ECALS: Loading Studies Interim Report October 2013

ACRCC Framework Item: 2.6.3
ECALS Project Management Plan Tasks: 3.2.1, 3.2.3 and 3.2.4
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Objectives:

Understand how diet affects the eDNA shedding rate of bigheaded carps

Introduction:

Here we follow up the loading studies interim report from July 2013 and include results from laboratory studies assessing the effects of diet on eDNA shedding rates by bigheaded carps (silver and bighead carp). In order to understand how eDNA behaves in the environment, we must understand how it enters the system. In our July interim report, we addressed three of our four hypotheses that could influence the shedding rate of eDNA by these fish (Table 1; hypotheses A, B and D). We now provide results from studies that tested the fourth hypothesis (C), **cellular debris from the gut-lining shed via excrement is a major source of shed eDNA.**

Methods:

A. Experimental set up
    a. Juvenile fish (60-100mm) were placed in 40 L glass aquaria and sub-adult fish (100-300mm) were housed in 379 L plastic, round tanks. The small tanks were set at a flow rate of 2 L / Hr and large tanks at 19 L/ Hr.
    b. For the diet study, four experiments were run: silver sub-adults, silver juveniles, bighead sub-adults, and bighead juveniles. Each experiment had four treatments: no food, low feeding rate of algae (soft food), high feeding rate of algae (soft food), and low feeding rate of brine shrimp (rough crustacean food). No high feeding rate of brine shrimp diet was used due to space limitations. Each treatment had three replicates. Three fish were placed in each tank. Daily feeding amounts were calculated as a percent of the average fish body mass.

Feeding rate percentages differed between the sub-adult and juvenile tests (see x-axis Figure 1). Fish were trained on their diet type (algae or shrimp) before the experiment began. Studies were run from October 2012 through August 2013. Each study ran approximately four weeks.

B. qPCR

a. Sample processing:

i. Water samples (50 ml) were taken every other day for 2.5 weeks in duplicate using either a clean serological pipette for experiments run in the 40 L aquaria, or using a siphon to sample from the 379 L plastic aquaria. All samples were taken below the surface but not from the bottom.

ii. Samples were then centrifuged for 30 minutes at 5000 RCF at 4°C. Afterwards, the water was decanted off, and samples were left to dry for at least 10 minutes before adding 250 ul of the extraction TDS0 buffer (AutoGen Inc., Holliston, MA). Samples were then frozen until extracted.

iii. Samples were digested using Proteinase K (AutoGen Inc. Holliston, MA) and left overnight in 55°C water bath.

iv. Samples were extracted with an AutoGen245 (AutoGen Inc. Holliston, MA) automated robot, using a phenol chloroform extraction method.

b. qPCR assay: Samples from the second to fourth experimental week were then run using the appropriate species' primer/ probe set designed by USGS Upper Midwest Environmental Science Center (UMESC) (Table 2). Samples were run in triplicate and each plate included a standard curve. Quantifications of eDNA were converted from copies per reaction to copies per liter (eDNA amount) or copies per hour (eDNA shedding rate).

C. Analysis

Average shedding rates determined from previous experiments (effects of temperature and biomass) were calculated from 8 subsamples per tank over the 2.5 week period. However, due to equipment complications during the diet experiments, the number of subsamples per experiment varied. Averaged

shedding rates for each tank were calculated from: 7 subsamples for bighead sub-adults; 6 subsamples for silver sub-adults; 5 subsamples for bighead juveniles and 3 subsamples for silver juveniles. In the bighead sub-adult test, fish mortality led to the removal of one tank (replicate) for both the unfed treatment and the high feeding rate of algae treatment.

Box plots of the subsamples per tank were used to identify extreme outliers in the dataset. Outliers were defined as points 1.5 times the inter-quartile range of the data. Extreme outliers were classified as those that cause a 2-fold or higher change in the mean. Extreme outliers were removed from data sets and the means for each treatment were calculated. Data (average eDNA shedding rates per tank) were log transformed to fit the assumption of normality. ANOVAs and subsequent pairwise tests with Bonferroni corrections were used to look for statistically significant treatment differences. Statistical significance was defined at $p = 0.05$.

We also looked at the average percentage of body weight gained or lost in each treatment. Figure 1 shows these weight gain/loss percentages above the average weights in the box plot.

Results:

Preliminary analyses revealed that the polymerase chain reaction was inhibited in samples from the tanks with the algae-fed juvenile fish. 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. The algae-fed sub-adults had a lower percentage of food added to their tanks, and subsequently, we detected no inhibition in these samples. 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. The quantification from these diluted samples was then multiplied by 10 to correct for the dilution factor.

As shown in Figure 1, unfed fish still shed DNA into the water; however, fed fish generally had higher eDNA shedding rates, particularly the fish fed an algae diet. The difference between fed and unfed fish was approximately a 10-fold increase or higher in average DNA shedding rates among silver sub-adults and bighead juveniles. 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 3). Brine-fed fish generally had shedding rates similar to the unfed fish, except for silver juveniles.

We found statistically significant differences among treatments in all four experiments ($p \leq 0.05$; Table 4). 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. For the sub-adult bighead carp no pairwise differences were significant; however, results followed a similar trend to results from the bighead juveniles and silver sub-adult experiments in that the brine shrimp-fed or unfed treatments shed less eDNA relative fish from the algae-fed treatments. Finally, although the ANOVA results showed significant ($p= 0.05$) differences for the juvenile silver carp experiment, none of the post-hoc pairwise comparisons were significantly different at the $p = 0.05$ level. Unlike the previous three experiments the brine shrimp-fed treatment and the algae fed-treatments were not different from one another ($p = 1.00$). Unfed silver carp juveniles shed less DNA that fed fish, however differences were not statistically significant.

Discussion:

Overall, fish fed the soft, algae diets shed more DNA than unfed fish. In general, fed fish shed about one order of magnitude more DNA than non-fed fish (Figure 1). We accept our hypothesis that gut cells shed via feces is a major source of shed DNA. Non-fed fish shed detectable amounts; however, feeding leads to higher shedding rates, and statistically higher rates in two of the experiments (silver sub-adults and juvenile bigheads).

Could shedding rate differences be due to size of the fish rather than the actual differences in diet? As previously shown, greater biomass (more fish or larger fish) leads to a greater amount of detectable DNA (previous July Interim report; Takahara et al. 2012). We tried to use fish of similar length and weight in each treatment; however, differences did exist. If fish in the non-fed and brine shrimp-fed treatments were smaller in size, they would be expected to shed less due to this size difference. However, figure 1 shows that in the sub-adult experiments, fish in the unfed and brine shrimp-fed treatments had higher average weights than either of the algae-fed treatments, yet shed less than the smaller fish from the algae-fed treatments. This suggests that the increase in shed DNA was likely attributed to an increase in excrement and sloughed off cells from the gut. Similarly, in the juvenile studies, the average initial weight of the low algae and no food treatments were similar, and yet the algae-fed treatments still had higher shedding rates. Size differences among treatments may also have contributed to a lack of statistical significance in the bighead sub-adult experiment. Although the algae-fed fish shed at higher rates, there was not a significant difference with the larger unfed and brine-shrimp fed fish. The large size of the unfed and brine shrimp fed fish may have resulted in higher shedding rates due to greater body mass, masking effects of diet. In fact, size difference of fish among treatments is greatest in the bighead sub-adult experiment relative to the other three experiments.

We hypothesized that rough crustacean food may 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. We do not have evidence to support this; and in fact, the brine shrimp-fed treatments generally had lower average shedding rates more similar to the non-fed treatments. It is possible that the brine shrimp food was less available to the fish than the algae food, leading to less excretion. Unlike the algae food which stayed in the water column until filtered out or eaten, the brine shrimp only stayed in the water column for a couple of hours before dying and dropping to the bottom. Although amount of feces was not recorded, 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. The lower shedding rates generally observed in the brine shrimp-fed treatments is likely a result of lower feeding rates due to limited availability of the crustacean food. Alternatively, silver carp may have a reduced gut length when on a zooplankton diet relative to when eating less nutritious phytoplankton (algae), which requires more digestion and thus a longer gut. Such environmentally induced phenotypic plasticity in gut length has been reported in perch (Olsson et al., 2007) and in silver carp (Ke et al., 2008). Because we did not measure gut length after the experiment, we cannot draw conclusions about this potential factor from this study.

Summary:

Fish do shed DNA at higher rates when fed, likely due to cells sloughed off in the excrement. Non-fed fish still shed detectable amounts of DNA but at approximately 10-fold lower rates compared to the fed fish (especially those fed algae). Non-fed silver carp juveniles and bighead sub-adults shed lower amounts of eDNA relative to algae-fed fish, but differences were not statistically significant. Lack of significance may be due to the fewer replicates for each treatments that we had in the silver juvenile experiment (n = 3). For the bighead sub-adults, shedding rates trended similarly to the bighead juveniles and sub-adult silvers, but did not show significant pairwise differences in shedding rates. This may be due in part to the unequal average size of the fish among treatments, with unfed and brine shrimp fed fish being larger than the algae-fed fish. The lower shedding rates found in most of the brine shrimp-fed treatments is likely due to the limited availability of the food to the fish during these experiments; however, the potential for gut length change dependent on diet type may also have an influence.

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Literature Cited

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Olsson, J., Quevedo, M., Colson, C., and Svanbäck, R. 2007. Gut length plasticity in perch: into the bowels of resource polymorphism. Biological Journal of Linnean Society. 90: 517-523.

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Table1. Tested hypotheses of the ECALS eDNA loading studies

Factors Influencing DNA shedding rates of Bigheaded carps

Hypothesis A: DNA shedding rate will increase with increasing fish density.

Results: We observed a positive correlation between eDNA loading and fish density (# fish/ tank).

Hypothesis B: Fish in warmer water may be more active and thus shed more DNA compared to fish in cooler water.

Results: We found no correlation between water temperature and eDNA shedding rates.

Hypotheses C: Sloughing of cells from the gut lining is a major source of shed eDNA, thus fish fed more food will also shed more eDNA.

Results: In this report

Hypothesis D: Spawning events can lead to a strong eDNA signal due to the high amounts of gametes released.

Results: We quantified the amount of eDNA in water samples given a known amount of sperm that had been added to the water. The peak loading (amount of eDNA) was detected one to two days after the initial addition of sperm. After 4 days, 99% of the eDNA quantified from the first day was undetectable. Some eDNA (< 1%) however, was still detectable until day 21of the experiment.

Table 2. Primer set and Probes used for the qPCR analyses.

Primers	Species	Region	Forward	Reverse	Probe	*Annealing Temperature (°C)	Amplicon Length (bp)
UMESC	SC	D-loop	GGTGGCGCAGAATGAACTA	TCACATCATTTAACCAGATGCC	CCATGTCCGTGAGATTCCAAGCC	58.0	108
	BH	D-loop	GGTGGCGCAAAATGAACTAT	GCAAGGTGAAAGGAAACCAA	CCCCACATGCCGAGCATTCT	58.0	190

SC-Silver Carp
BH-Bighead Carp
*Annealing Temperature according to Thermal Gradient qPCR

Table 3. Average log 10 eDNA shedding rates of each treatment, standard deviation in parentheses.

	Silver Carp Sub-Adult	**Silver Carp Juvenile**	**Bighead Carp Sub-Adult**	**Bighead Carp Juvenile**
High Algae	7.13 (0.20)	5.80 (0.14)	7.98 (0.16)	6.23 (0.21)
Low Algae	7.14 (0.41)	5.87 (0.23)	7.78 (0.32)	5.87 (0.43)
Low Brine Shrimp	6.03 (0.30)	5.73 (0.32)	7.14 (0.29)	4.77(0.35)
No Food	6.10 (0.19)	4.96 (0.59)	7.19 (0.02)	4.27(0.25)

Table 4. ANOVA and post-hoc pairwise comparison (with Bonferonni correction) statistics for each of the four diet experiments.

	Silver Carp Sub-Adult	Silver Carp Juvenile	Bighead Carp Sub-Adult	Bighead Carp Juvenile
number of subsamples	6.00	3.00	7.00	5.00
number of treatments ǂ	4.00	4.00	4.00	4.00
ANOVA				
F (degrees of freedom)	13.83 (3,8)	4.18(3,8)	6.28 (3,8)	24.74 (3,8)
p	< 0.01*	0.05*	0.03*	< 0.01*
Post-Hoc Pairwise Comparisons				
High Algae v Low Algae	1.00	1.00	1.00	1.00
High Algae v Low Brine Shrimp	0.01*	1.00	0.08	<0.01*
High Algae v No Food	0.01*	0.13	0.14	< 0.01*
Low Algae v Low Brine Shrimp	0.01*	01.00	0.14	0.02*
Low Algae V No Food	0.01*	0.09	0.28	<0.01*
Low Brine Shrimp v No Food	1.00	0.18	1.00	0.55

ǂthree 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.

* Significant (p =0.05)

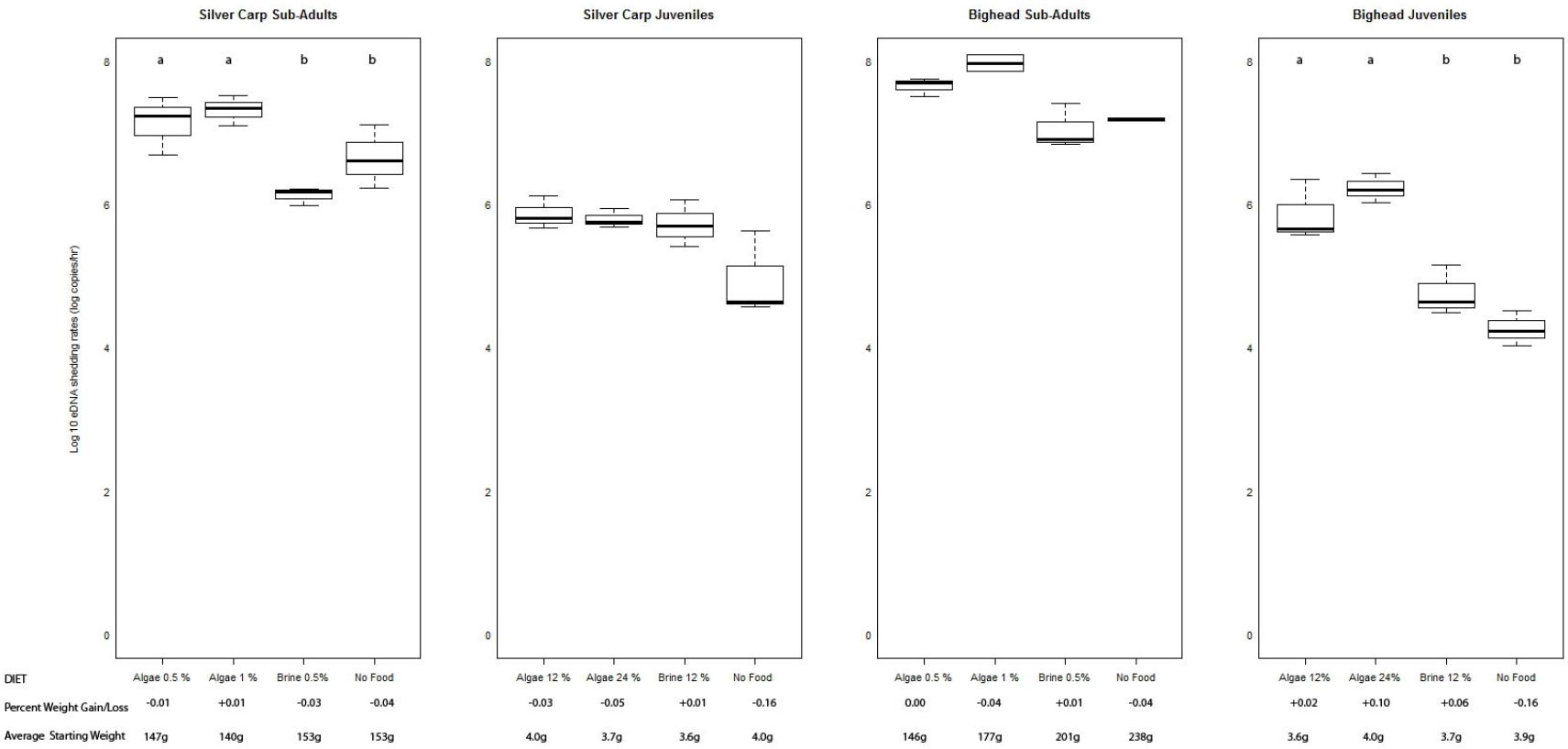

Figure 1. Box plots of treatment (Diet) averages for eDNA shedding rates in four experiments. Percent weight gain or loss and average starting weights are also shown below each plot.