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Assessment of small tributaries as possible habitats for larvae and juveniles of Japanese giant salamanders, *Andrias japonicus*, by coupling environmental DNA with traditional field surveys

Brianna Bjordahl[1], Sumio Okada[2] & Mizuki K. Takahashi[1,2]

[1)] Department of Biology, Bucknell University, Lewisburg, Pennsylvania, USA
[2)] The Hanzaki (Japanese Giant Salamander) Research Institute of Japan, Asago, Hyogo, Japan

Corresponding author: Mizuki K. Takahashi, e-mail: mt027@bucknell.edu

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Abstract. Demographic assessments of all four cryptobranchid salamander species have continued to indicate declines over the past several decades. One of the conservation challenges facing all cryptobranchid salamanders is the paucity of information about larvae and juveniles. Larvae and juveniles have only rarely been encountered during field surveys, even in streams where adults have commonly been found. In the case of the Japanese giant salamander (*Andrias japonicus*), several lines of evidence imply that larval and juvenile age classes use different habitats than adults such as small tributary streams, which have been overlooked by conservation monitoring surveys in Japan. We examined small tributary streams as possible habitats for young *A. japonicus* by integrating eDNA analysis with traditional field surveys. During the summer of 2018, we surveyed three first-to-third order tributaries of the Ichi River in Hyogo Prefecture, Japan, and collected water samples from each stream (Stream A: 465 m stretch, N=8; Stream B: 955 m stretch, N=21; Stream C: 2,331 m stretch, N=22) for eDNA analyses. Although no *A. japonicus* were observed during the eDNA water sampling, we repeatedly detected *A. japonicus* eDNA in all streams. Given this result, we conducted field surveys in the summer and fall of 2019, consisting of a daytime survey and a nighttime survey for each of the three streams. During the daytime surveys, we found no *A. japonicus* in Streams A and B, whereas in Stream C we found one larva, one juvenile, and one new nest with a large adult male actively guarding, from sampling sites that showed notably higher eDNA concentrations. During the nighttime surveys, we found five adults and one juvenile from Stream A, one adult from Stream B, and 13 adults from Stream C. These results suggest the importance of small tributary streams for *A. japonicus*, especially for smaller breeding adults and likely for larval and juvenile development. There are numerous previously unsurveyed small tributary streams throughout the range of *A. japonicus*. Our results suggest that the coupling of eDNA analysis with field surveys provides an efficient monitoring tool to examine those overlooked habitats, which would further emphasize the importance of including small tributaries in the conservation management of *A. japonicus* and potentially the other cryptobranchid salamanders.

Key words. Amphibia, Caudata, Cryptobranchidae, declines, conservation, eDNA, Japan, stream salamanders.

Introduction

The family Cryptobranchidae consists of four giant salamander species in North America and Asia: the hellbender (*Cryptobranchus alleganiensis*), the Chinese giant salamander (*Andrias davidianus*), the South China giant salamander (*A. sligoi*; Turvey et al. 2019), and the Japanese giant salamander (*A. japonicus*). The giant salamander group is one of the most imperilled families in the Order Caudata. The International Union for Conservation of Nature (IUCN) Red List currently lists *A. davidianus* as Critically Endangered (Gang et al. 2004; no Red List listing of *A. sligoi* yet), while *C. alleganiensis* (Hammerson & Phillips 2004) and *A. japonicus* (Kaneko & Matsui 2004) are listed as Near Threatened. Furthermore, *A. davidianus* is a Class II Protected Species in China, which makes hunting illegal (Gang et al. 2004). The United States Fish and Wildlife Service lists one of the two subspecies of the hellbender, the Ozark hellbender (*C. a. bishopi*), as Endangered (U.S. Fish and Wildlife Service 2011). The Japanese Ministry of the Environment recently changed the conservation status of *A. japonicus* from "Near Threatened" to "Vulnerable" (Ministry of the Environment 2006). Additionally, *A. japonicus* has been given the highest protection as a "Special Natural Monument" by the Japanese Agency for Cultural Affairs since 1952 (Agency for Cultural Affairs 1952). Despite the protections granted by their conservation status, population declines of cryptobranchids continue due to a combination of multiple anthropogenic threats such as habitat destruction and fragmentation by dams and concrete banks, pollution, poaching, and introduced species (Gang et al. 2004, Hammerson & Phillips 2004, Kaneko & Matsui 2004).

One of the common conservation challenges for all cryptobranchid salamanders is the paucity of information on the ecology of larvae and juveniles in the wild (Okada et al. 2008b, Foster et al. 2009, Hecht-Kardasz et al. 2012). It is rare to find young cryptobranchids even in the streams where large adults are commonly found (Wheeler et al. 2003, Okada et al. 2008b, Foster et al. 2009, Burgmeier et al. 2011). A few notable exceptions include the Little River, Tennessee, USA, for *C. alleganiensis* (Nickerson et al. 2003, Hecht-Kardasz et al. 2012) and one stream in Hiroshima, Japan, for *A. japonicus* (Okada et al. 2008b), where more larvae were found than any other age classes. The limited number of young cryptobranchid records has been of great concern to conservation biologists, as it is often indicative of low recruitment, which could lead to population extinctions when the long-lived current generations expire (e.g., Wheeler et al. 2003, Okada et al. 2008b, Burgmeier et al. 2011, Unger et al. 2013).

Low recruitment is one convincing explanation for the limited number of records on larvae and juveniles in some cases. However, another possible reason is that researchers have yet to fully identify the habitats used by larvae and juveniles during their development. In *C. alleganiensis*, a few studies have noted that larvae and juveniles use gravel beds, stream banks, and small rocks whereas adults typically hide under large rocks (Nickerson et al. 2003, Foster et al. 2009, Hecht et al. 2019). This ontogenetic shift in microhabitat use has likely prevented researchers from finding young hellbenders during field surveys.

In contrast to the research progress made on young hellbenders, there is little information on larvae and juveniles of the *Andrias* species in the wild. In China, finding any wild individuals of *A. davidianus* has become extremely difficult, largely because of overharvesting for human consumption and farming (Wang et al. 2004, Tapley et al. 2015, Cunningham et al. 2016, Pan et al. 2016, Turvey et al. 2018). As for *A. japonicus*, there are several studies implying habitat use by larvae and juveniles. Kobara (1985) and Taguchi (2009) note that adults typically migrate upstream to breeding sites, and as a result, nests are commonly found in the upper reaches of stream basins (Kawamichi & Ueda 1998, Okada et al. 2008b). Accordingly, Okada et al. (2008b) hypothesize that small tributaries may act as habitats for larvae and juveniles. Subsequently, little additional research has been done to test this hypothesis. This lack of research is presumably due to the conventional belief that small tributaries appear to be insufficient in size for adult *A. japonicus* to inhabit. Historically, conservation efforts for *A. japonicus* in Japan have prioritized finding and marking adult salamanders thus focusing searches on typical adult salamander habitats (Japan Giant Salamander Association 2016).

To examine the possibility of small tributaries serving as habitats for larval and juvenile *A. japonicus*, we surveyed three first-to-third order tributaries (Strahler stream order; Horton 1945) of the Ichi River in Hyogo Prefecture, Japan, where the Hanzaki (the regional name for the Japanese giant salamanders) Research Institute of Japan is located. The main-stem of the Ichi River has been well surveyed since 1975 and more than 1,700 different individuals have been recorded by the institute. On the other hand, small tributaries had rarely been surveyed. The recorded individuals in the Ichi River were almost exclusively adults; larvae and juveniles have been encountered infrequently (S. Okada unpubl. data). We conducted the first part of this study during the summer of 2018 by collecting and analysing water samples from unsurveyed tributaries for environmental DNA (eDNA) analysis. An eDNA survey allows researchers to identify the distribution of target species and is more efficient in terms of time and effort when compared to traditional field survey techniques of the giant salamanders (Fukumoto et al. 2015, Spear et al. 2015, Pitt et al. 2017, Takahashi et al. 2018). The results of the eDNA survey informed the design of the second part of the study where we used daytime and nighttime field survey techniques during the summer and fall of 2019 to physically locate *A. japonicus*.

Materials and methods

Study area

We conducted our research in the headwaters of the Ichi River in Ikunocho Kurokawa, Hyogo Prefecture, Japan. The Ichi River is 73 km long with a drainage area of 496 km^2 and runs into the Seto Inland Sea. Our study was focused on three small tributaries, henceforth referred to as Streams A, B, and C. Streams A and B are first-to-second order streams while Stream C is a second-to-third order stream (Strahler stream order; Horton 1945; Fig. 1). Elevations of the study sites along these streams ranged from 514 to 609 m asl. These streams were located within the temperate zone and surrounded by mixed forests with natural deciduous trees as well as commercially-planted coniferous trees. Stream A lay within an almost entirely forested landscape with some private streamside residences, while Streams B and C were situated within a mixed landscape of forest, streamside residences, and agricultural land mainly consisting of rice paddies, especially for Stream C. The studied stretch along Stream A was roughly 465 m (stream distance), for Stream B it was roughly 955 m, and for Stream C it was roughly 2,331 m. During typical streamflow, the sections close to the mouths of Streams A and B were 50 to 100 cm wide and 5 to 20 cm deep with several deeper (~50 cm) pools. That of Stream C was 100 to 200 cm wide and 10 to 30 cm deep with several deeper (~100 cm) pools. The average annual precipitation of the region, recorded between 1981 and 2010, was 2,021.2 mm, and the average annual temperature, recorded during the same time frame, was 13°C. There are no formal conservation management strategies employed to protect *A. japonicus* in the area.

eDNA field sampling

During late July and early August of 2018, we collected a total of 51 water samples from Streams A (N = 8), B (N =

21), and C (N = 22). We chose the sampling period based on the weather conditions and other logistical constraints. We started our sample collection at each tributary confluence with the main-stem of the Ichi River, and then hiked upstream until there was no visible water (Stream A and B) or until the stream became less than 50 cm wide and consistently less than 10 cm deep (Stream C). We collected water samples every 25 to 100 m in Streams A and B and 60 to 170 m in Stream C in places where the stream widened into a pool, at the confluence between two tributary streams, or in places where natural embankments and deeper water were present. We selected these sites for water sampling because individuals of *A. japonicus* tend to be found in these places during nighttime surveys and also because we attempted to include even smaller branches flowing into Streams A, B and C in the eDNA survey. The average distances between two consecutive sampling points for Streams A, B, and C were 61.4 m, 47.3 m and 110.4 m respectively. Samples were collected from Stream A over the course of one day. Sampling runs were completed over the course of multiple days for streams B (three days) and C (two days) because of logistical difficulties.

At each sampling site, we used a plastic syringe to collect five 200 ml surface water samples laterally across the width of the stream, combining the samples into a 1 l container. We used the same syringe for the sites along the same stream. To ensure no cross contamination occurred between sampling sites, the syringe was cleaned by washing it six times with stream water from each new location before new samples were collected. We verified that this cleaning method prevented cross contamination by conducting a controlled laboratory experiment at Bucknell University, USA. In this cleaning experiment, we created three treatments with different *A. japonicus* eDNA concentrations

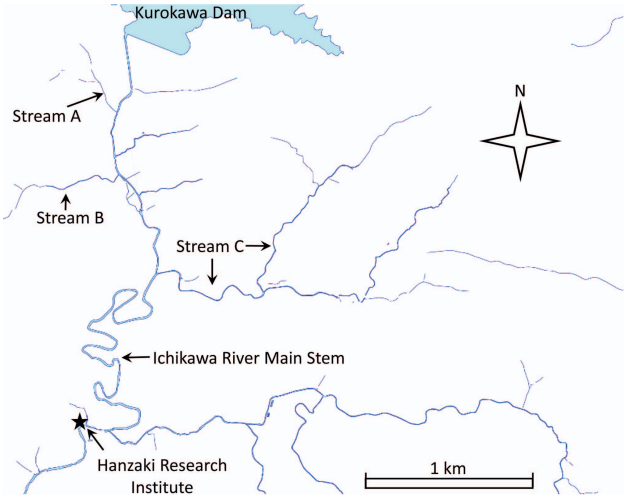

Figure 1. Study area map of the upper drainage basin of the Ichi River, Hyogo Japan. We surveyed the three small tributary streams A, B and C for *Andrias japonicus* during the summer and fall of both 2018 and 2019. The Ichikawa River runs from north to south with Stream A being the most upstream tributary.

(0.02 ng/l, 2.99 ng/l and 6 ng/l) through dilution of DNA extracted from a tissue sample and measured with a Qubit 4 fluorometer™. The lowest and the highest concentrations corresponded to the lowest and the highest concentrations we detected in the field respectively. The intermediate treatment was created based on the average of these two concentrations. In the laboratory, we pumped water from each treatment five times with the same syringe, simulating the field sampling. We then cleaned the same syringe by pumping tap water six times, after which we sampled tap water with the cleaned syringe for eDNA analysis. We processed the water samples (i.e., filtration and DNA extraction) and ran qPCR following the same procedures we used for the field samples (see below), with four replicates per treatment. We did not detect any residual eDNA from any of the replicates for all the treatments, confirming the effectiveness of our cleaning method between field samplings.

eDNA laboratory procedures

Within 24 hrs of collection, we filtered each water sample through 0.45 µm nitrocellulose filters (47 mm in diameter; Whatman™) using a pair of vacuum pumps. Additionally, we added one negative control to the Stream A samples, three negative controls to the Stream B samples, and two negative controls to the Stream C samples, dependent upon on how many days it took to complete sampling for each tributary. Stream water was clear and we did not have issues with turbidity and suspended organic material. After the filtration of each sample, we cleaned the equipment in purified water, a 50% ethanol-water solution, a 50% bleach-water solution, and again with purified water. In addition, we cleaned the top of each filtering device with DNA Away™ (Thermo Fisher Scientific, Inc.) before placing the filter paper for the next filtration. After filtration, we cut each filter in half, one to use for DNA extraction, the other to save as a backup. All work was conducted in a laboratory provided by the Hanzaki Research Institute of Japan that was closely located to the surveyed tributaries.

We extracted DNA from the filters using the standard protocol of the DNeasy Blood and Tissue Kit (Qiagen, Inc.). We conducted qPCR in accordance with the qPCR protocol developed by Spear et al. (2015), along with primers and a probe developed for *A. japonicas* by Fukumoto et al. (2015), and estimated the concentration of *A. japonicus* eDNA in each sample. We ran 24 µl qPCR reactions using 0.96 µM each of species-specific primer (Forward: 5' CGGCGTTCTTCAACCATTG 3'; Reverse: 5' AGCTCAAATTATTAAGGAGGTGGTTAA 3') and 0.48 µM of probe (5' 6FAM – ACACTCTTTTTAATTGCCCCAGT – MGB-NFQ 3') per individual well. Additionally, we used 12 µl of Qiagen QuantiTect Multiplex PCR Mix (Qiagen, Inc.), 0.96 µl of TaqMan Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems, Inc.), 0.48 µl of TaqMan Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems, Inc.), and 0.16 µl of dH_2O per individual well. We ran each sample and negative controls in quadruplicate

Table 1. Summary of the 2019 field surveys of the three small tributary streams A, B & C for *Andrias japonicus* in the headwaters of the Ichi River, Hyogo, Japan.

	Daytime Survey	Nighttime Survey	Additional Survey
Stream A	Sep. 6 (2 persons × 2 h). Walking upstream from the main-stem along the entire stretch covered by the eDNA survey with a special effort to look for larvae and juveniles in areas where rocks and leaves were piled up	Jul. 22 (2 persons × 2 h). Walking upstream from the main-stem along the entire stretch covered by the eDNA survey	None
Stream B	Sep. 6 (2 persons × 2 h). Walking upstream from the main-stem along the entire stretch covered by the eDNA survey with a special effort to look for larvae and juveniles in areas where rocks and leaves were piled up	Jul. 22 (1 person × 2 h). Walking upstream from the main-stem along the entire stretch covered by the eDNA survey	None
Stream C	Sep. 19 & 20 (2 persons × 2 h). Looking for larvae and juveniles in two eDNA sampling sites where extremely high eDNA concentrations were detected	Sep. 19–20 (2 persons × 5 h). Walking upstream from the main-stem along the entire stretch covered by the eDNA survey	An additional survey was done on Oct. 19 to examine the den master of the newly discovered nest (2 persons × 0.5 h).

with *A. japonicus* DNA standards ranging from 0.0001 to 1 ng/µl. These standards were created from a series of dilutions of DNA extracted from an *A. japonicus* liver tissue, which we measured using a Qubit 4 fluorometer™ (Thermo Fisher Scientific, Inc.). qPCR cycling began with 15 min at 95°C, followed by 50 cycles of 94°C for 60 s and 60°C for 60 s. To visualize our data, we used the LightCycler® 96 System (Roche Life Science, Inc.). This system allows users to set manual thresholds, and we manually set thresholds to exclude false positives, amplifications of which started towards the end of the cycles and thus had visibly different amplification curves from the standard curves. For all qPCR reactions, standard curve R^2 values were ≥ 0.95 and PCR efficiencies ranged between 92.5% and 101.5%.

eDNA statistical analysis

To examine overall differences in eDNA concentrations among the three tributaries, we conducted analysis of covariance (ANCOVA) by stream, controlling for the stream distance from the confluence as a covariate. We included stream distance as a covariate because we predicted greater concentrations of *A. japonicus* eDNA at sampling sites closer to the main-stem of the Ichi River for two reasons. First, given the sampling frequency at relatively short distances, we predicted the rate of eDNA accumulation downstream would be greater than the rate of eDNA dilution via degradation and diffusion. Second, we predicted greater *A. japonicus* biomass resulting from the increased likelihood that more individuals as well as individuals of a larger size would be distributed downstream of each stream due to the greater width and depth of the streams at these sites.

Traditional field survey

The eDNA survey results informed subsequent traditional field surveys; we conducted field surveys to search for individuals of *A. japonicus* between July and October 2019. We conducted two field surveys, a daytime survey and a nighttime survey at each of the three streams (Table 1). For the daytime surveys, we waded in a stream carefully looking for salamanders by visually covering the entire width of the stream as well as both sides of the stream bank. We turned rocks that appeared to provide suitable hiding spaces. We also made special efforts to look for young salamanders in leaf packs and rock piles. For the nighttime surveys, we followed the same procedure as in the daytime surveys but with flashlights and without turning any rocks, leaf packs, and rock piles. We conducted the daytime surveys between 10:00 and 18:00 hrs and nighttime surveys between 21:00 and 02:00 hrs. We spent a total of 29 person hrs searching for individual salamanders in the three streams, resulting in an average of 1 hr of search time for every 129 m of stream length. *Andrias japonicus* encountered in the field were captured by hand or hand net. Specimen data collection followed the protocol of the Hanzaki Research Institute of Japan, with the following morphometric data collected: head width, tail height, snout–vent length (SVL) and total length (TL), as well as body mass. We classified a captured *A. japonicus* into an adult, a juvenile or a larva based on the presence of external gills (larvae), the presence of swelling around a cloaca (reproductive male), the presence of abdominal swelling (gravid female), and body size. According to Kobara (1985), the smallest reproductive male was 30 cm in TL and the smallest reproductive female was 40 cm in TL. We used these body-size criteria and classified a metamorph larger than 30 cm in TL with a swelled cloaca as an adult male, a metamorph larger than 40 cm in TL without a swelled cloaca but with abdominal swelling as an adult female, and a metamorph with TL 30 to 40 cm without a swelled cloaca and abdominal swelling as a juvenile. One exception to these criteria was an individual of 40.1 cm in TL that we caught during one of the nighttime surveys. We classified this individual as a juvenile female because of the lack of swelling around a cloaca as well as the lack of abdominal swelling. Captured

animals were scanned for Passive Integrated Transponder (PIT) tags and photographed. When animals were not PIT tagged, we inserted PIT tags (Trovan ISO ID-100/R [2.1 × 11.5 mm] for metamorphs; Trovan ISO Mini ID-100/1.4/R [1.4 × 8 mm] for larvae) into left shoulders of metamorphs and tails of larvae that were 15 cm or larger in TL. Although we did not capture larvae smaller than 15 cm in TL during the surveys, such small larvae were too small for PIT tagging. We also took notes on apparent external injuries (wounds and scars) and missing body parts. In this study we reported only body mass, SVL and TL.

Field data analysis

In order to compare the body size of *A. japonicus* found in the main-stem Ichi River with those found in the tributaries, we examined the Hanzaki Research Institute of Japan's database and extracted data on adults of known sex that were captured between 2015 and 2019 from the stretch of the main-stem where the three study tributaries flew into. Prior to 2015, little survey was done in the section of the main-stem, and thus, data prior to 2015 were scarce. When there were recaptures of the same individuals from the main-stem, we used the data from the most recent captures. From our field surveys, we also selected data on adults of known sex for analysis. We then log-transformed the data and ran a two-way analysis of variance (ANOVA), testing the effects of stream identity (main-stem vs. tributary) and sex on SVL. We did not analyse migratory distance data because the data in the main-stem were collected by multiple researchers and many of them did not have GPS coordinates but with simple site descriptions.

Results

eDNA survey

Out of 204 total qPCR reactions (51 samples with 4 replicates), 10 reactions failed internal positive controls (194/204 success rate). The 10 failed reactions were distributed across 10 different samples, and thus, we averaged the values for those 10 samples based on triplicates instead of quadruplicates. All negative controls were negative, indicating that there was no issue of contamination. Although we did not observe any *A. japonicus* during water sample collection, we detected *A. japonicus* eDNA from all three of the tributaries surveyed (Fig. 2). At Stream A, *A. japonicus* eDNA was detected at all sampling locations with the exception of Site 5 (88% positive). At Stream B, Sites 13, 15, 17, 18, and 21, had no eDNA detected, while at the rest of the sites it was detected (76% positive). At Stream C, we detected *A. japonicus* eDNA at all sites except the final two upstream Sites 21 and 22 (91% positive). Overall, 43 out of 51 samples were positive with *A. japonicus* eDNA (84%). Stream A had an average eDNA concentration of 0.0199 ± 0.0165 (SE) ng/l, Stream B had an average concentration of 0.0119 ± 0.0144 (SE) ng/l, and Stream C had

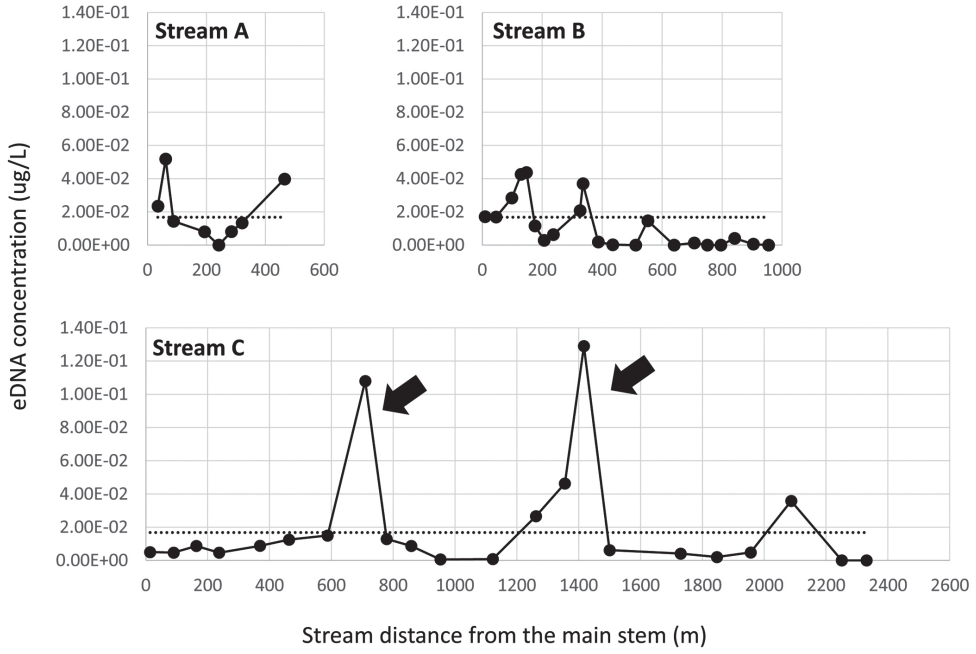

Figure 2. Spatial profiles of *Andrias japonicus* eDNA concentrations along the three small tributary streams (Streams A, B and C) located in the headwaters of the Ichi River, Hyogo, Japan. Black arrows in Stream C indicate sites with notably high eDNA concentrations where we conducted subsequent traditional field surveys.

an average concentration of 0.0202 ± 0.0332 (SE) ng/l. The result of ANCOVA showed that the three tributaries had similar eDNA concentration ($F_{2, 47}$= 0.761, P = 0.473) and that stream distance was not a significant covariate ($F_{1, 47}$ = 0.243, P = 0.624). There were two sampling sites (Sites 8 and 15) along Stream C where we detected notably higher concentrations of eDNA (Fig. 2C).

Traditional field surveys

Despite no sighting of *A. japonicus* during the eDNA water sampling, we encountered a total of 23 individuals, consisting of one larva, two juveniles, and 20 adults during the field surveys conducted between July and October 2019 (Fig. 3). The average body mass, SVL, and TL ± SE of the adults were 1335.2 ± 197.5 g, 385 ± 13 mm, and 582 ± 22 mm respectively. Sixteen out of the 20 adult salamanders (80%) were similar in size with SVLs between 300 and 400 mm. Overall, the SVLs of the adults of known sex captured in the three tributaries (N = 18, 383 ± 15 [SE] mm) were significantly smaller than those captured in the corresponding main-stem stretch of the Ichi River (N = 37, 483 ± 14 mm, $F_{1, 51}$ = 21.523, P < 0.001, Fig. 4). There were no effect of sexual differences on body size ($F_{1, 51}$ = 2.152; P = 0.149) nor the interaction between sex and stream ($F_{1, 51}$ = 0.297; P = 0.588). Of the 23 individuals, 13 were new individuals while 10 were recaptures from previous surveys in the main-stem, affirming that *A. japonicus* migrates between the main-stem and its tributaries.

During the daytime surveys, we found no salamanders in Streams A and B. We found three individuals in Stream C from the sites with notably high eDNA concentrations detected. We found the larva (body mass = 27.0 g, SVL = 108 mm, TL = 161 mm) and one of the two juveniles (body mass = 383.0 g, SVL = 249 mm, TL = 382 mm) in a pile of rocks covered with mosses (Fig. 5), which was located roughly 15 m upstream from Site 8 of Stream C (~710 m upstream from the main-stem of the Ichi River). We intentionally limited the survey time to 30 min at this site and left the majority of the rock pile untouched to avoid excessive habitat disturbance. We found a nesting site that was previously unknown, roughly 10 m upstream from Site 15 of Stream C (Fig. 6A&B, ~1420 m upstream from the main-stem of the Ichi River). While checking the new nesting site on 20 September 2019, we encountered a den master (a brood guarding male) actively guarding the nest and found healthy embryos that we estimated to be ~2 weeks post-fertilization (Fig. 6C). We conducted an additional survey on 19 October 2019 and captured and measured the den master (Fig. 6D, body mass = 2140.0 g, SVL = 534 mm, TL = 833 mm). This den master had the greatest body length of all the salamanders captured during our field surveys. During the nighttime surveys, we found five, one, and 13 *A. japonicus* in Streams A, B, and C, respectively. The other juvenile was caught in Stream A during the nighttime survey (body mass = 450.0 g, SVL = 259 mm, TL = 401 mm).

Discussion

Cryptobranchid salamanders are experiencing ongoing population declines (Gang et al. 2004, Hammerson & Phillips 2004, Kaneko & Matsui 2004), and it has be-

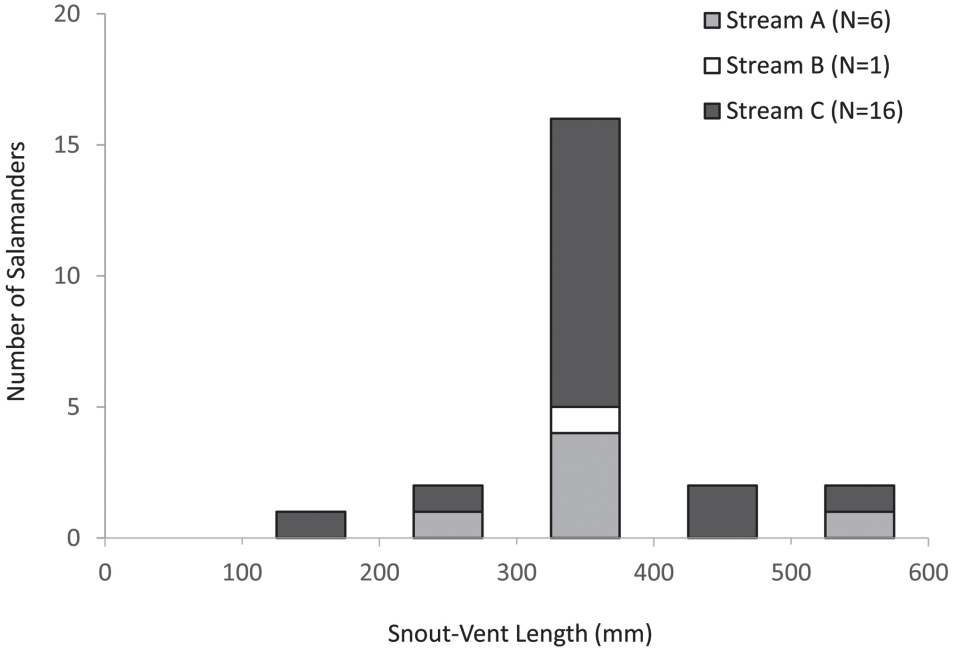

Figure 3. Body size histogram of *Andrias japonicus* in the three small tributary streams (A, B and C) located in the headwaters of the Ichi River, Hyogo, Japan.

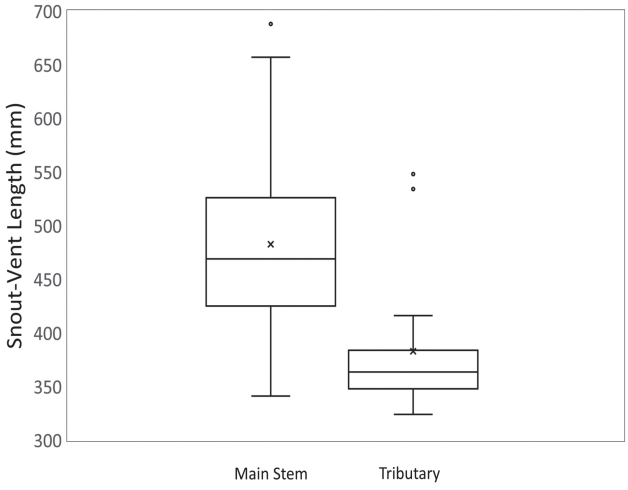

Figure 4. Body size comparison of adult *A. japonicus* between the main-stem of the Ichikawa River and the tributary streams (A, B and C) located in the headwaters of the Ichi River, Hyogo, Japan.

come critical now more than ever to monitor their population trends. However, without first identifying all core habitats for all life stages of these secretive salamanders, it is nearly impossible to monitor their populations and to determine population viability. The results of our eDNA analysis and field surveys confirm the use of small tributary streams by *A. japonicus* and advance the current knowledge of *A. japonicus* habitat use, migration, and demography accumulated by previous studies (Kobara 1985, Tochimoto 1993, Kawamichi & Ueda 1998, Okada et al. 2008b, Taguchi 2009, Taguchi & Natsuhara 2009). Results from our eDNA analysis were effective in informing us on the presence of *A. japonicus* as well as specific sites to search along tributary streams. Finding *A. japonicus* nests and the young in the wild is difficult (Okada et al. 2008b), especially in streams where we have no prior knowledge of salamander occurrence. Given this difficulty, it is compelling that the analysis of the spatial profile of eDNA concentrations, which indicated notably high eDNA concentrations in Stream C, led to the discoveries of the new nest-

Figure 5. Newly discovered microhabitat (A) and a captured larva (B) and a juvenile (C) of *Andrias japonicus* in the small tributary Stream C located in the headwaters of the Ichi River, Hyogo, Japan. The microhabitat is a pile of rocks covered with mosses, indicating its stability.

ing site and the microhabitat for young giant salamanders. These discoveries are underpinned by positive correlations between eDNA concentrations and abundance/biomass of aquatic organisms that have been well documented in lentic systems (Takahara et al. 2012, Pilliod et al. 2013, Klymus et al. 2015, Lacoursière-Roussel et al. 2016) and more recently in lotic systems (Jane et al. 2015, Doi et al. 2017).

We were concerned that accumulation of eDNA via transport would confound eDNA signals at downstream sites. Additionally, we considered that eDNA concentrations might be higher at downstream sites due to greater biomass of larger individuals entering from the main-stem. On the contrary, the result of our ANCOVA showed that stream distance from the confluence was not a significant covariate, suggesting not only that giant salamanders were distributed throughout the entire tributary, but that eDNA was rapidly degrading and diffusing as well. The results indicate that we were able to obtain an informative eDNA spatial profile due to the combination of this apparent rapid eDNA degradation/diffusion and frequent water sampling.

It is important to note that our eDNA water sampling was done before the onset of the *A. japonicus* breeding season in late August and early September. Therefore, the high eDNA concentration detected at the Stream C nesting site was not due to breeding activities as found in previous eDNA studies of *C. alleganiensis* (Spear et al. 2015, Takahashi et al. 2018). Instead, the high eDNA concentration most likely resulted from a large number of young salamanders that had hatched in previous years which were present in the pool. This pool in particular provided an isolated habitat for larvae and small juveniles because the concrete pipe laid underneath the forest road prevented the upstream migration of salamanders and the concrete floor and banks downstream created an inhabitable section for salamanders (Fig. 6A&B).

The discovery of a new nesting site and adult *A. japonicus* during and immediately before the breeding season suggest that small tributary streams may provide breeding sites for adults. It appears that small streams are especially important for smaller adults that are likely less com-

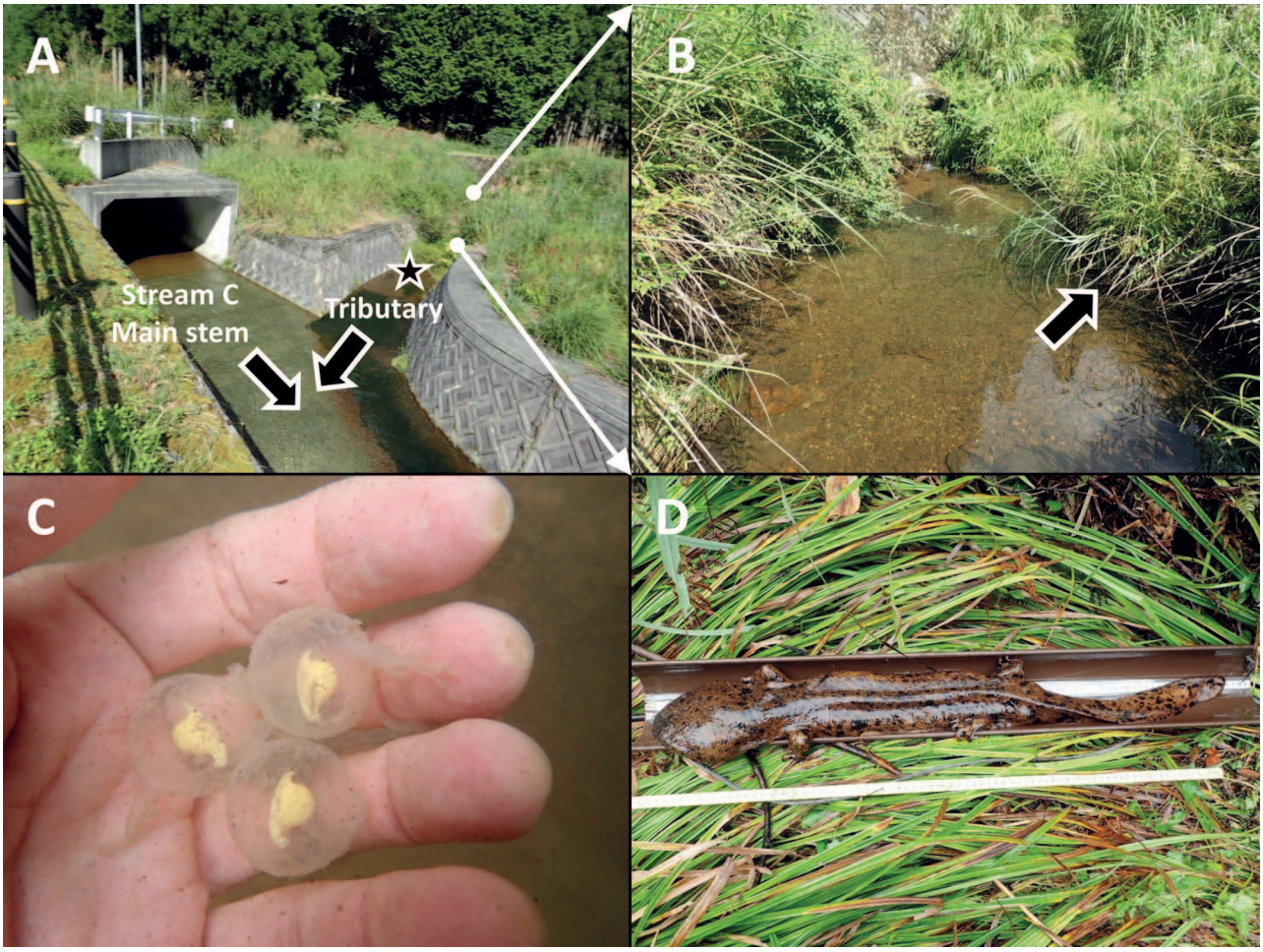

Figure 6. Newly discovered nesting site (A & B) and embryos (C) and a den master (D) found in the nesting site in the small tributary Stream C located in the headwaters of the Ichi River, Hyogo, Japan. The star in Panel A indicates the location of water sampling for the eDNA survey in 2018 where we detected a notably high eDNA concentration. The arrow in Panel B indicates the location of the nesting site.

petitive in securing reproductive success when competing with larger adults in larger streams. Small tributaries, with relatively fewer large predators including conspecific adults (Kawamichi & Ueda 1998, Okada et al. 2008a), are also likely to provide favourable habitats for young *A. japonicus*. During the field surveys of Streams A and B, two experienced field researchers (OS and MKT) carefully examined piles of leaves and rocks, the microhabitats where larval and juvenile *A. japonicus* are typically found (S. Okada unpubl. data). Despite the careful search, we found only one larva and two juveniles and did not find any larvae in Streams A and B. It is possible that young *A. japonicus* use different microhabitats than traditionally known leaf packs and rock piles within small tributaries. For example, in a small agricultural canal connected to a nearby river in Hiroshima Prefecture, a large number of larvae were observed in a narrow gap under the root system of a large tree growing next to the canal (K. Kuwabara pers. comm.). Furthermore, Nickerson et al. (2003) hypothesized that a reason for the extremely low number of larval hellbenders found in the North Fork of the White River, Missouri during their study, was that larval hellbenders were hidden in large, deep, gravel beds to avoid cannibalistic adults. In the tributary streams we surveyed, rock beds were prominent along with shallow gravel beds, and the risk of cannibalism is likely low because the shallow streams are unlikely to provide enough food and water for large adults to inhabit for extended periods. Yet, it is possible that small larvae of *A. japonicus* also hide in gravel beds. Thus, future studies should target exploration of small gaps within root systems of riparian vegetation as well as gravel beds in small tributaries. These microhabitats require careful investigation with equipment not typically employed in adult *A. japonicus* surveys, such as waterproof endoscopes and excavation tools.

Another possible reason for not detecting larvae in Streams A and B was that record heavy rainstorms in Western Honshu in September 2018 (Typhoon 21, 24 and 25; Japan Meteorological Agency 2019) potentially destroyed microhabitats and flushed young *A. japonicus* down to the main-stem of the Ichi River. The precipitation during September 2018 in the study area was roughly twice as much as the average September precipitation recorded between 1981 and 2010 (490.5 mm in 2018 vs. 246.2 mm on average; Japan Meteorological Agency 2019). We noticed changes in stream morphology in Streams A and B during the 2019 field surveys, and unlike those in Stream C, there were no mossy rock piles observed in these streams, indicating recent disturbances. The slopes of Streams A and B were much steeper (0.078 m and 0.097 m elevation change per meter of stream distance respectively) than that of Stream C (0.027 m elevation change per meter of stream distance). These lines of evidence suggest that the disturbances resulting from the 2018 storms were also likely responsible for the unsuccessful discovery of larvae and nesting sites in Streams A and B.

The results of our study highlight the importance of small tributary streams as vital habitats for *A. japonicus*. In Japan, the construction of concrete banks and numerous agricultural, flood-control, and sand-control dams have

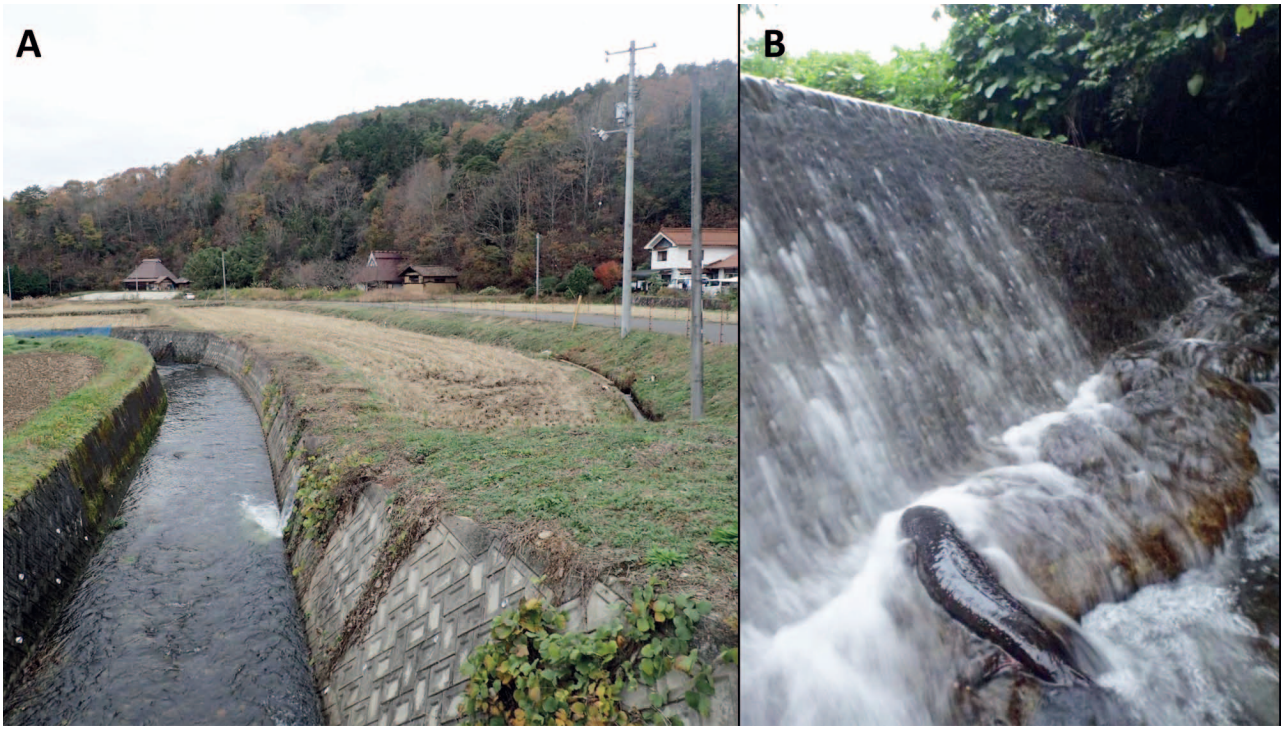

Figure 7. Typical examples of habitat fragmentation in Japanese rivers preventing migration of *Andrias japonicus*. Concrete banking cutting off the connectivity between the main-stem and its tributary (A). Agricultural dam preventing breeding migration of an adult *A. japonicus* (B).

caused habitat fragmentation and prevent *A. japonicus* from making breeding migration to small tributaries (Fig. 7A&B; Taguchi & Natsuraha 2009). Concrete banks have also destroyed nesting sites and prevented males from constructing new burrows along stream banks (Tochimoto 2005, Okada et al. 2008b, Takezawa et al. 2008). With growing concerns around changing global climate, the predicted increase in frequency and intensity of rainstorms in Japan (Ministry of the Environment et al. 2018) will likely result in further stream alterations, which would pose a serious conservation challenge for *A. japonicus*. We encourage future surveys on the use of small tributaries by *A. japonicus*. The database generated from these surveys should further confirm the need for the inclusion of small tributaries in future conservation strategies.

Finally, the present study suggests that the combination of eDNA analysis with field surveys provides an effective survey tool for elusive stream amphibians including the other cryptobranchids. For example, recent studies emphasize the urgent conservation need for each of the distinct lineages recently revealed within the Chinese *Andrias* species complex (Turvey et al. 2018, Yan et al. 2018, Turvey et al. 2019). However, extensive search using routine ecological survey methods failed to detect giant salamanders in the wild in China (Tapley et al. 2015). In such cases, we recommend the use of cost- and time-efficient eDNA survey covering entire stretches of candidate streams, which should inform researchers on specific sites for physical field surveys.

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References

Agency for Cultural Affairs (1952): Nationally Designated Important Cultural Properties. Database. – Available at https://kunishitei.bunka.go.jp/heritage/detail/401/3129.

Burgmeier, N. G., T. M. Sutton & R. N. Williams (2011): Spatial ecology of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. – Herpetologica, **67**: 135–145.

Cunningham, A. A., S. T. Turvey, F. Zhou, H. M. Meredith, W. Guan, X. Liu, C. Sun, Z. Wang & M. Wu (2016): Development of the Chinese giant salamander *Andrias davidianus* farming industry in Shaanxi Province, China: conservation threats and opportunities. – Oryx, **50**: 265–273.

Doi, H., R. Inui, Y. Akamatsu, K. Kanno, H. Yamanaka, T. Takahara & T. Minamoto (2017): Environmental DNA analysis for estimating the abundance and biomass of stream fish. – Freshwater Biology, **62**: 30–39.

Foster, R. L., A. M. McMillan & K. J. Roblee (2009): Population status of hellbender salamanders (*Cryptobranchus alleganiensis*) in the Allegheny River drainage of New York State. – Journal of Herpetology, **43**: 579–588.

Fukumoto, S., A. Ushimaru & T. Minamoto (2015): A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. – Journal of Applied Ecology, **52**: 358–365.

Gang, L., G. Baorong & Z. Ermi (2004): *Andrias davidianus*. The IUCN Red List of Threatened Species 2004: e.T1272A3375181. – Available at https://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T1272A3375181.en, downloaded on 31 January 2020.

Hammerson, G. & C. Phillips (2004): *Cryptobranchus alleganiensis*. The IUCN Red List of Threatened Species 2004: e.T59077A11879843. – Available at https://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T59077A11879843.en, downloaded on 31 January 2020.

Hecht, K. A., M. J. Freake, M. A. Nickerson & P. Colclough (2019): Hellbender salamanders (*Cryptobranchus alleganiensis*) exhibit an ontogenetic shift in microhabitat use in a Blue Ridge physiologic region stream. – Copeia, **107**: 152–159.

Hecht-Kardasz, K. A., M. A. Nickerson, M. Freake & P. Colclough (2012): Population structure of the hellbender (*Cryptobranchus alleganiensis*) in a Great Smoky Mountains stream. – Bulletin of the Florida Museum of Natural History, **51**: 227–241.

Horton, R. E. (1945): Erosional development of streams and their drainage basins: hydro-physical approach to quantitative morphology. – Geological Society of America Bulletin, **56**: 275–370.

Jane, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. H. Lowe, B. H. Letcher & A. R. Whiteley (2015): Distance, flow and PCR inhibition: e DNA dynamics in two headwater streams. – Molecular Ecology Resources, **15**: 216–227.

Japan Giant Salamander Association (2016): Individual registration manual for Japanese giant salamanders (in Japanese).

Japan Meteorological Agency (2019): Notification on the Typhoons in 2018.

Kaneko, Y. & M. Matsui (2004): *Andrias japonicus*. The IUCN Red List of Threatened Species 2004: e.T1273A3376261. – Available at https://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T1273A3376261.en, downloaded on 31 January 2020.

Kawamichi, T. & H. Ueda (1998): Spawning at nests of extra-large males in the giant salamander *Andrias japonicus*. – Journal of Herpetology, **32**, 133–136.

Klymus, K. E., C. A. Richter, D. C. Chapman, C. Paukert (2015): Quantification of eDNA shedding rates from invasive bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*). – Biological Conservation, **183**: 77–84.

Kobara, J. (1985): O-sansyo-uo [The Japanese Giant Salamander]. – Dobutsu-sha, Tokyo, Japan (in Japanese).

Lacoursière-Roussel, A., G. Côté, V. Leclerc &L. Bernatchez (2016): Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. – Journal of Applied Ecology, **53**: 1148–1157.

Ministry of the Environment (2006): Japan Ministry of the Environment Endangered Species Search. – Available at https://ikilog.biodic.go.jp/Rdb/env.

Ministry of the Environment, Ministry of Education, Culture, Sports, Science and Technology, Ministry of Agriculture, Forestry and Fisheries, Ministry of Land, Infrastructure, Transport and Tourism, & Japan Meteorological Agency (2018): Climate change in Japan and its impacts. – Synthesis Report on Observations, Projections and Impact Assessments of Climate Change, 2018.

Nickerson, M. A., K. L. Krysko & R. D. Owen (2003): Habitat differences affecting age class distributions of the hellbender salamander, *Cryptobranchus alleganiensis*. – Southeastern Naturalist, **2**: 619–629.

Okada, S., K. Ichisawa & Y. Kawakami (2008a): Records of dead Japanese giant salamanders *Andrias japonicus* from the Hino and the Shiokawa Rivers between 2001 and 2006, Tottori Prefecture, Japan. – Bulletin of the Tottori Prefectural Museum, **45**: 11–16 (in Japanese with English abstract).

Okada, S., T. Utsunomiya, T. Okada, Z. I. Felix & F. Ito (2008b): Characteristics of Japanese Giant Salamander (*Andronias japonicus*) populations in two small tributary streams in Hiroshima Prefecture, Western Honshu, Japan. – Herpetological Conservation and Biology, **3**: 192–202.

Pan, Y., G. Wei, A. A. Cunningham, S. Li, S. Chen, E. J. Milner-Gulland & S. T. Turvey (2016): Using local ecological knowledge to assess the status of the Critically Endangered Chinese giant salamander *Andrias davidianus* in Guizhou Province, China. – Oryx, **50**: 257–264.

Pilliod, D. S., C. S. Goldberg, R. S. Arkle, L. P. Waits (2013): Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. – Canadian Journal of Fisheries & Aquatic Sciences, **70**: 1123–1130.

Pitt, A. L., J. L. Shinskie, J. J. Tavano, S. M. Hartzell, T. Delahunty & S. F. Spear (2017): Decline of a giant salamander assessed with historical records, environmental DNA and multiscale habitat data. – Freshwater biology, **62**: 967–976.

Spear S. F., Groves J. D., Williams L. A. & L. P. Waits (2015): Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. – Biological Conservation, **183**: 38–45.

Taguchi, Y. (2009): Seasonal movements of the Japanese giant salamander (*Andrias japonicus*): evidence for possible breeding migration by this stream-dwelling amphibian. – Japanese Journal of Ecology, **59**: 117–128 (in Japanese with English abstract).

Taguchi, Y. & Y. Natsuhara (2009): Requirements for small agricultural dams to allow the Japanese giant salamander (*Andrias japonicus*) to move upstream. – Japanese Journal of Conservation Ecology, **14**: 165–172 (in Japanese with English aummary).

Tochimoto. T. (1993): Ecological studies on the Japanese Giant Salamander, *Andrias japonicus*, in the Ichi River in Hyogo Prefecture. V. Movements and permanent residences (Part 2). – Journal of Japanese Association of Zoological Gardens and Aquariums, **33** (4): 85–90 (in Japanese).

Tochimoto, T. (2005): Conservation of Japanese Giant Salamander. – pp. 204–215 in: Matsui, M. (Ed.): Directions in Batrachology. – Shokabo, Tokyo, Japan (in Japanese).

Takahara T., T. Minamoto, H. Yamanaka, H. Doi & Z. Kawabata (2012): Estimation of fish biomass using environmental DNA. – PLOS One, **7**: e35868.

Takahashi, M. K, M. J. Meyer, C. Mcphee, J. R. Gaston, M. D Venesky & B. F. Case (2018): Seasonal and diel signature of eastern hellbender environmental DNA. – Journal of Wildlife Management, **82**: 217–25.

Takezawa, M., Y. Maeno, Y. Yamashiki, H. Gotoh, M. Kawashima & T. Tanaka (2008): Restoration projects and mitigation of post-flood hazards – a case study on the Maruyama River in Japan. – WIT Transactions of Ecology and the Environment, **118**: 187–196.

Tapley, B., S. Okada, J. Redbond, S. T. Turvey, S. Chen, J. Lü, G. Wei, M. Y. Wu, Y. Pan, K. F. Niu, A. A. Cunningham (2015): Failure to detect the Chinese giant salamander (*Andrias davidianus*) in Fanjingshan National Nature Reserve, Guizhou Province, China. – Salamandra, **51**: 206–208

Turvey, S. T., S. Chen, B. Tapley, G. Wei, F. Xie, F. Yan, J. Yang, Z. Liang, H. Tian, M. Wu, S. Okada, J. Wang, J. Lü, F. Zhou, S. K. Papworth, J. Redbond, T. Brown, J. Che, A. A. Cunningham (2018): Imminent extinction in the wild of the world's largest amphibian. – Current Biology, **28**: R592–R594.

Turvey, S. T., M. M. Marr, L. Barnes, S. Brace, B. Tapley, R. W. Murphy, E. Zhao, A. A. & Cunningham (2019): Historical museum collections clarify the evolutionary history of cryptic species radiation in the world's largest amphibians. – Ecology and Evolution, **9**: 10070–10084.

Unger, S. D., T. M. Sutton & R. N. Williams (2013): Projected population persistence of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) using a stage-structured life-history model and population viability analysis. – Journal for Nature Conservation, **21**: 423–432.

U.S. Fish and Wildlife Service (2011): Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*). – Available at https://ecos.fws.gov/ecpo/profile/speciesProfile?sID=647.

Wang, X. M., K. J. Zhang, Z. H. Wang, Y. Z. Ding, W. Wu & S. Huang (2004): The decline of the Chinese giant salamander *Andrias davidianus* and implications for its conservation. – Oryx, **38**: 197–202.

Wheeler, B. A., E. Prosen, A. Mathis & R. F. Wilkinson (2003): Population declines of a long-lived salamander: a 20 + - year study of hellbenders, *Cryptobranchus alleganiensis*. – Biological Conservation, **109**: 151–156.

Yan, F., J. Lü, , B. Zhang, Z. Yuan, H. Zhao, S. Huang, G. Wei, X. Mi, D. Zou, W. Xu & S. Chen (2018): The Chinese giant salamander exemplifies the hidden extinction of cryptic species. Current Biology, **28**: R590–R592.