ADAPTIVE RADIATIONS

A key metabolic gene for recurrent freshwater colonization and radiation in fishes

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Colonization of new ecological niches has triggered large adaptive radiations. Although some lineages have made use of such opportunities, not all do so. The factors causing this variation among lineages are largely unknown. Here, we show that deficiency in docosahexaenoic acid (DHA), an essential ω-3 fatty acid, can constrain freshwater colonization by marine fishes. Our genomic analyses revealed multiple independent duplications of the fatty acid desaturase gene *Fads2* in stickleback lineages that subsequently colonized and radiated in freshwater habitats, but not in close relatives that failed to colonize. Transgenic manipulation of *Fads2* in marine stickleback increased their ability to synthesize DHA and survive on DHA-deficient diets. Multiple freshwater ray-finned fishes also show a convergent increase in *Fads2* copies, indicating its key role in freshwater colonization.

Empty niches can provide organisms with ecological opportunities to diversify (*1*–*3*). Many of the known large adaptive radiations followed invasion of underutilized habitats (*1*, *2*). However, not all lineages appear to take advantage of such opportunities. For example, habitat shifts from marine to freshwater environments have repeatedly triggered radiations, but only in a limited number of fish lineages (*4*–*6*). The physiological and genetic factors causing this variation are unknown.

One of the underappreciated constraints for freshwater colonization by marine animals is the poor nutritional quality of food in freshwater ecosystems. Generally, the food chain in marine environments is rich in ω-3 long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA) (fig. S1A) (*7*), which is essential for animal development and health (*8*, *9*). However, freshwater ecosystems contain very little DHA (fig. S1A) (*7*). Here, we tested and confirmed that DHA deficiency can constrain freshwater colonization by marine fishes and identified genetic changes that appear to have enabled some lineages to overcome this constraint.

Three-spined stickleback (*Gasterosteus aculeatus* species complex) are primarily marine or anadromous (hereafter, we call both marine) fishes, but when new freshwater habitats emerged after glacial retreat, they successfully colonized freshwater habitats and radiated into diverse ecotypes on multiple continents (*10*) (Fig. 1, fig. S2, and tables S1 and S2). By contrast, the closely related Japan Sea stickleback (*G. nipponicus*), which diverged from *G. aculeatus* ~0.68 to 1.5 million years (Ma) ago (*11*), failed to colonize freshwater environments and remains phenotypically homogeneous (Fig. 1). Although *G. nipponicus* co-occur with Pacific Ocean populations of *G. aculeatus* (Pacific Ocean stickleback) in some localities in Japan (*12*–*14*), have geographical access to many freshwater habitats, and can use freshwater environments for spawning (*14*), our phylogenomic analysis showed that all known Japanese freshwater populations belong to *G. aculeatus* (Fig. 1C and fig. S3) (*15*–*17*).

Because stickleback prey differs in DHA levels between marine and freshwater habitats (fig. S1B), we tested the hypothesis that Japan Sea stickleback may have a lower physiological ability than Pacific Ocean stickleback to survive on DHA-free diets (*18*). Our rearing experiments showed that, irrespective of salinity, Japan Sea stickleback had higher mortality than Pacific Ocean stickleback starting ~40 days after fertilization when fed DHA-free *Artemia* ($P < 0.01$) (Fig. 2A, fig. S4, and table S3); this age is close to the timing of seaward migration in nature (*19*). Marine-derived diets or *Artemia* enriched with several fatty acids, including DHA, significantly improved survival of Japan Sea stickleback ($P < 0.01$) (Fig. 2, A and B). Further, Japan Sea stickleback had a lower DHA content than Pacific Ocean stickleback when fed DHA-free *Artemia* ($P < 0.01$ for both the brain and eye) (Fig. 2C and fig. S5), suggesting that they have either lower DHA biosynthetic capabilities or higher rates of DHA degradation or secretion.

Our whole-genome resequencing revealed that *Fatty acid desaturase 2* (*Fads2*), a gene encoding a key enzyme catalyzing desaturation in DHA biosynthesis (fig. S6 and table S4) (*20*–*22*), has a higher copy number in Pacific Ocean stickleback than in Japan Sea stickleback (Fig. 2D and fig. S7) ($F_{1,12} = 79.8$, $P < 0.01$). Higher *Fads2* copy numbers in females compared with males ($F_{1,12} = 11.7$, $P < 0.01$) (Fig. 2D and fig. S7) are due to the X linkage of *Fads2* (see below). RNA sequencing further revealed that Pacific Ocean stickleback express *Fads2* at higher levels than Japan Sea stickleback ($F_{1,12} = 5.3$, $P < 0.05$ for brain; $F_{1,12} = 7.0$, $P < 0.05$ for eyes) when fed only DHA-free *Artemia* (Fig. 2E and fig. S8).

To directly demonstrate the effects of *Fads2* copy number on survival, we made transgenic Japan Sea stickleback overexpressing *Fads2*. When fed only DHA-free *Artemia*, the *Fads2*-trangenics showed higher survival rates (Fig. 2F) and higher DHA content at 40 days after fertilization than the control *GFP*-transgenics ($P < 0.01$) (Fig. 2G and fig. S9). Analysis of an F_2 intercross between Pacific Ocean and Japan Sea sticklebacks further showed that hybrids with higher *Fads2* copy number had higher survival rates at 40 to 60 days after fertilization ($P < 0.01$: 10.0 to 12.1% of variance explained) and longer overall life span ($P < 0.05$) (fig. S10). The higher survival rate of females compared with males in Japan Sea stickleback is consistent with the higher *Fads2* copy number in females ($P < 0.01$) (fig. S11). These data

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suggest that the lower *Fads2* copy number may be a constraint to colonization of DHA-deficient freshwater niches by Japan Sea stickleback.

Fluorescence in situ hybridization (FISH) revealed that *Fads2* was located only on the X chromosome [linkage group (LG) 19] in Japan Sea stickleback, but on LG12 and LG19 in Pacific Ocean stickleback (Fig. 3A). This result was confirmed by linkage analysis of *Fads2* copy number using an F_2 intercross (fig. S12). Genes flanking *Fads2* on LG19, but not on LG12, showed conserved synteny with other teleosts (fig. S13). Furthermore, an outgroup, *G. wheatlandi*, another marine stickleback with no known freshwater populations (*10*), has *Fads2* on LG19 but not on

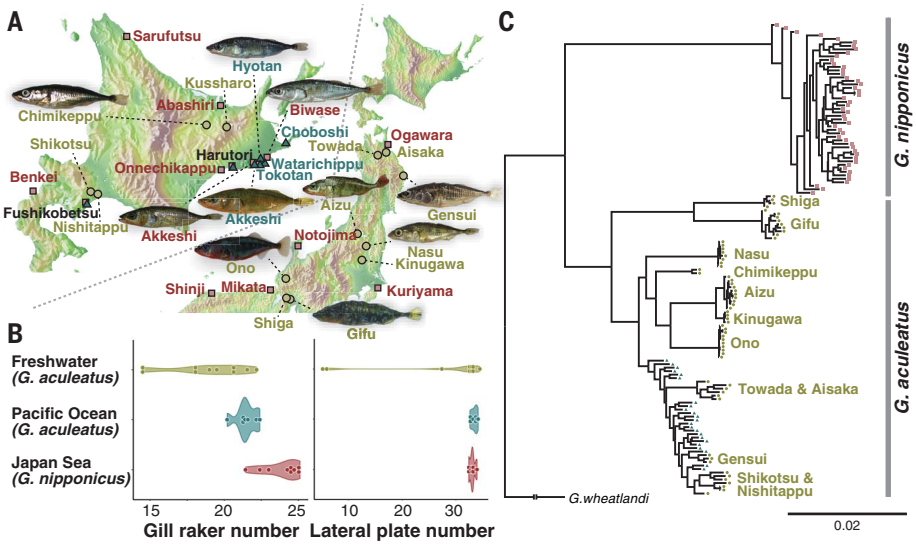

Fig. 1. Freshwater colonization and diversification in *G. aculeatus* but not in *G. nipponicus*. (**A**) Sampling sites in Japan: pink squares, *G. nipponicus*; blue triangles, Pacific Ocean populations of *G. aculeatus*; green circles, freshwater populations of *G. aculeatus*. (**B**) Diversification of key foraging (gill raker number) and armor traits (lateral plate number) in freshwater populations. (**C**) Double-digest restriction site–associated DNA sequencing (ddRAD-seq) phylogeny of Japanese *Gasterosteus* indicating that all freshwater populations (green circles) belonged to *G. aculeatus* rather than *G. nipponicus* (pink squares). Blue triangles indicate Pacific Ocean marine populations of *G. aculeatus*. Bar shows the substitution rate. For bootstrap values, see fig. S3.

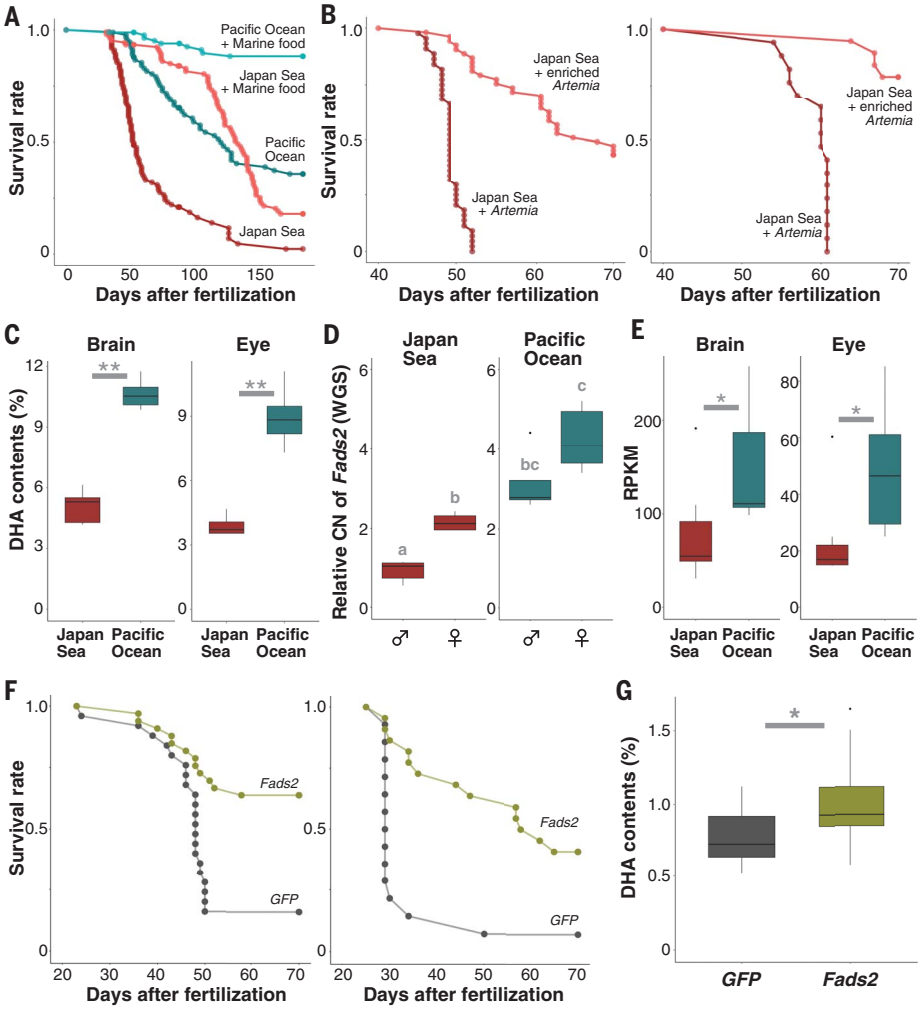

Fig. 2. Contribution of higher *Fads2* copy numbers to survival with DHA-free diets. (**A**) Survival curves of Japan Sea (red) and Pacific Ocean (blue) stickleback fed only DHA-free *Artemia* and Japan Sea (pink) and Pacific Ocean (light blue) stickleback fed marine-derived diets. (**B**) Survival curves of Japan Sea stickleback fed DHA-free *Artemia* (dark red) or *Artemia* enriched with several fatty acids, including DHA (pink). The two panels indicate independent replicate crosses. (**C**) DHA contents in the brain and eye of two species fed only DHA-free *Artemia*. $^{**}P < 0.01$. (**D**) Relative copy numbers of *Fads2* in males and females of Japan Sea and Pacific Ocean sticklebacks estimated from whole-genome resequencing (WGS) data. Different letters above the boxes indicate significantly different pairs ($P < 0.05$). (**E**) Expression levels of *Fads2* at 40 to 60 days after fertilization. $^{*}P < 0.05$. (**F**) Survival curves of *Fads2*-transgenic (yellow-green) and *GFP*-transgenic (gray) Japan Sea stickleback fed only DHA-free *Artemia*. The two panels indicate independent replicate crosses. (**G**) Whole-body DHA content of *Fads2*-transgenics (yellow-green) and *GFP*-transgenics (gray) at 40 days after fertilization, when Japan Sea stickleback start to die on DHA-free diets. $^{*}P < 0.05$. RPKM, reads per kilobase per million reads.

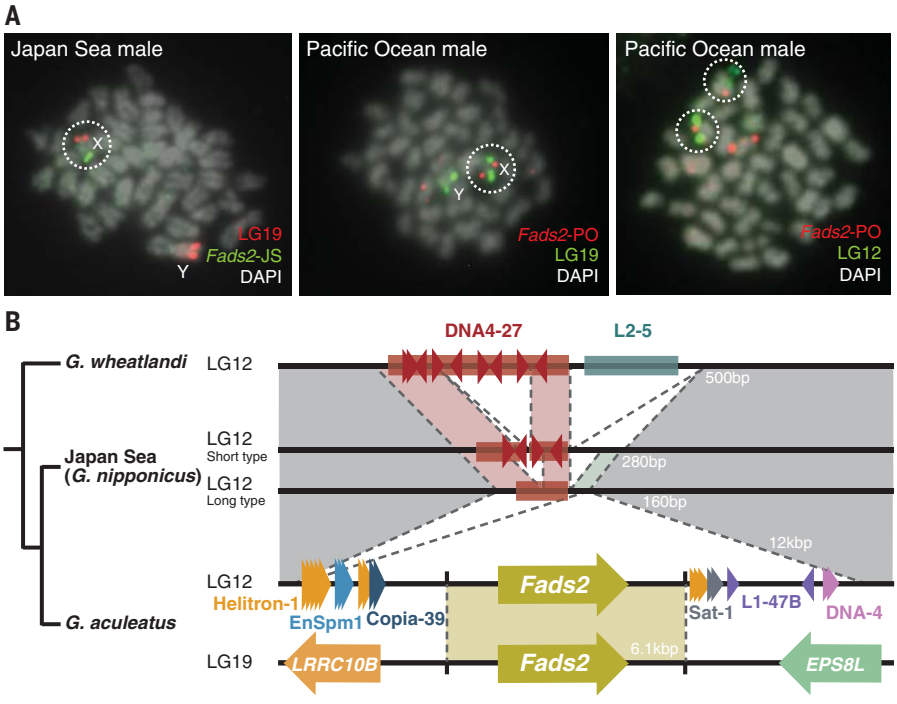

Fig. 3. Extra copy of *Fads2* on LG12 in *G. aculeatus*. (**A**) FISH results with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining. The left panel indicates a Japan Sea stickleback male with one *Fads2* copy (green) on the X chromosome (circled). In a Pacific Ocean stickleback male, *Fads2* (red) was located on the X chromosome (circle in the middle panel) and both copies of autosomal LG12 (circles in the right panel). The colors for *Fads2* and LG19 or LG12 are flipped between the Japan Sea and Pacific Ocean males. Note that LG19 is either the X or Y chromosome, and the LG19 probe detects the region retained on the Y chromosome. (**B**) Genome structure around *Fads2* on LG19 and LG12 of *G. aculeatus* and the corresponding region on LG12 of *G. nipponicus* and *G. wheatlandi*. Arrows and arrowheads indicate genes and repetitive sequences, respectively. White numbers indicate insertion size.

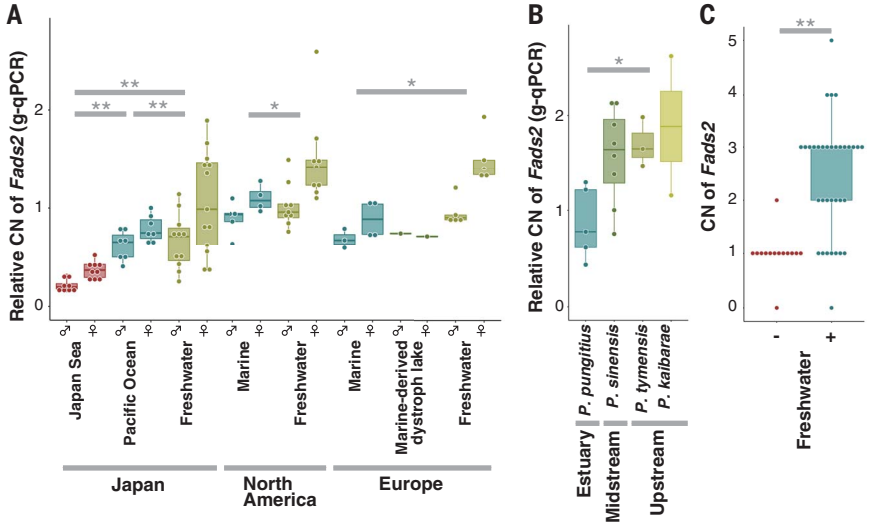

Fig. 4. Parallel increase in *Fads2* copies in freshwater fishes. (**A**) Relative copy numbers (CNs) of *Fads2* in males and females of *Gasterosteus* populations: red, Japan Sea; blue, Japanese Pacific Ocean, North American and European marine ecotypes; green, freshwater ecotypes. *$P < 0.05$, **$P < 0.01$. g-qPCR, genomic quantitative real-time polymerase chain reaction. (**B**) Relative CN of *Fads2* in *Pungitius*. A single dot indicates the median of a single population in (A) and (B). (**C**) Comparison of *Fads2* CNs among ray-finned fishes living in marine (−) or freshwater (+) niches.

LG12 (table S5) and copy numbers similar to those of Japan Sea stickleback (fig. S14). Thus, LG19 is the ancestral location of *Fads2*, and copy-and-paste transposition of *Fads2* from LG19 to LG12 increased the ability to synthesize DHA in *G. aculeatus*, but not in *G. nipponicus* or *G. wheatlandi*. At the LG12 locus, where a 12-kb insertion containing *Fads2* and several types of transposons exist in *G. aculeatus* (Fig. 3B and fig. S15), *G. wheatlandi* and *G. nipponicus* possess transposons without *Fads2* (figs. S16 to S18). This suggests that transposons might have mediated the *Fads2* transposition and/or that this locus is a hot spot of insertion–deletion mutations (*23*).

The estimated timing of *Fads2* duplication within *G. aculeatus* is 0.80 Ma ago (95% highest posterior density: 0.47 to 1.16 Ma ago) (fig. S19), which is much earlier than the end of the last glacial period (0.012 Ma ago), when most stickleback freshwater colonization occurred (*1*, *10*). Marine sticklebacks from western North America and Europe also repeatedly colonized freshwater habitats and radiated into diverse ecotypes (*10*). Our results show that they also have the extra copy of *Fads2* on LG12, with copy numbers similar to those of the Pacific Ocean stickleback in Japan (Fig. 4A and figs. S16 and S22C to S22F). These data confirm that transposition onto LG12 occurred before the split between the Pacific and the Atlantic Ocean lineages (0.3 to 0.5 Ma ago) (*24*). Thus, the preexisting duplication of *Fads2* has likely given *G. aculeatus* an advantage over other *Gasterosteus* species in colonizing freshwater habitats. However, our estimate suggests that *Fads2* on LG12 is younger than the oldest known freshwater *Gasterosteus* fossil (*10*). Ancient extinct freshwater species may therefore have possessed additional *Fads2* copies somewhere in the genome or adapted to DHA-deprived diets through other mutations.

To investigate whether there are any other loci involved in survivorship on DHA-deficient diets, we conducted quantitative trait locus (QTL) mapping of survival rates using an F_2 intercross between the Pacific Ocean and Japan Sea sticklebacks. In addition to a suggestive QTL overlapping the *Fads2* gene on LG12 (3.3% of variance in survival explained), one significant and two additional suggestive QTLs were found on different autosomes (fig. S20). The QTL on LG12, but not other QTLs, explained the *Fads2* copy-number variation. Two other QTLs, including a significant one, showed overdominance rather than additive effects on survival, which may reflect an epistatic interaction between interspecies alleles (*25*) (fig. S21). Although survival rate is a polygenic and complex trait, our unbiased QTL analysis confirmed that the additional *Fads2* copy of the Pacific Ocean stickleback on LG12 contributes to survivorship on DHA-deficient diets.

Because the Japanese Pacific Ocean stickleback also have increased survivorship with marine-derived diets compared with freshwater diets (Fig. 2A), additional *Fads2* duplications beyond the LG12 copy may further increase

DHA biosynthetic ability and be beneficial for permanent freshwater residency. Indeed, in Japan, freshwater stickleback populations had even higher *Fads2* copy numbers than Pacific Ocean populations (χ^2_2 = 17.1, P < 0.01) (Fig. 4A and figs. S22 and S23). Even within freshwater populations, those that had a longer evolutionary history in freshwater habitats had higher *Fads2* copy numbers (χ^2_3 = 35.3, P < 0.01) (Fig. 1C and fig. S22A to S22B). Additional copy number increases also occurred in North American (χ^2_1 = 4.4, P = 0.035) and European freshwater populations (χ^2_2 = 7.2, P = 0.028) (Fig. 4A and fig. S22C to S22F). We confirmed that freshwater populations with additional copies of *Fads2* had more DHA than the Pacific Ocean population or a freshwater population with fewer copies when fed only DHA-free diets ($F_{2,8}$ = 12.6, P < 0.01) (fig. S24). Both linkage analysis and long-read genome sequencing showed that tandem duplications on the X chromosome are responsible for additional copy number increase in both Japanese and Canadian freshwater populations (figs. S25 and S26). Transposons near *Fads2* might have facilitated these tandem duplications (fig. S26) (*26*).

To test the generality of the mechanism, we first investigated nine-spined sticklebacks (genus *Pungitius*). The freshwater species, *P. tymensis* and *P. kaibarae*, had higher *Fads2* copy numbers than *P. pungitius* (P < 0.05), which inhabits only brackish environments in Japan. *P. sinensis*, which inhabits both freshwater and brackish environments (*27*, *28*), had copy numbers intermediate between those of *P. pungitus* and freshwater nine-spined sticklebacks (Fig. 4B and fig. S27). Finally, we investigated *Fads2* copy numbers in the ray-finned fishes whose whole-genome sequences have been determined (fig. S28). Fish species that form freshwater populations had significantly higher *Fads2* copy numbers than entirely marine species (Fig. 4C and fig. S29; MCMCglmm accounting for phylogeny, pMCMC < 0.01), suggesting convergent increases of *Fads2* copies in diverse taxa that successfully colonized freshwater habitats.

Gene duplications not only enhance overall gene expression levels, but also allow duplicated copies to acquire new functions (*29*). Our yeast functional assay suggested that *Fads2* genes in the Pacific Ocean stickleback acquired an additional enzymatic function in the DHA synthetic pathway (fig. S6 and table S4). Some of the Pacific Ocean–specific amino acid changes were shared by other freshwater ray-finned fishes (table S6), suggesting that they may be responsible for the acquisition of new enzymatic function. In addition to amino acid changes, both cis- and trans-regulatory changes cause expression differences between *Fads2* haplotypes (fig. S30). Given that overexpression of *Fads2* rescued the lethality in Japan Sea sticklebacks (Fig. 2, F and G), differences in the copy number itself likely contribute to differences in survival on DHA-deficient diets, although the possible involvement of changes in *Fads2* protein sequence and regulation cannot be excluded.

Our data demonstrate that *Fads2* is a key metabolic gene important for overcoming the nutritional constraints associated with freshwater colonization in fishes. Intriguingly, *Fads* shows strong signatures of selection in human populations that colonized polar regions, suggesting the importance of *Fads* in even more diverse taxa, including humans (*30*).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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A key metabolic gene for recurrent freshwater colonization and radiation in fishes

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Well prepared

It is well known that species radiate into new niches by adapting to novel environments. But why do some species radiate in this way, while other, related, species do not. Ishikawa *et al.* looked across sticklebacks to determine why some, originally marine, lineages were able to colonize postglacial freshwater environments (see the Perspective by Weber and Tong). They found that a gene involved in fatty acid desaturation was duplicated in freshwater lineages. Transgenic manipulation of this gene allowed marine lineages to synthesize fatty acids and thus survive on fatty acid–deficient freshwater diets.

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