Female giant panda (*Ailuropoda melanoleuca*) chirps advertise the caller’s fertile phase

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Although female mammal vocal behaviour is known to advertise fertility, to date, no non-human mammal study has shown that the acoustic structure of female calls varies significantly around their fertile period. Here, we used a combination of hormone measurements and acoustic analyses to determine whether female giant panda chirps have the potential to signal the caller’s precise oestrous stage (fertile versus pre-fertile). We then used playback experiments to examine the response of male giant pandas to female chirps produced during fertile versus pre-fertile phases of the caller’s reproductive cycle. Our results show that the acoustic structure of female giant panda chirps differs between fertile and pre-fertile callers and that male giant pandas can perceive differences in female chirps that allow them to determine the exact timing of the female’s fertile phase. These findings indicate that male giant pandas could use vocal cues to preferentially associate and copulate with females at the optimum time for insemination and reveal the likely importance of female vocal signals for coordinating reproductive efforts in this critically endangered species.

**Keywords:** giant pandas; vocal communication; acoustic cues to oestrous stage

1. INTRODUCTION

Giant pandas (*Ailuropoda melanoleuca*) are solitary during most of the year, only associating briefly with other conspecífics during the annual breeding season (Schaller et al. 1985). Consequently, male and female giant pandas rely heavily on effective communication not only to locate one another in their natural environment, but also to negotiate the delicate courtship process leading to mating. Although olfaction is well established as a critical sensory modality in this species’ sexual communication, allowing giant pandas to recognize individuals and determine whether the signalling animal is reproductively mature, male or female, or of high competitive status (for review see Swaisgood 2004), vocal signals are also likely to be crucial for synchronizing male and female reproductive behaviour. Female giant panda chirps are high-pitched tonal vocalizations that are produced almost exclusively during their oestrous period and are theorized to solicit male attention (Kleiman & Peters 1990). Furthermore, these vocalizations increase in frequency as oestrous approaches (Lindburg et al. 2001) and could play an important role in providing information about short-term hormonal changes associated with oestrous.

Several non-human mammal studies have shown that female vocal behaviour can advertise fertility; however, the majority of this work only considered the occurrence of different call types (Buesching et al. 1998) and changes in calling rate across the female oestrous cycle (Matochik et al. 1992; Wielebnowski & Brown 1998; Leong et al. 2003; Schön et al. 2007) rather than the actual information content of female vocalizations. Other studies on non-human mammals that have considered structural variation within female calls and reported acoustic differences between female reproductive stages did not use objective criteria (i.e. hormone measurements) to quantify the exact period of oestrous (Semple & McComb 2000; Semple et al. 2002). It is important to assess whether the acoustic structure of individual female calls varies significantly around their fertile phase and hence, that males would be able to use them to determine the exact timing of the female’s fertile phase. Furthermore, it is crucial to consider the context in which female vocalizations are given, in order to determine whether changes in call structure are a result of female reproductive stage per se or other factors that could alter their motivational levels. Indeed, to our knowledge, only recent work on humans has shown that the acoustic structure of female mammal vocalizations varies significantly around their fertile period while using hormone data to quantify the exact period of oestrous and controlling for recording context (Bryant & Haselton 2009). Consequently, whether the acoustic structure of female non-human mammal vocalizations can signal the exact timing of the females’ fertile phase remains an open question.

In the current study, we used a combination of hormone measurements and acoustic analyses to determine whether female giant panda chirps have the potential to
directly signal the caller’s fertile phase, i.e. while statistically controlling for the social context of recording. In addition, we examine the response of male giant pandas to female chirp vocalizations produced during fertile versus pre-fertile stages of the female’s oestrous cycle. Female giant pandas are mono-oestrous and effective communication of reproductive stage is likely to be crucial for coordinating mating efforts in this species. Moreover, male giant pandas compete for mating opportunities with oestrous females and follow females for up to a month prior to copulating, yet show no tendency to mate guard after copulation (Schaller et al. 1985). These observations suggest either a first male advantage, or that male giant pandas can somehow assess female fertility and time their reproductive attempts to coincide with the female’s fertile stage. If male giant pandas can extract information about female reproductive state from chirp vocalizations, it makes adaptive sense for them to target their reproductive attempts to maximize the likelihood of conception. Accordingly, owing to the higher probability of obtaining a successful fertilization around ovulation, we predict that males will respond more strongly to female chirps produced during their fertile phase than those given by pre-fertile females still several days away from ovulating.

2. MATERIAL AND METHODS

(a) Study site and animals

The giant pandas involved in this study were housed at the China Research and Conservation Center for the Giant Panda (CRCCGP), Sichuan, China. The CRCCGP was located at Wolong Research Base, Sichuan, China, but relocated to Bi Feng Xia nature reserve (also in Sichuan, China) after the Sichuan earthquake of May 2008. Consequently, our recordings were captured during the 2008 breeding season at Wolong Research Base and the playback experiments to males were conducted at Bi Feng Xia nature reserve during the 2009 breeding season. All the animals were housed separately and were individually recognizable.

(b) Recordings

Female chirps were recorded between March and April 2008 at Wolong Research Base, Sichuan, China, from each of 14 female giant pandas during their oestrous cycle (for a spectrogram and waveform of a female chirp see figure 1). The recordings were captured using a Marantz PMD660 solid-state recorder and a Sennheiser MKH 70 P48 directional microphone (sampling rate of 44.1 kHz, 16 bits amplitude resolution) at distances of 1–15 m and uploaded onto a MacBook Pro laptop computer. We noted whether individuals in adjacent enclosures were vocalizing or not during the recording sessions and considered a male and female to be involved in a vocal interaction if they were both vocally active at the same time and no other individuals were vocalizing on the recording. For the acoustic analysis we only used chirps with low background noise when a female was either vocalizing alone or during a vocal interaction with a male in an adjacent enclosure: giving us 679 chirps from the 14 females (168 recorded when females were vocalizing alone and 511 recorded during vocal interactions with males). In addition, because vocalizations produced within the same bout are more likely to be acoustically similar than those emitted in different bouts, and therefore not statistically independent, we only used chirps recorded at least 10 s apart. Finally, in order to standardize the context for the comparisons, recordings captured during breeding introductions (when a female is placed in the male’s enclosure for breeding purposes) were not included in the acoustic analysis, nor did we use any recordings captured more than 9 days before female ovulation (see §2d for details on chirp classification according to female fertile phase).

(c) Acoustic analysis

We used PRAAT DSP package v. 5.0.29 (Boersma & Weenink 2005) for the acoustic analysis and focused on fundamental frequency (F0) parameters and F0 stability-related features of female vocalizations. To measure F0 parameters, we extracted the F0 contour of chirps using the ‘To pitch (cc)’ command (time step = 0.001 s; minimum and maximum F0 = 250 and 2000 Hz, respectively). Time-varying numerical representations of the F0 contour were compared with the F0 contour as visualized on a spectrogram and checked to see if PRAAT was correctly tracking the F0. In the cases where PRAAT incorrectly tracked a harmonic (or sub-harmonic), numerical representations of the F0 contour were adjusted using the ‘Edit’ window in PRAAT, before the extracted F0 contour was played back as a sine wave for subjective comparison with the original recording. We then applied a 25 Hz smoothing filter to remove any rapid variations in F0 before measuring mean F0 and the cumulative F0 variation across the call (sumvar) (for more information on this F0 measurement technique see Reby & McComb 2003). To quantify F0 stability, we measured the harmonics-to-noise ratio (HNR), which indexes the periodic distribution of
To construct fertile and pre-fertile chirp sequences, we ran.

Table 1. The number of recordings captured for each subject during their fertile and pre-fertile phases. We controlled for uneven subject participation and temporal pseudoreplication (repeated measures taken from the same individual) by entering subject identity as a random factor in the linear mixed models. Recordings from subjects in bold were used as playback stimuli.

<table>
<thead>
<tr>
<th>subject</th>
<th>fertile</th>
<th>pre-fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cao Cao</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Guo Guo</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>Long Xin</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Mao Mao</td>
<td>56</td>
<td>12</td>
</tr>
<tr>
<td>Mei Qing</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Na Na</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>Xi Mei</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>Xiao Xiao</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Ye Ye</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Ying Ying</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>Zhang Ka</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Zhu Yun</td>
<td>62</td>
<td>17</td>
</tr>
<tr>
<td>Xi Xi</td>
<td>112</td>
<td>74</td>
</tr>
<tr>
<td>Fei Fei</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>total</td>
<td>460</td>
<td>219</td>
</tr>
</tbody>
</table>

For the playback experiments, to control for temporal variation in motivation, each male was presented with a playback sequence of fertile or pre-fertile chirps followed 5 min later by the alternate condition. Each male subject was presented with a different female exemplar and to provide a balanced experimental design the stimulus order for each playback was alternated across subjects. Moreover, each male received both stimulus orders (fertile followed by pre-fertile and pre-fertile followed by fertile) on separate days so that they were exposed to each experimental condition twice. The timing of the playback presentation was determined by the current behaviour of the subject: playbacks were only initiated when males were active, not eating or interacting with any animals in adjacent enclosures, and in the side of their enclosure furthest away from the speaker position. The playback sequences were presented to males using an Anchor Audio Explorer Pro speaker at peak sound pressure levels sounding equivalent to that of naturally chirping females of 75 dB SPL peak (determined using a Radio Shack Sound Level Meter, set for C-weighted fast response and measured 1 m from the source) and at distances of approximately 25 m (measured using a Bushnell Yardage Pro laser rangefinder).

Energy within the call in dB (lower levels indicating increased aperiodicity resulting in a perceptually harsher call), and a measure of the cycle-to-cycle variability in F0 across the call termed jitter. HNR was measured using the ‘To Harmonicity (cc)’ command in Praat (time step = 0.01; minimum pitch (Hz) = 250; silence threshold = 0.1 and periods per window = 1). To increase the validity of our jitter measurements, and the applicability of our results, we averaged three measurement values (local, relative average perturbation and five-point period perturbation quotient).

(d) Definition of female fertile phase based on endocrine data

In order to determine ovulation and group recordings relative to this, female hormone levels were measured using assays of oestrone-3-glucuronide and enzyme-linked immunosorbent assay as previously described (Czekala et al. 2003) (full details of these methods and the oestrogen profiles used to determine ovulation dates for each female are provided in the electronic supplementary material). In humans, conception probably peaks just before ovulation and the median ovum survival time is approximately 12 h (Lynch et al. 2006). However, sperm can be fertile for up to 72 h (Wilcox et al. 1995) and hence, any copulations occurring up to 3 days before ovulation can still be fertile. Based on this, we defined the ‘fertile’ phase of the female oestrous cycle as the 3 days prior to ovulation and the date of ovulation itself (−3 to 0 days) and the ‘pre-fertile’ phase as the 6 days (−4 to −9) immediately preceding this; giving us 219 pre-fertile and 460 fertile chirps for the comparisons (table 1).

(e) Playback stimuli and experiments

The playback experiments were conducted during March and April 2009 on seven adult male giant pandas aged 6–19 years (mean = 12 years) housed at Bi Feng Xia nature reserve, Sichuan, China, and San Diego Zoo, USA. To construct fertile and pre-fertile chirp sequences, we randomly selected eight fertile chirps and eight pre-fertile chirps from each of seven females. The playback sequences consisted of these eight single chirps played consecutively and then repeated, so that males were presented with a total of 16 single chirps from either the female’s fertile or pre-fertile stages. The chirps in each sequence were separated by 4 s, constituting a realistic rate of delivery for these female vocalizations (R. Swaisgood, personal observation). The total mean duration for fertile and pre-fertile chirp playback sequences was 68.3 ± 2.4 and 66.1 ± 1.9 s, respectively. An acoustic analysis indicated that fertile chirp stimuli had longer duration ($P_{\text{1105}} = 39.280$, $p < 0.001$), higher jitter ($P_{\text{1105}} = 7.741$, $p = 0.006$) and tended to have lower HNR ($P_{\text{1105}} = 3.795$, $p = 0.054$) and hence, our playback stimuli were representative of this class of stimuli (see results of acoustic analysis; for details on analysis procedures see § 2c,d). Although the female chirps used as playback stimuli in this experiment were recorded at the base during the previous years breeding season, these individuals were not present at the breeding centre when the experiments were conducted. Moreover, we controlled for any pre-existing preferences that males might have had for particular exemplars by using a matched pairs design in which both experimental conditions from a given female exemplar (fertile and pre-fertile) were presented to each male.

For the playback experiments, to control for temporal variation in motivation, each male was presented with a playback sequence of fertile or pre-fertile chirps followed 5 min later by the alternate condition. Each male subject was presented with a different female exemplar and to provide a balanced experimental design the stimulus order for each playback was alternated across subjects. Moreover, each male received both stimulus orders (fertile followed by pre-fertile and pre-fertile followed by fertile) on separate days so that they were exposed to each experimental condition twice. The timing of the playback presentation was determined by the current behaviour of the subject: playbacks were only initiated when males were active, not eating or interacting with any animals in adjacent enclosures, and in the side of their enclosure furthest away from the speaker position. The playback sequences were presented to males using an Anchor Audio Explorer Pro speaker at peak sound pressure levels sounding equivalent to that of naturally chirping females of 75 dB SPL peak (determined using a Radio Shack Sound Level Meter, set for C-weighted fast response and measured 1 m from the source) and at distances of approximately 25 m (measured using a Bushnell Yardage Pro laser rangefinder).

(f) Behavioural analysis

Male behaviour was videotaped during the playback and for 1 min after the playback sequence had ended (the experimental period) using a Sony hard drive digital camera (model DCR-SR42) mounted on a tripod. To quantify male behavioural responses during the experimental period, the videotapes were analysed frame-by-frame (frame = 0.04 s) using the Gamebreaker 5.1 digital video analysis system for Mac OS 10 (SportsTec, Sydney) and male enclosures were divided up into three equally sized sections. This was done to determine the amount of time each male spent in the third of their enclosure nearest the playback speaker, termed the proximity zone. To further quantify the strength of the male’s response to the playback stimuli, we also measured the subject’s first look duration, number of looks and the total duration of looks given towards the
speaker while stationary, and noted whether males approached the speaker position or not during the actual playback. The subject was deemed to be looking towards the playback source when their head was oriented towards the speaker having previously faced away.

**Statistical analysis**

We used linear mixed effect models to examine the data in which we entered female oestrous stage (fertile or pre-fertile) as a fixed factor categorical-independent variable and subject identity as a random factor. By entering subject identity as a random factor, we controlled for repeated measures taken from the same individual. For the analysis of the acoustic data, our five acoustic measures (duration, mean F0, sumvar, HNR and Jitter) were entered as dependant variables in the linear mixed effect models, significance levels were set at 0.05 and two-tailed probability values are quoted.

### 3. RESULTS

**Acoustic analysis**

Female giant panda chirps given during their fertile phase were characterized by longer duration ($F_{1,677} = 5.893, \ p = 0.015$), greater jitter ($F_{1,674} = 13.805, \ p < 0.001$) and lower HNR/increased harshness ($F_{1,677} = 9.235, \ p = 0.002$) than those given during the pre-fertile phase (see figure 2a, d, e, respectively). No significant differences between female chirps given during fertile and pre-fertile phases were detected for mean F0 ($F_{1,665} = 1.653, \ p = 0.199$) and sumvar ($F_{1,677} = 0.016, \ p = 0.899$) (figure 2b and c, respectively).

**Playback experiment**

No difference in male looking response component to either playback condition was detected ($F_{1,21} = 0.073, \ p = 0.789$) (figure 3a). In contrast, movement response component was significantly higher when males were presented with fertile chirps than when they were presented with pre-fertile chirps ($F_{1,21} = 11.499, \ p = 0.003$); males were more likely to approach the speaker during the playback (18 times during the 28 playbacks) and spent more time in the proximity zone (54.99 ± 8.37 versus 26.16 ± 6.79 s) when presented with fertile chirps (figure 3b).
4. DISCUSSION

The results of this study indicate that female giant panda chirps have the potential to provide males with precise information about the timing of the caller’s fertile stage. Previous work on giant pandas suggested that the pitch and duration of female bleats increased as peak oestrus approached (Lindburg et al. 2001); however, these findings are not easily generalized because data from only one captive female was used and the acoustic measures were only subjective. Moreover, these reported changes in female bleats might have been a simple consequence of increased male–female interactions in the lead up to ovulation. By not using recordings captured during direct physical interactions with males, i.e. during breeding introductions, and statistically controlling for female involvement in vocal interactions, the results of the acoustic analysis presented here show unequivocally that female giant panda chirps produced during their fertile phase differ acoustically from those produced just prior to this period, and as such, constitute the first demonstration that structural variation within female non-human mammal calls can precisely signal the female’s fertile phase.

In particular, female giant panda chirps signalling fertile callers were of longer duration and characterized by higher jitter and harshness (lower HNR or tonality). The longer duration and increased harshness of chirps produced by fertile females could plausibly indicate greater arousal levels. Indeed, work on non-human mammals has shown that increased call duration is associated with increased urgency (Manser et al. 2002) and increased harshness is generally associated with high sub-glottal pressure and hence, likely to reflect a highly motivated caller (Fitch et al. 2002; Reby & McComb 2003). The higher jitter we found in fertile female chirps might be directly caused by the rise in oestrogen known to occur in female giant pandas just prior to ovulation (Lindburg et al. 2001). Human studies have shown that oestrogen increases vocal fold stiffness, by increasing oedema of the vocal fold mucosa (Abitbol et al. 1999), and decreased vocal fold elasticity/increased vocal fold stiffness is associated with increased jitter in human speakers (Ferreri 1959). It is conceivable, therefore, that a proximate cause for the increased jitter we found in fertile female giant panda chirps could be the rise in oestrogen occurring just prior to ovulation.

We also found that male giant pandas were more likely to approach and spend time in close proximity to playback speakers broadcasting fertile versus pre-fertile female callers. Although our sample size of seven males means we must exercise some caution when generalizing to the population, the magnitude of our results provide strong evidence that male giant pandas are more attracted to chirps produced by females during the fertile phase of their reproductive cycle. In humans, female voice pitch provides a reliable acoustic cue to ovulation and relevant pitch changes are perceptible (Bryant & Haselton 2009); however, whether male humans actually prefer female voices signalling fertile over non-fertile speakers is not known. In fact, the only previous mammal study to quantify male responses to female calls signalling different reproductive stages was conducted on Barbary macaques (Semple & McComb 2000). While this study revealed a male preference for female copulation calls given during late oestrus versus those produced on average 19 days prior to this time, whether male Barbary macaques can use these vocalizations to determine the exact timing of the female’s fertile phase is unknown.

Given the giant panda’s brief annual window for potential conception, selection should favour females that effectively signal fertility and males that can accurately assess this information. Indeed, male reproductive success in giant pandas may hinge on their ability to perceive fertility cues through several sensory modalities. Nevertheless, visual cues to fertility in this species, such as vulva colour and swelling, may be hard to gauge accurately and female anogenital marking and urine rates are typically highest prior to the fertile period (Lindburg et al. 2001), suggesting that female olfactory signals are more important for initially recruiting males and priming this solitary species for more direct sexual interactions. Our findings indicate that female giant panda chirps are important for coordinating mating efforts once potential mates have been located. We suggest that female chirps advertising fertility could facilitate mating, possibly by stimulating males or causing them to stay in close contact with females around their fertile period. Furthermore, female giant panda chirps advertising fertility are likely to evoke heightened intra-male competition, with the result that females gain the indirect benefits of more competitive offspring when mated by dominant/high-quality males that can outcompete rivals when the probability of conception is highest.

Figure 3. Error bar graphs to show estimated marginal means $\pm$ s.e. of male (a) looking and (b) movement responses to the playback stimuli. *$p < 0.01$. 

\[ \text{Figure 3. Error bar graphs to show estimated marginal means} \]

\[ \text{estimated marginal means} \]

\[ \text{pre-fertile fertile} \]

\[ \text{female chirp stimuli} \]
In conclusion, we have provided some important insights into the possible function of female giant panda chirps. In particular, we have shown that these female vocalizations have the potential to provide males with precise information on female fertility. Accordingly, female chirp vocalizations could provide the cues for dominant males to preferentially associate and copulate with females at the optimum time for insemination, when females are also increasingly likely to ‘cooperate’ with copulation attempts. This is an important distinction to make. For example, while the acoustic features of female Barbary macaque copulation calls vary when peak oestrous calls are compared with those given much earlier in the oestrous cycle (Semple & McComb 2000), they do not vary significantly across the 15 days spanning peak oestrous (Pfefferle et al. 2008) and, therefore, it is unlikely that males could use them to preferentially target fertile females during this period. By demonstrating that female giant panda chirps differ acoustically between fertile and pre-fertile callers and that males perceive these differences, our findings have not only furthered our knowledge of giant panda sexual communication, but also emphasize the likely importance of female vocal signals for coordinating reproductive efforts in this critically endangered species.

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