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RESEARCH ARTICLE

Cardiac performance and heat shock response variation related to shell colour morphs in the mudflat snail *Batillaria attramentaria*

Guodong Han*, Yinghui Du, Lina Du, Furui Qu and Zhenjun Zhao

ABSTRACT

Gastropods exhibit remarkable variation in shell colour within and among populations, but the function of shell colour is often not clear. In the present study, body temperature in the field and physiological and transcriptomic responses to thermal stress were investigated in different shell colour morphs of the mudflat snail *Batillaria attramentaria*. Using biomimetic models, we found that the body temperature of snails with a dark unbanded shell (D-type morph) was slightly higher than that of snails with a white line on the upper side of each whorl (UL-type morph) when exposed to sunlight. Despite no differences in upper lethal temperature among shell colour morphs, their Arrhenius breakpoint temperature (ABT) for cardiac thermal performance differed significantly, and the ABT of snails with the D-type morph was higher than that of snails with the UL-type morph. Transcriptomic analysis showed that D-type snails exhibit higher levels of four heat shock proteins (HSPs) than UL-type snails at control temperature. The unfolded protein response was activated in UL-type snails but not in D-type snails under moderate thermal stress. And 11 HSPs showed an increase in UL-type snails in contrast to 1 HSP in D-type snails, suggesting a 'preparative defence' strategy of the heat shock response in D-type snails under moderate thermal stress. When exposed to sublethal temperature, eight molecular chaperones were uniquely upregulated in D-type snails, suggesting these genes may allow D-type snails to improve their cardiac thermal tolerance. Our results suggest that the preparative defence strategies and higher ABT for cardiac thermal performance may allow the dark shell snails to adapt to rapid and stronger thermal stress in the field.

KEY WORDS: Shell colour polymorphism, Heart rate, Heat shock proteins, Moderate thermal stress, Thermal adaptations

INTRODUCTION

Animal coloration is one of the most conspicuous phenotypic traits in natural populations and has important implications for adaptation (Poelstra et al., 2015; Smith et al., 2016). Both intertidal and terrestrial gastropods exhibit remarkable variation in shell colour within and among populations, which is termed shell colour polymorphism. Shell colour polymorphism provides taxonomists with characters that can be used to recognise and distinguish species; however, their function for gastropods is sometimes less clear and has been the focus of many ecological and evolutionary studies (reviewed in Williams, 2017). In many cases, shell colours of gastropods are frequently associated with environmental stress, such as temperature (Etter, 1988; Harris and Jones, 1995; Miura et al., 2007; Phifer-Rixey et al., 2008), desiccation (Etter, 1988) and salinity (Sokolova and Berger, 2000). Additionally, long-term studies have shown that variability of climatic selection has driven the change of shell colour frequency within and among populations (Ożgo and Schilthuizen, 2012; Schilthuizen, 2013). These studies suggest that shell colour may affect fitness in gastropods and highlight the importance of understanding the selective mechanisms for the maintenance of shell colour polymorphism, notably in the context of climate change.

Temperature affects all physiological and biochemical processes, translating into effects on metabolic processes, fitness and ecological dynamics (Angilletta, 2009; Somero et al., 2017). Intertidal organisms frequently encounter extreme thermal stress during aerial emersion, and solar radiation is usually the dominant component of the surface energy balance during low tide (Helmuth and Hofmann, 2001; Seuront and Ng, 2016), causing mortality in summer (Chan et al. 2006). Shell colour is known to affect body temperature and survivorship in intertidal gastropods. For example, when exposed to sunlight over a range of ecologically relevant temperatures, the brown morph of the intertidal snail *Nucella lapillus* suffered much greater mortality than the white morph (Etter, 1988). However, when snails were instead placed in a drying oven, the survivorship curves of brown and white morphs were quite similar. Similarly, shell colour was found to be a significant predictor of survivorship in the flat periwinkle *Littorina obtusata* when exposed to solar radiation, and snails with dark shells exhibited greater mortality relative to snails with light-coloured shells (Schmidt et al., 2007; Phifer-Rixey et al., 2008). In a manipulative trial in which snails were painted with either yellow or black paint, the original shell colour had no detectable effect whereas the painted colour was a significant predictor of mortality patterns (Phifer-Rixey et al., 2008). These results suggest that dark morph snails could suffer stronger thermal stress when exposed to solar radiation, leading to greater mortality than light morph snails, while the upper lethal temperatures of dark and light morph snails may be similar. Therefore, tolerance to extreme temperature may offer a poor explanation for shell colour frequency within and among populations.

The body temperature of intertidal gastropods could be influenced by shell colour. Studies have shown that individuals with dark shell morphs are more rapidly heated by solar radiation, and can reach higher body temperatures than light shell morphs (Cook and Freeman, 1986; Phifer-Rixey et al., 2008; Miller and Denny, 2011). Consequently, individuals with dark shell morphs may suffer thermal stress more frequently than light shell morphs. Individuals subjected to thermal stress could employ both physiological and cellular mechanisms to reduce the negative impact of stress.

At the organism level, heart rate (f_H) increases with body temperature until the Arrhenius breakpoint temperature (ABT) is reached, after which f_H decreases rapidly. For intertidal molluscs,

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Received 19 July 2022; Accepted 10 October 2022

ABT is not acutely lethal, but does reflect cumulative damage to the cell during the heating process (Han et al., 2013, 2017), and is used as a proxy for sublethal (but stressful) temperature (Tagliarolo and McQuaid, 2015; Dong et al., 2022). The differences in ABT between molluscs reflect their distribution in both a large-scale temperature gradient (Tagliarolo and McQuaid, 2015) and microhabitats within a site (Li et al., 2021), and highlight the critical importance of differences in sublethal effects in physiology.

At the cellular level, thermal stress induces a set of transcriptomic responses that include the repair of DNA and protein damage, cell cycle arrest or apoptosis, the removal of cellular and molecular debris generated by stress, and an overall transition from a state of cellular growth to one of cellular repair (Sokolova et al., 2012). Transcriptomic studies thus are providing insights into shell colour-related differences under moderate thermal stress. The organism and cellular responses under moderate thermal stress are energetically costly and may divert energy flux and metabolic power from fitness-related functions, and may be a driving force shaping shell colour frequency in gastropods.

The mudflat snail *Batillaria attramentaria* (previous referred to as *Batillaria cumingi* in the literature) is widely distributed along the Northwestern Pacific coast (Ozawa et al., 2009; Ho et al., 2015). It is a dominant species in the tidal flats, and plays an important role in the ecosystem, because of its impact on ecosystem carbon flow (Kawasaki et al., 2019). *Batillaria attramentaria* exhibits remarkable variation in shell colour within and among populations (Miura et al., 2007). A dark unbanded shell type (D-type morph) and a shell type with a white line on the upper side of each whorl (UL-type morph) were found in *B. attramentaria* from the coast of China. A previous study found that geographical variation in shell colour polymorphism in *B. attramentaria* was significantly correlated with the temperature of the locality of the population, suggesting thermal selection was one of the significant factors maintaining shell colour polymorphism (Miura et al., 2007). In the present study, we hypothesized that snails with the D-type morph could reach a higher body temperature than snails with the UL-type morph, and strategies for coping with thermal stress may allow the D-type morph snails to adapt to the stronger thermal stress in the field. To test this hypothesis, we measured snail body temperature in the field and investigated the effects of acute changes in temperature on f_H and gene expression level of two morph type individuals. We also determined the effect of acute high temperature exposure on mortality to test whether the upper thermal limits differ between snails with D- and UL-type morph. Our results may help understanding of the function of shell colour in gastropods.

MATERIALS AND METHODS

Sample collection

Batillaria attramentaria (G. B. Sowerby II 1855) samples were collected from a muddy shore located at Yangmadao Island, Shandong province, China (37°27′N, 121°36′E) at daytime low tides. To collect snails randomly, an 18 cm×18 cm quadrat was thrown in several directions and all snails within the quadrat were collected. This operation was repeated 3–4 times in order to examine the colour variation of the shell. To investigate seasonal fluctuations in the frequency of shell colour patterns, sample collections were repeatedly conducted in summer (July 2021) and winter (January 2022). Differences in the frequency of shell colour patterns were analysed using the Chi-square test in R (http://www.R-project.org/). A total of 35 snails were randomly genotyped to verify their taxonomy. Genomic DNA was extracted from foot muscle tissue. A fragment of cytochrome oxidase subunit I mtDNA (COI) was amplified and sequenced using the following primers: LCO1490 – GGT CAA ATC ATA AAG ATA TTG G; and HCO2198 – TAA ACT TCA GGG TGA CCA AAA AAT CA (Folmer et al., 1994). Phylogenetic trees were constructed using MEGA11 (Stecher et al., 2020).

Shell colour and temperature

To evaluate the relationship between shell colour and temperature, the internal body temperature of D-type and UL-type morph shells was compared in the field. Eight D-type and eight UL-type morph snails were collected from Yangmadao Island to make biomimetic models (Marshall and Chua, 2012; Rolán-Alvarez et al., 2012). Shell length (mean±s.e.m. 27.46±1.97 mm) was measured to ensure that size did not vary significantly between colour morphs (t=−0.103, d.f.=14, P=0.920). Tissue was removed from the shells, the shells were cleaned, and thermocouples were affixed to the interior using thermally conductive epoxy. Shells were then placed on the dry surface of a mud flat inhabited by snails at Yangmadao Island, on mostly clear, sunny days in late June and early July 2022. Shells were placed in the same orientation and exposed to direct sunlight. Internal shell temperature was logged every 10 s using a Modbus data logger (LCES_UDR_V62, Songyue, Guangdong, China). The shell temperatures of D- and UL-type models logged at the same time were averaged as $T_{av,D}$ and $T_{av,UL}$, respectively. In order to avoid autocorrelation problems, a small sample size of $T_{av,D}$ and $T_{av,UL}$ for each day was drawn from the entire dataset (a time interval of 35 min). The *Box.test* function in R was performed to test the independence of the reduced dataset, and a paired *t*-test was then performed using the *t.test* function in R. A pyranometer (485 type, Puruisenzao, Shandong, China) was used to measure incident solar radiation (400–1100 nm), and data were logged every 10 s using a Modbus data logger. Data were pooled across all times in which incident solar radiation and body temperature were recorded. The correlation coefficient between incident solar radiation and the difference between $T_{av,D}$ and $T_{av,UL}$ for each day was analysed using the cross-correlation function (ccf) in R.

Survival analysis

The effect of acute high temperature exposure on mortality was determined using D- and UL-type morph snails collected in summer (July 2021) and winter (January 2022). Snails were held in air in test tubes (25 mm diameter) placed in a water bath (Grant TXF 200, Grant, Shepreth, UK) and heated at a rate of 6°C h^{-1}. Survival exposure to 43, 46, 49, 50, 51, 52 and 53°C was assessed from three groups of 4–5 snails. Following heat stress, the test tubes containing the snails were immersed in flow-through seawater at ambient temperature during a 3 day recovery period. Individuals that did not exhibit an opercular reflex (rapid and complete withdrawal into their shell) upon stimulation by a sharp probe on the foot were scored as dead. The median lethal temperature (LT50) at 3 days after heat shock was calculated with logistic analysis. The complete set of survival time data was analysed with a Cox proportional hazard regression model using the *survival* package in R (https://CRAN.R-project.org/package=survival).

Thermal sensitivity of f_H

Cardiac performance, which describes the relationship between body temperature and f_H, was determined using D- and UL-type morph snails collected in summer (July 2021). f_H was measured using a non-invasive method (Dong et al., 2021). Snails were attached to the bottom of a test tube and warmed by heating the bottom of the test tube in a water bath (Grant). Experimental

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Table 1. Summary of a reduced dataset drawn from the entire biomimetic data and the results of paired *t*-test of average model temperature

		Sample no. in reduced dataset		*P*-value of *Box.test*		Paired *t*-test	
Test date	Time of day	D-type	UL-type	D-type	UL-type	d.f.	*P*-value
22 June	10:17–15:00 h	9	9	0.725	0.911	8	0.526
24 June	10:00–15:00 h	9	9	0.287	0.444	8	<0.001
25 June	10:35–14:57 h	8	8	0.387	0.418	7	<0.001
2 July	10:09–15:00 h	9	9	0.430	0.259	8	<0.001
8 July	10:00–15:00 h	9	9	0.765	0.705	8	<0.001
9 July	10:00–15:00 h	9	9	0.070	0.067	8	0.008

All tests were performed in 2022.

temperature was increased from 28°C at a rate of 6°C h^{-1} in air until a temperature was reached where f_H fell to zero. To measure the snail's body temperature, a small hole (0.8 mm diameter) was drilled in the shell at a position above the heart, and a thermocouple was inserted. The heartbeat was detected by means of an infrared sensor fixed to the shell at a position above the heart (to the upper left of the aperture). Variation in the light-dependent current produced by the heartbeat was amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and a Powerlab AD converter (8/30, ADInstruments, Bella Vista, NSW, Australia). Data were viewed and analysed using LabChart v7 (ADInstruments). ABT for cardiac performance, the temperature at which the f_H (beats min^{-1}) decreases sharply with progressive heating, was determined using a regression analysis method that generates the best-fit line on either side of a putative break point for the relationship of ln-transformed f_H against the reciprocal value of absolute temperature. ABT was calculated using the *segmented* package (https://CRAN.R-project.org/package=segmented) in R. Comparisons of ABT between individuals of the D-type and UL-type morph were performed using *t*-test in R.

RNA sequencing

To analyse the transcriptomic response to thermal stress, snails of each morph were randomly selected and heated at a rate of 6°C h^{-1} in air from 28°C to 36°C (moderate thermal stress) or 46°C (sublethal thermal stress). After the heating step, three snails of each morph were dissected and foot muscle was immediately frozen in liquid nitrogen. Tissue samples were sequenced (Novogene, Tianjin, China) on an Illumina NovaSeq platform. All clean reads from 18 snail transcriptomes were aligned to the *B. attramentaria* genome (GenBank assembly accession: GCA_018292915.1) using *STAR* v2.7.9a (Dobin et al., 2013). The mapped reads of each sample were transformed into counts using *HTSeq* v1.99.2 (Anders et al., 2015). The differential expression analyses were conducted between the control (28°C) and the treatments (36°C or 46°C) for each morph. The absolute value of $\log_2$fold-change≥1 and adjusted *P*-value (P_{adj})<0.01 were set as the thresholds to screen out differentially expressed genes (DEGs). The differential expression analysis was also performed between the D-type morph and the UL-type morph at 28°C. Gene Ontology (GO) and KEGG enrichment analyses were applied using *clusterProfiler* v4.2.2 (Wu et al., 2021) in R to determine the significantly enriched GO terms and KEGG pathways of DEGs. Enriched GO terms and KEGG pathways were screened out with P_{adj}<0.05.

RESULTS

Abundance and frequency of shell morphs in the field

A 591 bp portion of COI was sequenced from 18 D-type and 17 UL-type individuals. The results of the phylogenetic analysis suggested that all individuals were *B. attramentaria* and there was no genetic differentiation among the two type morphs (Fig. S1). At the study site, the total abundance of *B. attramentaria* in summer averaged 1672±290 m^{-2} (mean±s.e.m.), of which the D-type morph

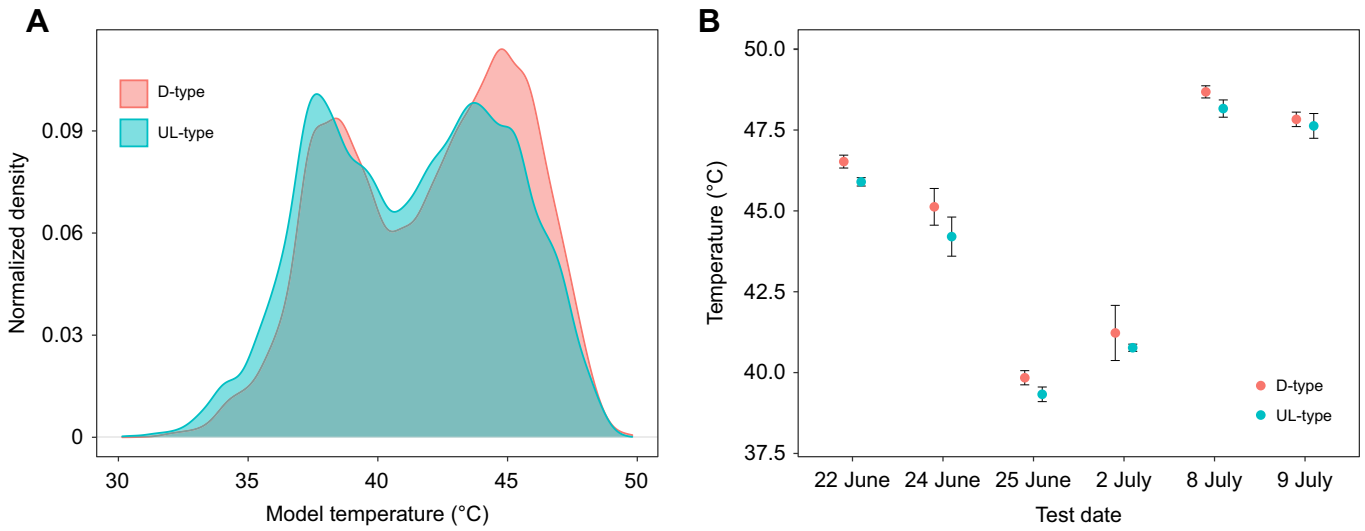

Fig. 1. *Batillaria attramentaria* shell colour and temperature. (A) Distribution of model temperature for D-type and UL-type snail shells on all test days. The horizontal axis shows the model temperature and the vertical axis shows the relative abundance (as normalized density). (B) Average maximal temperature for D-type shells and UL-type shells on each test day in 2022 (means±s.e.m., *n*=8).

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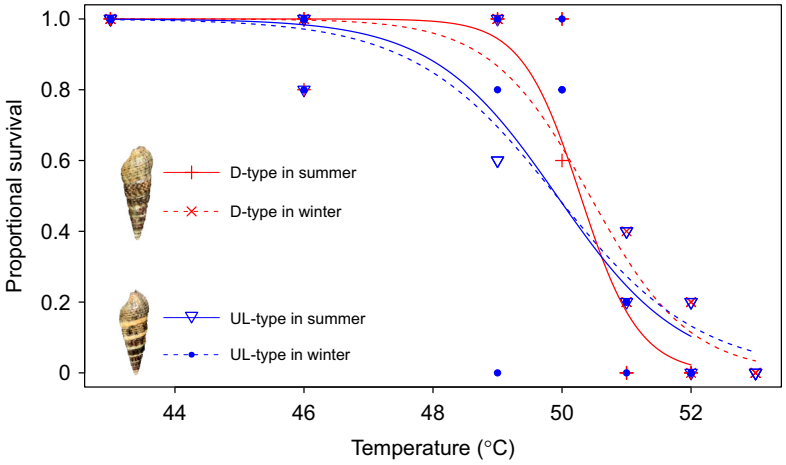

Fig. 2. Survival rate of *B. attramentaria* individuals after exposure to elevated body temperature. Data are for D- and UL-type body morphs collected in summer and winter. The curves were generated using logistic regression.

constituted 82.8% and the UL-type morph 17.2%. Lots of empty shells were found along the coast in winter, and live snails tended to aggregate together. The total abundance of snails in winter averaged 3138±1394 m^{-2}, of which the D-type morph constituted 85.2% and the UL-type morph 14.8%. There was no difference in frequency of shell morphs between summer and winter (n=675, χ^2=0.218, P=0.640).

Shell colour and temperature

The biomimetic data showed that model temperatures of D-type and UL-type morphs were different (Fig. 1A). Eight or nine pairs of $T_{av,D}$ and $T_{av,UL}$ were drawn from the entire biomimetic data on each test day to perform paired t-tests. The results showed that $T_{av,D}$ was higher than $T_{av,UL}$ on most test days, except for 22 June (Table 1). On each test day, the average maximal temperature of D-type models was higher than that of UL-type models (Fig. 1B). Results of cross-correlation analysis showed that the difference between $T_{av,D}$ and $T_{av,UL}$ was significantly correlated with incident solar radiation (Fig. S2). The maximal value of the correlation coefficient was 0.360, −0.269, 0.461 and −0.651 for 22 June, 25 June, 8 July and 9 July, respectively.

Survival following thermal stress

After 3 days of recovery, the LT50 values of snails with D- and UL-type morph in summer were 50.288±0.178 and 49.921±0.292°C, respectively (Fig. 2). In winter, the LT50 values of snails with D- and UL-type morph were 50.432±0.301 and 49.915±0.386°C, respectively. The proportional hazard assumption was tested for each variable (temperature, shell morph, season and shell morph×season) of the fitted Cox model by correlating the status (alive or dead) with time (Table 2). Survival did not vary among shell morph (P=0.5), season (P=0.4) or shell morph×season (P=0.8), but did differ among temperature (P<0.001).

Table 2. Statistical summary of the Cox proportional hazard model results for survival of snails after exposure to elevated body temperature

Model term	Wald statistic	d.f.	P
Temperature	75.03	6	<0.001
Shell morph	0.46	1	0.5
Season	0.61	1	0.4
Shell morph×season	1.19	3	0.8

Cardiac thermal performance

Under constant heating, the pattern of f_H was similar between the two morphs when the temperature was below 40°C (Fig. 3). There was a flattening of the curve for both D- and UL-type morphs. Cardiac thermal performance differed between the two morphs when the temperature was above 40°C, especially when the temperature reached the ABT. The mean±s.d. ABT for individuals with the D- and UL-type morph was 45.627±0.561 and 44.790±0.894°C, respectively (Fig. 4A). The ABT of individuals with the D-type morph was higher than that of individuals with the UL-type morph (two-sample t-test, t=2.631, d.f.=20, P=0.016). The mean (±s.d.) maximum f_H ($f_{H,max}$) was 136.727±17.281 beats min^{-1} for the D-type morph and 147.727±16.900 beats min^{-1} for the UL-type morph (Fig. 4B). There was no significant difference in $f_{H,max}$ between the two morphs (two-sample t-test, t=−1.509, d.f.=20, P=0.147).

DEGs under temperature stress

A total of 121.70 Gb of clean bases were obtained from 18 transcriptomes of *B. attramentaria*. The average mapping rates for

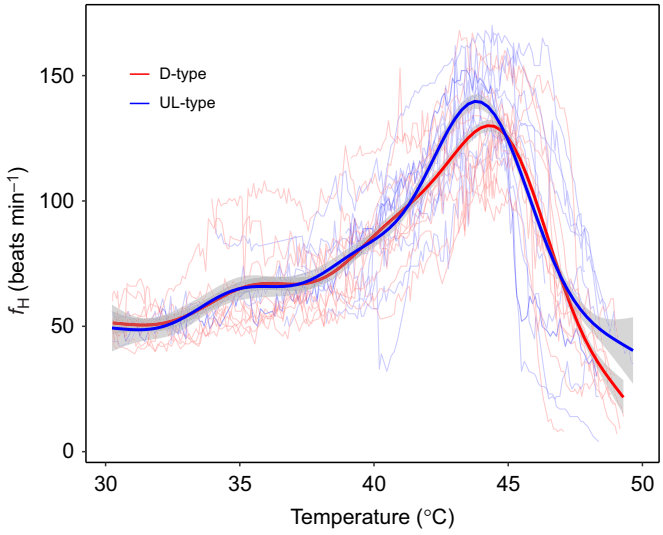

Fig. 3. Cardiac thermal performance of *B. attramentaria* individuals over an acute warming ramp. Each thin transparent line represents the cardiac performance of an individual with D- or UL-type morph over different temperatures. The thick curves were fitted from a GAM model for D-type (n=11) and UL-type (n=11) morph individuals, and grey regions are 95% confidence intervals.

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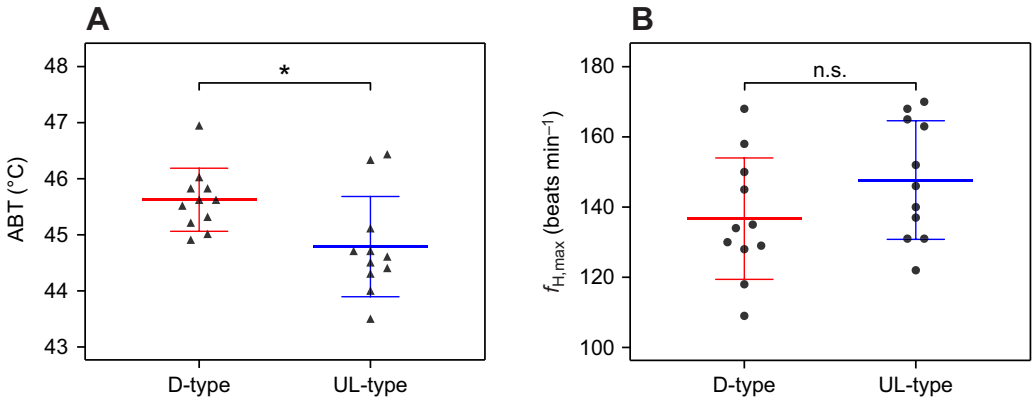

Fig. 4. Mean Arrhenius breakpoint temperature and maximum heart rate of *B. attramentaria* individuals. (A) Each triangle represents the Arrhenius breakpoint temperature (ABT) of an individual snail (n=11 for both D- and UL-type morph). Bars indicate mean±s.d. values. *Significant difference between morphs (P=0.016). (B) Each point represents the maximum heart rate ($f_{H,max}$) of an individual snail (n=11 for both D- and UL-type). Bars indicate mean±s.d. values. There was no significant difference between morphs (n.s., P=0.147).

D- and UL-type morph individuals were 85.39% and 84.09%, respectively. At the control temperature (28°C), the expression levels of 37 genes were higher in D-type than in UL-type morph individuals, while expression levels of 68 genes were lower in D-type than UL-type morph individuals (Fig. 5). In response to 36°C, there were 84 DEGs (46 upregulated and 38 downregulated) in D-type morph individuals in contrast to 37 DEGs (23 upregulated and 14 downregulated) in UL-type morph individuals. When the temperature was increased to 46°C, the number of DEGs was 1054 (641 upregulated and 413 downregulated) and 669 (475 upregulated and 194 downregulated) for D- and UL-type morph individuals, respectively.

Functional analysis of DEGs

GO annotations were performed for the DEGs under heat stress (Fig. 6A). In response to 36°C, 38 DEGs and 21 DEGs were annotated in the GO database in D-type and UL-type morph individuals, respectively. The terms ‘primary amine oxidase activity’, ‘amine metabolic process’, ‘quinone binding’ and ‘myosin complex’ were enriched in D-type morph individuals. The term ‘ATP hydrolysis activity’ was the most enriched GO term in UL-type morph individuals, followed by ‘unfolded protein binding’ and ‘protein folding’. When the temperature increased to 46°C, the terms ‘ATP hydrolysis activity’, ‘unfolded protein binding’ and ‘protein folding’ were enriched in both D- and UL-type morph individuals. GO terms ‘DNA integration’ and ‘calcium ion binding’ were uniquely enriched in D-type morph individuals, and ‘endopeptidase inhibitor activity’ and ‘signalling receptor activity’ were uniquely enriched in UL-type morph individuals.

In response to 36°C, KEGG annotations revealed that 32 DEGs and 15 DEGs were mapped to pathways in D-type and UL-type morph individuals, respectively (Fig. 6B). Among these pathways, ‘fluid shear stress and atherosclerosis’ was the most significantly enriched pathway in D-type morph individuals, containing six DEGs. ‘Chaperones and folding catalysts’ was the most significantly enriched pathway in UL-type morph individuals, containing 10 DEGs. When the temperature increased to 46°C, KEGG annotations revealed that 529 DEGs and 328 DEGs were mapped to pathways in D-type and UL-type morph individuals, respectively. The pathway ‘chaperones and folding catalysts’ was the most significantly enriched pathway in both D- and UL-type morph individuals.

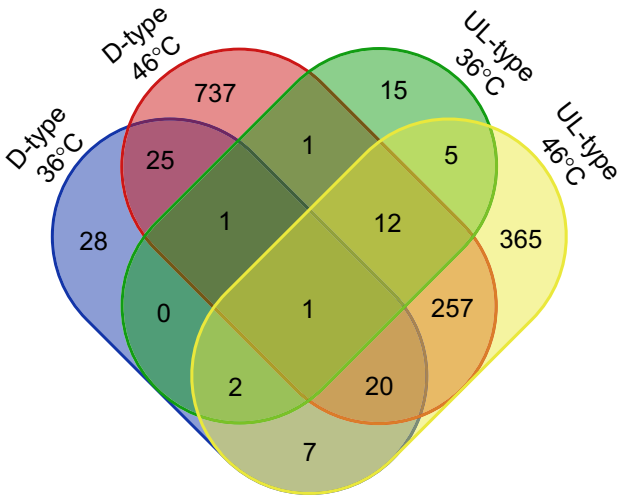

Fig. 5. Differential gene expression under temperature stress. Venn diagram comparing the number of differentially expressed genes (DEGs) in response to heat stress at 36°C and 46°C in D- and UL-type morph individuals.

Heat shock protein genes

The expression levels of five heat shock protein (HSP) genes, which were annotated as HSP70B2 and DNAJB1, were higher in D-type morph individuals than UL-type morph individuals at the control temperature (Fig. 7A). There was only one HSP gene (HSP90A1) that was upregulated in D-type morph individuals in response to 36°C in contrast to 11 HSP genes in UL-type morph individuals (Fig. 7B,C). These 11 HSP genes were annotated as DNAJA1, HSP90A1, DNAJB1, HSP70B2, CRYAA, HSPIV and HSPA8. When the temperature was increased to 46°C, a total of 35 HSP genes were upregulated in D- and UL-type morph individuals, of which 26 HSP genes were upregulated in both D- and UL-type morph individuals (Fig. 7D,E). There were eight HSP genes uniquely upregulated in D-type morph individuals in contrast to one unique HSP gene in UL-type morph individuals.

DISCUSSION

Our study was designed to examine the function of shell colour for the mudflat gastropod *B. attramentaria*. We asked whether body temperature and physiological responses to thermal stress were associated with shell colour polymorphism in gastropods. The

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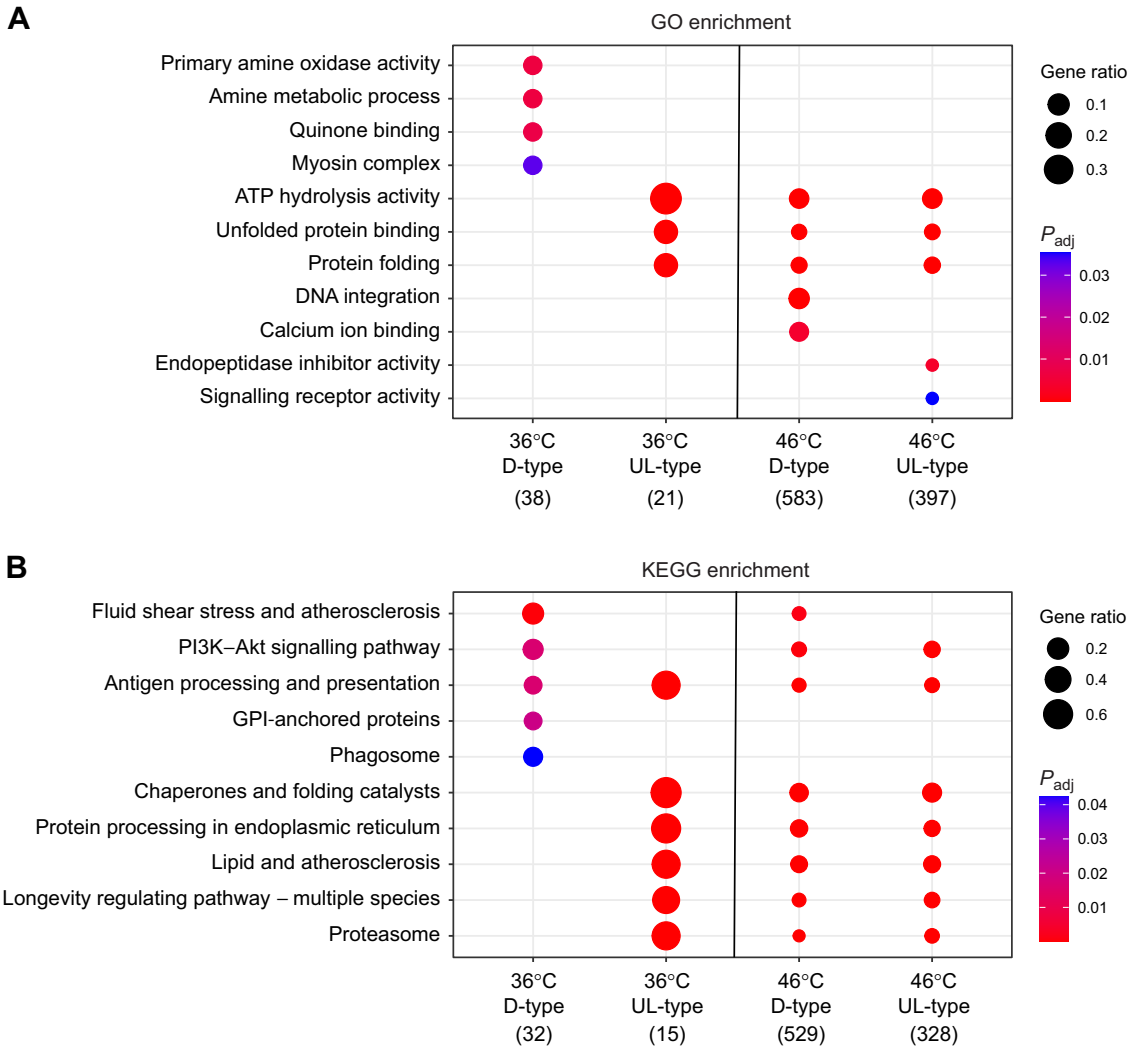

Fig. 6. Functional analysis of differential gene expression. DEGs enriched in the top GO terms (A) and KEGG pathways (B). The number in parentheses represents the total number of DEGs annotated in functional classes (GO terms or KEGG pathways) in response to heat stress at 36°C and 46°C in D- and UL-type morph individuals. The dot size represents the gene ratio between the number of DEGs enriched in each category and the total number of DEGs annotated in functional classes. The adjusted *P*-values (P_{adj}) are shown as different colours, with red being more significant than blue.

results of the biomimetic models showed that the body temperature of D-type morph individuals was consistently higher than that of UL-type morph individuals when exposed to sunlight. Using f_H, gene expression levels and mortality as proxies, we showed that responses to moderate and sublethal thermal stress, instead of lethal thermal stress, seem to be associated with shell colour morph in *B. attramentaria*.

The D-type snails frequently appeared at the study site (contributing 82.8% of total colour morphs in summer), suggesting a fitness advantage for D-type morphs at the study site. Differences in mortality rate of different morphs of polymorphic gastropods may be causally related to the variation in their shell colour (Köhler et al., 2021). However, the survivorship did not differ between D- and UL-type morph snails when exposed to acute heat stress in the laboratory. Our results are consistent with previous studies in the intertidal snails *Nucella lapillus* (Etter, 1988) and *Littorina obtusata* (Phifer-Rixey et al., 2008), which showed that upper lethal temperatures were not associated with shell colour. Although our experiments did not fully capture the range of thermal conditions snails experience during a year, our data represent the conditions snails experience during low-tide emersions on typical summer days. Our results suggest that body temperature may rarely exceed LT50 in the field. These results indicate that physiological selection imposed by extreme temperature conditions may not be a driving force shaping shell colour frequency of *B. attramentaria*.

The present study showed that D-type snails exhibited higher ABT than UL-type snails. Our data demonstrate that shell colour had a significant effect on model temperature, and D-type models reached a higher temperature than UL-type models (maximum difference 1.492°C). The results suggest that snails with the D-type morph shell could suffer stronger thermal stress than snails with the UL-type morph shell in the field. Our findings are similar to previous research in that they demonstrated that ABT of intertidal molluscs inhabiting hot conditions was higher than that of molluscs inhabiting benign conditions within a population (Moyen et al., 2019; Li et al., 2021). The ABT is not acutely lethal, but does reflect cumulative damage to the cells that is initiated during earlier stages of heating and gradually builds up to a level that causes heart dysfunction at the critical temperature (Han et al., 2013, 2017). The

Journal of Experimental Biology (2022) 225, jeb244796. doi:10.1242/jeb.244796

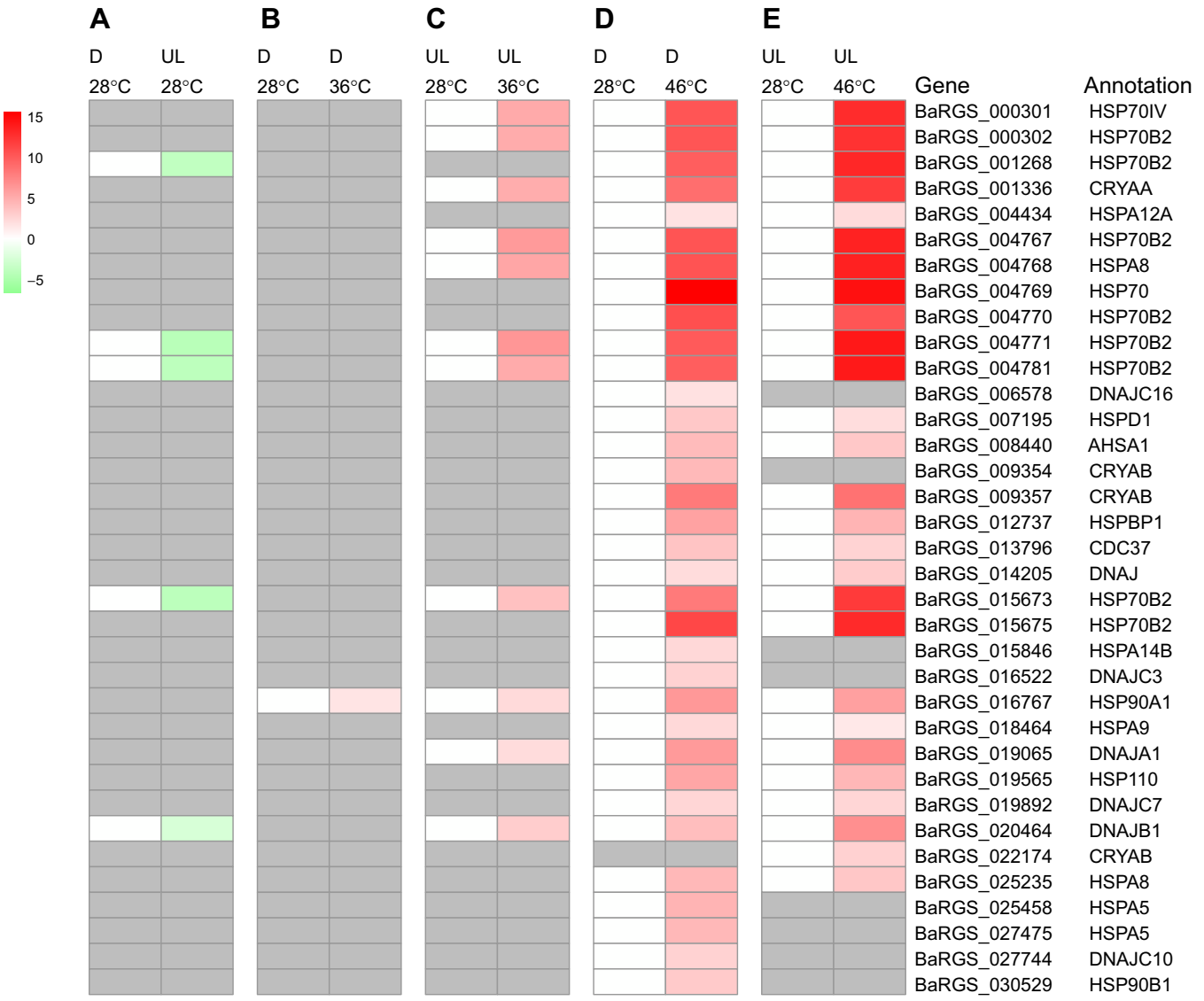

Fig. 7. Heatmap of differentially expressed heat shock protein genes. (A) Differential expression of heat shock protein (HSP) genes in D- and UL-type morphs at the control temperature (28°C). (B–E) Differential expression analysis conducted between the control (28°C; left column) and treatment (36 or 46°C; right column) group in D- and UL-type morphs. Each DEG is represented by a single row, and the $\log_2$fold-change values are shown in different colours: red corresponds to an upregulated gene product, green corresponds to a downregulated gene product and grey represents no significant difference.

ABT of cardiac function, while an index of organ-level dysfunction, thus can serve as an indicator that sufficient thermal damage of cellular structures has occurred to render the heart suboptimal in its performance (Moyen et al., 2019). The higher ABT indicates that D-type snails may be better adapted to thermal conditions around the sublethal temperature. The observed variability in cardiac thermal performance suggests that there are likely to be cellular and molecular changes allowing the snails with D-type shells improved cardiac thermal tolerance.

When exposed to moderate thermal stress (36°C), GO terms including ‘unfolded protein binding’ and ‘protein folding’ and KEGG pathways including ‘chaperones and folding catalysts’ and ‘protein processing in endoplasmic reticulum’ were significantly enriched in UL-type snails but not in D-type snails. These results suggest that the unfolded protein response was activated only in UL-type snails in response to moderate thermal stress. To ascertain fidelity in protein folding, cells regulate the protein-folding capacity in the endoplasmic reticulum according to need (Hetz, 2012). The endoplasmic reticulum responds to the burden of unfolded proteins in its lumen by activating intracellular signal transduction pathways (Walter and Ron, 2011). The lack of unfolded protein responses in D-type snails suggests that when these snails are exposed to moderate thermal stress, they do not mount transcriptome-wide intracellular signal transduction pathways (which may be very energy costly) and only induce genes essential to address immediate damage. The energy saving process may be a result of the timing of metabolic depression (Hui et al., 2020), which can allow intertidal gastropods to depress resting metabolism in response to moderate thermal stress (Marshall et al., 2011; Chen et al., 2021). Additionally, the decrease in expression for the gene encoding phosphoenolpyruvate carboxykinase (PEPCK) at 36°C observed by Tomanek and Zuzow (2010) is further evidence for a decrease in metabolism in response to moderate heat stress. Therefore, these snails may benefit from a temporal constraint on energy gain while experiencing high body temperature.

As observed in functional enrichment analyses, significant overexpression of several molecular chaperones occurs in response to thermal stress, which is in line with previous studies in intertidal gastropods (Sorte and Hofmann, 2004; Wang et al., 2014; Gleason and Burton, 2015; Han et al., 2017). We identified all differentially expressed HSPs and cofactors, and found distinct strategies of HSPs in *B. attramentaria* snails with different shell colours. At the acclimation (control) temperature, the expression levels of four HSP70B2 paralogues and DNAJB1 (HSP40) were significantly higher in D-type snails than in UL-type snails (Fig. 7). Dong et al. (2008) found that high-intertidal congeners of *Lottia* employ a 'preparative defence' strategy involving maintenance of high constitutive levels of Hsp70 in their cells as a mechanism for protection against periods of extreme and unpredictable heat stress. Our data suggest that *B. attramentaria* snails with D-type shell may benefit from such preparative defence strategies in response to moderate stress. Several molecular chaperones, including five HSP70B2 paralogues, HSP70IV, CRYAA, HSPA8, DNAJA1 and DNAJB1, were not differentially expressed in D-type snails under moderate thermal stress compared with UL-type snails. Our data generally agree with previous results showing that less thermotolerant gastropods under benign conditions have higher HSP protein expression following heat stress than congeners under warmer conditions (Tomanek and Somero, 1999, 2000; Tomanek, 2010). When exposed to 46°C, eight molecular chaperones – DNAJC16, CRYAB, HSPA14B, DNAJC3, two HSPA5 paralogues, DNAJC10 and HSP90B1 – were uniquely upregulated in D-type snails. This suggests that the evolution of elevated expression of these genes under extreme thermal stress adapts the D-type snails to the rapid and stronger thermal stress.

The patterns of shell colour polymorphism can be affected by a number of selective processes such as visual selection (Heller, 1975), sexual selection (Rolán-Alvarez et al., 2012), thermal regime (Schilthuizen, 2013) and balancing selection (Johannesson and Butlin, 2017). A previous study has found that thermal regime profoundly contributes to the maintenance of shell colour polymorphisms in *B. attramentaria* (Miura et al., 2007). Our study provides an example of the potential for physiological selection imposed by moderate and sublethal thermal stress to shape shell colour polymorphism. The potential impact of moderate temperature as a selective force is especially significant. Moderate thermal stress is not immediately lethal, but does divert energy flux from fitness-related functions such as reproduction and growth towards maintenance and repair (Sokolova et al., 2012; Han et al., 2013). *Batillaria attramentaria* is adapted to a broad range of temperatures throughout the intertidal regions of the Northwestern Pacific (Ozawa et al., 2009; Ho et al., 2015). The results of our study suggest that global warming has the potential to substantially change the frequency distribution of shell colour morphs of *B. attramentaria* along the Northwestern Pacific coast.

Conclusion

Our study provides an example of the potential functions of shell colour for gastropods. Average body temperature of snails with a dark shell was consistently warmer than that of snails with a light shell when exposed to sunlight. The mortality did not differ in snails with different shell coloration in response to lethal temperature. However, transcriptomic analysis suggested that the unfolded protein response was only activated in light shell snails under moderate thermal stress. Snails with a dark shell exhibit high levels of specific HSPs at the control temperature, and may employ a preparative defence strategy. The mean ABT was higher in dark shell snails than in light shell snails, indicating dark shell snails can maintain cardiac performance at higher temperature. When D-type snails were exposed to sublethal temperature, eight molecular chaperones were uniquely upregulated, indicating these genes may allow for improved cardiac thermal tolerance in the snails with D-type shells. Our results suggest that the preparative defence strategies and higher ABT of cardiac thermal performance may allow the dark shell snails to adapt to the rapid and stronger thermal stress in the field.

Acknowledgements

We thank Hua Tian for assistance in the field.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.H.; Methodology: G.H., Y.D., L.D., F.Q., Z.Z.; Software: G.H., Y.D., L.D., F.Q.; Validation: G.H., Y.D., L.D., F.Q.; Formal analysis: Y.D., L.D., F.Q., Z.Z.; Investigation: G.H.; Resources: G.H., Z.Z.; Data curation: Y.D., L.D., F.Q.; Writing - original draft: G.H.; Writing - review & editing: G.H.; Visualization: G.H.; Project administration: G.H.; Funding acquisition: G.H.

Funding

This work was supported by the National Natural Science Foundation of China (42006107) and the Modern Agricultural Industry Technology System of Shandong Province, China (SDAIT-14-05).

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