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Short Communication

Title: Microplastics ingestion induces plasticity in digestive morphology in larvae of *Xenopus laevis*

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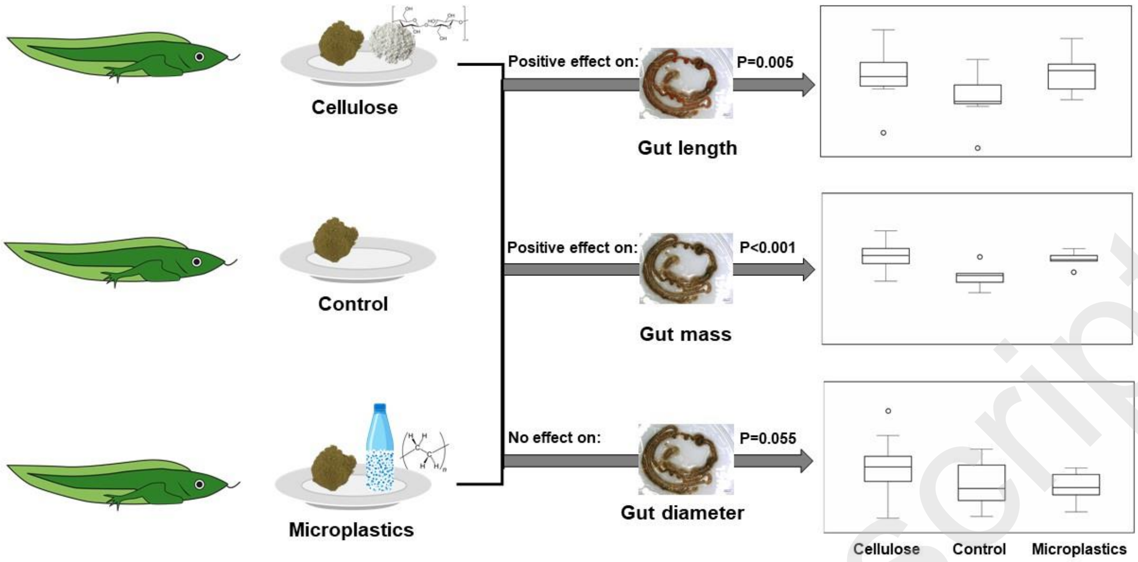

Graphical summary of the main findings related to the effects of natural fibers (cellulose) and artificial fibers (microplastics) on the digestive morphology in larvae of *Xenopus laevis*. Gut length and gut mass increased in response to microplastics and cellulose ingestion indicating that both types of fibers induced intestinal plasticity. However, there was no effect on average gut diameter.

Abstract

Global changes in temperature, predator introductions, and pollution might challenge animals by altering food conditions. A fast-growing source of environmental pollution are microplastics**.** If ingested with the natural food source, microplastics act as artificial fibers that reduce food quality by decreasing nutrient and energy density with possible ramifications for growth and development. Animals might cope with altered food conditions with digestive plasticity. We examined experimentally whether larvae of the African clawed frog (*Xenopus laevis*) exhibit digestive morphology plasticity (i.e., gut length, mass, and diameter) in response to microplastics ingestion. As natural systems contain non-digestible particles similar in size and shape to microplastics, we included cellulose as a natural fiber control group. Gut length and mass increased in response to microplastics and cellulose ingestion indicating that both types of fibers induced digestive plasticity. Body mass and body condition were similar across experimental groups, indicating that larvae fully compensated for low nutrient and energy density by developing longer intestines. The ability of a species to respond plastically to environmental variation, as *X. laevis* responded, indicates that this species might have the potential to cope with new conditions during global change, although it is uncertain whether this potential may be reduced in a multi-stressor environment.

Key words

intestinal plasticity, gut length, optimal nutrition theory, amphibians, gut adjustments, pollution

Global changes in temperature, predator introductions, and chemical pollution could challenge animals by altering food availability or quality (Rosenblatt & Schmitz 2016; Schmeller, 2018). Animals might cope with altered food conditions by exhibiting trophic (Carreira et al., 2016) and digestive plasticity (Secor, 2001). As the digestive tract represents the functional link between energy uptake and energy available for survival, development, and reproduction (Piersma and Lindström, 1997), digestive plasticity may be among the most important physiological adjustments necessary to cope with changes in food quantity and quality (Naya et al. 2007).

The capacity to adjust digestive features associated with food intake (i.e., oral morphology) (e.g., Pfennig and Murphy, 2002), food digestion (i.e., digestive system, enzyme activities) (e.g., Starck, 1996; Relyea and Auld, 2004) and nutrient transport (Sabat et al., 1995; Secor and Diamond, 2000) is found in many animal taxa (rev. in Naya and Bozinovic, 2004). Across vertebrate taxa, and consistent among species of mammals (Sabat and Bozinovic, 2000), birds (McWilliams and Karasov, 2001), reptiles (Naya et al., 2011), fish (Ke et al. 2008), and amphibians (Horiuchi and Koshida, 1989), herbivores exhibit longer digestive tracts than carnivores due to differences in food quality (rev. in Stevens and Hume, 2004). These differences in relative gut length can be explained by the *optimal digestion theory* (Sibly, 1981). It predicts that the consumption of food with a high content of non-digestible material and low protein and energy content, as is the case for plant-based food, results in an increase in gut dimensions (i.e., length and diameter; Sibly, 1987). Longer digestive systems allow for longer transit times and improved digestive efficiency, whereas larger gut diameters increase the amount of food that can be processed per unit body weight (Yang and Joern, 1994).

In amphibians, much work has investigated the effects of competition (Relyea and Auld, 2004, 2005), predation (Kehr and Gomez, 2004), aestivation (Cramp and Franklin, 2005), temperature (Castaneda et al. 2006), density of specimens (Bouchard et al., 2016), food quantity (Carabio et al. 2017), and chemical composition of food (Stoler and Relyea, 2013; Ruthsatz et al. 2019) on larval and adult digestive morphology and physiology. These studies found alterations in digestive performance, size of absorptive surface as well as gut length and mass in response to changes in food quantity and quality. For example, lower food quantity induced longer intestines in tadpoles of the wood frog (*Lithobates sylvaticus*, Relyea and Auld, 2004). Further, low food quality (i.e., low protein and energy content) increased gut length and stomach size in tadpoles of the European common frog (*Rana temporaria*; Ruthsatz et al., 2019) and decreased mouth size in tadpoles of the wood frog (*Lithobates sylvaticus*; Stoler and Relyea, 2013). However, studies investigating the capacity for digestive plasticity in response to pollutants that are ingested together with food are, so far, lacking.

Microplastics are an environmental pollutant that is increasingly gaining attention (Akdogan and Guven, 2019). Microplastics are defined as synthetic polymer particles below 5 mm in diameter (Horton et al., 2017) which originate from primary plastics (e.g., textiles, medicines, personal care products, and pellets for plastic production; rev. in Rochman et al., 2015) or secondary plastics (e.g., deriving from the debris of plastic items, such as fishing nets, films, and tires; rev. in Eriksen et al., 2014). Ingestion of microplastics has been demonstrated in a variety of terrestrial and aquatic organisms from numerous taxa (rev. in Ribeiro et al., 2019) including amphibians (Hu et al. 2016, 2022; da Costa Araújo et al., 2020a,b; Kolenda et al., 2020). Ingested microplastics are distributed to different tissues and organs and can lead to harmful, sometimes lethal, effects (re. in Prokic et al. 2019). If ingested together with the natural food source, microplastics act as artificial fibers that reduce food quality by decreasing the nutrient density with possible ramifications for growth, development, reproduction, and survival.

In this study, we examined experimentally whether larvae of the African clawed frog (*Xenopus laevis*, (Daudin, 1802)) exhibit phenotypic plasticity of the digestive morphology (i.e., gut length, mass, and diameter) in response to microplastics pollution and therefore, might be able to cope with changes in food quality associated with global change.

Xenopus laevis is the best studied amphibian species in terms of growth and development (Buchholz, 2017), providing solid physiological background for the patterns investigated in this study. Recently, *Xenopus* embryos and tadpoles have been used for studying the ecotoxicology of microplastics (rev. in Hu et al., 2016). Furthermore, as filter-feeders, *Xenopus* tadpoles are ideal models to explore the effects of microplastic ingestion in replicable experiments of dispersed particles in water.

Five clutches of *X. laevis* eggs were obtained from the Universitätsklinikum Hamburg Eppendorf. Each clutch was kept separately at 22°C until the embryos hatched and reached developmental stage NF45 (i.e., when exotrophic feeding occurs; Nieuwkoop and Faber, 1994). From each clutch, three larvae were randomly allocated to each of the 9 standard 12-L aquaria (i.e., 27 larvae from each clutch in total). Fifteen larvae were kept in an aquarium filled with 9-L of aged de-chlorinated water (15 larvae × 9 aquaria = 135 larvae in total; density: 1.66 larvae/L). The experiment was conducted in a climate chamber (Kälte-Klimatechnik-Frauenstein GmbH, Germany) with a 14:10 h light:dark cycle and a mean ± SD air temperature of 20 ± 0.2 °C. A water temperature of 25 (± 0.1) °C was achieved by adjustable heating elements (JBL GmbH & Co. KG, Germany, adjustable heating element, JBL PROTEMP S 25, 25 W). Three aquaria (i.e., replicates) were exposed to each the microplastics treatment, the fiber control group, and the control group (3 replicates × 3 treatments = 9 aquaria in total).

Larvae were fed high-protein powdered fish food (Sera micron breeding feed for fish and amphibians, Sera, Germany). The food was free of microplastics according to previous investigations and particle size was in the range of microplastics and cellulose particle size. *Ad libitum* rations were provided twice a day to guarantee that food was available in abundance. The size of the rations was continuously adjusted to account for changes in the size of the tadpoles and the number of individuals in each aquarium effectively avoiding any restricted feeding conditions. Any dead tadpoles were removed from the aquaria.

We used polyethylene particles (Sigma-Aldrich; polyethylene powder, CAS number 9002-88-4, particle size: 34-50 µm) as microplastics fiber because polyethylene is one of the most widely used polymers to produce plastic materials (Horton et al., 2017). Further, polyethylene has been identified as an environmentally relevant microplastics pollutant in amphibians (Karaoglu & Gül, 2020) and has been used in studies testing the effect of microplastics on amphibian behavior and health (da Costa Araújo et al. 2020a,b,c). We used a microplastics concentration of 60 mg/L according to the procedure of da Costa Araújo et al. (2020a). The selected concentration is environmentally relevant as such concentration is equivalent to $4.2^{4} \times 10^{-6}$ particles/m^3 and thus, within the range of surface water contamination with microplastics (1×10^{-2} to 10^{8} particles/m^3; Koelmans et al., 2019; da Costa Araújo et al., 2020a).

Natural systems contain a wide range of naturally occurring non-digestible particles similar in size and shape to microplastics fibers (Buss et al., 2021). We therefore included cellulose (Sigma-Aldrich; cellulose powder, CAS number 9004-34-6, particle size: 51 µm) as a natural fiber control group in our experimental design. We used a cellulose concentration of 60 mg/L, similar to the concentration used for the microplastics.

Microplastics and cellulose were directly added to the aquaria (i.e., 60 mg/L fibers × 9 L water = 540 mg fibers per aquarium). Every second day, water was changed completely by transferring the tadpoles gently to a new aquarium with fresh water at the same temperature and, if applicable, added microplastics or cellulose. The previously used aquarium was cleaned accurately to remove old fibers. Wooden-made air stones guaranteed continuous dispersion of fibers within the water. To avoid any microplastics contamination in the experimental climate chamber, only cotton-made tissues and clothes were used. We also used an air purifying system (TOPPIN TPAP003, CADR 260 m^3/h and Philips AC2889/10, CADR 333m^3/h) to filter possible air contamination.

When tadpoles reached developmental stage NF57 (i.e., all five toes separated; Nieuwkoop and Faber, 1994), five tadpoles were randomly selected from each aquarium. At this pro-metamorphic stage, the gut is largest and longest in *X. laevis* (Schreiber et al. 2005). The collected tadpoles were washed with filtered water and immediately anesthetized with 200 mg /Lof tricaine methanesulfonate (MS-222, Ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) buffered with 200 mg/L of sodium bicarbonate (Cecala et al., 2007), and subsequently euthanized for prolonged exposure to this solution.

We measured snout-vent length (SVL) and body mass directly after hatching (i.e., before the start of the experiment), and at developmental stage NF57 (i.e., at the end of the experiment).. Ontogenetic stage was determined every other day after hatching. Age was measured in days after hatching (dah). The SVL of the tadpoles was measured with a caliper to the nearest 0.5 mm. Specimens were dry blotted and weighed to the nearest 0.001 g with an electronic balance (Sartorius A200 S, Germany). Ontogenetic stage was determined by evaluating the status of key morphological features as detailed in Nieuwkoop and Faber (1994).

Body condition was determined using the scaled mass index (SMI) following Peig & Green (2009) once the tadpoles reached developmental stage NF57. The SMI accounts for the allometric relationship between mass and body length and is a standardized measure of the body condition that can be directly compared among individuals (Peig & Green, 2009; 2010). A high SMI suggests greater energy storages and, thus, a good body condition. The SMI slope is calculated from the regression of log transformed SVL and log transformed mass:

$$\text{SMI} = \left[individual\ Mass\ \times \left(\frac{mean\ SVL\ of\ population}{individual\ SVL}\right)^{slope\ of\ regression\ logMass\ \sim logSVL}\right]$$

After taking SVL and body mass measurements, specimens were preserved in an increasing ethanol series (30% for 24h, 50% for 24h, and 70% for 7d). Before dissection, the ethanol-preserved specimens were rehydrated in a decreasing ethanol series (70%, 50%, 30%, and water) to achieve their original wet weight. If there was a systematic error introduced by this procedure it would be identical for all specimens because all specimens were treated the same way; the conclusions should, thus, not be affected.

As intestinal structures, gut length (mm; from the end of the *Manicotto glandulare* to the vent; Ruthsatz et al., 2019), average intestinal diameter (mm; calculated from five measurements uniformly distributed over the length of the intestine), and gut mass (mg; dry blotted) were determined. Fixation in alcohol had the effect of turning the intestines rigid and breaking them into parts when dissecting. We carefully sorted (from anterior to posterior) and measured those parts for length and the other variables, and then added the measurements. All measurements were taken on a digital microscope (Keyence VHX-500F) using integrated measuring software tools. During measurements, tadpoles or dissected intestinal structures were placed in a petri dish.

All response variables (i.e., intestinal measures) are morphometric variables that are usually highly dependent on body size (Relyea and Auld, 2004; Shangling et al., 2013). To account for the effect of body size, therefore, we used second-order statistics, (specifically, we calculated residuals from linear regressions of the respective variable with SVL using the full dataset (i.e., all treatment groups). Accordingly, for example, a specimen with a positive residual of gut length represents one with a relatively long intestine (in respect to its body size). Kruskal-Wallis tests were initially conducted to determine the effect of treatments on age, SVL, mass, body condition, and gut morphology and, if significant, were followed by pairwise multiple comparisons between treatments and control group using Mann-Whitney-U test with Bonferroni correction. Results were pooled per aquarium, and average values of all dependent variables for each aquarium were used as unit for further analysis. The statistical unit was the single aquarium (n=9). For all statistical tests Cran R (Version 4.1.1, R Development Core Team 2021) for Windows was used.

Age at developmental stage NF57 significantly differed between the treatments and control group (Table 1). Larvae that were exposed to cellulose fibers were significantly the oldest, whereas exposure to microplastics fibers resulted in the youngest larvae. (Table 1). Thus, developmental rate was highest in larvae exposed to microplastics. Whereas SVL in cellulose- and microplastics-exposed larvae were significantly smaller than in the control group, mass and body condition did not significantly differ between treatments or between treatments and control group (Table 1). Survival was high across all treatments and aquaria (Microplastics=95.5%; Control=97.7%; Cellulose=95.5%).

Microplastics and cellulose were visible in feces indicating that larvae ingested both types of fibers. There were considerable differences in the relative length and mass of the intestinal tract between the treatments and the control group. The gut was significantly longer and heavier in larvae from both treatments compared to the control group (Fig. 1AB; Table 1). Larvae exposed to cellulose and microplastics fibers did not significantly differ in relative gut length or mass (Fig. 1AB; Table 1). However, there was no statistical difference in the relative diameter of the gut between the treatments and the control group (Fig. 1C; Table 1).

Table 1. Differences in age, body size, body condition, and intestinal dimensions of *X. laevis* larvae (NF 57; Nieuwkoop and Faber 1994) in response to artificial (microplastics) and natural fibers (cellulose) fibers that reduce food quality**.** Shown are means ± SD. See text for further details. MP = Microplastics. Total number of individuals = 45; total number of aquaria (i.e., replicates and statistical units) = 9.

Variable	**Control**	**Cellulose**	**MP**	**Kruskal-Wallis test (df=2)**		**Mann-Whitney-U test (pairwise comparisons)**					
				H	**P**	**Control - Cellulose**		**Control - MP**		**Cellulose - MP**	
						z	**P**	**z**	**P**	**z**	**P**
Age (dah)	25 (±0.37)	26 (±0.37)	23 (±0.53)	39.62	**<0.001**	-4.57	**<0.001**	-4.96	**<0.001**	-5.00	**<0.001**
SVL (mm)	15.5 (±0.68)	14.13 (±0.58)	14.06 (±0.65)	22.97	**<0.001**	-4.10	**<0.001**	-4.14	**<0.001**	-0.17	**<0.001**
Mass (mg)	507.93 (±109.32)	539.73 (±139.41)	533.40 (±95.64)	0.88	0.642	NA	NA	NA	NA	NA	NA
Body condition (SMI)	625.91 (±214.07)	509.66 (±188.86)	494.89 (±148.39)	3.81	0.149	NA	NA	NA	NA	NA	NA
Relative gut length	-0.62 (±0.92)	0.29 (±1.03)	0.33 (±0.72)	10.72	**0.005**	-2.72	**0.018**	-2.92	**0.009**	-0.18	0.870

Relative gut mass	-0.75 (±0.97)	0.43 (±0.94)	0.31 (±0.54)	15.32	**<0.001**	-3.13	**0.003**	-3.59	**<0.001**	-0.68	0.512
Relative gut diameter	-0.25 (±0.0.95)	0.51 (±1.20)	-0.25 (±0.55)	5.79	0.055	NA	NA	NA	NA	NA	NA

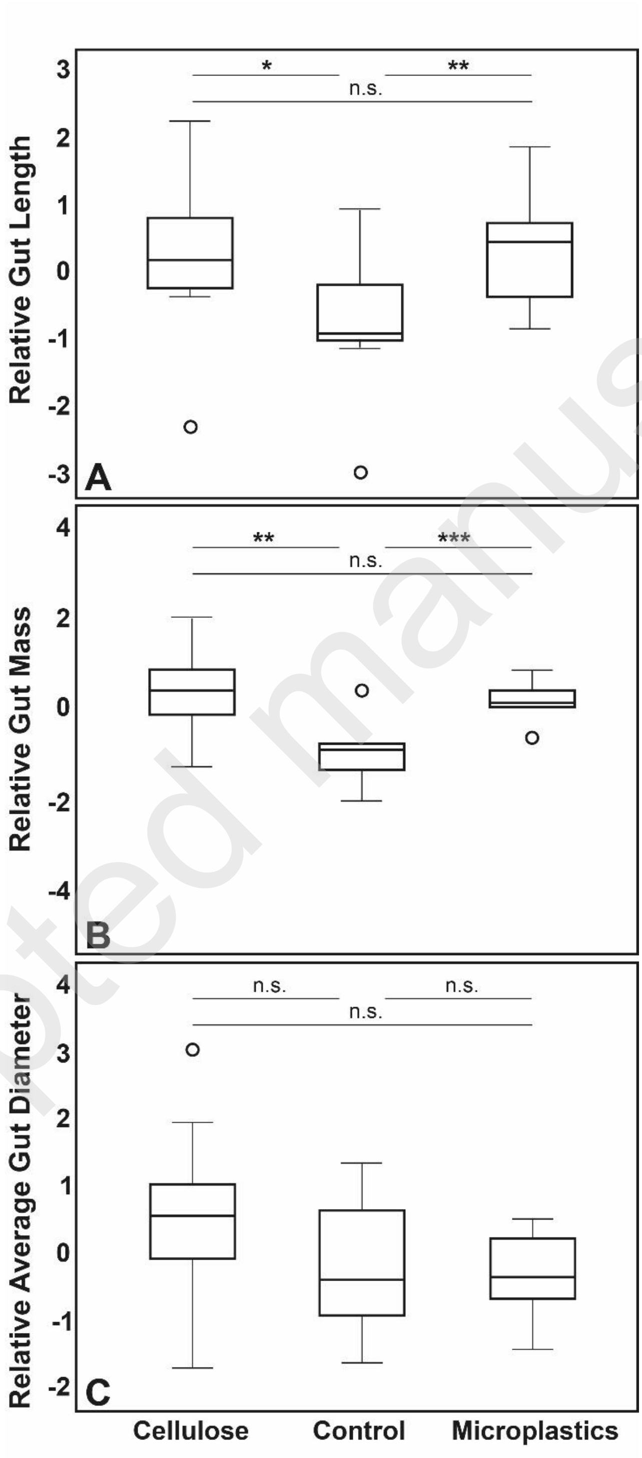

Fig. 1 Relative **A** gut length, **B** gut mass, and **C** average gut diameter of *Xenopus laevis* larvae in response to natural (cellulose) and artificial fibers (microplastics) that reduce food quality (i.e., nutrient and energy density). Shown are residuals versus snout-vent length. Boxes and whiskers show 25^{th} to 75^{th} and 10_{th} to 90^{th} percentiles, respectively; black lines indicate the median. Pairwise multiple comparisons were conducted using Mann-Whitney-U test with

Bonferroni correction. Asterisks indicate significant differences between cohorts within a nitrate concentration (*p<0.05; **p < 0.01; ***p < 0.001). Total number of individuals = 45; total number of aquaria (i.e., replicates and statistical units) = 9.

Digestive plasticity in response to environmental stressors directly or indirectly associated with global change such as changes in food quantity (Carabio et al. 2017), temperature (Curtis and Bidart, 2021), or predator introduction (Kehr and Gomez, 2004) is well studied for larval and adult amphibians. Pollutants are suggested to be among the five ultimate environmental stressors causing global amphibian decline (Hoffmann et al., 2010) and might also impact amphibians by modifying the quality of their food. The biological impacts of microplastics are still poorly known (Bosch et al. 2021), but their ingestion can interfere with physiological processes and might affect development, growth, metabolism, and survival in amphibians (Boyero et al., 2020; Buss et al. 2021; Lajmanovich et al. 2022). The present study is, to our best knowledge, the first investigating whether amphibian larvae might cope with reduced food quality due to microplastics pollution by exhibiting digestive plasticity in order to improve digestive efficiency. We demonstrate that differences in nutrient and energy density can induce a plastic response in gut length and mass. However, there was no such plasticity in the average gut diameter.

Based on optimal digestion theory (Sibly, 1981), digestive plasticity is suggested to correlate with digestion efficiency since longer guts extend the time it takes for food to pass through the intestinal tract (Yang and Joern, 1994; Relyea and Auld, 2004). Digestion in amphibian larvae is supported by microbial fermentation of intestinal bacteria (Altig and Johnston, 1989; Pryor and Bjorndal, 2005) which are more diverse in these larvae than in the purely carnivorous adult frogs (Vences et al. 2016). This is particularly important for cellulose fibers (Horiuchi and Koshida 1989), which no vertebrate can digest without its intestinal microbiome (McWilliams and Karasov, 2001). As microbial fermentation is very slow (Zimmermann and Tracey, 1989), longer retention times in the elongated digestive tracts of larvae raised on additional cellulose fibers supposedly led to more effective utilization of energy and allowed larvae to compensate for the lower nutrient and energy content of this diet.

In contrast to natural fibers such as cellulose, microplastics fibers are truly non-digestible and lack any nutrients or energy that could be assimilated. Nevertheless, both dietary fiber components induced a plastic response in gut length which allowed larvae to compensate (in terms of mass and body condition) for the lower nutrient and energy densities compared to the diet of the control group. We suggest that the elongated guts increased the efficiency of nutrient extraction from ingested food particles that also contain plant fibers which profit from a longer gut passage. This indicates that plasticity in digestive morphology is induced by low nutrient and energy density *per se* and is not related to the type of fiber. However, microplastics have recently been shown to alter the composition of gut microbiota in different fish species (rev. Lu et al., 2019) and caused gut inflammation in zebrafish (*Danio rerio*; Jin et al., 2018) with possible impacts on efficient microbial fermentation. Studies are needed to investigate whether microplastics might also modify the intestinal microbiome of amphibian larvae, and which consequences this may excert on their fitness. Furthermore, microplastics are known to have a high affinity toward aquatic contaminants including trace metals (Hildebrandt et al., 2021) as well as pesticides (Villegas et al., 2022) and softeners (Han et al., 2022). This *Trojan-horse effect* is considered to significantly change the potential health risks of microplastics for aquatic organisms such as amphibian larvae (rev. in Zhang and Xu, 2020) especially if contaminated microplastic fibers are ingested. Future studies need to address this effects in laboratory and field studies for a comprehensive understanding of microplastics pollution in the environment.Because the digestive tract represents the functional link between energy intake and allocation (Secor, 2001), digestive plasticity might not only affect larval growth and development (Lindgren and Laurila, 2005) but also has important implications for animal performance and survival in later life stages (Bouchard et al., 2016; Ruthsatz et al., 2019a,b). In anuran amphibians, larval duration and size at metamorphosis predict individual fitness (Berven, 1990): Specifically, individuals have a higher survival probability and reproduce earlier if (i) they reach metamorphosis earlier but at the same body size as other individuals, or (ii) reach metamorphosis later and at larger body size (Semlitsch et al., 1988; Beck and Congdon, 2000; Altwegg and Reyer, 2003). In this study, all larvae reached developmental stage NF57 at the same mass and body condition indicating that digestive plasticity allowed for completely balancing lower food quality against the higher energetic costs of generation and maintenance of larger digestive tracts (Cant et al., 1996). Consequently, there might be no size or body condition related post-metamorphic carry-over effects of low food quality in response to microplastics ingestion during larval development and digestive plasticity could therefore be adaptive (Relyea and Auld, 2004). Nevertheless, larvae could have the same mass and body condition but might suffer from further effects of microplastics such as neuro- or cytotoxic effects with possible ramifications for survival across metamorphosis (da Costa Araujo et al., 2020c). Since little is known about long-term effects of microplastics ingestion and how phenotypic plasticity might increase survival in later life stages, more long-term studies in natural environments are needed to understand how digestive plasticity might help amphibians cope with this growing-source of pollution.

Global change alters many environmental factors that might affect amphibians indirectly (e.g., altered food conditions) or directly (e.g., changes in temperature). The ability to exhibit phenotypic plasticity provides an advantage in unpredictable or variable habitats (Agrawal, 2001). However, as amphibians are ectotherms, environmental temperature determines their body temperature, and hence regulates the rates of all physiological and biochemical processes impacting growth, development, and metabolism (Hochachka and Somero, 1973, 2002; Huey and Stevenson, 1979; Angilletta et al., 2002). An increase in ambient temperature may, therefore, increase the metabolic rate resulting in more energy required to cover basal energetic demands (Dillon et al., 2010; Ruthsatz et al. 2019b) and less energy available for physiological adjustments such as plastic responses. Exhibiting phenotypic plasticity (DeWitt et al., 1998) and especially a plastic response of the digestive tract is energetically costly (Secor et al., 2001; Naya et al., 2008). The capacity to exhibit digestive plasticity might therefore be restricted if ambient temperatures increase and resources are limited. For example, Lindgren and Laurila (2005) found that the plastic response in intestinal length towards latitudinal differences was more pronounced at low temperatures in larvae of *R. temporaria*. Further, intestinal length and mass were smaller in larvae of the Chilean giant frog (*Caudiverbera caudiverbera*; Castañeda et al., 2006) and of the Northern leopard frog (*Lithobates pipiens*; Curtis and Bidart, 2021) when reared under higher temperatures. Future studies should therefore investigate whether the capacity for digestive plasticity in response to microplastics ingestion and other pollutants is reduced if larvae are exposed to higher temperatures.

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Author contributions

KR conceived and designed the study. KR and MD conducted the experiments. MD carried out the microdissections of intestinal structures. KR performed the statistical analysis and led the writing of the manuscript. All authors participated in manuscript editing and final approval.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Statement of Ethics

The authors have no ethical conflicts to disclose. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The experiments were conducted under permission from the *Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit*, Germany (Gz. 33.19-42502-04-21/3705).

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Figure legend

Fig. 1 Relative **A** gut length, **B** gut mass, and **C** average gut diameter of *Xenopus laevis* larvae in response to natural (cellulose) and artificial fibers (microplastics) that reduce food quality (i.e., nutrient and energy density). Shown are residuals versus snout-vent length. Boxes and whiskers show 25th to 75th and 10$_{th}$ to 90th percentiles, respectively; black lines indicate the median. Pairwise multiple comparisons were conducted using Mann-Whitney-U test with Bonferroni correction. Asterisks indicate significant differences between cohorts within a nitrate concentration (*p<0.05; **p < 0.01; ***p < 0.001). Total number of individuals = 45; total number of aquaria (i.e., replicates and statistical units) = 9.