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Small-scale population structuring results in differential susceptibility to pesticide exposure

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Abstract

Central European riverine networks are subject to widely varying local anthropogenic pressures, forcing species with limited dispersal abilities to adapt or become locally extinct. Previous catchment-wide studies have shown that some invertebrates tend to have pronounced population structuring throughout mountainous river networks, raising the question of whether this also translates into small-scale phenotypic differentiation and adaptation to local stressors. One such species is the headwater crustacean species *Gammarus fossarum* clade 11 (or lineage B), which we restudied in terms of population structure four years after first assessment. Our aim was not only to document the temporal stability/dynamics of the population structure, but we asked whether a small-scale genetic structuring also results in phenotypic differentiation and different susceptibility to a commonly applied pesticide. Therefore, we reassessed population structure based on COI haplotypes and their frequencies, and quantified key parameters related to morphological and life-history differentiation. Furthermore, we examined the difference in sensitivity towards the pyrethroid insecticide deltamethrin. COI haplotype patterns were found to be stable over time and confirmed the small-scale population structuring within the catchment, with isolated headwater populations and connected downstream populations. While little life-history differentiation was observed, marked differences in susceptibility to the pyrethroid insecticide were found. Populations from pristine sites responded significantly more tolerant than populations from anthropogenically impacted sites—showing that prior exposure to a spectrum of stressors does not automatically increase tolerance to a specific stressor. Therefore, our study demonstrates that limited dispersal capacity is reflected not only in population structure, but also in small-scale variation in susceptibility to anthropogenic disturbance. The system thus provides a suitable experimental landscape to test the impact of further stressors (e.g., other novel entities, including pesticides with other modes of action) on locally isolated populations. Based on these findings, important recommendations for the protection of riverine species and their intraspecific genetic variation can be developed.

Keywords: Amphipoda, *Gammarus fossarum*, Intraspecific genetic diversity, Novel entities, Chemical pollution, Deltamethrin

Introduction

Rivers and their spatial arrangement in the landscape are like the hierarchical structure of a tree, with an inherent unidirectional flow making them unique ecosystems. The branching-network of rivers significantly determine the physical properties as well as the chemical composition of ecosystems and thus also influence ecological

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and evolutionary dynamics [1, 2]. Compared to interspecific biodiversity, patterns of intraspecific diversity (i.e., within-species diversity) in river ecosystems are much less frequently studied and are rarely the target of global conservation efforts [1, 3]. Within a stream network, population structure of organisms is strongly influenced by the rates of genetic exchange between populations. Genetic exchange among populations can influence the genetic structure and diversity [4] and the extent of local adaptation [5, 6]. The rate of genetic exchange is determined by several factors, including species-specific life-history traits, the position within the dendritic structure of the river network and adaptations for dispersal [2, 7–9]. Non-insect aquatic invertebrates do not possess adult stages capable of flight and are thus constrained to the stream channel. Passive drift along the water current results in biased dispersal among populations and an asymmetric exchange of individuals [9]. In those hololimnic species, dispersal between streams is restricted to flooding events, transportation by larger animals or changes in watercourse [10, 11].

The genetic exchange of individual populations—especially in the upper reaches—is further hindered by anthropogenic modification of the river systems: changes in hydrological regime, construction of dams as well as the general degradation of aquatic habitats further disrupting the natural river ecosystem structure [12]. Due to anthropogenic influence, streams present the most modified ecosystem on Earth today [13, 14]. River ecosystem functioning is impacted by river regulation and chemical water pollution [15, 16]. Especially organic micropollutants and bioactive compounds that are only partially eliminated in wastewater treatment plants like pesticides and pharmaceuticals [17–19] accumulate along the stream gradient and impact aquatic species composition [20–23]. This increased exposure can lead to physiological adjustments and thus decrease sensitivity to disturbance [24, 25].

Owing to their ecological importance [26], ecotoxicological studies often use members of the family Gammaridae (Crustacea, Amphipoda) to study the impact of organic micropollutants on aquatic species (e.g., [27–29]). The common headwater species *Gammarus fossarum* Koch, 1836, is often used as indicator of good water quality [30], in consequence of its sensitivity to organic pollutants (e.g., [31]). In recent years a high degree of genetic diversity has been uncovered within the family Gammaridae (Crustacea, Amphipoda), especially within the *G. fossarum* morphospecies complex [32–35]. This genetic variation is thought to derive from the fragmentation of populations due to their limited dispersal potential [36–38] or environmental stress [39]. Similar to the continental scale patterns of diversity [34], significant differentiation within a clade can be found even on a regional scale [40, 41]. These small-scale patterns are more pronounced in the headwater species *G. fossarum* as compared to downstream species *G. roeselii* and *G. pulex* [34, 40].

The question whether genetic species, or isolated populations of a species, differ in their ecological characteristics has hardly been addressed so far. First studies, however, suggest differences in habitat selection [42] and deviations in sensitivity to chemical stressors [43]. Examples of different sensitivities to chemical stressors come from the insecticide group of pyrethroids, which are used worldwide and are frequently applied in urban and agricultural landscapes [44]. *Gammarus fossarum* and *G. pulex*, for example, show not only interspecific differences in tolerance to the pyrethroid deltamethrin, but also significant differences between populations within species [27]. Likewise, Weston et al. [45] found large differences in populations and cryptic lineages of the *Hyalella azteca* species complex when exposed to the pyrethroid cyfluthrin. In this context, this study focuses on combining genetic species identification, phenotypic differentiation, and ecotoxicological assessments to discuss the ecological role and importance of small-scale differentiation between populations of *G. fossarum* in a mountainous river basin.

The study was based on three main assumptions. The first aim was to analyse the spatial dynamics/stability of the mtDNA haplotype patterns of *G. fossarum* described by Weigand et al. [40] using DNA barcoding, assuming to (1) confirm the temporal stability of small-scale structured populations split between isolated headwater populations and connected downstream populations. Furthermore, (2) isolated upstream populations are expected to differ in phenotypic traits from the connected downstream populations. Finally, (3) the populations in pristine headwaters are expected to be more sensitive to anthropogenic disturbance than the populations regularly exposed to pollutants, including pesticides.

Material and methods

Study region and sampling

Our first aim was to re-evaluate COI (cytochrome c oxidase subunit I) haplotype patterns described by Weigand et al. [40] to investigate population dynamics/stability. Therefore, we revisited sampling sites from Weigand et al. [40] located within the 'Rhine-Main-Observatory' (RMO), a Long-Term Ecological Research site (LTER; [46]) (Fig. 1) encompassing the Kinzig catchment. With its ten most important tributaries, the Kinzig covers a wide array of land use intensity, ranging from undisturbed forests to densely populated areas [47]. Like many

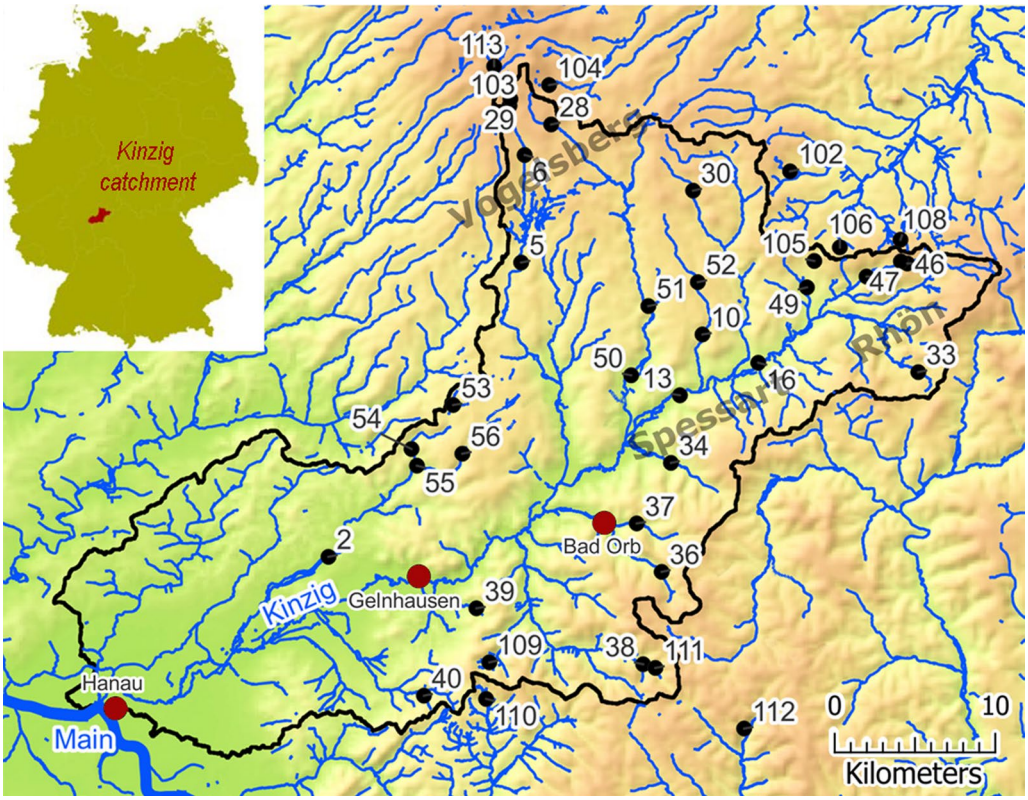

Fig. 1 Overview of sampling sites in the Kinzig catchment (i.e.,LTER site 'Rhine-Main Observatory') and adjacent catchments. The Kinzig river is a tributary of the river Main. The sampling sites are numbered according to Weigand et al. [40].

regions of Central Europe, the Kinzig catchment is characterized by a continuous increase in average annual temperature and increase in extremely hot days [48]. According to the German river classification system (LAWA; Dahm et al. [49]), the headwater and the northern tributaries with their coarse stony substrate of high silicate concentration belong to the LAWA river type 5, due to the influence of the low mountain ranges Rhön (northeast, mostly basalt) and Vogelsberg (north, mostly basalt) respectively. The southern and western tributaries are mostly characterised by a sandy substrate (LAWA river type 5.1), attributable to the influence of the low mountain range Spessart (south, mostly sandstone). The main river is classified as a secondary mountain river (LAWA river type 9).

To investigate dynamics of stability in local genetic diversity between seasons, we selected a total of ten sites that we sampled in May, August and November of 2020 and February of 2021. Of the original 56 sites described by Weigand et al. [40], sites 10, 28, 29, 33, 36, 37, 104, 105 and 109 were selected based on their population genetic structuring (for coordinates see Additional file 1: Table S1). Since no individuals were found at site 29 after the sampling in May 2020, we have sampled site 6 a few kilometres below, as a replacement site from August 2020 on. One new site (113), was sampled along the Nidder upstream of Sichenhausen, close to the northern boundary of the Kinzig catchment, because this site frequently served as a reference sampling site for *G. fossarum* in previous studies with little anthropogenic disturbance (e.g. [28, 29, 50, 51]).

All sites were sampled for amphipods using the kick-sampling method [52], taking multiple micro habitats such as macrophytes, leaves, roots, rocks and gravel into account. Specimens were captured using hand nets (1 mm mesh size), preserved on site in 96% ethanol and later stored at 10 °C to counteract DNA degradation. The catch per unit effort (CPUE) was used as a proxy for relative amphipod abundance. CPUE was calculated for each sampling site and defined as individuals taken per person and hour sampling time. Additionally, around 100 life specimens from sites 6, 33, 36, 37, 105 and 113 were collected to assess sensitivity to toxicants (see 2.4). Water parameters recorded (Table 1) include water temperature, oxygen concentration and saturation (Hach HQ40d multi, LDO101), conductivity (Hach HQ40d multi, CDC401), pH (Hach HQ40d multi, PHC201) and flow velocity (Dostmann electronic P670). Water samples

Table 1 Environmental factors at the sampling sites. Parameters are provided as mean values with standard deviation ($n=4$ sampling campaigns)

Site	Temperature [°C]	O_2 saturation [%]	pH	Conductivity [µS/cm]	Carbonate hardness [mg $CaCO_3$/L]	Total hardness [mg $CaCO_3$/L]
6	9.6 ± 5.3	94.6 ± 0.73	7.23 ± 0.23	185 ± 45.1	32.0 ± 0.00	58.7 ± 5.34
10	11.6 ± 4.6	95.9 ± 1.93	7.68 ± 0.37	248 ± 26.6	84.2 ± 18.2	115 ± 17.4
28	11.2 ± 5.0	93.1 ± 2.63	7.41 ± 0.16	127 ± 7.6	33.3 ± 1.60	60.5 ± 11.9
29	11.8 ± 6.0	73.3 ± 17.95	7.13 ± 0.21	201 ± 76.3	33.3 ± 3.92	63.5 ± 5.52
33	13.8 ± 5.2	96.6 ± 5.73	8.01 ± 0.29	426 ± 37.5	170 ± 20.6	195 ± 23.0
36	11.9 ± 4.1	95.6 ± 3.08	7.15 ± 0.12	157 ± 12.1	11.9 ± 5.52	39.2 ± 2.31
37	12.9 ± 4.2	96.5 ± 1.95	7.07 ± 0.08	92.3 ± 2.2	7.12 ± 2.31	32.0 ± 4.81
104	10.7 ± 5.1	92.9 ± 3.10	7.47 ± 0.21	82.8 ± 8.8	32.0 ± 0.00	44.5 ± 1.78
105	12.8 ± 4.2	96.0 ± 2.05	7.75 ± 0.24	432 ± 23.5	110 ± 14.2	192 ± 11.9
109	10.8 ± 4.2	95.1 ± 1.88	7.25 ± 0.26	86.2 ± 5.3	16.0 ± 7.12	34.4 ± 3.20
113	10.4 ± 5.0	95.8 ± 1.15	7.54 ± 0.19	82.9 ± 9.7	32.0 ± 2.31	47.5 ± 1.60

were analysed for nitrite and nitrate concentrations as well as carbonate and total hardness using colorimetric kits (Merck MColortests).

Effect-based assessment of chemical contamination at sampling sites

To characterize contamination level at our sampling sites, we first performed a series of in vitro assays, capturing baseline toxicity, mutagenicity, and endocrine activity in the water and sediment phase. This assessment of chemical pollution addresses different chemical contamination (e.g., compounds causing mutagenicity, dioxin-like activity, hormonal activity; Brack et al. [53]), than the pyrethroid insecticide tested in the acute toxicity tests (see Sect. "Acute toxicity test"), however, this assessment has repeatedly proven to be suitable for representing a general burden in a water body, including substances with a different mode of action [54, 55].

First, we carried out a solid phase extraction (SPE) following [56] in order to extract pollutants from the water. 1 L of native water sample from each site was solid phase-extracted with an Oasis HLB cartridge (Waters Corporation, Milford, MA, USA). The cartridges were then dried under a gentle stream of nitrogen and eluted in 200 µL DMSO, resulting in a 5.000-fold enriched extract. We further extracted pollutants from the sediment. Sediments give a better and time-integrated indication for the long-term pollution of a river than water samples, as sediments accumulate various environmental pollutants, especially hydrophobic organic contaminants [57]. Due to their benthic lifestyle, amphipods are constantly exposed to the sediment, making sediment analysis a good proxy for actual exposure to contaminants [58]. To this end, 10 g of fine sediment (< 20 µm) were shaken in 50 mL methanol for 1 h at 220 rpm on an orbital shaker (GFL 3017, GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) and eluted by sonication for 10 min (Sonorex RK 52 H, Bandelin electronic, Berlin, Germany). Subsequently methanol was removed using a rotary evaporator at 56 °C (Heidolph Laborota 4000-efficient, vacubrand CVC 2000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany; VWR RC-10 Digital Chiller, VWR International GmbH, Darmstadt, Germany). The remaining sample was captured in 500 µL DMSO. A Microtox assay (Table 2) with *Aliivibrio fischeri* was performed to analyse for baseline toxicity following a previously described procedure [29, 59]. The mutagenicity of the extracts was assessed using the Ames fluctuation test with and without metabolic activation (S9 liver homogenate from rats) with the *Salmonella typhimurium* strains YG1041 and YG1042 [60] according to Reifferscheid et al. [61]. The water and sediment extracts were also used in a series of yeast assays (Table 3) to screen for estrogenic (YES) and androgenic (YAS) activity according to Giebner et al. [56] as well as dioxin-like activity according to Stalter et al. [62]. The anti-estrogen (YAES) and anti-androgen (YAAS) screens used the sediment extract as well as unfiltered native water samples according to Giebner et al. [56]. These preceding analyses have shown that our sampling sites represent a gradient, from pristine sites (site 36), to semi-impacted sites (e.g., sites 37, 104, 113), to highly impacted sites (e.g., sites 6, 29, 33).

Life-history and morphometric analyses

Ethanol preserved individuals were first grouped into morphospecies according to characteristics described by Eiseler [63]. We collected information on male and female life-histories and morphological traits from 23 to 158 individuals per population (Additional file 1:

Table 2 Results of the Microtox assay and the Ames mutagenicity assays. Results are provided given as significance of difference to negative control ($^{+}p < 0.05$)

Site	Microtox		Ames YG 1041 without S9		Ames YG 1041 with S9		Ames YG 1042 without S9		Ames YG 1042 with S9	
	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment
6	+	–	+	+	+	+	–	–	–	–
10	+	–	+	–	+	+	–	–	–	–
28	–	–	–	+	–	+	–	–	–	–
29	+	+	–	+	–	+	–	–	–	–
33	–	–	–	+	–	+	–	–	–	–
36	–	–	–	–	–	–	–	–	–	–
37	–	+	–	–	–	+	–	–	–	–
104	–	–	–	–	–	–	–	–	–	–
105	+	–	–	–	–	+	–	–	–	–
109	+	+	–	–	–	–	–	–	–	–
113	+	+	–	+	–	–	–	–	–	–

Table 3 Results of recombinant yeast in vitro assays. Results given as significance of difference to negative control ($^{+}p < 0.05, ^{++}p < 0.01, ^{+++}p < 0.001$)

Site	YES		YAS		YDS		YAES		YAAS	
	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment
6	–	–	–	–	–	+	–	–	–	–
10	–	–	–	–	–	+	–	–	–	–
28	–	–	–	–	–	+	–	–	–	–
29	+++	–	–	–	–	–	–	–	–	–
33	–	+	–	–	–	+++	–	–	–	+++
36	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	++	–	–	–	–
104	–	+	–	–	+	–	–	–	–	–
105	–	–	–	–	–	+	–	–	–	–
109	–	+	–	–	–	–	–	–	–	–
113	+	–	–	–	–	–	–	–	–	–

YES, yeast estrogen screen; YAS, yeast androgen screen; YDS, yeast dioxin screen; YAES, yeast anti-estrogen screen; YAAS, yeast anti-androgen screen

Table S4). All measurements of distances or areas were conducted under a stereomicroscope (OLYMPUS SZX12; OLYMPUS, Germany), with an OLYMPUS SC30 camera connected to a computer. We used the software Cell^1 (Olympus) for all linear and area measurements.

The phenotypic (life-history and morphometric) characterization followed the detailed protocol provided in Jourdan et al. [64]. In short, we sexed specimens according to external sexual characteristics. Sex-ratios (number of females/number of males) were calculated based on the sampled specimens for each sampling site (Additional file 1: Table S4). We then determined each specimen's body length [mm] from the tip of the rostrum to the telson tip. We furthermore measured the gill surface areas in males, by carefully removing the gills from the right body side. The gills were photographed and their circumference measured. We summed data from all six gills per individual as a proxy of the respiratory surface area [mm^2]. We furthermore carefully removed both pairs of antennae of all individuals (males and females) at their base. Antenna length [mm] was assessed by measuring the distance from the base of the first pedunculus to the tip of the flagellum. For each female, we determined fecundity by carefully removing and counting all eggs within the brood pouch (the marsupium) and counting them. We identified the embryonic developmental stages (for pictures see Jourdan et al. [64]) and calculated the egg volumes [mm^3] using the longest and shortest axis based on an ellipsoid formula by Pöckl [65].

To analyse and visualise potential phenotypic differentiation between populations we performed sex-specific discriminant function analyses (DFA; Reisch et al. [66]). Since many of the measured phenotypic traits are strongly body size dependent, we first computed preliminary linear models that were used to calculate body size-corrected residuals for all phenotypic traits. In the case of egg volume, we additionally considered the egg stage (see Jourdan et al. [64]) in our initial model and corrected against it. All residual values, along with body size, were then used as independent factors to evaluate classification success based on population-level differentiation in two sex-specific DFAs. Site ID was used as grouping variable. The phenotypic trait data covers all four seasons, with the exception of population 29 (1 sampling; no individuals found after the first sampling) and site 6 (3 samplings from August, as replacement for 29). Statistical analyses were performed in SPSS statistics (IBM SPSS Statistics for Windows, 27, Armonk, NY, USA).

Acute toxicity test

To examine the vulnerability of each population to an acute exposure to pesticides, we selected the insecticide deltamethrin as a representative of pyrethroid insecticides, a group of fast acting insecticides used in agriculture, forestry, healthcare, and veterinary medicine [67]. The primary target site for pyrethroids are the voltage-gated sodium channels of the nervous system. Pyrethroids impede the closing of the channels, thus altering nerve function to cause repetitive firing and exhaustion of the nerve cells. These effects manifest as incoordination, convulsions, and paralysis of the organism [68, 69].

The specimens required for this purpose were sampled in May 2020 at sites 33, 36, 37, 105 and 113 (see Sect. "Study region and sampling"). Captured specimens were collected in coolers and brought to the laboratory. In the laboratory, the specimens were transferred into 54 L aquaria which were kept in a climatic chamber at 10 °C with a light–dark cycle of 16:8 h. Half the amount of water in the aquarium consisted of water from the sampling site and the other half of SAM-5S medium [70]. The water was gradually replaced over a few days by SAM-5S medium.

The acute toxicity assays were performed according to OECD guideline 202 [71], using the 5 populations of *G. fossarum*. The test organisms were randomly selected from the stock tanks, and only individuals that were clearly not parasitized with acanthocephalans were used. Single individuals were then introduced into 100 mL beakers, covered with a glass lid to prevent evaporation. The effect of eight nominal deltamethrin concentrations (200, 150, 100, 75, 50, 37.5, 25 and 12.5 ng/L), solvent- (200 µL/L DMSO = 0.02%) and negative control was investigated with 14 replicates per concentration, including solvent- and negative control. For the different concentrations a stock solution of 1000 µg/L deltamethrin was diluted in 1 L of medium. The test organisms were not fed during the test and the test solution was not renewed. The condition ('mobile', 'immobile', 'dead') of each amphipod was observed and recorded after 24 h, 48 h, 72 h, and 96 h by gently stirring each beaker for 30 s. Immobility rate was defined as the relative proportion of dead and immobile animals. For this analysis, individuals recorded as immobile after 96 h were considered dead. If the same individuals were consistently immobile at earlier points, they were retroactively considered dead. In a few cases, individuals classified as immobile at a particular measurement time point were classified as mobile thereafter. In such cases, individual amphipods were consistently classified as mobile individuals, even if they appeared immobile before their apparent recovery. All tests took place in a climatic chamber at 10 °C with a 16:8 h light–dark cycle.

The acute toxicity was expressed as median effective concentration (EC_{50}), which was taken as the concentration that killed or immobilised 50% of the amphipods. The EC_{50} of deltamethrin was compared between the different exposure times and the 5 populations of amphipods using a non-linear regression (four-parameter logistic models) and 95% confidence intervals (95% CI). GraphPad Prism (GraphPad Software Inc., 2009, version 5.03) was used for data analysis and data visualisation, including concentration–response curves. Significant differences between populations were evaluated based on non-overlapping 95% CI for the calculated EC_{50} values.

DNA isolation and amplification

For species identification and comparison to the data provided by Weigand et al. [40] the mitochondrial cytochrome c oxidase subunit I (COI) gene is sequenced. The cytochrome c oxidase is an enzyme in the respiratory electron transport chain of mitochondria and the subunit I (i.e., COI) has become a standard barcoding marker in animals [72]. The COI mutation rate is often high enough to detect closely related species or dynamics within species, while at the same time large parts of the sequence are conserved within closely related species, accordingly COI is regularly used for the identification of amphipods [34, 35, 73, 74]. DNA extraction was performed using the standard protocol for human or animal tissue and cultured cells of the NucleoSpin® Tissue Kit (Macherey–Nagel GmbH, Germany) on two specimens per site per sampling, for a total of 80 specimens (i.e., 8 COI sequences for each site). After removing excess ethanol with a clean paper tissue, four to eight pereiopods were removed using a scalpel and transferred to a 1.5 mL

reaction tube with forceps. To avoid cross-contamination of samples, the scalpel and forceps were rinsed in ethanol and flamed between each sample. For pre-lysis, samples were treated with 25 µL Proteinase K and 180 µL Buffer T1 according to protocol (NucleoSpin® Tissue Kit, Macherey–Nagel GmbH, Germany). Early tests showed no difference between methods of pre-lysis, thus, prelyses was carried out depending on the timeframe of the extraction at 56 °C for 4 h or at 37 °C overnight. Initial attempts of DNA amplification with Hot Start Mix Y (VWR, PEQL01-1599) produced only few useable reads and showed low reproducibility. For further amplification, 2 µL template DNA were added to a mix of 7.1 µL H_2O, 0.2 µL BSA, 1.8 µL $MgCl_2$, 1.5 µL Taq buffer, 1.2 µL dNTPs, 0.5 µL forward and 0.5 µL reverse primer and 0.2 µL Taq polymerase.

Initial testing revealed the primer pair LCO1490 and HCO2198 [75] to be the best suited. The PCR cycler was set to run the initial denaturation at 94 °C for 2 min, followed by 34 cycles of denaturation at 94 °C for 20 s, annealing at 46 °C for 30 s, elongation at 65 °C for 60 s and the final extension at 65 °C for 5 min. The PCR products were cleaned using the NucleoSpin® Gel and PCR clean-up Kit (Macherey–Nagel GmbH, Germany). Following the standard protocol for PCR clean-up, the sample volume was adjusted to 50 µL with low organic water (ROTIPURAN®, Carl Roth). Two washing steps were performed in preparation of two elution steps, each of the latter with 15 µL fresh buffer and a 5 min incubation period at room temperature. Fragments were quality checked for purity and length via Nanodrop measurements and gel electrophoresis before sequencing.

Sequencing and molecular analyses

DNA Sanger sequencing was conducted by Eurofins Genomics' GATC service "LightRun Tube" (Eurofins Genomics Germany GmbH, Germany). Raw sequences were manually checked and edited with the software MEGA X 10.1.8 [76]. The quality-checked sequences were compared to the NCBI nucleotide database via online BLAST search (Basic Local Alignment Search Tool) to verify the morphological identification and exclude contaminations. Forward and reverse sequences were combined into a consensus sequence using the online webtool Fragment Merger [77]. The detailed merger output was used as reference to visually check the chromatogram for ambiguous base signals.

The edited sequences were aligned to the data from Weigand et al. [40] using the standard settings of ClustalW [78], implemented in the software MEGA X. Trimming the alignment to the shortest sequence resulted in a 577 bp alignment. This alignment was used to analyse haplotype distribution as well as calculate haplotype and nucleotide diversity in DnaSP 6.12 [79]. When calculating haplotype distribution, gaps and missing sites were considered and invariable sites included. The resulting nexus file was used to construct a median joining haplotype network [80] in PopART 1.7 [81]. To allowing for tracking of seasonality and geological distribution, a trait column was manually added to the nexus file and individual haplotypes were colourized.

Results

Spatio-temporal genetic patterns

Our DNA barcoding revealed a total of 12 individual haplotypes for *G. fossarum* clade 11 across the ten sampling sites (Fig. 2a). By combining the data with the dataset provided by Weigand et al. [40], eight haplotypes were found to correspond to those previously described (H1, H2, H3, H5, H7, H8, H9 and H12; numbered according to [40]. The four previously unidentified haplotypes were consecutively numbered to those of Weigand et al. ([40], i.e., H19–H22). Haplotype diversity across all 22 haplotypes was 0.723 ± 0.017 (Additional file 1: Table S3). Most of the new haplotypes were found outside the Kinzig catchment, at the newly studied site 113 in the Nidda catchment (H19, H20 and H22). Apart from these exclusive haplotypes, site 113 shares the common haplotype H2, widespread in the Kinzig catchment.

Overall, the patterns observed within our study area are highly similar to those presented by Weigand et al. ([40]; Fig. 2b), with only minor changes in the relative proportion of the most common haplotypes (i.e., H1 and H2). For example, sites that were previously described as harbouring only H1 (sites 10 and 33) now also harbour H2, while at the previously only H2 harbouring site 6, H3 now co-occurs with H2. Besides this, the only notable addition is the novel occurrence of H21 at site 28.

Phenotypic differentiation

Our DFA for the male individuals correctly classified 24.5% of individuals as belonging to the respective population of origin. The most important traits contributing to the classification (i.e. had high loadings onto discriminant functions) were gill area (DF1), as well as antennae 1 and 2 (DF2; Fig. 3a; Additional file 1: Table S6). DF1 explains 73.7% while DF2 explains 16.5% of the total variance. The DFA for female individuals correctly classified 22.5% of individuals as belonging to the respective population of origin. The most important traits contributing to the classification were body length (DF1), as well as antennae 1 and 2 (DF2; Fig. 3b). DF1 explains 62.4% while DF2 explains 22.5% of the total variance (Fig. 3b).

Gill area proved to be the trait by which males could be most clearly distinguished between populations (Fig. 3a; Additional file 1: Table S6). Population differences in

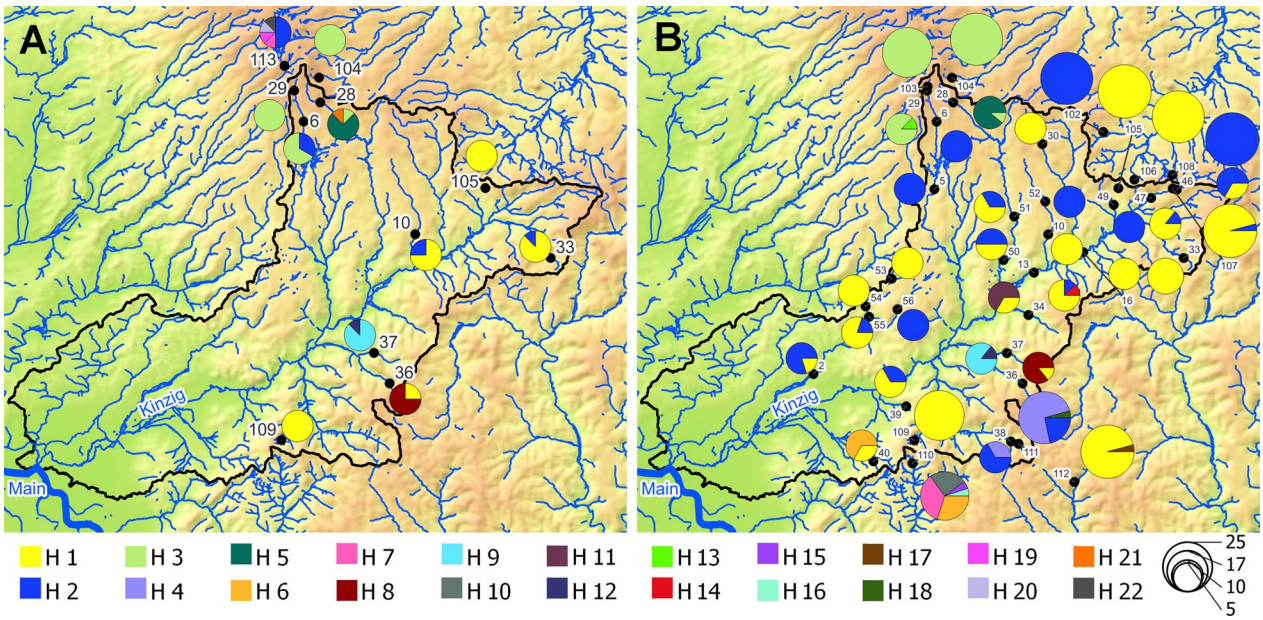

Fig. 2 Overview of comparison of the spatial distribution of *Gammarus fossarum* clade 11 haplotypes along the Rhine-Main Observatory between studies. (**a**) Data gathered between May 2020 and February 2021; (**b**) Original data as presented by Weigand et al. [40]. Haplotype titles and colours are consistent between studies

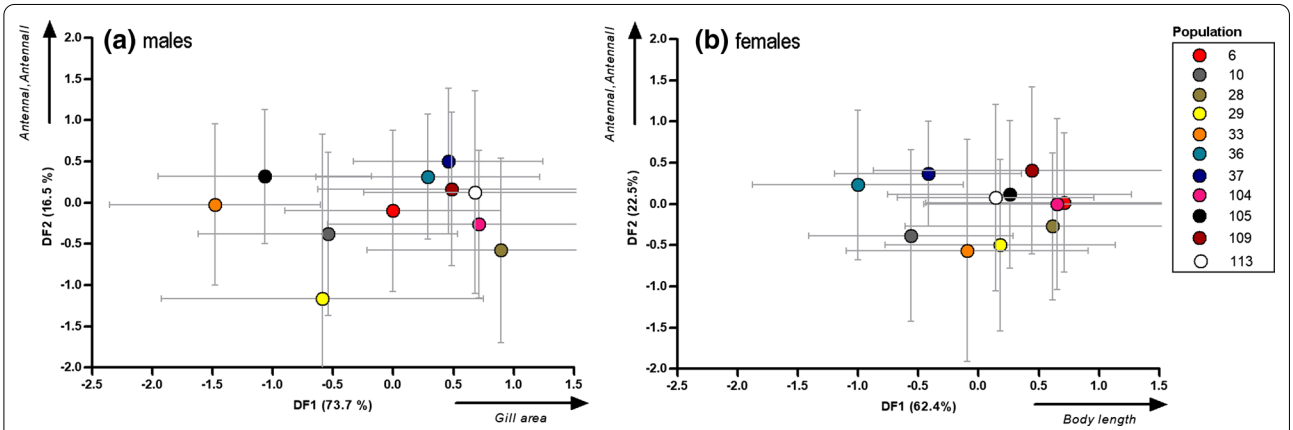

Fig. 3 Results of discriminant function analysis. Displayed are the mean value of the discriminant functions (DF ± standard deviation). Within populations, males (**a**) are discriminated by their gill area (DF1) and the length of both the first and second antennae (DF2), while females (**b**) are discriminated by their body length (DF1) and the length of both the first and second antennae (DF2). Correlations of phenotypic traits with each discriminant function are given in Additional file 1: Table S7.

females were found primarily in body size as well as length of both pairs of antennae (gill area was not measured in females; Fig. 3). Overall, however, a large overlap in the measured phenotypic traits could be observed, with no clear clustering of the populations. The measured population-specific phenotypic characteristics can be found in Additional file 1: Table S5.

Acute toxicity test

The acute toxicity tests have shown clear differences in sensitivity between populations. We found population dependent differences at all test time points (24–96 h; Fig. 3), as indicated by the non-overlapping 95% confidence intervals (Additional file 1: Table S3).

After 24 h of exposure, EC_{50} values ranged from 35.6 ng/L (30.7–41.4 ng/L, 95% CI) to 66.0 ng/L

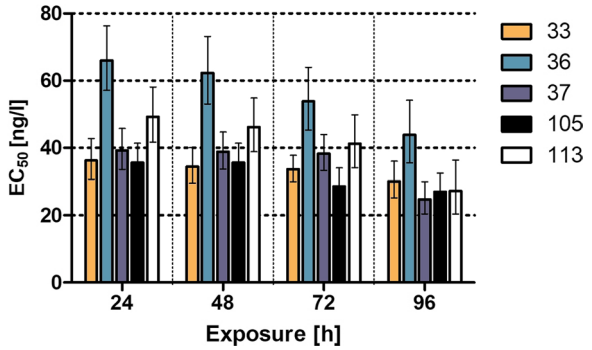

Fig. 4 50% effect concentrations (mortality rate and immobile individuals) for deltamethrin in *Gammarus fossarum* from different geographical locations. Displayed are mean EC_{50} values obtained after 24, 48, 72 and 96 hours of exposure to deltamethrin [ng/L] and the 95% confidence interval (CI). For detailed information see Additional file 1: Table S3

(57.1–76.3 ng/L, 95% CI) with populations 105 and 33 being the most and population 36 the least sensitive (Fig. 4). Populations 33, 37, 105, and 113 did not differ significantly from each other at all test time points. Population 36, on the other hand, was the most tolerant at all test time points, differing significantly from at least one other population (see Additional file 1: Table S3). After 96 h, the EC_{50} for population 36 was still 43.9 ng/L (35.5–54.2 ng/L, 95% CI). Mobility (i.e., survival) in the negative- and solvent controls was always $\geq$ 90%, therefore meeting the validity criterion for the toxicity test.

Discussion

Spatio-temporal genetic patterns are stable

Our study confirmed the presence and relative stability of diverse small-scale COI haplotype patterns of *Gammarus fossarum* clade 11 within the Kinzig mountainous river network as described by Weigand et al. [40]. While the main watercourse is dominated by the two most common haplotypes, the headwater regions display a large quantity of exclusive haplotypes. Similar small-scale COI haplotype patterns in *G. fossarum* clade 11 were previously described roughly 100 km to the northwest, in the mountainous region of the Sauerland [41].

The traditional view that rivers have a unimodal distribution of biodiversity, with a peak in mid-order streams and low diversity in headwaters and large rivers (river continuum concept; Vannote et al. [82]), has been challenged in recent years. Finn et al. [83] have shown in their meta-analysis that β diversity decreased along a stream-size gradient from headwaters to mid-order streams. The greater β diversity of headwaters (e.g., each branch in a stream network) contribute substantially to γ diversity in streams. The Kinzig river system provides an excellent model system for this assumption: Communities of *G. fossarum* show a distinct genetic structuring that follows zoogeographical models (combination of stream hierarchy model and headwater model; Hughes et al. [2]), with headwater populations showing exclusively small-scale local distribution suggesting headwater specialization; on the other hand, all downstream populations share the most common haplotypes indicating absence of genetic differentiation at lower parts of the river system. With a purely aquatic life cycle, the dispersal capabilities of *G. fossarum* between streams are limited to transportation by larger animals or changes in watercourse [10, 11]. Due to those limitations, small-scale population structure of *G. fossarum* was found to correspond to stream topography (this study; [84]). Headwater regions are ecologically unstable and consequently display an increased potential for allopatric diversification, thus genetic diversity is expected to be higher compared to the mid-order reaches [85].

Over the course of the studies in the Kinzig river catchment, several instances of ecological unstable conditions were observed in headwater regions. In September 2018 [40] and August 2020, drought events took place at several sites. At site 29 we could not find any individuals after May 2020, without recognizing any apparent reason for this, however, individuals carrying haplotype H3, previously described at site 29, were found further downstream (site 6). Repeated bottleneck events like this and the subsequent recolonisation and genetic drift might have formed the rather high haplotype diversity [41]. The haplotypes observed in the headwaters differed by only one or two mutations from the dominant haplotypes found within downstream populations. Although, single nucleotide polymorphisms were detected that result in amino acid substitutions (valine for isoleucine, in H2 and H14; alanine for valine in H10), the amount in which these occur is rather low and substituted amino acids are highly similar in size and charge and are therefore expected to behave neutral. In addition, a variation in amino acids in a DNA fragment as important as COI cannot vary freely and will most likely be lethal [86].

In general, passive drift could be expected to increase local genetic diversity downstream compared to the headwater sites. Yet, also during our resampling, no headwater haplotypes could be observed in the Kinzig mainstream which shows that even if individuals were drifted, these haplotypes do not establish in the lower reaches. One possible explanation is the local habitat specialization of headwater populations. The branching-network of rivers represents a unique spatial structure in which tips are always more isolated from one another than interior branches are [83]. Local climatic conditions, biogeographical features, flow regimes and environmental

conditions result in greater among stream heterogeneity in the tips than interior of the stream network [83, 87, 88]. This leads to the specialization of the local headwater populations which are subject to competitive exclusion at the intraspecific level [89, 90] in the case of drift—ultimately resulting in the non-establishment of these haplotypes at downstream sites. To shed some light on the mechanisms of isolation and to investigate the influence of competitive exclusion on the patterns observed in the Kinzig river catchment, a common garden experiment should be carried out. In addition, the recent changes in haplotype presence and frequency at site 6 should be monitored in the field to record whether one haplotype vanishes, or both remain present.

Haplotype patterns and phenotypic differentiation are not linked

The hypothesis of differences in phenotypes between the isolated headwater populations compared to the downstream populations could not be confirmed. While the studied populations differed in the length of the first and second antennae, body length and gill surface area, no clear pattern was found linking the displayed phenotypes to the haplotype groups. We hypothesized to observe a difference in life-history and phenotype of headwater populations sharing the same haplotype (e.g., sites 6, 29 and 104; H3) to populations sharing the common downstream haplotypes (i.e., sites 10, 33, 05 and 109; H1 and H2). However, while some of the populations sharing the common downstream haplotypes (i.e., H1 and H2) display similar life-history traits (sites 10 and 33), others display traits that were commonly represented in headwater haplotypes (sites 105 and 109).

There is still the possibility of differentiation in unsampled regions of the genome, so called genomic islands of differentiation [91, 92]. These islands are often associated with genes under divergent selection [93–95]. Genetic assignments between cryptic species lineages A and B of *G. fossarum* (defined as clades 12 and 11 respectively by Weiss et al. [35]) were found to conform to morphological variations, ecological niche preferences and differences in pollution sensitivity [38, 42, 96]. However, the morphological differentiation between the forms was negligible when compared to the genetic differentiation and was unable to discriminate the lineages completely [38]. With the focus on differentiation between populations within one lineage rather than between distinct lineages, any morphological differentiation is expected to be even less pronounced. Thus, the minor phenotypic differences between populations are likely not linked to underlying genetic differentiation. Instead, they could be explained by phenotypic plasticity and spatial variation of selective pressures, like abiotic factors and sexual selection.

Since the first pair of antennae is used to locate food sources based on chemosensory perception [97, 98], differences in food availability between sites can lead to differentiation. Both antennae are characterized by sexual dimorphism (longer antennae in males), with the second pair of antennae being used to locate potential mating partners [99]. In female-biased populations, males become more selective and choose partners of higher reproductive potential [99–101]. The variation in length of their antennae is probably a response to differences in population densities or sex-ratios that alter the need to find and assess mating partners [99], and not miss the short period after moulting in which copulation is possible [100].

The body length is influenced by several ecological factors, including predation pressure [102–104], competition at high population densities [105] and between congeneric species [106] as well as abiotic factors, like habitat structure [107] or temperature [64]. We found population-specific size differences especially in females that can likely be explained by an interaction of these environmental parameters.

As in previous studies on *G. roeselii* [64], the gill area proved to be one of the most variable traits in *G. fossarum*, even though all sites were characterized by high oxygen saturation values (Table 1). The difference in gill surface area appears to be influenced not by oxygen availability, but by conductivity. Populations at sites with low conductivity (sites 28, 104, 109 and 113; Table 1) display larger gills than populations from sites with high conductivity (sites 10, 29, 33 and 105). It remains unclear which components of the higher ionic load drive the reduction of gill surface area, but it could be an adaptive mechanism to reduce contaminant uptake (e.g., heavy metals; [108]). Linking contaminant uptake and vulnerability to pollutant exposure in conjunction with the individual gill surface area would be a promising upcoming study—even though the population tested here as the most tolerant (to deltamethrin) did not differ significantly in gill surface area. Since osmoregulation is an energy-intensive process [109, 110], reducing the gill surface area could also be an adaptation to save energy for osmoregulation in food-limited habitats (i.e., low energy environments; [109]). Furthermore, a reduction in gill surface area has been linked to presence of predators [111] and the resulting decrease in activity [112, 113].

Sensitivity of populations is linked with chemical contamination

We expected the upstream populations to be the most sensitive to the phyrethoride deltamethrin, compared

to downstream populations that are regularly exposed to various contaminants. Surprisingly, the population from the most pristine site (site 36; the only site without any effects in the effect-based assessment) proved to be the most tolerant. This finding is in contrast to previous studies, reporting that the occurrence of toxic pressure lead to increased tolerance to pesticides due to genetic and physiological adjustments [24, 25, 45]. By sequencing the primary pyrethroid target site, the voltage-gated sodium channel, Weston et al. [45] were even able to show the exact point mutations in the amphipod *Hyalella azteca* that led to increased tolerance to the pyrethroid cyfluthrin. Through these adaptations, some populations of *H. azteca* showed more than 550-fold variation in tolerance to the pyrethoid. We found about twice the tolerance in the population that had probably never been in contact with a pyrethroid. A microevolutionary response to this stressor can therefore be ruled out as an explanation for the higher tolerance.

Here, we put forward two possible—not mutually exclusive—explanations for this finding: First, the higher sensitivity at polluted sites can possibly be explained by exposure to bioaccumulating pollutants prior to the sampling [114]. Since many compounds have been shown to persist in tissue of *G. pulex* for weeks [115], it is possible that accumulated pollutants persisted in the tissue of the sampled *G. fossarum* even after acclimatization in the lab. In addition to differences in toxicodynamic recovery [116], this so called chronic toxic burden [114, 117] then results in earlier immobility and higher mortality rates in the acute toxicity test.

A second explanation would be that other characteristics/adaptations of the tolerant population 36 indirectly resulted in the high resilience of the population. For example, previous studies demonstrated interactions between dietary carotenoids and the antioxidant defence in *G. pulex* [118]. This relative increase in antioxidant defence could help to mitigate the deleterious effects of oxidative stress which has been observed, for example, in the black tiger shrimp (*Penaeus monodon*) after deltamethrin exposure [119].

Future studies should shed further light on the different explanatory attempts. For this purpose, oxidative stress biomarkers, i.e. total glutathione (tGSH), catalase (CAT), glutathione peroxidase (GPx) can be used [119]. In addition, the characterisation of pesticide body burdens [117] would provide valuable information on which pollutants may actually have had a negative impact on the low resilience of downstream populations.

Conclusion

Altogether this study confirms the small-scale population structuring of isolated headwater and connected downstream populations of *G. fossarum* clade 11 within the Kinzig catchment. This structuring is observed to be both temporally and spatially stable, based on the stability of the observed haplotype patterns. Populations display little differentiation in the investigated phenotypic characters, but significant differences in sensitivity to the pyrethroid insecticide deltamethrin as a representative of anthropogenic disturbance. Here, natural genetic variation and site-specific pesticide burden is suggested to influence the observed differences in sensitivity to deltamethrin. Contrary to the genetically homogenous, connected population structure of related species (*G. pulex, G. roeselii*; [40, 114]), the small-scale population structuring provides a suitable environment to test the impact of diverse stressors (e.g., other novel entities, including pesticides with other modes of action; [120]) on locally isolated populations. Sensitive headwater species and their small-scale structured population diversity is particularly threatened by the exponentially increasing use of synthetic chemicals [121]. A comprehensive documentation of the vulnerability of headwater species and their intraspecific diversity is therefore strongly recommended to protect species and their genetic variability.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00690-4.

Additional file 1. SI 1: Seasonal variation of haplotypes.**SI 2:** Additional information on sampling sites. **SI 3:** Additional results.

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Author contributions

M.G.: formal analysis, data curation, investigation, writing—original draft. L.P.: formal analysis, data curation, investigation. A.D.: formal analysis, data curation, investigation. J.K.: supervision, formal analysis. J.O.: supervision, resources, writing—review and editing. A.W.: conceptualization, writing—review and editing. J.J.: conceptualization, formal analysis, supervision, resources, writing—original draft. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are either directly uploaded as supplementary data, or for sequence data can be retrieved from the public Barcode of Life Data system (BOLD) project 'RMOGF'. Phenotypic data are available via figshare (https://doi.org/10.1186/s12302-022-00690-4).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors listed have approved the enclosed manuscript.

Competing interests
The authors declare no competing interests.

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