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Loss of Extra-pair Paternity is not Associated with Decreased Paternal Investment in Mixed-paternity Broods or Unrelated Nestlings in the Varied Tit, *Parus varius*

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W-D and L-D conceived the project, supervised the study and reviewed the manuscript. L-D and H-M designed the experiments and drafted the article. H-M, J-L and Z-L collected the samples and carried out Sex determination and paternity assignment. L-H, Y-JX assistanted in this research and revision of the paper.

Key words
Cuckoldry, Feeding rate, Genetic paternity, Linear mixed models, Microsatellite, Parental care

ABSTRACT

Males of socially monogamous bird species may be faced with providing costly care for unrelated offspring when nests have extra-pair young (EPY). Theoretical models predict that cuckolded males should lower their parental investment as the likelihood of paternity decreases. However, empirical data are not always in support of this prediction. Here, we explore parental behaviours within the context of extra-pair patenity (EPP) in a population of the varied tit *Parus varius* in China. The results showed that 39.5% of nests were cuckolded and 16.4% of chicks were sired by extra-pair males. We found no evidence that male and female varied tits reduced their feeding rates or relative feeding effort to EPP broods, or that they decreased provisioning for EPY in mixed broods. There was also no direct effect of EPP on the reproductive success of breeding adults and the body condition nestlings near fledging. The lack of reliable cues of EP copulations (EPC)s by social mates available for the males, and/or the absence of strictly environmental pressure on males that would favor discrimination may account for a lack of an adjustment in feeding effort. The absence of discrimination between own and EPP chicks in parental care suggests that females pay no fitness cost as a result of EPCs, which may explain the high frequency of EPY in nests.

INTRODUCTION

The phenomenon of extra-pair paternity (EPP), is widespread among socially monogamous birds (Griffith *et al*., 2002; Neudorf, 2004). In nests with EPP, the social male sires only a proportion, if any, of the young, while continued paternal provisioning benefits both the female mate and the mixed-paternity brood (Lack, 1968; Silver *et al*., 1985). In these cuckolded nests, the social male cares for unrelated offspring, which may be very costly for them (e.g. reduced adult survival; Owens and Bennett, 1994; Kokko and Jennions, 2008; review in Santos and Nakagawa, 2012) and may not translate into improved fitness (Westneat and Stewart, 2003). As a result, theoretical models of EPP generally assume that cuckolded males should adjust their parental investment in direct proportion to the likelihood or proportion of paternity (Trivers, 1972; Møller, 1988; Sheldon, 2002). To test this prediction, a considerable volume of empirical avian EPP research has focused on the inverse relationship between male parental effort and the proportion of sired offspring (Westneat and Sherman, 1993; Griffin *et al*., 2013). Several studies have identified strong negative correlations between paternal care and perceived paternity loss (e.g. Dixon *et al*., 1994; Chuang-Dobbs *et al*., 2001; Suter *et al*., 2009; García-Navas *et al*., 2013; Ball *et al*., 2017). Yet other studies have failed to find any such relationships (e.g. Whittingham and Lifjeld, 1995; Wagner *et al*., 1996; Yezerinac *et al*., 1996; Barati *et al*., 2018; Gao *et al*., 2019; Cousseau *et al*., 2020). Such conflicting empirical evidence has even been found amongst different populations of the same species (e.g. common reed bunting *Emberiza schoeniclus*, Dixon *et al*., 1994; Bouwman and Komdeur, 2005; Suter *et al*. 2009). Therefore, more empirical studies of different species and/or different populations of the same species are necessary to understand in greater detail whether males adjust parental investment in relation to perceived or actual EPP.

One way to assess paternal effort is through examination of provisioning rates of males in relation to their paternity. Critically, only a handful of studies have examined detailed feeding bouts by cuckolded males to

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individual nestlings in broods with mixed paternity, to determine whether males recognize and preferentially feed their kin (Kempenaers and Sheldon, 1996; Peterson *et al*., 2001). Currently, most evidence suggests that males do not bias their provisioning behaviour towards their own offspring compared with unrelated offspring within the same brood (e.g. Westneat, 1995; Whittingham *et al*., 2003). However, in several cooperatively breeding bird species, kin-biased feeding behaviour has been confirmed (Emlen and Wrege, 1988; Komdeur, 1994; Sharp *et al*., 2005). Therefore, more data are needed on the social father's provisioning changes between provisioning his entire brood versus only part of it that is related to him genetically, to understand in more depth the effect of EPP on the male provisioning decision rules.

Variation in reported male responses to cuckoldry may also result from a lack of standardized approaches to assess male parental effort since male parental investment can be represented by various factors such as nest defense, incubation, and food allocation (i.e. Kempenaers and Sheldon, 1996; Rytkönen *et al*., 2007; Matysioková and Remeš, 2012; Hoi *et al*., 2013; García-Navas *et al*., 2013). Male feeding rates at nests are commonly used to represent male parental effort, but the contribution of male provisioning to broods may be misleading if the female partners reduce their own parental care efforts following cuckoldry (e.g.Du *et al*., 2015). As a result, the relative parental effort (the level of parental care invested by either sex) should be used as an alternative measure, combined with the feeding rate, to characterise the balance between parental care and sexual conflict through EPP (Sheldon and Ellegren, 1998; Matessi *et al*., 2009).

Here, we explore sex-specific parental care behaviour within the context of EPP, using an individually marked population of the varied tit (*Parus varius*) in China, to assess whether male tits who lose paternity decrease their feeding effort. Specifically, we predict the cuckolded social male tits will lower their parental care to whole broods with extra-pair young EPY and/or specifically to unrelated offspring; we also compare the fledging success and the mass of nestlings near fledging between cuckolded and non-cuckolded nests to test the effect of male paternal investment.

MATERIALS AND METHODS

Study site and species

The study was conducted at the Xianrendong National Nature Reserve (40.02° N, 122.94° E), Liaoning Province, China, from 2011-2013 and 2016-2018. The reserve is characterized by a monsoon climate, with temperature ranging between -25°C and 36°C, and an annual mean rainfall of 79.9 cm (Li *et al*., 2012). Coniferous and broad-leaved mixed forests are the dominant habitats at the reserve, with Korean pine (*Pinus koraiensis*), Mongolian oak (*Quercus mongolica*), and Manchurian walnut (*Juglans mandshurica*) being the most abundant tree species (Li *et al*., 2012). The varied tit is a cavity nester species and ready to using the nest box. At this reserve, the varied tits begin nesting in early April and lay their first eggs by mid-April (mean clutch size =6.85 ± 0.78 SD, range 4-8 eggs) (Ju *et al*., 2014). The breeding season lasts through July and the number of active nests peaks in late April, and again in mid-May with some pairs producing two broods in one breeding season (Li *et al*., 2013). This species is socially monogamous with a high degree of EPP occurring in 40% of the broods and 14.7% of offspring (Ju *et al*., 2014). Both sexes are responsible for nest construction, incubation, and nestling feeding (Li *et al*., 2012).

Nest box checks

In 2009, 270 nest boxes were randomly positioned in a 170-hectare forest plot. The next boxes were fixed on trees about 2 m above the ground with a random direction. Approximately 10% of the nest boxes are used by varied tits, 20% by great tits (*Parus major*), and less than 1% by other passerine species annually. Beginning in early April of each field season, all nest boxes used by varied tits were systematically checked every 2-3 days to determine the clutch initiation date, hatching date and the fate of the nest.

Bird capture, banding and blood sampling

All adult birds were captured during the late incubation or early nestling stages (<4 days post-hatching) inside the next boxes with the assistance of an automatic trap, which was constructed inside the entrance of each nest box. When a bird enters the nest box, the trap will be triggered by pulling on a twig treadle and the door will close securely with a magnet. This technique has proven successful in catching adult birds breeding in nest boxes in our study area (Zhang *et al*., 2019). The captured birds were weighed using electronic scales (0.01g) and the lengths of their tarus were measured using calipers (0.01cm). All the captured birds were individually ringed with colored rings and a uniquely numbered metal ring, which were always the social parents (further confirmed by video recordings in this study) of nests where they were captured. A blood sample (20 μl) was taken from the brachial vein of each adult bird (under licence: LU201102), then absorbed onto sterile filter paper, allowed to dry and stored in a bag at -20°C (Ju *et al*., 2014). Handling of each individual bird was completed within 5 min of capture to minimize stress to the bird.

Parental provisioning behavior sampling

Parental feeding behaviour was recorded using a micro-video camera (Star-net V90-Q5, Xingwang Corporation, Fujian). During the video-recording, the lid of the nest box was temporally exchanged with a glass one and the camera was fitted under the glass for a good view of the nest (Supplementary Fig. R1). The use of this method compromises the need for necessary luminance for video recording and minimization of disturbance, and as well as for keeping warm environment inside the nest box. During our observation, this approach did not appear to disturb the birds since parent birds continued to feed their chicks in the presence of the video camera. The nests were repeatedly filmed during three nestling stages: early (4-5 days post-hatching), medium (9-10 days post-hatching) and late (13-14 days post-hatching) when possible and only during suitable weather conditions (i.e. no heavy rainfall or strong winds). Video recordings began at 6:00-6:30 hrs (local time) for a duration of 6 h for each nestling stage per nest to ensure there was sufficient daylight inside the nest boxes to capture sufficient image resolution.

To distinguish between individual offspring in the nests while recording, we used a combination of different colours and symbols applied to the head of the chicks using nontoxic colored dyes (Supplementary Fig. R1, in additional information) at the onset of the first video recording, and then reapplied the coloration before the subsequent video recording session during 2011-2013; we did not mark individual chicks with dye during 2016-2018 as a result of conflict with another experimental design. All blood samples for the chicks were collected from the brachial vein only during the medium nestling stage to minimize any potentially negative effects on the nestlings. Immediately after the video recording, we measured the body mass of each chick as the same method of adult. All the chicks from other non-filmed nests were also measured at the same three different nestling ages.

Sex determination and paternity assignment

DNA was extracted from the blood samples stored on the filter paper using a standard phenol-chloroform extraction protocol (Singer-Sam *et al*., 1989). Adults and offspring were sexed using molecular sex identification technology with P2/P8 primers (Griffith *et al*., 1998), and the nature of the parental relationship between social adults and chicks was assigned based on a molecular paternity identification method. Nestling paternity was analyzed using eight fluorescently labeled microsatellite markers: Pava06, Pava09, Pava10, Pava14, Pma22, Pma42, Pocc6, and Pma179 (Ju *et al*., 2014), and paternity assessed by Cervus (ver. 3.0) (Kalinowski *et al*., 2007). We employed the EPP identification criteria that have been widely used in other parentage studies (e.g. Kreisinger *et al*., 2010; E *et al*., 2017): a chick was considered to be an EPY if its genotype did not match the social male at ≥2 loci, and if at least one of these mismatches was not due to allele-specific issues, e.g. allelic drop-out or null alleles.

Parental feeding behavior extraction and defination

The recordings were viewed using KM player (3.5). Each recording was carefully examined to extract the sex of each feeding and the chicks that were fed. Furthermore, three measures were caculated to investigate parental provisioning of the brood: (i) male feeding rate, which was defined as the number of male feeding trips per hour; (ii) female feeding rate; (iii) male's relative feeding rate (or effort relative to the female), which was expressed as the proportion of male feeding effort relative to total rate. In addition, to investigate whether males biased their provisioning toward their kin relative to non-kin, the (i) male feeding rate per chick for each mixed brood (the average number of feeding trips per nestling per hour), (ii) female feeding rate per chick and (iii) male's relative feeding rate per chick were calculated for each EPY and within-pair nestling during each nestling stage (Peterson *et al*., 2001; Gao *et al*., 2019).

Statistical analysis

We used R. 3.6.0 (R Core Team, 2019) for the statistical analyses. Linear mixed models (LMMs) with Gaussian distribution and identity links were used to analyze variations in male and female provisioning both between and within broods using lmerTest package (Kuznetsova *et al*., 2017). All the variables were checked first for multicollinearity using variance inflation factors (VIF); which generally were <2.2, indicating there was no multicollinearity among the predictor variables (Dormann *et al*., 2013).

Firstly, we constructed three LMMs to analyze parental provisioning to the brood based on the following three response variables: (1) male feeding rate (square-root-transformed for normal distribution of the residuals), (2) female feeding rate (log-transformed for normal distribution of the residuals) and (3) male's relative feeding rate (this was normally distributed). The global LMMs included two fixed factors: (1) EPP (yes or no) and (2) nestling stage (early, medium or late), and their interaction in order to test how the effects of EPP vary between nestling stage. Year and nest box identity were entered as random factors to control for non-independent sample effects, and nestling stage was considered as a repeated factor among trials conducted at the same nest. We also entered male or/and female body condition (using the residuals from an ordinary least-squares linear regression of the body

mass on tarsus length), brood size, and clutch initiation date (using continuous values centered around the mean egg laying date within each year to exclude possible confounding effects caused by between-year variations).

Secondly, we constructed three additional LMMs to analyze parental provisioning to the chicks in extra-pair broods and to analyze the male feeding rate per chick (square-root-transformed), female feeding rate per chick and male's relative feeding rate per chick using EPY (yes or no), nestling stage (early, middle or late), and their interaction as fixed factors. Year and nest identity were entered as random factors, and the EPY ratio within the mixed brood was entered as a covariate.

All candidate models were generated from global models using the dredge function in the "MuMIn" package 1.43.6 (Barton, 2019). We used Akaike Information Criteria (AICc; Burnham and Anderson, 2002) corrected for low sample sizes to assess model fit. All the models were ranked using ΔAIC values, and models with ΔAIC≤2 were considered as equivalent alternative models. Akaike weights (w_i) were used to provide a quantitative measure of support for each model relative to the others. As there was no more than one alternative model with delta AICc≤2 (Supplementary Tables S1, S2), we calculated conditional model-averaged parameter values (β-values) and their 95% confidence intervals (CI) for the predictor variables using all the models by model averaging in "MuMIn" package. We used standardized (z-scores) values of the predictor variables to facilitate comparisons of β-values. Once model-averaged parameters were obtained, we used Wald test Z scores to make inferences about each parameter estimate. The parameter estimations for each factor of interest from the global models were also modeled with Restricted maximum likelihood (ReML), and Satterthwaite's method t-tests were used to compare the parameter estimation. This method generally yielded the same statistically conclusions as the model averaging and was not shown in the results but in supplementary materials (Supplementary Tables S3, S4). Post-hoc Tukey HSD was used to examine differences between broods or chicks in relation to the EPY in different nestling stages. The alpha threshold was set to 0.05 and the results are expressed as the means ± standard errors.

RESULTS

Extra-pair paternity

In total, 116 video-recording samples were collected from 80 varied tit nests. From these, data were obtained for all three nestling stages from 13 nests, data on two nestling stages were obtained from 10 nests, and data from just one nestling stage were obtained from the remaining 57 nests due to logistical constraints. Detailed information about the sample sizes for each nestling stage can be found in the Figure 1. Thirty-two nests (39.5%) included at least one EPY. There was no significant variation in the brood size between EPP nests (6.50±0.20, n=32) and non-EPP nests (7.00 ±0.16, n=48; t=1.833, df =78, p=0.09). In total 90 EPYs were recorded from the 32 EPP broods, which accounted for 43.3% (n=208) of chicks of mixed parentage broods and 16.4% (n=549) of all nestlings.

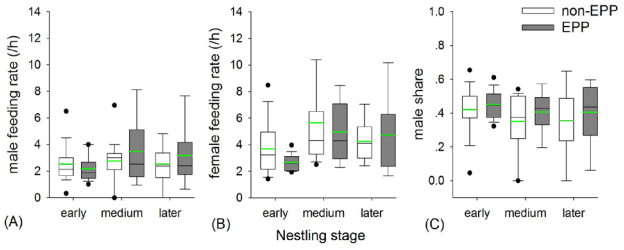

Fig. 1. Sex-specific social parental provisioning rates of adult varied tits directed towards broods in relation to EPP (sample sizes for early, medium and late nestling stages of non-EPP nests are 23, 24 and 18, and for EPP nests are 20, 18 and 13, respectively). Post hoc Tukey HSD tests show no significant differences in all values between EPP and non-EPP nests in each nestling stage (all p≥0.057). Boxplots and the whiskers show 25%, 75% and the 5%, 95% of the data, respectively. Black lines represent the median values and green lines the mean values.

Parental provisioning of broods

The best model was the null model with only the intercept when the male feeding rate was the response variable (△AIC ≤2; Supplementary Table S1). There was no significant difference in the male feeding rates between broods with or without EPY contained therein (estimates β ± se: 0.12 ± 0.15; z=0.64, p=0.442; Fig. 1a) or specially at any of the three different nestling stages (post hoc Tukey HSD: all ps≥0.33). Approaching significant differences (F=2.71, df=2, 44, p=0.078) in male feeding rates were found between nestling stages: male varied tits had much higher feeding rates during the medium stage than during the early stage (0.21 ± 0.07; z=2.91, p=0.004), but not between early and late stages (0.07 ± 0.08; z =0.78, p=0.435). All other variables had no significant effects on male feeding rate (Table I).

Only one model including the nestling stage satified ΔAIC ≤2 in the female feeding rate model (Supplementary Table S1). Female varied tits provided more feeding during the medium (0.21 ± 0.04; z=5.38, p<0.001) and late nestling stages (0.13 ± 0.04; z=3.13, p=0.002) than the early nestling stage. Female feeding rates at broods containing EPY were not significantly different from those

Table I. Effect estimates and standard errors (SEs) for each parameter from LMMs of parental provisioning of broods during 2011-2013 and 2016-2018.

Responses	Explanatory variables	Parameter β-values mean±SE	95% confidence interval		z value	P value
			Lower	Upper		
Male feeding rate	(Intercept)	1.50 ± 0.10	1.30	1.69	15.10	<0.001
	EPP	0.12 ± 0.15	-0.18	0.42	0.64	0.442
	Nestling stage: medium	0.21 ± 0.07	0.07	0.36	2.91	0.004
	Nestling stage: late	0.07 ± 0.08	-0.10	0.23	0.78	0.435
	Brood size	0.10 ± 0.13	-0.17	0.36	0.71	0.476
	Clutch initiation date	-0.18 ±0.16	-0.51	0.14	1.10	0.271
	Female body condition	-0.12 ± 0.13	-0.38	0.14	0.89	0.375
	Male body condition	-0.02 ± 0.14	-0.29	0.26	0.11	0.909
	EPP (yes): nestling stage (medium)	0.10 ± 0.14	-0.18	0.38	0.70	0.482
	EPP (yes): nestling stage (late)	0.31 ± 0.16	0.00	0.62	1.95	0.051
Female feeding rate	(Intercept)	0.46 ± 0.04	0.39	0.54	12.23	< 0.001
	EPP	-0.04 ± 0.04	-0.13	0.05	0.87	0.380
	Nestling stage: medium	0.21 ± 0.04	0.13	0.28	5.38	<0.001
	Nestling stage: late	0.13 ± 0.04	0.05	0.21	3.13	0.002
	Brood size	0.03 ± 0.04	-0.05	0.12	0.74	0.461
	First egg-laying date	0.00 ± 0.05	-0.10	0.10	0.02	0.982
	Female body condition	-0.02 ± 0.04	-0.11	0.06	0.51	0.61
	Male body condition	-0.06 ± 0.04	-0.14	0.03	1.34	0.18
	EPP (yes): nestling stage (medium)	0.03 ± 0.08	-0.12	0.18	0.39	0.696
	EPP (yes): nestling stage (late)	0.09 ± 0.08	-0.08	0.26	1.07	0.284
male share	(Intercept)	0.39 ± 0.03	0.34	0.44	14.32	< 0.001
	EPP	0.01 ± 0.04	-0.06	0.09	0.39	0.694
	Nestling stage: medium	-0.07 ± 0.02	-0.11	-0.02	2.85	0.004
	Nestling stage: late	-0.05 ± 0.02	-0.10	0.00	2.02	0.043
	Brood size	0.00 ± 0.03	-0.08	0.06	0.17	0.866
	Clutch initiation date	-0.02 ± 0.04	-0.10	0.06	0.51	0.612
	Female body condition	-0.03 ± 0.03	-0.09	0.04	0.86	0.393
	Male body condition	0.02 ± 0.03	-0.04	0.09	0.74	0.462
	EPP (yes): nestling stage (medium)	0.01 ± 0.04	-0.08	0.10	0.20	0.839
	EPP (yes): nestling stage (late)	0.04 ± 0.05	-0.06	0.14	0.72	0.474

Wald test Z scores were used to make inferences about each parameter estimate. Only *P* values ≤ 0.05 are highlighted in bold. The reference categories for "nestling stage" and "EPP" are "early" and "no," respectively.

containing none (-0.04 ± 0.04; z=0.87, *p*=0.380; Fig. 1b) as a whole and for any of the three different nestling stages (all *p*≥0.08), with no significant effects of other variables (Table II).

For the male's relative feeding rate, none of the fixed factors we looked at appeared in the model with the lowest AIC (ΔAIC ≤2). Varied tit fathers provisioned their young significantly less often during the medium (-0.07 ± 0.02; z =2.89, *p*=0.004) and late nestling stages (-0.05 ± 0.02; z=2.02, *p*=0.043) than during the early stage. All other variables (*p*s≥0.39), including EPP (0.01 ± 0.04; z=0.39, *p*=0.70; Fig. 1c), had no significant effects on the proportion of male feeding (Table II). There were no significant differences between broods containing some and no EPY at any of the three different nestling stages (all *p*s ≥ 0.32).

Table II. Effect estimates and standard errors (SE) for each parameter of the male varied tit strategy to feed EPY and their own nestling within mixed paternity broods (LMMs) during 2011-2013.

Responses	Explanatory variables	Parameter β-values mean±SE	95% confidence interval		z value	P value
			Lower	Upper		
Male feeding rate	(Intercept)	0.81 ± 0.18	0.46	1.16	4.52	<0.001
	EPY	-0.01 ± 0.05	-0.11	0.09	0.23	0.820
	EPY ratio	0.12 ± 0.12	-0.12	0.35	0.97	0.335
	Nestling stage: medium	0.15 ± 0.06	0.07	0.36	2.56	0.010
	Nestling stage: late	0.17 ± 0.06	0.04	0.29	2.60	0.009
	Brood size	-0.13 ± 0.09	-0.30	0.04	1.49	0.137
	Clutch initiation date	-0.22 ±0.16	-0.54	0.11	1.32	0.188
	Female body condition	0.03 ± 0.17	-0.31	0.38	0.19	0.846
	Male body condition	-0.09 ± 0.12	-0.32	0.15	0.71	0.478
	EPY (yes): nestling stage (medium)	-0.06 ± 0.12	-0.29	0.17	0.49	0.623
	EPY (yes): nestling stage (late)	-0.08 ± 0.12	-0.32	0.17	0.61	0.544
Female feeding rate	(Intercept)	0.61 ± 0.12	-0.27	0.84	5.11	< 0.001
	EPY	-0.10 ± 0.07	-0.24	0.05	1.30	0.192
	EPY ratio	-0.04 ± 0.21	-0.43	0.41	0.07	0.947
	Nestling stage: medium	0.11 ± 0.09	-0.08	0.29	1.13	0.259
	Nestling stage: late	0.12 ± 0.10	-0.08	0.32	1.18	0.238
	Brood size	0.07 ± 0.12	-0.18	0.33	0.57	0.572
	Clutch initiation date	-0.01 ± 0.25	-0.52	0.49	0.05	0.958
	Female body condition	0.14 ± 0.26	-0.38	0.67	0.54	0.59
	Male body condition	-0.00 ± 0.22	-0.45	0.44	0.01	1.00
	EPY (yes): nestling stage (medium)	0.03 ± 0.18	-0.33	0.38	0.15	0.884
	EPY (yes): nestling stage (late)	-0.11 ± 0.19	-0.49	0.27	0.58	0.565
male share	(Intercept)	0.51 ± 0.08	0.36	0.66	6.48	< 0.001
	EPY	0.05 ± 0.04	-0.04	0.13	1.05	0.294
	EPY ratio	0.09 ± 0.14	-0.20	0.38	0.62	0.533
	Nestling stage: medium	0.05 ± 0.06	-0.06	0.16	0.91	0.362
	Nestling stage: late	0.06 ± 0.06	-0.06	0.19	1.04	0.298
	Brood size	-0.14 ± 0.08	-0.29	0.01	1.83	0.068
	Clutch initiation date	-0.09 ± 0.17	-0.35	0.37	0.50	0.619
	Female body condition	0.01 ± 0.18	-0.35	0.37	0.06	0.953
	Male body condition	-0.06 ± 0.15	-0.35	0.23	0.41	0.679
	EPY (yes): nestling stage (medium)	-0.08 ± 0.11	-0.30	0.13	0.74	0.459
	EPY (yes): nestling stage (late)	-0.06 ± 0.11	-0.28	0.17	0.48	0.631

Wald test Z scores were used to make inferences about each parameter estimate. Only *P* values ≤ 0.05 are highlighted in bold. The reference categories for "nestling stage" and "EPY" is "early" and "no," respectively.

Parental provisioning of extra-pair and within-pair nestling in mixed parentage broods

The final models providing substantial support (ΔAIC≤2) for all three response variables (male feeding rate, female feeding rate and male's relative feeding rate) were all null models that did not include any of the global model's explanatory factors (Supplementary Table S2). There were no significant differences in the all three

response varibles between extra-pair and within-pair nestling in mixed broods (Fig. 2) or any other potential factors, except for nestling stage: the male fed broods more frequently during the medium (0.15 ± 0.06; z=2.55, p=0.010) and late stages (0.17 ± 0.06; z=2.66, p=0.009) than during the early stage (Table II).

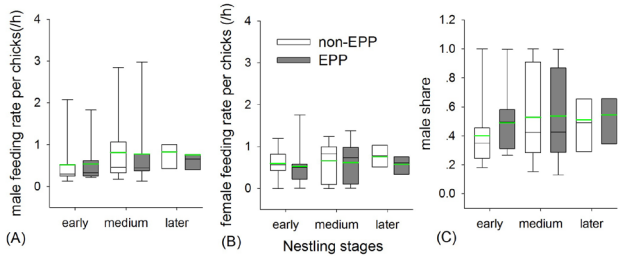

Fig. 2. Parental provisioning rates of varied tit toward individual chicks in relation to EPP (sample sizes for early, medium and late nestling stages are 9, 10 and 8 for both non-EPP and EPP nests). No significant differences between EPP and non-EPP group in each nestling stage (post-hoc Tukey HSD test (all $p \geq 0.339$). Boxplots and the whiskers show 25%, 75 % and the 5%, 95% of the data respectively. Black lines represent the median values and green lines the mean values.

Breeding success and body mass of near-fledging nestlings

There was no significant difference in the breeding success of birds feeding broods containing some (76.4%, n=72) or not EPY (84.5%, n=71; Chi-square Test: χ^2=1.025, df=1, p=0.311). Furthermore, no significant differences were found in the average body mass of nestlings in the late nestling stage (13-14 days post-hatching) between broods containing some (14.52 ± 0.30, n=54) and no EPY (14.17 ± 0.34, n=54; independent sample t-test: t =-0.30, df=106, p=0.763), or in the body masses of extra-pair (14.78 ± 0.26, n=52) and within-pair nestling (14.66 ± 0.28, n=52; t =-0.31, df=102, p=0.765).

DISCUSSION

Paternity and paternal care of broods

Theoretical models suggest that when cuckoldry lowers the probability of genetic relatedness of fathers to nestlings, cuckolded males should invest less in broods containing EPY due to the cost of male parental investment (Trivers, 1972; Møller, 1988; Sheldon, 2002). In contrast to these classical models' predictions, we found that cuckolded males did not change their parental care behaviour in response to relatedness to nestlings. This suggests that in this population at least, males either do not recognize that they have been cuckolded, or do not tend to adjust their provisioning strategy.

The prediction that cuckolded males should lower their food provisioning effort hinges on at least three key assumptions: (1) males can perceive loss of paternity; (2) that high feeding frequency reduces future adult fitness; and (3) that high loss of paternity this year is unrelated to prospects in later years (Westneat and Stewart, 1993; Lifjeld *et al*., 1998; also see Discussion in Barati *et al*., 2018). Firstly, any adjustment in male parental care may be attributed to the ability to perceive and assess the possibility of paternity loss (Whittingham *et al*., 1992; García-Navas *et al*., 2013). However, such clear clues of female infidelity, for example, pre-dawn extra-pair copulation (EPC) initiation outside the territory (Double and Cockburn, 2000; Ward *et al*., 2014), provided by females were usually difficult for the males to detect, but see García-Navas *et al*., 2013. In turn, males trying to use the strategy of mate guarding may react differently to EPP than males following a more frequent copulation strategy in that the mating access was easily monopolised (e.g. Komdeur, 2001; Valera *et al*., 2003; Kokko and Morrell, 2005). This may be an important reason for the huge difference among species ever found though it was still unclear which of these two strategies were most used by birds, including the varied tits in this study.

Although there is some evidence to support the second assumption in some taxa (Owens and Bennett, 1994; Kokko and Jennions, 2008; review in Santos and Nakagawa, 2012), if the third assumption were rejected, reducing investment in the current brood in order to have better prospects for the next breeding attempt would not be predicted for the tits, because then again the future prospects might be similarly poor as a result of environmental fluctuations in resource availability and nest predation (Zhang *et al*., 2014; Lee *et al*., 2016). In turn, if the expected survival rate to the next season is low for the birds, they should maximise investment in every breeding season because it might be their last. This predict seems to be much consistent with the situation of this tit speices, as the estimated annual adult survival rate is lower than 30% from long-term monitoring data. Alternatively, another prediction from the study of ground tits (*Pseudopodoces humilis*) that males may simply be doing 'the best of a bad job' and provide parental provisions to produce at least some related young even in EPP broods, might also fit well with the varied tits (Du *et al*., 2015).

Furthemore, there is a fourth possibility. The males did not reduce their feeding even though they can recognize EPY, because the cost of stopping their feeding of EPY is too high (Dickinson, 2003). There are three main reasons for this argument. First, the loudly begging calls of starving EPY will usually increase the nest predation rate for the whole brood by attracting predators (Leech and Leonard,

1997; Haff and Magrath, 2011). Secondly, if the birds die and decompose in the nest box due to low parental care, it will make their own chicks more vulnerable to infectious disease (Benskin *et al.*, 2009). Thirdly, if the starving EPY increase sibling aggression, the total survivorship of the social father's own nestling can also be reduced (Dickinson, 2003; Gao *et al.*, 2018).

The findings that the overall parental feedings rates of varied tits are significantly higher in medium and later stages was consistent with the previous study which implied that the age-difference parental provisioning may reflect on the varied energy demand of the nestlings (Li *et al.*, 2012; Gao *et al.*, 2018). However, the total feeding rate of varied tits was relative lower than that of other tit species (Hinde, 2006). It might be greatly related to the special provisioning strategy of this species that can bring multipe preys in each feeding bout (Jing *et al.*, 2019). The parental provisioning efforts of birds were believed to be largely regulated by the begging offsprings, as have been implied that the parents are usually the "loser" side to the chicks, either EPY or own, in the parent–offspring association (Budden and Wright, 2001; Kilner and Hinde, 2012). Howeve, due to the lack of begging behavioural data among the chicks within the mixed broods of varied tits, we can not decide what kinds of begging tactics do the EPYs chose to elimit the simialr feeding effort from both fosters.

Food provisioning and breeding success

Male varied tits with high EPP rates did not show any bias in their allocation of food to individual chicks, regardless of their genetic relationship with them. This result is consistent with several previous studies in altricial bird species indicating that there is generally a lack of kin-biased discrimination during parental care (e.g. Burke *et al.*, 1989; Dixon *et al.*, 1994; Kempenaers and Sheldon, 1996). This may be because the fitness advantage of biasing care for own vs. EPY is negligible when compared with the costs required for accurate kin recognition from the phenotypic characteristics in a fast-developing (and changing) brood (e.g. Johnstone, 1997). However, due to the logistical constraints, the sample size for this part of analysis was relative small (≤10), thus the underpowered statistically conclusion should be interpreted with caution.

We also did not find any effects of raising EPY on the the body mass of nestlings near fledging and breeding success. Research has shown that in some birds, the intensity of paternal care can positively affect reproductive output per breeding attempt (e.g. Moreno *et al.*, 1999). However, in our study there were no differences in parental care between pure and mixed broods and, in turn, a lack of change upon our metrics of nestling body condition. To address the theoretical requirements for male care for only related nestlings, further investigation of other factors, such as experimental approaches to alter perceived EPC risks and EPP cues with intruders (i.e. DiSciullo *et al.*, 2019), will be required.

CONCLUSION

In this study, male varied tits were not found to decrease their provisioning efforts in response to broods containing EPY. This is not consistent with current predictions of EPP theories. The cuckolded males did not bias their provisioning towards their own chicks and away from EPY. Thus, there is no evidence to suggest that varied tit males directly discriminate between related and unrelated offspring within a brood. The lack of reliable cues of EPCs by social mates available for the males, and/or the absence of strictly environmental pressure on males that would favour discrimination may account for a lack of an adjustment in feeding effort. Alternatively, the lack of direct linkage between male feeding rates on the body condition of near-fledging chicks may imply that feeding rate is not an accurate fitness-directing metric to be adjusted by cuckolded males. If males in this species adjusting other aspects of paternal behaviours (i.e. food quality) in response to EPY, merits further investigation.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200114210142

Statement of conflict of interest

All authors have declared no conflict of interest.

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