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Gill Na^+/K^+-ATPase in the threespine stickleback (*Gasterosteus aculeatus*): changes in transcript levels and sites of expression during acclimation to seawater

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ABSTRACT

Background: Na^+/K^+-ATPase (NKA) in the gills is a key contributor to osmoregulation in fishes.

Questions: Does gill NKA contribute to the regulation of salinity tolerance in stickleback? Do gill NKA expression patterns during acclimation to seawater differ among stickleback populations that differ in salinity tolerance?

Organisms: Freshwater threespine stickleback (*Gasterosteus aculeatus*) from populations in Ono and Gifu, Japan.

Methods: We gradually acclimated the stickleback to 100% seawater. During acclimation, we monitored changes in plasma Na^+ concentrations using atomic absorption spectrophotometry and gill NKA transcript levels using qPCR. We determined the sites of gill NKA expression by immunohistochemistry.

Results: Threespine stickleback from Ono were able to acclimate to 100% seawater. In contrast, stickleback from the Gifu population were able to survive in 50% seawater but not 100% seawater. Both ecotypes maintained a plasma Na^+ concentration of approximately 150 mM during the acclimation process. Patterns of change in NKA transcript and protein expression were similar between the two populations, although they differed in tolerance to salinity. In both populations, gill NKA-positive ionocytes were distributed on the primary filaments and secondary lamellae in fresh water, but during acclimation to seawater the NKA-positive ionocytes on the secondary lamellae disappeared. The cell sizes and densities of NKA-positive ionocytes were higher in fish from Ono than in fish from Gifu, which may explain the difference in salinity tolerance between these two stickleback populations.

Keywords: *Gasterosteus aculeatus*, gills, Na^+/K^+-ATPase, plasma Na^+, salinity, stickleback.

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INTRODUCTION

Selection against maladaptive migrants is an important mechanism in determining species distributions (Mayr, 1963; Nosil, 2012). In aquatic organisms, environmental salinity has a major influence on survival, since salt concentrations in the environment directly influence body fluid osmotic pressure as well as fitness (Schmidt-Nielsen, 1997).

To maintain homeostasis under high osmotic pressure (seawater), marine teleosts actively excrete excess sodium, chloride, and other ions. Conversely, freshwater teleosts, under low osmotic pressure (fresh water), actively take up these ions. Ion excretion and uptake are controlled by various ion transporters and channels present in gill ionocytes (Hiroi and McCormick, 2012). Among ion transporters and channels, the sodium-potassium pump Na^+/K^+-ATPase (NKA) is known to be the major driving force behind ion excretion/uptake in most animal systems (Marshall and Bryson, 1998; Hirose *et al.*, 2003; Lin *et al.*, 2004; Hwang and Lee, 2007). The NKA is localized in the plasma membrane, and pumps three sodium ions out of cells in exchange for two potassium ions (Kaplan, 2002) using energy derived from ATP hydrolysis. Thus, in teleosts, NKA is considered a major factor in osmoregulation.

The NKA is composed of two subunits, α and β. The α-subunit is responsible for the catalytic properties of the enzyme, while the β-subunit regulates the structural properties of the NKA (Blanco *et al.*, 1994). Multiple isoforms of both subunits have been identified and exhibit tissue-specific expression patterns (Lingrel *et al.*, 1990). The characteristics of the NKA α-subunits have been intensively studied since the functional properties of the NKA are largely regulated by the α-subunits. The isoforms of α-subunits in teleosts are more complicated than those of other organisms because teleosts underwent a third round of whole-genome duplication. Thus, the nomenclature of NKA α-subunits in teleosts has not yet been integrated. A recent comprehensive phylogenetic analysis of NKA α-subunits in teleosts showed that they can first be classified into α1-, α2-, and α3-subunits, with α1-subunits being further subdivided into α1a/b and α1c clades (Wong *et al.*, 2016). In the current study, we follow this nomenclature. In salmonids, the expression of α1a is high in fresh water and that of α1b is high in seawater, but this expression pattern switches when fish migrate between fresh water and seawater (Richards *et al.*, 2003; Madsen *et al.*, 2009; McCormick *et al.*, 2009). Consequently, the switching mechanism of multiple NKA isoforms is considered key for adaptation to diverse salinities, particularly in salmonids. Furthermore, the localization of NKA proteins has been reported to change in salmonids depending on environmental salinity; for example, NKA-positive ionocytes are distributed in both the primary filament and secondary lamellae of the gill in fresh water, but are only present in the primary filament in seawater (Uchida *et al.*, 1996; McCormick *et al.*, 2009).

Stickleback inhabit diverse environments with a variety of salinities, ranging from seawater to fresh water. Thus, the stickleback ought to be an excellent model to study the physiological mechanisms underlying variations in salinity tolerance and habitat isolation between populations (Heuts, 1946; Wootton, 1984; Kitano *et al.*, 2012). Variation in salinity tolerance has been reported among stickleback ecotypes (Kitano *et al.*, 2012; Ishikawa *et al.*, 2013; Kusakabe *et al.*, 2014, 2017; Divino *et al.*, 2016), and even parapatric ecotypes in the same river may differ in salinity tolerance. For example, a stream-resident threespine stickleback (*Gasterosteus aculeatus*) population lost its ability to adapt to seawater, while an anadromous ecotype that migrates to the same river to spawn is tolerant of seawater challenge (Ishikawa *et al.*, 2013; Kusakabe *et al.*, 2014, 2017). However, the mechanisms involved in loss of seawater adaptability remain largely unknown.

The stickleback reference genome contains four genes encoding NKA α-subunits: ENSGACG00000014324 (*Atp1a1*) on Chromosome 1 encodes α1, ENSGACG00000017683 (*Atp1a2*) on Chromosome 3 encodes α2, while ENSGACG00000001959 (*Atp1a3a*) on Chromosome 10 and ENSGACG00000009524 (*Atp1a3b*) on Chromosome 20 encode α3-subunits (Jones *et al.*, 2012; Wong *et al.*, 2016). ENSGACG00000014324 (*Atp1a1*) is composed of two genes resulting from a tandem duplication and produces at least two different transcripts, *atp1a1a.4-201* (ENSGACT00000018945) and *atp1a1a.4-204* (ENSGACT00000018961) (Wong *et al.*, 2016). A previous phylogenetic analysis showed that both transcripts belong to α1a/b, whereas no transcript corresponding to α1c has yet to be found in the stickleback genome (Wong *et al.*, 2016). Most previous studies on NKA expression in sticklebacks examined only *atp1a1a.4-201*, but showed inconsistent patterns; with increasing environmental salinity, the expression levels increased in both marine and lacustrine populations in British Columbia, Canada (Judd, 2012), or decreased in both marine and lacustrine populations in the St. Lawrence River and British Columbia (McCairns and Bernatchez, 2010; Gibbons *et al.*, 2017). The only study to examine these two isoforms separately found that, in a Mediterranean freshwater population, only the levels of *atp1a1a.4-204* increased in seawater, while levels of *atp1a1a.4-201* were unchanged (Rind *et al.*, 2017). Therefore, the general patterns of NKA response to seawater/freshwater challenges in sticklebacks remain unclear.

Our long-term goal is to understand how changes in osmoregulatory ability occur among stickleback populations inhabiting different salinity environments. As one of the first steps, in the present study we focused on variations in salinity tolerance and NKA expression between two Japanese freshwater threespine stickleback populations collected from Ono and Gifu. The two freshwater stickleback populations were acclimated to 100% seawater, and changes in plasma Na^+ concentrations were evaluated to examine the osmoregulatory ability of these two populations. We further examined NKA mRNA expression levels and protein localization in the gills to gain insights into how gill NKA contributes to osmoregulatory differences in these stickleback populations.

MATERIALS AND METHODS

Seawater acclimation experiments

We used freshwater threespine stickleback collected from Ono and Gifu, Japan, in July 2015 (Fig. 1) to test the salinity tolerance of wild-caught fish. The Ono population was collected from a freshwater pond of Hongan-shozu Itoyo-no-Sato (35.976°N, 136.491°E) in Ono City, Fukui Prefecture. The Gifu population was collected from a spring-fed pond located in Gifu-keizai University in Ogaki City (35.366°N, 136.616°E), Gifu Prefecture. Wild-caught stickleback were brought to the fish facility of the Atmosphere and Ocean Research Institute, University of Tokyo (Chiba, Japan), and maintained at 16°C in 60-litre tanks with a closed recirculating system.

Fish were first acclimated in fresh water (0.3‰) for 7 days, before being transferred to 10% seawater (3.3‰) for 1 day, 30% seawater (10.2‰) for 3 days, 50% seawater (15.2‰) for 3 days, and finally 100% seawater (34.5‰) for 8 days (Fig. 2). Plasma and gills were collected at the following sampling time points:

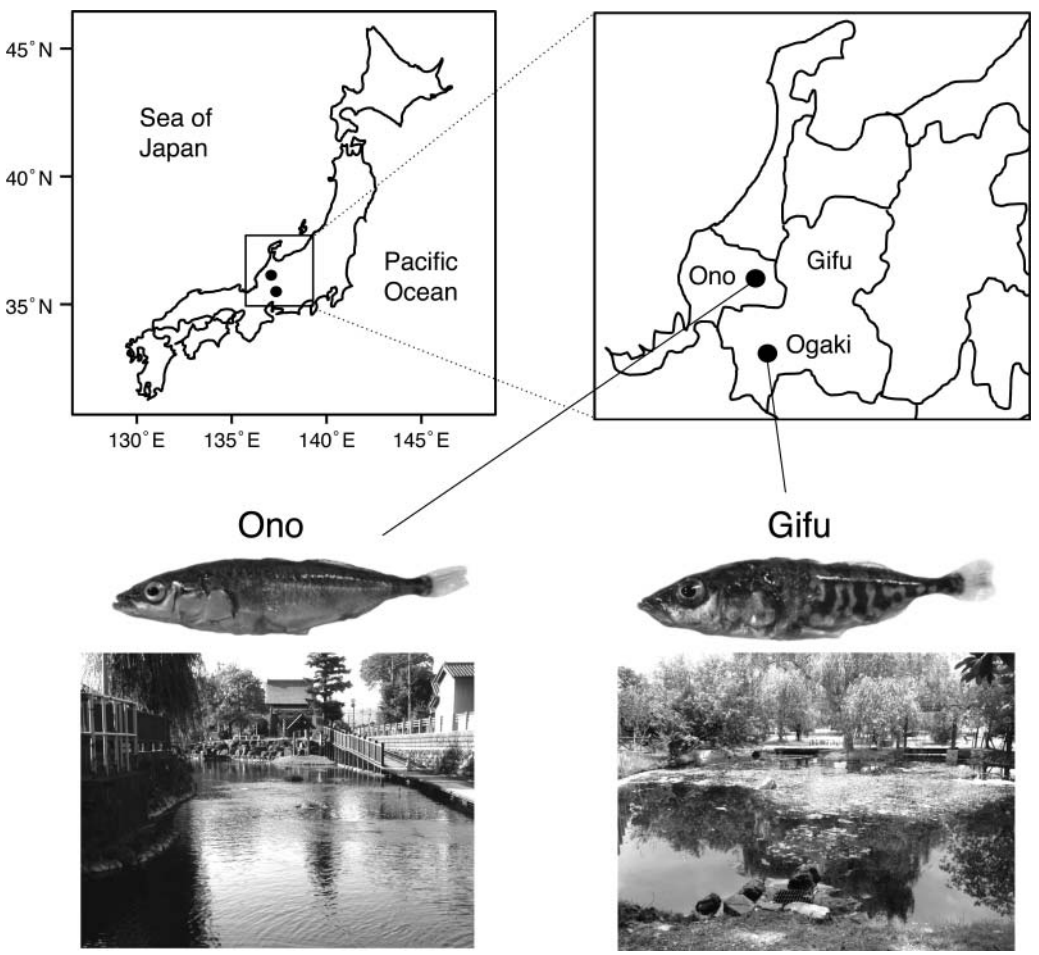

Fig. 1. Sampling locations for the Ono and Gifu threespine stickleback (*Gasterosteus aculeatus*) populations.

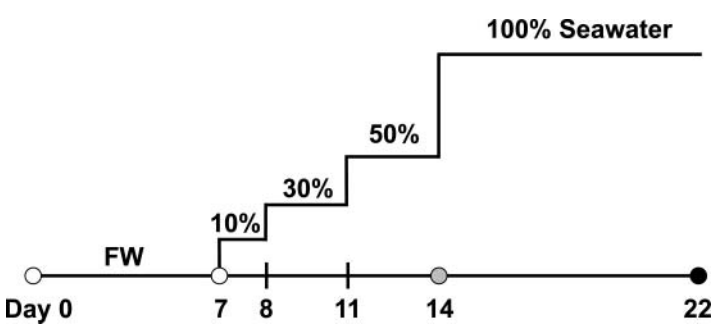

Fig. 2. Time course of the seawater acclimation experiment. Circles show the days when the plasma and gill samples were collected. Vertical lines indicate the days when tank salinity was changed but no samples were collected.

- immediately after the fish were collected from the wild (Day 0; $N = 10$ for Ono and $N = 10$ for Gifu);
- 7 days after acclimation to fresh water in the lab (Day 7; $N = 4$ for Ono and $N = 6$ for Gifu);
- after acclimation to 50% seawater (Day 14; $N = 3$ for Ono and $N = 6$ for Gifu); and
- after acclimation to 100% seawater (Day 22; $N = 6$ for Ono).

All the Gifu fish died within 10 hours of being transferred to 100% seawater ($N = 10$), and thus could not be sampled at Day 22.

For sampling, fish were euthanized using an overdose of MS-222 (tricaine methanesulfonate, 300 mg/L) buffered with sodium bicarbonate. Blood samples were collected from the caudal vasculature using heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). After centrifugation of the heparinized capillary tubes, plasma was obtained and stored at −80°C until required. Plasma Na^+ concentration was measured using an atomic absorption spectrophotometer (Z-5300 Hitachi, Tokyo, Japan) as described previously (Kusakabe *et al.*, 2014). The left halves of gills were removed and immersed in RNAlater reagent (Thermo Fisher, Waltham, MA) overnight at 4°C, and then stored at −80°C for RNA extraction. The right halves of gills were removed and fixed in 4% paraformaldehyde overnight at 4°C. The fixed gills were embedded in Paraplast-Plus® (Oxford Labware, St. Louis, MO). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Quantitative analysis of mRNA levels of the NKA α1-subunit in the stickleback gill

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was determined by measuring the absorbance at 260 nm using a BioPhotometer® D30 (Eppendorf, Hamburg, Germany), and RNA quality was assessed by A_{260nm}/A_{280nm} ratios, with ratios > 1.8 deemed acceptable. Total gill RNA (1 μg) was incubated with 2 units of TURBO™ DNase (TURBO DNA-free, Thermo Fisher, Waltham, MA) at 37°C for 2 hours to remove genomic DNA contamination. Single-strand cDNA was synthesized from the DNase-treated RNA using a High-Capacity cDNA archive kit as described by the manufacturer (Thermo Fisher, Waltham, MA). Real-time quantitative PCR with SYBR™ Green was performed to quantify the mRNA using a LightCycler® 480 (Roche, Basel, Switzerland). PCR was performed in a 10 μL reaction volume, using 1 μL of the 10-fold diluted reverse-transcribed cDNA. The primer pairs used for the detection of the stickleback NKA α-subunits and internal controls are listed in Table 1. The PCR conditions were as follows: 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. A standard curve was generated by a four-fold serial dilution of pooled cDNA. The transcript levels are indicated as relative mRNA levels. The stickleback genes encoding actin beta 2 (*actb2*), Ribosomal protein L13a (*rpl13a*), and Ubiquitin A-52 residue ribosomal protein fusion product 1 (*uba52*) were used as the internal controls. The primer sequences for *actb*, *rpl13a*, and *uba52* were obtained from a previous study of internal control genes in the stickleback (Hibbeler *et al.*, 2008). The mean expression levels of the three genes (*actb2*, *rpl13a*, and *uba52*) were used as internal controls and the expression levels of the *Atp1a* genes were divided by the internal control values. The mean transcript levels of the initial controls (Day 0) were assigned a value of 1.0.

Table 1. List of primers used for real-time quantitative RT-PCR assay

Gene name	UniGene ID	Description	Forward primer	Reverse primer
atp1a1a.2-201	ENSGACG00000014324	Na^+/K^+-ATPase α1-subunit	5′-TCAGAGTGATGTGGGACGAC-3′	5′-ACGATCTTTCTGCGCTCGTA-3′
atp1a1a.2-204	ENSGACG00000014324	Na^+/K^+-ATPase α1-subunit	5′-TATCGAGCTGTGCTGTGGAT-3′	5′-GTTTCAGGTGTGCCATCCTT-3′
atp1a2	ENSGACG00000017683	Na^+/K^+-ATPase α1-subunit	5′-ACAATCCCTCCGGTCACATT-3′	5′-CATTATGCTGCTGCACCTGT-3′
atp1a3a	ENSGACG00000001959	Na^+/K^+-ATPase α1-subunit	5′-TTCCTCGGCATGTCCTTCTT-3′	5′-CTCCGGCACATTAGCAACAA-3
atp1a3b	ENSGACG00000009524	Na^+/K^+-ATPase α1-subunit	5′-AAACATGGTGCCTCAGCAAG-3′	5′-CAACCAGATCTCCACCCACT-3′
actb2	ENSGACG00000007836	Actin, beta 2	5′-GCGTGGCTACTCCTTCACC-3′	5′-AGGACTTCATACCGAGGAAGG-3′
rpl13a	ENSGACG00000009310	Ribosomal protein L13a	5′-CACCTTGGTCAACTTGAACAGTG-3′	5′-TCCCTCCGCCCTACGAC-3′
uba52	ENSGACG00000008021	Ubiquitin A-52 residue ribosomal protein fusion product 1	5′-AGACGGGCATAGCACTTGC-3′	5′-CAGGACAAGGAAGGCATCC-3′

NKA immunohistochemistry in the stickleback gill

The embedded gill samples were sectioned into 6 μm slices and mounted on MAS-coated slides (Matsunami Glass, Osaka, Japan). Endogenous peroxidases were blocked in 0.6% H_2O_2 in methanol for 15 minutes at room temperature, and non-specific binding was blocked with 2% normal goat serum in phosphate-buffered saline at room temperature for 30 minutes. The primary antiserum against the NKA α-subunit of masu salmon (*Oncorhynchus masou*) (a gift from Dr. K. Ura, Hokkaido University) was raised in rabbits, and the antigen sequences are conserved among fish, including elasmobranchs (Ura *et al.*, 1996; Hyodo *et al.*, 2004; Takabe *et al.*, 2012). The antiserum was applied at a dilution of 1:1500. Sections were incubated with the primary antiserum for 40 hours at 4°C in a humidified chamber, and then incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) for 30 minutes and with an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) for 45 minutes at room temperature. The antibody–antigen complex was visualized by exposure to 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris buffer (pH 7.3) for 5 minutes at room temperature. The sections were counterstained with haematoxylin. The sizes and densities of ionocytes (NKA-positive cells) were analysed using the BZ-HIC software (Keyence, Osaka, Japan). For cell size measurement, 1735 NKA-positive cells of Ono fish and 1494 NKA-positive cells of Gifu fish were randomly selected from three fish of each population and area (μm^2) of the NKA-positive cells measured. For cell density measurement, 12 immunohistochemistry images were randomly selected from three fish of each population and area ratios of the NKA-positive cells against the entire gill area were calculated.

Statistical analysis

Differences in plasma Na^+ concentrations in fresh water (Days 0 and 7) were analysed by Student's *t*-test. Changes in plasma Na^+ concentrations during acclimation to seawater were assessed by two-way analysis of variance (ANOVA). Changes in mRNA levels of the gill NKA α1-subunits were assessed by one-way ANOVA, followed by Tukey's HSD *post-hoc* test. Differences in the sizes of the NKA-positive cells were analysed using Student's *t*-test. All statistical analyses were performed using R statistical software (v.3.0.2, R Foundation for Statistical Computing, Vienna, Austria). A *P*-value of 5% or less was considered statistically significant.

RESULTS

Plasma Na^+ concentrations during acclimation to seawater

There was no significant difference in the plasma Na^+ concentration between the Ono and Gifu sticklebacks immediately after collection in the field (Day 0, *t*-test, $P = 0.07$). After being brought to the laboratory, the plasma Na^+ concentrations of the Ono fish were significantly higher than those of the Gifu fish (Day 7, *t*-test, $P < 0.001$), but all the fish survived during acclimation to laboratory conditions (Fig. 3). There were no significant changes in the plasma Na^+ concentrations either in the Ono or Gifu fish during acclimation to seawater (two-way ANOVA, $P = 0.34$). However, the Gifu fish were unable to survive in 100% seawater even after gradual acclimation to 50% seawater. There was a marginally

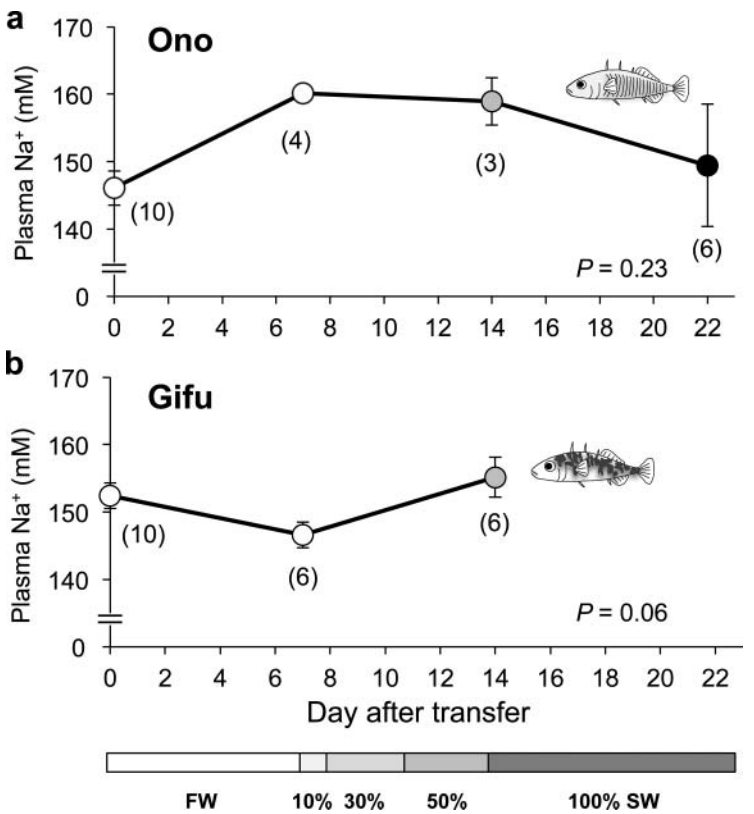

Fig. 3. Plasma Na^+ levels of (a) Ono and (b) Gifu threespine stickleback during acclimation to seawater. Plasma samples were taken immediately after fish were collected in the field (Day 0), and then after 7, 14, and 22 days. The number of samples per data point is shown in parentheses. Differences in plasma Na^+ levels between sampling points were analysed using one-way ANOVA.

significant interaction between population and salinity for plasma Na^+ concentrations (two-way ANOVA, $P = 0.05$): plasma Na^+ concentration tended to increase in Ono fish, whereas it appeared to decrease slightly in Gifu fish.

Transcript levels of the NKA α1-subunit during acclimation to seawater

The *atp1a1a.4-201* transcript levels of the Ono fish decreased significantly after transfer to 50% seawater ($P < 0.05$), but returned to baseline (Day 0) after acclimation to 100% seawater (Fig. 4a). The *atp1a1a.4-201* transcript levels of the Gifu fish also decreased significantly after transfer to 50% seawater (Fig. 4f, $P < 0.001$). There was no significant change in *atp1a1a.4-204* transcript levels either in the Ono (Fig. 4b, $P = 0.07$) or Gifu fish (Fig. 4g, $P = 0.36$) during acclimation to seawater. The *atp1a2* transcript levels of the Ono fish increased significantly after transfer to the laboratory, and transcript levels were maintained during acclimation to seawater (Fig. 4c, $P < 0.001$). The *atp1a2* transcript levels of the Gifu fish increased significantly after transfer to 50% seawater (Fig. 4h, $P < 0.001$). There was no significant change in *atp1a3a* transcript levels either in the Ono (Fig. 4d,

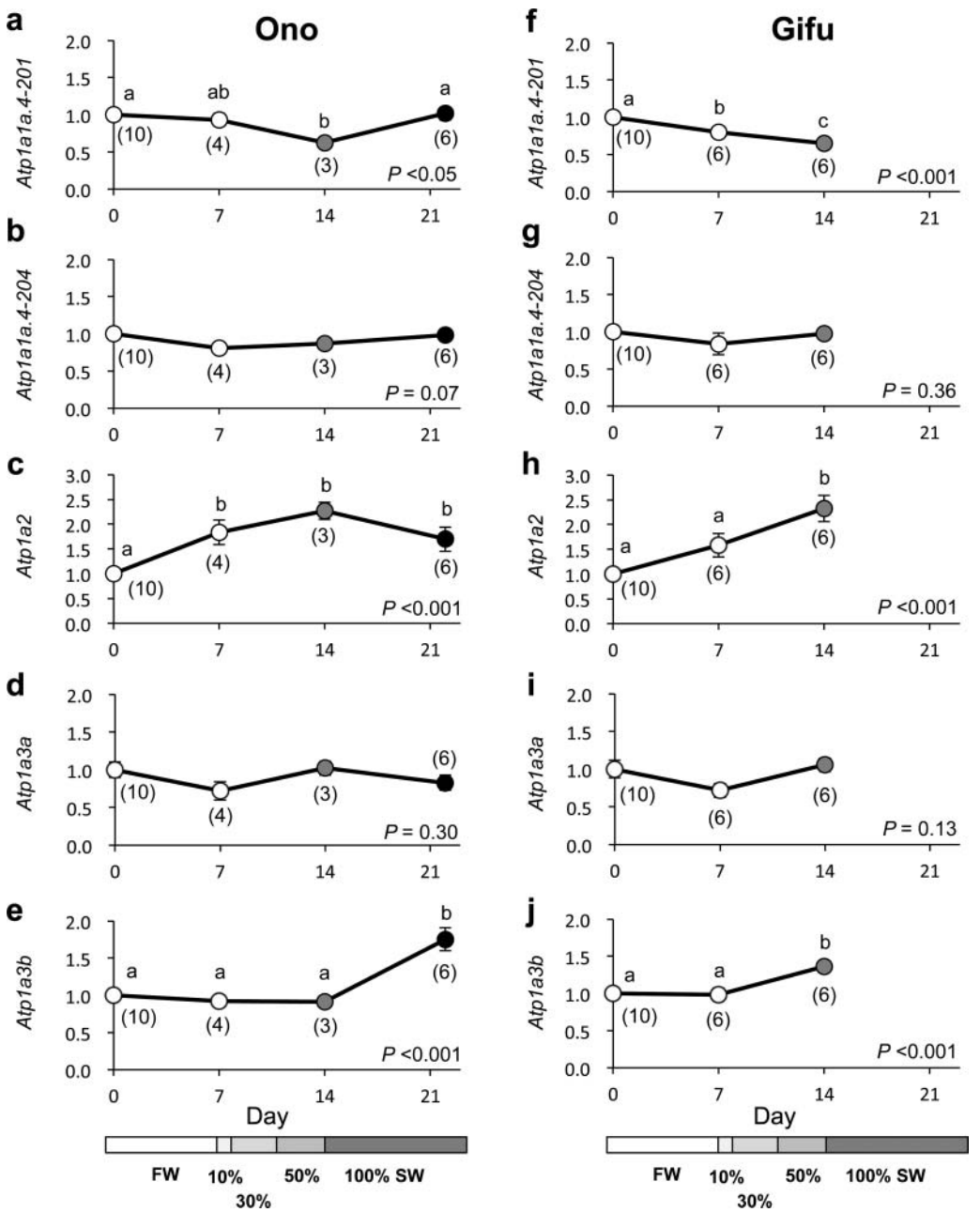

Fig. 4. Gill NKA α-subunit mRNA levels (*atp1a1a.4-201*, *atp1a1a.4-204*, *atp1a2*, *atp1a3a*, and *atp1a3b*) of the Ono (a–e) and Gifu (f–j) threespine stickleback populations during the seawater acclimation experiment. Gill samples were taken immediately after fish were collected in the field (Day 0), and then after 7, 14, and 22 days. The number of samples per data point is shown in parentheses. The values were corrected with internal controls and the relative expression values at Day 0 were set to 1.0 (see Materials and methods). Differences in transcript levels between sampling points were analysed using one-way ANOVA. Data points that do not share a letter are significantly different by Tukey's HSD ($P < 0.05$).

$P = 0.30$) or Gifu fish (Fig. 4i, $P = 0.13$) during the seawater acclimation experiment. The *atp1a3b* transcript levels of the Ono fish increased significantly after transfer to 100% seawater (Fig. 4e, $P < 0.001$). The *atp1a3b* transcript levels of the Gifu fish increased significantly after transfer to 50% seawater (Fig. 4j, $P < 0.001$). The qPCR crossing point (Cp) values, which indicate how rapidly the PCR products are detected and represent an approximation of the overall transcript levels, showed that *atp1a1a.4-201* was the most abundant transcript, and that the levels of the other transcripts were substantially lower in comparison (evolutionary-ecology.com/data/3177Appendix.pdf, Fig. S1). The predominance of *atp1a1a.4-201* in the gills is consistent with previous gill RNA-sequencing data for Russian populations (Appendix Fig. S2) (Artemov *et al.*, 2017).

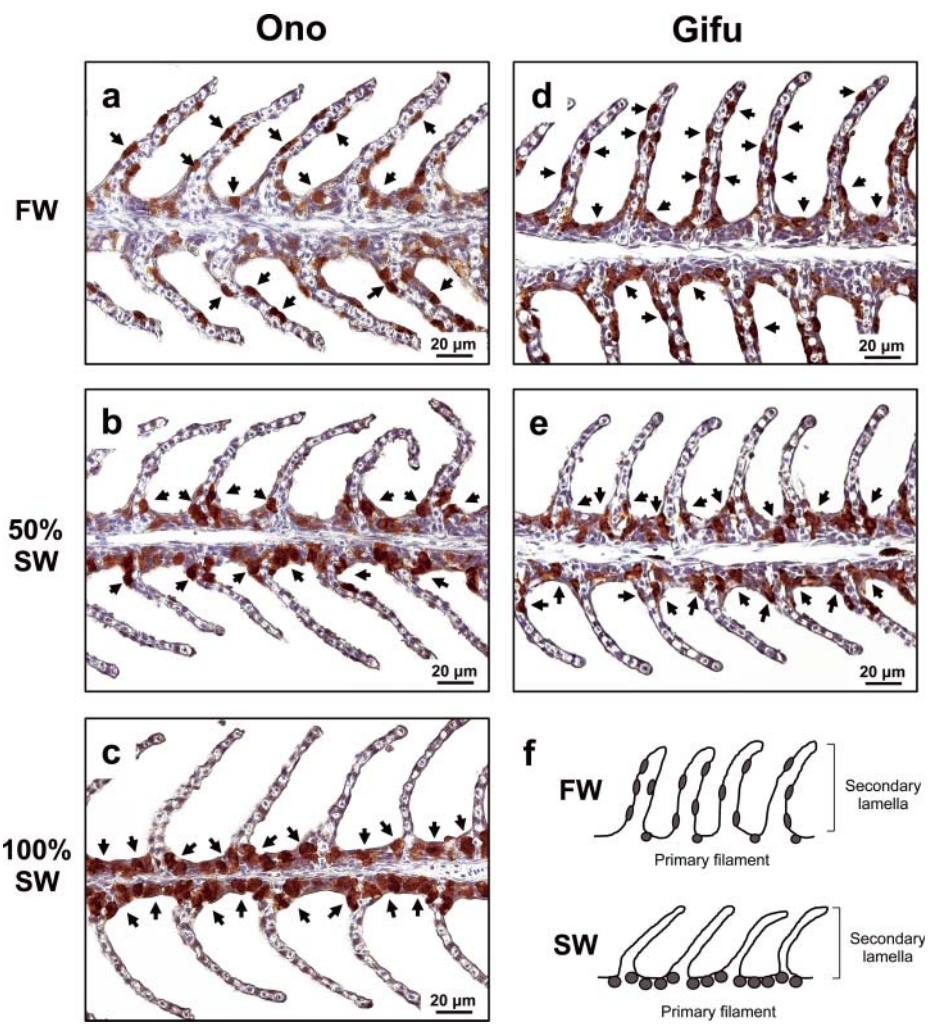

Fig. 5. Immunohistochemistry of the NKA in gills of the Ono (a–c) and Gifu (d, e) populations. Arrows indicate cells positive for anti-NKA antibody. Panel (f) summarizes the structures of stickleback gills and indicates sites of gill ionocyte localization after acclimation to fresh water or seawater (grey ovals).

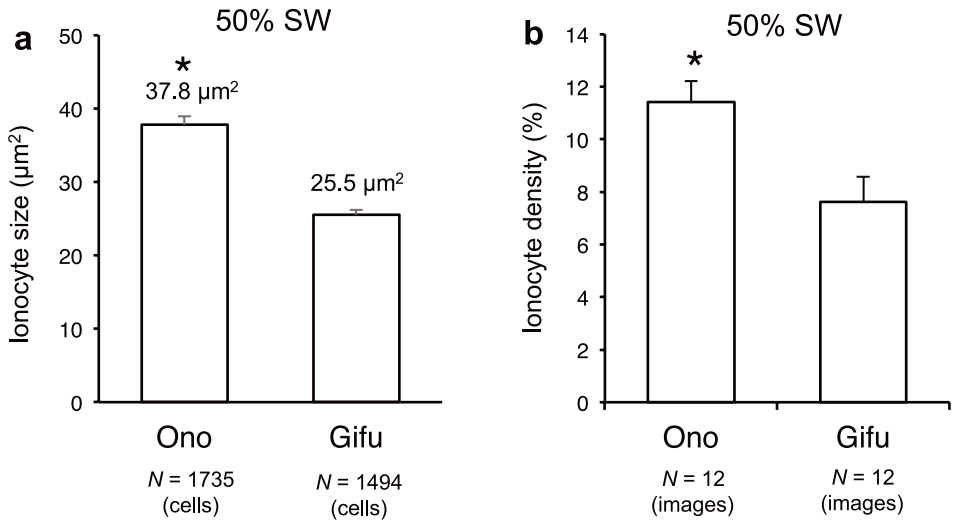

Fig. 6. Sizes (a) and densities (b) of gill ionocytes of fish acclimated to 50% seawater. Values are means ± S.E. Sample sizes are given. The asterisk indicates a significantly higher value (*$P < 0.05$).

NKA immunohistochemistry in the gill

The sites of NKA-positive cells were immunohistochemically detected using an antiserum specific for fish NKA. Under the freshwater condition, NKA-positive cells were detected in both the gill filament and secondary lamellae in both the Ono and Gifu sticklebacks (Fig. 5a, d). After transfer to 50% seawater (Day 14), the NKA-positive cells were detected only in the gill filament of both stickleback populations (Fig. 5b, e). The average size and density of NKA-positive cells of the Ono fish acclimated to 50% seawater were significantly larger than those of the Gifu fish (Fig. 6a, b). After transfer to 100% seawater (Day 22), NKA-positive cells could still be detected in the gill filament of the Ono fish (Fig. 5c). Since all Gifu fish died after being transferred to 100% seawater, Gifu fish gill samples could not be obtained. The localizations of NKA-positive cells in both freshwater and seawater conditions are summarized in Fig. 5f.

DISCUSSION

The two Japanese freshwater stickleback populations examined differed in salinity tolerance. The Ono population acclimated to 100% seawater, whereas the Gifu population was unable to do so even after gradual acclimation to such salinity (10% seawater for 1 day, 30% seawater for 3 days, and 50% seawater for 3 days, rising to 100% seawater). We repeated the acclimation experiment with members of the same Gifu freshwater population and once again all of them died after being transferred to 100% seawater ($N = 6$, data not shown). Mean plasma Na^+ concentration for both the Ono and Gifu populations during the seawater acclimation experiment was 140–160 mM, a physiologically normal range for plasma Na^+ concentration in teleosts (Schmidt-Nielsen, 1997). The plasma Na^+ concentration of the Gifu freshwater stickleback remained within the normal range at 50% seawater, but they could not survive in 100% seawater even after gradual acclimation to 50% seawater.

Comparison of NKA α-subunit mRNA levels did not reveal substantial differences between the two populations. Therefore, differential mRNA expression may not be the primary reason for the difference in salinity tolerance between the two populations. Switching between the NKA α1a- and α1b-subunits under different salinity conditions is a major osmoregulatory mechanism in salmonids. A similar switching system of different α1 isoforms has been reported in tilapia (Tipsmark *et al.*, 2011) and galaxiid fish (Urbina *et al.*, 2013). However, recent studies reported that Japanese medaka (Bollinger *et al.*, 2016) and Japanese eel (Wong *et al.*, 2016) did not show a switching pattern for any α1 isoform, suggesting that the switching of the NKA α1 isoforms is not a universal osmoregulatory mechanism for euryhalinity. In the current study, the two stickleback isoforms (*atp1a1a.4-201* and *atp1a1a.4-204*) did not show a clear switching pattern. In addition, we did not observe up-regulation of *atp1a1a.4-204* with the seawater challenge, which is different to that previously reported for a Mediterranean freshwater stickleback population (Rind *et al.*, 2017). It should be highlighted that the numbers sampled at some time points were low, particularly in the Ono population. This might have affected the results that showed no clear switching patterns of *atp1a1a* isoforms. However, previous gene expression analyses of *atp1a1a.4-201* with a seawater challenge also showed inconsistent results (McCairns and Bernatchez, 2010; Judd, 2012; Gibbons *et al.*, 2017). Changes in the transcript levels of the stickleback NKA α-subunits may not be as pronounced as for other euryhaline fish, such as killifish (Scott *et al.*, 2004), salmonids (Seidelin *et al.*, 2000; McCormick *et al.*, 2009), and Japanese eel (Wong *et al.*, 2016), indicating that differential NKA mRNA levels might not explain the variations in salinity tolerance among stickleback ecotypes.

Our immunohistochemical analysis showed clear changes in the localization sites of the NKA protein in the gills of both populations in response to the seawater challenge. In teleosts, NKA expression is found in ionocytes. In salmonids, ionocytes are found both in gill filaments and secondary lamellae in fresh water, whereas in seawater they are only found in gill filaments (Uchida *et al.*, 1996; McCormick *et al.*, 2009). In seawater, ionocytes are typically larger and embedded within the lamellae of filaments, whereas in fresh water they are flat and appear to be attached to the secondary lamellae (Uchida *et al.*, 1996; McCormick, 2001; McCormick *et al.*, 2009). We observed similar patterns in both stickleback populations. In fresh water, the ionocytes (NKA-positive cells) in the Ono and Gifu fish were found both in gill filaments and secondary lamellae. Following acclimation to 50% seawater, most ionocytes were located in the inter-lamellar region, and the ionocytes on the secondary lamellae could not be detected. Cell shape also appeared to change; ionocytes in fresh water were flatter, while those in seawater were more round. The cell measurement analysis showed that the mean size and density of the ionocytes in the gills of Ono fish acclimated to 50% seawater were both significantly greater than in the Gifu fish at 50% seawater (Fig. 6). Differences in ionocyte differentiation may be a possible mechanism underlying the differences in salinity tolerance between these two populations.

In summary, the two Japanese freshwater stickleback populations studied showed no significant changes in plasma Na^+ concentrations during the seawater acclimation process; however, the Gifu fish – but not the Ono fish – were unable to survive in 100% seawater. The transcript levels of the NKA α-subunit showed similar changes in response to seawater in both populations. The sites of NKA protein expression also showed similar patterns with up to 50% seawater, but the sizes of immunoreactive ionocytes differed between populations. The mechanism of ionocyte formation after mRNA synthesis could be key to determining osmoregulatory ability. Our results indicate that analysis of mRNA expression

levels is not sufficient on its own to investigate the physiological basis of variation in osmoregulatory ability, and highlight the importance of immunohistochemical analysis.

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