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Primary sex ratios in birds: problems with molecular sex identification of undeveloped eggs

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Abstract

Sex allocation studies seek to ascertain whether mothers manipulate offspring sex ratio prior to ovulation. To do so, DNA for molecular sexing should be collected as soon after conception as possible, but instead neonates are usually sampled. Here, we aim to identify and quantify some of the problems associated with using molecular techniques to identify the sex of newly laid avian eggs. From both fertilized and unfertilized chicken (*Gallus gallus*) eggs, we sampled (1) the blastoderm/disc, (2) vitelline membrane and (3) a mixture of (1) and (2). Thus, we replicated scenarios under which contaminated samples are taken and/or unfertilized eggs are not identified as such and are sampled. We found that two commonly used molecular sexing tests, based on the CHD-1 genes, differed in sensitivity, but this did not always predict their ability to sex egg samples. The vitelline membrane was a considerable source of maternal and probably paternal contamination. Fertile eggs were regularly assigned the wrong sex when vitelline membrane contaminated the blastoderm sample. The membrane of unfertilized eggs was always female, i.e. maternal DNA had been amplified. DNA was amplified from 47 to 63% of unfertilized blastodiscs, even though it was highly unlikely that DNA from a single haploid cell could be amplified reliably using these polymerase chain reaction (PCR) techniques. Surprisingly, the blastodiscs were identified as both males and females. We suggest that in these cases only maternal DNA was amplified, and that 'false' males, Z not ZZ, were detected. This was due to the reduced ability of both sets of primers to anneal to the W chromosome compared to the Z chromosome at low DNA concentrations. Overall, our data suggested that estimates of primary sex ratios based on newly laid eggs will be appreciably inaccurate.

Keywords: avian, CHD1-Z, CHD1-W, *Gallus gallus*, sex identification, W chromosome

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Introduction

Empirical tests of sex allocation theory are often made difficult because of the basic challenge of identifying the sex of individuals. In many taxa, such problems are exacerbated early in development and in adults that either lack sexual dimorphism, or are only morphologically distinguishable during the breeding season. The development of molecular techniques for identifying sex from small DNA samples has therefore greatly facilitated empirical work in this field (reviewed in Sheldon 1998). Consequently, a growing number of studies are being published testing theories of sex ratio manipulation in response to a range of social and environmental conditions (reviewed in Cockburn *et al*. 2002; West & Sheldon 2002). Often the aim is to determine the primary sex ratio, i.e. the sex of offspring at conception, and offspring are being sampled at the earliest possible stage of development. In avian studies, using blood samples from chicks to assign sex is often not early enough, given that sex-differential embryo (e.g. Whittingham & Dunn 2000, 2001) or chick mortality (Adkins-Regan 1998; Kilner 1998; Bradbury & Griffiths 1999; Williams 1999; Nager *et al*. 2000; Arnold & Griffiths 2003) may be occurring. Ideally, eggs should be sampled soon after ovulation i.e. before chick or embryo mortality can occur in order to gain an accurate estimate of primary sex ratio. This means that blastoderms,

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as opposed to obviously developing embryos, should be sampled to gain offspring DNA. Unless ova are collected in the oviduct, sampling will usual occur after an egg has been laid. Here, we explore some of the potential issues associated with sampling and sexing newly laid eggs and how this effects our interpretation of sex ratio data.

Currently, there are a number of molecular sexing techniques (e.g. Lessells & Mateman 1996; Griffiths *et al*. 1998; Fridolfsson & Ellegren 1999; Questiau *et al*. 2000), based on the chromosomal sex determination mechanism in birds. Females, the heterogametic sex in birds, possess one Z and one W chromosome and males two Z chromosomes. So, in contrast to mammals, the egg rather than the sperm determines the sex of offspring in birds (reviewed in Ellegren 2000; Kraak & Pen 2002). The two sexing techniques P2/P8 (Griffiths *et al*. 1998; Fridolfsson & Ellegren 1999) and 2550F/2718R (Fridolfsson & Ellegren 1999) that we assessed here are two of the most commonly used particularly by behavioural ecologists. They are both based on PCR coamplification of both chromobox–helicase–DNA-binding or CHD1 genes. The CHD1-Z gene occurs on the Z chromosome in both sexes, whilst the CHD1-W that is specific to the W chromosome identifies females. The introns in CHD1-W and CHD1-Z differ in length, so the polymerase chain reaction (PCR) products can be separated out using gel electrophoresis. So for both tests, a male (ZZ) results in a single band and a female (ZW) produces two bands. The P2/P8 test employs two PCR primers that anneal to conserved exonic regions but then amplify across an intron in both CHD1-W and CHD1-Z. The 2550F/2718R (Fridolfsson & Ellegren 1999) primers use the same CHD1 genes but amplify a slightly different and longer region.

The sensitivity of a molecular test determines the amount of DNA needed for it to work, which is important when working with newly laid eggs. In the case of freshly laid eggs, the isolation of an early embryo is more difficult than taking a blood or a feather sample from an adult. The tissue sample is taken by excising the blastoderm from the vitelline membrane that surrounds the yolk. DNA is then extracted from the blastoderm and a CHD1 test is often used to establish the embryo sex. Problems may arise when pieces of membrane are extracted with the blastoderm. The vitelline membrane consists of a structure of protein fibrils, and should not have a sex, but is open to contamination (Sasanami *et al*. 2002); for example, within the preovulatory follicle or reproductive tract maternal cells could adhere to the vitelline membrane (see also Pearce *et al*. 1997; Strausberger & Ashley 2001). In addition, varying numbers of sperm (between 29 and 164 000 per egg), i.e. paternal contamination, can be found trapped within the membrane (Birkhead *et al*. 1994).

Overall, we sought to determine whether newly laid eggs could be sexed reliably and accurately, allowing calculation of the primary sex ratio. Our first objective was to assess the relative abilities of two commonly used tests to identify sex from low DNA concentrations. Second, we aimed to determine how parental contamination affected the reliability of results from the sexing tests. If the sex of fresh eggs is misidentified frequently, this will have important consequences for the estimation of the primary sex ratio. In order to appraise these sources of error, DNA was amplified from samples taken from the blastoderm, the adjacent vitelline membrane surrounding the yolk, plus a mixture of both blastoderm and vitelline membrane from fertilized chicken (*Gallus gallus*) eggs. In this species, there are potentially very high numbers of sperm on the vitelline membrane surrounding the yolk (Birkhead *et al*. 1994), and thus may be prone to paternal contamination (Martinez & Burke 2003). We also tested whether the sex of different regions of the vitelline membrane varied. It was predicted that if any paternal contamination of the sexing sample occurred, then it would be more common around the germinal disc (Birkhead *et al*. 1994). Finally, we sexed blastodisc and membrane samples from unfertilized eggs. The molecular methodology used here is unlikely to reliably amplify a region from a single copy of haploid DNA (see Taberlet & Luikart 1999; Park *et al*. 2001). Therefore, we predicted that if unfertilized eggs were assigned a sex it would be due to maternal contamination.

Materials and methods

Molecular sexing techniques

DNA was extracted from tissue (liver or egg) samples using standard phenol/chloroform methodology (Sambrook *et al*. 1989). RNAse was added to each sample that was then re-suspended in TE buffer.

The P2/P8 test (Griffiths *et al*. 1998) was then carried out in a 10-μL reaction with the following chemical constituents: 6 pmol P2 and P8 primers (Griffiths *et al*. 1998), 200 μM of each dNTP, target DNA, 0.35 units *Taq* polymerase, 2.5 mM $MgCl_2$, 50 mM KCl, 10mM TrisHCl (pH 8.8 at 25 °C), 0.1% Triton X-100 (the last four are in Promega Mg/*Taq* buffer). The thermal cycling was carried out in a Biometra *Uno*II 94 °C/120 s, 30× (48 °C/45 s, 72 °C/45 s, 94 °C/30 s), 48 °C/60 s, 72 °C/300 s.

The 2550F/2718R test (Fridolfsson & Ellegren 1999) was run in a similar manner but used the primers 2550F and 2718R at 6 pmol, $MgCl_2$ at 2.5 mM with the following PCR conditions 94 °C/120 s, 30× (49 °C/60 s, 72 °C/60 s, 94 °C/45 s), 49 °C/60 s, 72 °C/300 s. All samples were run using both tests and visualized on a 33 cm × 41 cm × 0.4 mm denaturing polyacrylamide gel stained using silver stain (Promega). This gave the best separation and resolution of bands. For both tests, males (ZZ) result in a single band whereas females (ZW) result in two bands.

Variation in the sensitivity of two sex identification tests

To examine the sensitivity of the techniques, DNA was purified from liver samples from one male and one female chicken using the phenol/chloroform technique (Sambrook *et al*. 1989). The concentration of the samples was determined using a fluorometer (Bio-Rad Versa Fluor™ Fluorometer) and identical 1/2× serial dilutions, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005 ng/μL, were prepared. All samples were run using both techniques (see above). All gels were scored by K.J.O.

Sex identification of fertilized eggs

For this experiment we used 47 fertilized chicken eggs acquired from a local hatchery. In this facility, one rooster and eight chickens were housed together producing eggs with at least an 80% rate of hatching success. The blunt end of the egg was wiped with 70% alcohol before being cracked open. A 3-cm hole was made in the top of the egg. A small portion of the egg white was poured out. The blastoderm was then located, and a 1 cm × 1 cm region of vitelline membrane was cut out, with the blastoderm in the centre, using opthalmic scissors. The blastoderm and vitelline membrane were then transferred to a sterile Petri dish. Using watch-maker's forceps, the blastoderm was carefully peeled off the vitelline membrane and transferred to a new sterile Petri dish and cut in half. The 1 cm × 1 cm region of vitelline membrane from above the blastoderm was cut into two pieces, after removing any remaining albumen and yolk. For each egg, three scenarios were reproduced. The first sample consisted of vitelline membrane only. The second was blastoderm only. In the third sample, there was a mixture of vitelline membrane and blastoderm. DNA was extracted once from each of the three samples per egg and then used in both tests. All eggs used in this study were sampled within a few days of laying and had been stored at 15 °C in a cooling incubator.

Sex identification of three regions of the vitelline membrane

Next, to determine whether the sex identified is consistent across the vitelline membrane of an egg, i.e. if the levels of parental contamination vary, we sampled each of 12 fertilized eggs at four sites. Specifically, we sampled the blastoderm as well as three 1 cm^2 regions of the vitelline membrane: from the upper pole (above the blastoderm), the lower pole and the equator of the egg. Sex was identified using the above techniques.

Sex identification of unfertilized eggs

We attempted to assign sex to 19 unfertilized eggs laid by mothers that had not been housed with males within the previous few months. Using the same techniques, three samples per egg were extracted (vitelline membrane above the blastodisc, haploid blastodisc only and a mixture of equal parts blastodisc and vitelline membrane) and then tested using both techniques (Griffiths *et al*. 1998; Fridolfsson & Ellegren 1999).

Statistical analyses

The aim of our analyses was to ascertain if DNA samples came only from the offspring or were subject to parental contamination, i.e. did blastoderm, vitelline membrane and a mixture of the two concur in sex. Also, we aimed to determine whether the two primer sets produced significantly different sexing results under our distinct scenarios. Therefore, first sex and then amplification success were analysed as a function of egg region, i.e. blastoderm/disc, vitelline membrane or a mixture of blastoderm/disc and vitelline membrane, and sex identification primers, i.e. P2/P8 or 2550F/2718R. To control for the nonindependence of multiple samples from the same egg, we performed all analyses with a generalized linear mixed model (GLMM) with egg identity entered as a random factor (Krackow & Tkadlec 2001). The sex identification test used and region of egg sampled were entered as factors and the error distribution was binomial. Nonsignificant terms were excluded from the model starting with the least significant interaction. Nonsignificant main effects had to be retained in models if they were included in significant interactions. Significance was based on *F*-tests. All models used a Satterthwaite correction for degrees of freedom. Sample sizes vary among analyses because not all samples were sexed successfully.

Results

Variation in the sensitivity of two sex identification tests

Based on the serial dilutions of known sex samples, 2550F/2718R was twice as sensitive as P2/P8. Below DNA concentrations of 0.005 ng/μL the Z bands in males became indistinct and below 0.04 ng/μL the W band in females could not be scored reliably. For P2/P8 the cut-off for reliable scoring was 0.01 ng/μL for Z bands in males and 0.08 ng/μL for the W band of females. For both sets of primers, the W band disappeared first at low concentrations of female DNA (K. Orr, pers. obs.).

Sex identification of fertilized eggs

The sex ratio of the blastoderms did not deviate from parity, suggesting that in most cases we had sampled offspring DNA and not maternal contamination (Fig. 1). The two sets of primers were consistent in their assignment

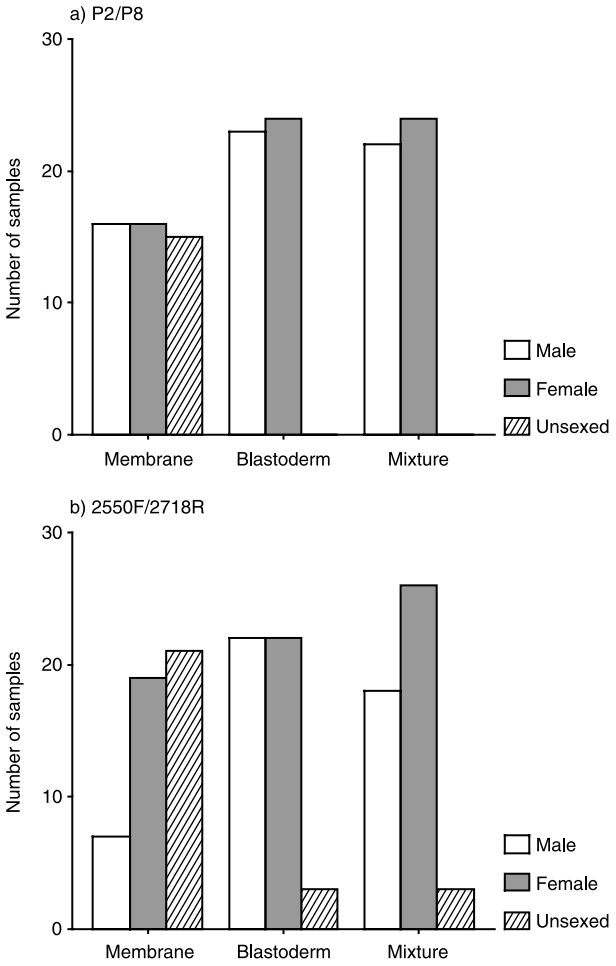

Fig. 1 The sex assigned to the vitelline membrane, blastoderm and a mixture of membrane and blastoderm from fertilized eggs by (a) P2/P8 and (b) 2550F/2718R. Samples producing either Z or ZZ bands were classified as males, those with ZW bands were females and those with no amplified bands were unsexed.

of blastoderm sex (44/44 samples sexed by both primer sets), but not for the vitelline membrane (14/18 samples sexed by both tests). Next, we sought to determine whether the two tests produced significantly different sexing results overall and if the sex of an egg was consistent across all regions sampled. Of the samples that amplified successfully, a GLMM analysis model revealed that 2550F/2718R were marginally ($P = 0.055$) more likely to identify samples as females than P2/P8 (Table 1a; Fig. 1). There were no significant differences in the sexes of the three samples from each egg. In 19 of 31 cases in which all three regions of the eggs were sexed by P2/P8, the sex of the blastoderm disagreed with that of the vitelline membrane, but always agreed with the blastoderm/vitelline membrane mix (Table 2a). Thus, the DNA on the vitelline membrane did not always originate from the offspring, but the DNA concentration in fertile blastoderms

Table 1 GLMM analysis of sexing data in relation to the region of the fertile egg sampled (blastoderm, membrane or a mixture of the two) and the test used (P2/P8 or 2550F/2718R): (a) sex of samples; (b) success of two sexing tests. Sex (female = Z and W bands present, male = Z or ZZ bands present) or amplification success (sexed/unsexed) was the binomial response variable. Egg identity was entered into the GLMM as a random factor. Test and region were entered as factors. Nonsignificant terms were removed stepwise from the model starting with nonsignificant interactions

Term	Deviance	d.f.	*F*	*P*
(a) Sex	167.74			
Test		1,187	3.72	= 0.055
Region				NS
(b) Success	119.92			
Test		1,230	10.71	= 0.0012
Region		2,242	39.10	< 0.0001

was higher than the degree of contamination on the vitelline membrane. Of course, this does not distinguish cases in which the contaminating parent was the same sex as the offspring.

The pattern was slightly different for fertile eggs sexed by 2550F/2718R primers. In 12 of 22 cases, there was a difference between the sex of the vitelline membrane and the blastoderm (Table 2b). Also, in three cases there was a mismatch between the sex of the blastoderm and the mixture of blastoderm and vitelline membrane, suggesting that contamination was present in sufficient quantities to produce an erroneous sexing result occasionally. Conversely, the vitelline membrane did not retain enough DNA to be detected in roughly 30% of eggs.

Among fertilized eggs, the vitelline membrane was less likely to be amplified and sexed successfully than the blastoderm or blastoderm/membrane mixture (Table 1b; Fig. 1a,b). Overall, P2/P8 were more likely to amplify DNA successfully than 2550F/2718R (Table 1b).

Sex identification of three regions of the vitelline membrane

Paternal contamination did appear to be present on the vitelline membrane but did not vary with distance from the germinal disc. A GLMM (deviance = 72.85) showed that the sexes assigned were significantly different using the two sets of primers ($F = 4.25$, d.f. = 1, 77.4, $P = 0.0425$) and between different regions of the egg sampled ($F = 7.40$, d.f. = 3, 78.2, $P = 0.0002$). The membrane was more likely to be assessed as female and the blastoderm as male (Fig. 2). P2/P8 were significantly more likely to identify regions of the vitelline membrane as male (Fig. 2a) compared with the other primers (Fig. 2b).

The 2550F/2718R primers were unsuccessful in identifying the sex of three samples, one blastoderm and two

Table 2 Estimated frequency of contamination by parental DNA when vitelline membrane was mixed with the blastoderm/disc sample. The sexes of the vitelline membrane, blastoderm/disc (Blasto) and a mixture of the two are shown for (a) fertilized eggs sexed using P2/P8; (b) fertilized eggs sexed using 2550F/2718R; (c) unfertilized eggs sexed using P2/P8; and (d) unfertilized eggs sexed using 2550F/2718R. Agree = the sex of the blastoderm/disc and mixture of vitelline membrane and blastoderm/disc were the same (i.e. no contamination has occurred unless the sex of the offspring and contaminating parent were the same). Disagree = the sex of the blastoderm/disc and mixture of vitelline membrane and blastoderm/disc were different (i.e. contamination has probably occurred). Equivocal = some samples could not be amplified. F(emale) = Z and W bands present. M(ale) = Z or ZZ bands present. X = bands could not be scored

	Agree						Disagree		Equivocal										
Vitelline	F	M	X	F	M	X	F	X	F	X	F	F	F	F	M	X	X	X	X
Blasto	F	F	F	M	M	M	M	M	F	F	X	X	X	M	M	M	X	X	X
Mixture	F	F	F	M	M	M	F	F	X	X	F	X	M	X	X	X	M	F	X
(a) Fertilized P2/P8	7	11	6	8	5	10	0	0	0	0	0	0	0	0	0	0	0	0	0
(b) Fertilized 2550F/2718R	8	4	11	8	2	7	2	1	0	0	1	0	0	0	1	0	0	0	2
(c) Unfertilized P2/P8	3	0	0	1	0	0	2	0	1	2	3	1	1	0	0	0	1	1	3
(d) Unfertilized 2550F/2718R	3	0	2	0	0	0	3	0	2	0	4	1	1	1	0	1	1	0	0

regions of vitelline membrane from the lower pole. P2/P8 produced a sexing result for of all samples. There was no significant difference in the success rates of the two tests or in the regions that could be sexed.

Sex identification of unfertilized eggs

The sex of the three samples from different parts of an unfertilized egg could not be analysed using a GLMM because the variance in the sex was so small, so the data were split by test and region of the egg and analysed using either a binomial or χ^2 test. The vitelline membrane always sexed as female (binomial test $P < 0.0001$ for both sexing tests) and tended to agree with the sex of the mixture (Table 2c and d). The blastodisc gave both male and female results. P2/P8 identified three males and six females ($\chi^2 = 1.0$, d.f. = 1, $P = 0.3$) and the 2550F/2718R primers five males and seven females ($\chi^2 = 0.33$, d.f. = 1, $P = 0.6$). In the 10 samples, from all parts of the eggs that could be sexed using both tests, the results were identical. With the mixture of blastodisc and vitelline membrane, male and female results were again detected, but the ratio was significantly female-biased with the 2550F/2718R primers ($\chi^2 = 7.14$, d.f. = 1, $P = 0.008$; Fig. 3b) and marginally significantly biased with P2/P8 ($\chi^2 = 3.0$, d.f. = 1, $P = 0.08$; Fig. 3a). Table 2c and d shows that although any DNA amplified should be maternal in origin, both the blastodisc and mixture could be a different sex from the vitelline membrane.

There was a nonsignificant tendency for the tests to differ in their success at identifying the sex of samples (deviance = 121.87, $F = 2.91$, d.f. = 1, 92.3, $P = 0.09$; Fig. 3). The blastodisc and vitelline membrane of the egg did not vary in their likelihood of being amplified by the sex identification primers.

Discussion

We determined that sex identification of avian eggs is inconsistent and unreliable when working with the small, amounts of DNA present within newly laid eggs. Similarly, we found that parental contamination of samples is an important and unpredictable source of error in calculating primary sex ratios. Also, we showed that two commonly used sexing techniques differed in their abilities to sex a sample and in some circumstances could classify the same sample as both male and female. Thus, in our analyses the 'population' sex ratio ascertained by each test reflected different subsets of eggs. Consequently, depending on the test used, quite different sex ratio results could be acquired from the same population.

The regularity (47–63% of samples) with which we could assign a sex to eggs that had not been fertilized either naturally or artificially was surprising. They should, in theory, have contained only the haploid DNA within the nucleus of the ova and possibly that of a polar body that separated from the ova during meiotic division, whereas newly laid fertile eggs contain roughly 60 000 cells (Simkiss 1991). Normal DNA extraction and PCR techniques were unlikely to be sensitive enough to amplify a single copy of haploid DNA (Taberlet & Luikart 1999; Wan *et al*. 2003). Most of these blastodiscs tended to be female, and are thus

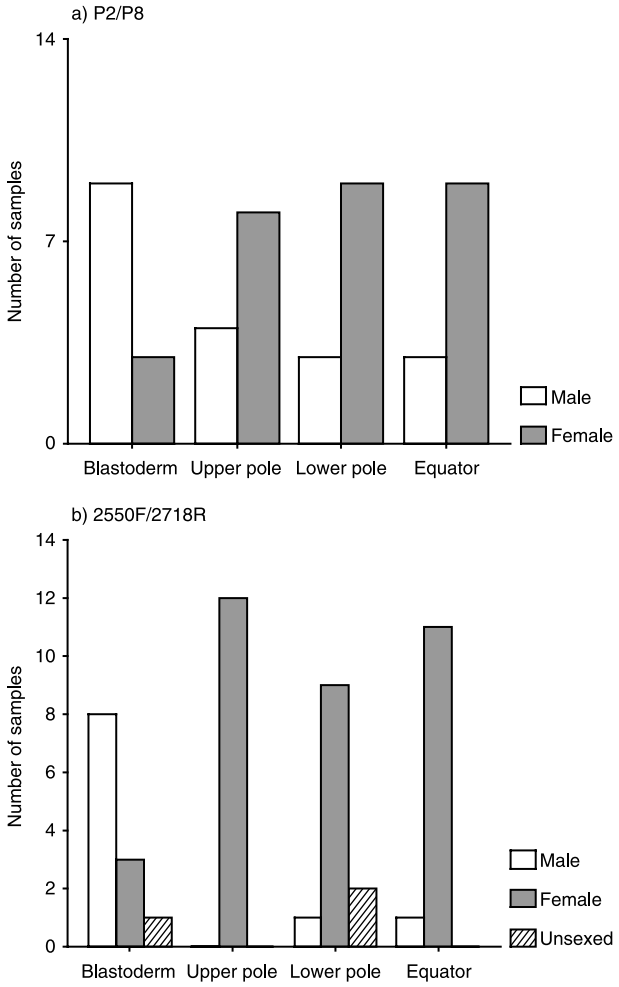

Fig. 2 The sex assigned to the blastoderm and the upper pole, lower pole and equator of the vitelline membrane of fertilized eggs by (a) P2/P8 and (b) 2550F/2718R. Samples producing either Z or ZZ were classified as males, ZW bands were females and no amplified bands were unsexed.

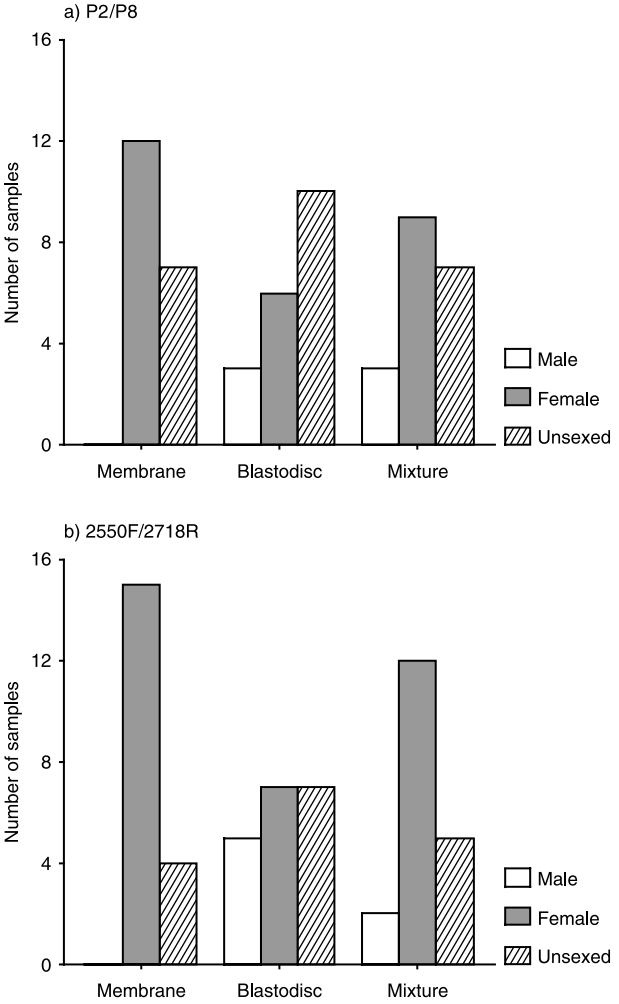

Fig. 3 The sex assigned to the vitelline membrane, blastoderm and mixture of membrane and blastoderm of unfertilized eggs by (a) P2/P8 and (b) 2550F/2718R. Samples producing either Z or ZZ bands were classified as males, ZW bands were females and no amplified bands were unsexed.

a result of maternal contamination because the samples scored as female definitely all had two bands, i.e. both Z and W, so could not have been amplified from haploid, either Z or W, blastodiscs. Strangely, some blastodiscs were sexed as male. In the nine cases in which both tests successfully sexed the same blastodisc the two tests agreed 100% (six females:three males). Either the samples sexed as males were the result of amplification of DNA from a single copy of a Z-chromosome in the blastodisc, which seems unlikely, or these were derived from maternal DNA. In other words, these were 'false males' (Z not ZZ) and were in fact females, but the CHD1-W could not be visualized on the gel. We suggest that these erroneous males are the result of a problem with the annealing of the primers to the W chromosome at low DNA concentrations. This is supported by the results of the serial dilutions in which both sets of primers successfully annealed to the Z chromosome at lower DNA concentrations than to the W-chromosome. Allelic dropout, when only one allele at a heterozygote locus is amplified, results in the scoring of a false homozygote, and has been found in a number of PCR studies using DNA extracts of a low quantity (< 0.05 ng/10 μL) and/or quality (Gagneux *et al*. 1997; Taberlet & Luikart 1999). One potential solution is to use a combination of primers differing in their relative amplification of the Z and W bands.

The two sets of primers, 2550F/2718R and P2/P8, varied in their sensitivity and ability to amplified DNA. Also they both differed in their relative abilities to be able to sex males and females, but as only one individual per sex was used in the serial dilutions the exact sensitivities cannot be derived here. The 2550F/2718R primers always sexed a greater proportion of the egg samples as female, suggesting

that 2550F/2718R anneal better to the W-chromosome than P2/P8 when the DNA concentration is low. However, a closer look at the data showed that in cases in which both sets of primers sexed regions of a fertilized ova, 77.8% produced the same result for the vitelline membrane and 100% for the blastoderm. The sex ratio biases tended to creep in only when we examined the samples that were only successfully amplified by one set of primers or the other. Thus, they may simply have been correctly sexing different subsets of the group that happened to have a sex bias. Unfortunately, this study suggests that we might not have been amplifying offspring DNA at all in some cases, but contaminant DNA from parents.

A sex could be frequently assigned to the vitelline membrane of both fertilized and unfertilized eggs. When we looked at the degree of mismatch in the sex of samples from the blastoderm and vitelline membrane of the same egg it seemed that the DNA found on the vitelline membrane was often not from the offspring (Table 2). The membrane was found to be both male and female suggesting that both paternal and maternal contamination were present. These two potential sources of contamination could result in inaccurate sex identification. First, if the membrane itself acquires maternal DNA, e.g. from granulosa or epithelial cells, at some point in the reproductive tract, this would provide both CHD1-Z and CHD1-W genes from the mother resulting in potential misidentification of embryo sex. A second source of contamination is after copulation, regardless of whether fertilization has succeeded, the vitelline membrane covering the blastoderm will also contain a number of sperm that have attempted to pierce the membrane but have failed to fertilize the ova (Birkhead & Fletcher 1994). The number of sperm that are present will vary enormously between species affecting the chance of paternal contamination being detected. Each sperm will contain its incumbent single CHD1-Z gene, so the band observed is the result of amplifying haploid (Z) genes not true male diploid (ZZ) genes. Unfortunately, we were unable to differentiate between paternal DNA and maternal DNA in which the CHD1-W had failed to amplify. We were confident that the amplification of CHD1-Z from the membrane of fertile eggs was truly paternal in origin and not due to vagaries of the primers (see also Martinez & Burke 2003). Support for this came from the unfertilized eggs because the vitelline membrane was always sexed as female. Therefore, maternal contamination on the vitelline membrane, if present, seemed to always be in sufficient quantities to anneal successfully to CHD1-W, so was not subject to allelic dropout.

For the 2550F/2718R primers, the vitelline membranes of fertilized eggs were significantly more likely to be sexed as female than with P2/P8, but again both parents seemed to be contributing DNA to the samples. Thus, contamination of the offspring DNA by parental DNA is possible if the vitelline membrane is included in the sample. So, for example, simply cutting the blastoderm off the yolk will probably result in a contaminated sample if the membrane is not peeled from the blastoderm. Under such conditions, there is a reasonable chance that the sex acquired from the fertile egg will be that of a parent rather than the offspring. Indeed, as our analyses of infertile eggs have shown, maternal DNA can adhere to the blastodisc/derm even when the membrane seems to have been removed cleanly. Clean excision of the blastoderm will be particularly difficult in unhatched eggs from the wild because after a couple of weeks of incubation, tissue degradation will have occurred.

In contrast to our original prediction, we found neither paternal nor maternal contamination were more common on any part of the vitelline membrane of a fertile egg (Fig. 2). Thus, although Birkhead *et al*. (1994) found a greater concentration of spermatozoa penetrated the perivitelline membrane around the germinal disc, they also detected spermatazoa in other regions, in line with our results. Although chicken eggs usually contain a great deal of sperm (Birkhead *et al*. 1994; K. J. Orr, unpubl. data), we found that haploid male (Z) contamination was relatively minimal compared with diploid maternal DNA (ZW). Again, we could not distinguish between maternal and paternal CHD1-Z genes.

In summary, low DNA concentrations resulted in molecular sexing tests producing unreliable results for a number of reasons. First, contaminant DNA was a key problem even when the sample seemed to have been cleanly taken. Depending on whether an ova was fertilized, parental, usually maternal, DNA influenced the sexing result of up to 63% of samples. In populations with an appreciable level of infertility, for example, analysis of newly laid eggs would yield a highly inaccurate, probably female-biased primary sex ratio. Second, we have highlighted that with highly sensitive molecular probes, when the target DNA is in low concentrations, even a small amount of contamination can result in false results. In such a case, no result from an insensitive set of primers would be better than a false one from very sensitive ones. Finally, when the sensitivity of the primers differs between the Z and W chromosome, e.g. because the match between the primer and the target sequence differs, then at low DNA concentrations females are likely to be mistaken for 'false' males (Z not ZZ). Again, this is a particular problem if fertilization has not occurred. Overall, our data demonstrate that estimations of primary sex ratios from newly laid eggs are likely to be inaccurate. Consequently, we recommend that eggs are incubated for at least 2 days so it can be visualized that they are fertile and contain enough cells for reliable DNA amplification (Arnold *et al*. 2003). Among unhatched incubated eggs, only those showing signs of development should be sampled. Data on the proportion of undeveloped, unsexed eggs in relation to relevant correlates should be presented to facilitate estimation of the error in calculating the primary sex ratio.

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