Introduction

The nature of the proximate mechanisms responsible for biased offspring sex ratios have been debated extensively in the past years (Krackow, 1995; Emlen, 1997; Oddie, 1998; Komdeur & Pen, 2002; Pike & Petrie, 2003). In birds direct modulation of offspring sex ratios can be expected as females are the heterogametic sex (ZW while males have ZZ) and sex-determining division in meiosis occurs prior to ovulation and fertilization (Pike & Petrie, 2003; Rutkowska & Badyaev, 2008). There is growing empirical evidence that avian sex ratios are biased at conception (Heinsohn et al., 1997; Badyaev et al., 2002; Komdeur et al., 2002; Polo et al., 2004; Pike & Petrie, 2005; Pryke & Griffith, 2009). This probably involves molecular and cytological mechanisms during avian meiosis such as biased segregation of sex chromosomes controlled by epigenetic factors, potentially hormones (Petrie et al., 2001; Pike & Petrie, 2003; Rutkowska & Badyaev, 2008). However, other authors emphasized the importance of sex-specific embryo mortality as cause of biases in sex ratios observed at hatching (Krackow, 2002; Cichon et al., 2005; Rutkowska & Cichon, 2006; Svensson et al., 2007). Embryo mortality might be the by-product of sexual dimorphism in susceptibility to early environmental conditions or embryonic growth rates (Krackow, 2002; Cichon et al., 2005). When one sex (the larger in sexually dimorphic species) is more susceptible to unfavourable condition, this should not be restricted to the egg stage (Whittingham & Dunn, 2001; Svensson et al., 2007) but should also manifest itself at later stages (Nager et al., 2000; Kalmbach et al., 2005; Müller et al., 2005).

Several problems may arise when egg sexes are used to determine primary brood sex ratios (sex ratio at conception). Sexing of eggs that show only early embryonic development has been shown to be rather unreliable (Arnold et al., 2003). Probes can be contaminated by parental DNA or if DNA concentrations are low the sensitivity of the primers may differ between the Z and W chromosomes resulting in false sex assignment (Arnold et al., 2003). As a consequence many studies only attempted sexing of eggs where a visible embryo was present (Polo et al., 2004; Cichon et al., 2005; Pike & Petrie, 2005, 2006) thus ignoring part of the real primary sex ratio. Another problem often neglected is that also sexing of eggs with visible embryonic
tissue often fails (Alonso-Alvarez & Velando, 2003; Polo et al., 2004; Rutkowska & Cichon, 2006). Sexing might have been unsuccessful due to degradation of embryonic tissue which will be more advanced the longer the incubation of “dead” eggs has taken place. DNA degradation is probably also higher in larger broods as more nestlings increase the temperature in the nest (Mertens, 1969). However, to our knowledge it its unknown how the degradation of tissue will affect the annealing of the sex-specific primers. The most commonly used sexing method amplifies stretches of DNA by polymerase chain reaction (PCR) that are 350–400 bp long (Griffiths et al., 1998). If the sample DNA is degraded into pieces < 350 bp this will interfere with the accurate amplification of the CHD-W and CHD-Z target regions for the P8/P2 sexing primers. These issues raise the question whether sex ratio biases found in eggs should be used to make inferences about primary sex ratio adjustment.

In this study we investigate sex ratio patterns and sexing success for dead collected offspring of great tits (Parus major) at the egg stage and two nestling stages, namely nestlings that died between hatching and day 2 and between day 2 and day 6. Furthermore, we investigate factors that might relate to sexing failure of eggs. We tested whether brood size, estimated hatching date or the time eggs remained in the nest after hatching related to sexing success of individual eggs.

Methods

Data collection and molecular sexing

From the beginning of April we checked nest boxes weekly to establish the start of egg laying (if necessary back calculated assuming one eggs was laid per day) and the clutch size was determined at the onset of incubation. Before the expected hatching date nest boxes were checked daily to determine hatching date (day 0). We sampled a small quantity of blood (ca 5–10 µl) from the tarsal vein of all nestlings when they were two days old. Blood was stored in 100% ethanol and was transported to the lab for immediate molecular sexing. All eggs that had not hatched until day 6 and nestlings that had died before day 2 were collected and directly frozen. Frozen eggs were cut in four quarter in the lab to see whether signs of embryonic development were visible (fig. C.1). In 2004 sexing was also attempted for eggs without visible embryonic development (n = 66) and sexing of those eggs always failed. Thus for eggs collected during the period 2005-2008, sexing was not attempted for those eggs in which no embryo could be visually confirmed. When an embryo was visible a small tissue sample was extracted. For both tissue and blood samples, DNA was extracted using the Chelex method described by Walsh et al. (1991). Sex of the young was determined following Griffiths et al. (1998) where part of the CHD-W and CHD-Z genes from the sex chromosomes were amplified using (PCR) with primers P8 and P2. The PCR products were separated by electrophoresis on a 2% agarose gel and were visualized with ethidium bromide staining under UV trans-illumination. When two
bands were visible (ZW) the sample was assessed to be from a female while one band indicated that the sample originated from a male (ZZ). For samples that had failed to show clear bands sexing was always tried one more time and in 2005 two additional attempts were made.

**Data analyses**

The reasons for not-hatching were numerous. Some eggs seemed either not fertilized or development had ceased at a very early stage and consequently for those eggs no tissue sample was taken (n = 326). However, of the eggs from which a tissue sample could be obtained, still 54% (117/217) could not be sexed successfully. Hatching failure of eggs with embryonic development could come about because some females had abandoned the brood before hatching for unknown reasons (7 broods with 47 eggs). Sometimes, some eggs in a brood were delayed since they were laid after females had started incubation (at least 4 broods with 6 eggs). Furthermore, females often ceased incubation after the first young in the brood hatched (personal observation). We tested whether brood size, estimated hatch date and the time eggs remained in the nest after hatching of the other nestlings affected the sexing success of individual eggs. For this we analysed sexing success as binominal dependent variable in a multilevel model (MLwiN 2.0; Rasbash *et al.*, 2004) with logit link function. We fitted plot, cohort (all broods within a year), brood and individual as random effects (levels) and permanently controlled for year differences in the model (the four years 2005-2008 with 2005 as reference category). We tested significance of the continuous variables “brood size at hatching”, “days collected after hatching” and “hatching date” (all centred on their population averages). For all other statistical analyses we used STATISTICA version 7 (StatSoft, Inc. 2004).

**Figure C.1** A) The left hand figure shows a great tit egg where no embryonic development could be visually detected. B) The right hand figure shows an egg with clear signs of embryonic development. a) egg shell, b) albumen, c) yolk, d) embryonic tissue.
Results

From 2005-2008 we had records of 7969 eggs laid in first broods in our study area of which 7426 (93%) had hatched. Unhatched eggs (n = 543) had remained in the nest after the estimated hatch date for on average 7.2 days ±0.44 SE (min = 0, max = 50). This shows that most eggs where collected during nestling measurements on day 6. In cases of abandoned broods, eggs were collected earlier (n = 60). In some cases (n = 27) eggs were collected at day 10, 14 or after nestlings had fledged and in case of one brood eggs (n = 3) were only collected much later (50 days after hatching). The sexing of unhatched eggs from first broods was not very successful with only 117/543 (21.55%) that could be sexed (see also fig. C.2). The sex ratio of those sexed eggs was biased towards males (44 females, 73 males; \( \chi^2 = 7.188, df = 1, p = 0.007; \) fig. C.3). The mortality of young between hatching and day 2 was 7.5 % (556/7426). Of the chicks collected dead at day 2, 95.7% could be sexed and the sex ratio was not biased to either sex (280 females, 252 males; \( \chi^2 = 1.474, df = 1, p = 0.225; \) fig. C.3). The mortality between day 2 and day 6 after hatching was 11.67% (797/6829). The sex of all chicks that had died in this period was known and the sex ratio was unbiased (405 females, 392 males; \( \chi^2 = 0.212, df = 1, p = 0.645; \) fig. C.3).

Sexing success was significantly lower for eggs from larger broods (brood size: \( \beta = -0.221 \pm 0.054, \chi^2 = 16.971, df = 1, p < 0.001 \)) and for eggs that had remained longer in the nest after hatching (days collected after hatching: \( \beta = -0.082 \pm 0.037, \chi^2 = 4.885, df = 1, p = 0.027; \) other terms in the model; intercept: \( \beta = 0.115 \pm 0.339, \chi^2 = 0.116, df = 1, p = 0.733; \) years: \( \chi^2 = 4.841, df = 3, p = 0.184 \)).

---

**Figure C.2** Agarose gel showing PCR gene products separated by gel electrophoresis from amplification with the P8/P2 sexing primers. Two bands indicate that the sample originated from a female (ZW) and one band from a male (ZZ). The samples 49–58 were obtained from eggs (embryos) while the samples 59-72 were obtained from hatched nestlings that were collected dead in the nest between days 0-2. Samples 58 and 71 were verified as males after a second and third trial respectively. Other samples that showed no result here also failed to show results in a second and third trial.
Hatching date showed no significant relation with sexing success ($\beta = 0.051 \pm 0.036$, $\chi^2 = 2.001$, df = 1, $p = 0.157$). The variation at nest level in the final model was not significant ($\beta = -0.221 \pm 0.054$, $\chi^2 = 1.694$, df = 1, $p = 0.193$) and the variation at cohort and plot level was zero.

**Discussion**

We could show that in our sample of dead collected eggs and nestling great tits the sex ratio was biased only among eggs but not among dead nestlings. However, the sexing success of eggs was considerably reduced compared to the sexing success of nestlings collected dead at day 2. Since dead eggs remained in the nest considerably longer than dead nestlings they probably suffered more from tissue and thus DNA degradation which affected sexing success and possibly also the unbiased annealing of sex-specific primers to the DNA. This reasoning is supported by our finding that eggs that had remained in the nest for longer time and eggs from larger broods were less successfully sexed. Low quality/quantity DNA samples are more likely to be wrongly scored as males, because the P8/P2 primers may fail to amplify the longer CHD-W fragment (Arnold et al., 2003). Many of our DNA samples from unhatched eggs were probably of low quality and hence 54 % of the sexing attempts failed completely. Therefore, it is conceivable that a part of the egg samples that did work were falsely scored as males.

Numerous studies have reported on average male biased sex ratios among unhatched eggs (Whittingham & Dunn, 2001; Cichon et al., 2005; Rutkowska & Cichon, 2006; Svensson et al., 2007), sometimes all unhatched eggs were exclusively
assigned to be male (Rutkowska & Cichon, 2006). Often the conclusion from those studies was that male embryos were more susceptible to adverse condition during incubation possibly because of sex differences in growth rates or size. Especially for those studies that could not find a male biased morality among nestlings (Whittingham & Dunn, 2001; Svensson et al., 2007) this seems to be an unlikely explanation. It is therefore possible that in these studies such as in ours, the biased sex ratio among the collected eggs was due to a bias in the (successfully) sexed sample or that females were erroneously detected as males due to biased primer annealing to the W chromosome (Arnold et al., 2003).

Our results show that inferences about egg sex ratios should be made with caution. Sex ratio biases among eggs might be caused by the sexing or sampling procedure itself and this leads to deduced primary sex ratio not representing the sex ratio at conception. This will affect conclusions drawn from such studies and may hamper the detection of the proximate and ultimate causes for sex allocation strategies.