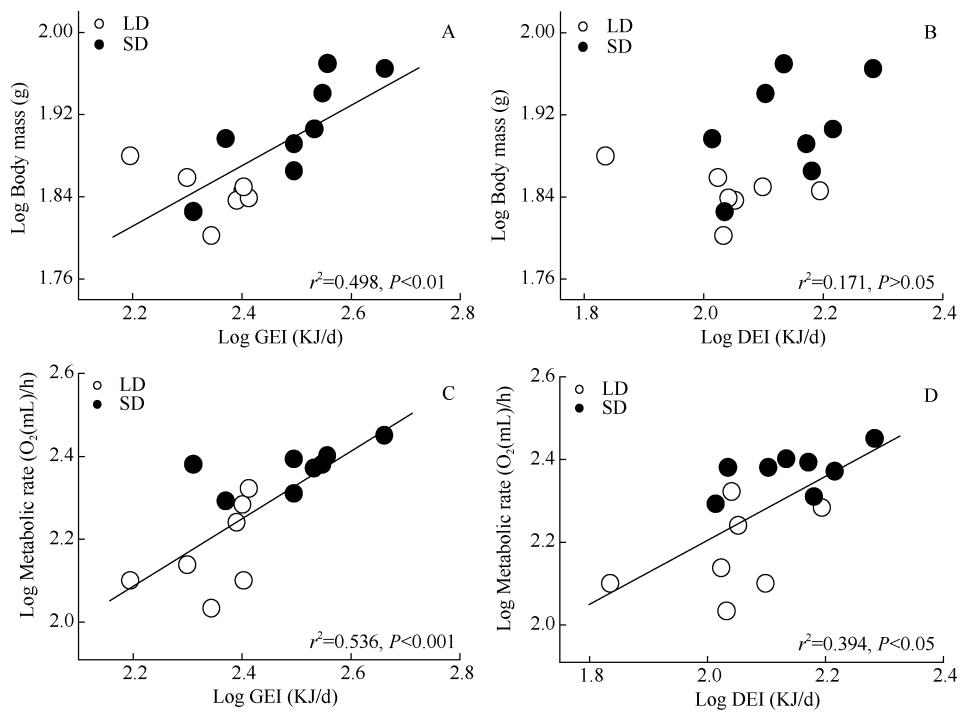
**Figure 4 Trends in GEI (A), FE (B), DEI (C), and digestibility (D) of silky starlings *Sturnus sericeus* acclimated to either a short, or a long, photoperiod for four weeks**

Data are shown as mean  $\pm$  SE, \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ ; SD: short photoperiod; LD: long photoperiod; GEI: gross energy intake; FE: feces energy; DEI: digestible energy intake.

#### Organ mass

ANCOVA (with body mass as the covariate) detected significant differences in the mass of several internal organs between the two treatment groups. These included differences in the wet and dry mass of the liver, gizzard, small intestine and digestive tract (Table 1). The average wet mass of the liver, gizzard, small intestine and digestive tract of the SD group was 24%, 26%, 22%, and 25%, respectively, higher than those of the LD group. The dry mass of the liver, gizzard, small intestine and digestive tract of

the SD group was 37%, 38%, 28%, and 32%, respectively, higher than those of the LD group. Other organs listed in Table 1 did not differ significantly in either wet or dry mass between groups. The partial relationships between log dry organ mass and log body mass (minus organ wet mass) were positive for all organs, however, only the dry mass of the heart, liver, kidney, gizzard, small intestine, and digestive tract were significantly correlated with body mass (Table 2). Residuals of liver dry mass were positively correlated with BMR residuals (Table 2).



**Figure 5 Correlations between body mass and GEI (A), body mass and DEI (B), basal metabolic rate and GEI (C), and between basal metabolic rate and DEI (D), in silky starlings *Sturnus sericeus* acclimated to either a short, or a long, photoperiod for four weeks  
SD: short photoperiod, LD: long photoperiod, GEI: gross energy intake, FE: feces energy, DEI: digestible energy intake.**

**Table 1 Mass (mean $\pm$ SE) of various internal organs of silky starlings *Sturnus sericeus* after four weeks acclimation to either a short (SD), or a long (LD), photoperiod**

	SD	LD	Significance
Sample size ( <i>n</i> )	7	8	
Wet mass			
Brain (mg)	1 689.1 $\pm$ 42.6	1 638.7 $\pm$ 46.0	$F_{(1,12)}=0.558, P>0.05$
Heart (mg)	849.3 $\pm$ 29.0	750.2 $\pm$ 31.3	$F_{(1,12)}=4.658, P>0.05$
Liver (mg)	2 082.3 $\pm$ 62.0	1 684.8 $\pm$ 67.0	<b><math>F_{(1,12)}=16.354, P&lt;0.01</math></b>
Spleen (mg)	116.3 $\pm$ 21.2	62.4 $\pm$ 19.6	$F_{(1,12)}=2.998, P>0.05$
Lung (mg)	625.7 $\pm$ 27.5	615.8 $\pm$ 30.0	$F_{(1,12)}=0.052, P>0.05$
Kidney (mg)	689.3 $\pm$ 31.9	648.6 $\pm$ 43.4	$F_{(1,12)}=0.647, P>0.05$
Gizzard (mg)	1 229.5 $\pm$ 25.2	907.7 $\pm$ 27.3	<b><math>F_{(1,12)}=64.566, P&lt;0.001</math></b>
Small intestine (mg)	2 120.1 $\pm$ 84.6	1 739.5 $\pm$ 91.4	<b><math>F_{(1,12)}=8.059, P&lt;0.05</math></b>
Rectum (mg)	235.8 $\pm$ 28.0	218.8 $\pm$ 30.2	$F_{(1,12)}=0.148, P>0.05$
Digestive tract (mg)	3 585.5 $\pm$ 111.5	2 865.9 $\pm$ 120.5	<b><math>F_{(1,12)}=16.567, P&lt;0.01</math></b>
Dry mass			
Brain (mg)	389.4 $\pm$ 9.6	362.4 $\pm$ 10.3	$F_{(1,12)}=3.179, P>0.05$
Heart (mg)	246.5 $\pm$ 29.3	223.4 $\pm$ 31.7	$F_{(1,12)}=0.247, P>0.05$
Liver (mg)	725.1 $\pm$ 19.4	545.9 $\pm$ 20.9	<b><math>F_{(1,12)}=34.011, P&lt;0.001</math></b>
Spleen (mg)	16.2 $\pm$ 4.7	28.4 $\pm$ 5.0	$F_{(1,12)}=2.711, P>0.05$
Lung (mg)	127.6 $\pm$ 6.2	118.0 $\pm$ 6.7	$F_{(1,12)}=0.967, P>0.05$
Kidney (mg)	191.9 $\pm$ 8.3	170.7 $\pm$ 8.9	$F_{(1,12)}=2.614, P>0.05$
Gizzard (mg)	389.8 $\pm$ 12.7	282.6 $\pm$ 13.8	<b><math>F_{(1,12)}=28.157, P&lt;0.001</math></b>
Small intestine (mg)	548.4 $\pm$ 29.8	428.3 $\pm$ 32.2	<b><math>F_{(1,12)}=6.480, P&lt;0.05</math></b>
Rectum (mg)	84.7 $\pm$ 11.8	63.6 $\pm$ 2.8	$F_{(1,12)}=1.264, P>0.05$
Digestive tract (mg)	1 022.9 $\pm$ 44.9	774.5 $\pm$ 48.5	<b><math>F_{(1,12)}=12.205, P&lt;0.01</math></b>

Values in bold type are statistically significant.

**Table 2** Linear regression statistics for partial and residual correlations of log dry organ mass versus log body mass (minus wet mass of the organ), and dry organ mass versus BMR in silky starlings *Sturnus sericeus* after 4 weeks acclimation to either a short (SD), or a long (LD), photoperiod

	Brain	Heart	Liver	Spleen	Lung	Kidney	Gizzard	Intestine	Rectum	Digestive mass
Partial Correlations										
$R^2$	0.479	0.623	0.608	<0.001	0.009	0.557	0.558	0.586	0.112	0.570
$P$	0.071	<b>&lt;0.05</b>	<b>&lt;0.05</b>	0.495	0.097	<b>&lt;0.05</b>	<b>&lt;0.05</b>	<b>&lt;0.05</b>	0.664	<b>&lt;0.05</b>
Residual Correlations										
$R^2$	0.063	0.079	0.321	0.173	<0.001	0.032	0.197	0.009	0.002	0.043
$P$	0.369	0.779	<b>&lt;0.05</b>	0.123	0.994	0.542	0.098	0.732	0.885	0.458

Values in bold type are statistically significant.

## DISCUSSION

Many small birds cope with seasonal stress in winter by adjusting their body mass (Dawson & Carey, 1976; Pohl & West, 1973; Swanson, 1991; Zheng et al., 2008a, 2014a), energy intake (Lou et al., 2013; Wu et al., 2014), metabolic rate (Klaassen et al., 2004; McKechnie, 2008; Zheng et al., 2008, 2014), and internal organ mass (Zheng et al., 2014b). The results of this study show that 4 weeks of acclimation to a short photoperiod is sufficient to cause significant changes in each of these variables in the silky starling; specifically, an increase in body and organ mass, BMR and energy intake associated with bodily metabolic functions. Collectively, these data suggest that the silky starling can change its thermogenic capacity in response to photoperiod, and provide further evidence to support the notion that small birds have high phenotypic plasticity with respect to thermogenic capacity (Liknes & Swanson, 2011; McKechnie et al., 2006; Swanson et al., 2014; Zhang et al., 2015).

### Effects of photoperiod on BMR and body mass

Seasonal changes in thermoregulation and body mass are important adaptive strategies for many small birds (Cooper, 2000; Swanson, 1990; Wu et al., 2015; Zheng et al., 2014b). Several environmental factors, such as temperature (Williams & Tieleman, 2000; Zheng et al., 2013), food quantity and quality (Wu et al., 2014), and photoperiod (Ni et al., 2011; Swanson et al., 2014) have been implicated in the regulation of seasonal variation in animals' thermogenic capacity and body mass. Short photoperiod could act independently and/or synergistically with lower temperature to enhance the thermogenic capacity of small birds (Ni et al., 2011; Saarela & Heldmaier, 1987; et al. Swanson et al., 2014). This notion is supported by the results of this study. SD starlings had significantly higher BMR compared to LD starlings after 4 weeks of photoperiod acclimation. Elevated BMR in response to short photoperiods, under either experimental, or natural, conditions, has been reported in other avian species (Ni et al., 2011; Saarela & Heldmaier, 1987; et al. Swanson et al., 2014). As BMR is directly related to the peak winter metabolic rate of thermogenesis in the wild, our data suggest that the effects of photoperiod on thermogenesis were both strong and significant. The same pattern was also found in body mass; SD starlings

underwent a gradual increase in body mass whereas LD starlings did not show any significant change in mass over the course of photoperiod acclimation. The time course data illustrate two interesting findings. First, no significant differences in body mass were found between the SD and LD starlings until day 21 of acclimation, indicating that a period of acclimation is required before short photoperiod exerted a significant effect on the body mass of silky starlings. Second, SD starlings displayed a steady increase in body mass during acclimation whereas LD starlings showed no significant change in mass. This suggests that the significant difference in body mass between the two groups occurred because LD starlings failed to increase their body mass during acclimation. Such increases in body mass will decrease the surface-to-volume ratio, which can reduce heat loss and thereby influence thermogenic demands and RMR (Christians, 1999; Swanson, 2010; Zheng et al., 2008). In addition, increased body mass is often the result of increases in fat deposits and/or metabolically active tissues (Williams & Tieleman, 2000; Wu et al., 2014; Zheng et al., 2014a), and is supported by increases in other parameters, such as GEI, DEI, and internal organ mass (see below).

### Effects of photoperiod on energy budget

In birds, body mass is an important indicator of their level of energy balance (Doucette & Geiser, 2008). Many birds display phenotypic flexibility in maintaining energy requirements and are capable of regulating their body mass up or down over a period of time in response to thermal acclimation (Vézina et al., 2006; Zheng et al., 2013, season (Petit et al., 2014; Swanson, 1990; Zheng et al., 2008a, 2014b) and photoperiod (Ni et al., 2011; Saarela & Heldmaier, 1987; Saarela & Vakkuri, 1982; Swanson et al., 2014)). Adjustments in energy intake and budget can compensate for the increased energy expenditure associated with thermogenesis under short photoperiod conditions (Ni et al., 2011; Saarela & Heldmaier, 1987). The significant increases in GEI and DEI observed in the SD group are consistent with the adaptive changes in energy intake and utilization in response to short photoperiod documented in many other small birds (Farner et al., 1961; Lou et al., 2013). Our repeated measurements of body mass and energy intake over the 4 week course of the experiment show the pattern of temporal change in these variables. The fact that the GEI of the

SD group was 42% higher than that of LD group suggests that the increased body mass of SD starlings was probably due to increased energy intake during the 4 week acclimation period. Increased GEI and DEI also contributed to the observed increase in body mass and BMR, as indicated by the positive correlation between these variables (Figure 5). One interesting finding of this study is the timing of adjustments made by birds in response to short photoperiod. A significant increase in body mass was apparent in the SD group after three weeks of acclimation. Moreover, a significant increase in GEI was apparent in the SD group after just two weeks of acclimation. These data provide further evidence that physiological responses to short photoperiod can occur relatively quickly (Heldmaier et al., 1989; Ni et al., 2011). The ability to make such physiological adjustments in response to changes in ambient photoperiod would clearly be advantageous for small birds (Swanson et al., 2014). The absence of a significant difference in digestive efficiency between the two experimental groups raises the question; "what is the benefit of developing a larger gut in response to short photoperiods?" Starlings consume more food during the short day of winter, which appears to stimulate the enlargement of digestive organs such as the gizzard, small intestines and the overall digestive tract (Table 1). Increasing gut size in response to increasing food quantity can yield several benefits. One is that it allows a constant mean retention time, thereby maintaining digestive efficiency if the ingestion rate increases (Karasov, 2011; Karasov et al., 2011).

#### Effects of photoperiod on organs

Short photoperiod has also been associated with changes in organ size and mass (Ni et al., 2011; Yang et al., 2009). One idea is that energetically challenged birds may increase their food intake, and at the same time, reorganize their internal organs to improve thermal and digestive efficiency (Liu & Li, 2006; Starck & Rahmaan, 2003; Williams & Tieleman, 2000). Several authors have suggested that much of the energy used in basal metabolism is consumed by visceral organs (Daan et al., 1990; Hansen et al., 2010; Piersma et al., 1996; Rolfe & Brown, 1997). The results of this study indicate that acclimation to a short photoperiod for 4 weeks was followed by significant increases in the mass of the liver, gizzard, small intestine and digestive tract, but not that of the heart and kidney. Increases in liver and small intestine mass are associated with thermogenic capacity. For example, the liver, kidneys, heart, and small intestine contribute to about 60% of total heat production (Clapham, 2012; Rolfe & Brown, 1997). Thus, the observed increase in the mass of the liver, gizzard, small intestine, and digestive tract in SD starlings may reflect adaptive regulation of organ morphology to accommodate increased food intake and digestion, ultimately contributing to an altered metabolic rate. Interestingly, only the dry mass of the liver was significantly correlated with BMR. This finding suggests that liver mass has a greater effect on BMR than that of other digestive organs. The liver is one of the largest and most metabolically active organs in birds. Under basal metabolic conditions, the liver has been shown to contribute 20%-25% of total heat production in

animals (Coutre & Hulbert, 1995). The liver's hepatocyte oxygen consumption is devoted to mitochondrial ATP production, mitochondrial proton leak and non-mitochondrial processes (Brand et al., 2003; Else et al., 2004). The mass of the liver is less often associated with BMR, however, positive correlations between BMR and liver mass have been documented in Eurasian tree sparrows and Chinese bulbuls (Zheng et al., 2013, 2014b). Thus, the positive correlation between liver mass and BMR in this study is not without precedent. The proportionately large nutritional liver mass of birds may facilitate the liver making a significant contribution to BMR (Coutre & Hulbert, 1995; Zheng et al., 2013).

In conclusion, environmental cues play important roles in the mediation of seasonal adaptation of body mass, thermogenesis, and energy intake in small birds. The results of this study indicate that the silky starling displays a general, elevated, whole-body response to short photoperiod, including increased body and organ mass, enhanced BMR and energy intake. The evident morphological and physiological flexibility in photoperiodic acclimation displayed by this species would be advantageous given the wide variation between the winter and summer climate in Wenzhou.

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# Diurnal brooding behavior of long-tailed tits (*Aegithalos caudatus glaucogularis*)

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## ABSTRACT

Brooding is a major breeding investment of parental birds during the early nestling stage, and has important effects on the development and survival of nestlings. Investigating brooding behavior can help to understand avian breeding investment strategies. From January to June in 2013 and 2014, we studied the brooding behaviors of long-tailed tits (*Aegithalos caudatus glaucogularis*) in Dongzhai National Nature Reserve, Henan Province, China. We analyzed the relationships between parental diurnal brooding duration and nestling age, brood size, temperature, relative breeding season, time of day and nestling frequencies during brooding duration. Results showed that female and male long-tailed tit parents had different breeding investment strategies during the early nestling stage. Female parents bore most of the brooding investment, while male parents performed most of the nestling feedings. In addition, helpers were not found to brood nestlings at the two cooperative breeding nests. Parental brooding duration was significantly associated with the food delivered to nestlings ( $F=86.10$ ,  $df=1$ ,  $193.94$ ,  $P<0.001$ ), and was longer when the nestlings received more food. We found that parental brooding duration declined significantly as nestlings aged ( $F=5.99$ ,  $df=1$ ,  $50.13$ ,  $P=0.018$ ). When nestlings were six days old, daytime parental brooding almost ceased, implying that long-tailed tit nestlings might be able to maintain their own body temperature by this age. In addition, brooding duration was affected by both brood size ( $F=12.74$ ,  $df=1$ ,  $32.08$ ,  $P=0.001$ ) and temperature ( $F=5.83$ ,  $df=1$ ,  $39.59$ ,  $P=0.021$ ), with it being shorter in larger broods and when ambient temperature was higher.

**Keywords:** Long-tailed tit; *Aegithalos caudatus glaucogularis*; Brooding; Daytime; Early nestling stage

## INTRODUCTION

A stable thermal environment is essential for egg hatching and nestling development during avian reproduction (Zhao et al., 2002). Brooding, whereby adult birds settle down with nestlings to provide heat (Johnson & Best, 1982), is an important investment in addition to feeding during the nestling period. For young nestlings, whose abilities to maintain body temperature are generally weak (especially that of altricial birds), brooding is extremely important to maintain a constant body temperature (Johnson & Best, 1982; Visser, 1998; Zheng, 2012).

The energy investments of caring for nestlings have profound effects on adult fitness (Monaghan, 2004). During the first days of the nestling period, brooding behavior can directly affect adults through loss of body mass, a phenomenon that has often been found in females of species in which the females undertake most of the brooding investment (Chastel & Kersten, 2002; Moreno, 1987, 1989a). Although brooding is important during the nestling stage, current studies on parental investment during this period have primarily focused on nestling feeding behavior. In some altricial birds, such as the long-tailed tit (*Aegithalos caudatus*) (MacColl & Hatchwell, 2003), twite (*Acanthis flavirostris*) (Zhao et al., 2003), and azure-winged magpie (*Cyanopica cyana*) (Valencia et al., 2006), the feeding frequencies of female parents are significantly less than those of male parents at the early nestling stage, though this difference gradually disappears by the late nestling stage. It is worth noting that brooding investment is often borne by the

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females in these species (Ru et al., 1997; Song, 1981; Zhao et al., 2003), and focusing on adult nestling feeding alone may not completely reflect parental investments and strategies.

The long-tailed tit is a small passerine bird in the family Aegithalidae (Zheng, 2006). Its breeding biology (Fang & Ding, 1997; Gaston, 1973; Li et al., 2012; Song, 1981) and nestling feeding behaviors (Hatchwell & Russell, 1996; Hatchwell, 1999; MacColl & Hatchwell, 2003) have been extensively investigated. Brooding behavior, however, has only been described briefly (Song, 1981). In this study, we investigated the differences in diurnal brooding investment between female and male long-tailed tits and analyzed the associations between diurnal brooding duration and nestling age, brood size, ambient temperature, breeding season, time of day and nestling feeding frequency during brooding.

## MATERIALS AND METHODS

### Study area and study population

This study was conducted in the Dongzhai National Nature Reserve ( $N31^{\circ}28'32''09'$ ,  $E114^{\circ}18' -114^{\circ}30'$ ) between January and June in 2013 and 2014. The reserve, characterized by its rich avian diversity, is located in the south of Henan Province and west of the Dabieshan Mountains, and is at the transitional region between subtropical and temperate zones. Annual mean temperature and precipitation are  $15.1^{\circ}\text{C}$  and 1 209 mm, respectively (Song & Qu, 1996).

There are three subspecies of long-tailed tits in China, that is, *A. c. caudatus*, *A. c. vinaceus* and *A. c. glaucogularis* (Zheng, 2006). The long-tailed tit population at our study site belongs to the subspecies *A. c. glaucogularis*. According to morphological characteristics and breeding isolation, the subspecies *A. c. glaucogularis* and *A. c. vinaceus* have recently been treated as the independent silver-throated tit, *A. glaucogularis* (Harrap, 2008). In this study, however, we followed the traditional classification (Zheng, 2006) and treated the Dongzhai National Nature Reserve population as a subspecies of the long-tailed tit. The long-tailed tits are resident in the study area and occur in conifer, broadleaf forests and shrubs. The breeding season is usually from late January to early June, and annual nesting success of this population is about 30% (Li et al., 2012). Helpers exist at about 30% of the nests during the nestling stage, with most helpers being male failed breeders (Li et al., 2012).

### Data collection and analyses

Each adult long-tailed tit involved in this study was banded with a metal ring and a unique combination of colored rings so that each individual could be identified. Banding was usually conducted at two periods: (1) before the breeding season (late December to January) when tits were still flocking and easily caught, and (2) during the breeding season when we continued to band the unbanded adults and nestlings (around 10 days old) born in that season (see Li et al., 2012 for banding method details). As we have studied the long-tailed tit population for several years, the birds involved in this study included those banded between 2011 and 2014 (2011:  $n=9$ ; 2012:  $n=27$ ; 2013:

$n=35$ ; 2014:  $n=16$ ). When banding a bird, we collected a 20-50  $\mu\text{L}$  blood sample via venipuncture of the brachial vein for molecular sexing (see Li et al., 2010 for details). During the breeding seasons, we looked for long-tailed tit nests by following adults carrying nesting material and food, and by searching potential nesting sites. When a nest was found, we gave it a unique nest number as its nest identity, and checked it every one to three days to ascertain its breeding status. After nestlings hatched, we used video cameras (Samsung SMX-F40, Sony HDR-XR160E, Sony HDR-SR10 and Sony HDR-HC5) placed 0.5-3.0 m from the nests to film the brooding and feeding behaviors of adults every one to three days. Filming was usually carried out between 0800-1800 h and usually lasted for at least 1 h. The tit banding and sampling methods used in this study are common in ornithological research and our operation strictly followed current regulations. Among the nests involved in the analyses of brooding behavior, no adults or nestlings were banded during the early nestling period (i.e., the period when brooding normally occurred) and therefore our analyses were not affected by banding or sampling. Camera concealment was also carefully considered and the tits resumed their activities soon after the cameras were set up (usually within 20 min), with no apparent adverse effects detected. No adults or nestlings were banded during the brooding stage.

To explore the factors affecting diurnal brooding duration, we analyzed whether brooding duration was related to nestling age, brood size, ambient temperature, relative breeding season, time of day or nestling feeding frequency during brooding. Nestling age was measured in days after hatching, with day 0 being the day of hatching (long-tailed tit broods hatch synchronously). Based on field observations, brooding behavior of the long-tailed tit population mostly occurred between nestling age of one and five days. We therefore included videos filmed for nestlings up to six days old. Brood size was the number of nestlings observed when filming the nest. We used the mean daily temperature recorded by the Xinyang Weather Station as the ambient temperature of the filming day. Xinyang Weather Station is the closest weather station to our study site (about 25 km) and the weather data were downloaded from the China Meteorological Data Sharing Service System (<http://cdc.nmic.cn/home.do>). Relative breeding season was the number of days after the hatching date of the first nest in each breeding season, with this variable used to reflect the relative position of the nest in the breeding season. When analyzing brooding duration in relation to time of day, we divided daytime (0800-1800h) into ten 1-h periods (0800-0900h, 0900-1000h, etc.) and assigned the brooding behavior into the corresponding time period based on the time of its occurrence. If the brooding behavior occurred across time periods, the time period was considered the period of longer brooding duration. For example, if brooding behavior occurred from 0940h to 1005h, its duration in period 0900-1000h (20 min) was longer than that in period 1000-1100h (5 min), and therefore the brooding behavior was assigned to period 0900-1000h.

The nests of long-tailed tits are dome-shaped, with an entrance hole on the side near the top (Li et al., 2012). We treated a behavior as brooding when an adult entered the nest

and settled down on the nestlings; it was not treated as a brooding behavior if an adult entered the nest for a short period of time without settling down. Brooding duration was the time between an adult's entry and leaving of the nest (min). Nestling feeding frequency included both feeding by the brooding adult who carried food to the nest and then brooded and feeding by non-brooding adults whose food was usually transferred by the brooding adult to the nestlings.

Data were analyzed using linear mixed models fitted by restricted estimation maximum likelihood. Linear mixed models can account for both fixed and random effects (Bolker et al., 2009). In the analysis, the duration of each brooding behavior was treated as the dependent variable. Year, nest identity, video identity and adult identity were included as random factors to control for non-independence between observations of the same year, same nest, same observation and same adult, respectively. Explanatory variables were the sex of parents, nestling age, square of nestling age (brooding duration may vary with age in an asymptote way), brood size, temperature, relative breeding season, time of day and nestling feeding frequency by adults during brooding. Two-way interactions of the above factors were also considered, except for that

between nestling age and square of nestling age (biologically meaningless). Significance of terms was assessed from type III F tests. The best fitting model was obtained by sequentially dropping nonsignificant terms from the initial model until all terms were significant (Crawley, 2007). All statistical analyses were performed in R v.2.14 (R Development Core Team, 2012) using package lme4 (Bates et al., 2014). Mean values were reported with standard deviation (mean $\pm$ SD).

## RESULTS

A total of 48 nests with 1-6 day-old nestlings were analyzed. Each nest was filmed  $1.54\pm0.71$  times, and each filming lasted for  $1.23\pm0.57$  h. Among all nests, two exhibited cooperative breeding, with each nest having a male helper. Among the 206 brooding behaviors recorded, female parents engaged in brooding behavior at a much higher rate (87.86%,  $n=181$  times). The brooding duration of female parents was also significantly longer than that of male parents (female parents:  $9.50\pm8.13$  min, male parents:  $4.70\pm5.38$  min) (Table 1 and Figure 1A). For the two cooperative breeding nests, helpers were not observed to brood nestlings.

**Table 1** Linear mixed model analysis results of parental brooding duration of long-tailed tits (*Aegithalos caudatus glaucogularis*)

Factors	Estimate	SE	df	F	P
Sex of parents	-3.86	-2.27	1, 20.92	8.30	0.009
Nestling age	-1.41	0.58	1, 50.13	5.99	0.018
Brood size	0.64	32.08	1, 32.08	12.74	0.001
Ambient temperature	-1.60	0.66	1, 39.59	5.83	0.021
Total feeding frequencies	3.91	1.34	1, 193.94	86.10	<0.001
Nestling age $\times$ total feeding frequencies	1.42	0.42	1, 198.10	11.35	<0.001

The best fitting model for fixed factors is shown above. Year, nest identity, video identity and adult identity were included in the model as random factors. Estimates of their variances $\pm$ SD were  $0.00\pm0.00$ ,  $1.25\pm1.12$ ,  $2.27\pm1.51$ , and  $9.99\pm3.16$ , respectively. The estimate $\pm$ SE of adults' sex was estimated for males, with females being the reference.

Female and male parents also differed in their investment in nestling feeding during brooding. In the non-cooperative breeding nests ( $n=46$ ), nestling feedings ( $n=22$ ) during brooding of male parents ( $n=24$  times) were all conducted by the male parents themselves who brought food when returning to the nest. However, during brooding of female parents ( $n=177$  times), only 39.94% ( $n=143$  times) of nestling feedings were performed by females carrying food back to the nest, with the remaining nestling feeding conducted by the male parents (60.06%,  $n=215$  times). In the two cooperative breeding nests, male parent brooding was only recorded once, during which the male parent and a male helper each fed the nestlings once; during female parent brooding ( $n=4$  times), nestlings were fed 21 times, with the brooding female parent, male parent and helper bird accounting for four, nine, and eight times, respectively.

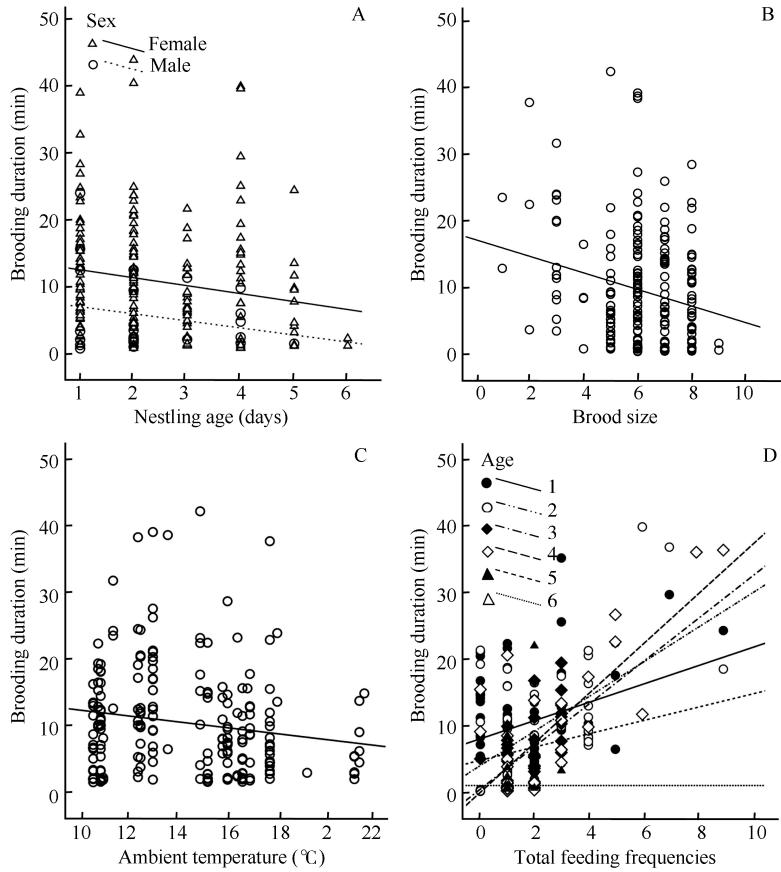
At the early nestling stage, brooding duration was negatively affected by nestling age, brood size and temperature (Table 1). As nestlings aged, brooding duration significantly declined (Figure 1A). Brooding duration was  $10.43\pm7.84$  min ( $n=58$ ) when nestlings were one day old, but only  $1.08\pm0.18$  min ( $n=2$ ,

based on 9 h of filming of seven nests) when nestlings were six days old. Brooding duration was significantly shorter in larger broods (Figure 1B) and when the ambient temperature was higher (Figure 1C).

In addition, brooding duration increased significantly with the increase in nestling feeding rates (Table 1 and Figure 1D). Brooding duration was also affected by the interaction between nestling age and adult feeding frequencies during brooding (Table 1). As nestlings aged from one to four days old, the effects of feeding frequency on brooding duration increased, as indicated by the slope of fitted lines (Figure 1D). When nestlings were five days old, the effect of feeding frequencies on brooding duration became smaller than that during the first four days, and had almost had no effect on brooding duration when nestlings were six days old (Figure 1D).

## DISCUSSION

During the early nestling stage, both brooding duration and brooding frequencies of the long-tailed tit female parents were



**Figure 1** Effects of nestling age (A), brood size (B), ambient temperature (C) and total feeding frequencies during brooding (D) on parental brooding duration in long-tailed tits (*Aegithalos caudatus glaucogularis*)

higher than those of the male parents, indicating that female parents bore most of the brooding investment. This is similar to that observed in most passerine birds, in which female parents assume all or most brooding investment (Chastel & Kersten, 2002; Moreno, 1989b).

Unlike female parents, long-tailed tit male parents invested more time in feeding nestlings during the early nestling stage. MacColl & Hatchwell (2003) also found that female long-tailed tits in a British population fed nestlings significantly less frequently than the male parents did during the early nestling stage, though these differences disappeared by the late nestling stage. They suggested that the differences might be due to the female taking on egg laying and incubation duties, and therefore investing less time in nestling feeding than that undertaken by male parents (MacColl & Hatchwell, 2003). Our study suggests that the division of labor (i.e., brooding or feeding) could be an alternative explanation to female and male differences in nestling feeding frequencies at the early nestling stage. The division of labor might be a breeding strategy formed during the long-term evolution of birds, and may be beneficial to female parents who can focus on brooding and protecting nestlings (Katzenberger et al., 2015).

Although long-tailed tits are a cooperative breeding species,

this study, like previous researches (Gaston, 1973; Hatchwell et al., 2004; Li et al., 2012), did not find helpers engaging in the brooding of nestlings. Again, this might be related to the division of labor between females and males. In long-tailed tits, most helpers are failed male breeders (Hatchwell et al., 2004; Li et al., 2012; MacColl & Hatchwell, 2002), with males more likely to engage in nestling feeding than brooding. Considering that only two cooperative breeding nests were involved in our study, however, the phenomenon that long-tailed tit helpers in the Dongzhai population do not participate in brooding needs to be confirmed with more observations in future studies.

During the early nestling stage in long-tailed tits, parental brooding duration declined as nestlings aged. This is probably related to the enhancement of the nestlings' ability to maintain their own body temperatures (Jia et al., 2001; Katzenberger et al., 2015; Zheng, 2012). According to our observations, the nestlings of the long-tailed tit population in Dongzhai National Nature Reserve likely developed the ability to maintain body temperature at six days old, a little earlier than that recorded from Changbaishan Mountain, which is ~7.5 days old (Song, 1981). The phenomenon that brooding duration declines with nestling age also commonly exists in other bird species, such as willow grouse (*Lagopus lagopus*) (Pedersen & Steen, 1979)

and black sparrowhawk (*Accipiter melanoleucus*) (Katzenberger et al., 2015).

Our results showed that the increase in nestling feeding frequency during brooding significantly lengthened brooding duration in long-tailed tits, implying that brooding adults may adjust brooding duration according to their partner's feeding investment and nestling demands. Moreover, the interaction between nestling age and adult feeding frequencies during brooding also significantly affected brooding duration. During the first four days after nestlings hatched, the effects of feeding frequencies on brooding duration increased with nestling age. This may be due to the increase in food demands of nestlings with age, with brooding adults being able to adjust brooding investment according to nestling demands, leading to stronger effects of feeding frequency on brooding duration. When nestlings were five days old, the effects of feeding frequency were smaller than those during the first four days, and almost no effects were observed when nestlings were six days old. This might be due to nestlings gaining the ability to maintain body temperature at five to six days old, with the importance of brooding and the relationship between brooding duration and other factors becoming weaker compared with that during the first four days. Alternatively, it might also be due to that fewer brooding behaviors were recorded when nestlings were five and six days old (13 and two times, respectively) as parents reduced their investment in brooding older nestlings.

In addition, brooding duration of long-tailed tit was shorter in larger broods, a pattern that has also been found in other birds (Chastel & Kersten, 2002; Koenig & Walters, 2011). The effects of brood size on brooding duration is probably related to the thermal preservation and production of nestlings. In larger broods, thermal preservation of nestlings is more effective (Royama, 1966), and total heat produced increases with nestling number (Clark, 1984), enabling larger broods to better regulate body temperature.

The brooding behavior of birds can be affected by ambient temperature. For example, brooding duration is negatively correlated with ambient temperature in species like blood pheasants (*Ithaginis cruentus*) (Jia et al., 2001) and acorn woodpeckers (*Melanerpes formicivorus*) (Koenig & Walters, 2011). In this study, daily mean temperature had similar effects on brooding duration of the long-tailed tit. This phenomenon could be related to nestling heat demands (Koenig & Walters, 2011): when ambient temperature is low, the temperature difference inside and outside the nest is greater and heat is lost quickly, resulting in adults spending a longer time in brooding behavior; by contrast, when the ambient temperature is high, the heat of the nest is lost slowly and adults can spend a relatively shorter time in brooding behavior.

In summary, long-tailed tit females and males exhibited different breeding behavior and division of labor in the early nestling stage: female parents assumed most of the brooding investment, while male parents engaged in more nestling feeding investment. The brooding behavior of long-tailed tits was related to many factors, with brooding duration affected by nestling age, brood size and ambient temperature.

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# Effects of osmotic pressure, temperature and stocking density on survival and sexual reproduction of *Craspedacusta sowerbii*

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## ABSTRACT

The effects of osmotic pressure, temperature and stocking density on medusae survival of *Craspedacusta sowerbii* were examined. The medusae were shown to be sensitive to the variations of osmotic pressure. And the survival time was <90 h at 34 mOsm/L and it declined rapidly with rising osmotic pressure. The peak survival time of >200 h was recorded at 0.2 mOsm/L. Comparing with 27 °C and 32 °C treatments, 23 °C treatment yielded lower activities at a range of 8–13/min. However, there was a longer survival time. A non-linear relationship existed between survival time and stocking density. Lower density resulted in larger body size. And sexual reproduction resumed after breeding for >22 days. Newly-formed polyps and medusae appeared subsequently but only in the higher-density groups of 10, 14 and 18 ind./L. It suggested that the number of newly-formed polyps and medusae was highly dependent on stocking density. That is, a higher stocking density produced more organisms. However, newly-formed medusae died within one month and none grew a diameter of >5 mm.

**Keywords:** *Craspedacusta sowerbii*; Osmotic pressure; Temperature; Stocking density

## INTRODUCTION

As a freshwater jellyfish, *Craspedacusta sowerbii* is widely distributed domestically in the Yangtze River (Dumont, 1994; Jankowski, 2001). Although several species of *Craspedacusta* have been identified, including *C. chuxiogensis*, *C. kawaii*, *C. kiatingi*, *C. kuoi*, *C. sichuanensis*, *C. sinensis* and *C. ziguiensis*, they were considered as the synonyms of *C. sowerbii* Lankester, 1880 (Bouillon & Boero, 2000a; Kramp, 1961; Lankester,

1880). Since the documentation of *C. sowerbii*, uni-sexual medusae organisms had been collected from many foreign habitats (Deacon & Haskell, 1967; Lytle, 1962). All Chinese medusae are composed of an almost equal number of females and males harvested from Zhejiang, Sichuan, Hubei and Yunnan Provinces (He, 2005). And it has survived as a successful invasive species on all continents except for Antarctica (Jankowski et al., 2008).

Previous studies of *C. sowerbii* have concentrated upon the four major aspects, including taxonomy (Bouillon & Boero, 2000b; Kramp, 1950), life cycle (Acker & Muscat, 1976; Lytle, 1959), distribution (Akçaaalan et al., 2011; Dumont, 1994; Lytle, 1957) and nutrition relationship & ecological impact (Boothroyd et al., 2002; Dodson & Cooper, 1983; Jankowski et al., 2005; Stanković & Ternjej, 2010). However, few studies have examined its survival rate under different environmental conditions because of its unpredictable occurrence and difficult artificial breeding.

Up to the present, osmotic pressure, temperature and population density have been proposed as three major ecological influencing factors of survival, reproduction and behaviors of some hydrozoan species (Ma & Purcell, 2005; Mills, 1984). And the invasive species have unique patterns of migration and distribution. The present study was intended to elucidate the effects of osmotic pressure, temperature and stocking density on the survival of *C. sowerbii* medusae so as to provide some valuable

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references for preventing its invasion.

## MATERIALS AND METHODS

### Animal specimens

The specimens of *Craspedacusta sowerbii* were collected from a research pond at Endangered Fish Conservation Center (EFCC), Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS) (Kunming, Yunnan Province of China). And the identity of each organism was confirmed by taxonomists at KIZ. Their morphological characteristics were observed under a dissecting microscope (ZEISS Stemi 2000-c, Edmund Optics, Barrington, NJ, USA). And the confirmation of *C. sowerbii* was based upon the following characteristics: a maximal umbrella diameter of 15 mm, 190–215 tentacles, tentacle order III, annular and papilla-shaped nematocyst warts and approximately 128 tubular statocysts & slightly yellowish gonads. The fishery pond (length×width×depth=60.0 m×50.0 m×1.2 m) was used for medusae breeding. Both genders of *C. sowerbii* appeared simultaneously and females slightly predominated.

### Experiment I: Effects of osmotic pressure on different growth stages of *C. sowerbii*

Medusae of three different growth stages (average diameters of 5.0, 8.0 and 15 mm respectively) were collected and then transferred into a 10 L laboratory fish tank filled with pond water for 2 h pre-adaptation. After 2-hour equilibration, each growth stage specimens (including 5 females and 5 males) were independently distributed at 7 gradients containing 1 L solution with duplicates respectively. Seven gradients of osmotic pressure (0.2, 34, 85, 170, 342, 684, 1 709 mOsm/L) were established by adding NaCl to a  $6 \times 10^{-4}$  mol/L CaCl<sub>2</sub> solution using underground water and the temperature was maintained at  $21 \pm 0.5$  °C (the same as pond). In each beaker, the diet was composed of ample shrimps *Eubranchipus vernalis* (40 ind./day, body size range of 1.5–2.5 mm). Survival time was recorded immediately after immersion and observation length determined according to osmotic pressure. Dead *C. sowerbii* medusae were removed immediately.

### Experiment II: Effect of temperature on *C. sowerbii*

The procedures of specimen collection and pre-adaptation were similar to those of Experiment I. Only mini-sized medusae (average diameter=5.0 mm) were used. Three temperature gradients (23 °C, 27 °C, 32 °C) were established with duplicates (a total of 6 fish tanks). Heating rod was used for stabilizing temperature. Twenty percent of water was replaced every 2 days with pond water. Thirty females and 30 males were placed into each tank after pre-adaptation and fed daily with 100 fairy shrimps (body size=1.5 mm). Survival numbers were recorded once daily and activity was checked twice daily.

### Experiment III: Effect of stocking density on *C. sowerbii*

A total of 10 tanks (10 L in volume) were suspended and immobilized by string at the corner of pond. Medium-sized specimens (average diameter=8.0 mm) were collected from pond and transferred into the tanks. And 5 stocking densities (2,

6, 10, 14, 18 ind./L) were established in duplicates (gender ratio=1:1). Ample fairy shrimps (body size=1.8 mm) were provided within each tank. Newly-formed polyps and medusae were checked daily for 76 days and new batches were transferred into a new tank suspended in pond. Average diameter (6 random specimens each time) and survival rate were measured every 3 days. One-third of water was replaced every 3 days by surrounding pond water.

### Statistical analyses

The specimens sinking to the bottom of fish tank, without swimming behavior and showing no response to any stimulation were judged as dead *C. sowerbii*. Survival time (T) was calculated by the equation of  $T=T_1-T_0$  where  $T_0$  was the starting time of study and  $T_1$  ending time when all specimens died. Activity was measured by recording the rising counts of *C. sowerbii* over half depth of water per minute in triplicates. Activity was calculated by the following formula:

$$\text{Activity}=\text{rising no./min} \quad (1)$$

All statistical analyses were conducted by SAS statistical software (SAS 9.1). And statistical significance was assessed at a level of  $P=0.001$ .

## RESULTS

### Experiment I: Stress tolerance of osmotic pressure

Osmotic pressure influenced greatly the survival time of *C. sowerbii* medusae (Table 1). There were significant variations of survival time among osmotic pressure gradients in all three growth stages (ANOVA,  $P<0.001$ ). In 0.2 mOsm/L treatment, medusae could survive over 200 h but activity and feeding rate declined obviously after 150 h. When osmotic pressure rose to 34, 85 and 170 mOsm/L, there was a rapid decline of survival time. At the osmotic pressure of 85 mOsm/L, none could survive over 24 h. At 342 and 684 mOsm/L, there was a quick body contraction and showed non-response to any stimulation after 4 min. At 1 709 mOsm/L, medusae died immediately and body sharply contracted to a minimum volume.

For different growth stages, osmotic pressure also affected its survival time (Duncan statistics based on observed data,  $P<0.0001$ ). Compared to the diameters of 15.0 and 8.0 mm, the 5.0 mm-diameter specimens were more vulnerable to the variations of osmotic pressure and had a shorter survival time (Table 1). When osmotic pressures rose to 34 and 85 mOsm/L, smaller medusae moved slowly and spent more time on the bottom of beaker than larger ones. When osmotic pressure rose from 342 to 1 709 mOsm/L, the medusae of all growth stages died quickly.

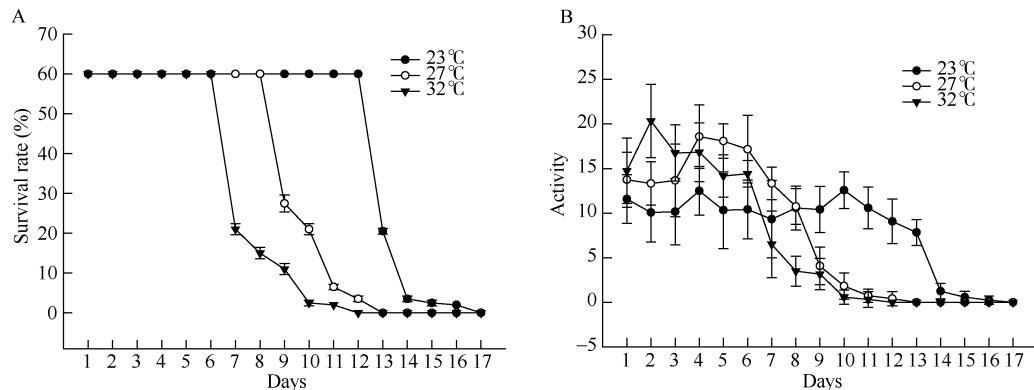
### Experiment II: Effect of temperature on *C. sowerbii*

The activities of medusae at 27 °C and 32 °C were significantly higher than those at 23 °C ( $P<0.001$ ) during the first 6 days (Figure 1B). However, more than half specimens died at Day 7 at 32 °C and at Day 9 at 27 °C. By contrast, average activity at 23 °C was the lowest in the first 6 days. However peak survival time was recorded for most specimens and death occurred at Day 13 (Figure 1 A). Prior to death, the non-feeding specimens idled on the bottom of tanks, wriggled bodies and could not

**Table 1** Effects of osmotic pressure on survival time of *Craspedacusta sowerbii*

Osmotic pressure (mOsm/L)	15.0 mm	8.0 mm	5.0 mm
0.2	216.000±1.414	218.000±2.121	240.000±4.243
34	89.680±0.014	87.680±0.141	47.930±0.339
85	15.500±0.071	19.650±0.042	4.230±0.184
171	0.275±0.007	0.400±0.085	0.230±0.014
342	0.045±0.007	0.055±0.007	0.045±0.007
684	0.015±0.007	0.030±0.000	0.030±0.000
1709	0.000±0.000	0.000±0.000	0.000±0.000

surface. All specimens died within 4 days. The variations of survival time were significant among these temperature treatments (ANOVA,  $P<0.001$ ). The peak survival time occurred at 23 °C at Day 17 while those at 27 °C and 32 °C were 12 and 13 days respectively. In summary, comparing to 27 °C and 32 °C treatments, 23 °C treatment showed lower activities (range, 8-13/min) with a longer survival time. During the study, no sexual reproduction occurred in all groups and no polyp was observed in each tank.

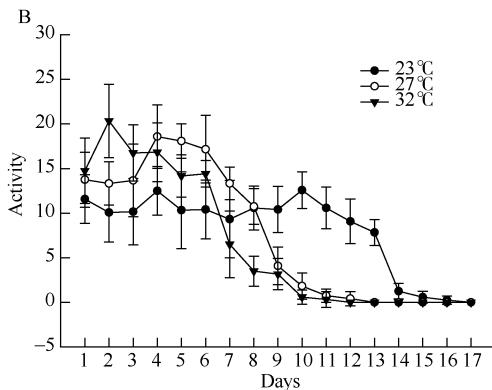
**Figure 1** Effects of temperature stress on survival (A) and activity (B) of *Craspedacusta sowerbii*

Sexual reproductive behaviors occurred simultaneously in the groups of 10 ind./L, 14 ind./L and 18 ind./L at Day 22. And the 18 ind./L group reproduced more vigorously while the 10 ind./L group showed the least reproduction. Correspondingly, the numbers of polyps at Day 7 post-breeding showed a similar trend in three treatments (35 in 18 ind./L, 25 in 14 ind./L, 5 in 10 ind./L). At Day 9 post-breeding, a few newly-formed medusae appeared in these groups and often surfaced. And then, the maximal numbers of newly-formed medusae were measured for a few more days and removed gradually. Significant variations existed in the maximal number of newly-formed polyps and medusae between each group (Duncan statistics based on observed data,  $P<0.0001$ ). All newly-formed specimens died within nearly 1 month and none grew a diameter of >5.0 mm. No reproductive behavior, newly-formed polyps or medusae were observed at the densities of 2 ind./L and 6 ind./L (Figure 2).

Based on the experimental data of the first 43 days, the average diameters of medusae changed marginally (Figure 3).

### Experiment III: Effect of stocking density on *C. sowerbii*

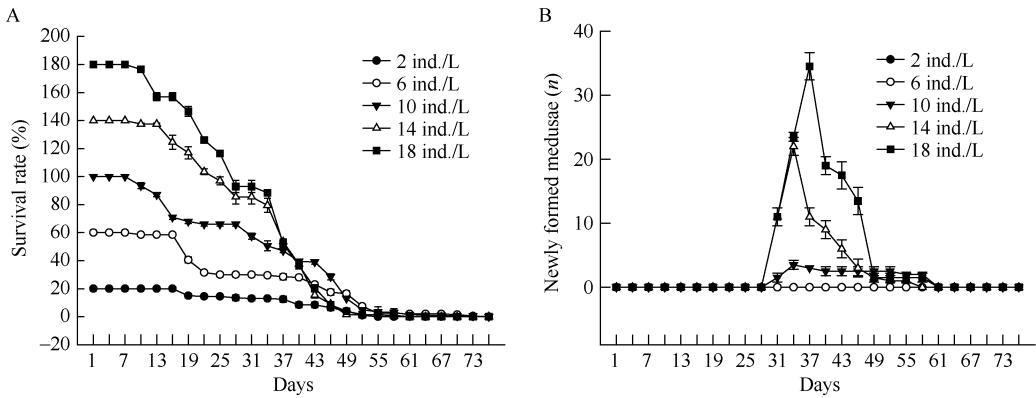
Based on daily measurements, the temperature range of pond water was 17 °C to 22 °C. More than half of specimens died at Day 34 in each treatment except for 2 ind./L density group. The mortality time of all individuals in each treatment was as follows: 2 ind./L, 55 days; 6 ind./L, 76 days; 10 ind./L, 76 days; 14 ind./L, 55 days and 18 ind./L, 61 days (Figure 2A). Non-linear relationship existed between survival time and stocking density.



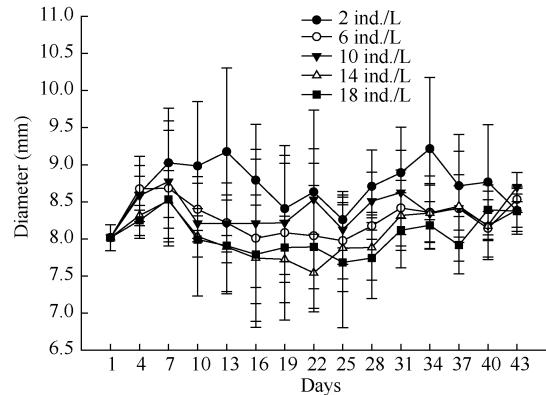
Low-density group attained a faster growth speed of average diameter than high-density group in the first 7 days. And then there was a general decrease of growth speed, the average diameter at a low stocking density was longer than that at a high stocking density.

### DISCUSSION

One of the most prominent features for *C. sowerbii* lied in its high water content. The medusae of freshwater jellyfish was presumed to have a water content of >98% of total body weight (Fleming & Hazelwood, 1971). Though hyper-osmotic to a pond water environment, the medusae had an appreciable permeability to sodium and water (Fleming & Hazelwood, 1967; Hazelwood et al., 1970) and could survive >200 h at 0.2 mOsm/L in underground water according to our study. However, under the stress concentration of 34 mOsm/L, its life span could not surpass 90 h. And its tolerability of osmotic pressure



**Figure 2** Effects of stocking density on survival (A) and sexual reproduction (B) of *Craspedacusta sowerbii*



**Figure 3** Effect of stocking density on diameter of *Craspedacusta sowerbii*

Up to Day 43, >6 specimens survived in each treatment.

was less than >120 hr at 92 mOsm/L in sea water as reported by Fleming. In our study, the tolerated osmotic concentration was <34 mOsm/L in pond water. It was lower than that of *Chlorohydra viridissimus* with a tolerated osmotic concentration of 40-50 mOsm/L (Lilly, 1955). At low osmotic pressures, younger specimens were more vulnerable to the variations of osmotic pressure. It suggested that younger medusae had not fully developed an osmoregulation system. Furthermore, for both old and young organisms, their volumes decreased with rising osmotic pressures. According to a previous study, gut acted as an osmoregulatory organ as well as a digestive system (Hazelwood et al., 1970). Thus medusae continued to excrete free water at a faster rate than an osmotic inflow of water through gut. Ultimately massive water loss disrupted the structure of gut and caused the death of organisms. In our non-published study, the organisms were lethal to larval fish through stinging. Therefore a slight elevation of osmotic pressure may be employed for preventing bloom of medusae and averting a depletion of fishery in pond.

On one hand, *C. sowerbii*, a globally distributed species, usually triggered the bloom of medusae at a temperatures

range of 21 °C to 24°C (Acker & Muscat, 1976; Fish, 1971; Kimmel et al., 1980; Stefani et al., 2010; Tresselt, 1950). On the other hand, due to a larger adaptive scope of temperature variations, polyps could live within a short temperature range (Acker & Muscat, 1976; Dunham, 1941; Kramp, 1950). In the present study, medusae could survive at a temperature range of 15 °C to 24 °C in pond, but disappeared under 15 °C. Some organisms of *C. sowerbii* bloomed at 22 °C. In the laboratory, warmer temperature promoted the activity of medusae but shortened their survival time (<10 days). Only at a temperature similar to pond water, a longer survival time was obtained. But the mechanism has remained illusive. Low-temperature treatments were not applied due to the difficulties of conditioning by pond water. However, at <17 °C, low activities reappeared and the organisms died quickly. So low temperature decreased activity while high temperature increased activity. However, there was a shorter life span. Based on the previous distribution studies (Dumont, 1994) and our results, it was assumed that the medusae of *C. sowerbii* failed to flourish under the temperature of 15 °C.

Acting as preys to polyps and medusae, zooplankton impacted greatly the life history of *C. sowerbii* (Jankowski & Ratte, 2002; Spadiner & Maier, 1999). In Experiment III, an ample supply of food was provided for eliminating the effect of prey deficiency. Initially the organisms grew normally in all groups but then death occurred gradually. Massive death was not found within a short period. Several specimens survived for up to 73 days in two treatments. A previous report had the similar findings (Pennak, 1956). However, the survival time was much longer in the present study. Except for particular cases, a downward trend of survival number was present among all treatments. Under our experimental conditions, the effect of stocking density on survival rate was not obvious under these settings. However, sexual reproduction and diameter were obviously influenced evidently in each treatment.

Sexual reproduction occurred at a stocking density of 10 ind./L. As shown in Figure 2, the number of polyps and newly-formed medusae increased with rising stocking density. Polyps tended to adhere to the tank bottom and some alga were difficult to discover. However, no polyp was found in each group at the

end. We surmised that temperature decrease might explain the disappearance of polyps. The peak number of newly-formed medusae occurred at around 21 °C while there was an onset of death at <19 °C. Thus younger organisms probably required a higher temperature for body growth. Besides, the population quantity of small dying zooplankton was low under a dissecting microscope. Previous study also showed mini-sized body of zooplankton was consumed that larger individuals were spared (Dodson & Cooper, 1983; Spadinger & Maier, 1999). No polyps was found in the groups of 2 and 6 ind./L. So only a high stocking density could induce sexual reproduction.

The diameter variations of medusae have been previously reported. However quantitative study has been scarcely performed (Fish, 1971; Pennak, 1956). The growth of medusae was negatively correlated with stocking density. After initial growth, morphological degeneration appeared after each treatment. So far the underlying reasons for morphological degeneration are still unclear. After the last treatment, secondary growth of medusae was probably due to the expansion of environmental space. In summary, low density enhanced growth but it had no effect on reproduction. By contrast, a high stocking density resulted in a lower average diameter. Yet medusae of a larger average diameter also exist and sexual reproduction may be triggered.

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# Molecular cloning, pathologically-correlated expression and functional characterization of the colony-stimulating factor 1 receptor (CSF-1R) gene from a teleost, *Plecoglossus altivelis*

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## ABSTRACT

Colony-stimulating factor 1 receptor (CSF-1R) is an important regulator of monocytes/macrophages (MO/MΦ). Although several CSF-1R genes have been identified in teleosts, the precise role of CSF-1R in ayu (*Plecoglossus altivelis*) remains unclear. In this study, we characterized the CSF-1R homologue from *P. altivelis*, and named it PaCSF-1R. Multiple sequence alignment and phylogenetic tree analysis showed that PaCSF-1R was most closely related to that of Japanese ricefish (*Oryzias latipes*). Tissue distribution and expression analysis showed that the PaCSF-1R transcript was mainly expressed in the head kidney-derived MO/MΦ, spleen, and head kidney, and its expression was significantly altered in various tissues upon *Vibrio anguillarum* infection. After PaCSF-1R neutralization for 48 h, the phagocytic activity of MO/MΦ was significantly decreased, suggesting that PaCSF-1R plays a role in regulating the phagocytic function of ayu MO/MΦ.

**Keywords:** Colony-stimulating factor 1 receptor; Pathologically-correlated expression; Monocytes/macrophages; Phagocytosis; Sequence analysis

## INTRODUCTION

The innate immune response, a fundamental defense mechanism in fish, is the first line of host defense against pathogens (Akira et al., 2006; Magnadottir, 2006). Cells involved in the innate immune system include monocytes/macrophages (MO/MΦ), neutrophils, and natural killer (NK) cells (Buchmann, 2014). MO/MΦ are critical effectors and regulators of inflammation and the innate immune response. Since this subset of immune cells is of primary

importance in combating infections in fish (Magnadottir, 2006), the function and development of MO/MΦ have been investigated in diverse teleosts (Chen et al., 2014; Hanington et al., 2009; Lu et al., 2014; Torracca et al., 2014; Wu et al., 2014).

Colony-stimulating factor 1 receptor (CSF-1R), also known as macrophage colony-stimulating factor receptor (M-CSFR) and cluster of differentiation 115 (CD115), is a member of the protein tyrosine kinase class III (PTK III) family. CSF-1R is structurally related to the prototypic platelet-derived growth factor receptor (PDGFR), mast/stem cell growth factor receptor (SCFR), and fms-like tyrosine kinase III receptor (Flt3) (Eleheert et al., 2011). Similar to other PTK III members, CSF-1R comprises five Ig-like extracellular ligand-binding domains joined by a single membrane-spanning hydrophobic helix to a cytoplasmic protein tyrosine kinase (PTK) domain (Lemmon & Schlessinger, 2010). Upon activation, CSF-1R dimerizes and autophosphorylates on a specific tyrosine residue, creating binding sites for several cytoplasmic SH2-containing signaling molecules that relay and modulate CSF-1R signals (Lemmon & Schlessinger, 2010). In mammals, CSF-1R has two ligands, CSF-1 and IL-34 (Ma et al., 2012). CSF-1R is critical for the proliferation, survival, and differentiation of macrophages, as knockdown of this gene results in large depletions of macrophages in most tissues (Dai et al., 2002; Dröin & Solary, 2010). In addition, CSF-1R signaling controls the development of the macrophage lineage under steady conditions and during certain inflammatory reactions (Lenzo et al., 2012).

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CSF-1R homologues have been identified in a number of teleost species, such as rainbow trout (*Oncorhynchus mykiss*) (Honda et al., 2005), goldfish (*Carassius auratus L.*) (Barreda et al., 2005), gilthead seabream (*Sparus aurata L.*) (Roca et al., 2006), and grass carp (*Ctenopharyngodon idellus*) (Chen et al., 2015). Furthermore, it has been reported that the CSF-1R protein is a specific marker of macrophages in goldfish (Katzenback & Belosevic, 2012), gilthead seabream (Roca et al., 2006), and grass carp (Chen et al., 2015). These studies showed that CSF-1R expression is confined to head kidney-derived MO/MΦ or only detected in purified macrophages. However, the function of CSF-1R in response to infection of teleost MO/MΦ remains unclear.

Ayu is an important commercial teleost widely cultured in Japan, China, and Korea. Recently, the development of ayu aquaculture in China has been severely challenged by *Vibrio anguillarum* infection, which has resulted in both production and animal welfare problems (Li et al., 2009). Considering the key role of MO/MΦ in the innate immune system of fish, it is important to determine their function in disease control. Due to its annual life cycle and accumulation of immunity knowledge, ayu was selected in the present study. We characterized a CSF-1R homologue (PaCSF-1R) from ayu, and analyzed the tissue and cellular distribution pattern before and after *V. anguillarum* infection. In addition, the effects of PaCSF-1R on MO/MΦ phagocytic activity were investigated.

## MATERIALS AND METHODS

### Fish maintenance

All fish were purchased from a fishery in Ninghai County, Ningbo City, China. Healthy fish, weighing 40–50 g each, were kept in freshwater tanks at 20–22 °C with regular feeding, as described previously (Chen et al., 2014). The fish were acclimatized to laboratory conditions for two weeks before the experiments were conducted. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

### Molecular characterization of PaCSF-1R cDNA

The cDNA sequence of PaCSF-1R was obtained from transcriptome data of ayu head kidney-derived MO/MΦ deposited in the NCBI SRA database with accession number SRX104781 using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The authenticity of the PaCSF-1R cDNA was confirmed by PCR, cloning, and sequencing. The cleavage sites of the signal peptides were predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>); ClustalW (<http://clustalw.ddbj.nig.ac.jp/>) was used for multiple sequence alignment; ligand-binding domains were predicted using the SMART web server (<http://smart.embl-heidelberg.de/>); phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011).

### Bacterial infection

The *V. anguillarum* challenge was carried out as previously

reported (Chen et al., 2014). Briefly, bacteria were grown in nutrient broth on a rotary shaker at 28 °C, and harvested in the logarithmic phase of growth, which was monitored by optical density assay. The *V. anguillarum* cells were washed, resuspended, and diluted to the appropriate concentration in sterile PBS. Head kidney-derived MO/MΦ were purified as described in the Materials and Methods and infected with live *V. anguillarum* at a multiplicity of infection (MOI) of 10. For tissues and peripheral blood leukocytes (PBLs), fish were challenged by intraperitoneal injection with  $1.2 \times 10^4$  colony forming units (CFUs) of live *V. anguillarum* (in 100 µL PBS) per fish, with PBS alone used as the control. At 0, 4, 8, 12, and 24 h post infection (hpi), the liver, head kidney, spleen, and MO/MΦ were collected and preserved at -80 °C until subsequent use. For the preparation of PBLs, blood was collected through puncture of the caudal vein using a heparinized syringe at 0, 4, 8, 12, and 24 hpi. PBLs were obtained by Ficoll-Hypaque PREMIUM (1.077 g/mL) (GE Healthcare, New Jersey, USA) density gradient centrifugation.

### Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed as described previously (Lu et al., 2014). Briefly, total RNA was extracted from fish tissue and MO/MΦ using RNAiso (TaKaRa, Dalian, China). After treatment with DNase I, first strand cDNA was synthesized using AMV reverse transcriptase (TaKaRa). Primers of PaCSF-1R were designed to amplify a 227 bp fragment, PaCSF-1Rtest(+): 5'-TGTACACCGTCCAGAGTGAC-3' and PaCSF-1Rtest(-): 5'-AATTGTTGGAAAGTGGGCC-3'. The primers: pActin2(+): 5'-TCGTGCGTGACATCAAGGAG-3' and pActin2(-): 5'-CGCACTTCATGATGCTGTTG-3' were used to amplify a 231 bp fragment from a housekeeping β-actin gene, which is a widely used internal control (Huang et al., 2011). RT-qPCR was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR premix Ex Taq II (Perfect Real Time; TaKaRa). The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Tissue samples were taken from four fish in each group. MO/MΦ samples were reproduced in three independent experiments.

### Prokaryotic expression

The partial sequence encoding a protein fragment at amino acid position 18–207 of PaCSF-1R (PaCSF-1R-Ex) was amplified using the primer pair, PaCSF-1Rp(+): 5'-GGAATTGCAGAATGGTCCGCCAG-3' and PaCSF-1Rp(-): 5'-GCTCGAGTCACTTCTGAATGACGTTGATGGA-3'. After digestion by EcoR I and Xho I, the amplicon was cloned into the pET-28a expression vector, and the constructed plasmid was subsequently transformed into *Escherichia coli* BL21 (DE3). After induction by IPTG, the recombinant protein (with an N-terminal His<sub>6</sub>-tag) was purified using a Ni-NTA column (Qiagen, Shanghai, China) according to the manufacturer's instructions.

### Antibody production and Western blot analysis

Antibody production was performed as previously reported (Wu et al., 2015). The purified PaCSF-1R-Ex protein emulsified with

Freund's incomplete adjuvant was used to immunize ICR mice (20-22 g) by intraperitoneal injection once every seven days for a total of four injections. Whole blood was collected and centrifuged to obtain sera. Control mice were injected with complete Freund's adjuvant. Anti-PaCSF-1R-Ex IgG (PaCSF-1R IgG) and control isotype IgG (IsolgG) were purified by Protein G HP SpinTrap columns (GE Healthcare, USA). The quality of PaCSF-1R IgG was tested by Western blot analysis, and visualization using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA).

#### Primary culture of ayu head kidney-derived MO/MΦ

Ayu head kidney-derived MO/MΦ cells were isolated and cultured as previously described (Zhang et al., 2015). Head kidney was isolated and washed in RPMI 1640 medium (Invitrogen, Shanghai, China) supplemented with 2% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/mL), streptomycin (100 µg/mL), and heparin (20 U/mL). The cells were separated using Ficoll-Hypaque PREMIUM (1.077 g/mL) (GE Healthcare) in combination with centrifugation according to the manufacturer's instructions. The cells were then seeded in 35 mm dishes at a density of  $2 \times 10^6$ /mL. Non-adherent cells were washed off, and the attached cells were incubated in complete medium (RPMI 1640, 5% ayu serum, 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) at 24 °C with 5% CO<sub>2</sub>. According to Giemsa staining results, over 96% of adherent cells were MO/MΦ.

#### Phagocytosis assay

Phagocytosis of ayu MO/MΦ was performed as described previously (Zhang et al., 2015). The *E. coli* DH5α in the logarithmic phase of growth were labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, USA) according to the manufacturer's protocols, and cells were thereafter designated FITC-DH5α. MO/MΦ were pre-incubated with PaCSF-1R IgG (250 mg/mL) for 4, 8, 12, 24, and 48 h. IsolgG (250 mg/mL) was added as a control. FITC-DH5α was added at a MOI of 20, and incubated with cells for 30 min. Cells were washed extensively with sterile PBS to remove extracellular particles. Trypan blue was used to quench the fluorescence that resulted from particles that were outside the cells or stuck to the surface of the cells. Adherent MO/MΦ were loosened by adding 500 µL trypsin-EDTA (0.05% Trypsin, Invitrogen) to each culture well, followed by a 5 min incubation. All cells were collected with no cells remaining in the wells. After centrifuging at 300 g for 5 min, the cells were suspended in FACS buffer (PBS, 5% FCS, 0.1% sodium azide). Cell counting using a hemocytometer showed nearly no loss of cells in this process. The engulfed bacteria were examined by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter, Miami, USA). The results were expressed as the relative mean fluorescence index (MFI) of the control in flow cytometric assay.

#### Statistical analysis

Results are presented as mean±SEM. All data were subjected to one-way or repeated-measures analysis of variance (ANOVA) with SPSS (version 13.0, Chicago, IL, USA).  $P<0.05$  were

considered statistically significant.

## RESULTS

### Molecular characterization of PaCSF-1R

The PaCSF-1R sequence was deposited in the GenBank Data Library under accession number KT692936. The cDNA of PaCSF-1R, which was 2 976 nucleotides (nts) long, possessed a large open reading frame that encoded a polypeptide precursor of 992 amino acids (aas). The protein precursor had a calculated molecular weight (MW) of  $111.10 \times 10^3$ , and its putative isoelectric point (*pI*) was 6.33. Similar to its mammalian counterpart, PaCSF-1R comprised a signal peptide (at aa position 1-17), five Ig-like domains (aa 33-500), a short single transmembrane domain (aa 522-543), and a cytoplasmic tyrosine kinase domain (aa 582-915). Multiple alignment with other known PaCSF-1R amino acid sequences revealed that the two Ig-like N-terminal domains, which are important for ligand binding to CSF-1R, were conserved in teleosts and mammals (Figure 1).

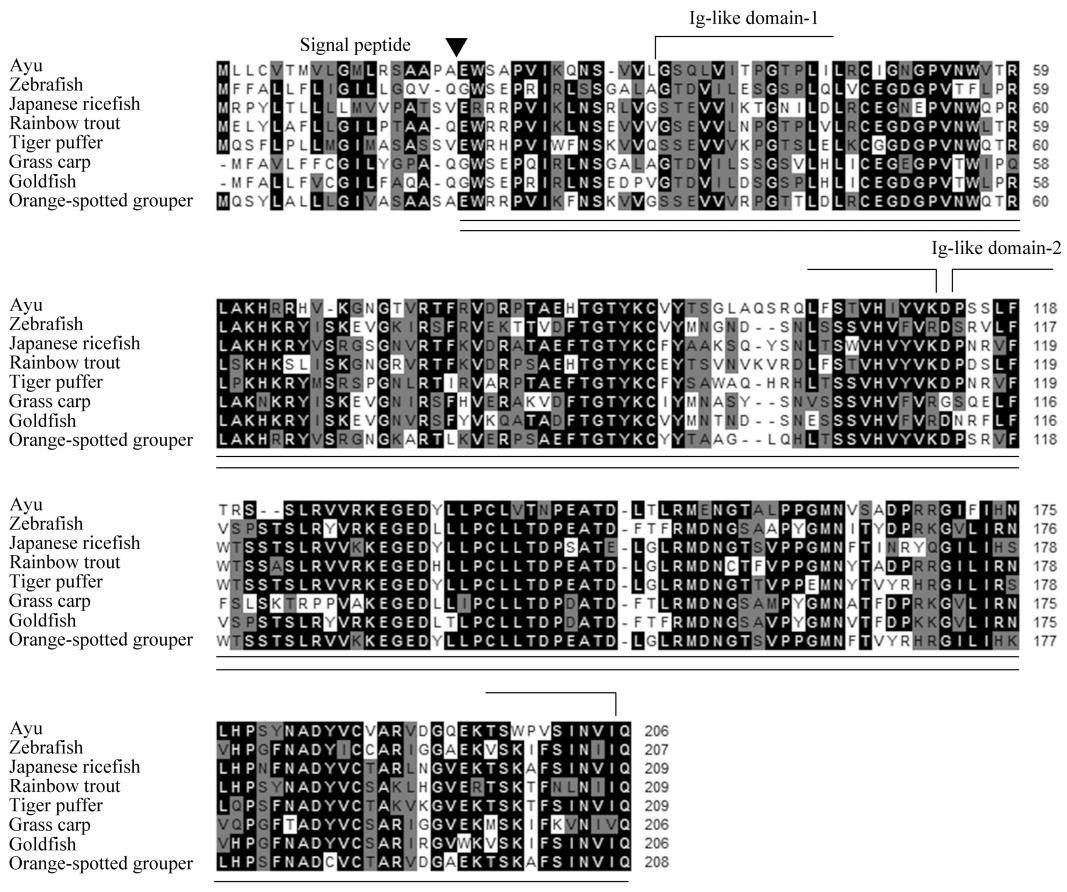
Sequence comparisons revealed that PaCSF-1R shared highest amino acid identity with that of Japanese ricefish (68.8%). Phylogenetic tree analysis showed that all fish CSF-1Rs were grouped together to form a distinct cluster that differed from the mammalian cluster (Figure 2), and PaCSF-1R was most closely related to that of Japanese ricefish.

### Alteration of PaCSF-1R mRNA expression in response to *V. anguillarum* infection

The mRNA expression of PaCSF-1R was detected in all tested tissues and in the MO/MΦ of healthy ayu, and was found to be extremely high in MO/MΦ, spleen and head kidney (Figure 3). Following infection with *V. anguillarum*, RT-qPCR was performed to analyze the changes in PaCSF-1R mRNA expression in various tissues and in MO/MΦ of ayu. The mRNA expression of PaCSF-1R significantly increased in the spleen, PBLs, and liver at 24 hpi, and decreased in the head kidney at 8 hpi, but not in the MO/MΦ (Figure 3).

### Prokaryotic expression of PaCSF-1R N-terminal region and IgG preparation

Previous analysis of human CSF-1R ligand-binding determinants showed that the three N-terminal Ig-like domains in the receptor's extracellular region contain the complete high-affinity CSF-1 binding site (Rieger et al., 2014; Wilhelmsen & Van Der Geer, 2004). Therefore, we selected the sequence comprising the first two N-terminal IgG-like domains of PaCSF-1R (PaCSF-1R-Ex) for prokaryotic expression. The sequence of PaCSF-1R-Ex was amplified from cDNA of MO/MΦ, and was subsequently cloned into a pET-28a vector. The recombinant plasmid pET-28a- PaCSF-1R-Ex was then transformed into *E. coli* BL21 (DE3), expressed by induction with IPTG, purified using the Ni-NTA column (Figure 4A), and used to immunize mice to produce antisera. Using this antibody, we found that the MW of mature PaCSF-1R protein from MO/MΦ was about  $1.5 \times 10^5$  by Western blot analysis (Figure 4B); the high MW of



**Figure 1** Multiple alignment of the amino acid sequences of two Ig-like N-terminal CSF-1R domains in several animals

Threshold for shading was 60%; similar residues are highlighted in gray; identical residues are highlighted in black; alignment gaps are denoted “-”. Inverted triangle indicates cleavage site of the CSF-1R signal peptide. The sequence for prokaryotic expression is double underlined. Ig-like domain-1 and Ig-like domain-2 are indicated. The sequences used in the analyses are ayu (*Plecoglossus altivelis*), KT692936; zebrafish (*Danio rerio*), NM\_131672; Japanese ricefish (*Oryzias latipes*), XM\_004073307; rainbow trout (*Oncorhynchus mykiss*), NM\_001124738; tiger puffer (*Takifugu rubripes*), XM\_004073307; grass carp (*Ctenopharyngodon idellus*), KP244336; goldfish (*Carassius auratus*); and, orange-spotted grouper (*Epinephelus coioides*), HQ594531.

native PaCSF-1R may be caused by post-translational modification, as previously reported (Wilhelmsen & Van Der Geer, 2004). Anti-PaCSF-1R IgG was subsequently purified from antisera using the Protein G HP SpinTrap (GE Healthcare), and was stored at -80°C for subsequent use.

#### PaCSF-1R mediation of ayu MO/MΦ phagocytosis

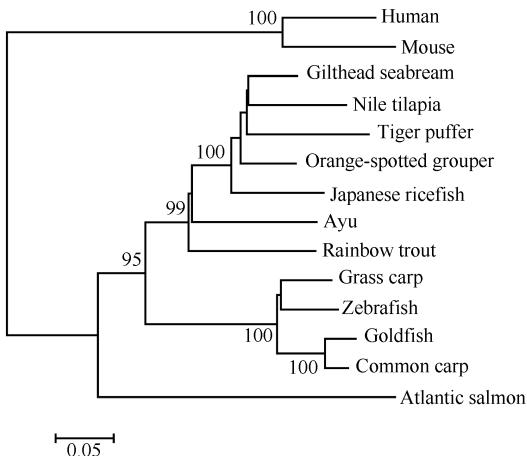
We analyzed the phagocytic activity of ayu MO/MΦ after PaCSF-1R IgG neutralization. After antibody neutralization for 4, 8, 12, and 24 h, the phagocytic activity of MO/MΦ was similar to that of the IsolgG-treated control (Figure 5A-D). However, the phagocytosis of MO/MΦ was significantly downregulated after antibody neutralization for 48 h (Figure 5E).

#### DISCUSSION

In mammals, CSF-1R is a specific marker of the MO/MΦ

lineage, and is critical for macrophage proliferation and development (Chitu & Stanley, 2006; Dai et al., 2002). However, the function of CSF-1R in teleost MO/MΦ remains unclear. In the present work, we characterized a CSF-1R homologue from ayu (PaCSF-1R). Multiple alignments revealed that PaCSF-1R contained the N-terminal ligand-binding domain, which was highly conserved in fish and mammals, suggesting that the function of CSF-1R may be conserved from teleosts to mammals.

A previous orange-spotted grouper study showed that CSF-1R mRNA expression was highest in the spleen, followed by the gill and kidney (Dan et al., 2013). RT-qPCR analysis in rainbow trout showed that M-CSFR mRNA was mainly expressed in the spleen, head kidney and kidney (Honda et al., 2005). In the present study, PaCSF-1R mRNA was expressed in all tested tissues, with the highest expression in MO/MΦ, followed by the spleen and head kidney, similar to that reported



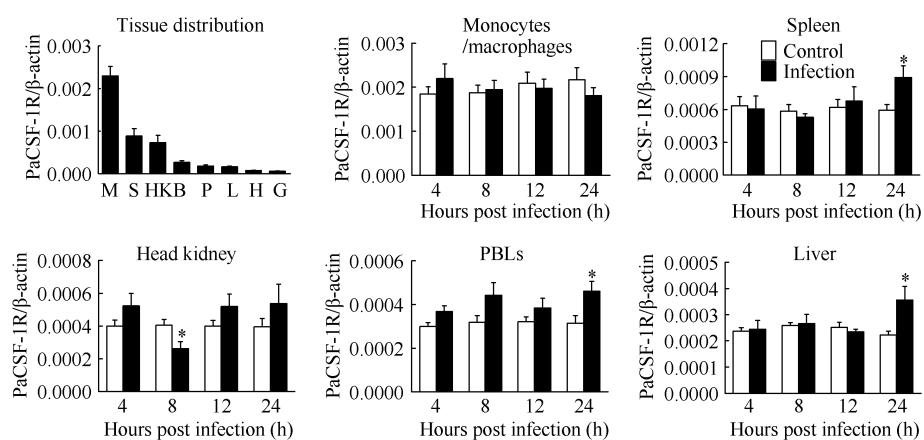
**Figure 2 Phylogenetic (neighbor-joining) analysis of the complete amino acid sequence of PaCSF-1R with other known CSF-1Rs using MEGA 5.0 software**

Values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1 000 replicates; shown only when >60%). Scale bar shows number of substitutions per base. In addition to those listed in Figure 1, accession numbers of the other sequences used are common carp (*Cyprinus carpio*), AB526448; zebrafish (*Danio rerio*), NM\_131672; orange-spotted grouper (*Epinephelus coioides*), HQ594531; gilthead seabream (*Sparus aurata*), CAJ18352; Nile tilapia (*Oreochromis niloticus*), XM\_003455186; and, Atlantic salmon (*Salmo salar*), NM\_001171807.

in other teleosts (Chen et al., 2015). Further bacterial infection results showed that PaCSF-1R mRNA expression in the spleen, PBLs, and liver were upregulated after *V. anguillarum* treatment, which is in consistent with the situation in orange-spotted grouper and gilthead seabream (Dan et al., 2013, Reyes-Becerril et al., 2011). Furthermore, the mRNA expression of PaCSF-1R in the head kidney was downregulated after *V. anguillarum* treatment. It has been reported that low doses of

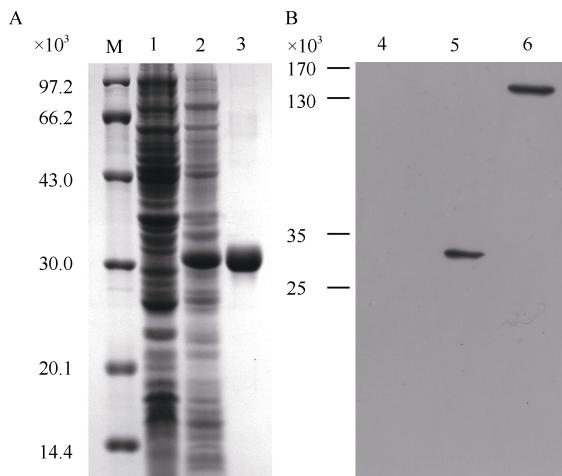
lipopolysaccharide (LPS) injected intravenously recruit monocytes from the bone marrow to the bloodstream 4 h after injection in mice (Ludin et al., 2012). Since the head kidney is the main hematopoietic and lymphoid tissue in teleosts, like mammalian bone marrow, the downregulation of mRNA expression of PaCSF-1R in the head kidney may be due to the decrease of MO/MΦ in the head kidney. These results suggested that PaCSF-1R may play an essential role in the anti-bacterial process. Considering its highest expression in MO/MΦ, the role of PaCSF-1R in MO/MΦ-mediated phagocytosis is at the top priority of function analysis.

In mammals, overexpression of CSF-1R on microglia accelerates the phagocytosis of aggregated amyloid beta (A $\beta$ ) through macrophage scavenger receptors and expression of Fc $\gamma$  receptors (Mitrasinovic et al., 2003). In the present study, after PaCSF-1R neutralization for 48 h, the phagocytic activity of MO/MΦ was significantly downregulated compared with that of the isotype control. Therefore, we suggest that PaCSF-1R may be involved in phagocytosis of ayu MO/MΦ through macrophage scavenger receptors and Fc $\gamma$  receptors. The function of CSF-1R in MO/MΦ may result from binding with its ligands, and neutralization of PaCSF-1R may block PaCSF-1R-mediated phagocytosis of MO/MΦ. In mammals, CSF-1 and IL-34 are two ligands of CSF-1R. CSF-1 competes with IL-34 for binding to CSF-1R (Wei et al., 2010), and may compensate for the absence of IL-34 in the brainstem and cerebellum (Wang et al., 2012; Wei et al., 2010). IL-34 does not control the recruitment of blood monocytic cells and their subsequent differentiation into Langerhans cells in inflammation, but is crucial for their maintenance in situ (Nakamichi et al., 2013). Macrophages are important participants in the phagocytosis of foreign material in most tissues (Pixley et al., 2004). Therefore, we speculate that PaCSF-1R may play a role in the regulation of MO/MΦ function in response to bacterial infection. Since the characterization of CSF-1R ligands in teleosts is still unclear, further investigation is needed to determine the detailed mechanism underlying the phagocytic function of PaCSF-1R.



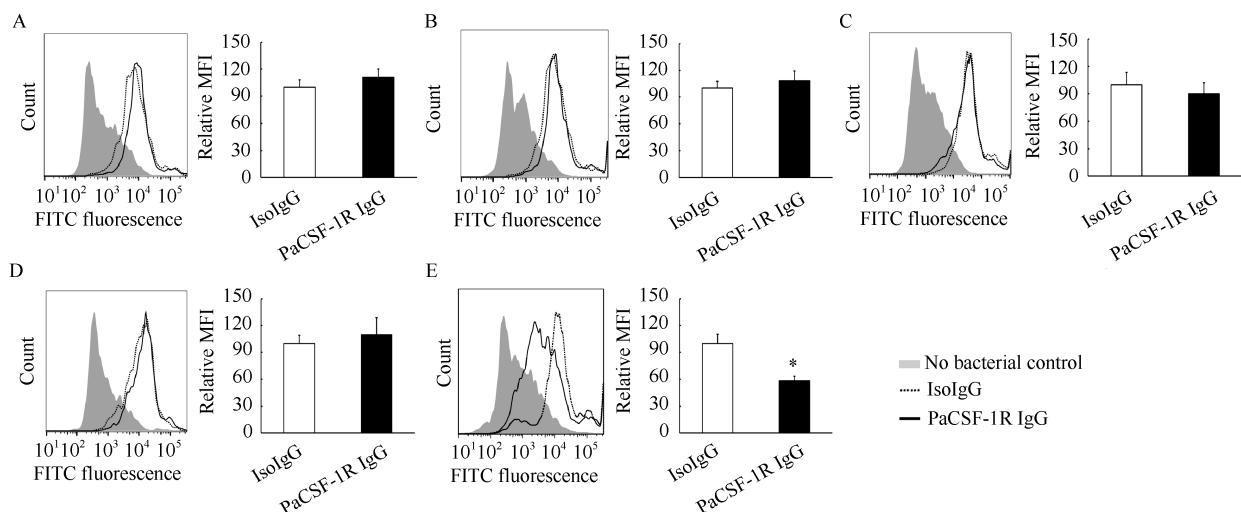
**Figure 3 RT-qPCR analysis of PaCSF-1R mRNA expression in various tissues and MO/MΦ of ayu**

Fish were sacrificed 4, 8, 12, and 24 h after intraperitoneal injection of *V. anguillarum* a; MO/MΦ were collected at 4, 8, 12, and 24 h post-infection. M, MO/MΦ, S, spleen, HK, head kidney; B, brain; P, PBLs; L, liver; H, heart; G, gill. PaCSF-1R transcript levels were normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  SEM of the results from four fish. \* $P$ <0.05.



**Figure 4 Prokaryotic expression of PaCSF-1R-Ex and Western blot analysis for PaCSF-1R**

A: SDS-PAGE analysis of prokaryotic expressed PaCSF-1R. Lane M: protein marker; 1 and 2: protein from BL21 (DE3) transformed with pET-28a-PaCSF-1R-Ex plasmid before and after IPTG induction; 3: purified recombinant PaCSF-1R-Ex. B: Western blot analysis of recombinant PaCSF-1R-Ex and native PaCSF-1R. Lane 4: negative control; 5: purified recombinant PaCSF-1R-Ex; 6: total proteins of MO/MΦ.



**Figure 5 Effect of PaCSF-1R neutralization on phagocytosis of FITC-DH5 $\alpha$  by ayu MO/MΦ**

MO/MΦ were incubated with PaCSF-1R IgG for 4 (A), 8 (B), 12 (C), 24 (D), and 48 h (E). IsoIgG was added as the control. Then, FITC-DH5 $\alpha$  was added at an MOI of 20, followed by incubation for an additional 30 min. The engulfed bacteria were also examined by flow cytometry. A total of 10 000 events were analyzed by flow cytometry. Relative mean fluorescence intensity (MFI) was presented as fold change over the control, which was assigned a unit of 100. Data are expressed as the mean $\pm$ SEM of three independent experiments. \*: P<0.05.

In summary, we identified a CSF-1R gene from ayu. PaCSF-1R expression was pathologically correlated with *V. anguillarum* infection, and may play a role in the regulation of MO/MΦ function in response to bacterial infection. Further investigation is needed to determine the detailed mechanism underlying CSF-1R function in teleosts.

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# Huangshan population of Chinese *Zacco platypus* (Teleostei, Cyprinidae) harbors diverse matrilines and high genetic diversity

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## ABSTRACT

Six main mitochondrial DNA (mtDNA) lineages have been described in minnow (*Zacco platypus*) samples obtained from northern, western and southern China. Perdices et al. (2004) predicted that further sampling of other tributaries might discover more lineages of this species. In this study, we collected 26 *Zacco platypus* individuals in the Huangshan area of eastern China and determined the cytochrome *b* (*cytb*) sequence variations. Combined with reported data in GenBank, we identified ten matrilines (*Zacco* A-J) in a total of 169 samples, with relatively high molecular divergence found among them. The Huangshan population had the greatest genetic variation among all sampled regions and hosted six of the ten matrilines. Our results highlight the significance of the Huangshan area for the conservation of *Zacco platypus*.

**Keywords:** *Zacco platypus*; Matriline; Huangshan; Phylogenetics; Diversity

## INTRODUCTION

*Zacco platypus* is a common minnow that occurs in sympatry with most Chinese cyprinids (Deng et al., 2013). Topographical barriers may restrict its life history and drive cryptic diversity. The species' distribution encompasses all major river systems in mainland China, as well as the Korean Peninsula and Japan

(Chen, 1998). Perdices et al. (2004) analyzed the genetic diversity of *Z. platypus* sampled in the upper and middle Changjiang (Yangtze River) and found four major matrilines that may harbor multiple species. Long-term interruption of dispersal is thought to have driven this diversity. Perdices and Coelho (2006) further studied samples from the Pearl River and northern drainages, and obtained six matrilines in China. Using nuclear DNA data, Berrebi et al. (2005) identified four genetic groups within *Z. platypus* from Sichuan, Hunan and Guangxi provinces in China.

Although Perdices et al. (2004) predicted that exhaustive sampling of other tributaries might discover other lineages of *Zacco*, few specimens have been sampled in eastern China. The Huangshan area in eastern China is a mosaic of mountains with elevations lower than 2 000 m, and exhibits a complex geological history that includes tectonic movements, orogenesis, and periodic climatic change (e.g., Ju et al., 2007; Rüber et al., 2004; Zhang et al., 1990). Based on patterns of intraspecific genetic variation and buffer-zone models, Huangshan hosts refugia of eastern Asian conifers, frogs, non-migratory birds and Asian salamanders (Gao et al., 2007; Li et al., 2009; Murphy et al., 2000; Wu et al., 2013; Zhang et al., 2008).

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In view of modern genetics, genetic diversity in a given species is closely related to its adaptability, variability, and evolutionary potentiality, with genetic variation considered a prerequisite for organisms to cope with environmental uncertainty (Conrad, 1983). Herein, we report on the genetic diversity of *Z. platypus* from eastern China based on extensive sampling of the Huangshan area together with prior *cytb* sequence data from mainland China, Taiwan (Perdices et al., 2004; Perdices & Coelho, 2006; Wang et al., 2007), and Japan (He et al., 2004; Kawamura et al., 2014; Kitamura et al., 2012; Sasaki et al., 2007; Wang et al., 2007). We further evaluated the matrilineal diversity of *Z. platypus* and revealed the possible ecological significance of the Huangshan area.

## MATERIALS AND METHODS

### Sampling

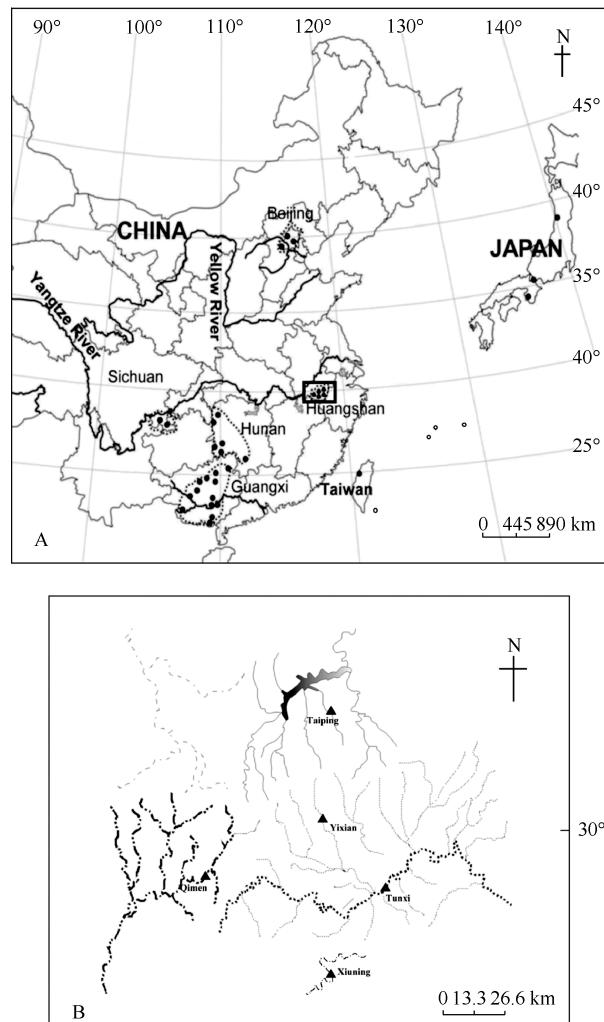
We evaluated 169 sequences in total, including 26 from five Huangshan counties (Table 1), 137 from Perdices et al. (2004) and Perdices & Coelho (2006), one from Wang et al. (2007), and five from Japan (AF309085, He et al. (2004); AB198972, Sasaki et al. (2007); AY958194, Wang et al. (2007); AB620130, Kitamura et al. (2012); and AB366543, Kawamura et al. (2014)). Our new samples were preserved and deposited in the Museum of Huangshan University (Voucher numbers: HUM201201–26). Sampling sites in this study are shown in Figure 1.

### PCR amplification and sequencing

Fresh dorsal muscle tissues were removed from the 26 Huangshan individuals and immediately preserved in 95% ethanol for sequencing complete mitochondrial *cytb*. Total DNA was extracted from tissues using standard phenol/chloroform techniques (Sambrook et al., 1989). *Cyt b* was amplified using polymerase chain reaction (PCR) with the following sets of primers: LCB1 (5'-AATGACTTGAAGAACCAACCGT-3') and HA (5'-CAACGATCTCCGGTTACAAGAC-3') (Brito et al., 1997; Schmidt & Gold, 1993). Reagents included 100 ng of template DNA, 1 µL of each primer, 5 µL of 10× reaction buffer, 2 µL dNTPs (each 2.5 mmol/L), and 2.0 U of Taq DNA polymerase. The reactions were cycled as follows: an initial preheating at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. We next obtained nucleotide sequences through fractionation, purification, and sequencing according to Tiangen's protocols. Newly obtained haplotype sequences were deposited in GenBank under Accession Nos. KM491716–35 (Table 1).

### Matrilineal genealogy and population structure

We used 916 bp out of 1 140 bp of the *cytb* sequences in the following analyses. The newly obtained sequences and those downloaded from GenBank were aligned using Clustal X (Thompson et al., 1997). For phylogenetic reconstruction, two closely related species, *Zacco temmincki* and *Candidia barbatus* (Mayden et al., 2007; Wang et al., 2011), were chosen as outgroups.



**Figure 1** Sampling localities (A) and main drainages of the Huangshan area (B)

Black dots refer to samples from GenBank, and triangles are from this study. This map shows the seven geographic units grouped according to geographic distances and similarities. Xiuning, Qimen, and Taiping drainages belong to the Yangtze River, while Tunxi and Yixian drainages belong to the Qiantang River.

Bayesian inference (BI) and maximum likelihood (ML) were used to reconstruct a bifurcating tree using MrBayes v3.0 (Huelsenbeck & Ronquist, 2001) and RAxML at the CIPRES Science gateway (<http://www.phylo.org/portal2/login!input.action>), respectively. JModelTest v. 0.1.1 (Posada, 2008) was used to find the best model of nucleotide evolution for ML based on the Akaike Information Criterion (AIC) and for BI based on the Bayesian Information Criterion (BIC). Analyses selected the TN93+G model. Bayesian posterior probabilities (BPP) and the frequencies of nodal resolution were obtained by Markov Chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. The BI analysis used 10 000 000 generations,

with sampling every 1 000 generations and discarding the first  $3 \times 10^6$  generations as burn-in. We ran four analyses starting

with random trees and a consensus of the resulting 36 000 trees was computed from all four runs.

**Table 1** Information for samples newly obtained in this study, including localities, rivers, sample sizes, haplotypes, coordinates, voucher specimens and GenBank accession numbers

Localities	Sample Size ( <i>n</i> )	Rivers	Coordinates	Haplotypes	Voucher specimens	GenBank Accession No.
Qimen	4	Chang Jiang	N29°84'51", E117°71'77"	Qimen1	HUM201212	KM491716
				Qimen2	HUM201213	KM491717
				Qimen3	HUM201214	KM491718
				Qimen4	HUM201215	KM491719
Xiuning	8	Jiangwan He	N29°43'20", E118°16'75"	Xiuning1	HUM201201	KM491733
				Xiuning2	HUM201202-03	KM491731
				Xiuning3	HUM201204-06	KM491732
				Xiuning4	HUM201207	KM491735
				Xiuning5	HUM201208	KM491734
Tunxi	3	Xinan Jiang	N29°70'43", E118°31'16"	Tunxi1	HUM201209	KM491728
				Tunxi2	HUM201210	KM491729
				Tunxi3	HUM201211	KM491724
Yixian	6	Xinan Jiang	N29°92'06", E118°10'13"	Yixian1	HUM201216	KM491730
				Yixian2	HUM201217	KM491727
				Yixian3	HUM201218	KM491726
				Yixian4	HUM201219-20	KM491725
				Yixian5	HUM201221	KM491723
Taiping	5	Taiping Hu	N30°36'16", E118°04'65"	Taiping1	HUM201222	KM491722
				Taiping2	HUM201223-25	KM491720
				Taiping3	HUM201226	KM491721

Sampling information of extant sequences is not listed in this table.

### Estimation of divergence time

Divergence times among the main lineages of *Z. platypus* were estimated using a Bayesian MCMC approach implemented in BEAST V.1.7.5 based on a strict molecular clock (Drummond & Rambaut, 2007). The parameters were: substitution model, TN93+G; tree prior, Coalescent: constant size; normal distribution; 10 million generations; parameters logged every 1 000; burn-in value=1 000. The molecular clock of cyprinids was assumed to be 1.52% site<sup>-1</sup> Ma (million years)<sup>-1</sup> (Doadrio et al., 2002) for *cyt b*.

### RESULTS

A total of 75 haplotypes were defined from all 169 in-group individuals. The topologies of the BI and ML trees were nearly identical (Figure 2). The haplotypes were grouped into main clade 1 and 2. Clade 1 hosted individuals from Huangshan, Sichuan, Hunan and Guangxi and clade 2 contained specimens from Huangshan, Beijing and Japan. We identified ten matrilines of *Z. platypus* according to the topology of the phylogenetic tree and the genetic variation between the ten matrilines.

Six of the ten lineages involved Huangshan individuals, and

four consisted entirely of Huangshan individuals. Moreover, samples from the same Huangshan location were grouped into different clades. For example, Qimen had samples from matrilines A, F and J, and Xiuning had samples from matrilines A and I. The genetic divergences of these samples were significant, and the maximum pairwise differences from the same counties reached 6.0% (Xiuning) and 21.9% (Qimen).

We grouped sampling localities into seven geographic units according to geographic distances and then calculated the nucleotide diversity within them. Huangshan showed remarkably high nucleotide diversity relative to other groups (2.5–111.7 times that of others) although the geographical area of Huangshan was less than that of the other units (Table 3).

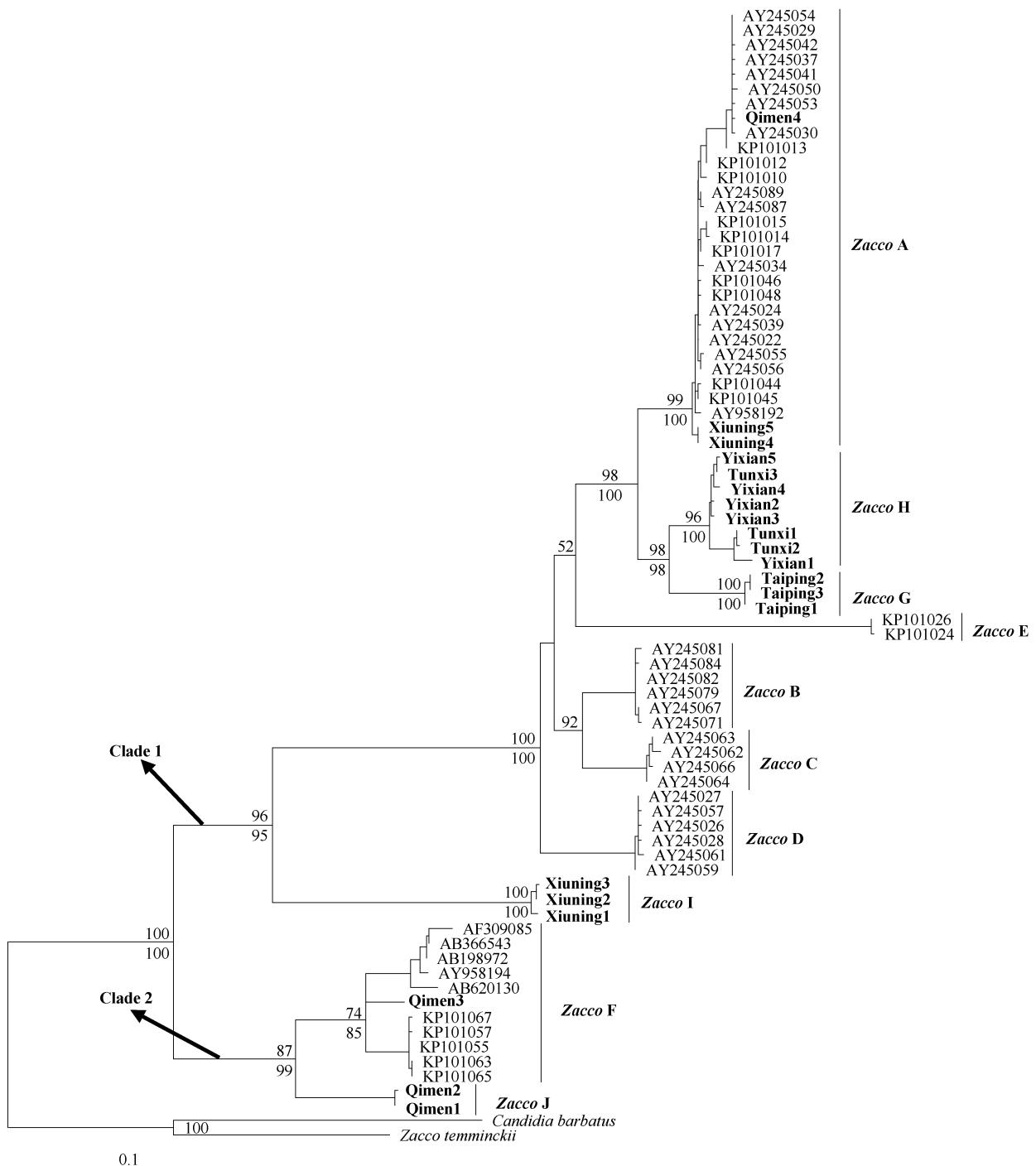
Divergence times estimated for the in-group nodes are shown in Figure 3. The initial divergence occurred at about 10.67 Ma.

### DISCUSSION

Perdices et al. (2004) and Perdices & Coelho (2006) divided *Z. platypus* sampled in southern, western and northern China into matrilines A–F. They suggested that the long-term interruption

of gene flow might have caused the diversification and an underestimation of the number of species. Our analyses identified ten matrilines of *Z. platypus* in Chinese and some Japanese populations. This confirms the prediction of Perdices

et al. (2004, page: 9) that "exhaustive sampling of other tributaries might evidence other *Zacco* lineages". This is also in accordance with that found for *Opsariichthys bidens*, a sympatric species of *Z. platypus* (Perdices et al., 2005).



**Figure 2 Phylogenetic tree derived from the maximum likelihood of the *cyt b* sequences**

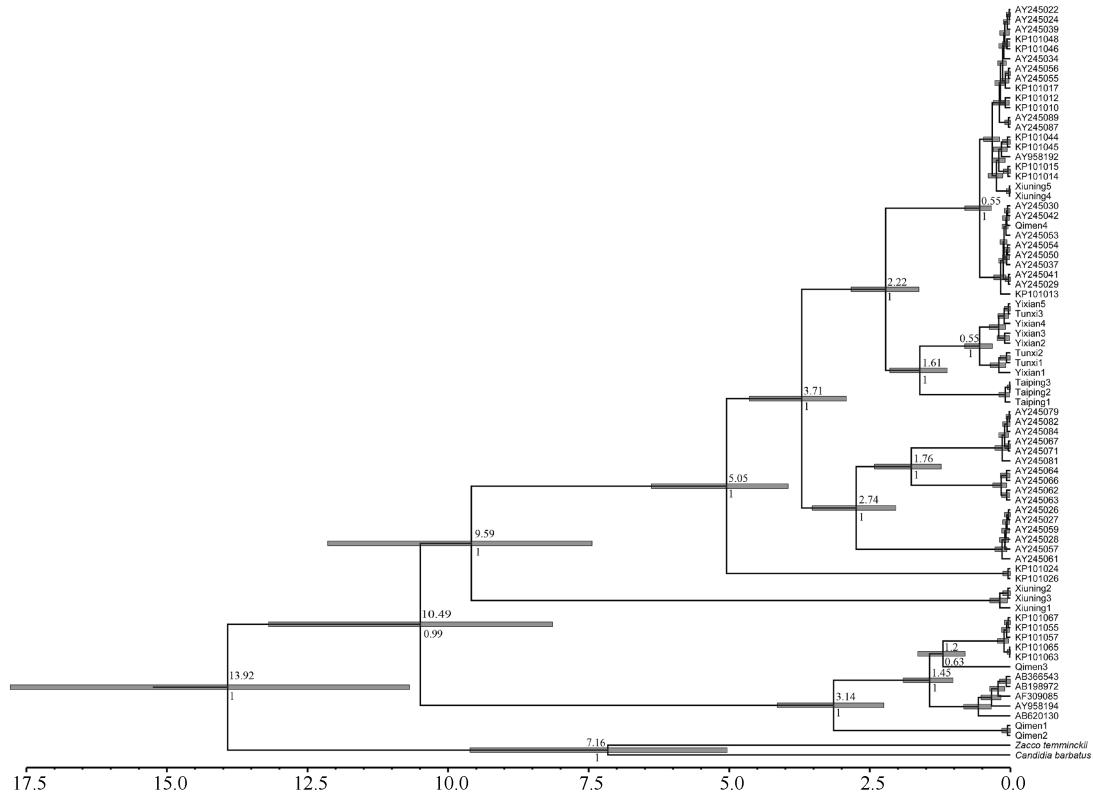
Values above branches represent the support level of ML (BSP) and values below branches represent the popularity rating of BI (BPP). Vertical bars indicate the mtDNA lineage assignment (A-J). Zacco A-F follow the nomenclature of Perdices & Coelho (2006) and the others follow the alphabet. Bold types are Huangshan populations.

**Table 2** Matrix of pairwise genetic variation between matrilines (A-J) of *Z. platypus*

	A	B	C	D	E	F	G	H	I	J
A										
B	0.067									
C	0.078	0.044								
D	0.076	0.059	0.065							
E	0.118	0.112	0.109	0.108						
F	0.146	0.148	0.163	0.158	0.174					
G	0.052	0.073	0.074	0.074	0.122	0.152				
H	0.059	0.076	0.091	0.08	0.128	0.158	0.044			
I	0.157	0.157	0.158	0.146	0.184	0.155	0.152	0.155		
J	0.157	0.151	0.156	0.151	0.181	0.067	0.153	0.164	0.135	

**Table 3** Matrilines, haplotype (*h*), and nucleotide diversity ( $\pi$ ) with standard errors (SE) for each geographic unit

Geographic unit	mtDNA lineage	No. of samples ( <i>n</i> )	<i>h</i> ±SE	$\pi$ ±SE
Huangshan	A, F, G, H, I, J	26	0.9631±0.0219	0.085751±0.042501
Beijing	F	23	0.6087±0.0761	0.000768±0.000670
Hunan	A, C, D	49	0.9092±0.0262	0.034648±0.017036
Sichuan	B	19	0.6374±0.1045	0.001098±0.000863
Guangxi	A, E	46	0.6963±0.0740	0.029668±0.014733
Taiwan	A	1	—	—
Japan	F	5	—	0.011681±0.007511

**Figure 3** Time tree of *Z. platypus*

Tree topology derived from BEAST analyses of all 77 haplotypes. Numbers above branches represent node age and values below are support rates. Gray bar represents 95% posterior credible intervals.

Some drainages still await sampling, such as the Yellow River, one of the most important drainages in China. Future research should detect additional matrilines of *Zacco*, while morphological analyses may help differentiate morphological differences of taxonomic significance.

Grant & Bowen (1998) interpreted four basic population history scenarios based on haplotype and nucleotide diversities, which can also be used to clarify the history of *Z. platypus* populations. Our results revealed a pattern of high haplotype and nucleotide diversity in the Huangshan population (Table 3), which likely indicates large stable populations with long evolutionary histories or secondary contact between differentiated lineages (Grant & Bowen, 1998). The highest levels of genetic variation may occur in the region of origin. For example, Savolainen et al. (2002) claimed an East Asian origin for the domestic dog in part due to the area having the highest level of genetic diversity.

Genetic variability in mtDNA has been reported in fish species. Several scenarios have been proposed to explain the maintenance of high haplotype diversity within populations, including large population size, environmental heterogeneity, and life history traits that favor rapid population increase (Han et al., 2008; Ju et al., 2013; Yang et al., 2012). Huangshan has a heterogeneous topography, with mountains of elevation lower than 2 000 m maintaining stable climatic conditions during the Pleistocene. This condition likely provided glacial refugia for many species (Gao et al., 2007; Li et al., 2009; Qian & Ricklefs, 2000; Wu et al., 2013; Zhang et al., 2008). At least three other species or species groups have high levels of nucleotide diversity in the Huangshan area, including the Chinese giant salamander, sharp-snouted pit viper and Asian salamander (Huang et al., 2007; Murphy et al., 2000; Wu et al., 2013). These co-occurrences indicate that Huangshan hosts old lineages.

We only used mtDNA for genetic analyses. Therefore, it will be necessary to gather and analyze nuclear DNA data in the future to assess population structure and gene flow and thus better inform the demographic history of this fish species.

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# ZIKA-How fast does this virus mutate?

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## ABSTRACT

The World Health Organization has declared the present Zika virus epidemic to be a 'Public Health Emergency of International Concern'. The virus appears to have spread from Thailand to French Polynesia in 2013, and has since infected over a million people in the countries of South and Central America. In most cases the infection is mild and transient, but the virus does appear to be strongly neurotropic and the presumptive cause of both birth defects in fetuses and Guillain-Barré syndrome in some adults. In this paper, the techniques and utilities developed in the study of mitochondrial DNA were applied to the Zika virus. As a result, it is possible to show in a simple manner how a phylogenetic tree may be constructed and how the mutation rate of the virus can be measured. The study showed the mutation rate to vary between 12 and 25 bases a year, in a viral genome of 10 272 bases. This rapid mutation rate will enable the geographic spread of the epidemic to be monitored easily and may also prove useful in assisting the identification of preventative measures that are working, and those that are not.

**Keywords:** Zika; Virus; Polyprotein; Mutation rate; Phylogenetic tree

## INTRODUCTION

On 01 February, 2016, the World Health Organization declared the emerging Zika epidemic to be a 'Public Health Emergency of International Concern' (PHEIC), highlighting that this epidemic is now considered to be a major threat to the whole world (WHO, 2016). Their statement of intent includes the lines: Appropriate research and development efforts should be intensified for Zika virus vaccines, therapeutics and diagnostics; and, National authorities should ensure the rapid and timely reporting and sharing of information of public health importance relevant to this PHEIC.

As a consequence, it can be expected that many research institutions will increase their studies into Zika and related viruses and many new scientific papers will appear in the coming months. At the same time, it is expected that

many more RNA sequences of the virus will appear in the public domain.

Also, as a result of the rapidly increasing importance of the Zika virus, it is likely that scientists and physicians who normally would not study the genetics of a virus might start examining the newly available data.

The Zika virus is a *Flavivirus* carried by mosquitoes and was originally found in a Rhesus monkey placed in the Zika forest of Uganda in 1947, as described by Haddow et al. (1964). Over the last 60 years it has been the cause of epidemics in several African countries. However, in 2010 the virus spread to parts of Asia, in particular to Thailand (Fonseca et al., 2014; Haddow et al., 2012), and by 2013 had reached French Polynesia (Baronti et al., 2014). Since then there has been an explosive epidemic affecting the populations of many countries in both South and Central America. At the present time this epidemic shows no signs of abating.

In the many small epidemics that have occurred, there has been no indication of the virus causing anything but mild and transient infections. In the recent epidemic in Polynesia, however, cases of central nervous system damage have been observed and described as being a form of Guillain-Barré disease (Korff, 2013; Winer, 2014). In the current Brazilian epidemic, the emphasis has been on the possibility of an association with birth defects, especially microcephaly, resulting from maternal infection with Zika in the first and second trimester of pregnancy (Mlakar et al., 2016). The presumptive link between Zika infection and microcephaly is now looking more and more likely. Further cases of Guillain-Barré syndrome have also been seen.

The Zika virus is closely related to the viruses of Yellow, Dengue and West Nile fever, all of which cause significant illness and mortality. However, there are many other flaviviruses that are less well known and their hosts include horses, sheep, bats, birds and many other animals. A paper in 1998 listed over 70 different flaviviruses (Kuno et al., 1998), with new ones continuing to be identified (Moureau et al., 2015).

All flaviviruses appear to have much the same structure. The mature virus particles, virions, are about 50 nm in diameter and icosahedral in shape. Modern electron microscopy can show

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virions in considerable detail (Zhang et al., 2013; Zhou, 2014). The outer part is formed by an envelope overlying a phospholipid bi-layer membrane and the core contains a single stranded RNA molecule of about 10k bases.

In the mature Zika virion, the RNA molecule, which encodes a polyprotein, is described as having 10 272 bases, or 3 424 3-base codons for specific amino acids. The translation of bases to functional codons is not perfect, but for analysis purposes it has become accepted to describe the structure of the molecule in this manner:

starting with MKN ... and ending with ... GVL  
(i.e. The codons for: methionine, lysine, asparagine .....  
..... glycine, valine, leucine).

The polyprotein is a linear assembly of both structural and non-structural genes. The structural genes are for the envelope, membrane and capsid, and the non-structural genes are usually NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Bollati et al., 2010). For the purposes of this paper, this simple explanation will suffice.

The envelope and membrane genes define how the outer part of a virion is conformed. This outer part is important as it acts as an antigen for antigen-antibody reactions and also in the interaction between the virus and entry receptors when a virion attempts to enter a cell. Consequently, mutations affecting the construction of the envelope and membrane are probably more significant than mutations in other parts of the genome, and perhaps of greater influence when it comes to possible changes in virulence.

It remains unclear as to which cells, if not all cells, in humans are susceptible to invasion with the Zika virus. However, the cells of the central nervous system do appear to be especially vulnerable (Mlakar et al., 2016). In all instances, the process appears to be the same, whereby a virion attaches itself to the entry receptors on the outer surface of the cell and then enters the cell in a process described as endocytosis (Perera-Lecoin et al., 2014).

Once inside a human cell, the envelope and membrane separate from the core. The virus then hi-jacks the cellular apparatus for its own purposes. The polyprotein is copied and cleaved into its constituent parts and daughter virions are produced, each containing its own copy of the polyprotein (Clark & Harris, 2006).

At present, there are few drugs that prevent replication of the Zika virus, and the older and well-established antiviral drugs, such as amantadine, which are active against the influenza virus, are not helpful against flaviviruses (Oxford et al., 1970). However, a great deal of work is being done to find new antiviral drugs (Sampath & Padmanabhan, 2009). It is interesting to note that a traditional Chinese remedy, Xiyapping, was used for its anti-viral properties in the treatment of a recent case of Zika (Deng et al., 2016).

In relation to the present epidemic, the ability of the Zika virus to enter cells of the placenta and the central nervous system is particularly important. For now, however, it is unclear whether or not these invasions are more dependent on the strain of the virus, the genetic make-up of the host, or some other factors. Once the virus has crossed the placental barrier to enter the

fetus or the blood-brain barrier to enter the central nervous system, it is likely that the usual antigen-antibody reactions are lessened and the virus is able to proliferate more easily. It is also unknown as to how long it might take for the virus to be cleared from the fetus or central nervous system, although it does seem likely that virus replication can continue in these areas for many months.

## MATERIALS AND METHODS

### GenBank database

The RNA sequences for the Zika virus in the public domain can be found in the GenBank database of the National Institute of Health (Benson et al., 2013). At present (March 2016), there are 16 complete sequences from viruses collected in Africa and Asia before the start of the present epidemic and 17 sequences produced since. Details of these sequences are given in Table 1.

The corresponding page on the GenBank database for a given sequence can be found by using a URL of the form: <http://www.ncbi.nlm.nih.gov/nuccore/KU744693>. Each page gives the amino acid list and nucleotide base FASTA file for the RNA sequence. However, a GenBank page contains no real explanation as to what each list or file might mean and for this reason the author has developed a pair of Zika virus utilities that allow the user to compare one sequence with another.

### Zika virus utilities

In conjunction with this paper, two simple utilities were prepared and can be found on the author's website in the form of two webpages ([www.ianlogan.co.uk/zikapages/zika.htm](http://www.ianlogan.co.uk/zikapages/zika.htm)). From there the user can choose either the Amino Acid Analyser or the Nucleotide Base Analyser

The Amino Acid Analyser has in its source file copies of the amino acid lists for all complete RNA sequences found in the GenBank database and a small JavaScript program that allows the user to compare any sequence against any other. The results are displayed as a list of amino acid changes.

The Nucleotide Base Analyser has in its source file copies of the FASTA files for the complete RNA sequences of all sequences in the current epidemic. Again, a small JavaScript program enables the user to compare two sequences and show the mutational differences between them.

Although the webpages can be viewed with any commonly used web browser, the author recommends MOZILLA FIREFOX as it allows the user to alter the size of the text area, if needed.

It is the author's intention to keep these webpages up-to-date as new Zika RNA sequences appear on the GenBank database.

## RESULTS

### Non-synonymous amino acid changes observed in the present epidemic

A mutation that causes a non-synonymous change of an amino acid is often considered to be significant. However, if the change is between amino acids of similar size and polarity, there is probably no effective change in the functioning of the target protein.

**Table 1** ZIKA RNA sequences in GenBank database (01 March 2016)

Accession no.	Country of origin	Date of collection
African sequences		
1 LC002520	Uganda	1947
2 HQ234499	Malaysia	1966
3 HQ234500	Nigeria	1968
4 KF383116	Senegal	1968
5 KF383115	CAR	1968
6 HQ234501	Senegal	1984
7 KF268948	CAR	1976
8 KF268949	CAR	1980
9 KF268950	CAR	1980
10 KF383117	Senegal	1997
11 KF383118	Senegal	2001
12 KF383119	Senegal	2001
Asian sequences		
13 EU545988	Micronesia	2007
14 JN860885	Cambodia	2010
15 KU681082	Philippines	2012
16 KU681081	Thailand	2014
Current epidemic		
Brazilian reference sequence		
17 KJ776791	Polynesia	2013
18 KU365779	Brazil	2015
19 KU365778	Brazil	2015
20 KU365777	Brazil	2015
21 KU365780	Brazil	2015
22 KU312312	Suriname	2015
23 KU501215	Puerto Rico	2015
24 KU509998	Haiti	2014
25 KU321639	Brazil	2015
26 KU527068	Brazil	2015
27 KU647676	Martinique	2015
28 KU501216	Guatemala	2015
29 KU501217	Guatemala	2015
30 KU707826	Brazil	2015
31 KU497555	Brazil	2015
32 KU740184	China	2016
33 KU744693	Venezuela	2016

The amino acid changes shown by the Zika RNA sequences in the present epidemic are listed in Table 2. The table demonstrates that by using this method the sequences can be split into 12 different strains with between 0 and 25 amino acid changes.

The mutation M2634V is common to all virus strains that have come from countries in South and Central America and is caused by the base mutation A7900G. However, as this mutation is found in the NS5 gene, it is unlikely to be of significance as to the virulence or general behavior of the Zika virus. The NS5 gene is involved in the replication of new virions and is not a structural gene (Zhao et al., 2015). It is perhaps too early to say that this mutation has absolutely no effect, but for the moment the M2634V mutation can be seen as a useful marker to the present epidemic.

**Base mutations in samples collected in the present epidemic**  
While there are relatively few non-synonymous mutations in the virus strains collected in the present epidemic, there are many more synonymous mutations (i.e., mutations that do not produce a change of amino acid), and as a result a phylogenetic tree can be constructed.

Figure 1 shows the phylogenetic tree produced by using the mutations from the 17 complete Zika sequences currently found in the GenBank database. This figure shows the virus samples can be separated into 15 different strains, considering the sequence pairs KU365777/KU365780 and KU365799/KU707286 as being from two strains.

#### Estimation of the Zika virus mutation rate

The data presented in Figure 1 show that actual mutations vary between 9 and 64 for the sequences collected during the present epidemic. This number was calculated by considering the mutations that have occurred since the outbreak of the epidemic in Brazil, and Figure 1 suggests the use of a hypothetical Brazilian Reference Sequence (BRS) to describe a possible sequence for the original strain arriving in Brazil.

The number of mutations found in a sequence appears to be proportional to the date on which the original sample was collected. The early sequences show between 9 and 30 mutations, whereas the two latest sequences, KU740184 and KU744693, show 30 and 64 mutations, respectively. The latter sequence is from a sample collected on 6 February, 2016, and shows that the Zika virus continues to mutate at a rapid rate.

As the present epidemic can be considered to have started in Polynesia in 2013 (Baronti et al., 2014) and has now lasted about 2.5 years (i.e., late 2013 to early 2016), the mutation rate appears to vary between about 12 to 25 mutations a year. The genome of the Zika virus is normally considered to be a polyprotein of 10 272 nucleotide bases, so the mutation rate can also be considered as changing between 0.12% and 0.25% of the RNA polyprotein each year.

It is not possible, using the data presently available, to provide a more accurate value for the mutation rate. However, the suggested rate of 12 to 25 mutations a year would appear to be a suitable starting point for further studies.

## DISCUSSION

#### Present epidemic

The decision by the World Health Organization to declare a Public Health Emergency in February 2016 due to the threat of

**Table 2** Non-synonymous amino acid changes found in sequences from the present Zika virus epidemic

GenBank accession no. -Country of origin-Date of collection	Amino acid changes				
KJ776791-Polynesia-2013					
KU365778-Brazil-2015	M2634V				
KU365779-Brazil-2015	M2634V				
KU707826-Brazil-2015	M2634V				
KU365777-Brazil-2015	M2634V	N2778D			
KU365780-Brazil-2015	M2634V	N2778D			
KU312312-Suriname-2015	M166T	T769A	M2634V		
KU501215-Puerto Rico-2015	I80T	A2611V	M2634V		
KU527068-Brazil-2015	K940E	T1027A	M1143V	T2509I	M2634V
KU647676-Martinique-2015	D107E	R1118W	I1226T	M2634V	T3353A
KU501216-Guatemala-2015	V346I	G894A	M2074L	M2634V	K2694R
KU501217-Guatemala-2015	V346I	G894A	M2074L	M2634V	K2694R
KU497555-Brazil-2015	S550T	L1259F	M2634V	E2831V	
KU740184-China-2016	D107E	D445G	I1285V	M2634V	T2749I
KU509998-Haiti-2014	Y916H	H1857Y	I2295M	I2445M	M2634V
KU321639-Brazil-2015	Y916H	H1857Y	I2295M	I2445M	M2634V
KU744693-Venezuela-2016	E76D	V323A	I442L	V503A	D520A
	H613D	V620G	A623G	F739I	A794G
	S970W	R1005W	T1050A	C1107S	R1118Q
	H1857Y	S1867R	D1938G	I2295M	A2313P
	D2419E	I2445M	M2634V	S2807A	H2809K
	P2833A	N2974I	M2975T	V3334A	E2831D

The change M2634V (Methionine to Valine), common to all sequences, occurs in the *NS5* gene and is the result of the base mutation: A7900G, which changes the codon from 'ATG' to 'GTG'.

a Zika virus pandemic may be thought a pessimistic move. However, the evidence appears to indicate that the Zika virus is no longer restricted to localized habitats nor largely dependent on the monkey as its host. It now covers a much larger area in South and Central America where it is wholly dependent on the human as its host. Unfortunately, there appears to be little, if any, herd immunity against the virus in the populations of these countries, despite the closely related Dengue virus being prevalent.

The sudden spread of Zika to South and Central America does not appear to have been due to any change in the mosquito vector or anything known to make the Zika virus more virulent. Rather, it appears related to the fact that infected people are now able to fly rapidly from country to country, thereby spreading the disease very easily. This means there is little to stop the epidemic continuing to spread to other populations that also have low levels of herd immunity against the virus.

The absence of mosquitoes and the low incidence of person-to-person spread of the virus will probably mean the epidemic will not spread in countries of the southern and northern latitudes. From evidence obtained so far, however, it would appear likely the epidemic is only at its earliest stage and any suggestions as to what might happen remain speculative (Bewick et al., 2016).

#### Zika phylogenetic tree

The two utilities prepared for this study show that many distinct strains of the Zika virus now exist, even though the present epidemic is less than three years old. When considering just the non-synonymous mutations in the RNA, it is possible to define 11 strains in the present epidemic. However, a more detailed

examination looking at the actual mutations of the available sequences distinguishes 15 strains. As more data are made available, it is expected that the number of identifiable strains will increase. The phylogenetic tree shown in Figure 1 suggests the beginning of a geographical spread of the associated virus strains, with distinct strains now coming from Martinique, Guatemala, Puerto Rico and Suriname, whilst Brazil continues to show a mix of strains.

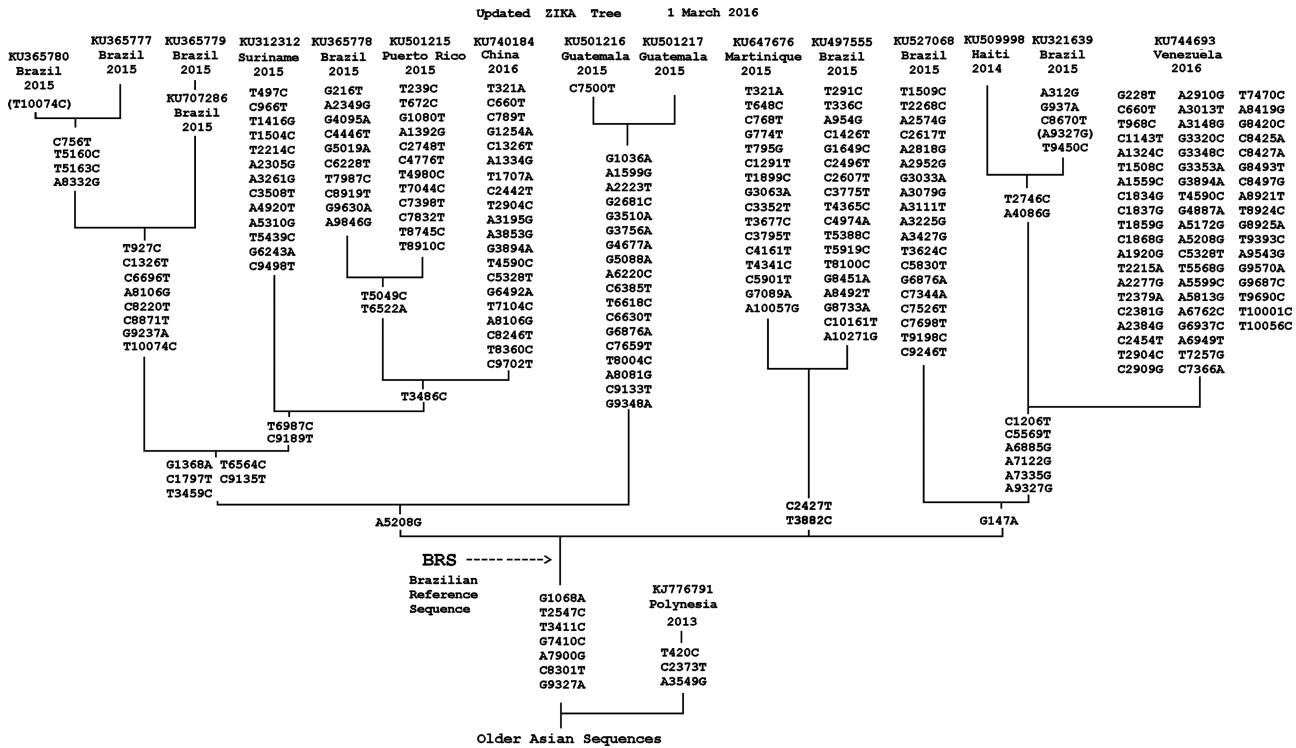
#### Estimation of the Zika mutation rate

The data used in building the phylogenetic tree can also be used to estimate the mutation rate of the Zika virus, which appears to vary between about 12 to 25 mutations per year, equivalent to 0.12%-0.25% of the RNA mutating each year. This rate is very high when compared to the human DNA mutation rate, where a period of perhaps 250 000 years might be expected (Logan, 2015). Nonetheless, it is not really appropriate to compare RNA mutations against DNA chromosomal mutations as DNA replication is a self-correcting process, whereas RNA duplication is liable to many sorts of errors.

However, the clear evidence of a high mutation rate in the Zika virus will allow for the present epidemic to be tracked in a fairly simple manner, and should be helpful in identifying where local mosquito prevention initiatives are working and where they are not.

#### Zika infection complications

A particular feature of the present epidemic is the presumptive link to the high incidence of fetal abnormalities and cases of Guillain-Barré syndrome. These two complications appear to be



**Figure 1** Phylogenetic tree of the 17 Zika RNA sequences from samples collected in the present epidemic

Two missing mutations - indicated by the brackets: C10074T in sequence KU365780-Brazil-2015 and G9327A in sequence KU321639-Brazil-2015, were probably caused by technical errors. The position of a hypothetical Brazilian Reference Sequence (BRS) is marked on the tree. The BRS is used in the two utilities, the Amino Acid Analyser and Nucleotide Base Analyser prepared for this paper and available at: [www.ianlogan.co.uk/zika-pages/zika.htm](http://www.ianlogan.co.uk/zika-pages/zika.htm).

caused in very different ways, with fetal abnormalities possibly being the result of direct fetal infection, and Guillain-Barré syndrome cases possibly due to an exaggerated auto-immune response (Cao-Larneau et al., 2016; Willison et al., 2016).

In the author's opinion, however, both conditions may result from the same underlying cause, in which the virus is able to cross the normally impenetrable placental and blood-brain barriers. How this happens is unclear, but it might just be a matter of a person getting a very high initial infection, possibly by having been bitten by a physically large carrier mosquito, or being bitten by several carrier mosquitoes in a very short period of time.

A study using the West Nile virus (Styer et al., 2007) showed that whilst most of the inoculum from a mosquito bite remains localized in the skin, there is always a significant initial viraemia. In this respect, a recent report from Slovenia (Mlakar et al., 2016) showed the X-rays of an affected fetus having numerous calcifications in the placenta and brain. Whilst it is unproven, it would seem possible that these lesions result from localized 'viral plaque formation' associated with an initial viraemic spread. A similar clinical picture is seen in tuberculosis. Although this disease is caused by a bacterium and not a virus, the resulting X-ray picture of localized calcifications is well-recognized and is termed miliary tuberculosis (Khan et al., 2011; Yang et al., 2015).

It is also possible that the risk of developing complications from the Zika virus may reflect the genetic differences between sufferers and the general population. At the present stage of our knowledge, however, there is no indication of what particular differences might be important.

## CONCLUSIONS

This study shows in a simple way how sequencing data from samples of the Zika virus available in the public domain can be collected and analyzed. Using this data, it is possible to construct a phylogenetic tree and show that in the present epidemic there are already many identifiable virus strains. The data also show that the Zika virus has a high mutation rate.

This short paper raises as many questions as it tries to answer. The present epidemic is from the Zika virus, but Yellow Fever cases are rising in Africa and Dengue affects millions of people each year. Thus, further pandemics caused by flaviviruses, other than Zika, pose a continuing threat.

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## Journal Correction

In the paper "The Australasian frog family Ceratobatrachidae in China, Myanmar and Thailand: discovery of a new Himalayan forest frog clade" (*Zoological Research*, 2016, 37(1): 7-14), all the scientific name of the species "*alpine*" should have been "*alpina*". Under Figure 3 on page 12, "*Liu. Alpine*" and "*Liu. Xizangensis*" should have been "*Liu. alpina*" and "*Liu. xizangensis*", respectively.

In the paper "A new genus and species of treefrog from Medog, southeastern Tibet, China (Anura, Rhacophoridae)" (*Zoological Research*, 2016, 37(1): 15-20), in Table 1 on page 17, the GenBank accession number of *Theloderma beibengensis*, *T. moloch* and *Nasutixalus medogensis* sp. nov. should have been KU243080, KU243081 and KU243082, respectively.

The online versions have been corrected. *Zoological Research* apologizes to the authors and readers for the mistakes.

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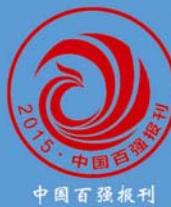
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