A key evolution in gene expression plasticity for freshwater colonisation in early life stage of fish

Yumeki Oto[1], Mari Kuroki[2], Midori Iida[3], Ryosuke Ito[4], Shota Nomura[5], and Katsutoshi Watanabe[4]

[1]National Fisheries University
[2]The University of Tokyo
[3]Niigata University
[4]Kyoto University
[5]National Institute for Basic Biology

July 14, 2022

Abstract

Colonisation of freshwater habitats by marine animals is a remarkable evolutionary event that has enriched biodiversity in freshwater ecosystems. For successful freshwater colonisation, high physiological plasticity is presumed to be necessary, but its evolutionary basis has not been detailed. Marine-originated amphidromous species, which regularly migrate between freshwater and marine environments, have repeatedly lost migratory behaviour in many lineages, which sometimes triggered species radiation in freshwater habitats. Since amphidromous species typically visit the sea during the larval period, the difficulty in the evolution of larval freshwater tolerance is a bottleneck for freshwater colonisation. To elucidate the key evolutionary changes that enhance the physiological plasticity for freshwater colonisation, we compared larval gene expression changes depending on salinity conditions among three congeneric amphidromous goby species (Gymnogobius) with varying dependences on freshwater habitats. First, an otolith microchemical analysis and rearing experiment under laboratory conditions confirmed the presence of freshwater residents only in G. urotaenia and higher larval survivorship of this species both in seawater and freshwater conditions than the obligate amphidromous G. petschiliensis and G. opperiens. Larval whole-body transcriptome analysis revealed that G. urotaenia exhibited the greatest differences in the expression levels of several osmoregulatory genes, including aqp3, which is critical for water discharge from their body during early fish development. Thus, we obtained the results that consistently support the importance of enhanced osmoregulatory plasticity for establishing freshwater forms, and further identified some important evolutionary changes for larval freshwater adaptation and colonisation in the goby group.

A key evolution in gene expression plasticity for freshwater colonisation in early life stage of fish

Running title: Evolution for freshwater colonisation in fish

Authors: Yumeki Oto[1, 4, *], Mari Kuroki[2], Midori Iida[3], Ryosuke Ito[1, 5], Shota Nomura[1, 6], Katsutoshi Watanabe[1, *]

Authors' affiliations:

[1] Division of Biological Sciences, Graduate School of Science, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto City, Kyoto Prefecture 606–8502, Japan

[2] Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo Metropolitan 113–8657, Japan

[3] Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, 87 Tassha, Sado City, Niigata Prefecture 952–2135, Japan

[4] Present address: Department of Applied Aquabiology, National Fisheries University, 2–7–1 Nagata-honmachi, Shimonoseki City, Yamaguchi Prefecture 759–6595, Japan

[5] Present address: Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto City, Kyoto Prefecture 606–8502, Japan

[6] Present address: Division of Evolutionary Developmental Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki City, Aichi Prefecture 444–8585, Japan

Corresponding authors: Y Oto and K Watanabe

E-mail: angler_yumeki@yahoo.co.jp

Tel and FAX: +81-83-242-5717

Key words: Life history evolution, Migration behaviour, Freshwater resident, Amphidromy, Osmoregulation, Transcriptome analysis

Abstract

Colonisation of freshwater habitats by marine animals is a remarkable evolutionary event that has enriched biodiversity in freshwater ecosystems. For successful freshwater colonisation, high physiological plasticity is presumed to be necessary, but its evolutionary basis has not been detailed. Marine-originated amphidromous species, which regularly migrate between freshwater and marine environments, have repeatedly lost migratory behaviour in many lineages, which sometimes triggered species radiation in freshwater habitats. Since amphidromous species typically visit the sea during the larval period, the difficulty in the evolution of larval freshwater tolerance is a bottleneck for freshwater colonisation. To elucidate the key evolutionary changes that enhance the physiological plasticity for freshwater colonisation, we compared larval gene expression changes depending on salinity conditions among three congeneric amphidromous goby species (*Gymnogobius*) with varying dependences on freshwater habitats. First, an otolith microchemical analysis and rearing experiment under laboratory conditions confirmed the presence of freshwater residents only in *G. urotaenia* and higher larval survivorship of this species both in seawater and freshwater conditions than the obligate amphidromous *G. petschiliensis* and *G. opperiens* . Larval whole-body transcriptome analysis revealed that *G. urotaenia* exhibited the greatest differences in the expression levels of several osmoregulatory genes, including *aqp3* , which is critical for water discharge from their body during early fish development. Thus, we obtained the results that consistently support the importance of enhanced osmoregulatory plasticity for establishing freshwater forms, and further identified some important evolutionary changes for larval freshwater adaptation and colonisation in the goby group.

1 | INTRODUCTION

Colonising novel environments is a significant event that increases biodiversity (Schluter, 2000; Losos, 2010). A variety of marine animals have repeatedly colonised freshwater habitats, leading to the diversification of species and life histories in snails (Abdou et al., 2015), crustaceans (Anger, 2013), fishes (Betancur-R et al., 2015) and mammals (Hamilton et al., 2001). Diadromous species regularly move between marine and freshwater habitats and are often regarded as evolutionary intermediates potentially undergoing freshwater colonisation (Gross, 1987; Dodson et al., 2009). The derivation of strictly freshwater species from diadromous species has promoted species radiation in freshwater areas (McDowall, 2001; Waters and Wallis, 2001; Burridge and Waters, 2020). This has contributed to the conspicuous biodiversity in freshwater habitats (Corush, 2019), which contains approximately 40% of teleost fishes despite freshwater habitats covering only 1% of the earth's surface (Lévêque et al., 2007). To deeply understand the origin of freshwater biodiversity, it is necessary to elucidate the underlying environmental, ecological, and physiological factors associated with the loss of diadromy (Burridge and Waters, 2020; Waters et al., 2020).

Physiological plasticity plays a crucial role in habitat expansion because its acquisition enables organisms to opportunistically utilise novel environments, providing the chance of evolutionary adaptation to the environments (Ghalambor et al., 2007; Lande, 2015). In teleost fishes, high osmoregulatory plasticity is presumed to be required for their habitat shift to novel environments with different salinities. Since teleosts maintain their body fluid at approximately one-quarter to one-third the osmotic concentration of seawater, they must discharge water and absorb salt ions (i.e. hyper-osmoregulation) to survive under freshwater conditions (Evans et al., 2005; Edwards and Marshall, 2013). To colonise freshwater habitats, marine fish need to acquire the ability to hyper-osmoregulate by developing osmoregulatory plasticity (Bamber and Henderson, 1988; McCairns and Bernatchez, 2010). Generally, early larval fish have poor osmoregulatory plasticity due to their undeveloped osmoregulatory organs such as gills, kidneys, and intestines (Varsamos et al., 2005). This circumstance potentially makes it difficult for larval fish to adapt to novel salinity environments than fish in later stages (McDowall, 1997; Lee and Bell, 1999), diadromous species actually tending to spend their early life stages in their ancestral environments (McDowall, 1997; Tsukamoto et al., 2002). Therefore, the acquisition of larval osmoregulatory plasticity is a key event to the completion of freshwater colonisation in fish, although the evolutionary mechanisms of plasticity has not been clarified.

Among the three types of diadromy (i.e. anadromy, catadromy and amphidromy), amphidromy provides the most obvious example where the difficulty in the evolution of larval physiology is a bottleneck to freshwater colonisation. Amphidromy can be found in various marine-originated lineages, including snails, shrimp and fishes, and is the most speciose (273 or more species) among the three types of diadromy (i.e. anadromy, 53 species; catadromy, 109 species; Augspurger et al., 2017). The marine-originated amphidromous animals visit the sea only during the short larval period because the larvae generally cannot survive or grow under the unusual freshwater conditions due to their physiological inadaptability (Rome et al., 2009; Iida et al., 2010; Abdou et al., 2015). Interestingly, in some groups, amphidromous species have repeatedly evolved landlocked or resident forms in freshwater areas (hereinafter collectively referred to as freshwater forms) (e.g. Augspurger et al., 2017; Delgado and Ruzzante, 2020). Phylogenetic studies have provided evidence that species radiation has occurred after the loss of amphidromy (i.e. the loss of marine larval dispersal) in some fish families, such as Galaxiidae (Waters and Wallis, 2001; Burridge and Waters, 2020), Gobiidae (Yamasaki et al., 2015) and Eleotridae (Stevens and Hicks, 2009). Thus, focusing on the freshwater forms derived from amphidromous species would help us to comprehend the role of physiological evolution in species diversification during freshwater colonisation. However, evolutionary studies have much less focused on amphidromy compared with the other types of diadromy (Shrimpton, 2013).

Among teleosts, the goby family Gobiidae includes more than a hundred amphidromous species (Augspurger et al., 2017; Delgado and Ruzzante, 2020). The marine-originated goby genus *Gymnogobius* , distributed in East Asia, includes at least three amphidromous, five freshwater species and eight marine and estuarine species (Ellingson et al., 2014; Chiba et al., 2020; Ito et al., 2022). The diversity in the life history and habitats makes this an attractive group to study the processes of freshwater colonisation (Fig. 1a). Especially, one clade in this genus contains three amphidromous species which differ in their dependences on freshwater environments; *Gymnogobius urotaenia* is known to contain multiple landlocked populations in freshwater lakes, while*Gymnogobius petschiliensis* and *Gymnogobius opperiens* are not (Aizawa et al., 1994; Harada et al., 2002; Stevenson, 2002; Sota et al., 2005). Here, we hypothesise that *G. urotaenia* have evolved a higher freshwater tolerance at the larval stage than the other two congeners through the enhancement of osmoregulatory plasticity, which enables *G. urotaenia* to flexibly establish freshwater forms.

To test the above hypothesis, we used comparative transcriptomic analysis to identify candidate genes that confer larval physiological plasticity essential for surviving and colonising freshwater environments in *Gymnogobius* (Fig. 1b). First, we confirmed the migratory history of the three *Gymnogobius* species by analysing the trace element concentrations in the otoliths, which vary with habitat salinity (Elsdon et al., 2008). We then quantified the larval survivorships in freshwater and seawater conditions in the laboratory to compare the extent of osmoregulatory plasticity among the three species. Finally, to identify the genetic basis responsible for larval survival in freshwater conditions, we analysed the whole-body transcriptomes of larval fish and sought differentially expressed genes between freshwater- and seawater-reared individuals in

each species. We investigated both the upregulation of genes responsible for hyper-osmoregulatory capacity and downregulation of genes responsible for water uptake and ion discharge (hypo-osmoregulation), which are similarly important for freshwater acclimation in teleosts (Velotta et al., 2017; Nakamura et al., 2020). We predicted that when the larvae were reared in freshwater, the expressions of some genes important for hyper- and hypo-osmoregulation were significantly up- and down-regulated, respectively, in *G. urotaenia* but not or less in the other amphidromous congeners. The results should enable us to confirm the importance of osmoregulatory plasticity and clarify key evolutionary changes for enhancing larval freshwater tolerance, which possibly enabled the species to establish freshwater forms.

2 | MATERIALS AND METHODS

2.1 | Sampling of male parents and egg clutches

For the otolith microchemical analysis and larval rearing experiment, male parents and egg clutches of three amphidromous *Gymnogobius*species were sampled from two or three populations per species inhabiting five rivers and one lake located on the Pacific and Sea of Japan coasts in Japan [PI and PA (population ID): *G. petschiliensis* ; OO and ON: *G. opperiens* ; UE, UA and UB: *G. urotaenia* ; 3–9 clutches for each; Fig. S1, Table S1]. We failed to capture male parents protecting eggs for the UE population, therefore other adult individuals captured at the same sampling site were used in the otolith analysis. The UE clutches were molecularly confirmed to be*G. urotaenia* (Tables S2, S3). All fish were handled according to animal welfare guidelines and policies of the Kyoto University animal welfare committee and Japanese Ministry of Education, Culture, Sports, Science and Technology (https://www.kyoto-u.ac.jp/en/research/research-compliance-ethics/animal-experiments).

2.2 | Analysis of fish migratory history

Otolith microchemical analysis was conducted to examine the migratory history of male parents from the seven populations of the three*Gymnogobius* species (using 2–9 individuals from each population; Table S1). We measured the strontium (Sr) and calcium (Ca) concentrations accumulated in the otolith specimens. The Sr/Ca ratio is influenced by environmental salinity, and is often used to reconstruct migratory patterns of various fishes, including amphidromous gobies (Tsunagawa and Arai, 2008; Iida et al., 2017). By measuring the ratio from the otolith core (formed when hatching) to the edge (formed just before sampling) along the transverse section of the otolith, the history of salinity habitat use can be traced. The specimens were read with an electron probe microanalyser (EPMA: JXA-8230, JEOL Ltd., Tokyo, Japan). Detailed specimen preparation and analysis protocols are available in the Supplementary Methods.

2.3 | Rearing experiment for larval freshwater tolerance

We conducted a rearing experiment under laboratory conditions using newly hatched larvae from 8–17 clutches for each species to compare the freshwater tolerance of the three *Gymnogobius* species. When more than half of the eggs had hatched from each clutch, exactly 18 individual larval fish were carefully transferred into 2-L experimental tanks filled with aerated freshwater (salinity 0.1; freshwater-reared group) or seawater (salinity 34.5–35.5; seawater-reared group) using pipettes. Under feeding conditions, surviving and dead individuals were counted daily until all individuals died. Detailed experimental protocol is available in the Supplementary Methods.

2.4 | Statistical analysis of the larval rearing experiment

To evaluate larval tolerance to freshwater in the three*Gymnogobius* species, we conducted a survival time analysis using the Cox proportional hazard model (Therneau and Grambsch, 2000), which accepts censored data caused by the sampling performed for other experiments. To compare the survivorship between the freshwater and seawater groups, Cox proportional hazard models were established for each species using the coxph function in the survival R package version 3.2-13 (Therneau, 2021). In these models, salinity and egg clutch ID were included as the variables explaining the survival (days) of individual larvae. Here, the positive/negative and significance of the coefficient of the salinity group were assessed.

In addition, to directly illustrate interspecific variations in the freshwater tolerance and test whether freshwater tolerant species also exhibit high survival in seawater (i.e. exhibit physiological plasticity), the survival in each salinity group was compared among the species by establishing the Cox proportional hazard models with the explanatory variable being species.

The Cox proportional hazard model assumes that the effects of the explanatory variables on the mortality are constant regardless of time (Therneau and Grambsch, 2000). Accordingly, if the survival curves estimated by the Kaplan-Meier method (Kaplan and Meier, 1958) intersect between different experimental groups (i.e. inversion of mortality occurred), careful interpretation of results is necessary with respect to the survival time analysis. R statistical software version 4.1.0 (R Core Team, 2021) was used for all analyses.

2.5 | RNA-Seq experiment

For the RNA-Seq analysis to compare transcriptional responses to different salinity conditions among the three species, three or more individuals of 7-day-old larvae were sampled from both freshwater- and seawater-reared groups of the PA (*G. petschiliensis*), OO (*G. opperiens*), UE, UA (*G. urotaenia* in the rivers) and UB (*G. urotaenia* in the lake) populations in the above experimental conditions. After total RNA was isolated from the larval whole bodies, the three samples with the highest RNA integrity number (4.0–8.5) for total RNA were selected per salinity group in each population (30 samples in total). Messenger RNA was isolated from the selected total RNA samples, and was converted into a cDNA library. Finally, libraries were sequenced by Macrogen Japan (Tokyo, Japan) using the Illumina HiSeq X sequencing platforms. This experiment generated a total of 1.4–3.8 million pair-end RNA-Seq reads (Table S4). For a detailed protocol on the larval sampling and library preparation, see Supplementary Methods.

2.6 | Differential expression analysis

To identify key candidate genes involved in larval adaptation to freshwater, we investigated differentially expressed genes (DEGs) depending on salinity conditions in larvae of the three species. Prior to the differential expression analysis, the trimmed 4.2–10.9 million pair-end RNA-Seq reads from each of the 30 samples were mapped to a reference draft genome of *Gymnogobius isaza* (closely related to the target species; Ito et al., registration in progress with DNA Data Bank of Japan) using STAR version 2.7.4a (Dobin et al., 2013; mapping results are listed in Table S5). Subsequently, significant DEGs between freshwater- and seawater-reared groups were listed for each species with the edgeR package in R version 4.0.2 (Robinson et al., 2010) using TCC-GUI (Su et al., 2019). All the DEGs were functionally annotated by referring to the annotation information for the genomes of the zebrafish (*Danio rerio*) and threespine stickleback (*Gasterosteus aculeatus*) in the online translated nucleotide Basic Local Alignment Search Tool (TBLASTN; Altschul et al., 1990). For a detailed protocol on the read mapping and DEG screening, see Supplementary Methods.

Among the significant DEGs, we first focused on those shared by all three populations of *G. urotaenia* but not included in the DEG lists of the other species. We predicted that the functions of those DEGs were important for hyper-osmoregulation because of the high freshwater tolerance of *G. urotaenia* larvae (see "Results"). The osmoregulatory importance of each DEG was determined by analysing whether its list of Gene Ontology (GO) terms describing biological processes included ion or water transport/homeostasis using the human genome database, GeneCards version 5.7.0 (Stelzer et al., 2016).

Second, we expanded our view to the entire list of significant DEGs (not limited to those found only in *G. urotaenia*). After identifying osmoregulatory DEGs using the GO term lists, we further extracted those which have been demonstrated to be responsible for osmoregulation in teleosts by exhaustively reviewing published researches. For those genes of apparent importance in teleost osmoregulation, the magnitude of expression changes was compared between the species with and without freshwater forms (i.e. *G. urotaenia* and the other two species). The differences in the magnitude were tested by investigating the significance of the interaction effect between the salinity and presence of freshwater forms in the species in LMMs using the lmer function in the lme4 R package (Bates et al., 2015). The variables in the models were as follows; the response variable: normalised read counts of each gene; the explanatory variables: salinity groups, presence/absence of freshwater forms and interaction term between these two explanatory variables; and the

random effect: parent ID. We predicted that DEGs critically responsible for hyper- or hypo-osmoregulation were more greatly up- or down-regulated in the freshwater-reared group of *G. urotaenia* than the other species. The DEGs found only in any one of*G. urotaenia* populations were eliminated from this analysis to exclude the effects of local adaptation within the species.

We also examined the expression of the gene of FADS2, which decisively influences larval growth and survival in freshwater habitats in stickleback and other fishes through docosahexaenoic acid (DHA) synthesis (Ishikawa et al., 2019) as another candidate gene involved in freshwater adaptation.

3 | RESULTS

3.1 | Migratory history estimation

The otolith microchemical analysis of adult fish from the targeted populations of the three *Gymnogobius* species revealed that all specimens of *G. petschiliensis* ($n = 7$) and *G. opperiens* ($n = 12$) showed high Sr/Ca ratios (5–8 $\times$ 10^{-3}) from near the core to the intermediate regions and low ratios (about 2 $\times$ 10^{-3}) in the outer regions, which aligns with the values in other amphidromous gobies (4–10 $\times$ 10^{-3} in the sea versus $< 4 \times 10^{-3}$ in freshwater areas; Tsunagawa and Arai, 2008; Iida et al., 2017). The results indicate that all specimens of these two species experienced amphidromous life histories (Figs. 2, S2). Similarly, five of six individuals in the UE population of *G. urotaenia* had otoliths showing amphidromous Sr/Ca patterns (near the core: 5–7 $\times$ 10^{-3}; outer region: 2 $\times$ 10^{-3}; Figs. 2, S2). Since amphidromous *Gymnogobius* species are observed to migrate from the sea to freshwater areas while juveniles (Oto, 2021), the increase in Sr/Ca ratios is considered to reflect this juvenile migration. On the other hand, otolith Sr/Ca ratios of one remaining UE individual and all the specimens from the UA ($n = 5$) and UB ($n = 9$) populations were constantly low (about 2 $\times$ 10^{-3}), suggesting that these individuals were freshwater forms.

3.2 | Comparison of larval freshwater tolerance

The intraspecific comparison of larval survivorship between different salinity treatments revealed that the survival rate of *Gymnogobius petschiliensis* was significantly higher in seawater than in freshwater (Cox proportional hazard model, $z = -9.4$, $P < 0.001$; Fig. 3a). In contrast, *G. opperiens* and *G. urotaenia* exhibited significantly higher survival rates in freshwater than in seawater ($z = 10.0$–17.3, $P < 0.001$).

The interspecific comparison of survivorship in each salinity condition supported that *G. urotaenia* larvae can survive well regardless of the salinity (highly plastic). Among the freshwater-reared groups, the survival rate of *G. urotaenia* larvae was significantly higher than those of *G. petschiliensis* and *G. opperiens* (Cox proportional hazard model, $z = 13.4$–18.5, $P < 0.001$; Fig. 3b). The magnitude relation of survivorships between the freshwater-reared *G. petschiliensis* and *G. opperiens*larvae could not be determined, due to the intersection of the estimated survival curves (i.e. the occurrence of survival rate reversal). For the seawater groups, the survival rate was significantly lower in *G. opperiens* than the two other species ($z = -15.5$—-14.4, $P < 0.001$; Fig. 3b). The mortality of *G. opperiens* was especially high immediately after the transition from freshwater to seawater, perhaps due to their vulnerability to salinity changes. The magnitude relation of the survivorships between seawater-reared *G. petschiliensis* and *G. urotaenia* larvae could not be determined because the estimated survival curves intersected. Intraspecific variations in larval survivorship in each salinity condition was sufficiently small compared with the variation between salinity groups or among species (Fig. S3).

The body size and starvation did not seem to critically affect the survival rate because notochord length and yolk sac size of *G. opperiens* (which showed poor survival in both salinity groups) and*G. urotaenia* (which displayed high survival in both salinity groups) was similar (Fig. S4; Table S6). Although *G. petschiliensis* larvae were slightly smaller than *G. urotaenia*(Table S6), an unclear difference in clutch size between the two species was unlikely to affect the survival rate (Fig. S5).

3.3 | Differential expression analysis

The gene expression patterns of the three *Gymnogobius* species clearly differed depending on species rather

than salinity conditions. The PCA for the RNA-Seq read count data of the most variably expressed 100 genes represented three clusters which corresponded to each of the three species (for analysis procedures, see Supplementary Methods; Fig. 4a). The proportion of the variance explained by PC1 and PC2 was 44.0% and 24.6%, respectively.

A total of 319 genes were detected as significant DEGs between the different salinity groups for at least one population (false discovery rate < 0.1). Within these, 86 and 233 genes were up- and down-regulated in the freshwater group, respectively (Fig. 4b; Table S7). The number of the DEGs tended to be larger in the *G. urotaenia* (246 and 30 in stream UE and UA, respectively, and 46 in lake landlocked UB) compared to *G. petschiliensis* (9) and *G. opperiens* (33) (Fig. 4b). Of all the 319 significant DEGs, 265 were found in any one of the three *G. urotaenia* populations alone and were excluded from the detailed expression change analysis among species.

The detected DEGs between the salinity groups included several osmoregulatory genes that were dramatically upregulated in freshwater-reared *G. urotaenia* larvae. Most notable were two significant DEGs which were shared exclusively by the three populations of *G. urotaenia* . These two genes were expected to be important for freshwater adaptation. However, the expression change of one of the two (*ca15b*) was not considered to be significant only in *G. urotaenia* because the significant DEG list of *G. opperiens* also included another transcript similarly derived from *ca15b* (Table S7). The only significant DEG shared exclusively by *G. urotaenia*populations was the transcript of *aqp3* , encoding aquaporin-3 (AQP3), which is responsible for hyper-osmoregulation through the enhancement of basolateral hydraulic conductivity for cell volume regulation (Cutler et al., 2007; Cerdà and Finn, 2010; Whitehead et al., 2011).

Exhaustive screening of all the significant DEGs detected several genes responsible for hyper- or hypo-osmoregulation that were more greatly up- or down-regulated in the freshwater-reared larvae of *G. urotaenia*than the other two species. After excluding the significant DEGs detected only from one *G. urotaenia* population, a total of 54 DEGs detected in any one (or more) species/populations remained, 49 of them being functionally annotated. These DEGs included the genes which have been demonstrated to be responsible for hyper-osmoregulation in teleosts encoding AQP3, solute carrier 13 (SLC13; Nakada et al., 2005) and solute carrier family 22 member 16 (SLC22A16; Lin et al., 2020) (Fig. 5, Table S7). Expression levels of these two genes were higher in the freshwater-reared *G. urotaenia* than in the other two species, but were similarly low in the seawater groups of all species. This was supported by the significant interaction effect between salinity condition and species' migratory pattern (presence of freshwater forms) on the expression levels of those genes (LMM, $P < 0.006$; Table S8).

The 49 DEGs also included the genes which have been demonstrated to be responsible for hypo-osmoregulation in teleosts. The genes encode serum and glucocorticoid-induced protein kinase-1 (SGK-1; Shaw et al., 2008) and Na^+-K^+-Cl^-cotransporter (NKCC; Flemmer et al., 2010). In these genes, the gene expression of NKCC was significantly more downregulated in freshwater-reared *G. urotaenia* than in the other species (LMM,$P = 0.023$). The DEG list also included the gene of claudin (CLDN) family, which is involved in hyper- or hypo-osmoregulation through tight junction pathways (Tipsmark et al., 2008; Guo et al., 2018). Its expression was more upregulated in freshwater groups of *G. urotaenia* than in the other species (LMM, $P = 0.029$). None of these DEGs, which are essential for teleost osmoregulation, exhibited greater expression changes in *G. petschiliensis* and *G. opperiens* than in *G. urotaenia* .

The gene expression of FADS2 was much lower in *G. opperiens* than in the other species, but did not significantly change between salinity groups in any species (Fig. S6).

4 | DISCUSSION

The main objective of this study was to elucidate key evolutionary changes for freshwater adaptation and colonisation in fish by comparing the larval gene expression responses to variations in salinity among three amphidromous goby species. As expected, *Gymnogobius urotaenia* was confirmed to flexibly establish freshwater-resident forms and exhibit high larval survival both in seawater and freshwater conditions. Furthermore, the species showed greater changes in the expression levels of some important osmoregulatory

genes than the obligate amphidromous *G. petschiliensis* and *G. opperiens* . The present results provide the first empirical evidence emphasising the importance of the evolution of larval freshwater tolerance by enhancing osmoregulatory plasticity for freshwater colonisation in diadromous fish, as discussed below in details.

4.1 | Variation in migratory pattern among amphidromous species

The otolith microchemical analysis confirmed variation in the occurrence of freshwater forms (remain in freshwater areas throughout life) among the three amphidromous *Gymnogobius* species. All *G.petschiliensis* and *G. opperiens* specimens exhibited amphidromous characteristics, with the hatchlings migrating from streams to the sea and returning to freshwater in the juvenile period. This result is consistent with the fact that no landlocked populations have previously been reported for these two species (Stevenson, 2002; Ishino, 2005). Similarly, the majority of *G. urotaenia* specimens from the Eiheiji River (UE) exhibited the amphidromous pattern. However, either all or some of the *G. urotaenia* specimens from all three populations were estimated to have remained in freshwater areas (both streams and lakes) throughout life. This result is consistent with previous findings that several putatively freshwater populations exist in this species (Stevenson, 2002; Ishino, 2005). Intriguingly, the individual variation in the migratory history of the UE population suggests that *G. urotaenia* can stay in freshwater habitats without being trapped in freshwater areas by physical barriers (e.g. naturally or artificially dammed lakes). This species may maintain the intra-populational polymorphism of migration as the alternative life-history strategy. The lack of female samples in this study necessitates the further examination of migration patterns in both sexes, although there have been no previous reports confirming sexual differences in the migration patterns in gobies.

4.2 | Variation in larval osmoregulatory ability

The rearing experiment supported the hypothesis that larval fish of*G. urotaenia* exhibit higher freshwater tolerance than those of the other two species. This tolerance has been acquired at the species level because only slight differences were found among the three geographically distant populations of this species. This physiological trait would partly explain the frequent occurrence of freshwater forms of this species. However, a longer-term rearing experiment is necessary to determine whether the larval freshwater tolerance identified in this study or other factors (e.g. those associated with subsequent growth) are critically responsible for landlocking because the larval period of amphidromous *Gymnogobius* species potentially lasts for a few months (Dotu, 1955; Ishino, 2005; Oto, 2021).

The present gene expression analysis provided evidence supporting that the evolution of gene expressions associated with hyper-osmoregulation improved freshwater tolerance of *G. urotaenia* larvae. Most of the DEGs (seawater vs. freshwater) responsible for hyper-osmoregulation were more greatly upregulated in freshwater-reared *G. urotaenia* than the other species. One of the upregulated genes was that of AQP3 (*aqp3*), which is a membrane water channel responsible for water discharge from the epithelial cells under hypotonic conditions (Cerda and Finn, 2010). In adult fish, it is well evidenced that the enhanced expression of *aqp3* is important for acclimation to freshwater in marine-originated species (e.g. the killifish *Fundulus heteroclitus* : Whitehead et al., 2011; the alewife *Alosa pseudoharengus* : Velotta et al., 2017). Remarkably, *aqp3* is practically the only significant DEG shared by all three *G. urotaenia* populations but not by the other species, which makes*aqp3* a particularly strong candidate gene for larval freshwater tolerance. Another was the gene of SLC13, which is known to contribute to the maintenance of sulphate homeostasis in freshwater-reared eels (Nakada et al., 2005). Similarly, the gene of CLDN, which can contribute to hyper-osmoregulation (Tipsmark et al., 2008; Guo et al., 2018), was more greatly upregulated in *G. urotaenia* . The enhanced expression of these hyper-osmoregulatory genes in larval fish would strengthen the freshwater tolerance of *G. urotaenia* , possibly facilitating landlocking or colonisation in freshwater areas. The importance of the evolution of hyper-osmoregulatory gene expression for freshwater residence has also been indicated in certain adult diadromous fish species (Velotta et al., 2015; Kusakabe et al., 2017; Velotta et al., 2017). The present study provides the first evidence of gene expression evolution of larval fish in a lineage colonising freshwater habitats.

The downregulation of gene expressions for water absorption and ion discharge (hypo-osmoregulation) can also be important for freshwater acclimation in teleosts (Velotta et al., 2015; Velotta et al., 2017; Nakamura et al., 2020). In our study, only one hypo-osmoregulatory gene (the gene of NKCC) was more greatly downregulated in freshwater-reared *G. urotaenia* than that in the other species. Although we cannot deny that some important hypo-osmoregulatory genes may have been missed, the upregulation of hyper-osmoregulatory genes may be particularly important for freshwater acclimation in *Gymnogobius* larvae.

The present results of the rearing experiment and gene expression analysis further suggest the importance of physiological plasticity for establishing freshwater forms at both individual and population levels. High physiological plasticity is considered to be commonly essential for colonising novel environments (Ghalambor et al., 2007; Lande, 2015). For instance, high osmoregulatory plasticity has possibly facilitated freshwater colonisation in the threespine stickleback (McCairns and Bernatchez, 2010). In the present study, *G. urotaenia* larvae appear to have higher osmoregulatory plasticity than the other species because the former species exhibited a relatively high survival rate in both freshwater and seawater conditions and could largely downregulate the expressions of hyper-osmoregulatory genes (e.g. *aqp3*) in seawater. The high osmoregulatory plasticity was also explained by the greater number of significant DEGs between salinity conditions in *G. urotaenia* (30–246) than that in *G. petschiliensis* (8) and *G. opperiens* (33). The acquisition of osmoregulatory plasticity in larval fish may allow *G. urotaenia* to flexibly choose a freshwater-resident life as an alternative strategy or establish freshwater populations when landlocked by geographic barriers.

The reason the larval habitats of amphidromous species are usually limited to the sea (i.e. ancestral environment) might be explained by their much lower osmoregulatory ability than that of the adult fish, as osmoregulatory organs (gills, kidneys and intestines) are poorly developed at this early life stage (Kaneko et al., 2002; Varsamos et al., 2005). Interspecific variations in the gene expression patterns among the *Gymnogobius* larvae were much larger than intraspecific variations between the salinity groups, implying that the number of molecular mechanisms involved in larval freshwater adaptation is not large. As in the case of *Gymnogobius* species, *aqp3* is already expressed at the early larval stage in some fish species (Deane and Woo, 2006; Cerda and Finn, 2010). The evolution of expression levels of such a few specific genes may be crucial to enhancing the physiological plasticity of early-stage larval fish.

4.3 | Other potential genetic bases for freshwater residence

In addition to our findings, there could be other molecular mechanisms which enhance larval osmoregulatory ability in freshwater conditions. Other studies analysing the transcriptomes in osmoregulatory organs (e.g. gill and intestine) detected >100 DEGs between freshwater- and seawater-reared adults of anadromous species of alewife and puffer fish (Velotta et al., 2017; Nakamura et al., 2020). The present study detected smaller numbers of DEGs between the salinity groups of larval fish from each of the *Gymnogobius* species (9–246, mostly < 50). This could be partly because the osmoregulatory organs of early larval fish are undeveloped as discussed above. Another possibility is that the efficiency of detecting DEGs was decreased by the highly expressed transcripts of non-osmoregulatory tissues in the whole-body transcriptome analysis. Further studies should therefore focus on direct quantification of the expression levels of commonly investigated genes controlling hyper-osmoregulation (e.g. Na^+, Cl^- cotransporter and Na^+/H^+ exchanger; Edwards and Marshall, 2013).

Factors other than the modification of osmoregulatory systems could also be essential for freshwater colonisation by larval fish. One known example is the duplication and enhanced expression level of the FADS2 gene (*fads2*) in marine-originated fishes, which increase their ability to synthesise DHA, dramatically improving larval survival in freshwater (Ishikawa et al., 2019). In the case of *Gymnogobius* ,*G. petschiliensis* larvae with low freshwater tolerance showed a constant, moderate *fads2* expression. Therefore, the DHA synthesis ability is unlikely to be a common restriction factor for freshwater colonisation. However, this might partly explain the absence of landlocked populations of *G. opperiens* within their natural environments, since the larvae exhibited fairly low *fads2*expression.

5 | CONCLUSIONS

In this study, otolith microchemical analysis, physiological experimentation and transcriptome analyses consistently supported the prediction that enhanced physiological plasticity of early larvae is crucial in establishing freshwater forms at both individual and population levels in a marine-originated amphidromous group (*Gymnogobius* gobies). As the genus *Gymnogobius* includes several obligate freshwater species that invade various habitats (Tabata and Watanabe, 2013; Chiba et al., 2020), the evolution of larval osmoregulatory plasticity may have played an important role in the ecological diversification of this genus. This study also found a strong candidate gene, *aqp3* , responsible for freshwater tolerance of*Gymnogobius* larvae, which is one of the few osmoregulatory genes already expressed during early developmental stages in teleost species (Deane and Woo, 2006; Cerda and Finn, 2010). Further experiments are needed to test whether the evolution of *aqp3* and other similar candidate gene expressions are responsible for larval freshwater colonisation by manipulating their expressions.

ACKNOWLEDGMENTS

We thank K. Shirai, Y. Kusaba, K. Kido and A. Goto for their help with the otolith microchemical analysis, S. Kunimatsu for his help with the molecular species identification, M. Nakamura, and Y. Morii for their advice on the manuscript, and members of Laboratory of Animal Ecology of Kyoto University for their helpful comments on the study design. This study was partially funded by JSPS KAKENHI (Grants No. 17H03720 and 18J21793).

REFERENCES

Abdou, A., Keith, P., & Galzin, R. (2015). Freshwater neritids (Mollusca: Gastropoda) of tropical islands: amphidromy as a life cycle, a review. *Revue d'ecologie* , 70, 387–397.

Aizawa, T., Hatsumi, M., & Wakahama, K. I. (1994). Systematic study on the *Chaenogobius* species (family Gobiidae) by analysis of allozyme polymorphisms. *Zoological Science* , 11, 455–464.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* , 215, 403–410.

Anger, K. (2013). Neotropical *Macrobrachium* (Caridea: Palaemonidae): on the biology, origin, and radiation of freshwater-invading shrimp. *Journal of Crustacean Biology* , 33, 151–183.

Augspurger, J. M., Warburton, M., & Closs, G. P. (2017). Life-history plasticity in amphidromous and catadromous fishes: a continuum of strategies. *Reviews in Fish Biology and Fisheries* , 27, 177–192.

Bamber, R. N., & Henderson, P. A. (1988). Pre-adaptive plasticity in atherinids and the estuarine seat of teleost evolution. *Journal of Fish Biology* , 33, 17–23.

Bates, D., Machler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* , 67, 1–48.

Betancur-R, R., Orti, G., & Alexander Pyron, R. (2015). Fossil-based comparative analyses reveal ancient marine ancestry erased by extinction in ray-finned fishes. *Ecology Letters* , 18, 441–450.

Burridge, C. P., & Waters, J. M. (2020). Does migration promote or inhibit diversification? A case study involving the dominant radiation of temperate Southern Hemisphere freshwater fishes. *Evolution* , 74, 1954–1965.

Cerda, J., & Finn, R. N. (2010). Piscine aquaporins: an overview of recent advances. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* , 313, 623–650.

Chiba, S., Kawasaki, T., Yamamoto, K., Chiba, S. N., Shibukawa, K., Mukai, T., & Kokita, T. (2020). Geographical distribution and population genetic structure of the gobiid fish *Gymnogobius* sp. 2, formerly known as *Gymnogobius* cf. *castaneus*"Toyama-endemic". *Ichthyological Research* , 67, 212–220.

Corush, J. B. (2019). Evolutionary patterns of diadromy in fishes: more than a transitional state between marine and freshwater. *BMC Evolutionary Biology* , 19, 168.

Cutler, C. P., Martinez, A. S., & Cramb, G. (2007). The role of aquaporin 3 in teleost fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* , 148, 82–91.

Deane, E. E., & Woo, N. Y. (2006). Tissue distribution, effects of salinity acclimation, and ontogeny of aquaporin 3 in the marine teleost, silver sea bream (*Sparus sarba*). *Marine Biotechnology* , 8, 663–671.

Delgado, M. L., & Ruzzante, D. E. (2020). Investigating diadromy in fishes and its loss in an-omics era. *iScience* , 101837.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T. R., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* , 29, 15–21.

Dodson, J. J., Laroche, J., & Lecomte, F. (2009). Contrasting evolutionary pathways of anadromy in euteleostean fishes. *American Fisheries Society Symposium* , 69, 63–77.

Dotu, Y. (1955). The life history of a goby, *Chaenogobius urotaenia* (Hilgendorf). *Science bulletin of the Faculty of Agriculture, Kyushu University* , 15, 367–374.

Edwards, S. L., & Marshall, W. S. (2013). Principles and patterns of osmoregulation and euryhalinity in fishes. In S. D. McCormick, A. P. Farrell & C. J. Brauner (Eds.), *Fish Physiology: Euryhaline Fishes* (pp. 1–44). New York, NY: Academic Press

Ellingson, R. A., Swift, C. C., Findley, L. T., & Jacobs, D. K. (2014). Convergent evolution of ecomorphological adaptations in geographically isolated Bay gobies (Teleostei: Gobionellidae) of the temperate North Pacific. *Molecular Phylogenetics and Evolution* , 70, 464–477.

Elsdon, T. S., Wells, B. K., Campana, S. E., Gillanders, B. M., Jones, C. M., Limburg, K. E., Secor, D. H., Thorrold, S. R., & Walther, B. D. (2008). Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. *Oceanography and Marine Biology: An Annual Review* , 46, 297–330.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews* , 85, 97–177.

Flemmer, A. W., Monette, M. Y., Djurisic, M., Dowd, B., Darman, R., Gimenez, I., & Forbush, B. (2010). Phosphorylation state of the Na^+–K^+–Cl^-cotransporter (NKCC1) in the gills of Atlantic killifish (*Fundulus heteroclitus*) during acclimation to water of varying salinity.*Journal of Experimental Biology* , 213, 1558–1566.

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments.*Functional Ecology* , 21, 394–407.

Gross, M.R., 1987. Evolution of diadromy in fishes. *American Fisheries Society Symposium* 1: 14–25.

Guo, B., Tang, Z., Wu, C., Xu, K., & Qi, P. (2018). Transcriptomic analysis reveal an efficient osmoregulatory system in Siberian sturgeon*Acipenser baeri* in response to salinity stress. *Scientific Reports* , 8, 14353.

Hamilton, H., Caballero, S., Collins, A. G., & Brownell Jr, R. L. (2001). Evolution of river dolphins. *Proceedings of the Royal Society of London. Series B: Biological Sciences* , 268, 549–556.

Harada, S., Jeon, S. R., Kinoshita, I., Tanaka, M., & Nishida, M. (2002). Phylogenetic relationships of four species of floating gobies (*Gymnogobius*) as inferred from partial mitochondrial cytochrome*b* gene sequences. *Ichthyological Research* , 49, 324–332.

Iida, M., Watanabe, S., Yamada, Y., Lord, C., Keith, P., & Tsukamoto, K. (2010). Survival and behavioral characteristics of amphidromous goby larvae of *Sicyopterus japonicus* (Tanaka, 1909) during their downstream migration. *Journal of Experimental Marine Biology and Ecology* , 383, 17–22.

Iida, M., Kondo, M., Tabouret, H., Maeda, K., Pecheyran, C., Hagiwara, A., Keith, P., & Tachihara, K. (2017). Specific gravity and migratory patterns of amphidromous gobioid fish from Okinawa Island, Japan.*Journal of Experimental Marine Biology and Ecology* , 486, 160–169.

Ishikawa, A., Kabeya, N., Ikeya, K., Kakioka, R., Cech, J. N., Osada, N., Leal, M. C., Inoue, J., Kume, M., Toyoda, A., Tezuka, A., Nagano, A. J., Yamasaki, Y. Y., Suzuki, Y., Kokita, T., Takahashi, H., Lucek, K., Marques, D., Takehana, Y., Naruse, K., Mori, S., Monroig, O., Ladd, N., Schubert, C. J., Matthews, B., Peichel, C. L., Seehausen, O., Yoshizaki, G., & Kitano, J. (2019). A key metabolic gene for recurrent freshwater colonization and radiation in fishes. *Science* , 364, 886–889.

Ishino, K. (2005). *Gymnogobius* . In H. Kawanabe, N. Mizuno & K. Hosoya (Eds.), *Freshwater Fishes of Japan* (3rd ed.) (pp. 618). Tokyo: Yama- to- keikoku- sha.

Ito, R. K., Harada, S., Tabata, R., & Watanabe, K. (2022). Molecular evolution and convergence of the rhodopsin gene in *Gymnogobius* , a goby group having diverged into coastal to freshwater habitats.*Journal of Evolutionary Biology* , 35, 333–346.

Kaneko, T., Shiraishi, K., Katoh, F., Hasegawa, S., & Hiroi, J. (2002). Chloride cells during early life stages of fish and their functional differentiation. *Fisheries Science* , 68, 1–9.

Kaplan, E. L., & Meier, P. (1958). Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association* , 53, 457–481.

Kusakabe, M., Ishikawa, A., Ravinet, M., Yoshida, K., Makino, T., Toyoda, A., Fujiyama, A., & Kitano, J. (2017). Genetic basis for variation in salinity tolerance between stickleback ecotypes.*Molecular Ecology* , 26, 304–319.

Lande, R. (2015). Evolution of phenotypic plasticity in colonizing species. *Molecular Ecology* , 24, 2038–2045.

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. *Trends in Ecology & Evolution* , 14, 284–288.

Leveque, C., Oberdorff, T., PAUGy, D., Stiassny, M. L. J., & Tedesco, P. A. (2007). Global diversity of fish (Pisces) in freshwater.*Hydrobiologia* , 595, 545–567.

Lin, G., Zheng, M., Gao, D., Li, S., Fang, W., Huang, J., Xie, J., Liu, J., Liu, Y., Li, Z., & Lu, J. (2020). Hypoosmotic stress induced tissue-specific immune responses of yellowfin seabream (*Acanthopagrus latus*) revealed by transcriptomic analysis.*Fish & Shellfish Immunology* , 99, 473–482.

Losos, J. B. (2010). Adaptive radiation, ecological opportunity, and evolutionary determinism: American Society of Naturalists EO Wilson Award address. *The American Naturalist* , 175, 623–639.

McCairns, R. S., & Bernatchez, L. (2010). Adaptive divergence between freshwater and marine sticklebacks: insights into the role of phenotypic plasticity from an integrated analysis of candidate gene expression.*Evolution: International Journal of Organic Evolution* , 64, 1029–1047.

McDowall, R. M., (1997). The evolution of diadromy in fishes (revisited) and its place in phylogenetic analysis. *Reviews in Fish Biology and Fisheries* 7, 443–462.

McDowall, R. M. (2001). Diadromy, diversity and divergence: implications for speciation processes in fishes. *Fish and Fisheries* , 2, 278–285.

Nakada, T., Zandi-Nejad, K., Kurita, Y., Kudo, H., Broumand, V., Kwon, C. Y., Mercado, A., Mount, D. B., & Hirose, S. (2005). Roles of Slc13a1 and Slc26a1 sulfate transporters of eel kidney in sulfate homeostasis and osmoregulation in freshwater. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* , 289, R575–R585.

Nakamura, M., Jiang, T., Xu, G., Yang, J., Xu, P., Watanabe, S., Kaneko, T., Koyama, T., Kikuchi, K., Tsukamoto, K., & Otake, T. (2020). Capacity for freshwater acclimation and differences in the transcription

of ion transporter genes underlying different migratory life histories of *Takifugu* fish. *Gene* , 145285.

Oto, Y. (2021). Distribution of adult fish and spawning nests of estuarine-dependent amphidromous goby (*Gymnogobius petschiliensis*) in two streams of central Japan. *Marine and Freshwater Research* .

R Core Team (2021). *R: A language and environment for statistical computing* . Vienna, Austria: R Foundation for Statistical Computing. R version 4.1.0.

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* , 26, 139–140.

Rome, N. E., Conner, S. L., & Bauer, R. T. (2009). Delivery of hatching larvae to estuaries by an amphidromous river shrimp: tests of hypotheses based on larval moulting and distribution. *Freshwater Biology* , 54, 1924–1932.

Schluter, D. (2000). *The ecology of adaptive radiation* . OUP Oxford.

Shaw, J., Sato, D., VanderHeide, J., LaCasse, T., Stanton, C., Lankowski, A., E. Stanton, S. E., Chapline, C., Coutermarsh, B., Barnaby, R., & Karlson, K. (2008). The role of SGK and CFTR in acute adaptation to seawater in *Fundulus heteroclitus* . *Cellular Physiology and Biochemistry* , 22, 069–078.

Shrimpton, J. M. (2013). Seawater to freshwater transitions in diadromous fishes. In S. D. McCormick, A. P. Farrell & C. J. Brauner (Eds.), *Fish Physiology: Euryhaline Fishes* (pp. 327–393). New York, NY: Academic Press

Sota, T., Mukai, T., Shinozaki, T., Sato, H., & Yodoe, K. I. (2005). Genetic differentiation of the gobies *Gymnogobius castaneus* and*G. taranetzi* in the region surrounding the Sea of Japan as inferred from a mitochondrial gene genealogy. *Zoological Science* , 22, 87–93.

Stelzer, G., Rosen, N., Plaschkes, I., Zimmerman, S., Twik, M., Fishilevich, S., Iny Stein, T., Nudel, R., Lieder, I., Mazor, Y., Kaplan, S., Dahary, D., Warshawsky, D., Guan-Golan, Y., Kohn, A., Rappaport, N., Safran, M., & Lancet, D. (2016). The GeneCards suite: from gene data mining to disease genome sequence analyses. *Current Protocols in Bioinformatics* , 54, 1.30.1–1.30.33.

Stevens, M. I., & Hicks, B. J. (2009). Mitochondrial DNA reveals monophyly of New Zealand's *Gobiomorphus* (Teleostei: Eleotridae) amongst a morphological complex. *Evolutionary Ecology Research* , 11, 109–123.

Stevenson, D. E. (2002). Discovery of the holotype of *Chaenogobius annularis* Gill (Perciformes: Gobiidae) and its taxonomic consequences.*Copeia* , 2000, 835–840.

Su, W., Sun, J., Shimizu, K., & Kadota, K. (2019). TCC-GUI: a Shiny-based application for differential expression analysis of RNA-Seq count data. *BMC Research Notes* , 12, 133.

Tabata, R., & Watanabe, K. (2013). Hidden mitochondrial DNA divergence in the Lake Biwa endemic goby *Gymnogobius isaza* : implications for its evolutionary history. *Environmental Biology of Fishes* , 96, 701–712.

Therneau, T. (2021). A package for survival analysis in R. *R package version 3.2-10* .

Therneau, T. M., & Grambsch, P. M. (2000). The cox model. In*Modeling Survival Data: Extending the Cox Model* (pp. 39–77). New York, NY: Springer.

Tipsmark, C. K., Kiilerich, P., Nilsen, T. O., Ebbesson, L. O., Stefansson, S. O., & Madsen, S. S. (2008). Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* , 294, R1563–R1574.

Tsukamoto, K., Aoyama, J., & Miller, M. J. (2002). Migration, speciation, and the evolution of diadromy in anguillid eels.*Canadian Journal of Fisheries and Aquatic Sciences*, 59, 1989–1998.

Tsunagawa, T., & Arai, T. (2008). Flexible migration of Japanese freshwater gobies *Rhinogobius* spp. as revealed by otolith Sr: Ca ratios. *Journal of Fish Biology* , 73, 2421–2433.

Varsamos, S., Nebel, C., & Charmantier, G. (2005). Ontogeny of osmoregulation in postembryonic fish: a review. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* , 141, 401–429.

Velotta, J. P., McCormick, S. D., & Schultz, E. T. (2015). Trade-offs in osmoregulation and parallel shifts in molecular function follow ecological transitions to freshwater in the Alewife. *Evolution* , 69, 2676–2688.

Velotta, J. P., Wegrzyn, J. L., Ginzburg, S., Kang, L., Czesny, S., O'Neill, R. J., McCormick, S. D., Michalak, P., & Schultz, E. T. (2017). Transcriptomic imprints of adaptation to fresh water: parallel evolution of osmoregulatory gene expression in the Alewife.*Molecular Ecology* , 26, 831–848.

Waters, J. M., & Wallis, G. P. (2001). Cladogenesis and loss of the marine life-history phase in freshwater galaxiid fishes (Osmeriformes: Galaxiidae). *Evolution* , 55, 587–597.

Waters, J. M., Emerson, B. C., Arribas, P., & McCulloch, G. A. (2020). Dispersal reduction: causes, genomic mechanisms, and evolutionary consequences. *Trends in Ecology & Evolution* , 35, 512–522.

Whitehead, A., Roach, J. L., Zhang, S., & Galvez, F. (2011). Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *Proceedings of the National Academy of Sciences of the United States of America* , 108, 6193–6198.

Yamasaki, Y. Y., Nishida, M., Suzuki, T., Mukai, T., & Watanabe, K. (2015). Phylogeny, hybridization, and life history evolution of*Rhinogobius* gobies in Japan, inferred from multiple nuclear gene sequences. *Molecular Phylogenetics and Evolution* , 90, 20–33.

DATA ACCESSIBILITY AND BENEFIT-SHARING

Data accessibility statement

The raw RNA-Seq data are deposited at DNA Data Bank of Japan (DDBJ) under BioProject PRJDB12996 (SAMD00442503–00442532). The registration of the raw data for the survival time analysis and osmoregulatory gene expression analysis are in progress in DRYAD (https://datadryad.org).

Benefit-sharing statement

This study was partially funded by JSPS KAKENHI (Grants No. 17H03720 and 18J21793).

AUTHOR CONTRIBUTIONS

Y.O. and K.W conceived the presented idea. Y.O. performed the fish sampling and rearing experiments. Y.O., M.I. and M.K. conducted the otolith microchemical analysis. Y.O., R.I. and S.N. conceived the detailed design of the gene expression analysis and executed this analysis. Y.O. and K.W wrote the manuscript in consultation with the other co-authors.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Figure captions

Fig. 1 Life history evolution in *Gymnogobius* . (a) Life history patterns of *Gymnogobius* gobies mapped on a phylogenetic tree based on nuclear genes (Aizawa et al., 1994; Ito et al., unpublished data with transcriptomes), which includes a clade with one freshwater (*G. isaza*) and three amphidromous species (*G.petschiliensis, G. opperiens* and *G. urotaenia*) with different freshwater dependences. Note that alternative phylogenetic relationships are also inferred from the mitochondrial gene sequences, in which *G. urotaenia* and *G. petschiliensis* are sister species and form a monophyletic group with *G. isaza* (Harada et al., 2002; Sota et al., 2005). (b) Schematic diagram of this study. Our goal was to define candidate

genes for larval physiological plasticity and freshwater colonisation in *Gymnogobius* . First, we compared the migratory patterns and larval survivorships linked to salinities among the targeted *Gymnogobius* populations. We then screened differentially expressed genes (DEGs) between freshwater and seawater conditions of each species by RNA-Seq and identified candidate genes for larval osmoregulatory plasticity from the two analytical perspectives.

Fig. 2 Migratory history of the three amphidromous species (2–3 populations for each species) inferred by otolith chemical analysis in this study (the data was smoothed by the loess function in R). The dashed line indicates an approximate boundary value of the otolith strontium/calcium (Sr/Ca) ratios when individuals were in marine (> 4) or freshwater environments (< 4) (Tsunagawa and Arai, 2008; Iida et al., 2017). For detailed information on sampling sites, see Table S1.

Fig. 3 Survival curves estimated by the Kaplan-Meier method, where larval survival was compared (a) between salinity conditions for each species and (b) among the three species for each salinity condition. The x- and y-axes provide the number of days elapsed since hatching and survival probability, respectively. Dashed and solid lines indicate the estimated survival curves in freshwater and seawater conditions, respectively. Coloured area around the estimated curve provides the 95% confidence interval.

Fig. 4 Summarised results from the gene expression analyses for larvae from three *Gymnogobius* species. (a) Overall gene expression patterns of the target species compared using principal components analysis (PCA) for the top 100 variably expressed genes among all 30 samples. The samples were clustered into the three groups based on PC1 and PC2 values by the k -means method (surrounded by broken lines). (b) Venn diagrams showing the commonality of differentially expressed genes (DEGs) between the two salinity groups for each species. Regions with no numbers indicate that there was no corresponding DEGs.

Fig. 5 Differentially expressed genes (DEGs) associated with hyper- and hypo-osmoregulation for larvae of three *Gymnogobius*species. The labels in parentheses next to the italicised gene names indicate the proteins encoded by each gene. Solid lines indicate significant DEGs for each species (broken lines, not significant). Asterisks indicate the statistical significance of the interaction between the presence of freshwater forms and the magnitude of the gene expression changes in each species (LMM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; see Table S8 for details).

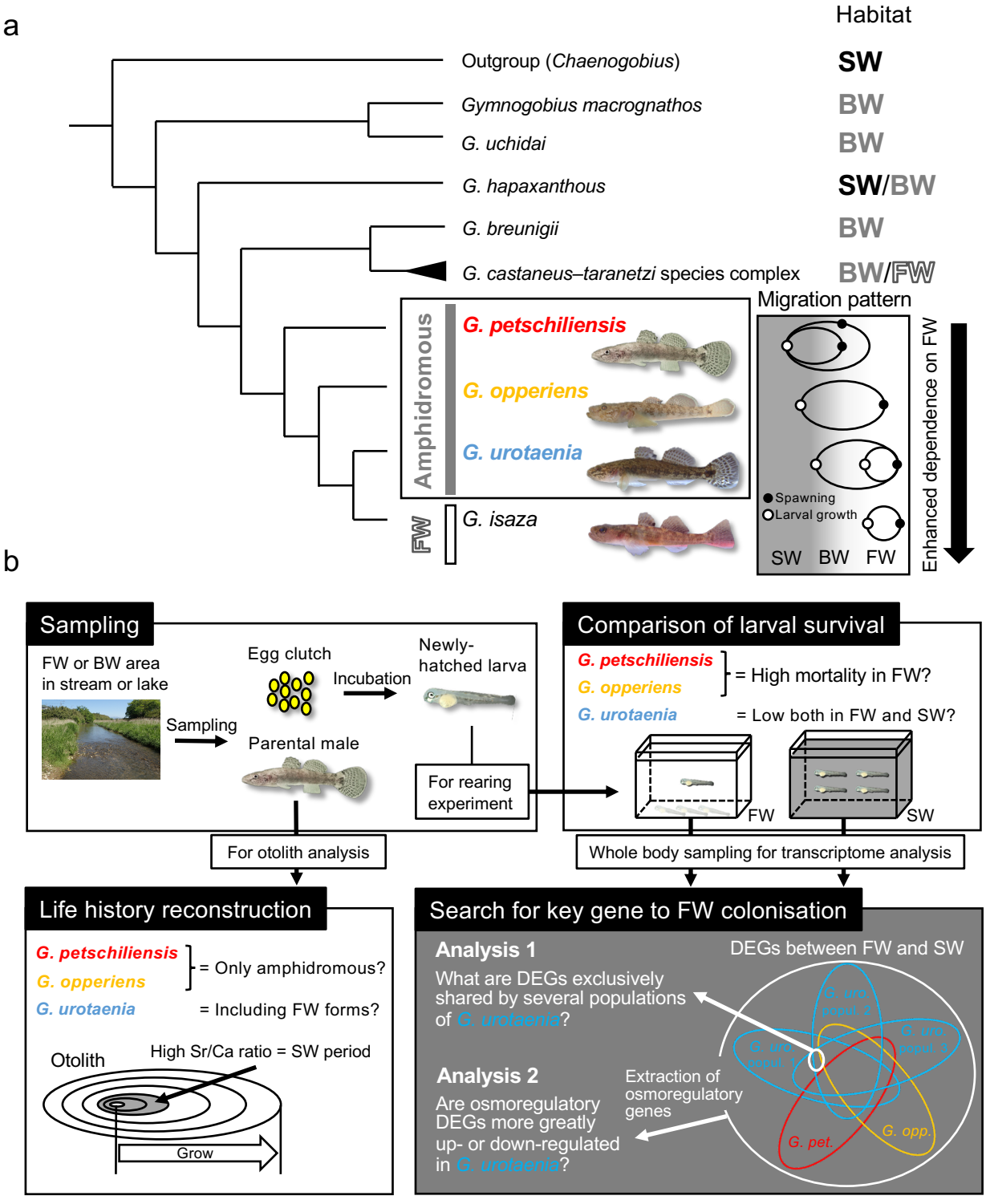
a
Habitat
Outgroup (Chaenogobius)
SW
Gymnogobius macrognathos
BW
G. uchidai
BW
G. hapaxanthous
SW/BW
G. breunigii
BW
G. castaneus–taranetzi species complex
BW/FW
Amphidromous
G. petschiliensis
G. opperiens
G. urotaenia
FW
G. isaza
Migration pattern
Spawning
Larval growth
SW BW FW
Enhanced dependence on FW
b
Sampling
FW or BW area in stream or lake
Sampling
Egg clutch
Incubation
Newly-hatched larva
Parental male
For rearing experiment
For otolith analysis
Comparison of larval survival
G. petschiliensis
G. opperiens
G. urotaenia
= High mortality in FW?
= Low both in FW and SW?
FW
SW
Whole body sampling for transcriptome analysis
Life history reconstruction
G. petschiliensis
G. opperiens
G. urotaenia
= Only amphidromous?
= Including FW forms?
Otolith
High Sr/Ca ratio = SW period
Grow
Search for key gene to FW colonisation
Analysis 1
What are DEGs exclusively shared by several populations of G. urotaenia?
DEGs between FW and SW
G. uro. popul. 1
G. uro. popul. 2
G. uro. popul. 3
G. pet.
G. opp.
Analysis 2
Are osmoregulatory DEGs more greatly up- or down-regulated in G. urotaenia?
Extraction of osmoregulatory genes

Fig. 1

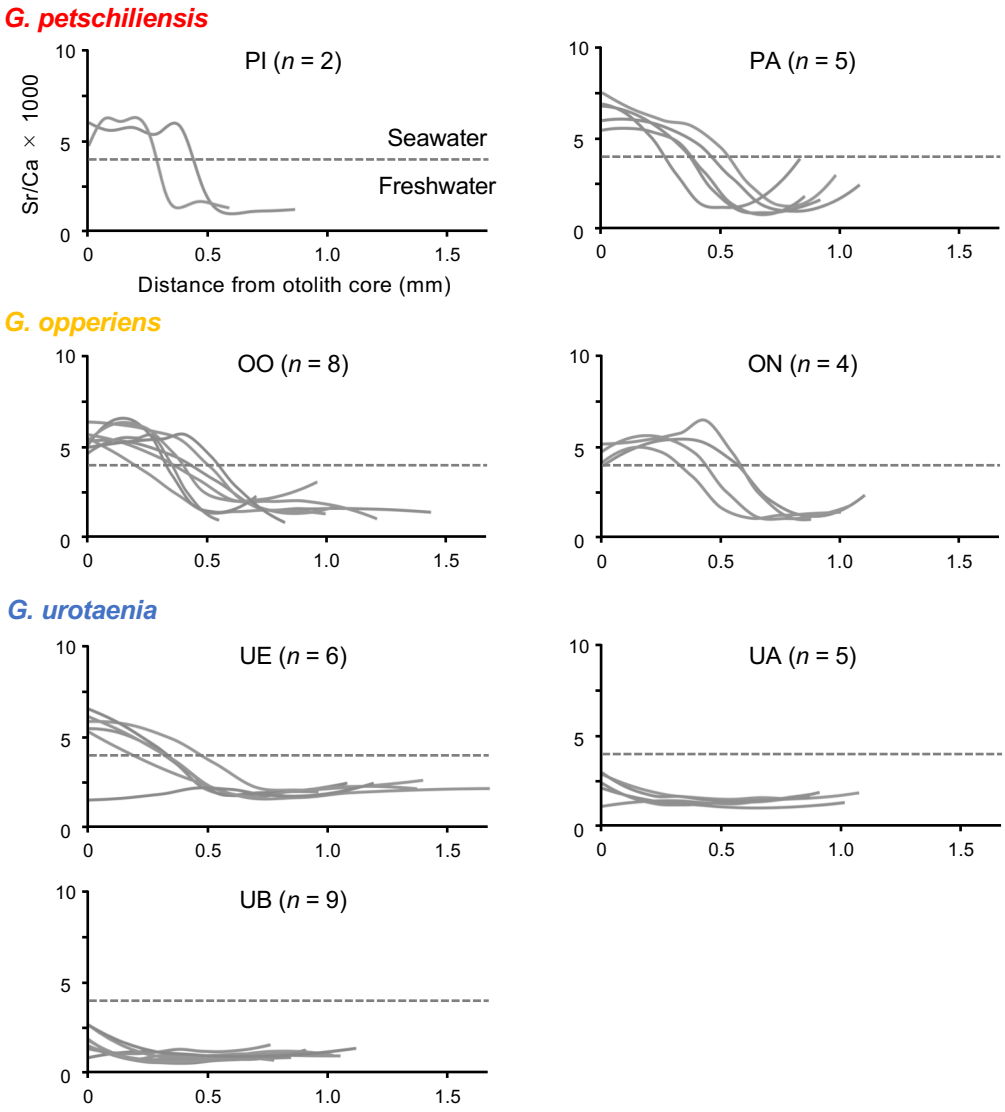
G. petschiliensis
PI (n = 2)
PA (n = 5)
Sr/Ca × 1000
Seawater
Freshwater
Distance from otolith core (mm)
G. opperiens
OO (n = 8)
ON (n = 4)
G. urotaenia
UE (n = 6)
UA (n = 5)
UB (n = 9)

Fig. 2

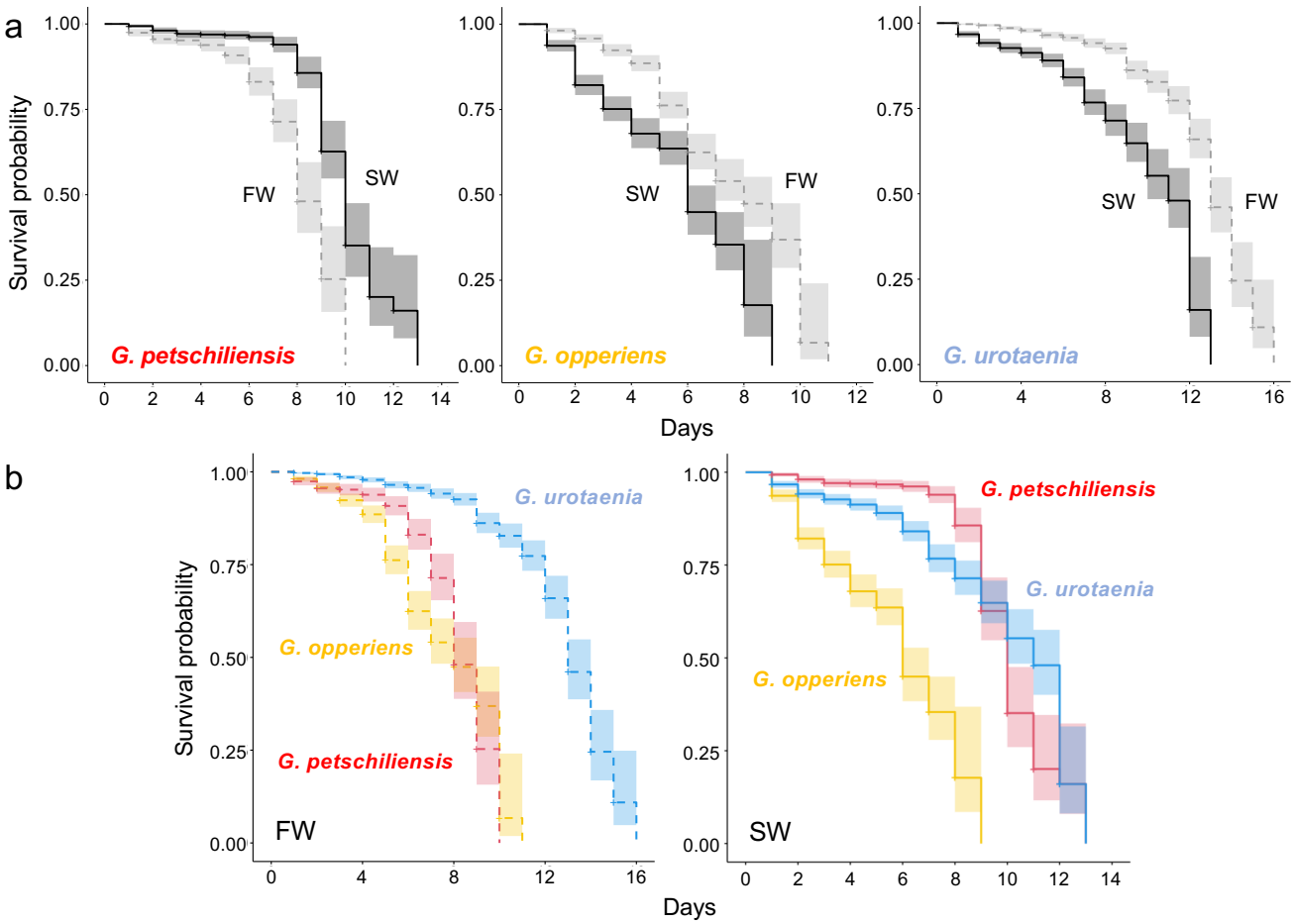
a
Survival probability
1.00
0.75
0.50
0.25
0.00
FW
SW
G. petschiliensis
G. opperiens
G. urotaenia
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12
0 4 8 12 16
Days
b
G. urotaenia
G. opperiens
G. petschiliensis
FW
SW
0 4 8 12 16
0 2 4 6 8 10 12 14

Fig. 3

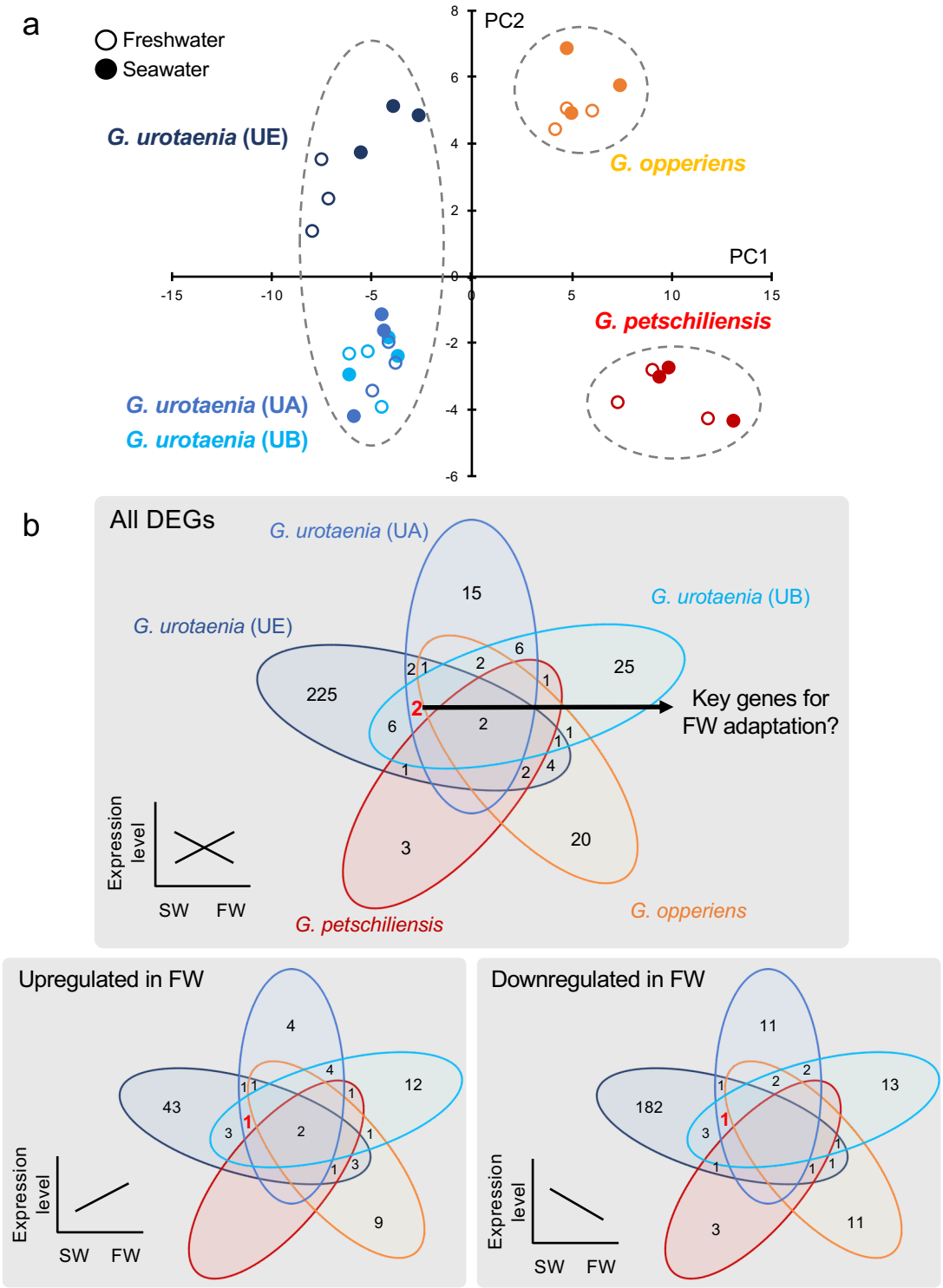
a
Freshwater
Seawater
G. urotaenia (UE)
G. urotaenia (UA)
G. urotaenia (UB)
G. opperiens
G. petschiliensis
PC1
PC2
b
All DEGs
G. urotaenia (UA)
G. urotaenia (UB)
G. urotaenia (UE)
G. petschiliensis
G. opperiens
Key genes for FW adaptation?
Expression level
SW
FW
Upregulated in FW
Downregulated in FW

Fig. 4

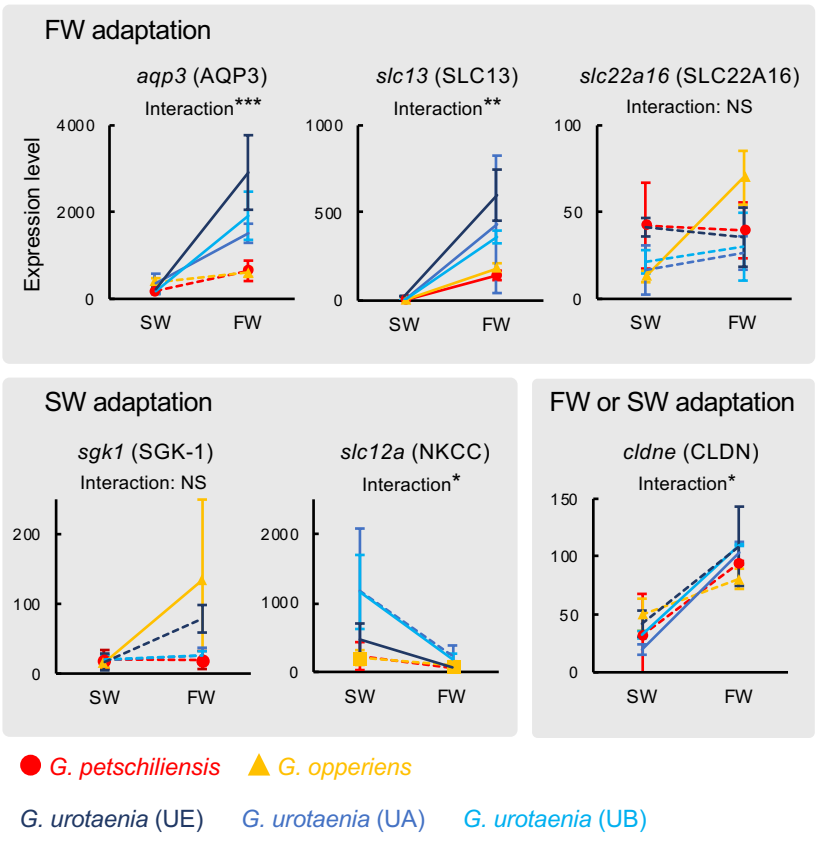
FW adaptation
aqp3 (AQP3)
Interaction***
slc13 (SLC13)
Interaction**
slc22a16 (SLC22A16)
Interaction: NS
Expression level
4000
2000
0
1000
500
100
50
SW
FW
SW adaptation
sgk1 (SGK-1)
Interaction: NS
slc12a (NKCC)
Interaction*
200
100
2000
1000
FW or SW adaptation
cldne (CLDN)
Interaction*
150
G. petschiliensis
G. opperiens
G. urotaenia (UE)
G. urotaenia (UA)
G. urotaenia (UB)

Fig. 5