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Utilization of energy substrates during calling activity in tropical frogs

Received: 7 January 1997 / Accepted after revision: 20 July 1997

Abstract Calling activity in frogs is energetically demanding to males because they usually perform at or near their physiological capacities. Metabolic fuel for muscle contractions during bouts of aerobic calling activity comes from carbohydrates and lipids that are stored in the trunk muscles. I monitored nightly calling performance in males of seven tropical frog species from two families, Hylidae and Leptodactylidae, and compared levels of glycogen and lipid in the trunk muscles from males collected before and after a three-hour period of calling activity. Trunk muscles from late-evening males in five species had up to 63% less glycogen than the trunk muscles from early-evening males; relatively little depletion was observed in two other species. Overall, glycogen reserves and rates of depletion were highest in species with very high calling rates. It was not possible to measure changes in the relatively large stores of lipid in the trunk muscles after only 3 h of calling. Nevertheless, intramuscular lipid stores probably provide a greater percentage of the energy needed for sound production than glycogen stores, and are largest in species with high calling rates.

Key words Amphibia · Anura · Reproduction · Energetics · Glycogen

Introduction

Male frogs attract females with vocalizations produced in part by contractions of the trunk muscles (external and internal obliques). Studies of the ultrastructure of these aerobic muscles show that, in contrast to highly glycolytic muscles involved in locomotion, they are composed entirely of fast-twitch oxidative fibers and rely principally on carbohydrates and lipids stored within the muscle to fuel contractions (Marsh and Taigen 1987; Pough et al. 1992). The fast oxidation rate and high-energy yield of glycogen, combined with the lower, sustainable rate of energy generation from lipids (Hochachka and Somero 1984) may, therefore, enable male frogs to maintain high rates of calling for several hours over several days during the breeding season. Frog choruses generally are most active during the early evening hours, rarely extending beyond midnight (e.g., Kluge 1981; Schwartz and Wells 1985; Cardoso and Haddad 1992; Runkle et al. 1994). In the tropics, environmental conditions remain relatively constant throughout the night and there is no obvious abiotic explanation for such limited chorus activity. Nightly calling performance by male frogs, however, may be energetically limited because they can store only a certain amount of carbohydrate and lipid in the trunk muscles to be used for sound production (Pough et al. 1992).

In vertebrates, sustained aerobic exercise often results in depletion of glycogen from working muscles, which can affect both muscle contraction and behavioral performance (Eichelberg and Obert 1976; Ianuzzo et al. 1986; Hoppeler and Billeter 1991; Fournier and Weber 1994; Schwartz et al. 1995; Wells et al. 1995; Wells and Bevier 1997). For example, rats exercised to exhaustion exhibited significant reductions of glycogen in aerobic muscles such as the diaphragm, intercostals, plantaris, and heart (Ianuzzo et al. 1986). In frogs, 4 h of stimulated contraction of laryngeal muscles resulted in significant declines of both glycogen and fat (Eichelberg and

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Obert 1976), and 2 h of natural calling activity resulted in significant glycogen depletion in the aerobic trunk muscles of the gray treefrog, *Hyla versicolor* (Wells et al. 1995).

Intramuscular lipid also contributes significantly to fueling call production in many frogs (Taigen et al. 1985; Marsh and Taigen 1987; Pough et al. 1992; Wells et al. 1995; Grafe 1996, 1997; Ressel 1996; Wells and Bevier 1997) but may not be measurably depleted on an hourly basis. Lipid reserves often are much larger than glycogen stores in vertebrate oxidative muscle fibers (Conley 1994). For example, fish store lipids in large quantities and, in high-performance fish, lipids represent a more important source of energy for sustained swimming than carbohydrates (Weber and Zwingelstein 1995; Weber and Haman 1996). Mammals also tend to store the majority of energy reserves as lipids (Pond 1981; Hochachka and Somero 1984; Bjorken 1986). In mammalian endurance exercise, the ability to use lipid as fuel is an important mechanism that postpones glycogen depletion and fatigue (Holloszy et al. 1986; Weber 1992). In fact, during early stages of highest-intensity aerobic work, at least in humans, glycogen and fat are simultaneously mobilized to obtain high and sustainable rates of energy generation. As endogenous glycogen stores are depleted, however, the importance of fat as an energy source rises and power output is reduced until, in long-duration human aerobic exercise, it is the sole fuel being utilized (Hochachka and Somero 1984).

Previous reports on many of the frog species included in the present study show that trunk muscles exhibit variation in aerobic characteristics (Bevier 1995; Ressel 1996). This variation is reflected in calling rate. The rate of call production, in turn, reflects the rate of muscle contraction, and is closely correlated with oxygen consumption in at least two neotropical species, *Hyla microcephala* (Wells and Taigen 1989) and *Physalaemus pustulosus* (Bucher et al. 1982). Neotropical frog species with high calling rates have trunk muscles with high mitochondrial densities and enzyme activity levels of catabolic enzymes such as citrate synthase. In contrast, species with lower calling rates have trunk muscles with relatively low mitochondrial densities and catabolic enzyme activity levels (Bevier 1995; Ressel 1996). These patterns suggest that metabolic fuel stored in trunk muscles is used to support calling activity, and concentrations may be related to calling performance.

The objective of this investigation was to examine variation in calling activity of several neotropical frogs and to relate this behavioral variation to differences in storage and use of glycogen and lipids in the muscles involved in call production. For example, species with high calling rates exercise their muscles at higher rates and should therefore deplete fuel reserves in those muscles more rapidly than species with low calling rates. The species included in this study, as in prior studies, live and reproduce in the same tropical environment but exhibit variation in both behavior and aerobic capacity of the trunk muscles.

Five species of the family Hylidae, *Hyla ebraccata*, *H. microcephala*, *Phrynohyas venulosa*, *Scinax* (= *Ololygon*) *boulengeri*, and *S. rubra*, and two species of the family Leptodactylidae, *Physalaemus pustulosus* and *Leptodactylus labialis*, were studied. All of these species breed in close proximity in Panama during the wet season and are active at temperatures of 24–26 °C; most use temporary ponds and shallow pools for breeding. The hylids are a particularly useful group for this study because most of the published work on calling energetics has been done on this family, including several of these same species (Pough et al. 1992; Bevier 1995, 1997; Ressel 1996). They also exhibit an unusually large diversity of calling strategies, nearly as great as that observed among all anuran amphibians. The two leptodactylids were included because they represent another family in which males call under similar conditions as the hylids. One of these, *P. pustulosus*, has been the subject of extensive behavioral and energetic studies (Bucher et al. 1982; Ryan et al. 1983; Ryan 1985).

Detailed information on the phylogenetic relationships of all species in this type of comparative study is ultimately required for a full analysis of variation in substrate use because closely related species may share behavioral or physiological traits simply because of their descent from a common ancestor (Felsenstein 1985; Harvey and Pagel 1991; Harvey 1996). Unfortunately, the relationships of the frogs in this study are poorly understood. Hylids and leptodactylids are non-ranoid, neobatrachian (derived) anurans, but the phylogenetic relationships of the neobatrachian families are poorly resolved (Ford and Cannatella 1993). *Hyla*, *Scinax*, and *Phrynohyas* are all in the subfamily Hylinae, but the monophyly of that group is questionable, and the precise relationships among genera are uncertain (Duellman and Wiens 1992). *Leptodactylus* and *Physalaemus* are thought to be relatively closely related genera within the large family Leptodactylidae, which may not be a monophyletic group as presently constituted (Heyer 1975; Ford and Cannatella 1993). Because a fully resolved phylogeny is not available for these species, I have tried to minimize the effect of phylogeny by making comparisons within families, and in some cases, within genera, as well as for all the species combined.

Materials and methods

Behavioral observations

Field work was conducted in Panama in June–August 1991, May–November 1992, and May–August 1993. These periods coincided with the tropical rainy season (late April–December) and the breeding season for most local frog species. The two study sites were a flooded field in Gamboa, where several other investigations on calling behavior have been conducted (e.g., Wells and Schwartz 1984), and a small pond in Summit Gardens about 9 km away. *H. ebraccata*, *H. microcephala*, *S. rubra*, *L. labialis*, and *P. pustulosus* were studied in the Gamboa field, and *Phr. venulosa* in a flooded gravel pit about 200 m away. All species except *L. labialis* are ex-

clusively nocturnal and observations of individual males were made between 1830 and 0300 h when temperatures ranged from 22–28 °C (average nightly temperature = 25.3 °C, SD = 1.1 °C, $n = 140$ nights). Male *L. labialis* were observed during periods of calling activity, between 1400 and 2400 hours. The activity of a population of *S. boulengeri* was monitored at a man-made pond at Summit Gardens between 1800 and 2400 h. Detailed descriptions of the study sites are reported in Bevier (1995).

Average nightly calling periods and variation in hourly calling rates were determined for six of the seven species by monitoring and repeatedly recording vocalizations of focal males throughout entire evenings of calling activity. Calling rates of males in the field were measured, either manually or with a Marantz PMD201 cassette recorder and a Realistic 33-1062 ultra-directional microphone, for 5–10 min periods two to four times each hour. The recordings of vocalizations were made between 1830 and 0200 hours and represent repeated samples of the same males throughout the night. Average calling rates for each species were determined from random, single-sample recordings of male vocalizations obtained throughout the entire study period. Activity of chorusing male *Phr. venulosa* was not possible to monitor adequately during their brief breeding season (three nights in 1992), so little information on the calling period for this species was collected. Male *L. labialis* were very difficult to follow for the duration of their calling bout, so five males were collected on different nights, allowed to acclimate in a large aquarium, and calling activity was monitored on the following day. Calling activity of two of the frogs was monitored from an adjacent room. Vocalizations of other males were continuously recorded for up to 8 h using a Uher tape recorder, and calling rates were determined from the recording later. Data from the five focal males were combined with calling rates from males observed for at least 1 h in the field to determine variation in nightly calling activity for *L. labialis*.

Finally, patterns of chorus attendance by males of the six species were studied using a mark-recapture census technique over seven months in 1992. To census a population, each calling male was located and captured. Body mass and snout-vent length were measured, then the frog was individually toe-clipped or identified if it was already marked. The frog was then released back to the chorus. These handling and census techniques are similar to those suggested by Heyer et al. (1994). Except for a 14-day period in early August, censuses were conducted regularly on at least four nights per week during the entire breeding season for each species. Male *H. ebraccata* were censused on 59 of 128 nights (46% of nights, 12 July–16 November), *H. microcephala* on 108 of 178 nights (61% of nights, 23 May–16 November), *S. boulengeri* on 135 of 203 nights (67% of nights, 4 May–22 November), *L. labialis* on 91 of 198 nights (46% of nights, 3 May–16 November), and *P. pustulosus* on 90 of 160 nights (56% of nights, 10 June–16 November). Choruses of *S. rubra* were episodic but censused when active on 41 of 169 nights (24% of nights, 8 May–23 October).

Tissue collection

Early-evening muscle tissue samples were collected just as chorus activity commenced (between 1800 and 2000 hours) from up to 21 male frogs of each species over several nights in 1991, 1992, and 1993. Up to three males of one species were collected for an early-evening sample on a given night. Each frog was captured by hand, placed on ice, immediately sacrificed by double-pithing, then dissected within 1 min to ensure that tissue glycogen levels did not change after the frog was captured. The internal and external obliques (trunk muscles) and liver were immediately frozen, along with the remaining carcass, in liquid nitrogen. Back at the chorus, calling activity of up to three males of the same species were then closely monitored for 2–3 h for a corresponding late-evening sample. These frogs were collected while they were still calling to avoid losing them if activity ceased for the night, and to standardize interspecific comparisons. The frogs were sacrificed, dissected, and frozen as described above. Tissue samples were transferred to a freezer and stored at –80 °C. To transport the samples to the

United States, the tissues were stored in a cooler with dry ice for 24 h, then placed in a freezer at –80 °C until assays were performed.

Glycogen assay

Half of the trunk muscles and the entire liver from each frog were removed from the freezer and weighed to the nearest 0.01 mg on a Sartorius analytical balance, then homogenized in 0.6 M cold perchloric acid on ice with a glass-glass homogenizer following methods of Keppler and Decker (1974) and Marsh and Dawson (1982). A portion (0.2 ml) of the crude homogenate was removed and used for glycogen digestion by amyloglucosidase to determine total glucose content. Supernatant from the remaining homogenate was neutralized and used to determine free glucose content. The concentration of glucose in the tissue was measured spectrophotometrically with a Gilford model 260 UV/VIS spectrophotometer equipped with a sipper unit and circulating water bath. Assays were conducted at 25 °C using a Sigma Glucose Diagnostic Kit (510A) at 450 nm, and two replicates for each tissue sample were analyzed. Values for each replicate were within at most 10% of each other. Mass-specific glycogen content of each tissue sample was calculated by subtracting free glucose from total glucose. The measurement was then converted from glycosyl units ($\mu\text{mol glycosyl units/g}$) by multiplying by the molecular weight of a glycosyl unit in glycogen (0.162 mg/ μmol).

Lipid extraction and stomach content analysis

The other half of trunk muscle tissue in some frogs was used to determine lipid content, following methods of Given (1988). The tissue was weighed, dried in an oven at 50 °C to constant mass, placed in petroleum ether for 24 h to remove triglycerides, then dried again to constant mass. This procedure was repeated until there was no change in mass. The amount of lipid in the tissue was determined as total mg and as the percentage of dry trunk muscle mass. The same procedure was used on the remaining frog carcasses. No fat bodies or other depots were observed.

Finally, the digestive tracts of several individuals of each species were examined. All bits of matter were removed from the stomach and dried to constant mass to quantify food intake. Food items were recognizably insects, but more specific identification was impossible because items were small and partially digested.

Estimation of relative utilization of glycogen and lipids

Average hourly calling rates, determined as described above, were combined with data on oxygen consumption and glycogen depletion during calling to estimate the extent to which two species, *H. microcephala* and *P. pustulosus*, rely on glycogen and lipids to fuel an hour of call production. The average rates of oxygen consumption during calling were calculated according to the following equations derived from previous measurements of oxygen consumption. For *H. microcephala* (Wells and Taigen 1989):

$$\dot{V}O_2 (\text{ml } O_2/\text{g h}) = 0.18 + 0.0004 \times 3132 \text{ notes/h}$$

For *P. pustulosus* (Bucher et al. 1982; K.D. Wells and T.L. Taigen, reported in Pough et al. 1992):

$$\dot{V}O_2 (\text{ml } O_2/\text{g h}) = 0.195 + 0.000656 \times 1680 \text{ notes/h}$$

Average body mass for frogs measured in the field (*H. microcephala*, 0.61 g; *P. pustulosus*, 1.69 g) was used to calculate hourly oxygen consumption and this figure was multiplied by 3 to determine the total amount of oxygen consumed in a 3-h period. This value was then divided by a conversion factor of 0.84 ml O_2 /mg of glycogen (Schmidt-Nielsen 1979) to estimate the amount of glycogen required to support calling activity if all energy came from glycogen. The actual amount of glycogen used and oxygen required

for glycogen oxidation were compared to this value. Any oxygen consumed that was not used to oxidize glycogen was presumably used to oxidize lipid; oxidation of each mg of lipid requires 2 ml O₂ (Schmidt-Nielson 1979) and yields 39.3 J of energy. Thus, the relative energetic contributions of glycogen and lipid to call production were estimated for the two species.

Results

Calling activity and chorus tenure

Nightly calling periods and average calling rates for each species are shown in Table 1. Calling rates taken throughout the night from focal males were pooled and categorized in 15-min intervals (Fig. 1a,b,c). *L. labialis* males called throughout the afternoon and evening, but data taken only after 1830 hours in the field and in the laboratory were included here. Average calling rate of male frogs sampled at 15-min intervals declined significantly throughout the night in four species, *S. boulengeri* (Spearman rank correlation, $r_S = -0.98$, $P < 0.001$, $n = 23$ males), *S. rubra* ($r_S = -0.56$, $P = 0.004$, $n = 24$ males), *L. labialis* ($r_S = -0.53$, $P = 0.010$, $n = 5$ males), and *P. pustulosus* ($r_S = -0.93$, $P < 0.001$, $n = 26$ males). Average calling rate for *H. ebraccata* decreased only slightly over time ($r_S = -0.36$, $P = 0.087$, $n = 19$ males). In contrast, average calling rate for *H. microcephala* slightly but significantly increased with time ($r_S = 0.42$, $P = 0.040$, $n = 25$ males), and peak calling activity occurred around midnight. Among the hylids, *H. microcephala* had the highest average calling rate, whereas *L. labialis* had the highest and *S. boulengeri* had the lowest average calling rate among all species (Table 1).

In general, individual male frogs were observed calling in the choruses on only a few nights during the breeding season (Fig. 2). Some male *S. boulengeri* were present on up to 38% of the census nights. In fact, several males were observed in the chorus on over 40 nights during the 7-month breeding season. Individuals of the other species were present on less than 30% of the census nights, and usually spent only two to four nights

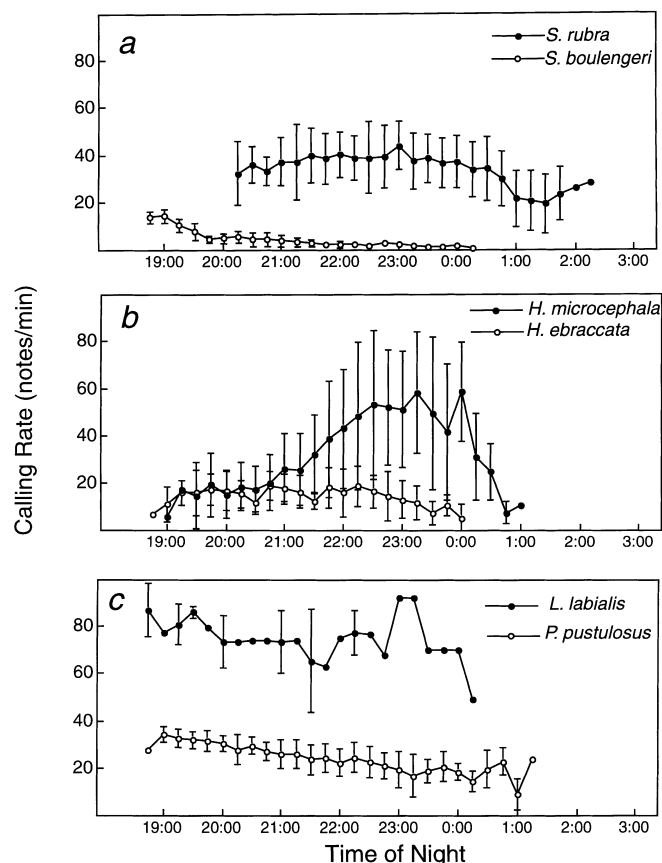


Fig. 1a–c Nightly calling activity in six tropical frogs. Species are paired by genus or family: **a** *Scinax*, **b** *Hyla*, and **c** *Leptodactylus* and *Physalaemus* (Family Leptodactylidae). Average calling rates \pm 1 SD are shown for each 15-min time interval

calling in a chorus during the breeding season. *P. venulosa* is a highly explosive breeder; males of this species appeared and called in choruses for only a few days after the first heavy rains at the beginning of the rainy season in April, then disappeared.

Table 1 Calling activity in tropical frogs. Values for calling rate and calling period are means \pm SD. The ranges of values and sample sizes are indicated for calling rate and calling period

Species	Calling rate			Calling period		
	Average (notes/h)	Range (notes/h)	<i>n</i> (males)	Average (h/night)	Range (h/night)	<i>n</i> (males)
Hylidae						
<i>Hyla ebraccata</i>	857 \pm 366	210–1944	38	4.6 \pm 0.71	3.0–5.5	19
<i>Hyla microcephala</i>	3132 \pm 1222	1340–5718	30	3.8 \pm 0.87	2.75–5.75	25
<i>Phrynohyas venulosa</i>	1585 \pm 861	756–2700	4	–	–	–
<i>Scinax boulengeri</i>	476 \pm 250	30–1188	83	3.2 \pm 1.02	1.45–5.0	23
<i>Scinax rubra</i>	2065 \pm 555	1020–3198	52	4.9 \pm 0.93	3.0–7.0	24
Leptodactylidae						
<i>Leptodactylus labialis</i>	4709 \pm 708	2960–5868	42	3.4 \pm 0.53	2.8–4.0	5
<i>Physalaemus pustulosus</i>	1680 \pm 388	1032–2328	33	4.7 \pm 0.96	3.0–6.75	26

Energy reserves

Glycogen levels in the trunk muscles were lower for late-evening males than for early-evening males in all seven species (Fig. 3). In *H. microcephala*, *P. pustulosus*, and *S. rubra*, differences in trunk muscle glycogen levels between early and late-evening males were significant (one-tailed Mann-Whitney *U*-test, $P < 0.05$). In contrast, only four of the seven species showed any decrease in liver glycogen, and none was statistically significant. A summary of glycogen reserves in liver and trunk muscles is presented in Table 2.

Other energy reserves included lipids stored in trunk muscles and the body, and food in the stomach. A summary of these reserves is reported in Table 3. Trunk-muscle lipid stores ranged from an average of 4.27% dry trunk muscle mass in *Phr. venulosa* to 16.35% in *S. rubra*. All species except *S. boulengeri* and *H. microcephala*

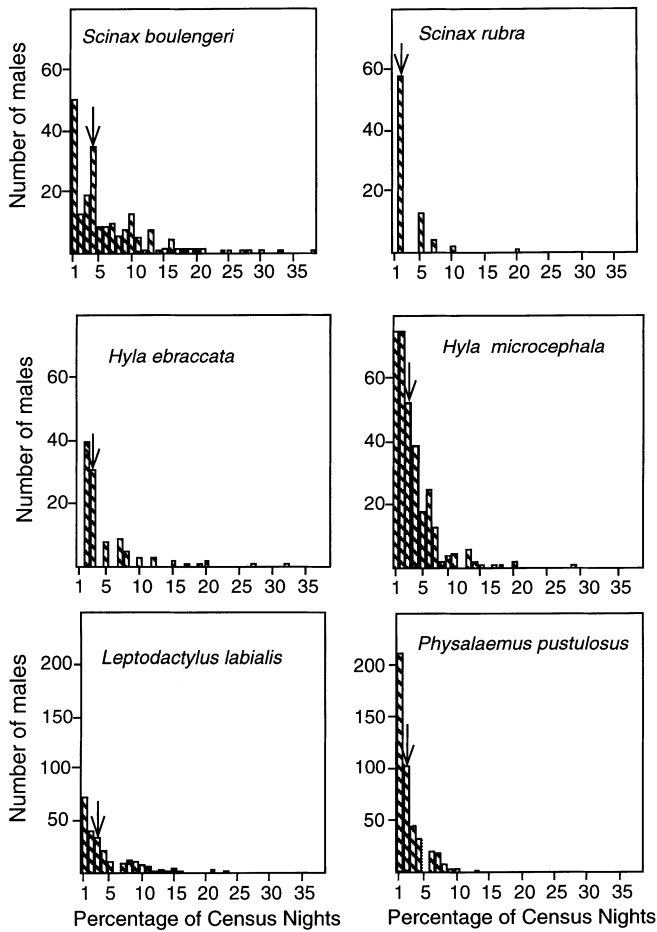


Fig. 2 Distribution of the number of nights of calling activity in 1992 for individual males from six tropical frog species. Bars show the number of males in each category. The number of nights of calling activity, relative to the number of total nights the chorus was censused, are represented on the X-axis. Arrows indicate median values. Sample sizes are as follows: 203 males, 135 census nights for *S.b.*; 74 males, 41 census nights for *S.r.*; 107 males, 59 census nights for *H.e.*; 326 males, 108 census nights for *H.m.*; 238 males, 91 census nights for *L.l.*; 457 males, 90 census nights for *P.p.*

Table 2 Glycogen content of liver and trunk-muscle of seven tropical frogs. Values are means \pm SD. Sample sizes are in parentheses

Species	Liver mass (g)	Trunk muscle mass (g)	Mass-specific liver glycogen (mg/g)	Mass-specific trunk-muscle glycogen (mg/g)	Total liver glycogen (mg)	Total trunk-muscle glycogen (mg)
Hylidae						
<i>H. ebraccata</i>	0.022 \pm 0.019 (17)	0.053 \pm 0.012 (26)	21.33 \pm 14.37 (17)	3.64 \pm 2.48 (30)	0.48 \pm 0.43 (17)	0.20 \pm 0.15 (26)
<i>H. microcephala</i>	0.015 \pm 0.005 (17)	0.052 \pm 0.008 (27)	24.44 \pm 11.44 (17)	7.40 \pm 4.77 (42)	0.37 \pm 0.21 (17)	0.35 \pm 0.22 (27)
<i>S. boulengeri</i>	0.054 \pm 0.020 (17)	0.245 \pm 0.050 (27)	16.83 \pm 7.94 (17)	3.50 \pm 1.68 (28)	0.97 \pm 0.59 (17)	0.87 \pm 0.43 (27)
<i>S. rubra</i>	0.080 \pm 0.018 (12)	0.428 \pm 0.066 (17)	19.58 \pm 7.64 (12)	6.78 \pm 3.71 (28)	1.64 \pm 0.82 (12)	3.10 \pm 1.82 (17)
<i>Phr. venulosa</i>	0.480 \pm 0.112 (6)	4.070 \pm 0.520 (6)	7.61 \pm 7.68 (6)	2.65 \pm 2.31 (6)	3.57 \pm 3.58 (6)	10.87 \pm 9.73 (6)
Leptodactylidae						
<i>L. labialis</i>	0.081 \pm 0.028 (17)	0.143 \pm 0.030 (17)	22.58 \pm 4.48 (17)	4.65 \pm 3.64 (18)	1.87 \pm 0.82 (17)	0.59 \pm 0.47 (17)
<i>P. pustulosus</i>	0.045 \pm 0.011 (18)	0.121 \pm 0.025 (28)	20.19 \pm 7.64 (18)	5.91 \pm 4.75 (30)	0.90 \pm 0.39 (18)	0.67 \pm 0.47 (28)

Fig. 3 Trunk-muscle glycogen levels in seven tropical frog species from two families, Hylidae and Leptodactylidae. Bars show medians and interquartile ranges. The percent difference between early- and late-evening males (as a percentage of early males) is shown for each species, and sample sizes for early and late samples are in parentheses at the top of each bar. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$ (one-tailed Mann-Whitney U -test)

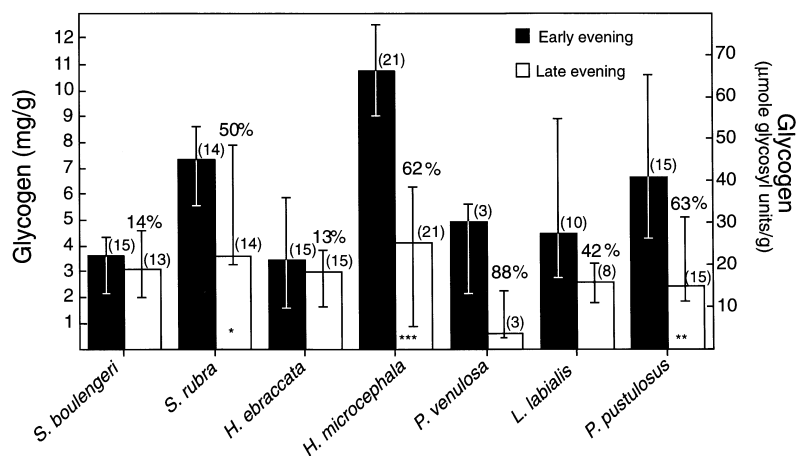


Table 3 Fuel types and quantities, including trunk-muscle and carcass lipid and stomach contents, in seven tropical frog species from two families, Hylidae and Leptodactylidae. Values are means \pm SD. Sample sizes are in parentheses

Species	Average trunk-muscle lipids (% dry trunk mass)	Average carcass lipids (% dry body mass)	Dry stomach contents (% dry body mass)
Hylidae			
<i>Hyla ebraccata</i>	9.59 \pm 4.93 (13)	12.57 \pm 6.80 (13)	0.93 \pm 0.78 (12)
<i>Hyla microcephala</i>	14.75 \pm 5.15 (15)	10.95 \pm 4.40 (15)	1.80 \pm 1.90 (15)
<i>Phrynohyas venulosa</i>	4.27 \pm 2.15 (6)	–	0.66 \pm 0.51 (6)
<i>Scinax boulengeri</i>	4.74 \pm 2.40 (12)	6.39 \pm 3.65 (11)	1.88 \pm 2.29 (10)
<i>Scinax rubra</i>	16.35 \pm 2.38 (11)	6.64 \pm 2.00 (11)	0.50 \pm 0.27 (11)
Leptodactylidae			
<i>Leptodactylus labialis</i>	6.11 \pm 1.93 (15)	4.69 \pm 1.30 (15)	4.06 \pm 3.81 (15)
<i>Physalaemus pustulosus</i>	5.73 \pm 2.78 (16)	10.66 \pm 6.24 (16)	1.38 \pm 1.88 (16)

exhibited smaller lipid stores in late-evening samples compared to early-evening samples; one species, *Phr. venulosa*, showed significant depletion, though only six males were sampled (one-tailed Mann-Whitney U -test, $P < 0.05$). Average carcass lipid concentrations ranged from $< 5\%$ dry body mass in *L. labialis* to nearly 13% in *H. ebraccata* (Table 3). There was no relationship between the average sizes of trunk-muscle lipid reserves and average carcass reserves ($r^2 = 0.04$, $P = 0.71$). Dried stomach contents composed $< 5\%$ of dry body mass for all frogs examined, but were slightly higher for more continuous breeders with high calling rates (*L. labialis*, *H. microcephala*) than for more opportunistic species (*P. venulosa*, *S. rubra*).

Calling activity and energy substrates

Rates of depletion of trunk-muscle glycogen (i.e., the difference in average glycogen levels between early and late males, divided by the time difference of 3 h) were positively but not significantly correlated with hourly calling rate among all seven species ($y = 0.0003x + 0.399$, $r^2 = 0.24$, $P = 0.27$). However, when *L. labialis*, an obvious outlier, was omitted from these analyses, the correlation was significant (Fig. 4a). For all species except *P. venulosa*, for which calling period data were not

available, the relationship between glycogen depletion and the total number of notes produced in an average night was also positive ($y = 0.00009x + 0.172$, $r^2 = 0.32$, $P = 0.24$) and more of the variation in glycogen depletion was explained when *L. labialis* was omitted (Fig. 4b). Average trunk-muscle lipid reserves for all species were positively, but not significantly, correlated with calling rate ($y = 0.0007x + 7.449$, $r^2 = 0.04$, $P = 0.68$) and total notes produced per night ($y = 0.0003x + 7.193$, $r^2 = 0.09$, $P = 0.57$). Again, more of the variation in trunk-muscle lipid reserves was explained by calling rate when *L. labialis* was removed, but the relationship was still not significant with either calling rate or total notes produced per night (Fig. 5).

Although glycogen stores were reduced considerably after only 2–3 h of calling in each species, glycogen apparently contributes much less than lipids to fueling calling activity. Calculated estimates of glycogen and lipid use from measurements of oxygen consumption and glycogen depletion suggest that *H. microcephala* would use about 1.04 mg of glycogen/h and *P. pustulosus* would use 2.61 mg of glycogen/h while calling if all energy came from glycogen. Glycogen depletion from trunk muscle reserves was actually only about 0.39 mg over 3 h in *H. microcephala*, or 13% of the glycogen needed to fully support call production, and about 0.48 mg, or 6%, in *P. pustulosus* (Table 4).

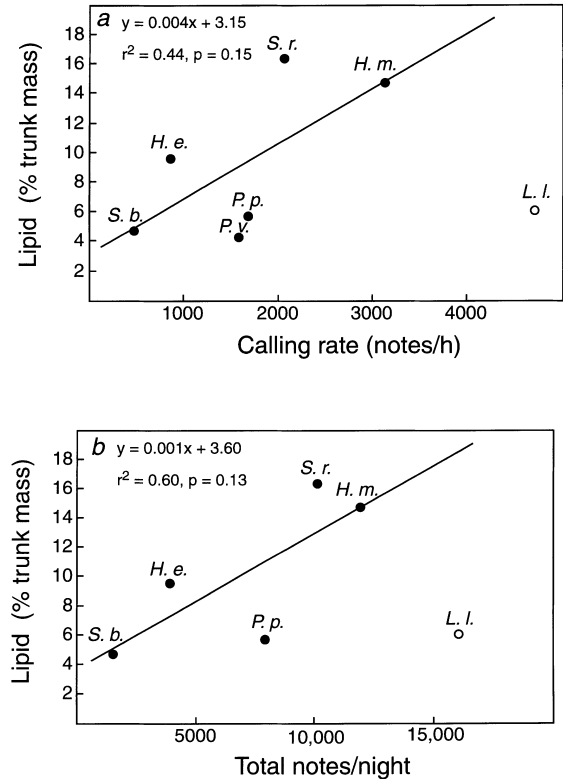
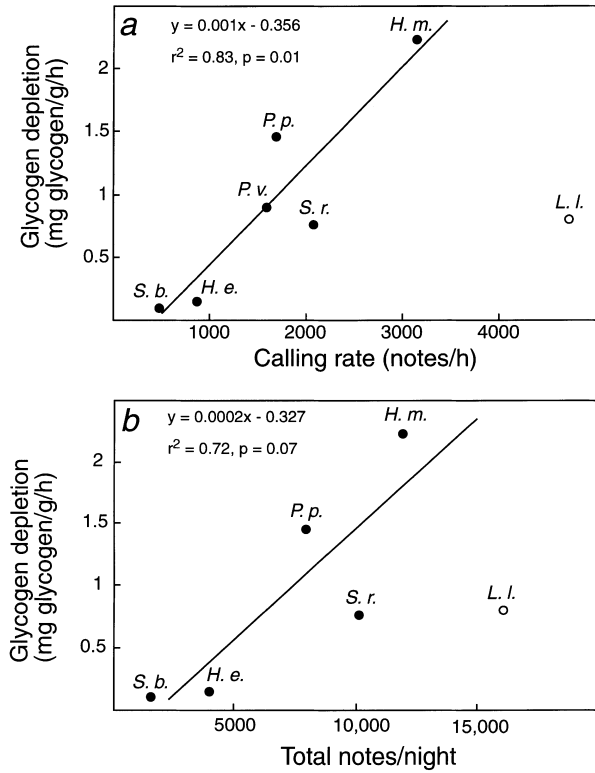


Fig. 4 Relationships of rates of trunk-muscle glycogen depletion to **a** calling rate and **b** total number of notes produced in an average night in seven tropical frog species from two families, Hylidae and Leptodactylidae. The regression line does not include *L. labialis*, but this species is plotted for comparison. *Phr. venulosa* is omitted from **b** because data on average calling period were not available (*L.l. Leptodactylus labialis*, *H.e. Hyla ebraccata*, *H.m. Hyla microcephala*, *P.p. Physalaemus pustulosus*, *P.v. Phrynohyas venulosa*, *S.b. Scinax boulengeri*, *S.r. Scinax rubra*)

Fig. 5 Relationships of average trunk-muscle lipid reserves to **a** calling rate and **b** total number of notes produced in an average night in seven tropical frog species from two families, Hylidae and Leptodactylidae. The regression line does not include *L. labialis*, but this species is plotted for comparison. *Phr. venulosa* is omitted from **b** because data on average calling period were not available (*L.l. Leptodactylus labialis*, *H.e. Hyla ebraccata*, *H.m. Hyla microcephala*, *P.p. Physalaemus pustulosus*, *P.v. Phrynohyas venulosa*, *S.b. Scinax boulengeri*, *S.r. Scinax rubra*)

Table 4 Estimated energetic contribution of glycogen and lipid to calling activity in *Hyla microcephala* and *Physalaemus pustulosus*

	Average oxygen consumption (ml O ₂ /g·h)	Substrate	Amount used (mg/h)	Oxygen consumed (ml/h)	Energy content (J/h)	Contribution (%)
<i>Hyla microcephala</i>	1.433	Glycogen	0.13	0.11	2.36	14
		Lipid	0.38	0.76	14.97	86
<i>Physalaemus pustulosus</i>	1.297	Glycogen	0.16	0.13	2.83	7
		Lipid	1.03	2.06	40.42	93

The remaining energy must come from lipids, so male *H. microcephala* would use an estimated average of 0.38 mg lipid/h (14.97 J) and *P. pustulosus* use 1.03 mg lipid/h (40.42 J). Hence both species derive about 90% of their energy for calling from lipids (Table 4). The estimated energy cost for *P. pustulosus* here is about 43% higher than that found in previously (Pough et al. 1992; Ryan et al. 1983) because the average calling rate reported here is higher (1680 versus 1000 calls/h).

Discussion

Calling frogs and human endurance athletes face similar problems in using highly aerobic muscles for prolonged periods of strenuous work. Energy can be delivered most rapidly to working muscle through oxidation of glycogen, but skeletal muscles have a limited capacity to store glycogen. Smaller reserves, therefore, may present a short-term limitation on muscle performance and the duration of intense muscular work (Guppy 1988; Weber

1992; Fournier and Weber 1994). The use of lipids often is required to support long-term aerobic muscle activity, and using a mixture of lipids and glycogen may increase the time over which the muscles can perform at peak levels (Hochachka and Somero 1984; Guppy 1988).

In mammals, species that engage in sustained periods of aerobic exercise, such as dogs, rely more heavily on intramuscular energy stores and utilize lipids to a greater extent than do species with relatively slow locomotion, such as opossums (Weber 1992; Fournier and Weber 1994). Similarly, insects with very high singing rates rely mainly on lipids to support song production, while those with lower singing rates rely almost entirely on carbohydrate reserves (Prestwich 1994). Calling frogs appear to fit this pattern as well. While the assay method used here for lipid analysis was somewhat crude and sample sizes were small, this study has shown that species with high calling rates also have larger reserves of lipids in their trunk muscles than do species with low calling rates (Fig. 5; see also Ressel 1996). With the exception of *L. labialis*, which has a much shorter and less intense call than any of the other species studied (Bevier 1995), *H. microcephala* has the highest calling rate, the largest number of notes produced per night, and the largest reserves of trunk muscle glycogen and lipids. At the opposite end of the spectrum is *S. boulengeri*, with the lowest calling rate, the smallest number of notes produced per night, and nearly the smallest trunk muscle glycogen and lipid reserves.

Furthermore, rates of glycogen depletion are positively correlated with calling rate among six of the seven species (Fig. 4). The general patterns observed from characteristics of these frogs also are evident in pairwise comparisons of species within genera, groups for which monophyly is not really in question. The relationships shown in Fig. 4 illustrate this well. For example, within *Hyla*, *H. microcephala* and *H. ebraccata* are closely related yet have very different rates of glycogen depletion in the trunk muscle. Similarly, within *Scinax*, *S. rubra* has higher rates of glycogen depletion and larger stores of trunk-muscle lipid than *S. boulengeri*. Moreover, the two species with relatively low calling rates (*S. boulengeri* and *H. ebraccata*) are closer to each other on the regression lines in Fig. 4 than they are to congeneric species with higher calling rates. This evidence clearly indicates that phylogenetic affinities are less important than differences in calling behavior as correlates of utilization of glycogen in muscles used for call production.

To study further the differences in utilization of fats versus carbohydrates, I estimated the relative proportion of energy derived from lipids and glycogen during call production for two species with relatively high calling rates, *H. microcephala*, and *P. pustulosus*. These estimates are based on the assumption that extramuscular energy reserves, such as glycogen in the liver, contribute little or nothing to call production. This seems reasonable, given the lack of any detectable change in liver glycogen during the course of one evening. In both cases, about 90% of the energy for calling appears to be de-

rived from lipids, even though glycogen reserves are depleted much more rapidly. This compares to an estimate of 75% lipids for the gray treefrog (*Hyla versicolor*), a temperate-zone species with a lower average calling rate (822 calls/h) than either *H. microcephala* (3132 calls/h) or *P. pustulosus* (1680 calls/h) (Wells et al. 1995). It seems likely that species with even lower calling rates, such as *S. boulengeri*, rely even less on stored lipid reserves to support call production. Unfortunately, data on oxygen consumption by calling males are not available for the other species, so calculations of energy substrate use are not yet possible.

Several species included in my study depleted more than 50% of their trunk muscle glycogen reserves after only 3 h of calling. However, most late-evening males of all species still had some glycogen left when they were collected, and none of the species appeared likely to completely deplete their glycogen reserves in one evening of calling. For all six species for which data on average calling period were available, the average percent of initial reserves depleted after 3 h of calling is less than the percentage of the average calling period represented by those 3 h (the period over which glycogen depletion was measured). For example, *H. microcephala* depleted trunk-muscle glycogen reserves significantly by an average of 62% over 79% of its average calling period (3 of 3.8 h). Assuming that males do not become energy-limited until they completely deplete their trunk-muscle glycogen, then all species appear to cease calling before they exhaust their trunk-muscle glycogen reserves. Therefore, the size of a male's glycogen reserve does not seem to limit the number of hours that a male calls within an evening.

Furthermore, there is considerable variation in nightly patterns of calling activity among species and among individuals of the same species. Some males called for only 1 or 2 h before stopping, while others called for up to 6 or 7 h. Average calling rates of five of the six species in my study declined during the evening, and most stopped calling shortly after midnight. Although depletion of energy reserves may contribute to this variation, it also may be related to variation in the timing of females arriving at the breeding site. Even when males have some energy reserves left, selection should favor reducing or ceasing calling activity at times when unpaired females are unlikely to be present to conserve energy reserves for another night.

In general, the arrival of females in the species included here coincided with peak periods of calling activity. For example, *S. boulengeri* females arrived early in the evening, and most mating occurred before 2000 h ($n = 90$). Males of this species called at high rates from 1815 to 2000 hours and then reduced their calling rates (Fig. 1). In *H. microcephala*, calling peaked at around 2300 h, which also coincided with the time that females were observed entering the chorus and choosing mates (2315 h, $n = 16$). Most males stopped calling within the next hour. The species that were most active after heavy rains (*S. rubra*, *P. pustulosus*), or concentrated their ac-

tivity into one explosive breeding event (*P. venulosa*), tended to have the longest calling periods each night (up to 7 h in *S. rubra* and *P. pustulosus*, all night in *P. venulosa*). This may be because arrival times of females are less predictable than in the more continuous breeders. However, such prolonged periods of calling would not be possible for species with high calling rates without extensive use of intramuscular lipid reserves, because glycogen reserves would be depleted after a few hours.

The ability of males to replenish energy reserves, especially their rapidly depleted glycogen stores, may be an important determinant of how many nights a male can remain in a chorus. Since these tropical frogs probably are relatively inactive during the heat of the day, they may use the hours after midnight to feed. If so, then extending the nightly period of calling activity would reduce the amount of time available for feeding (e.g., Woolbright and Stewart 1987). Among the strictly nocturnal frogs in my study, the relative mass of food in the stomach was lowest in *Phr. venulosa*, an explosive breeder that calls all night, and relatively low in *S. rubra*, an opportunistic breeder that calls for many hours per night after heavy rains. The largest amount of food, relative to body size, was found in *S. boulengeri*, a more continuous breeder with a shorter calling period. Males of this species may feed on many nights after chorusing stops. Among all the species, the largest relative amount of food was found in *L. labialis*, which is active during the day and may have more time to feed.

Green (1990) examined the effect of feeding on chorus participation in *P. pustulosus* by providing some males with supplemental food. Males held in captivity while feeding did not call for more nights than males held without food. Furthermore, when males were released into the field, the ones that had been fed were no more likely to return to the chorus and did not return sooner than males that had not been fed. Unfortunately, Green did not measure the effect of feeding on hourly calling rates or the number of hours a male called each night, but his general conclusion was that calling in this species was not constrained by food intake. Quite different results were obtained by Murphy (1994), who found that supplemental feeding of males of *Hyla gratiosa* in Florida resulted in males returning to the chorus sooner and for more nights than males that had not been fed. The difference in the results of these studies may reflect differences in calling strategies in the two species. *P. pustulosus* is a relatively opportunistic species, and it appears to rely on stored energy reserves (both lipids and glycogen) to sustain a long period of calling (Table 1). *H. gratiosa*, on the other hand, forms choruses nearly every night over a 5-month breeding period. While individual males often are present for only a small proportion of those nights, the more continuous pattern of breeding may favor continuous feeding throughout the season to maximize the number of nights a male can participate in a chorus. Hence, the energy use strategy of *H. gratiosa* may be more similar to that of *S. boulengeri* than to opportunistic species such as *P. pustulosus* and *S. rubra*.

Acknowledgements I thank K.D. Wells for help and support in planning and executing this project, and for many constructive comments throughout this study. I thank T.L. Taigen for careful guidance and use of laboratory space and equipment, and A.S. Rand for assistance in the field, helpful discussion, and support at the Smithsonian Tropical Research Institute (STRI). STRI provided housing and logistical support during the field portions of this project. Permission to study and collect frog species in Panama was granted by I.N.Re.Na.Re. I thank K.A. Robb, L.R. Bevier, J.M. Ellingson, and J.J. Roper for assistance in the field. K.D. Wells, C.S. Henry, A.S. Rand, C.G. Murphy, and an anonymous reviewer gave constructive comments on this manuscript. Financial support was provided by a Sigma Xi Grant-in-Aid of Research, fellowships from the University of Connecticut Graduate School, a STRI Short-Term Fellowship, a P.E.O. Scholar Award, and a grant to K.D. Wells from the University of Connecticut Research Foundation. Part of this work was done while C.R.B. was supported by an NSF Graduate Research Traineeship in the evolution, ecology and conservation of biodiversity (BIR-9256616).

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Communicated by W.A. Searcy