

Diversity of gill raker number and diets among stickleback populations in novel habitats created by the 2011 Tōhoku earthquake and tsunami

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ABSTRACT

Background: Catastrophic disasters often create novel habitats and offer an opportunity to investigate how rapidly phenotypic diversification occurs in nature. On 11 March 2011, a large earthquake and subsequent tsunami struck the downtown area of Otsuchi Town, Iwate Prefecture, Japan. This catastrophic event created new spring water-fed ponds that sticklebacks (genus *Gasterosteus*) quickly colonized. In 2014, there were 48 newly formed stickleback habitats.

Questions: Does trophic morphology and ecology vary among stickleback populations in newly created ponds? Is the morphological variation associated with any ecological or environmental factors in the newly formed habitats?

Methods: We sampled sticklebacks from four native and 17 novel habitats in 2014. We counted the number of gill rakers (an important trophic trait in fish) and analysed their stomach contents. We measured salinity, water temperature, pH, dissolved oxygen, and the density of benthos in the habitats and conducted principal component analysis on these environmental factors. Then, we tested whether any environmental factors or environmental principal components are associated with variation in gill raker number.

Results: The number of gill rakers varied significantly among the newly formed populations. Fish with fewer gill rakers had a higher proportion of benthos in their stomach, suggesting that gill raker number may contribute to the performance of benthic feeding. Furthermore, we found that sticklebacks in habitats with higher benthos density had fewer gill rakers.

Keywords: contemporary evolution, diet, empty niche, hybridization, rapid diversification, trophic ecology.

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INTRODUCTION

The emergence of new habitats provides organisms with opportunities to colonize empty niches and, potentially, diversify (Simpson, 1955; Schluter, 2000; Losos, 2010). Catastrophic events, such as volcanic eruptions, earthquakes, and tsunamis, can create new habitats and therefore play important roles in organismic evolution (Whittaker and Fernández-Palacios, 2007; Urabe and Nakashizuka, 2016). For example, volcanic activity has created new islands in the oceans, where birds have colonized from the nearby mainland and diversified (Lack, 1947; Grant and Grant, 2011). Because these catastrophic events are relatively rare and unpredictable, there are few opportunities to investigate the first stage of colonization of newly formed habitats.

A large-magnitude earthquake is a catastrophic event that can create new habitats by inducing landscape and hydrological changes and triggering massive tsunami waves (e.g. Wang and Manga, 2015; Urabe and Nakashizuka, 2016; Kume *et al.*, 2018). Charles Darwin experienced a large earthquake in Chile during the voyage of HMS *Beagle*, which led him to recognize the importance of crustal movements in organismic evolution (Darwin, 1839). Although recent studies have highlighted many examples of evolution over contemporary time-scales (Hendry and Kinnison, 1999; Reznick and Ghalambor, 2001; Bell and Aguirre, 2013), most have occurred in artificially introduced or human-disturbed populations (Hendry and Kinnison, 1999; Reznick and Ghalambor, 2001; Palumbi, 2002). Therefore, we know little about the effects of earthquakes on contemporary evolution.

Recently, an excellent example of contemporary evolution in earthquake-created habitats has been reported (Gelmond *et al.*, 2009; Lescak *et al.*, 2015; Bassham *et al.*, 2018). Uplift of islands by a large earthquake in Alaska in 1964 created new freshwater ponds in previously marine areas, where oceanic threespine stickleback (*Gasterosteus aculeatus*) colonized and diversified (Gelmond *et al.*, 2009; Lescak *et al.*, 2015; Bassham *et al.*, 2018). Genomic analysis of these populations showed that standing genetic variation in the ancestral oceanic populations enabled rapid diversification (Bassham *et al.*, 2018).

A more recent large earthquake and subsequent tsunami created new stickleback habitats in Japan (Kume *et al.*, 2018). On 11 March 2011, a large earthquake with a moment magnitude of 9.0 occurred off the coast of Tōhoku, Japan, where the Pacific Plate is subducting beneath the Okhotsk Plate (Urabe and Nakashizuka, 2016; Liu and Zhao, 2018). The earthquake triggered a massive tsunami, which struck stickleback habitats in Otsuchi Town, Iwate Prefecture, Japan (Fig. 1) (Kume *et al.*, 2018; Mori, 2018). Before the tsunami, three rivers in this area contained sticklebacks: the Gensui River (a tributary of the Otsuchi River) and Teranosawa River (a tributary of the Kozuchi River) provided habitat for freshwater *G. aculeatus* (Fig. 1), while the Namaisawa River (another tributary of the Kozuchi River) provided habitat for both freshwater *G. aculeatus* and anadromous Japan Sea stickleback (*G. nipponicus*) (Fig. 1). The huge tsunami and earthquake-induced ground subsidence created new ponds fed by spring water in a formerly urban area (orange area in Fig. 1). In 2012, we found sticklebacks in two of these newly created ponds (Fig. 2) (Kume *et al.*, 2018). Although the source populations in these novel habitats have yet to be identified, previous micro-satellite analysis by ourselves showed that these new populations contained hybrids between *G. aculeatus* and *G. nipponicus* (Kume *et al.*, 2018). Subsequently, we identified 48 stickleback habitats (Fig. 2). However, both variation in environmental factors in these habitats and stickleback phenotypes among these newly identified populations have yet to be investigated.

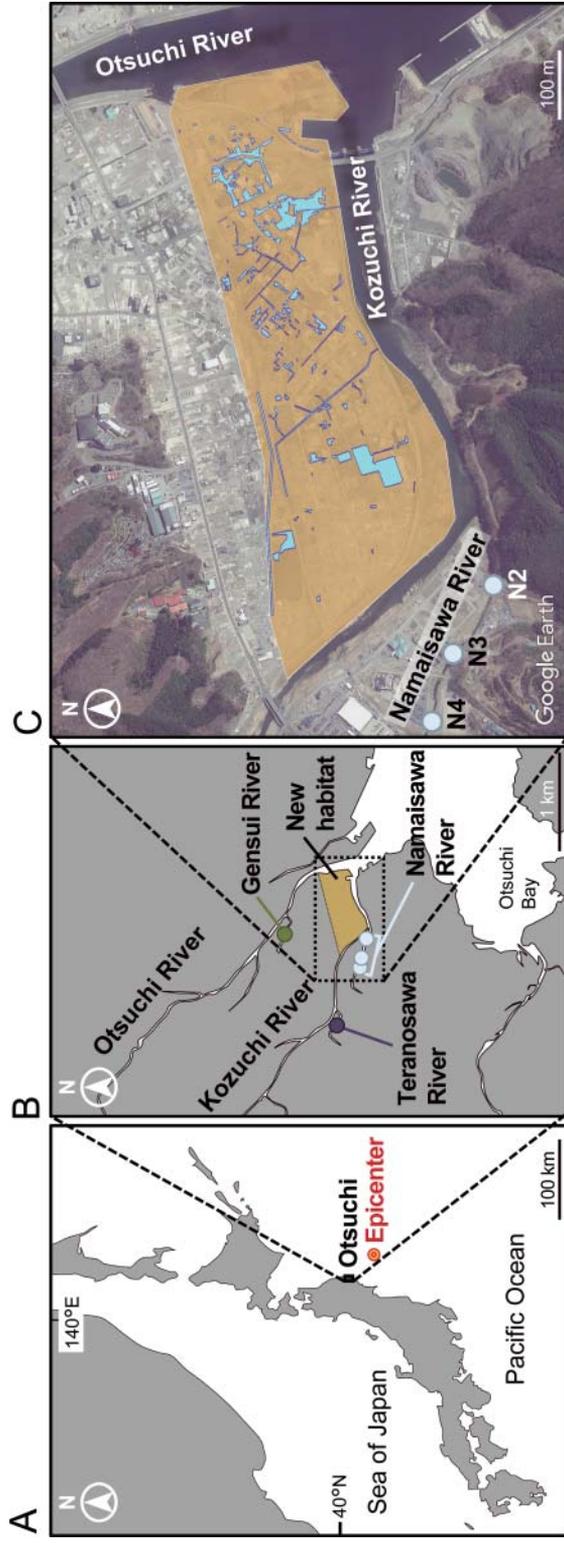


Fig. 1. Location of Otsuchi Town and the epicentre of the earthquake (A) and the southern urban area of Otsuchi Town (orange shaded area in B and C). New stickleback habitats were formed in the southern urban area (see Fig. 2 for details). The dots in (B) and (C) indicate study sites of the native habitats (green, Gensui River; light blue, Namaisawa River; dark blue, Teranosawa River). Satellite images used in this study were taken from Google Earth Pro (v. 7.3.2.5491, Map data: Google Earth).

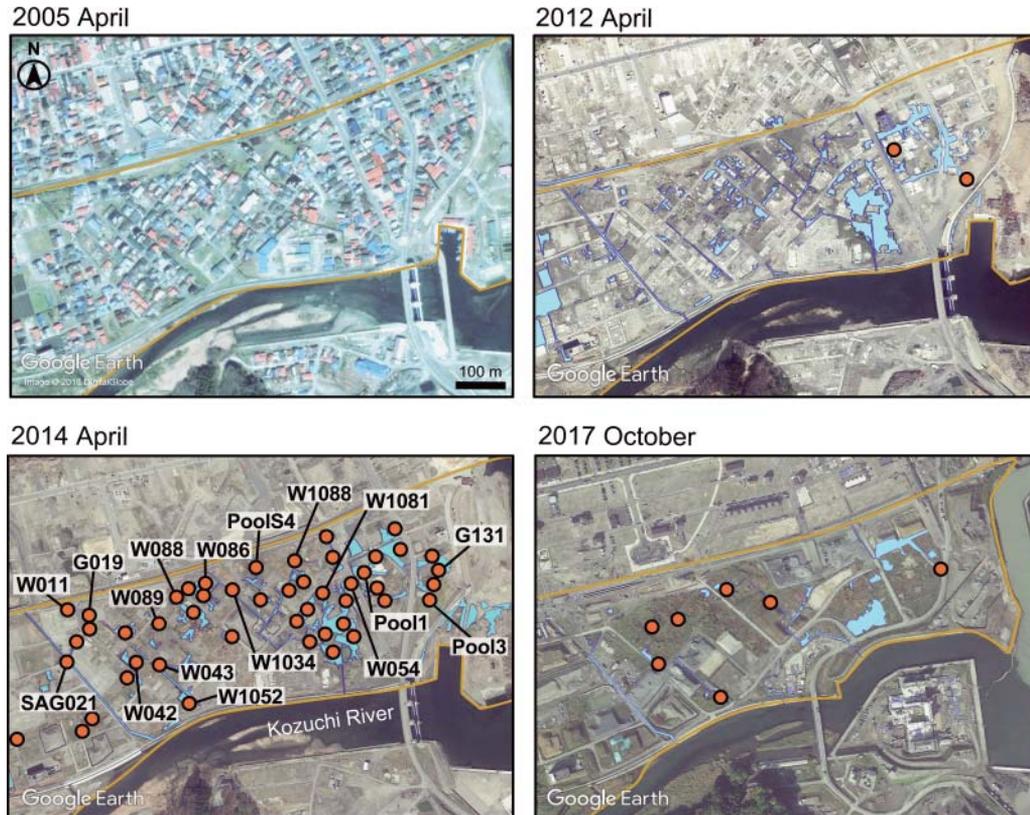


Fig. 2. Landscape of the southern urban area of Otsuchi Town before (2005) and after the tsunami (2012, 2014, and 2017). Blue areas indicate the tsunami-formed pools and channels. Orange dots denote the sites where stickleback were sampled. Satellite images used in this study were taken from Google Earth Pro (v. 7.3.2.5491, Map data: Google Earth and Digital Globe).

In the present study, we characterized the patterns of morphological variation among the newly created habitats, and tested whether the observed morphological variation can be explained by any environmental factors. We specifically focused on gill raker number. The number of gill rakers is known to be associated with trophic ecology in fishes: fish specialized for benthos feeding tend to have fewer gill rakers, while planktivores tend to have a large number of gill rakers (Schluter and McPhail, 1993; Robinson, 2000; Roesch *et al.*, 2013). Therefore, we first tested the hypothesis that fish with fewer gill rakers feed more on the benthos than fish with many gill rakers. Compared with the oceans, freshwater streams and shallow ponds are richer in benthic prey and poorer in planktonic prey, and sticklebacks in these freshwater environments generally have fewer gill rakers than marine or anadromous sticklebacks (Hagen and Gilbertson, 1972; Gross and Anderson, 1984; Schluter and McPhail, 1993). Therefore, we also tested the hypothesis that sticklebacks in the habitats with more benthos and/or less access to the sea have fewer gill rakers.

MATERIALS AND METHODS

Study sites

Our study sites were 17 locations in newly formed habitats (Fig. 2; Tables 1–3). As some habitats were difficult to access, environmental and benthos data are not available for all sites. For example, we were unable to conduct water measurements at G131 and W043 (Table 1), or benthos surveys at G019, G131, and W043 (Table 2). Although comparison with native sites was not the main focus of this paper, we similarly surveyed four native sites (Gensui River, Teranosawa River, and N2 and N4 sites in the Namaisawa River; Fig. 1) and present the data here.

Environmental and ecological surveys of stickleback habitats

To characterize the environmental variation among stickleback habitats, we measured water salinity, dissolved oxygen (mg/L), water temperature (°C), and pH (Table 1). At each site, measurements were conducted on the surface water using a multi-parameter water quality probe (WQC-24, DKK-TOA Co. Ltd., Tokyo, Japan) in August 2014, December 2015, and May 2016, and a glass electrode pH meter (WM-32EP, DKK-TOA Co. Ltd.) and dissolved oxygen meter (DO-24P, DKK-TOA Co. Ltd.) in September 2016. As we could not adjust tidal conditions, we conducted measurements multiple times at each site, except G019, to cover different tidal conditions (Table 1) and used the site-specific averages for statistical analysis.

For the analysis of benthos communities, benthic macroinvertebrates were collected by the Institute of Environmental Ecology IDEA Consultants (Shizuoka, Japan) in August 2014 (Table 2), three times at each site. Each sampling covered a quadrat of 25 cm × 25 cm, to a depth of about 3 cm (1875 cm³). We used a hand-net with a 500 μm mesh for collecting the benthos; thus, benthos smaller than 500 μm could not be assessed. Benthic macroinvertebrates were separated from sediments and debris, pooled, and stored in 99.9% ethanol. The total numbers of benthic invertebrates per three replicates (1875 cm³ × 3 = 5625 cm³) were used for subsequent analysis.

Morphological and stomach content analyses of sticklebacks

For morphological and stomach content analyses, adult fishes were collected with minnow traps and euthanized using an overdose of MS-222 (tricaine methanesulfonate). We preserved all samples in 99.5% ethanol. Sample sizes and ranges of standard length are shown in Table 3.

Under a stereomicroscope, we counted the number of gill rakers from the first gill arch on the right side of the fish, except for 10 individuals whose right gill rakers were damaged; for these 10 fish, the gill rakers on the left side were counted instead. Counting was conducted without dissecting the gill or staining it with alizarin red. Gill rakers on the ventral and dorsal arches were summed. Because body size is also known to be an important factor in determining the diets of sticklebacks (Schluter, 1993), we measured standard length with a vernier calliper and used it as a covariate in the statistical analysis (see below). For comparison, we used previously reported data for native populations collected before the earthquake (Kume *et al.*, 2018) and for anadromous sticklebacks from the Bekanbeushi River, Hokkaido, Japan (Kitano *et al.*, 2007) (Table 3). Because gill raker number was not sexually

Table 1. Sampling dates and locations

Population	Longitude	Latitude	Depth (m)	Date of measurement	Tidal range (m)
Gensui	39.364664	141.897731	2.0	Aug. 2014, Dec. 2015, May 2016	—
Namaisawa N2	39.352542	141.897410	2.5	Aug. 2014, Dec. 2015, May 2016	224–297
Namaisawa N4	39.353724	141.893194	2.9	Aug. 2014, Dec. 2015, May 2016	224–280
Teranosawa	39.356675	141.883638	4.9	Aug. 2014, Dec. 2015, May 2016	—
G019	39.356286	141.902237	0.7	Sept. 2016	226
Pool1	39.356939	141.906903	0.5	Aug. 2014, Dec. 2015, May/Sept. 2016	220–286
Pool3	39.356600	141.908151	0.6	Aug. 2014, Dec. 2015, May 2016	260–279
PoolS4	39.356910	141.905234	0.5	Aug. 2014, Dec. 2015	274–313
SAG021	39.355697	141.901815	0.7	Aug. 2014, Dec. 2015, May/Sept. 2016	214–303
W011	39.356382	141.901910	0.8	Aug. 2014, Dec. 2015, May/Sept. 2016	214–313
W042	39.355635	141.902956	0.8	Aug. 2014, Dec. 2015, May/Sept. 2016	210–271
W054	39.356672	141.906668	0.6	Aug. 2014, Dec. 2015	278–282
W086	39.356594	141.904195	0.4	Aug. 2014, Dec. 2015, May/Sept. 2016	210–270
W088	39.356493	141.903731	0.6	Aug. 2014, Dec. 2015, May/Sept. 2016	210–270
W089	39.356124	141.902999	0.7	Aug. 2014, Dec. 2015, May/Sept. 2016	214–277
W1034	39.356689	141.904639	0.5	Aug. 2014, Dec. 2015, May 2016	257–306
W1052	39.355222	141.903827	0.8	Aug. 2014, Dec. 2015, May/Sept. 2016	212–271
W1081	39.356674	141.906280	0.8	Aug. 2014, Dec. 2015, May/Sept. 2016	212–306
W1088	39.357035	141.905629	0.7	Aug. 2014, Dec. 2015	274–306

Note: Tidal range data were taken nearby at Ofunato City (39°1'N, 141°45'E), and provided by the Japan Meteorological Agency (<https://www.data.jma.go.jp/gmd/kaiyou/db/tide/suisan>).

Table 2. Variation in density of benthos among habitats

Population	Mean depth of sampling (cm)	No. individual benthic organisms/5625 cm ³
Gensui River	40	243
Namaisawa N4	70	464
Namaisawa N2	40	360
Teranosawa River	55	637
Pool1	25	149
Pool3	30	828
PoolS4	10	69
SAG021	40	156
W011	30	292
W042	30	111
W054	10	194
W086	25	167
W088	60	120
W089	15	918
W1034	5	266
W1052	30	349
W1081	15	258
W1088	25	246

dimorphic except at W1081 (Wilcoxon rank sum test, $W = 73.5$, $P = 0.0219$ for W1081 and $P > 0.05$ for other populations), we pooled the two sexes when testing the association between gill rakers and habitats.

For the stomach content analysis, prey items in the stomach of each fish were sorted into benthic and planktonic prey (Schluter and McPhail, 1992; Bell and Foster, 1994; Wetzel, 2001). Benthic prey included aquatic and terrestrial insects, Isopoda, Tanaidacea, Amphipoda, Ostracoda, and shells. Planktonic prey included Mysida, Cumacea, Copepoda, and Cladocera. For each fish, we measured the volume of benthic prey, planktonic prey, and others (digested prey and unidentified items) as well as the total volume of the stomach contents.

Statistical analyses

To determine whether the environmental variables, stomach contents, and gill raker number varied among newly formed stickleback populations, we conducted non-parametric Kruskal-Wallis tests, because the Shapiro-Wilk test showed violations of the assumption of normality for several variables. For analysis of gill rakers, including those of native populations, we conducted a Steel-Dwass test for multiple comparisons using both native and novel populations. As Spearman correlation tests showed that several environmental and ecological factors were correlated with each other (Table 4), we condensed these variables for four native sites and 14 new populations by principal component (PC) analysis using the *prcomp* function in R (Table 5). For PC analysis, we used site-specific means of log-transformed environmental variables.

Table 3. Sample sizes for morphological (N_{morph}) and stomach content (N_{stomach}) analyses, and the mean and the range of standard lengths (SL) of stickleback fishes

Population	Sampling date	N_{morph}	N_{stomach}	Mean (range) of SL (mm)
Gensui River	December 1998	29	—	50.88 (44.19–68.34)
Gensui River	June 2010	5	—	61.63 (39.83–77.81)
Gensui River	February 2014	20	20	56.77 (50.02–63.44)
Namaisawa N2	February 2014	20	20	52.56 (45.67–61.00)
Namaisawa N3	June 2010	43	—	55.89 (35.38–78.69)
Namaisawa N4	February 2014	20	20	53.21 (45.40–58.93)
Teranosawa River	February 2014	20	20	55.12 (40.35–64.62)
G019	July 2014	19	19	47.61 (42.36–53.09)
G131	August 2014	4	4	56.87 (51.48–62.69)
Pool1	February 2014	20	20	46.14 (40.27–55.52)
Pool3	February 2014	20	20	47.76 (41.54–59.79)
PoolS4	February 2014	20	20	45.67 (39.93–52.75)
SAG021	July 2014	23	23	52.02 (37.37–61.48)
W011	July 2014	5	5	51.03 (39.99–55.77)
W042	March 2014	5	5	49.91 (44.22–54.27)
W043	March 2014	20	20	50.62 (33.35–64.88)
W054	August 2014	3	3	57.71 (53.96–61.64)
W086	March 2014	20	20	36.18 (30.06–46.00)
W088	March/July 2014	27	27	47.28 (32.24–56.83)
W089	March 2014	10	10	46.57 (32.54–53.83)
W1034	February 2014	25	25	40.80 (33.26–60.54)
W1052	March 2014	21	21	46.46 (30.24–64.14)
W1081	February/March 2014	34	34	45.50 (29.81–57.83)
W1088	March 2014	24	24	43.49 (32.22–66.08)
Akkeshi <i>G. aculeatus</i>	May 2003	27	—	79.17 (70.58–84.47)
Akkeshi <i>G. nipponicus</i>	May 2003	26	—	64.40 (60.29–75.29)

Table 4. Correlations among environmental and ecological variables

	Salinity	Dissolved oxygen	Water temperature	pH	Benthos density
Salinity	—	−0.052	0.425	−0.104	−0.442
Dissolved oxygen	0.838	—	0.476	0.643	−0.445
Water temperature	0.079	0.046	—	0.455	−0.779
pH	0.682	0.004	0.058	—	−0.271
Benthos density	0.066	0.064	<0.001	0.276	—

Note: The matrix above the diagonal shows the Spearman's correlation coefficients, while the lower matrix shows *P*-values. Site-specific mean values were used for the analysis.

To test the effects of gill raker number on stomach contents, we used a generalized linear mixed model (GLMM) with stomach contents (either the volume of benthos or plankton in the stomach) as a response variable, gill raker number and standard length as explanatory variables, the total volume of stomach contents as an offset, and sampling site, sex, and sampling date as random factors. An offset term specifies that the volume of prey items in the stomach is proportional to the total volume of stomach contents (Zuur *et al.*, 2009; Kubo, 2012). For the GLMM, we used the *lme4* statistical package in R (Bates *et al.*, 2015). For statistical analysis of stomach contents, fish with empty stomachs were excluded.

To determine whether any environmental factors influenced gill raker number, we used a generalized linear model (GLM) with an environmental factor (either one of the PCs or site-specific mean of each environmental factor) as an explanatory variable and site-specific mean number of gill rakers as a response variable: each environmental factor was tested independently. A GLM with a gamma distribution and log-link function was used. For testing the effects of habitat benthos density on stomach contents, we used habitat benthos density as an explanatory variable and the site-specific mean of the proportion of either benthos or plankton in the stomach contents as a response variable in the GLM with a gamma distribution and log-link function.

RESULTS

Environmental variation among novel habitats

Novel habitats differed in salinity (Fig. 3; Kruskal-Wallis test, $\chi^2 = 32.87$, $P = 0.003$) and dissolved oxygen ($\chi^2 = 26.89$, $P = 0.020$) but not in water temperature or pH ($P > 0.05$). The density of benthos also showed variations among novel habitats (Table 2). These environmental variations can be summarized into two major principal components: PC1 and PC2 explain 48.6% and 33.0% of the variation, respectively (Table 5; Fig. 4). Larger PC1 values reflect higher values of water temperature, dissolved oxygen, pH and salinity, and lower density of benthos, while higher PC2 values reflect higher values of salinity and lower values of pH and dissolved oxygen (Table 5). PC1 was significantly higher in the new habitats than in the native habitats (Fig. 4) (Kruskal-Wallis test, $\chi^2 = 5.46$, $P = 0.020$), while PC2 did not differ between habitats (Kruskal-Wallis test, $\chi^2 = 0.18$, $P = 0.671$).

Table 5. Loading values and percentage of variance explained by principal components (PCs) of environmental and ecological variables

Environmental variable	PC1	PC2	PC3	PC4	PC5
Salinity	0.284	0.613	0.448	0.557	0.182
Dissolved oxygen	0.389	-0.518	-0.376	0.662	0.040
Water temperature	0.580	0.173	-0.056	-0.190	-0.770
pH	0.345	-0.535	0.726	-0.217	0.141
Benthos density	-0.559	-0.198	0.357	0.411	-0.593
% Variance explained	48.6	33.0	8.3	6.0	4.0

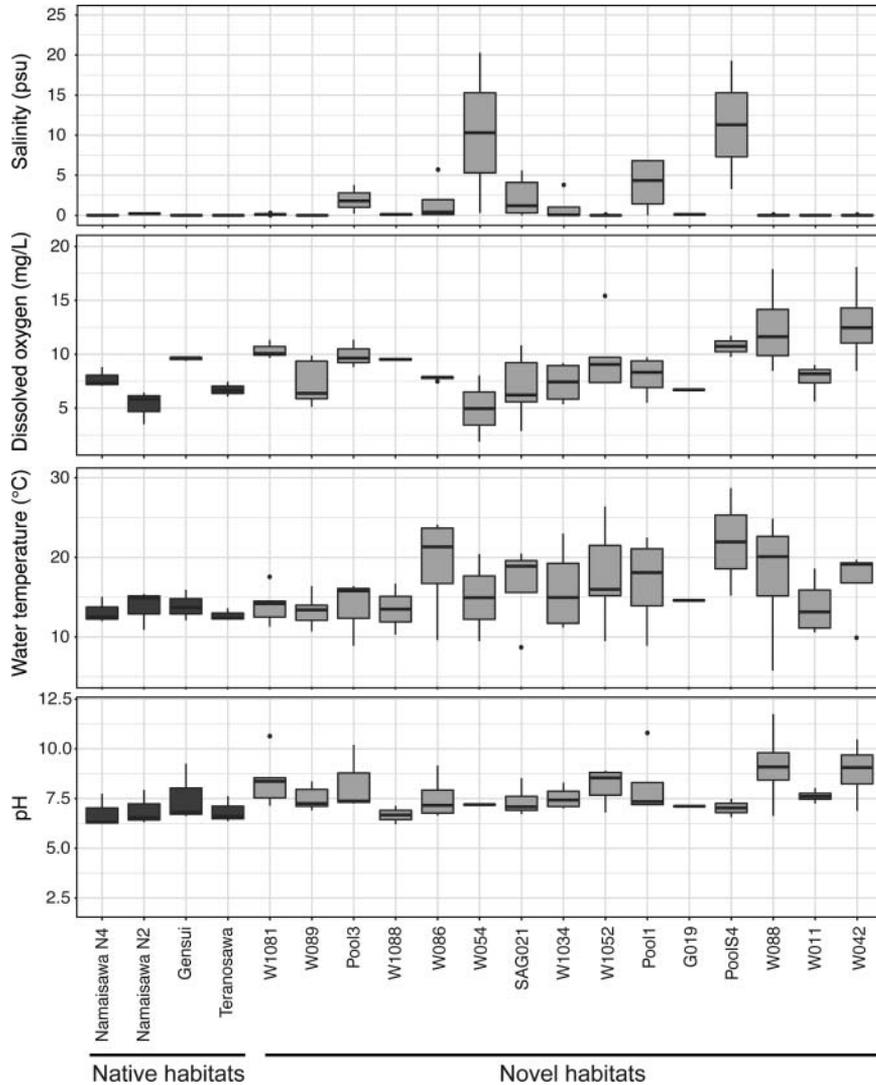


Fig. 3. Boxplots showing variation in water parameters among study sites. Environmental data were collected in 2014–2016. For comparison, we show the data from the native habitats.

Diversity in gill raker number and diets

Gill raker number varied significantly among novel habitats (Kruskal-Wallis test, $\chi^2 = 86.70$, $P < 0.001$) (Fig. 5). The proportions of benthos and plankton in the stomach contents also showed significant variation among novel habitats (Kruskal-Wallis test, $\chi^2 = 87.04$, $P < 0.001$ and $\chi^2 = 107.41$, $P < 0.001$ for benthos and plankton, respectively) (Fig. 6).

Fish with fewer gill rakers consumed higher proportions of benthic prey (GLMM: $P = 0.017$, coefficient estimate \pm S.E. = -3.72 ± 1.55) (Fig. 7). We also found that larger

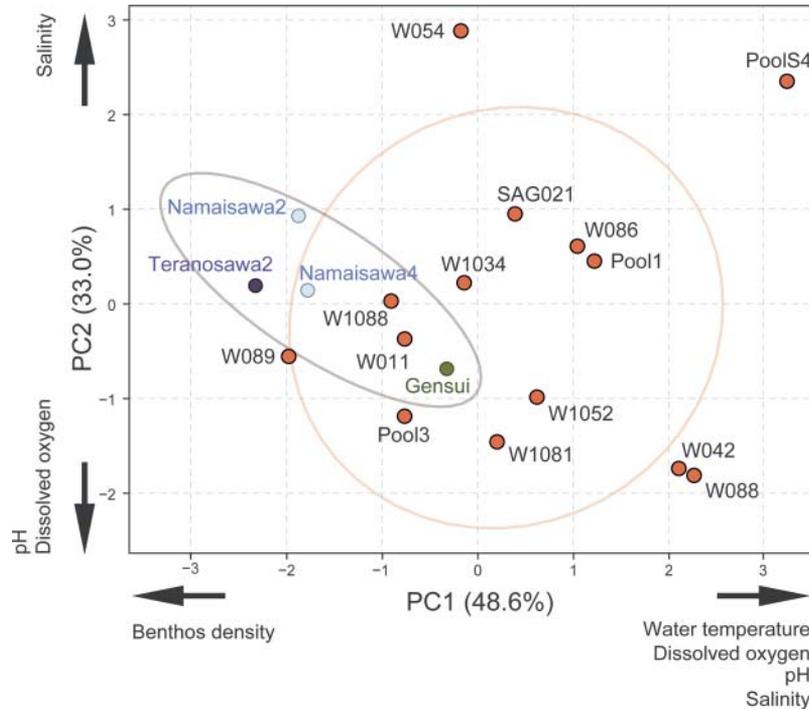


Fig. 4. Scatter plot of principal components (PC1 and PC2) of environmental and ecological variables. The percentage of variance explained by each principal component is shown in parentheses. Orange dots indicate novel habits, while other colours indicate native habitats (green, Gensui River; light blue, Namaisawa River; dark blue, Teranosawa River). The orange and grey circles indicate 95% confidence eclipses of novel and native sites, respectively.

fish foraged more benthos (GLMM: $P < 0.001$, coefficient estimate \pm S.E. = 2.15 ± 0.35) (Fig. 7). These data are consistent with the idea that fish with fewer gill rakers and larger body sizes have higher performance concerning benthos feeding (Schluter and McPhail, 1992; Schluter, 1993). The proportion of planktonic prey was not significantly associated with gill raker number (GLMM: $P = 0.486$, coefficient estimate \pm S.E. = -0.37 ± 0.54) or standard length (GLMM: $P = 0.854$, coefficient estimate \pm S.E. = -0.02 ± 0.12).

Next, we tested whether gill raker number is associated with any environmental factors. We found that fish in habitats with higher PC1 values had higher numbers of gill rakers (GLM: $P = 0.0175$, coefficient estimate \pm S.E. = 0.019 ± 0.007) (Fig. 8). PC2–PC5 had no significant effects on gill raker number. When we analysed each of the environmental factors, we found significant negative effects of benthos density (GLM: $P = 0.0347$, coefficient estimate \pm S.E. = $-0.0001 \pm 4 \times 10^{-5}$) and marginally significant positive effects of water temperature (GLM: $P = 0.0511$, coefficient estimate \pm S.E. = 0.009 ± 0.004) on gill raker number.

Finally, we tested whether stomach contents were associated with any environmental factors. Except for the effect of environmental PC5 on the proportion of benthos, we found no significant effects of any environmental variable on the proportions of benthos or

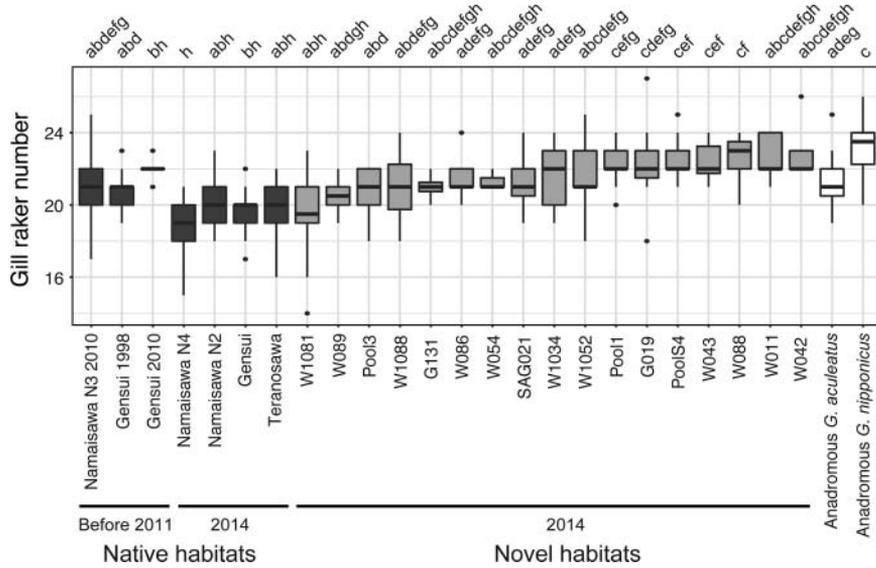


Fig. 5. Box plots showing variation in gill raker number. For comparison, we show the data for sticklebacks in the native habitats, if available, and anadromous *Gasterosteus aculeatus* and *G. nipponicus* collected from Hokkaido, Japan. Study sites with different letters indicate a statistically significant difference in gill raker number between them (Steel-Dwass test, $P < 0.05$).

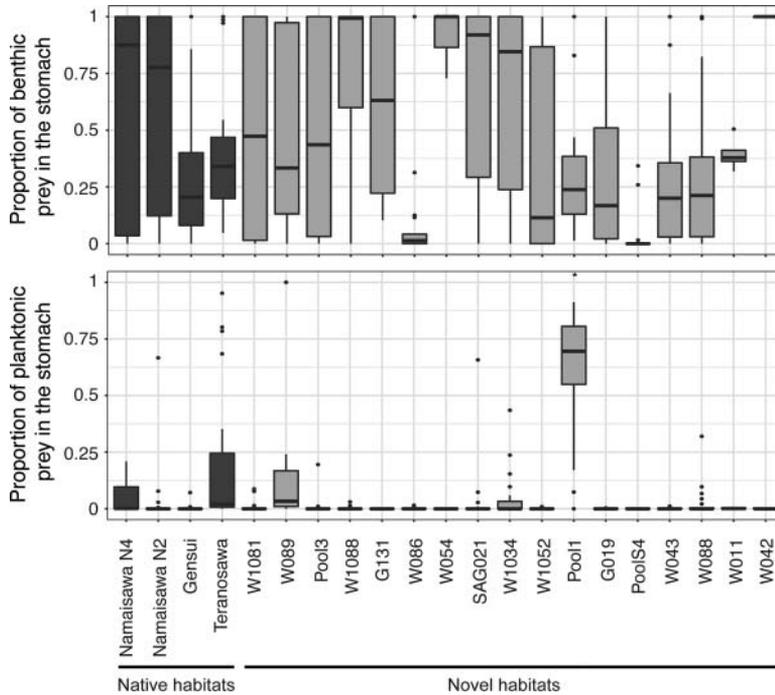


Fig. 6. Box plots of the proportions of prey items in stomach contents. Data are shown for benthic (upper panel) and planktonic prey (lower panel). All data were taken from fish collected in 2014. For comparison, we show the data from the native habitats.

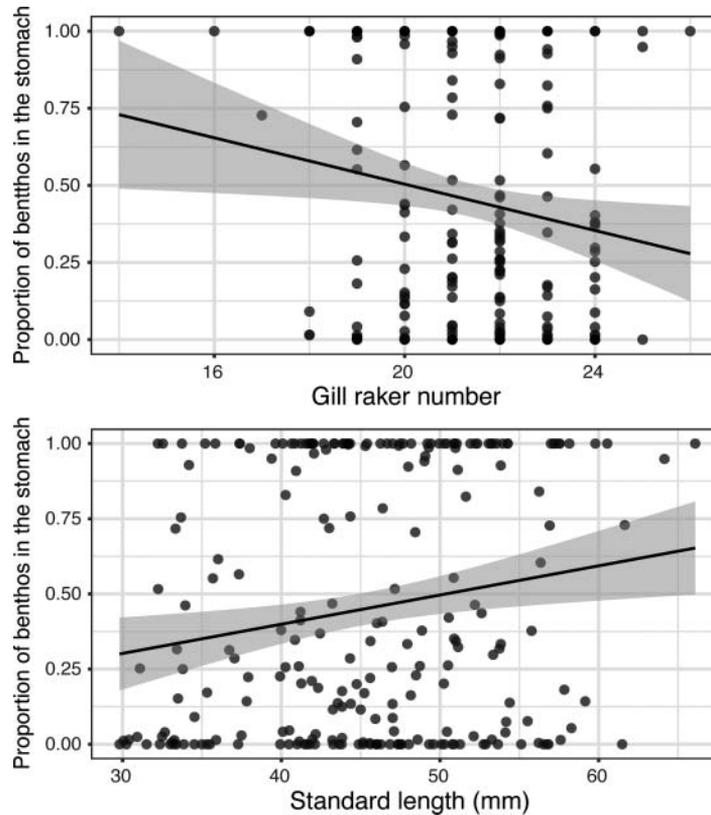


Fig. 7. Relationship between the proportion of benthic prey in the stomach contents and morphology (gill raker number in the upper panel and standard length in the lower panel, $N = 237$) in fish from newly formed habitats. The lines show linear regression relationships with 95% confidence intervals. Each dot represents a single fish. Note that the regression line is for visualization purposes only, and GLMM was used for statistical analysis (see Materials and Methods).

plankton in the stomach, regardless of whether any PC or single environmental factor was used for the analysis ($P > 0.05$). Fish from habitats with higher PC5, which reflects lower values of water temperature and benthos density (Table 5), tended to have more gill rakers (GLM: $P = 0.0184$, coefficient estimate \pm S.E. = 0.8152 ± 0.299), although PC5 explained only 4.0% of the environmental variations (Table 5).

DISCUSSION

We found diversity in gill raker number among new stickleback populations created by the 2011 earthquake and subsequent tsunami in Japan. Gill raker number is highly heritable (Hagen, 1973; Hermida *et al.*, 2002; Aguirre *et al.*, 2004; Glazer *et al.*, 2014) and rarely influenced by phenotypic plasticity in sticklebacks (Day *et al.*, 1994; Oke *et al.*, 2016). Therefore, the observed diversity in gill raker number is likely to have a genetic basis. What drove the diversification of gill raker number in the newly established populations?

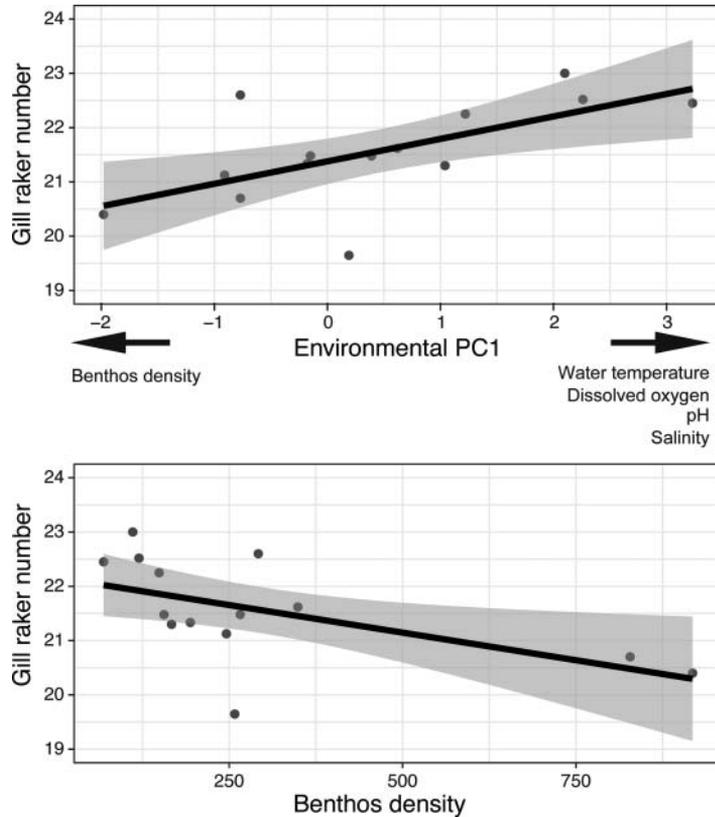


Fig. 8. Relationship between gill raker number and PC1 of the environmental factors (upper panel) and benthos density (lower panel). The lines show linear regression relationships with 95% confidence intervals. Each dot represents a single novel site.

One possibility is that this variation may reflect adaptation to diverse environments. Fish with fewer gill rakers were found in habitats with smaller PC1 (Fig. 8), which reflects a higher density of benthos (Table 5). Furthermore, we found that fish with fewer gill rakers had a higher proportion of benthos in their stomach contents (Fig. 7). These data are consistent with previous findings that benthos-feeding sticklebacks generally have fewer gill rakers than plankton-feeding sticklebacks (Gross and Anderson, 1984; Schluter, 2000; Kitano *et al.*, 2007; Berner *et al.*, 2009; Ravinet *et al.*, 2014). However, we did not find a significant association between the density of benthos in a habitat and stomach contents. This is contrary to the idea that the density of benthos in a habitat influences the diets of sticklebacks residing there. Thus, it remains unclear how much the density of benthos in a habitat contributes to the evolution of gill raker number. The results of the present study are based on few sampling time points. Further detailed long-term surveys across multiple seasons will be necessary to reach a clear conclusion on the relationships among gill raker number, diet, and habitat.

A second possible explanation is that different immigration rates of anadromous sticklebacks may cause variations in gill raker number. Anadromous *G. nipponicus*, which inhabits the sea around Otsuchi Town, has a larger number of gill rakers than native freshwater

G. aculeatus (see Fig. 5) (Kitano *et al.*, 2007; Kume *et al.*, 2018). Therefore, migration of the anadromous *G. nipponicus* into new habitats and hybridization with the fish there will result in an increase in gill raker number in habitats accessible to *G. nipponicus*. Our preliminary microsatellite analysis of two novel habitats in 2012–2013 showed that newly established populations contained hybrids between *G. aculeatus* and *G. nipponicus* (Kume *et al.*, 2018), suggesting that hybridization occurred in some of the new habitats. Genomic analysis of additional individuals in multiple newly formed populations will enable us to determine when and how much gene flow has occurred from anadromous *G. nipponicus* and whether gill raker number is associated with the rate of introgression.

The third possible explanation is that variation in gill raker number results from selection on other phenotypic traits. In North American populations, one of the genetic loci controlling gill raker number was found on Chromosome 4 (Miller *et al.*, 2014); the same chromosome contains genes important for other morphological, physiological, and behavioural traits (Colosimo *et al.*, 2005; Barrett *et al.*, 2009; Greenwood *et al.*, 2013; Peichel and Marques, 2017). Therefore, selection on other traits might change the allele frequency of genes regulating gill raker number through hitchhiking effects (Maynard Smith and Haigh, 1974; Barton, 2000). Using quantitative trait loci (QTL) mapping (Peichel *et al.*, 2001; Peichel and Marques, 2017), it will be possible to identify genetic loci important for variation in gill raker number and other adaptive traits in the Japanese stickleback populations.

Finally, gill raker divergence may occur by chance. The genetic composition of founder populations may differ among newly colonized habitats because of the random sampling of a limited number of alleles from the ancestral source populations (Whittaker and Fernández-Palacios, 2007). Even after colonization, genetic drift will continue to fix different alleles in different populations for every generation (Kimura, 1983). If we can identify the genetic loci underlying variation in gill raker number, we will be able to investigate the change in allele frequency at QTL and get better insight into the roles of founder effects and genetic drift in gill raker number diversification.

In conclusion, we have highlighted a clear case of a catastrophic earthquake creating new habitats for sticklebacks, which then diversified in gill raker number. Variation in gill raker number among habitats was associated with environmental factors. This is a good model system for investigating the ecological and genetic mechanisms by which phenotypic diversification occurs in the earliest stage of colonization of newly formed habitats over contemporary time-scales.

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