

THE EFFECTS OF GENDER AND BROOD SIZE ON
THE DEVELOPMENT OF FLAMMULATED OWL (*OTUS FLAMMEOLUS*) NESTLINGS

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ABSTRACT

Reversed sexual size dimorphism (RSSD), where females within a given species are larger than their male counterparts, is a phenomena observed across a few avian taxa including hawks and eagles (Accipitriformes), falcons (Falconiformes), waders (Charadriiformes), and owls (Strigiformes). While the mechanisms driving the evolution of this phenomenon are widely discussed, the proximate effects of RSSD on development and juvenile morphology are not well understood. Life history characteristics, such as brood size, influencing avian morphology are also important in understanding long-term patterns in development. I studied development of Flammulated Owls (*Otus flammeolus*), an RSSD species that tends to raise offspring in broods of 2-3 owlets, to better understand these relationships. I analyzed development using two measures: mass and wing feather length. First I determined the gender of all 2011 nestlings based on genetic analysis of blood samples collected from owlets captured and bled in 2011. Gender data since 2003 were already available. Growth analysis on a total of 189 owlets revealed that females reach a higher asymptotic mass than males. Broods consisting of three owlets reached a higher asymptotic mass than broods consisting of 2 owlets, an unexpected result based on previous research. The differences in maximum juvenile mass in broods of different sizes may be biased due to divergent sex ratios within broods; 57% of owlets in broods of three were female while 38% in broods of two were female. Even so, trends remained the same when males are compared with other males and females with other females in same-sized broods. Wing size differed little between the genders and broods. Juvenile body condition ultimately informs adult viability and fitness, thus it is important to understand these and other selective factors that influence avian development.

INTRODUCTION

In most mammalian and avian species, sexual selection has led to the evolution of size dimorphism favoring males as the larger sex due to male-male competition for females (Kruger 2005). Reversed sexual size dimorphism (RSSD), where females are the larger sex, is typically observed in four avian taxa: hawks and eagles (Accipitriiformes), falcons (Falconiformes), waders (Charadriiformes), and owls (Strigiformes) (Kruger 2005). Hypotheses seeking to explain the driving forces behind the evolution of RSSD in birds can be divided into three categories: ecological, sex-role differentiation, and behavioral (Mueller 1986).

Ecological hypotheses attempt to explain size dimorphism as the consequence of sexes evolving to capture different sized prey, thus reducing inter-sexual competition and allowing a single species to exploit a wider range of prey (Mueller 1986). If trophic structures allow, birds of different sizes will hunt prey of different sizes (Mueller 1986). Sex-role differentiation hypotheses assert that different sexes perform separate roles in activities related to reproduction, such as nest protection, territorial defense, and feeding, and have evolved different sizes to better fulfill these specialized responsibilities (Mueller 1986). Lastly, behavioral hypotheses generally contend that RSSD specifically has evolved to allow for female dominance over the male. Female dominance has a number of consequences, including anti-cannibalism against males; assertion of role-partitioning, including relegation of foraging responsibilities to the male; maintenance of pair-bonding; and female selection of high quality males to breed with (Mueller 1986). Mueller (1986), who summarized these three categories of hypotheses, identified one hypothesis that does not fit neatly into categorization, that cavity nesting inhibits the evolution of RSSD.

Though the above categorizations have been widely accepted since their inception, weaknesses have been identified. For example, hypotheses embedded within the ecologically-based theories fail to predict which sex will be larger (Kruger 2005). When discussing RSSD, it is more appropriate to divide competing hypotheses into those exploring the evolution of large females or of small males, or selective pressures that caused both of these evolutionary changes simultaneously (Massemin *et al.* 2000, Pleasants 2011). In a 2011 study evaluating RSSD in raptors based on the ratio of egg size to adult body mass, it was determined that the evolution of RSSD in strigiformes is consistent with all three aforementioned pathways (Pleasants 2011). Sex-role differentiation and behavioral hypotheses both incorporate elements of such discussions. The sex-role differentiation posits that males have evolved to be smaller to allow for increased agility for hunting, while the behavioral hypotheses postulates that females tend to be larger than males in order to exert dominance over them (Kruger 2005, Meuller 1986).

While the evolution of RSSD among and within various avian species continues to be contested, a number of studies have been carried out to better understand the physiological mechanisms of nestling growth that determine patterns of RSSD during early development. It is assumed that breeding adults of sexually-size dimorphic species invest more energy and resources provisioning for the larger sex (Magrath *et al.* 2007). If sex-specific allocation of resources does indeed exist in sexually dimorphic species, it should be reflected in comparative analysis of nestling development based on body mass and other morphological features, where differences in body mass between sexes reflect sex-specific food allocation by adults (Magrath *et al.* 2007). For example, a study of sexually-size dimorphic American Kestrels (*Falco sparverius*), a species in which females are approximately 9% larger than

males, determined that adult females consumed 7% more food than males, implying that parents of this RSSD species supply more food to the larger sex (Anderson *et al.* 1993). In discussing food-allocation hypotheses as they relate to RSSD as with the previous example, it is assumed that both sexes expend the same amount of energy in competing for food, and that both sexes allocate food resources to body growth and development in the same physiological proportions (Magrath *et al.* 2007). Thus, differences revealed in body mass during the developmental period reflect differential parental food allocation by gender (Magrath *et al.* 1998). Recent comparative studies on sexually dimorphic birds have revealed a positive correlation between the degree of differential allocation of food resources by gender, measured by body mass, and the degree of dimorphism between the sexes (Krijgsveld *et al.* 1998; Magrath *et al.* 2007).

Gender is not the only important characteristic influencing development of avian species and ultimate morphological properties that affect adult fitness. Brood size may also mediate developmental patterns in species that exhibit RSSD, by affecting juvenile and adult survivorship and fecundity. Little is known about the long-term effects of brood size on fitness of individual offspring in avian species, but such effects can ultimately influence survival and lifetime breeding success of individuals (de Kogel 1997).

The ecological theory of maximum reproduction, initially developed by David Lack (1947), postulates that birds will rear the maximum number of offspring that they can adequately nourish in a reproductive season (Skutch 1985). The size of the brood reflects the maximum reproductive effort of a breeding pair. During every reproductive effort, adult breeders confront the trade-off between number and quality of offspring, as well as the trade-

off between current and future reproductive effort, when striving to fulfill maximum reproduction (de Kogel 1997).

Offspring quality is difficult to measure, but size and mass at fledging is often used as an indicator of juvenile quality (Both *et al.* 1998). In his development of the *rapid-gain model*, David Lloyd (1987) used mass at fledging compared to number of offspring in a given clutch to address the quality-quantity compromise. Lloyd's model, reflective of the theory of maximum reproduction, suggested that the amount of parental care determines offspring viability and ultimately adult fitness, so increased quality of smaller clutches that receive more individual care compensates for the smaller number of offspring (Lloyd 1987). The evolutionary ramifications of this trade-off should thus be seen in a common trend for juveniles in smaller clutches of birds to reach a greater asymptotic mass and development at fledging than juveniles from larger clutches, a phenomena supported by a 1997 study on zebra finches (*Taeniopygia guttata*) where individuals raised in smaller broods were consistently heavier and had longer flight feathers at 12 months of age (de Kogel *et al.* 1997).

The trade-off between current and future reproductive effort is one made by adult breeders but nonetheless affects and is affected by brood size. Reproduction is a costly energy investment; reproduction benefits adult breeders by generating offspring but has costs associated with increased adult mortality or reduced fitness in future reproductive efforts (Stearns 2000). Optimal reproductive investment models propose that in addressing the compromise between current and future reproductive events, individuals invest in reproductive efforts that maximize lifetime reproductive success, which as Lack (1947) proposed in the theory of maximum reproduction is most often an intermediate level of investment (Stearns 2000).

Two theories have been proposed that address the theory of maximum reproduction and environmental causes of variation of within-species clutch size, both of which guide individual decision-making in regards to the life-history trade-offs previously discussed: the food limitation and nest predation hypotheses (Stryksy *et al.* 2005). The food limitation hypothesis proposes that food supply available to nestlings determines the number of offspring that a female can successfully raise, thus fulfilling the theory of maximum reproduction (Stryksy *et al.* 2005). The alternate hypothesis, nest predation, posits that clutch size can be measured as a function of nest predation, where larger broods attract more predators and compromise each individual's prospects of survival. Therefore smaller clutches will be selected for when predation is greater (Stryksy *et al.* 2005).

These theories address the evolutionary mechanisms driving brood size selection, but do not address the proximate effects of brood size on juvenile body condition that subsequently influences lifetime fitness. It is important to understand this relationship, as well as the relationship between gender and nestling body condition, in order to better comprehend proximate effects of life history strategy on early development. By modeling growth patterns of Flammulated Owl (*Otus flammeolus*) nestlings according to gender and brood size, I attempted to explore these relationships.

In order to understand the correlation between gender and development, I quantified development by comparing growth through mass and remige measurements, using body mass and the 5th left primary wing feather as the basis of comparison. Knowledge of the details of growth is essential to better understand the proximate mechanisms of sexual dimorphism for any species wherein ontological development directly informs adult morphology (Badyaev 2002). This study builds upon previous work dedicated to quantifying differential nestling

growth patterns of male and female Flammulated Owls by expanding on the data set and generating new growth statistics (Becker 2008). I followed examination of RSSD in Flammulated Owls through development analyses by an analysis of development based on brood size.

I expected that the tradeoff between the number and quality of nestlings, determined by brood size and development rates of mass and flight feathers, and brood size and parental investment will be evident through a comparison of individual growth patterns of Flammulated Owl nestlings reared in broods of two and those reared in broods of three. The same proxies of development used in the comparative gender analyses were used in this analysis. Additionally, by focusing on nestling development, predictions can be made about lifetime success of individual owls based on brood size.

Flammulated owls were chosen for this study for a number of qualifying reasons, including the species characteristic of RSSD, within-species variation of brood size, and their status as an important avian species of montane forests. The Flammulated Owl is a small, migratory North American Strigiform with a breeding range that includes the Rocky Mountains in the late spring and summer (Linkhart *et al.* 1998). Flammulated Owls preferentially select healthy, old-growth ponderosa pine/Douglas-fir nesting habitats maintained by frequent low-intensity fires (Linkhart *et al.* 1998). An insectivorous species, Flammulated Owls rely almost exclusively on invertebrates for food (Reynolds and Linkhart 1987, Linkhart *et al.* 1998). During the breeding season, breeding males are the primary food provisioners, hunting invertebrates that are delivered to the nestlings and breeding female (Linkhart *et al.* 1998).

Flammulated Owls, like many raptorial species, display reversed sexual size dimorphism (RSSD) wherein adult females are larger than adult males. It has been estimated that adult female Flammulated Owls are approximately 15% larger than their male counterparts (Linkhart and Reynolds 2006). The brood size in Flammulated Owl nests typically consists of two or three nestlings, with mean number of fledglings in Colorado estimated at 2.4 nestlings per breeding pair as compared with an average initial clutch size of 2.7 nestlings (Powers *et al.* 1996, Reynolds and Linkhart 1992).

In this study I sought to investigate two facets of life history as it pertains to the breeding strategy of Flammulated Owls and corresponding costs of reproduction: gender of offspring and brood size. Developmental data on individual nestlings with known gender and brood size have been collected since 2003. I predicted that females would grow larger in both mass and remige and that broods of two would grow larger than individuals in broods of three based on differential parental investment. It is important to understand growth patterns of Flammulated Owls, for nestling development directly informs adult morphology and subsequent competitive ability and viability.

METHODS AND MATERIALS

STUDY AREA

All study areas where data were collected are located in Teller County, CO and contain mature montane forests (Fig..1). Most data used in this study were collected in the 5.5 km² Hotel Gulch and 6 km² Missouri Gulch study areas (Linkhart, *pers. comm.*). In 2004, data collection began in small patches of unburned forest within the 200 km² Hayman Fire study area, and collection began in the 4.5 km² Trout Creek study area in 2008 (Linkhart,

pers. comm.). In owl territories, four different types of forest composition dominate: mature ponderosa pine (*Pinus ponderosa*) mixed with Douglas-fir (*Pseudotsuga menziesii*) on southern slopes and ridgetops; Douglas-fir on east and west facing slopes; Douglas fir mixed with blue spruce (*Picea pungens*) on higher drainage slopes and north-facing slopes; and quaking aspen (*Populus tremuloides*) mixed with blue spruce in low drainage bottoms. Study areas range in elevation from 2550 to 2855 m (Linkhart and Reynolds 2007). Hotel Gulch and Missouri Gulch study areas fit the above descriptions neatly. The Hayman Fire study area differs from other study areas due to the effects of the 2002 Hayman Fire, which impacted over 560 km² (Graham 2003). Trout Creek is similar in forest type to the Hotel Gulch and Missouri Gulch study areas but is dominated by younger-growth trees (Linkhart, *pers. comm.*).

Juvenile and adult Flammulated Owls were captured and processed at their nesting sites in the four aforementioned study areas. Flammulated owls are obligate secondary cavity nesters, most often utilizing cavities previously excavated by Northern Flickers (*Colaptes auratus*) and less frequently other members of the woodpecker family (*Piciformes*) for nests (McCallum and Gehlbach 1988; Reynolds and Linkhart 1992). Active nests in the four study areas were located beginning in the late spring and early summer between 1981 and 2011 by systematically identifying and checking every available known cavity in the study areas using small cameras mounted on fiberglass Hasting's poles (Fuhrman Diversified Inc, Seabrook, TX; Sandpiper Tech., Manteca, CA). Nests were also located at night when specific behavioral cues were observed and owl activity, including vocalizations used in the vicinity of nests and prey delivery to nests, could be traced to a specific cavity wherein pairs

were nesting. Territory delineation was determined through spot mapping of territorial males at night.

CAPTURING AND PROCESSING NESTLINGS

From 2003 to 2011, nestling development was monitored on a nearly daily basis during the 23 day nestling period (between 9 and 20 days throughout the season) at 20 nests representing 35 nestlings in the Hotel Gulch and Missouri Gulch study areas that were less than 6m high, accessible by ladder, and relatively close to roads. Mass and remige measurements were collected at these nests until nestlings fledged. Once during the season nestlings were also banded and bled. This was performed only when nestlings had reached a sufficient level of development, when eyes were open, nestlings were alert to surroundings, and juvenile plumage for the most part had replaced the white natal down characteristic of hatchlings. At all other nests, representing 153 nestlings in total, mass and remige data were taken between one and seven times, at least once during the time of banding and bleeding.

When nestlings were less than eight days old, they were processed at dusk when the female had flushed to feed and defecate so as not to disturb the female's brooding behavior; when nestlings became more homeothermic, owlets were measured midday between 11 and 3 pm. Before the nestlings were large enough to band, they were differentiated by marking each with a distinctive color of permanent marker. A Pesola® spring scale was used to weigh each owlet to the nearest 0.1 g, and remige measurements were taken on the 5th and 6th primary feathers of the left wing to the nearest millimeter when feathers began protruding from the shaft. To maintain consistency, primaries were flattened against a small ruler for measurement and measured from the base of the ventral side to the distal end of the feather.

Blood was drawn from the brachial vein of the left wing, or right wing if initial attempt failed, using a capillary tube to collect the blood and suspended in a Tris-EDTA buffer solution until time of analysis (Seutin *et al.* 1991). At least 200 μ L of blood were needed for gender determination, so excess of this was collected.

DETERMINING OWLET GENDER

While the sex of adult Flammulated Owls can, for the most part, be accurately determined by mass and behavioral differences, the sex of nestlings cannot be determined morphologically (Schmid *et al.* 2004). Thus genetic analysis of blood samples obtained during fieldwork is the most reliable way to determine sex of nestlings. Like all avian species, Flammulated Owl sex is determined by a ZZ/ZW sex chromosome inheritance system (Schmid *et al.* 2004). Individuals that inherit a Z chromosome from each parent are homogametic males, while heterogametic females inherit one Z chromosome and one W chromosome (Schmid *et al.* 2004). DNA primers have been previously developed to recognize a specific sequence of an intron on the W or Z chromosome of flammulated owls and can be used to amplify these introns through the polymerase chain reaction (PCR) following isolation and purification of DNA from nestling blood samples.

During the 2003-2011 field seasons, blood was collected from all banded owls, adults and nestlings alike, from the brachial vein and suspended in Tris-EDTA buffer. Over the past 4 years, an attempt has been made to sex all nestling blood samples. Using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), genomic DNA was isolated from the blood. Successful isolation of DNA was confirmed through visualization by gel electrophoresis; 10 μ l samples of DNA were run with 1 μ l loading dye were run on a 1% 1M TBE agarose gel

(Fisher Scientific, Pittsburgh, PA) stained with ethidium bromide at 90 V for approximately 1 hour. Samples with genomic DNA produced a visible band. During the fall of 2011, I extracted DNA from 65 blood samples, 38 adults and 25 owlets. Since 2003, the gender of a total of 200 nestlings has been successfully determined.

After DNA was successfully isolated, gender was determined for the 25 2011 owlets and 4 2010 owlets for which gender was not successfully determined through PCR amplification of the W and Z introns and visualization through gel electrophoresis. Primers 2550F (5'-GTTACTGATTCGTCTACGAGA) and 2718R (5' - ATTGAAATGATCCAGTGCTTG) were ordered from Sigma-Genosys (St. Louis, MO) and used in the PCR reactions. Reactions were performed in 25 µl volumes, consisting of 2 µl of purified and isolated DNA, 6.5 µl GoTaq Colorless Master Mix (Promega, Madison, WI), 2 µl (40 pmol) 2550F Primer, 2 µl (40 pmol) 2718R Primer and 12.5 µl sterile water. These solutions were placed in a Thermo Cycler (Bio-Rad, Hercules, CA) and followed a standardized protocol for amplification (Bertrand, *pers. comm.*). Amplification of introns on the W and Z chromosomes generates products of two different sizes, a 600 base pair fragment for the W chromosome and a 1200 base pair fragment for the Z chromosome. The PCR products were resolved in 1% 1M TBE agarose gel stained with ethidium bromide. After running for 1 hour at 90 V, gels were visualized under UV light. Blood samples from heterogametic females generate two visible bands of DNA with fragment sizes of 600 bp and 1200 bp; blood samples from homogametic males generate one visible band of DNA at a size of 600 bp (Fig. 2). Personal accuracy was confirmed by running PCR on 38 adult samples for which sex had already been determined in the field based on physical characteristics and corroborating the results genetically; accuracy was determined to be 98.5%.

DEVELOPMENT ANALYSES AND CALCULATIONS

Mass development data were fitted to logistic growth curves using Wolfram Mathematica software (*Mathematica* 8.0, 2010). The logistic equation was used because it best reflects the sigmoidal pattern of avian growth from post-natal nestlings to adult morphology, and allows for determination of growth rate and the asymptote of the growth curve estimated from a large number of data points (Rickelofs 1984). Raw mass data on owlets of known gender and known brood size gathered from 2003-2011 were input into Mathematica, where the logistic equation of the line, R^2 values, and associated confidence intervals were determined for all female and all male nestlings as well as nestlings raised in broods of three offspring and broods of two offspring. Additionally, a comparison was made between males and females in differing brood sizes independently to explore the potential bias gender ratio plays in influencing broods of different sizes. The data best fit the logistic equation, the form of which is:

$$y = (KY_0e^{rt}) / (K + Y_0(e^r - 1))$$

where K equals the carrying capacity, or maximum mass, Y_0 represents initial mass of nestlings, and r is the rate of growth measured in % per day (Hastings 1997). After the raw data were modeled, it was logistically transformed by taking the natural logarithm of raw mass data and again fitted to the logistic curve. By taking the natural logarithm of the raw data, the variation of residuals was minimized and equivariance could be assumed. This assumption of equivariance was made after a histogram of the residuals showed a normal distribution. Variation of data points away from the logarithmic curve was larger during later days of the nestling period than earlier days before the data were transformed. Because of

this, the estimated parameters were more influenced by latter days in the untransformed data; logarithmically transforming the data helped reduce this discrepancy.

After logistically transforming the data, equation 1 above becomes:

$$y = (KY_0e^{rt}) / (K + Y_0(e^r - 1))$$

where e^K equals the carrying capacity and r remains the rate of growth measured in % per day (Hastings 1997).

For remige data, average length of the 5th left primary on each day, 1 to 23, of development rather than total raw data were used to find best-fit lines and corresponding R^2 values. Daily averages were first converted into % maximum of the maximum remige length reached during the nestling period. The equation of the line used is:

$$t_L = (1/k) \ln(L / (1 - L)) + t_i$$

where k is proportional to the average growth rate of the 5th left primaries, L is the % maximum length of the primary at time t_L (day of nestling period), and t_i is equivalent to the number of days in the nestling period prior to the time at which the % maximum length of the primary is exactly 50% (Ricklefs 1968). Results are given in the more general form of a line,

$$y = mx + b.$$

Standard two-tailed T-tests were used to determine whether there was a significant difference in development based on both mass and remige growth on a daily basis.

Comparisons between daily growth of males vs. females and broods of 2 vs. broods of three were performed on each day of the development period, and significance was defined as a P value ≤ 0.05 , with P values $0.05 \leq P \leq 1$ considered trending.

RESULTS

OWLET GENDER AND SEX RATIOS

Sex was determined for a total of 189 owlets from 2003 to 2011. Of the 189 owlets, there were 94 females and 95 males, and 9 with unclear or ambiguous PCR results for which sex could not be determined (Table 1). In the summer of 2011, I participated in the collection of blood samples from 29 of these owlets; in the fall I determined that 10 of these owlets were male and 19 were female. Additionally, I processed all nestling blood samples with unclear gender results since 2003. The results of gender data for all nestlings processed, including gender ratios by year (Fig. 3) and gender ratios in broods of two and broods of three, are summarized in Table 1.

Sample size per year ranged from 2 nestlings in 2003 to 29 in 2005 and 2011. For brood size analysis, a total of 194 nestlings were raised in a brood of known size. Sample size ranged from 5 nestlings in 2003 to 31 in 2010. In broods consisting of two owlets, there were a total of 21 females and 34 males, 38% and 62%, respectively. In broods consisting of three owlets, there were 50 females and 38 males, 57% and 43%, respectively. The differential growth rates of male and female owls may influence growth patterns observed in broods of varying sizes based on the disparate proportion of males and females represented in broods of two and broods of three.

COMPARATIVE DEVELOPMENT OF OWLETS BASED ON GENDER

The equation of the logistic line that best fits the raw data of female nestlings is:

$$y = 526.56e^{0.22t} / (58.51 + 9(-1 + e^{0.22t}))$$

with an asymptote reached at 58.51 g +/- 0.78 and a growth rate of 0.22 % day⁻¹ +/- 0.005, where growth rate refers to maximum rate of growth during the exponential growth phase. The R² value for the line was 0.98. Raw daily mass data for male nestlings generated the following logistic growth equation:

$$y = 360.19e^{0.26t} / (55.41 + 6.5 (-1 + e^{0.26t}))$$

with an asymptote reached at 55.41 g +/- 0.65, a rate of 0.26 % day⁻¹ +/- 0.006, and an R² value of 0.98.

When female raw daily mass data are logged, the logistic equation for the plot becomes:

$$y = 8.57e^{0.19} / (4.08 + 2.1 (-1 + e^{0.19}))$$

with an asymptote at 4.08 +/- 0.02, a rate of 0.19 % day⁻¹ +/- 0.004, and an R² value of 0.998 (Fig. 4). The asymptote of the transformed data was biologically significant based on the relationship that e^k is equivalent to the maximum asymptotic mass that the nestlings reach during the growing period. For female owlets, $e^{4.08} = 59.15$; the maximum mass reached by female nestlings is thus 59.15 g. For the logistically transformed male data, the equation becomes:

$$y = 7.17e^{0.25t} / (3.98 + 1.8 (-1 + e^{0.25t}))$$

with an asymptote at 3.98 +/- 0.01, a rate of increase of 0.25 % day⁻¹ +/- 0.005, and an R² value of 0.999 (Fig. 4). The asymptote corresponds to a maximum male mass of 53.52 g.

Development of the 5th left primary was analyzed using a straight line, $y = mx + b$, to fit the data based on logistically converted average daily remige lengths for each sex. Average growth of female primaries over the nestling period is best modeled by the following equation of the line:

$$y = 0.27x - 4.22$$

with an R^2 value of 0.93 (Fig. 6). Female owlets reached an average maximum remige length of 72.8 mm on day 25 of the nestling period. The slope of the equation is proportional to the rate of growth and thus indicates that female owlets exhibited a 5th left primary growth rate of 3.71 mm day⁻¹. Average growth of male primaries is best fit by the line:

$$y = 0.28x - 4.34$$

with an R^2 value of 0.97 (Fig. 6). Male owlets reached an average maximum remige length of 75 mm on day 26 of the nestling period. This date, however, falls later than the mean number of days typical of the Flammulated Owl nestling period (23 days) and may reflect a sample-size related bias in the data. Male feather growth rate was 3.57 mm day⁻¹. After fitting these lines to the logistically converted data, there appears to be no difference in rate of growth for primaries based on gender (Fig. 6).

Thus the data indicate that females reach a greater average maximum mass, but males grow at a faster rate during the exponential growth phase of development. Average daily mass was found to be significantly different on days 0, 1, and 3 (Fig. 5). Between days 21 and 23 and onwards, average male mass exceeded average female mass (Fig. 5). Female owlets had already reached maximum average mass by day 21, following which mass loss prior to fledging was observed. During this period, male owlets continued to gain mass, accounted for by the fact that males were on average heavier than females on these days. Remige length was significantly different on days 14, 15 and 23 of the nestling period. Beginning on day 20, male owlets had consistently longer 5th left primaries.

Owlets with daily mass and remige data were isolated from all data and compared based on mean maximum mass and mean fledge date. Nineteen female owlets with daily data

reached a maximum mass of 62.37 g +/- 4.48; nine male owlets with daily data reached a maximum mass of 61.56 g +/- 6.77 with no significant difference from females. Both genders had an average fledge date of day 23 of the nestling period.

COMPARATIVE DEVELOPMENT OF OWLETS BASED ON BROOD SIZE

Owlets known to be raised in broods of two best fit the following logistic growth equation:

$$y = 440.80e^{0.24t} / (55.10 + 8.0 (-1 + e^{0.24t}))$$

with maximum mass reached at 55.10 g +/- 0.97 at a growth rate of 0.24 % day⁻¹ +/- 0.008 and an R² value of line of 0.98. Owlets raised in broods of three best fit the following logistic growth equation:

$$y = 379.72e^{0.25t} / (58.42 + 6.5 (-1 + e^{0.25t}))$$

reaching an average maximum mass at 58.42 g +/- 0.53 at a rate of 0.25 % day⁻¹ +/- 0.003. with an R² value of 0.98.

When the data for owlets from broods of two are logistically transformed, the equation becomes:

$$y = 8.00e^{0.22t} / (4.00 + 2.0 (-1 + e^{0.22t}))$$

with an asymptote reached at 4.00 +/- 0.02, a growth rate of 0.22 % day⁻¹ +/- 0.007, and an R² value of 0.998 (Fig. 7). This asymptote corresponds to a maximum mass of 54.60. The logistically transformed three brood data has the following equation:

$$y = 7.29e^{0.22t} / (4.05 + 1.8 (-1 + e^{0.22t}))$$

with an asymptote reached at 4.05 +/- 0.01, a growth rate of 0.22 % day⁻¹ +/- 0.003, and an R² value of 0.998 (Fig. 7). This asymptote corresponds to a maximum mass of 57.30 g.

Analysis of development of the 5th left primary based on brood size using logistically converted average daily remige lengths resulted in the following equation for broods of two:

$$y = 0.25x - 3.98$$

with an R² value of 0.96 and a growth rate of 4.0 mm day⁻¹, and the following equation for broods of three:

$$y = 0.26x - 4.09$$

with an R² value of 0.99 and a growth rate of 3.85 mm day⁻¹ (Fig. 9).

Average owlet mass differed significantly between brood sizes on days 5, 8, 19 and 21, and trending on day 9 (Fig. 8). Prior to day 14, the average mass of owlets in broods of three was greater than the average mass of owlets in broods of two (Fig. 8). From day 14 until the end of the nestling period, owlets belonging to broods of three had consistently higher average masses (Fig. 8). Brood size resulted in a significant difference in the average length of the 5th left primary on days 10 and 18, with a trend towards this difference on day 19.

Eight owlets raised in broods of two had daily mass and remige measurements taken; these owlets had a mean maximum mass of 56.78 g +/- 6.69. 27 owlets with daily measurements had a mean maximum mass of 58.79 g +/- 4.63. There was no significant difference between these data. On average, owlets from broods of two and three with daily data fledged on day 23 of the nestling period.

SAME-SEX COMPARISON OF INDIVIDUALS FROM DIFFERENT BROOD SIZES

To address the potential bias gender ratio may play on comparative development based on brood size, males from broods of two were compared with males from broods of three, as

were females from both brood sizes. Table 2 shows the gender ratios corresponding to each brood size; broods consisting of two owlets were 38.2% female and 68.1% male, while broods consisting of three owlets were 56.8% female and 43.2% male. All data were logistically transformed before fitted to a curve. Data for males raised in broods of two best fit the following equation:

$$y = 8.30e^{0.22t} / (3.95 + 2.1 (-1 + e^{0.22t}))$$

with an R^2 value of 0.999 (Fig. 10). An asymptote was reached at 3.95 +/- 0.03 and the growth rate was 0.22 % day⁻¹ +/- 0.010 (Fig. 10). The asymptote corresponds to a maximum weight of 52.94 g. Data for males raised in broods of three was best fit by the following logistic equation:

$$y = 7.24e^{0.24t} / (4.02 + 1.8 (-1 + e^{0.24t}))$$

with an R^2 value of 0.999 (Fig. 10). The asymptote was reached at 4.02 +/- 0.02 and the growth rate was 0.24 % day⁻¹ +/- 0.005; the maximum weight is approximated at 55.70 g (Fig. 10).

Females raised in broods of two are represented by the following logistic equation:

$$y = 8.91e^{0.20t} / (4.05 + 2.2 (-1 + e^{0.20t}))$$

with an R^2 of 0.998, an asymptote at 4.05 +/- 0.03, and a growth rate of 0.20 % day⁻¹ +/- 0.011 (Fig. 11). The asymptote corresponds to a maximum mass of 57.30 g. Females raised in broods of three best fit the following equation:

$$y = 9.07e^{0.17t} / (4.12 + 2.2 (-1 + e^{0.17t}))$$

with an R^2 of 0.998 (Fig. 11). The asymptote was reached at 4.12 +/- 0.02 and the growth rate was 0.17 % day⁻¹ +/- 0.004 (Fig. 11). The asymptote reflects a maximum mass of 61.56 g.

The data show that in comparing development of owlets from broods of different sizes, both males and females in larger broods grow to a larger maximum mass but at a lesser rate.

DISCUSSION

Modeling the development of Flammulated Owl nestlings during the nestling period confirmed that females reach a higher asymptotic mass than males, while males grew at a faster rate. Contrary to expectations, development analysis revealed that broods consisting of three owlets reached a higher asymptotic mass than broods consisting of two owlets. The potential effect of gender ratio within broods in biasing results, where a higher proportion of females in a given brood size would result in higher overall average maximum masses, was addressed by comparing development of males from broods of two with males from broods of three, and by comparing females from broods of two with females from broods of three. The results of these comparisons again indicated that larger broods produce larger individuals even though the sex ratio between brood sizes was disproportionate. Growth of flight feathers did not differ considerably in any comparison.

Although maximum asymptotic mass at fledging differed between every subgroup analyzed, there was little evidence that average daily masses differed considerably between subgroups. Males and females only differed statistically in average mass on days 0, 1, and 3. Brood sizes differed in mass statistically only on days 5, 8, 19, and 21, and trended towards a significant difference on day 9. Because significant differences in adult morphology between genders is the norm in this RSSD species, a larger number of days where average daily mass differed significantly based on gender was expected. Similarly, because literature addressing

the effects of brood size on developmental patterns across avian taxa generally suggests that smaller broods produce larger, higher quality individuals, a larger number of days where mass differed significantly with the ultimate result being a higher maximum mass of owlets from broods of two was expected (Lloyd 1987). None of these predictions was supported by the data. This possibly could have resulted from a relatively small sample size, but may also show that general patterns in avian development are more clear when data is analyzed on a whole rather than on a daily basis.

Breeding strategy trade-offs, specifically regarding quality vs. quantity and current vs. future reproductive effort, that fulfill Lack's (1947) theory of maximum reproduction do not predict the finding that, reflective of parental investment, broods of three reach a higher asymptotic maximum mass than broods of two (de Kogel 1997, Skutch 1985). The observed divergence from these theoretically-based expectations in Flammulated Owl nestling development may, however, be addressed by a number of species-specific alternative breeding strategies and life history characteristics. For example, broods of three may be associated with higher quality males that can provide more resources and protection to offspring than males raising broods of two. Numerous studies have shown that older male breeders are associated with larger clutches and tend to nest earlier in the season and have higher fledging success (Ferrer and Bisson 2003). Alternatively, females associated with larger broods may be foraging more at the end of the nestling period when owlets are becoming more homeothermic and require less incubation. Under this latter condition, the greater energetic cost females exert during more intensive foraging may compromise future reproductive effort, but maximizes the current effort.

The quality of parental care in influencing brood size and offspring body condition has a number of potential correlates and ecological ramifications. It has been demonstrated that parental quality, as measured by hatching success and fledging success of offspring, is correlated to the age, and thus experience, of adult breeding Tawny Owls (*Strix aluco*) (Sasvari and Heygf 2002). Reproductive performance increased over successive breeding years, with female experience affecting the number of eggs and hatching success and male experience affecting fledging success (Sasvari and Heygf 2002). A similar phenomenon may be at play in the determination of Flammulated Owl brood size, where larger broods may be associated with higher quality and more experienced breeding pairs. A brood size manipulation study of the reversed sexually size dimorphic Tengelman's Owl (*Aegolius funereus*), however, demonstrated that brood size, along with longevity of breeding males, is most strongly correlated with territory quality rather than parental quality, where larger broods are produced in higher quality territories (Korpimaki 1988). Within Flammulated Owls, parental quality and territory quality likely have synergistic effects on brood size and fledging success. It must be noted, however, that the range of brood sizes produced by Flammulated Owls is quite small compared with many other raptorial species, and differences seen between broods of two and broods of three may be quite different from those seen between broods representing range of sizes in other species. Nevertheless it is important to further explore the possible reasons underlying the observed developmental patterns of Flammulated Owls.

Territory fidelity of male Flammulated Owls is high, with most males apparently occupying one territory over the entire course of their reproductive lives (Linkhart and Reynolds 2007). It is most commonly assumed that males of territorial species relocate to

higher quality territories when they become available; the high territory fidelity of Flammulated Owls counters this assumption (Linkhart and Reynolds 2007). However, increased familiarity of foraging, singing, and roosting sites may benefit males to a degree that outweighs the costs of occupying a territory that is perceived as less productive, especially because females and broods rely exclusively on the male for prey for the majority of the nestling period (Linkhart and Reynolds 2007). Therefore, male parental quality may be enhanced in successive breeding seasons as experience and territory familiarity becomes more pronounced. Thus parental quality and territory quality may be acting synergistically in determining patterns of brood size and nestling development of Flammulated Owls. In order to better understand these relationships, long-term studies looking at reproductive fitness of individual adults over their entire breeding lifetime are necessary to correlate parental quality and experience with brood and offspring size.

Historical theoretical work on brood size determination and evolutionary trade-offs predicts that smaller broods will be of higher quality, and parents will maximize lifetime fitness by rearing sub-maximum brood sizes that will be of higher quality long-term (Dijkstra *et. al* 1990). The data presented on Flammulated Owl nestling growth counter these predictions, but due to lack of lifetime data on individuals reared in different sized broods, it is unclear whether perceived quality based on fledging weight accurately reflects ultimate fitness and viability. Numerous brood size manipulation studies have been conducted to address lifetime fitness of offspring and parents associated with different brood sizes, generating contradictory results among different species. Most studies on avian development have shown that larger broods are associated with reduced body condition at fledging compared to smaller broods (Pettifor *et. al* 2001). However, there have been few studies that

look at the post-fledging success of individuals reared in different sized broods, which may not directly reflect perceived quality at fledging (Dijkstra *et. al* 1990). A strong, though nonlinear, positive correlation between body condition, measured as mass at fledging, and subsequent survival has been identified in numerous avian species (Pettifor *et. al* 2001). In order to better understand the relationship between fledgling body condition, represented by asymptotic maximum mass, and lifetime success of Flammulated Owls, long-term studies on individuals raised in different sized broods is necessary.

It is clear that differences in mass between male and female Flammulated Owls emerge during the nestling period, reflecting adult patterns of RSSD. It is also clear that brood size affects the growth patterns of nestling Flammulated Owls. However, these effects appear in a surprising pattern, where larger broods produce larger offspring. Additional studies on lifetime success of individual birds from different sized broods and the relationship between adult experience and brood characteristics are necessary in order to determine the evolutionary and life history ramifications of this pattern. It does appear that within breeding populations of Flammulated Owls in Colorado, there is a strong selection for larger broods and larger offspring.

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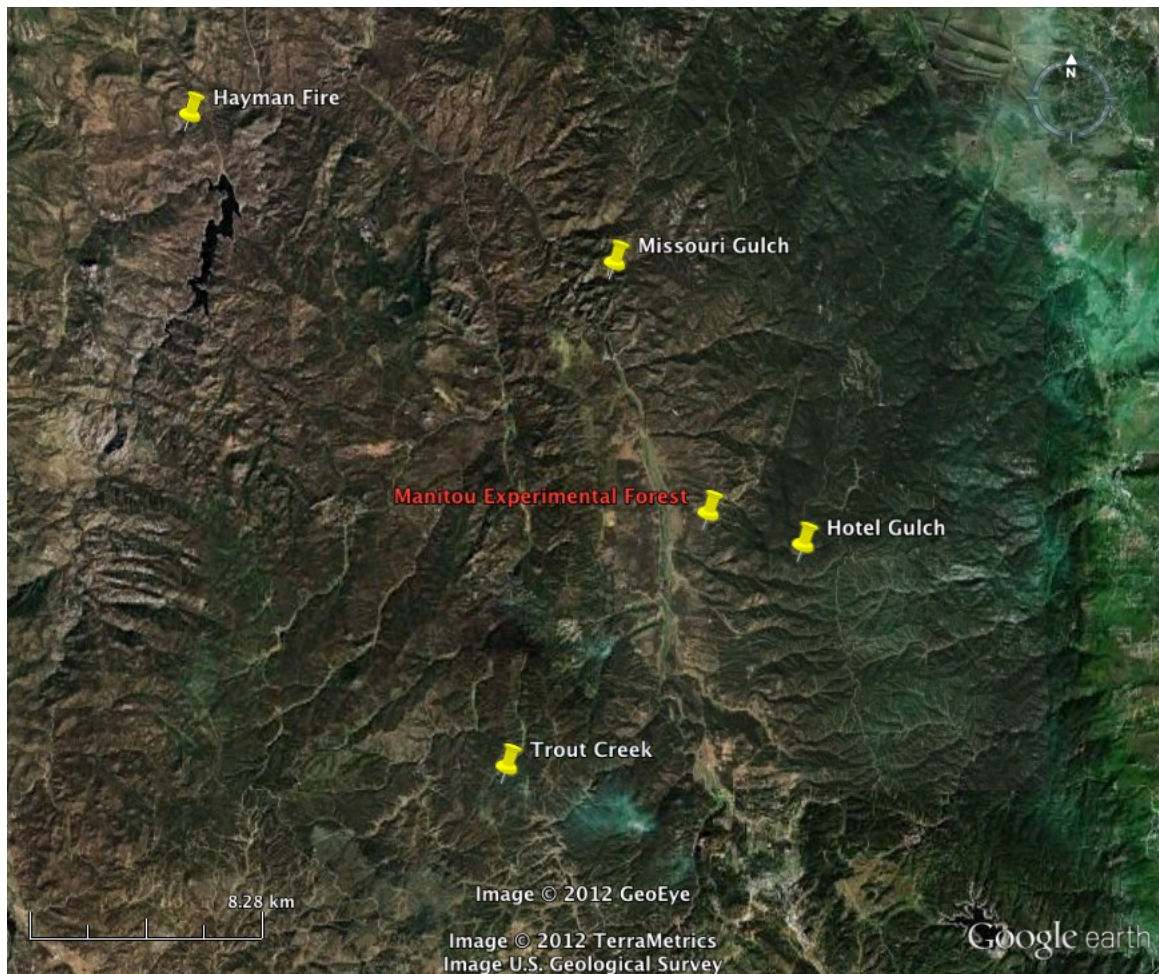


Fig. 1. Study areas in Teller and Divide Counties, CO. In Hotel Gulch and Missouri Gulch, breeding pairs of Flammulated Owls were identified and owlet data collected since 2003. Flammulated Owl studies began in Hayman Fire in 2004 and in Trout Creek in 2008.

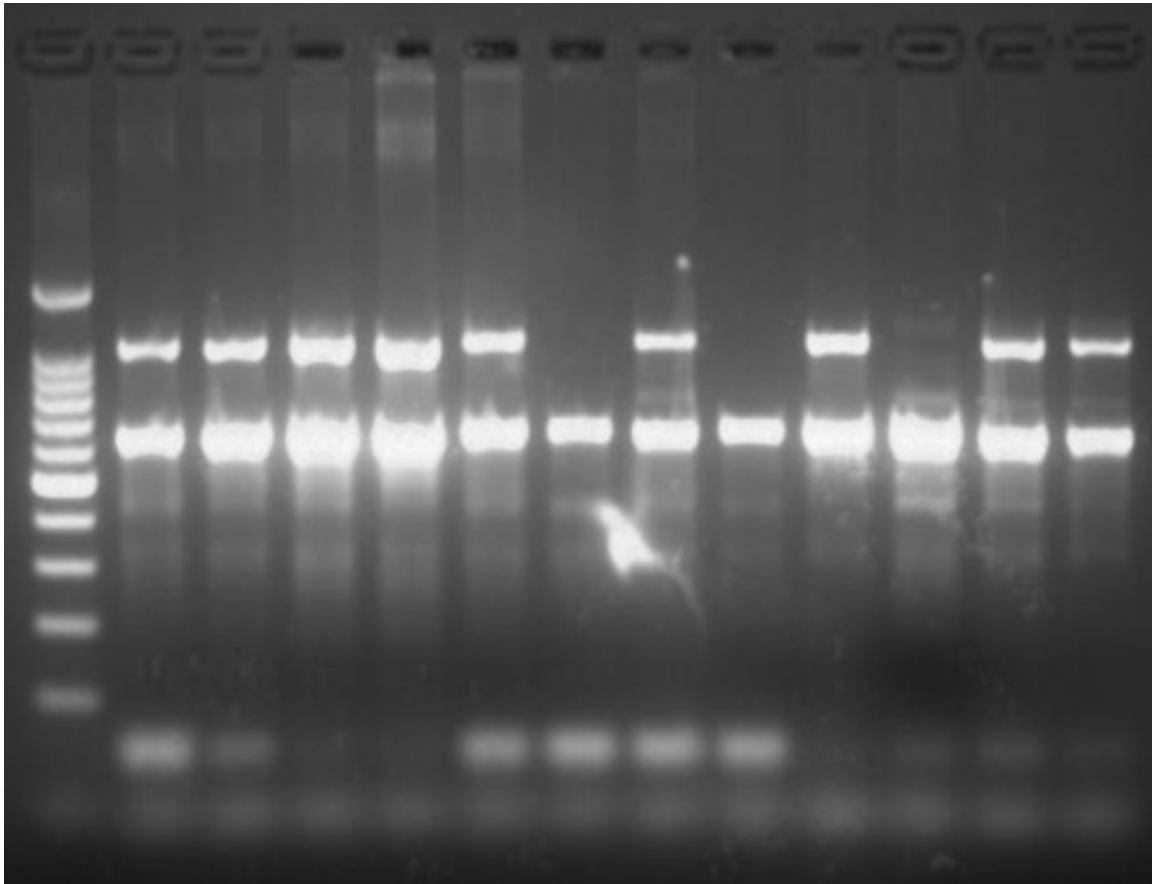


Fig. 2. Visualization of agarose gel displaying *ZZ/ZW* avian sex banding patterns used to identify gender of owlets from DNA extracted from blood samples; females are heterogametic *ZW* expressed as two distinct bands at 600 and 1200 base pairs, while males are homogametic *ZZ* expressed by a single band at 600 base pairs. The first well on the left represents the DNA ladder, with each band indicative of a certain number of DNA base pairs.

Table 1. Sample sizes and gender ratios of subgroups analyzed based on gender and size of brood.

	Males	Females	% Male	% Female
2003	3	2	60.00	40.00
2004	2	5	28.57	71.43
2005	14	15	48.28	51.72
2006	16	10	61.54	38.46
2007	14	8	63.64	36.36
2008	13	10	56.52	43.48
2009	9	8	52.94	47.06
2010	13	18	41.94	58.06
2011	10	19	34.48	65.52
Total	94	95	49.74	50.26
	Brood of 2	Brood of 3	% 3 Brood	% 2 Brood
2003	2	3	40.00	60.00
2004	2	10	16.67	83.33
2005	8	24	25.00	75.00
2006	15	10	60.00	40.00
2007	14	5	73.68	26.32
2008	5	16	23.81	76.19
2009	11	12	47.83	52.17
2010	17	14	54.84	45.16
2011	8	18	30.77	69.23
Total	82	112	42.27	57.73

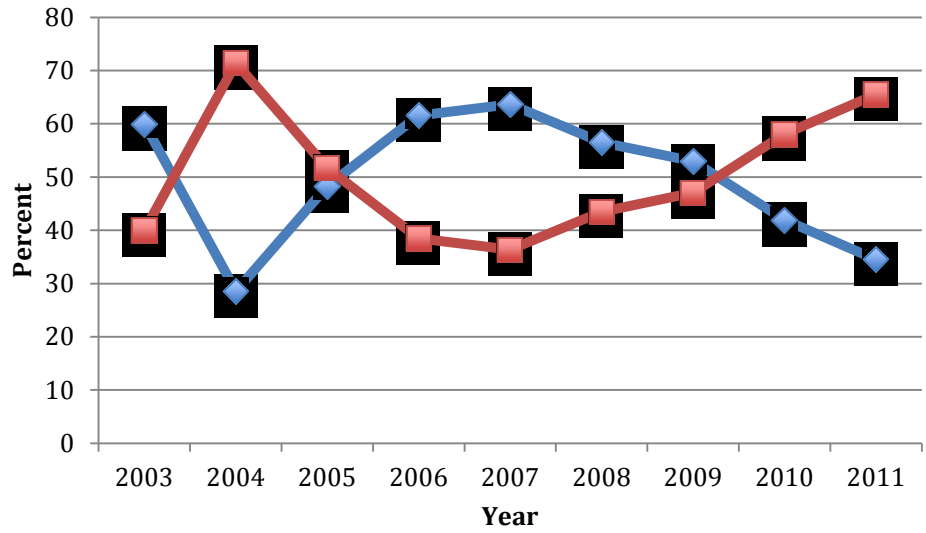


Fig. 3. Sex ratio by year of owlets processed from 2003-2011. Males are represented by blue diamonds and females by red squares. The overall sex ratio from 2003 to 2011 is 49.7:50.3 male to female.

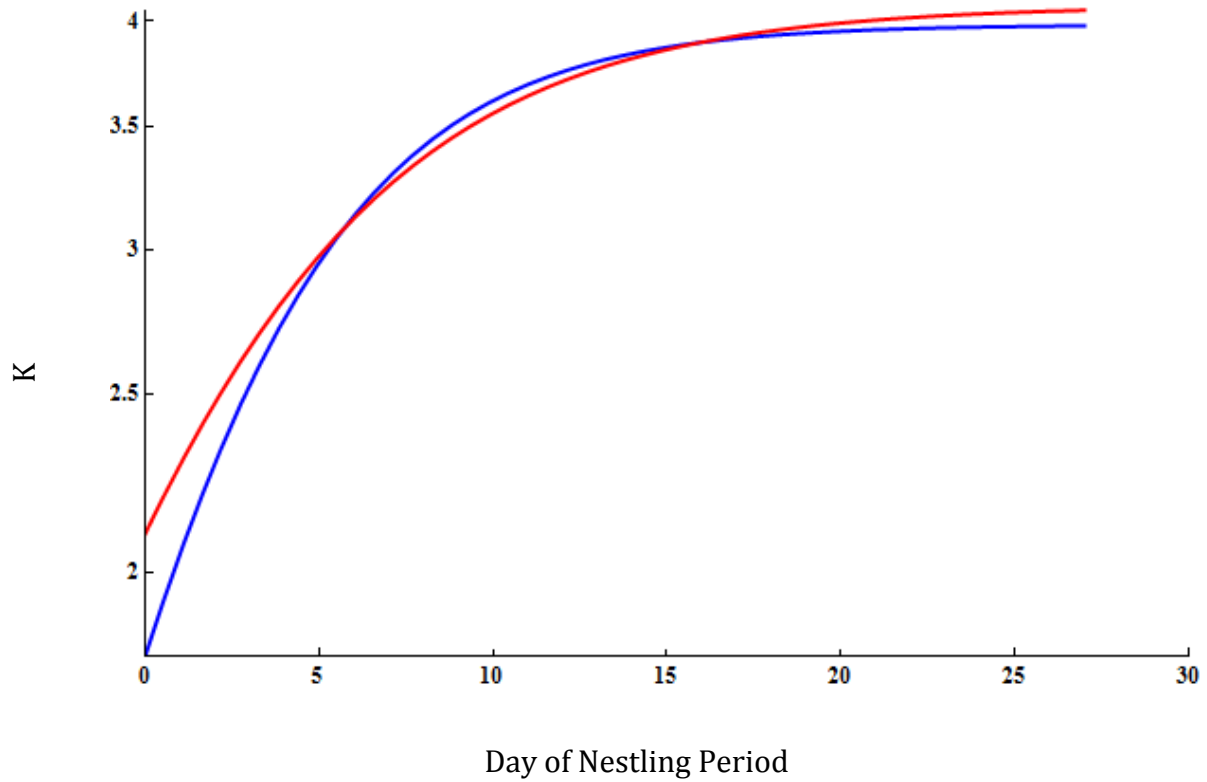


Fig. 4. Growth curves of male and female nestlings estimated from logistically transformed raw data. The blue line represents growth of male nestlings and the red line represents growth of female nestlings. Where K is maximum, e^K = maximum nestling mass in grams. Male nestling reached an asymptotic mass of 53.52 g at a rate of $0.25 \% \text{ day}^{-1} \pm 0.005$. Female nestlings reached an asymptotic mass of 59.15 g at a rate of $0.19 \% \text{ day}^{-1} \pm 0.004$.

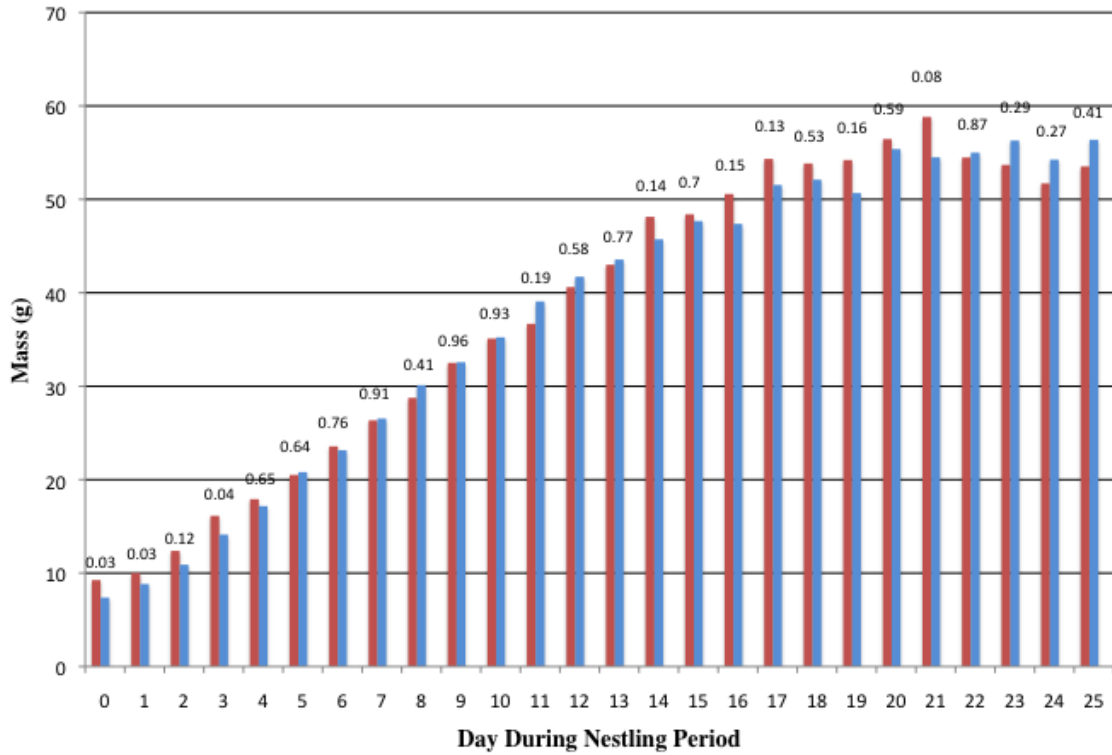


Fig. 5. Statistical significance of daily average mass of male and female nestlings with P-values shown above. Female nestlings are represented by red bars and males by blue bars. The difference in mass was significant on days 0,1, and 3. Females reached average maximum mass by day 21, after which males were heavier.

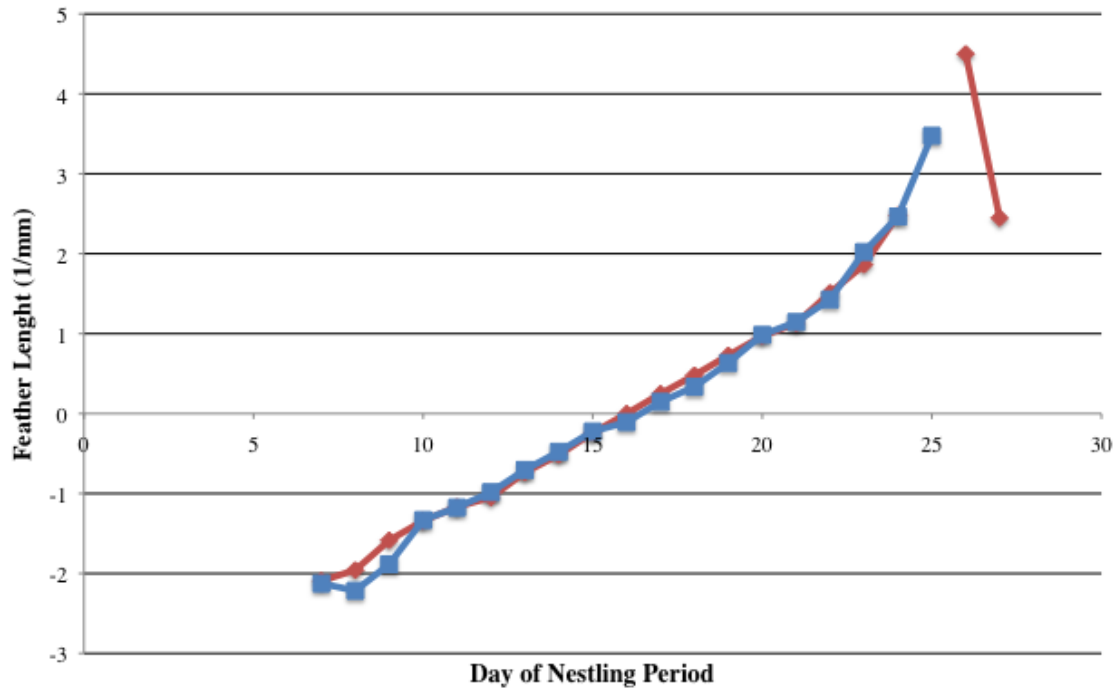


Fig. 6. Growth of male and female 5th left primaries. Males are represented by blue squares, females by red diamonds. Significant difference of remige growth was observed on days 14, 15, and 23.

Table 2. Proportion of males and females in broods of differing sizes

	Brood of 2	Brood of 3
Female	21	50
Male	34	38
% Female	38.18	56.82
% Male	61.82	43.18

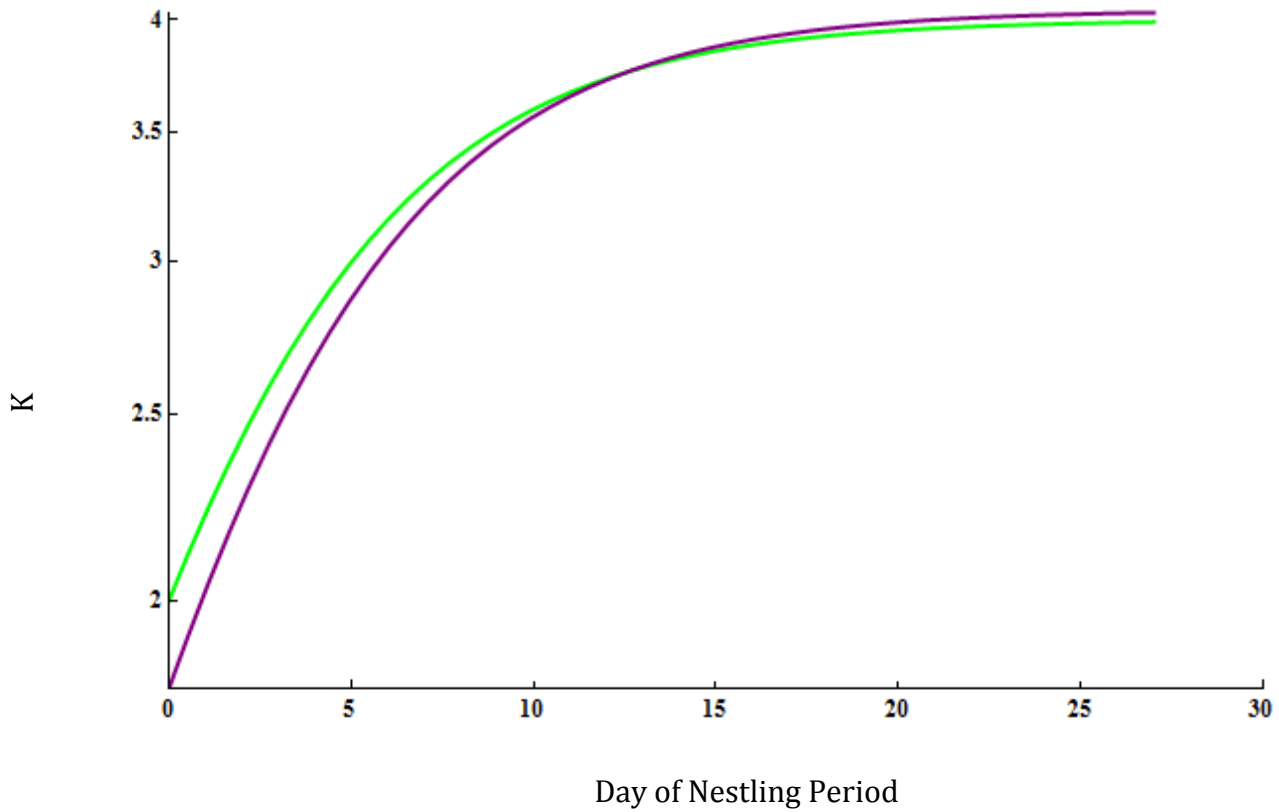


Fig. 7. Growth curves of nestlings in broods of 2 and broods of 3 offspring. Nestlings reared in broods of 2 are represented by the green line, those reared in broods of 3 by the purple line. Where K is maximum, $e^K =$ maximum nestling mass in grams. Nestlings reared in broods of 2 reached an asymptotic mass of 54.60 g at a rate of $0.22\% \text{ day}^{-1} \pm 0.007$. Nestlings reared in broods of 3 reached an asymptotic mass of 57.30 g at a rate of $0.22\% \text{ day}^{-1} \pm 0.003$.

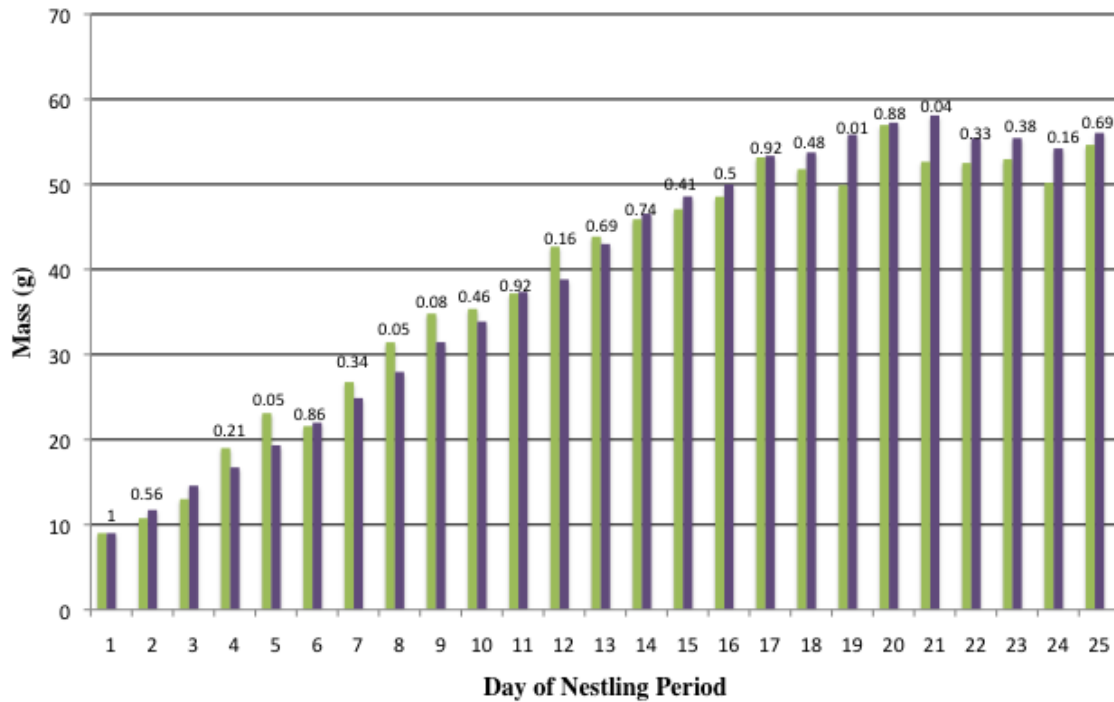


Fig. 8. Statistical significance of daily average mass of nestlings in broods of 2 and broods of 3 offspring with P-value and sample size. Daily mass averages of broods of 2 are represented by green bars, while daily mass averages of broods of 3 are represented by purple bars. Masses were significantly different on days 5, 8, 19 and 21, and trending towards significance on day 9. After day 14, owlets in broods of three had consistently higher masses.

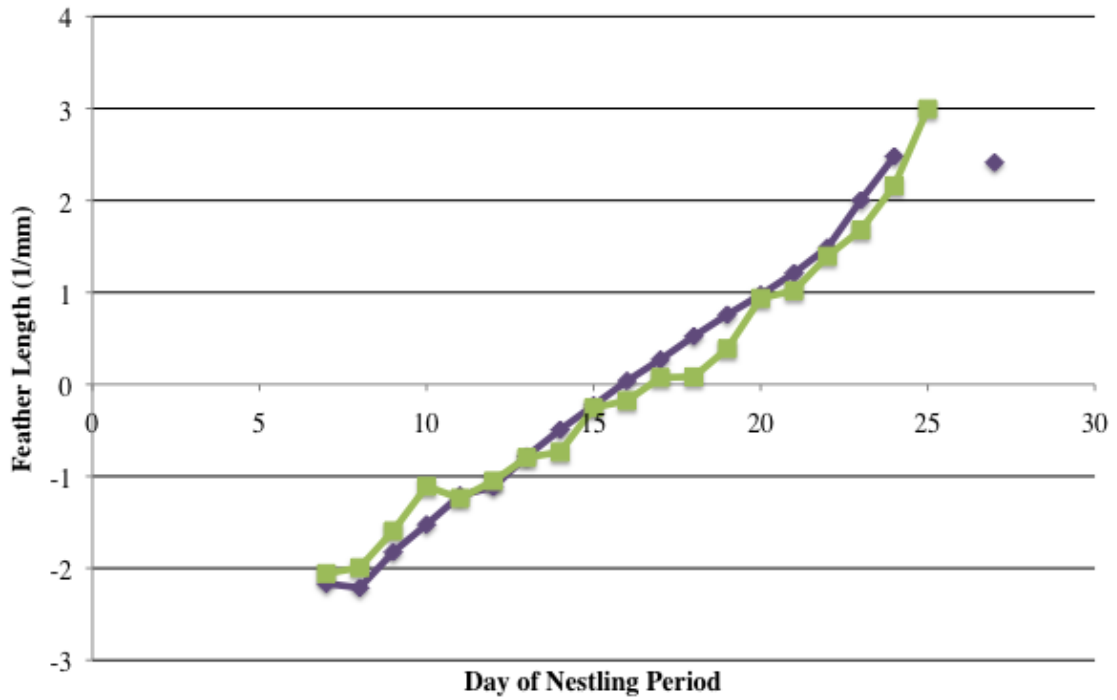


Fig. 9. Growth of 5th left primaries of nestlings from broods of 2 and broods of 3. Nestlings from broods of 2 are represented by green squares; nestlings from broods of three are represented by purple diamonds. Feather lengths were significantly different on days 10 and 18.

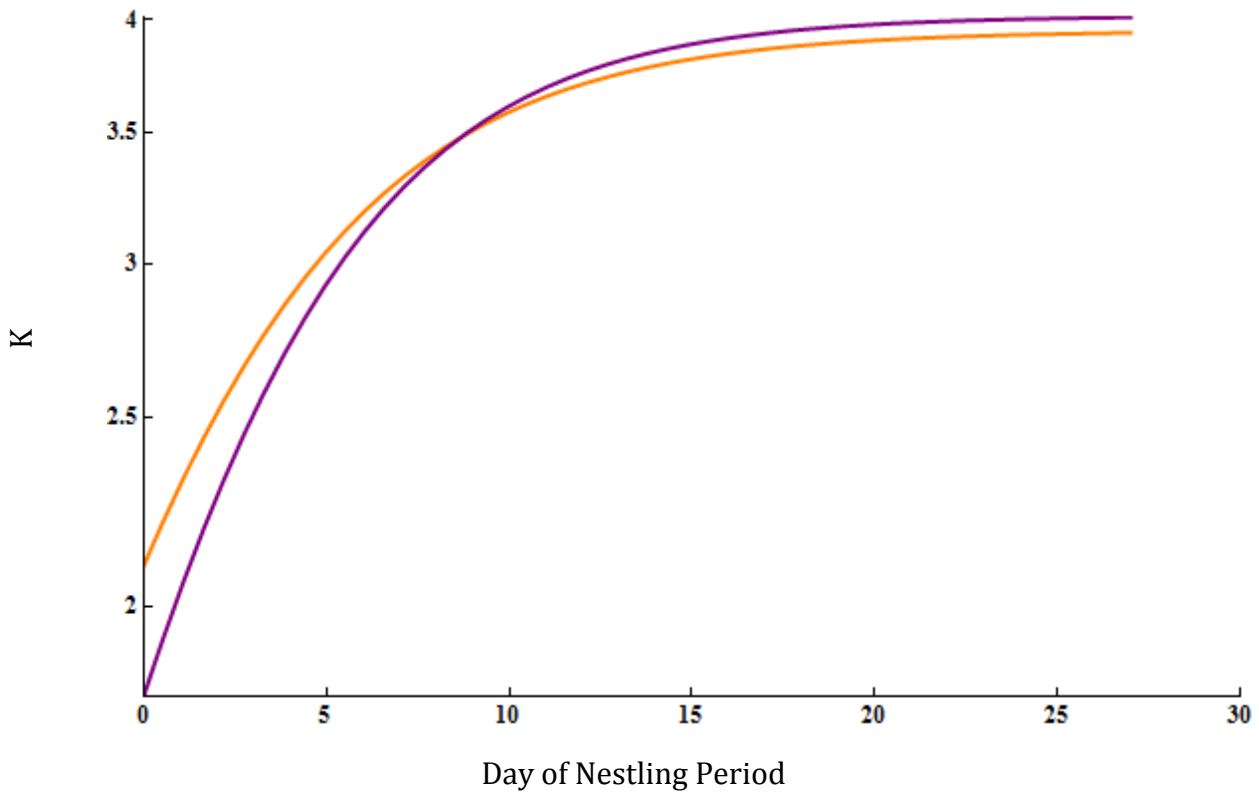


Fig. 10. Growth of male nestlings from broods of different sizes. The orange line represents male nestlings reared in broods of 2; the purple line represents male nestlings reared in broods of 3. Where K is maximum, e^K = maximum nestling mass in grams. Male nestlings from broods of 2 reached an asymptotic mass of 52.94 g at a rate of $0.22\% \text{ day}^{-1} \pm 0.010$. Males from broods of 3 reached an asymptotic mass of 55.70 g at a rate of $0.24\% \text{ day}^{-1} \pm 0.005$.

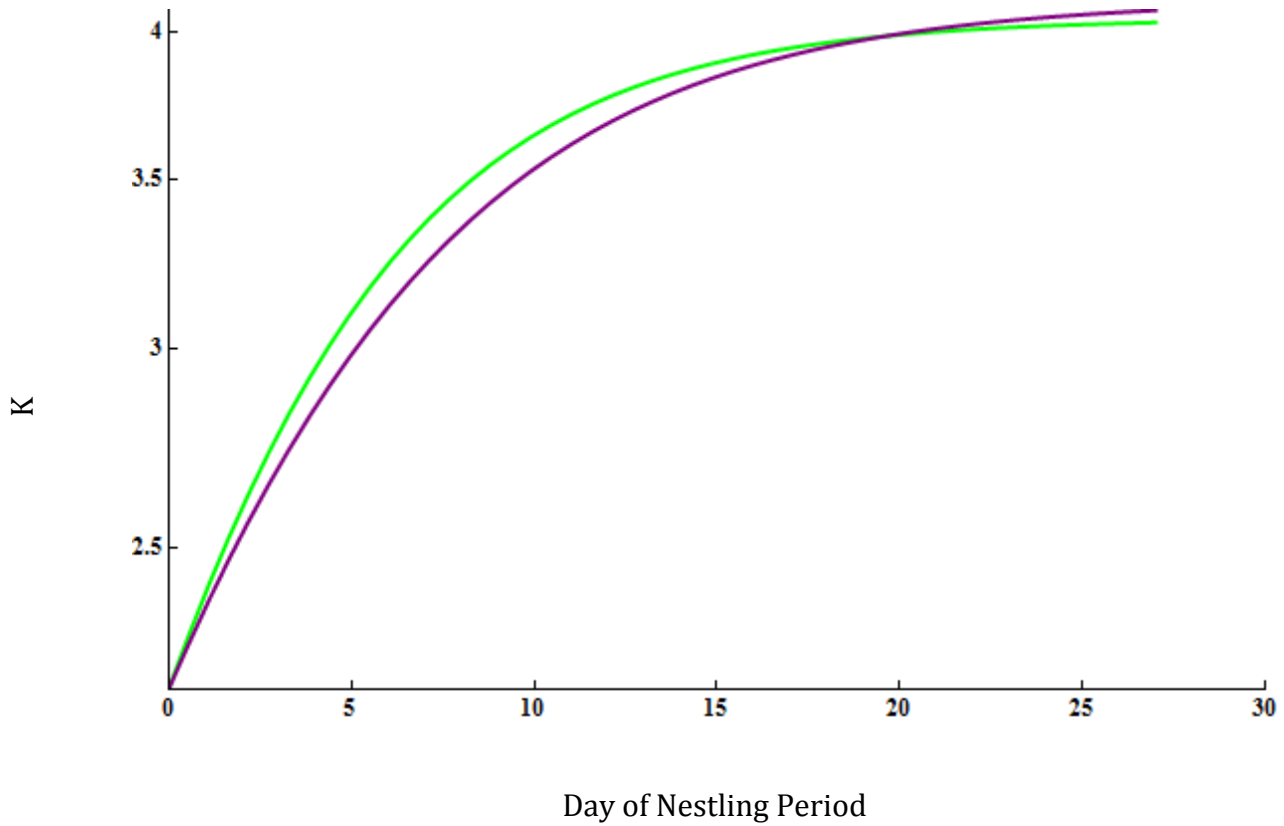


Fig. 11. Growth of female nestlings from broods of different sizes. The green line represents female nestlings reared in broods of 2; the purple line represents female nestlings reared in broods of 3. Where K is maximum, e^K = maximum nestling mass in grams. Female nestlings from broods of 2 reached an asymptotic mass of 57.20 g at a rate of $0.20\% \text{ day}^{-1} \pm 0.011$. Females reared in broods of 3 reached an asymptotic mass of 61.56 g at a rate of $0.17\% \text{ day}^{-1} \pm 0.004$.