

1 **Biomarker-based assessment of the muscle maintenance and energy status of**
2 **anurans from an extremely seasonal semi-arid environment, the Brazilian**
3 **Caatinga**

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5 **RUNNING TITLE: Anuran muscle maintenance and energy status**

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16 **Key words:** AKT; eIF2 α ; AMPK, Heat shock proteins, cytochrome c oxidase;
17 aestivation

18

19 **Summary statement**

20 We studied seasonal variation of key metabolic regulators in the muscles of anurans that
21 experience drastic variation in environmental conditions and differ in the seasonal
22 activity patterns.

23

24 **Abstract**

25 Strongly seasonal environments pose challenges for performance and survival of
26 animals, especially when resource abundance seasonally fluctuates. We investigated the
27 seasonal variation of expression of key metabolic biomarkers in the muscles of three
28 species of anurans from the drastically seasonal Brazilian semi-arid area, Caatinga. The
29 three studied anuran species (*Rhinella jimi*, *R. granulosa* and *Pleurodema diplolister*)
30 differ in their seasonal activity patterns. We examined the expression of proteins
31 regulating energy turnover (AMP-activated protein kinase [AMPK] and protein kinase
32 B [AKT]), protein synthesis and homeostasis (total and phosphorylated eukaryotic
33 initiation factor 2 α [eIF2 α and p-eIF2 α] and chaperone proteins [HSP 60, 70, and 90])
34 in muscles related to reproduction and locomotion. Cytochrome c oxidase (COX)
35 activity was also assessed as an index of the muscle aerobic capacity. Our results point
36 to the importance of metabolic regulators mediating the muscular function during the
37 drastic seasonal variation. The toads that remain active during the drought appear to
38 maintain muscles through more energy extensive pathways including elevated protein
39 synthesis, while the aestivating species employs energy conservation strategy
40 suppressing protein synthesis, decreasing chaperone expression and increasing
41 expression of AMPK. All three studied species activate cell survival pathways during
42 the drought likely to prevent muscle atrophy, and maintain the muscle capacity
43 throughout the year, despite the resource limitation. These strategies are important
44 considering the unpredictability of the reproductive event and high demand on muscular
45 activity during the reproductive season in these amphibians.

47 **Introduction**

48 Strongly seasonal environments characterized by large changes in conditions
49 such as temperature and food availability, pose challenges to the organisms that need to
50 adjust their functions to survive and complete their life cycles [1, 2]. These
51 physiological adjustments are dependent on biochemical regulators and cell signaling
52 pathways that mediate cell survival, ensure cell and organ integrity and prioritize the use
53 of energy to meet the (often conflicting) needs of survival, growth and reproduction
54 [1,3]. In vertebrate ectotherms, the skeletal muscle physiology and performance are
55 directly related to the overall fitness due to the muscles' involvement in courtship,
56 territorial defense, foraging, escape from predators, mating interactions and migration
57 [4]. In seasonally fluctuating environments, skeletal muscles can display phenotypic
58 changes such as atrophy and changes in the fiber number and type during extreme
59 environmental stress and/or resource limitation [5]. Nevertheless, anuran species that
60 aestivate display little or no changes in muscle morphology and performance [6,7]
61 suggesting that species living in arid and semi-arid environments may have molecular
62 regulatory mechanisms supporting the muscle maintenance and functionality under the
63 unfavorable environmental conditions.

64 There is an extensive literature on physiological and biochemical adjustments
65 associated with metabolic depression in anurans from environments with strongly
66 seasonal and/or unpredictable climatic variation [8,1,2]. However, possible biochemical
67 adjustments displayed by anurans that remain active during the periods of severe
68 environmental stress and resource limitation are not well understood. Furthermore,
69 different locomotor muscles can display distinct reduction in cross-sectional area and
70 mechanical proprieties in aestivating anuran species and mammalian hibernators,
71 prioritizing the maintenance of the muscles most relevant to performance [7,9]. Yet, it is
72 unknown whether seasonal adjustments differ between reproductive and locomotor
73 muscle in anurans, reflecting preferential investment into the maintenance and
74 reproductive effort. The possible molecular adjustments of locomotor and reproductive
75 muscles that help support individual's performance and survival [1] under
76 environmental stress and resource limitation are not well understood and require further
77 investigation.

78 Anurans from the Brazilian semi-arid area, the Caatinga, face drastic seasonal
79 changes in environmental conditions [10-12]. In the Caatinga, anurans depend on
80 unpredictable heavy rain events (during the short rain season between January and
81 April) to reproduce. Occasional small showers during the rainy season are not sufficient
82 to trigger breeding, yet make water and food more easily available to anurans. The rest
83 of the year is the dry season, characterized by scarce water and food resources. In some
84 years, there is no rain so that the anurans have no reproductive opportunity until next
85 rainy season. Anurans from the Caatinga show interspecific variation in behavioral
86 strategies to survive the drought. Some species such as *Rhinella jimi* and *R. granulosa*
87 remain active and foraging around humid areas [13]. In contrast, *Pleurodema diplolister*
88 aestivates and does not feed until the first rains start [12; 14]. Previous studies show that
89 these anuran species display adjustments in reproductive [13] and immune physiology
90 [14] throughout the year. During the reproductive period, they display elevated plasma
91 levels of androgens and higher immunological profile and response compared with the
92 dry season [14]. During the drought, anurans present lower steroid plasma levels and
93 lower immune parameters and performance, with the strongest suppression observed in
94 the aestivating species [13,14]. This stark seasonality of the physiology and behavior in
95 the anurans from the semi-arid Caatinga suggests a strong selective pressure to regulate
96 energy metabolism and muscle function to meet the seasonally variable demands of
97 reproduction, resource acquisition and activity (including the physiological challenge of
98 metabolic depression during aestivation).

99 To assess the potential mechanisms involved in the regulation and maintenance
100 of the muscle function in anurans from an extremely seasonal environment of Brazilian
101 semi-arid Caatinga, we investigated the seasonal variation of expression of key
102 metabolic regulators in the muscles of males from three species of anurans that differ in
103 their seasonal activity patterns: *Rhinella jimi* and *R. granulosa* that remain foraging
104 during drought and *P. diplolister*, which aestivates during this period. We studied
105 muscles predominantly specialized on reproduction (including the trunk and larynx
106 muscles used to sustain calling activity [15,16] and the flexor carpi radialis used in
107 amplexus (grasping) behavior [17]) as well as the muscles predominantly specialized on
108 locomotion (the plantaris muscle [18]). During dry season, we expected to see an
109 increase in the pathways that support cell survival and stress response in the muscle,
110 along with the suppression of the protein synthesis and catabolic pathways [3], and a

111 decrease of muscle aerobic capacity to conserve energy [19], especially in muscles
112 related to reproduction. The prioritization of the muscles integrity during drought might
113 ensure the rapid mobilization of these muscles during an unpredictable reproductive
114 opportunity, and thus would be adaptive. We also anticipated that the aestivating
115 species would display a more intense downregulation of metabolic functions, such
116 protein synthesis, compared with the species that remain active year round.

117 To test these hypotheses, we investigated the expression of two protein kinases
118 that play a key role in the regulation of energy turnover in the muscle (the AMP-
119 activated protein kinase and protein kinase B) and key proteins involved in the
120 regulation of the protein synthesis and homeostasis (eukaryotic initiation factor 2 α and
121 chaperone proteins). The cytochrome c oxidase (COX) activity was assessed as an index
122 of the mitochondrial aerobic capacity in the tissue [20]. The AMP-activated protein
123 kinase (AMPK) is an energy sensor of the cell responding to the AMP:ATP ratio
124 [21,22]. During periods that animals need to save energy, AMPK is activated to regulate
125 catabolic *versus* anabolic metabolism increasing ATP synthesis and suppressing ATP
126 consumption [21,22]. Protein kinase B (AKT) plays a central role in metabolism and
127 cell survival stimulating glucose uptake, glycogen synthesis, lipogenesis and protein
128 synthesis, and regulating the cell cycle and apoptosis [23]. Eukaryotic initiation factor
129 2 α regulates protein synthesis and plays a key role in the stress response and
130 suppression of the ATP-consuming protein synthesis under low energetic budget
131 scenarios [24]. Heat shock proteins are involved in the general stress response acting as
132 molecular chaperones and regulating folding of newly synthesized proteins or those
133 damaged by stressors [25]. Considering their key roles in regulation of the muscle
134 integrity and function, we expect to see different patterns of activation of the studied
135 signaling proteins across the season in different species. We also anticipate that the
136 stress-related pathways involved in energy conservation are upregulated and the aerobic
137 capacity (measured as the COX activity) is suppressed in anuran muscles during the
138 drought, when the species that remain active (*R. jimi* and *R. granulosa*) are facing food
139 and water shortage, and *P. diplolister* is aestivating.

140

141 **Materials and Methods**

142 **Field collections**

143 Field work was conducted at Fazenda São Miguel near the city of Angicos, in
144 the State of Rio Grande do Norte, Brazil (5°30'43''S. 36°36'18''W). The area is in the
145 domain of Brazilian Caatinga, and is characterized by high temperatures. January is the
146 hottest month with an average temperature of 27.4°C (minimum: 22.8°C, maximum:
147 32.0°C), and July is the coldest month, with an average temperature of 24.3°C
148 (minimum: 20.3°C, maximum: 28.3°C) (<http://pt.climate-data.org/location/312354/>).
149 The annual average temperature from 1950 to 2000 is 26.6 °C (worldclim.org) and there
150 are two distinct seasons: a rainy season (January to April, 96.4 mm of
151 precipitation/month) and a dry season (August to November, 2.5 mm of
152 precipitation/month). The intermediate months of June, July and December can be
153 considered dry or rainy depending on the extent of drought in the specific year. In
154 response to the challenges of the dry season, anurans from this locality have adopted
155 different behavioral strategies. *R. granulosa* and *R. jimi* remain active, foraging close to
156 humid areas and artificial water sources [14], while *Pleurodema diplolister* aestivate in
157 the sandy soil under the beds of temporary rivers [12]. For this study, animals were
158 collected during two different periods in 2015: (A) during the reproductive season
159 (March, 5–12th, 2015); (B) during the dry season (August, 10–16th, 2015).

160 During the reproductive period, males of *R. granulosa* (N = 13), *R. jimi* (N = 11)
161 and *P. diplolister* (N = 16) were located by visual inspection. During the dry period,
162 males of *R. granulosa* (N = 6), *R. jimi* (N = 7) were found by visual inspection, and *P.*
163 *diplolister* (N = 8) was found by excavating the known burrowing sites in the sandy
164 soil. Anurans were collected and individually maintained in plastic containers with
165 access to water, except the individuals of *P. diplolister* collected during the dry period,
166 which were maintained in plastic containers filled with humid sand collected in the
167 location they were found. After two days, animals were weighted (to the nearest 0.01g)
168 and euthanized with an injection of sodium thiopental solution (25 mg/ml)
169 (Thiopentax) while kept in an ice-cold dry bath. The muscles (plantaris from the
170 posterior limb, flexor from anterior limbs, larynx and trunk) were rapidly dissected.
171 Muscle samples were either immediately frozen in liquid nitrogen (for immunoblotting
172 analyses), or incubated for 1 to 2 minutes in a cryopreservation medium (10 mM
173 EGTA, 1.3 mM CaCl₂, 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM
174 K₂HPO₄, 9.5 mM MgCl₂, 5 mM ATP, 15 mM phosphocreatine, 10 mg/ml fatty acid-

175 free BSA, 20% glycerol, pH 7.1) [26] prior to freezing (for COX activity). Tissues were
176 stored in liquid nitrogen until their transport to the University of North Carolina at
177 Charlotte (UNC Charlotte), NC, USA on dry ice. At UNC Charlotte the muscle samples
178 were stored at -80°C until analyses. Fieldwork, maintenance of animals, and transport of
179 samples were conducted under the approved permissions of Comissão de Ética no Uso
180 de Animais do IB (CEUA) (Protocol number: 181/2013) and Ministério do Meio
181 Ambiente, ICMBio, SISBio (License to collect and transport animals: N°29896-1;
182 Export License number: 15BR017888/DF).

183 **Immunoblotting**

184 Muscle samples were homogenized (1:10 w:v) in ice-cold buffer (100 mM Tris,
185 pH = 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 10% glycerol,
186 0.1% sodium dodecylsulfate [SDS], 0.5% deoxycholate, 0.5 $\mu\text{g mL}^{-1}$ leupeptin, 0.7 μg
187 mL^{-1} pepstatin, 40 $\mu\text{g mL}^{-1}$ phenylmethylsulfonyl fluoride [PMSF], and 0.5 μg
188 mL^{-1} aprotinin), sonicated three times for 10 s each (output 69 Watts; Sonicator 3000,
189 Misonix Inc.) and centrifuged at $14000 \times g$ for 5 min at 4°C. The protein content of the
190 supernatant was measured using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA,
191 USA) with the bovine serum albumin (BSA) as a standard. Protein-containing
192 supernatant was mixed 3:1 (v:v) with a solution containing 4 parts of 4x Laemmli buffer
193 and 1 part of 1 M dithiothreitol (DTT), boiled for 5 minutes and frozen in -20°C until
194 further analysis.

195 Samples (20-50 μg protein per lane, depending on the antibody) were loaded
196 into 10% polyacrylamide gels and run at 72V for 3 hours at room temperature. After the
197 run, the gels were incubated for 30 min in 96 $\cdot\text{mmol}\cdot\text{l}^{-1}$ glycine, 12 $\cdot\text{mmol}\cdot\text{l}^{-1}$ Tris and
198 20% methanol (v/v). The proteins were transferred to a nitrocellulose (for HSP60,
199 HSP70 and HSP90) or polyvinylidene difluoride (PVDF) membrane (for all other
200 antibodies) using a Trans-Blot semi-dry cell (Thermo Fisher Scientific Inc., Portsmouth,
201 NH, USA). Membranes were blocked for one hour in 3% non-fat milk in Tris-buffered
202 saline, pH 7.6 with 0.1% Tween 20 (TBST) at room temperature, and incubated
203 overnight at 4°C with the primary antibodies diluted 1:1000 in 5% BSA in TBST. After
204 washing off the primary antibody with TBST, the membranes were probed with the
205 polyclonal secondary antibodies conjugated with horseradish peroxidase (Jackson
206 ImmunoResearch, West Grove, PA, USA) diluted 1:1000 with 3% non-fat milk in

207 TBST for one hour at the room temperature. After washing off the secondary antibody,
208 the proteins were detected using enhanced chemiluminescence according to the
209 manufacturer's instructions (Amersham Biosciences, Pierce, Rockford, IL, USA). The
210 signals were captured on X-ray film and relative optical density of protein bands was
211 digitalized with an image analysis software (Gel Doc EZ Imager, Bio-Rad, Hercules,
212 CA, USA) and quantified using Image Lab™ software (Bio-Rad Laboratories Inc.,
213 Hercules, CA, USA). The loading order of samples in the gels was randomized. The
214 protein loads per lane were identical for all muscle types and for both studied seasons
215 except for p-eIF2 α in *R. granulosa* collected during the dry season where the original
216 load of 30 μ g per lane did not produce a signal, and 50 μ g per lane was used. A single
217 sample (used as an internal control) was loaded on each gel and used to standardize the
218 expression of the target proteins and account for the potential gel-to-gel signal variation.
219 The following antibodies were used: AKT (AKT rabbit polyclonal IgG; Cell Signalling
220 Technology, cat. #9272, Danvers, MA, USA), total AMP-activated protein kinase
221 (AMPK) (AMPK α , Thr172, Rabbit mAb, Cell Signalling Technology, cat. #2535,
222 Danvers, MA, USA), phospho-EIF- 2 α (Ser51) (no. 07-760, Millipore, cat. #07-760,
223 Temecula, CA, USA), EIF-2 α (no. AHO1182, Life Technology, Grand Island, NY,
224 USA), HSP 60 (HSP60 [insect] polyclonal antibody, Enzo Life Science, cat.#ADI-SPA-
225 805-D, Farmingdale, NY, USA), HSP 70 (Heat Shock Protein 70 [HSP70] Ab-2, Mouse
226 Monoclonal Antibody, Thermo Fisher Scientific Inc., cat. # MA3-006, Portsmouth, NH,
227 USA), HSP 90 (Anti-HSP90 antibody, Rat [monoclonal], cat. #SPA-835, Stressgen
228 Bioreagents, Ann Arbor, MI, USA). All antibodies produced a single band of the
229 expected length (S1 Fig).

230 **Measurements of cytochrome c oxidase capacity**

231 Cryotubes containing the frozen plantaris or trunk muscles were incubated for
232 ~ 2 min at 35°C until the cryopreservation medium was completely thawed. The muscle
233 fibers were immediately washed in ice-cold medium containing 120 mM KCl, 10 mM
234 NaCl, 2 mM MgCl₂, 2 mM KH₂PO₄, 20 mM HEPES, 1mM EGTA Ca-free, 10 μ g mL⁻¹
235 PMSF and homogenized in 1-2 ml of the same media with several passes of a Potter-
236 Elvehjem homogenizer and a loosely fitting Teflon pestle at 200 rpm. The homogenate
237 was centrifuged at 2000 \times g and 4°C for 8 min to remove cell debris, and the
238 supernatant containing mitochondria was used to measure activity of cytochrome c
239 oxidase (COX).

240 COX activity was determined by measuring the oxygen consumption of
241 mitochondria-containing supernatant at 23°C in the presence of 5 µM antimycin A, 5
242 mM ascorbate and 10 mM *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD) as an
243 electron donor [27]. Oxygen concentrations were monitored using a fiber optic oxygen
244 sensor connected to the Microx TX3 oxygen monitor with temperature correction
245 (Precision Sensing, Dusseldorf, Germany) and Oxy Micro ver. 2.00 software (World
246 Precision Instruments, Sarasota, FL). A two-point calibration was performed prior to
247 each measurement. To correct for the potential autooxidation of TMPD, oxygen
248 consumption was measured after addition of 25 mM KCN to inhibit COX, and the
249 difference in the oxygen consumption rates in the presence and absence of KCN was
250 used to calculate COX activity. Concentrations of oxygen in the respiration chamber
251 were monitored using Logger Pro 3.2 with a Vernier LabPro interface (Vernier
252 Software and Technology, Beaverton, OR). Protein concentrations in mitochondrial
253 isolates were measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) in
254 the presence of 0.1% Triton X-100 to solubilize mitochondrial membranes, with BSA as
255 the standard. COX activity was expressed as µmol O₂ min⁻¹ g⁻¹ mitochondrial protein.
256 Each biological replicate represented an individual isolate obtained from the pooled
257 tissues of 2–3 animals.

258 **Statistical analysis**

259 Variables were log₁₀ transformed to improve normality. For the immunoblotting
260 results, a two-way ANOVA was used to compare the effects of the season, gel and their
261 interaction on protein expression (measured as a densitometric signal). When the effects
262 of the gel and/or gel x season interactions were not significant, a one-way ANOVA was
263 used to test for the effect of the season. To compare different muscles within a season, a
264 one-way ANOVA was used followed by Bonferroni post-hoc test. For COX activity, a
265 two-way ANOVA was used to compare the effects of species and muscles (COX ~
266 species + muscle); and species and season in each muscle (COX_{muscle} ~ species +
267 season). Assumptions of the ANOVA and t test (normality and equal variance) were
268 met and statistical significance was set at P < 0.05. All tests were run in R software,
269 version 2.10.0 (R Development Core Team, 2010). Data are shown as means ± the
270 standard error of the mean (S.E.M).

271 **Results**

272 Metabolic signaling

273 Total AMPK showed higher expression during the dry period compared to the
 274 reproductive period in all studied muscle types of *R. jimi* (larynx - $F_{1,6} = 0.25$, $P =$
 275 0.001 ; trunk - $F_{1,12} = 0.68$, $P = 0.001$; flexor - $F_{1,9} = 0.13$, $P = 0.04$; plantaris - $F_{1,12} =$
 276 0.65 , $P = 0.003$) (Fig 1). A similar trend was seen in *P. diplolister*, albeit it was only
 277 significant in the plantaris muscle ($F_{1,10} = 0.14$, $P = 0.002$) (Fig 1C). *R. granulosa*
 278 displayed similar AMPK expression levels across the two studied seasons in all muscle
 279 types (Flexor: $F_{1,8} = 2.74$, $P = 0.14$; trunk: $F_{1,12} = 0.84$, $P = 0.38$; plantaris: $F_{1,12} = 0.34$
 280 $P = 0.57$). When compared within the same season, AMPK levels in different muscle
 281 types were similar within *R. jimi* and *R. granulosa* ($P > 0.05$, Table 1). In *P. diplolister*,
 282 AMPK levels were similar in different muscle types during the dry period (Table 1).
 283 During the reproductive period, trunk muscles of *P. diplolister* showed higher AMPK
 284 expression compared with other muscles ($F_{2,20} = 4.23$, $P = 0.03$, Bonferroni $P = 0.03$)
 285 (S2 Fig).

286 **Fig 1. Expression of AMPK and AKT in different muscle types of *R. granulosa*, *R.***
 287 ***jimi*, and *P. diplolister* during reproductive and dry periods.** $n = 3-10$. AMPK
 288 expression is shown for (A) larynx, (B) trunk, (C) plantaris, and (D) flexor muscles.
 289 AKT expression is shown for (E) larynx, (F) trunk, (G) plantaris, and (H) flexor
 290 muscles. Asterisks indicate significant difference between the reproductive and dry
 291 periods ($P < 0.05$). ND - not determined because of the lack of samples.

292

293 **Table 1. ANOVA: comparison of protein expression in different muscles within a**
 294 **season. F ratios (with the degrees of freedom and the error shown as a subscript),**
 295 **P values are given, and significant effects ($P < 0.05$) are highlighted in bold.**
 296 **Missing analysis are either protein detection below limit or lack of samples.**

	<i>Rhinella jimi</i>	<i>Rhinella granulosa</i>	<i>Pleurodema diplolister</i>
	Reproductive	Reproductive	Reproductive
AKT	$F_{3,13} = 3.69$ $P = 0.04$	$F_{3,13} = 3.35$ $P = 0.053$	$F_{3,11} = 0.91$ $P = 0.47$
	Dry	Dry	Dry
	$F_{3,6} = 4.56$ $P = 0.06$	$F_{3,5} = 0.34$ $P = 0.80$	$F_{3,10} = 1.39$ $P = 0.30$
AMPK	Reproductive	Reproductive	Reproductive

297

	$F_{3,18} = 2.44$ P = 0.10	$F_{3,13} = 1.70$ P = 0.20	$F_{3,17} = 3.42$ P = 0.04
	Dry	Dry	Dry
	$F_{3,18} = 2.35$ P = 0.11	$F_{2,7} = 0.72$ P = 0.52	$F_{1,10} = 0.14$, P = 0.002
	Reproductive	Reproductive	Reproductive
p-eIF2α	$F_{3,14} = 2.21$ P = 0.13	$F_{3,6} = 0.87$ P = 0.51	$F_{3,15} = 1.59$ P = 0.24
	Dry	Dry	Dry
	$F_{3,14} = 0.74$ P = 0.55	-	$F_{3,6} = 11.53$ P = 0.01
	Reproductive	Reproductive	Reproductive
eIF2α	$F_{3,15} = 11.18$ P = 0.001	$F_{3,18} = 23.82$ P = 0.001	$F_{3,19} = 2.89$ P = 0.06
	Dry	Dry	Dry
	$F_{3,5} = 26.67$ P = 0.002	$F_{3,6} = 1.19$ P = 0.39	$F_{3,5} = 1.02$ P = 0.46
	Reproductive	Reproductive	Reproductive
p-eIF2α/ eIF2α	$F_{3,10} = 1.94$ P = 0.19	$F_{3,5} = 0.17$ P = 0.92	$F_{3,12} = 1.28$ P = 0.32
	Dry	Dry	Dry
	$F_{3,6} = 11.30$ P = 0.007	-	$F_{3,4} = 3.02$ P = 0.16
	Reproductive	Reproductive	Reproductive
HSP60	$F_{3,22} = 9.44$ P = 0.001	$F_{3,15} = 3.70$ P = 0.04	$F_{2,13} = 1.46$ P = 0.27
	Dry	Dry	Dry
	$F_{3,12} = 13.90$ P = 0.001	$F_{2,6} = 0.002$ P = 0.99	$F_{3,5} = 2.65$ P = 0.16
	Reproductive	Reproductive	Reproductive
HSP70	$F_{3,16} = 10.36$ P = 0.001	$F_{3,9} = 0.47$ P = 0.71	$F_{2,25} = 0.92$ P = 0.41
	Dry	Dry	Dry
	$F_{3,6} = 0.90$ P = 0.50	$F_{3,8} = 1.16$ P = 0.38	$F_{3,8} = 2.63$ P = 0.12
	Reproductive	Reproductive	Reproductive
HSP90	$F_{3,12} = 28.07$ P = 0.001	-	$F_{2,8} = 40.87$ P = 0.001
	Dry	Dry	Dry
	$F_{3,14} = 1.45$ P = 0.27	-	$F_{1,4} = 0.003$ P = 0.96

298

299 Expression of the protein kinase B (AKT) tended to be higher during the dry
300 period in all studied species and muscle types (except flexor for all species and the
301 larynx of *R. jimi*; Fig 1E-H). This trend was significant in the larynx muscles from *P.*
302 *diploister* ($F_{1,8} = 0.58$, P = 0.001), the trunk muscles from *R. granulosa* and *P.*
303 *diploister* ($F_{1,7} = 1.55$, P = 0.002; $F_{1,6} = 0.79$, P = 0.006), and the plantaris muscle from
304 all three studied species (*R. granulosa* - $F_{1,7} = 0.24$, P = 0.03; *R. jimi* - $F_{1,6} = 0.25$, P =
305 0.001; *P. diploister* - $F_{1,11} = 1.21$, P = 0.002) (Fig 1E-G). There were no significant
306 differences of AKT expression between seasons in the flexor muscle in the three studied
307 species (*R. granulosa* - $F_{1,6} = 3.57$, P = 0.11; *R. jimi* - $F_{1,8} = 0.19$, P = 0.68; *P.*

308 *diplolister* - $F_{1,4} = 5.58$, $P = 0.08$). AKT levels were the same across different muscle
309 types when compared within the same season in all three studied species ($P > 0.05$,
310 Table 1).

311 Protein homeostasis

312 Total expression levels of the eukaryotic initiation factor 2 α (eIF2 α) were lower
313 during the dry period than in the reproductive period in the larynx and flexor muscles
314 from *P. diplolister* ($F_{1,7} = 0.079$, $P = 0.016$; $F_{1,4} = 0.110$, $P = 0.04$) (Fig 2A-D), and in
315 the trunk and plantaris muscles from *R. granulosa* ($F_{1,14} = 1.06$, $P = 0.002$; $F_{1,12} = 0.132$,
316 $P = 0.02$) (Fig 2A-D). In all other muscle types (including the trunk and plantaris of *P.*
317 *diplolister*, the larynx and flexor of *R. granulosa*, and all muscle types of *R. jimi*),
318 season had no significant effect on eIF2 α expression ($P > 0.05$). In *R. granulosa*, during
319 the dry period, the flexor muscle had the highest levels of total eIF2 α when compare
320 with the other studied muscles types; during reproductive period, the flexor and trunk
321 muscles had elevated levels of eIF2 α compared larynx and plantaris (Table 1; S3A and
322 B Fig; Bonferroni $P = 0.001$ and 0.028 , respectively). For *R. jimi* collected in the
323 reproductive period, trunk and plantaris muscles showed lower total eIF2 α expression
324 compared with the larynx and flexor (Table 1; S3C Fig; Bonferroni $P = 0.042$). No
325 differences in total eIF2 α levels were found between different muscle types of *R. jimi*
326 during the dry period, or of *P. diplolister* during the dry and reproductive periods (Table
327 1).

328 **Fig. 2. Relative intensity of eIF2 α protein levels (A) larynx, (B) trunk, (C) flexor**
329 **and (D) plantaris; phosphorylate eIF2 α protein levels (E) larynx, (F) trunk, (G)**
330 **flexor and (H) plantaris; phosphorylate eIF2 α / eIF2 α ratio (I) larynx, (J) trunk,**
331 **(K) flexor and (L) plantaris in *R. granulosa*, *R. jimi*, and *P. diplolister* during**
332 **reproductive and dry period. n = 3–10, except when it is indicated. Zero indicates**
333 **protein levels below the detection limit. ND - not determined because of the lack of**
334 **samples. Asterisks indicate significant difference between reproductive and dry periods**
335 **($P < 0.05$).**

336

337 Phosphorylated eukaryotic initiation factor 2- α (p-eIF2 α) showed lower
338 expression during the dry period in trunk, flexor and plantaris muscles ($F_{1,13} = 0.94$, $P =$
339 0.003 ; $F_{1,9} = 0.23$, $P = 0.006$; $F_{1,10} = 0.47$, $P = 0.004$, respectively) (Fig 2E-H), but not in

340 the larynx from *R. jimi* ($F_{1,4} = 3.52$, $P = 0.13$). In contrast, p-eIF2 α levels were elevated
341 in the muscles of *P. diplolister* during the dry period compared to the reproductive one
342 (Fig 2E-H), and this trend was significant in the trunk ($F_{1,6} = 0.30$, $P = 0.003$) and
343 marginally significant in the flexor muscle ($F_{1,1} = 83.38$ $P = 0.069$) but not in the larynx
344 ($F_{1,8} = 0.673$, $P = 0.436$) or plantaris ($F_{1,12} = 0.506$ $P = 0.49$). Notably, p-eIF2 α levels
345 were below the detection limit in all muscles from *R. granulosa* during the dry period
346 (at 50 μ g of total protein per lane) (Fig 2E-H). Comparisons between different muscle
347 types within each species and each study season showed similar p-eIF2 α expression
348 among different muscle types in *R. jimi* and *R. granulosa* during the dry and
349 reproductive periods, and in *P. diplolister* during the reproductive period (Table 1).
350 However, during the dry period the trunk muscle of *P. diplolister* had significantly
351 higher levels than flexor and plantaris, and levels of p-eIF2 α in the plantaris tissue was
352 below that in all other studied muscle types (Table 1, S3D Fig).

353 The ratio of p-eIF2 α to total eIF2 α levels was lower during the dry period in all
354 muscle types of *R. granulosa* (reflecting the non-detectable levels of p-eIF2 α during the
355 dry period) and in the flexor and plantaris muscles *R. jimi* ($F_{1,3} = 0.518$, $P = 0.02$; $F_{1,4} =$
356 0.257 , $P = 0.034$), but not *P. diplolister* ($P > 0.05$) (Fig 2I-L). The ratio of p-eIF2 α to
357 total eIF2 α did not significantly vary among the different muscle types when compared
358 within the same season in *P. diplolister* and *R. granulosa* (Table 1). In *R. jimi* during the
359 dry period, larynx muscle showed higher p-eIF2 α / eIF2 α ratio when compared to the
360 other muscles (S3E Fig; Bonferroni $P = 0.05$); this difference was not significant during
361 the reproductive period (Table 1).

362 Heat shock protein 60 (HSP60) showed lower expression during the dry period
363 in the flexor from *R. jimi* ($F_{1,11} = 1.12$, $P = 0.001$), and in the larynx and trunk of *P.*
364 *dipolister* ($F_{1,7} = 0.33$, $P = 0.047$; $F_{1,8} = 0.11$, $P = 0.008$) (Fig 3A-D). In all other studied
365 tissue/species combinations, no significant differences in HSP60 levels were found
366 between the reproductive and dry periods ($P > 0.05$). Notably, the trunk muscles of *R.*
367 *jimi* and *R. granulosa* had lower HSP60 levels compared to other muscle types in the
368 reproductive season (Table 1; S4A and C Fig). During the dry period, HSP60 levels in
369 the trunk muscle of *R. jimi* were similar to that in the larynx and flexor while HSP60
370 levels in the plantaris muscle were higher than in the larynx and flexor (Table 1;
371 Bonferroni $P = 0.052$) (S4B Fig). In *P. diplolister*, HSP60 were similar among different
372 muscle types within each respective studied season ($P > 0.05$).

373 **Fig 3. Relative intensity of HSP 60 protein levels in (A) larynx, (B) trunk, (C)**
374 **flexor, (D) plantaris, HSP 70 protein levels in (E) larynx, (F) trunk, (G) flexor, (H)**
375 **plantaris, HSP 90 protein levels in (I) larynx, (J) trunk, (K) flexor and (L)**
376 **plantaris of *R. granulosa*, *R. jimi*, and *P. diplolister* during reproductive and dry**
377 **periods. Data are means \pm S.E.M., n = 3–11, except when indicated. ND indicates not**
378 **determined because of the lack of samples. Zero indicates protein levels below the**
379 **detection limit of the method employed. ND - not determined because of the lack of**
380 **samples. Asterisks indicate significant difference between reproductive and dry periods**
381 **(P < 0.05).**

382

383 Heat shock protein 70 (HSP70) showed lower expression during the dry period
384 compared to the reproductive season in the larynx and trunk of *P. diplolister* ($F_{1,9} =$
385 0.42 , $P = 0.03$; $F_{1,13} = 0.191$, $P = 0.02$) (Fig 3E-H). Similarly, the trunk muscles of *R.*
386 *jimi* had lower HSP70 levels during dry period compared to the reproductive one ($F_{1,7} =$
387 0.19 , $P = 0.02$). The flexor muscle of *R. jimi* showed higher HSP70 expression during
388 the dry period ($F_{1,6} = 10.27$, $P = 0.019$), while larynx displayed similar HSP70
389 expression along the year ($F_{1,5} = 2.05$, $P = 0.21$) (Fig 3E-H). *R. granulosa*'s trunk
390 muscles (but not the plantaris, flexor or larynx) showed higher HSP70 levels during the
391 dry period compared to the reproductive season ($F_{1,8} = 8.35$, $P = 0.02$) (Fig 3E-H).
392 Within each species and study season, only *R. jimi* showed differences in HSP70 levels
393 among different muscle types during the reproductive season, with the highest levels in
394 the trunk and the lowest levels in the plantaris muscle (Table 1; S4D Fig; Bonferroni P
395 = 0.006).

396 Heat shock protein 90 (HSP 90) showed lower expression during the dry period
397 compared with the reproductive period in the larynx and flexor muscles of *R. jimi* ($F_{1,8}$
398 = 0.27 , $P = 0.007$; $F_{1,11} = 0.37$, $P = 0.001$) and in the trunk muscles from *P. diplolister*
399 ($F_{1,1} = 1.02$, $P = 0.03$; $F_{1,6} = 0.44$, $P = 0.042$) (Fig 3I-L). In contrast, in the plantaris
400 muscle of *R. jimi* lower levels of HSP90 were found during the reproductive period
401 compared with the dry one ($F_{1,2} = 0.13$ $P = 0.045$). HSP90 could not be detected in *R.*
402 *granulosa* due to the lack of the cross-reactivity of the antibody. Comparison among
403 different muscle types within the same season showed elevated HSP90 levels in the
404 larynx and flexor compared to the trunk and plantaris of *R. jimi* during the reproductive
405 period (Bonferroni P = 0.013), and in the larynx of *P. diplolister* compared to all other

406 tissue types, also during the reproductive period (Bonferroni $P = 0.007$) (S4E and F
407 Fig, Table 1). In all other studied species/season combinations, no significant
408 differences in HSP90 levels were found between different muscle types ($P > 0.05$).

409 **Aerobic capacity**

410 Activity of cytochrome c oxidase (COX), which can serve as a marker of the
411 mitochondrial density and thus aerobic capacity of the tissue, was higher in the trunk
412 and plantaris muscles of *R. jimi* compared to *R. granulosa* and *P. diplolister* ($F_{2,22} =$
413 7.000 , $P = 0.005$ and $F_{2,43} = 9.616$, $P = 0.001$, for the dry and the reproductive period
414 respectively) (Fig 4). Notably, COX activity tended to be lower in the trunk muscle than
415 in the plantaris muscle across the species during the dry period (by 40-45% in *R.*
416 *granulosa* and *P. diplolister* and by 68% in *R. jimi*), although this difference was
417 significant only for *R. jimi* ($F_{1,22} = 5.334$, $P = 0.03$). Comparing within species, COX
418 activity was marginally higher during reproductive season than during the dry season in
419 the trunk *R. granulosa* ($t = 2.194$, $df = 5$, $P = 0.07$), but not in the plantaris ($t = 0.132$, df
420 $= 1$, $P = 0.90$). *Rhinella jimi* and *P. diplolister* did not display differences in the COX
421 activity between seasons ($P > 0.05$).

422 **Fig 4. Activity of cytochrome c oxidase (COX) in trunk (A) and plantaris (B)**
423 **muscles during reproductive and dry periods in *R. granulosa*, *R. jimi*, and *P.***
424 ***diplolister*.** $n = 3-11$, except when indicated. Asterisk indicates significant difference
425 between periods ($P < 0.05$).

426

427 **Discussion**

428 Our results indicate the importance of metabolic regulators mediating the muscle
429 maintenance and function during the drastic seasonal variation faced by the Caatinga
430 anurans. The toads that remain active during the dry period appear to maintain muscles
431 through more energy extensive pathways including elevated protein synthesis, while the
432 aestivating frogs employs energy conservation strategy that involves suppression of
433 protein synthesis, decrease in the chaperone expression and higher total expression of
434 AMPK. These adjustments are consistent with their lower metabolic rates [12] and need
435 for saving energy during aestivation. All three studied species activate cell survival
436 pathways during the dry period in the muscles likely to prevent muscle atrophy. All

437 three studied species thereby maintain the muscle capacity throughout the year, despite
438 the resource limitation. These strategies are important considering the unpredictability
439 of the reproductive event and the need to rapidly engage the muscular activity in
440 response to the rain event triggering reproduction.

441 **Cellular survival and protein synthesis pathways in the** 442 **muscle**

443 The protein kinase B (AKT) is an important signaling protein involved in
444 cellular survival pathways in the muscle [28]. AKT expression was elevated during the
445 dry period in the trunk and plantaris muscles from all studied species and in the larynx
446 in the aestivating species, but not in flexor. The most pronounced increases of AKT was
447 in the aestivating species, *P. diplolister*. Possibly, activation of AKT in the flexor may
448 occur later as the dry season progresses (similar to the delayed activation of the AKT
449 pathway in some skeletal muscle types of a hibernating mammal *Marmota flaviventris*
450 [29]; this hypothesis remains to be tested with regard to the Caatinga anurans. Similarly
451 to our present results, elevated expression of AKT was reported in the foot muscle and
452 hepatopancreas of estivating snails [30] and in the liver of a frog *Rana sylvatica*
453 exposed to freezing [31]. AKT promotes cell survival, downregulates pro-apoptotic
454 factors [32,30,33,31,34], and activates the cascade involved in cell cycle arrest and
455 quiescence [35]. Therefore, the upregulation of AKT in the Caatinga anurans during the
456 dry period could be important for preventing the muscle atrophy when resources are
457 limited. Notably, the upregulation of AKT during the dry period goes hand-in-
458 hand with an increase of the phosphorylated form of eIF2 α (p-eIF2 α) in the trunk
459 muscle and/or a decrease of the total eIF2 α in the larynx and flexor muscles of the
460 estivating species, *P. diplolister*. This agrees with the earlier findings showing that
461 phosphorylated eIF2 α can facilitate AKT activation thereby promoting cell survival
462 [36]. Furthermore, the eIF2 α is an essential initiation factor in protein synthesis which
463 controls the translation rates and becomes inactivated by phosphorylation [37]. Thus,
464 low levels of eIF2 α and/or elevated expression of p-eIF2 α indicate suppression of the
465 protein synthesis in the muscles of *P. diplolister* during aestivation. The stable level of
466 eIF2 α and p-eIF2 α in the other studied *P. diplolister* muscles might indicate that the
467 regulation of the protein synthesis in these tissues might be dependent on alternative
468 mechanisms such as control of the translation elongation [38] or ribosome

469 (dis)assembly [39]. Suppression of the protein synthesis is a common energy-saving
470 mechanism in estivating species [38] and has been observed in desert amphibians
471 *Neobatrachus centralis* [40] and in estivating snails *Otala lactea* [41,42]. Earlier studies
472 on estivating frogs (*Cyclorana alboguttata*) showed that muscles are protected against
473 atrophy during prolonged (9 months) estivation with no decline in muscle mass, cross-
474 sectional area or fiber number [7]. Our study in *P. diplolister* suggests a possible
475 mechanism for this protection involving the coordinated suppression of the protein
476 synthesis to conserve energy reserves and activation of the cell survival pathways to
477 prevent loss of the muscle cells.

478 The protein synthesis was activated during the dry period in the muscles of *R.*
479 *jimi* and *R. granulosa* (two species that remain active throughout the year) as indicated
480 by a decline in the amount of inactive p-eIF2 α in all studied muscle types, but not in the
481 aestivating species *P. diplolister*. This was especially notable in *R. granulosa* where p-
482 eIF2 α levels were below the detection limits of immunoblotting during the dry period.
483 The maintenance of foraging activity of *Rhinella* species during dry period might allow
484 higher protein synthesis and activation of the cell survival pathways, potentially helping
485 to build the muscle mass in preparation for the reproductive period in the two toad
486 species. The maintenance of the muscle mass during the dry period is important for the
487 Caatinga anurans, which start reproduction immediately after fairly unpredictable
488 rainfall events [13]. The reproductive behavior involves strenuous calling activity
489 (which engages the trunk muscles) in all three studied species [12]. In *P. diplolister*,
490 males must also energetically beat legs to build foam nests for eggs deposition [12].
491 Despite some variation in the AKT, eIF2 α and p-eIF2 α levels among the muscle types,
492 the seasonal patterns of expression of these proteins were generally consistent in
493 different muscles within each studied species. These results indicate that the molecular
494 mechanisms of the muscle maintenance during the resource-limited dry season are
495 similar in the locomotor muscles (i.e. plantaris) and the reproductively-related muscles
496 (such as trunk, flexor and larynx).

497 **Indices of energy status**

498 Elevated expression of AMPK in muscle tissues (indicative of the cellular
499 energy stress) was observed during the dry period in all muscles of *R. jimi* and in
500 plantaris muscle of *P. diplolister*. An increase in AMPK levels is common during the

501 resource- and energy-limited periods in many organisms including hibernating
502 mammals [43,44] and frogs exposed to hypothermia, hypoxia, freezing, dehydration or
503 anoxia [45,46,44]. Furthermore, AMPK suppresses energy-demanding metabolic
504 processes [47,22] and induces cell cycle arrest [12,22,44], which also contributes to
505 energy savings. The reasons for the differences in AMPK response between the two
506 non-estivating active species are not known. Furthermore, the expression of
507 phosphorylated and total AMPK might not always be in the same direction which limits
508 our conclusions on the potential changes in the active form of this protein (since the
509 phosphorylated form of AMPK could not be measured in our present study due to the
510 lack of antibody cross-reactivity with the anuran protein). Different responses regarding
511 AMPK activation in anuran species submitted to the same freezing condition has been
512 reported in *Rana perezi* and *Rana sylvatica* [45,46]. Additionally, during reproductive
513 period, AMPK expression was particularly elevated in trunk of *P. diplolister* compared
514 to other muscle types, which might indicate a metabolic stress due to high energy
515 demand of the trunk muscles due to calling activity [48,49,50]. However, this
516 hypothesis remains to be tested.

517 COX activity (indicative of the mitochondrial density) was considerably higher
518 in the muscles of the largest of the three studied species (*R. jimi*) compared to *P.*
519 *diploister* or *R. granulosa*. COX activity was especially high in the plantaris muscle of
520 *R. jimi*, which is consistent with higher locomotor capacity of large toads from *Rhinella*
521 *marina* group of species [51]. Generally, the mitochondrial COX capacity of the frogs'
522 muscles was maintained at the same level in the reproductive and dry period except for
523 a small but significant decline in the COX activity in the trunk muscle of *R. granulosa*
524 during the dry period. This indicates that the aerobic capacity of the locomotor as well
525 as the reproductive muscles is maintained throughout the year despite the energy and
526 resource limitation in the dry season.

527 **Expression of molecular chaperones**

528 Molecular chaperones involved in the folding of nascent and damaged proteins
529 (including a mitochondrial HSP60 and cytosolic HSP70 and HSP90) were expressed at
530 lower levels in the muscles of the estivating *P. diplolister* during the dry period. A
531 decrease in HSP expression in aestivating frogs goes hand-in-hand with the suppressed
532 protein synthesis in the muscles and may reflect lower protein turnover rates during

533 aestivation [41,52]. Similarly, other aestivating species such as land snails also decrease
534 HSP expression during quiescence [53, 54] and sharply increase it during arousal [55].

535 The higher expression of HSPs in the muscles trunk of *P. diplolister* during the
536 reproductive period might be attributed to the stress caused by the exercise from calling
537 behavior and high steroid receptor expression levels [56,57,58]. Calling is a highly
538 energetic demanding aerobic exercise for anuran males [59,48], and calling effort is
539 positively correlated with plasma levels of androgens and corticosterone [60, 65, 66].
540 Furthermore, anurans show increased expression of steroid hormones during
541 reproductive season [65,67], which are associated with HSP in the inactive state
542 [68,69,70]. Thus, elevated expression of HSPs during the reproductive season in *P.*
543 *diploister* might also reflect the high levels of steroids during this period. However, the
544 pattern of HSP levels in the muscles of *R. jimi* or *R. granulosa* males are less clear,
545 considering they also display high steroid levels during the reproductive season.
546 Overall, HSP levels of in the muscle tissues in the two anurans species that are year-
547 round active showed relatively little variation and no consistent pattern of seasonal
548 change between different muscle types. This indicates the lack of strong unfolded
549 protein response and thus maintenance of the protein homeostasis during the period of
550 reproductive activity as well as during times of resource limitation in these species.

551 **Conclusions and perspectives**

552 The differential responses of the cell signaling and stress response pathways
553 found in the muscles of the three studied species of desert anurans reflect differences in
554 their life habit and activity levels as well as the common need to maintain the muscle
555 capacity in an extremely seasonal environment of the Caatinga. Activation of the cell
556 survival pathways is the most consistent response in the muscles of all three studied
557 species during the dry period likely playing a role in preventing muscle atrophy during
558 the resource limitation. Expression of the regulators of protein homeostasis (including
559 chaperones and regulators of the protein synthesis) reflect different levels of resource
560 limitation, so that the less resource-limited active species upregulate protein synthesis
561 and maintain high levels of chaperones during the dry periods in the muscle while a
562 severely resource-limited aestivating species shuts down the protein synthesis to
563 conserve energy. Contrary to our prediction, we did not observe differential metabolic
564 regulation or trade-off between the reproductive and locomotor muscles. Future studies

565 are needed to determine whether the muscle maintenance during the dry period is
566 prioritized over that of other tissues and whether potential trade-offs exist between the
567 support of the muscle capacity throughout the year and other fitness-related functions in
568 desert frogs.

569

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573 **Competing interests**

574 The authors declare no competing or financial interests.

575 **Author contributions**

576 I.S., C.B.M and F.R.G. conceived the study, designed the experiments and contributed
577 substantially to interpreting the data. C.B.M and I.S. collected the data; C.B.M.
578 analyzed the data; C.B.M, I.S. and F.R.G. wrote the manuscript, and take full
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584

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763

764 **Supporting information**

765 **Fig S1. AMPK expression during (A) reproductive and (B) dry period for *R. jimi*,**
766 **AKT expression during (C) reproductive period for *R. jimi*; Total eIF2 α expression**

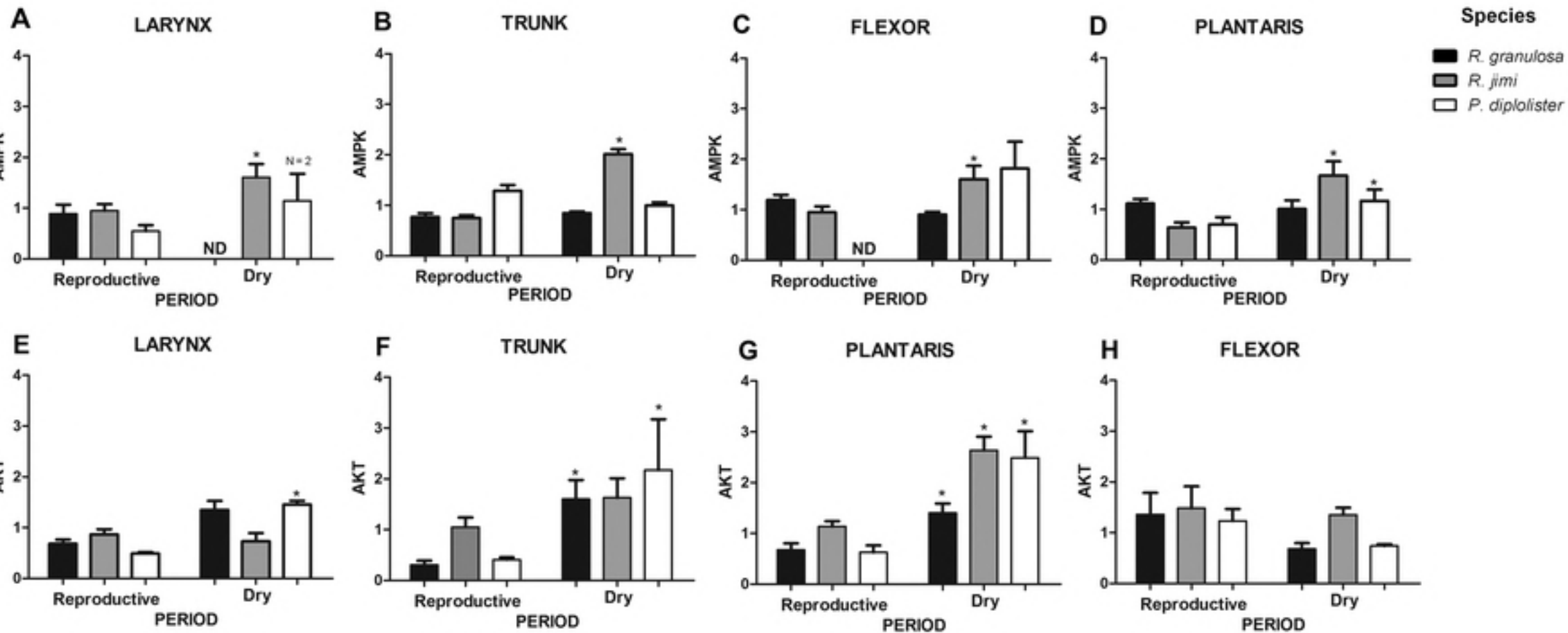
767 **during (D) reproductive and (E) dry period for *P. diplolister*; Phosphorylated**
768 **eIF2 α expression during (F) reproductive and (G) dry period for *R. jimi*; HSP60**
769 **expression during (H) reproductive and (I) dry period for *R. jimi*; HSP70**
770 **expression during (J) reproductive period for *R. jimi*; and HSP90 expression**
771 **during (K) reproductive period for *R. jimi*.**

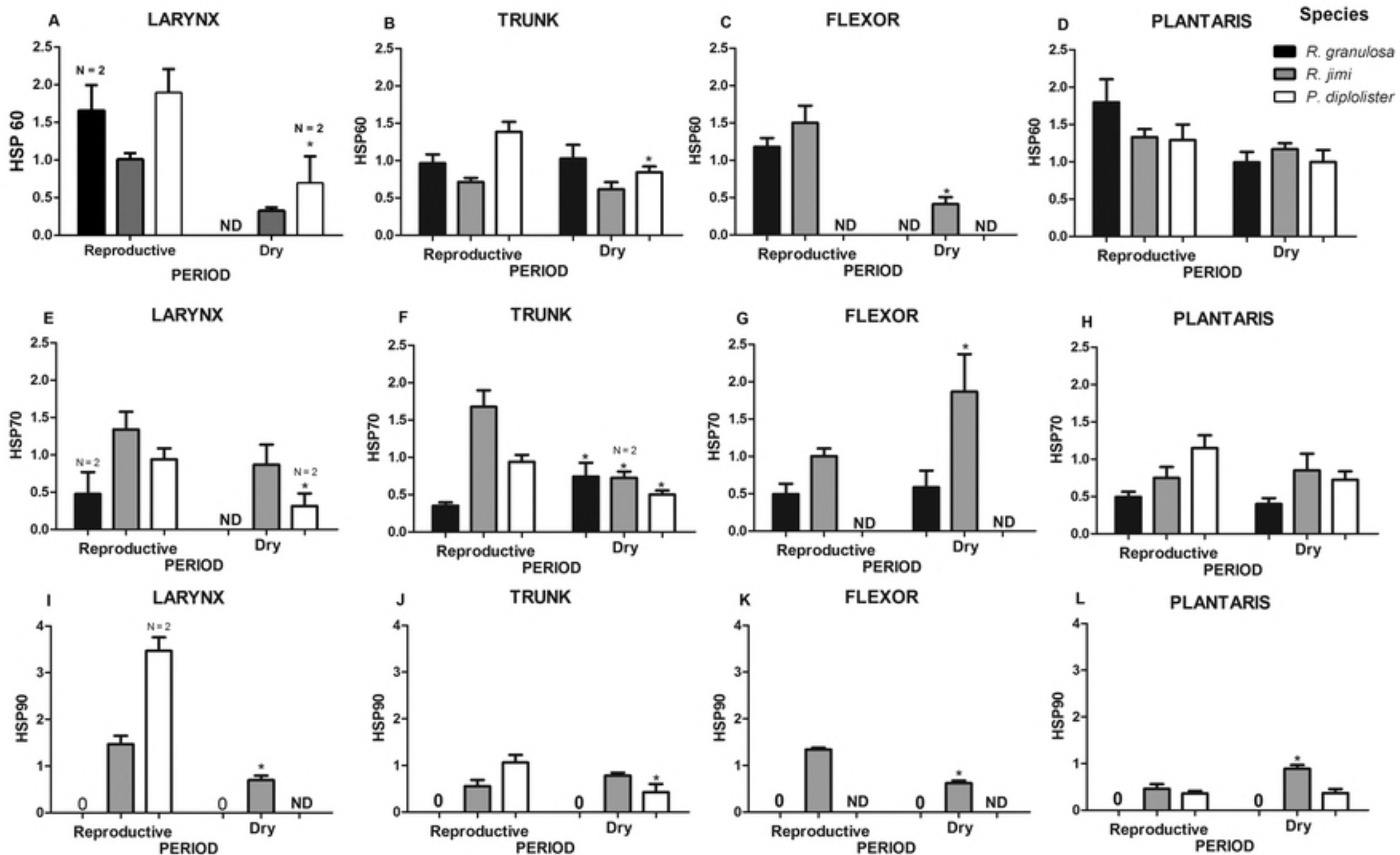
772 **Fig S2. Relative intensity of AMPK protein levels among different muscles in *P.***
773 ***diplolister* during reproductive period. Data are means \pm S.E.M., n = 7–10. ND**
774 **indicates not determined because of the lack of samples. Different letters indicates**
775 **significant difference (P < 0.05).**

776 **Fig S3. Relative intensity of eIF2 α protein levels among different muscles in *R.***
777 ***granulosa* during (A) reproductive and (B) dry period and *R. jimi* during (C)**
778 **reproductive period; phosphorylate eIF2 α among different muscles in *P. diplolister***
779 **during (D) dry period; and phosphorylate eIF2 α /eIF2 α ratio among different**
780 **muscles in *R. jimi* during (E) reproductive period. Data are means \pm S.E.M., n = 3–**
781 **10, except when otherwise indicated. Asterisks and different letters indicates significant**
782 **difference (P < 0.05).**

783 **Fig S4. Relative intensity of HSP 60 protein levels among different muscles in *R.***
784 ***jimi* during (A) reproductive and (B) dry period and in *R. granulosa* during (C)**
785 **reproductive period; HSP 70 protein levels in *R. jimi* during (D) reproductive**
786 **period; and HSP 90 in *R. jimi* during (E) reproductive period and in *P. diplolister***
787 **during (F) reproductive period. Data are means \pm S.E.M., n = 4–8, except when**
788 **otherwise indicated. ND indicates not determined because of the lack of samples.**
789 **Asterisks and different letters indicates significant difference (P < 0.05).**

790

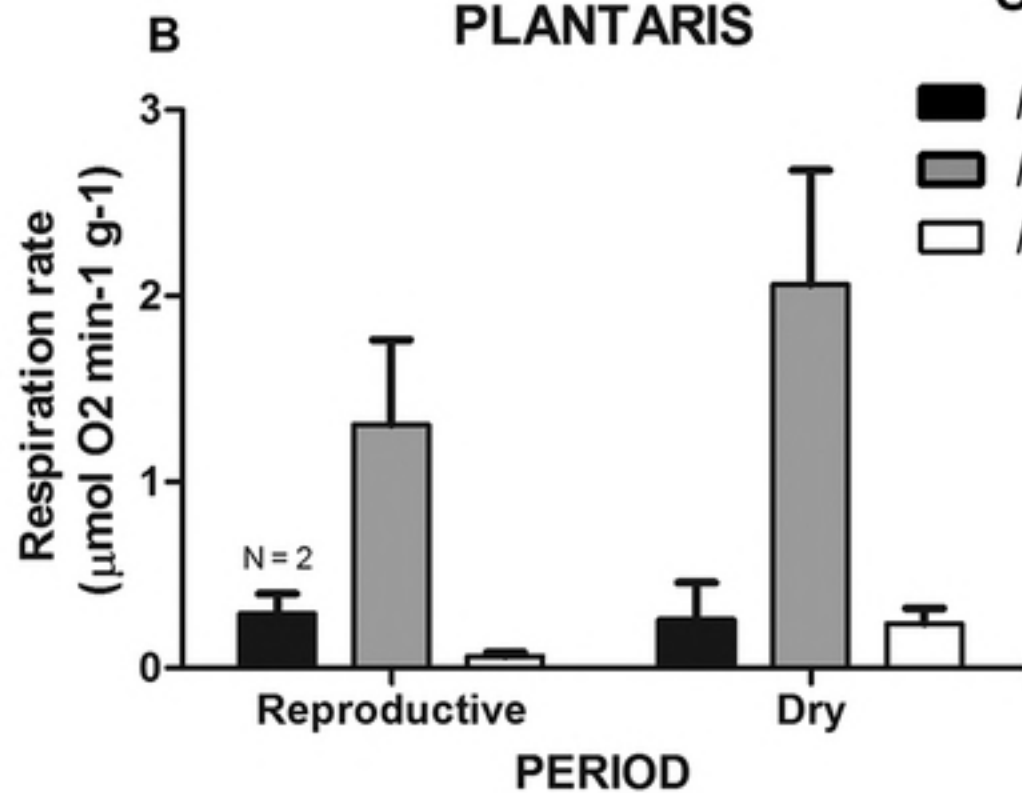




Species

- *R. granulosa*
- *R. jimi*
- *P. diplolister*

B PLANTARIS



A TRUNK

