Accurate Detection and Quantification of Seasonal Abundance of American Bullfrog (*Lithobates Catesbeianus*) Using ddPCR eDNA Assays

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Abstract

The invasive American bullfrog (*Lithobates catesbeianus*) imperils freshwater biodiversity worldwide. Effective management includes rapid detection and response to incipient invasions, as established populations are extremely difficult to eradicate. Although environmental DNA (eDNA) approaches provide a highly sensitive alternative to conventional surveillance techniques, extensive testing is paramount to generate reliable output. Here, we tested and compared the performance of two primer/probe assays to detect and quantify the abundance of bullfrogs *in silico* and *in situ* using digital droplet PCR. Although both assays proved to be equally specific and sensitive, one outperformed the other in detection resolution (*i.e.* distinguishing target and non-target droplets), and hence was selected for further analyses. Mesocosm experiments using density series of larval and juvenile bullfrogs revealed that eDNA concentrations could explain 99% of the larval abundance and biomass. Per individual eDNA emission rates did not differ significantly among life stages, indicating that eDNA concentrations can be used as a reliable proxy to asses bullfrog abundance in natural populations. Seasonal eDNA patterns in three infested ponds showed parallel fluctuations in bullfrog eDNA concentrations. A peak in late summer (August, September or October), coinciding with the breeding season, was followed by continuously low eDNA concentrations in winter and spring. These findings demonstrate that eDNA analyses can be used as a solid and reliable tool to detect the early stages of bullfrog invasions and to quantify temporal changes in abundance that will be useful in coordinating and evaluating large-scale bullfrog eradication programs.

Introduction

Freshwater ecosystems are biodiverse habitats that contain a large number of distinct animal and plant species, and fulfill important ecosystem functions. Unfortunately, many freshwater ecosystems worldwide have been exposed to a vast range of anthropogenic disturbances that jeopardize biodiversity [1-3]. Among these anthropogenic stressors are Alien Invasive Species (AIS)[4], which are recognized as one of the leading causes of animal extinctions worldwide[5,6], and as such contribute to the global biological homogenization[7]. Once successfully established, the complete eradication of AIS becomes exceedingly difficult[8]. Next to prevention, early detection of incipient invasions followed by rapid and appropriate eradication actions is thus considered to be the most effective and cost-efficient approach to counter AIS[9,10]. Reliable monitoring programs defined by a high detection resolution and large spatial scale employability are therefore a prerequisite for the development of successful and long-term extermination strategies[11,12]. However, since discovering the early stages of invasion can be challenging via conventional surveillance methods[9,13], more sensitive monitoring techniques are imperative to cope with freshwater invaders[14].

In recent years, environmental DNA (eDNA) approaches have emerged as promising non-destructive molecular tools for the detection of aquatic species[15,16]. These techniques are based on the detection of DNA traces that are continuously shed or excreted by organisms in their environment, serving as molecular fingerprints[17,18]. An aquatic eDNA survey typically involves the bulk collection of water samples, after which the comprised DNA, albeit fragmented and highly diluted (<200 pg/L), is concentrated, amplified,

and analyzed[19]. Given the profound sensitivity of eDNA approaches in combination with the confined longevity of eDNA in the water column and its constrained spatial diffusion in lentic systems, the target species' near presence can be inferred accurately and precisely[20]. Indeed, a myriad of studies have already demonstrated much higher detection probabilities obtained via eDNA analyses than realized with conventional methods[21,22]. Moreover, when elusive species present in low abundances are targeted, eDNA approaches can outperform conventional monitoring techniques in handling time and cost-effectiveness, especially in inaccessible habitats[18,23].

Prior to the implementation in large-scale and standardized surveillance programs, eDNA approaches, however, require extensive validation efforts to avoid methodological flaws that may potentially culminate in incorrect outcomes[19,23-25]. False positives (type I errors), for instance, can emerge from non-specific binding of primers, whereas deficient primer binding efficiency or non-target template competition, on the other hand, can culminate in false negatives (type II errors)[19,26,27]. Intensively testing the specificity and sensitivity of primer/probe assays *in silico*, *in vitro*, and *in situ* is therefore a crucial, yet frequently undervalued, step to avoid such errors[28-30].

Besides serving as a highly sensitive detection tool, eDNA analyses can also provide insights in the abundance of target species when combined with quantitative polymerase chain reaction (qPCR) or digital PCR (dPCR)[31,32]. Depending on the system and species, various studies have documented a positive correlation between the eDNA concentration measured and target species abundance or biomass, both under artificial experimental settings[33-35] and natural conditions[36,37]. However, to serve as a reliable tool to detect species at very low abundances in the field and to extrapolate eDNA concentrations into abundance estimates, temporal insights in eDNA patterns throughout the year are needed[38,39]. Only then, results generated by eDNA analyses can be appropriately interpreted and adopted to monitor aquatic populations in space and time in a standardized manner[24,38].

The American bullfrog (*Lithobates catesbeianus*; hereafter referred to as bullfrog) is a large amphibian that is native to eastern North America, but is currently invading freshwater ecosystems all around the globe, thereby reducing native biodiversity via competition, predation, and the transmission of novel pathogens[40-42]. The species is ranked as one of the 100 most adverse invaders in the world[43] and is acknowledged by the European Union as a species of concern (EU Regulation 1143/2014). Eradication of established bullfrog populations is a daunting endeavor to which an increasing amount of resources and efforts are being devoted worldwide, although chances of success remain slim[44-46]. This is reflected in the Belgian bullfrog invasion, which established in the nineties and is, despite eradication efforts, expanding ever-since[47,48]. Apart from a number of satellite populations, the main conservative concern is a well-established large metapopulation covering several hundreds of ponds and wetlands along the river Grote Nete (Antwerp), spanning an area of roughly 100 km² (Fig. 1a). Apparently, this stream functions as an artery for bullfrog displacement providing access to plenty of suitable ponds, mostly private, which opposes active control[48,49]. Disposing of a reliable and comprehensive overview of the precise distribution

and density of bullfrog populations, especially at very low abundances, in this and other cases is thus an absolute prerequisite for successful eradication, although currently one of the main shortcomings.

In this study we aim to (i) compare the specificity and sensitivity of two digital droplet PCR (ddPCR) eDNA primer/probe assays for the detection of bullfrogs in Western Europe, (ii) assess whether eDNA concentrations match bullfrog abundance and/or biomass in a mesocosm experiment, and (iii) investigate seasonal patterns in eDNA profiles in natural populations, in order to demonstrate that eDNA analyses targeting bullfrogs can be implemented as a robust and operational tool for routine use to complement large-scale eradication campaigns.

Results

Primer/probe assay validation

The *in silico* specificity analyses of both primer/probe assays showed multiple mismatches in the binding regions of the corresponding bullfrog sequence with most sympatric amphibian species in Western Europe (Supplementary Fig. S1). Each of the field samples taken from water bodies harboring the most common indigenous amphibian species, but no bullfrogs, showed no bullfrog eDNA amplification for either assay (Fig. 2). The droplet generation during preparatory ddPCR steps in the 25 bullfrog positive field samples and the positive reference sample containing bullfrog tissue resulted on average in 16,119 ± 95 (s.e.m.) accepted droplets, ranging between 8,686 and 19,450 droplets. The mean relative performance of the Goldberg and Lin assay on bullfrog positive field samples was 0.90 ± 0.043 and 0.84 ± 0.045 respectively, and did not significantly differ between both assays (t_{25} = 1.19, P = 0.24) (Fig. 3a). The mean resolution of the Goldberg assay (9,065.53 ± 982.32) was significantly greater (V_{25} = 3.00, P < 0.001) than that of the Lin assay (5,968.10 ± 589.7) (Fig. 3b), which was also the case for the mean resolution of the internal positive control (IPC) (V_{25} = 0.01, P < 0.001), resulting in a mean IPC resolution of 4,928.31 ± 21.9 and 4,855.77 ± 43.98 for the Goldberg and Lin assay, respectively (Fig. 3c). No positive target detection was obtained from each of the technical negative controls included in the workflow, while all positive reference samples were successfully amplified.

Mesocosm experiment

No bullfrog eDNA was detected in the water used for the mesocosm experiment prior to the introduction of bullfrogs, nor from the technical negative ddPCR controls included in the workflow, while the positive reference samples were successfully amplified. Although the mean number of accepted generated droplets was 15,287 ± 167, three measurements fell below the threshold of 8,000 generated droplets, and were consequently discarded. The eDNA concentration of caught bullfrog tadpoles and juveniles in the mesocosms significantly increased with their abundance (larvae: F_6 = 1122.00, R^2_{adj} = 0.99, P < 0.001; juveniles: F_5 = 8.65, R^2_{adj} = 0.56; P = 0.032) (Fig. 4a,b) and total biomass (larvae: F_6 = 721.00, R^2_{adj} = 0.99, P < 0.001; juveniles: F_5 = 12.34, R^2_{adj} = 0.65, P = 0.017) (Fig. 4c,d). The number of eDNA copies per liter filtered water ranged from 89,900 ± 3,686 copies for the lowest tadpole abundance (*i.e.* one individual) up

to 7,274,125 ± 463,510 copies for the highest abundance (*i.e.* 121 individuals), and between 53,832 ± 5,124 copies for the lowest juvenile abundance (*i.e.* one individual) up to 271,640 ± 19,444 copies for the highest abundance (*i.e.* 8 individuals). Across all mesocosms, the average eDNA emission rate from a single tadpole did not significantly differ from that of a single juvenile (W = 16.00, P = 0.19) (Fig. 5a), whereas at the per biomass level, larval eDNA emission rates were significantly lower relative to juvenile bullfrogs (W = 49.00, P = 0.014) (Fig. 5b).

Seasonal eDNA patterns

Across all seasonal samples analyzed, the total number of generated droplets was on average 15,935 ± 75, with a minimum of 8,511 droplets. Over the entire survey period (18 months), the mean bullfrog eDNA concentration (expressed as copies per liter filtered water) was highest in pond 2 (15,095 ± 10,683) and lowest in pond 3 (10,567 ± 4,902), with intermediate concentrations in pond 1 (12,329 ± 7,896). During summer time, bullfrog eDNA concentrations substantially increased towards distinct peaks in August for pond 1 (18,859 ± 822 and 144,969 ± 3,125 copies per liter water in 2019 and 2020 respectively) and pond 3 (75,226 ± 2,625 and 31,504 ± 1,711 copies per liter water in 2019 and 2020 respectively), and in September-October for pond 2 (65,482 ± 2,667 and 180,766 ± 500 copies per liter water in 2019 and 2020 respectively) (Fig. 6a-c). Bullfrog eDNA concentrations gradually decreased following these peaks, and reached the lowest concentrations from January to July for all three ponds during both years (on average 1,376 ± 92 copies per liter water). Although we could detect bullfrog year round in pond 1, even during winter and spring, zero detections were observed in pond 2 in November, May, and July 2019 and in March 2020, whereas in pond 3 bullfrogs were not detected in May 2019. Negative controls retrieved no bullfrog eDNA copies, whereas all positive reference controls were successfully amplified.

Discussion

As the performance of primer/probe assays can be inconsistent between study systems, formal validation, preferentially testing on local natural eDNA samples, should be the first step in developing large-scale eDNA-based surveillance protocols to ensure that the generated findings are reliable for the resulting decisions to be pertinent[29,30]. One of the most important aspects that has to be considered when evaluating the performance of a primer/probe assay is its specificity, *i.e.* the degree to which it can specifically detect the target sequence without interacting with genetic material of closely related sympatric non-target species. Specificity evaluation is especially important in the context of aquatic eDNA, as its highly degraded nature necessitates the use of shorter sequences as barcodes, which inherently harbors fewer species-specific regions[19,27]. Although primer specificity of the Goldberg and the Lin assay were previously excessively tested *in silico* for potential cross-amplification with co-occuring non-target species in the Western US and the Beijing area, respectively[29,30,50], it is imperative to repeat the analyses for amphibian species inhabiting a particular study area. The *in silico* tests performed here confirmed that both primer/probe assays specifically detected the bullfrog sequence without interfering with sympatric amphibian species indigenous to Western Europe (Supplementary Fig. S1).

Nevertheless, *in vitro* and *in situ* tests should complement *in silico* specificity tests, since primers can interact with non-target sequences regardless of nucleotide mismatches[26,51] and the outcome of amplification can also significantly depend on sample and system specific PCR conditions[52]. Although primer performance was already comprehensively evaluated *in vitro*[29,30,50], we conducted additional *in situ* tests on natural eDNA samples containing DNA originating from a mixture of sympatric indigenous amphibian species under variable environmental conditions (Supplementary Table S1), which provides a more pragmatic indication of an assay's specificity in the geographic area of interest relative to an *in vitro* approach[28]. When both assays were run on water samples collected from bullfrog-free water bodies harboring most of the co-occurring amphibian species in Western Europe, no bullfrog eDNA amplification could be observed for either of the two assays under study (Fig. 2), while the IPC and the bullfrog positive reference samples were always successfully amplified. Therefore, it can be safely stated that both assays specifically target and detect bullfrog eDNA in Western European water bodies, independent of sympatric non-target amphibian species.

A second aspect that determines primer/probe assay performance is its sensitivity, *i.e.* its ability to detect even the slightest traces of bullfrog eDNA. When the two primer/probe assays under study were subjected to a range of eDNA samples collected from water bodies that are currently invaded by bullfrogs with varying intensities, the two assays did not significantly differ in eDNA concentration quantified per sample (Fig. 2, Fig. 3a). However, in terms of detection resolution of both target and IPC (*i.e.* the separation of the target-positive and target-negative droplets), the Goldberg assay outperformed the Lin assay (Fig. 3b,c). Detection resolution is an important feature for ddPCR analyses, especially when water samples are supplemented with inhibitory compounds that can obstruct the differentiation of positive and negative droplets[31,53,54]. The higher detection resolution of the IPC when ran in duplex with the Goldberg assay was, at least partly, expected, as the optimal annealing temperature of the primers and probes targeting the IPC is equal to that of the Goldberg assay (*i.e.* 60 °C). Nevertheless, this implies that the correction factor based on IPC measurements, and thus final eDNA concentrations, can be more accurate when adopting the Goldberg rather than the Lin assay when combined with this particular IPC used in this work. Altogether, our results thus indicate that for the ddPCR approach applied, the Goldberg assay is more robust in detecting bullfrog eDNA in Western Europe compared to the Lin assay, which led us to select this assay for further analyses.

The controlled mesocosm experiment showed that the Goldberg assay was able to quantify eDNA signals according to bullfrog abundance and biomass. Especially at the tadpole life stage, the obtained eDNA concentrations predicted the tadpole abundance remarkably well (with 99% of the variance explained), whereas this relation was somewhat less pronounced at the juvenile life stage (Fig. 4a,b). This strong correlation observed at the tadpole life stage was well above the *in vitro* average of 0.82 as reported in a meta-analysis[32], suggesting relatively homogenous DNA discharge rates among bullfrog larvae and densities compared to other species. Besides, DNA quantification via ddPCR has been shown to be more precise than measurements via qPCR[31,54], the latter being overrepresented in the meta-analysis, and can additionally explain the higher predictive power observed, especially in combination with the inclusion of IPC's in our workflow. Juvenile bullfrogs, on the other hand, are not permanently submerged in the water

column and often reside on land near the water body or on aquatic vegetation as larval gills are functionally exchanged for lungs upon metamorphosis. Since our mesocosms were equipped with terrestrial islands for juveniles to dwell out of the water, this might have introduced more variation in the association between eDNA concentration and juvenile bullfrog abundance. Nevertheless, at a per individual basis, mean eDNA emission rates did not differ significantly between both life stages studied (Fig. 5a), indicating that, at least under controlled conditions, the Goldberg ddPCR primer/probe assay provides a relative accurate prediction of bullfrog abundance in the water column irrespective of life stage. The per gram eDNA emission rate, on the other hand, was significantly higher for the lighter juveniles relative to the heavier tadpoles (Fig. 5b), a pattern that also emerged in other studies such as with bluegill sunfish (*Lepomis macrochirus*), where heavier adults on average had a higher per individual eDNA release rate, whereas their per biomass eDNA release rate was lower relative to the lighter juveniles[55]. This finding, in conjunction with the highly variable biomass distribution among individuals in bullfrog populations, makes it more appropriate to predict bullfrog abundance rather than biomass from eDNA signals picked up in blind systems.

Under field conditions, however, relationships between abundance and eDNA concentration can be affected by other factors that introduce additional variation. A recent meta-analysis[32] showed that species abundance under natural conditions explained a substantially lower proportion of the variation in eDNA concentrations (0.57 on average). This can be attributed to the complex interplay of several factors affecting eDNA production and degradation rates, such as temperature, UV exposure, pH, microbial activity[50,56,57], animal behavior[37], and season- and age-dependent shredding rates[38,55]. In addition, the accumulation of particulate and dissolved substances (such as calcium ions, humic and tannic acids, polymers, *etc*.) and eDNA from non-target species can further hamper target DNA amplification[58], resulting in underestimations of the actual abundances of target organisms. However, the amplification in ddPCR appears to be less susceptible to inhibitory substances than qPCR, and is therefore expected to be more robust, especially when the target DNA template is scarce[33,35,59]. Besides, the inclusion of IPCs in our workflow may contribute to the standardization of variation in eDNA concentrations resulting from sample-specific suboptimal DNA extraction or ddPCR inhibition[28,60] (see[35] for more details). Altogether, these findings allow the *in vitro* established relation to be extrapolated to natural lentic systems in order to acquire rough indications of bullfrog abundance independent of their life stage, given an integrated and standardized sampling design. Nevertheless, it would be useful to apply this assay in parallel with conventional monitoring programs and eradication campaigns to further validate its ability to provide an approximation of local population density in natural ponds.

Since eDNA patterns in the field are expected to be influenced by seasonal alterations in both the ecology of the target species and its environment[37,56,57], we assessed year-round temporal variation in bullfrog eDNA concentrations for two subsequent years in three natural ponds. Over this eighteen month study period, the three ponds showed similar patterns in seasonal variation in eDNA concentrations: a consistently low concentration from December to July preceded one peak in late summer (between August and October), followed by a decrease during winter time (Fig. 6). These temporal eDNA patterns largely reflect the seasonal phenology of bullfrog populations in Western Europe, where mating peaks during late

spring and early summer and a new generation tadpoles most commonly emerges between July and September[61]. Reproduction in amphibian species has been documented to result in two peaks in eDNA concentrations in natural conditions, one representing the reproductive behavior of the adults and the subsequent mass release of gametes, followed by a second peak representing the emergence of a new generation of tadpoles[38,62,63]. Since we sampled our study ponds on a monthly basis whereas the timing between bullfrog breeding and larval emergence ranges between a few days up to one week[64,65], it is plausible that we could not differentiate these two peaks. Moreover, given that the breeding season of bullfrogs spans a few months, and that multiple breeding events can occur in the same water body[64,65], mating and emergence of bullfrog larvae presumably cannot be differentiated in time in terms of eDNA concentrations. On the other hand, it can also be assumed that the studied ponds served as refuges for first-year juveniles escaping competition and predation from congener adults in nearby breeding ponds, or as stepping stones or foraging sites[44,48,65,66]. Juveniles were indeed observed in large numbers in and around each of the three study ponds during summer in both years. A minimal influence of bullfrog adult presence on the observed peaks in eDNA concentrations was expected, since adults mostly reside out of the water on the shorelines of the water bodies during the breeding season[65]. It should also be noted that the observed peaks in eDNA concentrations could have been intensified by the extreme dry summers in 2019 and 2020, which resulted in strong declines of the water table.

As temperatures decrease in autumn and cross the threshold of 15 °C, which generally occurs from October onwards, bullfrogs enter winter torpor. They mostly hibernate in the water at the bottom of a pond, semi-immersed in mud, but winter lethargy or adopting terrestrial hibernacula instead is not exceptional[49,61]. Therefore, the decreased metabolic rate, and hence eDNA release, during hibernation[36], or the decreased abundance could explain the observed decrease in measured eDNA concentrations following the peak in late summer. Although previous research was unsuccessful in detecting eDNA of two hibernating endangered frog species in headwater streams during the winter season[39], we detected bullfrog eDNA all year round in the studied ponds. This suggests that our intense sampling strategy and efficient primer/probe assay could pick up even minute quantities of eDNA during the period bullfrogs are inactive or scarcely present. Altogether, these findings confirm previous research that has shown that eDNA concentrations closely track the seasonal phenology of the target species, and indicate that eDNA signals obtained from summer sampling most accurately predict bullfrog abundances present in these systems. The patterns observed here could thus provide crucial information on the timing when large-scale eDNA detection campaigns and eradication programs targeting the bullfrog would be most fruitful [36,39,63,67].

With this work, we report the validation process and exploratory research required for appropriately implementing species-specific eDNA approaches for reliable detection and quantification in large-scale monitoring campaigns. We tested two primer/probe assays specifically designed for bullfrog, and showed that one of them offered the highest detection resolution, which is an imperative feature for the early detection of this invasive species to be able to rapidly respond with the necessary eradication measures. The mesocosm experiments showed that the most robust ddPCR primer/probe assay provides a pragmatic approximation of bullfrog abundance independent of life stage, whereas under natural conditions

remarkable seasonal eDNA patterns could be revealed in some permanently infested natural ponds. The outcome of our validation process suggests that this protocol is ready to be implemented in large-scale monitoring campaigns, in order to coordinate, evaluate, and eventually fine-tune such eradication programs[67].

Methods

Primer/probe assay validation

In a first step, we tested the performance of available primer/probe assays specifically designed for bullfrog eDNA detection, both in terms of specificity and sensitivity. To the best of our knowledge, three primer pairs targeting bullfrog eDNA were published at the onset of this study, which are referred to as the Ficetola[15], Strickler[50], and Lin[30] assays. The Ficetola assay lacks a corresponding probe and was previously shown to have some weaknesses regarding its specificity[30], which led us to decide not to include this assay for further testing. In contrast, three probes of different length were available for the Strickler primers: the original 17 bp MGB probe[50] and two longer BHQ probes: an adapted 21 bp version[29] and an unpublished 27 bp version developed by C. S. Goldberg (personal communications) (Supplementary Fig. S1). Of the three aforementioned primer/probe combinations, we chose to further test the C. S. Goldberg assay (referred to as the Goldberg assay), since it was characterized by the longest probe (and thus the highest expected specificity) and an annealing temperature identical to the conditions needed for optimal co-amplification of the internal positive control used for quality testing (see section Laboratory protocol). The specificity and sensitivity of this Goldberg assay was compared to the assay designed by Lin (referred to as the Lin assay), which was already intensively screened and found to be very robust[30].

The Goldberg assay targets a 84 bp fragment of the mitochondrial cytochrome b gene and was specifically designed for the detection of bullfrogs in the Western US. The Lin assay, on the other hand, was designed for bullfrog detection in China, targeting a 120 bp fragment of the 16S rRNA gene (Table 1). We assessed the specificity of both assays to detect bullfrogs in Western Europe in a two-step process. First, *in silico* specificity tests for both assays were carried out using available sequences of the most commonly co-occurring amphibian species occurring in Western Europe from the International Nucleotide Sequence Database Collaboration (INSDC). The most common sequence for each species was used to visualize mismatches with the corresponding bullfrog sequence using Geneious (version 10.2.6) (Supplementary Fig. S1). Next, *in situ* specificity tests were performed for both assays by subjecting them to eDNA samples originating from thirteen ponds and lakes in Belgium that are known to be free of bullfrogs and to harbor the most common indigenous sympatric amphibian species. These ponds were previously screened via metabarcoding analyses, which confirmed this presumption (unpublished data, see Supplementary Table S1).

To investigate the sensitivity of both assays, we selected 25 eDNA samples collected from ponds that were invaded by bullfrogs, to obtain a gradient in natural bullfrog eDNA concentrations. Both primer/probe

assays were then run on each of these samples under optimal PCR conditions in triplicate via ddPCR (see Supplementary Methods). The relative performance in terms of bullfrog DNA concentration retrieved and ddPCR detection resolution (*i.e.* the difference in mean amplitude of positive and negative droplets[53]) was calculated for every sample along this gradient and contrasted among both assays.

Mesocosm experiment

To assess the extent to which eDNA concentrations can be related to the abundance and biomass of bullfrogs, a controlled mesocosm experiment was set up at the INBO facility in Linkebeek (Belgium). Sixteen outdoor polyester tanks (58 x 54 x 89 cm, volume: 200 L) were filled with 122.5 ± 2.58 L rainwater, that was tested for potential bullfrog contamination prior to the experimental setup by filtering 3 L over a similar filter as used in the mesocosm and temporal experiments (see below). Eight of these tanks were dedicated to bullfrog larvae (248 individuals in total), and seven tanks to juveniles (22 individuals in total). The tadpoles were partitioned over the mesocosms as follows: 1, 2, 4, 8, 16, 32, 64, and 121 individuals. Given the limited number of juvenile bullfrogs that could be obtained, the density range for this life stage was downscaled as follows: two mesocosms were stocked with one, two, and four juveniles, and one tank was stocked with eight juveniles. The per individual wet weight (larvae: 116.9 ± 11.3 g, juveniles: 50.5 ± 7.0 g) was measured, and the total wet biomass per mesocosm was calculated. Tanks dedicated to juvenile individuals contained one small artificial, similar sized island to prevent juveniles from drowning. 72 hours after introduction to the tanks, water samples were taken for eDNA analyses. From each experimental tank, three 1 L subsamples were collected and pooled into one merged sample. In a next step, each of these merged samples was filtered on a 50 mm diameter syringe disk filter with an integrated 5 µm glass fiber prefilter and a 0.8 µm PES membrane (NatureMetrics, Surrey, England) using a Vampire sampler pump (Buerkle, Bad Bellingen, Germany) with disposable silicone tubing. To minimize the likelihood of cross-contamination, sampling was performed in a low-to-high density direction using sterile disposable material. After filtration, the remaining water inside capsules was expelled by forcing air through the capsule. A total of 16 filters (15 filters from the mesocosms and one control filter) were capped at both ends, and stored at −21°C in anticipation of further analyses in the laboratory.

Seasonal eDNA patterns

Three permanent ponds that are representative for bullfrog infested water bodies in Belgium were selected to conduct a long-term temporal sampling survey. These ponds differed in shape and depth, and were simultaneously sampled each month between April 2019 and October 2020 for a total of 18 monthly sampling rounds (May 2020 being an exception). The ponds were located at the core of the Belgian bullfrog metapopulation (Fig. 1b,c) to guarantee that temporal eDNA patterns closely reflected the natural seasonal alterations in bullfrog abundance and activity. As eDNA is highly diluted and often patchy distributed in the water column[20], an integrated sample strategy was employed to maximize the total habitat coverage and as such to approach the true eDNA concentration as close as possible[68,69]. Therefore, ten to thirty 0.5 L subsamples were collected at five meter intervals around each pond and were pooled to obtain a single integrated, homogenous sample. Using a long sampling pole with a sterile Whirl-Pak bag (Sigma-Aldrich, Overijse, Belgium) attached at the end, water was sampled from just below the

water surface (±10 cm) as this appears the best section of the water column for eDNA detection in lentic systems[68]. The merged sample was filtered until the filter was saturated. The volume of filtered water was quantified and recorded for further calculation of target eDNA concentration per liter filtered water. Cross-contamination was impeded by wearing sterile nitrile gloves and using sterile sampling bags, which were discarded after each pond was sampled. All reusable field material was decontaminated between sites with 2% Virkon S (Antec - DuPont, Suffolk, UK) as a biosafety precaution and to avoid potential DNA cross-contamination. For all filters (3 ponds x 18 months = 54 in total), the remaining water inside was expelled until the capsule was dry, after which they were capped at both ends and immediately stored at -21 °C in a BlueLine box (delta T, Fernwald, Germany) for transportation to the lab for further storage and analyses.

Laboratory protocol

Prior to PCR, all eDNA samples were stored and processed in a PCR-free building dedicated to low copy number template extractions, with controlled DNA-free high-efficiency particulate air (HEPA)-filtered compartments with positive pressure to prevent eDNA sample contamination. An internal positive control (IPC) (KWR Watercycle Research Institute, Nieuwegein, Netherlands) was included in the first step of the extraction. This IPC consisted of a plasmid with a 149 bp insert sequence from Dengue virus type 2 (GenBank M29095.1) and was quantified with the primers and probe shown in Table 1 (NEN 6254, 2012) in duplex with the primer/probe assays used for bullfrog detection[35]. Using an IPC allows to standardize variation in bullfrog eDNA concentrations attributable to sample-specific differences in extraction or amplification efficiency and hence to increase the comparability of eDNA samples in space and time[28,60]. The DNA was extracted from the filters using Qiagen's DNeasy Blood & Tissue Kit according to manufacturer's instructions (NatureMetrics, Surrey, England), and was finally eluted in 100 µL Tris-EDTA (10 mM Tris-HCl, 1mM EDTA, pH 8.0) preheated at 70 °C[70]. DNA extracts obtained from the monthly sampled ponds were additionally purified with the DNeasy PowerClean Cleanup Kit (Qiagen) according to the guidelines provided by the manufacturer, and were eluted in 100 µL of TE. eDNA quantification with digital droplet PCR (ddPCR) is described in the Supplementary Methods.

Data analysis

The total number of bullfrog copies per liter filtered water (C_x) was calculated using the following formula[35]:

$$C_x = \frac{\left(\frac{C_{IPC\ initial}}{C_{IPC\ observed}}\right) \times C_{obs} \times \left(\frac{V_{PCR}}{V_r}\right) \times V_e}{V_w}$$

where $C_{IPC\ initial}$ is the concentration of IPC initially included in each sample (on average 1099 ± 55 copies per µL), $C_{IPC\ observed}$ is the obtained sample-specific IPC concentration in each ddPCR reaction, C_{obs} is the obtained sample-specific bullfrog eDNA concentration (in copies per µL) adjusted for 10% loss during droplet generation, V_{PCR} is the total ddPCR reaction volume (in µL), V_r is the volume of the eluted extract

undergoing ddPCR (in µL), V_e is the total elution volume after DNA extraction (in µL), and V_w is the total volume of filtered water (in L).

To quantify and compare the sensitivity of both primer/probe assays, the mean bullfrog eDNA concentration obtained per assay was calculated for the three ddPCR replicates run on each of the 25 positive field samples. Relative performance per sample was then determined based on the assay retrieving the highest concentration for that sample, and was compared among assays using a paired two-tailed Student's *t*-Test. The difference in resolution for both the target and the IPC quantification was tested between both assays with an nonparametric paired two-tailed Wilcoxon Signed Rank Test. Linear regressions were used to test the relationships between eDNA concentration, on the one hand, and abundance and total biomass on the other by applying the function *lm*. Differences in per individual and per biomass eDNA concentration among life stages were analyzed with a nonparametric two-sided Mann-Whitney test. All statistical analyses were carried out using an alpha-value of 0.05 and with the package *stats* in RStudio version 1.3.1073[71].

Ethics statement

All experimental protocols were approved by the Research Institute for Nature and Forest, and the mesocosm experiments were carried out according to the Institutional and International ethical guidelines.

Declarations

Data availability statement

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

R.B. designed the study and experimental set-up. T.E., N.D.R., D.H., and R.B. set up the mesocosm experiment and collected field samples. S.N. and T.E. performed the lab work, and T.E., R.B., and D.H. analysed the data. The manuscript was written by T.E. and R.B., and was reviewed by H.J., D.H., and S.N.

Competing Interests: The authors declare no competing interests.

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Tables

Table 1. Sequences of primers and probes for the two assays targeting bullfrog DNA at the mitochondrial cytochrome b gene (Goldberg) or the 16S rRNA gene (Lin) that were compared in this study, as well as for the internal positive control (IPC) being a 149 bp plasmid insert sequence from the Dengue virus type 2 (INSDC M29095.1).

Target	Assay	Primer/probe	Sequence (5’-3’)
L. catesbeianus	C. S. Goldberg	BullfrogF	TTTTCACTTCATCCTCCCGTTT
		BullfrogR	GGGTTGGATGAGCCAGTTTG
		Bullfrog BHQ Probe	(6FAM)TTATCGCAGCAGCAAGTATGATCCACC(ZEN/IBFQ)
L. catesbeianus	Lin et al. 2019	qLC16S Forward	GCAGAGATAACCTCTCGT
		qLC16S Reverse	GTCCCATAGGACTGTTCT
		qLC16S BHQ Probe	(6FAM)TGCCCTCCCGAAACTAAGTGAGC(ZEN/IBFQ)
IPC	NEN 6254, 2012	IPC-D2-F	ATGACAGCCACTCCTCCG
		IPC-D2-R	GGAACGAACCAAACAGTCTTC
		IPC-D2-Probe	(HEX)AGCAGAGACCCATTCCCTCAGAGC(ZEN/IBFQ)

Figures

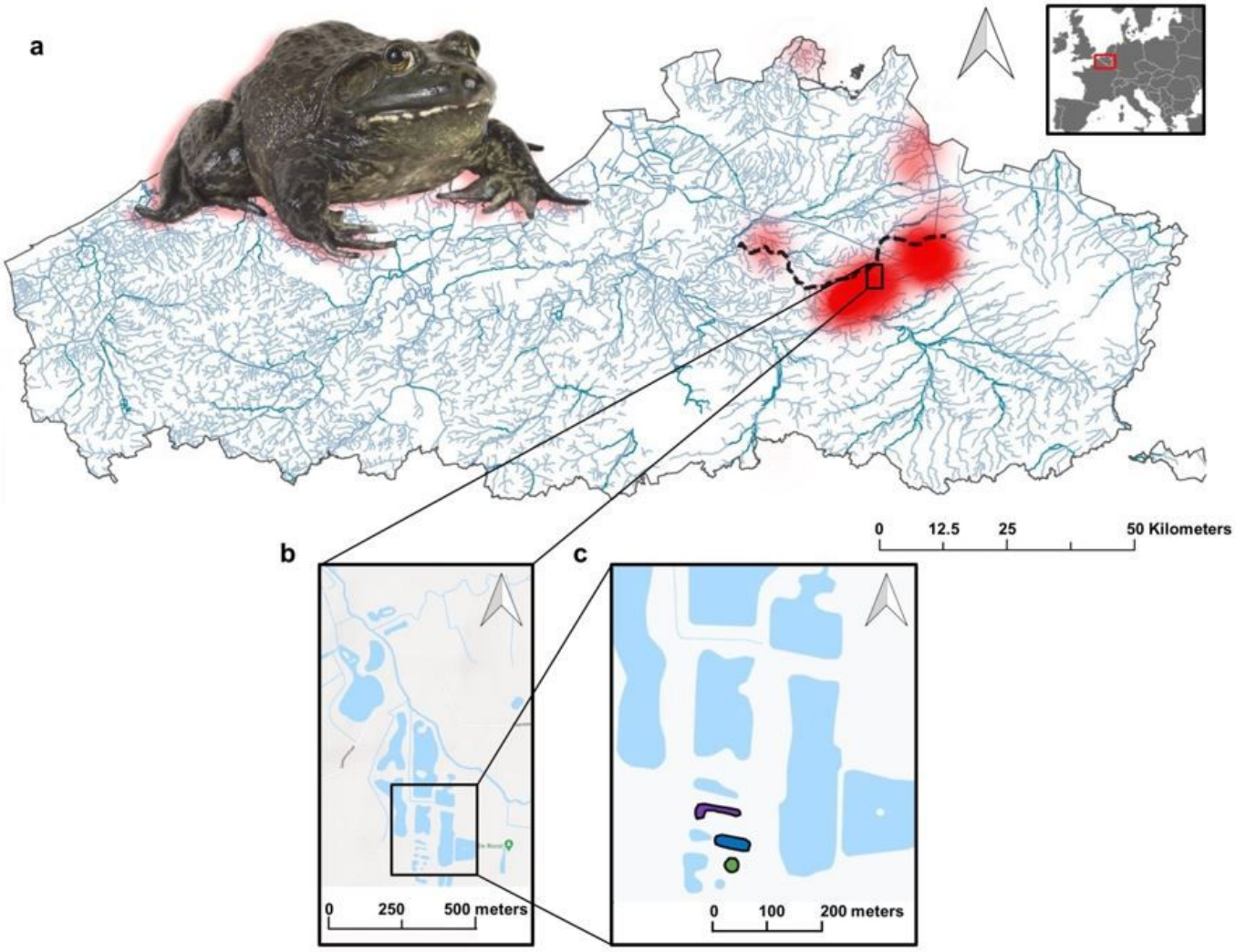
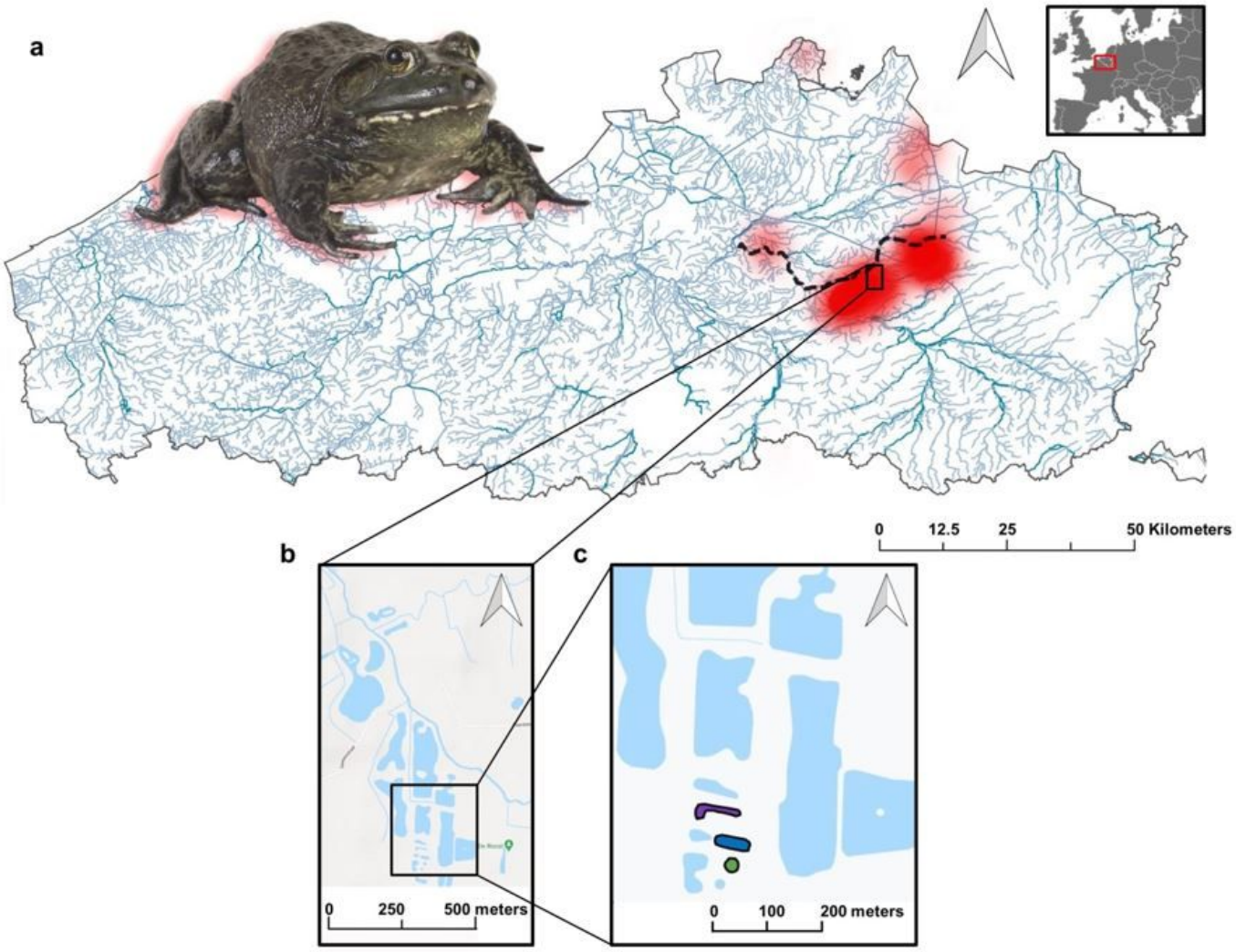


Figure 1

(a) A map of Flanders (Northern Region of Belgium, Western Europe) highlighting the contemporary bullfrog invasion in red (data derived from eradication campaigns and the open science data platform “waarnemingen.be”), with the river Grote Nete delineated by the black dotted line. (b) Location of nature reserve De Roost, where (c) the three permanent ponds were situated that were consecutively sampled for eighteen months, highlighted in green (pond 1), blue (pond 2), and purple (pond 3). Maps were created using map data derived from Google in QGIS version 3.10.10 (https://www.qgis.org/). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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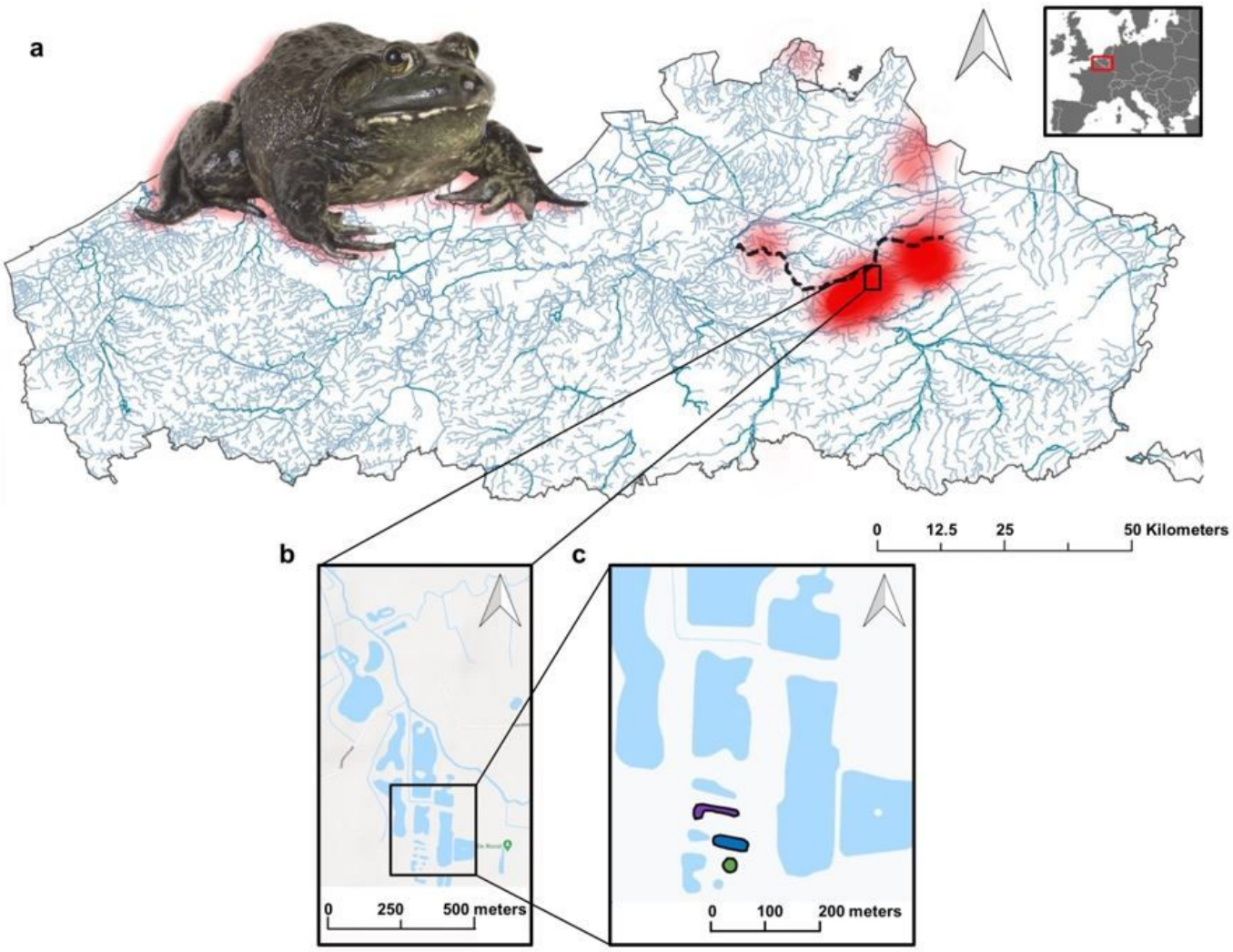

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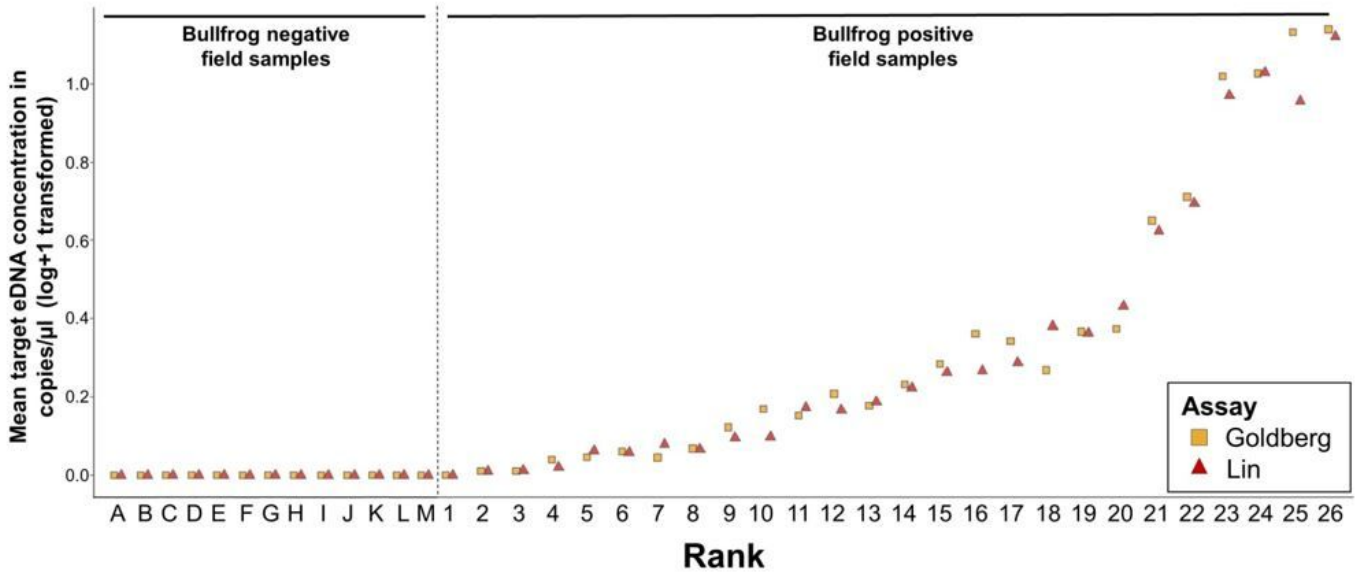
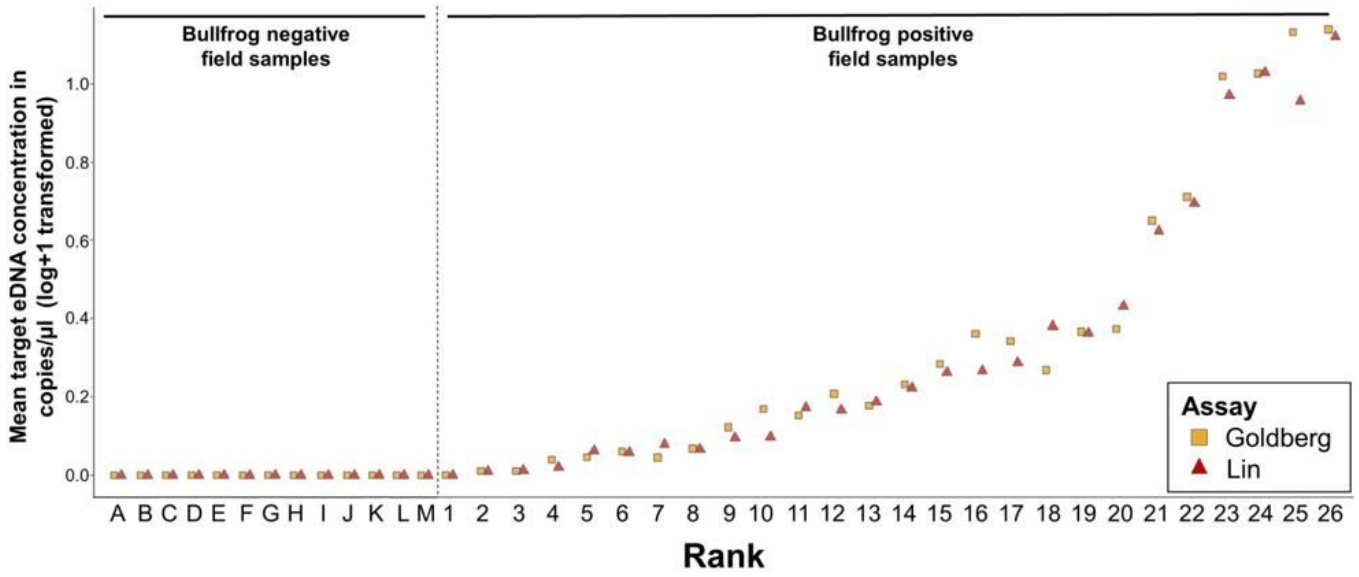


Figure 2

Mean bullfrog eDNA concentrations following in situ specificity (ranks A-M) and sensitivity (ranks 1-26) testing of the Goldberg and Lin primer/probes on field-collected samples. Samples corresponding to ranks A to M were collected from water bodies free from bullfrogs, but harboring the most common sympatric amphibian species native to Western Europe (see Supplementary Table S1). Ranks 1 (~ 0.025 copies/µL) to 26 (~ 12.45 copies/µL) reflect a ranking of increasing mean bullfrog eDNA concentrations of samples collected from natural water bodies, except sample 26, which represents a bullfrog tissue sample functioning as a positive reference in this study. Per sample, the mean log+1-transformed eDNA concentrations expressed as copies per µL of three technical replicates are plotted as orange squares for the Goldberg and as red triangles for the Lin assay.



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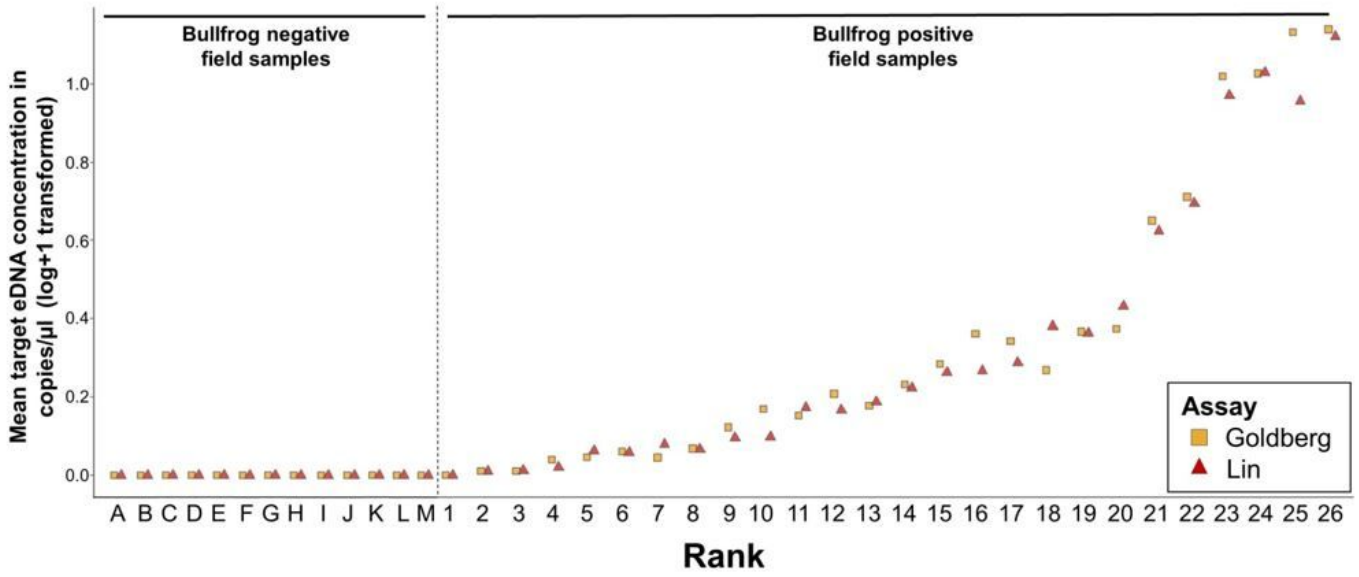

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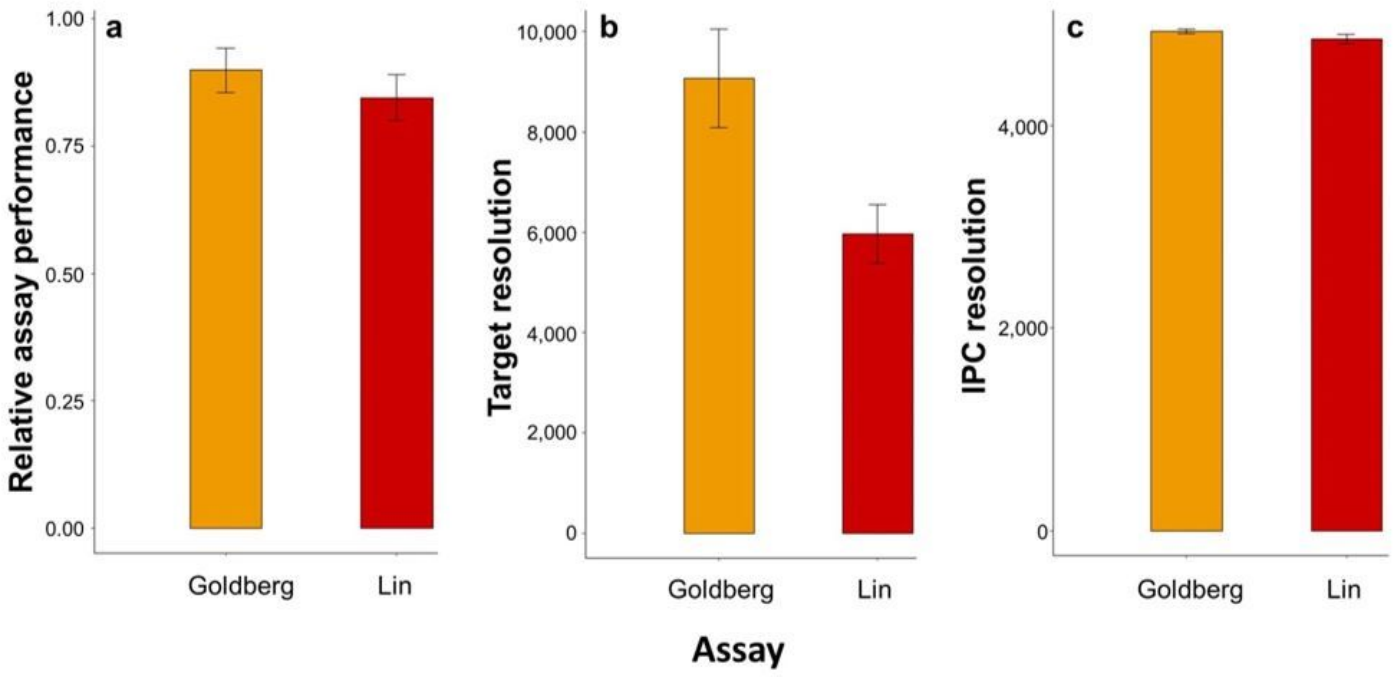
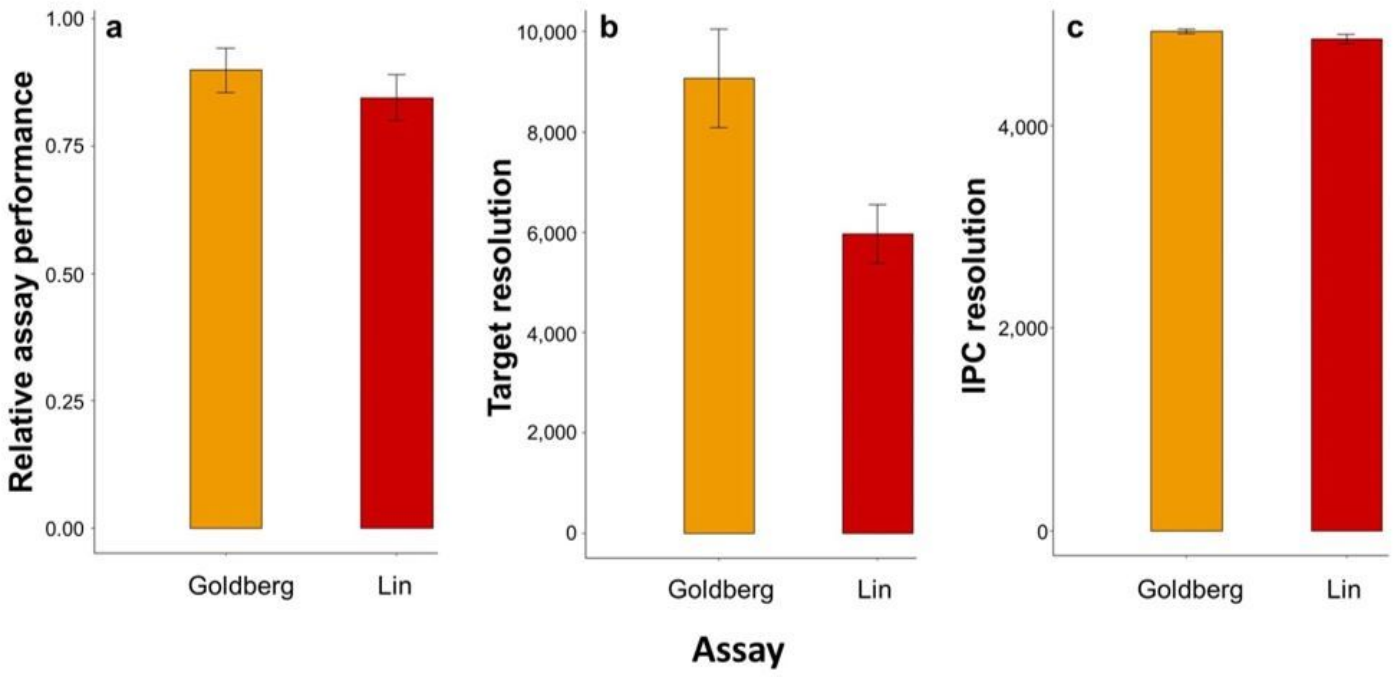


Figure 3

Results of the sensitivity tests of the Goldberg and Lin primer/probe assays using eDNA samples from natural water bodies (corresponding to Ranks 1-26 in Fig. 2). (a) Mean (± 1 s.e.m.) relative performance in bullfrog eDNA detection (in terms of eDNA concentration quantified per eDNA sample) and the mean (± 1 s.e.m.) (b) target and (c) IPC detection resolution. The detection resolution was calculated as the difference in fluorescence between positive and negative ddPCR droplets.



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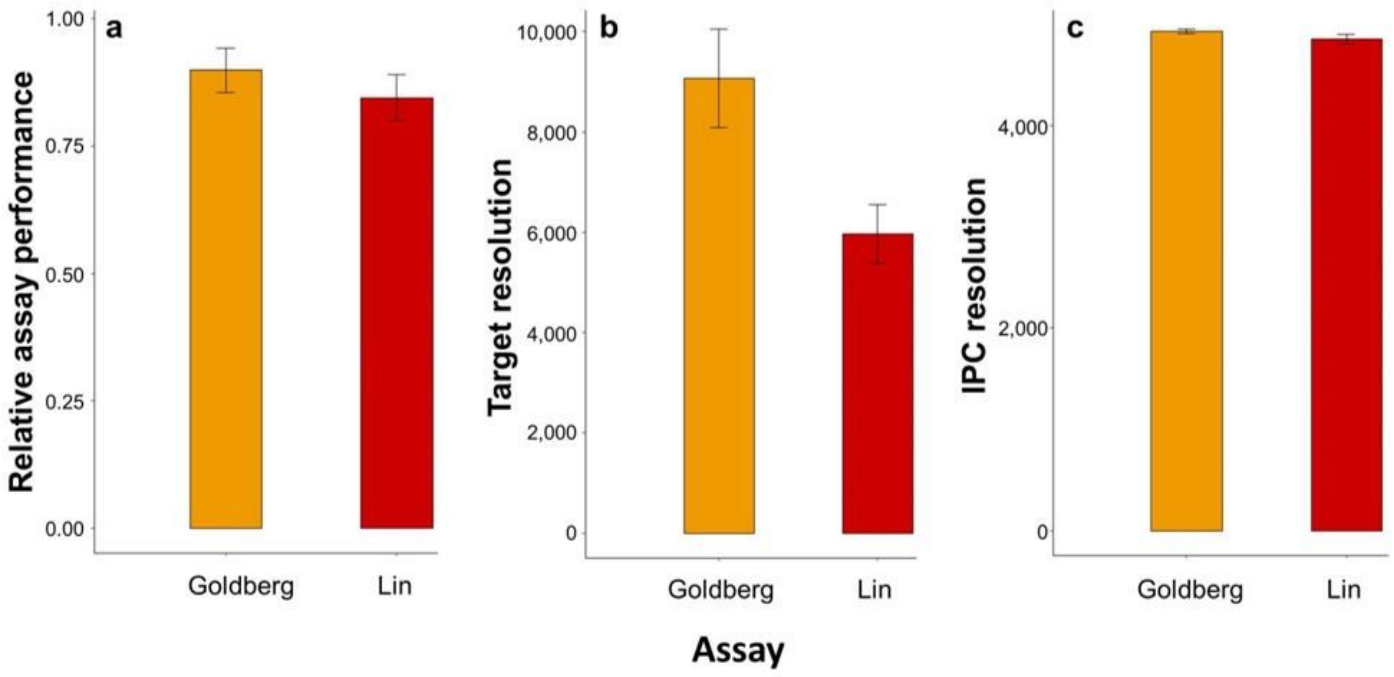

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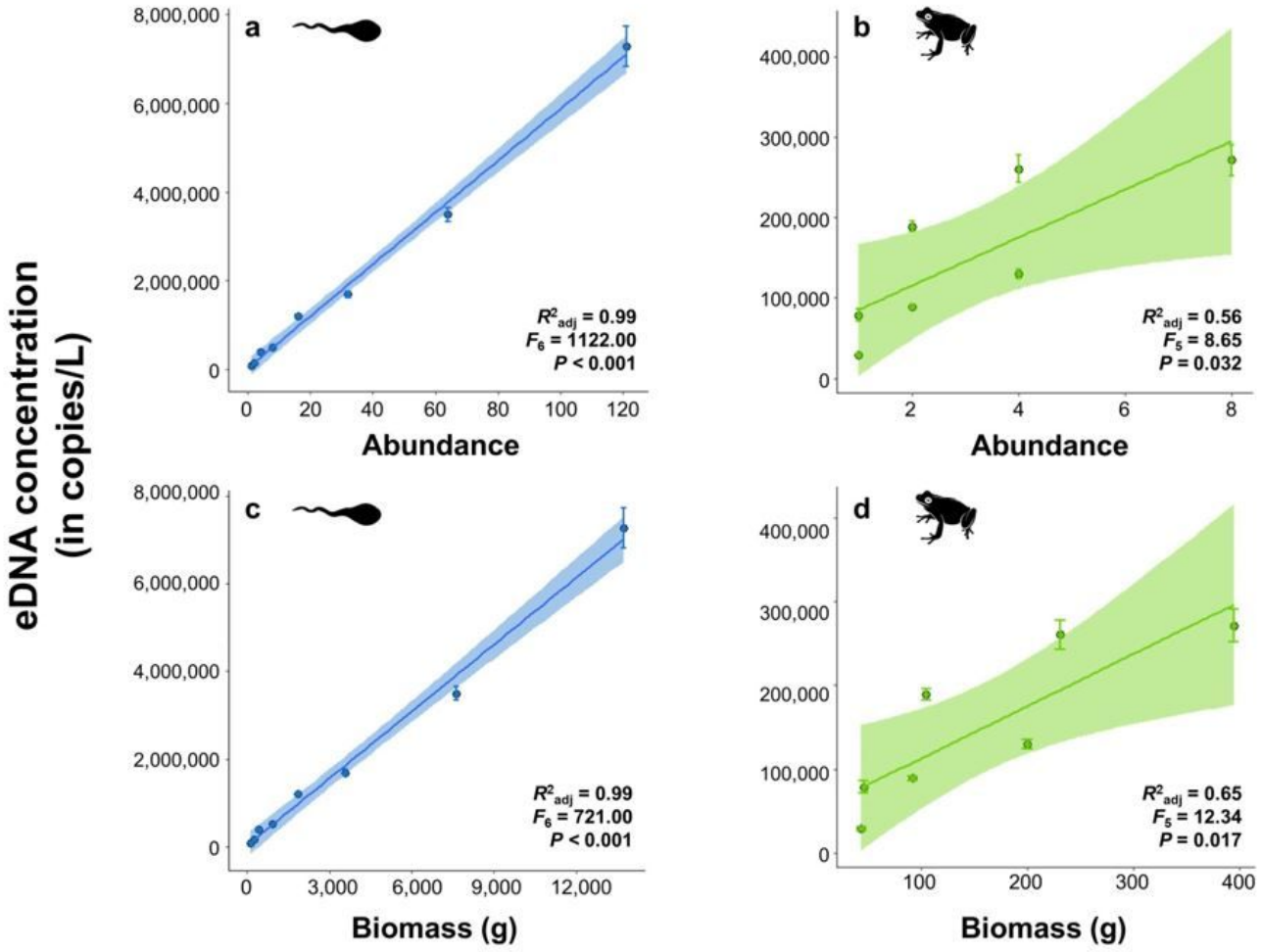
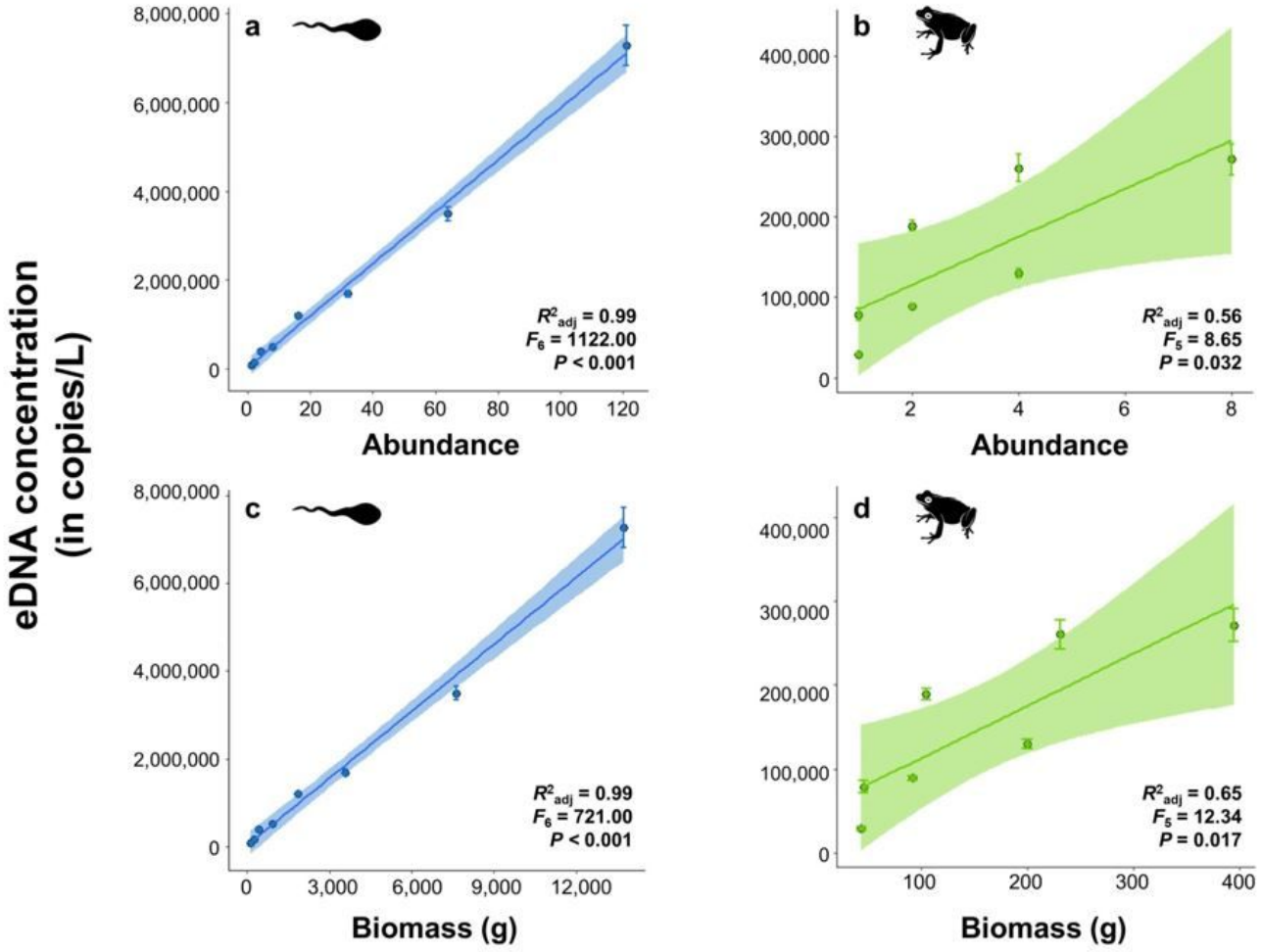


Figure 4

The relation between bullfrog eDNA concentrations and abundance (a, b) and total biomass (c, d) of larvae (blue) and juveniles (green) stocked in the mesocosms, expressed as copies per liter filtered water. Dots represent the means of the technical replicates with the shaded areas around the regression line indicating the 95% confidence intervals. R2adj values, effect sizes, and P values of each regression are given in the bottom right corner of the corresponding graph.



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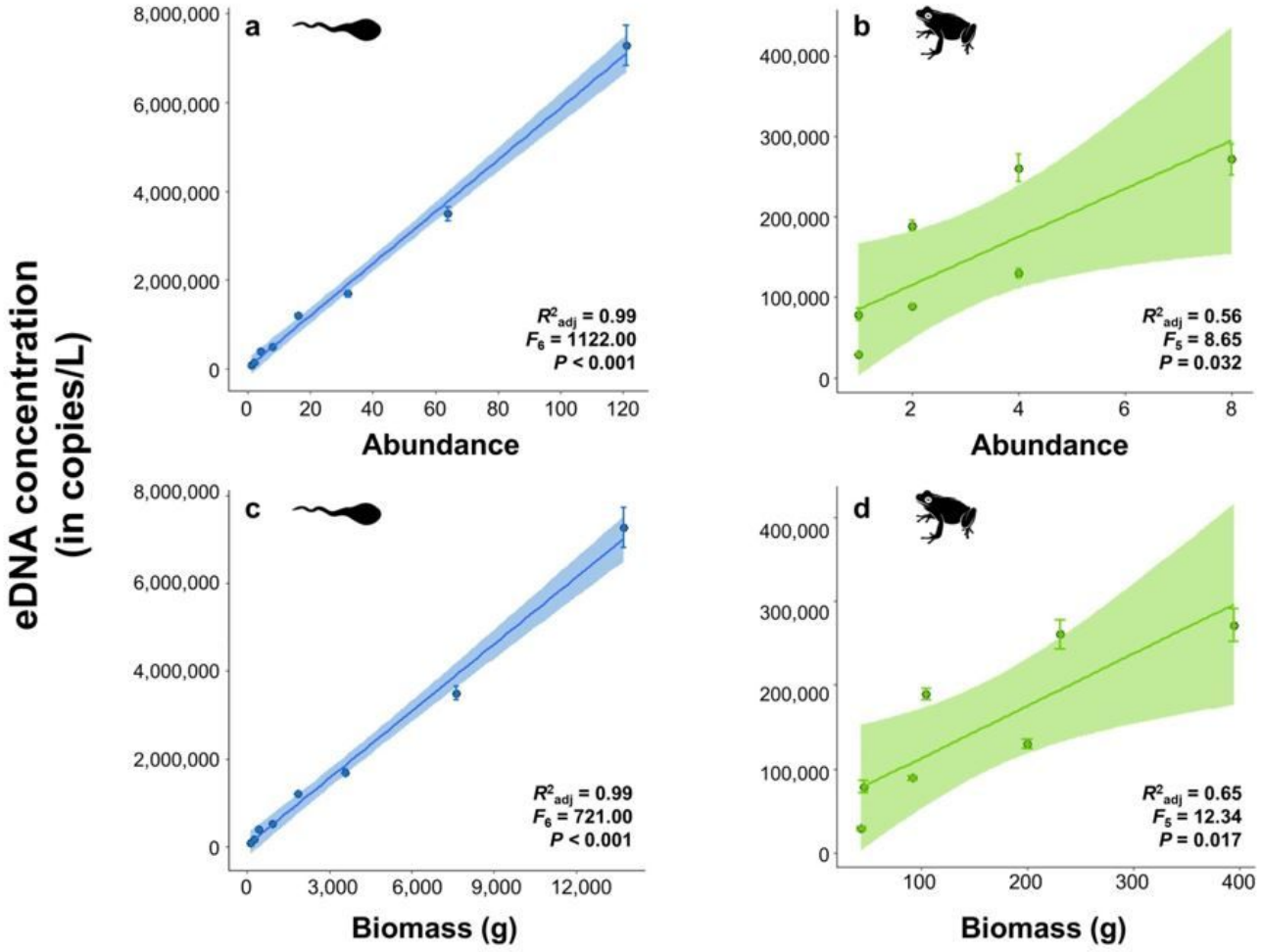

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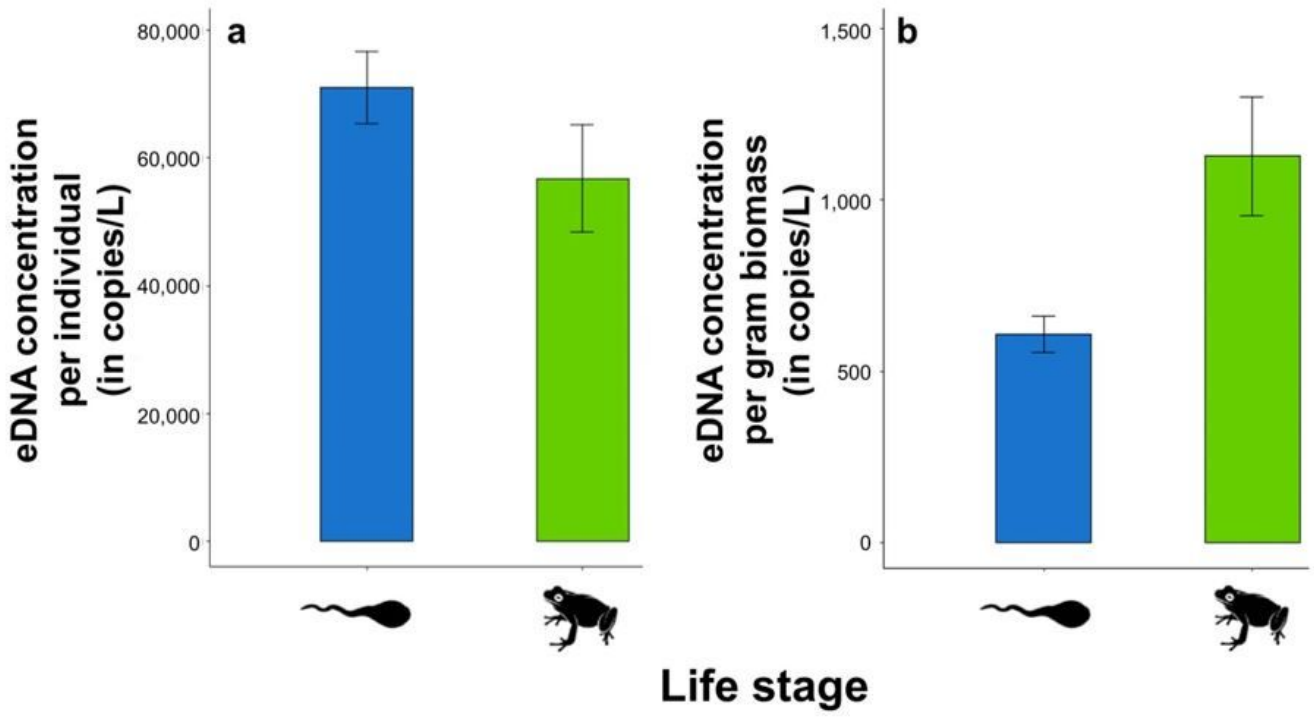
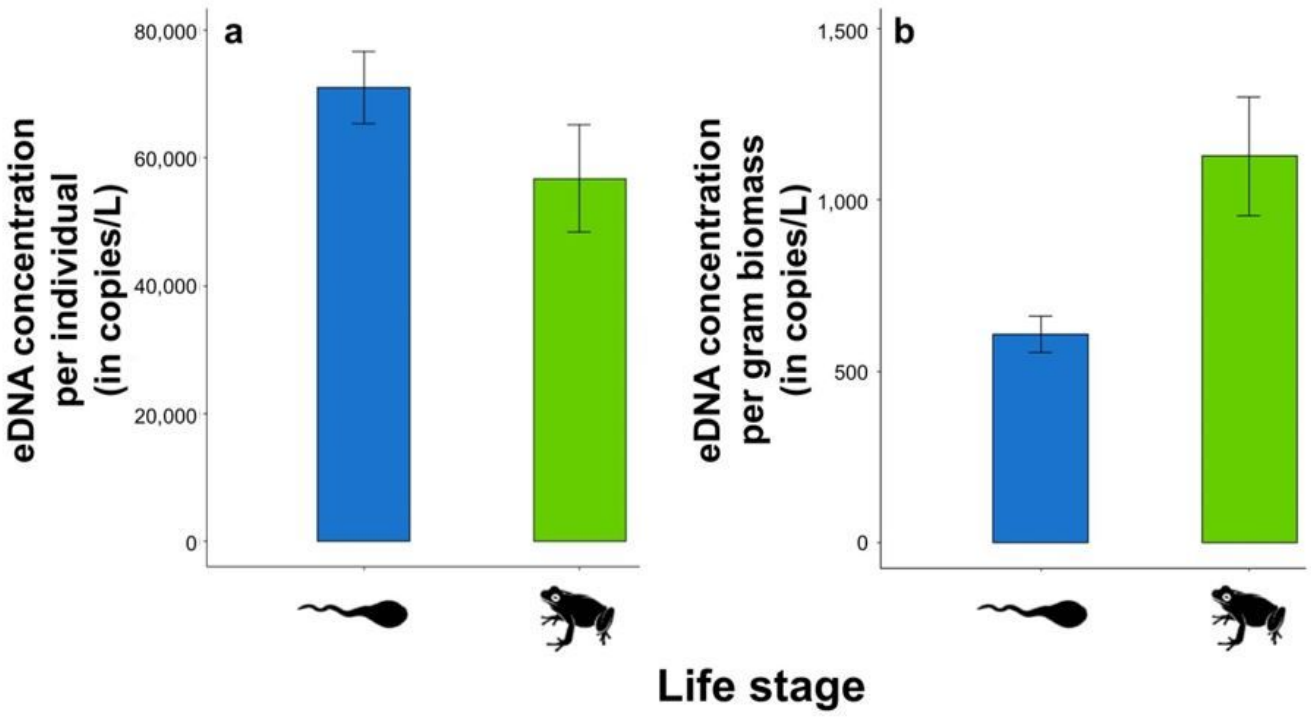


Figure 5

Average (± 1 s.e.m.) per individual (a) and per gram biomass (b) eDNA concentration of bullfrog larvae (blue) and juveniles (green) obtained from the mesocosm experiment and expressed as copies per liter filtered water.



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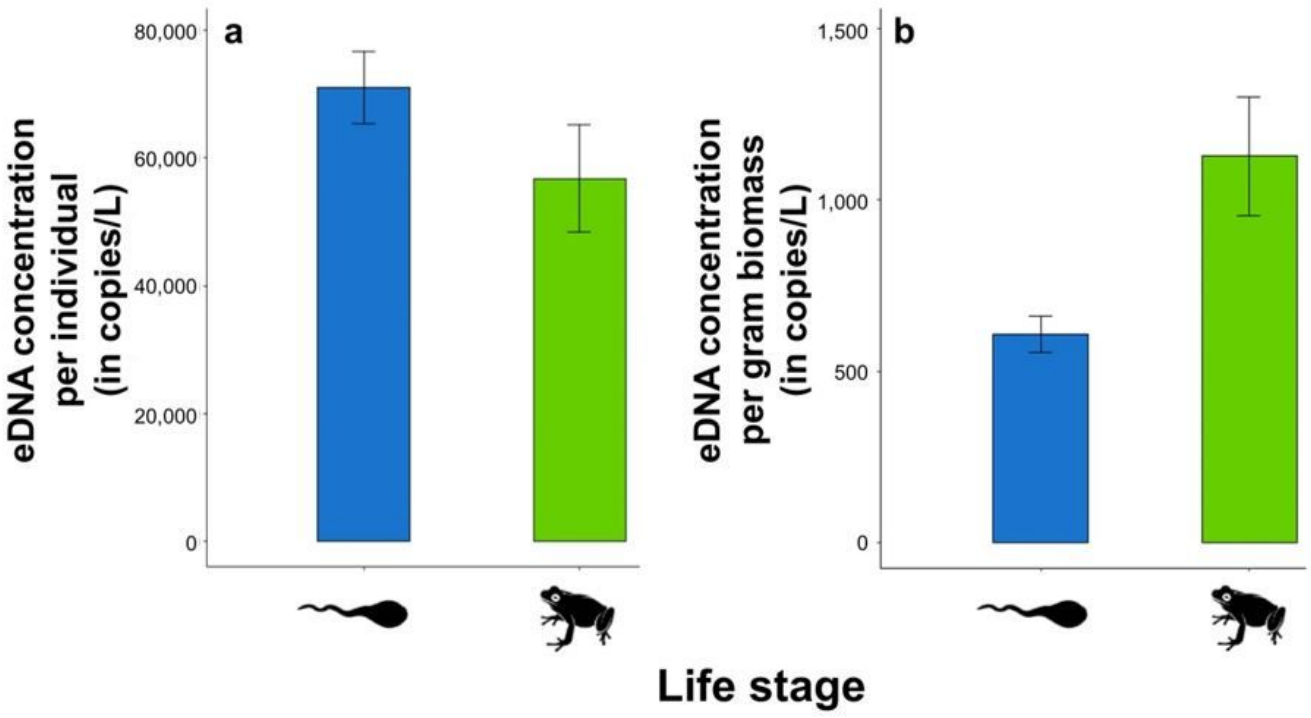

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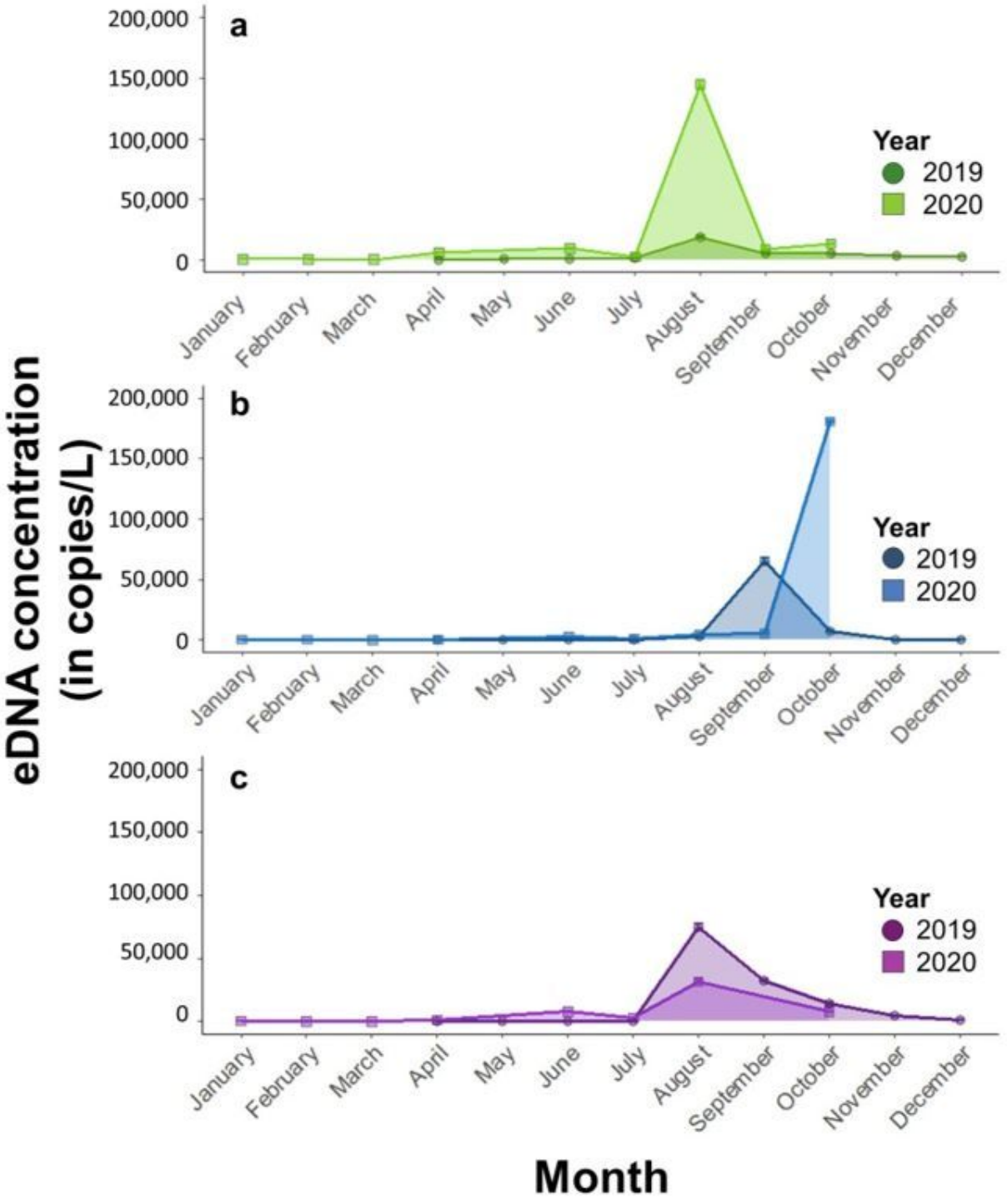
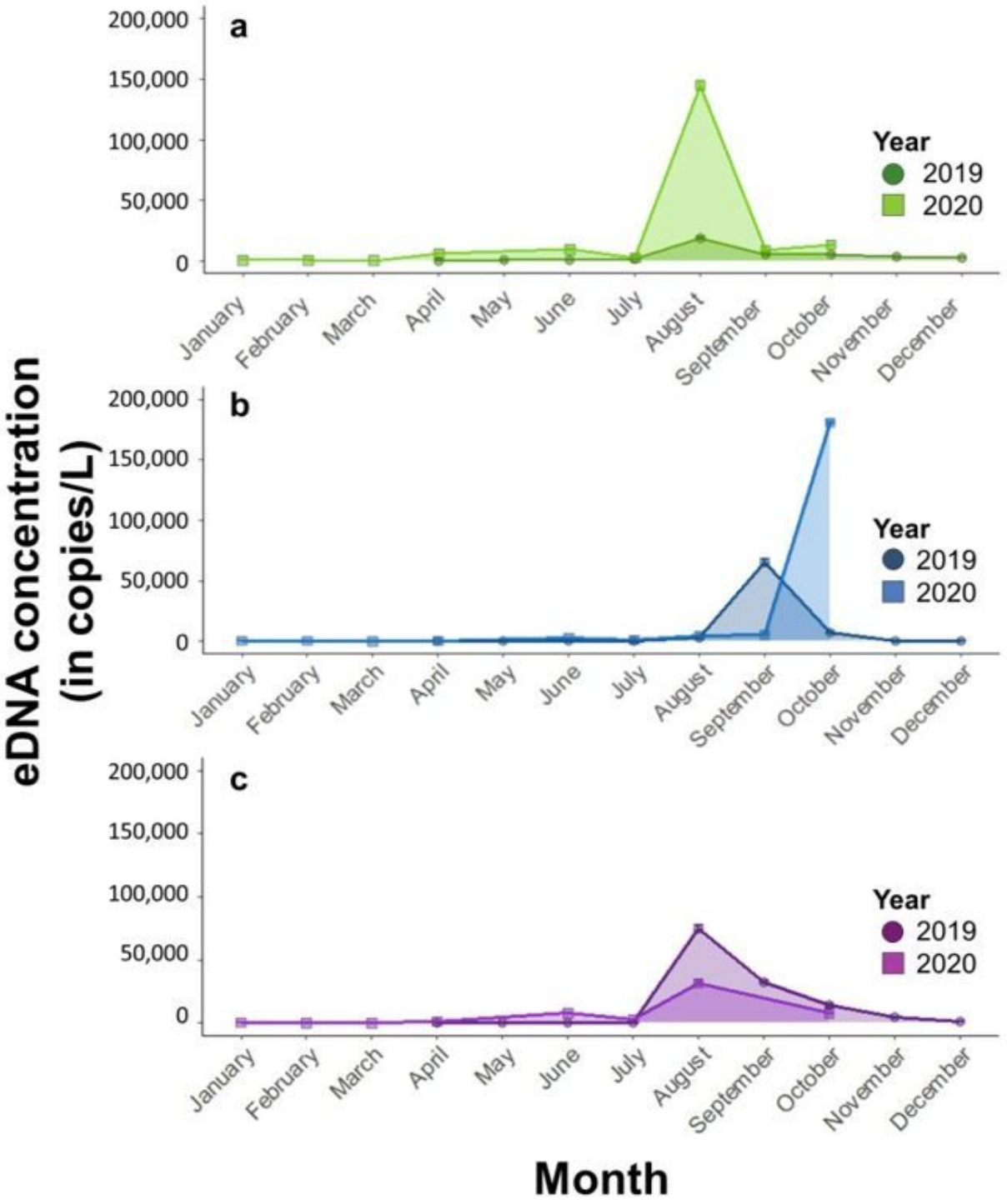


Figure 6

Monthly bullfrog eDNA concentrations, expressed as the average (± 1 s.e.m.) number of copies per liter filtered water of two technical replicates per filter in three permanent ponds known to be heavily infested by bullfrog, located at the core of the Belgian metapopulation (see Fig. 1): pond 1 (a), pond 2 (b), and pond 3 (c). Samples collected in 2019 and 2020 are represented by circles and squares, respectively.



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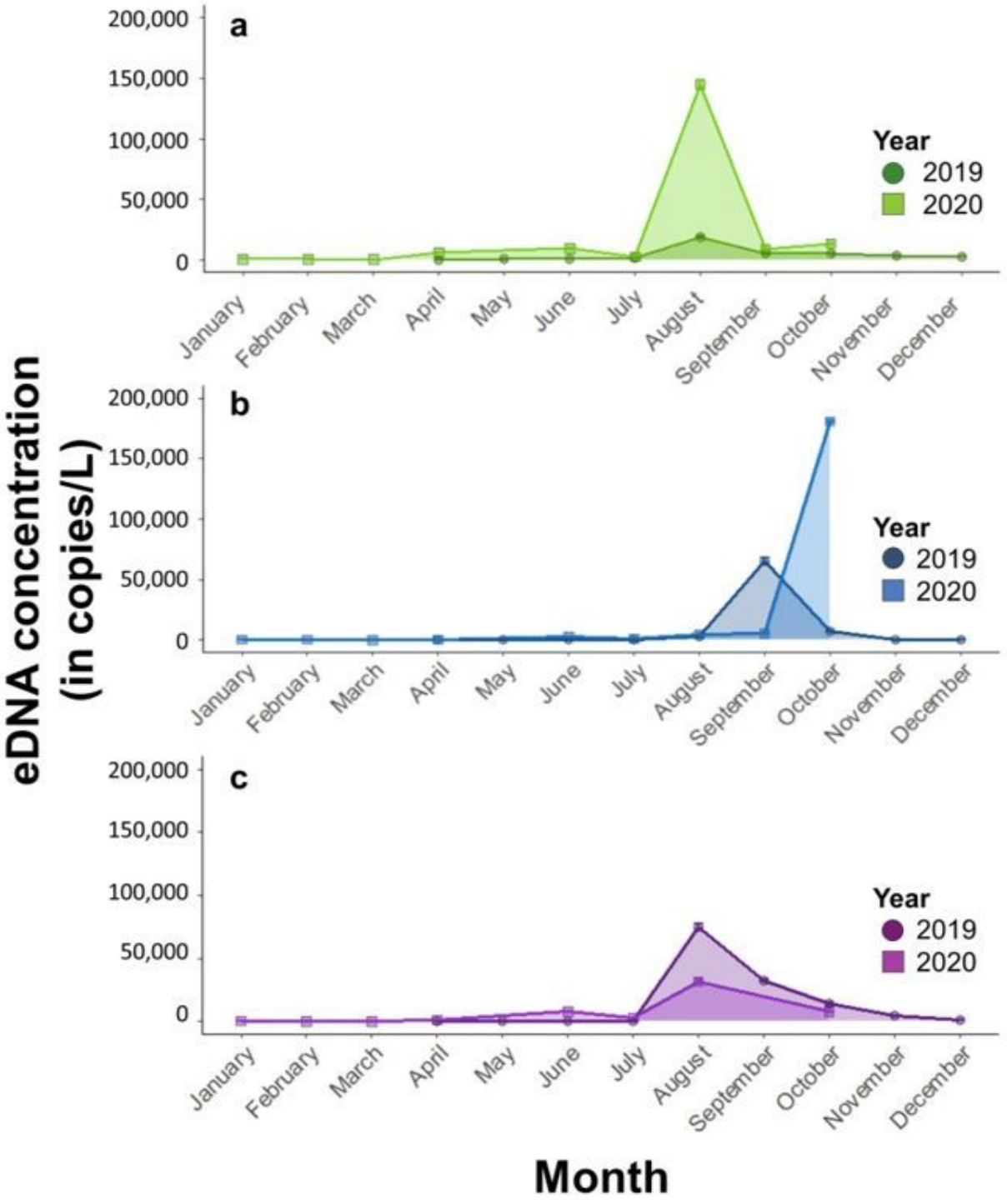

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryeDNABullfrog.pdf
- SupplementaryeDNABullfrog.pdf
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