SEASONAL CHANGES IN THE PHYSIOLOGY OF MALE VIRGINIA OPOSSUMS (*DIDELPHIS VIRGINIANA*): SIGNS OF THE DASYURID SEMELPARITY SYNDROME?

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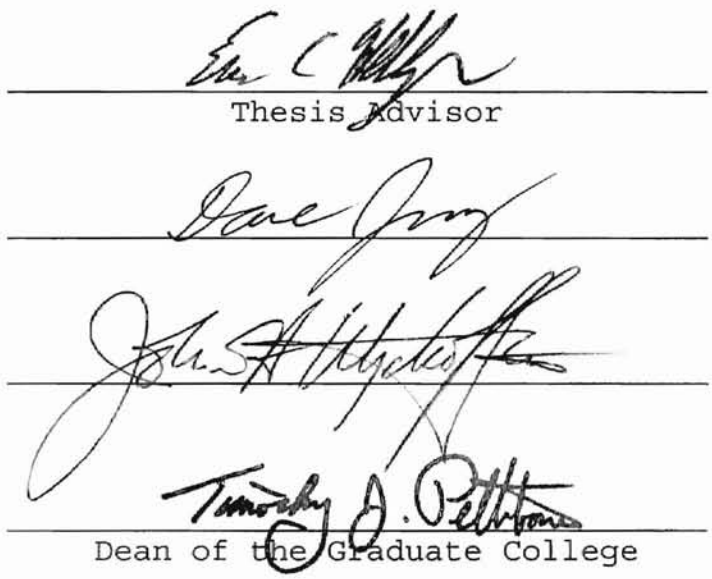

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INTRODUCTION

Investigations into explanations of population dynamics of small mammals have occurred for over 100 years (Macfadyen 1974). Since initiation of this research, many hypotheses have been proposed to explain the cyclic dynamics of many small mammal populations. The basis for these hypotheses included food (Lack 1954), predation (Pearson 1971), behavior (Watson and Moss 1970), and genetics (Chitty 1960). Christian (1950) broached a physiological-based hypothesis. He proposed that mortality and the resulting decline in high-density populations was the result of adrenal exhaustion brought on by increased social stimuli. Christian's hypothesis has had little support (Krebs and Myers 1974), but was an impetus to explain annual mortality in small mammals as founded on physiological alterations.

Semelparity among marsupials: physiological characteristics and demographic consequences.—Semelparity, defined by Cole (1954) as "multiplying once in a lifetime," is a reproductive strategy rarely present in mammals but found in plants, insects, and fish. Semelparity is found in animals that face low or variable probabilities of survival as adults (Roff 1992; Stearns 1992) and/or if juvenile survival is consistently higher in one season

al. 1981; Watt 1997; Wood 1970). This unique population syndrome does not include females, which are a synchronized monoestrous group and can survive to breed a second year (Wood 1970; Woolley 1966). Thus, post-mating mortality of males leaves only adult females and their young in the population during winter and early spring (Braithwaite 1979; Wood 1970; Woolley 1966).

Research into the cause of male mortality in these dasyurids revealed a sequence of events initiated with an increased concentration of plasma testosterone at the beginning of the mating season (Table 1). The testosterone increase is possibly stimulated by pheromones in male urine (Bradley et al. 1980; Millis et al. 1999; Toftegaard et al. 2002). As the concentration of testosterone rises, an unknown threshold is reached that results in the reduced concentration of corticosteroid-binding globulin (CBG; Bradley et al. 1976; McDonald et al. 1981). Due to the loss of CBG and impaired negative feedback of the pituitary-adrenal axis (Bradley 1990a; McDonald et al. 1986), free corticosteroid concentrations rise. Because cortisol is the dominant corticosteroid in marsupials, its concentration increases (Barnett 1973; Johnston et al. 1967).

Cortisol: physiological functions and effects.— Cortisol is a glucocorticoid produced by the adrenal cortex that is important in dealing with stressors. Results of stimulation from cortisol include increased blood glucose levels through the process of gluconeogenesis, inhibition of glucose uptake, and lipolysis (Sherwood 2001). Cortisol also suppresses the immune system, leading to lympholysis, reduced phagocytic and killing ability of macrophages and neutrophils, and reduction in spleen mass (Goldsby et al. 2000; Klein et al. 1996). If chronic supraphysiological levels of cortisol persist, other effects can include decreased tissue-repairing mechanisms, gastric ulceration, hypertension, and atherosclerosis (Sherwood 2001).

Physiological and morphological changes consistent with increased cortisol concentrations in semelparous, male dasyurids include enlarged adrenal glands (Barnett 1973), reduced spleen mass (Bradley 1987), reduced body mass, and negative nitrogen balance (Barnett 1973; Inns 1976; Wood 1970; Woollard 1971; Woolley 1966; Table 1). Changes in blood chemistry of semelparous, male dasyurids associated with increased cortisol included reduction in serum immunoglobulins (Bradley et al. 1980), lymphocytopenia and neutrophilia (Bradley 1990b; Bradley and Monamy 1990; Cheal et al. 1976), and decreased hematocrit (Cheal et al. 1976;

Table 1). Recently, similar physiological (e.g., cortisol concentrations, decreased hematocrit and decreased maximum corticosteroid binding capacity) and demographic changes (e.g., highly skewed post-mating sex ratio in favor of females) have been reported in male arctic ground squirrels (*Spermophilus parryii plesius*; Boonstra et al. 2001).

A suppressed immune system is extremely susceptible to infection. For example, gastric helminths can take advantage of the weakened immune system by evading a weak immune response and colonize the stomach (Cheng 1986; Noble and Noble 1982). Parasitic worms usually do not cause visible changes in the host when the worm burdens are low; however, when the worm burden is quite large (depending on the species of worm), physiological, mechanical, and tissue changes can occur (Matthews 1998). Physiological disturbances of helminth infection include inhibition of nutrition uptake, anorexia, and anemia (Matthews 1998). Mechanical damage can consist of intestinal villous atrophy and duct or lumen blockage (Matthews 1998).

Observations during postmortem examination of male *Antechinus* revealed increased endo- and ectoparasites, gastric ulcer hemorrhaging, and anemia (Table 1; Barker et al. 1978; Bradley et al. 1980; Dickman and Braithwaite 1992; Oakwood et al. 2001). These symptoms could be

replicated with injections of cortisol acetate (Bradley et al. 1980). These postmortem characteristics have been recorded in many species in the Dasyuridae family in which there is complete male mortality (Bradley 1987; Bradley and Monamy 1990; Braithwaite and Lee 1979; Dickman and Braithwaite 1992; Inns 1976; Wilson and Bourne 1984; Woolley 1981).

Life history and physiology of the Virginia opossum.— The Virginia opossum (*Didelphis virginiana;* Didelphidae), the only marsupial in North America, is distributed throughout most of the United States east of Colorado and some small areas along the west coast (Gardner 1982; Seidensticker et al. 1987). Cockburn (1997) described Virginia opossums as semelparous because they do not live much longer than 2 years; therefore, males usually only participate in one breeding season. During the breeding season, males can lose up to 23% of their average 2-kg body mass (Gardner 1982; Ryser 1992).

Virginia opossums are separated from members of the Dasyuridae family by millions of years. Didelphids originated in South America approximately 7.5×10^7 years ago, whereas the Dasyuridae family did not originate in Australia until approximately 4.5×10^7 years ago (Austad 1988). Despite the evolutionary time between origins, the

Virginia opossum and the above-mentioned dasyurids share the life-history trait of a short lifespan (Gardner 1982).

Almost every individual within a Virginia opossum population will die within 1 year; many investigators have reported annual mortality of opossums to be 90-100% (Austad 1993; Gehrt et al. 1997; Gillette 1980; Lay 1942; Llewellyn and Dale 1964; Petrides 1949; Seidensticker et al. 1987; Verts 1963). Populations tend to reach their minimum number during winter and early spring, consisting only of adults, whereas population peaks occur in autumn with inclusion of newly weaned individuals (McManus 1974; Seidensticker et al. 1987; Stout and Sonenshine 1974). Within an opossum population, both males and females succumb to early mortality, however, sex ratios are highly female-biased during late summer months (Kasparian 2002; Levesque 2001). As opposed to dasyurid females, female Virginia opossums are polyestrous, therefore males can have several opportunities of mating with an individual female (Gardner 1982).

Mating seasons within Oklahoma have been extrapolated from data collected in Missouri, Texas, and Louisiana (Gardner 1982; Kasparian 2002; Levesque 2001; Reynolds 1945). Depending on yearly environmental stochasticity, late January is the beginning of the first mating season,

with a peak in late February. The second season begins in late April and peaks in late May. Following a 13-day gestation (Gardner 1982), opossums are weaned after 100 days, with males reaching sexual maturity at approximately 8 months (Biggers 1966) and females approximately 6 months (Reynolds 1952). In early winter, male opossum's testes decrease in mass and begin to produce sperm at the same time as testosterone concentrations begin to rise (Biggers 1966; Chase 1939; Harder and Fleming 1986; Winegarner 1982). Litter size of opossums increase with latitude from an average of 6.0 young/female in the south to 9.2 young/female in the north (Gardner 1982). Density of the population depends on habitat. In a mesquite grassland and chaparral grassland the density was as low as 1.1/km^2, whereas in a hardwood-pine-savanna mix a density of 10.1/km^2 has been reported (Gehrt et al. 1997; Kissell and Kennedy 1992).

Immunological and physiological data on Virginia opossums are scarce, and there is no previous work on seasonal variation. Although limited studies have described levels of total serum protein (Rowlands and Dudley 1969) and hematological characteristics (Cutts and Krause 1980; Giacometti et al. 1972; Mays and Loew 1968; Timmons and Marques 1969; Youatt et al. 1961), animals in

these studies were laboratory maintained and may not reflect physiology of free-ranging populations. Other researchers have recorded data regarding immunoglobulin classes (Bell 1977), complement hemolytic activity (Ish et al. 1993), and spleen mass (Cutts and Krause 1982), but did not measure these parameters in free-ranging opossums nor at different times of the year. Gandolfi and Culbertson (1983) described a single account of lymphocytopenia and neutrophilia by administration of cortisol acetate to a captive injured opossum in a veterinary clinic. Research regarding digestive tract helminths in Virginia opossums has been restricted to surveys of species, with no investigation of helminth effects on opossum physiology. The dominant stomach helminth of the Virginia opossum is *Physaloptera turgida*. Other helminths such as *Lagochilascaris sprenti*, *Lagochilascaris turgida*, and *Gnathostoma* sp. have been found in stomachs of opossums (Alden 1995). Often associated with *Physaloptera turgida* are pinworms in the genus *Cruzia* (Ackerson 1992).

No explanation has been provided to describe the reason(s) for the short lifespan of the opossum. My study attempted to provide insight into seasonal physiological changes that may be associated with early mortality in the male opossum. The investigation involved collection of

physiological data from free-ranging male Virginia opossums during each season of a single year. Focusing on male Virginia opossums allowed comparison of the physiological profile of male Virginia opossums with semelparous, male dasyurids.

Objectives

1. To determine seasonal variation in serum cortisol concentrations of free-ranging male opossums.
2. To determine if seasonal changes in cortisol concentrations are associated with concomitant changes in adrenal mass, gastric helminth prevalence, lymphoid tissue mass, and hematological values.
3. To compare physiological changes in male Virginia opossums with those observed in male semelparous dasyurids.

Predictions

I predicted physiological changes would mimic those reported in dasyurid species (Table 1). Because May was the trapping season at the end of the opossum's second mating season, I expected to record parameter changes in May similar to the mating season changes of male dasyurids. During May, cortisol concentration should have been higher than other seasons; therefore, I expected other measured parameters in this particular season to change as a result

of exposure to supranormal cortisol concentrations. The WBC concentration, RBC concentration, PCV, spleen mass, total serum protein, serum immunoglobulin concentration, and complement activity should have decreased compared to other trapping seasons. Also, in May, I expected an increase in adrenal mass and gastric helminth numbers per individual.

The reason I predicted WBC concentration and immunoglobulin concentration to decrease was due to the lympholytic activity of cortisol. If lymphocytes were removed, then there could be reduced production of immunoglobulins by B-cells. If immunoglobulin concentration decreased then complement activity should decrease because immunoglobulins are used in initiation of complement activity. I predicted RBC concentration, PCV, and total serum protein to decrease due to the gluconeogenic effects of cortisol. This activity would remove protein to produce glucose, especially if the supranormal cortisol concentrations had persisted several weeks. Also, helminth numbers were expected to increase due to a suppressed immune system. A larger number of helminths feeding on the blood supply of the opossum would exacerbate the loss of red blood cells. Because the spleen is a major site for lymphocyte activity, spleen mass should

have decreased because removal of lymphocytes by cortisol would decrease the number of lymphocytes in the spleen. However, mass of the adrenal glands should increase, as hypertrophic adrenal glands would be needed to maintain supranormal cortisol concentrations.

I predicted that opossums would show changes similar to those seen during the dasyurid mating season in May because May is about four months into the breeding season, allowing sufficient time for the stressors of the breeding season to show their prolonged effects. February's trapping session was too early for stress to have mounted significantly. August was no longer the breeding season and only a few adult males were predicted to survive this long after breeding season. Other males would likely be recently weaned individuals from the mating seasons of 2001. During November, the majority of males were expected to be weaned males conceived in 2001 that had not lived through a breeding season.

Materials and Methods

Study area.—Free-ranging male Virginia opossums were collected using Tomahawk wire mesh traps (25 x 31 x 81 cm) at the Oklahoma State University Cross Timbers Experimental Range (CTER). The Cross Timbers ecoregion covers large parts of central Oklahoma and Texas. Livestock grazing is

the primary economic use of the region because the area produces few economically valuable timber products (Stritzke et al. 1991). CTER is located 11 km southwest of Stillwater, Payne County, OK (36°02'40" to 36°04'20"N, 97°09'30" to 97°11'39"W), and has been used since 1983 to study responses of livestock, wildlife (Boren et al. 1993; Lochmiller et al. 1991; McMurry et al. 1993, 1994, 1996; Schultz et al. 1992ab; Soper et al. 1993) and vegetation (Engle et al. 1991; Stritkze et al. 1991) to various brush management techniques. Climate is continental with an average frost-free growing period of 204 days from April to October. Mean annual temperature is 15°C and ranges from an average daily minimum of -4.3°C in January to an average daily maximum of 34°C in August. Average annual precipitation is 831 mm (Myers 1982; NOAA 1999).

Upland forest habitats were dominated by post oak (*Quercus stellata*), blackjack oak (*Q. marilandica*), and American elm (*Ulmus americana*) in the overstory, interspersed with a mosaic of tallgrass prairie. Understory was composed of eastern redcedar (*Juniperus virginiana* L.), American elm (*Ulmus americana*), redbud (*Cercis canadensis*), and rough-leaf dogwood (*Cornus drummondii*). Herbaceous ground cover was dominated by little bluestem (*Schizachyrium scoparium*), Indiangrass

(*Sorghastrum nutans*), rosette panicgrass (*Panicum oligosanthes*), and western ragweed (*Ambrosia psilostachya*; Ewing et al. 1984).

European land-use of CTER started in the 1930's with homesteading and cultivation of crops such as cotton (Ewing et al. 1984). CTER now encompasses 712 ha divided into 22, 32.4-ha pastures, each measuring 400 by 800 m. Beginning in 1983, 5 experimental conditions were applied randomly with 4 replicate pastures to produce a mosaic of vegetation types (Fig. 1). The treatments were tebuthiuron (*N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*,*N*`-dimethylurea, Dow Elanco, Indianapolis, Indiana, United States) applied aerially (2.2 kg/ha) in March 1983; tebuthiuron with late-spring prescribed fire (annually 1985-87, every 3 years 1990-present); triclopyr ([(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid, Dow Elanco) applied aerially (2.2 kg/ha) in June 1983; triclopyr with prescribed fire (annually 1985-87, every 3 years 1990-present) and 2,4-D and picloram; and control (no herbicides or burning). The triclopyr-alone treatment also had prescribed fire starting in 1996 on a 3-year cycle.

Prescribed fires were conducted by the Oklahoma State University Research Range Fire Crew in late March to early April using strip-headfires timed to coincide with initial

green-up of warm season grasses and leaf expansion of oaks and buckbrush. Conditions were about 18°C, wind speed greater than 10 km/h, and relative humidity between 30-50%. In 1985, burning was limited primarily to grassland sites due to fine fuel load. By 1986, some brush areas burned (mainly shallow savannah sites) and by 1987, about 25% of brush areas in the triclopyr-treated pastures and >50% of brush areas in the tebuthiuron-treated pastures burned.

Tebuthiuron, a soil-applied herbicide absorbed through the root system, resulted in die-off of the mature oak (*Quercus* spp.) forest and most of the other woody species except for eastern redcedar (Stritzke et al. 1991). Triclopyr is a foliar-applied herbicide absorbed through the leaf surface that caused die-off of mature overstory oak, but not other woody species. When either herbicide is combined with a spring headfire regime, remaining woody species are reduced (Engle et al. 1991; Stritzke et al. 1991).

Treatments resulted in heterogeneous study pastures dominated by 4 major habitat types: eastern redcedar forest in tebuthiuron pastures, derived grassland in tebuthiuron-with-fire and triclopyr-with-fire pastures, a mixed-brush community in triclopyr-with-recent-fire pastures, and mature oak forest in untreated pastures (Fig. 1). Habitat

types were classified based on vegetation composition in pastures observed in a 1998 aerial black-and-white photograph (scale 1:4,875) and extensive ground reconnaissance (Levesque 2001). Yearling cattle grazed all experimental pastures and stocking was adjusted annually to meet a goal of 50% use of annual forage production (Stritzke et al. 1991). No prescribed fires were conducted during this study.

Experimental animals.—Animals were collected during trapping sessions in February, May, August, and November of 2001. Each trapping session consisted of 2 groups of pasture arrangements (Fig. 1). The 1st group consisted of 4 pastures dominated by oak forest (n = 1), mixed-brush forests (n = 2), and derived grassland (n = 1). The 2nd group consisted of 3 pastures consisting of oak forest (n = 1), mixed-brush forest (n = 1), and derived grassland (n = 1). Traps were baited with sardines and checked daily over a 10-day period. Traps within each pasture were arranged in a grid of 6 perimeter traps and 2 interior traps. The perimeter traps were placed 100 m from the boundary of the pasture and 300-m apart along parallel transects with traps spaced at 200-m intervals, with the interior traps placed 180-m diagonally from the pasture corners.

Trapped males were immobilized with a cocktail of ketamine hydrochloride (15 mg/kg) and xylazine (7.5 mg/kg), or Telazol® (8 mg/kg). Once immobilized, mass was recorded followed by collection of blood via cardiac puncture. Blood was collected in 2 Vacutainer tubes (Becton Dickinson Company, Franklin Lakes, New Jersey) with EDTA and no-additive, respectively. After blood was collected, the opossum was euthanized with Beuthanasia®-D Special (80 mg/kg pentobarbital and 10 mg/kg phenytoin sodium; Schering-Plough Animal Health Corporation, Union, New Jersey), and transported in a pet carrier through the duration of that trap-checking day (≤ 4 hours). Procedures for opossum collection followed Institutional Animal Care and Use Committee protocol AS-50-719 at Oklahoma State University.

Hematology and serum chemistry.—I performed red blood cell (RBC) and white blood cell (WBC) concentrations from blood collected in the EDTA Vacutainer using a hemacytometer and Hayem's solution (RBC) or acetic acid (WBC). A heparinized, microcapillary tube and microcapillary centrifuge were used to determine packed cell volume (PCV). Serum was isolated after placing the no additive Vacutainer in a centrifuge at 2500 rpm (1250 x g) for 8 minutes at 8°C. Serum was stored in microcentrifuge

tubes at -70°C until appropriate assays were initiated. A smear was prepared for WBC differential counts. Diff-Quik (Baxter Healthcare Company, McGaw Park, Illinois) was used to stain whole blood smears. Differential WBC counts were conducted on the first 100 WBC's of a blood smear (1000x magnification, oil immersion lens). A microplate, colorimetric method using the biuret method as described in Kingsley (1942) was used to measure total serum protein. A total of 210 µl of biuret working reagent was added to 20 µl of sample serum or 20 µl of a bovine serum albumin standard. Following a 15-minute incubation at room temperature (RT), the absorbance of samples and standards were read at 550 nm.

A modified protocol of Bradford's (1976) ammonium precipitation assay was used to ascertain serum immunoglobulin levels. A 0.2-ml volume of serum was added to 4.8 ml of ammonium sulfate-sodium chloride reagent and centrifuged for 30 minutes at 2500 rpm (1250 x g) at RT. The supernatant was decanted, then the pellet was resuspended in 2.5 ml of 0.85% NaCl. A 5-µl sample of the resuspended solution was added to a microplate well and 25 µl of reagent A and 200 µl of reagent B were added to all standard and sample wells as instructed in the DC Protein Assay kit (Biorad, Hercules, California, United States).

After a 15-minute incubation, plates were read at 750 nm using a bovine serum albumin standard.

Morphology and helminths.—Following collection of hematological data, spleen, testes, and adrenal glands were removed and weighed. The opossum was then placed in a 4°C refrigerator overnight to allow helminths to detach. The next day, the gastrointestinal tract was excised, then helminths were removed and fixed in alcohol-formalin-acetic acid (AFA) for later identification (Ackerson 1992) and enumeration.

Immunological function.—The Davis et al. (1995) modification of De Waal et al.'s (1988) technique for assessing hemolytic complement activity was used. Serial two-fold dilutions (1/16 - 1/2048) of serum were placed in a 96-well plate. A 25-µl volume of 0.6% sheep red blood cells (SRBC; Colorado Serum Company, Denver, Colorado) in veronal buffered saline (VBS), modified for Virginia opossum complement activity (Wirtz and Westfall 1967), and 25-µl of a 1:80 dilution of rabbit anti-SRBC antibodies (Nordic Immunology, Tilburg, Netherlands) in VBS were added to each well. Incubation of plates occurred at 37°C for 1.5 hours. Following the incubation, the plates were spun at 1200 rpm (300 x g) and 60 µl of supernatant was transferred to a replicate plate. Absorbances of the supernatant at

414 nm were recorded for each known standard and sample. Using a standard curve of known amounts of lysed SRBC's, the hemolytic complement activity was expressed as CH_{50} units/ml serum, where 1 CH_{50} was the amount of complement required to lyse 50% of the SRBC (Mayer 1961).

Cortisol analysis.—A radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California) was used to measure concentrations of total cortisol in serum samples. A 25-µl sample of serum from each opossum was added to tubes coated with antibodies specific for cortisol. Next, a 1-ml volume of ^{125}I-Cortisol solution was added to compete with the cortisol in the serum sample. An incubation of 1.5 hours at 37°C followed the addition of the radiolabelled cortisol. After the incubation, the solution in each tube was aspirated and tubes allowed to dry. Once dry, the tubes were placed in a gamma counter. The counts from the samples were then graphed on a standard curve to determine the sample cortisol concentration. All samples were run in a single assay with each sample and standard run in triplicate. The recovery of cortisol added and intra-assay coefficient of variation were 80%-113% and 6.6%, respectively.

Testosterone analysis.—A radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California)

was used to measure concentrations of total testosterone in serum samples. A 50-µl sample of serum from each opossum was added to tubes coated with antibodies specific for testosterone. Next, a 1-ml volume of ^{125}I-Testosterone solution was added to compete with the testosterone in the serum sample. An incubation of 3 hours at 37°C followed the addition of the radiolabelled testosterone. After the incubation, the solution in each tube was aspirated and tubes allowed to dry. Once dry, the tubes were placed in a gamma counter. The counts from the samples were then graphed on a standard curve to determine the sample testosterone concentration. All samples were run in a single assay with each sample run in duplicate and each standard run in triplicate. The recovery of testosterone added and intra-assay coefficient of variation were 98%-116% and 3.3%, respectively.

Statistical analysis.—All data were first tested for normality using Proc Univariate in SAS 8.2 (SAS Institute 2001). If the data were found to be nonparametric, then ranks were assigned to the original data using Proc Rank (SAS Institute 2001), followed by a 1-way analysis of variance (ANOVA) of the ranks using Proc Mixed (SAS Institute 2001). If the data were found to be parametric, an ANOVA was conducted on the original data. Both

parametric and nonparametric analyses used season as the main factor.

Means and standard errors per trapping season for PCV, RBC concentration, WBC concentration, cortisol concentration, testosterone concentration, serum complement activity, immunoglobulin concentration, total serum protein concentration, helminths per individual, and WBC differentials were calculated using Proc Means (SAS Institute 2001). Log_{10}(spleen mass), log_{10}(total testes mass), and log_{10}(adrenal mass) were regressed against log_{10}(body mass). Only left adrenal masses were used in calculations because the right adrenal gland was not located until the May collecting session. Residuals of the regression equation were calculated for each datum point using Proc Reg (SAS Institute 2001). Residual data were analyzed the same as the parameters listed above.

I conducted 2 separate analyses, 1 with 4 seasons and another with 3 seasons only (February, May, and November). All males trapped in August were considered immature young of the year. Data analysis included pair-wise comparisons of seasonal means or ranks using least significant difference (LSD). Some samples could not be used in data collection; therefore, sample sizes for the appropriate parameter were adjusted accordingly.

Two testosterone concentration values were removed from analysis because they were extreme outliers according to box plot analysis (Mann 1998). The concentration of 1968 ng/dl was removed from February's data set and 1089 ng/dl was removed from August's data set. Both values were greater than 3X the interquartile range. Therefore, for testosterone analyses, February had a sample size of 6 for the 3- and 4-season analyses and August had a sample size of 8 for the 4-season analysis.

Results

Trapping.—During 4,462 trapnights, I collected 36 males: 12 in February, 7 in May, 9 in August, and 8 in November. Males captured in August were smaller in body mass ($F = 19.96$; $d.f. = 3, 32$; $P < 0.001$; Table 2) than males captured in other seasons. All males trapped in August were assumed to be juveniles from the spring 2001 cohort.

Hematology.—Lymphocytes, neutrophils, monocytes, and PCV varied seasonally (Table 2). Mean PCV during May ($F = 6.90$; $d.f. = 3, 30$; $P < 0.01$) was the lowest of all four seasons. During May and February, percent lymphocytes reached a minimum ($F = 16.72$; $d.f. = 3, 31$; $P < 0.001$), whereas percent neutrophils reached its maximum ($F = 22.08$;

d.f. = 3, 31; $P < 0.001$) in February and May (Table 2). The season with the lowest mean percent monocytes was May ($F = 3.23$; *d.f.* = 3, 31; $P < 0.05$). RBC concentration did not vary seasonally ($F = 0.86$; *d.f.* = 3, 30; $P = 0.47$) but was smallest in May (Table 2). Hematological results from analysis conducted without the August trapping season provided similar results (Table 3). Some blood samples in February and May were not included because of broken sample tubes, poor bloodsmear, or cell lysis after collection.

Morphology.—Residuals for spleen mass were smallest in November in the 4-season ($F = 5.84$; *d.f.* = 3, 32; $P < 0.01$; Table 2; Fig. 2) and 3-season analysis (Table 3). Testes mass residuals varied seasonally ($F = 5.97$; *d.f.* = 3, 26; $P < 0.01$), with both 4- and 3-season analysis showing residual means largest in May and smallest in November (Table 2 and 3). Residuals for left adrenal mass did not vary seasonally ($F = 1.85$; *d.f.* = 3, 31; $P = 0.15$; Table 2) in the 4-season analysis (Fig. 3). However, in the 3-season analysis, residuals for adrenal mass were largest in May (Table 3).

Helminths.—Males collected in February had more ($F = 27.75$; *d.f.* = 3, 32; $P < 0.001$) helminths than males trapped in the other 3 seasons (Table 2 and 3). Thirty-five of 36 males possessed gastric helminths and all

gastric helminths were *Physaloptera turgida*. Other helminths, collected from 5 males (4 in May and 1 in November), belonged to the genus *Cruzia*. These helminths were located in the caecum and/or large intestine.

Serum Chemistry and Endocrinology.—Immunoglobulin protein, complement and testosterone showed seasonal variation, whereas cortisol did not (Table 2 and 3). Immunoglobulin protein concentration was greatest in May ($F = 5.00$; $d.f. = 3, 32$; $P < 0.01$; Table 2). Complement activity varied seasonally ($F = 3.18$; $d.f. = 3, 32$; $P < 0.05$; Table 2) with a minimum of activity in May. Testosterone concentrations varied seasonally ($F = 2.79$; $d.f. = 3, 30$; $P = 0.057$), with testosterone concentrations largest in November (Table 2). Cortisol concentrations did not differ seasonally ($F = 2.41$; $d.f. = 3, 32$; $P = 0.08$) but were largest in August (Table 2). Three-season analyses for all variables provided similar results (Table 3).

Discussion

Male Virginia opossums experienced similar physiological changes to those of male dasyurids exhibiting semelparity. Opossum characteristics consistent with the dasyurid semelparity syndrome included PCV, helminth numbers, and adrenal mass. Minor lymphocytopenia and neutrophilia and testosterone concentration also were

consistent with characteristics of semelparous dasyurids. On the other hand, the lack of change in serum cortisol concentration and body mass, along with dynamics in immunoglobulin protein, serum protein, and testes mass were not consistent with previous reports of dasyurid physiology. Demographically, the lack of adult male opossums captured during August and a highly adult female biased sex ratio in two previous studies (1M:2.5F and 1M:4F) on the same study area in summer (Kasparian 2002; Levesque 2001) is evidence supporting male opossum semelparity.

Adrenal mass residuals were largest in May. Barnett (1973) also reported increased adrenal mass in *A. stuartii* during mating season, although Bradley et al. (1980) did not record a change in adrenal mass in mating season. Changes in adrenal mass during breeding season are species-specific. Reports of other mammals show wide seasonal variation in adrenal mass. For example, increases in adrenal mass during the breeding season have been reported in male meadow voles (*Microtus pennsylvanicus*; Sealbloom 1978); decreases have been reported in male bobcats (*Lynx rufus*; McKinney and Dunbar 1976), male pikas (*Ochotona princeps*; Millar 1970), and male muskrats (*Ondatra zibethica*; Beer and Meyer 1951); and no mass change has

been reported in male prairie deer mice (*Peromyscus maniculatus bairdii*; Christian 1967), male white-footed mice (*Peromyscus leucopus noveboracensis*; Christian 1967), male cotton rats (*Sigmodon hispidus*; Goertz 1965), and male golden-mantled ground squirrels (*Spermophilus lateralis lateralis*; Skryja and Clark 1970).

A rise in cortisol concentrations, during the breeding season, was not seen in male Virginia opossums, in contrast to results recorded in semelparous male dasyurids (Bradley 1987, 1990a; Bradley et al. 1975; Schmitt and Bradley 1989). The lack of increased cortisol in the male opossum is not unprecedented in medium-sized semelparous marsupials. *D. hallucatus*, a 1-kg marsupial, did not show significant changes in cortisol concentrations during the mating season (Oakwood et al. 2001).

The increase in cortisol for the August males is most likely due to the evasive activity of opossums in the trap (Dallman 1973). Acute increases in cortisol concentrations have been reported in handled and/or trapped mammals (Harlow et al. 1990; Hellgren et al. 1985; Osadchuk et al. 2001; Waas et al. 1999). August was the only month during which males performed such active behavior.

Given the lack of concomitant increase between seasonal cortisol concentration and adrenal mass, it is

unclear whether using the adrenal gland weight as an index for adrenocortical activity is useful in male opossums. Many influencing factors, such as age, sex, and body size, are important in size variation of the adrenal cortex. These factors are also the source of debate of whether the adrenal mass is a good index of adrenocortical activity in small mammals [reviewed in (Lee and McDonald 1985)].

An increase in cortisol concentrations of semelparous, male dasyurids may ensure sufficient glucose concentrations in the blood during the mating season. Male dasyurids spend enormous amounts of resources performing agonistic behavior or locating females or, in the case of *A. struartii*, possibly lekking (Barnett 1973; Braithwaite 1979; Inns 1976; Lazenby-Cohen and Cockburn 1988). These resource-demanding behaviors can place male dasyurids in negative nitrogen balance (Bradley 1997; Woollard 1971) due to protein catabolism for gluconeogenesis. Male Virginia opossums also will seek and defend a female in estrous. If multiple males approach a female in estrous, the largest male usually will win any male-male combat and the opportunity to copulate with the female (Ryser 1992). The large size (2-3 kg) of male Virginia opossums may allow for larger proportions of adipose deposition than smaller dasyurids. Therefore, protein may be less required for

gluconeogenesis. In contrast to the loss of body mass by male dasyurids during the mating season (Barnett 1973; Bradley 1997; Inns 1976; Wood 1970; Woolley 1966), male Virginia opossums did not show a decrease in body mass between February and May. Sustained body mass may be a result of males foraging and acquiring energy resources during the two long mating seasons.

An increase in testosterone concentrations before the mating season leads to increased concentrations of cortisol in semelparous, male dasyurids (McDonald et al. 1981; Millis et al. 1999; Oakwood et al. 2001; Wilson and Bourne 1984). Testosterone concentrations in the male Virginia opossum did not increase during the mating season; however, testosterone increased in November, the trapping season before the mating season. November is when sperm production of male Virginia opossums would be expected to begin in Oklahoma (Winegarner 1982). Harder and Fleming (1986) also reported an increased testosterone concentration in November for male opossums. Mean testosterone concentrations for February (180 ng/dl) and May (158 ng/dl) of this study were similar to the mean testosterone concentration from January to June (200 ng/dl) reported by Harder and Fleming (1986).

Reduction in testes mass during November is in agreement with Biggers' (1966) report of opossum testes mass, but is in disagreement with increased testes mass reported by Woolley (1966), the only report of testes mass in male dasyurids during the mating season. Winegarner (1982) described no seasonal variation of male opossum testes mass even in early winter, the time of sperm production in Florida. However, Winegarner's (1982) conclusion of no seasonal difference in testes mass was founded on testes mass only, not on a ratio or regression of testes mass with body mass.

The decline in PCV observed in May in opossums was concomitant with a nonsignificant decrease in RBC concentration. *D. hallucatus* (Oakwood et al. 2001) and *A. stuartii* (Cheal et al. 1976), both dasyurids, also exhibited a significant decrease in packed cell volume during the later portion of their breeding seasons. Authors of the dasyurid literature do not explain the decreased PCV, except for one instance where *Babesia spp.* (a blood parasite) was thought to be the culprit (Cheal et al. 1976). A possible explanation for seasonal PCV variation in male opossums could be the removal of damaged RBC's by the spleen (Ferrant et al. 1987; Grossman and Jollow 1988; Zocchi et al. 1987). Research has shown that

increased metabolic activity can increase the fragility of RBC's (Hanzawa and Watanabe 2000; Senturk et al. 2001). May is several months into the breeding season and male opossums have increased their searching, agonistic, and mating activity. This increased metabolic activity could produce damaged RBC's that have to be removed from the circulating population.

Minor lymphocytopenia and neutrophilia during both mating seasons (February and May) in opossums were consistent with results observed during the mating season of semelparous dasyurids (Bradley 1990b; Cheal et al. 1976). Previously, neutrophilia and lymphocytopenia had been recorded only in an opossum repeatedly injected with cortisol acetate (Gandolfi and Culbertson 1983). In this thesis, I report a 2:1 ratio of neutrophils to lymphocytes during the mating months of opossums. Boonstra et al. (2001) also reported a 2:1 ratio of neutrophils to lymphocytes during the breeding season for male arctic ground squirrels.

Low lymphocyte concentrations in male opossums may be a result of age and immunosenescence. Reports in mice and humans have shown decreases in populations of T-cells with age, possibly caused by involution of the thymus (Hirokawa 1992; McFarlane et al. 2001; Miller 1996). Chronic high

concentrations of cortisol indexed by increased adrenal mass, especially in May, also could be responsible for the decreased percent lymphocyte. The mean percent of lymphocytes in August (55.8) was similar to that reported by Cutts and Krause's (1980; 55.8) in juvenile opossums. However, Cutts and Krause (1980) reported a mean neutrophil percentage of 25.4 juveniles, whereas I report a mean of 3.4 in August.

The relationship between mating season and increased helminth populations in male *A. stuartii* (Bradley et al. 1980) was replicated in male opossums in February but not May. Blumenthal and Kirkland (1976) reported *P. turgida* in the stomach of Virginia opossums from Pennsylvania but did not find seasonal variation in burdens of *P. turgida*. *P. turgida* uses arthropods as an intermediate host and can use frogs, snakes, and small mammals as paratenic hosts (Anderson 1988). Arthropods and small mammals are important components of the opossum's diet during winter months (Kasparian 2002). A large proportion of worms during February was small adults or juveniles. During the other 3 trapping seasons, the majority of worms was large adults. The decrease in helminth numbers from February to May could be a reflection of diet change. Between February and May, worms excreted in the feces will not be replaced

if opossums are not consuming the intermediate host of *P. turgida*.

Male opossum serum protein concentrations did not vary seasonally, as reported for *A. stuartii* (Cheal et al. 1976). A lack of seasonal variation in male opossum's serum protein concentrations is contrary to increased plasma protein concentrations of male, semelparous dasyurids during mating, reported by Bradley (1990b). Elevated immunoglobulin protein concentrations during May for male opossums may be a consequence of a shift in T-cell classes (McFarlane 2001; Thoman and Weigle 1989). Increased immunoglobulin protein and serum protein seem to conflict with the decreased complement activity of male opossums during May. Immunoglobulins are used in initiation of the complement cascade (Goldsby et al. 2000); therefore, if immunoglobulin protein concentrations are largest in May, then possibly the concentration of complement proteins was decreased in May. Evidence to support the decrease in complement protein concentrations is the reduced number of monocytes in the May. Monocytes are a significant source of complement proteins (Goldsby et al. 2000).

A large proportion of lymphocyte activity occurs in the spleen (Goldsby et al. 2000), so spleen mass should

adjust to changing lymphocyte concentrations. This did not seem to be the situation for male Virginia opossums, as the highest percent lymphocytes and smallest spleen masses were observed in November. Spleen mass residuals for opossums were largest during May, which was also a time of lymphocytopenia. Bradley (1987) reported seasonal variation in spleen mass in male dasyurids during mating season, whereas Barker et al. (1978) reported no variation. Given these two conflicting reports, more dasyurids need to be studied to determine if seasonal variation exists for spleen mass. The large opossum spleen mass residuals during May could perhaps be better explained by increased removal of damaged RBC's (Ferrant et al. 1987; Grossman and Jollow 1988; Zocchi et al. 1987).

Differences in physiological changes in Virginia opossums and semelparous dasyurids may be partially explained by a difference in the length of their mating seasons. Mating seasons of dasyurids last about 1 month (Bradley 1987; Dickman and Braithwaite 1992; McDonald et al. 1981; Scott 1986; Wilson and Bourne 1984), whereas the Virginia opossum's mating season lasts about 5 months (Gardner 1982). Therefore, the process that leads to early mortality of opossums may occur at a more gradual pace in opossums than dasyurids to ensure that males have

sufficient opportunity to inseminate estrous females in both mating seasons. To understand short-term changes, investigating physiological and immunological parameters intra-seasonally and/or measuring other attributes of the male Virginia opossum should be the focus of future research. Life history and physiology of other marsupials similar in size and life span to the Virginia opossum also need to be investigated for more relevant comparisons to be performed.

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Table 1.—Physiological, morphological, and immunological changes observed in dasyurids exhibiting semelparity.

	Changes during mating season		
Parameter	Increase	Decrease	No Change
Body Mass	7	1,2,3,4,10,18,19,20	16
Testes Mass		1	
Adrenal Mass	4		9
Spleen Mass		14	8
Nitrogen Balance		3	
Cortisol Conc.	5,6,9,11,14,15,20	11	4,8
Androgen Conc.	9,11,12,14,17,18,20		
ACTH Conc.	9,15		
CBG Conc.		6,9,11,14	
Hematology		7,17,20	16
Parasites	9,17,20		8
Immunocompetence	9	14	
Hemorrhaging	Yes - 7,8,14	No - 17	

1) Woolley 1966
2) Wood 1970
3) Woollard 1971
4) Barnett 1973
5) Bradley et al. 1975
6) Bradley et al. 1976
7) Cheal et al. 1976
8) Barker et al. 1978
9) Bradley et al. 1980
10) Inns 1976
11) McDonald et al. 1981
12) Wilson and Bourne 1984
13) McDonald et al. 1986
14) Bradley 1987
15) Bradley 1990a
16) Bradley 1990b
17) Oakwood et al. 2001
18) Millis et al. 1999
19) Bradley 1997
20) Schmitt and Bradley 1989

Table 2.—Physiological and morphological characteristics of male Virginia opossums (*Didelphis virginiana*) over 4 seasons from Cross Timbers Experimental Range, Payne County, Oklahoma, 2001.

Parameter	February			May			August			November		
	n	$\bar{x}$	*SE*	*n*	$\bar{x}$	*SE*	*n*	$\bar{x}$	*SE*	*n*	$\bar{x}$	*SE*
White Blood Cells (10^3 /mm^3)	11	14.8	2.1	5	12.3	2.3	9	9.5	0.9	8	12.0	1.8
Red Blood Cells (10^6 /mm^3)	12	4.49	0.21	5	4.22	0.77	9	4.09	0.23	8	4.74	0.17
Packed Cell Volume (%)[c,e]	12	32B	1	5	26B	3	9	36A	1	8	36A	1
Lymphocytes (%)[d]	11	18.5C	2.4	7	24.8BC	5.4	9	55.8A	4.5	8	36.6B	4.8
Neutrophils (%)[d,e]	11	45.5A	4.9	7	56.1A	6.6	9	3.4C	1.4	8	30.0B	7.4
Eosinophils (%)[e]	11	2.9	1.2	7	1.7	0.8	9	4.6	1.4	8	3.3	1.3
Monocytes (%)[b]	11	33.1AB	4.3	7	17.3C	1.8	9	35.8A	4.7	8	31.0AB	4.5

Total protein (g/dl)	12	5.57	0.14	7	5.67	0.24	9	5.18	0.22	8	5.67	0.05
Immunoglobulin protein (g/dl)[c]	12	0.68AB	0.04	7	0.83A	0.09	9	0.54B	0.08	8	0.51B	0.04
Complement Activity (CH_{50}/ml)[c,e]	12	36B	12	7	16B	0	9	43A	7	8	60A	4
Cortisol (ng/ml)[e]	12	2.60	0.59	7	1.96	0.6	9	4.43	0.89	8	2.21	0.64
Testosterone (ng/dl)[e]	12	180.8AB	76.8	6	158.7AB	68.8	8	38.3B	13.4	8	434.3A	131.5
Body mass (kg)[d]	12	1.7B	0.1	7	1.7AB	0.1	9	0.9C	0.1	8	2.0A	0.2
Left adrenal[f]	11	-0.085	0.023	7	0.047	0.031	9	0.011	0.027	8	-0.043	0.027
Spleen[c,f]	12	0.107AB	0.077	7	0.123A	0.077	9	-0.033AB	0.039	8	-0.231C	0.041
Testes[c,f]	6	0.045AB	0.035	7	0.097A	0.030	9	-0.046BC	0.027	8	-0.066C	0.033
Helminths[d,e]	12	259A	51	7	61B	16	9	13C	5	8	29B	7

[a] Values with the same letter are not different ($P > 0.05$).

[b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$.

[e] One-way ANOVA of ranked data

[f] Residuals of regression of $\log_{10}$ of left adrenal, spleen, or testes mass against $\log_{10}$(body mass)

Table 3.—Physiological and morphological characteristics of male Virginia opossums (*Didelphis virginiana*) over 3 seasons from Cross Timbers Experimental Range, Payne County, Oklahoma, 2001. August was deleted due to the absence of adult males in the sample.

Parameter	February			May			November			ANOVA	
	n	$\bar{x}$	*SE*	*n*	$\bar{x}$	*SE*	*n*	$\bar{x}$	*SE*	*F*	*P*
White Blood Cells (10^3 /mm^3)	11	14.8	2.1	5	12.3	2.3	8	12.0	1.8	0.58	0.57
Red Blood Cells (10^6 /mm^3)	12	4.49	0.21	5	4.22	0.77	8	4.74	0.17	0.47	0.63
Packed Cell Volume (%)[b]	12	32B[a]	1	5	26C	3	8	36A	1	8.56	< 0.01
Lymphocytes (%)	11	18.5B	2.4	7	24.8AB	5.4	8	35.6A	4.8	4.92	0.02
Neutrophils (%)	11	45.5AB	4.9	7	56.1A	6.6	8	30.0B	7.4	3.99	0.03
Eosinophils (%)[b]	11	2.9	1.2	7	1.7	0.8	8	3.3	1.3	0.48	0.62
Monocytes (%)	11	33.1A	4.3	7	17.3B	1.8	8	31.0A	4.5	4.01	0.03
Total protein (g/dl)[b]	12	5.57	0.14	7	5.67	0.24	8	5.67	0.05	0.84	0.44

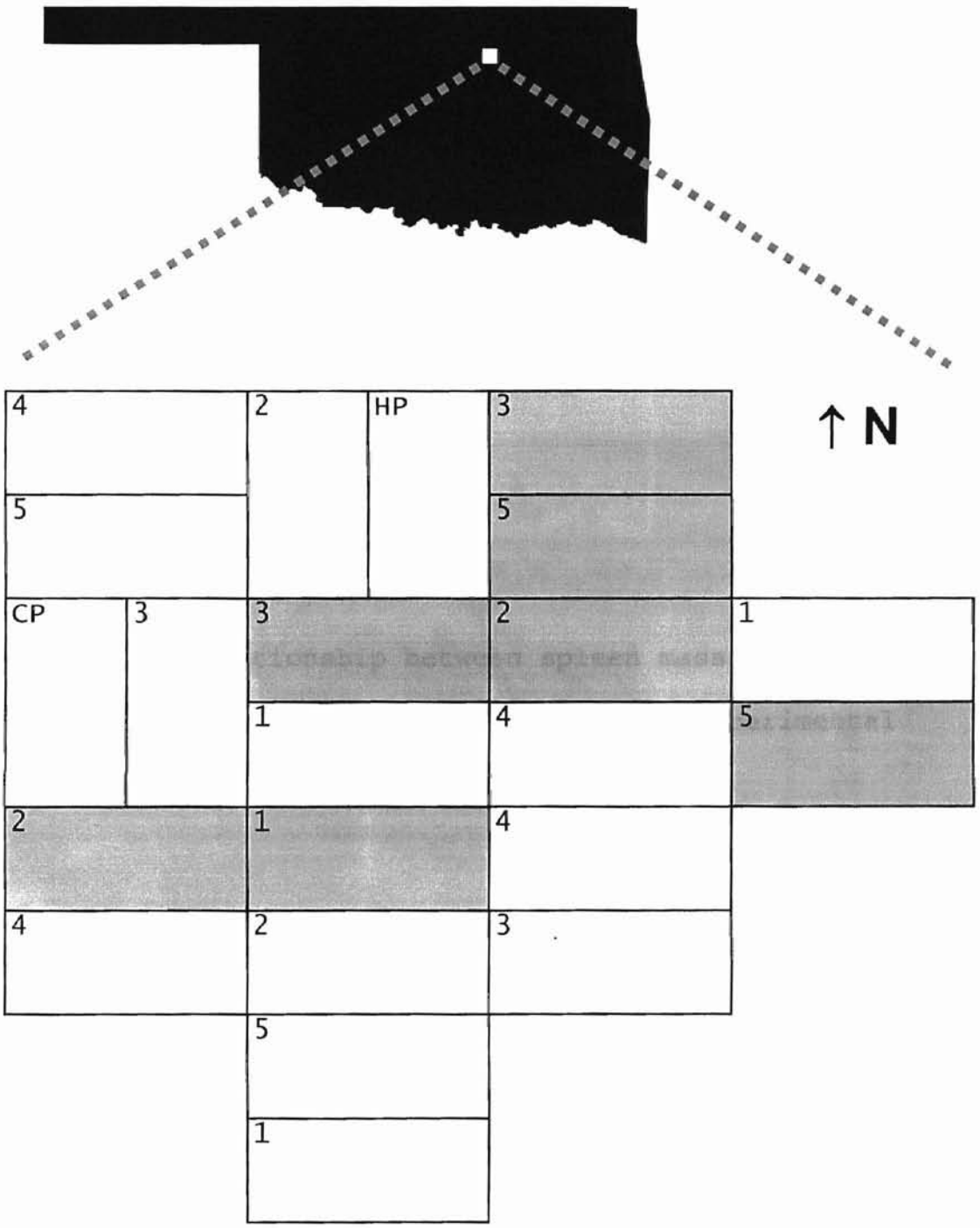
Immunoglobulin protein (g/dl)	12	0.68A	0.04	7	0.83A	0.09	8	0.51B	0.04	7.34	< 0.01
Complement (CH_{50}/ml)[b]	12	36B	12	7	16B	0	8	60A	4	14.35	< 0.01
Cortisol (ng/ml)[b]	12	2.60	0.59	7	1.96	0.6	8	2.21	0.64	0.31	0.74
Testosterone (ng/dl)[b]	12	180.8	76.8	6	158.7	68.8	8	434.3	131.5	1.74	0.20
Body mass (kg)	12	1.7	0.1	7	1.7	0.1	8	2.0	0.2	2.62	0.09
Left adrenal[c]	11	-0.001AB	0.022	7	0.053A	0.033	8	-0.045B	0.018	3.43	0.04
Spleen[c]	12	0.085A	0.082	7	0.108A	0.069	8	-0.221B	0.043	5.61	0.01
Testes[c]	6	0.012AB	0.034	7	0.061A	0.023	8	-0.062B	0.028	5.15	0.02
Helminths[b]	12	259A	51	7	61B	16	8	29B	7	22.69	< 0.01

[a] Values with the same letter are not different ($P > 0.05$).

[b] One-way ANOVA of ranked data

[c] Residuals of regression of $\log_{10}$ of left adrenal, spleen, or testes mass against $\log_{10}$(body mass)

Fig. 1.—Pasture arrangement at Cross Timbers Experimental Range, Payne County, Oklahoma including pastures trapped for male opossums, February - November, 2001.



1 = Cedar Forest
2 = Grassland
3 = Mixed Shrub
4 = Grassland
5 = Oak Forest
CP = Corral Pasture
HP = Holding Pasture
▭ = Trapped Pastures

Fig. 2.—Relationship between spleen mass and body mass in male Virginia opossums on Cross Timbers Experimental Range, Payne County, Oklahoma, 2001.

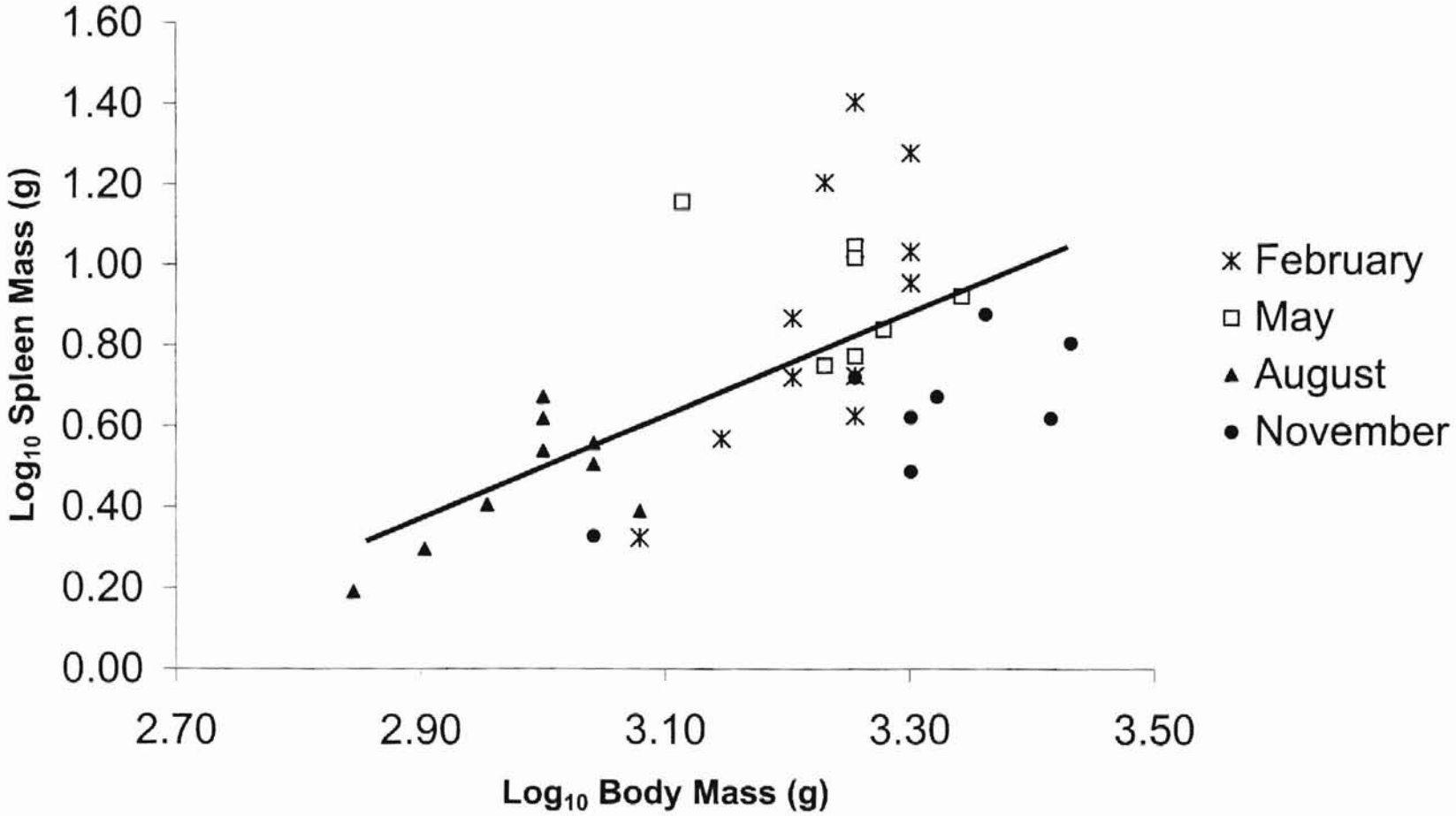
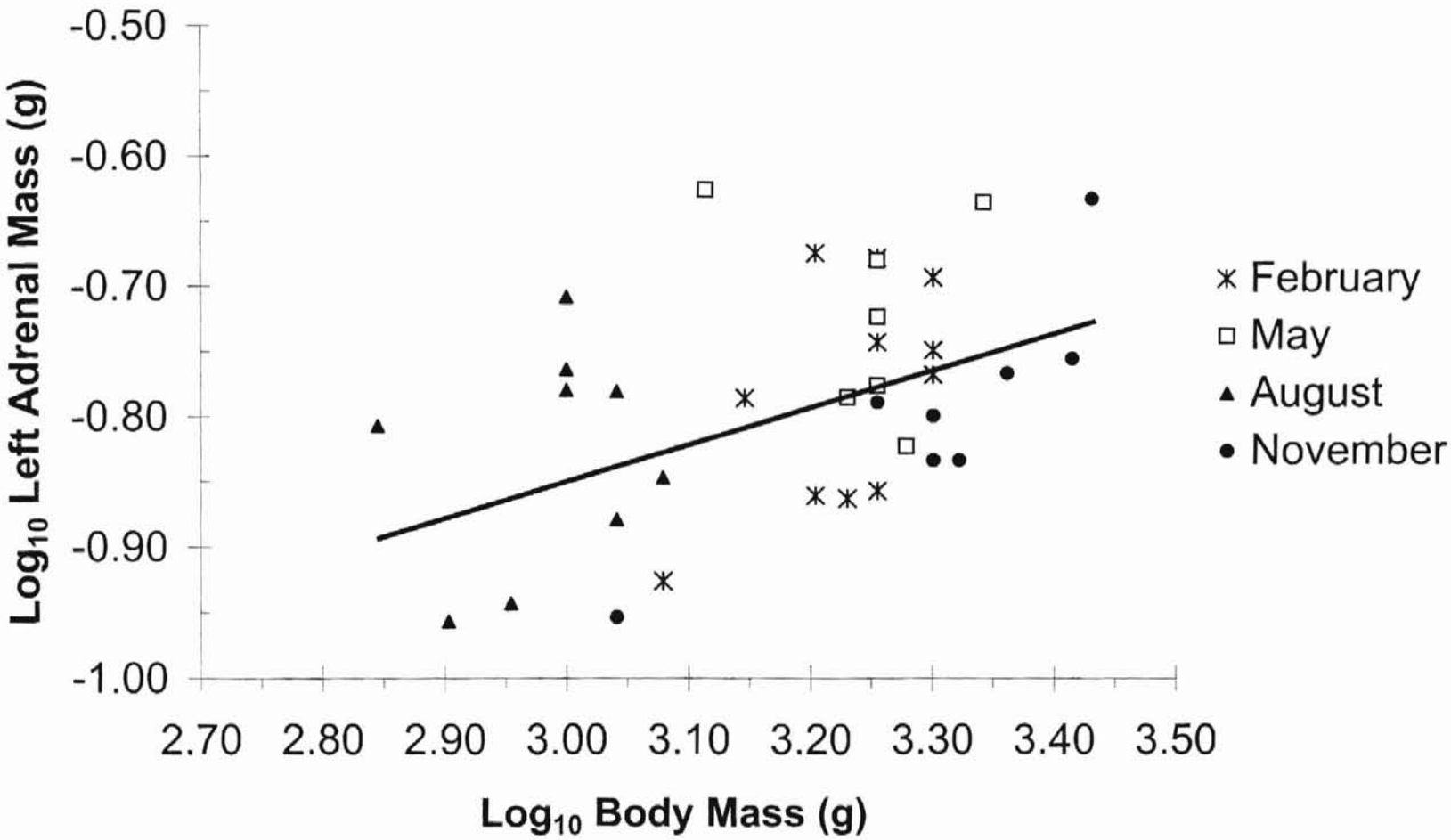

1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Log10 Spleen Mass (g)
2.70
2.90
3.10
3.30
3.50
Log10 Body Mass (g)
February
May
August
November

Fig. 3.—Relationship between left adrenal mass and body mass in male Virginia opossums on Cross Timbers Experimental Range, Payne County, Oklahoma, 2001.


-0.50
-0.60
-0.70
-0.80
-0.90
-1.00
Log10 Left Adrenal Mass (g)
2.70
2.80
2.90
3.00
3.10
3.20
3.30
3.40
3.50
Log10 Body Mass (g)
February
May
August
November

VITA

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Candidate for the Degree of

Master of Science

Thesis: SEASONAL CHANGES IN THE PHYSIOLOGY OF MALE VIRGINIA OPOSSUMS (*Didelphis virginiana*): SIGNS OF THE DASYURID SEMELPARITY SYNDROME?

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