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Alterations in brain morphology and HSP70 expression in lizard embryos exposed to thermal stress

Palma Simoniello [a], Maria Grazia Esposito [b], Francesca Trinchella [b], Chiara Maria Motta [b], Rosaria Scudiero [b,*]

[a] Department of Sciences and Technology, University of Naples Parthenope, Napoli, Italy
[b] Department of Biology, University of Naples Federico II, via Mezzocannone 8, 80134 Napoli, Italy

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ABSTRACT

The teratogenic effects of thermal stress were studied in the oviparous Italian wall lizard *Podarcis sicula*. To this purpose, the eggs were exposed to continuous or temporary cold (15 °C) or warm (30 °C) stresses and the effects were analysed at the cytological and molecular levels. The results demonstrated the lethality of the continuous regimes and of the warm temporary regime, no matter if given at early (5 days) or late (15 days) stages of development. Temporary cold stress also resulted in lethality, but only if given in the early stage; later, in fact, it resulted in an abnormal development, with marked alterations in the encephalic vesicles, in the eyes and the trunk organs. By *in situ* hybridization, it was demonstrated that these alterations were often correlated with changes in HSP70 expression. In conclusion, our data indicate that *Podarcis* embryos have a limited potential to tolerate thermal changes, especially warm ones. The average predicted temperature increase of 2–4 °C in the next few decades could therefore represent a real threat for lizard populations living in temperate areas.

1. Introduction

Human-induced climate changes are altering the patterns of mean and extreme temperatures across the globe, modifying the biogeographic distributions and abundance of species, especially of the ectothermic ones [1–3].

Among these, reptiles living at high latitudes or altitudes are particularly susceptible: their reproductive strategies in fact have become seasonal [4–6] and require fluctuating temperatures as 'zeitgeber' [7]. The importance of a suitable temperature is demonstrated by the careful selection of the nest-site by a gravid female [6,8,9].

Laboratory-based approaches have attempted to characterize the thermal limits and constraints behind a successful reproductive performance. The experimental results have demonstrated that environmental temperature controls performance-related traits, including morphology and behaviour [10–13]. Therefore, any dramatic thermal fluctuations or periods with extreme temperatures might have serious consequences on the reproductive fitness and, ultimately, on population survival [14–16].

In general, it has been demonstrated that juveniles and young adults display a wider thermal niche than embryos and adult spawners [17]. As an example, for the lizard *Podarcis muralis*, the optimal incubation temperature is 26 °C, and a critical threshold already occurs between 29 and 32 °C [10]. In this species, the low tolerance range is probably due to the effects that temperature changes exert on the egg incubation period and the size, locomotion and growth rate of the hatchling [10,18].

* Corresponding author.
E-mail address: rosaria.scudiero@unina.it (R. Scudiero).

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Although the fundamental role of environmental thermal parameters is known to determine offspring sex, behaviour and fitness in reptiles, little information, if any, is available about the onset of cell and tissue induced by thermal stress.

In this work, we examined the effects of continuous (from oviposition to day 20 post-oviposition [po]) or temporary (5 days from day 5 or 15 po) thermal stresses on the development of the wall lizard *Podarcis sicula*. Either cold (15 °C) or warm (30 °C) stresses were applied; the effects on morphogenesis and on the expression of HSP70 were analysed. HSP70 is a molecular chaperone involved in cellular stress response [19,20], and for this reason it is widely used as a biomarker for assessing thermal stress in a broad range of animals [21], including reptiles [22]. A *P. sicula* HSP70 cDNA fragment was cloned, sequenced and used in *in situ* hybridization analysis.

The results confirmed the influence of thermal stress on embryo development and indicated that HSP70 are involved in the molecular mechanisms activated by cold stress and have therefore an active part in the occurrence of the observed negative effects.

2. Materials and methods

2.1. Ethical procedures

All the procedures were conducted following the Guidelines for Animal Experimentation of the Italian Department of Health and were organized in order to reduce stress and the number of animals used. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II (Permit Number: 2011/0014483).

2.2. Animal housing and egg incubation

Gravid *P. sicula* females (30 specimens) were captured between May and June in the outskirts of Naples. Females were kept in a terrarium maintained under natural conditions of temperature (26 °C by day and 20 °C by night) and photoperiod, in accordance with the institutional guidelines for care and use of laboratory animals. The lizards were fed live mealworms three times a week and water was provided *ad libitum*. Daily check of the terrarium provided freshly laid eggs; each female laid an average of 18 eggs [23]. The eggs were pooled and randomly allocated in seven jars containing uncontaminated natural soil. For thermal treatments, the first two groups of eggs were incubated at constant temperatures of 15 and 30 °C, respectively, since the day of oviposition. The other four groups were exposed to thermal stress for 5 days, in accord to the convention that, for prolonged exposure, a 10% of the organism's life span is significant [24]. In *Podarcis*, embryo development lasts for about 55 days [25,26]. As a consequence, another two groups of eggs were incubated at natural thermal regime for 5 days and then exposed for 5 days at an incubation temperature of 15 °C or 30 °C. The last two groups of eggs were incubated at natural thermal regime for 15 days and then exposed for 5 days at an incubation temperature of 15 °C or 30 °C. Control embryos were incubated at 26 °C (day) and 20 °C (night). For each thermal treatment, 8 eggs were used. Soil water lost as vapour was reintroduced by daily nebulization with distilled water. Eggs were removed from terrariums and washed to remove soil traces. Embryos recovered from shells were checked for vitality (heart beating) or gross morphological alterations, staged [25,26] and processed for cytological investigations.

2.3. Light microscopy

Viable embryos were fixed in Bouin's solution and processed for paraffin wax embedding according to routine protocols [27]. Sections were stained with haematoxylin-eosin to show general morphology [27] or used for *in situ* hybridization analysis.

2.4. Cloning of lizard HSP70

Total RNA from 20-day-old embryos developed under natural conditions was extracted according to the Tri-Reagent (Sigma Aldrich) protocol. The concentration and purity of RNA dissolved in diethylpyrocarbonate (DEPC)-treated water were determined by UV absorbance spectrophotometry; RNA integrity was checked using 1% formaldehyde–agarose gel electrophoresis. An aliquot (5 μg) of this total RNA was reverse-transcribed. Briefly, RNA was denatured by heating at 65 °C for 5 min and then incubated in 20 μL of reverse transcriptase (RT) buffer (50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl) containing 0.5 mM of each deoxyribonucleoside triphosphate [dNTP], 10 mM dithiothreitol [DTT], 20 units of RNase inhibitor (Superase-In, Ambion), 1 mM of oligo(dT)-adaptor primer (5′-CGGAGATCTCCAATGT-GATGGGAAATTC$(T)_{17}$-3′), and 200 units of Superscript II reverse transcriptase enzyme (Life Technology), for 42 °C for 60 min, and successively at 70 °C for 15 min to inactivate the enzyme. The resulting first-strand cDNA was amplified by PCR using forward (5′-AGCCCAAGGTG-CAGGTGGAGTAC-3′) and reverse (5′-ACAGCTCTTTGC-CATTGAAGAA-3′) specific primers for HSP70. The primers were designed on a fragment of the nucleotide sequence of chicken HSP70 available at the EMBL Nucleotide Database. PCR reaction mixture contained 2 μL of first-strand cDNA, 1× PCR buffer [50 mM Tris–HCl, pH 8.3, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$], 0.2 mM dNTPs, 0.4 mM each of specific primers, and 2 units of Taq DNA polymerase (Euroclone). The reactions were carried out in a GeneAmp thermal cycler (Applied Biosystem), with an initial denaturation step at 95 °C for 4 min; 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final elongation step at 72 °C for 7 min. The PCR product was cloned using the StrataClone TA PCR cloning kit (Agilent), and multiple independent clones were sequenced by PRIMM. The identity of the clones was evaluated by matching the sequences to the nucleotide/protein sequences available at the EMBL Database. The *P. sicula* HSP70 mRNA fragment obtained by PCR is available in the GenBank database under the accession number LT219470.

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