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Population origin, development and temperature of development affect the amounts of HSP70, HSP90 and the putative hypoxia-inducible factor in the tadpoles of the common frog *Rana temporaria*

Mikko Nikinmaa[1,*], Lotta Leveelahti[1], Emma Dahl[2], Eeva Rissanen[1], Kalle T. Rytkönen[1] and Anssi Laurila[2]

[1]Centre of Excellence in Evolutionary Genetics and Physiology, Department of Biology, University of Turku, FI-20014 Turku, Finland and [2]Population and Conservation Biology/Department of Ecology and Evolution, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

*Author for correspondence (e-mail: miknik@utu.fi)

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SUMMARY

We raised *Rana temporaria* tadpoles from three different populations from southern, mid and northern Sweden (the total north-to-south distance between populations is approximately 1500 km) at two temperatures, and measured the differences in HSP70, HSP90 and putative HIF-1α levels (*Rana temporaria* HIF-1α was sequenced in the present study) with immunoblotting. The levels of the studied proteins increased with developmental stage. Also, the levels increased with latitude at the lower but not at the higher developmental temperature. This shows that there is a clear difference between the populations at the molecular level but that this difference can be modified by the environmental conditions experienced during development. The proteins analyzed may be involved in the regulation of developmental processes. If this is the case, the tadpoles from the northernmost population have the most advanced complement of regulatory proteins at developmental stages approaching metamorphosis.

Key words: amphibia, development, ecophysiology, population biology.

INTRODUCTION

Intraspecific variation in the physiology, morphology and life history traits of various poikilotherms may occur along climatic gradients (e.g. Spicer and Gaston, 1999; Angilletta et al., 2002; Angilletta et al., 2003; Blanckenhorn and Demont, 2004). More knowledge is needed on the cellular and molecular responses associated with these intraspecific variations, and on the intraspecific variation in general, especially in vertebrates (see Nikinmaa and Waser, 2007). One natural system where the effects of large-scale climatic variation on intraspecific variation can be studied conveniently is the development of the frog *Rana temporaria*. The species ranges from southern Europe to the northernmost parts of Scandinavia. Thus, populations along this latitudinal gradient encounter very different conditions in terms of both mean temperature and season length (Laugen et al., 2003).

This environmental variation has been well utilized in genetic and ecological studies, which have shown genetic differences in the rates of development and growth, and in associated traits such as energetics and behaviour (Merila et al., 2000; Laugen et al., 2003; Lindgren and Laurila, 2005). For example, when developing in the same conditions, the tadpoles from higher latitudes develop and grow faster than those from lower latitudes across a range of temperatures (Merila et al., 2000; Laugen et al., 2003; Lindgren and Laurila, 2005). This suggests that the more stringent time constraints in the north select for increased growth and development (Merila et al., 2000; Laugen et al., 2003; Lindgren and Laurila, 2005). *R. temporaria* tadpoles often develop in small ponds (e.g. Laurila, 1998), where both the temperature and the oxygen levels can vary markedly. In addition, *Rana temporaria* changes from an aquatic and gill-breathing tadpole to a lung-breathing frog at metamorphosis. This transformation is associated with a marked increase in ambient oxygen concentration. Both temperature and oxygen levels may affect regulatory proteins during development.

Although it has long been known that increases in temperature elicit a heat shock response (Feder and Hoffmann, 1999; Sørensen et al., 2003), it has recently become obvious that in poikilothermic vertebrates also a decrease in acclimation temperature can cause an accumulation of heat shock proteins (HSPs) (Rissanen et al., 2006). Furthermore, heat shock proteins and the regulatory protein of oxygen-dependent responses, hypoxia-inducible factor 1α (HIF-1α), interact [as originally described by Katschinski et al. (Katschinski et al., 2002)]. This interaction may be functionally significant in temperature acclimation of poikilothermic animals (Rissanen et al., 2006), since the DNA binding of HIF-1α, which is required for the transcriptional effects of the protein, is increased during acclimation of the crucian carp, *Carassius carassius*, to reduced temperature. Furthermore, the results show coprecipitation of Hsps with HIF-1α in an immunoprecipitation experiment, indicating that the proteins form a complex (Rissanen et al., 2006). Notably, vertebrate studies on Hsps or HIF have not concentrated on the development of specimens from natural populations.

In this regard, the stringent temperature conditions during development of northern *R. temporaria* populations may select for a tighter regulatory system than in more southern populations. If both HSPs and HIF-1α are required in the regulatory pathways of normal development, and their levels increase with the tightness of regulation, one would expect differences between populations, so that the northernmost populations are characterized by the highest levels of the proteins.

Whereas commercial (mammalian) antibodies function reasonably against some frog proteins, such as Hsps, our earlier experience with HIF-1α indicates poor cross-reactivity between

antibodies designed for species from other vertebrate groups. To date, *Rana temporaria* HIF-1α has not been sequenced, but another frog, *Xenopus laevis*, has five allelic variants of the upstream sequence before the HIF-1α (Sipe et al., 2004) gene in the tadpole, suggesting complicated regulation of the transcription factor during development.

In the present study we first cloned and sequenced *Rana temporaria* HIF-1α so that the data could be used to choose the most suitable antibody for later experiments. Second, we raised tadpoles from three different populations from southern, mid and northern Sweden (the total north-to-south distance between populations is approximately 1500 km) at two temperatures, and measured the differences in HSP70, HSP90 and putative HIF-1α levels (at constant total protein level) with immunoblotting followed by image analysis. In the results, we have evaluated the effects of developmental stage (before metamorphosis), temperature and population on the HSP and HIF-1α levels to see if there are population-level or temperature-induced differences in the levels of these regulatory proteins during tadpole development.

MATERIALS AND METHODS

Experimental animals and cultivation procedure

Freshly laid eggs from ten clutches from the following three populations were collected: (1) SK (Skåne) from Tvedöra in southern Sweden (Lund municipality, latitude 55°42′N, longitude 13°26′E; eggs collected on April 18, 2006), (2) UP (Uppland) from Tärnsjö in central Sweden (Heby municipality, 60°11′N, 16°53′E; eggs collected on May 2, 2006) and (3) NO (Norrbotten) from Björkliden in northern Sweden (Kiruna municipality, 68°25′N, 18°38′E; eggs collected on May 24, 2006). The location of the sites is given in Fig. 1.

The eggs were transported to the laboratory in Uppsala. They were evenly distributed in 3 l buckets with reconstituted soft water [RSW (American Public Health Association, 1985)] and kept at 19°C. At Gosner stage 25 [external gills fully absorbed (Gosner, 1960)] 80 individuals were taken at random from each population and placed into the experiment. The tadpoles were placed individually in opaque 1.0 l jars filled with 0.8 l RSW. The tadpoles were reared at two temperatures, 13 and 19°C, with 40 individuals from each population in both treatments. The group maintained at 19°C was placed in a temperature controlled room, and the group maintained at 13°C was placed on a water bath connected to a cooler. The photoperiod used in the experiment was 16 h:8 h light:dark. The water was changed completely every 4 days and the animals were fed *ad libitum* with chopped and lightly boiled spinach.

For molecular analysis, the tadpoles were snap frozen by placing them in test tubes and dipping them in liquid nitrogen, whereafter they were kept at –70°C. For analysis, the animals were sampled at the same developmental stage at both temperatures. Since development at the lower temperature is slower than at the higher temperature, the animals reaching a given developmental stage at the lower temperature were older than at the higher temperature. The whole development from Gosner stage 25 to 42 took approximately 30 days at 19°C (SK 32–36 days, UP 27–31 days and NO 20–24 days) and 60 days at 13°C (SK 55–65 days, UP 60–70 days and NO 48–58 days). Ten tadpoles in every population and treatment were sampled for molecular analysis at Gosner stages 26 (stage 1), 34 (2), 39 (3) and 42 (4) (Gosner, 1960). The analyses given in the Results consider stage 3 and especially 4, since there were fewer samples with adequate levels of antibody-binding protein for the analyses in the earlier developmental stages.

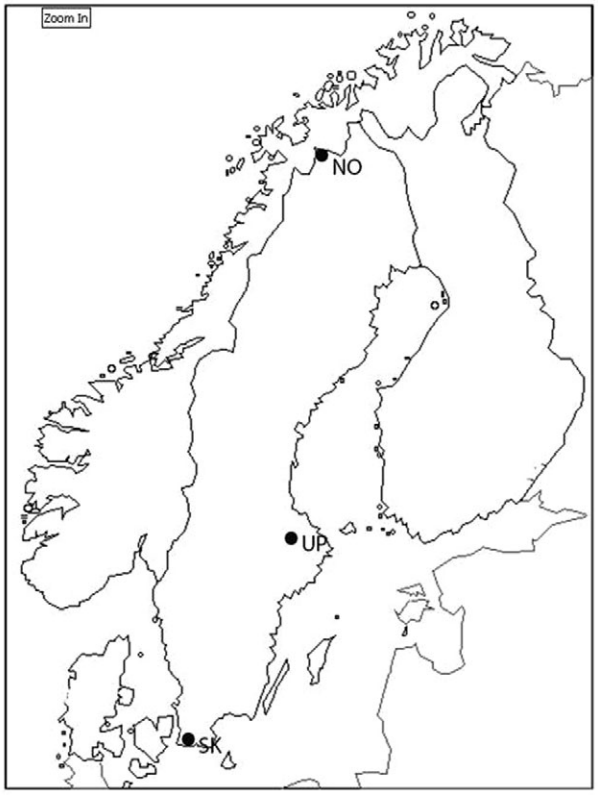

Fig. 1. A map of locations where frog eggs were collected. SK, Skåne; UP, Uppland; NO, Norrbotten.

Cloning and sequencing *Rana temporaria* HIF-1α

RNA extraction, cDNA synthesis and PCR reactions were conducted as described by Rytkonen et al. (Rytkonen et al., 2007) with the TRI reagent (Sigma, St Louis, MI, USA) and the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) from a whole *R. temporaria* tadpole preserved in –70°C. Alignments were conducted with ClustalW Multiple Alignment, version 1.4. (Thompson et al., 1994) and the primers were designed employing the internet-based Primer3 program (Rozen and Skaletsky, 2000). Forward 5′-CAARTCHGCYACVTGGAAGGT-3′ and reverse 5′-CCTCCWSRATGCCACTGAG-3′ universal primers designed from the alignment of HIF-1α sequences from *Homo sapiens* [GenBank: NM_001530], *Xenopus laevis* [GenBank: BC043769], *Danio rerio* [GenBank: AY326951] and *Oncorhynchus mykiss* [GenBank: AF304864] were used to obtain a primary fragment of the gene. Then, after a BLAST verification, the sequence of this primary fragment was used to design *Rana temporaria* HIF-1α-specific primers for 5′-RACE (rapid amplification of cDNA ends) (5′-TGGCGGCTCAGGAAGGTTTTACTGTCCA-3′) and 3′-RACE (5′-CCGCCACAGCCTTGACATGAAGTTTTCC-3′) reactions. The PCR fragments were run in agarose gels and HIF bands were excised and cloned with pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and sequenced using the ABI PRISM™ BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Immunoblot analysis

Nuclear extracts were prepared as described in Soitamo et al. (Soitamo et al., 2001) with some modifications. Protein concentration was determined spectrophotometrically using the Bio-

Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein (25 μg) was separated on 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked for 1 h in 5% nonfat dry milk in PBS with 0.3% Tween 20 at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies and the dilutions used were the following: polyclonal anti-HIF-1α recognizing human HIF-1α amino acids 432-528 (Novus Biologicals, Littleton, CO, USA) 1:1000, anti-HSP70, clone 3A3, raised against human recombinant HSP70 (Affinity BioReagents, Golden, CO, USA) 1:5000 and monoclonal anti-HSP90 against human HSP90 (Stressgen Biotechnologies, Ann Arbor, MI, USA) 1:500. Horseradish peroxidase-conjugated anti-rabbit (HIF-1α), anti-mouse (HSP70) and anti-rat (HSP90) antibodies (Amersham Biosciences, Piscataway, NJ, USA) were used as secondary antibodies. The proteins were detected using enhanced chemiluminescence according to the manufacturer's instructions (enhanced chemiluminescence; Amersham Biosciences). The signals were captured on X-ray film, and the relative optical density of protein bands was quantified with MCID 5+ image analyzer software (InterFocus Imaging, Cambridge, UK). Since gel-to-gel variation may bias western blot quantifications; every effort was made to avoid such bias in the present experiments. First, equal (protein) loading was confirmed by staining gels with Coomassie Brilliant Blue. Second, to account for differences in exposure, the bands obtained on every film were related to the background of the film. Third, the loading order of samples in the gels was randomized. Thus, samples from any group were divided among several gels (and consequently several films), and every gel (and film) contained samples from virtually all groups. Consequently, any differences between groups cannot be caused by gel-to-gel variation.

Statistics

For statistics, SPSS14 software (Chicago, IL, USA) was used. The equality of variances was tested with Levene's test. When two datasets were compared, *t*-test for independent samples was used. When more than two datasets were compared, the comparison consisted of an initial ANOVA (one-way or two-way as appropriate) followed by a *post hoc* LSD test.

RESULTS

Sequence of the hypoxia-inducible factor 1α

The sequence of HIF-1α has been deposited in a public data bank (GenBank: EU262663), and its major features are given in Fig. 2. The predicted *R. temporaria* HIF-1α protein sequence has 80% identity to *Xenopus laevis*, 70% identity to *Gallus gallus*, 65% identity to *Homo sapiens* and 53% identity to *Danio rerio* sequences. The molecule has 806 deduced amino acids. Thus, it is longer than the molecules of teleosts, supporting the notion that the molecules in teleosts have accumulated deletions which do not occur in tetrapods (Rytkonen et al., 2007). Since the molecule does not have major deletions or insertions as compared to the human molecule in the region of amino acids 432–528 (the equivalent sequences for human, *Xenopus* and *Rana temporaria* are given in Fig. 3), we used a polyclonal antibody, made to this amino acid region, to evaluate the changes in the level of the protein during development at the two temperatures. The antibody recognized a protein of the correct size, and thus we call the recognized molecule 'putative HIF'.

The levels of the studied proteins increase with developmental time

With tadpole development, there is an increase in the level of the measured proteins (antibody binding) for a given level of total protein (Fig. 4). It must be noted here that the data used in Fig. 4 contains tadpoles from different locations and temperatures. Since the location affects the levels of all the studied proteins and the temperature of development affects HIF levels (see below), these confounding factors must be considered when interpreting the data. However, the level of antibody-binding protein was higher at stage 4 than at stage 3 in samples from either the same location or temperature. Furthermore, the results in Fig. 4 are for only developmental stages 3 and 4 (Gosner 39 and 42), since no antibody-binding protein was found in samples of earlier

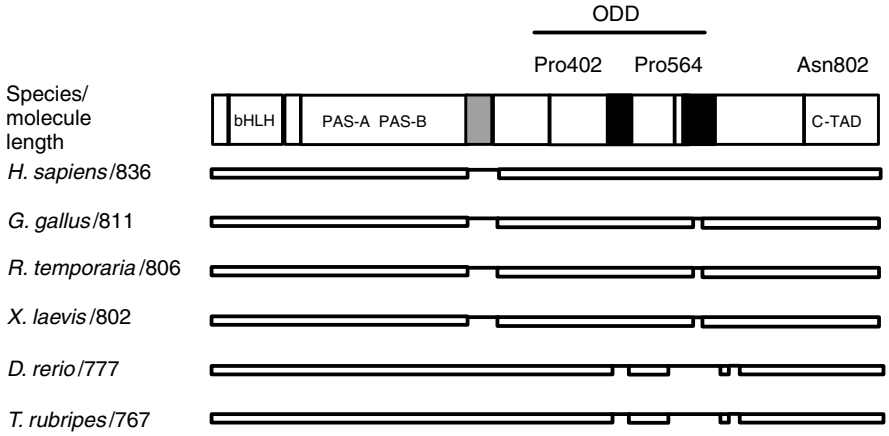

Fig. 2. A schematic representation of HIF-1α protein in *Rana temporaria* and other selected vertebrates. The crucial interaction domains and hydroxylation targets of the molecule are shown. The basic helix-loop-helix (bHLH) and the Per-ARNT-Sim (PAS) domains are involved in DNA binding and dimerization of the protein. The oxygen-dependent degradation (ODD) and the C-terminal transactivation domains (C-TAD) confer oxygen-dependent regulation. The figure also gives the length of the deduced HIF-1α protein and a comparison of its insertion/deletion patterns. In the block diagram, grey represents teleost-specific insertions that are absent from tetrapods. Black represents protein regions that are present in mammalian molecules, but to a variable degree absent from those of other vertebrates. In the schematic representation of the molecules, lines indicate gaps in the alignment. Accession numbers – *Homo sapiens*: GenBank, NM_001530; *Gallus gallus*: GenBank, AB013746; *Rana temporaria*: GenBank, EU262663; *Xenopus laevis*: GenBank: BC043769; *Danio rerio*: GenBank, AY326951; and *Takifugu rubripes*: Ensembl, SINFRUG00000154390.

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                                        432
H.sapiens      417 DTETDDQQLEEVPLYNDVMLPSPNEKLQNINLAMSPLPTAETPK--PLRSSADPALNQEV 474
X.laevis       418 .SDK---PY.D.........H.TSN..E--STPIT...AP.M..--....NV.....R.. 470
R.temporaria   418 .S.S---.YDDA........H.TSKPPE--.ILV....VP.K..SV....N......K.. 472
                                                                        528
H.sapiens      475 ALKLEPNPESLELSFTMPQIQDQTPSPSDGSTRQSSPEPNSPSEYCFYVDSDMVNEFKLE 534
X.laevis       471 VI.M.S...Q...A..I..LSKPD-....I.SS...T...T.-....D...E.AS....D 528
R.temporaria   473 VI.M.CSS.Q.G.A..I..LSKPS-...EI.SN...T..GT.A....DE.INVAP...MD 531
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Fig. 3. The predicted *Homo sapiens*, *Xenopus laevis* and *Rana temporaria* amino acid sequence of the area (amino acid residues 420–530, approximately) to which the antibody used for probing the HIF-1α was made. Arrows (at amino acids 432 and 528, human nomenclature) indicate the exact start and end of the peptide used for preparing the antibody.

developmental stages at either temperature or any location. Of specific note is the increase in the normoxic appearance of putative HIF, since it suggests that the molecule has a role in normal development.

The putative HIF, HSP70 and HSP90 levels increase with increasing latitude at 13° but not at 19°C

Fig. 5 gives the levels of the three measured proteins at developmental stage 4 (Gosner 42) in the different populations at 13°C and 19°C. There was a very significant effect (ANOVA) of latitude on all the proteins at 13°C. Pairwise comparisons with a *post-hoc* test (LSD test) indicated that the Skåne population differed significantly from the other two populations. At 19°C, there was an effect only in HSP90 levels, and the Skåne population again differed from the other two.

The temperature of development had a significant effect on the level of putative HIF, but not on the level of either HSP70 or HSP90

To check for the effect of temperature on the protein levels, the data for the last developmental stage (Gosner 42) were analysed. The results are shown in Fig. 6. The increase in the temperature of development had a significant effect on the level of putative HIF (even when the effect of location was taken into account), whereas neither of the HSPs was significantly affected. The possible effect of oxygen depletion from the jars as a result of oxygen consumed by the tadpoles was not taken into account.

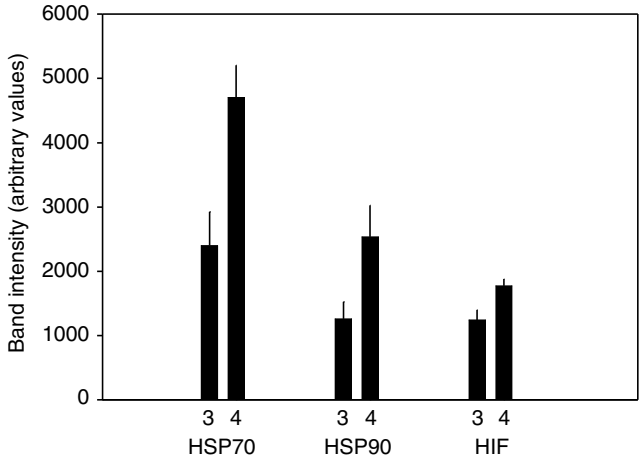

Fig. 4. The levels of HSP70 and HSP90, and putative HIF in the last two developmental stages studied (3, Gosner stage 39; and 4, Gosner 42). For all proteins the level increases significantly (P<0.05) from stage 3 to stage 4. Values are means and s.e.m. (indicated as lines above the bars). The number of tadpoles was seven for developmental stage 3 and ten for stage 4 in the case of HSP70; four and nine, respectively, for HSP90, and five and 37, respectively, in the case of putative HIF.

DISCUSSION

The major finding of the study is that there are clear population-specific differences in the levels of all the molecular markers studied at the end of the tadpole stage, i.e. at a similar developmental stage before metamorphosis, when the animals developed at the lower

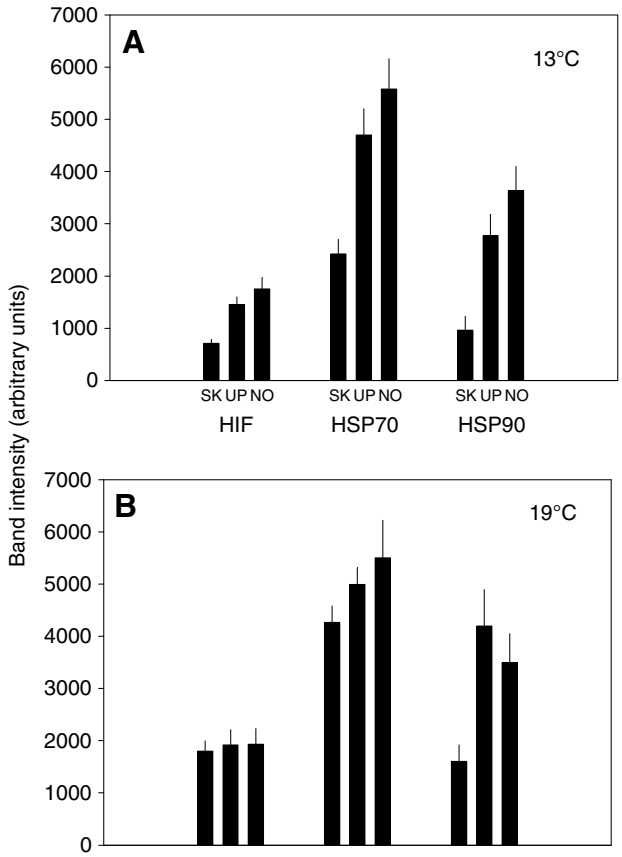

Fig. 5. The levels of the three measured proteins at developmental stage 4 (Gosner 42) in the different populations at 13°C (A) and at 19°C (B). SK denotes the southernmost and NO northernmost population (SK, Skåne; UP, Uppland; NO, Norrbotten). At 13°C for HIF N=7 for SK, 7 for UP, and 8 for NO; for Hsp70, N=9, 8 and 8; and for Hsp90 N=7, 9 and 6. At 19°C N=8, 8 and 7 for HIF; N=7, 10 and 9 for HSP70; and N=8, 7 and 8 for HSP90. There was a significant (P<0.01, ANOVA) effect of latitude on all the proteins at 13°C; pairwise comparisons with a *post-hoc* test (LSD test) indicated that the Skåne population differed significantly (P<0.01) from the other two populations. At 19°C, there was an effect only in HSP90, where the Skåne population again differed (P<0.01) from the other two.

temperature of 13°C, but not when the animals developed at 19°C. For all the molecular markers, the putative HIF, which represents a regulatory transcription factor (Bracken et al., 2003), total HSP70, which represents proteins involved in the control of native protein folding (Li and Mivechi, 1999), and total HSP90, which represents proteins involved in the control of cellular signalling (Neckers et al., 1999) the levels (at a constant amount of total protein) were higher in the tadpoles originating from the northernmost population than in the southern tadpoles. Data on growth and the rate of development have indicated that tadpole development and growth proceed faster in the northern than in the southern populations when tadpoles are raised under the same conditions (Merila et al., 2000; Laugen et al., 2003; Lindgren and Laurila, 2005). The available time for development is shorter at higher than at lower latitudes (Laugen et al., 2003). In the face of this, increased levels of regulatory proteins enable larger flexibility in development, and increase the effectiveness of controlling, for example any temperature-dependent response. The findings of the present study are also compatible with the observation that any genetic differences between populations can be masked by such environmental variables as temperature (Laugen et al., 2003), since we only found a clear population-dependent difference in the protein response in one of the two temperature treatments.

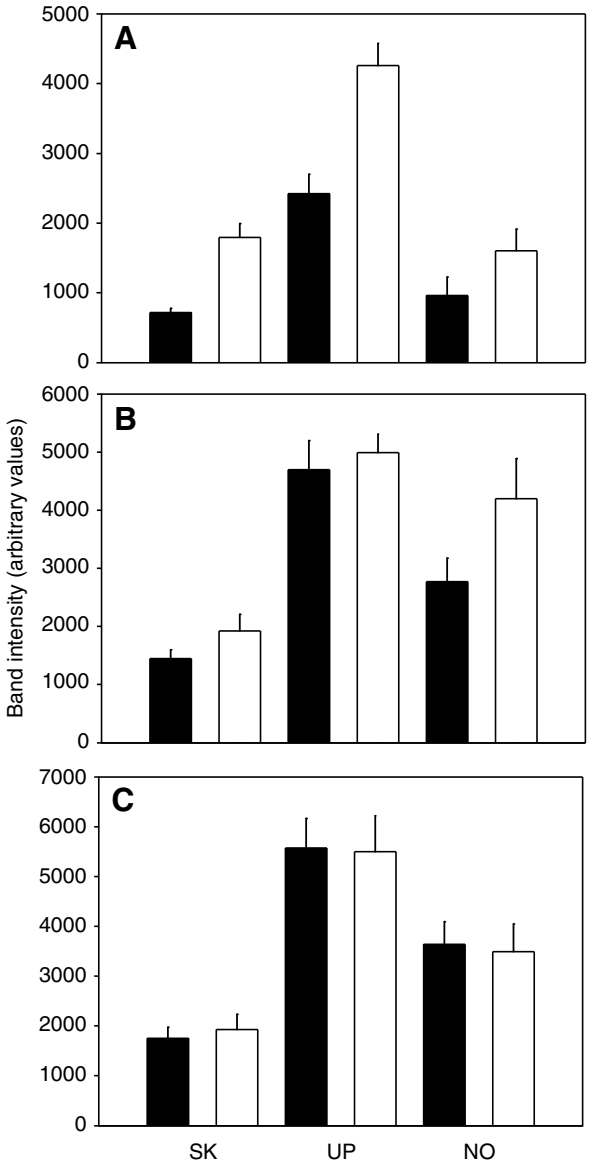

Fig. 6. The effect of rearing temperature on the measured protein levels (as indicated by antibody binding) at stage 4 (Gosner stage 42) for the populations studied. On the basis of two-way ANOVA, the effect of temperature was statistically significant for the putative HIF but not for HSP70 or HSP90 after the effect of sampling location was taken into account. Filled bars indicate 13°C, open bars indicate 19°C. SK, Skåne; UP, Uppland; NO, Norrbotten. (A) HIF. For SK N=7 at 13°C and N=8 at 19°C; for UP N=7 and 8, respectively; for NO N=8 and 7, respectively. (B) HSP70. For SK N=9 at 13°C and N=8 at 19°C; for UP N=8 and 10, respectively; and for NO N=8 and 9, respectively. (C) HSP90. For SK N=7 at 13°C and N=8 at 19°C; for UP N=9 and 7, respectively; and for NO N=6 and 8; respectively. Values are means and s.e.m. (indicated as lines above the bars).

In this study we did not try to separate the constitutive and inducible forms of HSPs. The constitutive functions, e.g. ensuring native protein folding, taking part in transmembrane movements of protein and binding to proteins involved in cellular signalling before activation, of both HSP70 and HSP90 are important. Thus, if these regulatory functions increase with development of tadpoles before metamorphosis, an increase in the total level of HSPs (per unit amount of protein) can be expected. Earlier reports on frogs (*Xenopus*) have indicated that the heat shock response can only be induced after a period of development (Krone and Heikkila, 1988), but we are not aware of published reports indicating an increase in the total HSP70 and HSP90 levels (per unit amount of protein) at temperatures which should not elicit heat shock response. In a way, it is not surprising that there is an increase in HSPs in tadpoles approaching metamorphosis, since metamorphosis is associated with massive restructuring of tissues, during which pronounced apoptosis must also occur (Nakajima et al., 2005). HSPs are intimately involved in the control of apoptosis (Garrido et al., 2001; Beere 2004; Zhang et al., 2006). In this regard it is interesting that there was no significant effect of temperature on the level of either heat shock protein in the last developmental stage studied, showing that, indeed, the temperatures employed did not induce the classical heat shock response, and suggesting that any response observed may be associated with the approaching metamorphosis. Further studies are clearly warranted to evaluate if the increased HSP levels at the developmental stage approaching metamorphosis are due to apoptosis (associated with tissue restructuring) or if they persist even in air-breathing juvenile and adult frogs. Provided that an increase in the level of HSPs indicates advanced development, our results fit with earlier observations of more rapid development of tadpoles from higher latitudes (Merila et al., 2000). Also the increased level of the putative HIF-1α protein in the northernmost population suggests that there is a need for a more effective regulation of transcription, which an increase in the level of, e.g. HIF enables, in an environment that approaches the limit of tolerance of the species.

Although temperature, as such, did not significantly affect the HSP levels, there was a significant increase in the putative HIF-1α level with temperature. As temperature increases, the water oxygen level decreases (Dejours, 1975), and since an increased HIF-1α level is associated with any responses to aquatic hypoxia (Nikinmaa and Rees, 2005), the result is as expected, provided that the putative HIF-1α is, indeed, the regulatory transcription factor involved.

The HIF-1α sequence shows, as expected, a reasonably high similarity to the *Xenopus* sequence. There are, in addition, a couple of important points with regard to the evolution of the transcription factor in vertebrates. First, it appears, as stated by Rytkonen et al. (Rytkonen et al., 2007), that the molecule is longer in tetrapods than

in teleost fish. Second, all vertebrates, apart from mammals, appear to have a short deletion after proline564 (human nomenclature). The amino acid is involved in the oxygen-dependent regulation of the stability of the molecule. Earlier studies on *Xenopus* have indicated that the molecule is transcriptionally regulated during the development of tadpoles (Sipe et al., 2004). This transcriptional regulation may be under the influence of environmental and genetic factors, since the apparent regulatory region preceding the transcribed gene shows marked allelic variation in *Xenopus* (Sipe et al., 2004). At present, it is not known if similar variation occurs in the control regions of the *Rana* HIF-1α gene, if different populations show differences in the control sequences, and if the different control sequences cause the transcription of the gene to be different under different environmental conditions. However, the studies of Sipe et al. (Sipe et al., 2004) on *Xenopus laevis*, those of Vuori et al. (Vuori et al., 2004) on *Salmo salar*, and the present one on *Rana temporaria* all indicate that there is clear normoxic appearance of HIF-1α during development, indicating the importance of this transcription factor in normal vertebrate development.

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REFERENCES

American Public Health Association (1985). *Standard Methods for the Examination of Water and Wastewater* (16th edn). Washington: American Public Health Association.

Angilletta, M. J., Niewiarowski, P. H. and Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *J. Therm. Biol.* **27**, 249-268.

Angilletta, M. J., Wilson, R. S., Navas, C. A. and James, R. S. (2003). Tradeoffs and the evolution of thermal reaction norms. *Trends Ecol. Evol.* **18**, 234-240.

Beere, H. M. (2004). 'The stress of ding': the role of heat shock proteins in the regulation of apoptosis. *J. Cell Sci.* **117**, 2641-2651.

Blanckenhorn, W. U. and Demont, M. (2004). Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? *Integr. Comp. Biol.* **44**, 413-424.

Bracken, C. P., Whitelaw, M. L. and Peet, D. J. (2003). The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell. Mol. Life Sci.* **60**, 1376-1393.

Dejours, P. (1975). *Principles of Comparative Respiratory Physiology.* Amsterdam: North-Holland.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.

Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. (2001). Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.* **286**, 433-442.

Gosner, K. N. (1960). A sinplified table for staging anuran embryos and larvae with notes of identification. *Herpetologica* **16**, 183-190.

Katschinski, D. M., Le, L., Heinrich, D., Wagner, K. F., Hofer, T., Schindler, S. G. and Wenger, R. H. (2002). Heat induction of the unphosphorylated form of hypoxia-inducible factor-1 alpha is dependent on heat shock protein-90 activity. *J. Biol. Chem.* **277**, 9262-9267.

Krone, P. H. and Heikkila, J. J. (1988). Analysis of hsp 30, hsp 70 and ubiquitin gene expression in *Xenopus laevis* tadpoles. *Development* **103**, 59-67.

Laugen, A. T., Laurila, A., Rasanen, K. and Merila, J. (2003). Latitudinal countergradient variation in the common frog (*Rana temporaria*) development rates – evidence for local adaptation. *J. Evol. Biol.* **16**, 996-1005.

Laurila, A. (1998). Breeding habitat selection and larval performance of two anurans in freshwater rock-pools. *Ecography* **21**, 484-494.

Li, G. C. and Mivechi, N. F. (1999). Heat shock protein 70. In *Stress Proteins* (ed. D. S. Latchman), pp. 43-68. Berlin, Heidelberg: Springer.

Lindgren, B. and Laurila, A. (2005). Proximate causes of adaptive growth rates: growth efficiency variation among latitudinal populations of *Rana temporaria*. *J. Evol. Biol.* **18**, 820-828.

Merila, J., Laurila, A., Laugen, A. T., Rasanen, K. and Pahkala, M. (2000). Plasticity in age and size at metamorphosis in *Rana temporaria* – comparison of high and low latitude populations. *Ecography* **23**, 457-465.

Nakajima, K., Fujimoto, K. and Yaoita, Y. (2005). Programmed cell death during amphibian metamorphosis. *Semin. Cell Dev. Biol.* **16**, 271-280.

Neckers, L., Mimnaugh, E. and Schulte, T. W. (1999). The Hsp90 chaperone family. In *Stress Proteins* (ed. D. S. Latchman), pp. 9-42. Berlin, Heidelberg: Springer.

Nikinmaa, M. and Rees, B. B. (2005). Oxygen-dependent gene expression in fishes. *Am. J. Physiol.* **288**, R1079-R1090.

Nikinmaa, M. and Waser, W. (2007). Molecular and cellular studies in evolutionary physiology of natural vertebrate populations: influences of individual variation and genetic components on sampling and measurements. *J. Exp. Biol.* **210**, 1847-1857.

Rissanen, E., Tranberg, H. K., Sollid, J., Nilsson, G. E. and Nikinmaa, M. (2006). Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*). *J. Exp. Biol.* **209**, 994-1003.

Rozen, S. and Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (ed. S. Krawetz and S. Misener), pp. 365-386. Totowa, NJ: Humana Press.

Rytkonen, K. T., Vuori, K. A. M., Primmer, C. R. and Nikinmaa, M. (2007). Comparison of hypoxia-inducible factor-1 alpha in hypoxia-sensitive and hypoxia-tolerant fish species. *Comp. Biochem. Physiol.* **2D**, 177-186.

Sipe, C. W., Gruber, E. J. and Saha, M. S. (2004). Short upstream region drives dynamic expression of hypoxia-inducible factor 1 alpha during Xenopus development. *Dev. Dyn.* **230**, 229-238.

Soitamo, A., Råbergh, C. M. I., Gassmann, M., Sistonen, L. and Nikinmaa, M. (2001). Characterization of a Hypoxia-Inducible Factor (HIF-1α) from rainbow trout: accumulation of protein occurs at normal venous oxygen tension. *J. Biol. Chem.* **276**, 19699-19705.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2005). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037.

Spicer, J. I. and Gaston, K. J. (1999). *Physiological Diversity and its Ecological Implications*. Oxford: Blackwell.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). Clustal-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Vuori, K. A. M., Soitamo, A., Vuorinen, P. J. and Nikinmaa, M. (2004). Baltic salmon (*Salmo salar*) yolk-sac fry mortality is associated with disturbances in the function of hypoxia-inducible transcription factor (HIF-1α) and consecutive gene expression. *Aquat. Toxicol.* **68**, 301-313.

Zhang, M. H., Lee, J. S., Kim, H. J., Jin, D. I., Kim, J. I., Lee, K. J. and Seo, J. S. (2006). HSP90 protects apoptotic cleavage of vimentin in geldanamycin-induced apoptosis. *Mol. Cell. Biochem.* **281**, 111-121.