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2 **Differential expression of two ATPases revealed by lipid raft isolation**

3 **from gills of euryhaline teleosts with different salinity preferences**

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28

29

## Abstract

30

31 In euryhaline teleosts, Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA) and V-type H<sup>+</sup>-ATPase A (VHA  
32 A) are important ion-transporters located in cell membrane. Lipid rafts (LR) are  
33 plasma membrane microdomains enriched in cholesterol, sphingolipids, and proteins  
34 (e.g., flotillin). Flotillin is a LR-associated protein, commonly used as the LR marker.  
35 Previous mammalian studies showed that LR may play a crucial role in ion exchanges.  
36 Meanwhile, studies on mammals and rainbow trout showed that NKA were found to  
37 be present mainly in LR. However, little is known about LR in fish. Therefore, the  
38 present study aimed to investigate the involvement of branchial LR in osmoregulation  
39 of tilapia and milkfish, two euryhaline teleosts with different salinity preferences, by  
40 (i) extracting LR from the gills of euryhaline teleosts; (ii) detecting the abundance of  
41 LR marker protein (flotillin-2) and ion-transporters (NKA and VHA A) in branchial  
42 LR and non-LR of fresh water- and seawater-acclimated milkfish and tilapia. The  
43 results indicated that the protein abundance of LR marker, flotillin-2, changed with  
44 environmental salinities in branchial LR of tilapia. In addition, flotillin-2 and NKA  
45 were only found in LR in both tilapia and milkfish gills, while VHA A were mainly  
46 present in non-LR. Relative protein abundance of NKA was found to be significantly  
47 higher in gills of freshwater milkfish and seawater tilapia, while VHA A was  
48 significantly higher in gills of freshwater tilapia and milkfish. This study illustrated  
49 differential distribution and salinity-dependent expression of NKA and VHA A in cell  
50 membrane of gill tissues of euryhaline teleosts with different salinity preferences.

51

## 52 **Introduction**

53 Lipid raft (LR) are membrane microdomains rich in cholesterol and  
54 glycosphingolipids involved in the organization and aggregation of lipid bilayer  
55 constituents including transmembrane proteins (Simons and Ikonen, 1997). LR have  
56 been reported to be involved in virus entry (Nguyen and Hildreth, 2000; Ono and  
57 Freed, 2001), protein trafficking (Brown and London, 1998), ion transport processes  
58 (Tillman and Cascio, 2003) and cell signaling (Varma and Mayor, 1998). Because of  
59 their high lipid content, i.e., high lipid-to-protein ratio (Brown and Rose, 1992) and  
60 relatively low density, the LR fraction can be extracted following non-ionic detergent  
61 treatment using density gradients. LR are highly dynamic, usually nanoscale  
62 structures that can form bigger raft by means of fusion of small entities (Lingwood  
63 and Simons, 2010). Levental et al. (2010) showed that S-acylation, referred to as  
64 'palmitoylation' plays an important role in regulating raft affinity. The binding of  
65 palmitate with cysteine residues of proteins promotes their insertion into the LR  
66 fraction and suggests that palmitoylation plays a critical role in membrane targeting  
67 mechanisms involving transmembrane proteins (Resh, 2006). According to Levental  
68 et al. (2010) and Contreras et al. (2011), among plasma membrane proteins, about  
69 65% were in the non-raft phase, whereas 12% required palmitoylation for raft phase  
70 inclusion, 11% were glycosylphosphatidylinositol (GPI)-anchored in the raft, and the  
71 other 11% could be bound to LR lipids such as cholesterol or sphingolipids (SLs).

72 Lipid compositions of the LR and non-LR fractions are considered to be different  
73 as shown in common carp (*Cyprinus carpio*), i.e., the LR fraction contained a higher  
74 percentage of cholesterol and non-polar lipids (Brogden et al., 2014). Non-ionic  
75 detergents are thus used to extract LR (also called detergent-resistant membranes  
76 (DRM)) from other cell membranes (Eckert et al., 2003; Dalskov et al., 2005;  
77 Lingwood et al., 2005). Commonly used detergents for LR extraction in mammalian

78 studies include CHAPS, Tween 20, Triton X-100, Lubrol WX, Brij96, and Brij98, and  
79 different detergents that may cause different result of lipid raft isolation due to the  
80 dissolvability (Drevot et al., 2002; Madore et al., 1999; Röper et al., 2000; Schuck et  
81 al., 2003). Among them, Triton X-100 is the most commonly used detergent for LR  
82 extraction (Foster and Chan, 2007; Pike, 2006; Schroeder et al., 1998). Triton X-100  
83 has also been applied in LR research with various protocols in skate (*Raja erinacea*;  
84 Musch et al., 2004), rainbow trout (Lingwood et al., 2005), Atlantic cod (*Gadus*  
85 *morhua*; Gylfason et al., 2010), goldfish (*Carassius auratus*; Garcia-Garcia et al.,  
86 2012), and common carp (Brogden et al., 2014). Previous studies on fish have not  
87 compared different detergents and have not quantified flotillin-2 following LR  
88 isolation. Brogden et al. (2014) found that lipid composition of plasma membrane in  
89 common carp was different from that in human, and the lipid components were  
90 organ-dependent, whether in LR or non-LR regions. In their results, even 0.1% of  
91 Triton X-100 cannot perfectly isolate flotillin-2 in LR from non-LR region in all  
92 tissue. As a result, it is important to test different concentration of detergents.

93 Flotillins are LR-associated proteins commonly used as markers for LR. Plasma  
94 membrane targeting and clustering of flotillins on LR mainly relies on acylation  
95 (myristoylation or palmitoylation) (Banning et al., 2011). Furthermore, they have also  
96 been shown to be involved in some basic functions such as trafficking and transport of  
97 membrane materials and proteins (Stuermer, 2010). Flotillins are divided into two  
98 isoforms. Flotillin-1 (previously named reggie-2) seems to associate with raft by  
99 means of the first hydrophobic domain (Liu et al., 2005) and has also been shown to  
100 be palmitoylated in Cys34, which is essential for flotillin-1 to locate on the  
101 cytoplasmic side of the plasma membrane (Morrow et al., 2002). Flotillin-2  
102 (previously named reggie-1) interacts with the plasma membrane through several sites  
103 of palmitoylations and myristoylations, and plays a significant role in the maintenance

104 of membrane raft (Neumann-Giesen et al., 2004, Langhorst et al., 2006). Evidence of  
105 flotillin function in fish is scarce. In zebrafish (*Danio rerio*), von Philipsborn et al.  
106 (2005) suggested that flotillin might be involved in development.

107 The LR plays important roles in ionoregulation and osmoregulation, as shown by  
108 proteins responsible for ion transport being localized to LR, including active transport  
109 pumps/enzymes (i.e., ATPase) (Tillman and Cascio, 2003; Murtazina et al., 2006). For  
110 example, the Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA) in fish branchial and renal cells (Lingwood et  
111 al., 2005; Welker et al. 2007) and vacuolar-type H<sup>+</sup>-ATPase (VHA) in mammalian  
112 cells (Lafourcade et al., 2008).

113 Membrane structure and transmembrane enzyme function are linked because the  
114 lipid environment of the enzyme can constrain protein motions required for catalysis,  
115 affecting the enzyme catalytic rate (Harris, 1985; Cossins et al., 1986). Among the  
116 enzymes/transporters, the NKA catalyzes the transport of Na<sup>+</sup> and Cl<sup>-</sup> across epithelia  
117 in both absorptive (fresh water; FW) and secretory (seawater; SW) modes in gills of  
118 euryhaline teleosts (Marshall, 2002; Perry et al., 2003; Hirose et al., 2003). Changes  
119 in branchial NKA activity in euryhaline fish are necessary for acclimation to  
120 environmental salinity (Marshall and Bryson, 1998; Kelly et al., 1999; Marshall, 2002;  
121 Mancera et al., 2002; Hirose et al., 2003; Lin et al., 2003; Scott et al., 2004).

122 Furthermore, NKA consists of  $\alpha$ - and  $\beta$ -subunits (Scheiner-Bobis, 2002). The  
123  $\alpha$ -subunit has a molecular weight of about 100 kDa and is considered the catalytic  
124 center of the NKA, with binding sites for cations, ATP, and ouabain (NKA inhibitor).  
125 The  $\beta$ -subunit, with a molecular weight of 40-60 kDa, can stabilize the structure and  
126 regulate the cations affinity of the  $\alpha$ -subunit on the plasma membrane (Skou and  
127 Esmann, 1992; Abriel et al., 1999). Moreover, reciprocal interactions between NKA  
128 and cholesterol or phospholipids have been proposed (Chen et al., 2011; Cornelius,  
129 2008; Cornelius et al., 2015; Haviv et al., 2013; Kravtsova et al., 2015) and NKA

130 distribution on LR has been reported in previous studies on mammalian tissues  
131 (Welker et al., 2007). Using the Brij 98 (as a non-ionic detergent) to extract the  
132 granulocytes in the brain of rat also revealed the presence of NKA  $\alpha$ -subunits in LR  
133 (Dalskov et al., 2005). However, there are few studies focused on fish NKA in LR. In  
134 gills of rainbow trout (*Oncorhynchus mykiss*), NKA are expressed in LR when  
135 transferred to SW but not detected in LRs in FW individuals, indicating different  
136 strategies in ionoregulation between FW- and SW-acclimated rainbow trout  
137 (Lingwood et al., 2005). Most reports on NKA  $\alpha$ -subunit, however, focused on their  
138 localization on the basolateral plasma membrane and their expression when fish  
139 encountered different environmental salinities (Lee et al., 2003; Lin et al., 2003). The  
140 NKA  $\alpha$ -subunit protein abundance in gills of FW-preference Mozambique tilapia  
141 (*Oreochromis mossambicus*) was significantly higher in SW than in FW (Lee et al.  
142 2003), while in gills of SW-preference milkfish (*chanos chanos*) it was significantly  
143 higher in FW than in SW (Lin et al., 2003).

144 The VHA is a multi-subunit complex organized into two domains: the 650 kDa  
145 cytosolic  $V_1$  domain and the 260 kDa membrane-embedded  $V_0$  domain (Nishi and  
146 Forgac, 2002; Forgac, 2007; Toei et al., 2010). In previous mammalian studies,  
147 deprivation of cholesterol from LR resulted in the decrease of electrogenic  $H^+$  efflux  
148 by VHA and in synaptic signaling deficiency (Yoshinaka et al., 2004). On frog and  
149 insect epithelial cells, VHA contributed to acid-base regulation and osmoregulation  
150 (Harvey et al., 1998). In the studies on rainbow trout gills, VHA activity and  
151 immunoreactivity decreased when FW trout were acclimated to SW (Lin and Randall,  
152 1993; Lin et al., 1994). In the Atlantic stingray (*Dasyatis sabina*) and killifish  
153 (*Fundulus heteroclitus*), VHA expressed on the cell membrane was significantly  
154 higher in FW than in SW (Piermarini and Evans, 2001; Katoh et al., 2003). As a result,  
155 this consistently higher expression of VHA in FW environments is linked to its role in



156 generating an electrical gradient favoring Na<sup>+</sup> uptake in FW-type ionocytes,  
157 additional to its role in acid secretion. In tilapia, bafilomycin, an inhibitor of VHA,  
158 has been shown to affect *in vivo* Na<sup>+</sup> influx (Fenwick et al., 1999), indicating a key  
159 role of this pump in freshwater osmoregulation in tilapia and suggesting an apical  
160 location in ionocytes.

161 To date, most studies on NKA and VHA in fish focus on their relationship with  
162 cell membrane, while the relationship between NKA and LR in fish was only reported  
163 in the rainbow trout (Lingwood et al., 2005). On the other hands, there is no reference  
164 reporting the associations of VHA and LR in fish. According to previous references,  
165 this study hypothesized that NKA and VHA, the major ion pumps in fish gills, may be  
166 mainly distributed in the LR to be involved in ionoregulation of euryhaline teleosts  
167 when acclimated to environments of different salinities. As a result, the present study  
168 aims to investigate the presence of LR and their exhibition of NKA and VHA in gills  
169 of tilapia (the FW euryhaline teleost) and milkfish (the marine euryhaline teleost), by  
170 (i) extracting membrane LR from gills of tilapia and milkfish, with fresh water (FW)  
171 and seawater (SW) preferences, respectively, and (ii) assessing differential NKA and  
172 VHA expression in branchial LR between FW- and SW-acclimated milkfish and  
173 tilapia. This study provides the evidence for expression of critical ion transporters in  
174 LR of euryhaline teleosts with different salinity preferences. Differential expression of  
175 flotillin-2 (the LR marker) and two ATPases, the ATP-consuming NKA and VHA, in  
176 gills of euryhaline fish will further clarify the ionoregulation roles of branchial LR in  
177 environments of different salinities.

178

179

180

## Materials and methods

### 181 Experimental fish and design

182 Mozambique tilapia (*Oreochromis mossambicus*), and juvenile milkfish (*Chanos*  
183 *chanos*) were obtained from laboratory stocks and a local fish farm, respectively, with  
184 average standard lengths of  $5.5 \pm 0.5$ , and  $10.6 \pm 0.4$  cm, respectively. Seawater (SW;  
185 35 ‰) and brackish water (BW; 15 ‰) used in this study were prepared from aerated  
186 tap water (FW) with artificial sea salt (Synthetic Sea Salt; Blue Treasure, Qingdao,  
187 China). The water was continuously circulated through fabric-floss filters and quarter  
188 of water was changed every two weeks. The ammonia contents were routinely  
189 examined using the commercial API<sup>®</sup> ammonia test kit. Quarter of water was changed  
190 when the ammonia level was higher than 0 ppm. Each species of fish was reared in  
191 four separated tanks at  $28 \pm 1^\circ\text{C}$  in BW for two weeks before transfer to either FW or  
192 SW, with a daily 12 h photoperiod and a daily diet of commercial pellets. The holding  
193 densities of tilapia and milkfish in each tank were 0.4 fish/L and 0.2 fish/L,  
194 respectively.

195 For establishing the protocol of lipid raft (LR) extraction, tilapia and milkfish  
196 were reared in FW and SW, respectively, for at least four weeks before sampling the  
197 gills. Differential expression of NKA and VHA in branchial LR was further assessed  
198 between FW and SW in each species after one-month acclimation. For subsequent  
199 analyses, six individuals of either FW or SW groups were used for experiments and  
200 four gill arches from the right opercular side of each fish were sampled. Before  
201 sampling, experimental fish were not fed for one day and anesthetized in 500  $\mu\text{L/L}$   
202 2-phenoxyethanol. The protocol used for the experimental fish was reviewed and  
203 approved by the Institutional Animal Care and Use Committee of the National Chung  
204 Hsing University (IACUC approval no. 102-09R and 105-130 to T.H. Lee).

205

## 206 **Preparation of crude membrane fractions, lipid raft and non-lipid raft fractions**

207 Gill tissues from the experimental fish were steeped in a mixture of  
208 homogenization buffer (SEI buffer, 150 mM sucrose, 10 mM EDTA, 50 mM  
209 imidazole, pH 7.4) with commercial protease inhibitor cocktail (Roche, Mannheim,  
210 Germany; 1:50, v/v). Homogenization was performed with a Polytron PT1200E  
211 (Kinematica, Lucerne, Switzerland). After the first centrifugation (1,500 g at 4°C for  
212 10 min), the pellets were discarded and the supernatants were centrifuged again  
213 (13,000 g at 4°C for 12 min). After the second centrifugation, the supernatants were  
214 retained and centrifuged a third time (20,800 g at 4°C for 60 min). Then after the third  
215 centrifugation, the supernatants as the cytosol was discarded. Meanwhile, the pelleted  
216 fraction containing large fragments of the plasma membrane was retained and  
217 considered as the crude membrane fraction (Tang and Lee, 2010). In this study, the  
218 crude membrane fraction was further dissolved to obtain the lipid raft using SEI  
219 buffer with 0.1% or 0.05% Triton X-100 or 0.05% Tween 20 (abbreviated as SEIT) at  
220 4°C (Brogden et al., 2014). After dissolution in detergents at different time points (30,  
221 60 and/or 120 min) to determine the best duration of digestion, the aliquots were  
222 centrifuged (20,800 g at 4°C for 30 min). The supernatants of the fourth centrifugation  
223 was considered as the detergent-soluble membrane (non-LR). The pellet as the  
224 detergent-insoluble membrane (LR) was then dissolved by the protease inhibitor  
225 cocktail (Roche) contained SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM  
226 imidazole, 0.5% sodium deoxycholate, pH 7.4) because LR can be dissolved in  
227 deoxycholate (McGuinn and Mahoney, 2014). Protein concentrations were measured  
228 with reagents from the Pierce™ BCA protein assay kit (Thermo Fisher, Rockford, IL,  
229 USA) using bovine serum albumin (Thermo Fisher) as standard.

230

## 231 **Antibodies**

232 The primary antibodies used in this study included (i) a mouse monoclonal  
233 antibody ( $\alpha 5$ , DHSB, Iowa City, IA, USA) raised against the avian NKA  $\alpha$ -subunit  
234 (Lin et al., 2003; Yang et al., 2015), (ii) a mouse monoclonal antibody (flotillin-2,  
235 Santa Cruz, CA, USA) raised against the amino acids 150-240 of human flotillin-2  
236 (XP\_016879883.1) with approximately 83% similarity with milkfish flotillin-2  
237 (XP\_030648347.1) and 80% similarity with tilapia flotillin-2 (XP\_003456181.1), and  
238 (iii) a rabbit polyclonal antibody (MDBio, Taipei, Taiwan) raised against the specific  
239 epitope (AEMPADSGYPAYLGARLA) of the pufferfish (*Tetraodon nigroviridis*)  
240 VHA A (ABX80240.1) with 100 % similarity with milkfish VHA A isoform1 and  
241 isoform 2, as well as tilapia VHA A (BAF94024.1). The sequences of two isoforms of  
242 VHA A found in the milkfish transcriptome database (Hu et al., 2015) were showed in  
243 Fig. S1. The specificities of the VHA A antibody to gills of tilapia and milkfish were  
244 tested and showed in Fig. S2. According to the primary antibodies, the secondary  
245 antibodies for immunoblots included the horseradish peroxidase-conjugated (i) rabbit  
246 anti-mouse IgG, (ii) goat anti-rabbit IgG, and (iii) rabbit anti-goat IgG (GeneTex,  
247 Irvine, CA, USA). The similarity between flotillin-2 with milkfish.

248

### 249 **Immunoblotting**

250 The sample mixture of 6x sample loading dye (0.06% bromophenol blue, 30%  
251 glycerol, 12% SDS, 0.6 M dithiothreitol, and 62.5 mM Tris with pH 6.8) and gill  
252 proteins (1:5, v/v) was heated at 65°C for 15 min to denature the proteins. Non-LR  
253 and LR samples were separated by electrophoresis on a 7% SDS-polyacrylamide gel  
254 (5  $\mu$ g of protein per lane) using a Mini-protein II electrophoresis cell (Bio-Rad,  
255 Hercules, CA, USA). Then, the separated proteins were transferred to polyvinylidene  
256 difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After incubation in  
257 the non-specific blocking solution using phosphate buffer saline with Tween 20

258 (PBST; 2.68 mM potassium chloride, 10.1 mM disodium phosphate, 137 mM sodium  
259 chloride, 1.76 mM potassium dihydrogen phosphate, and 0.2 % (v/v) Tween; pH 7.4)  
260 containing 5% (w/v) nonfat dried milk for 2 h at room temperature (RT), the blots  
261 were incubated overnight with the primary antibody (1:5000) at 4°C, followed by  
262 washing the membranes with PBST. Then the membranes were incubated with the  
263 secondary antibody (1:25000) for 1 h at RT. Images were developed with T-Pro  
264 LumiLong Chemiluminescent Substrate Kit (Ji-Feng Biotechnology, New Taipei,  
265 Taiwan) under a cooling-CCD (charge-couple device) camera (ChemiDoc XRS+,  
266 Bio-Rad) with associated software (Quantity One version 4.6.8, Bio-Rad). The blot  
267 bands were converted to numerical values by ImageLab 3.0 software (Bio-Rad) to  
268 quantify and compare relative protein abundance of the immunoreactive bands.

269

## 270 **Statistics**

271 In this study, comparisons in flotillin-2, NKA- $\alpha$  or VHA A abundance between (i) LR  
272 and non-LR fractions of gills in either SW or FW fish, or (ii) SW or FW groups in  
273 either LR or non-LR fractions, respectively, were analyzed by the Student's *t-test*,  
274 with  $p < 0.05$  was set as the significant level. Values are expressed as means  $\pm$  S.E.M.  
275 (standard error of the mean).

276

277

## 278 **Results**

### 279 **Separation and purification of LR from tilapia and milkfish gills**

280 In tilapia (Fig. 1), the crude membrane fraction was first reacted in the non-ionic  
281 detergent Triton X-100. After 30, 60, and 120 minutes of reaction with this detergent  
282 at 0.1% as well as 60 minutes of reaction at 0.05%, strong immunoreactive bands  
283 were found in LR for NKA  $\alpha$ -subunit and flotillin-2. However, faint immunoreactive  
284 bands of both proteins were found in non-LR, indicating that dissolvability of 0.1 and  
285 0.05% Triton X-100 may not be optimal for tilapia gill tissues (Fig. 1). Meanwhile,  
286 the supernatant separated by reacting with 0.05% Tween-20 for 60 minutes did not  
287 show immunoreactive bands of flotillin-2 in non-LR, indicating the better dissolution  
288 of this protocol for extracting LR from tilapia gills (Fig. 1). Moreover, no band for  
289 NKA  $\alpha$ -subunit was observed in the non-LR fraction.

290 In milkfish (Fig. 2), similar results were observed at 30-minutes treatments with  
291 the non-ionic detergent 0.1% Triton X-100 as tilapia reacted with Triton X-100 (Fig.  
292 1). However, after 60 and 120 minutes of reaction with 0.1% Triton X-100, there was  
293 no flotillin-2 and NKA  $\alpha$ -subunit immunoreactive band found in the non-LR  
294 membrane fraction, indicating that the LR of milkfish gills could be separated after  
295 0.1% Triton X-100 dissolution following 60 minutes of incubation. The NKA  
296  $\alpha$ -subunit abundance was high in the LR fraction (Fig. 2). Hence, extraction of LR by  
297 0.1% Triton X-100 for 60 minutes is an appropriate procedure for LR purification  
298 from milkfish gills.

299

### 300 **Comparisons of flotillin-2 and NKA in branchial LR and non-LR fractions** 301 **between FW and SW tilapia and milkfish**

302 As illustrated in Fig. 1, tilapia gills treated with 0.05% Tween-20 for 60 minutes  
303 revealed better dissolvability in LR membrane fractions. This condition has thus been

304 used to compare salinity effects on LR and non-LR gill fractions of tilapia. Flotillin-2  
305 and NKA  $\alpha$ -subunit protein levels were only detected in the LR of both FW and SW  
306 tilapia. The abundance of flotillin-2 in LR was significantly (2 times) higher in the  
307 FW group than in the SW group (Fig. 3A). On the contrary, the abundance of NKA  
308  $\alpha$ -subunit in LR of SW tilapia was 1.4 times higher than that of FW fish (Fig. 3B).

309 Similarly, flotillin-2 and NKA  $\alpha$ -subunit protein levels were only detected in the  
310 LR of both FW and SW milkfish using 0.1% Triton X-100 as detergent for 60 minutes  
311 (Fig. 4). In milkfish LR, no significant difference was found in the abundance of  
312 flotillin-2 between FW and SW groups (Fig. 4A). Significantly higher (4.4 times)  
313 NKA  $\alpha$ -subunit abundance was detected in LR of FW milkfish compared to the SW  
314 LR group (Fig. 4B).

315

#### 316 **Comparisons of VHA A abundance in branchial LR and non-LR fractions** 317 **between FW and SW tilapia and milkfish**

318 In tilapia, a specific immunoreactive band of 69 kDa was detected in branchial  
319 LR and non-LR fractions (Fig. 5A). Most VHA A was located in the non-LR (mem)  
320 compared to LR. In addition, significant higher VHA A abundance was detected in  
321 both non-LR (mem) and LR fraction of the FW group compared to those of the SW  
322 group. (Fig. 5B).

323 Two immunoreactive bands corresponding to 69 and 60 kDa were detected as the  
324 isoform 1 and 2, respectively, of the VHA A subunit of milkfish gills (Fig. 6A).

325 Similar to tilapia VHA A, relative abundance of milkfish VHA A isoform 1 and 2 was  
326 significantly higher in the non-LR fraction rather than the LR fraction (Fig. 6B, 6C).

327 Moreover, significantly higher amounts of both isoform 1 (about 2 times; Fig. 6B)

328 and isoform 2 (about 1.5 times; Fig. 6C) of milkfish VHA A subunit were found in

329 the non-LR fraction of the FW group compared to the SW group. In the LR fraction

330 of gills, however, no significant difference was found between FW and SW milkfish  
331 in either isoform 1 (Fig. 6B) or isoform 2 (Fig. 6C) of VHA A subunit.  
332



### 333 **Discussion**

334 This study revealed that Triton X-100 had better dissolvability for extracting LR  
335 from gills of milkfish rather than tilapia due to the weakly immunoreactive bands of  
336 flotillin and NKA found in the non-LR fractions of tilapia gills. LR and non-LR  
337 membrane regions of tilapia were isolated more efficiently using Tween 20 as  
338 detergent. LR extraction efficacy from Madin-Darby canine kidney (MDCK) cells has  
339 been previously reported for various surfactants. It was found that the membrane  
340 dissolving effects of Triton X-100 and CHAPS used to extract the LR-protein were 10  
341 times higher than using Tween 20, Brij58, and Lubrol WX. Moreover, the  
342 Madin-Darby canine kidney (MDCK) cell and MDCK cells expressing human PLAP  
343 (MDCK-PLAP) cell revealed better results in LR extraction by using Triton X-100 as  
344 a detergent compared to Tween 20, indicating that Triton X-100 had a higher ability to  
345 dissolve lipid structures than Tween 20 (Schuck et al., 2003). The detergent efficacy  
346 results in this study also showed that the strength of the detergents required for LR  
347 extraction in various fish species differed and thus had to be adjusted for each species  
348 in order to have optimal LR isolations. It has to be noted that when different  
349 detergents are used, LR composition might be slightly different (Schuck et al., 2003).  
350 Differences in SLs and cholesterol-rich fatty acids abundance found among various  
351 species may lead to different efficacy of detergents (Schuck et al., 2003; Lingwood et  
352 al., 2005; Brogden et al., 2014). As a result, different efficacy of detergents between  
353 tilapia and milkfish found in this study might be attributed to more abundant SLs and  
354 cholesterol-rich fatty acids in LR of milkfish than tilapia, requiring a more efficient  
355 detergent to extract LR from other portions of the membranes.

356 LR are known to act as signaling and sorting platforms for numerous molecules  
357 (Simons and Toomre, 2000; Smart et al., 1999), and proteins forming the scaffolding  
358 of LR (Babuke et al., 2009). Flotillins have generally been used as marker proteins for

359 LR (Bickel et al., 1997; Neumann-Giesen et al., 2004, 2007). In addition, specific  
360 GPI-anchored proteins have been observed to co-cluster with flotillins. So, flotillins  
361 have been proposed to represent centers for GPI-anchored proteins' communication  
362 with intracellular signal transduction molecules (Stuermer and Plattner, 2004). They  
363 have also been reported to play the roles in trafficking and transport of membrane  
364 proteins and materials (Stuermer, 2010). Our results in different species provided  
365 evidence that LR are differentially expressed in different euryhaline fish regarding  
366 salinity acclimation by assessing the expression patterns of the LR marker, flotillin-2.  
367 LR abundance (i.e., flotillin-2 abundance) in gills was significantly higher in FW than  
368 in SW tilapia while there was slightly but not significantly higher expression of  
369 flotillin-2 in gills of FW compared to SW milkfish. Differential LR abundance might  
370 affect transporter function, as the membrane lipid environment is essential for the  
371 functioning of transporters and channels. Until now, evidence on the link between LR  
372 and salinity acclimation was only reported in few species. In skate, the anion  
373 exchanger 1, for example, was present in intracellular vesicles in detergent-insoluble  
374 lipid raft in isotonic conditions and shifted to detergent-soluble plasma membrane  
375 regions through exocytosis following hypotonicity treatment (Musch et al., 2004).

376 NKA  $\alpha$ -subunit protein abundance (membrane fractions protein) in gills of  
377 FW-preference tilapia was significantly higher in SW than in FW (Lee et al. 2003; Lin  
378 et al., 2004), while in gills of SW-preference milkfish it was significantly higher in  
379 FW than in SW (Lin et al., 2003). This study further revealed that average LR  
380 abundance in tilapia gills was significantly higher (two folds) in the FW group than  
381 the SW group, while in milkfish gills LR abundance was similar between FW and SW  
382 individuals. In addition, the presence of NKA proteins in LR is positively correlated  
383 to NKA activities because they were also reported to be higher in gills of the FW  
384 milkfish (Lin et al., 2003) as well as SW tilapia (Uchida et al., 2000). Our results thus

385 echoed previous studies on NKA expression in membrane fractions of fish gills in  
386 tilapia and milkfish (Lee et al. 2003; Lin et al., 2003; Lin et al., 2004).

387 The immunoblots of this study clearly showed high and low abundance of VHA  
388 in the non-LR and LR fraction, respectively, of milkfish and tilapia. Contrary to NKA,  
389 VHA is more abundant in non-lipid raft fractions and salinity modulation affects only  
390 the expression of VHA in those membrane fractions. In FW non-LR fractions, VHA  
391 expression was increased compared to SW non-LR fractions in both species.  
392 Moreover, this study is the first to reveal the distribution of VHA in both LR and  
393 non-LR regions of cell membrane in gills of euryhaline teleosts. The presence of VHA  
394 mostly found in non-LR fractions may be due to differential membrane compositions  
395 between apical and basolateral membranes with remains to be clarified in future  
396 studies.

397

### 398 **Conclusion**

399 The present study set up the method of LR isolation by extracting the LR from  
400 two species of euryhaline teleosts with different salinity preferences. The results  
401 indicated that lipid raft isolation should be optimized in different species using  
402 different detergents or detergent concentration. Accordingly, NKA was found to  
403 distributed exclusively in the LR fraction of both tilapia and milkfish gills. The FW  
404 euryhaline species (tilapia) has more abundant (two folds) LR in its native media and  
405 about 25% lower NKA expression. The marine euryhaline species (milkfish), however,  
406 does not alter LR abundance following salinity changes but increases massive  
407 amounts of NKA in the LR fraction. Meanwhile, VHA was first found to be mostly  
408 distributed in non-LR fraction where salinity-mediated alterations also occurred in  
409 both euryhaline species. Relative VHA abundance in the non-LR fraction was  
410 significantly higher in the FW group than the SW group of both euryhaline teleosts

411 regardless of their salinity preferences. With successful separation of LR and non-LR  
412 fractions from the crude membrane of fish gills, this study deepened our  
413 understanding in distribution of two important ion transporters, NKA and VHA, in  
414 cell membranes of euryhaline fish gills under different salinities.

415

416

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426

#### 427 **Competing interests**

428 No competing interests declared.

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### Figure legends

653 **Fig. 1** Expression of NKA  $\alpha$ -subunit (NKA) and flotillin-2 protein in lipid raft of fresh  
654 water (FW) tilapia gills after digestion with 0.1% Triton X-100 for 30, 60,  
655 and 120 min, as well as 0.05% Triton X-100 and 0.05% Tween-20 for 60 min.  
656 Immunoblots revealed an immunoreactive band in each condition with a  
657 molecular mass of 100 kDa (NKA  $\alpha$ -subunit) and 48 kDa (flotillin-2). "mem",  
658 non-lipid raft; "raft", lipid raft. The tilapia were reared in brackish water (15  
659 ‰) for two weeks and then acclimated to FW for four weeks before  
660 experiments.

661 **Fig. 2** Expression of NKA  $\alpha$ -subunit (NKA) and flotillin-2 protein in lipid raft of  
662 seawater (SW) milkfish gills after digestion with 0.1% Triton X-100 for 30,  
663 60, and 120 min. The immunoblots revealed an immunoreactive band in each  
664 condition with a molecular mass of 100 kDa (NKA  $\alpha$ -subunit) and 48 kDa  
665 (flotillin-2), respectively. "mem", non-lipid raft; "raft", lipid raft. The  
666 milkfish were reared in brackish water (15 ‰) for two weeks and then  
667 acclimated to SW for four weeks before experiments.

668 **Fig. 3** Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA  $\alpha$ -subunit  
669 (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of  
670 seawater (SW) and freshwater (FW) tilapia. The representative immunoblots  
671 showed an immunoreactive band in each environment with a molecular mass  
672 of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA  $\alpha$ -subunit). The asterisk  
673 indicated the significant difference of protein abundance in branchial lipid  
674 raft (raft) fractions between the SW and the FW groups. The pound signs  
675 indicated significant differences of protein abundance between the lipid raft  
676 (raft) and non-lipid raft (mem) fractions of either SW or FW tilapia gills. N/A,

677 not detected. (n = 6, mean ± S.E.M., t-test, P < 0.05). The tilapia were reared  
678 in brackish water (15 ‰) for two weeks and then acclimated to either FW or  
679 SW for four weeks before experiments.

680 **Fig. 4** Relative protein abundance of (A) flotillin-2 (Flotillin) and (B) NKA  $\alpha$ - subunit  
681 (NKA) in lipid raft (raft) and non-lipid raft (mem) fractions in gills of  
682 seawater (SW) and freshwater (FW) milkfish. The representative  
683 immunoblots showed an immunoreactive band in each environment with a  
684 molecular mass of (A) 48 kDa (flotillin-2) and (B) 100 kDa (NKA  $\alpha$ -subunit).  
685 The asterisk indicated the significant difference of NKA abundance between  
686 the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions.  
687 The pound signs indicated significant differences of protein abundance  
688 between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW  
689 or FW milkfish gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, P <  
690 0.05). The milkfish were reared in brackish water (15 ‰) for two weeks and  
691 then acclimated to either FW or SW for four weeks before experiments.

692

693 **Fig. 5** Relative protein abundance of V-type H<sup>+</sup>-ATPase A subunit (VHA A) in lipid  
694 raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and  
695 freshwater (FW) tilapia. (A) The representative immunoblot showed an  
696 immunoreactive band in each group with a molecular mass of 68 kDa. (B)  
697 The asterisks indicated significant differences of VHA A abundance between  
698 the SW and FW groups in lipid raft (raft) or non-lipid raft (mem) fractions.  
699 The pound signs indicated significant differences of VHA A abundance  
700 between the lipid raft (raft) and non-lipid raft (mem) fractions of either SW  
701 or FW tilapia gills. N/A, not detected. (n = 6, mean ± S.E.M., t-test, P < 0.05).  
702 The tilapia were reared in brackish water (15 ‰) for two weeks and then

703 acclimated to either FW or SW for four weeks before experiments.  
704  
705 **Fig. 6** Relative protein abundance of V-type H<sup>+</sup>-ATPase A subunit (VHA A) in lipid  
706 raft (raft) and non-lipid raft (mem) fractions in gills of seawater (SW) and  
707 freshwater (FW) milkfish. The representative immunoblot of V-type  
708 H<sup>+</sup>-ATPase A subunit (VHA A) showed two bands at molecular weights of 69  
709 kDa (isoform 1) and 60 kDa (isoform 2) (A). Relative protein abundance of  
710 VHA A isoform 1 (B) and isoform 2 (C) revealed the significant difference  
711 indicated by the asterisk between the SW and FW groups in non-lipid raft  
712 (mem) fractions of gills. Meanwhile, the pound signs indicated significant  
713 differences in VHA A isoform 1 (B) and isoform 2 (C) between the lipid raft  
714 (raft) and non-lipid raft (mem) fractions of either SW or FW milkfish gills. (n  
715 = 6, mean ± S.E.M., t-test, P < 0.05). The milkfish were reared in brackish  
716 water (15 ‰) for two weeks and then acclimated to either FW or SW for four  
717 weeks before experiments.

718

719

720

721

Fig. 1

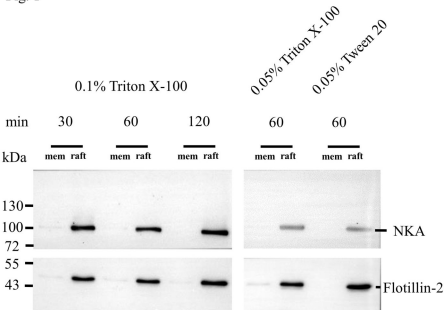


Fig. 2

0.1% Triton X-100

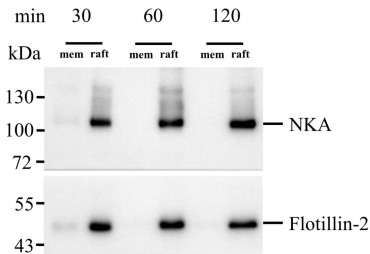


Fig. 3

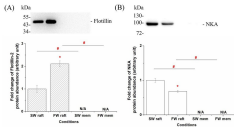




Fig. 4

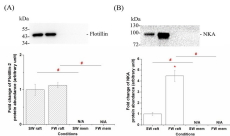


Fig. 5

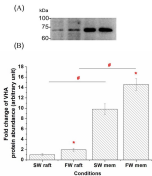
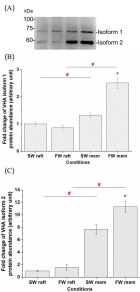


Fig. 6





Tilapia (*Oreochromis mossambicus*)



Milkfish (*Chanos chanos*)

Fresh water

VS

Seawater (35‰)



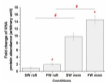
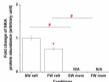
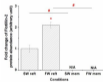
Gills

Establishing lipid raft (LR) isolation method

Flotillin-2  
(LR marker)

Na<sup>+</sup>, K<sup>+</sup>-ATPase  
(NKA)

V-type H<sup>+</sup>-ATPase  
(VHA)



Tilapia



Milkfish