Review

Title: Can mammalian mothers influence the sex of their offspring peri-conceptually?

Short title: Peri-conceptual influences on sex of offspring

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Abstract

Although controversial, growing evidence from evolutionary biology suggests that the mammalian mother may have a role in influencing the sex of her offspring. However there is competing information on the molecular mechanisms by which such influence could be manifested. The new initiatives are based on hypotheses from evolutionary biology: the ‘good condition’ hypothesis, which suggests that post-conception, higher levels of maternal glucose may differentially promote the development of male embryos; and the ‘maternal dominance’ hypothesis, which proposes that before conception, higher follicular testosterone may influence the development of the ovum so that it emerges already adapted to receive an X- or a Y-chromosome-bearing spermatozoon. Now it seems these hypothesised mechanisms could be operating in synchrony, each complementing and reinforcing the other. On the other hand, there are continuing problems in identifying a precise sequence of mechanisms as evidenced from research in sperm–sorting. Research on high fat diets and the sex ratio in polytocous species may indicate important differences in proximate mechanisms for sex allocation between polytocous and monotocous mammals.
Introduction

A growing body of evidence suggests there must be some form of adaptive regulation of mammalian sex ratios. Given that almost all other non-mammalian taxa have efficient adaptive control of the sex ratio of their offspring, and given that such control brings important evolutionary benefits, it seems likely that some kind of mechanism would also be present in mammals. In addition, it would make sense if this adaptive influence were at least partially under maternal control, given the mammalian mother’s disproportionate commitment to the production of the offspring. Following the logic of this argument and using the theoretical framework provided by Trivers and Willard (1973) evolutionary biologists have demonstrated strong (albeit often seemingly contradictory) links between maternal characteristics and statistically significant variations in offspring secondary (birth) sex ratios (Cameron 2004, Sheldon & West 2004, Grant 2007). Thus results from many studies in evolutionary biology suggest that even if current hypotheses are somewhat rudimentary, a proximate mechanism for adaptive sex allocation in mammals must surely exist. On the other hand, this thinking is in direct contradiction to the chance theory of sex allocation, which holds that the only influence on the sex of offspring is whether the fertilising sperm carries an X or a Y chromosome.

As a result of their findings on atypical secondary sex ratios, evolutionary biologists have offered three theoretically-derived clues to those searching for a mechanism. First, it is likely that the mammalian mother will have a role in the allocation of the sex of her offspring. Secondly, it is likely that in a highly conserved system of sex allocation in
sexually-reproducing animals, the most efficient and least costly time for such a modification to take place would be at, or near, conception. And thirdly, any such system of sex allocation would need to be able to respond to environmental stressors in a way that was evolutionarily advantageous.

Two main research streams have addressed the problems. One is based on the association between maternal dominance and offspring sex ratio, (the maternal dominance hypothesis) (Grant 1996), and the other between good condition in the mother and the subsequent sex of her offspring (Trivers & Willard 1973). The Trivers and Willard hypothesis suggested that mammalian parents would gain an evolutionary (fitness) advantage if they could manipulate the sex ratio of their offspring. They argued that a mother in good condition would gain maximal evolutionary advantage by producing a son, who, assuming he inherited her condition, would out-reproduce a sister in similar condition. In part because of difficulties in defining and measuring good condition, still unresolved (Wilson and Nussey, 2010) maternal dominance, under-pinned by testosterone, came to be seen by some researchers not only as a proxy for good condition, but possibly as the key component, over-riding environmentally-induced changes in condition.

The main factor in resolving the apparently conflicting results appeared to be whether or not the mothers were living under conditions of chronic stress e.g. food shortages, (Verme, 1969) population density increases (Kruuk et al, 1999) insufficient den or nesting sites (Clark, 1978). Under such conditions the atypical sex ratios were reversed,
that is, the mothers in poor condition had significantly more male offspring. This 
reversal may be accounted for by the fact that in females, testosterone (perhaps because 
in females it arises in peripheral tissues under the control of the adrenal glands – also 
associated with the stress response) rises under conditions of chronic stress (Christiansen 
1998). Stressful times also increase differential male vulnerability. Conceiving more 
males may offset this loss and thus be an integral part of frequency-dependent sex 
selection, which works to ensure there are equal numbers of males and females at the 
time of reproductive maturity. (See Grant 2003, 2007, 2009 for further details).

In the search for a proximate molecular mechanism which would fit the evolutionary 
findings, proponents of each hypothesis pursued a putative physiological correlate in the 
mother, predicted to have the potential to influence sex allocation. Recognizing that 
testosterone underpins a tendency to dominant behaviour (Bouissou 1978, Christiansen 
1998, Grant & France 2001), researchers who favoured the maternal dominance 
hypothesis sought an association between female testosterone and offspring sex ratio 
(Shargal et al 2008, Helle et al 2008, Grant & Irwin 2005, Grant et al 2008) while those 
opting for the good condition hypothesis sought links in the area of diet (Rosenfeld et al 
2003, Rosenfeld & Roberts 2004, Mathews et al 2008, Cameron et al 2008), and, more 
explicitly, unsaturated fats (Alexenko et al 2007), polyunsaturated fatty acids (Green et al 

Both streams of research have built on earlier work by reproductive biologists. There 
have been relevant studies of pre-conception maternal diet, investigating variations in
both quantity (Verme 1969) and quality of diet (Stolkowski & Lorrain 1980) for an effect on the sex ratio. There have also been studies of the timing of insemination (Guerrero, 1974; France et al, 1984; Weinberg et al, 1995), as well as maternal influences on the uterine environment and sex selective embryo loss. It is becoming clear that both genetic and environmental influences on the sex of the offspring in mammals are relevant to both prenatal (Navara & Nelson 2009) and peri-conceptual environments.

Evidence supporting the influence of maternal testosterone on offspring sex ratio

Independently, four research teams (Shargal et al 2008, Helle et al 2008, Grant & Irwin 2005, Grant et al 2008, Garcia-Herreros et al 2010) have investigated the hypothesized relationship between pre-conception maternal testosterone and the subsequent sex of the offspring. In one (Shargal et al 2008) the dominant females in a small captive herd of Nubian ibexes were found to have higher faecal testosterone and significantly more male offspring than subordinate females that had lower faecal testosterone and more female offspring. In the second, a much larger study, (Helle et al 2008) both glucose and testosterone were measured using serum samples from female field voles, taken a few days before conception. This study showed no effect of paternal testosterone or of maternal body condition, but higher levels of both glucose and testosterone in the mothers were significantly associated with higher offspring sex ratios (more males than females). Although this study demonstrated an association between high maternal circulating testosterone level and male-biased litter sex ratio, and was the first to do so in
a nondomestic mammal, the authors noted that “the exact mechanisms responsible for this association …remain unknown.” (Helle et al 2008).

The third and fourth studies (Grant & Irwin 2005, Grant et al. 2008) may however, offer a clue about mechanisms. In these studies we focused on follicular rather than serum or faecal testosterone. The fact that in the female’s follicular fluid testosterone occurs at much higher concentrations than it does in blood (in humans, 10,000 to 30,000 times more) and that there is wide variation both inter- and intra-individuals over time, provided the basis for the hypothesis that an ovum could emerge each oestrus or menstrual cycle already adapted to preferentially receive an X- or a Y- chromosome-bearing spermatozoon depending on the amount of testosterone it was exposed to at a crucial time during its development within the follicle. This hypothesized mechanism had the additional theoretical advantage of being the least costly mechanism for sex allocation of any so far suggested.

The two studies had a similar structure. As described elsewhere (Grant & Irwin 2005, Grant et al 2008), ova were removed from abattoir-derived bovine follicles. The follicular fluid in which the ovum had developed was collected and labelled to match the ovum, so that each egg could be traced back to the follicular fluid from which it originated. After in vitro fertilisation the sex of each embryo was determined by polymerase chain reaction, and the matched samples of follicular fluid were analysed for testosterone. Findings from the first study (Grant & Irwin 2005) suggested that the high level of testosterone had to be present earlier in the antral phase of development than first thought. In the second,
larger, study of 171 fertilised oocytes (Grant et al. 2008) we found that ova that had
developed in follicular fluid with high levels of testosterone were subsequently
significantly more likely to be fertilised by Y-chromosome – bearing spermatozoa,
particularly if the high testosterone levels were not simply the result of poor
aromatisation of the testosterone to oestradiol. These results suggested there may be a
critical time in the development of the zona pellucida (ZP) during which its molecular
composition may be subtly influenced by the high levels of follicular testosterone
rendering the oocyte more (or less) susceptible to fertilisation by a Y-bearing
spermatozoon.

The most recent study to investigate the relationship between maternal testosterone and
subsequent offspring sex was also relatively large and examined 140 fertilised bovine
ova, using methodology which closely replicated that of Grant et al. (2008). In that
study, Garcia-Herreros et al. (2010) demonstrated that median testosterone levels were
higher in follicles that resulted in subsequently male embryos (32.12ng/ml) than
subsequently female embryos (23.98ng/ml). This difference approached, but was not,
statistically significant (p = 0.06; Garcia-Herreros et al., 2010). Since the methodology of
Garcia-Herreros et al. (2010) is so similar to that of Grant et al. (2008) it is possible to
combine the summary data from the two studies. The data combined from both studies
show that there were 33 subsequently male embryos and 13 subsequently female embryos
that were fertilised from follicles with testosterone levels greater than 300 nM. This
demonstrates a significant (chi sq p = 0.003) bias for fertilisation of oocytes developing
in high testosterone to be fertilised by Y-chromosome-bearing spermatozoa. For a summary of the findings of these two studies see Table 1.

Although Garcia-Herreros et al (2010) did not achieve a significant effect in the conventional sense ($p = 0.06$ for a test of the difference between medians), nevertheless they did successfully replicate the results of Grant et al (2008) (see Table 1.). Garcia-Herreros et al's (2010) findings satisfy Killeen's (2005) criteria for a successful replication, namely that an equi-potent experiment obtains an effect of the same sign as that of the original experiment.

Evidence supporting the influence of maternal condition on offspring sex ratio

In 1973 Trivers & Willard [5] proposed that mothers in good condition could maximise fitness by producing male offspring (since a male in good condition could out-reproduce a sister in similar condition) thus providing a plausible context for studying maternal body condition and, more recently, the dietary pathway towards achieving good condition. In the decades that followed evolutionary biologists who found that superior maternal body condition was significantly associated with higher secondary sex ratios (more male offspring at birth) frequently interpreted their findings as evidence supporting the Trivers and Willard hypothesis. (For a review see Cameron, 2004.)

More recently, experimental interventions have demonstrated a significant effect of pre-conceptual maternal diet on the sex ratio (Alexenko et al 2007, Green et al 2008,
Fountain et al. (2008). Female mice fed a very high saturated fat diet had significantly more male offspring than either control or restricted fat diet females. For example, in Alexenko et al.’s (2007) study, 244 mice fed a very high saturated fat diet had 60% male offspring compared to 274 controls 48% male offspring ($p<0.0001$). Furthermore, since males fed similar diets had neither more Y-sperm, nor sired more sons than daughters, the authors concluded that the dietary effects were “manifested exclusively through the female” (Alexenko et al. 2007). Ewes fed a diet enriched with polyunsaturated fatty acids also had significantly more male offspring (Green et al. 2008) and a large study of human pre-conception diet appeared to demonstrate a similar effect (Mathews et al. 2008), although an alternative interpretation of this data has recently been offered by Young et al. (2009) on the grounds that multiple testing may result in false positives.

Although researchers have shown that pre-conception diets can be related to offspring sex ratio, the proximate (molecular) mechanisms thought to be associated with this effect have largely been thought to act post-conception. That is to say, although the nutritionally-induced high maternal glucose levels are likely to exist before conception, the researchers suggested they act post conception by differentially promoting the development of male embryos (Kimura et al. 2005, Kimura et al. 2008). These findings in turn are consistent with earlier work by reproductive biologists who found that male embryos developed more quickly than female embryos in both experimental (Mittwoch 1989, 1996) and clinical settings (Permagent et al. 1994, Ray et al. 1995).
Researchers pursuing the good condition hypothesis (Cameron 2004) have now suggested it might not be good condition per se that was the best predictor of offspring sex, but rather a change in condition, mothers whose condition was improving at the time of conception being more likely to bear males (Cameron & Linklater 2007). Studies with both murine (Cameron et al 2008) and bovine (Kimura et al. 2005) embryos showed that increasing amounts of glucose in the uterine environment post-conception were associated with the preferential development of males. These findings are consistent with earlier work which documented asynchronous development in embryos in several species, with males developing faster than females (Krackow 1995).

**A possible sequence of effects**

Thus, taken as a whole, recent work suggests these two influences (maternal testosterone prior to conception (Grant & Irwin 2005), and maternal glucose immediately post-conception (Kimura et al 2005) may have a combined effect on the sex ratio in mammals acting in sequence, and possibly in synchrony with one another. Such a system would have its own integrity, based initially on normally distributed female testosterone, with levels above the mean predisposing the female to more dominant behaviour, which in turn has a higher energy requirement (Rosenfeld & Roberts 2004). At the same time as higher testosterone levels in follicular fluid may be instrumental in modifying the ovum to preferentially (but not exclusively) receive either an X- or a Y- chromosome – bearing spermatozoon, variations in glucose levels in the uterine environment may be differentially supportive of either a male or a female embryo. Thus both pre- and post-
conception, behaviourally and physiologically, the mammalian mother may be specifically adapted at the time of fertilisation, to conceive and nourish either one sex or the other.

Furthermore, both hypotheses fulfil the evolutionary requirements. Each proposes a role for the mother and suggests a way in which she could influence the sex of her offspring. Both suggest proximate mechanisms which would minimise the costs of sex allocation, acting either immediately before, or immediately after, fertilisation. And each has the potential to be differentially responsive to changes in the environment. Regarding the good condition hypothesis, when resources are scarce, body condition deteriorates and glucose levels may decline. Regarding the maternal dominance hypothesis, since female testosterone rises in response to environmental stressors (Christiansen, 1998), there would be an opportunity for raised or lowered maternal testosterone to influence sex allocation at the exact time such influence was evolutionarily advantageous.

However, in spite of the neat theoretical fit, both hypotheses require both replication and further exploration. A comparatively simple yet potentially persuasive series of observations could be undertaken in either the field or the laboratory, preferably in different species, to assess the validity or otherwise of measuring pre-conception maternal hormones and glucose as an indicator of future sex of offspring. As cited above, five studies (three laboratory and two field studies) have shown that pre-conception hormone profiles may provide an indication of subsequent sex of offspring. One of the field studies measured serum testosterone and glucose, the other faecal testosterone; the
laboratory studies measured follicular testosterone. If further studies were undertaken, particular attention might be paid to both the timing of collection within the pre-conception cycle and the measurement of both testosterone and oestradiol in order to optimise the likelihood of capturing the full extent of the hypothesised hormonal influence.

A recent example of such a study is that by Perret (2005) who used a very small primate, the gray mouse lemur, as the animal model. Urinary oestrogen levels measured prior to oestrus were significantly predictive of offspring sex, with females that later conceived male offspring having significantly less urinary oestrogen. Since the ratio of oestrogen to testosterone in follicular fluid has long been thought to be relevant to successful oocyte development (McNatty et al 1984), it is possible to speculate that less oestrogen earlier in the follicular phase could be indicative of higher testosterone, and the high levels of oestrogen at the end of the follicular phase could reflect the rapid aromatization of testosterone towards the end of the antral phase of the oocyte development. However it would be preferable not to have to make this speculation and thus, testing for both oestrogen and testosterone early in the follicular phase may offer a relatively non-invasive method for exploring the hypothesized maternal influence on the sex of the offspring prior to conception.

Although the experiments described above offer tantalising evidence that mammalian mothers might be able to influence the sex of their offspring there remain significant unresolved problems.
Problems – 1. Issues of timing and oocyte development in the maternal testosterone hypothesis

In spite of the theoretical consistency in the current hypotheses, there remain several problems and inconsistencies. The first of these is exemplified in a study of sex-sorted spermatozoa for use in bovine reproduction (Bermejo-Alvarez et al. 2008). The problem is if, as suggested, an ovum is produced each oestrus cycle already adapted to preferentially receive an X- or a Y-chromosome-bearing spermatozoon, then exposure to any number of spermatozoa of the ‘wrong sex’ would mean fertilisation was less likely. Thus, as described elsewhere (Grant & Chamley 2007), the reported low fertility of animals fertilised with sex-sorted (X-chromosome-bearing) sperm was consistent with the hypothesis that only some of the mothers had produced ova adapted to receive X-chromosome-bearing spermatozoa.

In an elegant study Bermejo-Alvarez et al. (2008) reported for the first time, fertility rates and sex ratios produced by sex-sorted, sex-sorted then re-combined, and non-sorted spermatozoa. They found no differences in fertility rates from the three types of sex-sorted sperm and concluded that “the differences in cleavage and blastocyst development using sorted versus unsorted sperm” were “not due to the oocyte preferentially selecting sperm of one sex over another”…but were “more likely due to sperm damage caused by the sorting procedure”. These findings suggested that oocytes are not pre-adapted to preferentially accept spermatozoa that bear either an X or a Y chromosome. Therefore, if
mammalian mothers could influence the sex of their offspring, it was unlikely to be via the method of differential selection of an X- or Y- chromosome-bearing spermatozoon at the zona pellucida (ZP).

However, if the initial influence of a mother on the sex of her offspring is via an influence of testosterone levels on the developing ZP, then the timing of the recovery of oocytes is likely to be important since testosterone levels and the testosterone/oestradiol ratio change dynamically during follicular development. The extreme complexity of the cellular environment and production of hormones within the follicle have recently been described (Irving-Rodgers et al, 2003; Rodgers & Irving-Rodgers, 2010). Discriminating between antral and basal follicular atresia, the authors emphasized both the rate of follicular development (“on average a net 19 doublings in the surface area of the follicle from a primordial to an 18mm bovine follicle”) and the precise timing of the hormone sequences (for example, “a significant negative relationship between transforming growth factor … in follicular fluid and follicle diameter occurs at 6.5mm (before selection) but not at later stages”). Although in the past high levels of follicular testosterone were thought to indicate approaching follicular atresia, recent findings suggest a modification of this view which takes into account the precise stage of development of the follicle, as well as the differing characteristics of basal and antral atresia. For example, there was no statistical difference in the levels of testosterone between antral atretic and healthy follicles < 5mm in diameter (Irving-Rodgers et al 2003).
In the most recent studies examining the relationship between follicular testosterone and sex ratios, bovine follicles were selected according to size, diameters ranging from 2-8mm (Bermejo-Alvarez et al. 2008), from 4-10mm (Grant et al. 2008) and 2-12mm (Garcia-Herreros et al 2010). But gross measurement of follicle size is likely to be a poor indicator of the stage of follicular development and it may be necessary to further refine this in future investigations.

In our earlier studies (Grant & Irwin 2005; Grant et al. 2008) as well as in the more recently published study by Garcia-Herreros et al (2010) the distribution of testosterone in follicular fluid was markedly skewed to the right, with high levels of testosterone associated with fertilisation by Y-bearing-chromosome spermatozoa. As noted above, testosterone levels over 300 nMol/l were strongly predictive of subsequently male embryos, whereas the lower amounts of follicular testosterone did not discriminate between subsequently male and female embryos and this held true when the oestradiol to testosterone ratio was used to restrict our analysis to only those follicles at the same stage of development. Hence, we suggested it may not be simply the level of testosterone that is important but the timing of the testosterone peak, as well as the cause of the peak (i.e. increased testosterone synthesis rather than reduced aromatisation) that is relevant to the conditioning of the ZP for preferentially accepting an X- or a Y-chromosome-bearing spermatozoon.

**Problems - 2. The zona pellucida (ZP) in the maternal testosterone hypothesis**
If, as hypothesised, the ZP has a potential gate-keeping role in sex allocation, more information is needed about the form this role could take. Although the functions of the ZP may include responsibility for species specificity and facilitation of the acrosome reaction Wassarman (2008) wrote “many important issues related to the participation of the ZPGs [zona pellucida glycoproteins] in the fertilisation process remain unresolved or controversial”. At the same time he noted that such processes are likely to be highly conserved “during several hundred million years of evolution”. In the absence of a competing hypothesis, it continues to be logical to suggest that the ZP could be the site of a modifiable process involving pre-conceptual sex allocation. Such a modification might for example be a subtle variation in a carbohydrate (sperm-binding ligand) on the ZP proteins.

In the setting of human reproduction, there is some potentially corroborative evidence from clinical studies involving intracytoplasmic sperm injection (ICSI). When the sperm is injected directly into the ovum, it by-passes the ZP, thus also by-passing any putative role it might have in influencing sex allocation. Given equal numbers of X- and Y-chromosome-bearing spermatozoa are present in the ejaculate (Graffelmann et al 1999), it might be expected that the resultant birth (secondary) sex ratio would be no different to chance (100 males to 100 females). In fact, there are significantly lower than normal secondary sex ratios (i.e. fewer males) following ICSI. One study found a ratio of 96:100 (Luke et al. 2009) and another 101:100 (Fedder et al. 2007) males:females following ICSI. Although the normal primary (conception) sex ratio is considerably
higher (Hassold et al 1983), the normal secondary (birth) sex ratio in humans is 105 or 106 male births for every 100 female births.

Differential male vulnerability, which operates from conception onwards, has been demonstrated in animals (Forsyth et al. 2004), and extensively documented in humans (Kruger & Nesse 2004). It is said to account for the fact that more males than females are both conceived and born. In evolutionary terms it appears that one of the reasons more males are conceived in the first place is to counterbalance differential male vulnerability (Grant 2009). However, when births follow ICSI this normal conception or primary sex ratio (higher number of males) is by-passed leaving the most likely influence on the conception sex ratio to be the equal numbers of X- and Y-chromosome- bearing spermatozoa. In this setting therefore, only male vulnerability is left to modify the birth sex ratio. The consequence is that equal numbers of the sexes may well be conceived, but more males will be lost post- conception, resulting in fewer males than usual being born (Fedder et al 2007, Luke et al 2009). This suggests that in the absence of a potential influence of the zona pellucida during ICSI, random sex allocation does occur in humans but when the zona pellucida is present (i.e. in normal conceptions) more males are conceived.

Problems - 3. Seemingly contradictory results in studies of the good condition hypothesis
The seeming contradictions in studies of the good condition hypothesis are that first, researchers found that female mice fed a diet high in saturated fat produced significantly more male offspring (Rosenfeld et al. 2003). These results were consistent with the Trivers and Willard hypothesis providing experimental evidence in support of the proposition that mothers in good condition would produce more males. Next, researchers showed that it was the high fat content of the diet, rather than the number of calories which led to the production of more male offspring (Alexenko et al. 2007). At the same time it was reported that these changes in diet had led to changes in serum hormones (Whyte et al. 2007), but instead of the high fat diet being associated with increased serum testosterone around the time of conception, (which could have supported both the maternal dominance hypothesis and the good condition hypothesis) it was found instead to be associated with significantly lower serum testosterone. Half a day post-coitus, testosterone was substantially lower in the mice fed the very high fat diet (10.5 +/- 3.0 pg/ml) compared with the low fat group (32.7 +/- 8.4 pg/ml).

Then further investigation of the effect of high fat diets (which involved discriminating between diets high in the omega 3 and omega 6 components of polyunsaturated fatty acids) found the sex ratios were reversed (Fountain et al. 2008). The mice fed the high fat diet enriched with omega 6 gave birth to a significantly higher number of female offspring. In all these studies, the diet –induced changes in offspring sex ratio were not trivial. For example, in this study female mice fed the omega 6 – enriched diet had 213 female offspring and 133 male offspring (P < 0.001). Neither the omega 3-enriched nor the control diets had any effect on the birth sex ratio.
From the point of view of synthesizing the two main hypotheses, this latest result suggests a degree of compatibility. This is because results from the earlier work (Rosenfeld et al 2003) in which females fed a high fat diet conceived more male offspring, may have provided evidence for the good condition hypothesis as originally suggested because the particular kind of high fat diet was not so extreme as to overcome the body’s natural homeostasis; whereas in the later study, the more extreme diet resulted in lowered pre-conception serum testosterone which in turn resulted in the conception of significantly fewer male offspring. Thus further exploration of possible links and/or interaction effects between diets high in polyunsaturated fatty acids and their putative effects on serum testosterone may prove fruitful.

Problems - 4. Differences between polytocous and monotocous mammals

An alternative explanation for the seeming contradictions in research on both good condition and maternal testosterone on the secondary sex ratio is that there may be important species differences, in particular between monotocous and polytocous mammals. It is possible that putative mechanisms providing for adaptive allocation of sex of offspring in monotocous mothers may need to be more focused or specific than they are in polytocous mothers given that the monotocous mother has only one opportunity to get the sex of her offspring “right” in any particular breeding season.
Several lines of evidence support this suggestion. First, at the epidemiological level, James (2009) has pointed out that the distribution of the sexes in polytycous mammals is sub-binomial; that is, mathematically, “there are too many litters in which the sexes are equally balanced, and there are too few unisexual litters” (James, 2009). This he says is “most unusual in Nature” and requires an explanation. His suggestion is that the sub-binomial distribution arises because in polytycous mammals zygotes within a litter are formed across a period of time and hence may be differently influenced by the relevant maternal hormones.

Next, at the level of individual, behavioural and social characteristics Novokova et al. (2010) in a large study (n = 4048 spiny mice newborns) could find no difference in offspring sex ratio on a range of maternal attributes including parity, age in days, or litter size; nor in eight social variables including number of adult males present, time from founding of the breeding group, or maternal status (as defined by whether or not she was “first breeding founder”). It should be noted that these authors did not attempt any manipulation of the offspring sex ratio. They did however offer reasons why manipulation of the secondary sex ratio may not be necessary in these animals. Spiny mice in the wild, they wrote, “strictly avoid breeding whenever they perceive the environmental or social conditions as not fully favourable “; and “there may be reduced variance in body condition and consequently, no reason for maternal manipulation and/or any other maternal effect on the sex ratio of the progeny”. Both these reasons contribute to the debate about whether there would or would not be evolutionary incentives for
polytocous mothers to have an influence on the sex ratio of the offspring in the same way as monotocous mothers.

At the proximate level, there is evidence that in mice, oocytes are not selective towards X- or Y-bearing sperm (Zuccotti et al. 2005). By doubling the sperm concentration, these researchers were able to override natural barriers to polyspermy. Their results showed X- and Y-sperm fertilisations entirely consistent with a mathematically predictable distribution of the sexes on the basis of chance. The work was done with metaphase II oocytes, and whether this stage of development is sufficiently mature to allow an influence on the ZP in mice is unknown. However, as noted above, there is some evidence that the ZP2 in mice functions differently to that in other species (Hinsch et al. 1998). Therefore, a more parsimonious explanation for these results could be that no selection was taking place at the ZP.

Taken together, these multi-level findings (epidemiological, behavioural and physiological) suggest that putative maternal mechanisms influencing sex ratio manipulation in polytocous mammals could be somewhat different from those hypothesised to exist in monotocous mammals.

Furthermore, an example of a different mechanism for a maternal influence on offspring sex ratio in polytocous mammals was demonstrated by Clark et al. (1993) in gerbils and by Vandenberghe & Huggett (1994) in mice. Both studies showed that offspring sex ratios were affected by maternal testosterone. In both instances the higher maternal testosterone
was shown to have originated during the female’s development \textit{in utero}. The source of
the higher testosterone may have been from the neighbouring male foetuses. Since the
females thus androgenised not only produced significantly more male offspring when
they had their first litters, but more males throughout their reproductive lives, this effect
too warrants further exploration.

More recently it was found that bovine embryos and calves originating from the right
ovary were more likely to be male while those from the left ovary were more likely to be
female (Hylan \textit{et al} 2009). At the same time, the highly significant effects on the sex
ratio that were found in naturally bred cattle were not demonstrated in embryos
transplanted after in vitro fertilisation (IVF). But as the oocytes used in the IVF
programme measured between 2mm and 9mm, the same phenomenon could apply here as
in the sperm-sorting experiments. Unless the oocytes are studied at the appropriate stage
of development of the ZP, the ZPs may not demonstrate the ability to influence offspring
sex.

Problems - \textbf{5. Do males contribute to adaptive control of the sex ratio in mammals?}

Although the focus of this paper has been on ways in which the mammalian mother might
be able to adaptively influence the sex of her offspring, there remains a question about
whether or not there may also be a pre-conceptual paternal influence on the sex of the
offspring. In polygynous mammals a male in good condition will out-reproduce, often by
a wide margin, all the other males in the group. Providing the males disperse, thus
minimising in-breeding, there should be an adaptive benefit since the highest status male is also likely to be in the best condition. The sperm characteristics of such males have been described in detail, most recently by Malo et al (2005), and include measures of both quantity and quality (e.g. proportion of motile, morphologically normal spermatozoa). Recently the same team (Gomendio et al 2006) showed that semen from “more fertile” males (defined as impregnating a higher proportion of females) produced a higher number of male offspring. If replicated this would suggest that males too could have a role in the adaptive control of the sex ratio. On the other hand the experimenters reported a 50.39% fertility rate, and the hinds were synchronised in order to control for timing effects of insemination on the sex ratio. But if females are conceived later in the cycle (Weinberg et al, 1995) this manipulation may have countered the conception of female offspring.”

**Conclusion**

For many years the dogma has been that the sex of mammals is determined solely by chance, depending on whether an X- or Y- chromosome--bearing spermatozoon fertilises the oocyte. This thinking is now being challenged by growing evidence from field, clinical and laboratory studies, which suggests mammalian mothers may have some influence over the sex of their offspring. What is now required is the experimental confirmation of one or more molecular mechanisms demonstrating the existence (or not) of a testosterone-induced modification to the ZP (pre-conception) in monotocious females and/or a mechanism whereby male, rather than female zygotes are advantaged by the
presence of glucose in the uterine environment. Unless such mechanisms can be convincingly demonstrated, no matter how much behavioural and theoretical evidence accumulates, the hypotheses will remain nothing more than hypotheses. On the other hand, having the ability to modify natural sex selection processes, if they exist, would provide advantages to both livestock industries and mammalian conservation programmes, both good reasons to pursue these studies in addition to their academic interest.

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Table 1. Comparison of the main characteristics of studies of bovine follicular testosterone by Grant et al. (2008) and Garcia-Herreros et al. (2010).

<table>
<thead>
<tr>
<th>Study</th>
<th>N (embryos)</th>
<th>Follicular Size: Min-Max (mm)</th>
<th>Follicular Testosterone: Min-Max (nM)</th>
<th>Median Follicular Testosterone of Subsequent Embryos (nM)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grant et al. (2008)</td>
<td>171</td>
<td>4 – 10</td>
<td>11 – 977</td>
<td>Male 122.60 Female 90.75</td>
<td>0.032&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Garcia-Herreros et al. (2010)</td>
<td>140</td>
<td>2 – 12</td>
<td>13 – 893</td>
<td>Male 111.36 Female 83.13</td>
<td>0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes
- Two-tailed. Grant <i>et al.</i> reported a one-tailed value because a directional hypothesis was tested.
- Two-tailed. The <i>p</i>-value was reported for the Wilcoxon signed rank test, which is for related samples. We assume that the Wilcoxon rank sum test, which is for independent samples, was intended. (See Garcia-Herreros <i>et al.</i>, 2010, p. 535).