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Doping for sex: Bad for mitochondrial performances? Case of testosterone supplemented *Hyla arborea* during the courtship period

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ABSTRACT

Sexual selection has been widely explored from numerous perspectives, including behavior, ecology, and to a lesser extent, energetics. Hormones, and specifically androgens such as testosterone, are known to trigger sexual behaviors. Their effects are therefore of interest during the breeding period. Our work investigates the effect of testosterone on the relationship between cellular bioenergetics and contractile properties of two skeletal muscles involved in sexual selection in tree frogs. Calling and locomotor abilities are considered evidence of good condition in *Hyla* males, and thus server as proxies for male quality and attractiveness. Therefore, how these behaviors are powered efficiently remains of both physiological and behavioral interest. Most previous research, however, has focused primarily on biomechanics, contractile properties or mitochondrial enzyme activities. Some have tried to establish a relationship between those parameters but to our knowledge, there is no study examining muscle fiber bioenergetics in *Hyla arborea*. Using chronic testosterone supplementation and through an integrative study combining fiber bioenergetics and contractile properties, we compared sexually dimorphic trunk muscles directly linked to chronic sound production to a hindlimb muscle (i.e. *gastrocnemius*) that is particularly adapted for explosive movement. As expected, trunk muscle bioenergetics were more affected by testosterone than *gastrocnemius* muscle. Our study also underlines contrasted energetic capacities between muscles, in line with contractile properties of these two different muscle phenotypes. The discrepancy of both substrate utilization and contractile properties is consistent with the specific role of each muscle and our results are elucidating another integrative example of a muscle force-endurance trade-off.

1. Introduction

During the breeding season, a significant body-mass reduction linked with calling activities has often been observed in male frogs, particularly when breeding activity involves strong territorial defense or high calling activity (Wells, 1977; Eggert and Guyétant, 2003). This is especially the case in the European tree frog *Hyla arborea*, a species in which males alternate periods of vocalization at the pond to attract females with foraging on land, presumably to offset the elevated metabolic costs of calling (Grafe and Meuche, 2005; Meuche and Grafe, 2009). The energetic resources (*i.e.* lipid stores) acquired before returning to the pond strongly affect pairing success through modifications of vocal signal characteristics (Brepson et al., 2013). Lipid hoarding can therefore constitute a real fitness advantage, supporting a "sexy male" hypothesis based on body size (Bevier, 1997; Carvalho et al., 2008). Bigger males that are considered the "sexiest" possess larger amounts of muscle lipid and carbohydrate stores (Carvalho et al., 2008), allowing them to spend more time in chorus activity, which ultimately represents a key predictor of male pairing success (Ryan, 1988). Indeed, bigger males are capable of emitting the most attractive calls for females (Richardson and Lengagne, 2010) with reduced oxygen demand (Voituron et al., 2012). As a consequence, the energetic costs of calling have strong consequences on sexual selection processes, because males face a trade-off between calling in order to attract females and foraging to renew their energetic reserves.

Androgens, known to affect morphological and physiological traits, may also contribute to variations in performance (Huyghe et al., 2010; Guo et al., 2012). In amphibians, numerous sexually dimorphic neuromuscular structures that underlie reproductive behavior are known to be testosterone dependent, either in a developmental context or acutely (Brennan and Henderson, 1995; Kelley, 1986; Nagaya and Herrera, 1995; Sidor and Blackburn, 1998). In gray tree frogs, *Hyla*

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chrysoscelis, seasonal changes in testosterone levels enhance the *in vivo* contractile properties and the size of the trunk muscles involved in call production (Girgenrath and Marsh, 2003). Similarly, testosterone increases forelimb muscle dry mass by more than 150% under experimental conditions in *Rana pipiens* (Kim et al., 1998). The high sensitivity of neuromuscular structures to testosterone can be attributed to the expression of significantly higher testosterone receptors in these muscles (Emerson et al., 1999; Erulkar and Wetzel, 1987; Kelley, 1986; Kelley et al., 1989) compared to other neuromuscular structures. In addition, testosterone presumably triggers specific genes regulating both muscle size and contractile properties, but also the expression of metabolic enzymes and myosin isoforms in androgen-sensitive fibers (Catz et al., 1992; Melichna et al., 1972; Regnier and Herrera, 1993a,b; Rubinstein et al., 1983; Sassoon et al., 1987; Taigen and Wells, 1985). To our knowledge, testosterone's impact on mitochondrial functioning of muscles is poorly understood (reviewed in Traish et al., 2011, see also Usui et al., 2014). Moreover, little is known about short-term effects such as increases in testosterone that occur only during the breeding period.

Hyla arborea females show a significant preference for higher call rates and higher call amplitudes (Richardson et al., 2010). These male vocalizations are very costly (Voituron et al., 2012) and strongly subject to androgen influence (Desprat et al., 2015). As the cost of these calls can only be sustained by high quality males (Brepson et al., 2013), the acoustic signal is considered an honest signal of male quality. According to the Handicap principle (Zahavi, 1975, 1977), an honest signal is indeed energetically costly. The purpose of this study was therefore to measure bioenergetics parameters and to evaluate the contractile performances of two different muscles in *Hyla arborea* sustaining two widely different ecological activities. Anuran species typically have three sexually dimorphic muscles: the trunk and laryngeal muscles used for mate calling (Marsh and Taigen, 1987; review in Pough et al., 1992) and the *flexor carpi radialis*, the forelimb muscle used by males to grasp and control the female during amplexus (Melichna et al., 1972). Here, we chose to compare the trunk muscles highly developed in males during the breeding season (Girgenrath and Marsh, 2003) to a hindlimb locomotor muscle, the *gastrocnemius* muscle. The latter muscle, aka *Plantaris longus* is particularly adapted for jumping in frogs (Calow and Alexander, 1973) and seems not to be affected by seasonal cycles (Kirby, 1983).

In our study, we described the effects of chronic testosterone supplementation on the functioning of each muscle with respect to energetic substrate origin and on relative contractile properties, taking into account body mass variations. Due to their biological role and sexual dimorphism, we expected a more marked testosterone effect on trunk muscle function than on *gastrocnemius* function.

2. Material and methods

2.1. Frog sampling and experimental design

Forty *H. arborea* mature males were collected during nightly choruses in mid-April 2013 from a population located in France near Lyon according to the ethics committee of Lyon University (BE 2012-15) and the French government laws on the environment. Just after the capture, 8 individuals were used to fine-tune the experimental setup. The other 32 males were housed in individual terraria (25 × 17 × 15 cm) with a water-filled basin and a tree branch located in an amphibian facility, the EcoAquatron (University Claude Bernard, Lyon 1) approved by Veterinary Services (approval number 692661201). These males were randomly distributed into two groups: control males (Control) and testosterone-supplemented males (Testo). During all experiments, testosterone was delivered transdermally daily to each Testo-male following the method used by Desprat et al. (2015). Briefly, testosterone (number 86500, FLUKA analytical, Sigma-Aldrich) was diluted in commercial-grade sesame oil to obtain a 3 mg/ml hormone solution. Testo-males ($N = 16$) received 4.5 µl of hormone solution per day. Simultaneously, Control-males ($N = 16$) received an identical amount of sesame oil. After 10 days of treatment (D10), 8 individuals of each group were randomly sampled, weighed and a saliva sample was taken to measure testosterone levels. These individuals were directly killed by pithing and used for muscle withdrawal. The same protocol was used after 20 days of treatment, corresponding to the end of the experiment (D20). At this time, only 5 supplemented individuals with an effective high testosterone concentration (*i.e.* individuals with a testosterone concentration higher than Control group) were kept for results. Kmeans cluster analysis was utilized to assign saliva measurements to two groups, and any testo males that had salivary levels similar to the control group were not included in muscle analyses (K-means clustering, http://www.statmethods.net/advstats/cluster.html). Every night, males were stimulated using a recording of the chorus playback of their population (as described in Desprat et al., 2015). Male *H. arborea* produce advertisement calls in bouts containing on average of 25 calls and lasting an average of 4 s (Friedl and Klump, 2002; Richardson et al., 2010; Brepson et al., 2013). Each call has a dominant frequency—frequency with the highest energy—ranging from 2000 to 3000 Hz. Males produce on average 11,529 ± 8219 calls (mean ± SD, range: 0–47,627 calls) during a calling night (data from Brepson et al., 2013). During the experiment, males were force-fed with 2 domestic crickets (*Acheta domesticus*) every 2 days to guarantee consistent food intake.

2.2. Testosterone level analysis

A saliva sample was obtained with a cotton ball introduced directly into a frog's mouth for 20 s. Cotton balls were weighed before and after sampling saliva. Saliva was extracted from the cotton ball with the addition of 120 µL of phosphate buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA and 0.1% sodium azide) and centrifugation (10,000 rpm, 10 min). The testosterone analysis was performed in duplicate with a colorimetric 96-well testosterone Enzymo-Immuno Assay kit (EIA, number 582701, Cayman Chemical). The EIA used to measure testosterone in the saliva was previously validated for use with *H. arborea* saliva (Desprat et al., 2015).

2.3. Cellular muscle bioenergetics

Muscle fiber bioenergetics was investigated using a method described previously by Pesta and Gnaiger (2012) and adapted for amphibians in our laboratory. After euthanasia, half of each of the left trunk and *gastrocnemius* muscles was separately immersed and dissected in BIOPS solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Muscle strips were dissected to separate muscle fibers. Fiber bundles were transferred in a BIOPS solution containing saponin (50 µg/ml) for permeabilization and were shaken gently at 4 °C for 30 min. Then, permeabilized fibers were washed for 10 min at 4 °C in the Mir05 buffer (0.5 mM EGTA, 3 mM $MgCl_2.6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid BSA (1 g/L), pH 7.1).

Oxygen consumption and respiration rate of muscle fibers were measured using a high-resolution respirometer (Oxygraph-2k, Oroboros® Instruments, Innsbruck, Austria) at 20 °C, in the Mir05 solution using two different energetic substrates: a carbohydrate (CHO) substrate (pyruvate/malate/succinate, PMS, 5/2.5/5 mM) or a lipidic substrate (palmitoyl-carnitine/malate, PCM, 40 µM/2.5 mM). Note that we added succinate with pyruvate/malate to generate a convergent electron flow at the Coenzyme-Q junction of the electron transport chain, which would reconstitute the physiological citric acid cycle function in mitochondria, by generating simultaneously NADH

and succinate in the mitochondrial matrix (Gnaiger, 2009). This "fully-activated" state would reflect closer to what happens during calling effort at the whole-organism level (Reilly et al., 2014). Notwithstanding, these substrates initiate a non-phosphorylating state ("Basal" state). Adding ADP (1 mM) triggered an increase of oxygen consumption, corresponding to the "Phosphorylating" state. The ratio between Phosphorylating and Basal respiration rate illustrates the coupled state of the fibers, known as the RCR for "respiratory control ratio". The integrity of mitochondria within permeabilised fibers was systematically checked by the absence of the stimulation of respiration by cytochrome *c* (10 μM) addition. Finally, a titration of FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl hydrazine, up to 4 times 1 μL of 2 mM FCCP) was performed to characterize the maximal respiration rate of the electron transport chain exhibited by muscle fibers for a concentration of 1 μM. In reference to the classical *in vivo* aerobic scope (AS), this latter parameter allowed us to estimate the fiber aerobic scope, as the difference between FCCP respiration rate and basal respiration rate. We assumed that it represented the total aerobic capacity of the muscle fiber.

2.4. Muscle contraction properties

2.4.1. Muscle preparation

After euthanasia and skin removing, both the trunk and the hindlimb muscles were dissected. The trunk muscle consists of two obliquely oriented thin layers that are both inserted from the backbone to the breastbone. Depending on their position, muscle fibers have variable length to accommodate the curved surface of the trunk. Because of this particular morphology and for minimizing the cellular damage from the dissection leading to an underestimation of muscle fiber bioenergetics, we decided to use both layers of trunk muscle for this study. Concerning the hindlimb muscle, we decided to use the *gastrocnemius* muscle. It is the largest muscle of the hindlimb and the primary extensor of the ankle, therefore mainly involved in locomotion activity (Chadwell et al., 2002; Crockett and Peters, 2008; Moore, 1997). The *gastrocnemius* muscle was isolated with the knee articulation and Achilles tendon still attached. Bones and tendons let us to attach the muscles in the experimental setup described below.

After the contraction protocol, bones and tendons were removed and each muscle was weighed, to obtain the wet mass. Afterwards, they were lyophilized overnight and weighed again to estimate the total water content (TWC) and the ratio to body mass.

2.4.2. Contractile properties and fatigue measurement

The muscle sampled was completely immersed vertically in a polypropylene cylindrical chamber (D × h: 4.5 cm × 6 cm) with Ringer solution (110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2{-}6H_20$, 10 mM Hepes, pH 7.4) at 25 °C (Allard and Rougier, 1994) with a constant supply of oxygen. Muscles were attached to the bottom of the chamber with a plier grabbing either the backbone or the knee articulation. The upper part of the muscles (breastbone or Achilles tendon) was attached with another plier to the force sensor. Isometric contractile properties of both trunk and *gastrocnemius* muscles were recorded using a single column universal testing system (Instron 5940, Canton, MA, USA) as an isometric transducer (accuracy: Force ± 0.25% and repeatability threshold: 0.001% within this range of values). Muscle lengths were adjusted to obtain the optimal isometric twitch response.

The maximum twitch contractions were estimated by step-by-step increasing stimulus voltage output. Once no further increase was observed (between 25 V and 40 V), the voltage output was increased by 10% of this threshold value to ensure that the muscle was maximally stimulated. Two copper electrodes connected to a stimulator (model 6002, Harvard apparatus, UK) were placed within the chamber so that the delivered current (square wave stimulus of 0.5 ms at 20 Hz until exhaustion) would pass through the whole muscle.

The output signal from the transducer was recorded and analyzed using BlueHill® software (Instron, Canton, MA, USA) to collect muscle contractile properties and fatigue resistance during continuous stimulation. Recorded signal outputs followed the same pattern for both muscles, with three distinctive parts: a peak, a steady-state force phase ("plateau"), followed by a period of time to return to baseline. These three parts allowed us to characterize the contractile properties of each muscle through three parameters: first, the peak corresponds to the maximum developed force (F_{max}). To account for inter-individual variability, we expressed relative F_{max} as a ratio between measured forces relative to the maximal individual twitch force (as 100%) per gram of dry muscle ([N/N] g^{-1}). Second, we used the plateau duration (in seconds) as a proxy of muscle aerobic capacity. When the plateau phase could not be determined, plateau duration was counted as zero. Third, the fatigue resistance (in second) was calculated as the time for the muscle force to drop to 90% of the maximal developed force.

3. Statistical analysis

The data are expressed as the mean ± s.e.m. in all figures and tables. For organism, muscle and cellular levels, treatment (Control *vs.* Testo) and day (D10 *vs.* D20) effects were tested using two-way ANOVA or two-way ANOVA on ranks and when mentioned, three-way ANOVA were performed to test the effect of substrate (PCM *vs.* PMS) or muscle effects (Trunk *vs.* *Gastrocnemius*) using SigmaPlot v.12 software. Pairwise comparisons with adjustments for multiple comparisons (Holm-Sidak method) were conducted to detect further differences between treatments and respiration rates. All data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). When one of those conditions was not met, non-parametric ANOVA on ranks were performed. The level of significance was set at $P < 0.05$. All statistical parameters are summerized in Table 4.

4. Results

4.1. Testosterone level, body and muscle masses

Testosterone concentration in saliva was significantly increased in Testo-frogs from ~2.5-fold at D10 and up to ~13-fold at D20 compared to the control group (Tables 1, 4).

For both Testo- and Control- males, during the 10 first days of housing, frogs were losing an average ~70 mg of body mass per day, representing 1% of their initial body mass. During the second period (D10–D20), individuals lost on average ~150 mg of body mass per day, representing between 2.3% and 3.1% of their body mass. At the end of the experiment, frogs after 20 days weighed 33% less than frogs sampled at 10 days. Experimental duration was the reason for the nonlinear decrease in frog body mass, independent of treatment (Tables 1, 4).

Within the 20 days of frog captivity, trunk muscle wet mass decreased by ~42%. This decrease was significant only between D10 and D20 (Tables 1, 4) that represents a significant decrease in the proportion of trunk muscle relative to body mass (Tables 1, 4). Hence, because the total water content (TWC) was similar during the housing period (Tables 1, 4), this discrepancy is unlikely linked to different dehydration process.

Gastrocnemius muscle wet mass followed the same decrease pattern as the frog body mass, representing a mass loss around 38% without any treatment effect but contrary to trunk, the ratio of muscle mass to body mass stayed at the same value (Tables 1, 4).

4.2. Muscle bioenergetics

4.2.1. Basal and phosphorylating states of muscle fibers

a. Muscle effects

Table 1
Characteristics of *Hyla arborea* males used through this experiment. Values are mean ± s.e.m. of 5–8 individuals for each condition. TWC; Total water content (%). Two-way ANOVAs were performed for each parameter with treatment (Control *vs.* Testo) and day (D10 or D20) as fixed effects. No interaction was found, therefore the symbol $^{\$}$represents *P*-values < 0.05 for treatment effect. The symbol *represents *P*-values < 0.05 for day effect.

	D10		D20	
	Control (8)	Testo (8)	Control (7)	Testo (5)
[Testosterone] (pg/mg)	7.0 ± 2.1	17.0 ± 1.7$^{\$}$	3.2 ± 0.6	42.4 ± 8.4$^{\$}$
Body mass (g)	6.2 ± 0.4	6.6 ± 0.3	4.8 ± 0.2*	4.8 ± 0.1*
Trunk muscle				
Wet mass (mg)	379.1 ± 9.0	363.4 ± 25.3	223.3 ± 19.8*	202.7 ± 26.3*
TWC	79.0 ± 1.5	76.1 ± 0.8	77.9 ± 1.1	79.0 ± 1.9
Ratio to body mass	6.3 ± 0.3	5.5 ± 0.3	4.6 ± 0.4*	4.2 ± 0.5*
Gastrocnemius muscle				
Wet mass (mg)	52.1 ± 3.6	57.2 ± 3.9	40.8 ± 2.2*	39.1 ± 2.4*
TWC	80.9 ± 0.4	80.4 ± 1.2	81.7 ± 0.6	81.5 ± 0.6
Ratio to body mass	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.0

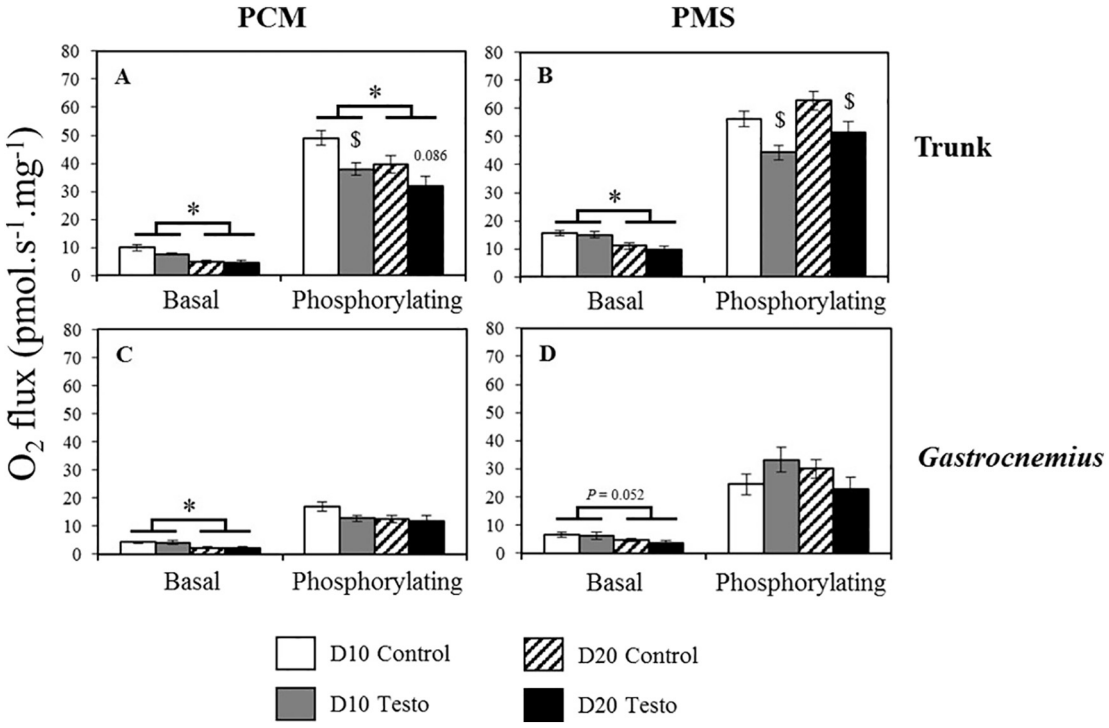

Fig. 1. Basal and phosphorylating respiration rates of trunk (Panel A and B) and *gastrocnemius* (Panel C and D) muscle fibers. Bars represent mean ± s.e.m. of 5–7 "Control" (open (D10), dash lined (D20) bars) or "Testo" (gray (D10) and black (D20) bars) individuals. Basal respiration rate was measured using either Palmitoyl/Carnitine-Malate (Panel A and C) or Pyruvate-Malate-Succinate (Panel B and D) without ADP. Phosphorylating respiration rate was obtained adding ADP. For each substrate (PCM or PMS) and each condition (basal or phosphorylating), muscle types were significantly different ($P < 0.05$). Effects of day are represented by the symbol (*) and effect of treatment by the symbol ($^{\$}$) for a *P*-value < 0.05 without any interaction.

Muscle fiber respiration rates, expressed in pmol $O_2\ s^{-1}\ mg^{-1}$ of wet tissue, were different depending on muscle type (Fig. 1). Indeed, trunk muscles (Fig. 1A and B) always exhibited a cellular respiration rate broadly higher than *gastrocnemius* fibers muscles (Fig. 1C and D), whatever the studied state and whenever the measurement occurred (Table 4). Origin of substrates used also affected the respiration rates associated with both states (phosphorylating and basal): Pyruvate-Malate-Succinate (PMS) (Fig. 1B and D) still induced a higher respiration rate than Palmitoyl-Carnitine-Malate (PCM) (Fig. 1A and C) for each day condition (Table 4). This difference was accentuated in *gastrocnemius* muscle compared to trunk muscle. Indeed, the ratio between PMS respiration and PCM respiration rates was around 2.5 for *gastrocnemius* and around 1.5 for trunk.

b. Testosterone effects

At D10, concerning the cellular basal O_2 flux, no effect of treatment was found, regardless of oxidized substrate and muscle phenotype (Fig. 1, Table 4). On the contrary, treatment induced variations in the phosphorylating states. Although there was no effect of testosterone supplementation on *gastrocnemius*, trunk phosphorylation respiration rate was significantly lower in Testo males than in Control for both day and substrate conditions (Fig. 1, Table 4).

c. Day effect

Basal respiration rates were significantly decreasing across the experimental duration, whatever the substrate or muscle (Fig. 1, Table 4). In contrast, oxygen fluxes of phosphorylating state did not vary throughout the experiment, except for Trunk muscle and PCM substrate where there were apparent declines between day 10 and day 20 (Fig. 1A, Table 4).

Table 2
PCM and PMS -induced aerobic scope (AS) and respiratory control ratio (RCR) of trunk and *gastrocnemius* frog muscle fibers. Values are mean ± s.e.m. of 5–7 individuals for each condition. AS was calculated as the difference between maximal respiration rate induced by FCCP and basal respiration rate obtained with Palmitoyl/Carnitine-Malate (PCM) or Pyruvate-Malate-Succinate (PMS) without ADP. We assume that it estimates the maximal range of aerobic activity the muscle fibers could sustain. The RCR is the ratio of the phosphorylating respiration rate upon the basal respiration rate of the fibers. It gives an estimation of the coupling state of the fibers. 2-WAY ANOVA were performed and the symbol *represents *P*-values < 0.05 for day effect and the symbol $illustrates the "treatment" effect with a *P*-value < 0.05. It is worth to note that a significant "substrate" effect was obtained for D10-Trunk RCR for both treatments but only for Testo-males at D10-*Gastrocnemius*. This effect was however significant for all data at D20. To simplify the table, we decided not to show this effect.

	D10			D20
	Control (7)	Testo (7)	Control (7)	Testo (5)
PCM (lipid-derived substrate)				
Trunk muscle				
AS	60.6 ± 1.8	56.5 ± 9.0	41.7 ± 3.9*	31.2 ± 3.8*
RCR	5.0 ± 0.3	5.0 ± 0.3	8.8 ± 1.0*	7.3 ± 0.8*
Gastrocnemius muscle				
AS	15.6 ± 2.5	13.3 ± 2.4	12.1 ± 1.2	9.4 ± 2.7
RCR	4.6 ± 0.8	3.4 ± 0.4	6.0 ± 0.7*	5.1 ± 0.9*
PMS (CHO-derived substrate)				
Trunk muscle				
AS	50.1 ± 12.3	61.0 ± 6.8	62.3 ± 5.3	37.6 ± 6.3*,$
RCR	3.6 ± 0.2	3.0 ± 0.1	6.0 ± 0.7*	5.4 ± 0.4*
Gastrocnemius muscle				
AS	NA	39.8 ± 4.6	38.0 ± 3.5	26.1 ± 3.2
RCR	4.0 ± 0.7	6.2 ± 1.0	6.5 ± 0.3	7.1 ± 1.6

4.2.2. Cellular aerobic scope and mitochondrial efficiency

a. Muscle effect

The fiber maximal aerobic capacity or cellular aerobic scope (AS) depended on muscle phenotype (Tables 2, 4). Indeed, trunk muscle fiber AS was similar for both substrates, PMS and PCM (~50 pmoles s^{-1} mg^{-1}, Table 2), whereas *gastrocnemius* muscle fibers exhibited a 2- to 3-fold higher AS induced by PMS (up to ~35 pmoles s^{-1} mg^{-1}) than that by PCM (up to ~15 pmoles s^{-1} mg^{-1}) (Tables 2, 4).

The respiratory control ratio (RCR), which is a classical proxy of mitochondrial efficiency, also depended on muscle phenotype and on substrate origin (Table 4). With PCM, trunk RCR was higher than *gastrocnemius* RCR regardless of the "treatment" and the "day" conditions. With PMS, trunk RCR remained lower for all day conditions (Tables 2, 4).

b. Testosterone effect

Testosterone supplementation only triggered a ~40% decrease in AS for trunk muscle when PMS substrates were being oxidized (Tables 3, 4).

Concerning the RCR, testosterone treatment was only involved in an interaction with substrate for *gastrocnemius* muscle: when PCM substrate is oxidized, RCR was lower in Testo males than in Control males, and RCR became higher for Testo males than for Control males with PMS (Tables 2, 4).

c. Time effect

For *gastrocnemius* muscle, PCM-AS did not vary through the different conditions. Note that because of an experimental issue, we were unable to estimate PMS-AS for this muscle. For trunk muscle, however, AS decreased at D20 (Tables 2, 4). Depending on the origin of substrates, at D20, RCR dropped for both groups (under PCM condition) or only for Testo-males (under PMS condition).

Table 3
Contractile properties of trunk and *gastrocnemius* muscles in tree frog control and testo-supplemented males. Relative maximal force ([N/N] g^{-1}) was calculated as the maximal value of the contraction reached during the tetanus divided by the highest twitch value obtained during the setup and then divided by the dry mass of muscle. Fatigue resistance was defined as the time for which the muscle forces drop to 90% of the maximal developed force. For more information, see M & M section. Contractile properties were significantly dependent on muscle type. Values are mean ± s.e.m. Sample size indicated within brackets corresponds to number of individuals exhibiting a plateau phase. Symbols represent either treatment effect ($) or day effect (*) without any interaction, with a *P*-value < 0.05.

		n	Relative F_{max} (N/N g^{-1})	Plateau duration (s)		Fatigue resistance (s)
Trunk muscle						
D10	Control	8	33.4 ± 6.15	(8)	16.3 ± 1.2	63.1 ± 4.5
	Testo	8	27.8 ± 2.1	(5)	8.2 ± 2.9$	63.6 ± 8.0
D20	Control	7	47.1 ± 5.8*	(6)	9.8 ± 2.3*	55.0 ± 5.2
	Testo	5	55.9 ± 12.8*	(5)	7.1 ± 0.1	42.0 ± 4.2*
Gastrocnemius muscle						
D10	Control	5	637.8 ± 151.8	(5)	5.1 ± 1.0	42.2 ± 4.2
	Testo	7	564.6 ± 109.6	(7)	8.3 ± 1.3$	50.2 ± 4.7
D20	Control	7	803.8 ± 121.7	(4)	2.8 ± 1.1	42.7 ± 3.2
	Testo	5	552.7 ± 81.1	(1)	0.6 ± 0.6*	32.9 ± 4.2

RCR increased significantly for both muscles following the same pattern for trunk muscle, while there was a significant interaction between day and treatment conditions for *gastrocnemius* muscle, as explained before (Tables 2, 4).

4.3. Muscle contraction properties

4.3.1. Maximal developed force

Comparison of the two muscles pointed out a sharp difference in contractile properties (Tables 3, 4) with a maximal force 10-fold higher for *gastrocnemius* than for trunk muscles. Relative maximal force (F_{max}) of trunk muscle significantly increased from ~31 N/N g^{-1} at D10 to ~55 N/N g^{-1} at D20 without any effect of treatment. *Gastrocnemius* muscle exhibited a relative F_{max} of ~600 N/N g^{-1} regardless of the day and without any effect of treatment (Tables 3, 4).

4.3.2. Plateau duration

In contrast with the force parameter, plateau duration was ~3-fold longer in trunk muscle than *gastrocnemius* muscle. Trunk plateau duration decreased significantly from 16.3 to 9.8 s and from 8.2 to 7.1 s between D10 and D20 for Control and Testo males, respectively (Table 3). Moreover, Testo males presented a shorter plateau duration that Control males at D10 (Tables 3, 4). On the other hand, *gastrocnemius* plateau duration decreased from 5.1 to 2.8 s and 8.3 to 0.6 s between D10 and D20 for Control and Testo males, respectively. It is worth noting that only 1 *gastrocnemius* muscle of testosterone-supplemented group exhibited a plateau at D20, the 4 others plateau durations were counted as zero seconds (Tables 3, 4).

4.3.3. Fatigue resistance

Fatigue resistance time clearly depended on muscle type and decreased within the time course of the experiments. This parameter however was not affected by testosterone treatment, except at D20 where it was reduced in the Testo group (Tables 3, 4).

5. Discussion

The ultimate goal of our work was to investigate the effect of testosterone upon the relationship between cellular bioenergetics and contractile properties of two skeletal muscles involved more or less directly in sexual selection in anurans. Indeed, trunk muscle is directly responsible for male vocalization, while the *gastrocnemius* muscle plays

Table 4
Parameters of 3-WAY or 2-WAY ANOVA used in the study. R (Ranks) means that ANOVA on ranks were performed.

Tested variable	Effect	df	F-value	AOV	*P*-value	REF
Frog characteristics						
[Testosterone]	TRT × Day	1; 24	6.317	2W R	0.019	Table 1
Body mass	Day	1; 24	27.550	2W	< 0.001	Table 1
Trunk wet mass	Day	1; 24	57.330	2W	< 0.001	Table 1
Trunk ratio to BM	Day	1; 24	16.533	2W	< 0.001	Table 1
Trunk TWC	None	1; 24		2W	NS	Table 1
Gastrocnemius wet mass	Day	1; 23	15.841	2W	< 0.001	Table 1
Gastrocnemius ratio to BM	None	1; 23		2W	NS	Table 1
Gastrocnemius TWC	None	1; 23		2W	NS	Table 1
Fiber bioenergetics						
Basal and phosphorylating state	Muscle			2W	< 0.001	
	Substrate			2W	< 0.001	
Trunk muscle						
PCM - basal	Day	1; 18	27.071	2W	< 0.001	Fig. 1A
PMS - basal	Day	1; 16	20.737	2W	< 0.001	Fig. 1B
PCM - Phosphorylating	TRT	1; 18	10.608	2W	0.004	Fig. 1A
	Day	1; 18	6.794	2W	0.018	
PMS - Phosphorylating	TRT	1; 16	13.316	2W	0.002	Fig. 1B
	Day	1; 16	4.727	2W	0.045	
Gastrocnemius muscle						
PCM - basal	Day	1; 19	12.772	2W	0.002	Fig. 1C
PMS - basal	Day	1; 17	4.372	2W	0.052	Fig. 1D
PCM - Phosphorylating	None	1; 19		2W	N·S	Fig. 1C
PMS - Phosphorylating	None	1; 17		2W	N·S	Fig. 1D
Aerobic scope	Muscle			3W	< **0.05**	
Trunk muscle						
PCM - AS	Day	1; 16	17.331	2W	< 0.001	Table 2
PMS - AS	TRT × Day	1; 16	4.738	2W	0.045	Table 2
Gastrocnemius muscle						
PCM - AS	None	1; 19		2W	> 0.05	Table 2
D20 - AS	Substrate	1; 26	70.124	2W	< 0.001	
Respiratory control ratio	Muscle			3W	< 0.01	
Trunk muscle						
RCR	Day	1; 34	43.936	3W	< 0.001	Table 2
	Substrate	1; 34	24.955	3W	< 0.001	Table 2
Gastrocnemius muscle						
RCR	Day	1; 36	7.877	3W	0.008	Table 2
	Substrate	1; 36	3.764	3W	0.06	Table 2
	TRT × Sub	1; 36	4.199		0.048	Table 2
Contractile properties						
Fmax	Day	1; 44	9.147	3W R	0.004	Table 3
	Muscle	1; 44	158.012	3W R	< 0.001	Table 3
Plateau duration	Day	1; 44	10.363	3W	0.002	Table 3
	Muscle	1; 44	22.555	3W	< 0.001	Table 3
	TRT × MSCL	1; 44	4.446	3W	0.041	Table 3
	Day × TRT × Muscle	1; 44	4.588	3W	0.038	Table 3
Fatigue resistance	Day	1; 44	8.713	3W	0.005	Table 3
	Muscle	1; 44	14.134	3W	< 0.001	Table 3
	Day × TRT	1; 44	4.550	3W	0.039	Table 3

a major role in locomotion, and therefore, in hunting ability, that is needed for both males and females. Our results show an effect of testosterone supplementation on trunk muscle bioenergetics, and especially on fiber respiration associated to ATP production. However, before discussing testosterone effects, we will focus on the difference between the two muscles, where we demonstrate a correspondence between oxidative capacity of different substrates and developed isometric force that corresponds with muscle phenotype and *in vivo* function.

5.1. Two bioenergetic patterns sustaining two specific contractile properties

As in mammals, anuran muscle fibers can be categorized according to fiber type and metabolic enzyme. The categories could be based on myosin-heavy-chain isoforms (Lutz et al., 1998, reviewed in Lutz and Lieber, 2000), as Type 1-, Type 2-, Type 3- twitch fibers and Type 4- and Type 5- tonic fibers (Crockett and Peters, 2008). These three twitch and two tonic types correspond roughly to another classification system based on the nature of oxidized substrates by the complexes of the

mitochondrial electron transport chain, such as slow-oxidative (SO), fast-oxidative-glycolytic (FOG) and fast-glycolytic (FG) fiber types (Marsh and Taigen, 1987). Therefore, Type 1 fibers can be described as large FG fibers, Type 2 and 3 as large and small FOG, Type 4 as an intermediate slow-twitch type and finally Type 5 as a true tonic fiber (Putnam and Bennett, 1983). Each isoform leads to specific contractile properties (Crockett and Peters, 2008; Lutz et al., 2002). Because of the lack of histochemical observation in our study, we purposely use FG and FOG denomination within the discussion.

As expected, our results show different bioenergetic patterns between trunk and hindlimb muscles, notably with respect to cellular aerobic scope (AS). Trunk muscle exhibited a higher lipid-induced AS (between 31.2 and 62.3 pmol s^{-1} mg^{-1}) than *gastrocnemius* muscle (between 9.4 and 15.6 pmol s^{-1} mg^{-1}) but this muscle type difference was reduced when CHO substrates were oxidized, likely because of the high values of AS for both types of muscle. This suggests that trunk muscle's high aerobic capacity is directly linked to its histochemical characteristics. Trunk oblique muscles are composed of 100% FOG fibers, with high capillary density and high mitochondrial content compared to *gastrocnemius* muscle (Marsh and Taigen, 1987). As well, a high oxygen transport must be combined with an efficient oxidative phosphorylation machinery to sustain a huge aerobic capacity. Our results show that trunk fiber of frog exhibited respiration rates induced by ADP addition comparable to those observed in rats (Picard et al., 2010).

In the same way, trunk muscle fibers are able to oxidize both substrates with approximately the same intensity. This result is supported by the enzymatic activities measured in *Hyla crucifer* (Taigen et al., 1985) or in *Limnodynastes peronii* (Rogers et al., 2007). Through high β-hydroxyacyl-CoA dehydrogenase (HOAD) activity in *H. crucifer* males, fatty acids are able to fuel trunk muscle during calling effort (Rogers et al., 2007; Taigen et al., 1985). This high enzymatic and functional ability to use lipids as fuel is reinforced by the high quantity of fat stored in trunk muscle (Bevier, 1997; Carvalho et al., 2008). The role of lipids as the main energy substrate during endurance effort is well-documented in mammals and birds (reviewed in Weber, 2011) and appears to operate similarly in anurans (reviewed in Navas et al., 2008). Catabolism of carbohydrate substrates, however plays the main role in *H. arborea* (Grafe and Thein, 2001). Our results are in line with this study, showing that trunk muscle fibers are obviously capable of oxidizing CHO derived substrates at high rates. But, even while *in vivo*, CHO substrates seem to be the main source of energy, cellular mechanisms are able to use both substrates indiscriminately.

Contrary to the trunk muscle, *gastrocnemius* muscle bioenergetic parameters (*i.e.* AS, respiration rate at basal and phosphorylating state) depend largely on the origin of the substrates. Compared to trunk, *gastrocnemius* muscle fibers exhibit a respiration rate approximately in the same range when fully activated by PMS, whereas lipid-induced bioenergetic parameters remain at a very low level. The inability to oxidize both substrate mixtures at the same rate may be explained by the fiber phenotype of this muscle. The *gastrocnemius* of hopping anurans is indeed composed by FOG, but also by FG fibers that are preferentially fueled by carbohydrates (Crockett and Peters, 2008; Marsh and Taigen, 1987; Mendiola et al., 1991; Moore, 1997; Putnam and Bennett, 1983). *Gastrocnemius* muscles of *Hyla arborea* are likely powered by rapidly and easily mobilized substrates through anaerobic metabolism (reviewed in Navas et al., 2008), such as in *Hyla versicolor* (Marsh and Taigen, 1987), *Rana perezi* (Mendiola et al., 1991), *Rana catesbeiana* (Crockett and Peters, 2008) or *Rana pipiens* (Putnam and Bennett, 1983). The large part of anaerobic catabolism is also supported by low activity of CS and high activity of glycolytic enzymes (phosphofructokinase, PFK and lactate dehydrogenase, LDH) in this muscle (Crockett and Peters, 2008; Given and McKay, 1990; Marsh and Taigen, 1987; Putnam and Bennett, 1983; Rogers et al., 2007; Taigen et al., 1985). In addition, a low HOAD activity (Marsh and Taigen, 1987; Taigen et al., 1985) combined with a low lipid storage (Carvalho et al., 2008) also gives clues about the low lipid use ability of *gastrocnemius* muscle.

These bioenergetics patterns are leading to different muscle contractile properties, directly linked to their specific role. To our knowledge, this is the first time that muscle contraction measurements were performed using a universal testing device (Instron) as an isometric transducer. Even if the Instron outputs may not be directly similar to former literature, we assumed that comparisons within our study, such as among days of experiment, treatment groups and muscle phenotypes, were reliable. Our results show a range of force values very different depending on the muscle type. Indeed, the *gastrocnemius* developed a relative maximum force (F_{max}) that was over 10-fold higher than the relative F_{max} of the trunk muscle. These results are coherent with the patterns obtained through more conventional devices (McLister et al., 1995; Marsh, 1999). The pattern of trunk contractile properties in this study is consistent with a stamina-designed muscle phenotype: a low relative force compared to *gastrocnemius*, but with a longer plateau duration.

Hylid locomotor muscles are not shaped for withstanding stamina due to the presence of the large FG fibers (Putnam and Bennett, 1983; Walton, 1993). The second characteristic of this kind of fiber is a very low fatigue resistance and our results support this description. Indeed, the plateau duration - defined as the time where contraction-developed force remains stable, is shorter for *gastrocnemius* than for trunk muscle. We assume that the plateau duration gives proxies i) about fatigue resistance: the shorter it is, the less resistant it is and ii) about the composition of the muscle fibers: the shorter it is, the less oxidative its nature. Concerning fatigue resistance, our results illustrate the trade-off between stamina and contraction speed and/or force (Marsh and John-Alder, 1994; Lutz and Rome, 1994, cited in Chadwell et al., 2002). It is worth mentioning that a plateau was only detected for 17 individuals out of a total of 24 for *gastrocnemius* muscles, with a dramatic drop at D20, where only 38% responded with any sustained force production. Concerning the low oxidative capacity of this muscle compared to trunk, it may be explained by the lower content of mitochondria in *gastrocnemius* than in oblique muscles, as well as in *sartorius* muscle compared to external oblique muscle (Marsh and Taigen, 1987). Indeed, large FG fibers take space at the sacrifice of oxidative machinery (mentioned in Girgenrath and Marsh 1999).

This relationship between oxidative capacities and muscle contractile functioning is clearly illustrated by the positive correlation between fiber lipid-induced aerobic scope and time of fatigue resistance in trunk muscle from among all the sampled frogs (Fig. 2A). This relationship was however not met for other conditions (*gastrocnemius*, Fig. 2B) and CHO-derived substrates (data not shown). All these results are in alignment with our hypotheses as we predicted that muscle bioenergetic performances depend on the metabolic substrate according to the ecological function of the muscles.

5.2. Testosterone supplementation mainly affects trunk bioenergetics

One striking result comes from the effect of testosterone on trunk bioenergetics, without any effect on *gastrocnemius* fiber respiration rate. Indeed, testosterone modulates the phosphorylating state of trunk fibers, under both substrate conditions. It was however surprising that testosterone decreased the phosphorylating state by ~20% compared to control group, leading to a significant decrease on the aerobic scope of the trunk at the end of the experiment. The decrease of the respiration rate associated with phosphorylating state in trunk muscle of testosterone supplemented frogs supports observations of old studies of Elliott's group (Eisenberg et al., 1949), showing an inhibition of oxygen uptake of rat skeletal muscle slices when testosterone was added *in vitro* in the solution.

This decrease in phosphorylating ability may represent a non-negligible energetic cost. Hence males with high testosterone may support a cost due to the inefficiency of phosphorylation. Testosterone

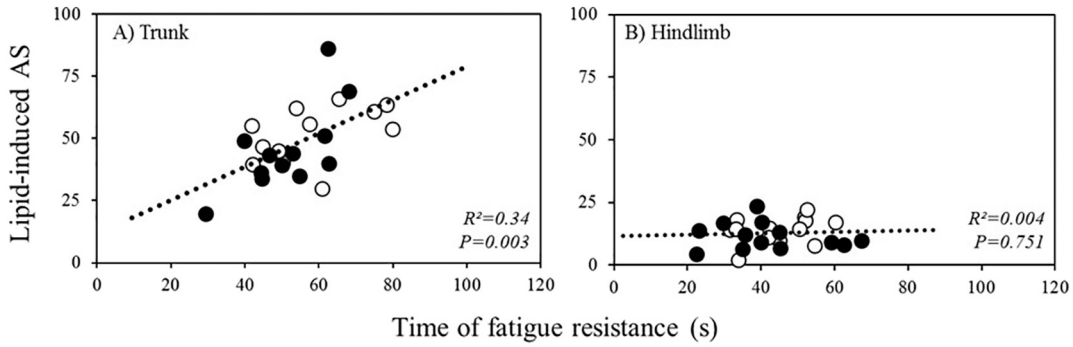

Fig. 2. Relationship between lipid-induced aerobic scope (PCM-AS) and the time of fatigue resistance of trunk (Panel A) and *gastrocnemius* muscles (Panel B). Treatment (*i.e.* Testo-males, dark circles, and Control-males, open circles) was not taken into account for the linear regression. For trunk muscle, the equation is: $y = 0.671x + 11.771$, $r^2 = 0.34$, $P = 0.003$ and for *gastrocnemius* muscle, the equation is: $y = 0.03x + 11.562$, $r^2 = 0.004$, $P = 0.751$.

was already thought to involve costs for organism particularly in a sexual selection context. Indeed, the immunocompetence handicap hypothesis suggests that testosterone serves a dual role in mediating both sexual signal expression and immunosuppression (Folstad and Karter 1992). Therefore, only high-quality males can afford to both fully express sexual traits and be able to resist parasite and pathogen attack. Our results suggested another form of handicap due to testosterone's effects on metabolic efficiency of muscles.

Contractile forces were not affected by testosterone supplementation in the trunk muscle nor in the *gastrocnemius* muscle. These results are surprising because of the numerous studies showing androgen effects on muscular force development in animals (for review, see Higham and Irschick, 2013). Our results show however an effect of testosterone on the contraction duration of the plateau in trunk muscle that was significantly shorter for Testo-males than for Control-males. The plateau duration could illustrate the contractile capacity of different fiber isoforms. It is however unlikely that the difference here comes from a direct influence of testosterone on fiber isoform transitions. This process was described in earlier metamorphic stages of the larynx in *Xenopus laevis* (Sassoon et al., 1987; Tobias et al., 1991).

5.3. Caloric restriction and inactivity may affect muscles functioning

Another main effect was highlighted through our experiment: the "Day" effect (see Table 4). This effect however was mainly observed on frog characteristics (Table 1) and bioenergetics parameters (Table 2 and Fig. 1). Following the housing protocol already published (Desprat et al., 2015), feeding frogs with two crickets led to a body mass loss, which characterizes a caloric restriction probably due to the experimental duration effect recorded in our study independent of the testosterone effect. During the breeding season in the wild, a significant body-mass decrease linked with calling activities has often been observed in males (Wells, 1977; Eggert and Guyétant, 2003; Girgenrath and Marsh, 2003; Meuche and Grafe, 2009). Our results (~40% mass loss of both muscles) suggest that muscle mass losses were actually due to intensive depletion of *in situ* substrates or protein catabolism, instead of dehydration process: total water content stays at the same level for all conditions, whatever the day and the treatment (Sidor and Blackburn, 1998). Despite this muscle mass loss, relative developed force did not decrease. Contrary to *gastrocnemius* muscle, the ratio of trunk muscle to body mass decreased during the experiment. This difference may be partially explained by a sparing mechanism induced by an energy constraint. Undergoing a food-scarcity period, frogs may preserve their locomotor muscles instead of trunk muscles, which are known to be atrophied during the non-breeding period (Girgenrath and Marsh, 2003). Such a prioritizing strategy toward locomotor muscle was previously described in other animals, such as birds (Dial and Carrier, 2012), when facing fasting periods (Monternier et al., 2015). It is also worth noting that trunk muscle contains more lipids *in situ* than *gastrocnemius*, and therefore during caloric restriction, the percentage of lipid depletion is likely more important in trunk than in *gastrocnemius* muscle. Even if we did not examine the nature and the amount of energetic substrates stored in the two muscles, the relationship between lipid-induced aerobic capacity and fatigue resistance only found in trunk muscle gives clues about the importance of lipid metabolism for sustaining endurance exercises (Fig. 2).

In the same way, our results show that the *gastrocnemius* muscle was generally less affected by the housing period duration than the trunk muscle. Due to a sexual dimorphism, trunk muscle exhibits a massive seasonal variation in terms of mass and contractile properties (Girgenrath and Marsh, 2003).

Notwithstanding these structural differences, it is interesting to note that both muscles followed a common pattern to adjust their bioenergetic parameters. Instead of increasing the ability of oxidizing substrates, frog muscle exhibited a lower basal respiration rate which result in increasing their mitochondrial efficiency. A low basal respiration rate would be a characteristic of a limited proton leakage across the inner membrane. This mechanism is known to be an energy sparing process in the mitochondria (Boutilier and St-Pierre, 2002).

6. Conclusion

Our results showed that trunk muscle and *gastrocnemius* have contrasted bioenergetics and contractile characteristics, and differentially responded to a strong elevation of testosterone. Hence, trunk muscle bioenergetics depends on aerobic oxidation of both lipid and CHO substrates, which was positively correlated with fatigue resistance, according to the high aerobic calling activity of these muscle groups. However, trunk muscle was more sensitive to testosterone supplementation. Indeed, males with high testosterone may incur a cost due to the lesser efficiency of phosphorylation. Our results suggested an energetic tradeoff results from testosterone.

This energetic cost could be harsher when combined with a period of caloric restriction, typical of the breeding period of *Hyla arborea*. Frogs indeed alternate vocalization sessions with foraging.

When animals face a caloric restriction and an inactivity period, trunk and *gastrocnemius* muscles followed the same pattern of energy sparing mechanisms, such as lowering their basal respiration rate. The understanding of these two energetics challenges deserves further investigation to accurately discriminate the role of each of them in muscle bioenergetics.

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