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Igor Krupnik, Michael A. Lang, and Scott E. Miller
Editors

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Environmental and Molecular Mechanisms of Cold Adaptation in Polar Marine Invertebrates

Adam G. Marsh

ABSTRACT. The under-ice environment places extreme selective pressures on polar marine invertebrates (sea urchins, starfish, clams, worms) in terms of the low temperature, oligotrophic waters, and limited light availability. Free-swimming embryos and larvae face inordinate challenges of survival with almost nonexistent food supplies establishing near starvation conditions at the thermal limits of cellular stress that would appear to require large energy reserves to overcome. Yet, despite the long developmental periods for which these embryos and larvae are adrift in the water column, the coastal under-ice habitats of the polar regions support a surprising degree of vibrant marine life. How can so many animals be adapted to live in such an extreme environment? We all recognize that environmental adaptations are coded in the DNA sequences that comprise a species genome. The field of polar molecular ecology attempts to unravel the specific imprint that adaptations to life in a polar habitat have left in the genes and genomes of these animals. This work requires a unique integration of both field studies (under ice scuba diving and experiments) and laboratory work (genome sequencing and gene expression studies). Understanding the molecular mechanisms of cold adaptation is critical to our understanding of how these organisms will respond to potential future changes in their polar environments associated with global climate warming.

INTRODUCTION: THE NECESSITY OF SCIENCE DIVING

Looking across the coastal margins of most polar habitats, one is immediately struck by the stark, frozen wasteland that hides the transition from land to sea beneath a thick layer of snow and ice. Standing on the sea ice surface along any shore line above 70° latitude, it is hard to imagine that there is fluid ocean anywhere nearby, and even harder to think that there is even a remote possibility of animal life in such an environment. Yet under the five meters of solid sea ice, a rich and active community of marine organisms exists. The real challenge is getting to them.

Scientists studying how these organisms are adapted to survive and persist near the poles are limited by the logistical constraints in getting access under the ice to collect animals and plants for study. The sea ice cover establishes an effective barrier to using most of the common collection techniques that marine biologists employ from vessel-based sampling operations. The time and effort that is invested in opening a hole in the ice greatly precludes the number of sampling sites

Adam G. Marsh, Professor, College of Marine and Earth Studies, University of Delaware, 700 Pilottown Road, Smith Laboratory 104, Lewes, DE 19958, USA (amarsh@udel.edu). *Accepted 28 May 2008.*

that one can establish. Thus, from one ice hole, we need to be able to collect or observe as many animals as possible. To this end, scuba diving is an invaluable tool for the marine biologist because the ability to move away from the dive hole after entering the water greatly expands the effective sampling area that can be accessed from each individual hole. There is just no way to drill or blast a hole in the ice and drop a "collection-device" down the hole and get more than one good sample. The first deployment would collect what is under the hole, and after that, there is little left to collect. At present, there is still no better method (in terms of observational data, reliability, and cost effectiveness) than scuba diving for providing a scientist the necessary access to collect and study benthic marine invertebrates living along the coastal zones of polar seas.

Although scuba diving under harsh, polar conditions is difficult and strenuous and not without risks, it is an absolute necessity for scientists to work in the water under the ice. We need to be able to collect, observe, manipulate, and study these unique polar marine invertebrates in their own environment. This is especially true for the developing field of environmental genomics, where scientists are attempting to decipher the molecular and genetic level changes in these organisms that make them so successful at surviving under extreme conditions of cold, dark and limited food. This paper will describe how important it is to ultimately understand how these adaptations work in the very sensitive early life stages of embryos and larvae, and how efforts to begin culturing these embryos and larvae under *in situ* conditions under the sea ice will contribute to this greater understanding.

ADAPTATIONS IN POLAR MARINE INVERTEBRATES

Antarctic marine organisms have faced unique challenges for survival and persistence in polar oceans and seas. The impacts of low temperatures and seasonally limited food availability have long been recognized as primary selective forces driving the adaptational processes that have led to the evolution of many endemic species in Antarctica (Clarke, 1991; Peck et al., 2004; Portner 2006; Clarke et al., 2007). Many elegant studies have demonstrated a wide-array of specific molecular adaptations that have been fixed in specific genera or families of Antarctic fish, including chaperonins (Pucciarelli et al., 2006), heat shock proteins (Buckley et al., 2004; Hofmann et al., 2005), red blood cells (O'Brien and Sidell, 2000; Sidell and O'Brien, 2006), tubulin kinetics (Detrich et al., 2000), and anti-freeze proteins (Devries and Cheng, 2000; Cheng et al., 2006). In contrast, the work with invertebrates has been less molecular and more focused on physiology and ecology, in terms of identifying adaptations in life-history strategies (Pearse et al., 1991; Poulin and Feral, 1996; Pearse and Lockhart, 2004; Peck et al., 2007), secondary metabolites (McClintock et al., 2005), energy budgets and aging (Philipp et al., 2005a; Philipp et al., 2005b; Portner et al., 2005, Portner, 2006), and an ongoing controversy over the ATP costs of protein synthesis (Marsh et al., 2001b; Storch and Portner, 2003; Fraser et al., 2004; Fraser and Rogers, 2007; Pace and Manahan, 2007).

Although much progress has been made over the last decade, we still barely have a glimmer as to the full set of adaptations at all levels of organization that have guided species evolution in polar environments. Each genetic description, each physiological summary provides a snapshot of a component of the process, but we are still much in the dark as to how all the expressed phenotypes of an organism are integrated into one functional whole, upon which selection is active. The survival of any one individual will depend upon the integrated effectiveness of "millions" of phenotypic character states ranging from molecular to organismal level processes. *How do you apply one snap shot to such a broad continuum spanning different organizational scales?* At present, there is a daunting lack of any specific indications of the "key" genetic adaptations in single gene loci of any marine invertebrate. This is in stark contrast to the literature that exists for adaptations in fish genes and microbial genomes (Peck et al., 2005). Without the guidance of knowing "where to look," we are faced with identifying the best strategy for surveying an entire genome to pinpoint any possible genetic adaptations to survival in polar environments.

The unique feature of cold-adaptation in polar marine invertebrates is that they are always exposed to a near-freezing temperature. Their entire lifecycle must be successfully completed at −1.8°C (from embryo development to adult gametogenesis). Cold water (−2 to 2°C) is an extreme environmental condition because of the complexity of the hydrogen bonding interactions between water molecules at the transition between liquid and solid phases. For a simple three atom molecule, the structure of water near freezing is very complex, with over four solid phases and the potential for two different liquid phases at low temperatures. In polar marine invertebrates, significant changes in the molecular activity of water at the liquid-solid inter-

face (−1.8°C) are likely to pose a strong selective force on biochemical and molecular function. Understanding how some organisms have adapted to this level of natural selection will provide information about the essential set of genetic components necessary for survival at low temperature margins of our biosphere.

Embryos in the Cold

The fact that polar marine invertebrates can maintain a complex program of embryological development at low temperatures has received much discussion in the literature in terms of life-history adaptations. Of these ecological studies, Thorson's rule has provided a focal point for numerous considerations of why development is so prolonged in marine invertebrates at high latitudes (Pearse et al., 1991; Pearse and Lockhart, 2004). The limited availability of food in polar oceans has lead to uncertainty regarding the relative importance of low food availability compared with low temperatures as the primary selective force limiting developmental rates (Clarke, 1991; Clarke et al., 2007). Overall, limits on metabolism have now received considerable attention in the literature and a general synthesis of the physiological constraints on organismal function in polar environments now appears to primarily involve cellular energetics at molecular and biochemical levels (Peck et al., 2004; Peck et al., 2006a; Peck et al., 2006b; Clarke et al., 2007). Recent work with the planktotrophic larvae of the Antarctic sea urchin, *Sterechinus neumayeri*, has demonstrated that changes in the nutritional state of this feeding larvae do not alter its rate of early larval development (Marsh and Manahan, 1999; 2000). Low temperatures are likely a primary selective force and these larvae exhibit unique molecular adaptations to conserve cellular energy (Marsh et al., 2001b). This may be a general characteristic of polar invertebrate larvae, and could account for the predominance of nonfeeding developmental modes found among benthic, macrofaunal invertebrates in Antarctica, particularly among echinoderms.

Embryo Energy Metabolism

One of the most striking characteristics of embryonic development in Antarctic marine invertebrates is the slow rate of cell division. In the sea urchin *S. neumayeri*, early cleavage has a cell cycle period of 12 h, which is an order of magnitude slower than in a temperate sea urchin embryo at 15°C. In the Antarctic asteroid *Odontaster validus* the embryonic cell cycle period is just as long, and in the Antarctic mollusk *Tritonia antarctica*, it is extended to almost 48 h (Marsh, University of Delware, unpublished data). In general we assume that cell division is linked or coordinated to metabolic rates and that the increase in cell cycle period results from an overall decrease in metabolic rate processing at low temperatures. Embryos of *S. neumayeri* are sensitive to changes in temperatures around 0°C. Between −1.5°C and +0.5°C, cell division exhibits a large change in the cycle period that is equivalent to a Q10 value of 6.2 (*i.e.*, a 6.2-fold increase in cell division rates if extrapolated to a Δ10°C temperature difference, Figure 1A). A Q10 greater than 3 indicates the long cell division cycles are determined by processes other than just the simple kinetic effects of temperature on biochemical reaction rates. In contrast, metabolic rates in *S. neumayeri* do not evidence a change over this same temperature gradient (Δ2°C; Figure 1B). There is no difference in oxygen consumption rates in embryos at the hatching blastula stage between +0.5°C and −1.5°C, despite large differences in cell division rates. This directly implies that the cell cycle period is not determined by a functional control or coordination to metabolic rates. The *S. neumayeri* results suggest that embryo development is not tied to metabolism at these low temperatures and the current notions of a selective mechanism that could favor patterns of protracted development by direct coordination to metabolic rates (ATP turnover) remain to be investigated.

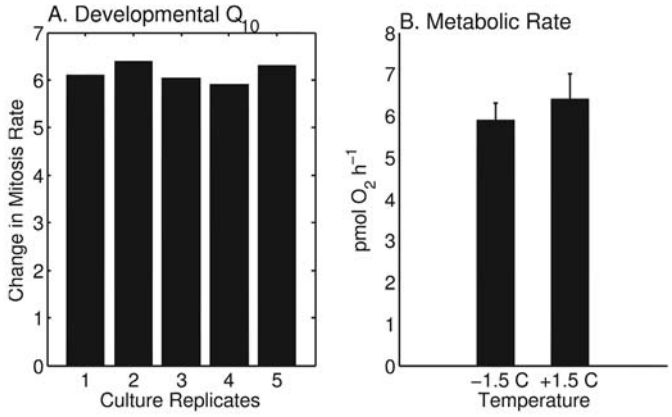

FIGURE 1. Temperature Q_{10} for developmental rates and energy utilization in the Antarctic sea urchin *Sterechinus neumayeri*. (A) Embryogenesis at +1.5°C was much faster than at –1.5°C and was equivalent to a 6-fold rate change per 10°C (i.e., Q10 estimate). (B) The metabolic rates of hatching blastulae at these two temperatures did not reveal any impact of temperature (i.e., Q10 ~ 0).

Embryo Molecular Physiology

Some embryos of polar marine invertebrates appear to have specialized programs of gene expression that suggest a coordinated system of activity as a component of metabolic cold-adaptation. Most notable are the recent findings in *S. neumayeri* embryos that mRNA synthesis rates are ~5-fold higher than in temperate urchin embryos (Marsh et al., 2001b). These data were determined from a time course study of whole-embryo RNA turnover rates and show that despite a large difference in environmental temperatures (~ Δ25°C) rates of total RNA synthesis are nearly equivalent. In comparing the rate constants for the synthesis of the mRNA fraction there is a clear 5x upregulation of transcriptional activity in the *S. neumayeri* embryos. What we need to know about this increased transcriptional activity is whether or not the upregulation of expression is limited to a discrete set of genes, or represents a unilateral increase in expression of all genes. In order to perform these kinds of studies, we need to be able to work with embryos and larvae in their natural environment under the sea ice.

In addition to deciphering the magnitude of changes in transcriptional activities, we are now just beginning to realize the importance of how transcript levels may vary among individuals within a cohort. Variation is a necessity of biological systems. We generally think in terms of point mutations when we conceptualize the underlying basis of how individual organisms differ from one another within a species, and how novel phenotypes arise through the slow incremental accumulation of changes in nucleotide sequence (evolution). At odds with this ideology is the observation that human and chimpanzee genes are too identical in DNA sequence to account for the phenotypic differences between them. This lead A.C. Wilson (King and Wilson, 1975) to conclude that most phenotypic variation is derived from differences in gene expression rather than differences in gene sequence. Microarray studies are now revealing to us an inordinate amount of variation in gene expression patterns in natural populations, and we need to understand both the degree to which that variance may be determined by the environment, and the degree to which that variance may be significantly adaptive.

Although it is clear that interindividual variance in gene expression rates is a hallmark of adaptation and evolution in biological systems, at present, only a few studies have looked at this variation and the linkages to physiological function in field populations. For Antarctic marine invertebrates, most of the molecular and biochemical work looking at adaptations in developmental processes has focused on trying to find "extraordinary" physiological mechanisms to account for the adaptive success of these embryos and larvae. But what if the mechanism of adaptation is not extra-ordinary for polar environments? What if the mechanism is just "ordinary" environmental adaptation: natural selection of individual genotype fitness from a population distribution of expressed phenotypes. Understanding adaptive processes in early life-history stages of polar marine invertebrates will ultimately require an understanding of the contribution that interindividual variance in gene expression patterns plays in determining lifespan at an individual level, survival at a population level and adaptation at a species level in extreme environments.

THE PROCESS OF ADAPTATION

Natural selection operates at the level of an individual to remove less-fit phenotypes from subsequent generations. However, it is clear that a hallmark of biological systems is the "maintenance" of interindividual variance among individuals at both organismal (Eastman 2005) and molecular levels (Oleksiak et al., 2002; Oleksiak et al., 2005; Whitehead and Crawford, 2006). Although early life-history stages (embryos and larvae) are a very good system for looking at selective processes because there is a continual loss of genotypes/phenotypes during development, they are difficult to work with in terms of making individual measurements to describe a population (cohort) distribution. Their small size limits the amount of biomass per individual and consequently most of what we know about molecular and physiological processes in polar invertebrate larvae is derived from samples where hundreds to thousands of individuals have been pooled for a single measurement.

However, methodological advances have allowed for quantitative measurements of molecular and physiological rate processes at the level of individual larvae in terms metabolic rates (Szela and Marsh, 2005), enzyme activities (Marsh et al., 2001a), and transcriptome profiling (Marsh and Fielman, 2005). We are now beginning to understand the ecological importance of assessing the phenotypic variance of characteristics likely experiencing high selective pressures. In Figure 2, the phenotype distributions of two species are presented to illustrate the significant functional difference between how a change in the mean metabolic rate of a cohort (A) could be functionally equivalent to a change in the variance of metabolic rates within a cohort (B), where a decrease in metabolic rates (equivalent to an increase in potential larval lifespan) could arise from either

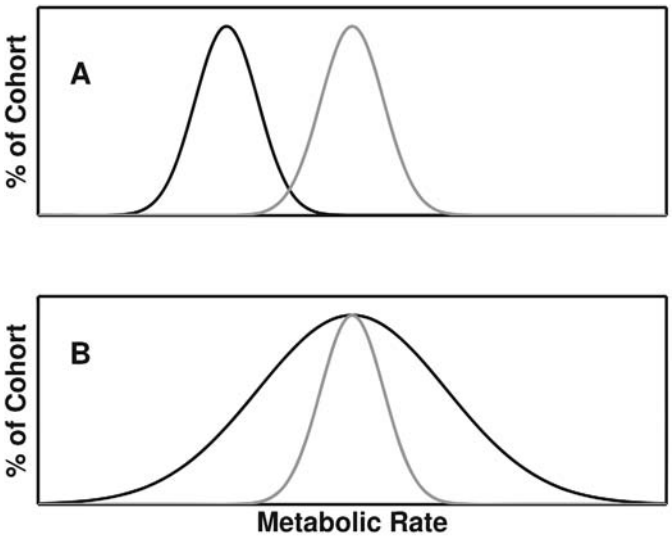

FIGURE 2. Illustrated selection shifts in the frequency distributions of larval metabolic rates in an environment favoring greater energy conservation (black) over one that does not (gray). A change in metabolic efficiency within a cohort could arises at either the level of: (A) the mean phenotype or (B) the interindividual variance around the same mean in the phenotype.

process. Natural selection acts at the level of the phenotype of an individual, not at the level of the mean phenotype of a population or cohort. Thus, in order to understand most of the fine-scale biological processes by which organisms are adapted to polar environments (integrated phenotypes from multiple gene loci), we must be able to describe the distribution of the potential phenotypic space encoded by a genome relative to the distribution of successful (surviving) phenotypes at a cohort (population) scale.

Hofmann et al.'s recent review (2005) of the application of genomics based techniques to problems in marine ecology clearly describes a new landscape of primary research in which it is possible to pursue the mechanistic linkages between an organism and its environment. One of the most interesting aspects of this revolution is the use of microarray hybridization studies for assessing gene expression profiles to identify the level of interindividual variance that does exist in gene expression activities within a population (Oleksiak et al., 2005; Whitehead and Crawford, 2006). Although there are clearly some primary responses that organisms exhibit following specific environmental stresses or cues in terms of gene up- or down-regulation (Giaever et al., 2002; Huening et al., 2006), the power of assessing the expression patterns of thousands of genes simultaneously has opened an intriguing avenue of biological research: *Why are gene expression patterns so variable, where is the source of that variation, what determines the adaptive significance of this variation?* Although the mechanistic linkage between gene expression events and physiological rate changes may yet remain obscure, it is clear that a significant level of biological variance is introduced at the transcriptome level, and the degree to which that variance may be significantly adaptive requires exploration (Figure 2).

Hypothesis

Overall, this research focus is attempting to describe a component of environmental adaptation as an integrative process to understand the mechanisms that may contribute to long lifespans of larvae in polar environments. *The over arching hypothesis is that embryos and larvae from the eggs of different females exhibit substantial variation in transcriptome expression patterns and consequently metabolic rates, and that these differences are an important determinant of the year-long survival of the few larvae that will successfully recruit to be juveniles.*

Big Picture

Variation is an inherent property of biological systems. We know that genetic variation generates phenotypic variation within a population. However, we also know that there is more phenotypic variation evident within a population than can be accounted for by the underlying DNA sequence differences in genotypes. Much recent attention has been focused on the role of epigenetic information systems in regulating gene expression events, but there has been no consideration yet of the contribution of epigenetics to the level of variation in a phenotypic character, whether morphological, physiological, or molecular. This idea focuses on a potential genome-wide control that could serve as a primary gating mechanism for setting limits on cellular energy utilization within a specific individual, while at the same time allowing for greater potential interindividual variance in metabolic activities within a larval cohort. Understanding the sources of variation in gene transcription rates, metabolic energy utilization, and ultimately lifespans in polar larvae (i.e., the "potential" range in responses to the selection pressures in polar environments) is essential for our understanding of how populations are adapted to cold environments in the short-term, and ultimately how some endemic species have evolved in the long term.

Within a cohort of larvae, it is now likely show that the variance at the level of gene expression events is

amplified to a greater level of expressed phenotypic variance. Thus, subtle changes in gene promoter methylation patterns (epigenetics) may have very pronounced impacts on downstream phenotypic processes (physiological energy utilization). Describing how genomic information is ultimately expressed at a phenotypic level is vital for our understanding of the processes of organismal adaptation and species evolution. Although phenotypic plasticity is documented in marine organisms (particularly with regard to metabolic pathways), what is absolutely novel in this idea is that we may be able to demonstrate how changes in the phenotype distribution within a cohort of a character such as metabolic rates can routinely arise independently of genetic mutations (*i.e.,* epigenetic controls). Conceptually, almost all studies of the regulation of gene expression events have been focused on a functional interpretation at the level of the fitness of an individual. Our work to describe the variance in the distribution of expression rates within a group of larvae opens up a new dimension of trying to describe adaptational mechanisms at the larger level of total cohort fitness.

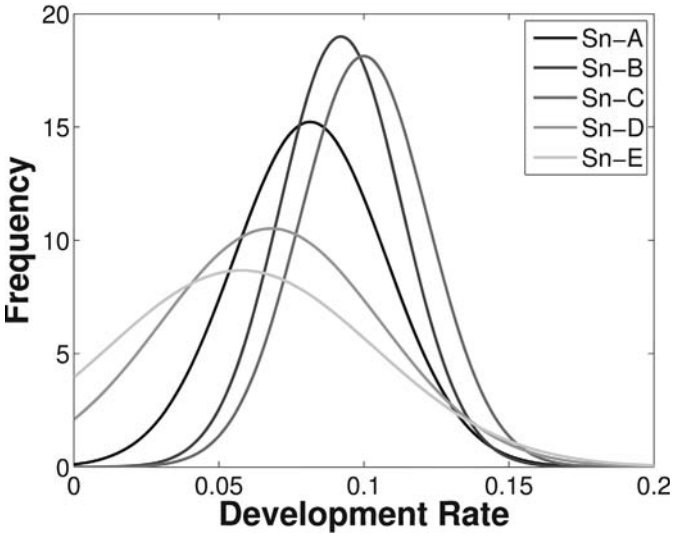

FIGURE 3. Normal Distributions of development rates in late morula embryos of *S. neumayeri* produced from different females. After fertilization, individual zygotes were transferred to 96-well plates and then scored individually for the time to reach 2-, 4-, 8-, 16-cell, and morula stages (n = 90 for each distribution).

INTERINDIVIDUAL VARIATION IN EMBRYOS AND LARVAE

Development

Embryos and larvae are rarely considered as populations of individuals. They are normally just cultured in huge vats and mass sampled with the assumption that there is negligible interindividual variance among sibling cohorts. In *S. neumayeri*, we have observed substantial functional differences in the distribution of "rate" phenotypes when the effort is made to collect data at the level of individual embryos and larvae. In Figure 3, eggs from five females were fertilized and individual zygotes of each were scored for their rate of development to the morula stage (~4 days at −1.5°C). Even in this short span of time, there was clearly a difference in the embryo performance from different females with an almost two-fold variance in the mean cohort rates and an order of magnitude difference in the rates among all individuals. We normally think faster is better, at least in temperate and tropical marine environments, but is that the case in polar environments? *Are the fastest developing embryos and larvae the ones that are the most likely to survive and recruit 12 months later?*

Energy Metabolism

A novel methodology for measuring respiration rates in individual embryos and larvae has been developed for measuring metabolism in many individual embryos or larvae simultaneously (Szela and Marsh, 2005). This high-throughput, optode-based technique has been successfully used to measure individual respiration rates in small volumes (5 ul) as low as 10 pmol O_2 x h^{-1}. The largest advantage of this technique is that hundreds of individuals can be separately monitored for continuous oxygen consumption in real-time. The most striking observations we have made so far with *S. neumayeri* embryos is that there are large differences in metabolic rates between individuals and that these differences appear to be influenced by the eggs produced by different females (Figure 4). Understanding the distribution of metabolic rates among individuals within a cohort of embryos or larvae is critical for understanding how metabolic rates may be "tuned" to polar environments.

In Figure 4, the most intriguing aspect of the distributions is the 2- to 3-fold difference in metabolic rates that can be found among individual embryos. Clearly there is a large degree of interindividual variance in the cellular rate processes that set total embryo metabolism, and we need to understand the mechanistic determinants of that variance. What we need to know now is how the biological variance at the level of the transcriptome (gene expression rates) impacts the variance at the level of physiological function (respiration rates). To date, the embryos and larvae of most polar invertebrates studied appear to evidence a strategy of metabolic down-regulation with the apparent

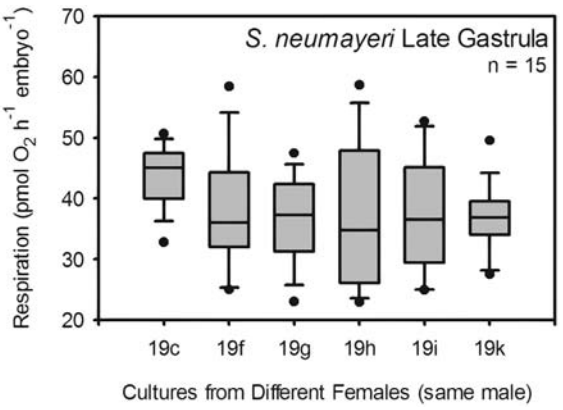

FIGURE 4. Distribution of respiration rates in late gastrula embryos of *S. neumayeri*. Eggs from 6 females were fertilized with the sperm from one male and maintained as separate cultures. The distribution of metabolic rates in these cultures is not equivalent (n = 15 for each culture; 90 individuals total).

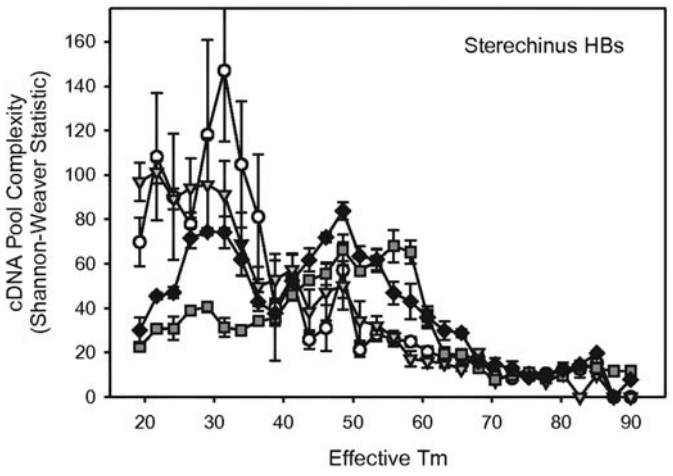

FIGURE 5. Individual cDNA libraries were constructed for four embryos and profiled for sequence complexity using a novel approach to measuring reannealing kinetics. Large differences in the sequence distribution and abundance of the transcriptome pool are evident among these sibling embryos.

effect of extending larval lifespan (Peck et al., 2004; Peck et al., 2005; Peck et al., 2006a; Peck et al., 2006b). That is the observation at a population level. At an individual level this could be achieved by one of two mechanisms: (1) embryos in a cohort could maintain the same relative distribution of metabolic rates around a lower mean rate (as in Figure 1A), or (2) embryos in a cohort could express a larger degree of interindividual variance (stochastic regulation) in respiration rates such that a larger fraction of the cohort would have lower metabolic rates that might concomitantly contribute to extended larval lifespans (as in Figure 1B).

Gene Expression

Physiological changes in metabolic rates must have an underlying basis in molecular events associated with gene expression rates. In order to compare changes within embryos or larvae to changes in gene activities, a novel methodology based on reannealing kinetics is employed for the rapid, high-throughput, efficient, and economical profiling of the sequence complexity of a transcriptome (Marsh and Fielman, 2005; Hoover et al., 2007a; b; c). Measuring renaturation rates of cDNA along a temperature gradient can provide information about the transcript sequence complexity of a nucleic acid pool sample. In our assay, the reannealing curves at discrete temperature intervals can be described by a second-order rate function. A full kinetic profile can be constructed by analyzing all the curves using an informatics statistic (Shannon-Weaver entropy) for individual *S. neumayeri* embryos (hatching blastula stage). In Figure 5, each point represents the total mRNA pool complexity at a given Tm class with a mean (sd) of 4 duplicate assays at each Tm. Our novel revelation is that these mRNA pools among embryos are not identical. We can detect discrete differences in the distribution and abundance of component transcripts at the level of these individuals, even though they are all apparently progressing through the same developmental program. Thus, there is a large variance component that arises at the level of gene expression within these Antarctic sea urchin embryos.

One of the most prominent mechanisms determining gene expression patterns is the system of chemical modifications on DNA that establish an epigenetic pattern of information. Epigenetic processes refer to heritable molecular structures that regulate gene expression events independent of any DNA nucleotide sequence within a genome. There is currently a clear recognition that a large component of gene regulation can operate at this level of local DNA structure and composition and that DNA methylation is one of the dominant mechanisms. Methylation of promoter domains is one of the key mechanisms that can account for temporal patterns of differential gene expression during embryological development (MacKay et al., 2007; Sasaki et al., 2005; Haaf, 2006). During cell division, this genome methylation pattern is re-established with high fidelity in daughter cells by a suite of methyl-transferases immediately after DNA synthesis. Consequently, an established regulatory "imprint" can be perpetuated during development and across generations. In this sense, methylation serves as a cell-based

memory system for maintaining a pattern of gene expression regulation.

Methylation is also evident in invertebrate genomes, although the distribution of methylated sites appears to be more variable than in vertebrates, being mainly concentrated within areas of gene loci (Levenson and Sweatt, 2006; Schaefer and Lyko, 2007; Suzuki et al., 2007). Some invertebrates exhibit mammalian-type levels of DNA methylation (up to 15 percent of all cytosine residues methylated). In many invertebrates, DNA methylases appear to be very active during development (Meehan et al., 2005) and we can measure ~4 percent methyl-cytosine levels in *S. neumayeri* (Kendall et al., 2005). More importantly, the presence/absence of specific methylation sites within a genome can be rapidly assessed using an Amplified Fragment Length Polymorphism derived assay. The key observations here are that methylation fingerprints can be rapidly assessed following experimental treatments, and that methylated sites in gene promoters can be identified and scored separately (Kaminsk et al., 2006; Rauch et al. 2007). The number and diversity of methylation sites we can identify in early *S. neumayeri* embryos indicates an active DNA methylation system that could be generating the variance in metabolic rates and developmental rates that we have observed.

DIVING WITH EMBRYOS AND LARVAE UNDER THE ICE

The previous sections have documented the prevalence of a high component of interindividual variance among individual embryos of *S. neumayeri*. In order to fully understand the implications for this variance in terms of its impact on the survival of a cohort of larvae and the persistence of a sea urchin population through time, we need to study lots of individual larvae under natural conditions. This is absolutely impossible to do in a laboratory setting because of the large volumes of sea water that would have to be maintained. As an alternative, a pilot program is underway to investigate the success of culturing embryos and larvae in flow-through containers under the sea ice in McMurdo Sound. The concept is simple: Let the embryos and larvae grow naturally without investing much time or effort in their husbandry.

Current efforts to mass culture sea urchin larvae utilize 200-liter drums stocked at densities of ~10 per ml. At those densities, the water needs to be changed every two days. One water change on one 200 liter drum can take 2 hours in order to "gently" filter all the larvae out first and then put them into another clean 200 liter drum. The word *gently* is emphasized here for irony, because there is nothing gentle about using a small-mesh screen to filter out all the embryos and larvae. It is a very physically stressful process, and mortality rates are significant.

An alternative approach utilizing in situ chambers could allow for the embryos and larvae to develop without human intervention. The under ice environment around McMurdo Station is very stable and the epiphytic fouling community of organisms is almost nonexistent. In Figure 6, photographs of under ice culture bags in use in McMurdo Sound are shown. These trial bags were fitted with two open ports having a 40 μm mesh Nytek screen coverings so that water could be freely exchanged through the bag. Low densities of embryos from different marine

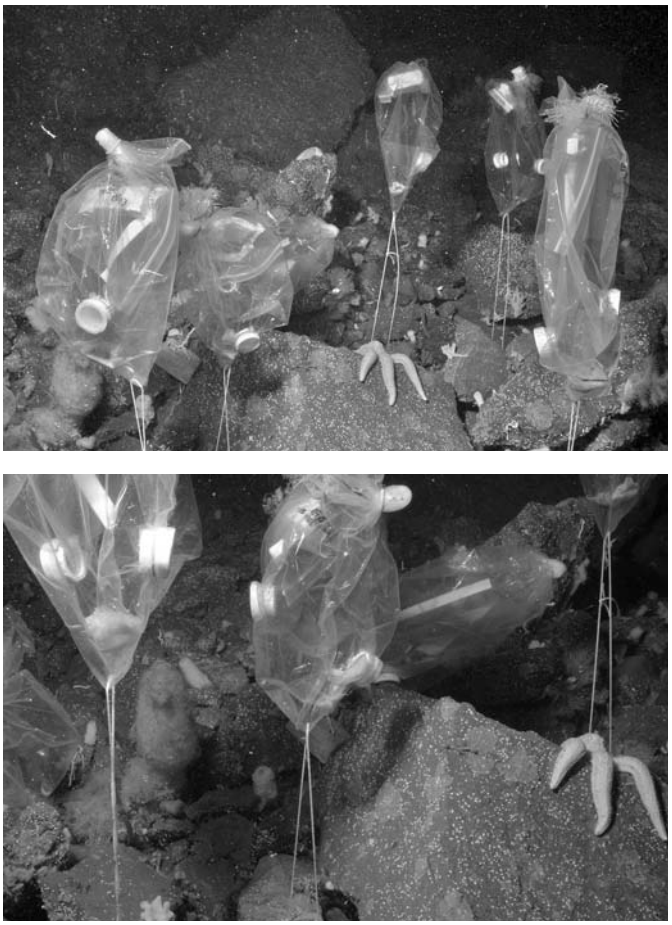

FIGURE 6. Culture bags with embryos and larvae of different marine invertebrates are tethered to the bottom rubble of the McMurdo Jetty under 6 m of solid sea ice and 25 m total water depth (photos by Adam G. Marsh).

invertebrates were placed in the bag and sampled at different time intervals. We have been successful at culturing larvae of the clam *Laternula eliptica* for over 13 months under the ice, and recovering fully metamorphosed juveniles at the end of that time period.

The ability to place embryos and larvae under the ice in culture containers in the Austral summer, then leave them in place through the winter season, means that we now have the potential to work with the full lifecycle of some marine invertebrate larvae. Because of the slow developmental rates of these larvae and protracted lifespans, there are relatively few studies that have been able to collect any data on the later life-stage as they approach metamorphosis. However, divers can now establish cultures in situ under the ice, then return at any time point later on to sample individuals. This increase in the time span for which we can now study will fill in the large gap in our current knowledge of what happens at the end of the Austral winter period when the larvae are ready to become juveniles.

In order to understand how larvae are adapted to survive in polar environments, we really need to make our best measurements and execute our most exact experiments on the individuals that have survived for the complete developmental period and are now ready to become juveniles. If only 10 percent of a cohort survives to this stage (+12 months development), then making early measurements on the other 90% (at 1 month) that were destined to die would essentially just give you information about what *does not* work. It is the survivors that hold the key to understanding how these organisms are adapted to persist in a harsh polar environment. In essence, all the existing studies on adaptations in polar marine larvae that have made measurements from bulk cultures at early developmental time points could be grossly misleading. All of those individuals are not likely to survive the full year to recruitment. Consequently, we need an experimental culturing system that will allow scientists to work with larvae that have survived the harsh polar environment. Those are the individuals that have the key to understanding adaptive processes.

Overall, the *in situ* culturing approach offers us three main advantages:

1. Large numbers of individuals can be cultured with relatively little husbandry effort. Once the culture containers are setup and stocked, then the only effort necessary is for a dive team to periodically sample and remove individuals. There is no feeding and little maintenance required.
2. Larvae can be cultured under very natural conditions without laboratory artifacts. The most important variable to control is temperature and by not having open culture containers in an aquarium room, there is no worry about the temperature in the vessels changing because of problems with electricity supply, pumps breaking down and losing the cold sea water supply rate, or someone just changing the thermostat within the aquarium room. The second most important variable is food, and under in situ conditions, the feeding larvae will receive a diet of natural species and in a natural supply.
3. Long term cultures can be maintained across the entire developmental period, which in most polar marine invertebrates can easily extend upwards of a year. This approach will provide access to larvae that have successfully survived their full lifecycle in a harsh polar environment.

CONCLUSION

The *S. neumayeri* distributions in developmental rate (Figure 3), respiration distributions (Figure 4), transcriptome profiles (Figure 5), and gene methylation (data not shown) have focused our attention on trying to understand the functional significance of interindividual variability at these levels of biological organization. In a life-history model that selects for a prolonged larval lifespan, it is intriguing to ask whether or not it is a reduction in individual metabolic rates (Figure 2A) or an increase in the cohort variance in metabolic rates (Figure 2B) that could account for the adaptation in metabolic phenotypes. The metabolic lifespans of polar invertebrate larvae could be under the same genetic determinants as other temperate species, but changes in patterns of gene regulation could substantially alter the distribution of physiological phenotypes within a cohort. Being able to study long-lived larvae that are ready to become juveniles holds the key for deciphering the adaptive mechanism that may be operative at the level of a full cohort to ensure that some percentage is capable of surviving. Selection is surely not operating to force the survival function of all individuals within a cohort. Only enough need to survive to keep a population established and stable.

Scientific diving will be an important component of discovering how these animals are adapted to survive. The opportunity to now work *in situ* with embryos and larvae will open new avenues of research and understanding. Even though the under ice work is not complex, it is nonetheless

rigorous and demanding. Any diver working on the bottom for more than 45 minutes will readily attest to the extreme nature of the cold that impacts all organisms in that environment. Being in the water gives one a unique perspective on the survival challenges that are facing the embryos and larvae of these polar marine invertebrates.

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