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Title: Similarities in temperature-dependent gene expression plasticity across time-scales in threespine stickleback (*Gasterosteus aculeatus*)

Running title: Gene expression plasticity across time-scales

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Abstract

Phenotypic plasticity occurs at a variety of time-scales, but little is known about the degree to which plastic responses at different time-scales are associated with similar underlying molecular processes, which is critical for assessing the effects of plasticity on evolutionary trajectories. To address this issue, we identified differential gene expression in response to developmental temperature in the muscle transcriptome of adult threespine stickleback (*Gasterosteus aculeatus*) exposed to 12, 18, and 24 °C until hatch and then held at 18 °C for nine months, and compared these results to differential gene expression in response to adult thermal acclimation in stickleback developed at 18 °C and then acclimated to 5 and 25 °C as adults. Adult thermal acclimation affected the expression of 7,940 and 7,015 genes in response to cold and warm acclimation, respectively, and 4,851 of these genes responded in both treatments. In contrast, the expression of only 33 and 29 genes were affected by cold and warm development, respectively. The majority of the genes affected by developmental temperature were also affected by adult acclimation temperature. Many genes that were differentially expressed as a result of adult acclimation were associated with previously identified temperature-dependent effects on DNA methylation patterns, suggesting a role of epigenetic mechanisms in regulating gene expression plasticity during acclimation. Taken together, these results demonstrate similarities between the persistent effects of developmental plasticity on gene expression and the effects of adult thermal acclimation, emphasizing the potential for mechanistic links between plasticity acting at these different life stages.

Introduction

Many organisms are able to respond to changes in environmental conditions by altering their phenotype, a phenomenon known as phenotypic plasticity. Phenotypic plasticity can be adaptive, maladaptive, or neutral (Ghalambor *et al.* 2007), and both maladaptive and adaptive phenotypic plasticity are thought to be important factors that can influence evolutionary trajectories (Ghalambor *et al.* 2015; Hendry 2016). However, there remains substantial debate as to whether phenotypic plasticity typically impedes or accelerates evolutionary change (Hendry 2016). The majority of phenotypic plasticity is ultimately the result of the differential regulation of gene expression (Schlichting & Smith 2002), and it has been suggested that understanding the molecular processes underlying this phenotypic plasticity is an important step in evaluating its effects in an evolutionary context (Schneider *et al.* 2014; Pfennig & Ehrenreich 2014).

Plastic responses can occur over a wide range of time-scales both across generations and within an individual organism's lifetime. Within-individual plasticity can, in turn, be divided into two broad classes acting at different life-history stages (Beaman *et al.* 2016). In the first class, which is typically termed developmental plasticity, the environment encountered during early life alters developmental trajectories. This type of plasticity is considered to result in a stable change in phenotype that lasts for the duration of the organism's lifetime, despite changes in the adult environment. The second class of phenotypic plasticity, which is often termed phenotypic flexibility (Piersma & Drent 2003), involves rapid and reversible changes in phenotype. This rapid and reversible plasticity includes processes such as physiological acclimation in adult organisms. There is substantial debate in the literature as to whether plasticity operating at different life stages operates via similar or different mechanisms (Kingsolver *et al.* 2002; Shintani & Ishikawa 2007; Kristensen *et al.* 2008; Colinet & Hoffmann 2012; Teets & Denlinger 2013). Thus, it is unclear whether developmental plasticity and phenotypic flexibility are mechanistically linked and whether these plastic responses across

different life stages are under similar selective constraints (Gerken *et al.* 2015; Beaman *et al.* 2016). The ability to comprehensively measure changes in gene expression through the use of high throughput sequencing technologies provides an unbiased and powerful approach to better understand the mechanistic relationship between plasticity at different life stages (Aubin-Horth & Renn 2009).

In ectothermic organisms such as fishes, body temperature closely mimics that of the environmental temperature. Consequently, changes in environmental temperature have been shown to have pervasive effects on biochemical and physiological processes, including profound changes in gene expression. In fishes, gene expression plasticity in response to changes in environmental temperature has been predominately investigated by manipulating juvenile or adult thermal environments and measuring gene expression patterns to detect thermal acclimation responses (Gracey *et al.* 2004; Scott & Johnston 2012; Morris *et al.* 2014; Shama *et al.* 2016; Kim *et al.* 2017a; Healy *et al.* 2017). A few studies have also examined the transgenerational effects of thermal exposure on the transcriptome of offspring, detecting effects of maternal or grandmaternal thermal exposure on offspring gene expression (Veilleux *et al.* 2015; Shama *et al.* 2016). Similarly, persistent effects of the temperatures experienced during early development (prior to hatch) on gene expression have been detected in adult fish held under common conditions (Scott & Johnston 2012; Oomen & Hutchings 2017). However, very little is known about whether plasticity at different life stages affects similar or different processes.

Epigenetic processes, such as DNA methylation, which result in chromosome bound, heritable changes to gene expression patterns that are not dependent on changes to the underlying DNA sequence (Deans & Maggert 2015), are thought to be important mechanisms regulating gene expression plasticity (Hu & Barrett 2017). For example, changes in DNA methylation patterns during development are thought to play an important role in cellular differentiation and in maintaining cell-type specific transcriptional activity through mitosis

(Monk *et al.* 1987; Li 2002). Although epigenetic effects are often considered to be relatively stable across the lifespan, or even heritable, recent studies (Baránek *et al.* 2015; Viggiano & de Pinto 2017; Metzger & Schulte 2017) indicate that DNA methylation can be rapidly altered by environmental change, and could be implicated in modulating more rapid and reversible plasticity such as is observed in phenotypic flexibility or acclimation (Bird 2007; Deans & Maggert 2015; Huang *et al.* 2017). Thus, DNA methylation has the potential to act as a mechanism underlying plasticity acting at different time scales.

Understanding the relationship between plasticity in response to thermal change at different life-history stages has important implications for predicting the resilience of populations to anticipated environmental change (Somero 2010). Within the next century climate change is expected to result in an increase in mean temperatures as well as an increase in the magnitude, frequency, and duration of extreme temperature events and these changes in environmental thermal regimes are predicted to impact the distribution and abundance of ectothermic organisms (Sunday *et al.* 2012; Bauerfeind & Fischer 2014; Thornton *et al.* 2014; Seebacher *et al.* 2015; Frainer *et al.* 2017). Understanding the relationship between gene expression plasticity at different life stages has the potential to be important in determining the capacity of organisms to cope with these changes (Donelson *et al.* 2017)

Here, we examine changes in gene expression in response to altered developmental temperature and adult thermal acclimation in the threespine stickleback fish (*Gasterosteus aculeatus*). Stickleback populations are found in both marine and freshwater environments throughout their Holarctic distribution. Post-glacial rebound following the retreat of the Pleistocene glaciers ~10-12 thousand years ago resulted in the colonization of newly formed freshwater habitats (followed by adaptation and reproductive isolation). Differences in the selective pressures of freshwater environments have resulted in the rapid, parallel morphological and behavioral divergence of freshwater populations from ancestral marine populations (eg. Jones *et al.* 2012b), including variation in DNA methylation patterns (Smith *et*

al. 2015; Artemov *et al.* 2017). Characterization of the underlying genetic divergence of marine and freshwater stickleback populations has established stickleback as a powerful system in which to investigate the genetic basis of adaptive evolution (Jones *et al.* 2012b). In addition, there is accumulating evidence of phenotypic plasticity in stickleback in ecologically relevant traits across a variety of time scales. For example, gene expression plasticity in response to temperature acclimation has been investigated in adult (Morris *et al.* 2014; Shama *et al.* 2016), and juvenile (Kim *et al.* 2017a) stickleback. Similarly temperature-dependent developmental plasticity has been detected in body shape (Ramler *et al.* 2014), reproductive strategies (Kim *et al.* 2017b), gene expression (Shama *et al.* 2016), and DNA methylation (Metzger & Schulte 2017), while significant transgenerational plasticity has been detected for hatching success (Shama *et al.* 2014), growth (Shama & Wegner 2014), and gene expression (Shama *et al.* 2016). Thus, stickleback not only present an ideal system in which to investigate the mechanisms underlying plasticity a different life stages, but to also help better understand the effects of plasticity on evolutionary processes.

Specifically, the objectives of this study were to 1) examine the persistent effects of temperature manipulations during development (from fertilization until hatch) on gene expression patterns in the muscle tissue of adults held under common conditions, 2) assess mechanisms associated with phenotypic flexibility by acclimating adult stickleback to warmer and colder temperatures and measuring the temperature-dependent effects of thermal acclimation on gene expression patterns in muscle tissue, 3) examine whether the persistent effects of thermal manipulations during development on gene expression are similar to adult acclimation effects on gene expression patterns, and 4) assess whether gene expression plasticity in response to developmental and adult temperature manipulations are associated previously characterized changes in DNA methylation patterns (Metzger & Schulte 2017).

Materials and Methods

Gasterosteus aculeatus rearing conditions

Adult threespine stickleback (*G. aculeatus*) of the fully plated "marine" ecotype were collected from Oyster Lagoon (British Columbia, Canada, GPS: 49.6121, -124.0314) in June 2014. In the lab, stickleback were separated into six 110-litre glass aquarium tanks (20 stickleback/tank) and acclimated to 20 ppt salt water (instant ocean) at 18 °C. A photoperiod of 14:10h light:dark was held constant throughout the experimental period. These conditions are similar to summer conditions in Oyster Lagoon at the time of collection. Stickleback were fed daily to satiation with Hakari Bio-Pure frozen Mysis Shrimp for three weeks prior to initiation of the breeding protocol.

Eggs were collected from a total of six different females as they became gravid. Testes were dissected from six different males, and used to generate six independent (unrelated) families. Fertilization and stickleback rearing were conducted as previously described (Metzger & Schulte 2017) and are summarized in Figure 1. Briefly each clutch was split across three separate 10 cm petri dishes. A single petri dish from each of the six families was held at 12, 18, or 24 °C until hatch. We chose these temperatures (hereafter, developmental temperatures) because they span the temperature range that might be experienced during the peak of breeding season of this population, which is typically May through July.

Fertilized eggs were kept in a monolayer and submerged in 15 mL of 20 ppt seawater. Petri dishes were partially covered to reduce water loss from evaporation while still allowing for surface gas exchange to insure adequate oxygenation. Eggs were monitored twice daily during which time unfertilized eggs and mortalities were removed and 10 mL of water was changed to prevent mold growth. Hatching success was determined based on the number of fertilized embryos that hatched compared to the total number of fertilized embryos. Developmental temperature did not have a significant effect on survival until hatch (Figure S1). Embryos that developed at 12 °C took approximately 13 days longer to hatch than embryos that

developed at 24 °C (Metzger & Schulte 2017). Once all the embryos in a given family had hatched they were transferred to 110-litre glass aquarium tanks and maintained at 18 °C at a salinity of 20 ppt. At eight months post-hatch, a random sample of stickleback that developed at 18 °C were mixed together from the six different families and then split between three different acclimation temperatures (5, 18, or 25 °C) and held at these temperatures for four weeks.

These acclimation temperatures were chosen because they represent the ecologically relevant extremes that populations in this region would experience as adults in the wild in the winter and summer respectively (Barrett *et al.* 2011), and because they are close to the maximum and minimum temperatures to which threespine stickleback can be acclimated for extended periods (Wootton 1984; Lefébure *et al.* 2011). We selected 18 °C as the "control" temperature because it is the typical temperature at which stickleback are held in the lab. The two "experimental" acclimation temperatures differ in their magnitude of temperature change from the "control" group of 18 °C because thermal performance curves are typically asymmetric, increasing gradually until reaching a peak and then rapidly decreasing (Dowd *et al.* 2015). Thus increasing and decreasing temperature by the same magnitude does not result in the same shift in thermal performance, a phenomenon known as Jensen's inequality (Denny 2017). Therefore, these acclimation temperatures were chosen to minimize the effects of Jensen's inequality by accounting for differences in slope along a thermal performance curve and choosing temperatures that would result in similar effects on performance rather than similar changes in temperature. We chose to use different thermal ranges at the two different life stages because these stages have different thermal sensitivities (Motani & Wainwright 2015), and also encounter different temperatures in nature. The selected temperatures thus were selected to represent similar extents of the thermal tolerance breadth at each life stage.

Following four weeks of acclimation to these temperatures, a total of six stickleback from each acclimation temperature were euthanized and muscle tissue samples were snap frozen in liquid nitrogen and stored at -80 °C until further use. A total of six stickleback from

each development temperature treatment were also euthanized at nine months post hatch, and muscle tissue samples were taken from each stickleback with sampling distributed across families (Table S1). One-way ANOVAs were used to determine whether there was a main effect of either developmental temperature or adult acclimation temperature on the stickleback length and weight. A Tukey's post-hoc analysis was performed to test for significant differences between temperatures. There was no difference in the length of the individuals from different temperature treatments (Figure S2 A/B). Developmental temperature had a significant effect on wet weight (ANOVA p-value = 0.0223). Stickleback that were exposed to 12 °C during development and had a significantly higher wet weight compared to stickleback exposed to 18 °C (p-value = .0459) or 24 °C (p-value = 0.0307) during development (Figure S2 C/D). However, because some individuals within a treatment are from the same family, we cannot rule out the possibility that the observed effects of developmental temperature on adult wet weight reflect heritable transgenerational temperature effects of the parental or grand-parental environment (Shama & Wegner 2014; Shama *et al.* 2016), or that the analysis is affected by pseudo-replication at the family level in some treatments but not others (Table S1).

We elected to examine gene expression in muscle tissue because previous work in a variety of species of fish suggests that both developmental temperature and adult thermal acclimation have substantial effects on muscle phenotype (Johnston 2006; Macqueen *et al.* 2008; Johnston *et al.* 2009; Finstad & Jonsson 2012; Salinas & Munch 2012; Scott & Johnston 2012; Schnurr *et al.* 2014; Shama *et al.* 2016). A muscle sample from the other side of the same individuals was used in a previous study that investigated the effects of developmental temperature and adult temperature acclimation on DNA methylation levels (Metzger & Schulte 2017).

RNA Isolation and sequencing

Total RNA was prepared from stickleback muscle tissue using TRIzol Reagent (Invitrogen Life Technologies). Approximately 20 mg of muscle tissue was homogenized in 1 mL of TRIzol in 1.5 mL Eppendorf® Safe-Lock micro centrifuge tubes containing approximately ten 1.0 mm ceria stabilized zirconium oxide beads (Next Advance) using a Bullet Blender24 (Next Advance). Total RNA was DNase treated using the Qiagen RNeasy DNase I on-column DNA digestion protocol. Total RNA was quantified using a QBit® RNA broad range assay kit (product # Q10210; ThermoFisher Scientific) and an Invitrogen™ Qubit® 2.0 Fluorometer. RNA quality was assessed using an Agilent RNA 6000 Pico Kit (product # 5067-1514) and an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA integrity numbers (RIN) were between 7.9 - 9.1 (mean = 8.6 ± 0.4 SD). Preparation of cDNA libraries and 100 base-pair paired end sequencing was performed at the UBC Nucleic Acid Protein Service Unit (NAPS) and UBC Biodiversity Research Center's next generation sequencing facility. Briefly, mRNA was purified using BIO-O NEXTflex® Poly-A beads. Sequencing libraries were prepared using the BIO-O NEXTflex® Rapid RNA-Seq kit. Each sample was individually barcoded and samples from different treatments were evenly distributed across 3 sequencing lanes of an Illumina HiSeq 2000 flow cell (10 samples/lane, two samples from each treatment/lane). Mean sequenced library size was 39,737,041 reads (± 7,227,925 SD; Table S2).

Sequence alignment and expression analysis

Reads were aligned to the stickleback genome (http://www.ensembl.org) using CLC genomics workbench v9.5. Average mapping efficiency of paired and broken reads was 88 %.

Analysis of total read counts was performed in R v3.3.1 with *edgeR* v3.14.0 (Robinson *et al.* 2010; McCarthy *et al.* 2012), and was based on the recommended guidelines in Lin *et al.* (2016). Genes with no reads were removed from the datasets. Counts were normalized using

the relative log expression (RLE) method. However, temperature acclimation has generally been shown to induce the up-regulation of a large proportion of genes in fish (Gracey *et al.* 2004; Healy *et al.* 2017). Many methods for normalization of RNA-seq data (e.g. TMM and RLE) assume that the majority of genes in an RNA-seq dataset are not differentially expressed (Dillies *et al.* 2013). Thus, normalizing factors can become problematic when a large proportion of the expressed genes are differentially expressed between treatments, particularly when the direction of change is biased in one direction (Dillies *et al.* 2013; Evans *et al.* 2017). Ideally, only those genes that are not differentially expressed should be used to calculate the normalization factors. Therefore, we applied the method describe in Healy *et al.* (2017), which utilizes a preliminary analysis of the dataset to identify and remove genes that are likely to be differentially expressed and then calculates standard RLE normalizing factors using the remaining dataset. To identify and remove putatively differentially expressed genes, two separate preliminary analyses were performed.

In the first preliminary analysis, putatively differentially expressed genes were identified without library normalization. Genes with low expression were filtered from the dataset. The minimum criterion for retaining a gene was at least 0.5 counts per million (~10 counts in the smallest library) in each of the six samples of each temperature. Tagwise dispersions were calculated using the robust method in edgeR. The data were then fit to a negative binomial generalized linear model using glmFit().

In the second preliminary analysis, sequencing libraries were normalized using the RLE method. Genes with low expression were filtered from the dataset using the same criteria as previously described. Tagwise dispersions were calculated using the robust method in edgeR. The data were then fit to a negative binomial generalized linear model using glmFit().

Genes that were identified as differentially expressed in each of these two preliminary analyses were then removed from datasets for the purposes of normalization. The majority of genes that remained following these steps are less likely to be differentially expressed in

response to temperature stimuli and are thus suitable to calculate normalizing factors for the rest of the dataset. Normalizing factors using the RLE method were then calculated for the dataset that contained these remaining genes. These normalizing factors were then used in a final analysis of the data. Separate preliminary analyses and normalizing factors were calculated in this way for each pairwise comparison of either a developmental or acclimation temperature treatment using stickleback that were held at 18 °C for the entire duration of the experiment as the control group.

For the final analysis of the data, differential expression was assessed using pairwise comparisons of the gene expression data for each developmental or acclimation treatment to stickleback that were held at 18 °C for the duration of the experiment. The minimum criterion for retaining a gene, following RLE normalization as described above, was for a gene to have at least 0.5 counts per million (CPM; ~10 counts in the smallest library) in each of the six samples within a temperature treatment. If a gene had a read count less than 0.5 CPM in at least one sample within a treatment then it was discarded from the analysis. After normalization and filtering of the datasets a total of 12,199 genes remained in the 5 °C acclimation dataset, 12,097 genes remained in the 25 °C acclimation dataset, 11,507 genes remained in the 12 °C development dataset, and 11,661 genes remained in the 24 °C development dataset for differential expression analysis. Tagwise dispersions were calculated using the robust method in edgeR. Differentially expressed (DE) genes were identified using the *glmFit*() function from *edgeR* to fit a negative binomial generalized linear model followed by a likelihood ratio test, *glmLRT*(). The resulting p-values were adjusted based on a false discovery rate (FDR) correction (Benjamini & Hochberg 1995), and the threshold for significance of these adjusted p-values (q-value) was set at 0.05. Gene ontology (GO) pathway enrichment analyses were conducted using the *goseq* (v1.22.0) R package (Young *et al.* 2010), with FDR correction as previously described.

Differential methylation analysis

Differentially methylated cytosines (DMCs) associated with differentially expressed genes were identified by filtering previously identified DMCs (Metzger & Schulte 2017) for those located within 5 kilobase pairs (kb) upstream or downstream of genes that were differentially expressed in stickleback from the same temperature treatment. Analysis of the genomic distribution of DMCs was conducted using the *annotateWithGeneParts*() function in the *genomation* v1.10 R package.

Results

Comparison of patterns of gene expression plasticity across time-scales

The expression levels of 10,140 genes were responsive to thermal acclimation (Figure 2). A total of 7,940 genes were differentially expressed in stickleback that were acclimated to 5 °C (Figure 2A/C) and 7,015 genes were differentially expressed in stickleback that were acclimated to 25 °C (Figure 2B/D). The majority of genes that responded to thermal acclimation changed by a $\log_2$ fold of less than two (i.e. an absolute fold change of less than four) for both cold (86 % of differentially expressed genes, Figure 2A) and warm (95 % of differentially expressed genes, Figure 2B) acclimated stickleback. In both cold and warm acclimated stickleback, substantially more genes were up-regulated than were down-regulated (cold-acclimated: 72% of all differentially expressed genes were up-regulated; warm-acclimated: 80% of all differentially expressed genes were up-regulated) (Figure 2 C/D).

Only 57 genes were differentially expressed in the muscle tissue of adult stickleback that were exposed to different developmental temperatures (Figure 3). A total of 33 genes were differentially expressed in stickleback that had developed at 12 °C (Figure 3 A/C) and 29 genes were differentially expressed in stickleback that had developed at 24 °C (Figure 3 B/D). Similar to the patterns of differential expression in response to adult thermal acclimation, the majority

of these genes had a $\log_2$ fold change less than two for stickleback that were exposed to colder (94 % of differentially expressed genes, Figure 3A) or warmer (90 % of differentially expressed genes, Figure 3B) temperatures during development. However, unlike the pattern that was observed in response to adult acclimation temperature, none of the genes affected by developmental temperature had a $\log_2$ fold change greater than five. As was the case for adult thermal acclimation, the majority of the differentially expressed genes in response to altered developmental temperatures were up-regulated (cold-development: 64 % of all differentially expressed genes were up-regulated; warm-development: 83 % of all differentially expressed genes were up-regulated)(Figure 3 C/D).

A complete list of all differentially expressed genes and the normalized counts per million of each gene for each sample can be found in supplementary file 1 in the online supplementary material.

Of the 10,140 genes that were differentially expressed in response to adult thermal acclimation, 4,851 were differentially expressed in both cold and warm acclimated stickleback (Figure 4A), and the majority of these genes (4,235 genes) were differentially expressed in the same direction between acclimation temperatures (Figure 4A). In contrast, of the 57 genes identified in stickleback from different developmental temperatures, only five were differentially expressed in stickleback from the cold and warm developmental temperature treatments (Figure 4B). The expression of all five of these genes changed in the same direction in stickleback exposed to cold or warm developmental temperatures.

To further assess the degree to which developmentally plastic responses to environmental temperature are consistent with adult thermal acclimation responses, we compared the list of genes that were differentially expressed between developmental treatments to those that were differentially expressed in adult stickleback acclimated to different temperatures. From this analysis, we identified 27 genes that were differentially expressed both in response to development at cold temperatures and in response to cold

temperature acclimation (Figure 4C, Table 1). The direction of differential expression for all 27 genes was conserved across developmental and adult treatments. Similarly, we identified 18 genes that were differentially expressed in response to development at warm temperatures and in response to warm temperature acclimation (Figure 4C). The direction of differential expression for 17 of these genes was conserved between developmental and acclimation treatments (Table 1). Comparison of the differentially expressed genes from all four analyses identified four genes (*irs2b, klhl38b, gadd45ga,* and *slc3a2a*) that were differentially expressed in all treatments and each of these genes was up-regulated in each treatment (Table 1).

In addition to comparisons of gene expression of the 12 °C and 24 °C developed groups to the group developed at 18 °C, we also examined the list of expressed transcripts in the stickleback that developed at 12 °C and 24 °C to determine whether there were any novel genes that were expressed at one temperature but not the other, because these genes would not necessarily be revealed by comparison to stickleback developed at 18 °C. However, there were no genes that fell into this category.

Identification of candidate biological processes affected by gene expression plasticity across timescales

Comparison of the most significantly enriched biological processes associated with genes that were differentially expressed in response to acclimation to low or high temperature revealed changes in the expression of genes involved in a common set of biological processes (Table 2; Supplemental file 2). Enriched biological processes for the up-regulated genes were generally associated with cell division, mRNA splicing, and protein degradation. Enriched biological processes for the down-regulated genes were generally associated with extracellular matrix organization and cell adhesion in both warm and cold-acclimated individuals.

There were no significantly enriched terms for genes that were uniquely up-regulated in cold-acclimated stickleback. Genes that were uniquely up-regulated in warm-acclimated stickleback were enriched for biological processes involved in protein translation and amino acid metabolism. Genes that were uniquely down-regulated in cold-acclimated stickleback were enriched for biological processes involved in muscle filament sliding, muscle contraction, oxidation-reduction, angiogenesis, and epidermis development. There were no significantly enriched processes associated with genes that were uniquely down-regulated in warm-acclimated stickleback.

There was no significant enrichment of biological processes for genes differentially expressed between stickleback that experienced different temperatures during development. This is likely due to the relatively small number of genes affected by developmental temperature; however, many of these genes are known to be involved in the same processes that were enriched among genes differentially expressed in adult stickleback acclimated to different temperatures. For example, transcripts encoding genes for the DNA damage inducible transcript 4 (*ddit4/redd1*) and *ddit4*-like (*ddit4l/redd2*) were differentially expressed in stickleback reared at 12 °C and are thought to be involved the attenuation of the mTORC1 protein synthesis which can result in muscle atrophy (Kelleher *et al.* 2013). In addition, several genes involved in muscle cell development, growth, aging and metabolism were also differentially expressed in stickleback that developed at 12 °C, including methyltransferase like 21C (*mettl21c*), CCAAT/enhancer binding protein delta (*cebpd)*, transferrin receptor 1a and 1b (*tfr1a, tfr1b*/*tfrc*), pim-1 proto-oncogene, serine/threonine kinase (*pim1*), B-cell CLL/lymphoma 2b (*bcl2b*)**,** FK506 binding protein 5 (*fkbp5*), Kruppel-like factor 2b and 13 (*klf2b*, *klf13*), transducer of ERBB2, 1 (*tob1*), and DNA-binding protein inhibitor ID-3 (*id3*).

A different set of genes that have been implicated in muscle development, growth and metabolism were differentially expressed in stickleback that developed at 24 °C including Ras protein specific guanine nucleotide releasing factor 1 (*rasgrf1*), alpha kinase 3 (*alpk3*), f-box

protein 32 (*fbxo32*), dual specificity phosphatase 8 (*dusp8*), tripartite motif containing 63b (*trim63b*/*murf1*b), endothelial lipase G (*lipg*), nuclear receptor subfamily 4 group A member 1 (*nr4a1*/*nur77*), and plexin A2 (*plxna2*).

Comparison of genes that were differentially expressed by either warm or cold development identified five genes whose expression was affected by both developmental temperatures (Figure 4 B/C), including the solute carrier family 3 member 2a (*slc3a2a*), growth arrest and DNA-damage-inducible, gamma a (*gadd45ga*), insulin receptor substrate 2b (*irs2b*), kelch-like family member 38b (*klhl38b*), and one unannotated gene ENSGACG00000008429. All but the last of these genes were also affected by thermal acclimation.

Comparison to patterns of DNA methylation

Because the samples used in this experiment were derived from the same individuals used to demonstrate that both altered developmental temperature and altered adult acclimation temperature result in changes in DNA methylation patterns in threespine stickleback muscle tissue (Metzger & Schulte 2017), it is possible to directly compare changes in DNA methylation patterns to changes in gene expression. In this analysis we compared the genes that we identified in this study as being differentially expressed to DMCs that were located near a gene (within 5 kb of either the transcription start site or the 3' end of the gene) identified in our previous study. Using this identification cutoff, none of the genes that were differentially expressed between developmental temperature treatments were associated with previously reported differential methylation, whereas 125 genes that responded to warm acclimation were associated with differentially methylated loci, and 199 genes that responded to cold acclimation were associated with differentially methylated loci (Supplemental file 3). Of these DMCs, the majority were located in intergenic regions within 5 kb upstream or downstream of the differentially expressed genes (Figure 5), and only 5-6% (depending on the acclimation temperature) were located in promoter regions (within 2 kb upstream of the

transcription start site). When considering the complete reduced representation bisulfite sequencing (RRBS) dataset, approximately 9% of sequence data were from promoter regions. In the subset of the data analyzed here, which excludes intergenic sequences located more than 5kb upstream or downstream of a gene (thus effectively excluding much of the intergenic sequence), the proportion of promoter sequences in the background sequence data is higher. Thus, the fact that we observe a lower percent of DMCs in promoter regions suggests that there is no evidence for enrichment of differential methylation in the promoter regions of differentially expressed genes.

We did not detect significant enrichment of gene ontologies among the differentially expressed genes associated with DMCs, but a number of these genes are involved in processes that are likely affected by thermal acclimation based on the GO enrichment analysis of the DE genes, including processes such as proper formation of the sarcomere (eg. *sh3bgr* and *ttn.2*), mRNA splicing (*aqr*, *sf3b6,* and *pus10*), the ubiquitin proteasome pathway (eg. *psmd1, psmd13, ube2e2*), muscle cell growth and development (eg. *col12a1a* and *relb*), mitochondrial proliferation (eg. *mdh1*, *hpc2*/*elac2* and *ugp2b*), and myogenesis (eg. *foxk1i*).

Discussion

Conserved effects of cold and warm temperature on gene expression

In this study we present evidence of similarities in temperature-induced gene expression plasticity at different life stages on adult gene expression patterns. In both the developmental and adult acclimation treatments, colder temperatures resulted in the differential expression of more genes compared to warmer temperatures, and there were more differentially expressed genes that were up-regulated compared to down-regulated in all treatments. While the effects of adult thermal acclimation on gene expression were much more extensive than the effects of developmental temperature on gene expression in adult stickleback

muscle, the majority of gene expression patterns that were affected by developmental temperature were also responsive to thermal acclimation, and these changes were generally in the same direction. Taken together these data suggest that there could be similar mechanisms that regulate plastic responses at these different life stages.

The effects of thermal acclimation on muscle gene expression have been examined in many fish species (eg. Gracey *et al.* 2004; Scott & Johnston 2012; Healy *et al.* 2016) including threespine stickleback (Morris *et al.* 2014; Shama *et al.* 2016); however, studies that contrast the gene expression patterns of cold- or warm-acclimated fish are less common (eg. Ikeda *et al.* 2017). In this study we characterized the transcriptional response to cold (5 °C) and warm (25 °C) temperature acclimation independently by comparing muscle gene expression patterns to those in individuals at a common control temperature (18 °C). By using this experimental design we are able to identify the common set of genes (Figure 4C) and biological processes (Table 2) that are differentially regulated in response to both warm and cold temperature acclimation, determine whether direction of regulation of these genes is similar at both temperatures (Figure 4A), identify genes that are uniquely regulated in one temperature but not the other (Figure 4A and 4C), and compare the overall magnitude of the transcriptomic response to warm and cold temperature acclimation (Figure 2). The striking similarity of the gene expression response to both cold and warm acclimation suggests that the observed patterns of changes in gene expression may reflect a generalized thermal stress response. For example, a variety of heat shock proteins are differentially regulated in response to both temperature acclimation conditions, and there is a substantial change in the expression of both aerobic and glycolytic metabolic genes in response to both warm and cold acclimation, as has been detected in other fish species (Logan & Buckley 2015). One gene family that has been consistently detected as differentially expressed in response to both warm and cold exposure are the high mobility group proteins, which are global regulators of transcription, and consistent with previous work (Logan & Buckley 2015), we also detect these genes as differentially expressed in response to both cold and warm acclimation in adult stickleback.

Another gene of note that we detected as differentially expressed in response to both cold and warm acclimation is the gene encoding the peroxisome proliferator activated receptor alpha (*pparaa*). This gene has been previously associated with a genetically divergent region of the stickleback genome that is under positive selection between marine and freshwater stickleback populations (Jones *et al.* 2012a), and has been shown to be have different responses to temperature acclimation in stickleback from a freshwater population compared to those from a marine population (Morris *et al.* 2014).

Disruption in the timing or magnitude of gene expression during development can result in permanent changes to adult phenotypes (West-Eberhard 2005). These effects have been previously demonstrated in zebrafish where warm temperatures during development have been shown to impact muscle cell fiber composition, swimming performance, and metabolism (Scott & Johnston 2012). Interestingly, in zebrafish these large changes in muscle phenotype and function were not associated with large changes in the transcriptional program of muscle tissue in adults. The magnitude of the transcriptional response in zebrafish muscle was modest (26 DEGs with a q-value < 0.05) and similar to what we observed in stickleback (33 and 29 DEGs with a q-value < 0.05 in stickleback exposed to either cold or warm temperatures during development, respectively). Genes that were differentially expressed between stickleback that developed at different temperatures are known to be involved in metabolism and muscle cell development (eg. *cebpd, tfr1, fkbp5,* and *klf2* in stickleback that developed at 12 °C, *nr4a1* in stickleback that developed at 24 °C, and *slc3a2* in both developmental treatment temperatures). Among this relatively small set of genes there were two genes, *fkbp5* and *slc3a2,* that were also affected by developmental temperature in zebrafish, These data suggest that altered developmental temperatures induce changes in muscle phenotype that could impact the performance of adults, and that the effect of developmental temperature on the regulation of these genes may be conserved across distantly related fish species.

Plasticity is thought to influence evolutionary processes by revealing previously hidden sources of variation and by facilitating phenotypic variation and divergence among populations that inhabit different environments (Schlichting 2008; Schneider & Meyer 2017). Natural selection can then act on this variation to refine the plastic phenotype closer to the optimum (Levis & Pfennig 2016). For example, in a "plasticity-first" hypothesis, developmental plasticity can induce phenotypes that increase fitness in stressful environments (Levis & Pfennig 2016). However, this type of analysis assumes the presence of beneficial plasticity, whereas maladaptive or non-adaptive plasticity can also affect evolutionary trajectories (Ghalambor *et al.* 2015). The differential expression of genes in stickleback reared at colder or warmer temperatures is consistent with the potential effects of developmental temperature on metabolism and muscle cell fiber composition that could impact adult performance, but whether these changes are likely to be adaptive or maladaptive is unknown.

Examination of the genes differentially regulated in response to all four of our temperature treatments strongly suggests that changes in the regulation of muscle growth are a core phenomenon uniting phenotypic plasticity in response to temperature change at different life stages and in response to both low and high temperature. Although there have been relatively few studies that directly compare plastic responses at the molecular level between life stages, one study comparing rapid cold hardening and exposure to altered developmental temperatures in *Drosophila melanogaster* (Gerken *et al.* 2015) detected effects on similar functional classes of genes at different life stages, although the genes themselves differed. This commonality in responses across life stages and temperatures could potentially be interpreted in two different ways. First, this pattern could reflect a common underlying mechanism regulating plasticity. An alternative, but not mutually exclusive, interpretation of this pattern is that these plastic responses at different life stages represent beneficial or adaptive responses to thermal change. For example, the persistent effects of developmental temperature on gene expression patterns could be adaptive by shifting gene expression levels closer to those

observed in fish at lower and higher temperatures or by reducing the cost of thermal acclimation.

Although the phenotypic consequences of the gene expression patterns observed here are unknown, a previous study in zebrafish (Schaefer & Ryan 2006) found that acclimation to increased temperature increased whole-organism thermal tolerance, and increased developmental temperature had a similar, but much smaller, effect. Taken together, this suggests that the changes in gene expression may have effects on a variety of traits at the whole-organism level.

Relationship to patterns of DNA methylation

Epigenetic mechanisms have been proposed to be important processes through which environmentally induced variation in phenotypic expression in one generation can impact subsequent generations and through which environmental conditions during development can have persistent phenotypic effects later in life. To explore the relationship between DNA methylation patterns and the effects of changes environmental temperature during development and in adults on gene expression, we compared the differentially expressed genes identified in this study to differentially methylated loci from the same stickleback reported in our previous study (Metzger & Schulte 2017). Differentially expressed genes in stickleback from different developmental temperatures were not closely associated with any of the differentially methylated loci that were previously described, suggesting a potential role of DNA methylation on trans-acting factors that regulate gene expression. Alternatively, differentially methylated regions could be located on regulatory elements that are not within 5 kb of a gene, or the persistent effects of developmental temperature on gene expression may not be regulated by DNA methylation and instead could be due to effects such as changes in histone acetylation or changes in miRNA activity. However, we cannot firmly rule out a potential role for changes in DNA methylation in regulating the changes in gene expression that we observed because

important regulatory regions located near differentially expressed genes may not have been assessed in the reduced representation analysis of DNA methylation (RRBS). For example, only ~10% of the differentially expressed genes were represented within the RRBS dataset, highlighting a potential limitation when deducing functional relationships that are based on a correlation between a reduced representation approach and a more comprehensive technique such as RNA-seq.

Analysis of differentially expressed genes and differentially methylated loci in adult stickleback acclimated to warm and cold temperatures identified genes involved in several processes that are known to be differentially regulated in marine ectotherms in response to changes in environmental temperatures including the ubiquitin-proteasome pathway, aerobic metabolism and mitochondrial proliferation, mRNA splicing, myogenesis, proper formation of the sarcomere, and muscle cell growth and development (Gracey *et al.* 2004; Scott & Johnston 2012) which suggests that modified DNA methylation levels are likely involved in many of the transcriptional responses of stickleback to variation in environmental temperatures. One potentially interesting candidate gene that was both differentially expressed and associated with at least one DMC in adult stickleback acclimated to different temperatures is phosphodiesterase 4B (*pde4ba*). Epigenetic mechanisms are thought to play an important role in an organism's capacity to adapt to environmental changes, particularly over shorter timescales, but there is little empirical evidence to support this hypothesis. The *pde4ba* gene has been previously associated with the genetic divergence of Baltic sea stickleback populations along a thermal gradient (Guo *et al.* 2015) and thus may play an adaptive role to changes in environmental thermal regimes. It is therefore a strong candidate for subsequent analysis of the effects of DNA methylation on adaptive evolutionary processes.

Taken together, these data demonstrate that changes in DNA methylation patterns are likely implicated in short-term, potentially reversible transcriptional response of adults to changes in environmental temperature.

Conclusions

The data presented here demonstrate that the temperature experienced during early stages of development (before hatch) can have persistent effects on gene expression patterns in adult stickleback muscle tissue. In addition, we demonstrate that the majority of differentially expressed genes in stickleback from different developmental temperatures are also differentially expressed as part of the adult stickleback thermal acclimation response. This pattern suggests that developmental plasticity and phenotypic flexibility in gene expression in response to temperature change share some common underlying mechanisms and may have similar functional consequences. However, adult acclimation resulted in a much larger overall change in the transcriptome than did developmental temperature exposure. Some genes that were differentially expressed as a result of the adult acclimation treatments were also associated with previously identified temperature-dependent effects on DNA methylation patterns, suggesting a potential role for epigenetic mechanisms in regulating plastic responses during acclimation. Overall, these results emphasize both the similarities and differences between developmental plasticity and phenotypic flexibility in adults and highlight the relationships between plasticity acting across different time scales.

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Data Accessibility

Supplemental figures and tables supporting this article can be found in supplementary material online. RNA-seq sequencing files can be downloaded from the NCBI sequence read archive (SRA study accession number SRP135801). The mapped read count data used for the RNA-seq analysis and the R-scripts used for data normalization and differential expression analysis have been submitted to the Dryad Digital Repository (doi:10.5061/dryad.8js678v)

Authors' contributions

D.C.H.M and P.M.S conceived and designed the study. D.C.H.M executed the experiments and analyzed the data. D.C.H.M and P.M.S. drafted the manuscript.

Figure legends

Figure 1: Experimental design. Six stickleback families were generated for use in this experiment. Each clutch was split between three developmental temperatures of 12 °C (blue), 18 °C (black), and 24 °C (red). Following hatch, all stickleback were transferred to aquaria at 18 °C and reared for 8 months. At this point, fish from the 18 °C development treatment were acclimated to either 5 °C (blue shaded aquaria), 18 °C (grey shaded aquaria), or 25 °C (red shaded aquaria) for 4 weeks, while fish from the other developmental treatments were maintained at 18 °C. This experimental design resulted in stickleback with 5 different thermal histories. At an age of nine months, muscle tissue was sampled for RNA-seq analysis (n=6 per treatment).

Figure 2: Differential expression in stickleback acclimated to either 5 °C (A/C) or 25 °C (B/D) relative to stickleback that were kept at 18 °C for the duration of the experiment. Panels A and B show the $-\log_{10}$ FDR corrected p-value (q-value) against $\log_2$ fold change for significantly ($q<0.05$) up-regulated (orange), down-regulated (blue) genes and non- differentially expressed genes (grey). Embedded plots are frequency histograms of $\log_2$ fold change (e.g. a bar above a $\log_2$ fold change of 2 represents the genes that range in a $\log_2$ fold change between 1 and 2). Panels C and D are heatmaps of differential expression in stickleback acclimated to 5 °C and 25 °C, respectively. Each row represents the expression value (log2 counts per million) for a single gene relative to the mean expression value for that gene across all individuals (orange representing higher expression and blue representing lower expression). Each column represents an individual stickleback. Columns 1-6 are stickleback that were kept at 18 °C for the duration of the experiment. Columns 7-12 are either stickleback that were kept at 18 °C for 8 months and then acclimated to 5 °C (C) or 25 °C (D) for four weeks.

Figure 3: Differential expression in stickleback exposed to either 12 °C (A/C) or 24 °C (B/D) during development and then kept at 18 °C until 9 months of age relative to stickleback that were kept at 18 °C for the duration of the experiment. Panels A and B show the $-\log_{10}$ FDR corrected p-value (q-value) against $\log_2$ fold change for significantly ($q<0.05$) up-regulated (orange), down-regulated (blue) genes and non- differentially expressed genes (grey). Embedded plots are frequency histograms of $\log_2$ fold change (e.g. a bar above a $\log_2$ fold change of 2 represents the genes that range in a $\log_2$ fold change between 1 and 2). Panels C and D are heatmaps of differential expression in stickleback exposed to 12 °C and 24 °C during development, respectively. Each row represents the expression value (log2 counts per million) for a single gene relative to the mean expression value for that gene across all individuals (orange representing higher expression and blue representing lower expression). Each column represents an individual stickleback. Columns 1-6 are stickleback that were kept at 18 °C for the duration of the experiment. Columns 7-12 are either stickleback that were kept at 18 °C for 8 months and then acclimated to 5 °C (C) or 25 °C (D) for four weeks.

Figure 4: (A) Comparison of up- and down-regulated genes in stickleback muscle tissue from adult stickleback acclimated as adults to 5 °C (blue) or 25 °C (red) for four weeks. (B) Comparison of up- and down-regulated genes in stickleback muscle tissue from stickleback exposed to 12 °C (blue) or 24 °C (red) during development and then kept at 18 °C until 9 months of age. (C) Comparison of differentially expressed genes in stickleback muscle tissue. Cold acclimation = fish developed and reared at 18 °C for eight months and then acclimated to 5 °C for four weeks. Warm acclimation = fish developed and reared at 18 °C for eight months and then acclimated to 25 °C for four weeks. Cold development = fish developed at 12 °C until hatch, and then held at 18 °C for nine months. Warm development = fish developed at 24 °C until hatch, and then held at 18 °C for nine months. All differential expression was identified relative to fish held at 18 °C for the duration of the experiment.

Figure 5: Genomic distribution of differentially methylated cytosines associated with differentially expressed genes (within 5 kilobase pairs) in muscle tissue from adult stickleback acclimated to 5 °C (cold acclimation) or 25 °C (warm acclimation).

Ensembl Gene ID	Gene Name	Log_2 Fold Change			
		Cold Development	Cold Acclimation	Warm Development	Warm Acclimation
ENSGACG00000003564	irs2b	0.80	1.06	1.03	1.76
ENSGACG00000006793	gadd45ga	0.95	0.89	1.52	2.75
ENSGACG00000006167	klhl38b	1.08	2.60	1.25	1.15
ENSGACG00000019745	slc3a2a	1.48	1.28	1.23	2.02
ENSGACG00000013368	bcl2b	-1.31	-1.04	-	-
ENSGACG00000007797	vkorc1	-1.27	-2.56	-	-
ENSGACG00000014656	ifi35	-1.24	-1.00	-	-
ENSGACG00000008237	EVC	-1.11	-0.97	-	-
ENSGACG00000004133	pim1	-0.92	-1.33	-	-
ENSGACG00000003021	klf2b	-0.81	-1.71	-	-
ENSGACG00000019700	DDIT4L	-0.81	-3.73	-	-
ENSGACG00000012452	IL16	-0.77	-2.23	-	-
ENSGACG00000009575	id3	-0.66	-1.04	-	-
ENSGACG00000005010	tob1a	0.57	0.70	-	-
ENSGACG00000010010	ezrb	0.89	0.61	-	-
ENSGACG00000008895	sesn1	0.94	1.79	-	-
ENSGACG00000006997	alas1 (1 of 2)	1.00	1.66	-	-
ENSGACG00000015298	ENSGACG00000015298	1.28	-1.53	-	-
ENSGACG00000015297	METTL21C (2 of 2)	1.33	-3.44	-	-
ENSGACG00000013859	zc3h12a	1.40	0.80	-	-
ENSGACG00000005398	tfr1b	1.56	3.73	-	-
ENSGACG00000017927	cebpd	1.65	1.15	-	-
ENSGACG00000002379	ddit4	1.70	1.54	-	-
ENSGACG00000016373	tfr1a	1.72	2.60	-	-
ENSGACG00000010739	klf13	1.79	1.42	-	-
ENSGACG00000001466	fkbp5	1.89	3.16	-	-
ENSGACG00000001632	samhd1 (2 of 3)	2.11	2.72	-	-
ENSGACG00000000049	rasgef1ba	-	-	1.20	2.52
ENSGACG00000001607	trim63b	-	-	0.96	1.96
ENSGACG00000006161	fbxo32	-	-	1.52	2.15
ENSGACG00000006480	ddit3	-	-	0.58	0.42
ENSGACG00000006908	ENSGACG00000006908	-	-	1.94	-1.01
ENSGACG00000008429	ENSGACG00000008429	-	-	3.48	1.10
ENSGACG00000010788	nr4a1	-	-	-0.70	-1.97
ENSGACG00000010861	RASGRF1 (1 of 2)	-	-	-1.89	-3.88
ENSGACG00000011050	DUSP8	-	-	0.85	0.51
ENSGACG00000011743	HIVEP2 (1 of 2)	-	-	0.74	0.75
ENSGACG00000013469	pptc7a	-	-	1.50	1.57
ENSGACG00000014133	irs2a	-	-	0.72	0.88
ENSGACG00000015066	camk2n1a	-	-	1.49	2.36
ENSGACG00000016438	ALPK3 (2 of 2)	-	-	1.83	1.08

	Cold Acclimation			Warm Acclimation		
	GO ID	GO term	Over represented p-value	GO ID	GO term	Over represented p-value
Up-regulated genes	GO:0000278	mitotic cell cycle	4.06E-21	GO:0010467	gene expression	1.41E-34
	GO:0010467	gene expression	1.53E-18	GO:0016032	viral process	4.78E-16
	GO:0000398	mRNA splicing, via spliceosome	1.08E-17	GO:0000278	mitotic cell cycle	2.47E-15
	GO:0008380	RNA splicing	3.51E-17	GO:0006364	rRNA processing	3.47E-15
	GO:0008033	tRNA processing	5.51E-13	GO:0008033	tRNA processing	4.40E-15
	GO:0006521	regulation of cellular amino acid metabolic process	1.03E-12	GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	5.97E-15
	GO:0043687	post-translational protein modification	4.59E-10	GO:0051439	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	1.16E-14
	GO:0006364	rRNA processing	1.51E-09	GO:0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	2.24E-14
	GO:0018279	protein N-linked glycosylation via asparagine	1.57E-09	GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	2.96E-14
	GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	1.76E-09	GO:0043488	regulation of mRNA stability	5.12E-14
Down-regulated genes	GO:0030198	extracellular matrix organization	8.88E-28	GO:0030198	extracellular matrix organization	1.19E-32
	GO:0030574	collagen catabolic process	5.56E-17	GO:0030574	collagen catabolic process	6.14E-22
	GO:0007155	cell adhesion	9.80E-15	GO:0022617	extracellular matrix disassembly	1.30E-18
	GO:0030049	muscle filament sliding	2.11E-14	GO:0007155	cell adhesion	5.68E-15
	GO:0006936	muscle contraction	1.28E-13	GO:0030199	collagen fibril organization	4.14E-14
	GO:0022617	extracellular matrix disassembly	3.78E-13	GO:0007160	cell-matrix adhesion	7.29E-09
	GO:0001525	angiogenesis	1.95E-09	GO:0001501	skeletal system development	1.95E-08
	GO:0030199	collagen fibril organization	2.55E-09	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	1.95E-07
	GO:0001501	skeletal system development	7.58E-09	GO:0051056	regulation of small GTPase mediated signal transduction	3.40E-07
	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	2.87E-07	GO:0005980	glycogen catabolic process	1.28E-06