Heritable variation in heat shock gene expression: a potential mechanism for adaptation to thermal stress in embryos of sea turtles

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Supplemental information

Supplemental methods

This research was conducted in accordance with the University of Western Australia (UWA) Animal Ethics Committee (RA/100/3/1195) and conforms to relevant guidelines for the care of experimental animals. Euthanasia protocols follow the American Veterinary Medical Association guidelines for embryonic reptiles. Fieldwork was conducted under permits SF009051 and SF009392 issued by the Western Australian Department of Parks and Wildlife (DPaW).

(1) Study species and sample collection

Caretta caretta is a globally distributed sea turtle that has a relatively narrow nesting range confined to temperate and sub-tropical latitudes [1,2]. In Western Australia a large temperate rookery occurs at Turtle Bay on Dirk Hartog Island (DHI; 25.49827 °S, 112.98719 °E) and smaller sub-tropical rookeries occur on the Australian mainland, including the Bungelup Beach rookery (BB; 22.282331 °S, 113.831570 °E) in Cape Range National Park (Fig 1). These rookeries, along with several other island and mainland rookeries, comprise a single genetic stock [3] and together are considered the world's third largest nesting population of

the *C. caretta* [1,4]. Nest temperatures are on average 2-3 °C cooler at DHI relative to BB during the respective peaks of summer nesting, and are subject to greater diel fluctuations of temperatures throughout the nesting season [5,6]. Embryos developing on these beaches can experience temperatures above 35°C near the end of incubation and lethal heating event [6], and incubation temperatures are likely to further increase as air temperatures rise with climate change [7].

We collected 1200 *C. caretta* eggs from Turtle Bay on DHI (80 eggs from each of 15 clutches) during peak nesting in late January 2013. This large collection was made to maximise the number of potential sires in each clutch sample, but when highly skewed paternity was detected [8] we opted to collect smaller samples from other sites. Consequently, 20 eggs were collected from each of four clutches deposited at Bungelup Beach near the end of peak nesting in early January 2014 (80 eggs in total). Although we had approval to sample up to 15 clutches from this site (300 eggs), this was not possible due to low densities of nesting females during our fieldwork. In all cases, eggs were collected while the females were nesting and were transported to the University of Western Australia following published protocols [9].

(2) Incubation and heat shock treatments

We distributed eggs among 1.5 L plastic containers half-filled with moist white sand; each contained a randomly selected egg from each female to limit clutch effects (DHI: 80 containers each with 15 eggs, BB: 20 containers each with 4 eggs). All eggs were held in one of two 500 L incubators (Steridium, Brisbane, QLD, Australia) at 29 °C (± 0.3 °C), which is the pivotal temperature that produces a balanced sex ratio for this population [6] and provides near optimum incubation conditions. Following a previously established protocol for

55 inducing upregulation of heat shock genes in *C. caretta*, embryos were incubated for 45 days
56 at 29 °C, and then either transferred to an incubator at 36 °C for 3 hrs (heat shock treatment),
57 or else moved to an incubator at 29 °C for 3 hrs (procedural control) [9]. The heat shock
58 temperature is one that causes 100% mortality in *C. caretta* at constant temperature
59 incubation [10], but is non-lethal to 45 day old embryos if exposure is limited to three hours
60 [9]. After a one-hour cool down period [9], embryos were removed from the egg, weighed,
61 and given a lethal injection of MS-222 (50 mg/kg, Sigma) [9,11] followed by decapitation to
62 ensure death.
63

64 **(3) RNA extraction and RT-qPCR**

65 We selected cardiac tissue for gene expression analysis, as it increases expression of *hsps* in
66 response to heat stress [9,12–14]. Whole heart was isolated and cut in half through the aorta,
67 atria, and ventricle. Each half was immediately stored in RNA*later* (AM7021, Invitrogen) at
68 –80 °C until RNA extraction. Paternity data from a concurrent study [8] was used as criterion
69 for selecting embryos from each family (mother-father pair) for gene expression assays. For
70 each family, expression assays were performed on 3-5 offspring in each of the procedural
71 control and heat shock treatments. Total RNA was extracted from each half of cardiac tissue,
72 and purity and quantity of RNA were measured following previously published protocols [9]
73 briefly described in-text.
74

(4) Detailed statistical analyses

Cycle Threshold (Cq) values (the number of PCR cycles required for the fluorescence signal to cross a threshold line) obtained from real-time PCR were converted to relative gene expression values ΔCq and ΔΔCq using the methods described in similar studies [9,15]. A mean ΔCq value, which is the difference between the mean Cq value of the target gene (*hsp60*, *hsp70* and *hsp90*) and the mean of the reference gene (*18s*), were calculated for each embryo in the procedural control and heat shock treatments. The mean ΔCq values for the procedural control samples represent baseline expression levels, whereas the mean ΔCq values for the heat shock treatment samples represent expression levels under thermal stress. The ΔΔCq values, which measure the change in relative gene expression between treatments, were calculated by subtracting the mean ΔCq value for the control samples for each clutch from the mean ΔCq value for each embryo in the heat shock treatment. These values represent the level of plasticity (up- or down-regulation) in gene expression in response to the heat shock treatment.

In the dataset made available on DRYAD, the reported Cq values are the average Cq for each reference and target gene for each individual offspring analysed from each clutch. The average Cq value for each offspring was calculated in this manner, as only one of the two independent RNA extractions was successful for some individuals. Data are organised by Sire ID, as more than one male sired three of the 18 clutches.

A linear mixed-effects model was used to estimate variance components between and within rookeries. The model included rookery and clutch nested within rookery as random factors. Comparing total variances explained by the full model with a model having one factor removed tested the significance of each level in the analysis. Variance components

were calculated using REML, and the *VarCorr* function in the R package nlme [16]. All ΔCq and ΔΔCq values were log-transformed with 2^{-X}, where X is the mean ΔCt or mean ΔΔCq value, prior to analysis.

The heritabilities of ΔCq values for *hsp60*, *hsp70*, and *hsp90* were estimated using an 'animal model' in ASReml 3.0 [17,18]. The animal model is a linear mixed model used for quantitative genetic analyses, which in our study can be used to estimate additive genetic variance and maternal effects, even when some pedigree information is absent [19]. A pedigree for these analyses was generated using available paternity information for each offspring [8]. We fitted separate animal models to each treatment (control and heat shock), with rookery, offspring identity, and dam as random factors. The term 'dam' is the maternal identity and estimates the genetic and environmental variance that is common to different mothers in the data set. Rookery and offspring identity measure genetic variance between rookeries and individual offspring, respectively. When the only pedigree term in the best-fitting model was dam, we estimated broad-sense heritability [20]. However, when both dam and offspring were included in the model we were able to estimate narrow-sense heritability. Narrow-sense heritability is of primary interest because it measures the extent to which phenotypes are determined by genes inherited from both parents, and is hence directly related to the ability of a population to respond to selection [20].

We also estimated heritability of expression across treatments (phenotypic plasticity) using the mean ΔΔCq values for *hsp60*, *hsp70*, and *hsp90*. Again, we fit the animal models to our data using the same parameters. For both the within treatment (ΔCq) and across treatment (ΔΔCq) estimates REML Likelihood-ratio tests (REML LRT [18]) were used to find the best

124 fitting model, by starting with a fully saturated model and then systematically reducing the
125 number of parameters.
126
127 We constructed a bivariate animal model to test for a genetic correlation between
128 levels of gene expression in the two different treatments, but models failed to converge.
129 Consequently, we used Spearman's rank correlation to analyse whether, at the clutch level,
130 the rank order of mean expression was correlated between treatments for each gene, and if
131 mean expression was correlated between genes for each treatment.

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Supplementary table 1.

Primers used for RT-qPCR. Efficiencies and coefficients of determination (R^2) are given for each primer pair.

Gene	Accession Number	Primers for RT-qPCR	Efficiency	R^2
18s (Reference)	KJ683738	F: GCTAATACATGCCGACGAG R: GGCCCGAGGTTATCTAGAG	110.77%	0.998
hsp60 (Target)	KJ683735	F: TACTGATGCACTGAACGCT R: TGGCGTTAAAGCATCTAGTG	103.15%	0.999
hsp70 (Target)	KJ683736	F: TCTCCGTACAGCTTGTGAAC R: CCACGGAACAGATCAGC	108.78%	0.998
hsp90 (Target)	KJ683737	F: GGATACTGGCATAGGGATG R: CAACACCAAACTGACCAATC	106.28%	0.996