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Levels of extra-pair paternity are associated with parental care in penduline tits (*Remizidae*)

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In most passerine birds, individuals attempt to maximise their fitness by providing parental care while also mating outside their pair bond. A sex-specific trade-off between these two behaviours is predicted to occur since the fitness benefits of extra-pair mating differs between the sexes. We use nest observations and parentage analysis to reveal a negative association between male care and the incidence of extra-pair paternity across three species of penduline tit (Remizidae). This provides evidence of a trade-off between these two behaviours, possibly due to the devaluing of paternal care by extra-pair offspring.

Parental care and mating systems are predicted to co-evolve because parental care is likely to enhance offspring fitness at the expense of future reproductive success (Williams 1966, Trivers 1972, Owens & Bennett 1994, Kokko & Jennions 2008). Males are predicted to enhance fitness by increasing their number of mates whereas females are constrained by their fecundity (Bateman 1948; Clutton-Brock & Vincent 1991; Kokko *et al.*, 2006). Theory therefore predicts that males are more likely to seek additional mates whilst females provide care (Trivers 1972). However, birds are often seen as an exception to this rule as most exhibit biparental care (Clutton-Brock & Parker 1992, Cockburn 2006, Kokko & Jennions 2008) and have a high prevalence of multiple matings by females (Parker 1970, Birkhead & Møller 1992, Yasui 1998, Arnqvist & Nilsson 2000). Research has therefore focussed on the benefits females might gain from multiple matings (Kempnaers *et al.* 1992, Jennions & Petrie 2000, Griffith *et al.* 2002). However, understanding the ramifications of this promiscuity for parental care investment

(Westneat & Sherman 1992, Sheldon 2002) is necessary if we are to understand the co-evolutionary relationships between these traits (Kokko & Jennions 2008).

Two factors have been identified that are likely to have implications for the evolutionary relationship between mating and parental care systems. The first is female promiscuity and its potential influence on the fitness benefits associated with paternal care. Female re-mating produces broods with multiple paternity and, in turn, reduces the fitness benefits to males associated with their paternal care; an equivalent devaluation due to extra-pair copulations (EPCs) does not apply to maternal care (Queller 1997). Thus, in most species, polyandry should select for a decrease in male, but not female, care. The second variable is the population sex ratio (Kokko & Jennions 2008), which is a central factor influencing mate acquisition; for example a female-biased sex ratio provides increased opportunities for males to re-mate, potentially at the expense of providing paternal care.

Few studies have investigated the evolutionary dynamics of the relationship between parental care and male and female promiscuity across species sets where suitable variation exists. Where such a comparative approach has been pursued, the predicted relationship of reduced male care in species with higher levels of extra-pair paternity (EPP) has been confirmed (Møller & Birkhead 1993, Arnold & Owens 2002). We aim to further this work by focussing on a group that has garnered attention for its unusual parental care behaviour. The penduline tits exhibit diverse parental strategies, with the Eurasian Penduline Tit *Remiz pendulinus* exhibiting unusual sex-specific patterns of care, which are consistent across populations and repeatable within individuals (Persson & Öhrström 1989, Pogány *et al.* 2008, van Dijk *et al.* 2010a).

The Eurasian Penduline Tit exhibits high levels of EPP (24% of offspring) and low levels of male parental care (van Dijk *et al.* 2010b). Care is always uniparental in this species and males are less likely to provide care than females (Szentirmai *et al.* 2007, van Dijk *et al.* 2007, 2010a, Pogány *et al.* 2008). Previous work has focussed on determining the factors that

influence this widespread, sex-biased desertion behaviour (Valera *et al.* 1997, Bleeker *et al.* 2005, van Dijk *et al.* 2008a, 2012, Pogány *et al.* 2012, 2015). Importantly, this unusual parental strategy is consistent across European populations despite variation in mating opportunities, suggesting that parental behaviour is unresponsive to local environmental cues (Pogány *et al.* 2008, van Dijk *et al.* 2008a, 2010a). Thus, we draw on existing knowledge of the Eurasian Penduline Tit and combine it with new data on two closely-related species, the Cape Penduline Tit *Anthoscopus minutus* and the White-crowned Penduline Tit *Remiz coronatus* to better understand the co-evolutionary relationship between promiscuity and parental care. We provide a detailed description of parental care systems, calculate offspring sex ratios and use molecular genetic analyses to characterise EPP to test the prediction that greater levels of EPP are associated with reduced paternal care.

METHODS

The Cape Penduline Tit was studied over a six-year period (2002–2007) at Koeberg Nature Reserve, Western Cape, South Africa (33° 38' S, 18° 26' E) and the White-crowned Penduline Tit was studied in 2008 in the Tien Shan foothills near Jabagly, Kazakhstan (42° 25' N, 70° 29' E). The Eurasian Penduline Tit population from which we leverage existing information was studied from 2002–2007 at Fehértó fishpond system, Hungary (46° 19'N, 20° 06'E).

All adult birds were ringed with a numbered metal ring and a unique combination of three plastic colour rings ('XF' size, A. C. Hughes, London, UK). The capture and sampling of Cape Penduline Tits were approved by the Animal Ethics Committee, University of Cape Town, and conducted under permits issued by SAFRING and the Western Cape Nature Conservation Board. The White-crowned Penduline Tit work was approved by the University of Bath Animal Ethics committee and conducted under the stewardship of the Association for the Conservation of Biodiversity of Kazakhstan. Blood samples (~10µl) were taken from the brachial vein and stored in 1 ml of Queen's Lysis Buffer (Seutin *et al.* 1991). Across all years the total number of

blood-sampled birds was 194 in the Cape Penduline Tit population (38 adults, 13 juveniles and 135 nestlings, plus an additional eight birds that were sampled as nestlings and recruited adults in subsequent years). For the White-crowned Penduline Tit population, blood samples were obtained from 63 birds (32 adults and 31 nestlings) and in the Eurasian Penduline Tit study 654 blood samples were genotyped (211 adults and 443 offspring; van Dijk *et al.* 2010b).

Parental care was recorded through observations made every two days at each nest, noting the identity and behaviour of adult birds (Cape Penduline Tit: 21 nests, White-crowned Penduline Tit: 18 nests, Eurasian Penduline Tit: 138 nests). Additionally, videos of parental behaviour during the latter stages of incubation (8–12 days after the last egg was laid) were recorded at six Cape Penduline Tit nests (299±44 minutes of footage per nest) and nine White-crowned Penduline Tit nests (173±7 minutes of footage per nest). The percentage of time that each parent spent in the nest was recorded as a measure of incubation investment. Parental provisioning rates were also calculated from videos taken in the latter stages of nestling care (12–15 days post-hatching) at five Cape Penduline Tit nests (317±38 minutes of footage per nest). Thus, our continual observations of colour-ringed birds throughout incubation and nestling care, supported by video footage, allowed us to identify the care provider(s) at each nest.

Parentage was assigned using 17 microsatellite markers for both the Cape Penduline Tit and White-crowned Penduline Tit populations (Table 1). Paternity was allocated using the program COLONY (v 2.0.5), which assesses the likelihood of sibships within the population and allocates paternity and maternity via a maximum likelihood method (Wang & Santure 2009, Jones & Wang 2010). Due to the conservative assignment of COLONY, birds were accepted as assigned correctly if they had a likelihood score ≥80% (as in Bergner *et al.* 2014, Gamero *et al.* 2014).

A Bayesian generalized linear mixed model (BGLMM, R Package 'blme') with binomial error structure was used to test whether paternal care (Binary response variable, 'care' or 'desert') was explained by EPP in our model. The explanatory variables of the model included the amount of EPP in a nest (the percentage of offspring unrelated to the social father) and species (Eurasian, White-crowned Penduline Tit or Cape Penduline Tit) as fixed effects. Male ID was included as a random term because the same male was occasionally observed across multiple nesting attempts. A 'normal'-prior was applied to the fixed effects when running the model to alleviate the problems of complete separation in the dataset. Sex differences in incubation and feeding rates were tested using a linear mixed model approach for both the White-crowned Penduline Tit and Cape Penduline Tit separately. Nest ID was included as a random term in all models and the null model (without sex) and the full model were compared using a likelihood ratio test to test for the effect of sex on either the proportion of time spent incubating or the provisioning rate of nestlings (visits/hour). A two-sided binomial exact test was used to test for any deviations from the expected equal sex ratio in the entire population of White-crowned Penduline Tit and Cape Penduline Tit nestlings. Additional details of field and laboratory methodology can be found in Supplementary Online Appendix S1.

RESULTS

Microsatellite genotyping and sex-typing

A total of 167 (34 adults, 13 juveniles and 120 nestlings) Cape Penduline Tits and 63 (32 adults and 31 nestlings) White-crowned Penduline Tits were genotyped, including a roughly similar number of adult males and females (Cape Penduline Tit: 16 males: 18 females, and White-crowned Penduline Tit: 19 males: 13 females). Details of the microsatellite loci genotyped for the parentage analyses are provided in Table 1. The overall sex ratio of 9–10 day old nestlings did not differ from parity in either Cape Penduline Tit offspring (62 males: 58 females, binomial

exact test, two-tailed; $P = 0.78$) or White-crowned Penduline Tit offspring (18 males: 13 females, binomial exact test, two-tailed; $P = 0.47$) using the *Z-002A* sexing marker.

Patterns of parental care

In the Cape Penduline Tit, parental care was provided by both males and females at the observed nests until fledging of offspring ($n = 21$). At six (29%) of these nests a helper was observed provisioning the nest in addition to the focal pair and in one case this was identified as a juvenile from a previous nest of the pair. Of the 18 nests observed in the White-crowned Penduline Tit population, both the male and female carried out incubation, and biparental care continued through nestling provisioning at 14 nests (78%). At the remaining four nests either the male (two nests; 11%) or the female (two nests; 11%) ceased parental care before nestling provisioning (Figure 1). The video footage supported the field observations of biparental care with no evidence for sex-specific care roles in the two studied species (Appendix S2). Contrastingly, in the Eurasian Penduline Tit nestling care is dominated by female-only care (109/138 nests; 79%) with the remaining nests exhibiting male-only care (29/138; 21%) (van Dijk *et al.* 2010b).

Parentage analysis

Of the 13 Cape Penduline Tit nests where the social mother and father were genotyped, two contained extra-pair young (15.4% of nests). These three extra-pair young were caused by EPP, resulting in three out of 42 (7.1%) nestlings having EPP (Figure 2). When including nests where a single social parent was genotyped, three of the 28 nests contained extra-pair young (10.7% of nests, a total of four nestlings). In these nests, three chicks of 97 (3.1%) were the result of EPP and one (1%) the result of intraspecific brood parasitism (IBP) i.e. one was unrelated to the caring female. This low level of IBP is consistent with the low rates observed across the majority

of bird species (Arnold & Owens 2002) including in the Eurasian Penduline Tit (6.6% of offspring result from IBP). In the Cape Penduline Tit, the average percentage of offspring that are unrelated to the social male per brood was 5.4% (across 28 nests). The probability that a female will be caring for unrelated offspring was 0.9%. In the White-crowned Penduline Tit no evidence of extra-pair offspring was found; all nestlings ($n = 29$) were assigned to both social parents with $\geq 97\%$ likelihood at the five nests at which both social parents were known (Fig. 2).

In our BGLMM, EPP was a weak negative predictor of paternal care across penduline tit nests (Table 2). The most important variable explaining paternal care was species, as paternal desertion was significantly more prevalent in the Eurasian Penduline Tit population (Table 2). However, as the Eurasian Penduline Tit exhibits substantially higher levels of young resulting from EPP (24% of 166 nestlings) than that observed in the mostly biparental Cape Penduline Tit or White-crowned Penduline Tit (7.1% of 42 and 0% of 29, respectively; Figure 2), the problem of collinearity complicates interpretation.

DISCUSSION

We provide evidence across three closely-related species of penduline tit that those with higher rates of male compared to female care exhibit lower rates of EPP. These results are consistent with a previous study that demonstrated that the level of male care during chick provisioning is negatively correlated with EPP (Møller & Birkhead 1993). Considering that biparental care is provided by most (c. 84%) passerine species (Cockburn 2006), highlighting this result in a family with diverse and atypical parental care strategies may provide future opportunities to disentangle the drivers of this relationship.

The adult or operational sex ratio was the second variable predicted to influence the mating and parental care system both within and between species, as it potentially skews the mating opportunities of each sex (Liker *et al.* 2014). In line with our prediction, we found no evidence of a bias in the sex ratios of nestlings in the two biparental species (White-crowned Penduline Tit and Cape Penduline Tit). Although we cannot rule out the possibility of skewed sex ratios amongst adults, the low rates of EPP combined with biparental care found in these two species would be consistent with unbiased adult sex ratios (but see van Dijk *et al.* 2008b showing parity of offspring sex ratio also in the polygamous Eurasian Penduline Tit). In most bird species, and especially small passerine birds, it is difficult to estimate adult sex ratios (Donald 2007). However, in a taxon of birds that facilitate more accurate estimations of the population sex ratio, an association between sex ratio and parental care behaviour has been observed (Liker *et al.* 2013). We therefore cannot rule out that biased adult sex ratios in the Eurasian Penduline Tit caused after fledging might be responsible for differing fitness costs associated with providing paternal care.

We acknowledge that this study would benefit from increased sample sizes of populations and nests because extrapolating observations from a single population limits species-wide inferences (Lott 1991), and because EPP estimates increase in accuracy with increased samples of nests (Griffith *et al.* 2002). However, we would argue that the parental care system in the single population of the Eurasian Penduline Tit is typical of that observed across geographically distinct populations (van Dijk *et al.* 2010a). Although this provides some support that parental care observed in our single Cape Penduline Tit and White-crowned Penduline Tit populations is typical of each species, we require definitive evidence for this. In addition, we acknowledge the need for increased sample sizes in EPP studies in general. Due to the intensive nature of this research, both in the field and lab, few studies have assessed EPP and parental care in multiple populations of the same species (see Brommer *et al.* 2010 for an exception). Nonetheless, our results highlight a key difference worth further investigation. For example, if we were to sample five nests in the Eurasian Penduline Tit population (29/55 nests

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contained chicks resulting from EPP) the likelihood of gaining only nests with within-pair young, as was the case in the White-crowned Penduline Tit, is only 3.4%. The proportion of nests containing EPP in the Cape Penduline Tit (18%) is also significantly lower than the 53% that contain young resulting from EPP in the Eurasian Penduline Tit (Pearson chi-squared test, $\chi^2 = 4.5$, $df = 1$, $P = 0.03$). Thus, even low sample sizes can reveal a significant difference in EPP rates between populations. However, we hope future research effort will focus on assessing intraspecific variation in EPP, the underlying reasons for this variation and how this may relate to the evolution of parental care behaviour.

Diversity in promiscuity rates remains one of the most puzzling topics in evolutionary biology and to tackle it will likely require a multifaceted approach (Arnold & Owens 2002, Griffith *et al.* 2002, Kokko & Jennions 2008). We would argue based on our findings that differences in mating and parental care among populations within species and among closely-related species, are likely to generate novel insights into the co-evolution of these critical life-history traits. Our study has confirmed an assortment of parental care systems across the penduline tit family that are associated with levels of EPP. The White-crowned Penduline Tit and Cape Penduline Tit exhibit biparental care and high levels of fidelity, in contrast to the high levels of EPP and reduced paternal care observed in the Eurasian Penduline Tit. The Remizidae may therefore provide an informative model system to test specific evolutionary hypotheses that could explain diversity in breeding systems more generally.

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Table 1. Microsatellite characterization in the White-crowned Penduline Tit and Cape Penduline Tit.

Locus	Species	# individuals Genotyped	Allele size range (bp)	# alleles	Ho	He	Estimated null allele frequency
<i>Remiz-01</i>	Cape Penduline Tit	148	107–269	20	0.29	0.75	0.442
	White-crowned Penduline Tit	67	169–190	6	0.63	0.73	0.064
<i>Remiz-07</i>	Cape Penduline Tit	168	206–214	3	0.23	0.23	-0.019
	White-crowned Penduline Tit	67	159–179	6	0.81	0.79	-0.016
<i>Remiz-09</i>	Cape Penduline Tit	166	113–168	11	0.37	0.74	0.334
	White-crowned Penduline Tit	67	108–137	9	0.45	0.79	0.269
<i>Remiz-10</i>	Cape Penduline Tit	168	177–206	5	0.46	0.48	0.030
	White-crowned Penduline Tit	67	187–211	7	0.78	0.77	-0.005
<i>Remiz-14</i>	Cape Penduline Tit	-	-	-	-	-	-
	White-crowned Penduline Tit	67	206–223	9	0.76	0.80	0.026

Locus	Species	# individuals Genotyped	Allele size range (bp)	# alleles	Ho	He	Estimated null allele frequency
<i>Remiz-17</i>	Cape Penduline Tit	-	-	-	-	-	-
	White-crowned Penduline Tit	67	142–164	6	0.78	0.71	-0.042
<i>Remiz-18</i>	Cape Penduline Tit	167	100–394	24	0.89	0.89	-0.005
	White-crowned Penduline Tit	67	83–114	9	0.85	0.80	-0.034
<i>CAM-10</i>	Cape Penduline Tit	172	183–194	7	0.68	0.72	0.036
	White-crowned Penduline Tit	67	179–187	4	0.43	0.42	-0.041
<i>CAM-13</i>	Cape Penduline Tit	172	213–218	5	0.51	0.50	-0.005
	White-crowned Penduline Tit	67	215–221	7	0.76	0.77	-0.001
<i>CAM-15</i>	Cape Penduline Tit	168	283–385	11	0.85	0.85	0.004
	White-crowned Penduline Tit	-	-	-	-	-	-
<i>CAM-17</i>	Cape Penduline Tit	173	203–211	5	0.37	0.39	0.045

Locus	Species	# individuals Genotyped	Allele size range (bp)	# alleles	Ho	He	Estimated null allele frequency
CAM-18	White-crowned Penduline Tit	67	203–204	2	0.46	0.49	0.028
	Cape Penduline Tit	167	334–340	4	0.50	0.54	0.041
CAM-20	White-crowned Penduline Tit	-	-	-	-	-	-
	Cape Penduline Tit	-	-	-	-	-	-
CAM-24	White-crowned Penduline Tit	66	198–211	11	0.86	0.83	-0.026
	Cape Penduline Tit	-	-	-	-	-	-
TG01-040	White-crowned Penduline Tit	67	97–102	3	0.22	0.35	0.208
	Cape Penduline Tit	171	291–293	2	0.35	0.33	-0.037
TG01-124	White-crowned Penduline Tit	-	-	-	-	-	-
	Cape Penduline Tit	149	404–406	2	0.16	0.18	0.059
	White-crowned Penduline	64	396–400	2	0.28	0.31	0.040

Locus	Species	# individuals Genotyped	Allele size range (bp)	# alleles	Ho	He	Estimated null allele frequency
	Tit						
TG03-098	Cape Penduline						
	Tit	169	233–234	2	0.43	0.49	0.065
	White-crowned Penduline						
	Tit	67	229–230	2	0.58	0.50	-0.076
TG04-012	Cape Penduline						
	Tit	171	136–141	3	0.40	0.42	0.013
	White-crowned Penduline						
	Tit	67	137–145	5	0.64	0.67	0.010
TG04-041	Cape Penduline						
	Tit	173	170–190	10	0.71	0.71	0.009
	White-crowned Penduline						
	Tit	-	-	-	-	-	-
TG04-061	Cape Penduline						
	Tit	173	184–195	7	0.67	0.77	0.066
	White-crowned Penduline						
	Tit	67	188–196	4	0.27	0.25	-0.065
TG05-046	Cape Penduline						
	Tit	171	326–332	3	0.58	0.53	-0.047
	White-crowned Penduline						
	Tit	-	-	-	-	-	-
TG05-053	Cape Penduline	173	201–295	9	0.79	0.78	-0.013

Locus	Species	# individuals Genotyped	Allele size range (bp)	# alleles	Ho	He	Estimated null allele frequency
TG11-011	Tit						
	White-crowned Penduline						
	Tit	67	231–240	9	0.88	0.83	-0.035
	Cape Penduline						
	Tit	-	-	-	-	-	-
	White-crowned Penduline						
TG12-015	Tit	67	210–216	4	0.64	0.63	-0.013
	Cape Penduline						
	Tit	170	279–281	2	0.09	0.10	0.032
	White-crowned Penduline						
TG13-017	Tit	-	-	-	-	-	-
	Cape Penduline						
	Tit	-	-	-	-	-	-
	White-crowned Penduline						
	Tit	66	308–320	4	0.42	0.43	0.004

bp, basepairs; Ho and He represent observed and expected heterozygosity, respectively. Ho, He and estimated null allele frequencies were calculated in CERVUS v 3.0 (Kalinowski *et al.* 2007).

Table 2. Parameter estimates and fit statistics of the Bayesian GLMM model used to predict paternal care ('care' or 'abandon') at nests (n = 74) of three penduline tit species. Male ID was included as a random term.

Fixed effects	Estimate	s.e.	z	P
EPP	-1.01	0.92	-1.10	0.27
Species (Eurasian)	-8.19	2.01	-4.08	<0.001
Species (White-crowned Penduline Tit)	1.31	1.94	0.67	0.50

Figure titles

Figure 1. Diversity of nestling provisioning behaviour in three penduline tit species. The identity and sex of the parent(s) provisioning the young post-hatching were recorded at each nest. The Eurasian Penduline Tit data are sourced from van Dijk *et al.* (2010b).

Figure 2. Extra-pair paternity (EPP) variation in three penduline tit species. The proportion of nestlings found to be either within-pair young (WPY), the result of EPP or intra-specific brood parasitism (IBP) are shown for each species. All data are based on offspring in nests where both social parents have been identified and the Eurasian Penduline Tit data are sourced from van Dijk *et al.* (2010b).

Figure 1.

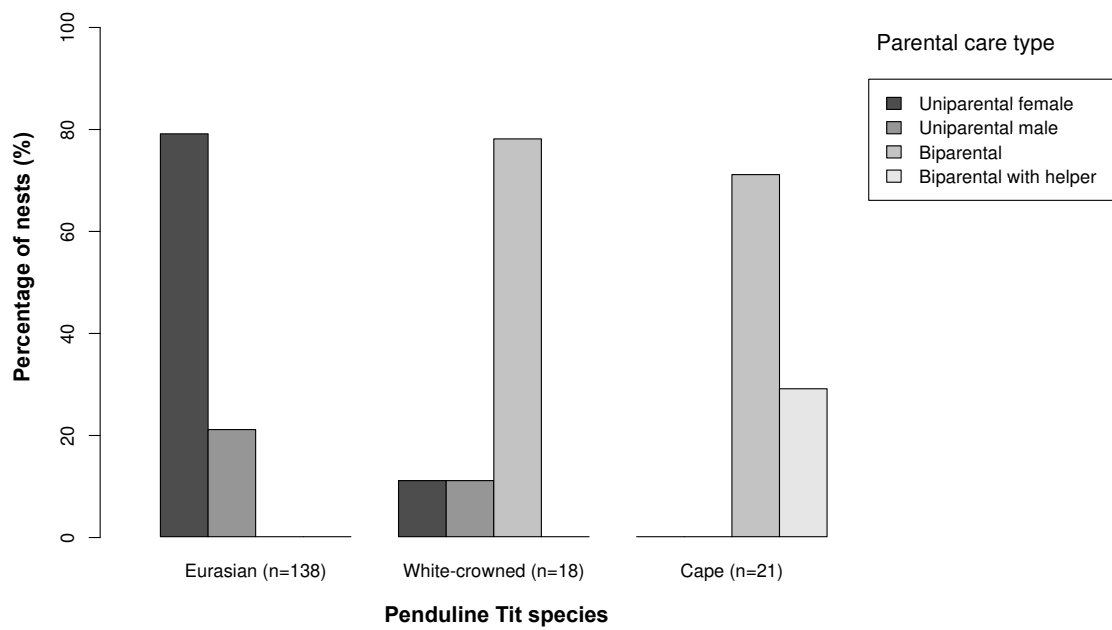


Figure 2.

