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Sex Ratios Provide Evidence for Monozygotic Twinning in the Ring-Tailed Lemur, *Lemur catta*

John St Clair,[1] Roisin Campbell-Palmer,[2] and Richard Lathe[1,3]
[1]*Pieta Research, Edinburgh, UK*
[2]*Animals, Conservation Programmes, and Education Department, Royal Zoological Society of Scotland, Edinburgh, UK*
[3]*State University of Pushchino and Institute of Bio-Organic Chemistry, Pushchino, Moscow Region, Russian Federation*

Monozygotic (MZ) twinning is generally considered to be rare in species other than human. We inspected sex ratios in European zoo-bred ring-tailed lemurs (*Lemur catta*), revealing a significant excess of same-sex twins. Of 94 pairs, 60 (64%) were either both males or both females ($p = .004$). Application of the Weinberg differential rule argues that 27% of all twins in this species are MZ pairs. In this protected species, where twinning is commonplace (~50% of newborns are twins), the probable existence of frequent MZ twinning has ramifications for breeding programs aimed to maximize genetic diversity, and suggests that twin studies in a species other than human could have potential as a medical research tool.

Keywords: conservation, *Lemur catta*, monozygotic, primate, seasonality, twinning

Classical twins studies, as originally pioneered by Galton (1875), are employed as a powerful tool in medicine for identifying the contributions of environment and genetics to human disease. They can also cast light on both nutrition and reproductive and population dynamics in humans. These are comparative studies and depend on the comparison between monozygotic (MZ) twins, who traditionally owe their sameness to sharing an identical genome, versus non-identical dizygotic (DZ) twins, which have elevated divergence because they harbor stochastic genomic contributions from each of their parents. However, the classical paradigm may not be completely secure. It assumes only two exclusive and exhaustive categories of twins: either non-identical (DZ) twins, who arise from independently fertilized eggs and are genetically no different from ordinary siblings, or 'identical' (MZ) twins, who arise from one egg fertilized by one sperm, which after the fertilized egg has split generates two genetically identical siblings. Observations that fail to fit within the classical MZ/DZ paradigm include the discovery of 'semi-identical' twins apparently genetically intermediate between MZ and DZ (Golubovsky, 2006; Souter et al., 2007) and 'genome-identical' human twins so dissimilar in construct as to be divergent for sex (Kurosawa et al., 1992; Perlman et al., 1990; Reindollar et al., 1987; Wachtel et al., 2000). The inheritance pattern of twinning in humans may also be more complicated than traditionally assumed because evidence has emerged of a direct paternal genetic factor in the production of both MZ and DZ twins (St Clair & Golubovsky, 2002).

A further problem with twin studies is that MZ twins are generally accepted to be the unique province of the human species, with rare exceptions, which means that classical twin studies are effectively confined to studies of humans, and a range of experiment-based research is thereby excluded. Although common in human, where there are approximately 3.8 twin births per 1,000 births (Levitan & Montagu, 1971), and of these ~39% are MZ pairs (Antoniou et al., 2011), MZ twinning is generally assumed to be rare in other species — with the exception of the nine-banded armadillo *Dasypus novemcinctus*, a South/Central American mammal, in which DNA typing has demonstrated that polyembryonic MZ twinning is the standard mode of reproduction in this species (Loughry et al., 1998; Prodöhl et al., 1996). Despite thorough investigation, identical twins are extremely rare in mice (McLaren et al., 1995), rabbits (Bomsel-Helmreich & Papiernik-Berkhauer, 1976), and livestock (Baker, 1964; del Rio et al., 2006). In primates, with the exception of humans, identical twins are generally restricted to the outcomes of manipulated reproduction such as in rhesus monkeys (Mitalipov et al., 2002). However, MZ twins are not entirely unknown in other primate

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ADDRESS FOR CORRESPONDENCE: Richard Lathe, Pieta Research, PO Box 27068, Edinburgh EH10 5YW, UK. E-mail: rlathe@pieta-research.org

TABLE 1

Twinning Frequencies in Zoo-Bred *Lemur catta*

Center	Total births (individuals)	Singletons	Triplets (individuals)	Twins (individuals)	Total pairs	Twins of different sex (pairs)	Twins of same sex (pairs)
Edinburgh	74	21	9	44	22	6	16
Blackpool	88	41	3	44	22	6	16
Chester	177	88	3	86	43	17	26
Emmen	35	21	0	14	7	5	2
Total	374	171	15	188	94	34	60

species; for example, one identical twin pair in chimpanzees has been described (Zhang et al., 2000) and a MZ twinning rate of 0.43% versus 2.36% for DZ pairs has been estimated in this species (Ely et al., 2006).

We report results of investigations on an endangered primate species, the ring-tailed lemur, a member of the *Lemuridea* family of true lemurs, to suggest that MZ twins may not be a rare or isolated occurrence in this species. *Lemur catta* is indigenous to the island of Madagascar, but colonies have been more widely maintained for decades in zoological institutes. In this species, twinning is commonplace and up to one-half of newborns are twins. For example, in one small survey 22/60 (37%) of *Lemur catta* offspring were members of twin pairs (Pasztor & Van Horn, 1976), and in the present analysis 50.4% of live births were members of twin pairs. It has generally been assumed that the majority (if not all) of lemur twin pairs are DZ. However, if MZ twins were found to exist on a significant scale, despite the caveats raised above as to the robustness of the twinning paradigm, this could have major ramifications for breeding programs designed to maximize genetic diversity, and potentially for medical research studies based on a species of primate in addition to humans.

To evaluate the possibility of MZ twinning in this species, we analyzed birth records for ring-tailed lemurs from multiple zoological centers in Europe for frequencies of same-sex twins. We present statistical evidence for an elevated proportion of same-sex twins that is indicative of a high frequency of MZ twinning in this species.

Methods

Ethical Approval

The analysis of twinning in *Lemur catta* was granted ethical approval (2011) from the Taxon Advisory Group (UK) and British and Irish Association of Zoos and Aquariums (BIAZA; http://www.biaza.org.uk/), an associate member of the European Association of Zoos and Aquaria (EAZA), the umbrella organization for European Endangered Species Programmes (EEPs).

Methods

Taxon data from all centers over the period 1988–2006 were kindly made available by the curators of different institutions. Data for individuals were excluded from specific twin analysis if any of the following conditions applied: (1) recorded birth dates of twins were greater than two days apart; (2) twin pairs where one or more individuals were of unknown sex; (3) twin pairs where evident recording errors were noted (e.g., discordance for recorded parental identities between the twin progeny); (4) incomplete or contradictory records; and (5) stillbirths. A total of 374 live births were analyzed. For specific statistical analysis of twinning, births of triplets were excluded. For triplets, in one set of multiplets born at Edinburgh, only two of three siblings survived and were recorded as twins. The triplet has been recorded as such (but not the stillborn individual), producing an $n = 1$ discrepancy (of 374) in summary data.

Results

To estimate the prevalence of identical twins, we evaluated sex ratios in all twin pairs. If all twins are non-identical (DZ), one-half of all twin pairs are inferred to be discordant in sex. Because identical twins are generally of the same sex, a surplus of same-sex twins would indicate that a significant proportion of live births are identical (Weinberg, 1901).

Edinburgh (UK) Twins

We analyzed *Lemur catta* records for the colony at Edinburgh, pertaining to 74 animals (including triplets) born over the period 1988–2006. Twinning was common (44 twin births vs. 21 singleton births). Of birth pairs, 16 were of the same sex and six were discordant (Table 1). The excess of pairs concordant for sex provided the first evidence for identical twinning in this species. Three sets of triplets were also recorded; all three sets were all-male, but the small sample size precluded statistical analysis.

Blackpool (UK), Chester (UK), and Emmen (the Netherlands)

We then sought records from other centers. The *Ring-Tailed Lemur Studbook* (Baistrocchi, 2005) was first consulted, but was found to be incomplete. We instead approached the three centers with the most extensive listings in the Studbook. Taxon records were analyzed for twins, again showing an excess of sex-concordant twins (Table 1).

Pooled Data from Four Centers

Combined data from all centers provided clear evidence for an excess of same-sex twins, with 34 discordant and 60

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TABLE 2

Gender Frequencies in Singletons Versus Twins

	Total births (individuals; singletons plus twins)*			Total twin births (individuals)		
Center	M	F	M/F (M%)	M	F	M/F (M%)
Edinburgh	43	31	58.9	20	24	45.45
Blackpool	55	33	62.50	32	12	72.72
Chester	89	88	50.28	37	49	43.02
Emmen	9	27	25.71	5	9	35.7
All	195	179	54.50	94	94	50.00

Note: *Triplets were excluded from this analysis.

TABLE 3

Estimated Proportions of MZ Versus DZ Twinning in *Lemur catta* According to Weinberg's Differential Rule

	Twin pairs	*N*	%
Observed	Total number of pairs	94	100
Observed	Different-sex pairs (inferred DZ)	34	36.2*
Inferred	Same-sex pairs (DZ)	34	36.2*
Observed	Same-sex pairs (MZ + DZ)	60	63.8
Inferred	Excess (60 - 34) of same-sex (inferred MZ) pairs	26	27.6*

Note: *Total inferred DZ twins, 72.4%, versus total inferred MZ twins, 27.6%.

TABLE 4

Seasonal Breeding: Mean *Lemur catta* Birth Dates

	All progeny		Twins	
Blackpool	Mean	82.20*	Mean	87.78
	SD	22.28	*SD*	28.62
Chester	Mean	82.72	Mean	82.78
	SD	24.97	*SD*	24.47
Edinburgh	Mean	87.82	Mean	86.82
	SD	25.72	*SD*	24.39
Emmen	Mean	86.42	Mean	67.43
	SD	58.62	*SD*	77.03
All centers	Mean**	84.23	Mean	83.78
	SD	30.10	*SD*	32.41

Note: *Birth dates given by yearday numbers.
SD: Standard deviation.
**Yearday 84 is March 25 (*SD* = 54–114: Feb 23–April 24).

concordant pairs (Table 1). Statistical analysis of the twins indicated that the likelihood such a result would occur by chance was $p = .004$. Across the entire dataset there was a small excess of male progeny (54.5%; Table 2); when considered separately the sex ratio in twins was balanced (50.0%), indicating that bias in sex ratio does not contribute to the excess of same-sex twins. Overall, the total number of same-sex male twins (60 individuals) was equal to the number of same-sex female twins (60 individuals), arguing that same-sex twinning is not gender-specific.

The inferred rate of identical twinning in this species, as a fraction of twin pairs, may then be calculated from the excess of same-sex twins to be approximately one-third (27.6%; Table 3).

Seasonal Breeding and Discordant Birth Dates

Analysis of the combined dataset confirmed highly seasonal breeding in *Lemur catta*. As shown in Table 4, births clustered around a midline date of March 25 (yearday 84) with *SD* = 54–114 for yeardays (February 23–April 24). There was no difference in the mean dates of singleton versus twin births (Table 4). Furthermore, the data indicated that a small number of birth dates were discordant between siblings (>2 days). In Edinburgh and Emmen all twins (and triplets) were recorded to have been born on the same day. In Chester, three pairs had discordant birth dates, with two females, two males, and two females being separated by respectively 5 days, 10 days, and 2 months. Birth date discordance was also observed in Blackpool (UK), where in a tetraplet of mixed sex one animal was born in March and three others were born in May (2 months discordance). A similar discordance was observed at this center for a triplet, where two females were born in early March and a third female was born in early June (3 months discordance). Finally, in one very exceptional pair of same sex (female), the first was born in mid-March and the second birth was delayed until mid-August (5 months discordance). In all these cases it is unknown whether the offspring resulted from a single mating and conception. Although excluded from the present analysis of MZ/DZ twin frequencies, the existence of twin pairs with widely discordant birth dates is notable.

Discussion

The primary indicator of MZ twinning is given by concordant versus discordant sex in twin births, according to Weinberg's differential rule (Weinberg, 1901). This rule has been challenged (Boklage, 1985; James, 1992), for example, by the finding that pregnancy loss in humans may be higher in same-sex compared with different-sex pairs (Rydhstrom, 1994) and that there could be an excess of like-sexed versus unlike-sexed pairs among DZ twins (James, 1971; Machin et al., 1995). However, these effects are not large, and recent reanalysis has pointed to the robustness and reliability of Weinberg's differential rule (Fellman & Eriksson, 2006).

Applying the rule to *Lemur catta* at four European centers, we have obtained evidence for MZ twinning in this species; specifically, a marked excess of same-sex twins. The excess achieved high statistical significance ($p = .004$). Same-sex twins were equally male or female, arguing that same-sex twinning is not gender-specific. At face value, the data suggest that approximately 28% of same-sex twins in this species are MZ.

In *Lemur catta*, an endangered species, twin births are prevalent. However, to date it has been assumed that the majority (if not all) of lemur twin pairs are DZ. The evidence reported here suggests that MZ twinning may be commonplace in this species (Figure 1).

DNA Typing Is Constrained by Legislation

A central objective of this project has been to obtain formal confirmation of MZ twinning by the use of DNA typing. However, procurement of tissue samples (blood, hairpluck)

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FIGURE 1

(Colour online) Same-sex *Lemur catta* female twins with suggestive similarity of facial features (inset). There is as yet no formal proof of MZ twinning because sampling appropriate for DNA typing in this species is constrained by legislation. (Note: Photograph kindly supplied by Donald Gow [RZSS, Edinburgh], with permission)

is subject to legislative control in this protected species, and sample collection has proved intractable. Therefore, in the absence of DNA typing analysis our conclusion regarding MZ twinning frequencies must be considered provisional.

Are Zoo-Bred Animals Representative of the Wild?

A further concern is that MZ twinning may not be representative of the species as a whole. It is possible that a degree of genetic homogeneity might have emerged among zoo-bred *Lemur catta* as a result of resource sharing between European zoological institutions. Studies on zoo-bred and potentially wild populations further afield will be necessary to determine whether the generation of MZ twins is a general feature of *Lemur catta* and, potentially, of closely related species.

Conservation and Seasonality

In the absence of other mechanisms capable of explaining the excess of same-sex twins, we surmise that MZ twinning is the most likely explanation. These data therefore suggest that MZ twinning is commonplace in this species; this finding urges caution in the selection of individuals in breeding programs designed to maximize genetic diversity. Conservation breeding programs at global and regional levels involve the selection of breeding pairs with the least relationship, based on minimizing mean kinship (Ballou & Lacy, 2013). Kinship values for siblings do not account for MZ twinning, potentially leading to a significant underestimate of relatedness among particular individuals throughout a breeding program where MZ twinning is prevalent.

Our data also confirm the well-known seasonality of reproduction in *Lemur catta*. In the present study the mean birth date was on yearday 84 (March 25) +/- *SD* of ~1 month. We also report several cases where individual offspring have been born to the same *Lemur catta* mother on different dates. Although generally under 10 days, cases were noted where single mothers produced offspring that were respectively 2 (two cases), 3, and 5 months discordant in their birth dates. It is not known whether this phenomenon results from developmental delay in one embryo, perhaps amplified by the bicornuate nature of the uterus in Lemur, or whether it might reflect multiple matings/ovulations in this species. This contrasts with the situation in human, where, although heteropaternal superfecundation is not entirely unknown, twin birth dates are predominantly concordant.

In conclusion, the present finding challenges the monopoly of *Homo* for frequent MZ twinning in a primate species. Excluding rare triplets, the distribution of progeny in *Lemur catta* is calculated to be 48% singleton, 38% DZ twin, and 14% MZ twin individuals. The frequency of inferred MZ twinning in this species, 27.6% of all twin pairs, is comparable with that observed in human, where in the East Flanders study ~39% of all twins were MZ (Antoniou et al., 2011).

The finding of probable MZ twinning in another primate species, *Lemur catta*, may cast light on aspects of primate evolution and on the contentious genetic mechanism that gives rise to identical twins.

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