Ocean-Warming During Embryogenesis Programs a Lasting Transcriptomic Signature in Fishes

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Abstract

Exposure to elevated temperatures during embryogenesis has profound acute effects on the cardiac performance, metabolism, and growth of fishes. Some temperature-induced effects may be retained into, or manifest in, later-life through a mechanism termed developmental programming. In this study, we incubated *Scyliorhinus canicula* embryos at either 15˚C or 20˚C before transferring the newly hatched sharks to a common set of conditions (15˚C) for 5 months. Lasting transcriptomic differences associated with the developmental environment were identified, and interactions between cardiac genes were investigated using hypernetwork modelling. Development at an elevated temperature caused changes in transcriptomic connectivity and entropy, parameters thought to relate to plasticity and fitness. We then validated these observations through a novel re-analysis of published *Danio rerio* and *Dicentrarchus labrax* muscle tissue datasets. Together, these data demonstrate a persistent, programmed effect of developmental temperature on the co-ordination of gene expression in three species of fishes, which may relate to altered plasticity and fitness in later-life.

1 INTRODUCTION

Developmental programming comprises changes to an organism's phenotype that are induced, or programmed, by the environmental conditions experienced during development (Bateson *et al*., 2014). These developmental conditions elicit changes in DNA and chromatin methylation patterns, with consequent alterations in gene expression (Fellous *et al*., 2015). Some of these changes in methylation pattern, and their concomitant influence on gene expression, can become fixed and retained into later life. For instance, changes in developmental temperature cause hypermethylation in threespine stickleback (Metzger & Schulte, 2017), and studies in rainbow trout (*Oncorhynchus mykiss*) demonstrate the ability of early-life hypoxic events (Liu *et al*., 2017) and dietary composition (Geurden *et al*., 2014) to alter glucose metabolism and homeostasis in later-life. Finally, the developmental temperature of zebrafish (*Danio rerio*) has been shown to program their gene expression and physiology, culminating in an enhanced thermal tolerance (Schaefer & Ryan, 2006) and acclimation capacity (Schnurr *et al*., 2014; Scott & Johnston, 2012) in adulthood.

As well as altering mean trait values, the embryonic environment can also program an organism's ability to acclimate to conditions experienced in later-life (Beaman *et al*., 2016). That is, the capacity to physiologically respond to and mitigate future environmental challenges can be enhanced or hindered by an individual's embryonic conditions (Beaman *et al*., 2016). Evidence in zebrafish (*Danio rerio*) (Scott & Johnston, 2012; Schnurr *et al*., 2014) and mosquitofish (*Gambusia holbrooki*) (Seebacher *et al*., 2014), amongst other species (Beaman *et al*., 2016), show that the thermal environment experienced during early-life can have lasting effects on the acclimation capacity of multiple physiological traits, including metabolic scope (Seebacher *et al*., 2014), critical thermal maximum (Healy *et al*., 2019), and swimming performance (Scott & Johnston, 2012).

Phenotypic changes arising from physiological plasticity are underwritten by changes in an organism's gene expression in response to environmental cues. Such plasticity may be affected by an individual´s embryonic environment, which may either facilitate or hinder changes in gene expression later in life (Beaman *et al*., 2016). Thus, there is a link between an organism's phenotype, gene expression, and plastic potential, which can be modulated by its embryonic environment (Beaman *et al*., 2016). Transcriptional entropy is a metric describing the order or predictability of gene-gene interactions within a transcriptional network. The more entropic a network, the less structured and less predictable its behaviour. Transcriptional entropy is known to correlate with the differentiation potency and phenotypic plasticity of single cells (Banerji *et al*., 2013; Teschendorff & Enver, 2017). The more entropic a cell, the greater its number of possible fates (Teschendorff & Enver, 2017), and thus the more plasticity it possesses. Given that several studies have shown that the environmental temperature experienced during development can alter plasticity in the adult organism (Beaman *et al*., 2016), we aimed to assess whether developmental temperature also alters gene-level plasticity by measuring the network entropy and connectivity of the transcriptome in later life.

Whilst many animals may experience adverse environmental conditions during embryogenesis, some species, owing to their ecology, are more prone than others. Oviparous elasmobranchs, because of their protracted, sessile developmental period, are susceptible to experiencing sub-optimal

environmental conditions for long durations. Oviparous elasmobranchs lay their eggs inside collagenous egg cases termed mermaid's purses, which the embryo remains within for several months to over a year, depending on the species (Benjamins *et al*., 2021; McLaughin & Ogower, 1971). Throughout embryogenesis the embryo is unable to move from, and thus may be consistently exposed to, unfavourable environmental conditions such as elevated temperatures. As the world's oceans continue to warm (IPCC, 2021), the likelihood of oviparous elasmobranch embryos developing in sub-optimal conditions is increasing. Representative pathway (RCP) 8.5, a worst-case scenario model, estimates a ~3˚C increase in ocean temperatures by the end of the century if greenhouse gas emissions continue to rise (Cai *et al*., 2014). The rising ocean temperatures, coupled with an increasing frequency and duration of marine heatwaves (Oliver *et al*., 2018), renders exposure to temperatures above animal's optima highly likely. Exposure to elevated temperatures is known to affect the growth and development of elasmobranch embryos (Hume, 2019; Musa *et al*., 2020; Rosa *et al*., 2014). However, no studies have investigated how embryogenesis at elevated temperatures may have lasting effects on elasmobranchs through developmental programming mechanisms.

Here, we incubate *Scyliorhinus canicula* embryos at 15˚C or 20˚C throughout embryogenesis before moving them to a common-garden environment (15˚C) upon hatching. Following 5 months in the common garden environment, we sacrificed the animals and performed RNA-seq on the ventricle tissue of six individuals to investigate whether the developmental environment could program lasting changes in the gene expression and organisation of the *S. canicula* ventricular transcriptome. Finally, to validate our findings, we re-analysed published datasets of temperature-induced developmental programming in zebrafish (*Danio rerio)* hypaxial fast muscle (Scott & Johnston, 2012) and European seabass (*Dicentrarchus labrax)* muscle (Anastasiadi *et al*., 2021) to test the consistency of our observations across multiple species of fishes.

2 MATERIAL AND METHODS

2.1 Experimental animals

Scyliorhinus canicula embryos were collected from a population of 7 adult individuals held at 15˚C at the Ozeaneum, Stralsund, Germany, and transported to the University of Manchester, UK. Upon arrival the health and developmental stage of each embryo was assessed using the Musa scale (Musa *et al*., 2018), and the egg cases were submerged in dechlorinated tap water for two minutes to remove any marine microorganisms. Only healthy embryos at stage 1 were used in the study (Musa *et al*., 2018). All regulated procedures received approval from the institution's ethical review board.

Following embryonic staging and two days in quarantine (15˚C), individuals were randomly assigned to a temperature treatment group (15 ± 0.3˚C or 20 ± 0.3˚C). 15˚C was chosen for the control condition as it falls within the range of temperatures experienced by *S. canicula* in the wild and is the holding temperature of the parent population at the Ozeaneum, Stralsund, Germany. The egg cases in each treatment group were hung vertically in two, well aerated, 55 l static seawater (35ppt salinity) tanks equipped with internal filters and left to continue their embryonic development.

Upon hatching, fin clips were taken to facilitate identification in later-life through microsatellite analysis. The fin clips were stored in 98% ethanol at -20˚C, and the sharks were moved into one of four well aerated 400 l static seawater tanks held at 15˚C ± 0.3˚C. The hatchlings from the 20˚C treatment group were lowered to 15˚C at a rate of 2.5˚C per day, and sharks from both treatment groups were mixed and randomly allocated to a tank. The sharks were fed a mixture of squid, crab and krill three times per week. The sharks from both treatment groups were held at 15˚C ± 0.3˚C for an average of 136 days prior to tissue sampling. During the entire experiment, saltwater changes were performed three times weekly to maintain ammonia, nitrite and nitrate below detectable levels.

2.2 Tissue sampling

Sharks at 4-5 months age (mean ± SEM = 136 ± 6.3days) were euthanised with an overdose of buffered tricane methanesulfonate and a fin clip was taken post-humous. The ventricle was excised from each individual, placed into RNAlater, and flash frozen in liquid nitrogen before being stored at -80˚C prior to RNA-sequencing.

2.3 Individual identification using microsatellite analysis

To identify which condition an individual was developed in, microsatellite analysis was performed on the fin clips taken at birth and at the end of the experiment to match each individual using the methods described in Hook *et al*. (2019). Fin clips were extracted using a BioLine Isolate Genomic kit with an extended proteinase K digestion to maximise DNA yield. DNA yield was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis. A one-primer cocktail containing 11 microsatellites and three tail dyes was used for DNA amplification (Griffiths *et al*., 2011) using the QIAGEN multiplex PCR kit. The thermocycling protocol consisted of initial denaturation cycle at 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 90 seconds and 72°C for 45 seconds, and finalised by one cycle at 72°C for 30 minutes. The products were visualised on a gel and then genotyped using an ABI sequencer. Positive control samples were added to each plate genotyped to account for possible allele slippage.

Genotypes were scored using GeneMapper© v4.1 (Applied Biosystems) and validated through Microchecker (van Oosterhout *et al*., 2004). Duplicates were found between the two batches using CERVUS (Marshall *et al*., 1998). In cases where some alleles within the genotype did not match, controls were used as a reference to identify possible allele slippage. Probabilities of the identity analysis (pID) were taken from CERVUS to confirm match identification between the two batches.

2.4 *Danio rerio* and *Dicentrarchus labrax* re-analysis

The computational analysis pipeline (sections 2.5-2.7 below) was also implemented on previously published RNA-seq datasets of temperature-induced developmental programming in zebrafish (*D. rerio*) hypaxial fast muscle (Scott & Johnston, 2012) and European seabass (*D. labrax*) muscle tissue (Anastasiadi *et al*., 2021, Anastasiadi & Piferrer, 2019). This analysis was performed to assess the robustness and between-species consistency of the temperature-induced effects observed in *S. canicula*.

Full details of the animals used in the re-analysis can be found in Scott & Johnston 2012 (*D. rerio*, age = 8-9 months, N = 5-8) and Anastasiadi *et al*. 2021 and Anastasiadi & Piferrer, 2019 (*D. labrax*, age = 36 months, N = 5) respectively. The *D. rerio* and *D. labrax* data were processed in an identical manner to the *S. canicula* data. Transcriptome assembly statistics for the three species are shown in table 1.

2.5 de-novo transcriptome assembly and differential expression analysis

The ventricle tissue from six individuals (N = 3 per treatment group), stored in RNAlater at -80˚C, was used for generating the transcriptome. The tissue samples were extracted using the Qiagen kit with homogenisation. Basic sample quality was assessed using a Nanodrop (ThermoFisher) and the high-quality ventricular RNA samples were sequenced using a 76 base pair long, pair-ended reads on the Illumina TruSeq system. Reads that mapped to human, bacterial, and viral sequences were removed using DeconSeq (Schmieder and Edwards, 2011) whilst reads mapping to ribosomal RNA were removed using sortmeRNA (Kopylova *et al*., 2012). Adaptor sequences and sequences with a low-quality score (regions averaging a score <5 over a 4bp sliding window, and leading/trailing sequences scoring <5) were then removed from the cleaned reads using Trimmomatic (Bolger *et al*., 2014) prior to transcriptome assembly. 264 million bases survived the quality control process and were used for transcriptome assembly in Trinity 2.8.4 (Grabherr *et al*., 2011; Haas *et al*., 2013). Default parameters were used in Trinity, except –normalize_reads, producing 347710 contigs with a N50 of 1422, and a BUSCO score of 88.8%, with 519 single copy, and 2458 duplicated copy, BUSCOs (BUSCO v5.2.2, Simao *et al*., 2015). Open reading frames (ORFs) were predicted from the transcripts using Transdecoder (https://github.com/TransDecoder/TransDecoder/wiki) with a minimum length threshold of 100 amino acids. The retained predicted protein sequences were annotated using a BLAST search against the SwissProt database. The quality-controlled reads were then mapped using Bowtie-2 (Langmead & Salzberg, 2012), and pseudo-aligned to the de novo transcript assemblies using Kallisto (Bray *et al*., 2016), allowing the relative abundance of each gene to be calculated. Where multiple transcripts mapped to a single predicted protein, the median transcript abundance was used. The abundance estimates were filtered by removing fragments that had zero mapped reads in any of the samples before differential expression analysis was performed with EdgeR (McCarthy *et al*., 2012; Robinson *et al*., 2010) in R 3.6.0 (R core team, 2019).

2.6 Hypernetwork analysis

The differentially expressed genes (DEGs) were then used for hypernetwork analysis to assess the network properties of the transcriptomes from the 15°C and 20°C treatment groups (supplementary figure 1). The expression of each DEG was tested for correlations against all the assembled transcripts within each treatment group. The correlation values were binarized using a cut-off of one standard deviation from the mean, such that strong positive or negative correlations were retained (denoted as 1) and weak correlations were discarded (denoted as 0). The binarized correlation matrix is square (DEGs x DEGs) and represents the incidence matrix of the hypernetwork. The binarized

correlation matrix was then multiplied by the transpose of itself ($M * M^t$) to return the adjacency matrix of the hypernetwork, where the values in each cell represent the number of correlations with non-DEGs shared between any pair of DEGs. These values also represent the dimensionality of the hyperedge connecting each pair of nodes. Supplementary figure 1 gives an overview of the hypernetwork analysis. The data were visualised as heatmaps to assess clustering of the genes, per-gene connectivity, and per-gene Shannon entropy (herein, entropy) using the R packages mixOmics (Rohart *et al*., 2017) and BioQC (Zhang *et al*., 2017). A highly connected, central cluster of genes was identified using hierarchical clustering on each heatmap and the connectivity and entropy of the genes in these clusters was calculated. Connectivity was calculated for each gene as the sum of shared connections made between the given gene and all other DEGs in the hypernetwork. Entropy per-gene was calculated with the same values in a column-by-column manner using BioQC (Zhang *et al*., 2017) in R 3.6.0 (R core team, 2019), and scaled to size of the central cluster by taking $\log_2 N^2$, where N is the length of the gene list.

Having identified differences in the connectivity and entropy of the hypernetwork models of DEG interactions, we extended this approach to assess the gene network properties across the whole transcriptome. To do this, we selected a random set of non-differentially expressed genes (N = 160, to match the size of the DEGs list) and subjected them to the hypernetwork analysis described above. Entropy and connectivity were calculated on the central cluster genes per iteration, and entropy scores were then scaled to gene list size by taking $\log_2 N^2$, where N is the length of the gene list. This hypernetwork approach was iterated 10,000 times to provide a whole-transcriptome assessment of connectivity and entropy for animals from each treatment group. Wilcoxon tests were used to contrast entropy and connectivity between the sharks incubated at 15˚C and 20˚C.

2.7 Over-representation analysis on the central cluster of transcriptional activity

Having generated the hypernetwork adjacency matrices from the DEGs, hierarchical clustering was used and identified a group of highly connected genes present in the 15°C, but not 20°C, incubated sharks. This refined set of DEGs share the largest number of correlations across the rest of the transcriptome and as such, the expression patterns of these genes are related. We identified the highly connected genes present in the cold incubated animals (yellow clusters, fig. 2a, 2c & 2e) and subtracted the few highly connected genes from the warm-incubated animals (yellow clusters, fig. 2b, 2d & 2f). This final per-species gene list represents the genes whose highly connected nature with the transcriptome is disrupted by elevated incubated temperature. Over representation analysis was performed on these genes in DAVID, using each species' full gene list as a reference, to predict possible biological function (Huang *et al*., 2009a; Huang *et al*., 2009b).

3 RESULTS

The expression of 160 genes in the ventricle of *S. canicula*, 125 genes from *D. rerio* muscle, and 341 from *D. labrax* muscle tissue, were significantly altered by temperature during embryogenesis (*S. canicula*: 90 upregulated and 70 downregulated, p-value range = $1x10^{-3}$ - $3.3x10^{-17}$; *Danio rerio*: 112

upregulated, 13 downregulated, p-value range = $1x10^{-3}$ - $7.2x10^{-7}$; *Dicentrarchus labrax*: 293 upregulated and 48 downregulated, p-value range = $1x10^{-3}$ – $9.56x10^{-11}$).

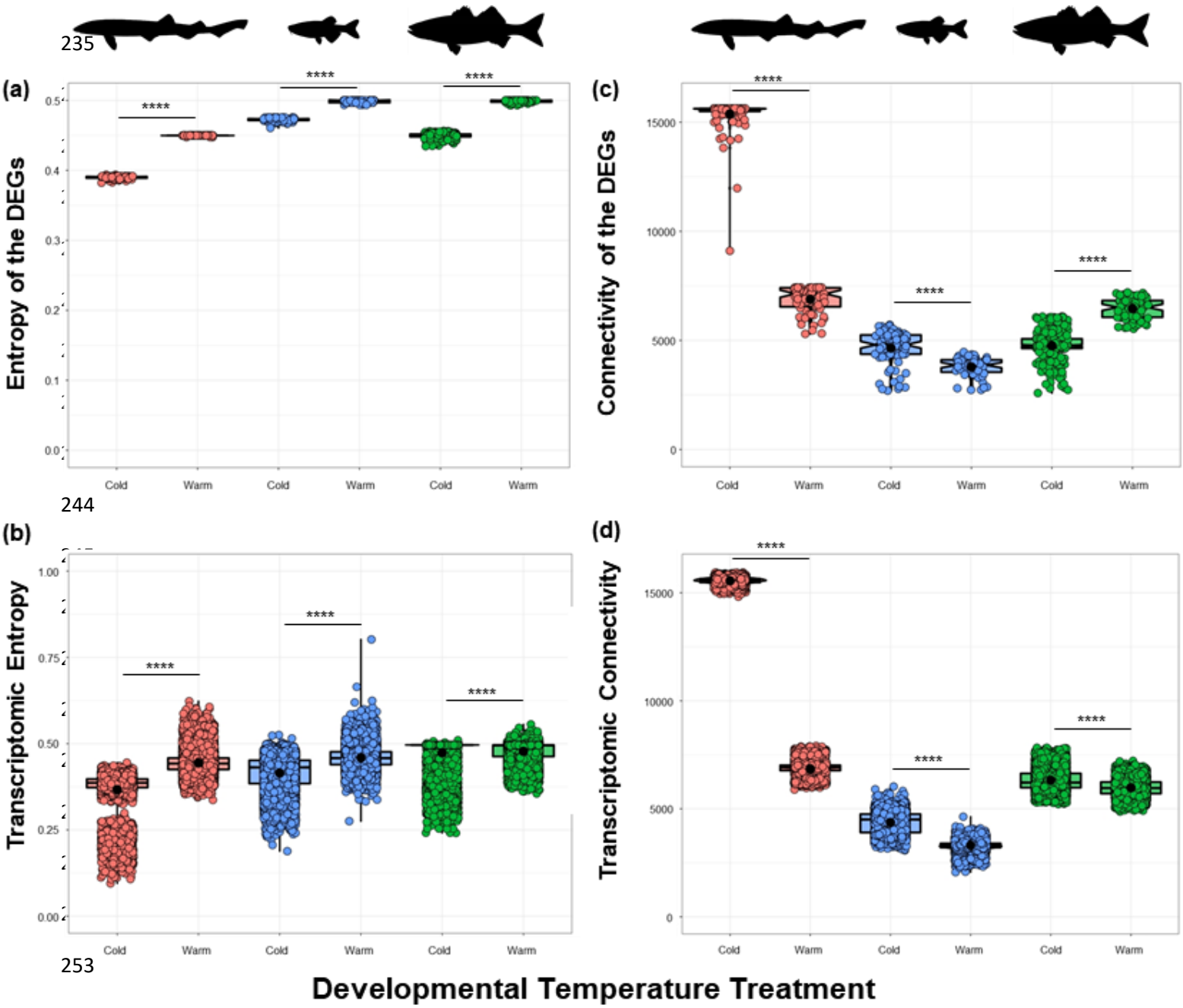

FIGURE 1 The entropy (A & C) and connectivity (B & D) of the differentially expressed genes (A & B) and entire transcriptome (C & D) of *Scyliorhinus canicula* (red, N = 3), *Danio rerio* (green, N = 5-8), and *Dicentrarchus labrax* (blue, N = 5) that experienced embryogenesis at different temperatures. Asterisks denote statistically significant differences between temperature treatment groups (Wilcoxon test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Hypernetwork analysis was performed on both the DEGs lists identified in each species, and on 10,000 permuted random subsets of the entire transcriptome. Embryogenesis at an elevated temperature significantly increased the entropy (Figure 1a; *S. canicula* $p < 2.2x10^{-16}$, *D. rerio* $p < 2.2x10^{-16}$, *D. labrax* $p < 2.2x10^{-16}$) of the DEGs in all three species, as well as reducing connectivity in *S. canicula* and *D. rerio*, whilst increasing it in *D. labrax* (Figure 1b; *S. canicula* $p < 2.2x10^{-16}$, *D. rerio* $p = 4.8x10^{-12}$, *D. labrax* $p = 2.2x10^{-16}$)

Connectivity was decreased in all three species across the entire transcriptome by an elevated incubation temperature (Figure 1d; *S. canicula* $p < 2.2x10^{-16}$, *D. rerio*, $p < 2.2x10^{-16}$, *D. labrax* $p < 2.2x10^{-16}$). However, entropy of the entire transcriptome was only increased in *S. canicula* and *D. rerio*, whilst the *D. labrax* transcriptome displayed a lower entropy following incubation at an elevated temperature (Figure 1c; *S. canicula* $p < 2.2x10^{-16}$, *D. rerio*, $p < 2.2x10^{-16}$, *D. labrax* $p < 2.2x10^{-16}$).

Scyliorhinus canicula, *D. rerio*, and *D. labrax* all had a clear cluster of highly connected DEGs (yellow clusters), when raised under control conditions *(*Figure 2a, c, e), which was not present in the animals raised at an elevated temperature (Figure 2b, d, f). Over-representation analysis of the genes present in these highly connected clusters highlighted a role for these genes in development and muscle contractile performance (Tables 2, 3 & 4), amongst other processes.

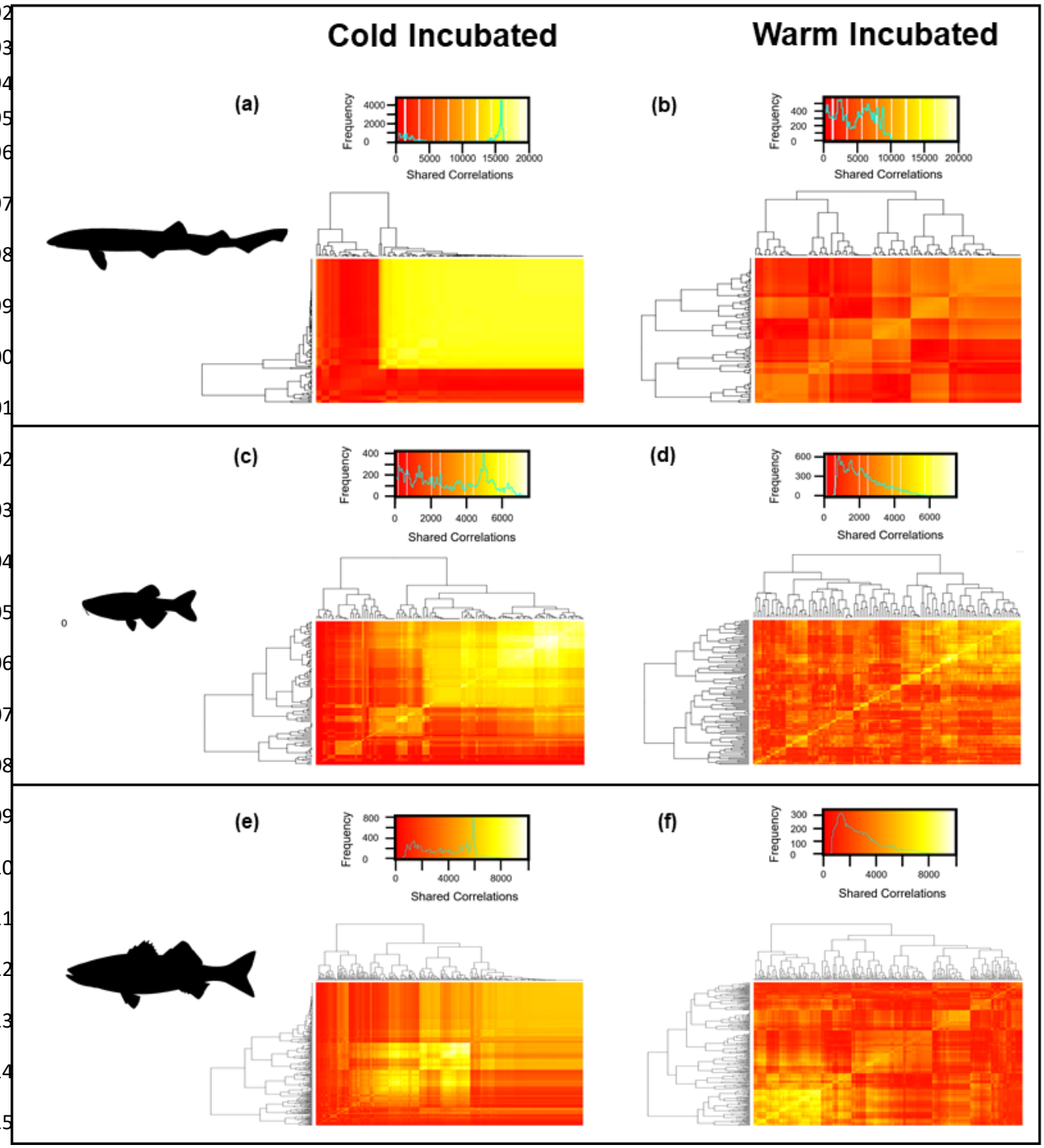

FIGURE 2 Heatmaps displaying the shared correlations between pairs of differentially expressed genes to the rest of the transcriptome in *Scyliorhinus canicula* (A & B, N = 3), *Danio rerio* (C & D, N = 5-8), and *Dicentrarchus labrax* (E & F, N = 5) that experienced embryogenesis at either control (A, C, E) or elevated temperature (B, D, F) conditions. The colour key, and a histogram of the data, are shown in the insets above each heatmap.

4 DISCUSSION

Previous studies have shown that the thermal environment experienced during embryogenesis can have persistent effects on the physiology, gene expression, and phenotypic plasticity of fishes (Anastasiadi *et al*., 2021; Beaman *et al*., 2016; Schnurr *et al*., 2014; Scott & Johnston, 2012). Here, we show that elevated embryonic temperature also programs lasting changes to the organisation of transcriptional networks in small-spotted catsharks (*S. canicula*), zebrafish (*D. rerio*), and European seabass (*D. labrax*), and suggest that this may play a role in the previously observed phenomena of developmentally programmed, temperature-induced changes in phenotypic plasticity (Beaman *et al*., 2016). Development at an elevated temperature resulted in differentially expressed genes (DEGs) with a significantly higher entropy, and altered connectivity, in all three species. The properties of the entire transcriptome were also significantly affected by developmental temperature. Transcriptome wide gene connectivity was significantly decreased by an elevated incubation temperature in all three species. Network entropy increased with developmental temperature in *S. canicula* and *D. rerio,* but decreased marginally in *D. labrax*. The magnitude of this change was greatest in *S. canicula*, followed by *D. rerio*, and then *D. labrax*. Interestingly, this order of effect sizes matches the times since exposure, which were 4-5, 8-9, and 34 months in *S. canicula*, *D. rerio*, and *D. labrax*, respectively. Whilst the DEGs identified in each species/tissue type showed little overlap, warming during embryogenesis produced consistent effects on the properties of both the DEG and transcriptome-wide gene networks. Such tissue independent transcriptomic signatures have been demonstrated during growth in mammals (Stevens *et al*., 2013), and may relate to warming-induced changes in ectotherm early-life growth pattern (Musa *et al*., 2020; Hume, 2019). These developmentally programmed changes in transcriptional entropy and connectivity will change the response of the transcriptome to future environmental challenges, which may result in altered phenotypic plasticity, and thus may have implications for both physiological and population-level resilience to future warming events.

Changes in the role of the DEGs in the transcriptional network, which manifest as an increase in entropy and changes in connectivity, may consequently alter the ability of these genes and thus the animal to mount a co-ordinated response to future environmental challenges. Recent studies have suggested that the developmental environment influences the capacity to acclimate to environmental changes in later-life (Beaman *et al*., 2016). For example, intertidal copepods (*Tigriopus californicus*) that undergo embryogenesis at 25˚C have the capacity to raise their critical thermal maximum as adults through temperature acclimation (Healy *et al*., 2019). However, embryos of the same species incubated at 20˚C show no acclimation capacity in critical thermal maximum as adults (Healy *et al*., 2019). Given that phenotypic plasticity is facilitated by co-ordinated changes in gene expression, these developmentally programmed changes in phenotypic plasticity may relate to the changes in transcriptional organisation observed in our study. Further studies support the link between embryonic temperature and later-life acclimation capacity. Mosquitofish (*Gambusia holbrooki*) produce multiple generations per year, with those born in summer experiencing a warm but constant environment, and

those born in spring experiencing cool, but steadily warming, conditions. Recent work has demonstrated that mosquitofish born in the more thermally variable spring environment have a greater capacity to acclimate their metabolic processes than mosquitofish from the more thermally stable summer conditions (Seebacher *et al*., 2014). Finally, studies of fruit flies (*Drosophila melanogaster*) have shown that heat tolerance is influenced not just by acclimation temperature, but by an interaction between acclimation temperature and embryonic temperature, further supporting the role of the early-life thermal environment in dictating an individual's response to future temperature challenges (Willot *et al*., 2021).

Studies of developmental programming resulting from environmental challenges in fish and reptiles have demonstrated a protective phenotype in later-life, in contrast to the negative effects often reported in mammals (Galli *et al*., 2021; Hellgren *et al*., 2021; Ruhr *et al*., 2019; Seeabacher *et al*., 2014). For example, work on the common snapping turtle (*Chelydra serpentina*) revealed that hypoxia exposure (50% air saturation) throughout embryogenesis improved the anoxia tolerance of the cardiomyocytes isolated from juveniles (Ruhr *et al*., 2019). Similarly, jacky dragons (*Amphibolurus muricatus*) raised with extended basking times (11 vs. 7 hours of daily heat lamp exposure) show a higher panting threshold than those from control conditions, implying a greater thermal tolerance (So & Schwanz, 2018). Such protective phenotypes, programmed by environmental challenges during embryogenesis, may be associated with the altered transcriptional landscape resulting from environmental stresses during early life. However, exposure to a stressor during embryogenesis does not always facilitate resilience to that stressor in adulthood. Cuban brown anole (*Anolis sagrei*) eggs incubated under cool, warm, and hot temperature fluctuations, and then raised in standard conditions after hatching, show no differences in thermal tolerance as adults (Gunderson *et al*., 2020). Thus, whilst developmental exposure to a stressor often influences the capacity to respond to that same stressor in later-life, it is not always the case.

One mechanism linking the developmental environment to later-life acclimation capacity is through the activity of DNA-methyltransferases (DNMTs) (Radford, 2018). DNA methylation by DNMTs can repress gene expression either directly, whereby the methylation prevents the interaction between a gene and its DNA binding proteins (Watt & Molloy, 1988), or indirectly, through recognition of the methyl cytosine by methyl cytosine binding proteins, and the consequent recruitment of transcriptional corepressors (Boyes & Bird, 1991; Klose & Bird, 2006). Through these mechanisms, DNMTs can alter gene expression. Changes in embryonic temperature have been shown to alter DNA methylation patterns in fish (Metzger & Schulte, 2017), with a recent study identifying DNMT3a as the mediator of developmental thermal plasticity in zebrafish (*Danio rerio*) (Loughland *et al*., 2021). Thus, by modulating DNMT3a's activity, changes in embryonic temperature can have persistent effects on the gene expression and plasticity of fishes (Loughland *et al*., 2021; Metzger & Schulte, 2017). Whilst the DEGs identified in our study show little overlap between the species/tissue-types (*S. canicula*; ventricle, table 2; *D. rerio*, hypaxial fast muscle, table 3; and *D. labrax*, muscle, table 4), the observed changes in entropy and connectivity span the entire transcriptome. This transcriptome-wide remodelling suggests that a mechanism basal to gene expression, such as DNA/chromatin

methylation, may be facilitating the developmentally programmed changes in gene network co-ordination.

As well as implications for an organism's plasticity, changes in transcriptional entropy are known to be a predictor of biological fitness (Zhu *et al*., 2020). As stress increases, gene expression patterns become more random and consequently transcriptional entropy increases (Zhu *et al*., 2020). The negative relationship between transcriptional entropy and fitness has been demonstrated in seven species of bacteria, across a range of contexts, and found to be robust (Zhu *et al.,* 2020). Therefore, within this framework, the increase in entropy caused by developmental exposure to elevated temperature in fishes could be interpreted as a decline in fitness. Whilst this interpretation is tentative, given that the negative relationship between entropy and fitness has only presently been demonstrated in bacteria (Zhu *et al*., 2020), studies in animals have shown later-life fitness costs of increased embryonic temperatures. *Drosophila subobscura* incubated at elevated temperatures throughout juvenilehood show a lower reproductive performance as adults than those incubated in control conditions, regardless of the temperatures experienced during later-life (Santos *et al*., 2021). Therefore, the link between the embryonic environment and later-life fitness is present in ectotherms, and future studies should probe the potential importance of transcriptional entropy in mediating these effects.

Climate change is a major threat facing animals. As the world's oceans and rivers continue to warm (Cai *et al*., 2014; Oliver *et al*., 2018), the physiological and population-level stresses exerted upon fishes will continue to grow. Embryogenesis is a sensitive period in many animal's life histories, and the conditions experienced during embryonic development influence their growth, physiology, and behaviour throughout their life. Consequently, to develop a comprehensive appreciation of how fishes will respond to climate change stressors, it is crucial that we develop an understanding, and incorporate into predictive models, the role of developmental programming in dictating an organisms' physiology and capacity to respond to future environmental challenges.

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Competing Interests

We declare no competing interests.

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Author Contributions

D.M.R co-ordinated the experiments, analysed the data, and drafted the manuscript. T.G contributed to the code and analytical method development. S.A.H and A.V performed the microsatellite analysis, and S.A.H identified the individual animals. B.G and T.M supplied *Scyliorhinus canicula* eggs. H.A.S, P.C, and A.S conceived the study, secured funding, and reviewed and revised the manuscript. All authors contributed to the manuscript and gave their approval for publication.

Data Availability

Data are available through NCBI's Gene Expression Omnibus (GEO, Edgar et al., 2002) at the GEO accession number GSE189976.

TABLE 1 Experimental details and transcriptome assembly statistics for *Scyliorhinus canicula*, *Danio rerio*, and *Dicentrarchus labrax*.

Parameter	Species		
	Scyliorhinus canicula	*Danio rerio*	*Dicentrarchus labrax*
time since exposure	4-5 months	8-9 months	34 months
developmental temperatures	15˚C and 20˚C	27˚C and 32˚C	17.2˚C and 20.8˚C
exposure period	until hatching (~15 weeks)	until hatching (~3 days)	6-63dpf (57 days)
N50	1422	1049	1601
median contig length	363	342	428
BUSCO score	88.8%	78.1%	50.7%
annotated transcripts	19306	18374	12607

TABLE 2 Over-representation analysis of the differentially expressed genes identified in the central cluster of the cold, but not warm, incubated *Scyliorhinus canicula*

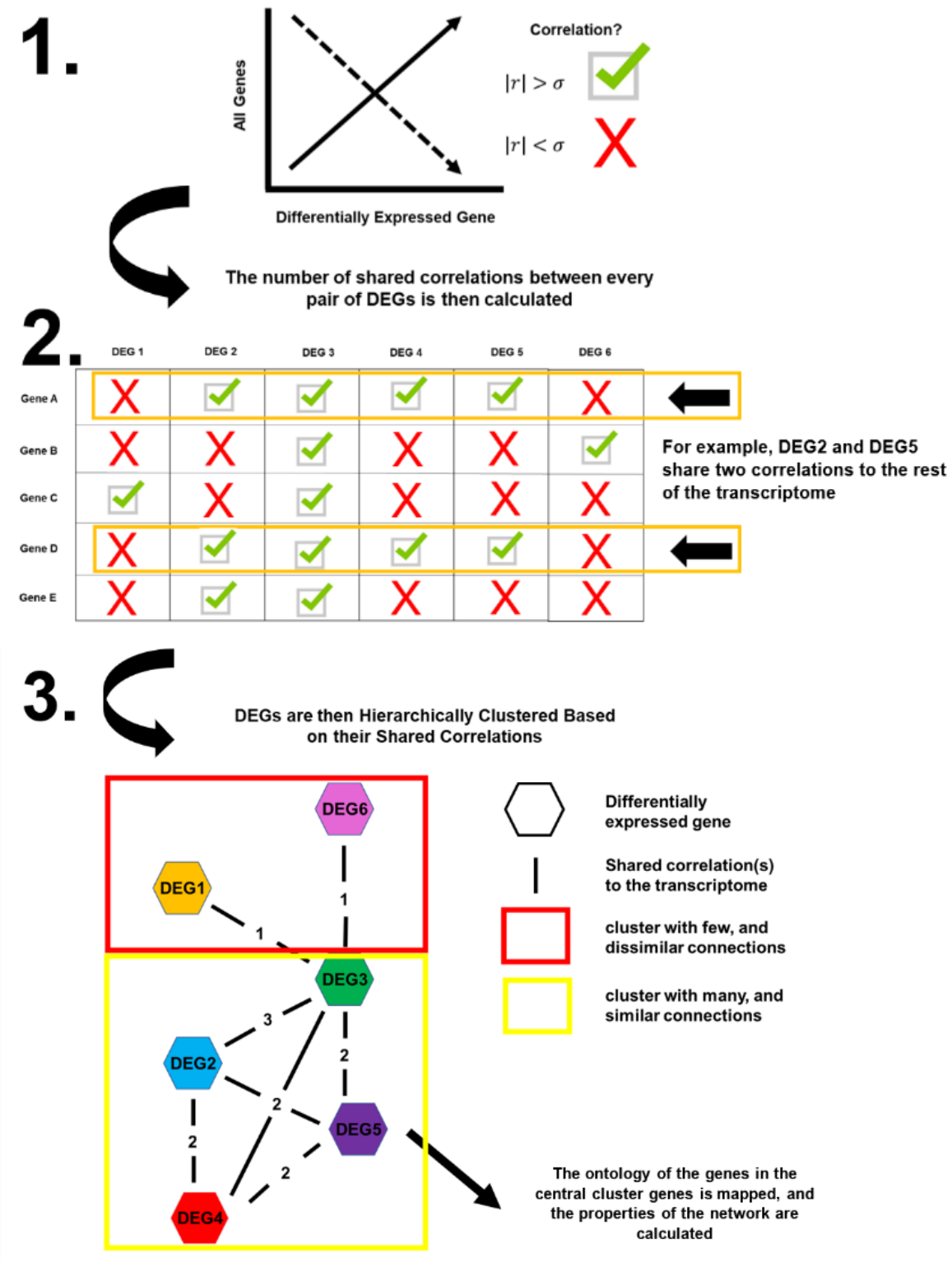
Biological Process	P-value
multicellular organism development	0.040
response to tumour necrosis factor	0.074
positive regulation of telomere maintenance via telomerase	0.092

TABLE 3 Over-representation analysis of the differentially expressed genes identified in the central cluster of the cold, but not warm, incubated *Danio rerio.*

Biological Process	P-value
cellular response to UV	0.061
positive regulation of smooth muscle cell proliferation	0.064
negative regulation of apoptotic process	0.069

TABLE 4 Top 10 results from over-representation of the differentially expressed genes identified in the central cluster of the cold, but not warm, incubated *Dicentrarchus labrax.*

Biological Process	P-value
ventricular cardiac muscle tissue morphogenesis	4.4×10^{-21}
muscle filament sliding	1.7×10^{-20}
regulation of the force of heart contraction	2.0×10^{-19}
cardiac muscle contraction	4.6×10^{-18}
transition between fast and slow fiber	2.5×10^{-15}
regulation of heart rate	3.0×10^{-14}
cardiac muscle hypertrophy in response to stress	1.5×10^{-12}
adult heart development	9.8×10^{-12}
skeletal muscle contraction	2.7×10^{-11}
ATP metabolic process	5.4×10^{-11}



Supplementary Figure 1: A schematic qualitatively describing hypernetwork analysis. **1.)** All the differentially expressed genes (DEGs) identified between the two treatment groups are tested for correlations against all other genes identified within the transcriptome. The relationships are binarized as 1s (correlation) or 0s (no correlation) based on a cut-off of one standard deviation. **2.)** The number of correlations to the rest of the transcriptome that are shared between each possible pair of DEGs is calculated. In this, any pair of DEGs that are correlated with many of the same genes will have a high shared correlations value, whilst any pair of DEGs that have no/few shared correlations will have a low shared correlations value. **3.)** DEGs are hierarchically clustered based on the similarity of their shared correlations allowing the identification of associated clusters of genes. This network can then be represented as a heatmap (Figure 2) and the mathematical properties of the network (for instance, connectivity and entropy) can be calculated per treatment group.

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