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4 **Title:**

5 Unique habitat and macroinvertebrate assemblage structures in spring-fed stream: a

6 comparison among clastic lowland tributaries and mainstreams in northern Japan

7

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24

25   **Abstract**

26   The stable flow and temperature regimes of spring-fed streams are distinct from the
 27   dynamic regimes of other streams. We investigated differences in habitat and
 28   macroinvertebrate assemblages among three stream types (spring-fed tributary,
 29   non-spring-fed tributary and mainstream) in a clastic lowland of northern Japan.
 30   Current velocity was the slowest in the spring-fed reach, where the percent of fine
 31   sediment deposition was also 3.8–11.4 times higher than in the other stream types. The
 32   standing stock of detritus was also greater in the spring-fed reach. These results
 33   suggest that the stable flow regime in the spring-fed stream leads to the accumulation
 34   of fine sediment and detritus on the streambed. Oligochaeta and chironomids, which
 35   are burrower-gatherers, were remarkably abundant in the spring-fed reach. Total
 36   macroinvertebrate abundance was 3.8–12.2 times greater in the spring-fed reach than
 37   in the other stream types. Sprawler-grazer ephemeropterans were the most abundant in
 38   the mainstream reaches, likely due to higher primary productivity. *Allomyia* sp, which
 39   depend on cool spring-fed habitats, were found only in the spring-fed reach. The
 40   indicator species analysis also indicated multiple taxa of detritivores and *Allomyia* sp.
 41   for the spring-fed tributary. The macroinvertebrate assemblage in the spring-fed reach
 42   was characterized by numerous burrowers, collector-gatherers, and crenobiont taxa,
 43   highlighting the uniqueness and its contribution to enhance beta diversity in river
 44   networks.

45

46   **Keywords** Community ecology • Functional feeding groups • Groundwater • Indicator
 47   species • Life-form types • River ecosystem

48 **Introduction**

49

50 Groundwater flow into river ecosystems depends on underground flow pathways and
 51 aboveground topographic features (Allan and Castillo 2007). Although apportioning
 52 stream water to runoff and groundwater sources is generally difficult, streams that are
 53 sustained by a significant amount of permanent groundwater discharge from a spring
 54 mouth are typically referred to as spring-fed streams (Cantonati et al. 2010). Because
 55 groundwater flow is rarely affected by rapid fluctuations resulting from rainfall,
 56 snowmelt, or seasonal and diurnal changes in air temperature, spring-fed streams are
 57 characterized by stable water flow and temperature regimes (Mattson et al. 1995; Sear
 58 et al. 1999; Lusardi et al. 2016). Thus, spring-fed streams are distinct from the dynamic
 59 flow and temperature regimes that characterize non-spring-fed streams.

60 Flow regime is one of the most influential factors determining the structure of
 61 streambed substrates (Davis and Barmuta 1989). The frequency and magnitude of high
 62 flows are directly related to sediment runoff; stream substrate components are
 63 sequentially flushed from smaller to larger particles under high flows (Richards 2004).
 64 Streams with frequent high flows typically have a coarse streambed structure relative
 65 to those with less variable flow regimes. The substrates of spring-fed streams are
 66 dominated by fine sediments, owing to their stable flow regimes (Sear et al. 1999).
 67 Spring-fed streams in lowland regions may have particularly fine streambeds due to
 68 low channel gradients. Streambed particle size is a major determinant of the benthic
 69 macroinvertebrate community and its structure (Richards and Bacon 1994; Wood and
 70 Armitage 1997). For example, the relative density of burrowers and gatherers may
 71 increase with fine sediment deposition (Rabeni et al. 2005). These groups are well

72 suited to depositional habitats because these habitats provide organic matter as well as
 73 burrowing spaces. Lowland spring-fed streams may therefore house unique community
 74 assemblages, dominated by burrowers and gatherers, relative to adjacent
 75 non-spring-fed streams that have less fine sediment content.

76 Water temperature in spring-fed streams is highly stable; it is typically cold in
 77 summer and warm in winter (Mattson et al. 1995). Previous studies have reported that
 78 these stable water temperature regimes provide refugia for aquatic fauna to avoid
 79 severe hot (Nakagawa et al. 2015) and cold seasonal conditions (Inoue and Ishigaki
 80 1968). Moreover, Sun et al. (2020) suggested that most indicator species of spring-fed
 81 habitats may be stenothermal. This further suggests that the narrow range of water
 82 temperatures in spring-fed streams may lead to the formation of unique assemblages of
 83 crenobiont and/or crenophilous species (Lusardi et al. 2016).

84 The stable flow and temperature regimes of spring-fed streams provide unique
 85 habitat and macroinvertebrate assemblage structures and subsequently contribute to
 86 beta diversity within river networks. However, studies on spring ecosystems have
 87 advanced mainly in carbonate geologies (e.g., karst) with dense spring habitats
 88 (Mattson et al. 1995; Wood et al. 2005; von Fumetti et al. 2017; von Fumetti and
 89 Blattner 2017), and the ecosystem functions are poorly understood in other geologies.
 90 This is perhaps related to sparse distributions of springs and difficulties to prepare
 91 replicable sampling design in such geologies. Although more than 16,000 spring-fed
 92 sites have been recognized in Japan (Ministry of Environment 2020), functional
 93 aspects of spring-fed streams are still elusive in any geological conditions. We assessed
 94 differences in habitat characteristics and macroinvertebrate assemblages between
 95 spring-fed and adjacent non-spring-fed streams in a clastic lowland, one of the

dominant geological conditions in Japan. Our aim was to investigate potential processes explaining these differences, detect potential indicator species of different stream types, and to contribute to the understandings of faunal and functional responses of macroinvertebrates to the unique habitats of spring-fed tributaries.

Materials and methods

Study area

We assessed streams in the Shubuto River system, located in Kuromatsunai, Hokkaido, Japan (42.64°N, 140.34°E). The Shubuto River basin comprises 367 km² of montane and lowland areas; our study focused on the lowland region (Fig. 1). Water quality is suitable for most freshwater organisms: dissolved oxygen > 95% in degrees of saturation, biochemical oxygen demand is 0.2-1.7 mg/L and ammonia concentration < 0.05mg/L (Miyazaki and Terui 2016). The geology is dominated by sandstone and mudstone with Cainozoic fossil seashells and tuff. Mean annual precipitation and air temperature, obtained from the Kuromatsunai automated meteorological data acquisition system station (located 4 km northwest of the site) were 1615.8 mm and 7.5°C, respectively, between 2009 and 2018. Dominant tree species in the riparian zones of the study area were *Salix* spp. and *Quercus crispula*, and dominant understory plants were *Sasa kurilensis* and *Reynoutria sachalinensis*. High flows generally occur in non-spring-fed streams during summer to autumn rainfalls and snowmelt season in early spring (Fig. 2).

The lowland region of the Shubuto River basin includes areas of groundwater

120 discharge such as flowing wells, seepages, and springs. The spring-fed tributary
 121 assessed in this study is fed by a significant amount of groundwater discharge
 122 (approximately 0.1 m³/s) that is directly connected to the Shubuto River (Sakai et al.
 123 2020). This tributary has stable flow regime and water temperature compared to
 124 adjacent mainstream (Fig. 2). We further selected a non-spring-fed tributary and three
 125 mainstreams that also drained into the lowland area (Fig. 1). The spring-fed and
 126 non-spring-fed tributaries were near to each other and had similar flow directions.
 127 Three mainstream areas were identified below the confluence with the spring-fed
 128 tributary (mainstream 1), between the confluences with the spring-fed and
 129 non-spring-fed tributaries (mainstream 2), and below the confluence with the
 130 non-spring-fed tributary (mainstream3) (Fig. 1). We used 40-m sections (reaches) in
 131 each study site to assess macroinvertebrates and habitat characteristics. The
 132 mainstream reaches had a typical fluvial geomorphology of Japanese midstream with
 133 riffle-pool structures whereas the tributary reaches rarely had heterogeneous structures
 134 like riffle-pools, step-pools, or cascades.

135   Although the river system has the only one typical spring-fed stream in the
 136 lowland area, similarly to other river systems in clastic geology, assumable unique
 137 habitat and community structures should be important for assessing local biodiversity
 138 even if focal river systems have limited numbers of spring-fed streams. This limitation
 139 may have hindered conceiving robust study design and advances in studies on
 140 ecosystem functions of spring-fed streams draining outside carbonate geologies. This
 141 study can therefore fill an important knowledge gap to understand the function of
 142 sparsely distributed spring-fed streams in structuring local biodiversity.

143

144 **Macroinvertebrate assemblage**

145 We used Surber nets (25 × 25 cm, 0.5-mm mesh) to sample macroinvertebrates from
 146 five randomly placed quadrats in each reach in June 2016. This period was chosen to
 147 avoid seasons of snow accumulation, snowmelt, and primary insect emergences. Each
 148 sample was collected along center lines of the streams to obtain representative
 149 assemblages. The sampling points for the mainstream reaches categorized as riffles,
 150 but those for the tributary reaches did not have any clear riffle nor pool structures.
 151 Samples containing macroinvertebrates, detritus, and inorganic sediments were placed
 152 in a tray and the macroinvertebrate and detritus portions were rinsed into unwoven
 153 bags, and immediately preserved in 70% ethanol. Macroinvertebrates were sorted and
 154 identified to the lowest possible taxonomic level using a stereomicroscope (SZ61,
 155 Olympus, Tokyo, Japan) and keys published by Kawai and Tanida (2018) and Merritt
 156 et al. (2008). Following identification, we classified macroinvertebrates by life-form
 157 type (burrowers, sprawlers, clingers, and swimmers) and functional feeding group
 158 (shredders, collector-gatherers, collector-filterers, predators, and grazers) based on
 159 information from Takemon (2005) and Merritt et al. (2008) as well as our own
 160 unpublished data.

161

162 **Habitat characteristics**

163 Stream width was measured with a measuring tape under base flow conditions at five
 164 points in each study reach. We measured water depth and current velocity (repeated 5-s
 165 measurements) using a folding scale and an electromagnetic current meter (VE10,
 166 Kenek, Tokyo, Japan), respectively, at 15 points in each study reach during
 167 macroinvertebrate sampling. Hemispherical photographs were taken in June 2016 at 10

168 m intervals along the 40 m reaches (five points) to estimate canopy openness.

169 Photographs were taken with a digital camera (D40, Nikon, Tokyo, Japan) equipped

170 with a fisheye lens (4.5 mm F2.8 EX DC Circular Fisheye HSM, Sigma, Kanagawa,

171 Japan) that was fixed horizontally at 1 m above the streambed. Canopy openness (%)

172 was estimated using CanopOn2 software (Takenaka 2009) from these photographs.

173 We applied the methods of Sakai et al. (2013) to estimate fine sediment

174 deposition in each study reach by visually estimating the percent cover of fine

175 sediment (< 2 mm) on the streambed in 40×40 cm quadrats with 20 rectangles

176 separated by wires (5% increments, ten quadrats per reach). We also estimated the

177 periphyton biomass on upper exposed surfaces of five submerged large cobbles in each

178 study reach. Periphyton was collected by brushing material from a 4 cm^2 patch on the

179 upper cobble surface and filtering the resulting slurry through pre-combusted

180 glass-microfiber filters (Whatman GF/F, Maidstone, United Kingdom). Prior to

181 periphyton sampling, each cobble was thoroughly rinsed in stream water to remove

182 attached fractions of nonalgal materials as much as possible. Periphyton dry mass and

183 ash-free dry mass (AFDM) were determined by drying the periphyton samples in an

184 oven (FC-610, Advantec, Tokyo, Japan) at 70°C for 24 h and then weighing them,

185 followed by combustion in a muffle furnace (FO300, Yamato Scientific Co., Ltd.,

186 Tokyo, Japan) at 500°C for 2 h and re-weighing. Detritus samples remained after

187 macroinvertebrate collections and identifications (five quadrat samples per reach) were

188 dried at ambient temperature for three days, followed by oven drying at 70°C for three

189 days and combustion in a muffle furnace at 500°C for 4 h. The AFDM of periphyton

190 and detritus samples were used as measures of periphyton biomass and standing stock

191 of detritus (mg/cm^2), respectively.

192

193 **Statistical analyses**

194 To test for differences in environmental variables, total abundance and taxa richness of
 195 macroinvertebrates, and abundance of the most dominant taxa (> 1% of the individuals
 196 in all samples) among the three stream types (spring-fed and non-spring-fed tributaries
 197 and mainstream), we first used one-way analysis of variance (ANOVA) with
 198 permutation test (Legendre 2007) to test for a significant difference of the response in
 199 at least one stream type. When significant, we used the same permutation ANOVA test
 200 for *post hoc* comparisons among the pairs of stream types. The permutation test used
 201 allowed us to correctly handle the unbalanced design of the study (mainstream has
 202 three times more observations than the tributaries). We adjusted *p*-values for multiples
 203 tests using the false discovery rate correction (Benjamini and Hochberg 1995), to avoid
 204 inflated type I error rates.

205 To visualize differences in macroinvertebrate assemblage structures among
 206 stream types and associated environmental variables, we performed a principal
 207 coordinate analysis (PCoA) based on the Chao index (Chao et al. 2005), followed by a
 208 permutational multivariate analysis of variance (PERMANOVA), testing for significant
 209 differences in macroinvertebrate assemblage structure among the stream types. Chao
 210 index was calculated from the total numbers of individuals of each taxon in each
 211 quadrat. We then superimposed *a posteriori* the environmental vectors (fine sediment
 212 deposition, canopy openness, current velocity, stream width, water depth, standing
 213 stock of detritus and periphyton biomass) using the weighted averages of the species,
 214 and environmental variables (Borcard et al. 2018). The PERMANOVA was tested
 215 using 999 permutations. In order to test for the presence of significant patterns of

216 spatial autocorrelation in the macroinvertebrate communities across our study reaches,
 217 we generated spatial eigenvectors using Moran's eigenvector maps (MEM; Dray et al.
 218 2006), a powerful multivariate approach allowing the detection of multiscale spatial
 219 structures in ecological data. To obtain the spatial eigenvectors, we used a spatial
 220 weighting matrix based on a Gabriel graph and weighted by a decreasing linear
 221 function of the distance among streams (details in Bauman et al. 2018a), following
 222 Bauman et al. (2018a, b). A redundancy analysis of the reach-scale community matrix
 223 was performed against the whole set of spatial eigenvectors associated to positive
 224 spatial autocorrelation patterns, for each spatial weighting matrix, and was tested by
 225 permutations (9,999).

226 We additionally tested whether some of the macroinvertebrate taxa were
 227 significant indicators of some of the stream types, that is, displaying both high fidelity
 228 and specificity to one of the habitats. To do so, the *IndVal* index was calculated for all
 229 species in the three stream types (Dufrêne and Legendre 1997) and was tested using
 230 10,000 Monte Carlo permutations. All statistical analyses were performed in R version
 231 3.6.3 (R Core Team 2020) using the packages *rcompanion* (Mangiafico 2020),
 232 *adespatial* (Dray et al. 2020), *vegan* (Oksanen et al. 2019), *indicspecies* (Cáceres et al.
 233 2020) and the function *anova.lway* (Legendre 2007).

234

235

236 **Results**

237

238 Stream width and water depth were significantly greater in mainstream reaches and
 239 smallest in the non-spring-fed tributary (Table 1). Correspondingly, canopy openness

240 was relatively greater in the mainstreams compared to the tributaries. Current velocity
 241 was significantly slower in the spring-fed tributary and faster in the mainstreams
 242 (Table 1). The percent cover of fine sediment was significantly higher in the spring-fed
 243 tributary; mean cover was approximately 11.4 times greater than those of the
 244 mainstreams and 3.8 times greater than that of the non-spring-fed tributary (Table 1).
 245 The periphyton biomass was not significantly different among the reaches, whereas the
 246 standing stock of detritus was greatest in the spring-fed tributary (Table 1).

247   The total abundance and taxa richness of macroinvertebrates were both
 248 significantly greater in the spring-fed tributary and smaller in the non-spring tributary
 249 (Table 1; Supplementary Table S1). For life-form types, the four most abundant
 250 burrower taxa (*Oligochaeta*, *Pagastia* spp., Chironomidae gen. spp. and Chironominae
 251 spp.) and Tanypodinae sp. were more abundant in the spring-fed tributary. Sprawlers
 252 were typically the most abundant group in the mainstreams, except for *Ordobrevia* sp.
 253 (Table 1). For functional feeding groups, the four most dominant collector-gatherer
 254 taxa (*Oligochaeta*, *Pagastia* spp., Chironomidae gen. spp. and Chironominae spp.)
 255 were most abundant in the spring-fed tributary (Table 1). Excluding two trichopteran
 256 grazers that were dominant in the spring-fed tributary (*Apatania* sp. and *Allomyia* sp.),
 257 all grazer taxa reached their maximum abundance in the mainstreams. We noted that
 258 *Allomyia* sp. was only found in the spring-fed tributary (Table 1). In this study, some
 259 individuals of Chironomids could not be identified into subfamily or lower taxonomic
 260 levels, so that Chironomidae gen. spp. may potentially influence the total number of
 261 taxa, particularly in the spring-fed tributary that housed numerous Chironomid midges
 262 (Table 1).

263   The MEM analysis did not detect any significant patterns of spatial

autocorrelation in the macroinvertebrate assemblage composition ($P = 0.68$), indicating spatial independence among the communities. The first two axes of the PCoA captured 97.6% of the total variation in the community Chao index matrix (Fig. 3), and the PERMANOVA indicated that macroinvertebrate assemblage structure significantly differed among the stream types ($P = 0.001$). After overlapping the environmental variables to the PCoA, the first PCoA axis indicated that the mainstreams presented greater water depth, stream width, periphyton biomass, current velocity and canopy openness than the spring-fed and non-spring-fed tributaries. The second PCoA axis mostly separated the non-spring-fed tributary from the spring-fed tributary, the two presenting very contrasted community composition as well as fine sediment deposition and standing stock of detritus (higher for the spring-fed tributary). The indicator taxa for the spring-fed tributary were mostly detritivores (shredders, collector-gatherers and collector-filterers) of Chironomidae, Nemouridae, Ceratopogonidae, Lepidostomatidae, Limnephilidae, Pisidiidae and Eusiridae, but also included one herbivorous trichopteran (*Allomyia* sp.) (Table 2). The indicator taxa for the mainstreams were mainly grazers (*Caenis* sp., *Epeorus* sp., *Drunella* sp., *Setodes* sp. and *Ecdyonurus viridis*) and partly contained predators (*Drunella* sp. and *Rhyacophila nipponica*), collector-gatherer (*Ephemerella setigera*) and collector-filterer (*Cheumatopsyche infascia*) whereas those for the non-spring-fed tributary were collector-gatherer and/or grazer (*Agapetus budoensis*) and collector-filterer (Simuliidae gen. spp.) (Table 2).

Discussion

288 Spring-fed streams in lowlands rarely have steep, deep channels (Sear et al. 1999) and
 289 their flow regimes are stable (Mattson et al. 1995; Lusardi et al. 2016). Sakai et al.
 290 (2020) showed that the spring-fed tributary assessed here maintained a stable flow
 291 regime even during rainfall events, implying that sediment runoff during floods likely
 292 occurs less in the tributary than in other non-spring-fed reaches. Because stable flow
 293 regime can settle fine particles in streambed (e.g., Kiffney et al. 2000), the significantly
 294 greater cover of fine sediment and higher standing stock of detritus observed in the
 295 spring-fed tributary are attributable to its stable flow regime. The observed slower
 296 current velocity in the spring-fed tributary could further contribute to the settlement of
 297 fine particles. The relatively closed canopy openness in the spring-fed tributary might
 298 additionally enhance accumulations of submerged detritus deriving from litter inputs.

299 Oligochaeta and most of the chironomid taxa were remarkably abundant in the
 300 spring-fed tributary. As a result, the total macroinvertebrate abundance was 3.8–12.2
 301 times greater in this tributary than in the other reaches. The relative abundance of
 302 burrowers increases with fine sediment deposition (e.g. Zweig and Rabení 2001;
 303 Rabení et al. 2005), and our findings suggest that the fine substrate found in spring-fed
 304 streams provides attractive habitat for burrower species. Moreover, the stable flow
 305 regimes of spring-fed streams may relate to less disturbance to macroinvertebrates
 306 during flood events (e.g., Fritz and Dodds 2004; Robinson et al. 2004). Because the
 307 burrower species that dominated in the spring-fed tributary typically gather and feed on
 308 fine detritus (i.e., collector-gatherers), spring-fed environments clearly provide
 309 adequate food resources for this group. The assumable long retention of submerged
 310 detritus and associated consumptions and dominance of detritivores may thereby form
 311 detritus-based food webs in spring-fed streams. Meanwhile, the strong variation of

312 macroinvertebrate abundances among the quadrats of a given stream type (e.g.,
313 Oligochaeta) suggest potential undetected microhabitat heterogeneity at finer spatial
314 scale, within the relatively homogeneous habitats found in the spring-fed tributary.

315 In contrast to our results, previous studies have reported that spring-fed
316 streams often have lush macrophytes (e.g., Sherwood et al. 2000; Katagiri et al. 2011)
317 and are accordingly dominated by herbivorous invertebrates (Takemon 2010).
318 Although obligate herbivores feeding on macrophytes are generally rare, macrophytes
319 may supplement food resources for grazers that usually consume algal masses and
320 provide spatially complex habitats. The streambed of the spring-fed stream we studied
321 lacked macrophytes perhaps because of limited light conditions. Food web and habitat
322 structures in spring-fed streams are probably site-specific and tightly associated with
323 environmental factors that affect the growth of macrophytes.

324 Wider streams with more open canopies generally induce higher primary
325 productivity (e.g., Vannote et al. 1980), but periphyton biomass was not significantly
326 different between the mainstreams and tributaries in this study. However,
327 ephemeropteran grazers significantly predominated in the mainstream reaches. This
328 contradiction is attributable to greater occupancy by grazers in reaches with higher
329 periphyton productivity. Feeding pressure of grazers is one of primary factors reducing
330 periphyton biomass (Feminella et al. 1989; Hill et al. 1995), and reaches with higher
331 productivity often have lower or similar periphyton biomass compared to those with
332 lower productivity (Feminella and Hawkins 1995). Therefore, grazing-based food webs
333 may be more pronounced in mainstreams and the dominance of grazer taxa and
334 associated consumption of periphyton may be responsible for the lack of differences in
335 periphyton biomass among reaches. The dominance of sprawlers in mainstreams is

336 attributable to their adaptability to fast-current habitats.

337   In the PCoA, the mainstream 2 had somewhat similar macroinvertebrate
338 assemblages to those in the spring-fed tributary. This result suggests that mainstream
339 reaches may have more heterogeneous habitats compared to tributaries, and partially
340 possess spring-fed-like macroinvertebrate communities even though the samples were
341 collected from riffles. For example, patches with upwelling water within reaches can
342 have such communities (Milner et al. 2001). Meanwhile, the macroinvertebrate
343 assemblages in the non-spring-fed tributary were clearly different from those in the
344 spring-fed tributary. Also, *Agapetus budoensis*, which was an indicator taxon of the
345 non-spring-fed tributary, was only found there. This indicates that the non-spring-fed
346 tributary also has unique habitats and macroinvertebrates assemblages compared to the
347 spring-fed tributary and mainstreams. Although taxa richness was lowest in the
348 non-spring-fed tributary, combinations of mainstream, spring-fed and non-spring-fed
349 tributaries can be important for maintaining the beta and gamma diversities of the river
350 network.

351   Given that the basal food resources and macroinvertebrate communities
352 differed between the spring-fed tributary and the adjacent mainstream reaches, we
353 suggest that the confluence of these streams may tangle the structure of the food web
354 such that the abundant macroinvertebrates in the spring-fed tributary may subsidize
355 predatory animals, such as fishes, inhabiting surrounding streams (cf. Uno and Power
356 2015). Moreover, the stable flow regime and water temperature in spring-fed streams
357 provide refugia during flood events (Sakai et al. 2020) and thermal stresses (Inoue and
358 Ishigaki 1968). Terui et al. (2018) reported that complex branches in river networks
359 can increase metapopulation stability for fish. Spring-fed streams may also contribute

360 to such stabilities in stream animal communities. Given that spring-fed habitats are
361 ubiquitous and distributed across Japan, examining the ecosystem functions of these
362 streams in nutrient cycling and as refugia is an important topic in river ecosystem
363 conservation. Given that the climate crisis relates to extreme runoff events (Yin et al.
364 2018) and severe thermal stress (van Vliet et al. 2013), these streams may be vital to
365 conservation efforts.

366 Two sprawler-grazer trichopteran species (*Allomyia* sp. and *Apatania* sp.) that
367 can be considered crenobiont and/or crenophilous were dominant in the spring-fed
368 tributary. In Japan, *Allomyia* species occur only in Hokkaido, and are preferentially
369 found in cool spring-fed habitats (Nishimoto and Kuhara 2001; Kawai and Tanida
370 2018). *Allomyia* sp. was found only in the spring-fed tributary in this study, similarly to
371 another study conducted in a montane spring-fed stream (Sueyoshi et al. 2014). At the
372 global scale, many of the crenophilous invertebrates are stenothermal, which is related
373 to their evolutionary origins (Sun et al. 2020), and the stable thermal environments of
374 spring-fed streams may provide refugia for these coldwater taxa (Lusardi et al. 2016).
375 The taxa that were dominant in the spring-fed tributary included stenothermal species,
376 implying that unique assemblages may be found in such streams. This may be an
377 important function of spring-fed streams; their unique habitats may contribute to the
378 beta diversity of focal river networks (Reiss et al. 2016).

379 Intensive researches conducted in carbonate geologies revealed that locations
380 of spring habitats are largely influential on local faunas. For example, faunal
381 characteristics tend to be similar between alpine springs and headwaters (Wigger et al.
382 2015), and substrate and macroinvertebrate assemblage structures vary with altitudinal
383 gradients (von Fumetti and Blatter 2017). Additionally, regions that receive small

384 amount of precipitations need to consider flow permanence of lotic systems. Some
 385 studies evaluated faunal characteristics in between intermittent and perennial spring
 386 habitats indicated similarity between them in high flow seasons (Wood et al. 2005;
 387 White et al. 2018) but life cycle adaptations of some taxa in the intermittent habitats
 388 (Wood et al. 2005). In lowland regions, Reiss et al. (2016) reported that crenobiont
 389 taxa which can be considered as glacial relicts inhabit in spring habitats, corresponding
 390 to this study. Although intermittent spring habitats may be relatively less under the
 391 moist climate of Japan, a concern regarding impact of anthropogenic disturbance such
 392 as land modifications and climate change on spring habitats (Cantonati et al. 2012) is
 393 common across locations and geologies.

394   Our comparisons of spring-fed and non-spring-fed streams revealed unique
 395 habitat characteristics and macroinvertebrate assemblage structure in the spring-fed
 396 tributary. The macroinvertebrate assemblage was characterized by numerous burrowers
 397 and collector-gatherers, and possibly crenobiont and/or crenophilous taxa. This unique
 398 assemblage may therefore enhance the beta-diversity of this river network. Owing to
 399 the high cover of fine sediments, the abundance of burrower taxa was remarkably high
 400 in the spring-fed tributary. These taxa may further play significant roles as prey for
 401 carnivores in the surrounding area. Although spring-fed streams are sparsely
 402 distributed under clastic geology, ideal site selections that include multiple spring-fed
 403 and non-spring-fed streams would further improve our understandings how spring-fed
 404 habitats structure animal communities. Given that spring-fed streams may act as flow
 405 and temperature refugia (Sakai et al. 2020, Lusardi et al. 2016), further studies that
 406 examine availabilities of the refugia in spring-fed streams can inform biodiversity
 407 conservation in river ecosystems. Here, we highlight the ecosystem function of

408 providing unique habitats and housing unique macroinvertebrate assemblages.

409

410

411   **Acknowledgments** Dr. Izumi Washitani provided invaluable comments on the study
412   plan. We thank the field assistance by Dr. Kosei Takahashi, Mr. Hitoshi Saito, Nobuo
413   Hatai and Kengo Ebihara.
414
415

416 **Funding** A portion of this study was supported by JSPS KAKENHI Grant numbers
 417 2629181 and 19K20491, and Kuromatsunai Biodiversity Conservation Research Grant
 418 (2017) to Masaru Sakai. David Bauman is supported by the Belgian American
 419 Educational Foundation.

420

421 **Competing interests** The authors declare no conflict of interest.

422

423 **Availability of data and materials** The data that support the findings of this study are
 424 available from the corresponding author upon reasonable request.

425

426 **Authors' contributions** MS, KI and DB conceived and designed the study. MS and KI
 427 conducted the field investigations, and MS and DB performed the statistical analyses.
 428 MS and KI wrote the first draft, and all authors contributed to the writing of the final
 429 manuscript.

430

431 **Ethical approval** Not applicable.

432 **Consent to participate** Not applicable.

433 **Consent for publication** Not applicable

434 **Code availability** Not applicable.

435

436

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Table 1 Environmental characteristics, total abundance, taxa richness, and the abundance of each dominant taxa in study reaches. LFT: life form type, B: burrower, Sp: sprawler, C: clinger, Sw: swimmer. FFG: functional feeding group, C/G: collector-gatherer, P: predator, G: grazer. Bold characters indicate statistically significant difference among stream types by one-way analyses of variances with permutation tests ($P < 0.05$), and different alphabetical letters indicate statistically significant differences by *post hoc* pairwise tests ($P < 0.05$). *P*-values are corrected for multiple tests (see Methods).

Variable	LFT	FFG	Spring-fed tributary			Non-spring-fed tributary			Mainstreams			F*	P
			Mean	±	SD	Mean	±	SD	Mean	±	SD		
Environmental													
Stream width (m)	-	-	3.8	±	0.3 ^b	1.0	±	0.2 ^c	12.7	±	2.4 ^a	87.40	<0.01
Water depth (cm)	-	-	16.8	±	3.8 ^b	9.4	±	1.9 ^c	32.0	±	2.7 ^a	421.21	<0.01
Current velocity (cm/s)	-	-	12.7	±	3.0 ^c	24.5	±	5.5 ^b	41.8	±	16.0 ^a	32.90	<0.01
Canopy openness (%)	-	-	34.3	±	12.1 ^b	45.5	±	17.4 ^{ab}	55.1	±	15.0 ^a	3.77	0.043
Fine sediment deposition (%)	-	-	83.5	±	10.6 ^a	22.0	±	7.2 ^b	7.3	±	3.7 ^c	554.89	<0.01
Periphyton biomass (mg/cm ²)	-	-	1.4	±	0.4	1.5	±	0.3	1.6	±	0.4	0.53	0.589
Standing stock of detritus (mg/cm ²)	-	-	1.7	±	0.9 ^a	0.7	±	1.0 ^{ab}	0.3	±	0.2 ^b	9.14	<0.01
Macroinvertebrates													
Total abundance	-	-	416.4	±	210.9 ^a	34.0	±	18.2 ^c	108.5	±	53.4 ^b	22.46	<0.01
Taxa richness	-	-	23.8	±	3.8 ^a	10.0	±	3.9 ^c	17.9	±	3.2 ^b	20.03	<0.01
Oligochaeta	B	C/G	116.6	±	141.5	0.6	±	1.3	20.3	±	34.2	4.84	0.018
<i>Pagastia</i> spp.	B	C/G	116.2	±	52.9 ^a	0.0	±	0.0 ^b	0.4	±	0.7 ^b	52.81	<0.01
<i>Drunella ishiyamana</i>	Sp	G, P	2.6	±	2.1 ^b	0.2	±	0.4 ^c	31.8	±	20.3 ^a	10.53	<0.01
<i>Apatania</i> sp.	C	G	34.8	±	21.7 ^a	7.0	±	4.6 ^b	0.1	±	0.5 ^c	25.18	<0.01
Chironomidae gen. spp.	B	C/G	40.0	±	41.5 ^a	0.0	±	0.0	0.0	±	0.0 ^b	10.20	<0.01
Chironominae spp.	B	C/G	30.2	±	31.9 ^a	0.0	±	0.0	0.0	±	0.0 ^b	9.86	<0.01
<i>Caenis</i> sp.	Sp	C/G, G	0.0	±	0.0 ^b	0.0	±	0.0 ^b	8.7	±	8.3 ^a	5.23	0.023
<i>Baetis</i> sp.	Sw	G	3.0	±	3.2	2.6	±	3.4	4.9	±	4.2	0.86	0.440
<i>Dicranota</i> spp.	B	P	5.4	±	2.6	4.4	±	3.4	2.9	±	3.3	1.24	0.296

<i>Allomyia</i> sp.	C	G	18.4 ± 11.7 ^a	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	27.22	<0.01
<i>Epeorus</i> sp.	Sp	G	0.2 ± 0.4	0.0 ± 0.0	6.0 ± 5.6	5.24	0.013
<i>Ordobrevia</i> sp.	Sp	C/G	1.8 ± 1.3	4.2 ± 1.3	4.1 ± 5.8	0.49	0.625
<i>Ephemerella setigera</i>	Sp	C/G	0.4 ± 0.5	0.0 ± 0.0	5.6 ± 6.4	3.33	0.049
<i>Ecdyonurus viridis</i>	Sp	G	0.4 ± 0.5	0.0 ± 0.0	3.9 ± 4.4	3.45	0.044
Tanypodinae sp. 1	B	P	12.2 ± 9.9 ^a	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	16.59	<0.01
<i>Drunella</i> sp.	Sp	P, G	0.2 ± 0.4	0.0 ± 0.0	3.5 ± 3.3	5.18	0.018
Orthocladiinae sp.	B	C/G	2.0 ± 1.9	1.2 ± 0.8	2.1 ± 1.4	0.70	0.526
<i>Paraleptophlebia japonica</i>	B	C/G	2.4 ± 3.7	0.6 ± 1.3	1.7 ± 1.7	0.88	0.422

599 * $F_{2,72}$ for water depth and current velocity, $F_{2,47}$ for fine sediment deposition, and $F_{2,22}$

600 for the other variables.

601

602

603 **Table 2** Statistically significant indicator taxa for each stream type ($P < 0.05$; adjusted P
604 for multiple tests). LFT: life form type, B: burrower, C: clinger, Sp: sprawler, Sw:
605 swimmer. FFG: functional feeding group, C/G: collector-gatherer, G: grazer, P: predator,
606 S: shredder, C/F: collector-filterer.

Taxon	LFT	FFG	Stream type	IndVal	P
Chironomidae gen. spp.	B	C/G	Spring-fed tributary	1.000	0.0001
<i>Allomyia</i> sp.	C	G	Spring-fed tributary	1.000	0.0001
Tanypodinae sp. 1	B	P	Spring-fed tributary	1.000	0.0001
<i>Pagastia</i> spp.	B	C/G	Spring-fed tributary	0.998	0.0001
<i>Nemoura</i> sp.	Sp	S	Spring-fed tributary	0.995	0.0001
Chironominae spp.	B	C/G	Spring-fed tributary	0.894	0.0009
Tanypodinae sp. 2	B	P	Spring-fed tributary	0.894	0.0009
Ceratopogonidae gen. spp.	B	C/G	Spring-fed tributary	0.894	0.0009
<i>Lepidostoma japonicum</i>	Sp	S	Spring-fed tributary	0.824	0.0052
<i>Nothopsyche</i> sp.	Sp	C/G, S	Spring-fed tributary	0.775	0.0101
<i>Pisidium japonicum</i>	B	C/F	Spring-fed tributary	0.775	0.0080
<i>Stenomoera yezoensis</i>	Sw	C/G, S	Spring-fed tributary	0.764	0.0068
<i>Agapetus budoensis</i>	C	C/G, G	Non-spring-fed tributary	1.000	0.0002
Simuliidae gen. spp.	C	C/F	Non-spring-fed tributary	0.764	0.0124
<i>Caenis</i> sp.	Sp	C/G, G	Mainstream	0.966	0.0001
<i>Epeorus</i> sp.	Sp	G	Mainstream	0.950	0.0004
<i>Drumella</i> sp.	Sp	G, P	Mainstream	0.940	0.0006
<i>Ephemerella setigera</i>	Sp	C/G	Mainstream	0.899	0.0028
<i>Setodes</i> sp.	Sp	C/G, G	Mainstream	0.856	0.0044
<i>Ecdyonurus viridis</i>	Sp	G	Mainstream	0.852	0.0154
<i>Cheumatopsyche infascia</i>	C	C/F	Mainstream	0.775	0.0193
<i>Rhyacophila nipponica</i>	Sp	P	Mainstream	0.730	0.0464

607

608

609 **Figure captions**

610

611 **Fig. 1** Study site location within the Shubuto River system in Kuromatsunai, Hokaido,
612 Japan. Open circles indicate the study reaches.

613

614 **Fig. 2** Flow and temperature regimes of the spring-fed tributary and adjacent
615 mainstream. Water levels were initially set at 0 cm at the onset of the monitoring.

616

617 **Fig. 3** Results of the principal coordinate analysis based on the abundance of all
618 macroinvertebrate taxa and the environmental variables. Significant indicator taxa for
619 each stream type are shown nearby the plots.

620

Fig. 1

Fig. 2

Fig. 3