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Comparing techniques for measuring corticosterone in tadpoles

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Abstract Glucocorticoids play a key role in mediating stress responses in vertebrates. Corticosterone (CORT) is the main glucocorticoid produced in amphibians, birds, and reptiles, and regulates several metabolic functions. The most common methods for quantifying CORT are competitive binding immunoassays: radioimmunoassay (RIA) and enzyme immunoassay (EIA). RIA has been broadly used since the 1980's but it requires radioactivity. Commercial EIA kits permit quantifying hormone levels without radioactivity although the requirement for a larger sample volume may be a strong limitation for measurements involving larval amphibians. Here we used *Xenopus laevis* tadpoles to compare the performance of three commonly used procedures for determination of CORT: RIA on a chloroform extract of whole-body homogenate, EIA on plasma, and EIA on supernatant of whole-body homogenate. We treated tadpoles with exogenous CORT at 0, 25, 50, and 100 nM. RIA could distinguish between 0 and 25 nM, and EIA on plasma between 0 and 50 nM, whereas whole-body homogenate EIA only detected significant differences between 0 and 100 nM. Each procedure presents advantages and disadvantages regarding sensitivity, the use of radioactivity, sample size, handling time, and economic cost. RIA is preferred when studying small-bodied animals from which blood samples cannot be obtained. When CORT level differences are intermediate and blood sampling is possible, EIA on plasma is a good non-radioactive alternative. EIA on whole-body homogenates may be useful to assess qualitative changes in CORT levels when considerable differences are expected. Finally, we discuss our findings in the context of previous studies on CORT in amphibians [*Current Zoology* 61 (5): 835–845, 2015].

Keywords Corticosterone, Radioimmunoassay, Enzyme immunoassay, Glucocorticoids, Amphibians, Stress

Organisms make physiological adjustments in response to environmental fluctuations, whether natural or anthropogenic, through hormonal regulation (Johnson et al., 1992; Nussey and Whitehead, 2001; Becker et al., 2002). In vertebrates, the main hormonal response to environmental perturbations is based on the activation of the hypothalamic-pituitary-adrenal (HPA) axis to release corticotrophin-releasing hormone (CRH) (Miller and O'Callaghan, 2002; Denver, 2009). CRH stimulates the anterior pituitary to secrete the adrenocorticotropin hormone (ACTH) (Raffin-Sanson et al., 2003) followed by release of glucocorticoids (GCs) from the adrenal gland (Aguilera, 1994). Although the activation of this endocrine pathway is rapid, the GC level increase varies among species and individuals and can take around 3–5 min in vertebrates (Cash et al., 1997; Sockman and Schwabl, 2001; Romero and Romero, 2002). Corticosterone (CORT) is the main GC involved in the stress response in reptiles, birds, and amphibians whereas cortisol is the main one in fish and mammals (Sapolsky et al., 2000; Romero, 2004; Denver, 2009). Moreover, GC levels are associated with health condition, developmental rate, metabolism, and immune function (Denver et al., 2002; Walker et al., 2005; Wikelski and Cooke, 2006; DuRant et al., 2008; Davis et al., 2008), and they are commonly studied in developmental, ecological, and conservation studies (Romero, 2004; Busch and Hayward, 2009).

The most common methods for quantifying hormones are competitive binding immunoassays: radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Sherrif, 2011; Narayan, 2013). Other techniques such as gas or liquid chromatography coupled with mass spectrometry have been successfully, though less frequently, used (Webb et al., 2007). RIA has been used extensively for over three decades and is a very sensitive technique to

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determine the concentration of antigens (e.g. hormones). RIA, however, requires the use of radioactive isotopes, specialized equipment, and a laboratory classified for handling radioactive materials. Moreover, radioactivity is increasingly avoided in laboratories and institutions because of the health risk and paperwork it entails.

In the 1970's, the development of commercial EIA kits allowed hormonal assays without the use of radioactivity. Commercial EIA kits are commonly designed for plasma or urine samples, providing information on circulating hormones (Wada et al., 2007; Mills et al., 2010) at competitive prices. However, when studies involve small species or life stages (e.g. embryos or larvae from amphibians, reptiles, and fish), obtaining the minimum required amount of plasma or urine to conduct EIA may prove challenging. Consequently, some researchers are attempting to conduct EIA using commercial kits on supernatant of whole-body homogenate instead of plasma (McMahon et al., 2011; Burraco et al., 2013). Clearly this would yield a diluted hormone sample compared to blood, and intracellular hormone may be included in the measurement. It is unclear whether this would yield comparable data to the other procedures or has a similar sensitivity to detect differences among study groups (e.g. species, populations, experimental treatments). Reduced specimen size may render whole-body supernatant EIA the only viable alternative to RIA, unless plasma from a large number of individuals could be pooled together. However, losing the possibility to study CORT variation at the individual level may be undesirable because increasing the minimum required number of experimental individuals raises practical and bioethical issues. The choice of hormonal assay is therefore subject to various considerations depending on the study system, experimental design, infrastructure, and budget availability.

Amphibians are ideal to compare different procedures for determining GC levels as they have been used as a model group to analyze the role of GCs in multiple physiological processes, especially regarding the timing of metamorphosis (Denver et al., 2002; Gomez-Mestre et al., 2013). However, determining CORT levels in larval amphibians often poses difficulties derived from the small size of individuals of some species.

Here we compare the performance of three procedures for measuring CORT using tadpoles of the African clawed frog *Xenopus laevis*: RIA on chloroform extract of whole-body, EIA on plasma, and EIA on supernatant of whole-body homogenate. We compare CORT estimates obtained from tadpoles treated with different amounts of exogenous CORT and discuss the suitability of each procedure with respect to sensitivity and discrimination power, but also to bioethical, logistic, and budgetary criteria. We also review and discuss the methodology used for CORT determination in anurans over the last 20 years.

1 Material and Methods

1.1 Experimental setup

We collected egg clutches from three *Xenopus laevis* pairs from a breeding colony at Centro Andaluz de Biología del Desarrollo (Seville, Spain). After hatching, 240 tadpoles were reared for 60 days in 2.7 L buckets (5 tadpoles/bucket) until they reached Nieuwkoop and Faber developmental stages 52–54 (Nieuwkoop and Faber, 1994). At this stage range, tadpoles show fore and hind limbs in paddle stage and the hind limbs without feet and longer than broad (mean length 55 mm, Nieuwkoop and Faber, 1994). During this rearing period, water was renewed twice a week and tadpoles were fed 30 mg of ground rabbit chow every other day. The experimental units were distributed across shelves in a walk-in chamber set at constant 20°C, and a 12:12 light-dark cycle.

When the tadpoles reached NF 52–54 we treated them with exogenous CORT (0 nM, 25 nM, 50 nM, or 100 nM). Each CORT treatment was replicated 12 times for a total of 48 experimental units. Water was changed daily, and at each water change tadpoles were fed 30 mg of ground rabbit chow and CORT (C2505, SIGMA) was added. Tadpoles have permeable skin (Bentley, 1971; Parson, 1994), so CORT diluted in water is directly uptaken via skin absorption. To reach the experimental concentrations, we prepared stock solutions of CORT diluted in absolute ethanol so that an addition of 200 μL of any of them to 2.7 L of water in the experimental containers would result in the target concentrations of 25, 50, and 100 nM. We first prepared a 1350 μM stock solution (23.5 mg of CORT in 50 mL of ethanol) from which the 100 nM experimental concentration would be obtained, and then prepared two stocks at lower concentrations by serial 1:1 dilutions with absolute ethanol. Buckets in the control (no CORT) received 200 μL of ethanol each time. The stock concentrations are based on previous studies (Glennemeier and Denver, 2002) and were kept at -80°C. Three days after applying the hormonal treatments we collected tadpoles from each bucket for CORT measurements, randomly assigning them to RIA (one tadpole per bucket), EIA on plasma (three tadpoles pooled per bucket) and EIA on

whole-body homogenate (one tadpole per bucket). We did not find differences in weight among treatments at the end of the experiment, and tadpoles weighed an average of 223 ± 9 mg (*SE*).

Plasma samples for EIA were obtained through heart puncture. Tadpoles deeply anesthetized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma), were placed under a dissecting scope, and blood was extracted via cardiac puncture with a non-heparinized insulin syringe (BD Micro-Fine Insuline U-100 0.5 ml). Blood from all three individuals (ca. 20 μL per tadpole) from each bucket was pooled in heparinized tubes and then centrifuged at 4000 rpm, at 4°C for 20 minutes to obtain plasma (Gomez-Mestre et al, 2013). Plasma samples were stored at -80°C in 0.5 ml eppendorf tubes until assayed. The average handling time per individual for the blood extraction procedure from immersion in anesthetic to complete collection of the blood sample was 193 s ± 7.5 *SE*.

Individuals assigned to RIA were dipnetted from their containers, euthanized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate), dissected to remove the gut, snap-frozen in liquid nitrogen, and preserved at -80°C until used. For whole-body homogenate EIA, tadpoles were euthanized with MS-222, dissected to remove the gut, ground up with a homogenizer (MICCRA D-1), and centrifuged at 4,000 rpm at 4°C for 15 min. The resulting supernatant was preserved at -80°C until used (Burraco et al., 2013).

1.2 Radioimmunoassay

Specimen samples were homogenized (except the gut) as described above centrifuging and extracting the hormone from the supernatant with chloroform and ion-exchange chromatography prior to RIA following previous protocols (Denver, 1993). Lower detection limit was 0.20 pg/mL/mg.

1.3 Plasma and whole-body homogenate EIAs

We took 50 μL of either plasma or homogenate (supernatant) from each sample and conducted EIA with a commercial kit following manufacturer specifications (Cayman Chemical Company – catalog n° 500655). The CORT determination with this EIA kit is based on the competition between a CORT-acetylcholinesterase conjugate and CORT, for a limited number of CORT-specific sheep antiserum binding sites which bind to the rabbit polyclonal anti-sheep IgG that previously was attached to the well. For this, each plate was incubated for two hours at room temperature on an orbital shaker. Ellman's reagent, which contains a substrate for acetylcholinesterase, was added to the wells to develop the assay. After incubating for 60–90 minutes to achieve a stable end point in an orbital shaker in the dark, absorbance was read at a wavelength of 412 nm, and CORT concentration was determined based on standard curves run in duplicate on each plate. According to the manufacturer, the detection limit (80% B/B_0) is approximately 40 pg/mL, and cross reactivity with other tested steroids is below 1%. It was not the aim of this study to compare the efficacy of the various commercial kits available, and differences among kits from different suppliers are likely to affect the outcome due to differences in their antibody specificity or detection limits. Moreover, most of these kits are optimized for use with human samples, and in our experience not all work well with amphibian samples.

1.4 Statistical tests

We assayed 38 tadpoles through RIA, with 9–10 replicates per treatment. For EIA on plasma, we extracted blood from 135 tadpoles. We obtained the required 50 μL pooling plasma from three tadpoles per bucket, yielding 6–7 replicates per treatment. For some pooled samples, the total volume obtained was insufficient to run the assay. In whole-body homogenate EIA we obtained 9–11 replicates per treatment and we used 40 tadpoles in total. All statistical analyses were conducted in R version 2.14.1 (R Development Core Team 2007). We checked the parametric assumptions testing normality of the data via analysis of residuals distribution and homoscedasticity using Barlett's tests (bartlett.test). Data from RIA and whole-body homogenate EIA were heteroscedastic and not normally distributed, and hence we fitted generalized linear models with a negative binomial distribution (function glm.nb in MASS package, version 7.3–22) to test for differences among CORT treatments. We then ran post-hoc tests using the glht function (multcomp package, version 1.2–13). Plasma EIA data, however, met parametric assumptions and therefore we fitted instead a general linear model (aov function) followed by post-hoc Tukey tests. We tested the effect of handling time including it as a covariate in the model. We calculated intraclass correlations to determine the reliability and consistency of the measurements within treatments for each procedure with the ICC function (ICC package, version 2.2.1).

2 Results

Handling time for individuals preserved for whole-body homogenate (RIA or EIA) was limited to ~30 s in the anesthetic (MS-222). Handling time during blood sample collection had no significant effect on CORT

levels (coefficient of regression: 0.19, $P = 0.355$, $R^2 = 0.037$).

We detected significant changes among treatments in CORT concentration, regardless of the procedure used (Figure 1; overall tests: $\chi^2 = 50.605$, $df = 3$, $P < 0.001$ for RIA; $F_{3,22} = 10.08$, $P < 0.001$ for EIA on plasma; $\chi^2 = 7.89$, $df = 3$, $P = 0.017$ for EIA on whole-body). For RIA data, post-hoc tests (Table 1) showed significant differences among the control treatment and each exogenous CORT treatment (25, 50 and 100 nM; all $P < 0.001$). The CORT levels increased on average by 6.1-fold between 0 nM and 25 nM treatments, and by up to 9.52 and 9.47-fold in the 50 and 100 nM treatments in comparison to the control treatment. Plasma EIA showed that CORT increased 4.12 times in the 100 nM treatment with respect to the control ($P = 0.001$). We also found significant differences between 25 nM and 50 nM ($P = 0.045$), and 25 nM and 100 nM ($P < 0.001$), but not between the control and 25 mM. We found noticeable changes in the CORT levels between 0 nM-50 nM and 50 nM-100 nM but they were marginally not significant (Table 1). Overall EIA on plasma showed the same number of significant pairwise differences among treatments that RIA did, and in both cases we found significant differences between treatments that differed in 25 nM of CORT. However, RIA detected significant differences between the lowest concentrations of corticosterone (0 nM-25 nM) and EIA on plasma detected better than RIA differences when higher concentrations were added (50 or 100 nM). The model fit on whole-body homogenate EIA indicated a significant effect of hormonal treatments. Post-hoc tests only found significant differences between 0 nM and 100 nM treatments ($P = 0.002$) although differences between 0 nM–50 nM and 25 nM–100 nM neared statistical significance ($P \sim 0.07$).

Comparing the absolute values (in pg/ml) obtained in plasma EIA with those estimated from whole-body homogenate EIA (Fig. 1), we found diluted levels in the homogenate, yielding values in the range of 2.1 to 4.6 times lower concentrations than in plasma. This comparison is not possible with the RIA values because these are given per mass unit (pg/ml/mg).

The intraclass correlation coefficient (ICC) provides an idea of the consistency of the data within experimental treatments. ICCs indicated a higher consistency of data from EIA on plasma (ICC = 0.56) and RIA (ICC = 0.40) than EIA on supernatant of whole-body homogenate (ICC = 0.14).

3 Discussion

The comparison of RIA on whole-body extract, EIA on plasma, or EIA on supernatant of whole-body homogenate, revealed that RIA was a more sensitive procedure for detecting low CORT values than the EIA procedures. RIA on whole-body extract was the only procedure capable of detecting differences between control tadpoles and tadpoles treated with 25 nM CORT (Table 1). RIA, however, was rapidly saturated and could not distinguish tadpoles treated with more than 50 nM. Diluting the samples prior to the assay, however, could solve this problem of RIA saturation. EIA on plasma samples was less sensitive than RIA at low CORT levels but also allowed detection of differences between treatments that differed by 25 nM (i.e. 25 nM *vs* 50 nM). Moreover, EIA showed no indication of saturation in our study, indicating a larger dynamic range for detecting significant differences. This characteristic of EIA on plasma may be advantageous when species have unknown basal CORT levels and limited sample volumes preclude use of dilution series to assess assay saturation. EIA on whole-body homogenate supernatant showed the lowest sensitivity since we could only detect significant differences between tadpoles treated with 100 nM CORT and those with no CORT added. Nevertheless, EIA on whole-body homogenate supernatant showed the same trend as EIA on plasma (Fig. 1) but with rather dampened values, most likely a consequence of dilution with CORT-free body fluids. The most consistent procedure (i.e. the one with higher intraclass correlation coefficient) was EIA on plasma, followed by RIA.

CORT concentrations measured in previous studies in natural or semi-natural conditions reported values that were within the range in which the three procedures can

Table 1 Post-hoc Tukey tests for differences among treatments in RIA, EIA on plasma, and EIA on whole-body homogenates

Post-hoc (*P*-adjusted)	0–25 (nM)	0–50 (nM)	0–100 (nM)	25–50 (nM)	25–100 (nM)	50–100 (nM)
RIA	<0.001	<0.001	<0.001	0.093	0.093	0.980
EIA (plasma)	0.7497	0.0627	0.0011	0.0454	<0.001	0.0615
EIA (homog)	0.475	0.071	0.023	0.229	0.071	0.4988

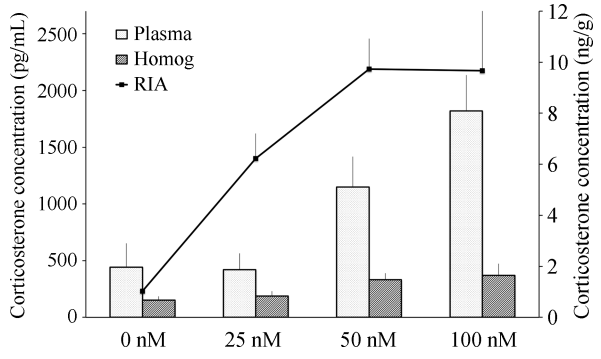

Fig. 1 Corticosterone concentration measured applying EIA plasma (light grey bars, left Y axis), EIA whole-body homogenate (dark grey bars, left Y axis), or RIA (black line, right Y axis)

Error bars indicate standard error of the mean.

detect significant differences. There is much information about CORT levels obtained via RIA on whole-body extracts. For example, Glennemeier and Denver (2002) obtained values between 0.4 and 0.8 ng/g in *Rana pipiens* differentially treated with exogenous CORT; Middlemis-Maher et al. (2013) measured CORT in *R. sylvatica* tadpoles and juveniles differentially exposed to predator presence and obtained values between 0.5 and 4 ng/g. Likewise, Belden et al. (2010) measured CORT in *R. sylvatica* tadpoles repeatedly to estimate capture stress in natural ponds (0.2–0.5 ng/g), and in *Scaphiopus holbrooki* raised in 1000 L mesocosms (less than 0.1 ng/g). Regarding CORT values measured with EIA on plasma, pre- and postmetamorphic *Pelobates cultripes* individuals exposed to pond drying reported CORT concentrations between 0.1–0.4 ng/mL (Gomez-Mestre et al., 2013). Burraco et al. (2013) measured CORT on supernatant of whole-body homogenate and values were around 0.1 and 0.2 ng/mL in tadpoles exposed to predators and herbicide.

The study of stress responses in amphibians has required conducting corticosterone assays on several species. We found 56 studies that determined CORT in anurans in the last two decades (Table 2). Some of these studies included several species or different life stages, which we considered as separate corticosterone measurements (n = 70). The majority of such measurements were obtained using RIA (n = 46). Within these RIA studies, we found 11 instances where CORT was measured on plasma: six in adults, four in juveniles, and only one in tadpoles (which included one or two tadpoles per sample; Wright et al, 2003). This bias towards adult and juvenile stages in the analysis of plasma samples likely reflects the difficulty in obtaining blood samples in tadpoles due to its reduced size. Consequently, most tadpole studies opted for whole-body chloroform extracts (n = 31, Table 2).

Studies with EIA used different organs or fluids or else incorporated various modifications of the technique. For example, we found 18 instances where CORT levels were assayed in urine of adult amphibians and just one where CORT was determined in plasma. Studies on tadpoles applied EIA in various ways. Two studies ran assays on whole-body homogenates (McMahon et al., 2011; Burraco et al., 2013) whereas another study determined CORT levels from pond water (Gabor et al., 2013).

RIA has thus been the preferred technique for quantifying CORT in anurans in the last two decades, most often applied to extracts from whole-body tadpole homogenates. EIA was conducted on various sample types, and was preferred when assaying urine or blood samples. Various factors need to be taken into account in order to decide which method to use (Table 3). RIA is widely used but requires radioactivity, which is not implemented in many research institutions. Regarding budgetary aspects the use of EIA can be favored over the use of RIA, as EIA commercial kits are available at competitive prices that do not require additional disposal costs as with radioactive materials generated by RIA. Our results indicate that EIA on plasma samples is a reasonable non-radioactive alternative to RIA, with two caveats. First, EIA has lower sensitivity at low CORT levels. Second, many studies will focus on study systems where individuals are too small for collecting sufficient plasma samples for commercial kits (typically requiring ca. 50 μL), especially so in early ontogenetic stages. Thus, obtaining sufficient plasma samples from small organisms may require pooling blood from several individuals into a single sample, hence requiring the sacrifice of many more individuals than are otherwise required for either RIA or EIA on whole-body homogenate. In our study, each plasma sample for EIA required pooling blood from three *X. laevis* tadpoles whereas single tadpoles were allocated to either RIA or EIA on whole-body supernatant. This of course raises conservation and bioethical concerns, especially when dealing with threatened or vulnerable species, which in the case of amphibians includes ca. 43% of all known species (Hoffmann et al., 2010; LesBarrères et al., 2014). Moreover, an increase in the number of tadpoles in the study can pose some logistical problems in laboratories as well as during field campaigns. Thus, studies will have to either increase the total number of replicates to account for the need to pool samples, or increase the nu-

Table 2 Overview of corticosterone studies in anurans from 1993 to 2013

Techniques	Body parts	Stages	Species	References
RIA				
	plasma	adult	*B. terrestris*	Hopkins et al., 1997
		adult	*B. terrestris*	Hopkins et al., 1998
		adult	*B. terrestris*	Ward and Mendonça, 2005
		adult	*R. catesbeiana*	Wright et al., 2003
		adult	*R. sylvatica*	Crespi and Warne, 2013
		adult	*X. laevis*	Hayes et al., 2006
		juvenile	*S. hammondii*	Crespi and Denver, 2005
		juvenile	*X. laevis*	Kloas et al., 1997
		juvenile	*X. laevis*	Crespi et al., 2004
		juvenile	*X. laevis*	Hu et al., 2008
		tadpole (1-2 per sample)	*R. catesbeiana*	Wright et al., 2003
	whole-body	juvenile	*R. sylvatica*	Belden et al., 2007
		juvenile	*R. pipiens*	Glennemeier and Denver, 2002
		juvenile	*R. sphenocephala*	Peterson et al., 2009
		juvenile	*X. laevis*	Glennemeier and Denver, 2002
		tadpole	*B. boreas*	Hayes, 1997
		tadpole	*B. boreas*	Hayes and Wu, 1995
		tadpole	*H. regilla*	Belden et al., 2005
		tadpole	*H. versicolor*	Chambers, 2011
		tadpole	*R. cascadae*	Belden et al., 2003
		tadpole	*R. clamitans*	Fraker et al., 2009
		tadpole	*R. pipiens*	Glennemeier and Denver, 2002
		tadpole	*R. pipiens*	Glennemeier and Denver, 2002 (2)
		tadpole	*R. pipiens*	Glennemeier and Denver, 2001
		tadpole	*R. pipiens*	Glennemeier and Denver, 2002 (3)
		tadpole	*R. sphenocephala*	Peterson et al., 2009
		tadpole	*R. sylvatica*	Belden et al., 2010
		tadpole	*R. sylvatica*	Fraker et al., 2009
		tadpole	*R. sylvatica*	Chambers, 2011
		tadpole	*R. sylvatica*	Warne et al., 2011
		tadpole	*R. sylvatica*	Belden et al., 2007
		tadpole	*R. sylvatica*	Crespi and Warne, 2013
		tadpole	*R. sylvatica*	Middlemis et al., 2013
		tadpole	*R. sylvatica*	Reeve et al., 2013
		tadpole	*R. temporaria*	Dahl et al., 2012
		tadpole	*S. bombifrons*	Ledón-Rettig et al., 2009
		tadpole	*S. hammondii*	Denver, 1998
		tadpole	*S. hammondii*	Crespi and Denver, 2004
		tadpole	*S. hammondii*	Crespi and Denver, 2005
		tadpole	*S. hammondii*	Denver, 1997
		tadpole	*Sc. couchii*	Ledon-Rettig et al., 2009
		tadpole	*Sc. couchii*	Ledon-Rettig et al., 2010
		tadpole	*X. laevis*	Kloas et al., 1997
		tadpole	*X. laevis*	Glennemeier and Denver, 2002

Continued Table 1

Techniques	Body parts	Stages	*Species*	References
		tadpole	*X. laevis*	Boorse and Denver, 2004
		tadpole	*X. laevis*	Hu et al., 2008
EIA				
	plasma	adult	*R. marina*	Brown et al., 2011
		tadpole	*P. cultripes*	Gomez-Mestre et al., 2013
	whole-body	tadpole	*O. septentrionalis*	McMahon et al., 2011
		tadpole	*P. cultripes*	Burraco et al., 2013
	urinary CORT	adult	*L. wilcoxii*	Kindermann et al., 2012
		adult	*L. wilcoxii*	Kindermann et al., 2013
		adult	*M. Fasciolatus*	Graham et al., 2013
		adult	*P. vitiana*	Narayan et al., 2010
		adult	*P. vitiana*	Narayan et al., 2010 (2)
		adult	*P. vitiana*	Narayan and Hero, 2011
		adult	*P. vitiana*	Narayan et al., 2012
		adult	*P. vitiana*	Narayan et al., 2013
		adult	*P. vitiana*	Narayan et al., 2013 (2)
		adult	*P. vitiana*	Narayan et al., 2013 (3)
		adult	*R. marina*	Narayan et al., 2011 (2)
		adult	*R. marina*	Narayan et al., 2012 (2)
		adult	*R. marina*	Narayan et al., 2012 (3)
		adult	*R. marina*	Narayan et al., 2012 (4)
		adult	*R. marina*	Narayan et al., 2012 (5)
		adult	*R. marina*	Narayan et al., 2012 (6)
		adult	*R. marina*	Narayan et al., 2013 (4)
		adult	*R. marina*	Narayan et al., 2013 (5)
	in water pond	tadpole	*A. obstetricans*	Gabor et al., 2013
		tadpole	*A. muletensis*	Gabor et al., 2013

We have only included studies that used RIA or EIA to determine the corticosterone levels. Some studies have multiple entries because they included several analysis, e.g. with different species or individuals' stages. The full references are included in the "Suplementary material".

Table 3 Main characteristics of RIA, EIA on plasma, EIA on homogenate

	Ind/sample (in *X. laevis* tadpoles)	Freeze prior to measure	USD / sample	Radioactivity	Use in field studies	Use in small individuals	ICC-value
RIA	1	YES	3 *	YES	+++	++	++
EIA on plasma	3 (at least)	NO	2.8	NO	+	+	+++
EIA on homogenate	1	YES	2.8	NO	+++	+++	+

*plus radioactive disposal costs. + symbols indicate the viability of each procedure.

mber of tadpoles per experimental unit. Besides, rearing more tadpoles per unit requires larger containers since larval density in itself directly results in increased CORT levels (Hayes, 1997).

Another potential confounding factor in determining CORT levels is the effect of handling time. GCs levels can increase inordinately after only 3–5 minutes from the first contact with the individual (Wingfield et al., 1982; Cash et al., 1997; Romero and Romero, 2002) and usually reach peak levels after 15-30 min after exposure to the stressor (De Kloet et al, 2005). Handling times are not a concern for procedures like RIA and whole-body homogenate EIA in which animals can be quickly (under 3–5 min) euthanized and preserved (e.g. through immersion in MS-222 followed by snap freezing of the whole body). Obtaining blood samples from tadpoles, however, is a more laborious procedure and some training is required to reduce handling times under 3–5 min.

Therefore, handling times may become a likely source of error and need to be accounted for in the analyses. For this reason, RIA and whole-body homogenate EIA are the best method to measure GC in field studies. EIA on supernatant of whole-body homogenates has the advantages of not requiring radioactivity, avoiding potential error from increased handling times, and requires fewer individuals than plasma EIA. Conversely, it constitutes a more conservative test of differences among test groups (experimental treatments, populations, species) (Burraco et al., 2013), as it is less sensitive than either RIA or plasma EIA.

The choice of procedure for each study should thus be made considering methodological limitations and tractability for each study system. RIA is still the golden standard in eco-physiological studies because it is most sensitive, it is appropriate for small larvae, and can be used to compare CORT levels using different tissues or on whole-body homogenates. However, plasma EIA is a good non-radioactive alternative that has been used to obtain significant results in response to natural perturbations such as pond drying (Gomez-Mestre et al., 2013). EIA on whole-body homogenate supernatant could detect qualitative differences in CORT levels among experimental groups, and may be of use if individuals are small and radioactive assays cannot be implemented.

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References

Aguilera G, 1994. Regulation on pituitary ACTH secretion during chronic stress. Frontiers in Neuroendocrinology 15: 321–350.

Becker JB, Breedlove SM, Crews D, McCarthy M, 2002. Behavioral Endocrinology. 2nd edn. Cambridge: MIT Press.

Belden LK, Wingfield JC, Kiesecker JM, 2010. Variation in the hormonal stress response among larvae of three amphibian species. Journal of Experimental Zoology, Part A, Ecological Genetics and Physiology 313: 524–31.

Bentley PJ, 1971. Endocrine and Osmoregulation: A Comparative Account of the Regulation of Water and Salt in Vertebrates. New York, Heidelberg, Berlin: Springer Verlag.

Burraco P, Duarte LJ, Gomez-Mestre I, 2013. Predator-induced physiological responses in tadpoles challenged with herbicide pollution. Current Zoology 59(4): 475–484.

Busch DS, Hayward LS, 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142: 2844–2853.

Cash WB, Holberton RL, Knight SS, 1997. Corticosterone secretion in response to capture and handling in free-living red-eared slider turtles. General and Comparative Endocrinology 108: 427–433.

Davis AK, Maney DL, Maerz JC, 2008. The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. Functional Ecology 22: 760–772.

De Kloet ER, Sibug RM, Helmerhorst M, Schmidt M, 2005. Stress, genes and the mechanism of programming the brain for later life. Neuroscience & Behavioral Reviews 29: 271–281.

Denver RJ, 1993. Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. General and Comparative Endocrinology 91: 38–51.

Denver RJ, Boorse GC, Glennemeier KA, 2002. Endocrinology of complex life cycles: Amphibians. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Moss R et al. ed. Hormones, Brain and Behavior. San Diego: Academic Press, 469–513.

Denver RJ, 2009. Structural and functional evolution of vertebrate neuroendocrine stress systems. Annals of the New York Academy of Sciences 1163: 1–16.

Durant SE, Romero LM, Talent LG, Hopkins WA, 2008. Effect of exogenous corticosterone on respiration in a reptile. General and Comparative Endocrinology 156: 126–133.

Gabor CR, Fisher MC, Bosch J, 2013. A non-invasive stress assay shows that tadpole populations infected with *Batrachochytrium dendrobatidis* have elevated corticosterone levels. PLoS ONE 8: e56054.

Glennemeier KA, Denver RJ, 2002. Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127: 16–25.

Gomez-Mestre I, Kulkarni S, Buchholz DR, 2013. Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. PLoS ONE 8:e84266.

Hayes T, 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. American Zoologist 37: 185–194.

Hoffmann M, Hilton-Taylor C, Angulo A, Böhm M, Brooks T et al., 2010. The impact of conservation on the status of the world's vertebrates. Science 330: 1503–1509.

Johnson EO, Kamilaris TC, Chrousos GP, Gold PW, 1992. Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. Neuroscience and Biobehavioral Reviews 16: 115–30.

Lesbarrères D, Ashpole SL, Bishop CA, Blouin-Demers G, Brooks RJ et al., 2014. Conservation of herpetofauna in northern landscapes: Threats and challenges from a Canadian perspective. Biological Conservation 170: 48–55.

McMahon TA, Halstead NT, Johnson S, Raffel TR, Romansic JM et al., 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environmental Health Perspectives 119: 1098–1103.

Middlemis-Maher J, Werner EE, Denver RJ, 2013. Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. Proceedings of the Royal Society B, Biological Sciences 280: 1758.

Miller DB, O'Callaghan JP, 2002. Neuroendocrine aspects of the response to stress. Metabolism 51: 5–10.

Mills SC, Mourier J, Galzin R, 2010. Plasma cortisol and 11-ketotestosterone enzyme immunoassay (EIA) kit validation for three fish species: The orange clownfish *Amphiprion percula*, the orangefin anemonefish *Amphiprion chrysopterus* and the blacktip reef shark *Carcharhinus melanopterus*. Journal of Fish Biology 77: 769–777.

Narayan E, 2013. Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. Conservation Physiology 1: 1–16.

Nieuwkoop P, Faber J, 1994. Normal Table of XenopusLaevis (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg Until the End of Metamorphosis. New York: J. Garland Publishing Inc.

Nussey E, Whitehead S, 2001. Endocrinology: An integrated Approach. Oxford: BIOS Scientific Publishers.

Parsons RH, 1994. Amphibian Biology, The Integument. In: Heatwole H, Barthalmus GT ed. Chipping Norton, Australia: Surrey Beatty & Sons, 132–146.

Raffin-Sanson ML, de Keyzer Y, Bertagna X, 2003. Proopiomelanocortin, a polypeptide precursor with multiple functions: From physiology to pathological conditions. European Journal of Endocrinology 149: 79–90.

Romero LM, Romero RC, 2002. Corticosterone responses in wild birds: The importance of rapid initial sampling. The Condor 104: 129–135.

Romero LM, 2004. Physiological stress in ecology: Lessons from biomedical research. Trends in Ecology & Evolution 19: 249–255.

Sapolsky RM, Romero LM, Munck AU, 2000. How do glucocorticoids influence stress responses? Integrating permissive, supressive, stimulatory, and preparative actions. Endocrine Reviews 21: 55–89.

Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R, 2011. Measuring stress in wildlife: Techniques for quantifying glucocorticoids. Oecologia 166: 869–887.

Sockman KW, Schwabl H, 2001. Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition. General and Comparative Endocrinology 122: 205–212.

Wada H, Hahn TP, Breuner CW, 2007. Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low. General and Comparative Endocrinology 150: 405–413.

Walker BG, Wingfield JC, Boerma PD, 2005. Age and food deprivation affects expression of glucocorticoid stress response in Magellanic penguin *Sphenicus megallanicus* chicks. Physiological and Biochemical Zoology 78: 78–89.

Webb AH, Allert JA, Kappenman KM, Marcos J, Feist GW et al., 2007. Identification of plasma glucocorticoids in pallid sturgeon in response to stress. General and Comparative Endocrinology 154: 98–104.

Wikelski M, Cooke SJ, 2006. Conservation physiology. Trends in Ecology & Evolution 21: 38–46.

Wingfield JC, Smith JP, Farner DS, 1982. Endocrine responses of white-crowned sparrows to environmental stress. The Condor 84: 399–409.

Wright ML, Guertin CJ, Duffy JL, Szatkowski MC, Visconti RF et al., 2003. Developmental and diel profiles of plasma corticosteroids in the bullfrog *Rana catesbeiana*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 135: 585–595.

Supporting Information: References of Table 2

Belden LK, Moore IT, Mason RT, Wingfield JC, Blaustein AR, 2003. Survival, the hormonal stress response and UV-B avoidance in cascades frog tadpoles *Rana cascadae* exposed to UV-B radiation. Functional Ecology 17: 409–416.

Belden LK, Moore IT, Wingfield JC, Blaustein AR, 2005. Corticosterone and growth in pacific treefrog *Hyla regilla* tadpoles. Copeia 2: 424–430.

Belden LK, Rubbo MJ, Wingfield JC, Kiesecker JM, 2007. Searching for the physiological mechanism of density dependence : Does corticosterone regulate tadpole responses to density? Physiological and Biochemical Zoology 80: 444–451.

Belden LK, Wingfield JC, Kiesecker JM, 2010. Variation in the hormonal stress response among larvae of three amphibian species. Journal of Experimental Zoology, Part A, Ecological Genetics and Physiology 313: 524–31.

Boorse GC, Denver RJ, 2004. Expression and hypophysiotropic actions of corticotropin-releasing factor in *Xenopus laevis*. General and Comparative Endocrinology 137: 272–82.

Brown GP, Kelehear C, Shine R, 2011. Effects of seasonal aridity on the ecology and behaviour of invasive cane toads in the Australian wet-dry tropics. Functional Ecology 25: 1339–1347.

Burraco P, Duarte LJ, Gomez-Mestre I, 2013. Predator-induced physiological responses in tadpoles challenged with herbicide pollution. Current Zoology 59: 475–484.

Chambers DL, 2011. Increased conductivity affects corticosterone levels and prey consumption in larval amphibians. The Society for the Study of Amphibians and Reptiles 45: 219–223.

Chambers DL, Wojdak JM, Du P, Belden LK, 2011. Corticosterone level changes throughout larval development in the amphibians *Rana sylvatica* and *Ambystoma jeffersonianum* reared under laboratory, mesocosm, or free-living conditions. Copeia 4: 530–538.

Crespi EJ, Vaudry H, Denver RJ, 2004. Roles of corticotropin-releasing factor, neuropeptide Y and corticosterone in the regulation of food intake in *Xenopus laevis*. Journal of Neuroendocrinology 16: 279–288.

Crespi EJ, Denver RJ, 2004. Ontogeny of corticotropin-releasing factor effects on locomotion and foraging in the Western spadefoot toad

Spea hammondii. Hormones and Behavior 46: 399–410.

Crespi EJ, Denver RJ, 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comparative Biochemistry and Physiology, Part A, Molecular & integrative Physiology 141: 381–90.

Crespi EJ, Warne RW, 2013. Environmental conditions experienced during the tadpole stage alter post-metamorphic glucocorticoid response to stress in an amphibian. Integrative and Comparative Biology 53: 989–1001.

Dahl E, Orizaola G, Winberg S, Laurila A, 2012 Geographic variation in corticosterone response to chronic predator stress in tadpoles. Journal of Evolutionary Biology 25: 1066–76.

Denver RJ, 1997. Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Hormones and Behavior 31: 169–179.

Denver RJ, 1998. Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad *Scaphiopus hammondii*. General and Comparative Endocrinology 110: 326–336.

Fraker ME, Hu F, Cuddapah V, McCollum SA, Relyea RA et al., 2009. Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. Hormones and Behavior 55: 520–529.

Gabor CR, Fisher MC, Bosch J, 2013. A non-invasive stress assay shows that tadpole populations infected with *Batrachochytrium dendrobatidis* have elevated corticosterone levels. PloS ONE 8: e56054.

Glennemeler KA, Denver RJ, 2001. Sublethal effects of chronic exposure to an organochlorine compound on northern leopard frog *Rana pipiens* tadpoles. Environmental Toxicology 16: 287–297

Glennemeier KA, Denver RJ, 2002. Developmental changes in interrenal responsiveness in anuran amphibians. Integrative and Comparative Biology 42: 565–573.

Glennemeier KA, Denver RJ, 2002. Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127: 16–25.

Glennemeier KA, Denver RJ, 2002. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. Journal of Experimental Zoology 1: 32–40.

Gomez-Mestre I, Kulkarni S, Buchholz DR, 2013 Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. PLoS ONE 8: 0084266

Graham C, Narayan E, McCallum H, Hero J-M., 2013. Non-invasive monitoring of glucocorticoid physiology within free-living highland and lowland populations of native Australian great barred frogs *Mixophyes fasciolatus*. General and Comparative Endocrinology 191: 24–30.

Hayes TB, Wu TH, 1995. Interdependence of corticosterone hormones and thyroid hormones in toad larvae *Bufo boreas*. 2. Regulation of corticosterone hormones and thyroid hormones. Journal of Experimental Zoology 271: 103–111.

Hayes T, 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. American Zoologist 37: 185–194.

Hayes TB, Case P, Chui S, Chung D, Haeffele C et al., 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environmental health perspectives 114: 40–50.

Hopkins WA, Mendonça MT, Congdon JD, 1997. Increased circulating levels of testosterone and corticosterone in southern toads *Bufo terrestris* exposed to coal combustion waste. General and Comparative Endocrinology 108: 237–46.

Hopkins WA, Mendonça MT, Congdon JD, 1999. Responsiveness of the hypothalamo-pituitary-interrenal axis in an amphibian *Bufo terrestris* exposed to coal combustion wastes. Comparative Biochemistry and Physiology Part C, Pharmacology, Toxicology & Endocrinology 122: 191–196.

Hu F, Crespi EJ, Denver RJ, 2008. Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog *Xenopus laevis*. Endocrinology 149: 5470–5481.

Kindermann C, Narayan EJ, Hero JM, 2012. Urinary corticosterone metabolites and chytridiomycosis disease prevalence in a free-living population of male Stony Creek frogs *Litoria wilcoxii*. Comparative Biochemistry and Physiology Part A, Molecular & Integrative physiology 162: 171–176.

Kindermann C, Narayan E, Wild C, Wild F, Hero J-M, 2013. The effect of stress and stress hormones on dynamic colour-change in a sexually dichromatic Australian frog. Comp. Biochem. Physiol. A: Molecular and Integrative Physiology 165: 223–227.

Kloas W, Reinecke M, Hanke W, 1997. Stage-dependent changes in adrenal steroids and catecholamines during development in *Xenopus laevis*. General and Comparative Endocrinology 108: 416–426.

Ledon-Rettig CC, Pfennig DW, Crespi EJ, 2009. Stress hormones and the fitness consequences associated with the transition to a novel diet in larval amphibians. The Journal of the Experimental Biology 212: 3743–3750.

Ledon-Rettig CC, Pfennig DW, Crespi EJ, 2010. Diet and hormonal manipulation reveal cryptic genetic variation: Implications for the evolution of novel feeding strategies. Proceedings of The Royal Society B: Biological Sciences 277: 3569–3578.

McMahon TA, Halstead NT, Johnson S, Raffel TR, Romansic JM et al., 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environmental Health Perspectives 119: 1098–1103.

Middlemis-Maher J, Werner EE, Denver RJ, 2013. Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. Proceedings of The Royal Society B, Biological Sciences 280: 1758.

Narayan EJ, Molinia F, Christi K, Morley C, Cockrem J, 2010. Urinary corticosterone metabolite responses to capture and annual patterns of urinary corticosterone in wild and captive endangered Fijian ground frogs *Platymantis vitiana*. Australian Journal of Zoology 58: 189–197.

Narayan EJ, Molinia F, Christi K, Morley C, Cockrem J, 2010 (2). Annual cycles of urinary reproductive steroid concentrations in the

endangered Fijian ground frog *Platymantis vitiana*. General and Comparative Endocrinology 166: 172–179.

Narayan EJ, Hero JM, 2011. Urinary corticosterone responses and haematological stress indices in endangered Fijian ground frog *Platymantis vitiana* during transportation and captivity. Australian Journal of Zoology 59: 79–85.

Narayan EJ, Molinia FC, Kindermann C, Cockrem JF, Hero JM, 2011 (2). Urinary corticosterone responses to capture and toe-clipping in the cane toad *Rhinella marina* indicate that toe-clipping is a stressor for amphibians. General and Comparative Endocrinology 174: 238–45.

Narayan EJ, Cockrem JF, Hero JM, 2012. Urinary corticosterone metabolite responses to capture and handling in two closely related species of free-living Fijian frogs. General and Comparative Endocrinology 177: 55–61.

Narayan EJ, Molinia FC, Cockrem JF, Hero JM, 2012 (2). Individual variation and repeatability in urinary corticosterone metabolite responses to capture in the cane toad *Rhinella marina*. General and Comparative Endocrinology 175: 284–289.

Narayan EJ, Molinia FC, Cockrem JF, Hero J-M, 2012 (3). Changes in urinary testosterone and corticosterone metabolites during short-term confinement with repeated handling in wild male cane toads *Rhinella marina*. Australian Journal of Zoology 59: 264–269.

Narayan EJ, Cockrem JF, Hero J-M, 2012 (4). Effects of temperature on urinary corticosterone responses to capture and handling stress in the cane toad *Rhinella marina*. General and Comparative Endocrinology 178: 301–305.

Narayan EJ, Hero J-M, Cockrem JF, 2012 (5). Inverse urinary corticosterone and testosterone responses to different durations of restraint in the cane toad *Rhinella marina*. General and Comparative Endocrinology 179: 345–349.

Narayan EJ, Cockrem JF, Hero JM, 2013. Sight of a predator induces a corticosterone stress response and generates fear in an amphibian. PloS ONE 8: e73564.

Narayan EJ, Cockrem J, Hero J-M, 2013 (2). Repeatability of baseline corticosterone and short-term corticosterone stress responses, and their correlation with testosterone and body condition in a terrestrial breeding anuran *Platymantis vitiana*. Comparative Biochemistry and Physiology. Part A: Molecular & Integrative Physiology 165: 304–312.

Narayan EJ, Hero J-M, 2013 (3). Repeatability of baseline corticosterone and acute stress responses to capture, and patterns of reproductive hormones in vitellogenic and non-vitellogenic female Fijian ground frog *Platymantis vitiana*. Journal of Experimental Zoology A: Ecological Genetics and Physiology 319: 471–481.

Narayan EJ, Hero J-M, Cockrem JF, 2013 (4). Are baseline and short-term corticosterone stress responses repeatable in free-living amphibians? Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 164: 21–28.

Narayan EJ, Cockrem J-F, Hero J-M, 2013 (5). Changes in serum and urinary corticosterone and testosterone in adult male cane toad (*Rhinella marina*) during short-term capture and handling. General and Comparative Endocrinology 191: 225–230.

Peterson JD, Peterson VA, Mendonça MT, 2009. Exposure to coal combustion residues during metamorphosis elevates corticosterone content and adversely affects oral morphology, growth, and development in *Rana sphenocephala*. Comparative Biochemistry and Physiology Toxicology & Pharmacology 149: 36–39.

Reeve BC, Crespi EJ, Whipps CM, Brunner JL, 2013. Natural stressors and ranavirus susceptibility in larval wood frogs *Rana sylvatica*. EcoHealth 10: 190–200.

Ward CK, Mendonça MT, 2006. Chronic exposure to coal fly ash causes minimal changes in corticosterone and testosterone concentrations in male southern toads *Bufo terrestris*. Archives of Environmental Contamination and Toxicology 51: 263–269.

Warne RW, Crespi EJ, Brunner JL, 2011. Escape from the pond: Stress and developmental responses to ranavirus infection in wood frog tadpoles. Functional Ecology 25: 139–146.

Wright ML, Guertin CJ, Duffy JL, Szatkowski MC, Visconti RF, Alves CD, 2003. Developmental and diel profiles of plasma corticosteroids in the bullfrog, *Rana catesbeiana*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 135: 585–595.