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Dear Sharon Adams:

We have made substantial progress on our NRAC minigrant titled “Aquaculture Biotechnology for the Enhancement of Live Feed Production.” The goals of the proposed research have not changed from those outlined in the original submission. In summary, the goals of the project are 1) to develop optimal transfection conditions for introducing CRISPR-Cas12a into rotifers for genetic modification and 2) to generate a repair template that expresses eGFP under the *B. plicatilis* GAPDH promoter.

We chose to target CRISPR-Cas12a to the *B. plicatilis* IGFR1 locus, as the IGFR1 gene has been shown to be non-essential in other invertebrate animals and knockout is demonstrated to increase life span in other model organisms, including the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. In order to investigate whether known longevity-promoting factors are conserved in marine rotifers, we chose to test the effect of 5 μM rapamycin on the life span of *B. plicatilis* (**Figure 1**). We found a robust extension of the median life span (defined as the age at which 50% of the population is still alive) when treated with 5 μM rapamycin. In WT cultures, the median life span was ~7.5 days, while cultures treated with rapamycin exhibited a median life span of ~12.5 days (a 40% increase in median life span). This concentration of rapamycin, however, did not extend the maximal life span of rotifers (15 days in both experiments).

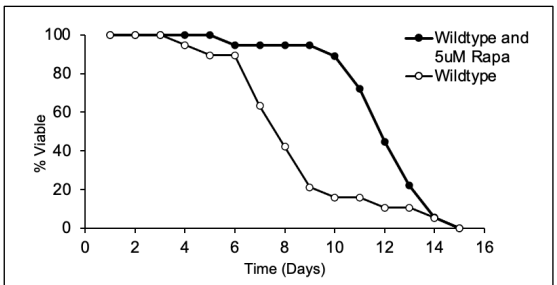

Figure 1. 5μM rapamycin extends the median life span of *B. plicatilis* by 40%.

gRNA Sequence	Position
TTTAGTCTCACGACTCTACCATGCC	+456
TTTAAATAACAACGAGTGTTTGAC	+543
TTTGACAGATTGTACCCAAAATTGC	+561

Table 1. Design of 3 Cas12a spacer sequences to target *B. plicatilis* IGFR1 gene. The PAM sequence in orange is not part of gRNA design. Position is in reference to the start codon.

These data suggest that knockout of the IGFR1 locus could also extend the *B. plicatilis* life span. With regards to Goal 1, we have developed 3 guide RNAs which target the CRISPR-Cas12a endonuclease for digestion at three discrete regions in the *B. plicatilis* IGFR1 gene (**Table 1**). Each of these guides were independently tested for their activity to create a double-strand DNA break *in vitro* using a 518bp PCR amplicon as template (**Figure 2**). Incubation of the PCR product with Cas12a alone produced no cleavage products (**Figure 2**, -gRNA lane). However, when the PCR product was treated with Cas12a pre-incubated with each of the three designed gRNAs, expected cleavage products were observed (**Figure 2**).

Our *in vitro* data suggest that gRNA 456 results in the most complete digestion of the full-length PCR product (**Figure 2**). We are now testing optimal delivery strategies, including both electroporation and lipofectamine. We have determined that we can recover live rotifers from amictic eggs following electroporation at 200V using electroporation media (10% glycerol), as well as lipofectamine in 4ppt artificial seawater. Our protocol for egg harvest yields approximately 100-400 amictic eggs per harvest. Since verifying that we can recover live animals from eggs treated with our electroporation and lipofectamine protocols, we have used GFP mRNA as a reporter for positive transfection but have yet to observe a GFP signal from our experiments. We are still in the process of developing robust transfection strategies, and plan to explore transfection with both resting cysts and amictic eggs.

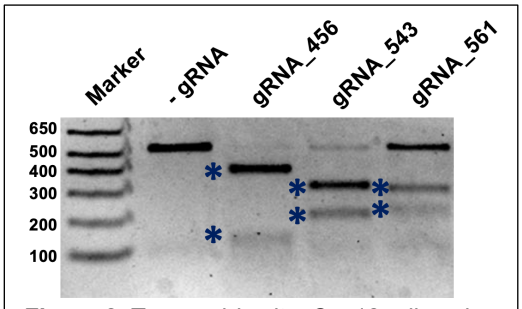

Figure 2. Targeted *in vitro* Cas12a digestion of a 518bp PCR product of the *B. plicatilis* IGFR1 locus. -gRNA, no gRNA control. Asterisks mark expected cleavage products.

Additionally, for Goal #2, we are currently developing our repair template for CRISPR-Cas12a homologous recombination. The repair template is being constructed in a plasmid and will contain 1) 500bp of homologous sequence upstream of Cas12a cut site in IGFR1; 2) the *B. plicatilis* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter (1000bp of genomic sequence upstream of the GAPDH start site); 3) the coding sequence for eGFP; 4) the GAPDH terminator sequence (500bp downstream of the GAPDH stop codon); and 5) 500bp of homologous sequence downstream of the Cas12a cut site of IGFR1 (**Figure 3**). This repair template can be amplified by PCR for co-transfection along with the Cas12a-gRNA complex. Successful transformants will be GFP-positive and can be isolated by pipetting from a culture under a fluorescence stereoscope.

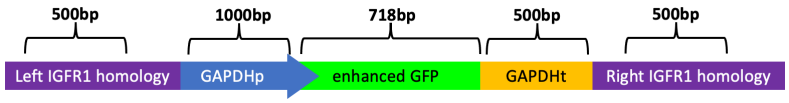

Figure 3. Repair template to incorporate a GFP gene into the IGFR1 cut site in *B. plicatilis*. The left IGFR1 homology, GAPDH promoter, and eGFP have been successfully subcloned. The GAPDH terminator and right IGFR1 homology arm remain to be cloned into the repair template.

Sincerely,

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