Deliverable Report

Deliverable No:	D7.4	**Delivery Month:**	24
Deliverable Title	Protocol for shipping grey mullet eggs		
WP No:	7	**WP Lead beneficiary:**	P4. IOLR
WP Title:	Reproduction and Genetics-grey mullet		
Task No:	7.5	**Task Lead beneficiary:**	P25. DOR
Task Title:	Establish a shipping protocol for grey mullet eggs		
Other beneficiaries:	IOLR		
Status:	Incomplete (an update will be submitted on Mo 36)	**Expected month:**	24
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Lead Scientist preparing the Deliverable: B. Koven (IOLR)

Other Scientists participating: H. Rosenfeld (IOLR), G. Shafran (DOR)

OBJECTIVE: Establish a shipping protocol for grey mullet eggs.

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DELIVERABLE DESCRIPTION (from the DOW)

Protocol for shipping grey mullet eggs: Establishment of procedures for handling grey mullet eggs in order to allow their transport to various larval rearing facilities. The deliverable will present procedures for the optimal (a) egg disinfection, (b) shipment conditions including: egg density, duration, temperature and pH. The deliverable will summarize the results from repeated trials addressing different environmental parameters in order to optimize the procedure and maximize larval survival.

INTRODUCTION

The development of a protocol for the transferring of grey mullet eggs to the various partners in the DIVERSIFY project was based on methodology developed earlier by P4. IOLR for shipping Atlantic bluefin tuna (BFT) eggs to different Mediterranean partners (Greece, Spain, Malta, Italy) in the EU 7th framework projects SELFDOTT (212797) and TRANSDOTT (311904) (De la Gándara 2012, Bridges 2014). In brief, this protocol recommends the stocking of 10 l of filtered (10 µm) seawater with 10-15,000 gastrula-stage bluefin tuna eggs/l in 20 l cubitainers (stiff plastic 6 sided, square bottom cubitainers used in the wine industry), which is placed in a Styrofoam container (**Fig. 1a**). Pure oxygen is added to supersaturate the container seawater as well as flushing the air layer in the upper part of the cubitainer. One to two ice packs, wrapped in cardboard, are placed adjacent to the air-oxygen layer (not against the water layer) and the package closed (**Fig. 1b**). Transport by air from Spain or Malta to Israel, which was the longest transit time among the partners, included two flights, custom documents arranged prior to arrival that facilitated entry at Ben Gurion International airport and taxi transport that brought the shipment from Tel Aviv to the IOLR (4-5 h) in Eilat. Total transit time from egg collection at sea to arrival at the Eilat facility during the SELFDOTT and TRANSDOTT projects ranged between 26-29 h. The eggs did not hatch in transit. During the TRANSDOTT program BFT egg shipments consistently arrived at the Eilat institute in excellent condition where oxygen (>200%), pH (6.5-7.0) and temperature (ca 22-23°C) were within acceptable limits.

(a) (b)

Figure 1. (a) The 20-l cubitainer and (b) styrofoam box with ice pack in cardboard, used for the shipment of grey mullet eggs.

The eggs were temperature acclimated in the tanks, pH was incrementally increased by dripping a solution of 0.1 N NaOH into the cubitainers to reach a pH of 7.5 to 8.0, the percent of dead sinking and live floating eggs was calculated and the eggs stocked in the experimental system. The following day the percent (%)

hatching and survival to 1 day after hatching was high (ca 79-90 %) provided good quality eggs were sent. Based on the success of this methodology from these previous EU projects, this transport protocol was also tested when transporting grey mullet eggs within Israel (ca 10-11 h in transit) and a simulated transport for longer periods (23 h) was attempted. Importantly, higher grey mullet egg densities (55,000-84,000 eggs/l) were sent compared to the BFT egg (10-15,000 eggs/l) shipments.

MATERIALS AND METHODS

2014-Deliveries to P25. DOR fish farm

During 2014, three shipments of mullet eggs were made to P25. DOR fish farm from P4. IOLR and one shipment was made from the kibbutz Ma'agan Michael to the P4. IOLR. A modified BFT protocol was employed where the final volume of the water in the 20 l cubitainer was 15 l and egg density ranged from 55-84,000 gastrula-stage eggs/l. Pure oxygen was added to supersaturate the container seawater as well as flushing the air layer in the upper part of the cubitainer. One to two ice packs, wrapped in cardboard, was placed adjacent to the air-oxygen layer (not against the water layer) and the package closed. Table 1 lists shipping conditions as well as percent oxygen and hatching results at final destination for grey mullet eggs in 2014 from the P4. IOLR facility to the P25. DOR fish farm. Shipment results of eggs sent from kibbutz Ma'agan Michael to the IOLR were also included, which has a slightly longer transit time of 11h. In contrast, the eggs shipped from the Ma'agan Michael hatchery to P4. IOLR utilized similar high egg densities (66-110,000 eggs/l), but were sent using thick plastic bags filled with pure oxygen. Domestic shipments necessitated only one internal flight and vehicle transport to the destination.

2015- Testing longer transit times

In order to test longer transit times and to monitor water quality more closely, a controlled simulation trial was set up. Two gastrula-stage egg densities (10 and 15,000 eggs/l) were tested in replicates of 4 and 3 Styrofoam containers for each treatment, respectively, over a period of 23 h (**Table 2**). The protocol used in this trial was identical to the transport protocol developed in SELFDOTT and TRANSDOTT. Pure oxygen was added to the cubitainer, followed by securely closing the container. The cubitainers were then placed into Styrofoam containers (30x30x39 cm) where two icepacks, wrapped in cardboard were placed on the upper part of the container next to the air pocket. The Styrofoam boxes were placed in a temperature controlled room (24°C) for 23 h. During this period all boxes were gently shaken every few hours. A data logger was placed in Styrofoam container 7 to measure temperature variability throughout the trial (**Table 2**).

Single eggs from the same batch were taken and carefully placed in each of 12 wells (3 ml) in each of 3 plastic plates to determine hatching success, after incubation in a controlled temperature incubator. Unfortunately, this batch of eggs was poorly fertilized and hatching did not occur in any of the 12 well plates nor in the Styrofoam containers. In fact, this study was repeated, but egg quality remained poor and no hatching was observed in the second trial either. Nevertheless, the pH, oxygen and ammonia were measured at the end of the trial in order to determine if water quality was compromised during the 23 h simulation.

2015- Nitrofurazone treatment of eggs at destination.

A further step of reducing the bacterial load of transported eggs upon arrival was investigated in 2015. Polydine disinfectant treatment of eggs, which was the selected disinfectant with the eggs of other species, was not successful in treating grey mullet eggs as this approach frequently resulted in >70% mortality of live and highly buoyant eggs. On the other hand, no disinfection commonly leads to the development of a red bacteria on the tank walls, which has recently been identified at the IOLR as a potential cause of newly hatched larval mortality. Consequently, a number of preliminary toxicity trials were conducted with

nitrofurazone, an antibiotic that appears to successfully treat this bacterial strain. In Israel, it is not legal to use nitrofurazone to treat fish during grow-out for the market but this antibiotic can be used to treat eggs. These studies were carried out by stocking 1-2 gastrula-stage grey mullet eggs in each well of three 24 well plastic plates for the control (no antibiotic) and two nitrofurazone concentration treatments (2.5 and 5.0 mg/l of ambient 40 ‰ seawater). All nine 24 well plastic plates were placed in an incubator at 25.5 ^{0}C. After 33 h, hatching occurred where the percent (%) hatching and the survival of the pre-larvae immediately following hatching were noted. The latter parameter was measured as mullet larvae frequently die upon hatching. In addition, survival of the pre-larvae following a further 3 h exposure to the antibiotic was determined. Although the use of nitrofurazone is not true disinfection, it was hypothesized that the reduced bacterial load might be beneficial.

RESULTS AND DISCUSSION

The results of **Table 1** show that despite higher egg densities in the cubitainers, compared to the BFT egg shipping protocol, the shipments arrived at their domestic destinations in 9-10 h where oxygen levels (265-339 %) and hatching rates (85-90 %) were very good. In fact, eggs that were sent in plastic bags from Ma'agan Michael to the IOLR arrived in excellent condition after 11 h and also demonstrated hatching rates of 96-97%. Taken together, these results suggest that short term shipping of gastrula stage grey mullet eggs, provided that egg quality is very good, can be readily carried out using cubitainers or strong plastic bags as long as pure oxygen is added and that the shipment does not encounter temperature extremes.

Table 1. Shipping conditions as well as percent oxygen and hatching results of grey mullet eggs sent from P4. IOLR to P25. DOR in 2014, and from Ma'agan Michael to P4. IOLR, which were sent in plastic bags in Styrofoam boxes with no ice packs.

Spawning date	Box no.	Egg vol. sent (ml)	Egg number	Total Vol. sent (l)	Eggs/l	% O_2 *	Time in transit (h)	% hatching*
	Eggs sent from IOLR to DOR							
16.8.14	1	350	1.26×10^6	15	84000	272	9.0	85
16.8.14	2	250	0.825×10^6	15	55000	280	9.0	85
3.10.14	1	250	1.6×10^6	15	55000	330	10.5	90
3.10.14	2	250	1.6×10^6	15	55000	330	10.5	90
16.10.14	1	350	1.26×10^6	15	84000	265	9.0	85
	Eggs sent from Kibbutz Ma'agan Michael to IOLR							
14.8.14	1	500	1.65×10^6	15	110000	270	11	96
14.8.14	2	300	99000	15	66000	265	11	97

*Measured at destination

Despite the failure of the eggs to hatch due to poor fertilization, **Table 2** shows that water quality was conserved, in terms of pH, O_2 and NH_3 over the 23 h test period while temperature fluctuated between 20-24 ºC (**Fig. 3**). This suggests that implementing the BFT transport protocol in sending eggs to the other partners would be sufficient at the two egg concentrations tested.

Table 2. The effect of transit simulation on water quality (pH, O_2, NH_3) in cubitainers, 23 h after trial. Poor quality eggs with low fertilization rates were used and no hatching was observed.

T0				23 h		
Box no.	pH	O_2	NH_3 (mg/l)	pH	O2	NH_3 (mg/l)
1	8.02	365	<0.05	8.01	285	<0.05
2		390		8	290	
3		287		8	297	
4		314		7.98	235	
5		257		7.96	242	
6		307		8.01	256	
7		296		8	290	

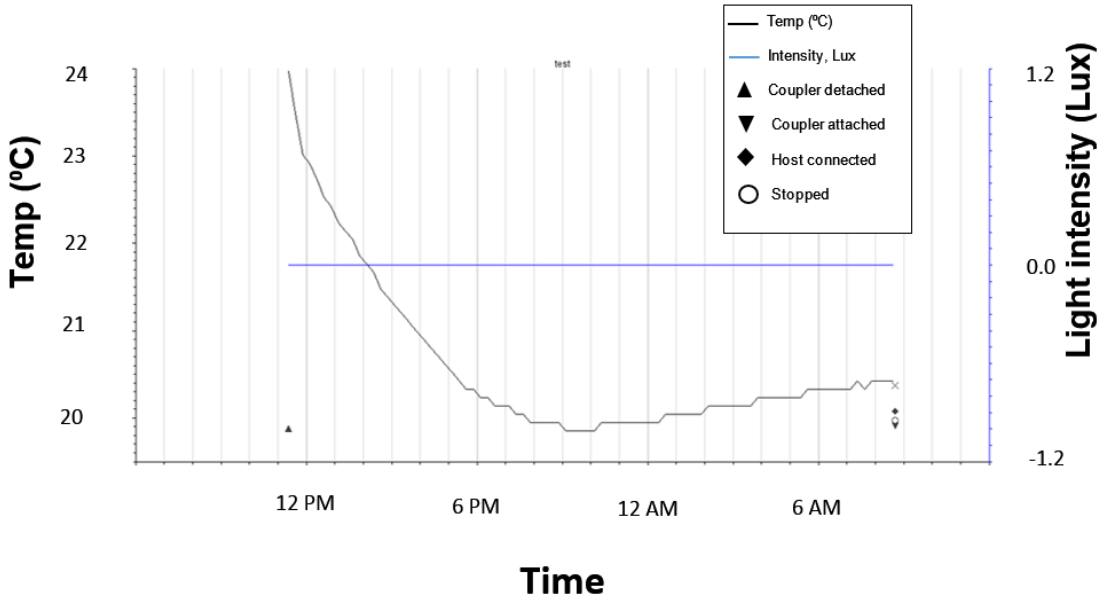

Figure 3. Temperature (^{0}C; black line) and Light intensity (Lux; blue line) measurements (data logger) in Styrofoam container 7 during the 23 h simulation of transporting grey mullet eggs using the protocol developed in the EU SELFDOTT project. Temperature decreased from approximately 24 to 20 ^{0}C during the simulation.

In **Figures 4** and **5**, exposing the eggs for 33 h to a 2.5 mg/l nitrofurazone concentration significantly ($P<0.05$) increased the hatching success of grey mullet eggs compared to the control and the 5 mg/l concentration. In addition, there was no difference in the percent (%) of surviving larvae following hatching ($P>0.05$). On the other hand, if the pre-larvae continued to be exposed to the antibiotic for a further 3 h, mortality was total (**Fig. 6**). Nevertheless, further studies are necessary on nitrofurazone due to the use of poor quality eggs in these studies as well as the need to examine shorter exposure times (<33 h) and other antibiotic candidates. Having said that, the preliminary results suggested that nitrofurazone may be an effective egg treatment approach that can markedly improve hatching rate after transport from the spawning tank or to another facility.

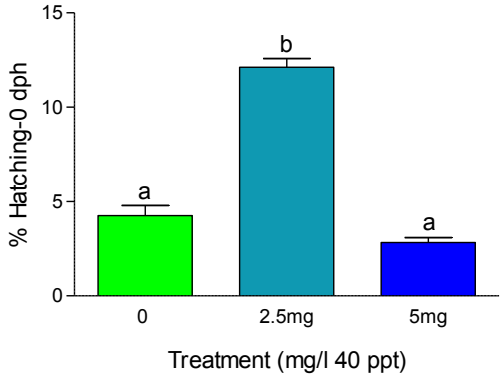

Figure 4. The effect of nitrofurazone treatment concentration on percent (%) hatching in grey mullet eggs following 33 h of immersion. Values having different letters were significantly (P<0.05) different.

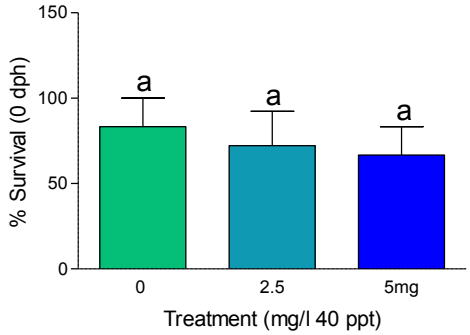

Figure 5. The effect of nitrofurazone treatment concentration on percent (%) larval survival following hatching in grey mullet eggs. Values having the same letter were not significantly (P>0.05) different.

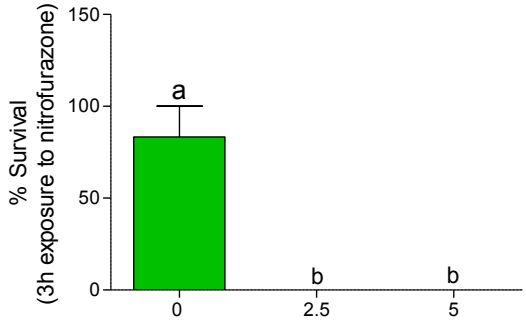

Figure 6. Survival of larvae after a further 3 h exposure to nitrofurazone following the 33 h of exposure up to hatching. Values having different letters were significantly (P<0.0%) different.

CONCLUSIONS

1. Short term shipping (≤11 h) of gastrula stage grey mullet eggs, provided that egg quality is very good, can be readily carried out using cubitainers or strong plastic bags together with the addition of pure oxygen. One or no Freezer packs may be sufficient as long as the shipment does not encounter temperature extremes.

2. Water quality, in terms of temperature (20-24°C), pH, O_2 and NH_3,, was stable after 23 h of simulated egg transport, even when eggs of poor quality were used.

2. Exposure of eggs only to nitrofurazone (2.5 mg/l sea water) is a promising treatment to reduce bacterial load and improve hatching rate. However, further studies to determine the most effective egg exposure times in this and other antibiotics are necessary.

REFERENCES

De la Gandara, F. 2012. Final Report on Project 212797: From capture based to SELF-sustained aquaculture and domestication of bluefin tuna, *Thunnus thynnus*. FP7-CP-FP

Bridges, C. 2014. Final Report on Project 311904: Translation of domestication of *Thunnus thynnus* into an innovative commercial application.

DEVIATIONS:

Due to poor egg quality and fertilization rates in 2015, the egg shipment protocol was not thoroughly tested during the present reporting period and these studies should be repeated. Nevertheless, the data collected so far does indicate that the SELFDOTT transport protocol can be readily adapted to shipping grey mullet eggs to the partners in DIVERSIFY. An updated version of the Deliverable will be submitted in Mo 36.

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