Ecologically relevant stress resistance: from microarrays and quantitative trait loci to candidate genes – A research plan and preliminary results using *Drosophila* as a model organism and climatic and genetic stress as model stresses

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We aim at studying adaptation to genetic and environmental stress and its evolutionary implications at different levels of biological organization. Stress influences cellular processes, individual physiology, genetic variation at the population level, and the process of natural selection. To investigate these highly connected levels of stress effects, it is advisable – if not critical – to integrate approaches from ecology, evolution, physiology, molecular biology and genetics. To investigate the mechanisms of stress resistance, how resistance evolves, and what factors contribute to and constrain its evolution, we use the well-defined model systems of *Drosophila* species, representing both cosmopolitan species such as *D. melanogaster* with a known genome map, and more specialized and ecologically well described species such as the cactophilic *D. buzzatii*. Various climate-related stresses are used as model stresses including desiccation, starvation, cold and heat. Genetic stress or genetic load is modelled by studying the consequences of inbreeding, the accumulation of (slightly) deleterious mutations, hybridization or the loss of genetic variability. We present here a research plan and preliminary results combining various approaches: molecular techniques such as microarrays, quantitative trait loci (QTL) analyses, quantitative PCR, ELISA or Western blotting are combined with population studies of resistance to climatic and genetic stress in natural populations collected across climatic gradients as well as in selection lines maintained in the laboratory.

[Loeschcke V, Sørensen J G and Kristensen T N 2004 Ecologically relevant stress resistance: from microarrays and quantitative trait loci to candidate genes – A research plan and preliminary results using *Drosophila* as a model organism and climatic and genetic stress as model stresses; *J. Biosci.* **29** 503–511]

1. Introduction

There is a growing awareness that environmental stress has played and still plays a significant role in the evolution of biological systems, from the level of the gene to that of the ecosystem. In this context environmental stress is regarded as an 'environmental factor causing a change in a biological system, which is potentially injurious' (Hoffmann and Parsons 1991) and which has some fitness consequences (e.g. Bijlsma and Loeschcke 1997). We consider genetic stress as stress that arises from inbreeding or a disturbance of co-adapted gene complexes, e.g. by hybridization. Genetic stress seems to share a lot of similarities with environmental stress as they both lead to an

Keywords. Environmental stress; gene expression; genetic stress; stress resistance

Abbreviations used: EEPG, Ecological and evolutionary functions genomics; qPCR, quantitative PCR; QTL, quantitative trait loci.

up regulation of molecular chaperones (Kristensen *et al* 2002).

In the last century, increasing levels of environmental stress have been inflicted on the biosphere at a global scale by the increasing human population and the demands for space and resources that this creates. This has caused and will increasingly cause major environmental changes, such as climatic shifts, and habitat destruction, reduced population sizes and increased rates of inbreeding. The size and rate of these changes continue to threaten life on this planet. Habitat destruction and fragmentation has caused an accelerating rate of species extinction, and global warming may exert thermal and desiccation stress. Understanding the nature and consequences of environmental stress at a global level from an ecological and evolutionary perspective, and its potential synergistic interaction with genetic stress, is of paramount importance for the development and evaluation of countermeasures. To gain this understanding, in depth investigations of the mechanisms that allow organisms to cope with environmental and genetic stresses are essential.

In order to investigate stress response mechanisms, collaboration between evolutionary biologists, physiologists and molecular geneticists is required. These disciplines are concerned with the same questions, asking how organisms adapt to environmental stress and what mechanisms exist to cope with genetic stress. Traditionally these disciplines are not integrated and therefore there is a considerable potential for major advances in the understanding of stress adaptation by bringing these approaches together, using the same model organism.

We base our studies on experience in conducting microarray studies using commercially available gene chips from Affymetrix, performing quantitative PCR (qPCR), doing quantitative trait loci (QTL) analyses, DNA sequencing of candidate genes and utilizing an existing set of selected *Drosophila* lines for heat, cold, desiccation and starvation resistance, at present having undergone > 15 generations of selection at relatively high effective population sizes. We also maintain lines that are inbred to different levels of inbreeding and which differ in the rates at which these levels were reached. We also have experience with performing population assays of stress resistance and related fitness traits, and in manipulating and maintaining the model organism in the laboratory. Additionally, we have access to material from collections of flies originating from various environmental (climatic) gradients. Thus we have the required starting conditions to reach the described goals of identifying genes that matter for stress resistance, based on thorough studies using various complementary molecular methods as well as testing of hypotheses in natural populations. Recent developments in molecular genetics have even further stimulated interest in stress responses, and detailed studies of the stress responses have revealed that most organisms have evolved sophisticated mechanisms to cope with stress.

2. Research plan

Climatic stress is used as the model environmental stress system. This stressor is ecologically relevant as most organisms are exposed to varying temperatures in time and space, and even to thermal environments that directly induce a stress response. In addition, it appears that stress responses are similar for very different forms of stress and thus results obtained for a climatic stress model system can be extrapolated to other environmental, and, even genetic stresses (Hoffmann *et al* 2003; Sørensen *et al* 2003). Using *D. melanogaster* as the main model organism for this multidisciplinary investigations has the advantage that we can draw on a wealth of knowledge from the molecular to the population level.

The *Drosophila* genome project was completed in 2000 (Adams *et al* 2000) and provides the full genome sequence along with annotations to a large number of genes. Since its publication, gene chips have become commercially available (Affymetrix). Alternatively, having the *Drosophila* genome mapped raises the option of spotting chips with a subset of probe sets of particular interest for the question being asked – and this technology is now widely used.

We will study the evolutionary genetics of stress acclimation and adaptation, as well as the physiological consequences of environmental stress. Besides protein stability and functioning, maintenance of membrane fluidity will be important for flies to withstand temperature and desiccation stress (Hazel 1995).

Study of gene expression patterns of the stress response will provide an overview of the primary cellular responses to stress. By comparing the immediate and the evolutionary genomic response, we will not only be able to identify yet unknown mechanisms, but also be able to judge which physiological processes do evolve in response to both artificial selection in the laboratory and natural selection. By investigating which parts of the genome contribute most strongly to stress adaptation in artificial selection, we will be able to judge which of the stress response genes are likely to respond evolutionarily and which may underlie genetic or functional constraints that in turn may constrain the evolutionary trajectory. Genetic variation in the stress resistance genes is necessary if populations are to evolve adaptively by natural selection. However, Rutherford and Lindquist (1998) have shown that existing genetic variation may be canalized and only manifest itself in the phenotype under environmental stress. Some studies have shown that the increase in the phenotypic variance under stress has a genetic basis (Hoffmann and Merilä 1999;

Bubliy and Loeschcke 2001). Therefore, we will investigate the influence of stress on evolutionary rates to test the hypothesis that stress promotes or constrains adaptive evolutionary processes and impacts on mutation rates.

The approach outlined here, with its high degree of subproject integration, defines the newly emerged field of ecological and evolutionary functional genomics (EEFG). EEFG is a multidisciplinary approach to study mechanisms of and constraints on evolution. It reunites genetics, molecular biology, biochemistry, physiology, evolutionary biology, and ecology and will bring ecology and evolution into the genomics and post-genomics era. The quest of EEFG is to investigate which genes are accountable for phenotypic traits, and here more specifically, which genes affect acclimation and adaptation on both ecological and evolutionary time scales.

To achieve our research goals, we will do studies of gene regulation in stress selected and inbred lines, map QTL responsible for stress resistance, study variation in candidate genes and the relation of candidate gene variation to variation in resistance traits in natural populations and selection lines, study rates of evolution under stress, identify biochemical changes that contribute to resistance, and investigate the proteomic responses under stress as well as the cellular physiology of whole flies in selection lines.

With our work we intend to contribute to the body of knowledge on one of today's major questions in most, if not all, biological disciplines: the relationships between genes, functions of their protein products, interactions of proteins in biochemical pathways and cellular structures, and the intracellular interactions contributing to the fitness of an individual in its specific environment.

To obtain a high ecological and evolutionary relevance of our investigations we plan to carry out most studies with whole flies and to investigate the genetic and physiological kinetics of the response aspects that have been largely neglected in the field. Using natural and artificially selected genetic variants we want to establish gene to function relations for genes involved in stress resistance.

3. Preliminary results

We will focus on the following main projects to study the implications of environmental and genetic stress on different levels of biological organization using a multidisciplinary approach. We aim at contributing to a broad understanding of the evolution of stress response mechanisms and their role for evolution of other traits.

We will identify candidate genes and relate variation in these genes to variation in stress resistance traits. Candidate genes will either be those known to have some role for resistance as e.g. the well-known heat shock protein genes and other 'housekeeping' genes, or previously unknown genes identified as interesting by gene expression profiling (microarrays) of selection lines or as potentially responsible for peaks revealed in QTL studies. Quantitative PCR, RNA-interference or genetically modified lines (e.g. knock-out or extra copy lines) lines will be used as follow-up steps to verify and further study genes revealed by the previous methods. Hypotheses generated on the basis of the molecular data will be complemented by population studies to test if candidate genes in natural populations behave as predicted from the molecular data from laboratory studies. These studies will be undertaken using flies caught along altitudinal and latitudinal transects – or flies from well-defined climatic gradients. Finally, physiological studies of the same selection lines and natural populations will be used to study physiological adaptation and other non-genetic modes of adaptation such as maternal effects and phenotypic plasticity.

3.1 *Studies of gene regulation*

Studies of gene regulation in selection and control lines of *D. melanogaster* via Affymetrix gene chips will be performed. Simple designs will be used, and well-defined questions asked; something which we believe is important to be able to interpret microarray results in a clear way. The objective is to elucidate which genes or group of genes are up or down regulated in stress selected lines (and in which life stages) and determine the time course of regulation after short-term acclimation (mild and severe stress exposure) in order to identify potential candidate genes for resistance to particular stress types, and to identify genes that are common stress genes and genes that are specific to certain stress types. Previous results have shown that the time curve of up regulation of one specific candidate gene, hsp70, does not coincide with the curve describing the beneficial effects of the short-term acclimation in terms of increased resistance to stress (figure 1, Dahlgaard *et al* 1998). This has created a need for a better time resolution of the various genes involved in the stress response system.

Preliminary results show a drastic change in gene regulation in response to a short-term hardening treatment, and a different time resolution of different genes. Up regulated genes include the well-known heat shock genes, and also many other genes involved in cellular defence, immune response and metabolism are being up regulated (Leemans *et al* 2000; Kayo *et al* 2001; Lee *et al* 2001; Pletcher *et al* 2002; Seroude *et al* 2002; Sørensen J G, Nielsen M M, Kruhøffer M, Justesen J, Boriss H and Loeschcke V, unpublished results; Kristensen T N, Sørensen P, Kruhøffer M, Pedersen K S and Loeschcke V, unpublished results). However, care has to be taken in interpreting results from

microarray analyses for various reasons. Not only do general statistical considerations suggest a lot of false positive results due to the many thousand tests involved, but we should also be aware that differences in RNA expression levels not necessarily correlate strongly with protein expression levels (Efron and Tibshirani 2002; Mootha *et al* 2003). Microarray results are followed up with investigations by qPCR, ELISA or Western blotting techniques. While ELISA and Western blotting are used to quantify protein levels, qPCR is targeting the mRNA level like the arrays. All techniques are employed to verify and further investigate the regulation and function of candidate genes as identified by the arrays.

Studies of gene regulation in inbred and hybrid lines of *D. melanogaster* and possible other *Drosophila* species via Affymetrix gene chips shall reveal which genes are up or down regulated under inbreeding and hybridization, and how different rates of increase of the inbreeding coefficient affect the outcome.

First results from this project have shown that, in accordance with earlier predictions, genes involved in housekeeping functions are up regulated with inbreeding (Kristensen *et al* 2002). Also, other genes of importance for the stress response and metabolism are affected by inbreeding (Kristensen T N, Sørensen P, Kruhøffer M, Pedersen K S and Loeschcke V, unpublished results). Our first result from array analyses on inbred lines also reveals that the up or down regulation of genes involved in stress resistance is more pronounced with fast compared to slow inbreeding (inbred to the same inbreeding level at different rates). This result is in support of the purging hypothesis stating that slow inbreeding reduces the inbreeding depression as deleterious alleles have been purged. Our hypothesis is that slow inbred lines therefore have a lower 'need' for gene products related to stress resistance.

3.2 *Identification and analysis of QTL for stress resistance*

Identification and analysis of QTL for stress resistance and their possible impact on stress resistance traits or longevity in *D. melanogaster* is another important aim of our work. The objective of this project is to identify QTL for

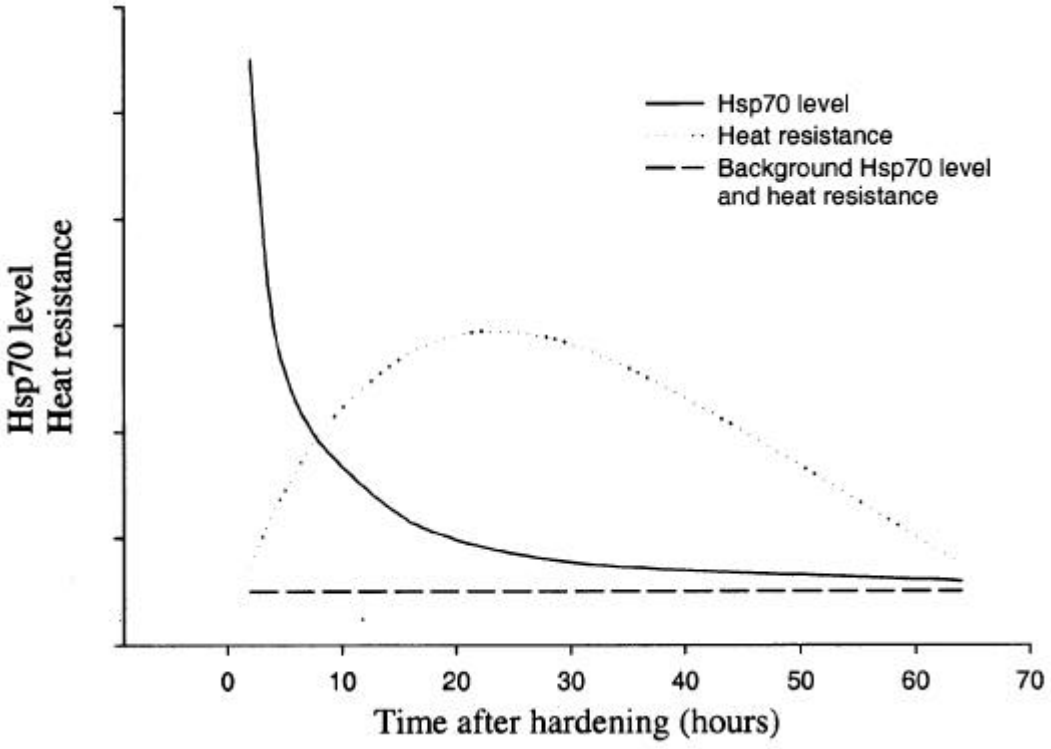

Figure 1. Hsp70 expression level and heat stress resistance after exposure to high but non-lethal temperature (time 0 to 1) in *D. melanogaster*. The benefits from this hardening treatment and Hsp70 level were measured at various times after hardening. Hardening increased survival and the Hsp70 level. However, the two processes do not coincide as Hsp70 level decreased much faster with time than heat stress resistance. Hsp70 does therefore only explain a part of the increased thermotolerance obtained by the hardening treatment (modified after data from Dahlgaard *et al* 1998 from Hoffmann *et al* 2003).

stress resistance and, additionally, to test if these QTL affecting specific stress resistance traits also affect other resistance traits or longevity, which is known to be highly correlated with resistance to environmental stress (Norry and Loeschcke 2002, 2003).

First analyses of QTL for knockdown resistance reveal a number of peaks (QTL) in regions of the chromosome that are known to carry candidates of importance for stress resistance, including several well-know heat shock genes (Norry *et al* 2004). Identified peaks usually contain many loci, on average between 50 and 100 per cM in *Drosophila*, so the identification of a peak does not necessarily imply that the most important gene(s) causing this peak has/have been identified. Fine scale mapping would reduce this problem and maybe followed up by studies of gene regulation using gene chips; however, QTL studies can complement other studies in supporting their conclusions as well as identify regions of importance that may not have received attention before.

3.3 *DNA-sequence variation in candidate genes*

Another level of biological organization besides gene regulation is variation in the DNA sequence. We intend to study DNA-sequence variation in candidate genes and its relation to variation in stress resistance. The objective of this project is to elucidate the significance of genetic variation at specific loci for stress resistance and test for a relation to 'natural' stress exposure in lines collected from different climatic gradients and by a comparison with laboratory selection lines.

Some earlier results of our group have shown ample variation in promoter and coding regions of some of the small heat-shock proteins (Frydenberg *et al* 2002) and latitudinal variation at two genes along a north-south gradient in eastern Australia (figure 2, Frydenberg *et al* 2003). These studies will be followed up by studies of other known or newly identified candidate genes, and hypotheses generated from results of laboratory selection lines will be tested on natural populations. Identification of clinal variation in expression levels or frequencies of naturally occurring alleles is a strong support for evolutionary importance of the genes and gene variants in question. Flies collected along latitudinal and altitudinal gradients in eastern Australia, Argentina and Canary Islands will be used in this part of the project.

3.4 *Population studies*

Population studies on quantitative genetic variation in stress resistance traits and related life history traits in populations originating from different climatic regions (e.g. Hoffmann *et al* 2002) will complement the molecular studies described above. The objective of these studies is to identify intraspecific variation in stress resistance traits and to relate this variation to the origin of the populations. We will test along altitudinal and latitudinal gradients, using independent sample sites. First results show that several resistance and life history traits show altitudinal and latitudinal variation. A screening of the natural populations showing differences in resistance will be performed in the projects mentioned above.

An interesting issue regarding climatic adaptation is which climatic characteristics have the largest impact on

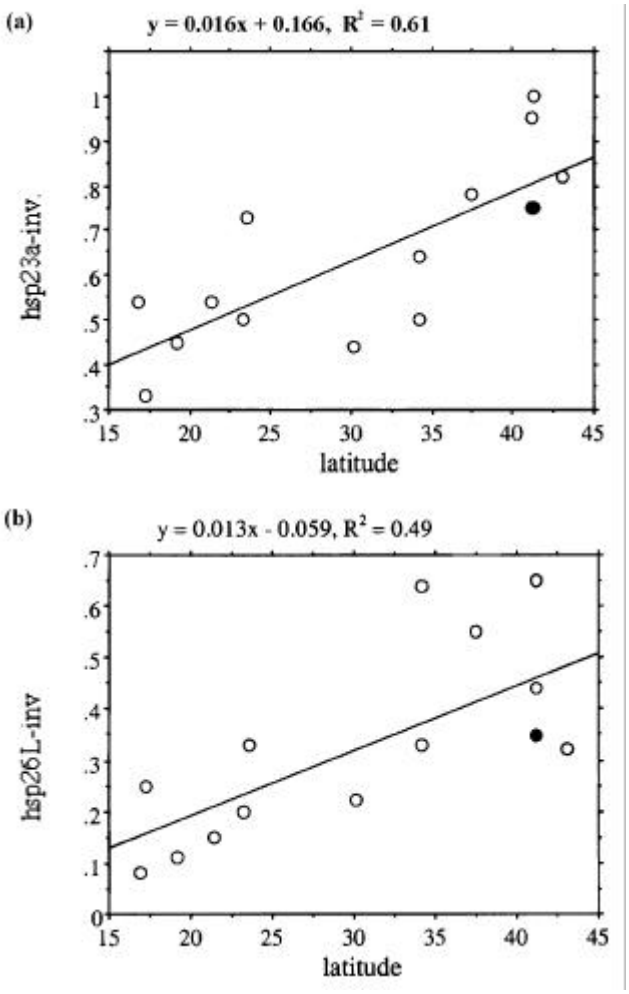

Figure 2. Haplotype frequencies in standard chromosomes of flies from 15 populations from the east coast of Australia and linear regression with latitude (degrees South). Individuals with inverted chromosomes of *In(3L)P*, in which the here studied loci are located, are not included, as the inversion frequency is also correlated with latitude. Filled black points show where two populations at similar latitude had the same allele frequency: (**a**) haplotype without a GGA insertion at *hsp23* and (**b**) haplotype with a nine base pair insertion at *hsp26* (modified from Frydenberg *et al* 2003, see also Frydenberg *et al* 1999).

populations and thereby constitute the most potent evolutionary force. While annual means in temperatures are well described and correlate well with latitude, this variable probably has little meaning for the adaptation of populations. More likely, the extreme events during days, seasons and years and the variation between time points provide the most difficult challenge for populations. We have made geographical collections in areas where ample knowledge of many climatic characters are available. From data relating population performance to different resistance tests and climatic variables, we can identify which laboratory measures of adaptation and which climatic factors are most ecologically relevant for environmental adaptation (Sarup P, Sørensen J G, Barker J S F, Dimitrov K and Loeschcke V, unpublished results).

Further, we wish to undertake population studies on the role of inbreeding, inbreeding depression and purging of the genetic load in stressful environments (Bijlsma *et al* 1999; Kristensen *et al* 2003) and on the role of hybridization, hybrid digenesis and its underlying genetic basis in stressful environments. Here the objective is to characterize the role of the rate of increase of the inbreeding coefficient, the role of purging and the accumulation of environment specific sensitive genes and to elucidate the effects of a break-up of co-adapted gene complexes on fitness in stressful environments. Optimised designs described by Wang *et al* (1999) and Frankham *et al* (2001) will be used for this purpose.

3.5 *Rates of evolution under stress*

In a number of experimental papers, stressful environmental conditions have been shown to affect the additive genetic variance (Hoffmann and Parsons 1991). Often an increase in additive genetic variance is found in stressful compared to non-stressful conditions, but the results are not consistent (Hoffmann and Merilä 1999). We want to investigate further the effect of environmental and genetic stress on evolutionary potential.

The response to selection (rates of evolution) for traits of adaptive importance such as heat stress and desiccation tolerance will be investigated under single or multiple stress conditions. Furthermore, we intend to look into whether correlated responses in traits associated with heat and desiccation stress resistance (such as longevity) depends upon the stress level under which selection is performed. Further, microsatellites will be used as markers to analyse genomic stability under environmental stress (such as heat) and genetic stress (such as inbreeding).

Apart from considering the importance of environmental stress on the evolutionary potential of populations, we also will investigate whether inbreeding and genetic drift increase the environmental sensitivity (as suggested by Lerner 1954) and reduce the additive genetic variance and heritability of fitness and non-fitness traits (fertility, heat stress resistance and sternopleural bristle number) in the manner suggested from population genetic models assuming neutrality and additive gene action (also investigated by Robertson 1952; Goodnight 1988; López-Fanjul *et al* 1989). Preliminary results suggest that the additive genetic variance does not behave as expected even for the trait sternopleural bristle number which is traditionally thought of as being additive and neutral, and that the environmental variance increases with inbreeding (Kristensen T N, Sørensen A C, Sorensen D, Pedersen K S, Sørensen J G and Loeschcke V, unpublished results).

3.6 *Physiological responses during acclimation to climatic stress*

The study of physiological responses during acclimation to climatic stress is done with the aim to perform an investigation of the physiological changes in *Drosophila* during acclimation to desiccation, drought, cold and heat stress using various techniques, including HPLC and NMR. Here, we will focus on membrane lipid composition and secondary metabolites. This will add information on the changes in and adaptive role of these traits, and complement data on the levels of DNA, RNA and protein. The physiological responses to genetic stress will also be studied using the same techniques.

3.7 *Applications in animal breeding and medicine*

The application of our results and the accumulating knowledge on mechanisms behind the stress response system may be useful in agricultural sciences/animal breeding. Using the stress response system as an indicator of the health status of domestic animals may allow developing a fast and early warning test system. Furthermore, animals may be selected based on levels of proteins known to be involved in stress resistance. This may be an efficient way to breed for more robust farm animals. Another interesting task is to bridge the existing gap to scientists outside Natural Sciences to getting the dialogue going with people working on psychic stress, gerontology and hereditary diseases related to protein misfolding. Psychophysiological or psychosocial stress also induce the heat shock response (Fukudo *et al* 1997; Lewthwaite *et al* 2002), and thus has important biological similarities with environmental stresses. The same applies for ageing and a number of diseases caused by misfolding of proteins. A number of 'housekeeping' genes are up regulated under such conditions (Bross *et al* 1999; Gregersen *et al* 2001, 2003; Pletcher *et al* 2002; Morrow and Tanguay 2003).

4. Summary and conclusions

With our work we intend to contribute to the growing body of knowledge on a highly relevant topic of modern biology, namely to relate variation at the DNA level to variation in function and phenotype, and ultimately to variation in fitness. We chose a model organism, *D. melanogaster*, whose genome is mapped, which allows us to apply a large suite of new molecular technologies for reaching our goals. As models of external and internal factors that affect phenotype and thereby fitness, we chose climatic stress and inbreeding/hybridization – which are relevant stresses in a globally changing world. Results from these models may also be relevant to other stresses, which, at least partly, induce similar mechanisms in response to stress as e.g. crowding (Sørensen and Loeschcke 2001), presence of predators (Kagawa and Mugiya 2000) and parasitism (Arif *et al* 1999; Rinehart *et al* 2002). Also from an ecological and experimental viewpoint the choice of our model organism has some strong advantages, as *Drosophila* is a higher organism that easily can be manipulated in the lab – and that embraces many species with known taxonomy and well-described and distinct ecologies, from cosmopolitan to specialist species.

Using natural and artificially selected genetic variants we are on the way to establish gene to function relations for genes involved in stress resistance. We have chosen either ecological gradients in the field, as latitudinal or altitudinal gradients, which are usually related to climatic gradients and to variation in life history traits. Flies collected along these gradients are screened for variation at candidate loci, as e.g. variation in the well-known heat shock protein genes or other genes with important known functions that relate to climatic gradients, as e.g. the Turandot and antibacterial peptide genes (Ekengren and Hultmark 2001). When finding allelic differences across ecological/climatic gradients, we look for comparative evidence in testing if these differences also can be seen in independent systems, so any explanation that involves genetic drift and founder effects can be excluded as the main factor of shaping these differences. Testing hypotheses on different islands or even continents provides strong support, if similar relations between allele frequencies and climatic variables can be shown. By mixing local populations collected along climatic gradients and forming a mass population, we can select for resistance to climatic variables that resemble natural conditions, and test if genetic variants that we related to certain climatic variables also get selected in the laboratory. To identify further candidate genes for climatic stress resistance a couple of molecular techniques can be used: one is especially common in modern animal breeding and aims at identifying QTL, pieces of the chromosome that have a major impact on a trait of interest and that statistically show up as peaks in the analysis. The technique requires that one has lines or populations that differ significantly with respect to the trait to be studied (e.g. > 3 SD), and identifies a piece of the chromosome that often contains still a high number of genes. In *D. melanogaster* one cM may still contain around 50–100 genes, and to maintain a fine scale mapping of this level is very laborious. QTL work can be complemented by gene expression profiles using gene chips that are commercially available, for *Drosophila* from Affymetrix. We have successfully applied this technique and identified a large number of putative candidate genes, which then again can be verified and further studied for significance in laboratory and natural populations. However, even though the *D. melanogaster* genome is mapped, the functions of more than 50% of the gene products are not well described. No doubt, groups of genes identified by gene chips will also contain a proportion of false positives – and we are working on the RNA level and not with gene products. But our experience supports the application of these molecular techniques that complement each other and population studies of stress resistance tests, and we expect that much progress will be made in coming years combining population studies with studies of DNA variation, creating hypotheses to be tested. This will not only contribute to our understanding of the role of the relation between variation at the DNA level and variation in phenotype and function (fitness), a classical and yet rather unsolved problem of evolutionary biology, but also enable us to identify genes of importance for coping with climatic and genetic stress that may be crucial for populations to survive global change.

In a second phase of the project, we will interact with scientists in agriculture, medicine and psychology for testing some of the implications of our findings. Questions regarding if the stress resistance genes in *Drosophila* also are relevant for domestic animals and whether heat shock protein expression levels are good indicators of the health status of such animals will be addressed. We will look at the relation between stress resistance and longevity as well as the role of mild stress in *Drosophila* and investigate if these relations have implications for human health and disease resistance. There might be strong similarities between psychophysiological or psychosocial stress on the one side and genetic and environmental stress on the other in that both induce heat shock proteins to prevent damage of the biological system. It seems that damaging effects are similar in that permanent stress is destructive, while short bouts of mild stress do not seem to be harmful and may even have positive effects.

Acknowledgements

We thank J S F Barker, H Boriss, O Bubliy, J Dahlgaard, K Dimitrov, J Frydenberg, J Justesen, M Hercus, A

Hoffmann, M Holmstrup, M Kruhøffer, M M Nielsen, F M Norry, K S Pedersen, C Pertoldi, S Rattan and P Sarup for collaboration on the discussed stress projects, for comments on previous versions of parts of this text and/or for discussion, to Doth Andersen for running the fly lab so smoothly, and for invaluable help over so many years, and the Danish Natural Sciences Research Council for continued financial support of our research via frame grants and a centre grant to VL.

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*e*Publication: 15 October 2004