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Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* ☆

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ABSTRACT

Wildlife managers can more easily mitigate the effects of invasive species if action takes place before a population becomes established. Such early detection requires sensitive survey tools that can detect low numbers of individuals. Due to their high sensitivity, environmental DNA (eDNA) surveys hold promise as an early detection method for aquatic invasive species. Quantification of eDNA amounts may also provide data on species abundance and timing of an organism's presence, allowing managers to successfully combat the spread of ecologically damaging species. To better understand the link between eDNA and an organism's presence, it is crucial to know how eDNA is shed into the environment. Our study used quantitative PCR (qPCR) and controlled laboratory experiments to measure the amount of eDNA that two species of invasive bigheaded carps (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*) shed into the water. We first measured how much eDNA a single fish sheds and the variability of these measurements. Then, in a series of manipulative lab experiments, we studied how temperature, biomass (grams of fish), and diet affect the shedding rate of eDNA by these fish. We found that eDNA amounts exhibit a positive relationship with fish biomass, and that feeding could increase the amount of eDNA shed by ten-fold, whereas water temperature did not have an effect. Our results demonstrate that quantification of eDNA may be useful for predicting carp density, as well as densities of other rare or invasive species.

1. Introduction

The use of environmental DNA (hereafter referred to as eDNA) for detection of rare or hard to observe species is increasing (Lodge et al., 2012). Although the technique of taking water, soil or organism excretion/secretion samples and extracting DNA from organisms or their shed cellular material has been used for decades in microbial diversity studies (metagenomics – Handelsman, 2004; Tablerlet et al., 2012) and characterization of ancient macroorganism populations (ancient DNA – Willersev & Cooper, 2005); recent work addresses the utility of eDNA to examine current macroorganism diversity and ecology (Foote et al., 2012; Nichols et al., 2012; Thomsen et al., 2012a,b). For a detailed history of eDNA methods and applications, see Thomsen and Willerslev (2015).

An important application of the method is in early detection surveys for invasive species (Ficetola et al., 2008; Jerde et al., 2011). Early detection can be difficult as the expansion of a species' range may be initiated by only a few individuals, and thus observations of individuals will be rare. Furthermore, there is often a lag time between initial colonization and expansive population growth, resulting in low detectability (Crooks and Soulé, 1999; Sakai et al., 2001). However, eradication efforts by management may be most successful at eliminating invasive species early in an invasion (Hulme, 2006), thus increasing the sensitivity of detection methods is important. Studies have demonstrated an increased detection sensitivity with eDNA methods compared to traditional surveying methods for aquatic species (Jerde et al., 2011; Dejean et al., 2012; Thomsen et al., 2012a), because polymerase chain reaction (PCR) technology can theoretically detect a single molecule of DNA.

Early studies of eDNA used conventional PCR to detect DNA presence or absence (e.g., Ficetola et al., 2008; Jerde et al., 2011). Successful amplification of DNA, as determined by a band on an agarose gel, is then used to infer a target species' presence in the system. However, the quantification of eDNA via quantitative PCR (qPCR), digital PCR or next generation sequencing can provide

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estimates of relative eDNA abundance; suggesting that target species abundance in a system may also be inferred. Several studies have found that eDNA concentration correlates with abundance of macroorganisms in aquatic systems (fish – Takahara et al., 2012; amphibians-Thomsen et al., 2012b; Pilliod et al., 2013; and snails – Goldberg et al., 2013). Nevertheless, many other factors could affect the amount of eDNA detected. Studies addressing what influences eDNA production, transportation and degradation will improve our understanding of how eDNA quantification relates to presence and distribution of the organism as well as the errors associated with this technique (Darling and Mahon, 2011). Our study begins to address these questions by understanding how much eDNA is produced and shed into the water by two species of invasive Asian carps.

Environmental DNA is being used to monitor for bighead (*Hypophthalmichthys nobilis*) and silver carps (*Hypophthalmichthys molitrix*) in the Laurentian Great Lakes and the Chicago Area Waterways System, which is a water connection between the Great Lakes and the Mississippi River Basin (ACRCC, 2013). Since the initial escape of these species, and their movement up the Mississippi Basin, the potential for invasion of the Great Lakes by silver and bighead carps has been a major concern to stakeholders (i.e. shipping industry, fisheries, recreationists; Kolar et al., 2007). Despite several seasons of monitoring with conventional (non-quantitative) PCR resulting in positive eDNA detections of both species in the area (Jerde et al., 2011; USACE, 2012; USFWS, 2013), no bighead or silver carp have been found in subsequent rapid response netting efforts. One bighead carp was netted by commercial fisherman above the electric barrier in an area where eDNA had been detected, but not during a rapid response event. These monitoring results suggest a complex relationship between positive eDNA detections and the presence of live organisms.

Our goal was to obtain information on what influences the shedding of eDNA from both silver and bighead carps. Ultimately our data will be incorporated into a probabilistic model to better inform management about how positive eDNA results might reflect population size and occurrence in specific areas. To obtain these goals, qPCR was used to quantify the amount of carp DNA in water samples from a series of experiments that tested the influence of fish biomass and temperature on eDNA shedding rates. We were also interested in investigating the main source of shed cellular debris that contains the eDNA because it is currently unknown. However, likely sources are from the exterior epithelial cells shed through sloughed skin and mucus or from cells lining the gut and shed through excrement. If the gut lining is the source of most eDNA, then we predict that the amount and quality of food consumed will alter eDNA shedding rates.

We specifically addressed three hypotheses concerning factors that could influence the shedding rate of eDNA by these fish, including differences between species and age classes:

1. We hypothesized that shedding rate (amount of DNA released to the water per hour) increases as biomass (g) of fish increases.
2. We hypothesized that fish shed more DNA as water temperature increases.
3. We hypothesized that unfed fish would shed less eDNA than fish being fed, and that fish fed a rough food, brine shrimp (*Artemia* sp.) nauplii, would shed more eDNA than fish fed green algae.

2. Methods

2.1. Experimental set up

Studies were run in a temperature-controlled lab from July 2012 through August 2013. Bighead and silver carp from two size classes were used in the experiments, and individual fish were only used once throughout all studies. Bighead and silver carp sub-adults were captured as fingerlings from Missouri River tributaries and floodplain environments in central Missouri, and raised in captivity in ponds and in tanks at USGS facilities in Columbia, Missouri. Juvenile fish were obtained from an aquaculture facility in Lake Ozark, Missouri (Osage Catfisheries). For the studies, juvenile fish (60–100 mm total body length) were placed in 40 L glass aquaria and sub-adults (101–300 mm total body length) were housed in 379 L plastic, round tanks. All tanks and aquaria were aerated with air stones and filled with untreated well water. Additionally, all containers had water flowing in and out of them with a 20 h turnover rate. The 40 L aquaria had a water flow rate of 2 L/h using a Mount–Brungs proportional diluter apparatus (Mount & Brungs, 1967) to provide water at pulsed intervals of 0.5 L per 15 min. The large tanks had a continuous flow rate of 19 L/h measured by a Cole–Parmer flowmeter. Because the flow rates led to a water turnover rate of less than 24 h, and sampling occurred every 48 h, we assume that DNA quantification estimates represent the rate of eDNA shed by the fish as opposed to accumulated amount of eDNA that would be sampled in a closed system.

Prior to each study a water sample from each tank was tested for the presence of bighead or silver carp DNA. All tanks were found to be clear of any carp DNA before addition of fish. Fish were anesthetized with MS-222, individually weighed to the nearest 0.1 g and total body length measured to the nearest millimeter before being placed in tanks. The number of fish added to each tank depended on the experiment (see below).

Fish were fed daily with an algae diet that consisted of *Spirulina* spp. and *Chlorella* spp. (Bulkfoods.com, U.S.A.), rotifers (Brine Shrimp Direct, Inc., U.S.A.), and other microalgae (Reed Mariculture, Inc., U.S.A.) but contained no carp material. Except in the diet studies, sub-adult fish were fed approximately 1% of their body weight and juveniles were fed 10–30% of their body weight. Temperature, dissolved oxygen and pH were measured daily, while total ammonia was tested twice a week.

All study plans were approved by the Columbia Environmental Research Center Institutional Animal Care and Use Committee and conforms to relevant national and international guidelines.

2.1.1. Quantification of eDNA variability

We ran a preliminary study to assess how variable eDNA measurements were over time before testing factors that could influence shedding rates. Three 40 L glass aquaria, each with a different water flow rate (1 L/h, 2 L/h, 3 L/h) were used, and one juvenile silver carp was placed in each aquaria for a six week period. Water samples for eDNA analysis were taken daily.

2.1.2. Biomass study

To assess the effect of fish biomass and potential interactions between species and size class on eDNA quantification, four separate experiments were conducted with two different size classes (juvenile or sub-adult) of both species. Each experiment included three treatments: 1, 3, or 6 fish. Each treatment had four replicates, and tanks were randomly assigned treatments. Fish were kept in tanks for 25 days and samples were taken every other day starting from the second week. A total of eight samples per tank were collected over the course of the experiment.

2.1.3. Temperature study

For the temperature assay two experiments were run, testing the effect of water temperature on the eDNA shedding rate of sub-adult silver carp and sub-adult bighead carp. One fish was kept in each large tank. Tank water was maintained at one of three treatment temperatures: low (19 °C), medium (25 °C), or high

(31 °C). Each temperature treatment had three replicates. The experiments were run for 25 days and samples were taken every other day starting from the second week. As in the biomass studies, a total of eight samples per tank were collected.

2.1.4. Diet study

Like the biomass study, we conducted four separate experiments in the diet study, each with a different combination of species (silver or bighead) and size class (juvenile or sub-adult). Each experiment had four treatments which were randomly assigned to tanks: no food, low feeding rate of algae (0.5% body weight of sub-adults; 12% bodyweight of juveniles), high feeding rate of algae (1% body weight of sub-adults; 24% body weight juveniles), and low feeding rate of brine shrimp (0.5% body weight sub-adults, 12% body weight juveniles). Each treatment included three replicates. Because these fish feed better in groups, three fish were placed in each tank. To ensure fish would eat their diet during the eDNA sampling portion of the experiment, fish were trained on their diet type (algae or brine shrimp) during the first week that fish were in the tanks. As in the previous studies, fish were maintained in the tanks for 25 days and samples were taken every other day starting from the second week.

2.2. Sample processing and qPCR analyses

2.2.1. Sampling

Fifty milliliter water samples were taken using either a clean serological pipette for experiments run in the 40 L aquaria, or using a tank-attached siphon to sample from the 379 L plastic tanks. Samples were taken below the water surface, 10–15 cm from the aquaria bottom. Water samples were then centrifuged for 30 min at 5000 RCF at 4 °C. Afterwards, the water was decanted off, and samples were left to dry for at least 10 min before adding 250 µl of the extraction TDS0 buffer (AutoGen Inc., Holliston, MA). Samples were kept frozen until extracted.

Before DNA extraction, samples were digested using proteinase K (AutoGen Inc. Holliston, MA) and left overnight in a 55 °C water bath. DNA was extracted with an AutoGen 245 system (AutoGen Inc. Holliston, MA), using a phenol chloroform extraction method according to the manufacturer's protocol, and resuspended in 50 µl nuclease-free water.

2.2.2. qPCR assay

Samples were run using the appropriate species' primer/probe set (Coulter et al., 2013). Primer/probe sets were initially tested using DNA extracted from both tissue and environmental (water) samples, and sequencing of amplification product verified their specificity. Each 20 µl reaction contained 375 nM of each primer (forward and reverse), 125 nM of the probe, 1X PCR mix (SsoFast™ Probe Supermix, BIO-RAD®), and 5 µl of DNA. Reactions were run on a CFX96 BIO-RAD® thermal cycler with the following: 2 min at 95 °C, 40 cycles of 5 s at 95 °C, 10 s at 58 °C. The standard curve was made from six ten-fold serial dilutions of a non-linearized plasmid that includes the target amplicon. The original plasmid concentration was quantified with a SPECTRAmax® 190 spectrophotometer (Molecular Devices, LLC) and copies/µL were calculated. Because our low concentration plasmid standards degraded within two days, we made new serial dilutions of our standard curve for each plate. All standards and samples were run in duplicate (preliminary study) or triplicate. Each qPCR run included wells containing no DNA template to test for contamination. The amplification efficiencies from all plates ranged between 88.4% and 107.7%. Quantity of eDNA estimates were converted from copies per reaction to copies per liter and then copies per hour given the water flow rate. The limit of quantification (LOQ) was 20 copies per reaction and was determined using a method adapted from Bustin et al. (2009). A standard curve dilution series with eleven replicates was run, and the LOQ was designated as the lowest standard dilution at which 90% of replicates amplified. Any samples in which DNA was detected below this threshold were assigned a quantity of half the LOQ, because quantification was not possible even though amplification of target DNA occurred.

PCR was inhibited in samples from the algae-fed treatments of the juvenile fish diet studies. No amplification was observed in these samples, even after spiking the samples with a known amount of DNA before running the reaction. Samples from the tanks of unfed fish and brine shrimp-fed fish were not inhibited. Samples from the algae-fed sub-adults, which had a lower percentage of food added to their tanks, were also not found to be inhibited using the same spiking test. We found that a 1:10 dilution of the inhibited samples (all samples from the algae-fed juvenile fish) allowed for recovery of the polymerase chain reaction and DNA quantification. Quantification from these diluted samples were then multiplied by ten to correct for the dilution factor.

Upon recognition that samples from the other assays could have been inhibited, a subset of twelve samples from each of the biomass and temperature studies were spiked with a known amount of DNA, and tested for inhibition. None of these samples showed signs of inhibition.

2.3. Statistical analysis

Because of the large variability observed in eDNA quantifications from the preliminary analyses, shedding rates per tank for the other experiments were calculated as averages of the quantification of the eight 50 ml samples from each tank. Box-plots of the qPCR quantifications of eight water samples per tank were used to identify outliers as points being 1.5 times the inter-quartile range of the data for each tank. Extreme outliers were then classified as those points that cause a 2-fold or higher change in the average. These outliers likely represent the heterogeneous distribution of eDNA containing particles (Pilliod et al., 2013). To reduce variability in the tank estimates of shedding rates (8 replicates samples), the extreme outliers were removed before calculating the average eDNA shedding rate for each tank. Results from data sets that include the outliers are also presented in supplemental materials (Tables A1, A2; Fig. A1). We ran Shapiro–Wilk tests to assess the distribution of the data, and found that data had a non-normal distribution unless log transformed. Mean eDNA shedding rates per tank were therefore $\log_{10}$ transformed to better fit the assumption of normality for subsequent statistical analyses. Linear regressions were then used to assess the relationship between eDNA shedding rate and continuous variables (fish biomass and water temperature). One-way analyses of variance (ANOVA) and subsequent pairwise tests with Bonferroni corrections were used to assess treatment differences in the diet study. Statistical significance was defined at $p = 0.05$.

Due to water pump failures during the diet experiments, the number of tank samples that could be used to estimate shedding rates, as described above, varied from the originally planned eight. The mean shedding rates for each tank were calculated from: seven samples for the bighead sub-adult diet experiment; six samples for the silver sub-adult diet experiment; five samples for the bighead juvenile diet experiment and three samples for the silver juvenile diet experiment. Furthermore, during the bighead sub-adult diet experiment, fish mortality led to the removal of one tank (replicate) from both the unfed treatment and the high feeding rate of algae treatment.

3. Results

3.1. Quantification of eDNA variability

Results from our preliminary analysis are presented as untransformed data, whereas data from all other experiments are $\log_{10}$ transformed. We found that the amount of eDNA shed is variable among samples taken from the same individual, and that 10–100 fold differences occurred through the six weeks of sampling (Fig. 1). Because of this variation, we sampled multiple times throughout our other experiments and used the mean of these tank samples as our estimate of eDNA shedding amount. Furthermore, we observed relatively higher amounts of eDNA the day that the fish were introduced, likely due to fish being stressed from handling. Therefore, we did not include samples from the first week in our mean eDNA shedding rates for all subsequent experiments. As might be expected the amount of eDNA quantified in the low water flow tank was higher than the amount quantified from tanks with a faster flow rate, as less DNA is being flushed out of the tanks (Fig. 1). However, after adjusting the amount of DNA for the flow rate, shedding rates are similar among tanks (or fish), indicating that fish shed similar rates under the same conditions (mean copies/h ± sd; 1 L/h tank: 50,000 ± 43,000; 2 L/h tank: 62,000 ± 61,000; 3 L/h tank: 42,000 ± 71,000 *not including data from the first week but including the removal of the outlier in the 1 L/h tank from the second week-see Fig. 1). The large standard deviations relative to the means, suggest that the untransformed data are not normally distributed and/or that outliers are having a strong effect on the mean. Therefore, data from subsequent studies were log transformed and extreme outliers were removed.

3.2. Biomass study

Data from the four biomass experiments were combined and a linear regression on transformed data showed that eDNA shedding rates increased with fish biomass (g) ($F = 468.4$, DF = (1,46), $p < 0.01$, $R^2 = 0.91$) (Fig. 2). Results of a linear regression using the data set that included all outliers were similar but had a slightly lower R^2 value ($F = 234.7$, DF = (1,46), $p < 0.01$, $R^2 = 0.84$) (Fig. A1).

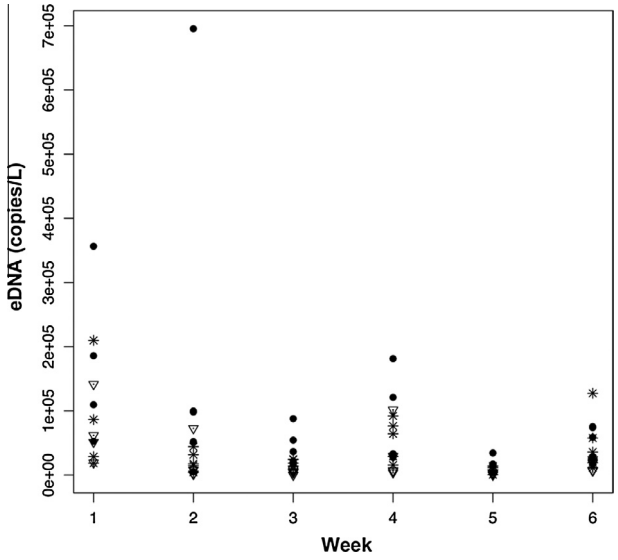

Fig. 1. Variation in eDNA concentration of water samples for individual silver carp juveniles over time. Closed circles- fish in the 1 L/h flow tank, asterisks-fish in the 2 L/h flow tank and upside down triangles, fish in the 3 L/h flow tank.

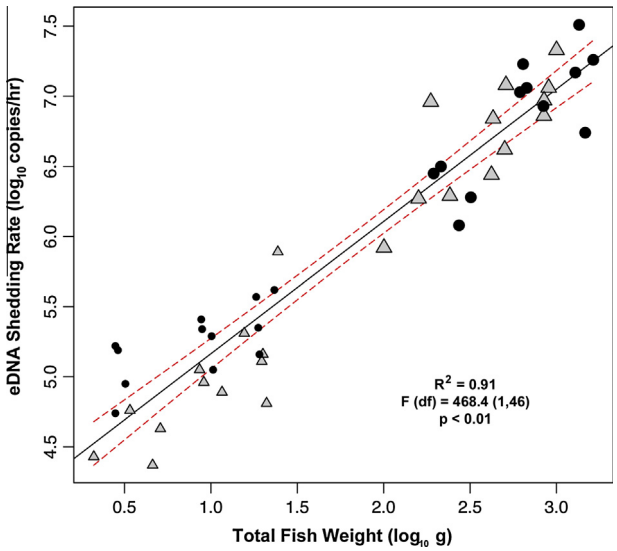

Fig. 2. Combined scatter plots of eDNA shedding rate against biomass of fish in tanks. (Bighead carp sub-adults – large, black circles; bighead carp juveniles –small, black circles; silver carp sub-adults – large, grey triangles; silver carp juveniles – small, grey triangles). Dashed line indicates 95% CI.

3.3. Temperature study

Shedding rate was not related to water temperature for sub-adult bighead carp ($F = 0.69$ DF = (1,7), $p = 0.43$, $R^2 = 0.09$) or sub-adult silver carp ($F = 0.49$, DF = (1,7), $p = 0.51$, $R^2 = 0.07$), and inclusion of outliers did not affect the outcome (Table A1). Mean transformed shedding rates ranged from 5.78 to 6.01 $\log_{10}$ copies of DNA/h for sub-adult bighead carp, and from 5.87 to 6.47 $\log_{10}$ copies of DNA/h for sub-adult silver carp, regardless of water temperature (Fig. 3).

3.4. Diet study

The difference between fed and unfed fish was approximately a 10-fold increase in mean shedding rates among silver sub-adults and bighead juveniles (Fig. 4). Similarly, bighead sub-adults and silver juveniles had shedding rate increases between non-fed and algae-fed treatments, but not to the same degree (Table 1).

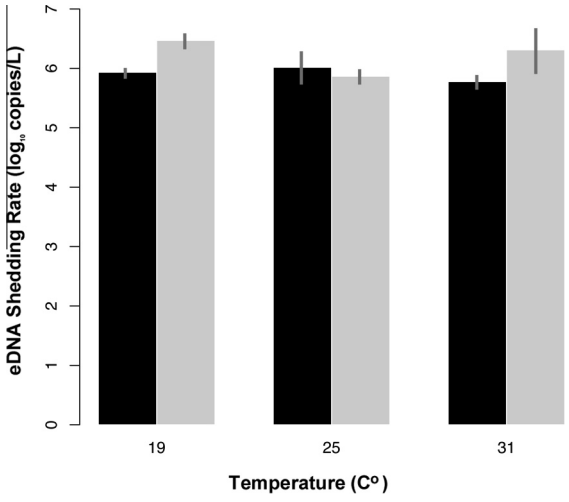

Fig. 3. Mean log shedding rates and standard deviations for bighead carp sub-adults (black bars) and silver carp sub-adults (grey bars) across three temperature treatments.

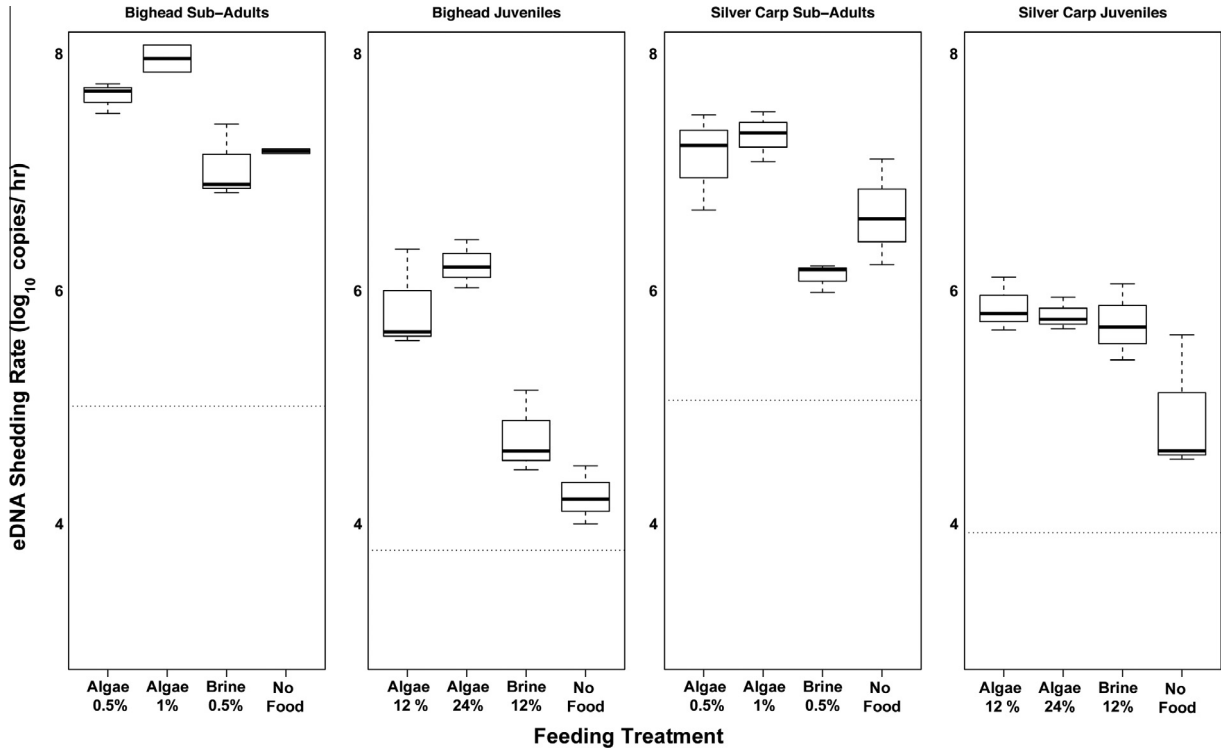

Fig. 4. Box plots showing the median, 25th and 75th quartiles for log transformed eDNA shedding rate among different diet treatments for all four diet experiments. Whiskers indicate 1.5 times the interquartile range. Percent weight gain or loss and average starting weights are shown in Table 2. Dashed line represents the limit of quantification adjusted for flow rate.

Brine-fed fish generally had shedding rates similar to the unfed fish, except for silver carp juveniles.

We found statistically significant differences among treatments in all four experiments ($p \leqslant 0.05$; Table 1). Pairwise *t*-tests indicate that for silver sub-adults and bighead juveniles, algae-fed groups were significantly different from brine shrimp-fed and unfed groups. No pairwise differences were significant among the sub-adult bighead carp treatments nor among the juvenile silver carp treatments; however, unfed fish shed less eDNA relative to fish from the algae-fed treatments.

4. Discussion

4.1. Quantification of eDNA variability

We found that quantification of eDNA samples can be highly variable even when sampling from the same individual under controlled conditions. Similarly, Pilliod et al. (2014) detected high variability of shed eDNA among salamanders in lab experiments. We believe this variability could be due to the heterogeneous nature of eDNA as masses of tissue, cells or fecal debris that contain high amounts of DNA are not evenly dispersed in the environment. This is consistent with the findings of Turner et al. (2014) which found a highly skewed distribution of eDNA concentrations as well as a heterogeneous distribution in the size of eDNA containing particles. Nevertheless our preliminary study showed that similarly-sized fish shed eDNA at similar rates under the same conditions, suggesting that we should be able to detect differences in shedding rates caused by different conditions. Furthermore, averaging the quantification estimates of many samples may minimize the effects of inherent sampling variability, and reduce the effect of outliers that occur simply due to the clumped nature of eDNA. Pilliod et al. (2013) suggest that taking multiple replicates of the same sampling unit and/or removing outliers will improve the precision of eDNA quantification.

4.2. Biomass study

As we hypothesized, the amount of eDNA (or eDNA shedding rate) increased linearly with fish biomass (both variables being log transformed). Such a relationship has been demonstrated in other organisms as well (Takahara et al., 2012; Thomsen et al., 2012b; Goldberg et al., 2013; Pilliod et al., 2013), suggesting that quantification of eDNA can be used to estimate a targeted organism's abundance or density in the field. However, field experiments will be necessary to confirm this relationship and assess factors that may confound it (e.g., Pilliod et al., 2013; Spear et al., 2015). Our work extends previous studies, by estimating actual eDNA shedding rates relative to fish biomass. Estimating shedding rate per gram of fish may be useful for future modeling of eDNA distributions in natural settings.

4.3. Temperature study

A factor that could confound the relationship between fish biomass and eDNA amount in field samples might be water temperature. We hypothesized that warmer water would lead to more active fish which we predicted would lead to increased shedding of DNA. However, we found no relationship between water temperature and shedding rate. This is analogous to the findings of Takahara et al. (2012) in a similar study using common carp. In both studies, samples from tanks with different water temperatures did not have significantly different amounts of eDNA. Interestingly, in field collected samples Takahara et al. (2012) detected more eDNA in samples from warmer stretches of water than in samples taken from cooler water. They suggested that that carp prefer to congregate in warmer waters, thus resulting in a

Table 1
ANOVA and post hoc pairwise comparison (with Bonferroni correction) statistics for each of the four diet experiments.

	Bighead carp sub-adult	Bighead carp juvenile	Silver carp sub-adult	Silver carp juvenile
Number of subsamples	7.00	5.00	6.00	3.00
Number of treatments[a]	4	4	4	4
ANOVA				
F (degrees of freedom)	6.28 (3,8)	24.74 (3,8)	13.83 (3,8)	4.18 (3,8)
p	0.03[b]	<0.01[b]	<0.01[b]	0.05[b]
Post-hoc pairwise comparisons				
p				
High algae v low algae	1.00	1.00	1.00	1.00
High algae v low brine shrimp	0.08	<0.01[b]	0.01[b]	1.00
High algae v no food	0.14	<0.01[b]	0.01[b]	0.13
Low algae v low brine shrimp	0.14	0.02[b]	0.01[b]	01.00
Low algae v no food	0.28	<0.01[b]	0.01[b]	0.09
Low brine shrimp v no food	1.00	0.55	1.00	0.18

[a] Three replicates per treatment; except for the bighead carp sub-adult study which had 3 replicates for the low algae and low brine shrimp treatments but only 2 replicates for both the no food and the high algae treatments.
[b] Significance set at $p = 0.05$.

stronger eDNA signal from such locations. So although water temperature does not appear to directly affect the shedding rate, it may dictate where fish are likely to be found in the field, and subsequently, where more eDNA will be collected in the field.

4.4. Diet study

Finally, we hypothesized that feeding should increase the amount of eDNA that a fish sheds due to increased metabolism and excretion. Overall, fish fed the soft, algae diets shed one order of magnitude more eDNA than non-fed fish which is consistent with our hypothesis that gut cells shed via feces are a major source of eDNA. Because the size of the fish will also contribute to the amount of eDNA that is shed, we tried to use fish of similar length and weight in each treatment. Despite our efforts to minimize the impact of this potentially confounding factor, size differences among treatments did exist (Table 2). However, sub-adult fish in the algae-fed treatments were smaller in size relative to those in the unfed and brine shrimp-fed treatments, yet they shed more eDNA. This suggests that the increase in shed eDNA was likely attributed to more excrement and sloughed gut cells because of higher food intake.

We also hypothesized that rough crustacean food would lead to more sloughed off gut cells and thus higher shedding rates; as the crustacean exoskeletons would be more abrasive on the digestive tract compared to the softer algae diet. However, the brine shrimp-fed treatments generally had low shedding rates more similar to the non-fed treatments than to the algae-fed fish. It is possible that the fish fed the brine shrimp diet did not feed as much relative to fish that were fed the algae diet. Brine shrimp may have been successful in evading capture by these filter-feeding fishes. Kolar et al. (2007) noted that zooplankton evasion of bigheaded carp feeding influences carp diets. Furthermore, unlike the algae food which stayed in the water column until filtered out of the tank or eaten, the brine shrimp only stayed in the water column for 1–2 h before dying from hyposalinity stress and dropping to the bottom where they may have been less fed upon by the carp. Although amount of feces was not quantified, the unfed tanks were observed to have little to no feces, and the brine tanks had some feces but less than the algae treatment tanks, suggesting that the fish in these tanks were not feeding as much. The lower shedding rates generally observed in the brine shrimp-fed treatments is likely a result of lower feeding rates due to limited consumption of the crustacean food. Additionally, it should be noted that bigheaded carps sometimes exhibit reduced gut length when feeding on zooplankton diet, and thus may shed less eDNA because less cellular material is shed from a smaller gut. Ke et al. (2008) found that silver carp fed less nutritious phytoplankton (algae), which requires more digestion time, had longer gut lengths than when fish had a mixed diet of zooplankton and phytoplankton. Such environmentally induced phenotypic plasticity in gut length has also been reported in perch (Olsson et al., 2007). Because we did not measure gut length after the experiment, we cannot draw conclusions about this potential factor from this study.

4.5. Removal of outliers

Results between data sets that removed the extreme outliers and those that retained all data points did not cause a change in overall conclusions for each study. In the biomass experiments the data set with outliers removed did have a higher R^2 value compared to that from the data set that included outliers (Figs. A1 and 2). In the temperature studies, neither analysis indicated any relationship between temperature and shedding rates. Finally in the diet study, results did not differ to a large degree between the data sets. However, in the silver carp sub-adult study the significant pairwise difference between the algae-fed fish and non-fed fish disappeared when including outliers (Table A2). We believe that

Table 2
The average percent body weight gain or loss of fish during the diet studies, and the average initial starting weights.

	Bighead sub-adult				Bighead juvenile				Silver sub-adult				Silver juvenile			
Diet	0.5% Algae	1.0% Algae	0.5% Brine	No Food	12% Algae	24% Algae	12% Brine	No Food	0.5% Algae	1.0% Algae	0.5% Brine	No Food	12% Algae	24% Algae	12% Brine	No Food
% Weight gain/loss	0.00	−0.04	+0.01	−0.04	+0.02	+0.10	+0.06	−0.16	−0.01	+0.01	−0.03	−0.04	−0.03	−0.05	+0.01	−0.16
Average starting weight (g)	146.0	177.0	201.0	238.0	3.6	4.0	3.7	3.9	147.0	140.0	153.0	153.0	4.0	3.7	3.6	4.0

the extreme outliers are likely due to the heterogeneous nature of eDNA in the water and may have resulted from sampling of clumps of fecal material or tissue. We feel that these extreme outliers are not representative of the average shedding rates, and that they may mask underlying patterns. For instance, in the preliminary study eDNA quantification estimates were higher during the first week and a half of sampling, but afterwards, shedding amount leveled off at lower concentrations. We hypothesize that this spike in eDNA at the beginning may be due to stress from handling and a new environment, leading to higher eDNA production. If we only used samples from this first week, our estimated shedding rates would likely have been higher.

4.6. False negatives and PCR inhibition

During the juvenile fish diet studies we detected the presence of PCR inhibitors in all of our algae-fed samples, likely due to PCR inhibitory compounds in the algae. Although the identity of the exact inhibitory compounds is unknown, we only had evidence for PCR inhibition from samples taken during the juvenile diet studies. Later studies indicated what algae concentration levels led to PCR inhibition, and these were concordant with the algae concentrations in the juvenile diet algae-fed treatments. We mitigated the effects of these inhibitors by diluting our samples, and we were able to recover quantification estimates for those samples. Nevertheless, our experience exemplifies the importance of testing samples for PCR inhibition, because inhibition leads to false negatives or lower quantifications. Regarding monitoring efforts for these two invasive carp species, the possibility of PCR inhibition in field collected samples is potentially high, because these fish are planktivorous and likely will select areas of high phytoplankton density.

Monitoring efforts that use eDNA as a species detection tool should incorporate an internal amplification control in the PCR to identify sample inhibition (see Hoorfar et al., 2004 for a review of internal amplification control methods). Kinetic outlier detection methods are also being developed to identify sample inhibition (Bar et al., 2012). To alleviate inhibition effects, dilutions and further purification of samples allow for recovery of the PCR, but both of these options also lead to a loss of sensitivity. Given the detrimental impact that false negatives might have on field surveying efforts, proper identification and treatment of inhibited samples is important.

4.7. Conclusion

Although PCR is theoretically very powerful, the limit of quantification for any PCR assay (qPCR or conventional) will affect the interpretation of field eDNA samples. Most of our samples from the laboratory studies were above the PCR assay's limit of quantification (LOQ), and thus eDNA amounts were accurately quantified. Low fish densities that would lead to samples with signal below this assay's LOQ will occur in the field, especially when sampling at the invasion front which generally has fewer individuals (Travis and Dytham, 2002; Pilliod et al., 2014). Thus error in detection and quantification of small amounts of DNA via PCR (quantitative or conventional) may need to be addressed for field monitoring programs. Although our study did not assess variability in eDNA concentrations at a spatial scale, Pilliod et al. (2013) found evidence that concentrations did not vary even between sites separated by 450 meters for stream dwelling amphibians. Understanding the relationship between eDNA quantification and distance from the source are important in determining the spatial volume in which eDNA sample data can be interpreted, and should be measured for any species that is being monitored with this method.

In conclusion, our laboratory studies are an important step in understanding how eDNA detection relates to an organism's presence and abundance by measuring how certain factors influence the shedding of eDNA by the invasive bighead and silver carp. Further steps in understanding this relationship include studying degradation and particle movement of eDNA. Several studies have measured relatively rapid degradation, with no eDNA detection within four weeks in a non-flowing system (Dejean et al., 2011; Thomsen et al., 2012a; Barnes et al., 2014). In flowing systems, detection of eDNA will also be affected by eDNA movement. Pilliod et al. (2014) found no eDNA signal one hour after removal of the target organism from a lotic system. Thus, the dynamics of eDNA production, persistence and movement will differ by system and species, emphasizing the importance of running species specific studies to better inform the interpretation of field monitoring. Overall, eDNA holds promise for species monitoring programs, but further research is needed to fully understand the relationship between an organism and the detection and quantification of the eDNA that it releases.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.11.020.

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