Warming During Embryogenesis Induces 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 embryonic 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 small-spotted catshark (*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 genes were investigated using network modelling. Development at an elevated temperature caused an increase in transcriptomic entropy, a parameter thought to relate to plasticity. We then validated this observation through a novel re-analysis of published zebrafish (*Danio rerio*), European seabass (*Dicentrarchus labrax*), and three-spined stickleback (*Gasterosteus aculeatus*) datasets and show that developmental temperatures influence the transcriptional response to future thermal challenges by, in part, altering the organisation of gene networks.

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 (Anastasiadi *et al*., 2017, Fellous *et al*., 2015). Some of these changes in methylation pattern, and their concomitant influence on gene expression, can be retained into later life. For instance, changes in developmental temperature cause hypermethylation in threespined 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, hindered, or simply altered 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 can 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). Shannon entropy (herein entropy) of the transcriptome is a metric describing the order or predictability of gene-gene interactions within a transcriptional network. These gene-gene interactions can either be direct (pairwise) or indirect (higher order interactions) (Sanchez, 2019). 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 of the transcriptome in later life.

Whilst many animals may experience adverse environmental conditions during embryogenesis, some species, owing to their ecology, are more susceptible than others. Oviparous elasmobranchs,

because of their protracted, sessile developmental period, are susceptible to experiencing sub-optimal environmental conditions for long durations. Oviparous elasmobranchs develop 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 the purse, and thus may be consistently exposed to unfavourable environmental conditions such as elevated temperatures. As the world's oceans continue to warm (IPCC, 2021, Cai *et al*., 2014), and the frequency of marine heatwaves is increasing (Oliver *et al*., 2018), the likelihood of oviparous elasmobranchs developing in sub-optimal conditions increases too. 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, Ripley *et al*., 2021). However, no studies have investigated how embryogenesis at elevated temperatures may have lasting effects on elasmobranchs through developmental programming.

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. Ventricle tissue was chosen for sequencing owing to the ventricle's key role in mediating thermal performance and tolerance (Pörtner and Knust, 2007). 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), European seabass (*Dicentrarchus labrax)* muscle (Anastasiadi *et al*., 2021), and threespine stickleback (*Gasterosteus aculeatus*) muscle (Metzger and Schulte, 2018) to test the consistency of our observations across multiple species of fishes and other striated muscle tissues.

2 MATERIAL AND METHODS

2.1 Experimental animals

Details of the experimental design can be found in figure 1. *Scyliorhinus canicula* embryos were collected from a population of 7 randomly mating 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). 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 and were performed under the Home Office License P005EFE9F9 held by HAS.

Following embryonic staging, individuals were randomly assigned to a temperature treatment group (15 ± 0.3˚C or 20 ± 0.3˚C). For the control condition, 15˚C was chosen 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. For the treatment group, +5˚C (20˚C) was chosen

as is it represents the increase in ocean temperature predicted by the end of the century, whilst also being within the current range of temperatures experienced by some *S. canicula* populations in the wild (Pegado *et al*., 2020). 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 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. As *S. canicula* lay multiple eggs, microsatellite analysis was also used to confirm that no samples used for RNA-sequencing were taken from full siblings.

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159 **2.4 *Danio rerio, Dicentrarchus labrax,* and *Gasterosteus aculeatus* re-analysis**

160 The computational analysis pipeline (sections 2.5 and 2.6 below) was also implemented on previously
161 published RNA-seq datasets of temperature-induced developmental programming in zebrafish (*D.*
162 *rerio*) hypaxial fast muscle (Scott & Johnston, 2012), European seabass (*D. labrax*) muscle tissue
163 (Anastasiadi *et al*., 2021), and threespine stickleback (*G. aculeatus*) muscle tissue (Metzger and
164 Schulte, 2018). This re-analysis was performed to assess the robustness and between-species
165 consistency of any temperature-induced effects observed in *S. canicula*.

166 The *D. rerio, D. labrax*, and *G. aculeatus* data were processed in an identical manner to the *S.*
167 *canicula* data. Developmental conditions and transcriptome assembly statistics for the four species
168 are shown in table 1. Full details of the animals used in the re-analysis can be found in Scott &
169 Johnston 2012 (*D. rerio*, N = 4), Anastasiadi *et al*. 2021 (*D. labrax*, N = 5), and Metzger and Schulte
170 2018 (*G. aculeatus*, N = 6 respectively).
171

172 **2.5 de-novo transcriptome assembly and differential expression analysis**

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

Schulte, 2018. Significance levels were adjusted for multiple testing using a false discovery rate (FDR) correction.

2.6 Entropy analysis

Entropy of both pairwise and higher-order interaction networks was determined in each treatment group. See figure 2 for a conceptual overview. The expression of each gene was tested for correlations against all the assembled transcripts within each treatment group. The correlation values were binarized such that strong positive (top 10% of R-values) or strong negative (bottom 10% of R-values) correlations were retained (denoted as 1) and weak correlations were discarded (denoted as 0). This binarized correlation matrix is square and represents the pairwise interaction network.

To generate the higher order interaction networks (hypernetworks), the binarized correlation matrix was multiplied by the transpose of itself ($M \times M^t$) to return the adjacency matrix of the hypernetwork, where the values in each cell represent the number of shared correlations any given pair of genes have to the rest of the transcriptome. These values also represent the dimensionality of the hyperedge connecting each pair of nodes.

To assess whole-transcriptome properties, we selected subsets of 200 genes from the network (either the binarized network or the hypernetwork) and calculated the entropy of each gene using the R packages mixOmics (Rohart *et al*., 2017) and BioQC (Zhang *et al*., 2017) in R 3.6.0 (R core team, 2019). The mean gene-level entropy was then calculated for each iteration. This approach was iterated 1000 times for both the binarized and hyper-networks to provide a whole-transcriptome assessment of both pairwise (binarized network) and higher order (hypernetwork) entropy for animals from each treatment group. Hypernetwork entropy was then scaled to one by taking $\log_2 N^2$, where N is the length of the gene list. Wilcoxon tests were used to contrast entropy between the sharks incubated at 15˚C and 20˚C. This same approach was applied to re-analysed zebrafish, seabass, and stickleback datasets.

2.7 Gene ontology analysis

Gene ontology analysis, leveraging the pairwise and higher-order network entropy, was used to identify specific gene-pathways whose organisation was influenced by developmental temperature. Gene pathways (geneontology.org) of interest were selected *a priori* and each treatment group was analysed separately. Gene networks (both pairwise and higher-order) were created (as described in section 2.6) from a subset (N = 20) of genes within each gene ontology term, and an entropy score was calculated. This approach was iterated 1000 times per gene pathway to generate a distribution of entropy scores for each gene ontology term. A Bayesian approach was used to assess the differences between groups; entropy distributions for each gene pathway contrasted between developmental temperatures and β entropy scores were calculated. These β values represent the difference in entropy between warm and control incubated fish for a given gene pathway. The posterior of β entropy values were plotted and the 89% credible intervals calculated. Differences in

pathway entropy were defined as significant where the 89% credible interval did not cross 0, per standard protocol (McElreath, 2018).

2.8 Zebrafish acclimation analysis

A subset of zebrafish from both developmental temperatures in the Scott and Johnston (2012) paper were cold acclimated (16˚C for 28-30 days) in adulthood prior to RNA-sequencing. We re-analysed these data (transcriptome assembly, read quantification and annotation as described in sections 2.5 and 2.6 above) to look at the effects of developmental temperature on temperature-induced gene expression changes in later-life. The proportion of genes whose expression changed due to cold acclimation was compared using Fisher's exact test, and the magnitude of change in the differentially expressed genes (DEGs) was contrasted with a Wilcoxon test. Finally, we created a logistic regression model (R package 'lme4' (Bates *et al*., 2015)) to assess whether a gene's response (differentially expressed or not, as defined by a false discovery rate corrected p-value (FDR) cut-off of 0.05) to cold-acclimation was influenced by its pre-acclimation entropy in both the pairwise and higher-order interaction networks. Pairwise network entropy, higher order network entropy, developmental condition, direction of the change (positive or negative fold change), and all their interactions were included in the initial logistic regression model. Model simplification was then performed to remove redundant predictors using stepAIC from the package 'MASS' (Venables and Ripley, 2002). Predictors retained into the final model were tested for significance using a Wald test (package 'aod', Lesnoff and Lancelot, 2012), and their effect size calculated (package 'oddsratio', Schratz, 2020).

3 RESULTS

Developmental warming caused the differential expression of 163 (FDR < 0.05) genes in juvenile *S. canicula*, compared to 66 (FDR < 0.05), 68 (FDR < 0.05), and 21 (FDR < 0.05) in adult *D. labrax*, *D. rerio*, and *G. aculeatus* respectively (figure 3). The DEGs showed little overlap between species (figure 3).

Entropy of the pairwise interaction networks was increased due to embryonic warming in all species (*S. canicula*; figure 4a, $p < 0.0001$. *D. labrax*; figure 4b, $p < 0.0001$. *D. rerio*: figure 4c, $p < 0.0001$. *G. aculeatus*; figure 4d, $p < 0.0001$), whilst cooling caused an entropy decrease in *G. aculeatus* (figure 4d, $p < 0.0001$). Gene ontology revealed warming-induced entropy differences in 11/14 (*S. canicula*), 10/14 (*D. labrax*), 13/14 (*D. rerio*) and 7/14 (*G. aculeatus*) of the pathways investigated (figure 5). Developmental cooling altered the entropy of 14/14 pathways in *G. aculeatus*.

Entropy of the higher-order interaction networks was also increased by developmental warming in *S. canicula* (figure 6a, $p < 0.0001$), *D.labrax* (figure 6b, $p < 0.0001$) and *D. rerio* (figure 6c, $p < 0.0001$), but not in *G. aculeatus*, where entropy of the higher-order interactions was highest in the control animals, and reduced by both developmental warming and cooling (figure 6d, $p < 0.0001$ & $p < 0.0001$). Gene ontology revealed warming-induced entropy differences in 13/14 (*S. canicula*), 9/14 (*D.*

labrax), 10/14 (*D. rerio*) and 4/14 (*G. aculeatus*) of the pathways investigated (figure 7). Developmental cooling altered the entropy of 5/14 pathways in *G. aculeatus*.

D. rerio that were incubated at an elevated temperature showed a greater number (figure 8a, $p < 0.0001$) and magnitude (figure 8b, $p < 0.0001$) of gene expression change following 28-30 days cold acclimation (16˚C) than *D. rerio* developed in control conditions. Of the cold-induced DEGs, 44.1% were shared across both developmental groups. Genes that were programmed by the developmental environment were more likely to respond to cold acclimation in adulthood than genes that were not developmentally programmed (figure 8c, $p < 0.0001$). The gene-level entropy of both the pairwise ($p = 0.031$) and higher order ($p = 0.044$) interaction networks before cold-acclimation were associated with the probability of a gene responding to cold-acclimation in later-life (pseudo $R^2 = 0.091$). An increase in higher order entropy was associated with a reduced likelihood of a gene's expression changing (one-unit increase in higher order network entropy: odds ratio & 95% CI – 0.816, 0.671-0.996), whilst an increase in pairwise network entropy was positively associated with the probability of a gene's expression changing (one-unit increase in pairwise network entropy: odds ratio & 95% CI – 6.344, 1.184-33.699).

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, Metzger and Schulte 2017). Here, we document temperature-induced developmental programming for the first time in elasmobranchs and show that elevated embryonic temperature programs lasting changes to the organisation of transcriptional networks in small-spotted catshark (*S. canicula*) ventricular tissue. Furthermore, we demonstrate the consistency of this observation across species and muscle types through a novel re-analysis of previously published datasets on zebrafish (*D. rerio*), European seabass (*D. labrax*) and three-spined stickleback (*G. aculeatus*) muscle tissue. Such transcriptomic signatures, seen here in both ventricle and muscle tissue, begin to suggest a tissue independent effect such as those previously demonstrated during growth in mammals (Stevens *et al*., 2013, Lui *et al*., 2010, Lui *et al*., 2008). Finally, we suggest that these entropy changes may play a role in the previously observed phenomena of developmentally programmed, temperature-induced changes in phenotypic plasticity (Beaman *et al*., 2016).

Developmental warming programmed few (21-163) differentially expressed genes in later-life, with little overlap between species (figure 3). However, a consistent increase in the entropy of pairwise gene interaction networks was observed across each species with increasing developmental temperature (figure 4). These pairwise interaction networks represent gene-gene correlations, and the consistent differences in the pairwise network entropy across species gives strong support that developmental temperature influences the organisation of these simple gene-gene connections in fishes. Gene ontology analysis of these pairwise networks revealed that pathways involved in ribosome assembly, response to hypoxia, DNA repair, protein folding, organismal growth, and insulin

receptor signalling were affected by developmental temperature in all four species, with insulin receptor signalling showing a consistent direction of change (figure 5). The insulin receptor signalling pathway is a key modulator of growth and metabolism. Environmental temperature is known to have both immediate (Musa *et al*., 2020) and delayed (compensatory growth, Mortensen and Damsgard, 1993) effects on growth, which may relate to changes in insulin receptor pathway signalling (Won and Borski, 2013).

Entropy of the higher order interactions was also affected by developmental warming, increasing in the catshark, seabass, and zebrafish, but decreasing in the stickleback (figure 6). Only the DNA repair and protein folding pathways showed warming-induced changes in all four species, with neither sharing a consistent between-species direction of change (figure 7). These higher order interactions represent networks of shared connections between gene pairs, and thus capture more nuanced and complex structure within the transcriptome than the simple gene-gene connections. Given this, it is perhaps unsurprising that the effects of developmental warming on the structure of these more complex (hyper)networks are less consistent across species. Whilst it is tempting to speculate on the reason that the higher order interaction networks of the stickleback differ to those of the catshark, zebrafish, and seabass, especially given the stickleback's complex evolutionary history (McKinnon and Rundle, 2002), the data required to disentangle this question does not yet exist. Nevertheless, these developmentally programmed changes in transcriptional entropy, of both the pairwise and higher order interaction networks, may change the response of the transcriptome to future environmental challenges, potentially resulting in altered phenotypic plasticity. Indeed, our zebrafish re-analysis shows that fish incubated in warmer conditions exhibit increased transcriptional entropy and a greater number and magnitude of gene-expression changes in response to later-life thermal challenges (figure 8). Interestingly, genes that were programmed by the developmental environment (figure 3) were more likely to change in response to later-life cold-acclimation than those not affected by developmental warming (figure 8c). Thus, there is a degree of consistency in temperature-sensitive genes throughout ontogeny in zebrafish, which aligns with findings from previous studies on three spined stickleback (*G. aculeatus*, Metzger and Schulte, 2018). Furthermore, the pre-cold acclimation entropy of individual genes in both the pairwise and higher order interaction networks was associated with the likelihood of a gene's expression changing in response to later-life cold acclimation. Whilst the R^2 of the logistic regression was modest, both the pairwise and higher order entropy had significant effects on the probability of gene expression change. Furthermore, the odds ratios and their associated confidence intervals did not cross 1, demonstrating that there is a significant effect of entropy on the transcriptomic response to temperature acclimation. Whilst this effect may be small and noisy at the level of individual genes, the cumulative effects across the whole transcriptome may be marked. Thus, the thermal environment experienced during embryogenesis may influence an individual's responsiveness to future temperature challenges via changes in entropy of the pairwise and higher order gene interactions within the transcriptome.

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 often show a protective phenotype in later-life, in contrast to the negative effects typically 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 (figure 3), the observed changes in entropy span the entire transcriptome. This transcriptome-wide remodelling suggests that a mechanism upstream of 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). Although the same relationship remains to be tested in fish, the increase in entropy caused by developmental exposure to elevated temperature could have similar effects on fitness. *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, it is crucial that we expand our understanding of the mechanisms by which the developmental environment can influence 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.

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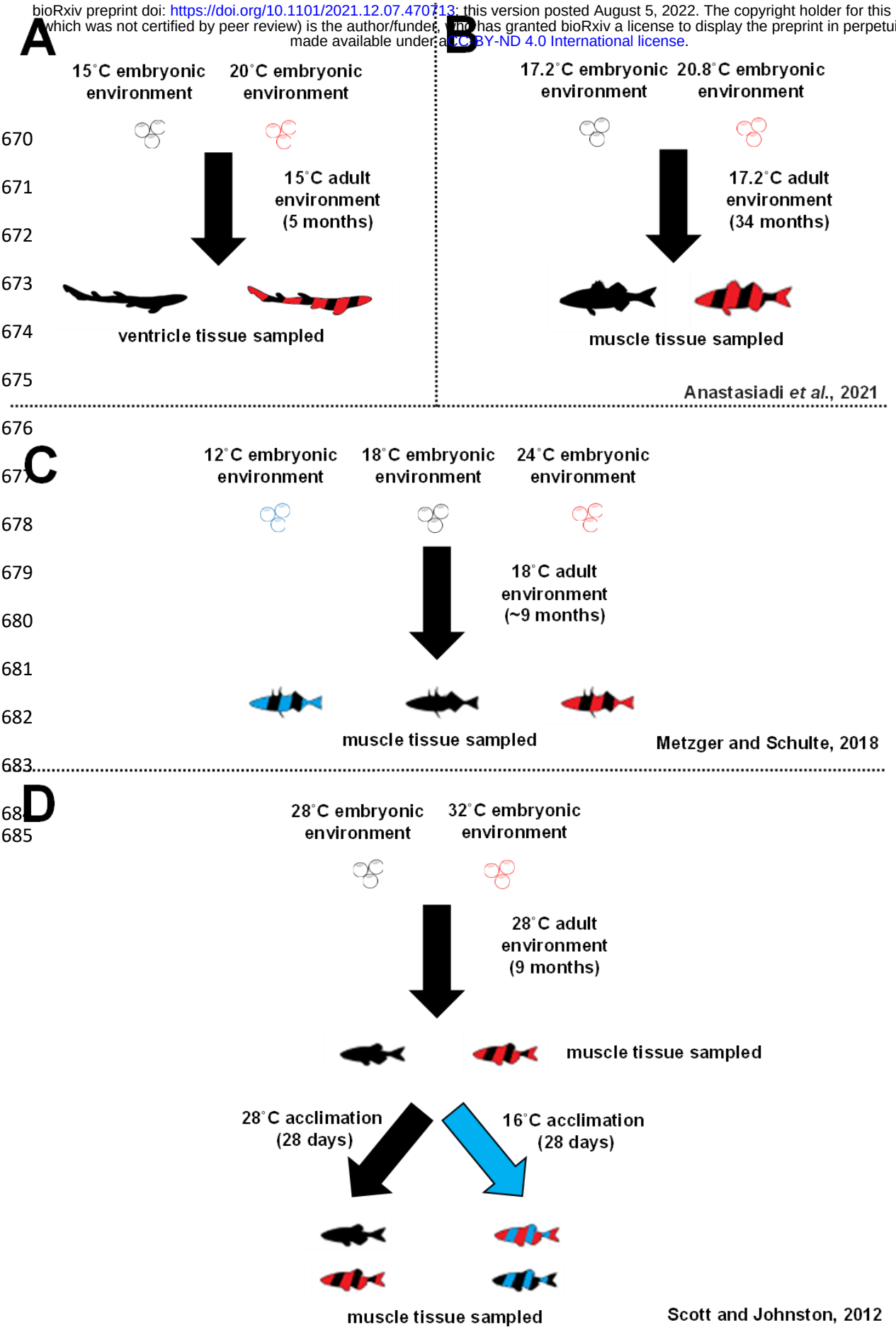

Figure 1: An overview of the experimental design for the RNA-seq data collected from **A)** *Scyliorhinus canicula*, and data re-analysed from **B)** *Dicentrarchus labrax* (Anastasiadi *et al*., 2021), **C)** *Gasterosteus aculeatus* (Metzger and Schulte, 2018), and **D)** *Danio rerio* (Scott and Johnston, 2012).

TABLE 1 Experimental details and transcriptome assembly statistics for *Scyliorhinus canicula*, *Danio rerio* (Scott and Johnston 2012), *Dicentrarchus labrax* (Anastasiadi *et al*., 2021) and *Gasterosteus aculeatus* (Metzger and Schulte, 2017).

Parameter	Species			
	Scyliorhinus canicula	*Danio rerio*	*Dicentrarchus labrax*	*Gasterosteus aculeatus*
time since exposure	4-5 months	8-9 months	34 months	~9 months
developmental temperatures	15˚C and 20˚C	27˚C and 32˚C	17.2˚C and 20.8˚C	12˚C, 18˚C and 24˚C
exposure period	until hatching (~15 weeks)	until hatching (~3 days)	6-63dpf (57 days)	until hatching (5-23 days)
life stage at sampling	juvenile	adult	adult	adult
tissue sampled	ventricle	hypaxial fast muscle	muscle	muscle
N50	1422	916	1601	459
median contig length	363	325	428	338

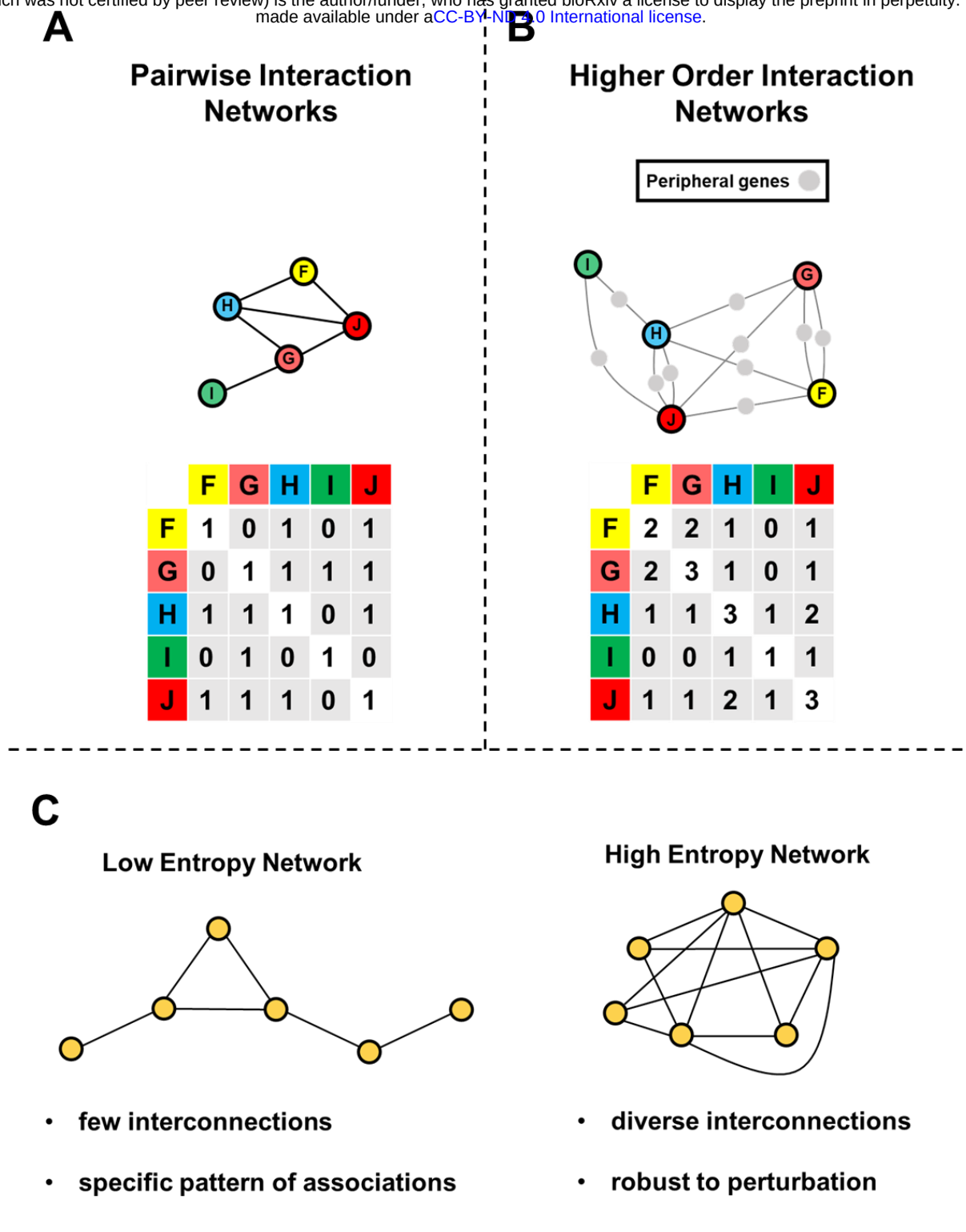

Figure 2: An example **A)** pairwise gene interaction network. Correlations between pairs of focal genes are shown by a solid black line, and denoted by a 1 in the associated matrix. Pairwise gene network entropy is calculated from the pairwise interaction matrix and represents the networks' information content (complexity). **B)** an example higher order interaction network. Focal genes (the two being assessed for their interaction) are associated via their shared connections to peripheral (not in the focal network) genes, denoted by the grey lines. An interaction between two focal genes can occur via one (gene H – gene I) or multiple (gene I – gene J) shared connections to the peripheral genes. The adjacency matrix denotes the number of shared connections to peripheral genes each pair of focal genes has. Higher order network entropy is calculated from this adjacency matrix and represents the networks' information content (complexity). **C)** an example low and high entropy network.

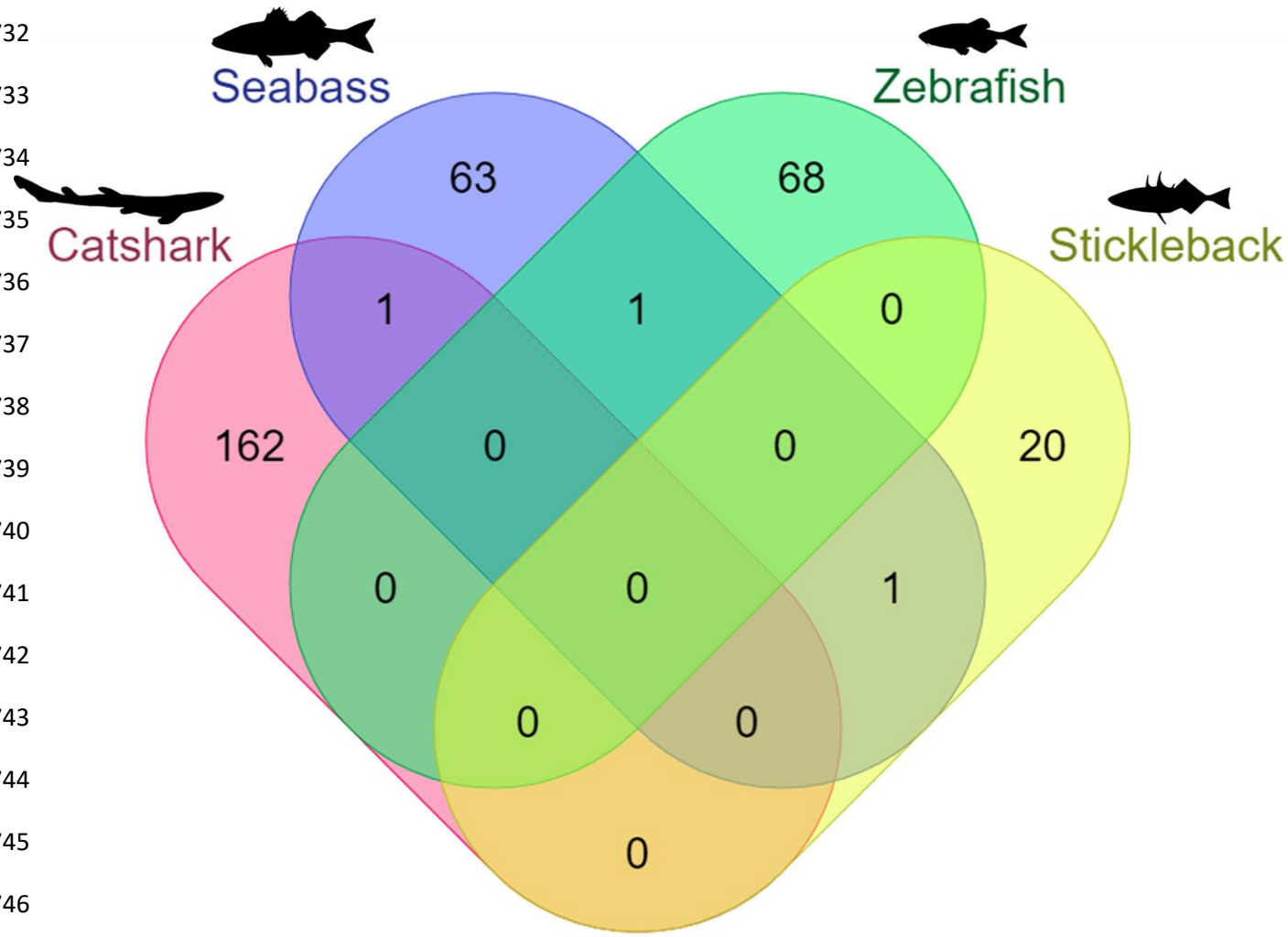

Figure 3: Overlap of genes in *Scyliorhinus canicula* (red), *Dicentrarchus labrax* (blue), *Danio rerio* (green), and *Gasterosteus aculeatus* (yellow) that are differentially expressed (FDR < 0.05) in later-life due to an increased embryonic temperature. *D. labrax, D. rerio, and G. aculeatus* data are re-analysed from Anastasiadi *et al*., 2021, Scott and Johnston 2012, and Metzger and Schulte 2017 respectively.

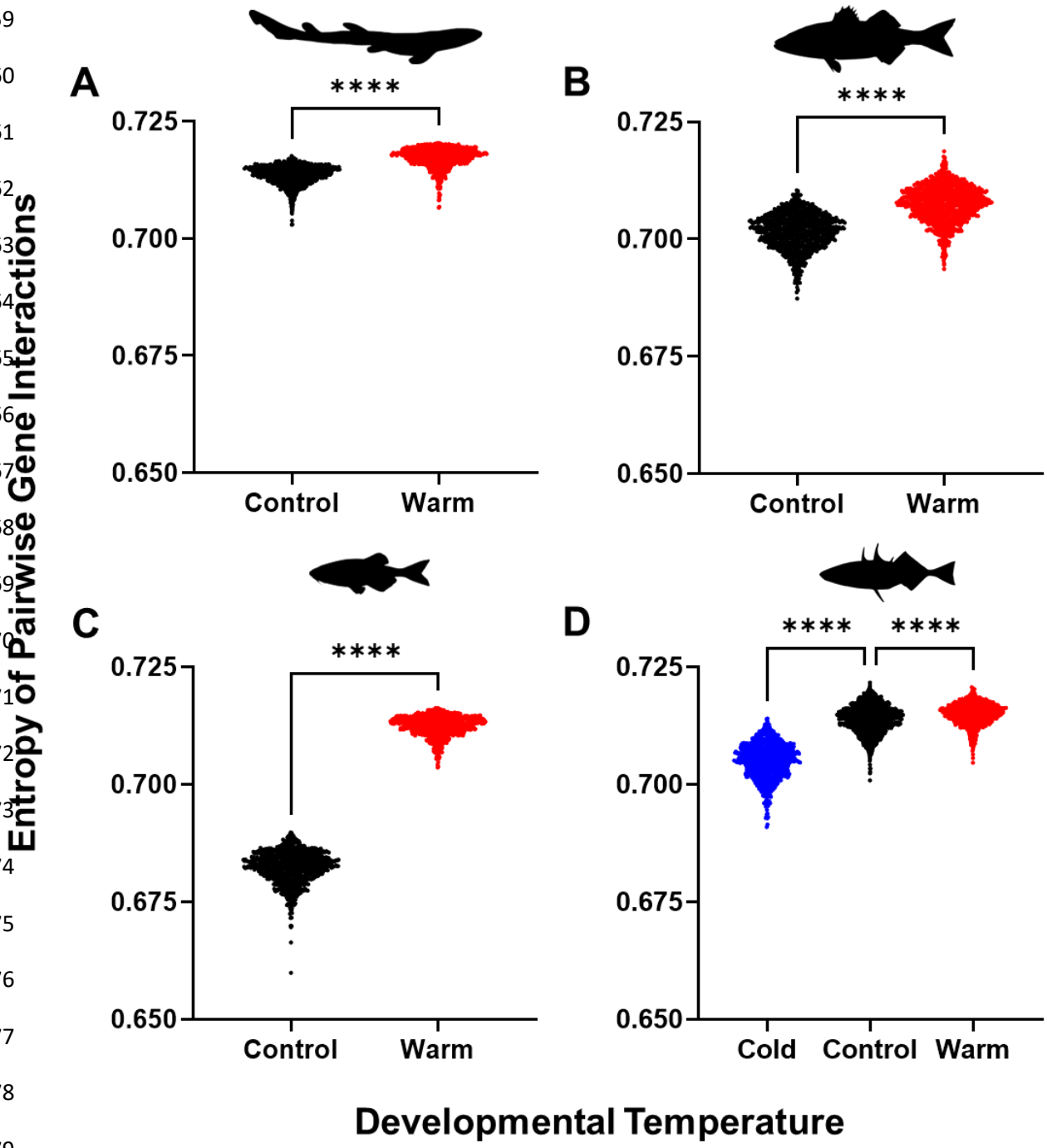

Figure 4: Entropy of pairwise gene interaction networks in **A)** *Scyliorhinus canicula*, **B)** *Dicentrarchus labrax,* **C)** *Danio rerio*, and **D)** *Gasterosteus aculeatus* that underwent embryogenesis at different temperatures. *Scyliorhinus canicula*; Wilcoxon test, $p < 0.0001$, N = 3 per group. *Dicentrarchus labrax*; Wilcoxon test, $p < 0.0001$, N = 5 per group. *Danio rerio*; Wilcoxon test, $p < 0.0001$, N = 4 per group. *Gasterosteus aculeatus*; Kruskal-wallis test and Dunn's multiple comparisons test, $p < 0.0001$ (cold vs. control) and $p < 0.0001$ (warm vs. control), N = 6 per group. *D. labrax, D. rerio, and G. aculeatus* data are re-analysed from Anastasiadi *et al*., 2021, Scott and Johnston 2012, and Metzger and Schulte 2017 respectively.

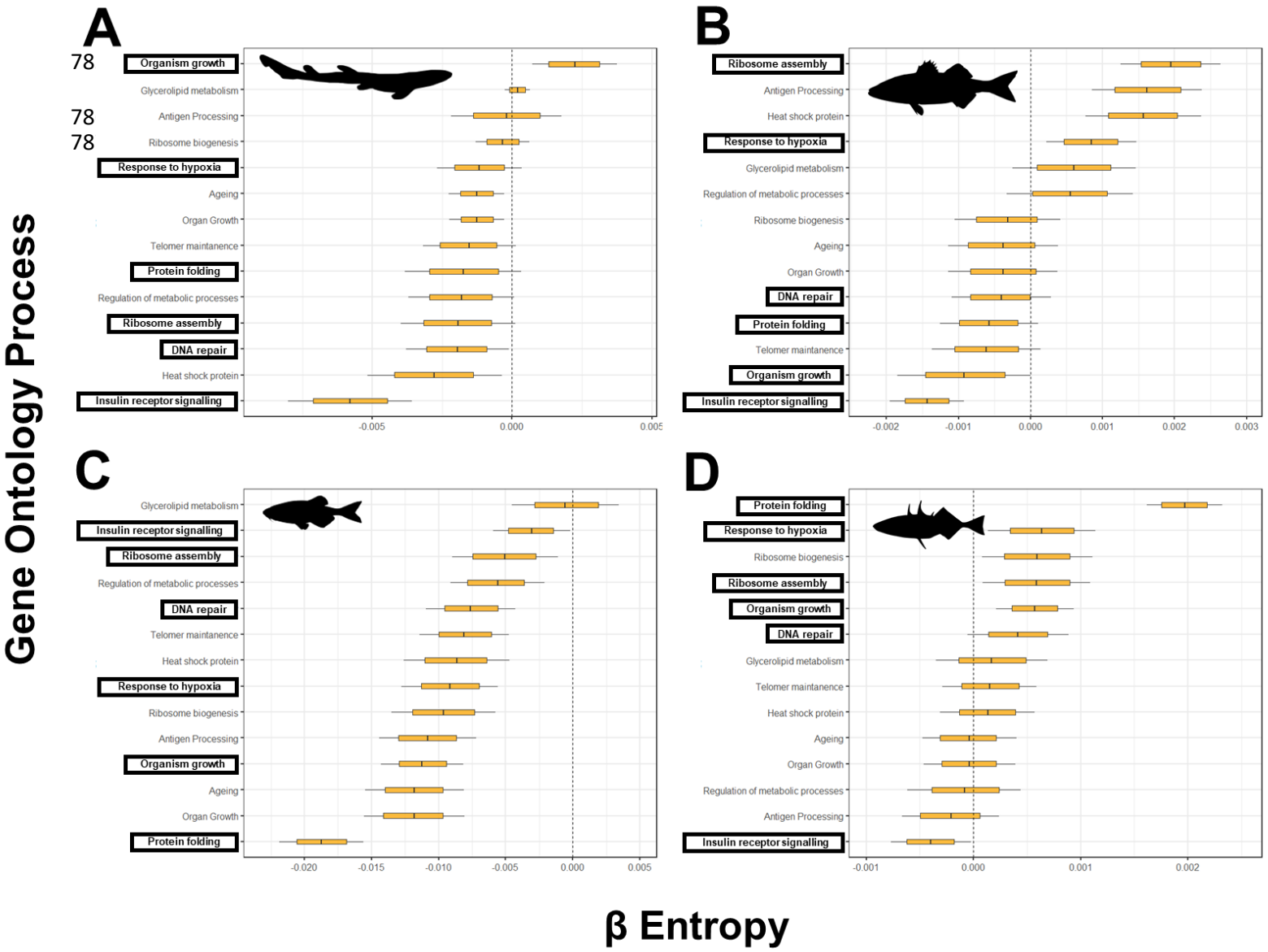

Figure 5: Median and 89% credibility intervals for pairwise network entropy of gene ontology pathways between control and warm incubated **A)** *Scyliorhinus canicula* (N = 3), **B)** *Dicentrarchus labrax* (N = 5), **C)** *Danio rerio* (N = 4), and **D)** *Gasterosteus aculeatus* (N = 6). β entropy represents the difference in entropy between warm and control incubated animals. Pathways that are significantly altered by developmental temperature in all species are highlighted in bold. *D. labrax, D. rerio, and G. aculeatus* data are re-analysed from Anastasiadi *et al*., 2021, Scott and Johnston 2012, and Metzger and Schulte 2017 respectively.

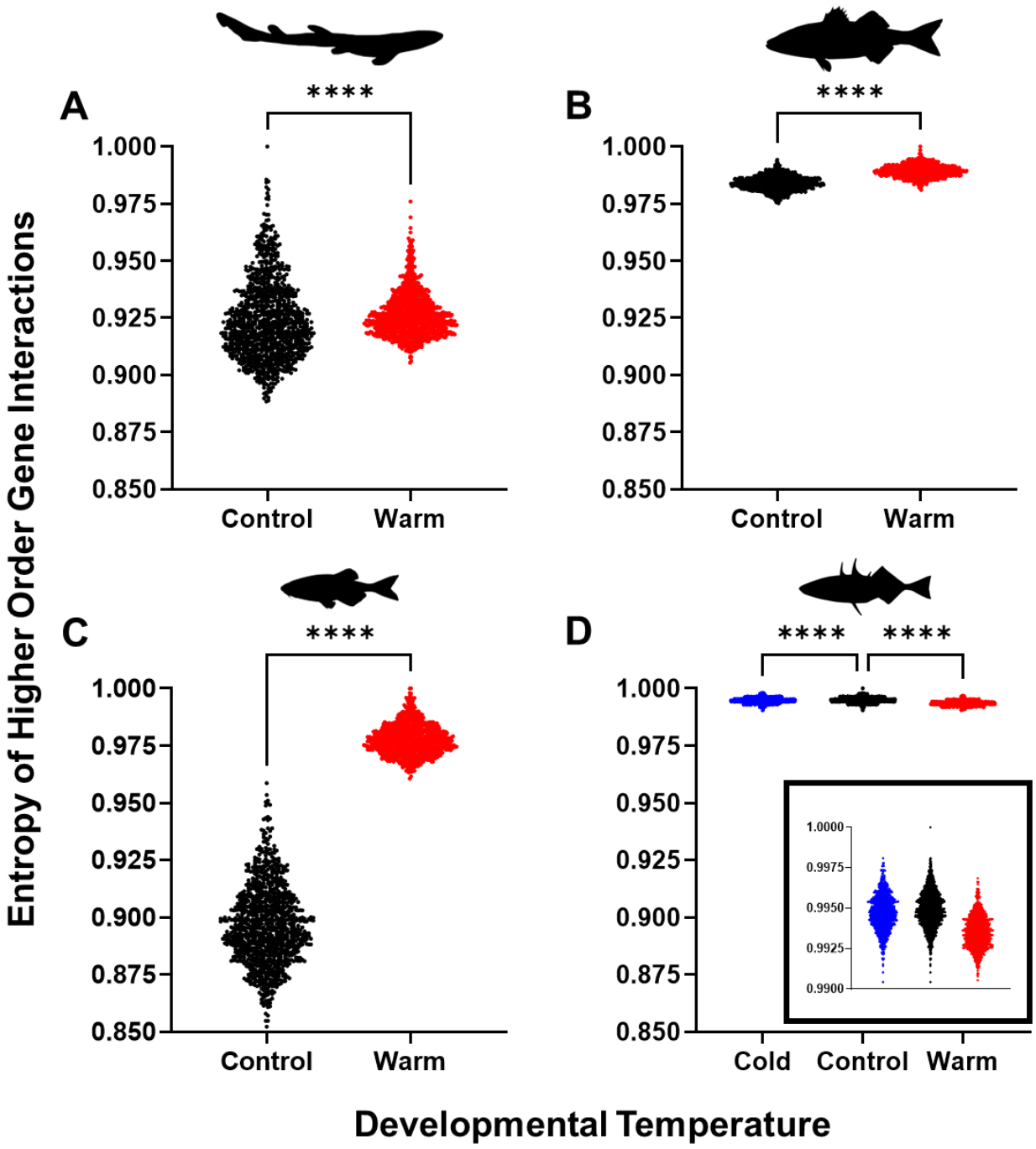

Figure 6: Entropy of higher-order gene interaction networks in **A)** *Scyliorhinus canicula*, **B)** *Dicentrarchus labrax,* **C)** *Danio rerio*, and **D)** *Gasterosteus aculeatus* that underwent embryogenesis at different temperatures. *Scyliorhinus canicula*; Wilcoxon test, $p < 0.0001$, N = 3 per group. *Dicentrarchus labrax*; Wilcoxon test, $p < 0.0001$, N = 5 per group. *Danio rerio*; Wilcoxon test, $p < 0.0001$, N = 4 per group. *Gasterosteus aculeatus*; Kruskal-wallis test and Dunn's multiple comparisons test, $p < 0.0001$ (cold vs. control) and $p < 0.0001$ (warm vs. control), N = 6 per group. *D. labrax, D. rerio, and G. aculeatus* data are re-analysed from Anastasiadi *et al*., 2021, Scott and Johnston 2012, and Metzger and Schulte 2017 respectively.

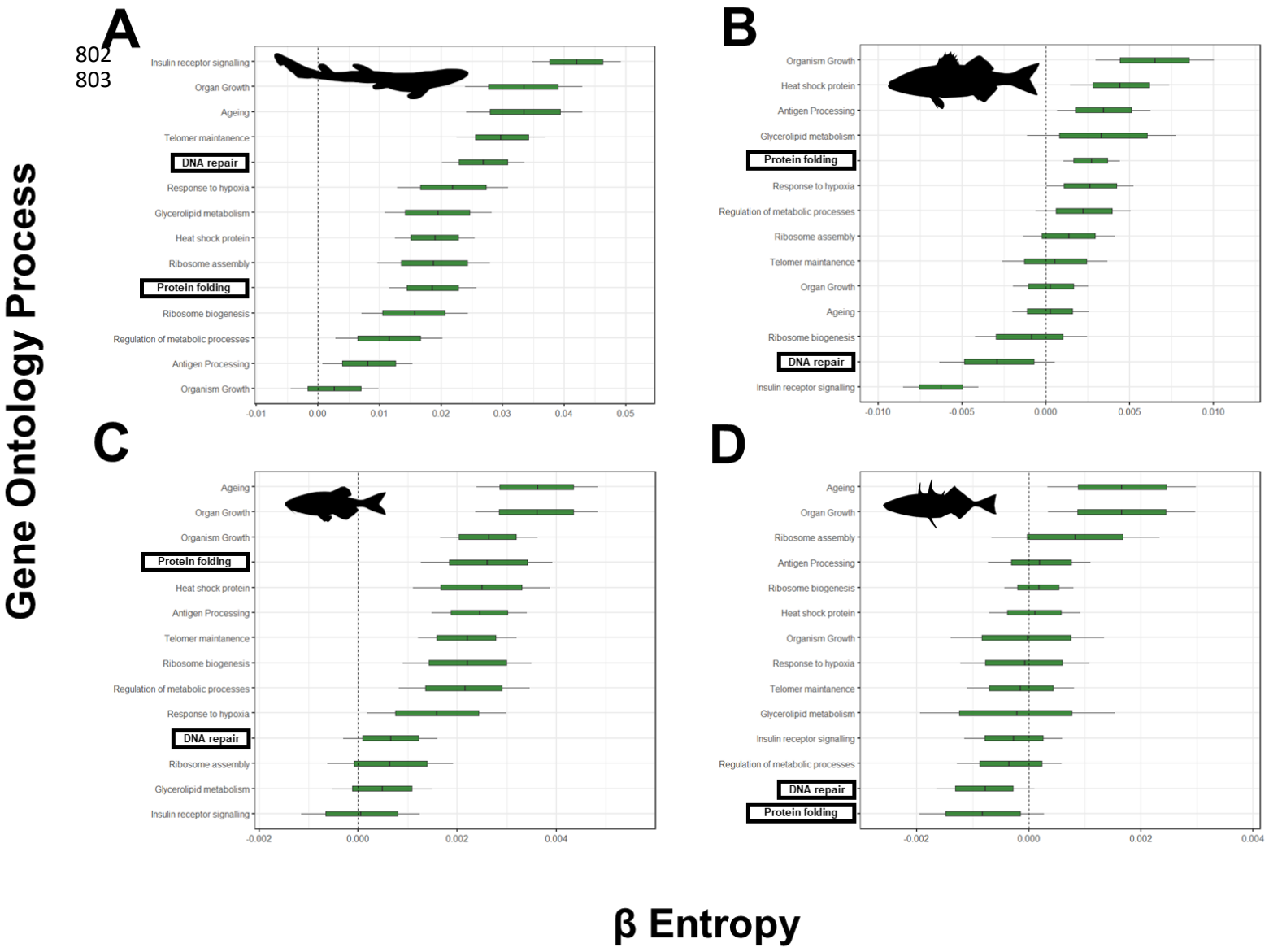

Figure 7: Median and 89% credibility intervals for hypernetwork entropy of gene ontology pathways between control and warm incubated **A)** *Scyliorhinus canicula* (N = 3), **B)** *Dicentrarchus labrax* (N = 5), **C)** *Danio rerio* (N = 4), and **D)** *Gasterosteus aculeatus* (N = 6). β entropy represents the difference in entropy between warm and control incubated animals. Pathways that are significantly altered by developmental temperature in all species are highlighted in bold. *D. labrax, D. rerio, and G. aculeatus* data are re-analysed from Anastasiadi *et al*., 2021, Scott and Johnston 2012, and Metzger and Schulte 2017 respectively.

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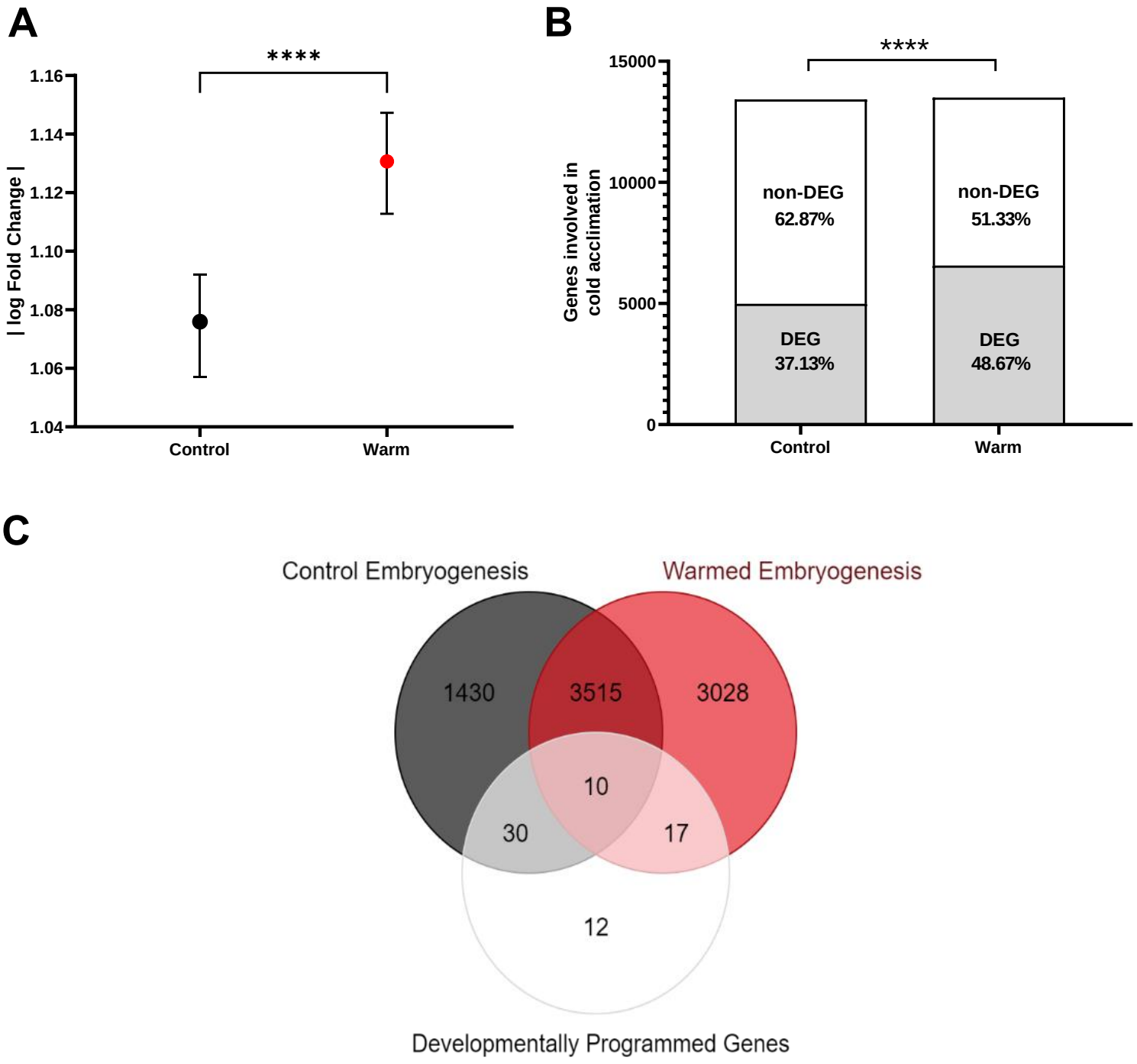

Figure 8: The cold-acclimation response of zebrafish (*Danio rerio*) that underwent embryogenesis in either control or warmed conditions. **A)** median ± 95% CI of the modulus fold change of the differentially expressed genes (Wilcoxon test, $p < 0.0001$, N = 4 per group), **B)** the number and proportion of gene expression changes (Fisher's exact test, $p < 0.0001$, N = 4 per group), and **C)** the similarity of gene expression changes between developmental groups (Fisher's exact test, $p < 0.0001$, N = 4 per group). *D. rerio* data are re-analysed from Scott and Johnston 2012.