FIELD-TESTING OF A RAPID LAMP ASSAY TO DETECT THE MARINE PARASITE *AMYLOODINIUM OCELLATUM* IN COMMERICAL AQUACULTURE FACILITIES

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OBJECTIVES:

1) Evaluate the newly developed LAMP assay in commercial aquaculture facilities, assessing:
   a. time to diagnosis,
   b. sensitivity as compared to standard microscopic evaluation,
   c. cost,
   d. disease incidence/production outcomes, and
   e. false positives and negatives using positive and negative controls.
2) Develop a standard operating procedure (SOP) for *A. ocellatum* surveillance utilizing the LAMP assay and incorporating findings from Objective 1.

ANTICIPATED BENEFITS:

The dinoflagellate *Amyloodinium ocellatum* is a major constraint in warm water marine fish culture because it rapidly produces large numbers of infectious stages and is difficult to detect prior to the onset of morbidity. Current, farm-level diagnostics rely on microscopic identification of adult trophonts on the gills and skin. However, microscopic diagnosis is most likely to be accomplished when the trophonts are numerous or at the onset of morbidity, at which point options for treatment and control are limited. Field-testing of a LAMP assay that could detect single parasites in water or fish tissue, and thereby provide more sensitive and earlier diagnosis, will transition this technology to commercial application and provide enhanced opportunities for improving commercial outcomes.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:

Objective 1. Evaluate the developed LAMP assay in commercial aquaculture facilities
Subobjective 1a. Evaluate the LAMP assay in commercial aquaculture facilities, assessing time to diagnosis
Auburn University, USM, and University of Florida

Because of *Amyloodinium ocellatum's* (AO) ability to produce large numbers of dinospores, each of which can attach and transform into a trophont, the ability to detect the parasite before lethal loads of trophonts develop is critical to achieving control of the parasite. Microscopic examination of wet-mounted gill tissue is the standard diagnostic technique. However, microscopic techniques can detect only trophonts, typically once they are numerous. Also, trophonts may superficially resemble other fish parasites such that false positives are recorded. The LAMP assay markedly reduces the likelihood of false positives, eliminates the subjectivity associated with parasite morphological identifications, and potentially reduces the time to a confirmed diagnosis thereby providing a treatment advantage.

We partnered with two clownfish farms in central Florida (Oceans, Reefs, and Aquariums (ORA), Ft. Pierce, FL and Proaquatix, Vero Beach, FL) to obtain samples from their production facilities which periodically experience outbreaks. Both facilities produce numerous species including clownfish for the aquarium hobby industry and operate multiple recirculating systems that may range in size from individual tanks on flow through systems to large multiple tank recirculating aquaculture systems (RAS) for grow-out. The University of Florida representative met with staff from both facilities several times over the course of the study to discuss concerns related to AO, current facility-specific methods for control, diagnostic protocols and treatment limitations, and treatment effectiveness. The staff at both facilities was familiarized with project details, and production history and data needed for the study were discussed. Sample collection schema and shipping protocols were coordinated. Farm visits for investigators from USM and Auburn were coordinated to help familiarize producers with the other members of the research team and allow the other members to better understand general disease and production concerns of the producers.

Water samples were collected from the two systems at each farm prior to stocking to ensure the absence of the parasite. Following stocking, water samples and fish were collected from at least one system at each farm up to twice per week for 12 weeks. Water and fish samples were shipped fresh and live to Auburn University. Water samples were filtered through a 200 µm mesh followed by a 125 µm mesh and then a 3-µm pore size Nucleopore filter (Nalgene Nunc International, Rochester, NY, USA). Filters were air dried and stored in Allprotect for future processing. Fish were killed using an overdose of MS-222. One dextral and one sinistral gill arch (or a 2-mm section of said gill arches) from each fish was extracted, wet-mounted on a microscope slide, and scanned in its entirety using first the 4x objective of a compound microscope, then the 10x, and finally the 40x objective. From the remaining gill tissue from each fish, a haphazard sample of approximately 100 mg of tissue was collected and stored in RNALater tissue reagent (Qiagen) at room temperature for eventual homogenization and suspension in lysis buffer for analysis. An additional gill arch (or 2-mm section) was fixed in 10% formalin for archival purposes. Additional samples of the parasite were used for studies on freeze tolerance. Jack-knife fish were exposed to the parasite in the laboratory. Tomonts were isolated from the aquarium sediment, skin, and gill of the dead jack-knife fish, placed in glass dishes to observe development and dinospore emergence, or used to infect 6 naive clown anemonefish. Upon mortality, infected clown anemonefish were frozen at -20°C for 36 hrs. Upon thawing, gills were removed and tomonts were harvested to individual petri dishes where they were observed for development. Developing tomonts from the frozen material were used to expose additional clown anemonefish to test the freeze tolerance of the parasite. Filters from the

water samples and buffered homogenized fish tissue will be processed according to the protocol of Picon-Camacho et al. (2013, Veterinary Parasitology 196:265-271). Briefly, DNA extraction will be carried out using the QIAmp stool kit (Qiagen). DNA will be extracted from filters directly using the same kit. DNAs will be quantified using nanodrop and the PCRability of the samples tested to rule out inhibitors. Sensitivity assays will be conducted as previously described (Picon-Camacho et al., 2013) to determine both analytical and diagnostic sensitivities at the Arias' lab. Given appropriate sample sizes, LAMP and microscopy will be compared with respect to time to detection. Time to diagnosis will be examined with respect to sampling interval. Results from different water samples and replicates from the same system will the tracked to assess the degree to which they agree. Once a LAMP positive test occurs, microscopy will continue to assess the difference in time to detection and the correlation between positive tests and morbidity. Concordance correlation coefficients between results obtained by LAMP and microscopy and other appropriate statistical tests will be used to determine the overall agreement between both methodologies. Treatment initiation in positive systems will follow farm-specific protocols.

A total of 61 samples of water and fish (14 shipments from ORA comprising 28 samples and 19 shipments from ProAquatix comprising 32 samples; Table 1) were received at Auburn University and processed. Only two samples of fish tested positive for AO by microscopy. Positive results were confirmed by bioassay. All jack-knife fish died within 72 hrs. All clown anemonefish were readily susceptible to infection and died within 72 hours. Fish exposed to parasites subjected to freezing became heavily infected with AO and died within 5 days. Two molecular diagnostic methods for AO were set up at Auburn University: the PCR protocol described by Levy et al. (2007) and the LAMP assay described by Picon-Camacho et al. (2013). Both protocols target specific AO regions in the 18S rDNA. Positive control was generated from AO infected jack-knife fish using universal 18S primers. Regions flanking both LAMP and PCR targets were sequentially amplified and cloned. Several clones (positive controls) for each assay were sequenced to confirmed sequenced targets. The resulting 250 bp fragment was compared to others in GenBank for AO, revealing a small degree of polymorphism. Analytical sensitivity using purified plasmids containing PCR and LAMP target sequences was determined by serially diluting the target to extinction. Original analytical sensitivity for PCR was 9 pg/reaction, similar to what was described by Picon-Camacho et al. PCR primers AO18SR1 and AO18SF1 were redesigned and PCR conditions optimized in an attempt to increase analytical sensitivity. After those modifications, our PCR analytical sensitivity increased to 9 fg/reaction. This was in the same range as the analytical sensitivity reported for the LAMP assay previously. With respect to the LAMP assay, we obtained positive controls for the original PCR primers (targets LSU) and external primers (targets SSU) and demonstrated repeatedly in 10-fold dilutions that the analytical sensitivity for the original PCR primers using PCR was 4.2 pg/ul and that the analytical sensitivity level for the external primers using PCR was 9.9 fg/ul. We ran the LAMP assay 3 times on different days and demonstrated consistent results.

Table 1. Samples (fish and water) received for *Amyloodinium ocellatum* analysis at Auburn University. ORA = Oceans, Reefs, and Aquariums; Proaquatix = Proaquatix; N = negative; Y = positive.

Date	Producer	System	Water	Fish
3/6/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8		N
		C-9		N
3/9/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8		N
		C-9		N
3/14/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8		N
		C-9		N
3/16/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8		N
3/20/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8		N
		C-9		N
3/23/2018	ORA	Tank 1		Y
		Tank 2		N
	Proaquatix	C-8		N
3/27/2018	Proaquatix	C-8 Tank 1		N
		C-9 Tank 2		N
3/28/2018	ORA	Tank 1		N
		Tank 2		N
4/3/2018	Proaquatix	C-8		N
		C-9		N
4/6/2018	Proaquatix	C-8 tank 1		N
4/10/2018	Proaquatix	C-8 Tank 1		N
		C-9 Tank 2		N
4/13/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8 Tank 1		N
4/17/2018	Proaquatix	C-8 Tank1		N
		C-9 Tank 2		N
4/24/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8 Tank 1		N
		C-9 Tank2		N
4/27/2018	ORA	Tank 1		N
		Tank 2		N
	Proaquatix	C-8 Tank 1		N
5/1/2018	ORA	Tank 1		N
		Tank 2		N

	Proaquatix	C-8 Tank 1	N
		C-9 Tank 2	N
5/8/2018	Proaquatix	C-8 Tank 1	N
		C-9 Tank 2	N
5/11/2018	Proaquatix	C-8 Tank 1	N
5/15/2018	Proaquatix	C-8 Tank 1	N
		C-9 Tank 2	N
5/18/2018	ORA	Tank 1	Y
		Tank 2	N
	Proaquatix	C-8 Tank 1	N
5/21/2018	Proaquatix	C-8 Tank 1	N
		c-9 Tank 2	N
6/1/2018	ORA	Tank 1	N
		Tank 2	N
6/8/2018	ORA	Tank 1	N
		Tank 2	N

Previous to this study, no information was available on freeze-tolerance of the pathogen. Our work fulfilled Koch's postulates with frozen material and demonstrated that frozen fish can vector viable, infective parasite life history stages that can establish infections in relatively biosecure facilities. Marine aquaculture operations should adapt biosecurity SOPs to incorporate this knowledge. Cloned controls are currently archived at Auburn University, but are available to aquatic animal health laboratories and researches interested in AO. Using these controls we could determine the exact copy number threshold for detection of the parasite using qPCR.

Overall, the occurrence of AO on the farms was lower than average. Only two instances of positive fish were identified. Additionally, fewer samples than anticipated were received for a variety of reasons including outbreaks of other diseases. However, because of the low prevalence of infection, it is not clear that more samples would have produced additional positive results. Additionally, we were unable to determine the analytic sensitivity for the LAMP assay. Negative controls were positive and despite many attempts to eradicate the contamination issue it was not possible to eliminate it. As such, we were unable to establish the diagnostic sensitivity of the LAMP assay or accomplish the statistical comparison of LAMP and microscopy due to the lack of positive fish and/or water from the farms. Therefore, reports on subsequent subobjectives are limited.

Objective 1. Evaluate the developed LAMP assay in commercial aquaculture facilities
Subobjective 1b. Evaluate the LAMP assay in commercial aquaculture facilities, assessing sensitivity as compared to standard microscopic evaluation (AU, UF)

No additional report (see entry for Subobjective 1a)

Objective 1. Evaluate the developed LAMP assay in commercial aquaculture facilities
Subobjective 1c. Evaluate the LAMP assay in commercial aquaculture facilities, assessing cost (AU)

Auburn University

Because it is difficult to detect *Amyloodinium ocellatum* prior to the onset of morbidity using standard microscopy, alternative methods such as the LAMP assay are being developed. The latter method, once perfected, should detect tomonts and trophonts in the culture system water and fish tissue samples earlier than waiting for them to be detected through microscopy once they show up on the fish gills. The ability to detect the parasite before lethal loads of trophonts develop is critical to achieving control of the parasite. The economic consequences of early detection of tomonts and trophonts and treatment with Cupramine is paramount to improved fish survival and resulting numbers of clownfish to sell.

A cost/benefit analysis comparing the LAMP assay to standard microscopy was conducted through the development of enterprise budgets. However, because only two minor AO infections occurred during this study at the commercial businesses, the cost-benefit analysis was not possible to conduct directly. However, indirectly we were able to compare investment costs of the microscopy, LAMP and also for a PCR detection method. Secondly, we were able to estimate an enterprise budget for clownfish production using the three AO detection methods. We compared the investment and enterprise budgets for these three methods as if the business purchased the equipment necessary to do the AO detection in-house and in the case where they sent the water or tissue out to third parties for analysis. Further, we conducted the enterprise budgets at varying clownfish survival levels that experts provided and which took into account earlier tomonts/trophont detection by LAMP and PCR methods and later detection from the microscopy method.

The costs of applying the LAMP assay in commercial clownfish facilities was estimated and compared to the standard microscopy examination on the commercial clownfish operation. The two collaborating clownfish operations were visited to understand the commercial production process, AO frequency and its impact on survival levels and with Cupramine treatment, and cost of clownfish production. The cost of applying the LAMP assay and PCR methods were compared to the standard microscopy examination on the commercial clownfish operations.

This analysis looks at the cost of equipment required for each detection method (LAMP, PCR and Microscopy). Total investment was calculated and from that annual depreciation costs were calculated, using straight line depreciation methods with zero salvage value and 10-year economic life. Depreciation was broken down to a per sample level by dividing by 1000 samples analyzed per year. In like manner, the annual maintenance cost, skilled labor cost and reagent costs were calculated on a per sample basis.

The sample size differs among the methods. For instance, the minimum number of water samples that must be run in a LAMP or PCR method is 20 and each run requires 4 and 8 hours respectively. For this per sample cost calculation, the hours were converted to minutes and divided by the minimum 20 samples. The microscopy hours required was based on running 7 to

10 samples in an hour or 6 minutes per sample (using 10 samples per hour). Adding up the per sample calculated costs for investment depreciation, maintenance labor and reagents provided a cost of sampling per method. Secondly, an estimated cost per sample was established through discussions with fish health research experts (Cova Arias, Roy Yanong) of what their labs would charge if they were asked to conduct the same AO detection analyses. On a per sample basis, they provided a cost per sample for the cases of sending the water sample or the fish gill tissue sample to their labs for analysis.

Enterprise budgets were developed showing the cost/benefit effects of using the three detection methods. Receipts varied by detection method and expected survivorship levels for clownfish if an AO outbreak occurred. Expert opinion from the two commercial clownfish operations was used in selecting these survival levels. For microscopy, in the event of an AO outbreak, survival could be from 0-100%, but commonly in the 50-80% survival range once detected and therapeutic Cupramine was applied and cost $200 for a 21-day period treatment period. The chelated copper sulfate treatment is hard on the fish so operators do not like to use it more than once per crop cycle. A sales price of $10 per clownfish was used.

Variable costs included AO detection costs, calculated from the cost per sample multiplied by the number of samples taken per crop (20 samples was chosen as this was the minimum required to run the LAMP and PCR methods and would give plenty of health check during the early stages of the clownfish life). A standard Cupramine treatment of 21 days was used for each method. A labor cost was calculated using 180 days per crop and 2 hours per day devoted to a single 200-gallon tank, paid at a $12/hour rate. Not precisely knowing the other costs incurred per production cycle, such as feed, electricity, etc., a $3,000 per crop amount was chosen to cover these 'other costs'. This charge was applied equally to all enterprise budget detection method and survivorship levels. An income above variable costs was calculated by subtracting total variable costs from receipts and is a short-term indicator of profitability.

Enterprise budget fixed costs were based on annualized equipment depreciation costs divided by two production cycles per year to give a cost per crop. Additional fixed costs for equipment/building loan interest and repairs/maintenance were not calculated as a full investment analysis was not the focus of this work. In any case, these other fixed costs would be equal and thus a constant if anyone wanted to add them to this analysis.

Total costs were calculated by adding the total variable costs to the total fixed costs. Total costs were subtracted from the total receipts to obtain a net return to management. Secondly, if the clownfish AO water and tissue samples were sent out to a third party laboratory for analysis was incorporated into a second set of enterprise budgets. Instead of using AO detection methods cost from the in-house operation, the cost per outside lab was substituted in its place. All other receipts and variable costs of production were the same as in the prior enterprise budget. Fixed costs did change in this scenario as there were no AO detection pieces of equipment purchased and thus the prorated depreciation and annual equipment maintenance costs were zero. otal receipts, total variable costs, income above variable costs, total fixed costs, total costs and net returns were calculated for this scenario as well.

Again, to be clear, the enterprise budgets were developed based on whether the business purchased the equipment and conducted the AO detection methods themselves using the three methods or if they sent the samples out for analysis by third party laboratories using the three methods (microscopy, LAMP, PCR).

Table 2 presents the cost of equipment required for each detection method (LAMP, PCR and Microscopy). Total investment for the microscopy, LAMP and PCR was $1500, $6000 and $15800, respectively. Itemization of equipment/supply items (filtration, centrifuge, water bath, pipettes and miscellaneous, electrophoresis, microscope and slide/slip cover supplies) is shown in Table 1. Other items such as refrigerator and other items are assumed and the same for all three cases. Annual equipment depreciation costs, on a per sample basis was $0.15, $0.60 and $1.58 on a per sample basis, respectively. The combined annual equipment maintenance cost ($0.05 $0.30 and $0.50), skilled labor cost ($1.50, $3.00, $6.00) and reagent costs ($1.70, $28.90. $23.08), on a per sample basis were $1.70, $28.90 and $23.08, respectively. Secondly, the estimated cost per sample for each detection method if sent to a third party lab would be approximately $9, $50 and $50, respectively, Table 1. NOTE: it is highly unlikely that a commercial aquaculture facility would purchase LAMP or PCR equipment (but they do currently have microscopes and the ability to detect the AO parasites on gill tissue). First, they are quite expensive, secondly, it takes skilled labor to operate them, and third a quality dry lab is required to prevent contamination during their operation. However, these methods do provide a much elevated and earlier detection ability of tomonts/trophonts in the clownfish culture water than microscopy. A strong caution is required at this point as the LAMP assay was plagued with contamination issues and did not perform as expected, so additional work on this protocol is needed.

Enterprise budgets for clownfish production using three purchased AO detection methods are presented in Table 3. Receipts varied by detection method and expected survivorship levels. Microscopy had a 50% and 80% survivorship after an AO outbreak and Cupramine treatment and had sales receipts of $10,000 and $16,000, respectively for one 200-gallon production tank. Higher survival (90%) for the LAMP and PCR methods resulted in receipts of $18,000 for both. Variable costs for AO detection costs were $2, $29 and $23 per sample for the microscopy, LAMP and PCR methods and for the 20 samples were $34, $578 and $462, respectively. A standard AO Cupramine treatment lasting 21 days cost $200 and was used for each method. Labor cost per 180-day production cycle was $4,320 for each detection method. As mentioned in the methods section a constant $3,000 was included for “other” costs incurred per production cycle, such as feed, electricity, etc.

Table 2. Cost of Clownfish *Amloodinium ocellatum* (A.o.) sampling and detection

	Microscopy	LAMP	PCR
I. Cost of the equipment If business is to conduct the detection operations.			
Investment			
- Equipment cost and type			
1 - Filtration equipment		$500	$1,000
2 - Centrifuge		$3,000	$3,000
3 - Water bath		$700	
4 - Pipettes & miscellaneous		$800	$800
5. PCR thermocycler			$10,000
6. Electrophoresis equipment		$1,000	$1,000
7. Microscope	$1,000		
8. Slides/slip cover supplies	500		
Total equipment cost	$1,500	$6,000	$15,800
Depreciation cost, 10-year economic life			
- Annual depreciation cost	$150	$600	$1,580
- Samples run per year	1,000	1,000	1,000
- Annual depreciation cost per sample	$0.15	$0.60	$1.58
Annual equipment maintenance costs	$50	$300	$500
- Per sample	$0.05	$0.30	$0.50
Labor, per sample			
- Skilled laboratory labor, minutes per sample*	6	12	24
- Skilled laboratory labor wage, $/hour	$15	$15	$15
- Labor cost per sample	$1.50	$3.00	$6.00
- Reagents, list below, per sample			
- LAMP reagents		$20	
- PCR reagents			$10
- DNA extraction kit		$5	$5
Total cost per sample	$1.70	$28.90	$23.08
II. Cost of sending water or fish tissue sample out for analysis.			
- Cost per sample	$9	$50	$50

Table 3. Clownfish enterprise budget, if conducting Microscopy, LAMP or PCR on-site, per 200-gallon tank.

I. Budget when A.o. detection equipment is purchased by business is to conduct the detection analyses in-house.					
Receipts		Microscopy	Microscopy	LAMP	PCR
- Stocking	2000				
- Survival, total for crop		50%	80%	90%	90%
- Sales quantity		1,000	1,600	1,800	1,800
- Sales price, per fish		$10	$10	$10	$10
- Total receipts/sales per tank		$10,000	$16,000	$18,000	$18,000
Variable Costs					
A.o. detection cost, in-house lab		$34	$34	$578	$462
- Costs for detection, per sample		$2	$2	$29	$23
- Number of samples per crop		20	20	20	20
Cupramine cost per 21 day treatment	$200	$200	$200	$200	$200
- Number of treatments per cycle		1	1	1	1
Labor Cost per cycle		$4,320	$4,320	$4,320	$4,320
- Hours per cycle, 180 days x 2 hrs/d	360				
- Labor rate, $/hr	12				
Other Costs per cycle		$3,000	$3,000	$3,000	$3,000
Total Variable Costs		$7,554	$7,554	$8,098	$7,982
Income Above Variable Costs		$2,446	$8,446	$9,902	$10,018
Fixed Costs					
- Equipment cost		$1,500	$1,500	$6,000	$15,800
- Annual depreciation		$150	$150	$600	$1,580
- Cycles per year		2	2	2	2
- Prorated depreciation per cycle		$75	$75	$300	$790
- Annual equipment maintenance		$50	$50	$300	$500
Total Fixed Costs		$125	$125	$600	$1,290
Total Cost		$7,679	$7,679	$8,698	$9,272
Net return to Management		$2,321	$8,321	$9,302	$8,728

The income above variable costs for microscopy, LAMP and PCR was $2446, $8,446, $9902 and $10018, respectively, Table 3. The differences are attributed to lower survivor and lower sales receipts for the microscopy detection methods and higher receipts but also higher detection cost for the LAMP and PCR detection methods. In the latter case the higher sales receipts more than offset the increased AO detection costs.

Enterprise budget prorated depreciation costs per crop cycle for the microscopy, LAMP and PCR detection methods were $75, $300 and $790 respectively and annual equipment maintenance costs were $50, $300 and $500 respectively. Total fixed costs for the three methods were $125, $600 and $1,290 respectively. Variable plus fixed costs equal total costs and they were $7679, $8698 and $9272 for the microscopy, LAMP and PCR detection methods respectively.

Subtracting total costs from receipts provided net returns of $2,321 for the microscopy method having a 50% clownfish survivorship and $8,321 for this method when an 80% survivorship occurred. The LAMP and PCR methods had 90% fish survivorship and net returns of $9,302 and $8,728 respectively.

Thus, LAMP and PCR AO detection methods show high net returns with high clownfish survival rates. Microscopy net returns are comparable if survivorship is high as well, but not if mortality levels are high. As stated earlier, it is not likely that commercial operations will purchase LAMP and PCR equipment, build research-level laboratories and hire highly skilled labor for their farms. Commercial farms are more likely to stick to the microscopy method which is applied when clownfish begin to behave erratically. Often, collection of these fish and gill inspection with microscopes can quickly tell if AO is present. Fast action through a Cupramine treatment or freshwater baths (could lose 10-50% or up to 100% with this method) can often stop the AO outbreak or at least prevent large near 100% losses.

Secondly, if the clownfish AO water and tissue samples were sent out to a third party laboratory for analysis, the net return for the microscopy detection method with 50% fish survival was $2,310 and with 80% survival was $8,310, Table 4. Net returns for the LAMP and PCR methods were $9,480 for both treatments as there were no equipment depreciation costs and no equipment maintenance costs. Total receipts, total variable costs, income above variable costs, total fixed costs, total costs and net returns for each method and survival level are shown in Table 4.

The cost comparisons between the microscopy, LAMP and PCR detection of AO was hindered by lack of AO outbreaks. However, equipment and annual maintenance costs to implement the three detection methods were calculated. Overall, the microscopy (~$1,500 to 3,000) method was the least expensive, the LAMP assay (~$6,000) cost a little more than the microscopy, and both cost less than the PCR (~$15,800+). On a per sample basis (including reagents, DNA extraction, and labor), the microscopy method required none of these costs but had a cost of $ 1.70 per sample, while the LAMP assay cost $28.90 per sample and the PCR method cost $23.08 per sample. Third party laboratories might charge the equivalent of $9, $50 and $50 to analyze fish gill tissue using the microscopy method and water samples for the LAMP and PCR methods, respectively.

Benefits or receipts/sales of surviving clown fish were not determined for a specific production cycle but inferred from interviews with the two collaborating businesses which reported typical 50-80 percent survival with the microscopic AO detection methods and a Cupramine treatment. Early detection of AO by molecular methods (PCR and/or LAMP) might improve survival to 90%, which would increase the net returns per tank by $1,000 to $7,000 to the 50% and 80% survival rates resulting from later microscopy detection of AO gill parasites. Therefore, ***IF*** the LAMP assay could be performed without contamination issues, it could effectively pay for itself by increasing the revenue per tank, but it would require major equipment investment, hiring of skilled laboratory technicians and building research-level laboratories, which is not likely at the farm business level. The sophistication of each detection method varies greatly, with the PCR method requiring the highest skill level followed by LAMP detection method and much easier operation by the microscopy method.

Table 4. Clownfish enterprise budget, if conducting Microscopy, LAMP or PCR on-site, per 200-gallon tank.
II. Budget when A.o. detection is obtained through sending water or fish tissue sample out for analysis.

	Microscopy	Microscopy	LAMP	PCR
Survival, percent	50	80	90	90
Receipts				
- Sales total per tank	$10,000	$16,000	$18,000	$18,000
Variable Costs				
- A.o. detection cost, third party	$170	$170	$1,000	$1,000
- Cupramine cost per 21 day treatment	$200	$200	$200	$200
- Labor Cost per cycle	$4,320	$4,320	$4,320	$4,320
- Salt for system				
- Other Costs per cycle	$3,000	$3,000	$3,000	$3,000
Total Variable Costs	$7,690	$7,690	$8,520	$8,520
Income Above Variable Costs	$2,310	$8,310	$9,480	$9,480
Fixed Costs				
- Prorated depreciation per cycle	$0	$0	$0	$0
- Annual equipment maintenance	$0	$0	$0	$0
Total Fixed Costs	$0	$0	$0	$0
Total Costs	$7,690	$7,690	$8,520	$8,520
Net return to Management	$2,310	$8,310	$9,480	$9,480

Objective 1. Evaluate the developed LAMP assay in commercial aquaculture facilities
Subobjective 1d. Evaluate the LAMP assay in commercial aquaculture facilities, assessing disease incidence/production outcomes (UF, AU)

No additional report (see entry for Subobjective 1a)

Objective 1. Evaluate the developed LAMP assay in commercial aquaculture facilities
Subobjective 1e. Evaluate the LAMP assay in commercial aquaculture facilities, assessing false positives and negatives using positive and negative controls (AU, USM)

University of Southern Mississippi

The ability of a diagnostic test to accurately identify the infection status of an organism or system is critical to the successful implementation of a test into a monitoring protocol. False positives (see 1b above) result in expenses related to unnecessary treatments. False negatives result in losses to due to the disease. This subobjective will assess the ability of the LAMP assay to accurately classify systems and animals as positives or negatives in production situations.

A sample of AO from the University of Florida was used to establish a laboratory infection at USM/GCRL which is now maintained in a variety of wild and cultured fish including spotted seatrout and Atlantic croaker juveniles from the Thad Cochran Marine Aquaculture Center. To evaluate the diagnostic power of the LAMP assay in comparison to microscopy in the laboratory setting, forty-two 20L tanks

divided into seven groups of six each were filled with artificial salt water. Each of the seven groups was spiked with either 0, 1, 10, 100, 1000, 5000, or 10,000 dinospores. Following agitation, three tanks in each group were stocked with four spotted seatrout. The remaining three tanks in each group remained fish free. Ten 1-L aliquots of water were then taken from the fish-free tanks, vacuum filtered through a membrane, and stored at -80°C. For the fish tanks, after seven days (or at the onset of morbidity, whichever came first), fish were removed, gills were excised, and two gill arches (one from each side) were examined using the dissecting microscope. Additionally, ten approximately 100mg samples of gill tissue were taken randomly from fish in each dosage and stored in RNALater. Directly examined gill tissue was recorded as positive if any single trophont is identified visually. Frozen membranes and/or tissue samples in RNALater were stored for molecular analysis along with positive and negative controls. The experiment will be repeated four times. Molecular analyses using the methods of Picon-Camacho et al. (2013) will be achieved once contamination issues are resolved. In the event that contamination issues remain, standard PCR will be used.

We will statistically evaluate the diagnostic power of the LAMP assay by comparing it to the gold standard of microscopic identification using a 2X2 contingency table analysis. Counts of known (spiked) true positives and negatives and false positives and negatives from each test will be used to calculate sensitivity and specificity and their 95% confidence intervals. McNemar's test for paired data (i.e., both tests performed on same specimen) will be used to test whether or not the sensitivity and specificity of the two tests are significantly different. Further, we will estimate the reproducibility of the test by evaluating replicates from each prepared sample of known (spiked) positive and known negative prepared samples. The ratio of positive and negative tests in the ten tests from each dosage will demonstrate whether or not the test accurately identifies all positive and negative samples.

To date, because of unresolved contamination issues with the LAMP assay, only the samples collected for microscopy have been processed. Infection is not identifiable microscopically at dosages less than 100 dinospores (5 dinospores L^{-1}). At that dosage, a mean of approximately 0.67 fish per replicate were positive (~25% prevalence). At the 1000 dinospore (50 L^{-1}) dose, a mean of approximately 2.0 fish per replicate was positive (50% prevalence). By the 5000 dose, virtually all fish were classified as positive (mean of 3.3 fish per tank). Mortality was observed only in the 10,000 (500 L^{-1}) dose over the 7 day period (2 fish out of 12). The time to which an infection at the lower dosages, particularly those at the dosages in which the parasite was undetected by light microscopy, would proceed to mortality is unknown at this time and requires investigation.

Our work has quantified the dinospore density in the water required for subsequent microscopic detection of the parasite on fish. Because the parasite can escape microscopic detection at dinospore densities of 10 L^{-1} and infections in no more than half the fish are likely to be detected at dinospore densities up to 50 L^{-1}, methods for better detecting these early infections would be of substantial benefit to the producer.

Objective 2. Develop a standard operating procedure (SOP) for A. ocellatum surveillance utilizing the LAMP assay and incorporating findings from Objective 1.

Unable to produce an SOP. See Subobjective 1a.

IMPACTS:

- Optimized primers for both PCR and LAMP that achieve analytical sensitivities comparable to that described for the original LAMP assay are available to other researchers at no cost.
- Cloned controls, which could serve as a basis for quantifying the threshold of detection using qPCR, are available to aquatic animal health laboratories at no cost.

- The cost of the LAMP assay falls in between that of microscopy and PCR, but is not necessarily prohibitive because improved survival as a result of early detection potentially recovers the cost required to run the assay.

PUBLICATIONS, MANUSCRIPTS OR PAPERS PRESENTED:

Oral Presentations

Gonzales, RD, CR Arias, RB Blaylock, & SA Bullard. Detecting *Amyloodinium ocellatum* in cultured fish: efficiency comparisons between direct observation of trophonts using light microscopy and detection of waterborne DNA using loop-mediated isothermal amplification (LAMP). Southeastern Society of Parasitologists, Athens, Georgia, 11−13 April 2019.

Dutton, HR, R Yanong, W Cai, CR Arias, & SA Bullard. *Amyloodinium ocellatum* infections in marine aquacultured fishes: A new host record and anecdotal observations that indicate freeze tolerance. Southeastern Society of Parasitologists, Starkville, Mississippi, 19–21 April 2018.

Dutton, HR, R Yanong, W Cai, CR Arias, & SA Bullard. *Amyloodinium ocellatum* infections in marine aquacultured fishes: A new host record, a novel DNA extraction method, and anecdotal observations that indicate freeze tolerance. 43rd annual Eastern Fish Health Workshop, Chattanooga, Tennessee, 9–13 April 2018.

Blaylock, RB, JM Lotz, SA Bullard, C Arias, T Hanson, & RPE Yanong. Field testing of a new diagnostic assay for *Amyloodinium ocellatum*. World Aquaculture 2017, Cape Town, South Africa, 26-30 June 2017.

RESULTS AT A GLANCE:

Amyloodinium ocellatum can survive freezing in a domestic freezer. Thus, marine aquaculture operations should adapt biosecurity protocols to prevent spread in otherwise relatively biosecure facilities.

The threshold exposure density for early (within 7 days of infection) microscopic detection is 5 dinospores L^{-1}. Only about 50% of fish exposed at a dinospore density of 50 L^{-1} display infections detectable by microscopy. Exposure at 500 dinospores L^{-1} produced less than 20% mortality in 7 days. Thus, we experimentally demonstrated the parasite can easily escape detection by microscopy and confirmed the notion that a considerable population of the parasite is present prior to the onset of morbidity.

Although we were ultimately unable to field test the LAMP assay due to the general absence of AO outbreaks on the farms and contamination issues in the lab, we generated and optimized cloned, positive controls that are available to the research community for further research.

While microscopy is the cheapest detection method (and PCR the most expensive, with LAMP falling in between), an increase in survival by as little as 10%, which might be achieved by early

detection of AO, would generate enough additional revenue to offset the added cost of running the LAMP assay.