Prenatal bias in sex ratios in a marsupial, *Antechinus agilis*

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Biased sex ratios of young of birds and mammals clearly occur and may have an adaptive significance, but we rarely know the stage at which a bias is generated, or the mechanism. If a bias is generated prior to birth, studies of marsupials may be insightful because gestation is short and neonates are relatively undifferentiated. This study investigated whether biased sex ratios in *Antechinus agilis* are generated in the brief period between birth and the attachment of young to the mother's teats. When all young born, or just pouch young, or supernumerary young were considered, litters were strongly biased towards females (0.32 males), and there was no significant difference across the groups, so a bias is generated before birth in this species. Evidence from counts of corpora lutea suggests that embryo loss during gestation cannot account fully for the level of bias observed. Therefore, prefertilization mechanisms must contribute to the generation of sex-biased litters in this marsupial.

Keywords: *Antechinus*; marsupial; prenatal; sex allocation; sex bias; sex ratios

1. INTRODUCTION

Many ecological studies of mammals have reported biases in the sex ratios of offspring at birth (Clutton-Brock & Iason 1986; Cockburn 1990). This has generated enormous interest and prompted many investigations of sex allocation (see, for example, Clark 1978; Maynard Smith & Stenseth 1978; Clark *et al.* 1990), particularly in long-lived monotocous species of eutherian mammals (Clutton-Brock *et al.* 1986), and more recently in birds (see, for example, Komdeur 1996; Heinsohn *et al.* 1997; Oddie 1998). Biased sex ratios have been found in several field studies of marsupial mammals, leading some to suggest that such biases may occur more frequently in marsupials than in eutherians or birds (Clutton-Brock & Iason 1986; Cockburn 1990). However, there are few experimental studies of sex ratios in marsupials (but, see Austad & Sunquist 1986) and no studies have investigated the stage of development at which such biased ratios are generated.

In both birds and mammals the sex of an individual is genetically determined, but there is a fundamental difference between the two groups: the heterogametic sex of birds is female and that of mammals is male. In theory, female birds may influence sex ratios very early by manipulating the size or survival of oocytes (Oddie 1998). In mammals, the sex of the sperm determines the sex of the zygote, and the avenues for maternal influence of sex ratios are very different. Some mechanism of sperm separation or selection within a female's reproductive tract may operate before fertilization, or sex-differential embryonic success or foetal mortality may operate *in utero* after fertilization (Krackow 1995). Marsupial neonates are born at a relatively early stage of development after a

brief period of organogenesis, so the role for postfertilization mechanisms is even more limited than in eutherian mammals.

The mechanisms for altering sex ratios of bird and mammal young before birth are currently speculative and none has been identified operating in a system of adaptive sex allocation. That we know of no low-cost mechanism for sex-ratio manipulation in birds or mammals is currently an important stumbling block to those arguing for adaptive significance of sex-ratio bias (Clutton-Brock & Iason 1986; Krackow 1995). It is clear that the first step in the identification of a mechanism for sex-ratio manipulation is to determine the stage at which it operates.

(a) *Antechinus* and sex-ratio bias

This study investigates sex bias in litters of agile antechinus, *Antechinus agilis*, a small (20–40 g) nocturnal insectivorous marsupial that inhabits forests and woodlands in south-eastern Australia. *Antechinus* spp. have a simple life-history strategy and population structure (Braithwaite & Lee 1979; Lee *et al.* 1982). They have a short, predictably timed annual breeding season with one litter born each year of a female's life (Woolley 1966a), and all males die at the end of the mating period. Females ovulate spontaneously at the end of the mating period (Selwood 1985) and give birth to a litter 27 days later. Generally more young are born than there are teats; because each neonate requires exclusive access to a teat for a period of at least one month, supernumerary neonates do not survive (Woolley 1966b; Selwood 1982b).

The young of *A. agilis* are born at an early stage of development, weighing 17.7 ± 1.1 mg (M. J. Davison and S. J. Ward, unpublished data) after only 5–6 days of organogenesis (Selwood 1980). At this stage, most organ systems are at an early stage of development (Hughes & Hall 1988) and gonad differentiation has yet to occur

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(Short *et al.* 1988). Thus, the production of primary sex hormones has not started at this stage and so there are no obvious steroidal cues present that would allow maternal recognition of the neonatal sex.

Sex-ratio bias has been reported in litters of *Antechinus* spp. at the stage of permanent attachment to teats. Cockburn *et al.* (1985) examined sex ratios of pouch young across populations of three species of *Antechinus* and found that their sex ratio was positively correlated with the degree of iteroparity. In more semelparous populations female-biased litters occurred, and this was typical of *A. agilis*. In more iteroparous populations, male-biased litters were more common in the first year of breeding. Cockburn *et al.* (1985) concluded that the sex ratios of litters of first-time mothers were being influenced by the probability of competition between mothers and daughters in the next breeding season (Clark 1978). This has also been suggested for some eutherians (Armitage 1987; Johnson 1988), and selection may favour an inverse relationship between female age and sex ratio (Clutton-Brock & Albon 1982; Cockburn *et al.* 1985).

In all previous studies reporting sex-ratio bias in marsupials, information has come from counts within the period when young are permanently attached to their mother's teats. As less than 5% of *Antechinus* litters experience brood reduction, we know that a bias is established before or immediately after birth, before attachment to teats (Cockburn *et al.* 1985; Austad & Sunquist 1986; Cockburn 1990, 1994). Therefore, the time at which sex-ratio bias may be generated in marsupials is restricted to one, or more, of three very different developmental stages: (i) between insemination and conception, by differences in the number or viability of X and Y sperm that reach and fertilize the ova (Cockburn 1990; Krackow 1995); (ii) during gestation, when loss of embryos may be sex biased (Krackow 1995); and (iii) in species where there are more young born than teats to attach to, a bias may be generated by sex-related differences in success at reaching and attaching to teats.

The first step in any investigation of the mechanisms of sex-ratio bias is to identify the stage at which the bias is generated. This study is, to our knowledge, the first investigation of the stage of sex-ratio bias in *A. agilis*, and concentrates on the brief period between birth and attachment of young to the mother's teats.

2. MATERIALS AND METHODS

(a) Study populations

We identified a population of *A. agilis* in the Spargo Creek area of the Wombat State Forest (altitude 600 m, 37°29'S, 148°08'E) that exhibited a low proportion of females surviving to breed in a second season (0.26) and so was expected to show the strongest sex-ratio bias (Cockburn *et al.* 1985). Pregnant females were collected from the Spargo Creek population after the period when males die off. *A. agilis* has recently been recognized as being taxonomically distinct from *A. stuartii* (Dickman *et al.* 1998) and many previous studies of antechinus can now be attributed to *A. agilis* based on the location of study sites. Such studies cited in this paper include Cockburn (1994), Cockburn *et al.* (1985), Dickman (1988), Selwood (1980, 1982a,b, 1983, 1985), Taggart & Temple-Smith (1991), and Woolley (1966a,b).

(b) Maintenance of captive animals and collection of neonates

Pregnant females were housed individually in glass-fronted stainless steel cages (Selwood 1982b) and were handled daily to inspect pouches. The stages of pouch development have been documented previously, and this allowed the date of parturition to be estimated (Woolley 1966b; Selwood 1982a). Once the first female had given birth, the cages of all females were inspected every half-hour around the clock. This frequency of observation was necessary to minimize cannibalism by mothers (Cockburn 1994) and/or desiccation of supernumerary young (M. J. Davison and S. J. Ward, personal observation). Females predicted to be close to birth were handled and inspected more often than once per day.

Once a female had given birth she was removed from her nest-box and the nest material was searched for supernumerary young. Pouch young were then removed from the mother's teats by means of blunt-nosed forceps. Each neonate was placed on a numbered dissecting tray, immobilized on ice and bisected transversely above the abdomen. The posterior half was fixed and stored in neutral buffered formalin for light microscopy.

(c) Determining the sex of neonates

Antechinus young cannot be sexed visually at birth; however, mammary anlagen (a female character) and scrotal bulges (in males) can be detected subdermally on histological investigation (Bolton 1983). The posterior half of each neonate was processed for histology, serially sectioned at 8 µm and stained with haematoxylin and eosin. The sections were inspected under a microscope at ×100 to identify the presence of mammary anlagen or scrotal bulges. In didelphid marsupials, mammary anlagen are present in both females and males at birth; however, no mammary anlagen have been found in male Australian marsupials (Robinson *et al.* 1991; Ullmann 1993), and Bolton (1983) found no evidence of mammary tissue in male *Antechinus*.

(d) Counts of corpora lutea

A randomly chosen subset of ten female *A. agilis* were killed with CO₂ and dissected within a few hours of giving birth. The ovaries were observed fresh under a dissecting microscope to identify and count corpora lutea. The number of corpora lutea was used as an estimate of the number of ova ovulated from that ovary, and therefore the potential number of embryos. Females not killed were released at their point of capture at the end of the birth period.

(e) Statistical analysis

Statistical analyses were performed on sex ratios of total litters, litters of pouch young and supernumerary young. Sex ratios were analysed by χ^2 tests, binomial probability tests and paired *t*-tests using SYSTAT 5.2 (Wilkinson 1992). In all cases the null hypothesis being tested was a sex ratio of 0.5, and was rejected when $p < 0.05$. Paired *t*-tests were performed on the sex ratios of each category of young to detect differences in the level of bias between categories. Sex ratios within litters were tested for significance by χ^2 and binomial probability tests. Correlations between maternal body mass, sex ratios and litter size were analysed by means of SYSTAT 5.2.

3. RESULTS

(a) Collection of females

A total of 20 females were collected from the Spargo Creek area between 29 August and 11 September 1995.

Table 1. *Details of individual litters of Antechinus agilis*

litter identification	date of birth	time of birth	total litter size	no. of pouch young	no. of super-numerary young	sex ratio in total litter
A	14/9/95	19.20	9	9	0	0.11
B	16/9/95	07.30	17	10	7	0.43
C	16/9/95	17.00	10	9	1	0.40
D	17/9/95	04.20	10	10	0	0.56
E	18/9/95	11.00	10	10	0	0.10
F	18/9/95	16.40	12	9	3	0.42
G	19/9/95	10.30	15	10	5	0.13
H	19/9/95	11.35	10	10	0	0.20
I	20/9/95	20.45	10	10	0	0.50
J	21/9/95	00.45	10	10	0	0.40
K	21/9/95	06.00	12	10	2	0.50
L	21/9/95	07.15	9	9	0	0.22
M	21/9/95	11.10	12	10	2	0.17
N	21/9/95	11.10	12	10	2	0.42
O	22/9/95	18.15	15	10	5	0.33
P ^a	22/9/95	23.25	10	9	1	0.10
Q	25/9/95	15.15	14	10	4	0.21
R	26/9/95	07.00	15	10	5	0.39
S	26/9/95	07.00	11	9	2	0.45

^aThe mother of this litter had nine teats (all others had ten).

All females were in their first breeding season, there was little variation in body mass (28.6 ± 2.6 g, mean $\pm$ s.d.), and all but one female had ten teats (one had nine).

(b) *Birth and litter sizes*

Nineteen females gave birth in captivity (one did not). Births occurred between 14 and 26 September, and occurred both during the day and during the night (table 1). These females produced a total of 223 young, of which 183 were pouch young and 40 (18%) were supernumerary young. Mean total litter size was 11.7 ± 2.4 (range 9–17). There was no correlation between maternal body mass and litter size (Pearson's coefficient = 1.38, $n=18$, $p=0.24$) (figure 1a). Five females did not have all teats occupied; however, no more than one teat was left unoccupied, and the mean number of pouch young was 9.7 ± 0.5 . Twelve females produced supernumerary young, with a mean of 3.25 (± 1.91 , range 1–7) when present. The mean proportion of total young born that successfully attached to teats was 0.85 ± 0.14 .

(c) *Sex ratios*

Accurate sexing of some young was impossible, owing either to tissue damage during histological preparation or to desiccation of some supernumerary young. Consequently, eight neonates, 3.6% of the total young born (four pouch young and four supernumerary), could not be sexed.

A total of 69 males and 146 females (sex ratio 0.32) were produced by the 19 mothers. Of these, 57 males and 123 females (0.32) were pouch young and 12 males and 23 females (0.34) were supernumerary young. Eighty-three and 84% of all male and female neonates, respectively, successfully attached to teats. The sex ratios of total young born and pouch young were significantly different from parity ($\chi^2 = 24.2$ and 27.6 , respectively, $p < 0.001$ for both). The sex ratio of supernumerary young approached significance ($\chi^2 = 3.5$, $p = 0.063$); the lack of significance was probably due to the reduced statistical power of the

test when only a small number of supernumerary young was produced.

Within litters, the mean ($\pm$ s.d.) number of males and females per total litter was 3.6 ± 1.8 and 7.7 ± 2.2 , respectively. The mean sex ratio of total litters was 0.32 ± 0.15 ; this ratio differed significantly from parity ($t_{18} = 5.23$, $p = 0.01$). Female-biased litters were produced by 16 (84%) females (table 1); two litters were unbiased, and one litter was male biased, but not significantly (5 males, 4 females, 1 unsexable). Only six female-biased litters were significantly biased (χ^2 and binomial tests). A χ^2 test of homogeneity revealed no significant difference across the sex ratios of the 19 litters ($\chi^2_{17} = 25.23$, $p > 0.05$). Biased sex ratios could not be explained by maternal body mass, because there was no correlation between total litter sex ratio and maternal body mass (Pearson's coefficient = 2.54, $n=18$, $p=0.11$) (figure 1b). Similar patterns were observed when pouch litters were analysed; however, numbers of supernumerary young per litter were too small for similar analyses of these young.

There was no correlation between total litter size and total litter sex ratio (Pearson's coefficient = 0.19, $n=19$, $p=0.66$). The combined sex ratio of all young born in litters of ten or fewer young (i.e. up to and including the maximum pouch litter size) was 0.29 (25 males:60 females, from 9 litters), compared with 0.34 (44:86, 10) for neonates in large litters (more than ten young). There was no significant difference between the sex ratios of total litters of these two groups ($\chi^2_1 = 0.463$, $p > 0.05$).

(d) *Counts of corpora lutea*

The mean ($\pm$ s.d.) number of corpora lutea (both ovaries combined) was 15.3 ± 2.4 ($n=10$). If we take the number of corpora lutea as the potential number of embryos, $78 \pm 9.5\%$ of embryos survived to birth, and $65 \pm 8.5\%$ attached to teats. The sex ratio of all young born was 0.26; if 100% of lost embryos were male, the maximum sex ratio for embryos was 0.42 ($\chi^2_1 = 3.46$,

Figure 1. Relations with maternal body mass in *Antechinus agilis*. (a) Total litter size. (b) Sex ratio of the total litter. Neither correlation was significant: litter size, Pearson's coefficient = 1.38, $n = 18$, $p = 0.24$; sex ratio, 2.54, 18, 0.11.

$p = 0.063$), so a male-biased loss of embryos could have contributed a maximum of only 66.7% of the sex bias observed. All ten litters were female-biased at birth, but under the scenario above, only one of the ten litters could have been male biased at the embryo stage, and at least six were female biased at fertilization.

4. DISCUSSION

(a) Sex ratios

In this study, litters of *A. agilis* were strongly female biased. Previous studies have reported biased sex ratios in pouch litters of *Antechinus* spp. (Cockburn *et al.* 1985; Dickman 1988) and *Didelphis* spp. (Austad & Sunquist 1986; Sunquist & Eisenberg 1993), and more generally in pouch young of other marsupials (Hughes 1962; Caughley & Kean 1964; Hope 1972; Johnson & Jarman 1983; Poole *et al.* 1985; Sunnucks & Taylor 1998). However, the present study is, to our knowledge, the first to record the sex of all young born. The sex ratio observed in pouch young of *A. agilis* was almost exactly the same as the ratios observed for supernumerary young and for all young born, so sex-related success in attaching to teats immediately after birth is not the mechanism by which sex bias in *Antechinus* spp. is generated. A mechanism (or mechanisms) prior to birth must be operating to produce the biased sex ratios of litters in *Antechinus* spp.

(b) Sex allocation

In the source population for animals in this study, relatively few females survived to breed in their second year, so the observed female bias in litters was consistent with the sex ratio predicted from the relationship presented by

Cockburn *et al.* (1985). In the present study there was no evidence of a facultative response in *A. agilis*; virtually all females produced female-biased litters, and neither the mother's body mass nor the size of the litter influenced the sex ratio. Similarly, in their study of common opossums, *Didelphis marsupialis*, Austad & Sunquist (1986) reported that sex-ratio bias was independent of both maternal body mass and litter size. These results therefore provide no evidence for the Trivers–Willard effect on early sex allocation in *Antechinus* (Trivers & Willard 1973; Haig 1990). However, Dickman (1988) found in his study of food provisioning in *A. agilis* that the proportion of males in litters was greater in heavier mothers, which he interpreted as supporting the Trivers–Willard hypothesis. Perhaps the level of provisioning in Dickman's study was sufficient to elevate maternal condition to a level at which such effects could be detected statistically without very large sample sizes. Furthermore, Cockburn (1994) has shown a substantial postnatal manipulation of sex ratios in *A. agilis*, indicating that Trivers–Willard effects are probably operating at later stages of development.

(c) The mechanism

Overproduction of young, if combined with a selective sorting system, is potentially a low-cost mechanism for the adaptive manipulation of sex ratios (Meikle *et al.* 1993). Our results show that this is clearly not the mechanism operating in *A. agilis*, and that the bias is generated prenatally. Losses of potential offspring occur between ovulation and birth in *A. agilis* (Selwood 1983), but most occur during early embryonic stages, well before any major sexual differentiation. In the present study, all females mated naturally in the wild before collection, so natural numbers of corpora lutea were recorded. It is possible that captivity and the handling regime influenced the number of embryos reaching birth. The mean number of corpora lutea per female was in excess of the number of embryos reaching birth, and if a 100% fertilization rate is assumed, less than three-quarters of embryos survived to birth, and only two-thirds became pouch young. These rates are comparable with those for *A. agilis* reported by Selwood (1983), who found 74% and 69%, respectively. Sex-biased loss of embryos could be a partial mechanism for producing sex-biased litters of pouch young in these populations, but it cannot be the only mechanism. Even if all the lost embryos were male, they could have contributed only two-thirds of the sex bias observed here. Because gonadal differentiation occurs after birth in marsupials (Short *et al.* 1988), any sex-biased loss of embryos must be operating on genetic rather than hormonal cues. A more likely mechanism is the assortment of sperm resulting in sex bias in the fertilization of ova (Cockburn 1990). It is possible that the period of sperm storage between copulation and fertilization in *A. agilis* (Selwood 1982b; Taggart & Temple-Smith 1991) contributes to such assortment. Mechanisms at both prefertilization and embryonic stages need to be considered in future studies of sex bias in litters of *A. agilis*.

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