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ORIGINAL PAPER

Population variability in heat shock proteins among three Antarctic penguin species

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Abstract Heat shock proteins (HSPs) are synthesised under stressful conditions such as exposure to elevated temperatures, contamination, free radicals, UV light or pathophysiological states resulting from parasites and/or pathogens. HSPs function to protect cells by means of modulation of protein folding. In Antarctica, these proteins have been studied in such organisms as protozoa and fishes, without attention to geographical variation. We studied the variation of HSP70 and HSP60 levels in Gentoo, Adelie and Chinstrap penguins among different populations along the Antarctic Peninsula from King George Island (62°15′S) to Avian Island (67°46′S). Our results show that the northern population of Gentoo penguin showed higher levels of HSP70 and HSP60 than the southern population. High temperature, human impact and immunity as a proxy for parasites and diseases in northern locations could explain such variation. Adelie penguin only showed significant geographical variation in HSP70, increasing north to south, a pattern perhaps related to increased UV radiation and decreased temperatures from north to south. Chinstrap penguin shows no population differences in the variation in neither HSP70 nor HSP60, although HSP70 showed marginally significant differences. Sexual differences in the level of these proteins are also discussed.

Keywords Antarctica · Ecophysiology · Environmental gradient · Heat shock protein · *Pygoscelis adeliae* · *Pygoscelis antarctica* · *Pygoscelis papua* · Stress

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Introduction

Heat shock proteins (HSPs) are synthesised by cells under a variety of stressful conditions. These proteins were discovered by Ritossa (1962) as a response to severe heat shock in *Drosophila*, giving this compound its name. HSPs have now been found in many organisms from bacteria to plants and animals (Morimoto 1991) and are highly conserved from an evolutionary standpoint (Schlesinger 1990). This suggests their importance to cells as a protection against stress (Linquist 1986). These proteins are part of the Protein Quality System (PQS), which is involved in protein quality control operating to maintain the homeostasis under normal cellular conditions. The function of this system is both to secure correct folding of proteins and to assist in degradation of denatured or aggregated proteins. HSPs function as molecular chaperones that provide an environment in which protein that have folded incorrectly due to stress can be properly folded (Parsell and Lindquist 1993; Gregersen et al. 2001). There are several kinds of HSPs that can be classified by molecular weight into five major groups, HSP100 (100–105 kDa), HSP90 (82–90 kDa), HSP70 (68–75 kDa), HSP60 (58–65 kDa) and the small HSP group (15–30 kDa). Among them, the more commonly studied are HSP70 and HSP60 (e.g. Fader et al. 1994; Merino et al. 1998; Carey et al. 1999; Morales et al.

2004). A high number of stressors promoting the expression of HSPs have been described as, for example, high temperature, UV radiation, heavy metals, parasitism or bacterial and viral infection (Collins and Hightower 1982; Trautinger et al. 1996; Werner and Nagel 1997; Merino et al. 1998; see review in Sorensen et al. 2003).

Studies on HSPs, mainly HSP70, in Antarctic organisms include algae (Vayda and Yuan 1994), protozoa (La Terza et al. 2001) and fish (Hofmann et al. 2000; Place and Hofmann 2005). For example, Antarctic algae expressed HSPs in response to temperature variation (Vayda and Yuan 1994), while some fishes like *Trematomus bernacchii* lack the ability to up-regulate HSP70 due to a mutation in the HSF1promotor (Buckley et al. 2004). This is likely due to the absence of positive selection during evolution at stable subzero temperatures.

Variation in the levels of HSPs among different populations of the same organism has not been addressed in Antarctica. Here, we report results of a study of such variation in different populations of three species of penguins along the Antarctic Peninsula. The latter region has been showing strong latitudinal changes in several factors, which could influence HSP levels (see Sorensen et al. 2003), such as variation in temperature (Turner et al. 2004), UV radiation (Madronich et al. 1994), contamination and human pressure (Hofman and Jatko 2001; Bargagli 2005). Moreover, a latitudinal variation in immunological parameters has been reported in this area, a pattern perhaps indicating latitudinal variation in infection by parasites, pathogens or diseases (Barbosa et al. 2007). The Antarctic Peninsula has also experienced faster and higher temperature change than elsewhere in the world (King et al. 2003). Moreover, a delay in ozone hole recovery is predicted (Shindell and Grewe 2002); contamination levels (Bargagli 2005) and a change in the range, abundance and virulence of parasites is predicted under a scenario of temperature increase (Sutherst 2001).

Our aim was to study variation on HSP levels in several populations of three penguin species along the Antarctic Peninsula to establish a baseline for future comparisons.

Materials and methods

The study was carried out in several locations on islands along the west coast of the Antarctic Peninsula (see Table 1).

Three species of pygoscelid penguins were studied: chinstrap penguin (*Pygoscelis antarctica*), gentoo penguin (*Pygoscelis papua*) and adélie penguin (*Pygoscelis adeliae*). Chinstrap penguins range from 56° to 65°S, gentoo penguin from 46° to 65°S and adélie penguins from 54° to 77°S (Williams 1995). Therefore, our study covers the intermediate part of the adélie penguin range, and the southern part of the ranges of chinstrap and gentoo penguins.

During January and February 2003, we visited several penguin breeding localities along the Antarctic Peninsula region (Table 1). Adult penguins were captured on shore in order to minimise disturbance in the breeding colonies. To make comparisons among different localities, adults were chosen instead of chicks due to likely differences in chick development when sampling was done. Anyway, we sampled the penguin populations when chicks were in guard phase, thus precluding the likely effect on variation by the breeding period.

From each individual, we measured body mass and took a blood sample from the foot vein using a needle and a heparinised capillary tube. Blood was later centrifuged at 12,000 rpm for 10 min to separate plasma from red blood cells. Hematocrit or packed cell volume was measured before separation of plasma and cell fractions. After centrifugation, cell fraction was frozen for subsequent analyses.

Heat shock protein determination was carried out from the blood cellular fraction by means of the Western blot technique using the same amount of protein for all individuals. Samples of soluble proteins (70 μg/well) were separated by SDS-PAGE; this amount of total protein is in the linear range of the antibody-antigen response for the species and antibodies studied (Fig. 1a, b). The primary monoclonal antibodies used were anti-HSP70 (clone BRM22, Sigma H-5147) diluted 1/5,000 and anti-HSP60 (clone LK2, Sigma H-3524) diluted 1/1,000. These antibodies

Table 1 Localities, species studied and sample sizes

Localities		Species	Sample size	Region
Point Thomas (King George I.)	62°10′S 58°29′W	*P. adeliae P. papua*	25 (9F, 4M) 10 (7F, 3M)	1
Miers Bluff (Livingston I.)	62°43′S 60°26′W	P. antarctica	25 (6F, 17M)	1
Baily Head (Deception I.)	62°58′S 60°30′W	P. antarctica	25 (10F, 13M)	1
George Point (Ronge I.)	64°40′S 60°40′W	*P. Antarctica P. papua*	25 (10F, 10M) 25 (11F, 9M)	2
Torgersen I.	64°46′S 64°04′W	P. adeliae	25 (5F, 18M)	2
Avian I.	67°46′S 68°43′W	P. adeliae	25 (5F, 15M)	3

F females, *M* males

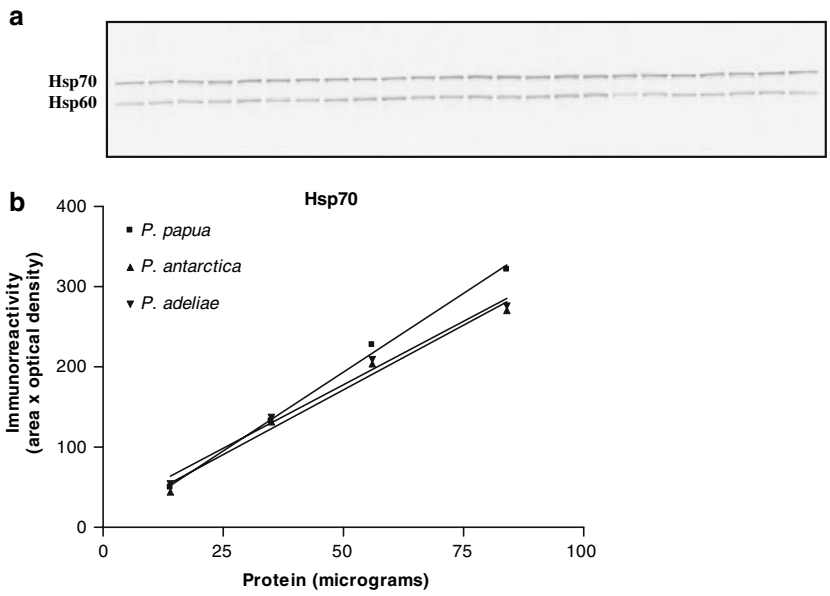

Fig. 1 **a** Gel/Blot set showing equal amounts of proteins loaded per lane. **b** Correlation between observed HSP bands and the arbitrary measures of optical density per area in the three studied species

react specifically with HSP70 and HSP60, respectively, as shown by the immunoreactive bands of appropriate molecular weights obtained. These antibodies recognise both constitutive and inducible forms of the HSPs under study. The peroxidase-conjugated secondary antibody was goat anti-mouse specific for the Fc region (Sigma A-0168) at 1/6,000 dilution. Immuno-reactivity of blots was measured by means of densitometric quantification using a digital image system (Scion Image for Windows, Scion Corporation, Frederick, MD, USA). Results are expressed as arbitrary measures of optical density per area (OD/area) (see details in Moreno et al. 2002).

To account for potential sexual variation in the level of HSPs within each species, we sexed the individuals by means of molecular markers (Ellegren 1996). In the case of the adélie penguin, we used a PCR-RFLP method (Boutette et al. 2002).

Data were analysed with generalised linear models (GLM) with region and sex as factors and body mass and haematocrit as covariates. We used backwards stepwise selection procedures to determine which variables accounted best for variation in the dependent variable. The criterion to remove a variable was set a $P = 0.05$. All means are expressed ±SE.

Results

The HSP70 and HSP60 were detected in the three species of penguins studied and showed geographical differences in their levels. Levels of HSP70 showed differences in both adélie penguin ($F_{2,62} = 5.25$, $P = 0.007$; Fig. 2a) and gentoo penguin ($F_{1,20} = 30.58$, $P < 0.0001$; Fig. 2b), but not the Chinstrap penguin ($F_{2,59} = 2.47$, $P = 0.09$). The pattern of HSP70 variation was different for each species. Among adélie penguins HSP70 increased from north to south, with the southern-most population (Avian Island) showing the highest level. However, gentoo penguin showed the opposite pattern with higher levels in the northern-most population (King George Island). Gentoo penguin showed marginally significant sexual differences in HSP70 ($F_{1,19} = 3.51$, $P = 0.07$), with males showing higher values (172.00 ± 4.86) than females (160 ± 3.62). Otherwise, no statistically significant difference in HSP70 was evident between sexes. Body mass and haematocrit did not explain a significant percentage of the variation found in HSP70 level ($P > 0.05$, results not shown).

With respect to HSP60, only gentoo penguin populations showed significant variation in the level of this protein ($F_{1,20} = 6.4$, $P = 0.01$; Fig. 2c), with higher levels in the northern population. Sex accounted for a significant variation of HSP60 in chinstrap penguins ($F_{1,60} = 5.03$, $P = 0.028$); females (111.07 ± 4.49) showed higher values of this protein than males (98.19 ± 3.57). For gentoo penguins, males exhibited marginally higher HSP60 levels than females ($F_{1,19} = 4.10$, $P = 0.056$; males = 125.45 ± 11.72, females = 95.79 ± 8.75). Body mass and haematocrit did not show any significant relationship with HSP60 levels in any species ($P > 0.05$ results not shown).

We also compared levels of HSP70 and HSP60 between species in the same locality as it is expected that the environment acts similarly on them: adélie versus gentoo penguin at Point Thomas and chinstrap versus gentoo penguin at George Point (Ronge Island). In the first case, we found no significant differences between adélie and gentoo penguins in either HSP70 nor HSP60 ($F_{1,39} = 1.40$, $P = 0.24$, $F_{1,39} = 1.36$, $P = 0.25$, respectively). However, in the second, we found significant differences in levels of both HSP70 and

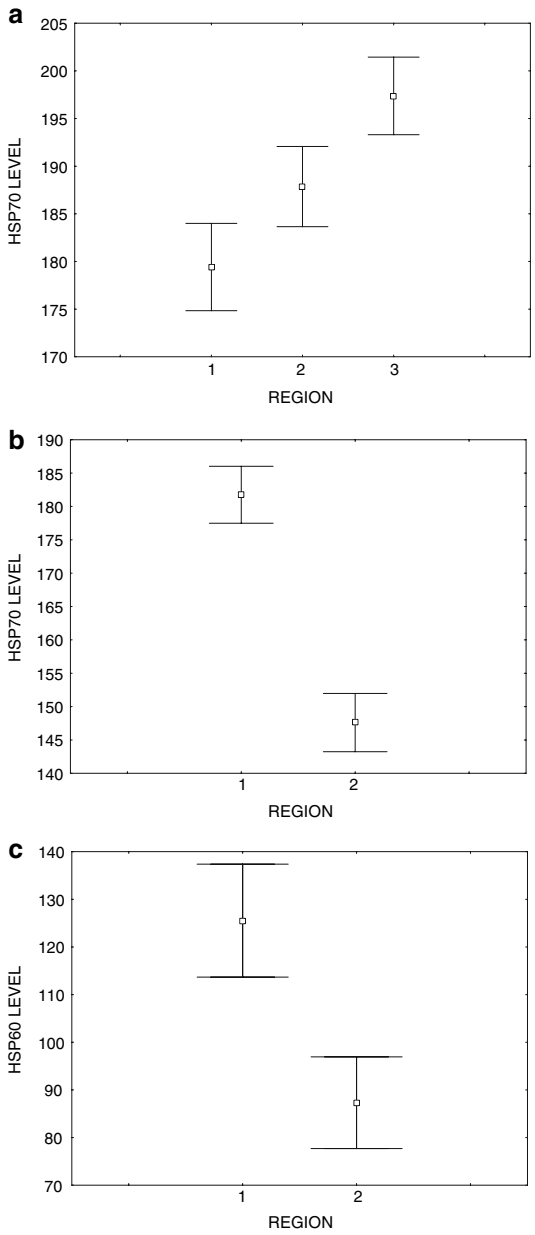

Fig. 2 **a** Geographical differences in HSP70 levels in the adélie penguin. **b** Geographical differences in HSP70 levels in the gentoo penguin. **c** Geographical differences in HSP60 levels in the gentoo penguin

HSP60 ($F_{1,27} = 8.25$, $P = 0.007$, $F_{1,27} = 5.68$, $P = 0.02$, respectively) between the chinstrap and gentoo in both HSP70 (chinstrap = 170.68 ± 4.76, gentoo = 147.60 ± 5.99) and HSP60 (chinstrap = 110,57 ± 5.58, gentoo = 87.31 ± 7.02).

Discussion

Our results revealed: (1) no general pattern of geographical variation in levels of HSPs among the three species of penguins; (2) population differences, south to north, in HSP70 and HSP60 levels of gentoos; (3) a significant population differences, north to south, variation in HSP70 but not HSP60 in adélies; and no pattern in the chinstrap penguin.

Although, interspecific differences on HSP levels should be taken cautiously as each species may show differences in the affinity to the monoclonal antibody used to detect HSPs, the comparison of the levels of HSP70 and HSP60 between chinstrap versus gentoo penguin in George Point showed a significant difference. As the same environment should act on all penguins present in the same location, this result shows that some species-specific differences exist in the level of HSPs. Differences in the stress threshold among penguin species might well be involved. Some authors have found similar results comparing different species of Antarctic and non-Antarctic notothenioid fishes (Carpenter and Hofmann 2000).

Heat shock proteins respond to a wide variety of environmental factors, from UV radiation (Trautinger et al. 1996), contaminants (Werner and Nagel 1997), temperature (Sonna et al. 2002), bacterial and virus infection (Collins and Hightower 1982), and parasitism (Merino et al. 1998), among others. Some of these factors vary by latitude in Antarctic Peninsula region (Table 2). Temperature (Turner et al. 2004), human activities that can promote the increase of contamination levels such as the establishment of scientific bases and tourism (Hofman and Jatko 2001; Bargagli 2005), and levels of immunity that can be explained by the presence of parasites or pathogens are higher in northern locations (see Gardner et al. 1997; Kerry et al. 1999; Gauthier-Clerc et al. 2002; Barbosa et al. 2007). Such variation could explain the intraspecific differences found in HSP70 and HSP60 in the gentoo penguin. Although all the species should be affected by these factors as well, species-specific differences in the stress threshold as suggested above could explain such results.

The pattern of latitudinal variation found in the adélie penguin, opposite that of the gentoo, could be related to changes of UV radiation, although nothing is known about the direct effects of UV radiation on penguins (Karentz and Bosch 2001). On the other hand, temperature decreases from north to south, and low temperatures can increase the levels of HSPs (Martinez et al. 2001) even in homeotherms

Table 2 Changes in environmental factors in the different regions studied (see Table 1)

Region	Temperature	Human impact	Parasites and/or diseases[a]	UV radiation
1	++	++	++	–
2	+	++	+	+
3	–	–	–	++

[a] Suggested by variation in immunoglobulin levels (Barbosa et al. 2007)

(Sonna et al. 2002). It is possible that we detected an effect of HSP70 in its role of avoiding protein denaturation in response to cold (Sonna et al. 2002; Place and Hofmann 2005). However, if this explains variation in HSP in adélie penguins, why was there no similar pattern among the other species? Perhaps such a difference in adélie penguins could be related to the lower temperatures it experiences in its more southern distribution. Specific differences in response to cold stress have been found in other organisms as well (Bosch et al. 1988; Sanders et al. 1991).

Only the chinstrap penguin showed significant differences in HSP60 levels between sexes, with higher levels being found for females; sexual differences among gentoo penguins were the opposite for both the HSP70 and HSP60. Sexual differences in HSP60 levels have also been found in other birds such as barn swallows (Merino et al. 2002). These differences could be linked to levels of stress experienced by these birds (Merino et al. 2002). For example, sexual differences are apparent in the susceptibility of males to the effect of parasites or the differential exposure to pathogens (Zuk and McKean 1996), with the differences operating in either direction with respect to contaminants depending on circumstances (Mateo and Guitart 2003; Taggart et al. 2006). Obviously, additional investigation is needed.

Climate change in the Antarctic Peninsula is a plausible scenario (King et al. 2003). Considering the interactions between climate change and environmental factors affecting the level of HSPs, our results on the variation of HSPs in penguins should be considered as a baseline for future comparisons under a scenario of temperature increase in the area.

In summary, our results show for the first time the detection of HSPs in Antarctic penguins. We also show that HSP level varies geographically within species in this continent although in different ways in the three species.

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