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A Comparative Developmental Genetic Study of Branchiopods: Measuring Hedgehog Gene Expression Across Embryonic Development of *Daphnia* and *Artemia*.

AUGSBURG UNIVERSITY

Todd Fairbanks[1], Laura Fricke[1], Brittany Stokes[1], Abigail Garofalo[1], Olivia Fitch[1&2] and Matthew L. Beckman[1]

[1]Augsburg University, Minneapolis, Minnesota; [2]Michigan State University, East Lansing, Michigan

Abstract

Daphnia magna (branchiopoda), a freshwater microcrustacean, possesses a unique morphological feature: a cyclopic eye. Within class Branchiopoda, we observe one-eyed and two-eyed species with varying degrees of eye fusion. Importantly, we find a correlation between the degree of eye fusion and branch distance from a basal member (*Artemia franciscana*) in the branchiopod phylogenetic tree. The molecular genetic program underlying the development of cyclopia has not been elucidated, but models of cyclopia have been created using teratogens and genetic manipulations. These models, along with the analysis of the hedgehog (*hh*) gene sequence in humans with holoprosencephaly have implicated the Hedgehog signaling pathway in cyclopia and other midline defects. The Hedgehog signaling pathway is highly conserved across animal taxa playing an important function in directing midline and other features of embryonic development for both invertebrates and vertebrates. We sought to determine whether the *D. magna* and *A. franciscana* hedgehog gene sequences and expression profiles play a role in cyclopic development through a comparative genetic study of one-eyed and two-eyed organisms. Our sequence analysis shows that *Daphnia* spp. *hh* genes share unique amino acid substitutions that correlate to known mutations in human families exhibiting midline defects, while the *A. franciscana* sequence and all two eye organism sequences do not. We performed RT-qPCR on four stages of eye development in *D. magna* and *A. franciscana* and found hh expression decreases over developmental time in *Daphnia* but increases during *Artemia* development. In order to further determine the spatial and temporal expression of hh we have created a series of plasmids and made digoxigenin-labeled probes for in situ hybridization in *Daphnia* and *Artemia*. Preliminary data from *Daphnia* demonstrate strong expression in the midline head region in early stages of embryonic development. Continued in situ hybridization experiments will establish the expression profile for stages that match those studied by RT-qPCR in both *Daphnia* and *Artemia*.

Branchiopod Eye Morphology

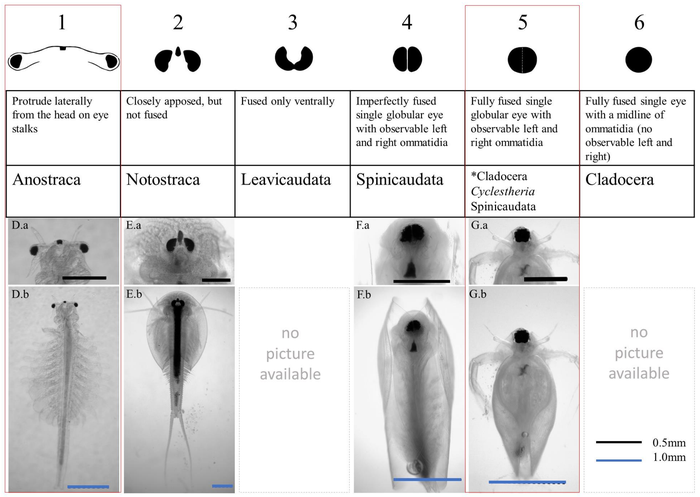


Figure 1: Two members of the Class Branchiopoda exhibit very different adult eye morphologies. The diagrams show adult eye morphology in schematic and photographic form along with whole animals. For Leavicaudata and Cladocerans with fully fused eyes no photographs were available. Note the extremes of eye types between Anostracas and the cyclopic Branchiopods (red boxes). The photomicrographs were made from specimens reared in the laboratory. Scale bars are designated on the image (black bar=0.5mm and blue bar=1.0mm.

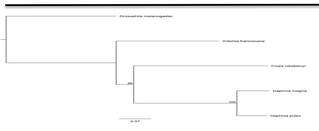


Figure 2: Gene tree for Branchiopoda based on a Hedgehog protein sequence alignment. Neighbor-Joining consensus tree of *Drosophila melanogaster*, *Artemia franciscana*, *Triops newberryi*, *Daphnia magna*, and *Daphnia pulex*. *D. melanogaster* is used to root the tree. The tree is supported by currently accepted molecular based trees for Branchiopoda. Geneious version 11.0.3.[2]

Research Questions

1. Is altered expression or function of the *Daphnia* hedgehog gene during early development in the eye field responsible for cyclopic eye development?
2. Can we learn about the developmental genetic basis for cyclopia by comparing the spatial and temporal profile of hedgehog gene expression in *Daphnia* versus *Artemia*?

These mechanisms are hypothesized to potentially contribute to cyclopia in *Daphnia magna*:

- Lowered function of the Hedgehog protein due to mutations resulting in:
  - ❑ lower expression or altered developmental time-course of expression
  - ❑ defects in proper protein trafficking
  - ❑ defects in the signaling function of the hedgehog protein

Furthermore, these mechanisms may conspire to cause the development of cyclopia.

Hedgehog Sequence Comparison

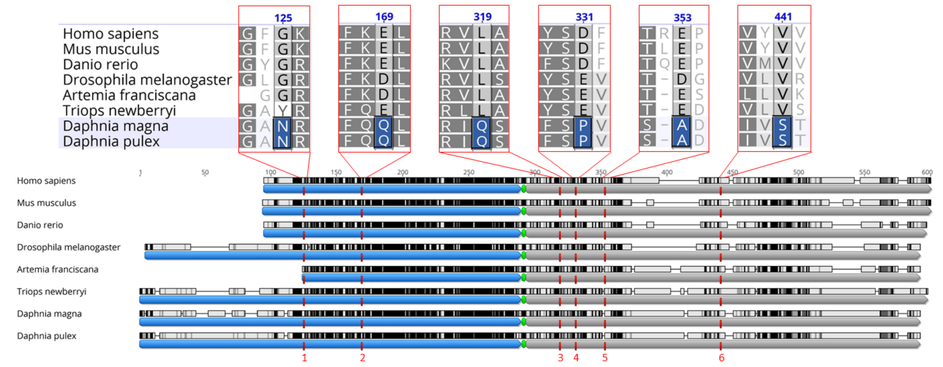


Table: Analysis of Cyclops Distinct Amino Acid Sites

Site #	Conserved Amino Acid	Cyclops Amino Acid	Conserved Property	Cyclops Property	Position (Human)	Known HPE Family Mutation
1	Glycine	Asparagine	Aliphatic, Nonpolar	Amidic, Polar	Gly31Asn	Gly31Arg
2	Glutamic/Aspartic Acid	Glutamine	Acidic	Amidic, Polar	Glu75Gln	-
3	Leucine	Glutamine	Aliphatic, Nonpolar	Amidic, Polar	Leu225Gln	Val224Glu, Ala226Thr
4	Glutamic/Aspartic Acid	Proline	Acidic	Aliphatic, Nonpolar	Asp237Pro	-
5	Glutamic/Aspartic Acid	Alanine	Acidic	Aliphatic, Nonpolar	Glu259Ala	-
6	Valine	Serine	Aliphatic, Nonpolar	Hydroxilic, Polar	Val317Ser	-

Figure 3: Hedgehog sequence alignment of *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Drosophila melanogaster*, *Artemia franciscana*, *Triops newberryi*, *Daphnia magna*, *Daphnia pulex* and protein structure analysis. Analysis of the protein alignment shows six sites where the *D. magna* and *pulex* sequences differ from the rest of bilateralia. These sites are highlighted in red. Blue region: amino terminal domain, green region: cleavage site, gray region: carboxy terminal domain. Geneious version 11.0.3.[3] B. Hedgehog protein models made in SWISS MODEL are the normal *D. magna* Hh and an "Artemized" *D. magna* Hh with all cyclops amino acids replaced with conserved amino acids. Trace view shows an example of one SNP found in Daphnia $E_{two\text{-}eyes} \rightarrow P_{cyclops}$. The right panel shows QMEAN quality scores. SWISS MODEL license link: https://creativecommons.org/licenses/by-sa/4.0/legalcode

Eye Stages Studied: Daphnia and Artemia

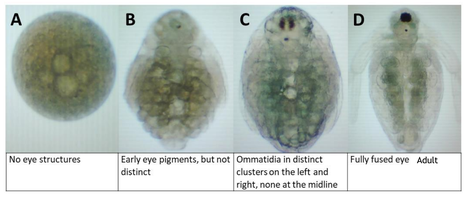


Figure 4: *Daphnia* eye stages studied. The top panel of four Daphnia stages, designated A-D here correlate with a standard staging scheme developed by Mittman, et al, 2014[3] as follows. The following stages A-D roughly correlated with numbered stages in in the developmental staging scheme proposed by Mittman et al. Stage A roughly correlates to stage 4, stage B roughly correlates to stage 8, stage C roughly correlates to stage 10 and stage D is an adult animal that is past stage 12 (first instar) of the Mittman scheme. *Daphnia* were cultured as described in Barrozo et al., 2015[1].

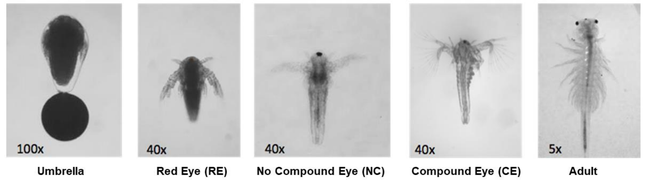


Figure 5: Artemia eye stages studied. Animals were staged based on eye development. The above figure shows the first stage as the umbrella stage, the second red eye (RE), the third is no compound eye (NC), and the fourth is compound eye growing (CE). The final stage is a fully grown *Artemia (Adult)*.

Daphnia magna in-situ hybridization

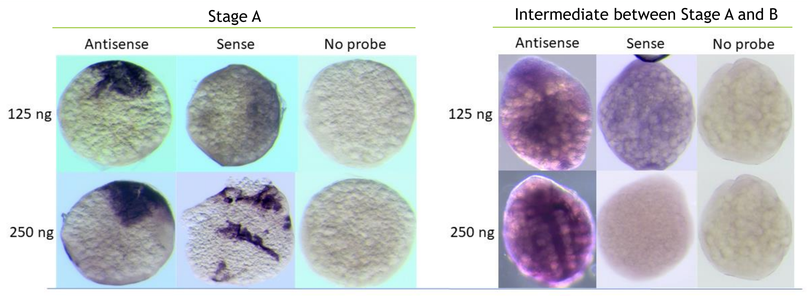
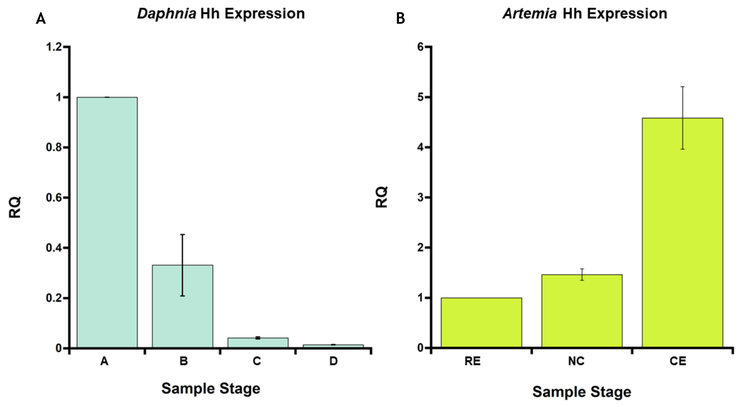
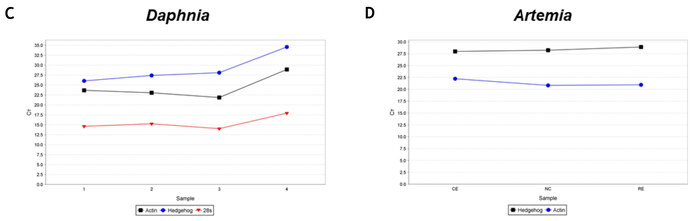


Figure 6: In-situ hybridization in *Daphnia magna* shows hedgehog expression in two early stages of Daphnia development Daphnia embryos from the stages designated above were extracted from pregnant females and fixed in paraformaldehyde, stored at -20°C in methanol till processing. Digoxigenin-labeled probes were synthesized by in vitro transcription from linearized plasmid (pCR-TOPO 2.0) containing cloned *D.magna* Hh fragments. In situ hybridization was performed using Dig-labeled probes and an anti-Dig-Alk-Phos Fab fragment followed by NBT-BCIP staining to detect probe:Ab complex. Higher and lower probe concentrations (62.5ng and 500ng) yielded poor results compared to the data presented here.

Hedgehog Gene Expression





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Gene	Primer Sequences	Amplicon Length (bp)	Efficiency (%)	R^2	Linear Regression Equation
Actin	F: CCGGCCCTGGCAATTG R: CGGAAAGCGCCAGATGATT	53	100.9	0.995	Y=-3.299x + 17.485
Hedgehog	F: CGCCCATTTGGCCTTTT R: CCAAACTCTTGTGCACGTTGTT	56	101.4	0.995	Y=-3.288x + 25.404
28s	F:CACCGAACCCGTCTTGAA R:GACGACTCGCGCACATGTTA	55	90.7	0.998	Y=-3.568x + 9.507

F

Gene	Primer Sequence	Amplicon Length	Efficiency(%)	R^2
hedgehog	F:GACAGGGACAGA TCCAAGTATG R:GCAGACGATGAT TCGGACTTA P:	22 21	-3.769	0.997
actin	F:AGGCTCAGAGCA AACGTGGTA R:GTGACAACACCG TGCTCGAT	22 24	-3.994	0.999

Figure 7: Determining changes in *hedgehog* expression during *D. magna and A.franciscana* development using comparative C_T method. The developmental stages from which total RNA was extracted are shown in Figures 4 and 5. qRT-PCR was performed using TaqMan Probes from Thermo Fisher in a Quant Studio 3.0 instrument using Therm Fisher PCR reagents. RNA extraction was done with the PureLink Mini RNA extraction kit. Reverse transcription on equal amounts of total RNA for all samples was performed with the SuperScript Vilo IV RT kit using DNAse. Panels A and B show relative quantification (RQ) performed using the ddCT method. For Panel A the data represents the average +/- S.D. of three separate experiments, each with technical triplicates. For Panel B this data is from one experiment with technical triplicates. Panels C and D show the endogenous control and Hh Ct values for each developmental stage. For panel A we show the RQ using the 28s probe as reference, while in B we are showing the Actin probe as reference. Panels E and F show details for our primer and probe sets.

Future Work

Mutational analysis shows unique amino acids found only in daphnids and humans with holoprosencephaly. Further, qRT-PCR studies show the Daphnia hedgehog gene expression rapidly declining in early development while the Artemia hedgehog gene expression profile is quite different—increases greatly over early development. Yet, our understanding of hedgehog gene expression in both *Daphnia* and *Artemia* is still incomplete. We will apply in situ hybridization to additional stage of *Daphnia* development, begin the in situ hybridization studies in *Artemia* and perform additional replicates of the qRT-PCR for *Artemia*. One limitation of the qRTPCR is that the samples are whole body samples. We hope to micro-dissect embryos and perform RNAseq on heads of *Daphnia* and *Artemia* in the future.

References

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