Expression and Localization of PAX6 during Embryonic Development of *Caridina pseudogracilirostris*

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ABSTRACT:

The *Caridina pseudogracilirostris* shrimp, known for its herbivorous nature and adaptability to both wild and laboratory conditions, has gained popularity as an ornamental fish in aquariums due to its unique features such as transparency, color, and elongated rostrum. Understanding the reproductive biology and embryonic development of this species is crucial for its conservation and aquaculture management. In this study, the researchers focused on the expression pattern of the pax6 protein, which plays a critical role in tissue and organ formation during embryonic development. The embryos at various developmental stages were quantified in wet weights. The results indicated a gradual decrease in protein concentration as the embryos progressed from the cleavage stage to the pre-hatching stage, suggesting that the proteins were being utilized for organogenesis. The researchers then investigated the expression of pax6, a gene known for its involvement in eye development and central nervous system regulation in both vertebrates and invertebrates.

Immunohistochemistry using a pax6 antibody revealed the localization and expression pattern of the protein during different embryonic stages. The highest expression of pax6 was observed in the first post-nauplius stage, coinciding with the development of the eyes and nerve ganglia. This finding supports the crucial role of pax6 in organ maturation. The expression of pax6 decreased in the pre-hatching embryo stage, indicating its maintenance function in developed organs. To validate the protein expression levels observed through immunohistochemistry, an enzyme-linked immunosorbent assay (ELISA) was conducted. This quantitative analysis confirmed the protein concentration changes observed during embryonic development. The findings contribute to our understanding of the role of pax6 in invertebrate embryonic development and highlight the need for further investigation into the presence and function of Pax6 in larval and adult stages of this species.

Keywords: ELISA, pax6, Immunostaining, imaging, *Caridina pseudogracilirostris*.

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1. INTRODUCTION

Caridina pseudogracilirostris, a brackish water shrimp, has been widely utilized as a developmental model to gain insights into the genetics of organogenesis. To study the expression levels of transcription factors involved in various developmental processes, the Enzyme Linked Immunosorbent Assay (ELISA) has proven to be a powerful tool, particularly in *Caridina pseudogracilirostris* embryos. Transcription factors, also known as sequence-specific DNA-binding factors, play a crucial role in controlling the transcription of specific genetic information by binding to enhancer or promoter sequences of DNA (Phillips 2014), (Dynan and Tjian 1985). These factors are involved in diverse processes such as morphogenesis, cell fate determination, and cellular differentiation (Lobe 1992). One significant transcriptional protein involved in the development and maintenance of the eye and brain is Pax6. Apart from its role in embryonic developmental stages, Pax6 is also expressed in adult neural stem cells, predominantly in astrocytes and neurons located in regions such as the olfactory bulb, thalamus, and cerebellum(van Heyningen 2002) The Pax family comprises several types of transcription factors, and the vertebrate genes contain a conserved sequence motif called the paired box, which encodes the DNA-binding domain (Mansouri, Goudreau, and Gruss 1999) Pax6 acts as a transcriptional activator and promotes neurogenic cell fates during development(Jang and Goldman 2011). It possesses a Pax6-transactivation domain responsible for its function.

Notably, a novel PAX6 mutation has been identified in a patient with aniridia, characterized by a genetic duplication within the PAX6 gene (Winegarner et al. 2017). The expression level of Pax6 has been investigated in zebrafish during embryonic development stages using a reliable assay for quantifying Pax6 transcription factor, namely the ELISA method (Kannan and Vincent 2015). In this study, our main objective is to detect and quantify Pax6 expression in different developmental stages of *Caridina pseudogracilirostris* embryos using an indirect ELISA approach. This research represents the first report on the quantification of Pax6 using the ELISA method during the embryonic development of *Caridina pseudogracilirostris*.

2. METHODOLOGY

2.1. Breeding and maintenance of *Caridina pseudogracilirostris*

Adult *Caridina pseudogracilirostris* were collected from Rajakkamangalam Estuary near the Centre for Marine Science and Technology, Rajakkamangalam. The shrimps were promptly packed and transported to our laboratory. Upon arrival, they were acclimatized by placing them in a 1:1 ratio of fresh and native water. Subsequently, the shrimps were transferred and housed in 30 L tanks filled with fresh tap water. The tanks were maintained at a controlled room temperature ranging from 26°C to 28°C, following a 14:10 hour light-to-dark cycle. The shrimps lay their eggs in clusters, typically consisting of around 200 to 500 eggs. To extract the eggs without causing any damage to the abdominal region, a delicate paintbrush was used manually. The extracted eggs were then carefully maintained in E3 medium, which is composed of 5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl2, and 0.16 mM MgSO4 in 1 L of distilled water(“Shrimp Culture” 2020).

2.2. Total Protein extraction and quantification

A total of 100 embryos from each developmental stage were subjected to homogenization using a homogenization buffer consisting of 50 mM Tris-HCl with a pH of 8.0, supplemented with 100 mM NaCl (TBS). Following homogenization, the samples were centrifuged at 10,000 rpm for 5 minutes to reduce the lipid content. The resulting pellet was then resuspended in a solution containing 10 mM MgCl2 and 7 mM β-mercaptoethanol. To extract the protein pellet, a 200 mM NaCl

solution in 20 mM Tris (pH 8) was utilized, and subsequent precipitation was achieved by adding 70% ammonium sulfate. The resulting sample was stored at -20˚C in an equilibration buffer consisting of 10 mM Tris-HCl (pH 8.0) with 1 mM EDTA, until further use.

To quantify the protein content, the Pierce™ BCA Protein Assay Kit from Thermo Scientific was employed. The quantification was performed by measuring the absorbance at 280 nm.

2.3. Enzyme Linked Immunosorbent Assay of Pax6

ELISA-based quantification of Pax6 expression was conducted for five different developmental stages of Caridina. In each stage, 100 embryos were homogenized in a solution consisting of 50 mM Tris-HCl with a pH of 8.0, supplemented with 100 mM NaCl (TBS). The homogenized samples were then centrifuged at 10,000 rpm for 5 minutes at 4˚C. For the ELISA assay, a microtitre plate was utilized and coated with 100 µL of embryo extracts from each developmental stage in triplicates. The plate was covered and incubated overnight at 4˚C to allow binding of the antigens. To remove the excess antigen solution, the plate was inverted and dried by shaking. Subsequently, 200 µL of blocking buffer, which consisted of 1% BSA in TBS, was added to each well and incubated overnight at 4˚C. After removing the blocking buffer, the plate was washed twice for 3 minutes with 200 µL of wash buffer, containing 0.1% Tween 20 in TBS, per well. The primary antibody, AD1.5 sc-53106 (Santa Cruz, USA), specific to Pax6, was diluted at a ratio of 1:1000 in blocking buffer. A volume of 100 µL of this diluted antibody solution was added to each well and incubated for 1 hour at room temperature. Following the incubation, the plates were washed thrice with 200 µL of wash buffer per well.

To detect the bound primary antibody, a secondary antibody, Goat anti-mouse IgG labeled peroxidase (Genei), was used. This secondary antibody was diluted at a ratio of 1:2500 in blocking buffer, and 100 µL of the diluted solution was added to each well. The plate was then incubated for 1 hour at room temperature with shaking. After the incubation, the plates were washed five times for 3 minutes with 200 µL of wash buffer per well. To initiate the color development, 100 µL of the substrate solution TMB/H2O2 was added to each well and incubated for 5 minutes at room temperature. Following color development, 90 µL of 1N HCl (stop solution) was added to each well to halt the reaction. The absorbance of the wells was measured at 450 nm using a Multimode Plate reader (Perkin Elmer) to obtain the results of the ELISA assay (Kannan and Vincent 2015).

2.4. Whole mount Immunostaining and Imaging

The embryos were fixed in a paraformaldehyde solution for 24 hours. Following day the specimens were washed with PBS or TBS to remove any residual fixative. To block non-specific binding sites, the specimens were then incubated in a blocking solution. The Pax6 primary antibody was diluted in the ratio of 1:1000 using the blocking solution according to the manufacturer's instructions and incubated with the embryos overnight at the temperature of 4°C. After the primary antibody incubation, thorough washing with PBS or TBS was performed to remove unbound primary antibody. The HRP-conjugated secondary antibody was diluted at the radio of 1:500 using blocking solution as per manufacturer's instructions and incubated. Subsequent washing steps were carried out to remove unbound secondary antibody. The DAB substrate solution was prepared and incubated with the embryos until the desired staining intensity was achieved. The reaction was terminated by rinsing the embryos with distilled water. The embryos were then dehydrated using an ascending alcohol series and cleared in an appropriate clearing agent, such as xylene. Finally, the specimens were mounted on glass slides using a mounting medium. The immunostained specimens were imaged using a stereo microscope. Image

analysis was conducted to assess Pax6 expression patterns (Soundharapandiyan, Thanumalayaperumal, and Rajaretinam 2022).

3. RESULTS AND DISCUSSION

*Caridina Pseudogracilirostri*s found to be herbivorous habitat and they mostly feed on plant vegetation's in brackish water. But it has good adaptation capacity to survive even in lab conditions which is totally different when compared to wild environment. This shrimp has been used as ornamental fish in aquarium, due to its transparency nature, color and its long rostrum. Breeding takes place during the early dawn or dusk after getting molted. Once the breeding takes place the matured eggs move from ovary to brood sac through oviduct and gets attached to the female abdomen and forms brood sac within the pleopods. The embryos were collected directly from those pleopods using brushing techniques for all five stages. Wet weight of the collected embryos from Cleavage Stage, Germinal Disc, Embyonized nauplius stage, first post nauplius stage and Pre hatching embryo stage were 31.5, 19.47, 18.96, 27.77 and 58.11 mg respectively.

Before moving to expression studies of pax6, the total protein concentration in each stage has been quantified using BCA method. The protein quantity decreases gradually from Cleavage stage to pre hatching embryo stage (Table 1). The decrease in protein concentration is proportional to the protein utilization by the embryo for organ formation or organogenesis.

Table 1. Protein concentration in different developmental stages of *Caridina pseudogracilirostris* embryos.

S I.No	Stages	Protein µg/mg
1	Cleavage Stage	185±7
2	Germinal Disc	174±5
3	Embyonized nauplius stage	155±3
4	First post nauplius stage	110±8
5	Pre hatching embryo stage	97±4

The PAX6 genes play a critical role in the formation of tissues and organs during and embryonic development and maintenance during later stages. The expression of Pax6 genes have been observed in eye development and in central nervous system of both vertebrates and invertebrates. Pax6 negatively regulates telencephalon proliferation and it promotes proliferation in both vertebrate (Marquardt et al. 2001) and invertebrate (Dominguez et al. 2004)eye. For Morphogenesis of eye pax6 is considered as only master controller (Gehring 1996).The Pax6 genes of eight different animal phyla are structurally and functionally similar (Niimi et al. 1999). Pax6 expression is found in developing eye, nose, pancreas, and central nervous system ((Ton et al. 1991), (Walther and Gruss 1991), (Grindley, Davidson, and Hill 1995), (Davis and Reed 1996)). When the function of PAX6 is lost it leads to severe brain early postnatal death, abnormalities, microencephaly, and the absence of eyes and nose in rodents ((Matsuo et al. 1993), (Schmahl et al. 1993)) and humans (Glaser et al. 1994). Moreover, PAX6 is essential for α-cells differentiation and islets in the pancreas formation((St-Onge et al. 1997), (Sander et al. 1997)). Many studies stated the importance of pax6 for embryonic development as well as in adult vertebrate organisms. But studies are very limited in case of invertebrates.

This study has proven the expression of pax6 in different embryonic stages of

invertebrate model *Caridina pseudogracilirostris s*and maximum expression has been observed in first post nauplius stage (Fig.1). The gradual increase in pax6 expression was observed from cleavage stage to first post nauplius stage; this is due to the development of eye and other nerve ganglion. Most of the organs including eye and ganglion are well developed during the first post nauplius stage and due this maximum expression of pax6 was observed. Following first post nauplius stage the expression of pax6 declines in pre hatching embryo stage. In order to maintain the functions of organs pax6 is expressed in low concentration after pre hatching embryo stage. Further studies have to be done with developing larval stages and adult *Caridina pseudogracilirostris* in-order to find the presence and role of Pax6.

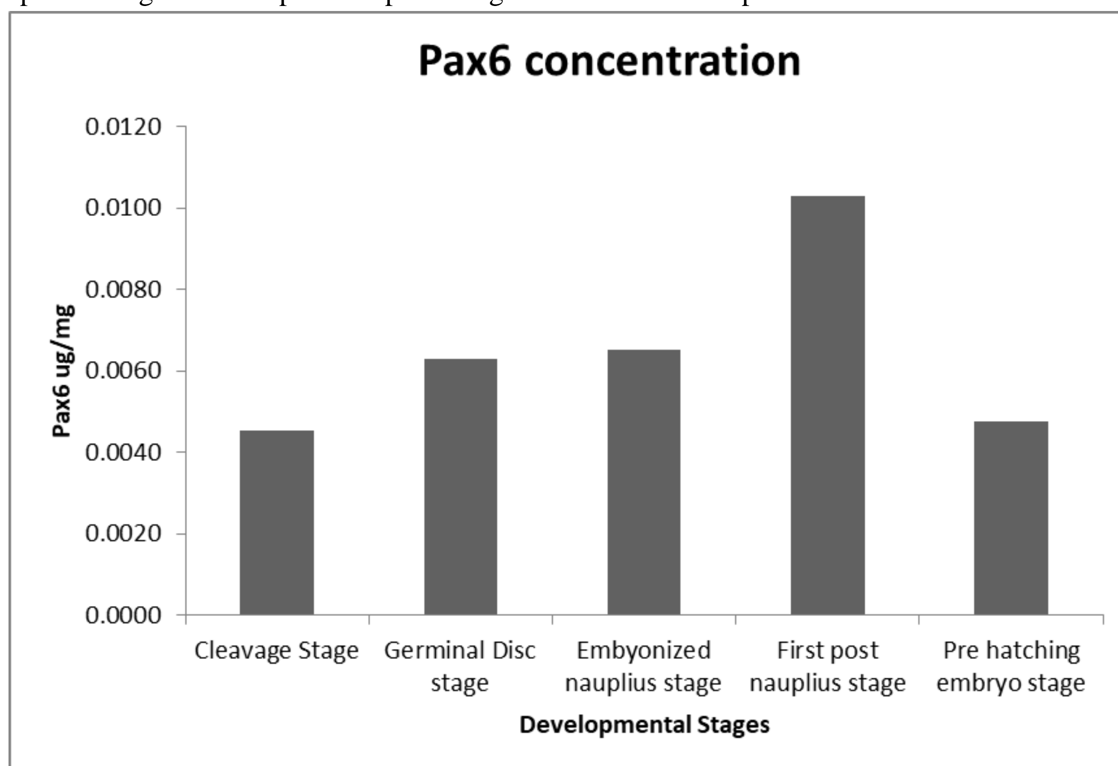

Figure 1. Pax6 Expression in different developmental stages.

The study aimed to investigate the time and site of protein function in *Caridina pseudogracilirostris* embryos using immunohistochemistry with a pax6 antibody. Additionally, the researchers examined the expression pattern of the protein at different time points (Day-1, Day-3, and Day-5) to understand its role in the development and maturation of various organs. The study also employed ELISA-based confirmation to validate the protein expression levels.

The findings of the study revealed that major expression of the pax6 protein was observed on Day-5 (Fig 2). This time period was identified as a crucial stage when maturation occurs in all previously formed organs. These results are consistent with previous studies that have implicated pax6 in the regulation of organ development and maturation. Pax6, a highly conserved transcription factor, plays a crucial role in eye development and has been extensively studied in various organisms, including zebrafish and mice. It is involved in the specification of ocular tissues and the regulation of genes necessary for eye morphogenesis (Sinn and Wittbrodt 2013; Fuhrmann 2010). Studies in zebrafish have shown that pax6 is expressed in the developing eye primordia and continues to be expressed throughout eye development(Nornes et al.

1998). These findings highlight the importance of pax6 in eye development and support the notion that its expression during Day-5 in *Caridina pseudogracilirostris* embryos signifies a critical period for maturation.

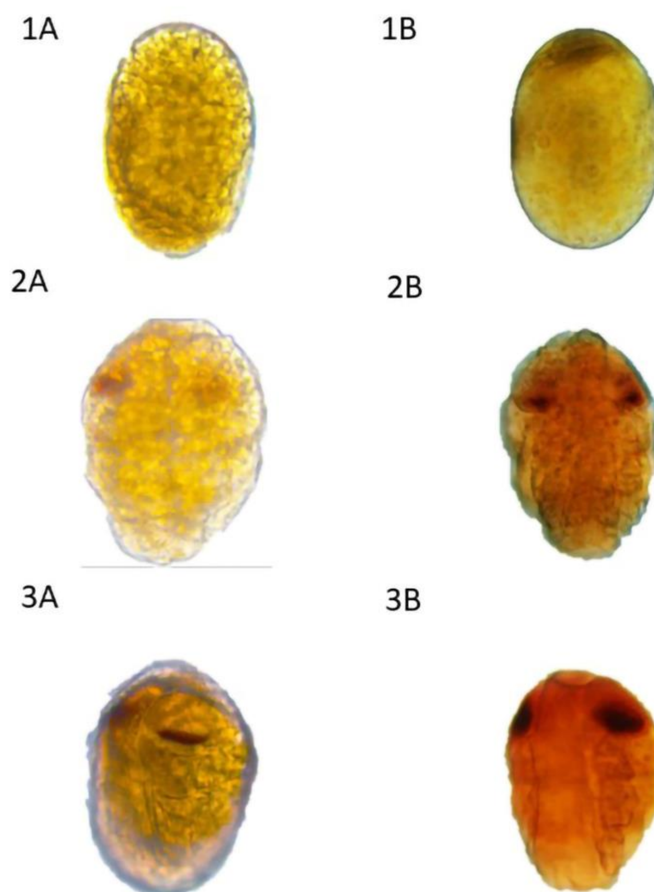

Figure 2. Whole-mount immuno-stained images of embryos showing the expression of Pax6 at different time points. The left column (1A, 2A, 3A) represents control embryos collected on day 1, day 3, and day 5, respectively. The right column (1B, 2B, 3B) shows antibody-treated embryos collected on day 1, day 3, and day 5, respectively.

Immunohistochemistry is a powerful technique that allows for the visualization of protein expression in specific cell types and tissues(Magaki et al. 2019). It provides valuable information regarding the localization and distribution of proteins within an organism. In this study, immunohistochemistry using a pax6 antibody enabled the researchers to precisely determine the expression pattern of the protein during different developmental stages of *Caridina pseudogracilirostris* embryos. The ability to visualize small alterations in the development of specific structures or cell types, such as neurons and axons, further emphasizes the utility of immunohistochemistry in screening zebrafish lines with random mutations(Hammond-Weinberger and Zeruth 2020). Staining intensity alone may not accurately reflect the amount of protein present(Watzlawik et al. 2021). To address this limitation, the researchers employed an ELISA-based confirmation assay to quantify the protein expression levels(Saleem and Kannan 2018). ELISA (enzyme-linked immunosorbent assay) is a widely used method for detecting and quantifying specific proteins in biological

samples. It offers high sensitivity and specificity, allowing for the accurate measurement of protein concentrations(Yang et al. 2022). By performing an ELISA assay, the researchers were able to validate the protein expression levels observed through immunohistochemistry, strengthening the reliability of their findings(Gunawan et al. 2018).

4. CONCLUSION

The study investigated the expression pattern of the pax6 protein in *Caridina pseudogracilirostris* embryos at different developmental stages. The results showed that pax6 expression was highest in the first post-nauplius stage, which corresponds to the maturation phase of various organs, including the eye and nerve ganglia. This finding is consistent with the known role of pax6 in regulating organ development and maturation in other organisms. Previous studies have demonstrated the critical role of pax6 in eye development and its expression in ocular tissues. The present study provides evidence for the expression of pax6 in *Caridina pseudogracilirostris* embryos and suggests its involvement in organogenesis.

Immunohistochemistry using a pax6 antibody allowed for the visualization and localization of the protein within specific cell types and tissues. This technique provided valuable insights into the expression pattern of pax6 during different stages of embryonic development. Additionally, the researchers utilized an ELISA-based confirmation assay to validate the protein expression levels observed through immunohistochemistry. The combination of these techniques strengthened the reliability of the findings by quantifying the protein concentration accurately.

The study contributes to our understanding of pax6 expression and its potential role in *Caridina pseudogracilirostris* embryonic development. However, further investigations are necessary to explore the presence and function of pax6 in developing larval stages and adult *Caridina pseudogracilirostris*. Additional studies focusing on different developmental time points and tissues would provide a comprehensive understanding of pax6's involvement in organogenesis and maintenance.

Overall, the findings of this study shed light on the expression pattern of pax6 in *Caridina pseudogracilirostris* embryos and highlight its potential significance in the development and maturation of organs. Further research in this area will contribute to our knowledge of the role of pax6 in invertebrate embryonic development and its potential implications in adult organisms.

5. FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

6. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. ETHICAL APPROVALS

Because invertebrates, such as prawns, are not subject to ethics committee approval, this study was exempt.

8. DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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