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Peroxiredoxin 6 from the Clawed Frog *Xenopus laevis*: cDNA Cloning, Enzyme Characterization, and Gene Expression during Development

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Abstract—The *Xenopus laevis* 1-Cys-peroxiredoxin (peroxiredoxin 6, Prx6) gene was cloned and expressed in *Escherichia coli*. The enzymatic properties of the recombinant protein were characterized and compared to those of human Prx6. *Xenopus laevis* Prx6 has 224 amino acid residues including five Cys, one of which, Cys47, is located in the active center determining peroxidase activity. The stability and activity of *X. laevis* Prx6 relative to hydrogen peroxide and *tret*-butyl hydroperoxide are very similar to corresponding values for human Prx6. Both enzymes have temperature optimum at 37°C, but the clawed frog enzyme retains no less than 50% of activity over a wider temperature interval (10-50°C) than the human one (25-50°C). The expression of *X. laevis prx6* at different stages of development was investigated. The level of gene expression increased during development, especially at stages 33-43 during formation of the lungs, when heartbeat and hatching begins.

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Peroxiredoxins (Prx(s)), which form a superfamily of selenium-independent peroxidases, were discovered about 10 years ago [1]. Prxs carry out fermentative degradation of H_2O_2, organic hydroperoxides, and peroxynitrite [2, 3]. Prxs were discovered in various pro- and eucaryotic organisms. There are six types of Prx: Prxs 1-5 have two active cysteine residues and use thioredoxin and glutathione as reducing substrates, and Prx6 has one active cysteine residue and uses glutathione in the reaction of catalytic reduction of H_2O_2 and different organic hydroperoxides. Unlike other peroxiredoxins, Prx6 reduces phospholipid hydroperoxides and possesses phospholipase A2 activity [4, 5]. Prx6 apparently plays a unique role in antioxidant protection in mammals, and its function cannot be compensated by expression of other genes. For instance, *prx6* knocked-out mice appeared to have low degree of survival, high level of protein oxidation, and considerable injury of many organs. At the same time, it should be noted that the expression level of other antioxidative enzymes, like catalase, glutathione peroxidase, and superoxide dismutase did not differ from that of wild-type mice [6, 7]. The *prx6* gene is activated in newborn rats and primates [8, 9], as well as in adult animals with hyperoxia [10].

The clawed frog *Xenopus laevis* is one of the best-studied objects in developmental biology. In the present work, we studied the properties of *X. laevis* Prx6 and the expression of the *prx6* gene at early stages of development.

MATERIALS AND METHODS

Object of study. Clawed frogs *X. laevis* were obtained from the Institute of Developmental Biology, Russian Academy of Sciences. To get embryos, females were injected with 500 IU of human chorionic gonadotropin, and in 16 h the derived eggs were impregnated with sperm suspension. More than 80% of eggs were successfully impregnated. Embryos were kept in an aquarium at 20°C. Stages of development were determined with a binocular microscope using the description from Nieuwkoop and Faber [11]. Embryos at different stages were frozen in liquid nitrogen and kept at −80°C.

Abbreviations: DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; Prx6, peroxiredoxin 6; t-BHP, *tret*-butyl hydroperoxide.

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RNA extraction from *X. laevis*. Embryos (200 mg) were frozen in liquid nitrogen and homogenized in a ceramic vessel. Two milliliters of buffer D (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 4.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol), 0.2 ml of 2 M sodium acetate, 2.2 ml of water-saturated phenol, and 2.2 ml of chloroform–isoamyl alcohol (49 : 1 v/v) were then added to homogenate. The final suspension was shaken for 20 sec and centrifuged at 18,000*g* for 15 min at 4°C. The aqueous phase was collected followed by a second extraction with an equal volume of chloroform. Samples were centrifuged for 15 min at 18,000*g*, and the aqueous phase was collected with subsequent RNA precipitation with an equal volume of isopropanol for 16 h at –20°C. The solution was then centrifuged at 18,000*g* for 15 min at 4°C, the sediment was washed with 70% ethanol, dried, dissolved in 0.3 ml of buffer D, and RNA was repeatedly precipitated with isopropanol (for 3 h at –20°C). Finally, the RNA was dissolved in 100 μl of deionized water (RNA concentration 500 ng/μl). The RNA solution was stored at –80°C.

Reverse transcription and PCR. RNA solution was treated with DNase RQ1 (Promega, USA) according to the manufacturer's instructions and purified on Qiagen (Germany) columns before the reaction of reverse transcription. RNA quality was evaluated by electrophoresis (by bands of 18S and 28S rRNAs) in 1% agarose gel and by spectrophotometry using the ratio of light absorption at 260/280 nm (2.57) and 260/230 nm (2.22). The resulting RNA solution was used for reverse transcription (RT). Total RNA (1 μg) from Fermentas (Lithuania), reverse transcriptase M-MLV, and standard oligo-dT primer were used in RT assay. The resulting cDNA was then used in PCR with gene-specific primers for the *prx6* gene (containing NdeI and XhoI restriction sites): direct, 5′-TT*CATATG*CCTGGAATCCTGCTAGGAG-3′; reverse, 5′-AA*CTCGAG*TTGTGGCTGTGCAGTGTATCTC-3′. PCR was run in a MJMini thermocycler (BioRad, USA) in 0.2 ml tubes under the following conditions: denaturation at 94°C for 2 min; then 30 cycles: denaturation at 94°C for 30 sec; primer annealing at 56°C for 30 sec; DNA synthesis at 68°C for 1 min, and final DNA elongation at 68°C for 5 min. High Fidelity PCR Enzyme mix (Fermentas) was used in the reaction.

Cloning and expression of *X. laevis prx6*. The obtained PCR fragment was exposed to NdeI and XhoI restrictase treatment, refined from nucleases by means of electrophoresis in 0.8% agarose, and extracted from gel using a QIAquick Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. Plasmid pET-23b(+) (Novagen, USA) was treated with the same enzymes, and the linear form was extracted from agarose gel after electrophoresis. Ligation and transformation of BL21(DE3) [pRIL] cells (Novagen) were carried out by a standard protocol [12]. Clones were selected on ampicillin-containing agar, and the presence of the required insertions was tested by PCR using standard primers for pET plasmids (T7 forward and T7 reverse). Clones containing the *prx6* gene were verified for gene induction and target protein synthesis. At the final control step, clones were sequenced using T7 plasmid primers. Later on we used the clone containing the insertion exactly coinciding with the sequence of *X. laevis prx6* gene taken from GenBank (Accession No. BC054309).

Extraction and purification of *X. laevis* Prx6. As a result of *X. laevis prx6* cloning, a construction was obtained in which the gene is orientated so as to contain six histidine residues (His-tag) on the C-terminus upon translation. Previously it has been shown on human Prx6 that the presence of C-terminal His-tag does not affect enzyme activity [5]. His-tag simplifies recombinant protein purification due to specific binding to Ni-NTA-agarose.

One hundred milliliters of LB medium containing 100 μg/ml ampicillin was inoculated with 1 ml of fresh night culture of the clone and grown on a shaker (120 rpm) at 37°C to optical density of 0.6 at 600 nm. After that, IPTG was added to final concentration of 1 mM and cell growth continued for 3 h. The bacteria were harvested by centrifugation for 20 min at 1500*g*. The bacterial pellet was resuspended in 8 ml of buffer: 12 mM Tris-HCl, pH 7.8, and 10 mM imidazole. The bacteria were then lysed by ultrasound in a UZDN-2T ultrasonic disintegrator (Russia) at 4°C. Fragments of disrupted cells were removed by 20 min centrifugation at 2000*g*, and the supernatant was transferred to a Ni-NTA-agarose column (Invitrogen, USA). One hundred milliliters of 12 mM Tris-HCl, pH 7.8, with 20 mM imidazole was used for washing, and elution was carried out using 5 ml of the same buffer containing 250 mM imidazole. The protein was concentrated with a VivaScience membrane concentrator (Germany) and dialyzed against 7 mM phosphate buffer, pH 7.3, containing 150 mM NaCl. Usually about 5 mg of electrophoretically pure protein (≥95% purity) was obtained as the result of this procedure. Enzyme was stored at concentration of 10-20 mg/ml in 7 mM phosphate buffer (pH 7.3) containing 150 mM NaCl at –20°C. During two months storage no decrease in enzymatic activity was registered.

Determination of Prx6 peroxidase activity. Hydroperoxide from Calbiochem (USA) and *tret*-butyl hydroperoxide (t-BHP) from Sigma-Aldrich (USA) were used as substrates. Dithiothreitol (DTT) from Sigma-Aldrich was used as a reducing agent. Peroxidase activity was determined according to a slight modification of a protocol described by Kang et al. [13]. The reaction mixture (150 μl) contained 7 mM phosphate buffer, pH 7.3, 150 mM NaCl, 2 mM DTT, 100 μM hydrogen peroxide (or 100 μM t-BHP), and 35 μg of the enzyme. The reaction was carried out at 25°C. Each 30 sec samples were taken and the reaction was stopped by addition of 50 μl of 0.6 M HCl followed by introducing of 100 μl of 10 mM

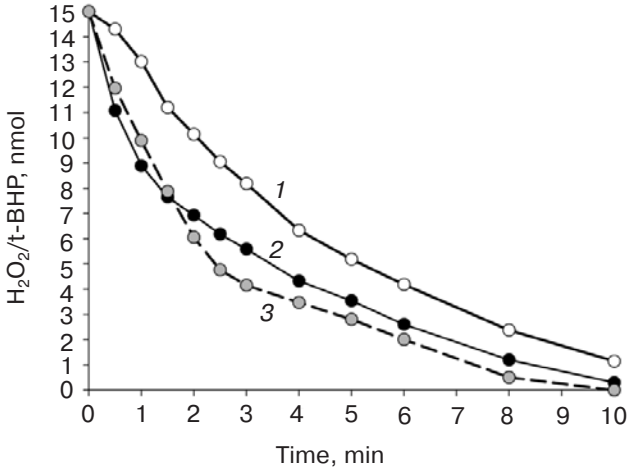

Fig. 1. Activity of clawed frog Prx6 against t-BHP (*1*) and H_2O_2 (*2*) and of human Prx6 against H_2O_2 (*3*).

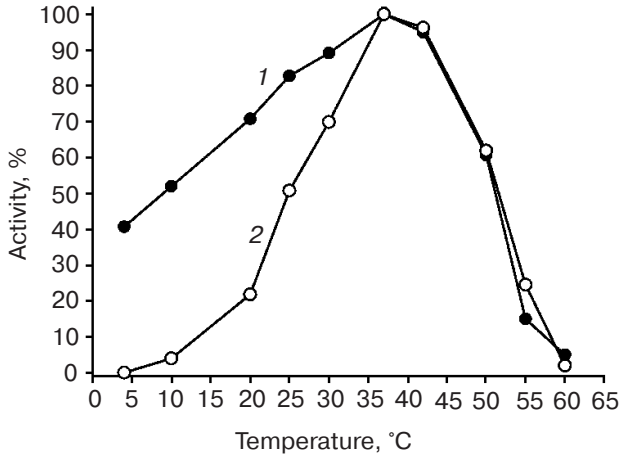

Fig. 2. Temperature optimum for clawed frog (*1*) and human Prx6 (*2*).

$Fe(NH_4)_2(SO_4)_2$ and 50 μl of 2.5 M KSCN resulting in formation of a red complex of iron. Concentration of peroxide is proportional to color intensity measured at 492 nm. Three independent experiments were made, the error being ± 4% of mean value.

Determination of Michaelis constant (K_m). K_m was calculated from peroxidase reaction ratio dependence on substrate concentration. Reaction was carried out as described above at constant concentration of the enzyme for 5 min at 25°C.

Determination of thermostability and temperature optimum of Prx6. The enzyme was incubated at 37-65°C taking samples at regular intervals, and then the reducing agent was added (2 mM DTT) and residual activity against t-BHP was evaluated at 37°C. The temperature optimum of activity against t-BHP was defined in the presence of 2 mM DTT with incubation time of 10 min.

Comparison of *X. laevis prx6* expression levels at different developmental stages. Total RNA (1 μg) extracted at different stages of development (stages 10-14, 33-34, 42-43, 47-48, 63-64) was taken for reverse transcription with oligo-dT primer. The obtained cDNA was used in PCR with primers for the *prx6* gene. PCR for cytoskeletal actin (type 5) was used as a control since its expression does not change substantially during the development of *X. laevis* embryos [14]. The following primers for gene of cytoskeletal actin (Accession No. AAA49638) were used: direct, 5′-ATCATGTTTGAGACTTTCAA-3′; reverse, 5′-TTGCGTTCAGGCGGGGCAAT-3′. Samples were collected after 20-35 PCR cycles followed by electrophoresis in 1% agarose gel. Intensity of bands containing PCR products was evaluated with ImageJ software (www.rsb.info.nih.gov/ij/). Comparison of intensity for corresponding bands was used for semiquantitative determination of *prx6* expression level.

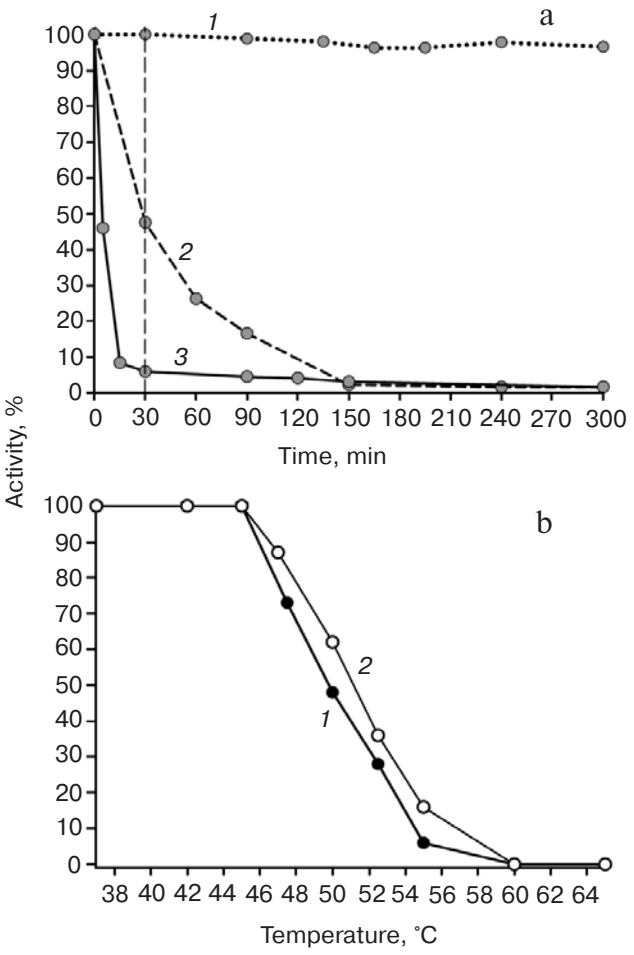

Fig. 3. Thermostability of Prx6 of clawed frog and human. a) Residual activity of clawed frog Prx6 upon incubation at different temperatures: *1-3*) 45, 50, and 55°C, respectively. b) Residual activity of clawed frog (*1*) and human (*2*) Prx6 upon 30 min incubation at different temperatures.

RESULTS AND DISCUSSION

The sequence of the *X. laevis prx6* gene was obtained as a result of research work in the context of the "*Xenopus* initiative of NIH USA" project [15]. The gene was identified based on pronounced homology with genes of the best-studied peroxiredoxins 6, particularly, with human *prx6*. The sequences of *prx6* genes in human and clawed frog are 72.9% identical, and the amino acid sequences coincide by 80.4%.

The secondary and tertiary structures of the above-mentioned enzymes are apparently also rather similar. For instance, the secondary structure of human Prx6, predicted with APSSP2 software (www.imtech.res.in/raghava/apssp2/), contains 29 (29.5)% of α-helices and 17 (16.1)% of β-structures, while clawed frog Prx6 contains 28% of α-helixes and 19% of β-structures (data of X-ray analysis for human Prx6 are given in parentheses). Clawed frog Prx6, as well as human Prx6, has a thioredoxin-like fold typical for all Prxs, a conservative PVC(47)TTE motif, and a triad of amino acid residues forming the active center: His39–Cys47–Arg132 [16].

Activity and K_m. *Xenopus laevis* Prx6 is more active against H_2O_2 in comparison to t-BHPas as can be seen from Fig. 1. Its affinity to H_2O_2 is apparently lower than to t-BHP, as K_m for H_2O_2 (165 μM) is higher than for t-BHP (130 μM). Activity of clawed frog Prx6 against t-BHP does not differ from that of human Prx6, but clawed frog Prx6 is less active against H_2O_2 than human Prx6 (Fig. 1). H_2O_2 affinity (K_m = 125 μM) for human Prx6 is also somewhat lower than t-BHP affinity (K_m = 110 μM).

Thermostability and temperature optimum. As seen from Fig. 2, the temperature optimum for both human and clawed frog Prx6 is around 37°C, but it should be noted that the enzyme from clawed frog retains quite high activity at low temperature (no less than 50% activity over the range of 10-50°C) in contrast to human Prx6 (no less than 50% activity over the range of 25-50°C). The clawed frog is a cold-blooded animal and can live at temperatures much lower than 37°C, so their antioxidative system must be active at low temperatures. On the contrary, the temperature of the human body is constantly kept near 37°C, which, most probably, explains the fact that human Prx6 is active in a narrower temperature range. Figure 3 (a and b) shows that Prx6 from clawed frog and human are rather close in thermostability.

Expression of *X. laevis prx6* gene. To evaluate the amount of *prx6* mRNA, we used cDNA obtained from equal portions of total RNA extracted from embryos at different developmental stages. PCR for the gene of *X. laevis* cytoskeletal actin (type 5) was used for comparison since its expression does not change considerably during development [14]. We did not observe substantial change in expression level for this gene, yet the expression level for *prx6* changed significantly. Thus, *prx6* expression level increases approximately four times at developmental stage 33-34, 20 times at stage 42-43, and more than 40 times at stages 47-64 compared to the expression level at stage 10-14 (Fig. 4). The evaluation is certainly quite rough, but it shows that *prx6* mRNA level sharply increases as an adaptive reaction to the appearance of active forms of oxygen at the developmental stages when active

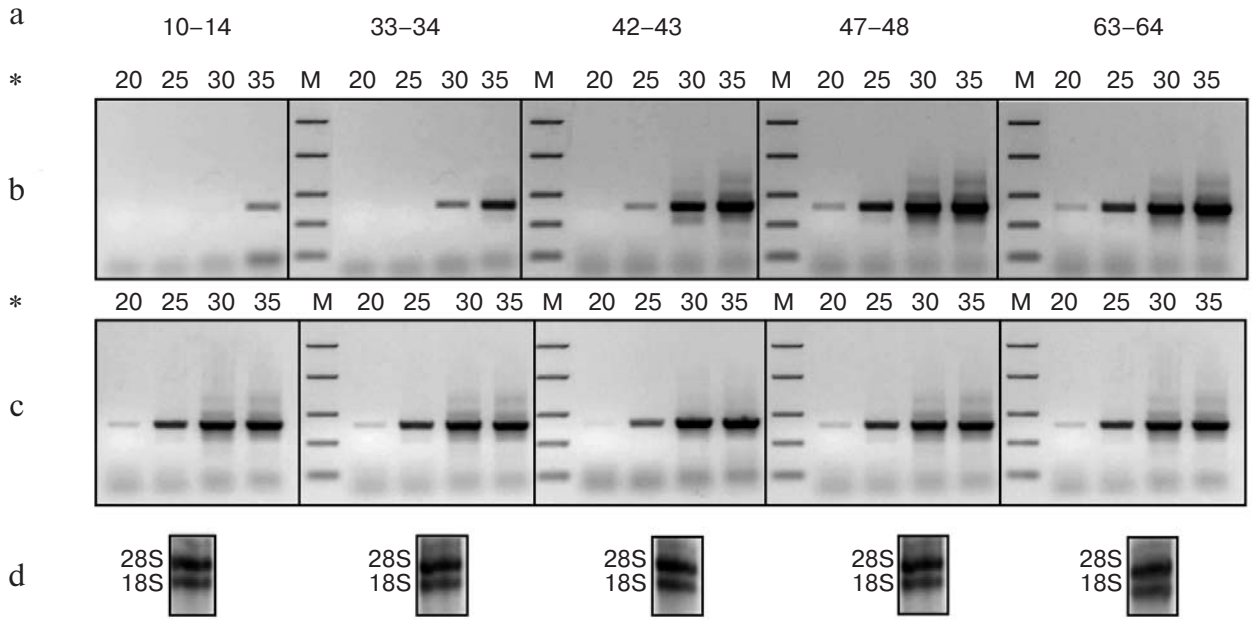

Fig. 4. Expression of clawed frog *prx6* gene at different developmental stages. a) Clawed frog developmental stages; b) *prx6* gene; c) actin gene; d) total RNA used for RT-PCR. Asterisk indicates PCR cycles. M, DNA markers (from top to bottom: 5000, 2000, 850, 400, 100 bp).

respiration begins. This result correlates well with the data obtained by Rizzo et al. [17], who found that the level of some antioxidative enzymes (superoxide dismutase, catalase, glutathione transferase, and glutathione reductase) in *X. laevis* increases significantly at developmental stages 44-47 (in comparison to stage 22), i.e. when active respiration begins.

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REFERENCES

1. Flohe, L., and Harris, J. R. (2007) *Subcell. Biochem.*, **44**, 1-25.
2. Hofmann, B., Hecht, H.-J., and Flohe, L. (2002) *J. Biol. Chem.*, **383**, 347-364.
3. Wood, Z. A., Schroder, E., Harris, J. R., and Poole, L. B. (2003) *Trends Biochem. Sci.*, **28**, 32-40.
4. Manevich, Y., and Fisher, A. B. (2005) *Free Rad. Biol. Med.*, **38**, 1422-1432.
5. Chen, J. W., Dodia, C., Feinstein, S. I., Jain, M. K., and Fisher, A. B. (2000) *J. Biol. Chem.*, **275**, 28421-28427.
6. Wang, X., Phelan, S. A., Forsman-Semb, K., Taylor, E. F., Petros, C., Brown, A., Lerner, C. P., and Paigen, B. (2003) *J. Biol. Chem.*, **278**, 25179-25190.
7. Nagy, N., Malik, G., Fisher, A. B., and Dipak, K. D. (2006) *Am. J. Physiol. Heart Circ. Physiol.*, **291**, 2636-2640.
8. Kim, H. S., Manevich, Y., Feinstein, S. I., Pak, J. H., Ho, Y. S., and Fisher, A. B. (2003) *Am. J. Physiol. Lung. Cell Mol. Physiol.*, **285**, 363-369.
9. Das, K. C., Pahl, P. M., Guo, X. L., and White, C. W. (2001) *Am. J. Respir. Cell. Mol. Biol.*, **25**, 226-232.
10. Mitsumoto, A., Takanezawa, Y., Okawa, K., Iwamatsu, A., and Nakagawa, Y. (2001) *Free Radic. Biol. Med.*, **30**, 625-635.
11. Brodsky, V. Y., Dyban, A. P., Lopashev, G. V., and Tokin, B. P. (1975) *Objects in Developmental Biology* (Dettlaff, T. A., ed.) [in Russian], Nauka, Moscow, pp. 392-441.
12. Sambrook, J., Fritsch, E. E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
13. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) *J. Biol. Chem.*, **273**, 6303-6311.
14. Mohun, T. J., and Garrett, N. (1987) *Development*, **101**, 393-402.
15. Klein, S. L., Strausberg, R. L., Wagner, L., Pontius, J., Clifton, S. W., and Richardson, P. (2002) *Devel. Dynamics*, **225**, 384-391.
16. Karplus, P. A., and Hall, A. (2007) *Subcell. Biochem.*, **44**, 41-60.
17. Rizzo, A. M., Adorni, L., Montorfano, G., Rossi, F., and Berra, B. (2007) *Comp. Biochem. Physiol.*, **146**, 94-100.