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Comparison of sHSP2 Expression in Hydra oligactis Before and After the Induction of Aging

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Comparison of sHSP2 Expression in *Hydra oligactis* Before and After the Induction of Aging

By

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Abstract

The invertebrates *Hydra oligactis* and *Hydra vulgaris* are valuable models for research on aging. *H. oligactis* show senescence after sexual reproduction is induced, reducing their lifespan to about 90 days, while *H. vulgaris* remain alive indefinitely. One possible explanation for the difference in lifespan is that *H. oligactis* have a less robust heat shock response than *H. vulgaris*. Among the proteins rapidly produced during the heat shock response are small heat shock proteins (sHSPs). Past research in our lab characterized expression of sHSP genes in *H. vulgaris* in the absence of heat shock. All five of the *H. vulgaris* sHSP genes examined were expressed in stem cells in adult *H. vulgaris*, suggesting a role in maintaining protein quality control in stem cells. The current project sought to determine whether sHSPs are also expressed in *H. oligactis* stem cells and if so, whether expression is maintained once sexual reproduction and aging are induced. Expression of a *H. oligactis* sHSP gene corresponding to one studied in *H. vulgaris*, sHSP2, was characterized using RNA *in situ* hybridization. Expression was examined in *H. oligactis* before and both 27 and 42 days after the induction of aging. We found that even after induction, *H. oligactis* show expression of sHSP2 in epithelial stem cells. This preliminary data provides evidence that sHSP2 is not critical for aging in *H. oligactis.*

Introduction

Data suggest that *Hydra vulgaris* do not go through senescence (Schaible et al., 2015). Another species, *Hydra oligactis*, can stay alive for years when reproducing only asexually. However, when *H. oligactis* are induced to sexually reproduce, they undergo senescence and die within 90 days (Martínez & Bridge, 2012). One group of proteins whose regulation may help to explain the difference in lifespan between *H. vulgaris and H. oligactis* are heat shock proteins.

Vital for dealing with stress is the heat shock response, which involves a rapid increase in transcription of heat shock protein genes (Brennecke et al., 1998; Haslbeck & Vierling, 2015). Heat shock proteins control levels of denatured proteins, by binding to unfolded proteins and allowing them to remain functional (Richter at al., 2010). The heat shock response can be induced by temperature, oxidative stress, and other environmental stressors, which lead to denaturation of proteins (Haslbeck & Vierling, 2015; Sami et al., 2017).

It has been shown that *H. oligactis* have a poor heat shock response compared to *H. vulgaris* (Bosch et al., 1988; Brennecke et al., 1998). Preliminary data on the transcriptome of each species before and after thermal stress are consistent with these results; indicating that *H. vulgaris* have greater upregulation of genes in response to stress compared to *H. oligactis* (Nawrocki, personal communication).

Therefore, the senescence might be explained by a decreased heat shock response in *H. oligactis* after induction to sexually reproduce. This claim is logical because a decreased heat shock response is associated with aging in different invertebrates, such as *Caenorhabditis elegans* and vertebrates, including humans (Calderwood et al., 2009). An increased lifespan has

also been associated with greater expression of heat shock proteins (Calderwood et al., 2009). Specifically, *C. elegans* with elevated levels of heat shock factor 1, a transcription factor important for expression of heat shock proteins, have a longer lifespan (Calderwood et al., 2009).

One set of proteins that are part of the heat shock protein class are small heat shock proteins (sHSPs), which are found in Archaea, Bacteria, and Eukarya (Carra et al., 2017). These proteins have a N-terminal domain, a C-terminal sequence, and a conserved α-crystallin domain (Haslbeck & Vierling, 2015) These three domains play a role in the oligomerization of sHSPs, which regulates the activity of these proteins (Haslbeck & Vierling, 2015). sHSPs of hydra are unusual because they often have two α-crystallin domains instead of one (Nicosia et al., 2014). sHSPs act as molecular chaperones, binding to early unfolding intermediates of proteins (Haslbeck & Vierling, 2015). Protein aggregation harmful to cells can occur after misfolding due to exposure of hydrophobic amino acids to solvent (Sami et al., 2017). Activity of sHSPs can therefore both assure that proteins remain functional and protect cells from aggregation of misfolded proteins (Haslbeck & Vierling, 2015). As with other heat shock protein genes, transcription of small heat shock proteins increases during the heat shock response (Haslbeck & Vierling, 2015). Some sHSPs are expressed constitutively, in organisms not undergoing thermal stress (Sarkar et al., 2009). sHSPs expressed constitutively are present in a cell-type specific manner (Jagla et al., 2018).

sHSP expression both before and after induced sexual reproduction has been characterized in both *H. vulgaris and H. oligactis*. There was work done specifically on the expression of sHSPs after thermal stress in both of these organisms. It was shown that three

sHSPs have an increase in transcription after stress in *H. vulgaris* that have no change in expression in *H. oligactis* (Nawrocki, personal communication). This was not the case in the other two sHSPs investigated, sHSP2 and sHSP5, which had increased transcription in both species after stress (Nawrocki, personal communication). sHSP2, specifically, is unique in the fact that it is upregulated more due to thermal stress in non-aging *H. oligactis* then *H. vulgaris*. This protein has been shown to be expressed in the epithelial stem cells of the body column (Nawrocki, personal communication).

These epithelial stem cells are present in the fairly simple body plan for a hydra. Hydra are a tube that have both a head and a foot on opposite sides (Bode, 1996). They are comprised of two epithelial layers, the ectoderm and the endoderm (Bode, 1996; Hobmayer et al., 2012). In the body column, epithelial cells act as stem cells, differentiating to produce cells of the tentacles or basal disk when they are displaced toward the ends of the body (Bode, 1996). The other stem cell system is the interstitial cells, which are present in between ectodermal cells, and give rise to neurons, gametes, secretory gland cells, and stinging cells (Bode, 1996).

In the laboratory setting, *H. vulgaris* are able to live indefinitely (Schaible et al., 2015). *H. oligactis*, if they are not induced to sexually reproduce, can remain alive for years (Martínez & Bridge, 2012). *H. oligactis* become sexual (meaning they produce either eggs or testes) if they are in a 10 °C environment (Martínez & Bridge, 2012). A study by Yoshida and colleagues showed that when *H. oligactis* were moved to 10 °C they became sexual after approximately four weeks (Yoshida et al., 2006). Before the death of these hydra there are various physiological changes (Tomczyk et al., 2015). These include prey capture and the movement of food to the gastric cavity. Beyond physiological function, it has been shown that there is a

decline in interstitial stem cells, which eventually leads to almost all of these cells not being present by day 30 of being sexually induced (Tomczyk et al., 2015).

One goal of this project was to determine whether sHSP2 shows similar expression in *H. oligactis* before induction of aging as in *H. vulgaris*, with expression in the body column where epithelial stem cells are present. A second goal was to determine whether *H. oligactis* showed a decrease in sHSP2 expression after induction of sexual reproduction and aging. It was expected that there would be decreased expression of sHSP2 in the *Hydra oligactis* induced to age compared to the non-aging *Hydra oligactis*.

Materials & Methods

Hydra Strains and Culture Conditions

The organisms used for this project were *H. oligactis* (Swiss male strain) and *Hydra magnipapillata* (Strain 105). Both strains consist of genetically identical animals produced through asexual reproduction. The hydra were fed freshly hatched brine shrimp 3 times a week and were cultured at a temperature of either 18 °C or 10 °C. They were maintained in hydra medium, which is composed of 1 mM $CaCl_2$, 1.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.08 mM $MgSO_4$, and 0.03 mM KNO_3. *H. oligactis* induced to reproduce and age were cultured for either 27 days or 42 days at 10 °C.

Production of Labeled Probes for in situ hybridization

Twenty-five *H. oligactis* were used for the synthesis of cDNA. Animals were heat shocked for 25 minutes at 29 °C. The Qiagen RNeasy kit was used to isolate total RNA. First

strand cDNA was produced from the total RNA with the Invitrogen Gene Racer Kit, using the GeneRacer Oligo dT Primer.

A 488 base pair portion of the sHSP2 gene was isolated using PCR, with the first strand cDNA as a template. Primers were designed based on *H. oligactis* sequence data kindly provided by Annalise Nawrocki, Pomona College. The sequence of the primers used were 5' GGA CAA GTT CTC GAA GTA TGT GG 3' and 5' CTA TTC TTC CAT TTT GAT CTC AAG TTT GAG 3'. The PCR program used was as follows: 95 °C for 2 minutes, three initial cycles of 94 °C for 30 seconds, 54 °C for 1 minute, and 68 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 1 minute, 68 °C for 2 minutes, and 68 °C for 10 minutes before being held at 4 °C.

The PCR product was gel purified using the Qiagen QIAquick Gel Extraction kit and cloned using the Promega pGEM-T Easy Vector System.

One-Shot Chemically Competent *E. coli* were transformed with the ligation reaction. Plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep kit.

To produce the digoxygenin-labeled antisense probe, the plasmid was linearized by digestion with NcoI-HF. A phenol:chloroform extraction was performed to produce RNAse-free DNA. In vitro transcription was performed using the Roche DIG RNA Labeling kit, with Sp6 RNA polymerase.

RNA in situ hybridization

In situ hybridization was performed as described in Bridge et al., (2010), with an overnight pre-blocking step. As positive and negative controls, previously synthesized antisense (positive control) and sense (negative control) probes corresponding to an 891 base pair portion of the *Hydra magnipapillata* Hyzic gene were used. The Hyzic gene is expressed in differentiating nematocytes (Lindgens et al., 2004). For the positive control, *H. magnipapillata* were used, since the antisense probe is complementary to the Hyzic mRNA for this species. For the negative control, the Hyzic sense probe should not be complementary to any mRNA. *H. oligactis* were used with this probe, so that any non-specific staining of *H. oligactis* tissue could be detected. The *in situ* hybridization procedure was performed twice, details are given in Tables 1 and 2.

Table 1: The components for the first *in situ* hybridization.	
Animals used	Probe
12 *H. oligactis*, 18 °C	sHSP2 antisense
12 *H. oligactis*, 10 °C for 27 days	sHSP2 antisense
12 *H. magnipapillata*	Hyzic antisense
12 *H. oligactis*, 18 °C	Hyzic sense
12 *H. oligactis*, 10 °C for 27 days	Hyzic sense

Table 2: The components for the second *in situ* hybridization.	
Animals used	Probe
24 *H. oligactis*, 18 °C	sHSP2 antisense
12 *H. oligactis*, 10 °C for 42 days	sHSP2 antisense
12 H*. magnipapillata*	Hyzic antisense
12 *H. oligactis*, 18 °C	Hyzic sense
12 *H. oligactis*, 10 °C for 42 days	Hyzic sense

The pictures for both *in situ* hybridizations were obtained using a Nikon Eclipse 80i light microscope. The software used for imaging was NIS-Elements.

Results

The positive control *H. magnipapillata* cultured with the Hyzic antisense probe showed the expected expression pattern (Figure 1) (Lindgens et al., 2004).

In *H. oligactis* cultured at 18 °C, sHSP2 expression was detected in body column epithelial cells, as well as in the basal disk (Figure 2). Light expression was also seen at the bases of the tentacles. No expression was detected in most of the length of the tentacles.

H. oligactis induced to age through incubation at 10 °C for 27 days showed sHSP2 expression similar to the expression seen in non-aging animals. Staining was present in body column epithelial cells but not in tentacles (Figure 3). However, the basal disk staining seen in animals cultured at 18 °C was not present (Figures 3B, D). The negative control of *H. oligactis*

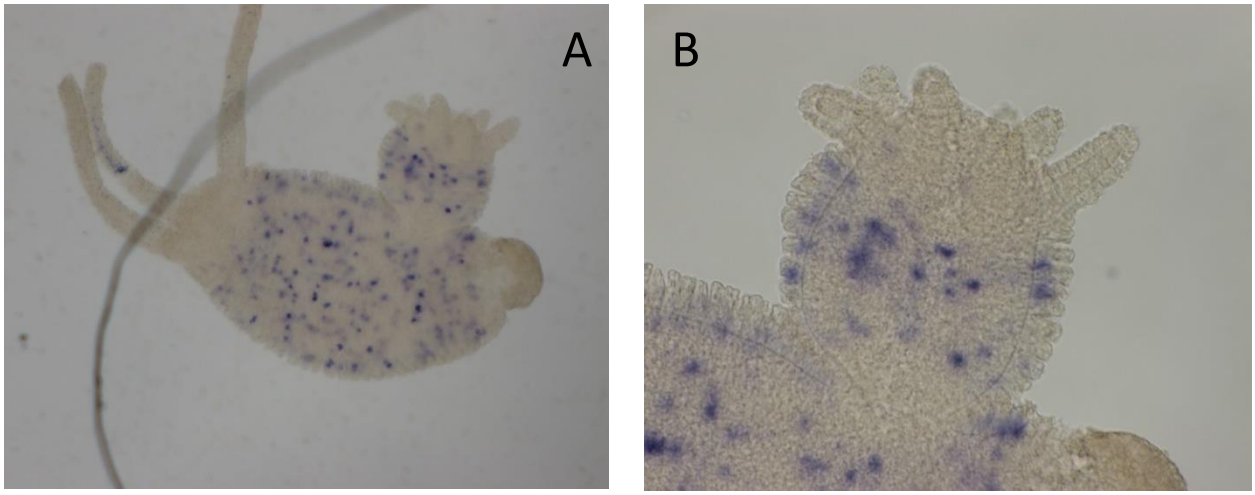

Figure 1: *H. magnipapillata* hybridized with a Hyzic antisense probe as a positive control. (A) Staining in a whole animal, 50X magnification. (B) Staining in a bud, 125X magnification.

incubated at 10 °C for 27 days showed staining in the testes. However, this pattern was distinct from the one produced by the sHSP2 antisense probe (Figures 3B-D).

H. oligactis incubated at 10 °C for 42 days showed sHSP2 expression similar to that seen in animals incubated at 10 °C for the shorter period. Staining was present in body column epithelial cells but not in tentacles or in the basal disk (Figure 4). Of note, the basal disks of these aging *H. oligactis* were visibly larger than those of the *H. oligactis* maintained at 18 °C (Figures 1D, 4D).

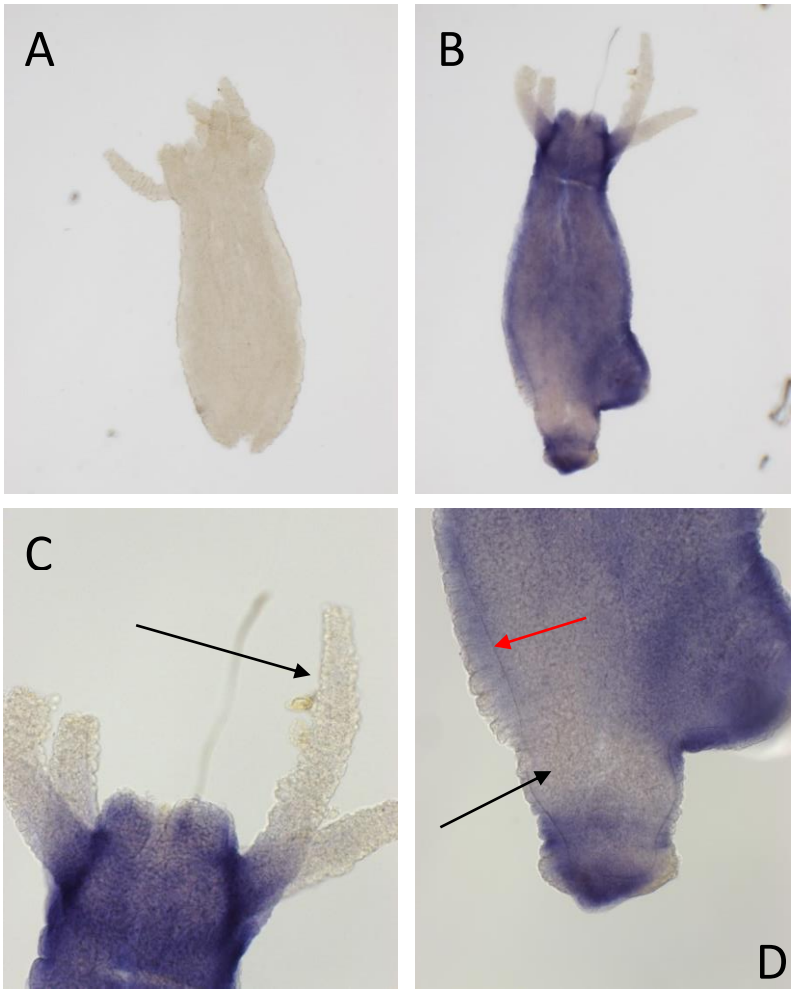

Figure 2: sHSP2 expression in *H. oligactis* maintained at 18 °C. (A) Negative control produced using a labeled probe with the same sequence as a portion of the *H. magnipapillata* Hyzic gene, 40X magnification. (B) Staining in a whole adult animal, 40X magnification. (C) Staining in the head region, 100X magnification. Arrow indicates tentacles. (D) Staining in the basal region, 100X magnification. Black arrow indicates the basal disk, and red arrow indicates the border between the ectoderm and endoderm layers.

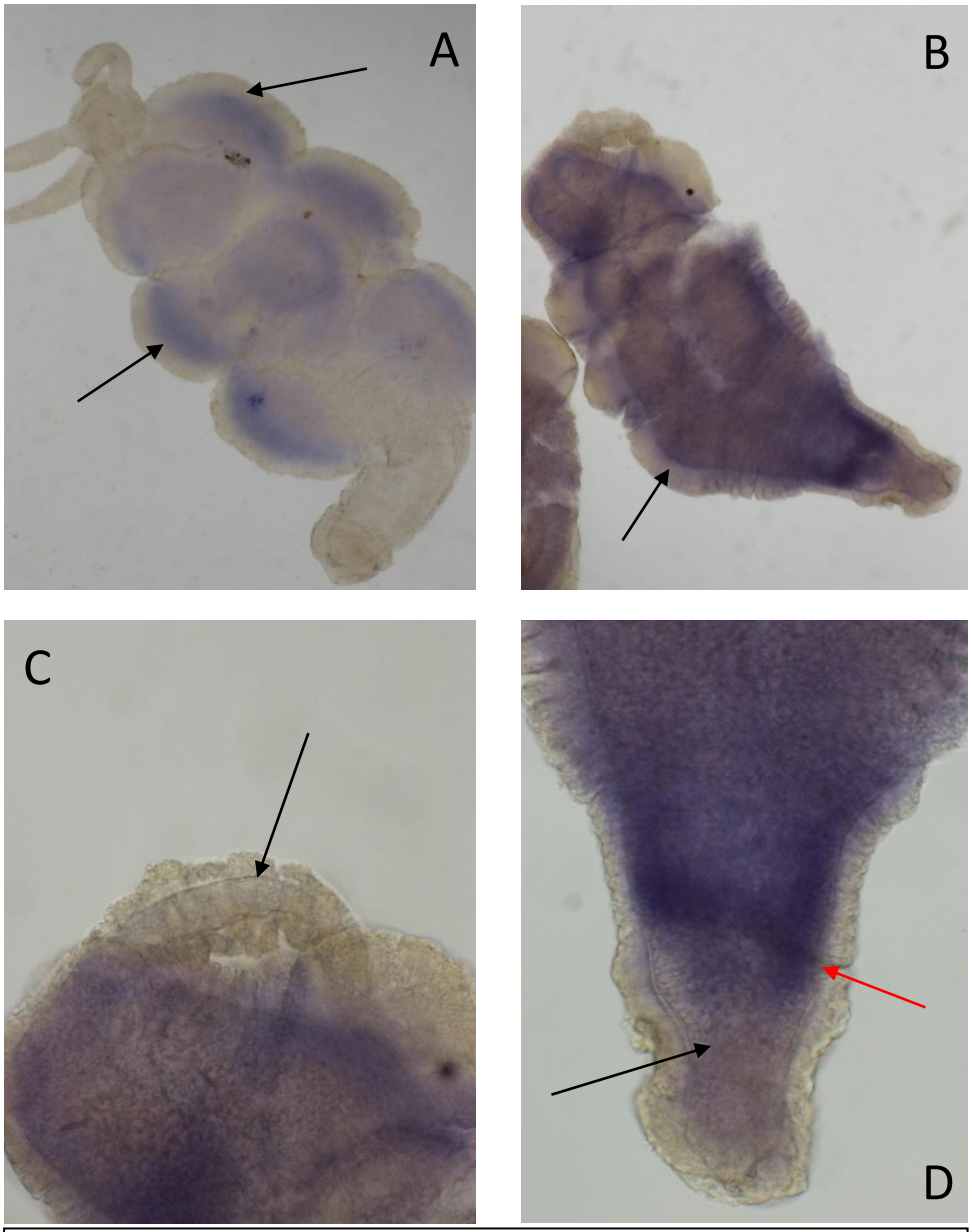

Figure 3: sHSP2 expression in *Hydra oligactis* incubated at 10 °C for 27 days. (A) Negative control, showing background staining, 50X magnification. Arrows indicate testes. (B) Staining in a whole animal, 50X magnification. Arrow indicates teste. (C) Staining in the head region, 125X magnification. Arrow indicates tentacles. (D) Staining in the basal region, 125X magnification. Black arrow indicates the basal disk, and red arrow indicates the border between the ectoderm and endoderm layers.

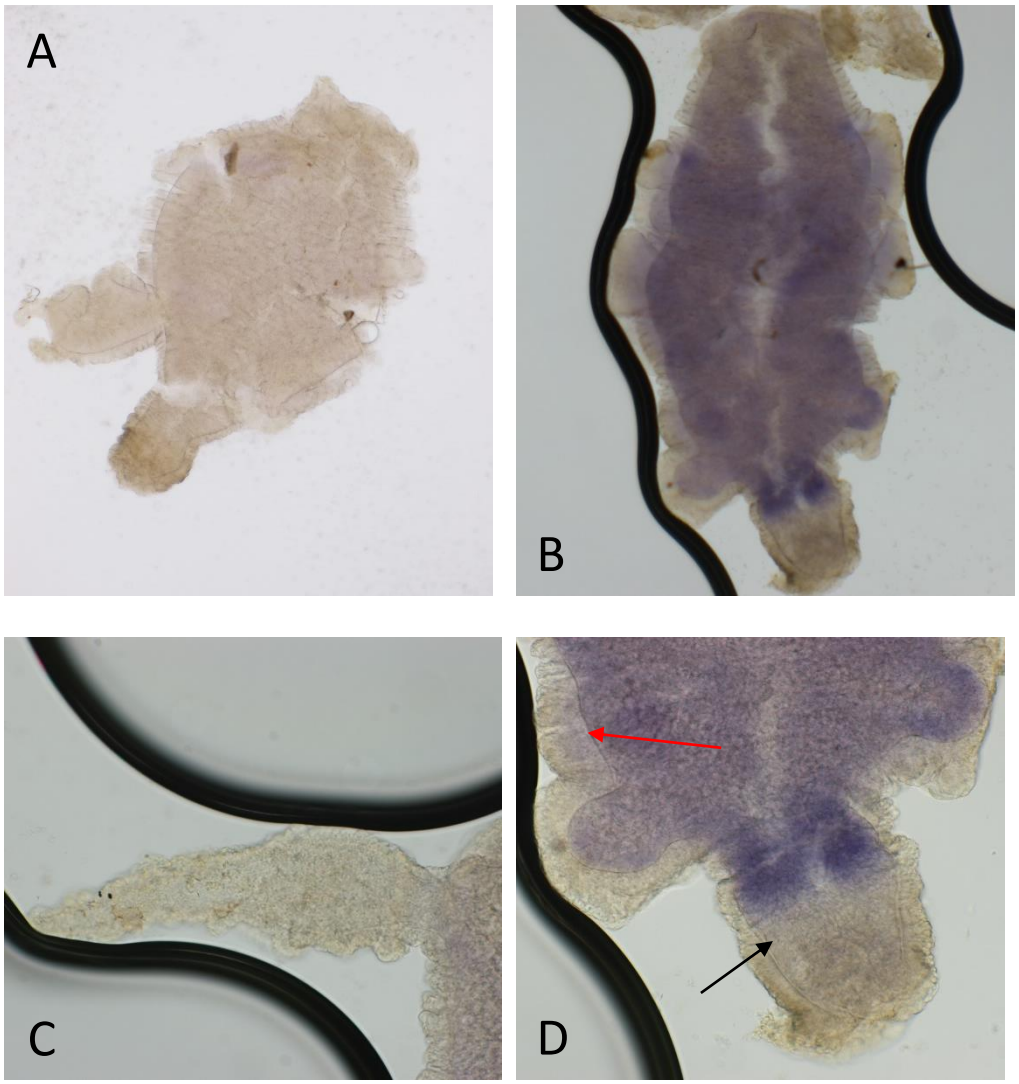

Figure 4: sHSP2 expression in *H. oligactis* incubated at 10 °C for 42 days. (A) Negative control, 40X magnification. (B) Staining in a whole animal, 40X magnification. (C) Tentacle, 100X magnification. (D) Staining in the basal region, 100X magnification. Black arrow indicates the basal disk, and red arrow indicates the border between the ectoderm and endoderm layers.

Discussion

The *in situ* hybridization procedures show that non-aging *H. oligactis* cultured at 18 °C have a pattern of sHSP2 expression similar to the expression patterns seen in the non-aging species *H. vulgaris* for sHSP2 and four other sHSP genes (Altares, personal communication; Graver, personal communication). Expression is not uniform within the body but is higher in epithelial stem cells of the body column than in the differentiated cells in the distal tentacles. As in *H.* vulgaris, the expression pattern of sHSP2 is consistent with a role in maintaining proteostasis and preventing harmful aggregation of denatured proteins in stem cells.

Even though the overall sHSP2 expression pattern is similar in *H. vulgaris* and non-aging *H.* oligactis, a difference was observed in the *H. oligactis* pattern. Strong expression was detected in the ectoderm of the basal disk in *H. oligactis* cultured at 18 °C (Figure 2). One possible explanation for this could be a response to bacteria present on the surface of culture dishes. The expression of certain sHSPs has been shown to increase due to bacterial infection in various organisms, including channel catfish (Xie et al., 2015). sHSP2 might be upregulated in response to specific bacteria in a similar fashion, leading to the pattern shown in the 18 °C *H. oligactis*. The basal disk ectoderm is the point of contact with the substrate and therefore with bacteria growing on the substrate. This possibly could be further investigated by examining sHSP2 expression in *H. oligactis* grown at 18 °C in sterile culture dishes and by placing non-basal disk tissue, like tentacles, in contact with the surface of a normal *H. oligactis* glass culture dish.

We found that expression of sHSP2 is maintained in the body column epithelial cells of *H. oligactis* once sexual reproduction and aging are induced. *H. oligactis* cultured at 10 °C for

both 27 days and 42 days showed expression of the gene in body column epithelial cells (Figure 3, 4). Complete loss of sHSP2 expression is thus not a feature of aging in *H. oligactis*.

One difference between the *H. oligactis* cultured at 18 °C and those cultured at 10 °C is that those at 18 °C showed slight expression of sHSP2 in the base of the tentacles while those cultured at 10 °C did not (Figure 2,3,4). This difference could be due to a higher rate of cell division in the body column in the *H. oligactis* at a higher temperature. An increased rate of cell division would be expected to displace cells into the tentacles more quickly (Bode, 1996). As cells pass into the tentacles and differentiate, sHSP2 mRNA might not be degraded until cells are in the distal region of the tentacles. In the 10 ° *H. oligactis* cell proliferation would be expected to be slower, leading to a slower rate of cell displacement into tentacles. This might allow degradation of sHSP2 mRNA at a more proximal position in the tentacles.

Of note, the hydra that were aging for 42 days appear to have an increased basal disk size compared to the 18 °C *H. oligactis* and the other 10 °C *H. oligactis* group (Figure 2,3,4). This morphological change has not been previously noted in aging *H. oligactis.* Along with this, the sHSP2 expression was altered by stopping higher up on the body column compared to the other two experimental groups. More information is needed to determine if an increased foot size is a physical change that accompanies aging in *H. oligactis* and, if it is, when it happens during the aging process.

Besides the slight changes, there appears to be the similar expression of sHSP2 in the three experimental groups that received the sHSP2 probe. Therefore, loss of sHSP2 expression in stem cells does not appear to lead to aging in *H. oligactis*. This would need to be confirmed using qPCR on sHSP2, which would provide quantitative data. The role of sHSP2 could also be

elucidated from creating transgenic hydra that express hairpin RNA for sHSP2 (Juliano et al., 2014). Hairpin RNA causes knockdown of the gene of interest (Juliano et al., 2014). If the lack of sHSP2 leads to aging in *H. oligactis*, then that would provide evidence that sHSP2 does contribute to the process of aging. Transgenic hydra that increase the expression of sHSP2 could also provide insight on the importance of sHSP2 in aging (Juliano et al., 2014).

In addition to sHSP2, other aspects of the heat shock response need to be explored as a possible explanation for aging in *H. oligactis*. This could possibly include another sHSP that has been looked at in both *H. vulgaris and H. oligactis*, sHSP5. This is the other sHSP that has been shown to be upregulated due to thermal stress in *H. oligactis*, so therefore it might be of significance in the heat shock response and aging.

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