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The Developmental and Genetic Basis of Differences in Cave and Surface-dwelling Forms of the Crustacean, *Asellus aquaticus*

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**The developmental and genetic basis of differences in cave and surface-dwelling
forms of the crustacean, *Asellus aquaticus***

By

Hafasa Mojaddidi

A culminating thesis submitted to the faculty of Dominican University of California in
partial fulfillment of the requirements for the degree of Master of Science in Biology

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Certification of Approval

This thesis, written under the direction of candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree of Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

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Table of Contents

CERTIFICATION OF APPROVAL.....	II
LIST OF FIGURES.....	V
LIST OF TABLES	VII
ABSTRACT	VIII
ACKNOWLEDGEMENTS.....	IX
BACKGROUND.....	1
CHAPTER 1.....	5
SPECIFIC AIM I	5
MATERIAL AND METHODS.....	6
RESULTS	8
<i>Embryonic development of surface-dwelling A.aquaticus.....</i>	<i>8</i>
<i>Comparison of eye and pigment development in cave and surface embryos.....</i>	<i>9</i>
<i>Comparison of antennal article number in both surface and cave hatchlings.....</i>	<i>13</i>
<i>Comparison of aesthetasc morphology in both surface and cave hatchlings</i>	<i>15</i>
<i>Comparison of duration of embryonic development in cave and surface forms.....</i>	<i>16</i>
<i>Comparison of effects of starvation on both cave and surface forms after embryogenesis</i>	<i>17</i>
DISCUSSION	17
CHAPTER 2.....	24
SPECIFIC AIM II.....	24
MATERIALS AND METHODS.....	25
RESULTS	28
<i>RT-qPCR.....</i>	<i>28</i>
<i>In-situ hybridization images of stage 4-6 Drosophila melanogaster embryos of stage 4-6</i>	<i>34</i>
<i>of embryogenesis and A. aquaticus embryos.</i>	<i>34</i>
<i>DP311 and DP312 antibody staining of D. melanogaster and A. aquaticus embryos.....</i>	<i>36</i>
DISCUSSION	39
CONCLUSION.....	44
REFERENCES	45

List of Figures

- Figure 1. Differences between adult surface and cave individuals of *A. aquaticus*.** Adult surface individual (A) and adult cave individual (B). Pigmentation and eye presence in the surface adult (C) compared to the absence of pigmentation and eye in the cave adult (D). Left eye with pigmentation and units of the eye (E) compared to the left side of the cave adult with no pigmentation or units of the eye (F)..... 3
- Figure 2. Embryonic development of the surface-dwelling form of *Asellus aquaticus*.** A. An embryo at 6 days post fertilization. B. At 10 days post fertilization, the head (H) and the tail (T) are distinct. Yolk is (Y). C. Lateral view of embryo at 12 days post fertilization with the chorion (M) starting to shed off. D. Lateral view of embryo now extending while membrane (M) continues to be shed. E. Lateral view of embryo with defined head (H) and tail (T) and three membranes remaining. F. At 26 days, the second membrane (M) is shed, the yolk size is reduced, and eye pigmentation (E) first appears. G. Lateral view after second membrane is removed and there is an increase in eye pigmentation (E). H. At 32 days, the embryo sheds the third membrane and the eye and head have increased pigmentation..... 9
- Figure 3. Comparative embryonic development of surface and cave embryos of *A. aquaticus*.** Surface embryos (A-C) A. The head (H) and tail (T) are now distinct. B. The embryo is comma-shaped. Legs are labeled with (L) C. Appendages are freed from the membrane and eye pigmentation (E) is observed. Cave embryos (D-F) D. The head (H) and tail (T) are now distinct. E. The embryo is comma-shaped. F. Appendages are freed from the membrane and pigmentation is not observed. 11
- Figure 4. Eye region of surface and cave forms of *A. aquaticus*.** A-C. Surface embryos from RS population ultimately showing 3 pigmented spots (ommatidia are difficult to see because of pigmentation). D-F. Surface embryos from a light colored variant from an adjacent surface population first develop small circular fragments, which then combine into ommatidia by stage 3. G-I. Cave embryos (Z) at similar stages with no ommatidia (or forming ommatidia) observed. J, K. Infernus cave embryos from Romania (ROM) observed at the last two stages of development..... 12
- Figure 5. Antenna I article number in cave and surface *A. aquaticus* upon hatching.** A. Article count in left and right antenna I of 20 surface (S) hatchlings and 22 cave (C) hatchlings of *A. aquaticus*. LAI = left antenna I and RAI = right antenna I. Wilcoxon rank sum (Mann-Whitney U-test) yielded the following p-values comparing cave and surface: 0.7888 left antenna 1 (LAI) and 0.4864 right antenna 1 (RAI). B. Antenna II article number in cave and surface *A. aquaticus* upon hatching. Article count in left and right antenna II of 20 surface (S) hatchlings and 22 cave (C) hatchlings of *A. aquaticus*. LAII = left antenna II. Wilcoxon rank sum (Mann-Whitney U-test) comparing cave and surface values yielded significant p-values: 0.008736 left antenna II (LAII) and 1.473 e-05 right antenna 2 (RAII). 14
- Figure 6. Aesthetasc morphology in *A. aquaticus* upon hatching.** A. Tapered aesthetasc morphology. B. Bulbous aesthetasc morphology. C,D. Schematics of the tapered and bulbous aesthetasc morphologies. E. Comparison of the two morphologies, tapered and bulbous, in 17 cave and 14 surface individuals. Fisher's exact test yielded a

<i>p</i> -value of 0.0109 showing that the association between population and morphology was statistically significant. C= cave and S= surface	15
Figure 7. A. Duration of embryogenesis in cave and surface <i>A. aquaticus</i> embryos. Amount of days from 24 hours after extraction until hatching compared in 50 surface (S) and 21 cave (C) embryos. <i>P</i> value = 0.2622 B. Survival rates in cave and surface <i>A. aquaticus</i> hatchlings in nutrient-deficient conditions. Amount of days hatchlings lived without nutrients. The effects of starvation were compared in 38 surface (S) and 25 cave individuals (C). <i>P</i> value=0.5106	16
Figure 8. Checking for DNA contamination and confirming reverse transcription. A. Actin (lanes 2-5), <i>pygopus</i> (lanes 6-9), <i>pax2</i> (lanes 10-13), <i>sine oculis</i> (lanes 14-15). B. <i>Sine oculis</i> (lanes 2-3), retinal dehydrogenase 11 (lanes 4-7), eyes absent (lanes 8-11), and hedgehog (lanes 12-15) were tested with reverse transcriptase, without reverse transcriptase and similarly with an extra DNase treatment.	30
Figure 9. Identifying intron-boundary primer pairs. New sets of primer pairs designed for different regions of retinal dehydrogenase, eyes absent, <i>pygopus</i> , RPL13A, tubulin α , tubulin β , actin, GAPDH, and <i>ef1α</i> were tested for lack of bands indicating no presence of an intron.	31
Figure 10. Even-skipped (eve) hybridization of <i>D. melanogaster</i> embryos. RNA in situ hybridization was performed for eve using a control probe from another lab (A) and a newly synthesized probe using reagents in our lab (B).	35
Figure 11. Sense and anti-sense hybridization of <i>A. aquaticus</i> surface embryos. Late stage surface embryos were probed with sense T7 (A) and anti-sense SP6 (B) probes and both produced non-specific staining. H= head	35
Figure 12. Modified protocol of sense and anti-sense hybridization of <i>A. aquaticus</i> surface embryos. Surface embryos fixed for a shorter period of time were probed with T7 sense (A) and SP6 antisense (B) eve probes and produced non-specific staining. H= head.....	36
Figure 13. Hybridization of DP311 of <i>D.melanogaster</i> embryos. Detection and localization of the pair-rule pattern gene DP311 seen in embryos of stage 4-6 of embryonic development. Blue = DAPI, Green = DP311	37
Figure 14. Hybridization of DP312 of <i>D.melanogaster</i> embryos. Detection and localization of the pair-rule pattern gene DP312 seen in embryos of stage 4-6 of embryonic development. Blue = DAPI, Green = DP312	37
Figure 15. Hybridization of DP311 and DP312 in <i>A.aquaticus</i> embryos. Detection and localization of DP311 (left) and DP312 (right) seen in <i>A. aquaticus</i> late stage surface embryos.....	38

List of Tables

Table 1. <i>Primer sequences for RT-qPCR. Primer sequences that successfully flanked an intron and were used to amplify controls or candidate genes involved in eye development.....</i>	<i>32</i>
Table 2. <i>RT-qPCR Ct values for surface and cave embryonic samples performed in triplicates with reverse transcriptase (RT) or without (noRT).....</i>	<i>33</i>

Abstract

Currently, there are many gaps in our understanding of the genetic mechanisms responsible for human diseases. One novel method to bridge these gaps is to investigate the naturally occurring variation of wild populations, which is arguably more similar to the genetic complexity present in human disease than artificially induced mutations in model species. Species adapted to subterranean environments often share phenotypic characteristics such as the reduction or complete absence of eyes, reduced pigmentation, and enhanced sensory systems. In order to understand the evolution of these morphological changes, we selected an invertebrate model system the freshwater isopod crustacean *Asellus aquaticus*, which contains both surface-dwelling and blind cave-dwelling forms. Our goal was to investigate how and when during embryonic development all of these morphological differences came about. In addition, we wanted to investigate the molecular mechanisms of eye degeneration and to develop methods to identify differential expression between cave and surface forms in the candidate genes *hedgehog*, *sine oculis*, *pax2*, *pygopus*, and *retinal dehydrogenase 11*. The results from this work have helped address evolutionary questions that have been historically difficult to dissect and have generated a much-needed animal model system to better understand when and how cave characteristics develop.

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Background

One goal of developmental biology research is to understand how differences between species or populations are established. Unfortunately, few species are conducive to both comparative embryology and genetic analysis. Therefore, a largely unanswered question is how and when various differences between species are established during development. Specifically, one can examine whether different characteristics present in adults are established embryonically or postembryonically.

The diversity in morphology in different taxonomic levels has been linked to differences in groups of regulatory genes during development [1]. For example, the development of digits in bats (*Carollia perspicillata*) is initially comparable to the development of digits in mice (*Mus musculus*) but ultimately, bat digits elongate further with the increase of expression of *Bmp2* [2]. Another study showed that artificially decreasing *Bmp* signaling in chicken generated webbed feet analogous to that of ducks. This finding suggested that *Bmp* signaling plays a role in phenotypic variation between species [3]. Comparisons have also been done between more closely related species such as the Galapagos finches, investigating the importance of the transcription factor *Bmp4* in different beak morphologies [4].

Comparative embryological studies within populations of a species can also be performed, but they are often limited by the lesser amount of morphological differences between populations. However, cave animals are an excellent system to study the embryological basis of morphological differences because populations of the same species can vary drastically in morphology.

Previous research in cave animals has predominantly focused on *Astyanax mexicanus*, a vertebrate cavefish [5]. Many diverged phenotypes have been noted in surface versus cave forms of this species including eye loss, pigment loss, and metabolic differences. Regarding eye loss, many years of work have shown that cave embryos start developing eyes that then arrest and degenerate [6-8]. Yamamoto and Jeffery have shown that lens apoptosis in the cavefish is an indirect consequence of an increase in midline *Hedgehog* signaling [8]. This finding is supported by results in which an overexpression of *Hedgehog* in surface fish embryos results in the degeneration of the eyes and apoptosis of the lens. Similarly, in another cave model system, the amphipod crustacean *Gammarus minus*, changes in expression of the *Hedgehog* pathway also appear to be associated with morphological differences in eye size [9]. Therefore, changes in the *Hedgehog* pathway might be a common mechanism of eye size variation in cave animals.

The development of pigmentation has also been investigated in *A. mexicanus*. Surface embryos develop pigmentation embryonically but cave embryos never develop pigmentation though they have been shown to contain tyrosinase positive cells [10]. Genes responsible for pigmentation differences subsequently were identified [11, 12]. Metabolic differences have also been examined and a candidate gene approach identified coding mutations in *melanocortin 4 receptor (mc4r)*; individuals homozygous for these mutations showed a greater appetite and starvation resistance [13], a common adaptive cave characteristic.

Much of the genetic and developmental research has focused on *A. mexicanus* because most cave animals are not amenable to laboratory experiments. However, little can be said about morphological evolution in cave animals, as a whole, by studying just a single species. Fortunately, multiple recent studies have investigated additional cave-dwelling species. Genomes of surface and cave individuals of the fish genus *Sinocyclocheilus*, found in China, were sequenced and compared [14]. In addition, transcriptomes of the cave beetle, *Ptomaphagus hirtus* have been sequenced and analyzed [15]. All of these studies have allowed for a greater understanding of cave biology and evolution. However, what has been lacking is a species similar to *A.*

mexicanus with both cave and surface forms that can interbreed and be raised in the lab, thereby allowing for both developmental and genetic studies.

Therefore, we decided to investigate an emerging model, *A. aquaticus*, a freshwater isopod crustacean that consists of both surface and cave forms (Fig. 1).

Figure 1. Differences between adult surface and cave *A. aquaticus* individuals. Adult surface individual (A) and adult cave individual (B). Pigmentation and eye presence in the surface individual (C) compared to the cave individual (D). Left eye in a surface adult (E) compared to the same region in a cave adult (F).

Surface populations are found in freshwater lakes and streams throughout much of Europe, while cave populations are found in several countries including Slovenia and Romania [16-18]. Advantages of this species include the ability to raise the animals in the laboratory using limited space and resources, the ability to culture embryos *in vitro*, a 6-month generation time, the existence of multiple, independently evolved cave populations, and the ability to interbreed the two forms [16, 17, 19-21]. Features of some cave populations of *A. aquaticus* include absence of ommatidia (units of the eye in arthropods), absence of pigmentation, elongated antenna II, shortened antenna I, and longer (in relation to antenna I) chemosensory sensilla, i.e., aethetasci [22-24]. These morphological characteristics have predominantly been compared in adults (Fig. 1) although some late-stage cave and surface embryos have been examined [25, 26]. The eye phenotype is particularly interesting because the cave form previously investigated, the Planina population, has intrapopulation variation, in which some individuals have no visible external eye structure and are referred to as “eyeless” while others have a “fragmented” phenotype with variation in shape and size of ommatidia fragments [20, 21]. The cave population we will be focusing on, the Zelske population, appears to be uniformly eyeless.

Multiple genetic tools have been generated for *A. aquaticus* such as genetic markers and a linkage map [21, 27]. These tools have been used in conjunction with backcrosses between the cave and surface individuals to map regions responsible for eye and pigmentation traits [21]. Regarding the eye phenotype, a single locus has been identified as responsible for the majority of variation in the qualitative trait of eye presence versus absence and another region responsible for variation in the quantitative

trait of eye size. Moreover, transcriptomes of adult individuals have been sequenced and analyzed, placing additional genes on the linkage map and identifying many more genetic markers [28]. Furthermore, much information is known about the phylogenetic history of multiple populations of this species [16-18].

Although there is much existing genetic information for *A. aquaticus*, knowledge of the embryonic development of this species has been limited to late embryos of the surface and cave forms [25, 26]. Therefore, one of our goals was to perform a descriptive analysis of embryonic development in both cave and surface forms, which would allow us to examine the developmental time frame of eye degeneration and other cave characteristics. Our second major goal was to investigate another unanswered question in these animals: what are the genes, pathways, or mutations responsible for eye degeneration? Our experiments will help to establish a promising model system for cave evolution and allow us to better understand the embryonic development of unique morphological characteristics in cave animals. In addition, studying naturally occurring eye variation will yield a better understanding of eye degeneration and may provide insights into eye degeneration in other systems, including humans.

Chapter 1

Specific Aim I

- I. Establish a time frame for embryonic eye, pigmentation, and antennal development in the surface and cave form and investigate when any differences in development between surface and cave embryos arise.

Hypothesis: I. The morphological differences between adult surface and cave animals are all established during embryonic development.

II. Eye development specifically begins in cave embryos similar to eye development in the surface population and then degenerates, ultimately leading to the absence-of-eyes phenotype in adulthood.

The first objective is to determine whether morphological differences between cave and surface animals are established embryonically or post-embryonically. It is expected that these experiments will generate an understanding of the time frame of both surface and cave embryonic development and a foundational basis for further genetic and developmental studies.

Material and Methods

Animal husbandry. *Asellus aquaticus* were collected from the Rak channel of the Planina cave (Z), the Rakov Škocjan (RS) surface location, and the Planina Polje (PP) surface location in Slovenia. An additional population, *Asellus aquaticus infernus*, was collected in Romania (ROM) from wells outside of the Movile Cave. The animals were kept in breeding tanks of artificial freshwater [29] or spring water (Crystal Geyser Alpine Spring Water) in an incubator set at 12°C [21] and fed decaying leaves collected from Strawberry Creek (Berkeley, CA). The incubators did not have internal lights, and animals were exposed to light only when the water and food were being changed.

Embryo collection. To track the morphological changes in both populations during development, embryos were extracted within 24 hours after they were first observed in the female's brood pouch (48 hours post conception). Females with embryos were then

anesthetized in a clove oil solution (20 μ L clove oil extract in 50 mL of artificial water) for about 30 minutes and then washed twice with artificial freshwater. Females with embryos were then placed in a Petri dish, ventral side facing up, and the embryos were extracted with forceps using a compound light microscope. Post-embryo removal, females were placed in new artificial freshwater for recovery prior to being returned to the breeding tanks ready to mate again post shedding of the brood pouch. Embryos were then transferred into new Petri dishes with fresh artificial water, placed in the incubator at 12°C, and checked for mortality daily.

Live imaging throughout embryonic development. Two embryos per 10 cave and 20 surface female brood pouches were tracked throughout embryonic development. Images were taken biweekly using LAS Core software and Image Builder (Leica S8AP0 brightfield microscope) live images. Images were taken right after extraction from the female and continued until hatching. Additional images were taken using a Zeiss Axiovert 40 CFL inverted microscope to get a closer look at the development of ommatidia (regions of the eyes in arthropods), closer to hatching.

Nutrient-poor environment effects. To observe other differences in the two populations, rates of survival without nutrients were observed as follows. Hatchlings of both surface and cave forms that have not been previously observed or tracked were placed into a Petri dish with artificial freshwater or spring water at 12°C. These hatchlings were observed daily for mortality. If an individual did not survive for at least 14 days, it was excluded from the analysis.

Measurements of article numbers of antennae. Number of articles of all four antennae were counted in 20 surface and 22 cave hatchlings from 10 surface broods and 11 cave broods, respectively. They were placed in 100% ethanol and kept at -20°C.

Phenotyping of aesthetascs. Photos of the aesthetascs of the same 20 surface and 22 cave hatchlings were taken. Photos were randomized, and two people determined phenotype (either tapered or bulbous) for an individual. Individuals were removed from the analysis if there were no aesthetascs, if at least one person was unable to tell if the aesthetasc was tapered or bulbous, if both people did not call the same phenotype for the same animal, or if the hatchling differed in phenotype for its two antennae.

Results

Embryonic development of surface-dwelling *A.aquaticus*

To provide a framework for studying development in this species, first we followed surface embryos throughout embryonic development. Initially, the surface embryo is mostly yolk. After around a week, the germ band becomes visible on the surface of the embryo (Fig. 2A). Ultimately, the germ band elongates, and a separation is seen between the anterior and posterior ends (Fig. 2B). The chorion begins to shed off shortly after the separation is seen, while the second and third membranes are still intact (Fig. 2C). Three to four weeks post fertilization, embryos then shed the second transparent membrane. During this period, faint red pigmentation is observed in the eye region of the embryo, the limbs are continuing to extend, and the body of the embryo is becoming straighter and less comma-shaped (Fig. 2F). About a month after fertilization, the third membrane is shed, and the embryo extends its appendages (Fig. 2H). Soon after,

the embryo hatches out of the final membrane and resembles a smaller version of the adult with a pair of legs missing.

Figure 2. Embryonic development of the surface-dwelling form of *A. aquaticus*. **A.** An embryo at 6 days post fertilization. **B.** At 10 days post fertilization, the head (H) and the tail (T) are distinct. Yolk is (Y). **C.** Lateral view of embryo at 12 days post fertilization with the chorion (M) starting to shed off. **D.** Lateral view of embryo now extending while membrane (M) continues to be shed. **E.** Lateral view of embryo with defined head (H) and tail (T) and three membranes remaining. **F.** At 26 days, the second membrane (M) is shed, the yolk size is reduced, and eye pigmentation (E) first appears. **G.** Lateral view after second membrane is removed and there is an increase in eye pigmentation (E). **H.** At 32 days, the embryo sheds the third membrane and the eye and head have increased pigmentation.

Comparison of eye and pigment development in cave and surface embryos

Next, we examined when embryonic development differs in the cave embryos, specifically looking at eye formation and pigmentation. The first morphological difference seen between cave and surface embryos was in pigmentation (Fig. 3); the surface embryos developed pigmentation in the eyes, but the cave embryos did not. As embryos approached hatching, the surface embryos became more pigmented, but the cave embryos never developed pigmentation (Figs. 3 and 4).

We then looked at the ommatidia. Surface adults possess four ommatidia [30], but cave adults lack ommatidia or have degenerate ommatidia [20]. Our goal was to determine whether this difference in morphology occurred during embryonic development. The eye regions of cave and surface embryos were examined daily using a Zeiss Axiovert microscope from a little over halfway through embryonic development until hatching. Three pigmented spots were seen developing in surface embryos from the Rakov Škocjan population (RS) (Fig. 4A–C). The eye pigmentation in the surface form made it difficult to visualize forming ommatidia; therefore, a naturally occurring surface variant with light pigmentation from the adjacent surface population Planinsko Polje (PP) was also tracked (Fig. 4D–F). In this light surface variant, three ommatidia are clearly seen developing with initial formation of many small circles that combined to form three larger circles (Fig. 4D–F). On the other hand, cave individuals from the Zelske (Z) cave population never showed any evidence of developing ommatidia (Fig. 4G–I) while in contrast, the few individuals that could be examined from a second cave population from Romania (ROM) were compared and showed development of ommatidia at a similar embryonic timepoint to the surface embryos (Fig. 4J, K).

Figure 3. Comparative embryonic development of surface and cave embryos of *A. aquaticus*.
Surface embryos (A-C) **A.** The head (H) and tail (T) are now distinct. **B.** The embryo is comma-shaped. Legs are labeled with (L) **C.** Appendages are freed from the membrane and eye pigmentation (E) is observed. **Cave embryos (D-F)** **D.** The head (H) and tail (T) are now distinct. **E.** The embryo is comma-shaped. **F.** Appendages are freed from the membrane and pigmentation is not observed.

Figure 4. Eye region of surface and cave forms of *A. aquaticus*. A-C. Surface embryos from RS population ultimately showing 3 pigmented spots (ommatidia are difficult to see because of pigmentation). D-F. Surface embryos from a light colored variant from the adjacent surface population first develop small circular fragments, which then combine into ommatidia by stage 3. G-I. Cave embryos (Z) at similar stages with no ommatidia (or forming ommatidia) observed. J, K. Infernus cave embryos from Romania (ROM) observed at the last two stages of development.

Comparison of antennal article number in both surface and cave hatchlings

Next, we investigated another common cave characteristic, elaboration of antennae. This feature is thought to be an adaptive characteristic that allows cave individuals to better find one another and/or food in the dark cave environment. In *A. aquaticus* adults, the number of antennal articles in antenna I is generally smaller in cave individuals as compared to surface individuals, and the number of antennal articles in antenna II is greater in cave individuals as compared to surface individuals [22, 24, 31]. The one exception is the cave population that we examined, Zelske cave, that does not have a smaller number of antennal articles in antenna I although it does have a greater number of antennal articles in antenna II (Prevorčnik, pers. comm.). Article number for all antennae were counted for 20 surface and 22 cave individuals right after the completion of embryogenesis (Fig. 5). Wilcoxon rank-sum (Mann–Whitney U) test yielded the following p-values: 0.7888 left antenna I (LAI), 0.4864 right antenna I (RAI), 0.008736 left antenna II (LAII), and 1.473×10^{-5} right antenna II (RAII). Based on these data, we concluded that the cave hatchlings have more antennal articles on antenna II (Fig. 5B) than the surface hatchlings and that the right side has a more significant difference. In addition, the number of antennal articles of antenna I (Fig. 5A) did not significantly differ between cave and surface hatchlings.

Figure 5. A. Antenna I article number in cave and surface *A. aquaticus* upon hatching. Article count in left and right antenna I of 20 surface (S) hatchlings and 22 cave (C) hatchlings of *A. aquaticus*. LAI = left antenna I and RAI = right antenna I. Wilcoxon rank sum (Mann-Whitney U-test) yielded the following p-values comparing cave and surface: 0.7888 left antenna 1 (LAI) and 0.4864 right antenna 1 (RAI). **B. Antenna II article number in cave and surface *A. aquaticus* upon hatching.** Article count in left and right antenna II of 20 surface (S) hatchlings and 22 cave (C) hatchlings of *A. aquaticus*. LAII = left antenna II. Wilcoxon rank sum (Mann-Whitney U-test) comparing cave and surface values yielded significant p-values: 0.008736 left antenna II (LAII) and 1.473 e-05 right antenna 2 (RAII).

Comparison of aesthetasc morphology in both surface and cave hatchlings

Our goal was to determine whether there were any obvious differences in aesthetascs comparing cave and surface individuals at the end of embryonic development. Upon hatching, most individuals only had a single aesthetasc (Fig. 6). We observed two aesthetasc morphologies (tapered and bulbous) in 17 cave and 14 surface individuals (Fig. 6). Animals with the tapered morphology had a tapered end and looked more similar to adult aesthetascs (Fig. 6A, C). The bulbous morphology looked more like a bulb with a stalk (Fig. 6B, D). We found that surface individuals were equally likely to have either tapered or bulbous aesthetascs whereas the majority of cave individuals had tapered aesthetascs (Fig. 6E). Fisher's exact test yielded a p-value of 0.0109.

Figure 6. Aesthetasc morphology in *A. aquaticus* upon hatching. **A.** Tapered aesthetasc morphology. **B.** Bulbous aesthetasc morphology. **C,D.** Schematics of the tapered and bulbous aesthetasc morphologies. **E.** Comparison of the two morphologies, tapered and bulbous, in 17 cave and 14 surface individuals. Fisher's exact test yielded a p-value of 0.0109 showing that the association between population and morphology was statistically significant. C= cave and S= surface

Comparison of duration of embryonic development in cave and surface forms

Prolonged embryogenesis is also a common cave characteristic. Therefore, we next compared the duration of embryonic development in 20 cave embryos and 50 surface embryos (Fig. 7A). Embryos of the surface populations completed embryonic development within 27–40 days while similarly the cave population completed it within 25–36 days. Wilcoxon rank-sum (Mann–Whitney U) test yielded a p-value of 0.2622. Therefore, no significant difference between the duration of embryonic development in cave and surface embryos was found.

Figure 7. A. Duration of embryogenesis in cave and surface *A. aquaticus* embryos. Amount of days from 24 hours after extraction until hatching compared in 50 surface (S) and 21 cave (C) embryos. P value = 0.2622 **B. Survival rates in cave and surface *A. aquaticus* hatchlings in nutrient-deficient conditions.** Amount of days hatchlings lived without nutrients. The effects of starvation were compared in 38 surface (S) and 25 cave individuals (C). P value=0.5106

Comparison of effects of starvation on both cave and surface forms after embryogenesis

Cave animals commonly live in nutrient-deficient environments and might be especially adapted to them [32]. It is not currently known whether the cave form of *A. aquaticus* is better adapted to nutrient-poor environments than the surface form. To investigate this characteristic, both cave and surface hatchlings were raised without nutrients after they completed embryonic development. On average, cave individuals survived for a couple more days without nutrients (Fig. 7B) although the difference was not statistically significant (Wilcoxon rank-sum (Mann–Whitney U) test yielded a p-value of 0.5106).

Discussion

We compared the embryonic development between surface and cave forms so that we could investigate whether characters either known or predicted to be different between adult cave and surface *A. aquaticus* were established during embryonic development. This work provides a foundation for future developmental studies using this promising model system.

To generate a framework for studying the embryology of this species and identify when particular developmental events occur, we tracked multiple surface embryos throughout embryogenesis. Our results showed developmental events occurring at stages similar to that of the terrestrial isopod, *Porcellio scaber*, which has been studied most intensely [33, 34]. Embryos at the germ band stage and later looked fairly similar between *P. scaber* and *A. aquaticus* (before germ band stage, we were not able to see cell divisions in *A. aquaticus*). Pigmentation also develops embryonically in *P. scaber*, first in

the body region and then in the eye [34], at a comparable stage to that in *A. aquaticus*. Similar to *A. aquaticus*, the pigmentation in *P. scaber* is first orange-red and then darkens closer to hatching [34].

After achieving an understanding of the timeline of embryonic development in surface individuals, we compared it to the timeline of embryonic development in cave individuals. We did not detect any obvious differences until around the third quarter of embryogenesis when the surface-dwelling form began developing pigmentation in the eye region but the cave form did not (Figs. 2 and 3). Upon hatching, the cave form still did not have pigmentation in both the Zelske (Z) and Romanian infernus (ROM) cave populations. Parallel to these observations, a study using embryos from wild-caught ovigerous cave females also reported absent pigmentation in late cave embryos [26]. Therefore, the loss of pigmentation in adult animals stems from a lack of formation of pigmentation rather than the development and subsequent degeneration of pigmentation. This mimics what is seen in albino populations of the cavefish *A. mexicanus* where pigment is never present [10]. The mechanism of how or why pigmentation is absent in embryonic development of the *A. aquaticus* cave embryos is still unknown. Possible reasons are the absence of pigment cells, a defect in one of the genes involved in pigment production, or a defect in pigment transport.

Regarding ommatidial development, surprisingly, the two cave populations behaved differently even though their adult phenotype is similar. In the Zelske cave form of *A. aquaticus*, by an external view, no evidence of forming ommatidia were ever seen in embryogenesis. However, in the Romanian infernus cave form, by an external view, developing ommatidia was observed in later stages of embryonic development.

Development of ommatidia in the surface form was similar to that in the Romanian infernus cave form: towards the end of embryonic development, small fragments of the eye were seen that ultimately merged together to form three ommatidia right before hatching (Fig. 4).

The lack of ommatidia development in the Zelske cave is very different from what happens in cave individuals of *A. mexicanus* where the eye begins to develop similarly to surface embryos and then degenerates over time [35]. There are several possible interpretations for this difference. First, it is possible that the eye does begin to form in *A. aquaticus*; photoreceptors could develop internally, and then mechanisms similar to that present in *A. mexicanus*, such as progressive apoptosis and degeneration, could result in the eyeless phenotype. There is some evidence for this from sections performed on adult cave *A. aquaticus*, which show degenerate eye regions described in the publication as eye nuclei, though it is unclear exactly what these eye nuclei are [20]. The existence of a degenerate eye can be tested in future studies using antibody staining, *in situ* hybridization, RT-qPCR, and transcriptomics investigating candidate genes in eye development and eye structure. If an eye starts to form internally in the cave form, we would expect to see expression of these genes. There is precedent for this idea that a described “blind” cave species in fact does have a degenerate and even somewhat functional eye; transcriptomic studies of the cave beetle *P. hirtus* showed expression of phototransduction genes and structural photoreceptor genes, although this species was previously described as being blind [15].

Another possibility is that the formation of the eye is halted very early in development and then is almost completely lost in *A. aquaticus*, aside from the eye nuclei

described by Kosswig and Kosswig [20]. There are several categories of eye size in cave animals including macrophthalmic, microphthalmic, and anophthalmic (either throughout development or exclusively as adults) [36]. Anophthalmic (throughout development) is the most extreme and appears to describe the Zelske population of *A. aquaticus*, at least outwardly.

In reference to the Romanian infernus cave population, the few individuals that were tracked mimicked the situation in *Astyanax mexicanus* where eye structures develop then possibly degenerate resulting in an adult form with no external eyes. However, few individuals were tracked for this study, and more individuals would be needed to confirm that all embryos in the population form ommatidia embryonically. Because of the limited number of Romanian infernus embryos, the embryos perished shortly after hatching, and further tracking of the eye structure was not possible. We hope to obtain more of these animals and attempt to examine them past embryonic development.

Another common characteristic of cave inhabitants is elaboration of appendages; which is thought to allow animals to better find food or mates in the dark cave environment [32]. In adults of *A. aquaticus*, the cave population has a greater number of antennal articles [24] (Prevorčnik, pers. comm.). In addition, comparisons of adults of most cave populations with the surface adults have shown that the cave population has a smaller number of antennal articles in antenna I although the particular population we were examining did not. Our goal was to determine if cave and surface hatchlings showed any difference in these measures. Our comparisons indicated that cave hatchlings did not have statistically significantly fewer articles in antennae I (Fig. 5A). This result was expected because, again, the particular population we examined did not show a

difference in number of antennal articles of antenna I in the adult (Prevorčnik, pers. comm.). Our comparisons, however, did show that cave hatchlings had statistically significantly more articles in antenna II than surface hatchlings (Fig. 5B). Therefore, the adult difference in article number of antenna II between cave and surface individuals is at least partially established in embryonic development.

Another interesting result was that the difference between article number in antenna II was more significant for the right antenna rather than the left antenna. In other arthropods, for example in honeybees, the right antenna is used preferentially in social interactions and olfaction [37-39]. Therefore, it could be that the right antenna II in *A. aquaticus* is preferentially used for particular functions and therefore its article number is under greater selection than the left antenna II. Another possible reason for the left–right differences is that *A. aquaticus* was previously shown to have fluctuating asymmetry in number of antennal articles upon temperature stresses [40]. The asymmetry observed in our experiments could result from some sort of stress during the rearing of the individuals.

An important distinction in our measurements was that we were not measuring relative length. Therefore, if the cave individuals have more but shorter antennal articles in antennal II, the overall length of the antennae would be similar. However, the previous study examining embryos from wild caught ovigerous cave females reported longer antennae in cave embryos than surface embryos [26]. Another possible reason that the cave hatchlings have more antennal articles in antenna II upon hatching is that the cave hatchlings could be larger at hatching. Finally, it is possible that cave individuals are more developed before hatching, which influences the number of articles of antenna I.

However, our experiments showed that the duration of embryonic development was not statistically significant, so that possibility is unlikely.

Another characteristic that is often elaborated in cave animals is sensory structures that help the animals better find food and mates in the dark cave environment. Sensory structures, aesthetascs, are found on the first antennae of *Asellus aquaticus*. At hatching, both cave and surface individuals generally had only one aesthetasc per antenna I and therefore the number of aesthetascs could not be compared. However, we did note two general morphologies present in aesthetascs. One was a tapered morphology with a tapered and extended end. The other was a bulbous morphology, which looked more like a bulb on a stalk. Interestingly, we found that cave individuals were more likely to have aesthetascs with the tapered morphology and that the surface individuals were equally likely to have aesthetascs with either the bulbous or tapered morphology (Fig. 6). One possible explanation is that the bulbous morphology is an immature aesthetasc and that the aesthetascs mature more quickly in the cave hatchlings rather than the surface hatchlings. Future studies will compare aesthetasc development in both surface and cave individuals both embryonically and post hatching to see if a difference in maturation of aesthetascs explains the different morphologies upon hatching.

Because pigmentation, eye size, aesthetasc morphology, and antennal article number differences are all established already in embryonic development, we want to determine if any of these features are genetically associated. Future studies include QTL (quantitative trait loci) mapping studies investigating whether mapped regions responsible for the multiple phenotypes coincide. There is precedent for “loss of function” and “gain of function” traits being genetically linked, similar to the hyperactive

signaling of *Shh* causing an increase in oral and taste bud amplification in *A. mexicanus* in expense to their eye loss [41].

Other common characteristics present in cave animals are an extended duration of embryonic development and the ability to live in starvation conditions [32]. Regarding duration of embryonic development, both populations showed a similar average amount of days taken to complete embryonic development (Fig. 7A). A previous study examined the duration of embryonic development in the cave form and showed that broods took between 19 and 47 days to develop [42], which was similar to our data. However, these experiments were performed at temperatures ranging from 13–16°C, higher temperatures than our experiments, which could account for differences.

Although we observed no significant difference in starvation endurance of cave and surface hatchlings, it is impressive that both groups survived for 3-4 weeks without any nutrients (Fig. 7B). Therefore, *A. aquaticus* seems tolerant to starvation conditions. There also might be some variation in our experimental conditions, as individuals could have been eating bacteria present in the Petri dish, which might not have been uniform. One surface individual greatly surpassed the average survival time, spending 102 days without nutrition, again indicating that there is either intrapopulation genetic variation in ability to resist starvation or that perhaps this individual had more bacteria in the plate, which allowed it to survive for longer. Maybe both cave and surface hatchlings have low nutrition demands and differences in resistance to starvation would only be expressed in juvenile/adult individuals. Also, there are various strategies that allow animals to be adapted to nutrient poor conditions. One is being able to survive on less nutrients, but the other is to be better able to find food more easily in nutrient poor conditions. Feeding

behavior was previously compared between a cave population of *A. aquaticus* from Romania and a surface population, and the cave form moved more and fed less than the surface form [43]. Perhaps the cave form is more active and therefore better able to find food than the cave form. Starvation experiments at various ages of cave and surface individuals and food competition experiments between cave and surface individuals will demonstrate how and whether the cave animals are adapted to nutrient poor conditions. Another possibility to consider is that this particular cave environment is not actually nutrient poor as they consume microbial biofilms (Trontelj, pers. comm.).

Chapter 2

Specific Aim II

- I. Investigate and identify specific genes(s), pathway(s), and mechanism(s) responsible for eye loss in the cave form of *Asellus aquaticus*.

Hypothesis: The *hedgehog* pathway plays a crucial role in eye loss of cave populations of *Asellus aquaticus*.

The objective of the second aim is to investigate possible candidate genes and/or pathways responsible for eye loss in the cave form in comparison to the sighted surface form. This comparison will be made by RT-qPCR and confirmed using *in situ* hybridization and/or immunohistochemistry of both surface and cave embryos. Differential expression of genes in the developing surface embryo in comparison to the cave embryo could indicate genes and pathways responsible for the reduction or absence of eyes in the cave form.

Materials and Methods

Animal and embryo collection. Animal breeding tanks and embryo collection occurred similarly to the experiments described in Chapter 1.

RNA extraction and cDNA synthesis. Total RNA was extracted with TRIzol (Life Technologies) and treated with TURBO DNA-free Kit (Invitrogen) from embryos of different stages from both populations. RNA was quantified on a Nanodrop spectrophotometer, and 250 ng of RNA was reverse transcribed with SuperScript III (Invitrogen) or without transcriptase using random hexamers.

RT-qPCR Primer design. Primer 3 (<http://frodo.wi.mit.edu>) was used to design primers from sequences available for candidate genes provided from adult and embryonic transcriptomes [28]. Controls for RT-qPCR were created with a minus reverse transcriptase control DNA sample. Gene identity for *pax2*, *pygopus*, *hedgehog*, *retinal dehydrogenase 11*, and *GAPDH* were confirmed by BLAST search. Forward and reverse primers were designed with the following parameters: product size 100–150 bp, T_m values 65–73°C, and GC% 35–80. Primers were diluted to a working dilution of 10 μ M. An extra step was taken for samples with unwanted PCR amplification to instead amplify a portion of the sequence that spanned an exon–intron boundary and avoid amplifying DNA.

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR). Embryo cDNA samples were pipetted into 96-well plates and placed in an Applied Biosystems Real-Time PCR instrument (Foster City, CA) with ViiA7 software, using Applied Biosystems Fast SYBR Green PCR Master Mix (Thermo Fisher Scientific), an

annealing temperature of 62°C, and an extension time of 30 s. Before testing comparative samples, a set of tests were performed to create a standard curve from a series of diluted concentrations of late staged surface (RS) embryos. Embryonic surface and cave cDNA generated from the above reverse transcription protocol were then amplified using PCR. PCR products were then purified using the MinElute Gel Extraction Kit (Qiagen) and then tested at dilutions of 1×, 1:10, 1:100, 1:1000, 1:10000, 1:100000, and 1:1000000 to determine PCR efficiency and amplification through a standard curve. To perform a comparative RT-qPCR analysis between cave and surface embryonic samples, cDNA at an initial 1:5 dilution was used from a single time point (60% of development completed) in embryonic development.

Statistical analysis of RT-qPCR data. C_t values were compared between samples including biological triplicates, technical triplicates, and samples generated as a control without reverse transcriptase.

DNA template and cloning for *in situ* hybridization protocol. RNA was extracted and cDNA synthesized from a surface adult head sample according to the protocol described previously. *Dll* was amplified by PCR with the following primers *dllF* (5' TGGGGGTTTCATATCTCCCCACTGA 3') and *dllR* (5' GAACTAGCGGCCTCTTTGGGCCTTA 3'). The fragment was cloned using a pDrive Ligation Kit (Qiagen) and transformed into DH5α *E. coli*–competent cells. Colonies were screened for fragment insertions. Clones were sequenced using the Elim Biopharmaceutical (Hayward, CA) sequencing service and confirmed through BLAST.

DIG-labeled T7/SP6 RNA probes. Following washes with sodium acetate and ethanol at 4°C to precipitate the probe, an RNA labeling mix (Roche Diagnostics) was used to transcribe digoxigenin-UTP-labeled RNA sense and anti-sense probes using T7 and SP6 RNA polymerases, respectively.

Embryo fixation. To compare both surface and cave population specific differences in RNA expression, embryos of both populations were fixed in 9 parts artificial freshwater to 1 part 37% formaldehyde at room temperature for one minute. Membranes were then removed using Tungsten needles, following a protocol used in the crustacean *Parhyale hawaiiensis* [15]. For the second round of in situ hybridization, the protocol was modified as follows: leaving embryos in fixation solution for no more than 20 min to reduce potential background. Fixed, membrane-less embryos were dehydrated through a series of methanol (MeOH)/PBS washes (5 minutes in 1×PBS, 50% MeOH in 1×PBS, 70% MeOH in 1×PBS, 90% MeOH in 1×PBS, 100% MeOH) and stored in 100% MeOH at -20°C. Embryos were then rehydrated right before use for any of the tests and then ready for use.

Establishing whole-mount in situ hybridization (WISH) protocol for *Asellus*

aquaticus embryos. The *Parhyale hawaiiensis in situ* protocol [44] was used with the following modifications: hybridization of whole-mount embryo was performed in 1 mL Eppendorf tubes with hybridization mix (50% formamide, 5×SSC, and 0.25% Tween (Bio-Rad), 50 µg/µL heparin, 100 µg/mL salmon sperm DNA) for ~15 h at 65°C. BCIP/NBT (Sigma) reactions were used to visualize where the probe of interest hybridized using brightfield microscopy (Leica S8AP0).

DP311 and DP312 detection of *D.melanogaster* and *A.aquaticus* embryos using immunohistochemistry. Antibody staining was carried out on both *D.melanogaster* and *A.aquaticus* embryos using previously established protocols in *Parhyale hawaiiensis* [45] with minor modifications to preparation and fixation of the animals for mounting including for the *A.aquaticus* embryos: leaving embryos in fixation solution for no more than 10 min to reduce potential background. For detection of domains of Pax3/7 and non-Pax3/7 protein in *Drosophila melanogaster*, DP311 and DP312 were investigated using monoclonal antibodies at a 1:1000 dilution on stage 4-6 *Drosophila* embryos [46]. The stained embryos were then imaged using confocal laser scanning microscopy (Leica LMD6500).

Results

RT-qPCR

Our goal was to use RT-qPCR to identify differentially expressed genes in eye development in cave versus surface embryos. Candidates were selected from a comparative RNA sequencing experiment of cave and surface embryos (Protas, unpublished), from mutant phenotypes in *Drosophila melanogaster*, or from experiments in cavefish. *Retinal dehydrogenase* and *pygopus* were candidates from RNA sequencing, while *pax2*, *sine oculis*, and *eyes absent* were selected from *Drosophila* studies [47]. *Hedgehog* was selected from cavefish experiments [8] and *Actin* was initially selected as a control gene.

Samples were generated with reverse transcriptase (RT) and also without reverse transcriptase (noRT). Ultimately, the noRT samples were used as a control to test for DNA contamination. The values considered for this control test were the C_t values provided by the qPCR machine. The C_t (threshold cycle) value is a point on the graph produced by this machine that indicates the intersection between the threshold line and the amplification curve. This intersection value indicates the number of cycles it took to detect a real signal from the nucleic acids in samples. These controls were expected to produce high C_t values or even surpass the maximum value of the instrument by resulting with an undetermined C_t value. Additionally, both RT and noRT samples were treated with DNase in an attempt to eliminate any potential DNA contamination. Initial comparisons of the RT and noRT samples using *hedgehog*, *eyes absent*, *sine oculis*, *pax2*, *retinal dehydrogenase*, *pygopus*, and *actin* showed bands in the noRT controls as it did in the RT samples, demonstrating that there was DNA contamination. To avoid DNA contamination, an extra DNase treatment was performed on the same RT and noRT samples, but the extra treatment did not eliminate the DNA contamination issue (Fig. 8). The only gene that behaved as desired, *hedgehog*, amplified in the RT sample (lane 11) but not in the noRT sample (lane 12). We therefore concluded that *hedgehog* primers likely flanked a large intron and consequently did not amplify from DNA and instead only amplified from cDNA.

All other primer pairs that did show amplification in both RT and no RT samples were discarded and redesigned. The single control gene *actin* was unsuccessful; therefore, primers for other control genes – *ef1a*, *tubulina*, *tubulin β* , *RPL13A*, and *GAPDH* – were designed using the previously mentioned transcriptome sequence.

Figure 8. Checking for DNA contamination and confirming reverse transcription. **A.** *Actin* (lanes 2-5), *pygopus* (lanes 6-9), *pax2* (lanes 10-13), *sine oculis* (lanes 14-15). **B.** *Sine oculis* (lanes 2-3), *retinal dehydrogenase 11* (lanes 4-7), *eyes absent* (lanes 8-11), and *hedgehog* (lanes 12-15) were tested with reverse transcriptase, without reverse transcriptase and similarly with an extra DNase treatment.

Since DNA contamination was observed, primer pairs needed to be redesigned to amplify across an intron boundary to prevent amplification from DNA in the cDNA samples (Fig. 8). Primers were initially designed to amplify a fragment of 400–500 base pairs (bp). PCR was performed with DNA as a template, and after electrophoresis of the PCR products, if we saw a band of expected size, we knew that there were no introns. Those primer pairs that produced a band of expected size using DNA as a template (Fig. 9) were *pygopus* (lane 6), *tubulin β* (lane 9), *actin* (lanes 10), and *GAPDH* (lane 11-12) and were discarded from further testing. Other larger fragments from *GAPDH* and *pygopus* sequences were used to find alternative primer pairs that did successfully flank an intron.

Figure 9. Identifying intron-boundary primer pairs. New sets of primer pairs designed for different regions of *retinal dehydrogenase*, *eyes absent*, *pygopus*, *RPL13A*, *tubulina*, *tubulin β* , *actin*, *GAPDH*, and *ef1 α* were tested for lack of bands indicating no presence of an intron.

Primer pairs that lacked a band possibly contained an intron. However the fragments were too large to be used in the RT-qPCR protocol and need to be reduced in base pair size to 100–150 bp. Therefore, primers were redesigned to amplify several blocks internal to the initial fragment. For testing, again DNA was used as a template. Introns were not found for the following genes: *ef1 α* , *tubulina*, *tubulin β* , *RPL13A*, and *α -actin*. Primers flanking putative introns were successfully designed for the following experimental genes: *sine oculis*, *eyes absent*, *hedgehog*, *retinal dehydrogenase 11*, *pygopus*, and a single control gene, *GAPDH* (Table 1). These primer pairs were used for the remainder of the experiments, including for the comparative RT-qPCR analysis.

Table 1. Primer sequences for RT-qPCR. Primer sequences that successfully flanked an intron and were used to amplify controls or candidate genes involved in eye development.

Gene Name	Forward Sequence	Reverse Sequence
<i>hedgehog</i>	5' GCAGGGAGTTGGGGGCGTGT 3'	5' GCACAGGCGCAGATAGGCTCA 3'
<i>pax2</i>	5' CGATGACCCCTGCTTTGTAGCTTCC 3'	5' GGATAGTCGAGTTGGCCCACAATGG 3'
<i>sine oculis</i>	5' GAGGGCACGCACCCACTCTCCTC 3'	5' CGTGAACCCGAAGGAAGGCAACATA 3'
<i>retinal dehydrogenase 11</i>	5' TGCCCACAAGATTCCAACATTGAT 3'	5' TGTTGCAATCCGCAAAATGTGTAGC 3'
<i>eyes absent</i>	5' CGGGCTCACCTCTACAAGTTC 3'	5' GCCAGTTTCCTCATCCAATCC 3'
<i>pygopus</i>	5' GGCCCTGGAGCTATGGTTCATCCTT 3'	5' TGACCACCCATTCCCATGTTCTTCA 3'
<i>GAPDH</i>	5' TCTGTTGGAAGGTGGAGAGG 3'	5' TGCTGACAGAATGCAGAAGG 3'

To further confirm that these genes were amplifying from cDNA and not DNA, RT-qPCR was performed. All experimental and control genes were tested in triplicate with surface embryonic cDNA from embryos at one stage where approximately 60% of development was completed and pigmentation was present in the eye. The results showed C_t values ranging from 21–30 cycles for samples with reverse transcriptase, and either undetermined or high C_t values (30+), indicating no specific amplification in the samples without reverse transcriptase. This result confirmed that the primers were successful in flanking large introns and could be used to amplify solely from cDNA and not from DNA (Table 2).

These validated primer pairs were then tested with both surface and cave embryonic cDNA to compare any potential expression differences to the surface samples tested previously. Experimental results suggested that there were no differences in expression with all the samples containing a 1:5 concentration of cDNA (Table 2). However, one of the triplicate cave cDNA samples did not behave as expected, so the sample could not be normalized to the control gene, and the triplicate value was

discarded and new cDNA at that time point in embryonic development was synthesized to be further tested.

Table 2. RT-qPCR Ct values for surface and cave embryonic samples performed in triplicates with reverse transcriptase (RT) or without (noRT).

Gene	Surface C_t value	Cave C_t value
<i>hedgehog (RT)</i>	21.3	n/a
<i>hedgehog (RT)</i>	28.0	28.3
<i>hedgehog (RT)</i>	33.9	30.0
<i>hedgehog (noRT)</i>	Undetermined	Undetermined
<i>retinal dehydrogenase 11 (RT)</i>	26.6	n/a
<i>retinal dehydrogenase 11 (RT)</i>	28.8	27.7
<i>retinal dehydrogenase 11 (RT)</i>	29.3	30.3
<i>retinal dehydrogenase 11 (noRT)</i>	Undetermined	Undetermined
<i>pygopus (RT)</i>	25.4	n/a
<i>pygopus (RT)</i>	27.5	26.8
<i>pygopus (RT)</i>	26.6	28.9
<i>pygopus (noRT)</i>	Undetermined	Undetermined
<i>pax2 (RT)</i>	27.4	n/a
<i>pax2 (RT)</i>	28.9	28.4
<i>pax2 (RT)</i>	29.6	30.6
<i>pax2 (noRT)</i>	Undetermined	Undetermined
<i>sine oculis (RT)</i>	28.2	n/a

<i>sine oculis</i> (RT)	29.2	29.3
<i>sine oculis</i> (RT)	29.9	30.2
<i>sine oculis</i> (noRT)	38.1	37.4
<i>GAPDH</i> (RT)	20.8	n/a
<i>GAPDH</i> (RT)	23.4	22.5
<i>GAPDH</i> (RT)	23.2	24.7
<i>GAPDH</i> (noRT)	Undetermined	37.4

***In-situ* hybridization images of stage 4-6 *Drosophila melanogaster* embryos of stage 4-6 of embryogenesis and *A. aquaticus* embryos.**

Next, our goal was to establish an RNA *in situ* protocol for *A. aquaticus* to examine the expression of genes involved in eye development and further validate the results from RT-qPCR.

First, we tested our reagents using fixed *Drosophila* embryos and a probe to even-skipped (*eve*) while using a standard *in situ* hybridization protocol for these *Drosophila* embryos, both provided by Kasia Okataba (UC Berkeley, CA). In addition, using DNA, which was also provided by Kasia Okataba, a new test probe was created with our reagents. Images showed that the hybridization reaction was successful (Fig. 10) for the control probe and the probe made in our lab. Fly embryos had the hallmark *eve* stripe pattern across the embryos, indicating that they were between stages 4 and 6 of development (Berkeley Drosophila Genome Project [BDGP], 2016).

Figure 10. Even-skipped (*eve*) hybridization of *D. melanogaster* embryos. RNA in situ hybridization was performed for *eve* using a control probe from another lab (A) and a newly synthesized probe using reagents in our lab (B).

Next, we moved on to *A. aquaticus* embryos. After dissection, they were fixed, dehydrated, and stored in methanol. The gene *Dll* was chosen as a control gene because it is robustly expressed in developing limbs in arthropods and commonly used as a control gene for *in situ* hybridizations [48]. The probes did not hybridize specifically and instead showed high background (Fig. 11) This non-specific binding could be potentially due to the fact that one of the membranes of the embryo could not be removed by dissection and/or that the probe was binding to cuticle on the embryo, producing dark purple stains.

Figure 11. Sense and anti-sense hybridization of *A. aquaticus* surface embryos. Late stage surface embryos were probed with sense T7 (A) and anti-sense SP6 (B) probes and both produced non-specific staining. H= head

The protocol was altered to increase the removal of more membrane in a shorter time prior to probe hybridization and also to use embryos of younger stage embryos to limit the amount of cuticle present. Images were taken again of embryos stained with the sense and anti-sense probes. Embryos probed with sense (Fig. 12A) and anti-sense probes (Fig. 12B) showed similar non-specific staining.

Figure 12. Modified protocol of sense and anti-sense hybridization of *A. aquaticus* surface embryos. Surface embryos fixed for a shorter period of time were probed with T7 sense (A) and SP6 antisense (B) eve probes and produced non-specific staining. H= head

DP311 and DP312 antibody staining of *D. melanogaster* and *A. aquaticus* embryos.

Because *in situ* hybridization proved to be a challenging technique, we decided to see if antibody staining would be more successful. Once again, we started off with *D. melanogaster* embryos of stage 4–6 as controls. Fly embryos and DP311 and DP312 antibodies [46] were provided from the Patel Lab (Berkeley, CA). These antibodies detect proteins of the Pax3/7 family, which are responsible for various early developmental patterning and segment polarity [49].

Figure 13. Hybridization of DP311 of *D.melanogaster* embryos. Detection and localization of the pair-rule pattern gene DP311 seen in embryos of stage 4-6 of embryonic development. Blue = DAPI, Green = DP311

Figure 14. Hybridization of DP312 of *D.melanogaster* embryos. Detection and localization of the pair-rule pattern gene DP312 seen in embryos of stage 4-6 of embryonic development. Blue = DAPI, Green = DP311

DP311 (Fig. 13) and DP312 (Fig. 14) were expressed in the body segments of the embryo at various stages, as expected and as seen in the merged fluorescent images. After a successful staining protocol was confirmed with the flies, both antibodies were tested using the same protocol on *A. aquaticus* late stage embryos. However, the embryos stained non-specifically (Fig. 15). Separate panels of two stains alongside the merged image show both tissue stains overlaid for DAPI and DP311 in a later-stage *A. aquaticus* embryo with high background and non-specific staining (Fig. 15A). A merged image shows non-specific staining of DAPI and DP312 in another later-stage *A. aquaticus* embryo (Fig. 15B).

Figure 15. Hybridization of DP311 and DP312 in *A. aquaticus* embryos. Detection and localization of DP311 (left) and DP312 (right) genes seen in *A. aquaticus* late stage surface embryos.

Discussion

In order to determine the molecular basis of the differences observed throughout embryogenesis in the cave and surface form of *A. aquaticus*, we attempted to use three different methods, RT-qPCR, *in-situ* hybridization, and antibody staining. To our knowledge, of these three methods, only *in-situ* hybridization has been attempted [25] in *A. aquaticus* and only for a couple of genes. To further the potential of *A. aquaticus* as a model system, it is imperative to have a viable method to examine gene expression differences. To quantitatively detect differences in eye development with experimental genes in a non-model system, RT-qPCR will allow the understanding and verification of gene expression differences. Further verification can be achieved by performing *in-situ* hybridization and antibody staining.

RT-qPCR was initially used to look at expression levels of various candidate genes in eye development. However, DNA contamination of cDNA samples was an issue (Fig. 8), and to address this, an extra treatment with DNase was used to remove the contaminated DNA from the RNA samples that were then synthesized into cDNA. However, the DNA contamination still persisted (Fig. 8) possibly because it was difficult to digest all the DNA without degrading the RNA as well. In order to prevent the contaminated DNA from being amplified, we designed primers flanking an intron. A series of newly designed primer pairs were created starting from larger regions and then were ultimately designed to both flank introns and amplify a fragment between 100 and 150 bp, in order to comply RT-qPCR testing parameters. Unfortunately, this was unsuccessful for several genes: *ef1 α* , *tubulina*, *tubulin β* , *RPL13A*, and *α -actin* because no

introns have been identified. Further experiments to identify introns are necessary prior to assaying these genes by RT-qPCR.

By performing RT-qPCR for our five newly designed primers that did flank introns using 1:5 concentration of surface and cave embryonic cDNA, results showed amplification in samples with reverse transcriptase and no specific amplification in the samples lacking reverse transcriptase (Table 2). This indicated that cDNA was the template, not DNA, from the no-RT samples. One cave cDNA sample behaved unexpectedly with all genes and was resynthesized into cDNA again from the original RNA sample. However, the sample behaved unexpectedly a second time and was then discarded from any further use. A new cave embryonic sample was collected, RNA extracted, and synthesized into cDNA to serve as the new biological triplicate for further analysis. Explanations for this sample behaving unexpectedly could include that the cDNA sample may not have been homogenously mixed, the actual sample could have been of low concentration of cDNA template, or there could have been a problem with pipetting. Overall, the samples for all the experimental and control genes suggested that amplification was occurring only from cDNA and not DNA when the newly designed primers flanking introns were used. This information confirmed that the DNA contamination issue was resolved and primers were ready to be tested for efficiency by performing a standard curve.

In the initial comparison of gene expression between cave and surface embryos of the same stage, no significant difference was seen for any of the experimental genes. This outcome could have resulted from the amount of cDNA template in the samples having

been too low to detect differences or inhibitors could have been present, causing similar early amplification in the majority of the samples regardless of the population.

To investigate any potential expression differences throughout different stages in embryonic development between the cave and surface populations, a standard curve was necessary to determine the efficiency of the assay and accurately interpret data. PCR and primer efficiency was tested using the newly designed primer pairs with a series of six 10-fold dilutions of cDNA samples. The results of the first standard curve showed low PCR efficiency, indicating that the template of interest was not doubling with each cycle throughout the series of concentrations and producing a lot of early amplification and signal. Following the same 10-fold dilution series, instead of cDNA serving as our template, PCR products for GAPDH were purified and then tested starting with a 1:10000 dilution and tested through six more 10-fold dilutions. Results showed no differences in C_t values for GAPDH indicating that the same amount of template was in each serial dilution. Considering the values were in the mid-20s, a difference of template amount should have produced differences in cycles between C_t values. This could have resulted to do low quality of PCR products from multiple freezing and thawing steps. Therefore, another candidate gene was tested using newly purified PCR products and tested through the same six 10-fold dilutions. The results from this test produced C_t values of 3 cycle differences within the first four dilutions and showed overlapping values in the last two dilutions. In order to further conclude PCR efficiency through a standard curve, testing the serial diluted PCR products can be tested using another master mix (Power SYBR Green, ThermoFisher) which could possibly be highly sensitive to DNA quantification and enable indication of low copy number in samples. Furthermore, in order to increase

cDNA yield from challenging embryonic RNA samples, using another cDNA synthesis kit (SuperScript IV Reverse Transcriptase, ThermoFisher) designed to be super efficient and super sensitive could allow for overall better quality of samples and sensitivity to any potential differences within the experimental genes. Once PCR efficiencies are indicated, testing the candidate genes with an optimized qPCR protocol to compare surface and cave embryos at four stages throughout development could be performed next. These four stages would respectively represent the periods after approximately 40% of development is completed, after approximately 60% of development is completed, and after 90% pigmentation of the eye is present in the surface but not in the cave, and at hatching. By looking at additional timepoints, we should have an increased chance of detecting time-sensitive expression differences in eye development.

To have another method to investigate differences in gene expression between surface and cave embryos, we attempted to generate a protocol for *in situ* hybridization in *A. aquaticus*. We used a whole-mount *in situ* hybridization protocol established for the crustacean *P. hawaiiensis*. Initial results after following the established protocol indicated non-specific hybridization. Some modifications were made to the protocol, which also resulted in non-specific staining, indicating that further modifications are necessary. Possible reasons the *in situs* did not result in specific hybridization include incomplete removal of membranes of the embryos, probe degradation, or incomplete fixation of the embryos prior to hybridization.

While optimizing a better *in situ* protocol, antibody staining was attempted to try another method to examine expression differences. Antibody staining was done for DP311 and DP312 in *D. melanogaster* to validate reagents in our lab (Figs. 13 and 14).

Subsequently, *A. aquaticus* embryos were tested using the same antibodies previously shown to be cross-reactive with other arthropods [49] and the *P. hawaiiensis* antibody staining protocol [45] (Fig. 15). However, only non-specific staining resulted. Possibly, the membrane removal and fixation steps were not performed successfully on these embryos, leading to generalized staining. Another possibility is the cross-reactivity of the antibody, leading to amplification of background and non-specific staining. Further optimization of the protocol, again focusing on membrane removal, is likely necessary for successful and specific binding of the antibodies.

Overall, attempting to visualize expression differences in *A. aquaticus* embryos has proven very challenging. Many of these challenges stem from using an organism that is not well studied from a molecular standpoint. One of the difficulties that likely affected the last two techniques was the extra membrane that was challenging to remove. In order to address this challenge, preparing *A. aquaticus* embryos through a methanol cracking protocol, similar to that of *D. melanogaster*, might allow for removal of that final and difficult membrane. Hopefully, this method would remove the final membrane and allow for successful probing and staining steps in these additional techniques. Additionally, other steps in the methanol cracking protocol can be optimized to fit best to *A. aquaticus* embryos. However, by using three different techniques, we hope that at least one will prove successful and allow for the examination of differences in candidate gene expression. We expect that gene expression differences between cave and surface populations result in the morphological differences between them. Therefore, optimization of these molecular techniques will provide the necessary methods to

investigate the genetic mechanisms responsible for the embryonic differences in cave and surface forms.

Conclusion

The comparative studies of *Asellus aquaticus* provided significant insight into the development of variation between the two forms. Eye, pigmentation, and antennal differences in the cave and surface forms were seen in embryogenesis. However, duration of embryonic development, and time to starvation post hatching did not show a significant difference between the two forms. With this better insight into the developmental progression of these phenotypes, we initiated studies focusing on identifying the genetic mechanisms responsible for these differences in phenotype. However, most of the experiments focused on developing molecular techniques to investigate the genetic basis of developmental morphological differences. Future experiments will use these molecular techniques to help determine and visually confirm any differential expression of genes between the surface and cave embryos at different stages throughout embryonic development.

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