Does Environmentally Contingent Variation in the Level of Molecular Chaperones Mirror a Biochemical Adaptation to Abiotic Stress?

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1. Introduction

As Hochachka and Somero emphasized in their seminal book "Biochemical Adaptation: Mechanism and Process in Physiological Evolution", the key question to be posed in the study of biochemical adaptation is: "How have living systems, which are based on a common set of biochemical structures and processes and subject to a common set of physical-chemical lows, been able to adapt to the enormously wide spectrum of environmental conditions found in the biosphere?" (Hochachka & Somero, 2002). Given that the biosphere encompasses habitats with tremendously diverse combinations of physical, chemical, and biotic environmental factors, it seems reasonably to believe that the diversity of life forms that are observable in these habitats is the outcome of *adaptations* which have evolved to permit organisms the exploitation of nearly all land and water areas around the globe (Hochachka & Somero, 2002).

1.1 Conceptual approaches to study adaptation

Evolutionary biologists are well conscious that the term adaptation refers to both a process and its product; however, there still exists the controversy about the perception of that biological phenomenon. Amudson pointed out that "Natural selection, the mechanism universally regarded as the primary causal influence on phenotypic evolutionary change, is first and foremost an explanation of adaptation"(Amudson, 1996). In this context, adaptation is a process, which creates the *generic state* of adaptedness, as well as *specific traits* that - because arising by adaptive modifications - are referred as adaptations. However, irrespective of being a process, a generic state, or an individual trait, adaptation is a relational concept. Thus, if it is viewed as a process, adaptation signifies how an entity fits to the other one. The generic state reflects the functional efficiency of a given trait in an environment adapted to it, whereas *an* adaptation is a modified part of an organism that fulfills a biological function for that organism. Notably, in all of these definitions, natural selection is the principal mechanism that simultaneously generates both adaptation and adaptation(s) (Amudson, 1996).

Gould & Vrba (1982) called attention to the presence of another two distinct concepts of adaptation, one designated as *historical* and one as *nonhistorical*. According to the historical

definition of adaptation, an existing trait is an adaptation for a current function only if it was *improved by natural selection* for that function. On the nonhistorical definition, however, a trait is an adaptation just in the case if it *provides the current fitness benefits.* As proponents of the historical concept of adaptation, they introduced the term "aptation" to modify the nonhistorical definition of adaptation. Thus, aptation is a trait that confers fitness advantage irrespective of its origin. They also contrasted the concept of adaptation with the term "exaptations", which refers to aptations that are nonadaptations. In other words, exaptations are traits currently showing beneficial effects for which they were not selected.

Vermeij (1996) advocated a *comparative* concept of adaptation, defining adaptation as a "heritable attribute of an entity that confers advantages in survival and reproduction of that entity in a given environment" (Vermeij, 1996). His definition implicates the superiority of one trait over the alternative ones. Yet, since its advantage is context dependent, it implies that under different environmental conditions an alternative trait will be adaptive. As was previously pointed out, the term adaptation refers to both the beneficial trait and the process that generates and maintains it. Vermeij's concept of adaptation assumes that *adaptive traits have to be heritable*, as well as that *there must be a selective factor*, which discriminates among heritable alternatives, favoring those with greater reproduction or survival. At the organismal level, inheritance occurs by means of transmission of nuclear genes, although maternal effects, repeated infection, etc. may influence it. Since the mechanisms of inheritance in ancestral organisms cannot be precisely determined, Vermeij (1996) has recommended to make a difference between *functional benefit* or adaptation and the mechanism of *transmission* of the trait in question. Effectively, there are two possibilities for doing such comparisons. One of them is to contrast co-occurring individuals that bear a hypothetical adaptation with those individuals that possess alternative traits. Accordingly, any difference in the performance among compared individuals is the evidence of the presence or the absence of that adaptation. However, when some traits influence the fitness of populations, species or higher clades, it is better to compare the sister-groups instead of co-occurring individuals (Lauder, 1981).

1.1.1 Biochemical adaptation

Any biological entity that satisfies three necessary conditions for natural selection to operate: has the ability to vary, has continuity (heritability), and differs in its success relative to another co-occurring entity, can be adapted and can produce adaptations (Frank, 1996; Vermeij, 1996). In line with this statement, a frequently addressed question is whether the amazing adaptive diversity discovered in morphology, habitat preferences, mode of life and other attributes of contemporary organisms is comparable with the degree of their adaptive differentiation at the biochemical level. Unfortunately, the literature data indicate that the answer to that question is ambivalent (i.e., a mixture of "yes" and "no").

Because natural habitats occupied by different life forms exhibit a great diversity in physicochemical parameters, biochemical systems must confront with numerous environmental challenges during the process of adaptation. All of these challenges are focused exclusively to the two most important sources: (i) the essential biochemical constituents of every organism, including nucleic acids, enzymatic and structural proteins, and lipoprotein structures, and (ii) the competence of cells to maintain an adequate level of energy turnover to sustain life. Given that the core biochemical structures (and the interactions among them) are highly susceptible to direct perturbations from external

physical and chemical factors, each of them must remain a delicate balance between stability and instability to preserve its functional uniqueness, even in the face of environmental forces that may danger their integrity. However, many environmental factors can damage the cell indirectly by influencing its ability to maintain sufficient rates of energy turnover. In such cases, cells can modulate activities of the existing biochemical systems by redirecting metabolic flux in the manner that compensates for particular environmental insult (Hochachka & Somero, 2002). The unity of cellular biochemical design was found in all organisms and in all environments, presumably as the result of adaptive processes that allow conservation of the core set of structures and processes that form their biochemical architecture. Moreover, there is consensus that genes encoding the core components of cellular function and structure are highly conserved in most species as well, including genes for direct sensing the extracellular environmental changes, and those ones for transdusing these informations to corresponding intraracellular targets. However, the latter kind of genes is fundamental for the evolution of physiological diversity and species specificity. In this context, Hochachka and Somero pointed out "Underlying biochemical unity is preserved at the same time that diversity is generated"(Hochachka & Somero, 2002).

One of the classical examples of biochemical adaptation defined in the restrictive sense of the word (Gould & Vrba, 1982) is molecular chaperones. Molecular chaperones are a highly conserved group of proteins that occur universally in all prokaryotes and eukaryotes (Pearl & Prodromou, 2006). Chaperones assist in various cellular functions, such as *de novo* folding, refolding of stress-inactivated proteins, oligomeric assembly, protein transport within cell and proteolityc degradation, but without becoming part of a protein final structure (Frydman, 2001; Hartl & Hayer-Hartl, 2009; Rutheford, 2003; Wegele et al., 2004). Although essential under physiological conditions, protein chaperones are indispensable for survival under environmental conditions that interfere with protein folding, including extreme temperature or other environmental stresses (Frydman, 2001). Importantly, protein-folding chaperones do not modify genotype; instead, they influence "target-protein activity by allowing structurally unstable mutant protein sequence to fold into active configuration" (Rutherford, 2003). Because highly induced under conditions of conformational stress, a large number of structurally unrelated chaperones are denoted as stress proteins or "heat shock proteins" (Parsell & Lindquist, 1993); that is, in the case when the amount of aggregation-prone folding intermediates amplifies in the cell (Hartl & Hayer-Hartl, 2009). Molecular chaperones form different families according to their molecular weight (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and so-called small Hsp proteins). Since their current beneficial properties in all organisms are very likely the same as the benefits that initially favored their evolution, there is no doubt that protein-folding chaperones arose and are maintained by natural selection (Feder & Hofmann, 1999). Here, we test this statement by exploring spatial and temporal variation in the cellular level of the two heat shock proteins, Hsp70 and Hsp90, which exhibit chaperone properties.

The 70-kDa heat shock proteins (Hsp70s) are a large family of chaperones that plays diverse role in the cell. In addition to the folding of *de novo* translated and recovery of stress-denatured proteins, they assist the post-translational unfolding and translocation of nuclear-encoded proteins through the lipid bilayers of organelles (Bukau & Horwich, 1998; Rutheford, 2003). The Hsp70 proteins are present in all eukaryotic cells, in eubacteria, as well as in many archaea (Macario et al., 1999). Recently, Albanèse at al. (2006) have applied a system biology approach that combines genomic and functional analyses to examine

whether there is a difference between chaperone-mediated *de novo* folding and polypeptide refolding following stress, in yeast *Saccharomyces cerevisiae*. Their global analyses, together with previous studies investigating the functional organization of individual cytosolic chaperones, suggest that the eukaryotic Hsp70 chaperone machinery consists of two robust networks with highly specialized functions. One of them, denoted as the Hsp network, facilitates the protection of the cellular proteome from environmental stress by refolding stress-denatured proteins or by directing misfolded proteins to the ubiquitin-proteosome system for degradation (Parsell & Lindquist, 1993). The other network, called the CLIPS (Chaperones Linked to Protein Synthesis) network, is transcriptionally coregulated with the translational apparatus and functions co- and post-translationally to mediate *de novo* folding (Albanèse at al., 2006). Yet, whereas the stress-inducible chaperones dedicated to refold stress-denatured proteins reside within the bulk cytosol (Thulasiraman et al., 1999), the stress-repressed chaperones devoted to folding of nascent polypeptides occur in a sequestered environment, which is physically linked to the translational apparatus (Frydman & Hartl, 1996; Thulasiraman et al., 1999). It has also been revealed that both chaperone networks consist of distinct chaperones, which exhibit different but partially overlapping functions. For example, a small number of Hsp70 chaperones, including *SSA1* and *SSE1*, that are upregulated during highly proteotoxic heat shock, while repressed by most other stresses, are categorized into the CLIPS subset, because performed phenotypically, and bound newly made polypeptides as did the CLIPS proteins. According to Albanèse at al. (2006), "these chaperones function at the interface of both CLIPS- and HSP networks by fulfilling a dual function: normally assisting *de novo* folding but also contributing to the rescue and/or quality control of stress denatured proteins". This stress-induced subset of cytosolic chaperones is also indispensable for termotolerance (Parsell & Lindquist 1993).

In addition to Hsp70s, the 90-kDa heat shock proteins (Hsp90s) are also essential components of the chaperone system in all eukarya and eubacteria, with exception of the archaea, were they are no detected (Pearl & Prodromou, 2006; Wegele et al., 2004). In contrast to Hsp70, which is less selective to its substrates, Hsp90 is restricted to the conformational regulation of highly specific cell-cycle- and developmental regulators, including cyclin-dependent kinases, tyrosine kinases, steroid hormone receptors, transcription factors or mitochondrial membrane components (Buchner, 1999; McClellan et al., 2007; Picard, 2006; Rutherford, 2003; Young et al., 2004). It is likely that Hsp90 recognizes its client proteins on the basis of their hydrophobic surface features that are found on nearly mature proteins in normal condition (Richter & Bucher, 2001) or at the initial stage of unfolding on proteins damaged by stress (Freeman & Morimoto, 1996). Although each of the two Hsps is indispensable for viability, they fulfill non-overlapping functions in the cell (Frydman, 2001; Young et al., 2004). Moreover, regardless of an elevated content of both Hsps during environmental stress, there is no data to demonstrate that the Hsp90 chaperone is required for thermotolerance and/or disaggregation of heat-denatured proteins, as was found for Hsp70.

To elucidate the functions of Hsp90 more deeply, McClellan et al. (2007) have applied a genome-wide chemical-genetic screen in *S. cerevisae* combined with bioinformatic analyses. They revealed a number of new Hsp90-dependent cellular function under physiological (normal) conditions and in response to environmental stress. In general, at stress-induced high temperatures, Hsp90 is essential for cell-cycle progression, meiosis, and cytokinesis,

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whereas at normal growth temperatures, Hsp90 facilitates protein trafficking and assembly or stabilization of oligomeric complexes (Frydman, 2001; McClellan et al., 2007; Young et al., 2004). Because several important cellular processes, such as transcription factor activity or mitochondrial function, are similarly expressed at 30°C and 37°C, it suggests that Hsp90 plays a housekeeping role in daily activities of the cell as well (McClellan et al., 2007). There is growing evidence that the functionally active Hsp90 is an evolutionary conserved capacitor of hidden genetic and epigenetic variation that accumulated in wild-type phenotypes (Manitašević et al., 2007; Queitsch et al., 2002; Rutherford & Lindquist, 1998; Salathia & Queitsch, 2007; Sangster et al., 2004). However, if the function of Hsp90 is impaired either by environmental stress or by genetic or pharmaceutical treatments, this variation usually releases, providing the raw material for natural selection to operate (Salathia & Queitsch, 2007).

2. Testing hypotheses about local adaptation to environmental conditions

The phrase "*local adaptation*" refers to patterns and processes detected across conspecific populations associated by gene flow (Kawecki & Ebert, 2004). The intensity of natural selection within local populations is usually specific to habitat conditions, leading to genotype x environment interaction for individual fitness. If there are no other forces or constraints, such spatially heterogeneous or *divergent selection* affects each population to develop traits which are functionally advantageous under specific composition of environmental factors prevailing in its own habitat, not considering the effects of these traits for fitness in other habitats. Consequently, local genotypes in each population would express the elevateted mean fitness relative to genotypes stemming from the other habitats. According to Williams (1966), the process resulting in such a pattern is defined as *local adaptation*. In this context, local adaptation would be the outcome of two evolutionary factors that operate antagonistically to each other: the spatially heterogeneous natural selection with potentially differentiating effect and the homogenizing effect of gene flow. This conception regards populations either as discrete entities within fixed environmental patches or random sampling units in a continuous species range. Similarly, the spatial environmental variation can be also disconnected and composed of a number of different habitat types, or, as in the case of a continuous environmental gradient, a "habitat" can represent an arbitrary point along this gradient with a specific combination of environmental factors (Kawecki & Ebert, 2004).

2.1 Reciprocal transplant experiments

Experimental biologists interested in testing hypotheses about local adaptation to native environments have used reciprocal transplant experiments for more than a half-century (see Emms & Arnold, 1997; O'Hara et al., 2004; Schluter, 2000). The method requires reciprocally transplanting two or more populations among their natural habitats in the wild and, within a single generation, comparing the relative performance of a sample of genotypes from the "local" population and those one from the "foreign" population(s) under the same environmental conditions, *i.e.*, in the same habitat. An alternative approach involves comparing the performance of the same genotype among contrasting habitats; that is, "at home" and "away". Currently, there is a controversy over which of the two criteria can be used as diagnostic to local adaptation. The "local *vs.* foreign" criterion accentuates the

contrast between populations within habitats, predicting that in each habitat local populations should exhibit greater fitness relative to populations from the foreign habitats. Conversely, the "home *vs.* away" criterion gives emphasis to the contrast between a population's fitness among alternative habitats. In this case, local adaptation is expected to occur if each population exhibits a higher performance in its native habitat ("at home") in comparison to other habitats ("away") (Kawecki & Ebert, 2004).

2.2 Chaperone Hsps as biochemical adaptation

There is growing evidence on adaptive variation in thermal resistance in natural populations, indicating that stressful environmental conditions select for adaptations in natural populations (Hoffmann et al., 2003). For example, in laboratory experiments, very small heat induction of Hsps results in measurable effects on development, life span, fecundity, and stress resistance in plants and animals (Queitsch et al., 2002; Rutherford & Lindquist, 1998). In the field, adaptive changes in the Hsp level over days (Nguyen et al., 1994) and/or over season (Hoffmann & Somero, 1995; Manitašević et al., 2007) appeared to be the ecologically significant for natural populations as well. Hovewer, in contrast to laboratory conditions where the importance of Hsps for survival following heat shock is evident, under field conditions their ecological significance is less explicit and rarely directly explored (Gehring & Wehner, 1995; Manitašević et al., 2007). Recently, the ecological significance of Hsps for adaptation in natural populations have been confirmed using data from latitudinal and climatic clines (Frydenberg et al., 2003; Hemmer-Hansen et al. 2007; Sørensen et al., 2009), which documented that natural selection affects *hsp* variation, which was found to be very low in the coding regions of the *hsp* genes (Sørensen, 2010)

To this end, the goals of this study were to corroborate the statements: (i) that variation in the endogenous relative level of Hsp70 and Hsp90 chaperones is a biochemical adaptation to fluctuating environmental conditions, and (ii) that the amount of plasticity in the relative level of these two chaperones is protein-specific and dependent on the habitat type that the examined plants were derived from. The model-organism used was *Iris pumila*, a perennial herb, naturally growing in lowlands of central and southeastern Europe, including the Deliblato Sands in northern Serbia (Randolph, 1955).

3. Material and methods

3.1 Studied are and species

The Deliblato Sands is an isolated district of sand masses located between the western Carpathian slopes and the Danube River in southern Banat (Serbia). The relief of this area is of eolian origin and therefore has an undulating dune shape. The sand dunes extend in a straight south-east-north-west direction, as does the complete sandy district (44° 47' 39'' N / 21° 20' 00'' E to 45 ^{o} 13' 10'' N / 28 ^{o} 26' 08''E). Surface water streams are completely absent in the Deliblato Sands. The uniqueness of its relief combined with shortage of ground water generated the specific ecological conditions and, as a result, the diversity of habitats and wildlife therein.

Iris pumila L. (Iridaceae) is a rhizomatous perennial monocot, which is very abundant in the dune system at the Deliblato Sands (Gajić, 1983). Within its natural habitats, the species forms circle-chapped clones that differ in size, depending on the clone age (Tucić et al., 1989). *Iris* clones result from the development of horizontally growing and tightly packed

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rhizome segments that spread radially from the center of each clone toward its margin. Populations of *I. pumila* are markedly polymorphic for flower colour due to segregation at several gene loci. Consequently, each of the flower colour variants commonly found in an *Iris* population represents a unique clonal genotype (Tucić et al., 1988).

3.2 Experimental setup

To detect local adaptation in the level of Hsps in natural populations of *I. pumila*, we conducted a reciprocal transplant experiment in the Deliblato Sands. For this experiment, we choose two populations of *I. pumila* naturally growing in contrasting light habitats. One at an open dune site, where plants experienced multiple abiotic stresses, and one in a more "friendly" environment under the canopy of a *Pinus silvestris* stand. In April 2001, samples of adult genotypes from each of the two populations (developed from rhizome modules raised under controlled ambient conditions in a growth-room from May 1998 to April 2001), we transplanted reciprocally between their original habitats. During 2007, when most transplants produced several new leaves, we cut one fully developed leaf from each genotype between 15:00 and 16:00 h, once in each of two seasons (spring and summer), for determining the endogenous level of Hsp70 and Hsp90 chaperones. After cutting leaves were immediately frozen in liquid nitrogen, transported to the laboratory, and stored at -70°C until preparation.

3.2.1 Leaf extracts preparation

We pulverized frozen leaf tissue under liquid nitrogen and resuspended in two volumes (w/v) of extraction buffer (0.1 M Tris, pH 7.6, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF). After sonification for 3 x 15 s on ice, at 1A and 50/60 Hz, with 30% amplitude (Hielscher Ultrasound Processor), the homogenates were centrifuged twice at 12000g at 4 °C, for 15 min and the supernatants analyzed for total protein content by the method of Spector (1978) with bovine serum albumin (BSA) used as a standard.

3.2.2 SDS-polyacrilamide gel electrophoresis and Western blot analysis

Tissue extracts, mixed with equal volumes of 2X SDS-sample buffer (0.125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), were boiled for 5 min at 100°C. Samples containing 40 µg proteins were loaded onto 7.5% SDS-PA gels (Laemmli, 1970) and separated by electrophoresis at 120 V and 4 °C, using Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). We applied a Page Ruler Prestained Protein Ladder (Fermentas International Inc., Canada) for precise molecular weight determinations. After electrophoresis, the separated proteins were transferred from the gels to nitrocellulose membranes (Hybond-C, Amersham) and electroblotted overnight at 135 mA and 4°C in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Unbound sites on the membranes we blocked by incubation in PBS (1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH 7.2) containing 1% nonfat dry milk (GE Healthcare Bio-Sciences) for 1.5 h at room temperature. Proteins of interests were detected using appropriate monoclonal antibodies; Hsp70 by SPA-820 (1:1000; StressGen, Canada) and Hsp90 by SPA-830 (1:2000; StressGen, Canada). After washing with PBS containing 0.1% Tween 20, we incubated the membranes for 1h with alkaline phosphatase-conjugated secondary antibody (1:20000). The immunoreactive bands we visualized and quantified by

enhanced chemifluorescence (ECF) detection system using STORM Imager and ImageQuant image analysis software (Amersham Biosciences Limited, UK). To make quantitative comparisons between multiple immunoblots reliable, i.e. to exclude the inter-gel variation, an internal reference sample consisting of a mixture of all samples was run simultaneously on each gel. Prior to any comparison, the intensity of each analyzed immunospecific band we normalized to the intensity of the respective internal reference band on the same blot. The representative immunoblots display the presence of one immunospecific band for Hsp70 and two different bends for Hsp90, in all leaf samples of *I. pumila* plants from an exposed and a shaded habitat across spring and summer (Figure 1).
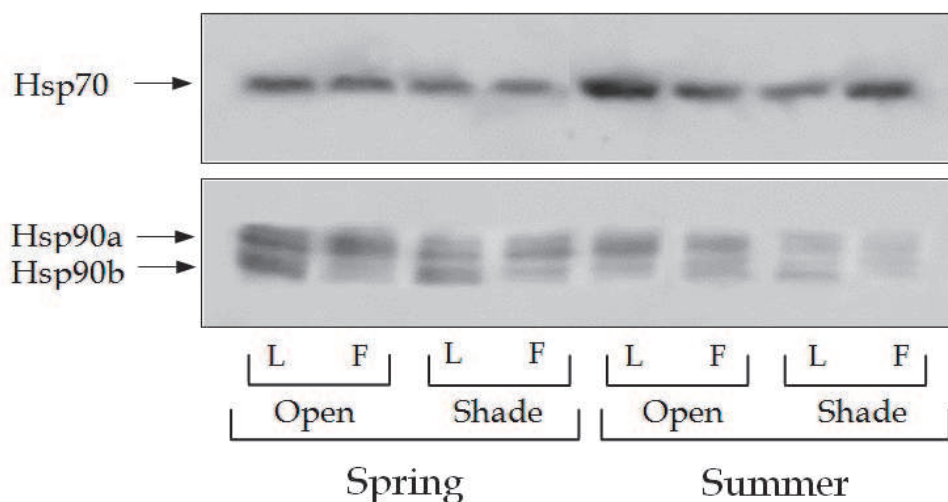

Fig. 1. Immunoblot detection of Hsp70 and Hsp90 in the foliage of *Iris pumila* genotypes from an exposed (Dune) and a shaded (Woods) habitat over spring and summer. L = local habitat; F = foreign habitat. Proteins from whole cell extracts (40 μg) were separated by 7.5% sodium dodecyl sufate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Hsp70 was detected by N27F3-4, whereas Hsp90 isoforms (Hsp90a and Hsp90b) were revealed by AC88 monoclonal antibody. Immunoreactive bands were visualized by enhanced chemifluorescence reaction.

4. Statistical analyses

All analyses were conducted using 22 *Iris* genotypes: 11 from the Dune and 11 from the Woods population. The reaction norm plots were depicted for each Hsp genotype using its individual value expressed within each habitat and season. To examine the effect of environmental conditions in different light habitats as well as within the same habitat over seasons, a factorial ANOVA was implemented using GLM procedure in SAS (SAS Institute 2003). The full ANOVA model included the following sources of variation: habitat (amount of phenotypic plasticity between habitats), season (amount of phenotypic plasticity between seasons), population (genetic variation in trait means between populations), habitat-by-population interaction (genetic variation for plasticity to habitat conditions

between populations), season-by-population interaction (genetic variation for plasticity to seasonal changes between populations), and habitat-by-season-by-population (season-dependent genetic variation for plasticity to habitat conditions between populations). In these analyses, we specified light habitat, season and population as fixed factors. Since all traits (i.e. Hsps relative level) were normally distributed and had equal variances, all ANOVAs were computed on row data. Apart from producing environmentally contingent differences between populations in the mean value of a trait, natural selection can influence the trait plasticity between different habitats and/or within the same habitat over season. In this study, we estimated the amount of plasticity of a clonal genotype for the relative level of each Hsp by calculating an index of plasticity, PI_C (Valladares et al., 2006):

$$PI_C = |(X_1 - X_2)| / |(X_1 + X_2)|,$$

where X_1 is the Hsp level of a clone (c) in the open habitat, or in the spring within that (open) habitat, whereas X_2 refers to the Hsp level of the same clone in the shaded habitat, or in the summer within that (shaded) habitat. The index of plasticity, PI_C, measures the changes in a trait induced by spatial and/or temporal variation in the environmental states. Wilcoxon 2-sample test (SAS Institute, 2003) was used as a nonparametric procedure to compare the mean plasticity to seasonal variation in abiotic factors between the local and foreign genotypes within the same habitats for different Hsps. The same test we used to determine whether the plasticity to contrasting light conditions in the sample of Dune genotypes varied for a given Hsp between the sample of the Woods genotypes.

5. Results

5.1 Habitat and seasonal variation in the mean relative level of Hsp70 and Hsp90 chaperones

In the sun-exposed habitat during both seasons, the mean relative level of all tree foliar chaperones, Hsp70, Hsp90a and Hsp90b, was generally greater in the local (Dune) genotypes relative to their foreign (Woods) counterparts (Table 1).

Considering individual Hsps, in spring, Hsp70 exhibited the lowest (0.63 in the Dune, and 0.29 in the Woods population), while Hsp90a had the highest mean value (0.91 in the Dune, and 0.53 in the Woods population) among all chaperones studied. Yet, when Hsp90a and Hsp90b were compared one to another, the relative level of Hsp90a was greater than that of Hsp90b in both populations of *I. pumila* (0.91 *vs*. 0.78 in the Dune, and 0.53 *vs*. 0.50 in the Woods population). In summer, however, the mean level of Hsp70 was more than twofold greater compared for its spring value (1.46 *vs*. 0.63 in the Dune, and 0.96 *vs*. 0.29 in the Woods population). Conversely, the amount of the chaperones Hsp90a and Hsp90b appeared to be twofold lower than that observed during spring (Table 1).

In the shaded habitat during spring, the mean relative level of all tree foliar chaperones, Hsp70, Hsp90a and Hsp90b, was generally greater in the local (Woods) genotypes as compared to the foreign (Dune) genotypes (Table 1). Again, the mean relative level of Hsp70 chaperone appeared to be the lowest in the Dune genotypes (0.33). In contrast to the open habitat, the average relative level of Hsp90b chaperone in the Woods genotypes was appeared to be the greatest (0.79). In the summer, the foreign (Dune) genotypes produced a higher relative amount of all heat shock proteins (Hsp70, Hsp90a and Hsp90b) than the local (Woods) genotypes.

	OPEN HABITAT					
	Spring			Summer		
Trait	Mean	SD	CV%	Mean	SD	CV%
DUNE (n=11)						
Hsp70	0.63	0.39	62.29	1.46	0.32	21.71
Hsp90a	0.91	0.13	14.06	0.56	0.18	32.82
Hsp90b	0.78	0.03	4.46	0.39	0.06	14.77
WOODS (n=11)						
Hsp70	0.29	0.12	39.46	0.96	0.45	46.94
Hsp90a	0.53	0.15	27.81	0.30	0.04	13.28
Hsp90b	0.50	0.12	23.98	0.24	0.05	20.42
	SHADED HABITAT					
	Spring			Summer		
Trait	Mean	SD	CV%	Mean	SD	CV%
DUNE (n=11)						
Hsp70	0.33	0.21	63.56	0.56	0.10	17.93
Hsp90a	0.58	0.10	17.92	0.65	0.22	34.22
Hsp90b	0.68	0.11	16.28	0.54	0.20	37.08
WOODS (n=11)						
Hsp70	0.34	0.23	67.45	0.50	0.20	39.41
Hsp90a	0.67	0.24	36.70	0.51	0.17	33.59
Hsp90b	0.79	0.26	33.21	0.40	0.07	17.57

Table 1. Sample size (n), mean value (in AU = arbitrary units), standard deviation (SD) and coefficient of variation (CV%) for the relative level of three heat shock proteins, Hsp70, Hsp90a, and Hsp90b, measured during spring and summer in different *Iris pumila* genotypes from a sun-exposed (Dune) and a shaded (Woods) natural populations transplantated in each of their alternative light habitats

The three-way ANOVA applied to each of the three Hsps revealed a highly significant main effects of abiotic environmental conditions on all but one of these traits, the mean relative level of Hsp90a between contrasting light habitats (Table 2). These results suggest that *I.pumila* genotypes possess the capability to alter the level of the two chaperones in accordance with abiotic environments conditions they have happened to experience. A significant main effect of population was obtain for all three chaperones (Hsp70, Hsp90a and Hsp90b), indicating that the exposed and shaded population of *I. pumila* are genetically

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differentiated one from the other for these biochemical traits. The two-way interaction between habitats and populations were highly statistically significant for all three chaperones studied (all $P < 0.001$), implying the presence of genetic differences between populations in plasticity to environmental conditions within contrasting light habitats. The season-by-population interaction appeared to be insignificant for the relative level of all Hsp chaperones. Conversely, the three-way interaction was highly significant for each of the Hsp (all $P < 0.001$), indicating that genetic differences in plasticity to habitat conditions between the Dune and the Woods population are dependent upon season (Table 2).

Source of variation	d.f.	Hsp70		Hsp90a		Hsp90b	
		F	P	F	P	F	P
Habitat	1	46.36	**0.0001**	0.55	0.4623	19.57	**0.0001**
Season	1	62.68	**0.0001**	21.57	**0.0001**	103.79	**0.0001**
Population	1	13.79	**0.0004**	24.13	**0.0001**	16.07	**0.0001**
H x P	1	11.39	**0.0011**	16.99	**0.0001**	11.42	**0.0011**
S x P	1	1.01	0.3183	0.74	0.3929	1.15	0.2871
H x S x P	2	10.89	**0.0001**	9.00	**0.0003**	5.86	**0.0042**

Table 2. Factorial ANOVA results for the relative level of three heat shock proteins, Hsp70, Hsp90a and Hsp90b, measured *in situ* during spring and summer in leaves of distinct *I. pumila* genotypes, stamming from a sun-exposed (Dune) and a shaded (Woods) population, which were reciprocitally transplanted between their local habitats in the Deliblato Sands. Significant F values are given in bold face

5.2 Individual variation (CV %) in the relative level of Hsp70 and Hsp90 between habitats and season

Apart from exhibiting seasonal and habitat-specific differences in the average relative level of Hsp70 and Hsp90, our study provides evidence that the individual variation among genotypes, expressed in term of a coefficient of variation (CV %), also changed over seasons and between the populations from which they originate. In general, Hsp70 expressed the greatest individual variation in both populations across both seasons, with only exception during summer (21.17%). In addition, the lowest level of CV% was observed for Hsp90b during spring (4.46%) in the Dune population, as well (Table 1).

The individual variation (CV%) in the mean relative level of the three analyzed chaperones in the shaded habitat displayed similar trend to that revealed at the open Dune site. However, the percentage of individual variation for each chaperone analyzed appeared to be greater for the local (Woods) genotypes compared to that revealed for the foreign (Dune) genotypes.

Again, in the summer, the Dune genotypes exhibited the lowest individual variation for the mean relative level of Hsp70 (17.93%), as was revealed for the individual variation of the mean relative level of Hsp90b in the Woods genotypes (17.57%) (Table 1).

At the open habitat, the univariate ANOVAs revealed a significant difference in the endogenous level of all Hsps (Hsp70, Hsp90a and Hsp90b) between the Dune and the Woods genotypes in both seasons (all $P < 0.001$; Table 3A). Conversely, in the shaded habitat, a significant difference appeared exclusively for Hsp90b chaperone in the summer ($P < 0.05$; Table 3B).

A.		OPEN HABITAT											
		Spring						Summer					
Source of variation		Hsp70		Hsp90a		Hsp90b		Hsp70		Hsp90a		Hsp90b	
	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
POPULATION	1	7.43	0.013	41.85	0.000	55.27	0.000	9.17	0.007	21.43	0.000	43.48	0.000

B.		SHADED HABITAT											
		Spring						Summer					
Source of variation		Hsp70		Hsp90a		Hsp90b		Hsp70		Hsp90a		Hsp90b	
	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
POPULATION	1	0.03	0.859	1.29	0.269	1.58	0.223	0.73	0.404	3.04	0.097	5.09	0.035

Table 3. ANOVA exploring the effect of population origin in a single generation during spring and summer on the relative level of Hsp70, Hsp90a and Hsp90b chaperones in leaves of *Iris pumila* genotypes grown at an open (A.) and a shaded habitat (B.). The *F*-value for each effect is reported

When the mean relative level of all three Hsps (Hsp70, Hsp90a and Hsp90b) in genotypes stemming from the two populations were compared between alternative light habitats over seasons, a univariate ANOVA revealed that the Dune genotypes expressed significantly different level of all these Hsps in both season (all $P < 0.05$), with only exception of Hsp90a in summer (Table 4A). An inverse trend exhibited the Woods genotypes. In summer, their mean relative level for all three Hsps differed significantly between alternative light habitats (all $P < 0.001$; Table 4B), in contrast to their spring counterparts that differed significantly only for the mean relative level of Hsp90b chaperones between the contrasting light habitats ($P < 0.001$; Table 4A).

We presented the reaction norm plots to habitat type for the level of three Hsps (Hsp 70, Hsp90a and Hsp90b) in leaves of *I. pumila* clonal genotypes from the Dune and the Woods population during spring (Fig. 2A and 2B) and summer (Fig. 2C and 2D).

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A.		SPRING											
		Population Dune						Population Woods					
Source of variation		Hsp70		Hsp90a		Hsp90b		Hsp70		Hsp90a		Hsp90b	
	d.f.	F	P	F	P	F	P	F	P	F	P	F	P
HABITAT	1	5.15	0.034	44.77	0.000	6.94	0.016	0.40	0.533	2.62	0.121	11.41	0.003

B.		SUMMER											
		Population Dune						Population Woods					
Source of variation		Hsp70		Hsp90a		Hsp90b		Hsp70		Hsp90a		Hsp90b	
	d.f.	F	P	F	P	F	P	F	P	F	P	F	P
HABITAT	1	80.96	0.000	1.07	0.313	5.90	0.025	9.51	0.006	15.44	0.001	37.96	0.000

Table 4. ANOVA exploring the effect of habitat type in a single generation during spring and summer on the relative level of Hsp70, Hsp90a and Hsp90b chaperones in leaves of *Iris pumila* genotypes from the Dune (A.) and the Woods population (B.). The *F*-value for each effect is reported

The plots of reaction norms over seasons are shown for the level of three Hsps (Hsp 70, Hsp90a and Hsp90b) in leaves of *I. pumila* clonal genotypes from the Dune and the Woods population at an open (Fig. 3A and 3B) and a shaded habitat (Fig. 3C and 3D).

The mean reaction norms were steep for all Hsps measured (Figs. 2 and 3), suggesting a general ability of *I. pumila* clones for plastic adjustment of their leaf biochemistry to spatial and temporal variation of environmental conditions. Pattern of reaction norms was rather complex in both populations, with some genotypes exhibiting reversals of ranking in different seasons and/or habitat. Crossed-reaction norms indicate that there was genetic variation for plasticity in the leaf Hsp level within and between populations, corroborating the factorial ANOVA results (Table 2).

The plasticity means between seasons or habitats (average PI_C) appeared to be strongly trait-specific (Table 5). The relative level of Hsp70 chaperone was found to be the most plastic, whereas the relative level of Hsp90a appeared to be the least plastic in both populations of *I. pumila*. Plastic response of Hsp90b chaperone was intermediate in the magnitude.

A Wilcoxon 2-sample test revealed that the amount of plasticity for identical *Iris* genotypes was similar between distinct light habitats or between seasons within the same habitat for any of the three chaperones, in each of the two populations studied. Regarding contrasting light habitats, however, in the summer, the mean plasticity was significantly greater for Hsp70, while in the spring, Hsp90b expressed the lowest mean plasticity, when the Dune and the Woods genotypes were compared.
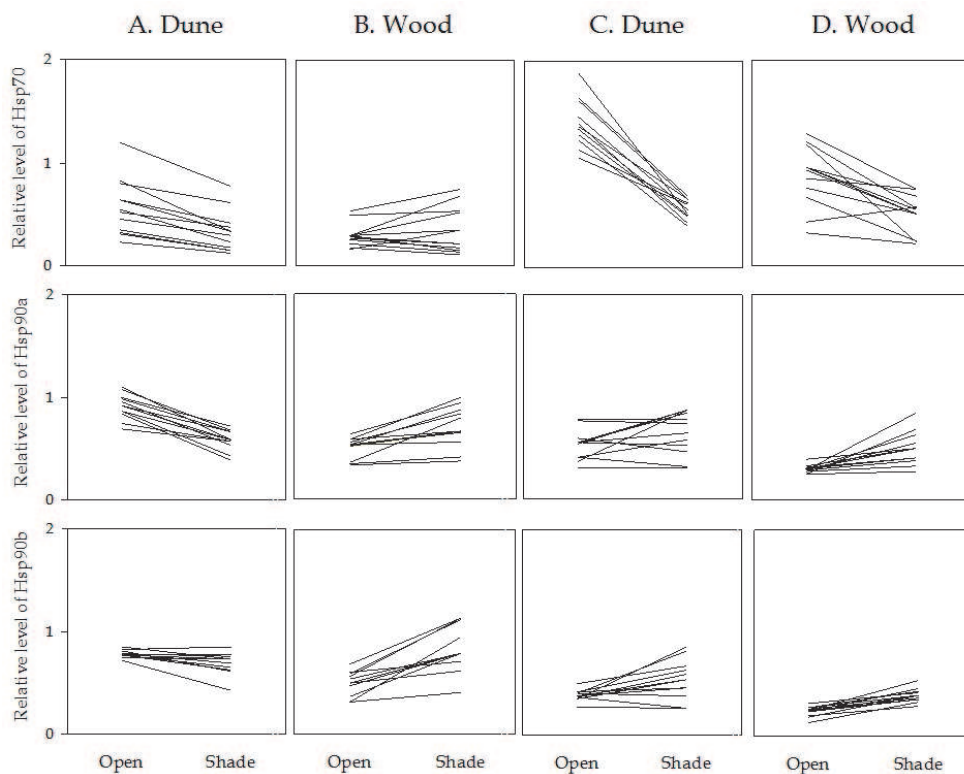
Fig. 2. Reaction norm plots for 22 *Iris pumila* clonal genotypes, native to an exposed (Dune) and a shaded (Woods) population, which were reciprocally transplanted between their original habitats. The relative level of leaf Hsp chaperones in 11 Dune genotypes and 11 Woods genotypes was observed at an open and a shaded habitat during spring (A and B) and summer (C and D)
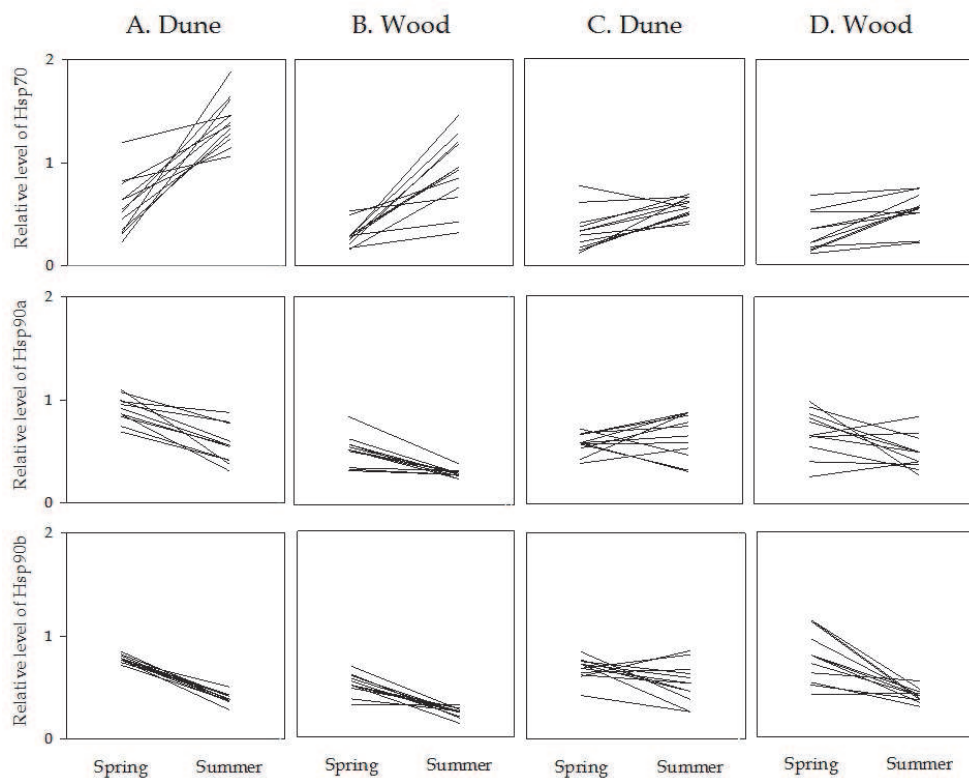
Fig. 3. Reaction norm plots for 22 *Iris pumila* clonal genotypes, native to an exposed (Dune) and a shaded (Woods) population, which were reciprocally transplanted between their original habitats. The relative level of leaf Hsp chaperones in 11 Dune genotypes and 11 Woods genotypes was observed during spring and summer at an open (A and B) and a shaded (C and D) habitat

	SPRING *vs.* SUMMER				OPEN HABITAT *vs.* SHADED HABITAT			
Trait (n = 11)	Open habitat		Shaded habitat		Spring		Summer	
DUNE	PI_C	SD	PI_C	SD	PI_C	SD	PI_C	SD
Hsp70	0.444	0.222	0.340	0.201	0.315	0.130	0.439	0.106
Hsp90a	0.249	0.138	0.185	0.105	0.225	0.093	0.122	0.129
Hsp90b	0.335	0.069	0.189	0.139	0.074	0.070	0.171	0.127
WOODS	PI_C	SD	PI_C	SD	PI_C	SD	PI_C	SD
Hsp70	0.484	0.224	0.324	0.223	0.224	0.106	0.315	0.178
Hsp90a	0.261	0.131	0.221	0.148	0.143	0.091	0.231	0.153
Hsp90b	0.337	0.182	0.307	0.168	0.213	0.136	0.250	0.097

Table 5. Sample size (n), mean plasticity index (PI_C), and standard deviation (SD) for the relative level of three heat shock proteins, Hsp70, Hsp90a and Hsp90b, measured during spring and summer in leaves of different *I. pumila* genotypes from a sun-exposed (Dune) and a shaded (Woods) populations transplanted in each of their alternative light habitats

6. Discussion

During the course of evolution, plants have evolved a variety of different biochemical mechanisms for preventing fitness reduction under adverse environmental conditions (Bazzaz, 1996; Lambers et al., 2008; Tucić et al., 2009; Vuleta et al., 2010). One of such mechanisms is molecular chaperones – a group of proteins that respond to sudden increase in temperature or exposure to other environmental stresses (Feder & Hofmann, 1999; Salathia & Queitsch 2007; Sangster et al., 2004; Sørensen et al., 2003). Molecular chaperones, particularly Hsp90, also restrict stochastic phenomena within cells, by minimizing developmental perturbations, thereby canalizing the organism's development (Samakovli et al., 2007). Results presented in this study provide evidence that in *Iris pumila* plants the relative level of heat shock proteins Hsp70 and Hsp90 (Hsp90a and Hsp90b isoforms), varied significantly in a single generation, between the samples of local and foreign genotypes reciprocally transplanted to their original light habitats ("local *vs.* foreign" approach), and in the same genotype grown under alternative light conditions ("at home *vs.* away" approach), or among different seasons in the same habitat ("spring *vs.* summer"). In general, local genotypes exhibited significantly higher relative amounts of all three chaperones (Hsp70, Hsp90a and Hsp90b) compared to the foreign genotypes. Similarly, the relative level of all the three Hsps in each genotype from both populations of *I. pumila* was greater "at home" (within its native habitat) than "away" (within the non-native habitat). Theoretically, a higher performance under native environmental conditions, and a lower performance under non-native environmental conditions can be interpreted as an evidence of local adaptation (Kawecki & Ebert, 2003). Based on the results obtained using the two

experimental approaches, it seems reasonable to conclude that the analyzed populations of *I. pumila* are genetically differentiated for the mean relative level of the two Hsp chaperones, Hsp70 and Hsp 90, most probably due to divergent natural selection operating within alternative light habitats. Although the two criteria can frequently be simultaneously satisfied, Kawecki and Ebert have the preference to the "local *vs.* foreign" criterion as diagnostic for the pattern of local adaptation. They believe that "This criterion is directly relevant to the driving force of local adaptation – divergent natural selection - which acts on genetic differences in relative fitness within each habitat. In contrast, the "home *vs.* away" criterion confounds the effects of divergent selection with intrinsic differences in habitat quality" (Kawecki & Ebert, 2003). The term divergent selection is closely related to the phenomenon of ecological speciation – the process by which reproductive isolation between populations evolves as a results of ecologically based divergent selection (Rundle & Nosil, 2005). According to Schluter (2000), divergent selection can arise due to differences between populations in their environmental conditions, including habitat structure, climate, resources, and the predators or competitors present. Indeed, in the studied populations of *I. pumila*, the ambient air temperature and the instantaneous light intensity markedly differed between contrasting light habitats, as well as over seasons in the same habitat. For example, 2004 measurements, at an open habitat in the spring, the mean air temperature amounted 19.5 ± 0.5°C and light intensity 1797 ± 16 µmol m^{-2} s^{-1}, while in the summer, the average air temperature appeared to be 29.7 ± 0.6°C and light intensity 1378 ± 16 µmol m^{-2} s^{-1}. In the forest shade, however, the spring temperature mean was 21.6 ± 0.6 °C and the mean light intensity 136 ± 1.8 µmol m^{-2} s^{-1}, whereas in the summer, the average air temperature reached 19.5 ± 0.5°C and the mean light intensity 45 ± 3.1 µmol m^{-2} s^{-1} (Vuleta et al., 2010). It has been recently reported that the relative level of the two leaf Hsp chaperones, Hsp70 and Hsp90, varied significantly across seasons in the same clonal genotypes native to an exposed and a shaded population of *I. pumila*, and between different naturally growing genotypes in these *I. pumila* populations experiencing contrasting light conditions as well (Manitašević et al., 2007). Our study provides evidence that the average relative level of Hsp70 chaperone was significantly higher at an exposed site than under the forest understorey, reaching its maximum in the summer, especially in plants exposed to full sunlight. Of note, in the open habitat, the relative level of Hsp70 in the local (Dune) genotype appeared to be significantly greater in spring- and summer-collected leaves (0.63 *vs.* 1.46, respectively) than in their foreign (Woods) counterparts (0.29 *vs.* 0.96, respectively) (Table 1; Fig. 2), presumably due to ecologically based divergent selection.

6.1 The role of Hsp70 chaperones for adaptation

It is well known that molecular chaperones play a crucial role at two stages in the life of a protein: throughout *de novo* folding following translation, and during denaturation imposed by environmental stress (Morimoto et al., 1997; Parsell & Lindquist, 1993). Although many chaperones are highly elevated during stress, the investigations of eukaryotic cells revealed a significant difference between the folding of newly translated polypeptides and stress-denatured proteins. Regarding Hsp70 protein family, the former - stress-repressed chaperones are located in a sequestered cell environment close to the translational apparatus, while the latter - stress-induced chaperones occur in the bulk cytosol (Thulasiraman et al., 1999). Recently, Albanèse et al. (2006) have applied a systems biology approach to elucidate the functional organization of cytosolic chaperones in *Saccharomyces*

cerevisiae. They revealed that the eukaryotic chaperone machinery comprises two networks with specialized functions. The first, stress-repressed network consists of chaperone linked to protein synthesis, and, therefore, is denoted as CLIPS, and the second, stress-inducible or the Hsp chaperone network, which includes components that either renature or clear misfolded proteins. These authors proposed that Hsp70 chaperones, particularly the Hsp70 Ss1/2p, plays a central role early during polypeptide synthesis, i.e., in *de novo* folding of newly made polypeptides, but also in response to stress.

Because the essential role of Hsp70 in stressful environments is to prevent aggregation, and to facilitate refolding and/or proteolytic degradation of nascent proteins (Wang et al., 2004), the elevated level of Hsp70 in sun-exposed plants, especially during summer, might be viewed as a kind of "anticipatory" phenotypic plasticity to increasing chances of heat stress in that habitat. In the forest understory, however, where thermal fluctuations are fewer and less frequent, a lower relative level of Hsp70 are sufficient to maintain native protein structure and occasional refolding of damaged proteins (Manitašević et al., 2007). Given that increased level of Hsp70 chaperone correlates well with greater thermotolerance in many plant species and that heat stress jointly occurs with high irradiance, the *I. pumila* genotypes naturally exposed to multiple abiotic stresses could be though as more stress–tolerant compared to those ones inhabiting a more "benign" vegetation shade.

6.2 The role of Hsp90 chaperones for adaptation

Contrary to Hsp70 proteins, which achieved their maximal relative level in the summer, the average level of both Hsp90 isoforms (inducible-Hsp90a and constitutive-Hsp90b) were highly suppressed in the summer compared to their spring counterpart, especially Hsp90b isoform (Table 1; Fig. 3). In the open habitat, genotypes from both populations differed significantly in the average level of the two Hsp90 isoforms (Fig. 3A and 3B). Conversely, in the shaded habitat, their relative level was similar between local and foreign genotypes in both seasons, with only exception of Hsp90b isoforms in the summer, which relative amount appeared to be greater in the Dune than in the Woods population (0.54 *vs.* 0.40, respectively; Table 1; Fig. 3C and 3D).

In the eukaryotic cytosol, Hsp70 and Hsp90 chaperones are each essential for cell viability under all growth conditions, implying that they fulfill non-overlapping function (Frydman, 2001; Young et al., 2004). Hsp90s are highly conserved group of molecular chaperones, which constitute about 1-2% of all cytosolic proteins in most cells under non-stress conditions (Parsell & Lindquist, 1993). Hsp90 is not a chaperone for newly synthesized proteins, but, instead, its cellular function is restricted to the conformational regulation of the limited group of substrates or "clients" (McClellan et al., 2007). In higher eukaryotes, Hsp90 work together with a large set of co-chaperones to mediate the conformational regulation of tyrosine kinases and steroid hormone receptors (Picard, 2006), but also to prevent phenotypic variation of these signaling molecules in the face of gene mutation (Sangster et al., 2004). The current understanding of Hsp90 function in tyrosine kinase and steroid hormone receptors maturation suggests that Hsp90 binds to "clients" that are substantially folded, facilitating their conformational remodeling.

Recently, McClellan et al. (2007) have used a genome-wide chemical-genetic screen combined with bioinformatic analyses to elucidate more deeply the Hsp90 functions. They identified several *unanticipated function* of Hsp90 under normal conditions and in response to stress. One of new informations obtained from these studies is the modular nature of the

Hsp90 interaction network. The Hsp90 network consists of two major functional modules, one dedicated to cellular trafficking and transport, and the other dedicated to the cell cycle regulation. Hsp90 functions in almost all aspects of the exocytic and endocytic secretory pathway through direct physiological interaction with their components. It was found that the Hsp90 targets tend to interact with each other, and, surprisingly, the average distance between them was found to be smaller than expected from random chance, as well as that each of these targets contains higher than expected number of hubs (proteins with more than 25 interaction partners) (McClellan et al., 2007). Under normal environmental conditions, Hsp90 is essential for vesicular transport and protein trafficking, which require the ordered assembly and disassembly of large multisubunit complexes. Hsp90 stabilizes or assists in the development of these oligomeric complexes by stabilizing their subunits prior to assembly or by assisting in their conformational transition. During environmental stress, however, Hsp90 appears to stabilize unstable conformations of many proteins, and has a key role in the continued function of the cell cycle machinery (McClellan et al., 2007).

Our study provides evidence that at the sun-exposed habitat the relative level of inducible and constitutive Hsp90 isoforms of Hsp90 chaperone was lower in the summer-collected leaf samples from both local and foreign *Iris* genotypes, compared for their spring value, and, as a rule, was greater in the former than in latter ones. The same trend was detected in the shaded habitat, but more conspicuously during summer, in both populations studied. According to Sørensen (2010), it is not easy to decide "when the level of constitutive and inducible HSP expression should be interpreted as reflecting the capacity or ability to mount a strong defense (i.e. as a benefit) or when it should be interpreted as reflecting the need to mount a strong response as the organism is stressed (i.e. as a cost)" (Sørensen, 2010). Since at the open habitat, the local genotypes had an increased level of constitutive Hsp90b over seasons in general than the foreign genotypes, it suggests that this chaperone might be important for adaptation to usually higher temperature prevailing there. The finding that the relative level of both Hsp90 isoforms was lower in summer than in spring ought to have special attentions because it opposed the general prediction that the amount of Hsps is elevated by stressful stimuli. It is well known that heat shock response is energetically costly, since both the protein production and chaperoning activity of Hsps require energy supply. The costs may also arise from stress-related destruction of normal cellular functions, extensive use of energy by antioxidants, and as well as from negative influence of Hsps on fitness (Feder & Hofmann, 1999; Heckathorn et al., 1996; Manitašević et al., 2007; Sørensen, 2010). Contrary to Hsp90, the Hsp70 dramatically increased in the summer, but about 50% more in the Dune than in the Woods populations, suggesting than this Hsp might be most important during rare and unpredictable environmental stress episodes and not for continuous or regularly occurring stress exposure (Sørensen, 2010).

Apart from producing environmentally contingent differences between populations in the mean value of a trait, natural selection can influence the trait plasticity within the same habitat as well. We quantified the degree of plasticity in the level of Hsps in *I. pumila* using an index of plasticity, PI_C (Valladares et al., 2006). Among the three chaperones analyzed, Hsp70 exhibited the greatest amount of plasticity, regardless of the habitat type and/or season. However, a statistically significant difference in plasticity to habitat light conditions was observed between the Dune and the Woods populations for the relative level of Hsp70 (PI_C = 0.437 and 0.315, respectively) in the summer, and for Hsp90b (PI_C = 0.074 and 0.213, respectively) in the spring (Table 5). Unexpectedly, the degree of plasticity to seasonal variation in abiotic environment

conditions for the relative level of all three Hsps (Hsp70, Hsp90a and Hso90b) appeared to be similar between the two *I. pumila* populations, in each of their native habitats. The observed results could be interpreted as the outcome of divergent selection on Hsp plasticity; one, operating within the thermally unstable sun-exposed dune sites, and the other, generated by more variable light conditions prevailing under the forest understory. In addition, our results corroborate the hypothesis that thermal variation occurring within a generation time scale likely selected for increased Hsp chaperone level and, consequently, for greater inducible thermotolerance.

7. Conclusions

There is a consensus among evolutionary biologists that the phenomenon of adaptation has dual meaning: as a process and as a product of that process, which mechanism is natural selection. In this context, any biological entity that satisfies three necessary conditions for natural selection to operate: has the ability to vary, has continuity (heritability), and differs in its success relative to another co-occurring entity, can be adapted and can produce adaptations. When defined in a restrictive sense of the word, the term "adaptation" refers to a "trait (i) that enhances the fitness of an organism, and (ii) whose current beneficial characteristics reflect the selective advantage of the trait at its time of origin" (Hochachka & Somero, 2002). Molecular chaperones are a highly conserved set of functionally defined proteins that are involved in the folding and degradation of stress-demaged proteins. Because the role they play in all contemporary organisms is the benefit, which is very likely to be the same as the benefit that initially favoured the evolutionary development of these proteins, molecular chaperones are viewed as "adaptations". Among the molecular chaperones, the heat shock proteins Hsp70s are involved in the folding of newly translated and stress-denatured proteins. In addition to stress-inducible chaperone networks, eukaryotes contain a stress-repressed chaperone network that is dedicated to protein biogenesis. Although the Hsp90 molecular chaperones are highly abundant under normal conditions, they are restricted on a limited set of nearly mature, but inherently unstable, signaling proteins. Thus, under normal conditions, Hsp90 plays a key role in various aspects of the secretory pathway and cellular transport, while during environmental stress, Hsp90 is necessary for the cell cycle, meiosis and cytokinesis. In this study local adaptations for the relative level of three heat shock proteins, Hsp70, Hsp90a and Hsp90b in leaves of *Iris pumila* genotype native to contrasting light habitats were tested using a reciprocal transplant experiment conducted in the wild. Two experimental approaches, "local *vs* foreign" and "at home *vs* away", were applied to find out which of them can be used as diagnostic for local adaptation (Kawecki & Ebert, 2004). At a sun-exposed site, local genotypes were found to produce a higher amount of all three Hsps than did the foreign genotypes transplanted from a shaded habitat. Similarly, each of the genotypes exhibited a greater level of all three Hsp chaperones "at home" than "away", indicating that both criteria are satisfied for testing local adaptations. The obtained results indicate that the revealed genetic differentiation between populations from the exposed and the shaded habitats could be ascribed to divergent selection operating within their natural habitats. Apart from producing environmentally contingent differences between populations in the mean value of a trait, natural selection can influence the trait plasticity within the same habitat as well. Among the three chaperones analyzed, Hsp70 exhibited the greatest amount of plasticity, regardless of the habitat type and/or season. However, a statistically significant difference in plasticity to

habitat light conditions was observed between the Dune and the Woods populations for the relative level of Hsp70 in the summer, and for Hsp90b in the spring. The observed results could be interpreted as the outcome of divergent selection on Hsp plasticity; one, operating within the thermally unstable sun-exposed dune sites and the other, generated by more variable light conditions prevailing under the forest understory. In addition, our results corroborate the hypothesis that thermal variation occurring within a generation time scale likely selects for increased Hsp chaperone level and, consequently, for greater inducible thermotolerance.

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