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Research article

Effects of salinity level on the activity of chloride cell and mucus secreting cell in the gill of the female Shortfin molly, *Poecilia mexicana* Steindachner, 1863

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Abstract

Ovoviviparous poeciliid fishes have been relatively well studied in the unique reproductive strategy, but their osmoregulatory system largely remains unknown. In this study, we conducted a short-term (7 days) lab experiment to investigate the effect of different salinity levels from 0 (freshwater) to 50 ppt (mesosaline) on the number of chloride cells and mucus secreting cells of female *Poecilia mexicana*. Chloride cells were found at high density along the epithelial lamellae, whereas mucus secreting cells were also concentrated in the gill raker epithelium. More interestingly, the average density of chloride cells and the mucus secreting cell were significantly increased at high salinity levels ($P < 0.05$). While further validation by immunohistochemistry is warranted, integrative data from our study suggested that the potential function of the osmoregulatory mechanism/strategy was supported by chloride and mucus secreting cells of female *P. mexicana* gill.

Keywords: Chloride cell, Histology, Molly fish, Osmoregulation

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INTRODUCTION

Gills are the primary osmoregulatory organ in teleosts (Brown, 1992; Laurent et al., 1994a; Laurent et al., 1994b). The general structure of gill is composed of gill rakers, gill arches and gill filaments where the epithelial cells are located (Senarat et al., 2018). Morphological, histological and physiological characterizations of the gill epithelial cells (or respiratory epithelium) have demonstrated that these cells are designed for gas-exchange surface, systemic maintenance of blood acid-base balance and ionic regulation (Goss et al., 1998; Sturla et al., 2001; Garcia et al., 2015; Neurasteh et al., 2017).

Chloride cells (or mitochondria-rich cells) play a specific role in trans-epithelial Na^+, Ca^{2+} and Cl- influxes as well as an integral role in acid-base regulation (Perry, 1997; Güner et al., 2005). Chloride cells are thus important in the adaptive process to various osmotic and ionic environments (Caberoy and Quinitio, 2000; Fielder et al., 2007) and are necessary for uptake and excretion of ions under freshwater and saltwater environments (Evans et al., 2005; Pisam et al., 1991). The number of chloride cells is known to increase after exposure to high salinity (Carmona et al., 2004) associated with the increase in the Na^+-K^+-ATPase activity (Caberoy et al., 2000). This change in chloride cells has been also reported in other fishes for example the guppy *Poecilia reciculata* (Pisam et al., 1995) and the milkfish Chanos chanos (Lin et al., 2003).

Mucus-secreting cells are another important type of gill epithelial cells associated with the protection against abrasive injuries, pathogenic bacteria, parasites (Dezfuli et al., 2003; McCahon et al., 1987), and pollutants (Ledy et al., 2003; Roberts and Powell, 2005; Singh and Banerjee, 2008). Because the mucus-secreting cell activity changes in response to the salinity level (Roberts and Powell, 2003), this cell is suggested to play a role in the osmoregulation (Powel, 2007; Roberts and Powell, 2003). Indeed, a significant increase in the density of mucus-secreting cells in response to changes in the ion concentrations (sodium, calcium and chloride) in water has been well documented (Laurent and Hebibi, 1989; Perry and Wood, 1985).

The ovoviviparous molly fish *Poecilia mexicana* is one of the important invasive species in Thailand. Since *P. mexicana* in Hawaii can dwell in habitats with a wide range of salinity from 0 to 40 ppt (Englund, 1999), it is interesting to investigate whether and how this fish has adapted to different salinity levels in Thailand. In this study, to gain a better understanding of the osmoregulation of *P. mexicana*, we experimentally tested the effect of salinity change from 0 to 50 ppt on the chloride and mucus-secreting cells in the gill structure of female *P. mexicana* using short-term exposure (7 days).

MATERIALS and METHODS

Fish and experimental design

Live healthy *P. mexicana* individuals [n = 270 individual fish with mean standard length (SL) of 22.81 ± 4.24 mm] were obtained from the small canal at Samut Prakan province in October 2019, transported to the laboratory and acclimated to the dechlorinated tap water (20-liter aquaria filled with dechlorinated tap water) at room temperature for 14 days. This experiment was performed at the Department of Marine Science, Faculty of Science,

Chulalongkorn University during October to December 2019. All fish were fed with commercial feed pellets (C.P. optimum). After one week acclimation, the female fish were randomly divided into six group in triplicate (n = 15 each, total 270 individuals). Each group was placed in a closed recirculation system with one of the six different salinity levels; 0 ppt (freshwater condition), 10 and 20 ppt (hyposaline condition) and 30, 40 and 50 ppt (mesosaline condition). The salinity levels were adjusted by adding appropriate amounts of purified NaCl to dechlorinated tap water. The fish were fed the same commercial pellet two times a day (07:00 am and 04:00 pm), which were cultured under natural photoperiod. At the end of the experiment (7th day), the fish were euthanized using a rapid cooling method for 30 min (Wilson et al., 2009). They were measured for the total length to nearest 0.1 mm. All fish were then fixed in Davidson's fixative (Dietrich and Krieger, 2009). The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Review No. 1923026).

Histological analysis of gills

After dissection, gill tissues form experimental fish were decalcified with the decalcifying agent (Leica, Biosystems) for a period ranging from 3 hr to 6 hr. They were processed by appropriate histological processing methods (Presnell and Schreibman, 1997; Suvarna and Layton, 2013; Senarat et al., 2020a; Senarat et al., 2020b). The paraffin-embedded tissue blocks were sectioned (4 um thick) and stained with hematoxylin–eosin (H&E) to allow histological examinations. All histological images were taken by a light microscope coupled with a camera for photography (Leica DM 750). The illustration diagram of the density of chloride cell and mucus-secreting cell was created by the Adobe Illustrator CS6.

Cell counting protocol and statistical analysis

The densities of chloride cells in the epithelia of filaments and lamellae were documented, whereas the mucus-secreting cells in the gill raker were determined as follows. Three random pieces of gill filament from the section were observed at 40x magnification following the adapting guideline of Ghahremanzadeh et al. (2014). All data were analyzed using one-way ANOVA followed by the Tukey test (SPSS software version 17).

RESULTS

Observation of chloride cells

Overall distribution patterns and the density of chloride and mucus-secreting cells are shown in Figures 1. All fish living in freshwater (0 ppt salinity) showed normal gill morphology without any sign of abnormality (Figure 2). A few chloride cells were present mainly at the base of the interlamellar space of primary lamella (Figure 2). Gills of fish in the hyposaline condition (10 and 20 ppt salinity) (Figures 1, 2B-2C) and mesosaline condition

(30, 40 and 50 ppt salinity) (Figures 1, 2D-2H) showed a marked difference in overall appearance, and the number of chloride cells was dramatically increased as salinity increased (Table 1, Figures 1-2).

Quantitative data on the density of the chloride cells of 50 ppt of salinity water had developed to a significantly larger and more numerous than those of 0-50 ppt of salinity water ($p < 0.05$) (Table 1).

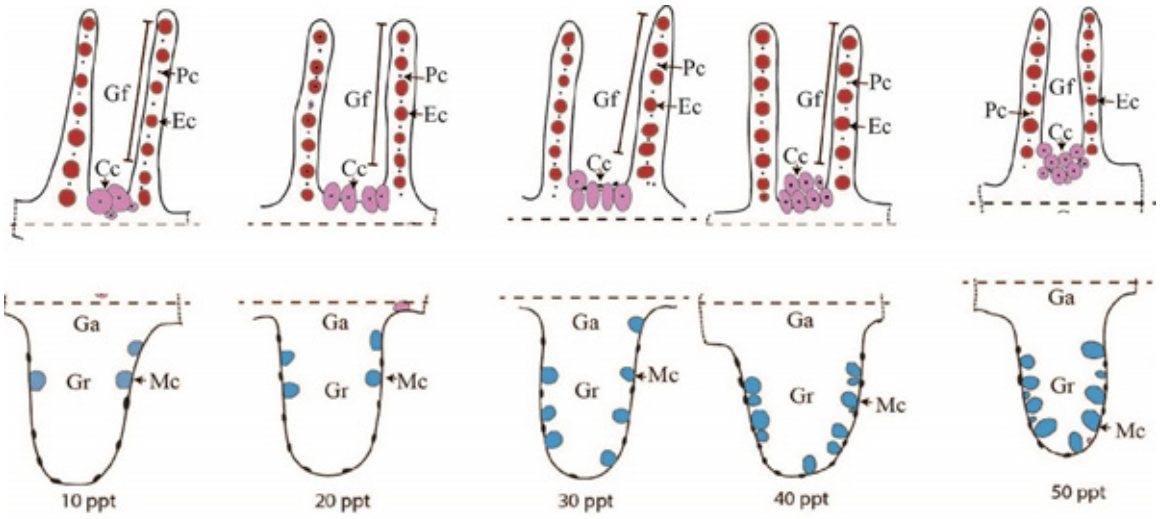

Figure 1 Schematic diagram of *Poecilia mexicana* gill showing the distribution of chloride cell (Cc) and mucus secreting cells (Mc) at different salinity levels (10 to 50 ppt). Ec = erythrocytes, Ga = gill arches, Gf = gill filaments, Gr = gill rakers, Pc = pillar cells.

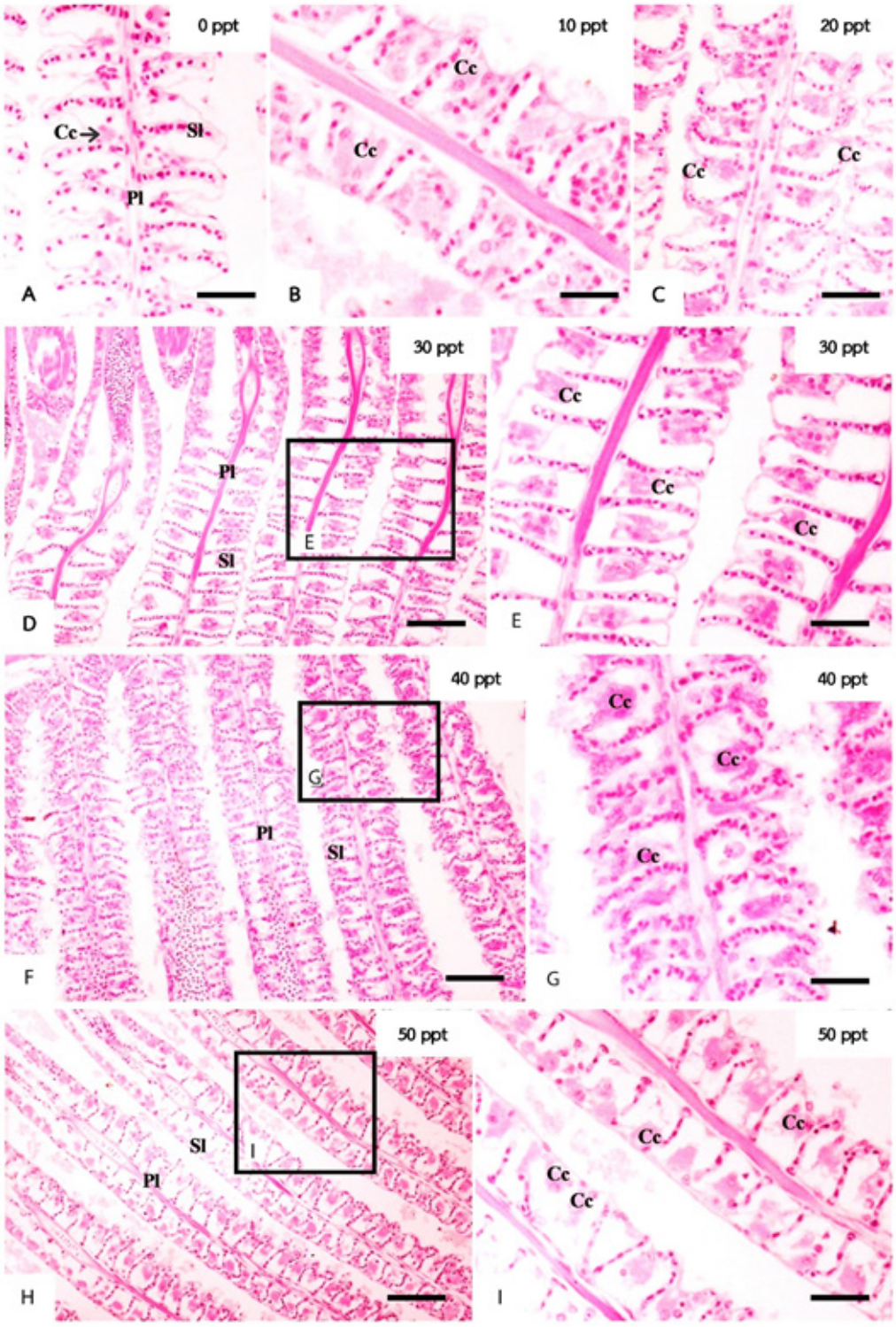

Figure 2 Light micrographs of *Poecilia mexicana* gill filaments showing chloride cells (Cc). The Cc were located on gill filaments and the base of primary lamellae (Pl) at different density depending on salinity levels. Scale bars; A, B, C, D, E, G, I = 50 µm; D, F, H = 100 µm, SI = secondary lamellae.

Table 1 The number of chloride cell and mucus-secreting cells in the gill of the female shortfin molly, *Poecilia mexicana* at different treatments.

Salinity levels (ppt)	Conditions	Number of Chloride cell per field (Mean ± SE)	Number of mucus-secreting cell per field (Mean ± SE)
0	**Freshwater**	13.37 ± 0.18^{a}	4.93 ± 0.15
10	**Hyposaline**	$30.17 \pm 0.41^{a,b}$	4.95 ± 0.20
20		$36.28 \pm 0.53^{a,b,c}$	6.97 ± 0.18^{a}
30		$41.24 \pm 0.26^{a,b,c,d}$	$8.40 \pm 0.20^{a,b}$
40	**Mesosaline**	$52.24 \pm 0.33^{a,b,c,d,e}$	$9.28 \pm 0.16^{a,b,c}$
50		$54.82 \pm 0.15^{a,b,c,d,e,f}$	$10.42 \pm 0.19^{a,b,c,d}$

Note: ^{a,b,c,d,e,f} show a statistically significant difference

Observation of mucus-secreting cells

The routine H&E staining identified a basal nucleus of an irregular shape and a clear vacuolar cytoplasm of mucus-secreting cells in gill sections (Figure 3A). The mucus-secreting cells were commonly found along the epithelium of gill raker borders. A few of this cell were also found in the gill arch epithelium. The density of mucus-secreting cells was dramatically increased under high salinity (Figures 1, 3A-3F), in which most differences were statistically significant (Table 1; $P < 0.05$).

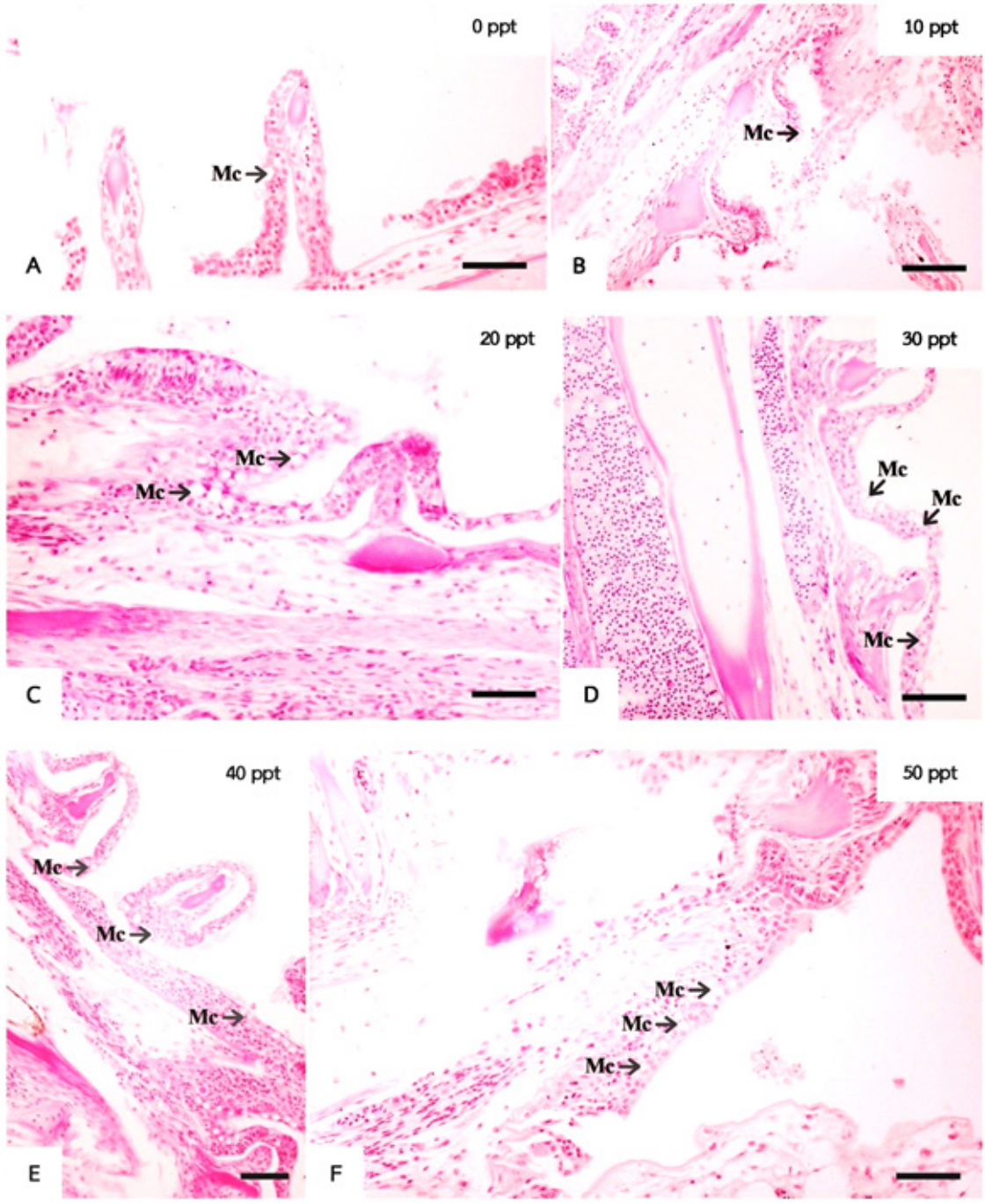

Figure 3 Light micrographs of *Poecilia mexicana* gill raker showing the mucus-secreting cells (Mc) at different salinity levels. Scale bars: A-F = 100 µm.

DISCUSSION

Our observation showed that the density of chloride cells changes in response to salinity changes in *P. mexicana*. The different number of chloride cells were observed in experimental fish, suggesting that the experimental period of 7 days is sufficient to induce the change in chloride cell density. This should contribute to fish adaptation to different salinity since chloride cells regulate the transport of major ions (i.e., Na^+/Cl^- and Ca^+) to maintain a biologically suitable hydro-mineral balance (Flik et al., 1984; Laurent and Dunel, 1980). Consistent with this concept, several documents showed apparent increase in the density of gill chloride cells in economically important fishes such as the killifish *Fundulus heteroclitus* (Lima and Kültz, 2004), the Japanese eel *Anguilla japonica* (Wong and Chan, 2001) and the sturgeon Acipenser naccarii (Martínez-Álvares et al., 2005). Together, we conclude that *P. mexicana* is able to adapt to a wide range of salinities from 0 to 50 ppt and hence could be designated as a "euryhaline fish", and the morphological adaptations of gill chloride cells likely contributes to the ability. The importance of chloride cells in euryhaline fish has been documented by Uchida and Kaneko (1996) and Uchida et al. (2000).

The mucus production in gills is known to be stimulated under certain environmental conditions (Laurent, 1984; Handy and Eddy, 1991) as well as in response to acidic stresses (Berntssen et al., 1997) and salinity (Roberts and Powell, 2003). Our observation showed that the density of mucus-secreting cells of *P. mexicana* increases in response to the salinity change from freshwater to mesosaline conditions. It is possible that these cells support the ionoregulation of chloride cells in *P. mexicana*. Shephard (1989) suggested that mucus secreting cells trap neighboring cations, creating an ionic gradient. Franklin (1990) also showed that the increased number of mucus cells in the sockeye salmon, *Oncorhynchus nerka* after transfer to seawater. In addition to the potential function of supporting chloride cells, mucus-secreting cells had been suggested to play a role in the increase of the blood-to-water diffusion barrier for respiratory gas exchange (Fernandes et al., 1998; Fernandes and Perna-Martins, 2002), and consequently reduces oxygen uptake (Sakuragui et al., 2003) and carbon dioxide excretion (Powell and Perry, 1997). In this study, experimental fish had a significantly increased number of mucus-secreting cells following to level of salinity. This should be further confirmed by the ultrastructural approach.

A limitation of this study is that we identified chloride cells only by the traditional H&E staining. Although several previous reports have used the same method focusing of the histological features of chloride cells such as the ovoid shape, acidic cytoplasm and rounded nuclei (Moghadam et al., 2013; Smith et al., 2018), these results should be confirmed by immunohistochemistry or electron microscopy in future.

CONCLUSION

The results of our study showed for the first time that the increased salinity levels provoke morphological alteration in the gill chloride cells and mucus-secreting cells in *P. mexicana*. It is likely that these cells have the osmoregulatory function. More importantly, we claim that *P. mexicana* is a euryhaline fish, which can tolerate a wide range of salinity from freshwater to marine waters.

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AUTHORS CONTRIBUTION

Conceptualization, S.S., S.Se. and J.K; Methodology, S.S., S.Se., J.K. Formal Analysis, S.Se. and S.S; Investigation, S.Se.; Resources, S.S.; Writing – Original Draft Preparation, S.Se.; Writing – Review & Editing, S.S., G.K., W.J. and K.W; Supervision, S.Se.; Project Administration, J.K.; Funding Acquisition, S.S. and J.K.

CONFLICT OF INTEREST

The authors declare that no conflict of interest.

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