

DO *ODOCOILEUS VIRGINIANUS* MALES PRODUCE Y-CHROMOSOME-BIASED EJACULATES? IMPLICATIONS FOR ADAPTIVE SEX RATIO THEORIES

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The theory of adaptive sex ratio variation assumes that parents can increase their fitness by investing in the offspring sex that will gain the greatest lifetime reproductive success. Many adaptive sex ratio hypotheses imply or predict a female-mediated physiological mechanism that facilitates parental control. However, the ability of females to control offspring sex ratio could be significantly affected if males provided X- or Y-chromosome-biased ejaculates, as was recently discovered in domestic artiodactyls. To determine whether this occurs in white-tailed deer (*Odocoileus virginianus*), we examined semen sex ratio of captive deer using DNA fluorescence flow cytometry. We also used microsatellite markers to assign paternity of fawns sired in previous years to determine if temporal patterns existed in fawn sex relative to conception date. The ratio of X- and Y-sperm did not differ from an expected 1:1 among deer or between collection periods. Median conception dates of male and female fawns sired in previous years were similar, and there were no apparent temporal trends in offspring sex. We find no evidence that skewed ejaculates occur in white-tailed deer.

Key words: adaptive, Cervidae, DNA, flow cytometry, *Odocoileus virginianus*, reproductive physiology, sex allocation, sex ratio, sperm, white-tailed deer

It has long been recognized that offspring sex ratios in vertebrate populations may deviate from a 1:1 male:female (M:F) ratio at birth (Clutton-Brock and Iason 1986). This variation in offspring sex ratios has drawn considerable interest from vertebrate ecologists because of the possible existence of parental strategies for sex ratio allocation. For example, if offspring fitness differed by sex under different environmental or demographic conditions, then 1 or both parents could gain increased fitness by investing in offspring of that sex. Many hypotheses have been proposed to predict situations where offspring sex ratio should change if parents possess the ability to control offspring sex ratios (Clark 1978; Fisher 1930; Hamilton

1967; Hiraewa-Hasegawa 1993; Silk 1983; Trivers and Willard 1973; Wright et al. 1995). Ungulates are commonly used to test these hypotheses about adaptive sex ratio allocation because of their typically polygynous mating systems, high variation in male reproductive success, and sexually dimorphic body size and growth patterns (Kojola 1997). Despite much research on the subject, however, the conclusions of many studies are contradictory, and the adaptive nature of offspring sex ratios remains controversial both within and across ungulate species (Burke and Birch 1995; Caley and Nudds 1987; DeGayner and Jordan 1987; Hewison and Gaillard 1996; Hewison et al. 1999; Kojola 1997; Verme 1983; Wauters et al. 1995).

The interpretation of some adaptive sex ratio allocation studies may be complicated by extrinsic sources of variation. Long-term studies on birds and mammals have concluded that sex ratios are influenced by multiple factors (Cockburn et al. 2002). Recent evidence suggests that climate (Post et al. 1999) and population density (Kruuk et al. 1999) may affect offspring

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sex ratio independently from hypothesized parental strategies. Factors such as these may partially account for the discrepant support for competing sex ratio allocation hypotheses reported within and among ungulate species. Lack of consensus regarding a physiological mechanism that could allow adaptive parental control of offspring sex further complicates hypothesis testing. Researchers have presented several plausible physiological mechanisms for offspring sex ratio control, but no concrete evidence for any specific mechanism exists (see Hardy 1997 for a detailed review). Most proposed mechanisms for adaptive control of offspring sex are assumed to be defined by the female (Hardy 1997). Increasing offspring fitness, however, would be adaptive for both parents (McCullough 1979), so a physiological mechanism to influence or control offspring sex could be present in either or both sexes.

A male influence on offspring sex through variation of ejaculate composition is possible since males are the heterogametic sex in mammals. Proposed female physiological mechanisms for controlling offspring sex involve the differential motility or passage of X- and Y-sperm in the reproductive tract or differential acceptance of X- or Y-sperm by the ova under certain conditions (Hardy 1997). The ability of a female to control offspring sex could be severely limited (or greatly enhanced) if males provided X- or Y-biased ejaculates.

The amount of X- and Y-sperm in domestic cattle and swine ejaculates may vary considerably between individuals and collections (Chandler et al. 1998, 2002). The human male population as a whole produces X- and Y-sperm at about a 1:1 ratio, but individual male Y-sperm percentages may vary widely (27–68%—Beckett et al. 1989; 42–57%—Lobel et al. 1993). Therefore, since sex chromosome composition varies among ejaculates and individuals in some species, the ability of females to control offspring sex could be affected. In addition, these changes in ejaculate sex ratio may indicate a previously unsuspected male influence in adaptive sex ratio control.

The investigation of alternative strategies for offspring sex ratio allocation has long been limited by the lack of technology that could reliably measure parameters of interest. Recent development and refinement of techniques such as flow cytometry (Johnson and Welch 1999) allow separation and quantification of X- and Y-sperm and can address variations in primary (at fertilization) sex ratio. In addition, microsatellite DNA markers enable parentage determination with a high degree of accuracy (Anderson et al. 2002), providing an opportunity for assessment of secondary (at birth) sex ratios.

We investigated the potential of the sire to influence offspring sex ratio in a captive group of white-tailed deer (*Odocoileus virginianus*), a species that has been the subject of many sex ratio allocation studies. Specific objectives were to evaluate the feasibility of relative DNA content analysis for determining the ratio of X- to Y-chromosome sperm in white-tailed deer, to compare sex chromosome ratios of ejaculates among sexually mature (≥ 1.5 years) males at different time periods, and to investigate possible temporal trends in sex chromosome ratio by examining offspring sex in relation to conception date.

MATERIALS AND METHODS

Deer handling and sedation.—Captive white-tailed deer were housed at the Mississippi State University Captive Animal Facility. The facility consists of 6 pens (0.4–1.3 ha in size). Deer were allowed ad libitum access to water and feed consisting of a commercial pelleted ration. Deer density within pens varied but did not exceed 20 deer/ha. Nineteen sexually mature males (≥ 1.5 years) were available for semen collection in fall 2000. Fourteen males (≥ 2.5 years) were housed in a single all-male pen and sexually rested during the study period. The remaining 5 males were allocated to 2 breeding pens with 6–8 adult females and allowed to compete for mating opportunities.

Blood samples were taken from adult deer while either manually restrained or sedated. Sedation was achieved using a Telazol–xylazine mixture (4.4 mg/kg Telazol [Fort Dodge Animal Health, Fort Dodge, Iowa] plus 2.2 mg/kg xylazine hydrochloride [Phoenix Scientific, St. Joseph, Missouri]), antagonized with 0.125 mg/kg yohimbine hydrochloride [Abbott Laboratories, North Chicago, Illinois]) delivered by cartridge-fired dart (Pneu-Dart Inc., Williamsport, Pennsylvania) or pole syringe. Blood (3–5 ml) was obtained by venipuncture and stored at 4°C in vacuum tubes containing EDTA (Vacutainer, Becton-Dickson and Company, Franklin Lakes, New Jersey). Fawns were manually restrained within 1–3 days postpartum. Circular plugs of ear tissue (0.5 cm in diameter) were taken and preserved in 70% ethanol. Birth dates of fawns were recorded, and conception dates were estimated by subtracting 200 days (mean of reported white-tailed deer gestation periods—Adams 1960; Haugen 1959; Haugen and Davenport 1950; Verme 1965).

Semen collection and analysis.—Semen was collected from sedated males using an electroejaculator (Pulsator IV, Lane Manufacturing Inc., Denver, Colorado) and a 3-pronged ram probe according to the methods of Jacobson et al. (1989). The voltage delivered by the device was adjusted until ejaculation was obtained (ejaculates occurred between 75 and 200 mV). Ejaculates were transferred to 1.5-ml cryogenic vials (Nalge Company, Rochester, New York), stored on ice, and analyzed within 48 h. Two collections were performed: during 16–17 October 2000, after antler hardening but prior to breeding, and during 5 February 2001, after peak breeding and prior to antler casting.

Percentage of X- and Y-sperm in ejaculates was determined by DNA fluorescence flow cytometry, where sperm are separated by relative difference in DNA content (Johnson and Welch 1999). Sperm concentration of each ejaculate was determined using a spectrophotometer (Spermaque, Minitube, Verona, Wisconsin), adjusted to 150×10^6 /ml in 0.5 ml phosphobuffered saline. Sperm were sonicated to obtain sperm nuclei, centrifuged, and resuspended in phosphobuffered saline.

Sperm nuclei were then stained using bisbenzimidazole Hoechst 33342 (Calbiochem, La Jolla, California) at a final concentration of 80.1 μ M, incubated for 1 h at 35°C, and filtered to remove clumps. Sperm nuclei were then analyzed on a high-speed cell sorter (MoFlo, Cytomation Inc., Fort Collins, Colorado) modified for sperm sorting (Johnson and Pinkel 1986) for separation and detection. The cell sorter was equipped with a laser (Coherent Inova 307, Coherent Inc., Palo Alto, California) operating in the multiline UV spectrum (351, 364 nm) at a power output of 150 mW (Johnson and Welch 1999). The instrument sheath pressure was 50 psi, and event rate was about 2,000 sperm/s during analysis. Fluorescence emitted by sperm nuclei was collected from forward- and side-angle detectors (0° and 90°, respectively) and stored as 256-channel distributions (histograms). A minimum of 10,000 sperm from each ejaculate was recorded, and histograms for X- and Y-sperm were fitted to Gaussian distributions, whose means, coefficients of variation, and relative areas were adjusted to give the

best least-squares fit to the data. Percentage difference in DNA content of the 2 peaks was calculated using the equation

$$\text{difference} = 100 \left[\frac{(X - Y)}{0.5(X + Y)} \right],$$

where X and Y represent channel means for the peaks from X- and Y-sperm, respectively.

We tested each ejaculate for departure from an expected 50% X-sperm using a chi-square test for specified proportions implemented in SAS (Statistical Analysis System, SAS Institute Inc., Cary, North Carolina).

DNA isolation and amplification.—We isolated DNA from whole blood through either phenol–chloroform extraction or a commercial kit (Puregene DNA isolation kit; Gentra Systems Inc., Minneapolis, Minnesota). For the extraction protocol, we mixed 0.5–1 ml of the blood-EDTA mixture with 2× sucrose-Triton X buffer (0.32 M sucrose, 0.01 M Tris pH 7.6, 0.005 M MgCl₂, 1% Triton X-100) and centrifuged (2,000 × g for 20 min) for white cell collection. This procedure was repeated 2–3 times. White blood cells were then placed in 500 μl of Laird's buffer (Laird et al. 1991) and digested overnight with Proteinase K (20 mg/ml) at 37°C. We extracted DNA using an equal volume of phenol–chloroform–isoamyl alcohol, precipitated with 1/20 volume of 5 M NaCl and 2 volumes of 95% ethanol, then pelleted by centrifugation (14,000 × g for 5 min). Samples were vacuum-dried and redissolved in double-distilled H₂O or Tris-EDTA.

For ear tissue, a small (about 0.25-cm) section was excised, and sections were further reduced with a razor blade to increase surface area before tissue lysis. DNA was isolated using Qiagen minispin columns as recommended by the supplier (DNeasy Tissue Kit; QIAGEN Genomics Inc., Bothell, Washington), except tissue lysis was performed using 40 μl Proteinase K (20 mg/ml) and overnight incubation. DNA concentration was determined using a fluorometer and DNA standards.

Anderson et al. (2002) described a 21-locus microsatellite panel optimized for use in white-tailed deer, and a full description of the panel and reaction conditions is contained therein. We used 17 of the loci described by Anderson et al. (2002), omitting the BM203, BM415, ETH152, and OCAM loci. Fragments were amplified via polymerase chain reaction using fluorescent-tagged primers in single or multiplexed reactions on a PE Gene Amp 9600 thermocycler (Applied Biosystems Inc., Foster City, California). For each individual, the products from 3–4 polymerase chain reactions were mixed together (3 μl of each reaction), and 1 μl of this mixture was added to a denaturing formamide and size standard mix (Genescan *rox* 500; Applied Biosystems). The reaction product-denaturing mixes were loaded on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) for separation and detection. DNA fragments were quantified and analyzed with GeneScan software, and alleles were assigned using Genotyper software (both by Applied Biosystems) followed by visual verification.

Parentage assignment.—We assigned parentage for all fawns using the computer program CERVUS 2.0 (Marshall et al. 1998). The likelihood ratio methods implemented by the CERVUS software have demonstrated paternity assignment confidence consistent with simulated values (Slate et al. 2000). DeYoung et al. (2002) previously used this procedure with the Anderson et al. (2002) microsatellite panel to assign parentage in the Mississippi captive herd.

Temporal variation in offspring sex ratio.—Sperm sex ratio in domestic cattle varies with collection frequency and appears to fluctuate on a sinusoidal rhythm in bulls collected on a weekly or 3-week schedule (Chandler et al. 2002). We were unable to conduct frequent semen collections to empirically evaluate any temporal changes in

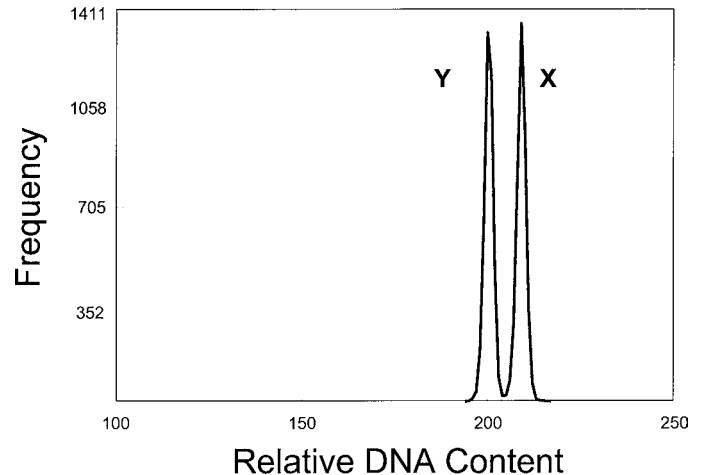


FIG. 1.—Separation and incidence of X- and Y-sperm in the semen sample of 1 white-tailed deer. For this particular animal, the ratio of X- and Y-sperm was 50:50, and the difference in DNA content (of X- and Y-bearing sperm) was 4.4%.

sperm sex ratio. To investigate the possibility of temporal patterns in ejaculate sex ratios, we compared offspring sex versus conception date. We collected blood or tissue samples from all fawns and their parents born in the Mississippi State University captive herd during 1997–2001. DNA isolation, microsatellite amplification, and parentage analyses were performed as described previously. Our analysis considered only those fawns sired by bucks that produced ≥9 fawns within a single year. Several captive males sired 1–6 offspring within a single year during this period, but we used an arbitrary lower limit of 9 fawns to achieve a balance between stochastic variation in offspring sex ratio among individual males and sample size. We assumed that these males (which produced the most offspring) had to copulate more frequently. If sperm sex ratios became skewed during the breeding period, a plot of offspring sex should reveal any important temporal changes. The number of male and female fawns produced was tallied at weekly intervals, and a Smirnov 2-sample procedure (Conover 1980) was used to test the equality of the distributions of male and female fawns throughout the breeding season.

RESULTS

We obtained 32 ejaculates from 19 individual captive males in the Mississippi State University facility. Ejaculates were collected from 18 males during 16–17 October 2000 and from 14 males during 5 February 2001. The mean difference in relative DNA content of X- and Y-sperm was 4.4% ($SE = 0.04$, range: 4.0–4.7). This difference in DNA content was sufficient to permit reliable high-speed separation and quantification of the 2 chromosome types (Fig. 1).

We observed a mean X:Y sperm ratio of 50.1:49.9 (range: 48:52–52:48; Table 1). Sperm sex ratio did not differ from an expected 50:50 ratio for any ejaculate ($\chi^2 = 0.04$ –0.16, $df = 1$, $P > 0.6$). Thus, there were no significant changes in sperm sex ratio among individual males or between collection periods. Three of the 5 males that were allocated to breeding pens were successful breeders, producing a slightly male-biased progeny group (13:11 M:F). Male breeding success was variable, with 1 male siring a slightly male-biased progeny

TABLE 1.—Sperm sex ratio (X:Y) of captive white-tailed deer at 2 collection periods, percentage difference between relative DNA content of X- and Y-sperm, and offspring sired by 5 males that had access to breeding opportunities.

Buck	Buck age	X:Y		Mean difference (%)	Offspring (M:F) ^a
		October	February		
1	7.5	50:50		4.5	
2	3.5	50:50	51:49	4.3	
3	3.5	50:50	52:48	4.2	
4	3.5	52:48	50:50	4.5	
5	3.5	50:50	49:51	4.5	2:2
6	2.5	50:50	48:52	4.1	
7	2.5	51:49	50:50	4.3	
8	2.5	49:51		4.4	11:8
9	2.5	50:50	51:49	4.5	
10	2.5	49:51	51:49	4.2	
11	2.5	49:51	51:49	4.4	
12	2.5	49:51	51:49	4.7	
13	2.5	49:51	50:50	4.5	
14	1.5	51:49	48:52	4.5	0:0
15	1.5	49:51		4.6	0:0
16	1.5	49:51		4.6	0:1
17	3.5	51:49		4.4	
18	3.5	51:49	52:48	4.3	
19	3.5		51:49	4.0	

^a All males were sexually rested at first collection. Only these 5 males had access to breeding opportunities prior to 2nd collection.

group (11:8 M:F), 1 producing an even sex ratio (2:2), and the other siring a single female fawn. The remaining 2 males did not produce offspring. Conception dates ranged from 14 November to 1 January, with median conception occurring on 11 December.

Fawn sex ratio in the captive facility was male biased during 1997–2001. Seven individual males sired ≥ 9 fawns in a single year (range: 9–18) and totaled 89 fawns (50:39 M:F). Median conception dates for male and female fawns were similar (M:16 December, F:11 December). Although more males were produced, the distributions of male and female fawns did not differ ($T = 0.255$, $d.f. = 10, 11$, $P > 0.2$; Fig. 2). Each sex was equally likely to be conceived at any time during the breeding season.

DISCUSSION

Flow cytometry exploits relative differences in DNA content between X- and Y-sperm for separation. This difference varies among species (e.g., humans: 2.8%; chinchilla [*Chinchilla lanigera*]: 7.5%—Johnson 1995) and affects the efficiency with which sperm may be sorted by sex chromosome. Since an X-Y difference of $\geq 3\%$ is preferred for the sorting to be reliable (Johnson and Welch 1999), we first had to determine if the difference was sufficient to accomplish separation in deer. Relative differences in DNA content between X- and Y-sperm we observed in white-tailed deer (4.4%) permitted efficient, high-speed separation using flow cytometry. Therefore, this technique could be used for separation of live sperm intended for artificial insemination. We are not aware of any previous applications of flow cytometry to wildlife research, but the technique could be a powerful tool for investigating mating strategies.

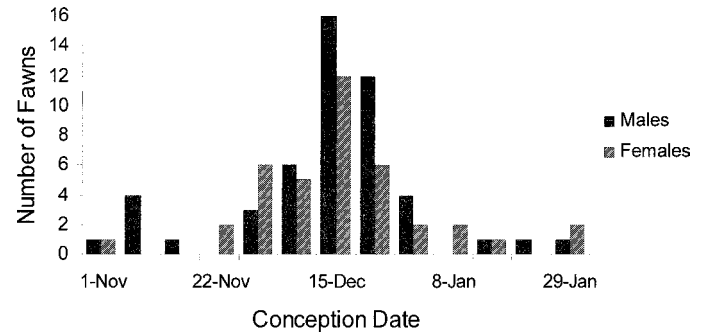


FIG. 2.—Offspring sex ratio plotted versus conception date for 89 fawns (50 males, 39 females) sired by 7 white-tailed deer males in the Mississippi State University Captive Facility, 1997–2001. Offspring sex is plotted at weekly intervals. The distributions of male and female fawns are not significantly different ($P > 0.2$).

Flow cytometry also may prove to be a useful tool to facilitate the management of endangered populations. A number of recent advances in the control of mammalian reproduction enable biologists to manipulate the amount of genetic diversity in small or endangered populations (Santiago and Caballero 2000). These techniques do not address progeny sex ratio, however, which is especially important in small populations where stochastic variation may result in a decreased effective population size or an inefficient investment of reproductive effort. Thus, the ability to sort sperm by sex chromosome also has implications for wildlife conservation programs where preselection of offspring sex would be advantageous.

We found no differences in sperm sex ratio among individuals or collection periods; all males essentially had a 1:1 ratio. In contrast, sperm sex ratio of sexually rested bulls varied widely (17–71% Y-chromosome sperm—Chandler et al. 2002). Our sample cannot be considered large, but we examined more individuals than in the livestock study (deer: $n = 19$; cattle: $n = 9$ —Chandler et al. 2002). In addition, differences in sperm sex ratio among males in other species were relatively easy to document (Beckett et al. 1989; Chandler et al. 1998, 2002; Lobel et al. 1993; Rorie 1999). The lack of variation in sperm sex ratio of deer among collections or individuals is a good indication that sperm sex ratios are much less variable in deer than in some other species. We were unable to obtain ejaculates for 3 of the 5 males in the breeding pens during the 2nd collection period because of logistical constraints. The progeny sex ratios produced by the 3 successful males did not greatly deviate from 1:1, however, suggesting that sperm sex ratios also remained near 1:1.

We could not achieve the collection frequency of Chandler et al. (2002) with white-tailed deer. This prevented us from rigorously investigating the possibility of rhythms in deer sperm sex chromosome content that were detected in cattle (Chandler et al. 2002). Unlike Chandler et al. (2002), however, there was essentially no variation in sperm sex ratio among individuals or collections. Chandler et al. (1998) observed skewed sex ratios in litters of calves and piglets conceived via artificial insemination using semen straws constructed from single ejaculates. This suggests that biased ejaculates produce

biased progeny sex ratios. Thus, we would expect to observe differences in the sex of offspring during the breeding season if there were any important temporal fluctuations in sperm sex ratio. We found no tendency for males to sire a greater proportion of either sex at any point in the breeding season. This method for detecting temporal variation is indirect and could be affected by female-mediated mechanisms. However, the advantages of conducting this type of study in captive conditions are that all females could be maintained on identical rations, randomly assigned to pens, and held at similar densities. Therefore, we could minimize the effects of nutritional and social factors known to affect progeny sex ratios in captive deer (Verme 1983).

Why does sperm sex ratio fluctuate in cattle and swine but not in deer? Chandler et al. (2002) speculated that the rhythmic variation in bovine sperm sex ratio was due to testicular or epididymal physiology or anatomy. Since there was no evidence for any changes in deer sperm sex ratio, there may be some anatomical or physiological differences between deer and cattle that do not permit fluctuations of sperm sex ratio in deer. We cannot completely rule out the possibility that the captive conditions may have influenced our results and that differences in ejaculate composition among white-tail males may occur under other conditions. However, this seems unlikely considering that the largest differences from a 1:1 sperm sex ratio in bulls were seen in sexually rested animals (Chandler et al. 2002). We found no differences in sperm sex ratio in the sexually rested deer in 2 separate collections. If sperm sex ratios vary among male white-tails, one would expect to detect this using our methods.

Although there is no evidence for a male influence on offspring sex ratio control in white-tails, percent Y-sperm differs between ejaculates in cattle, swine, and humans (Beckett et al. 1989; Chandler et al. 1998, 2002; Lobel et al. 1993; Rorie 1999). These findings raise the possibility that male strategies exist for influencing offspring sex ratio in some species. It is interesting to note that cattle and swine, species where sperm sex ratio fluctuates, have large testicles relative to body size, which is considered to be an adaptation for sperm competition (Kenagy and Trombulak 1986). It is important, however, to emphasize that there is no evidence that the variations in sperm sex ratio observed in these studies could be adaptive in nature. Rather, differences in sperm sex ratio in these species may simply be a by-product of their reproductive anatomy or physiology that permits physical separation to occur. This is supported by the observation that sperm sex ratio underwent regular sinusoidal fluctuations when cattle were collected frequently (Chandler et al. 2002). If the changes in sperm sex ratio were adaptive, responding to behavioral factors such as copulation frequency, one would expect a unidirectional shift in sperm sex ratio.

Biased ejaculates seem to be an unlikely source of extrinsic variation for sex ratio allocation studies in white-tailed deer. The fact remains that the interpretations of offspring sex ratios in white-tailed deer are often contradictory, providing support for competing hypotheses (Burke and Birch 1995; Caley and Nudds 1987; DeGayner and Jordan 1987; Verme 1983; Verme and Ozoga 1981). Thus, the ability of white-tailed deer to exert

adaptive control of offspring sex ratio or the conditions under which they do so remain uncertain. Designed experiments are needed both to elucidate the physiological mechanisms that cause variation in offspring sex ratio in deer and to confirm or refute its adaptive nature. Many sex ratio allocation studies are conducted on free-ranging animals where reliable assessments of body condition, dominance, and so on are difficult to achieve. Captive studies in controlled environments are lacking and may prove informative for testing hypotheses related to sex ratio allocation theories. It seems likely that adaptive sex ratio control in white-tailed deer, if indeed this occurs, is due to changes in female reproductive physiology.

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