Development of germ cell transplantation technology for the Australian aquaculture industry

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Project No. 2011/730

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

May 2014

This project was conducted by University of the Sunshine Coast, Flinders University and Queensland Department of Primary Industries and Fisheries in collaboration with Clean Seas Tuna Ltd.

Title: Development of germ cell transplantation technology for the Australian aquaculture industry

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ISBN: 978-0-9804744-3-5

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Program. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

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Australian Government
Fisheries Research and Development Corporation

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Table of Contents

Non-Technical Summary

Project 2011/730: Development of germ cell transplantation technology for the Australian aquaculture industry

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The project was designed to explore the application of surrogate technology as an alternative broodstock system for the Southern Bluefin Tuna (SBT). Surrogate technology, also known as germ cell transplantation, uses germ cells from a donor species, in this case SBT, and transplants them into a host species, the surrogate. The germ cells can migrate and form part of the host's gonad, resulting in the production of the donor sperm and egg by the host gonad.

We have explored the suitability of the Yellowtail Kingfish (YTK) as a surrogate for SBT. Over 12,000 YTK larvae were injected with SBT germ cells, and 3-4 weeks after transplantation we could observe the migration and colonisation of the SBT cells (which are labelled with red florescence dye for ease of detection) to the YTK genital ridge, confirming SBT cells responded to YTK migration cues. Transplanted larvae were raised and samples assessed a few months later, however so far we could not detect SBT cells in the maturing YTK, indicating that whilst SBT germ cells respond to the YTK migration cues we cannot confirm proliferation of the germ cells in the YTK host at this stage. About 100 YTK approaching one year of age are maintained at CST and will need to be examined for the presence of SBT sperm or eggs once they reach sexual maturity.

We have also started culturing two other potential hosts, from evolutionary closer species. These hosts (the Mackerel Tuna and the Blue Mackerel) both belong to the Scombridae fish family and therefore should provide a more compatible environment for SBT cell proliferation. Techniques for capture and live transportation of these fish were established, however we did not yet achieve spawning.

We have also isolated a suite of molecular probes, which clearly identify SBT sequences in the YTK genomic environment.

This project has established the protocols and know-how to embark on a challenging yet potentially game changing new technology that can revolutionise aquaculture of large species in general and SBT in particular.

PROJECT OBJECTIVES

1. To develop and optimise protocols for the collection and dissociation of SBT germ cells for transplantation
2. To assess and optimise cryopreservation procedures of SBT germ cells
3. To investigate and establish potential surrogate host for SBT
4. To develop molecular tools for the identification of SBT germ cells in surrogate host
5. To develop strategies for sterilization of surrogate host to obtain only SBT eggs and sperm for transplanted fish

OUTCOMES ACHIEVED

The project will directly benefit the closed lifecycle aquaculture of Southern Bluefin (SBT) Tuna, in particular Clean Seas Tuna Ltd; however, it will have flow on effects to other finfish industries and the conservation efforts of endangered species.

The planned outcome of this project was to produce tuna gametes in a surrogate fish that can be housed in a small and cheap to maintain facility and will have short generation time. This outcome was partially achieved. We have been successful in transplanting Yellowtail Kingfish (YTK) with SBT germ cells and have shown that these cells have migrated and colonised. We do not yet have a definite answer as to whether these cells have also proliferated, which is not assured given the large evolutionary distance between the two species.

We have established robust protocols for the preparation and cryopreservation of SBT cells and those cells are now available for transplantation into either YTK or alternative tuna species so a further outcome from this research is likely to be further research to develop surrogacy of SBT (and potentially other Australian aquaculture species) across a range of potential host species.

LIST OF OUTPUTS PRODUCED

1. Optimised protocols for the collection, dissociation and cryopreservation of SBT germ cells were established.
2. Molecular tools (genes expressed specifically in primordial germ cells) for identification of SBT germ cells in surrogate host developed.
3. Yellowtail Kingfish juveniles (n = 100) with transplanted SBT primordial germs cells with potential to produce SBT gametes.
4. Germ cell transplantation technology identified as an attractive option for giant grouper (*Epinephelus lanceolatus*) aquaculture and work began in collaboration with Northern Fisheries Centre and ACIAR.

ACKNOWLEDGEMENTS

The authors would like to thank:

- Dr Craig Foster, CEO of Cleanseas Tuna Ltd, who was a key supporter of this work and without it this project would not have been possible.
- Staff of Cleanseas Tuna Ltd, Dr Bennan Chen, Mr David Poppi and Mr Adam Miller, for their technical assistance throughout the project and for access to their facilities, staff and larvae.
- Mr Robert Staunton and the harvest crew from Australian Tuna Fisheries for allowing access to their Southern Bluefin Tuna for sampling during commercial harvests.
- Staff and students from the University of the Sunshine Coast. In particular, Dr Scott Cummings, Dr Tomer Ventura, and other Genecology Research Centre members for providing helpful advice to Mr Ido Bar while implementing new molecular techniques, especially protein related issues. Also, Dr Scott Cummings, Mr Jorge Fernandez, Ms Adi Bar and Mr David Bright for traveling to Arno Bay to provide critical technical assistance to Mr Andre Smith and Mr Ido Bar during transplantation experiments and Dr Nyugen Nyugen for assisting Mr Andre Smith with his statistical analysis.
- Mr Luke Dutney, Mr Dave Nixon, Mr Matthew Campbell, Mrs Hazra Thaggard and Dr Peter Lee from Bribie Island Research Centre, for providing the aquaculture facilities and research vessel for the capture, handling and maintaining of the alternative host fish.
- All recreational fishermen who provided observations and samples of wild fish.
- Prof Goro Yoshizaki, Prof Yutaka Takeuchi, Dr Ryosuke Yazawa and their students at the Tokyo University of Marine Science and Technology, for allowing Prof Abigail Elizur, Dr Erin Bubner and Mr Ido Bar to visit their laboratories in Japan, for sharing their methodologies and knowledge and providing essential training in advanced germ cell transplantation techniques.

1. Introduction and Background

Breeding Southern Bluefin Tuna (SBT) (*Thunnus maccoyii*) in captivity is proving to be a major challenge. While Clean Seas Tuna Ltd (CST) successfully produced tuna offspring in the last six years in their onshore breeding facility at Arno Bay, the number of broodstock that can be held is limited due to their size (~150kg) and the availability of 10-12 year old fish, the age of sexual maturity. Further to this, SBT broodstock cannot be handled for reproductive assessment, and the spawning season in captivity is short, thus limiting work on larval rearing and juvenile stages. Therefore, in order to secure a steady supply of SBT seed, both for research and development and commercial applications, improvement in SBT reproductive performance is needed.

One of the most important recent discoveries that may revolutionise SBT aquaculture has been the development of surrogate broodstock technology, pioneered by Professor Goro Yoshizaki and his research group in Japan (Takeuchi et al. 2004). This refers to the technique whereby immature germ cells or spermatogonia from a target fish species are transplanted into larvae from a related species (surrogate) that is easy to raise and mature, so that the germ cells colonise the surrogate gonad, to produce sperm and eggs of the target species. This was elegantly demonstrated in the production of rainbow trout from masu salmon surrogates (Takeuchi et al. 2004). This technique offers the potential to significantly reduce the time, cost, rearing space and intensive labour that are normally necessary for the seed production of large donor species. In this manner, it is possible to not only conserve endangered species, but also drastically improve the supply efficiency of marine products. The application of surrogate broodstock technology could significantly improve the efficiency of seed production for tuna to counter the rapidly decreasing numbers of sexually mature fish seen in wild captures in the recent years. Maintaining and spawning SBT in captivity is a major challenge and a key researchable constraint to developing tuna aquaculture based on captive bred and reared stock. SBT take up to 12 years to mature, and since mature adults weigh about 150 kg, they are difficult to handle and farming requires large facilities, manpower and costs. This project, in collaboration with Professor Goro Yoshizaki, provided us an unprecedented opportunity to extend on his ground-breaking work with salmonids and other species, to the benefit the Australian tuna aquaculture industry, by generating SBT surrogates.

Surrogate technology is already used across the medical and agricultural disciplines, when embryos from high value cows or mares are implanted in less valuable surrogates. The extension of this technology to fish opens up the exciting possibility of transplanting SBT germ cells in a small and fast growing host (such as Yellowtail Kingfish (YTK), *Seriola lalandi*), and obtaining a surrogate that develops gonads with SBT eggs and sperm. Surrogates for SBT would transform broodstock management, reducing dramatically the cost of facilities, enable year round SBT seed production, and open the door for the development of a genetic selection program for SBT, as it would effectively cut the generation time to 2-3 years.

1.1 Need

Currently SBT is being bred in an expensive on-shore facility at Arno Bay, where a single tank holds a limited number of broodstock, which spawn for a limited period of time. In order to expand on the production of SBT seed, additional facilities/tanks at the costs of millions of dollars will be required in addition to the need to source additional 12 year old broodstock. Therefore, there is a need to look at alternative approaches for SBT broodstock management. This project explored the application of a highly innovative approach - that is the use of fish surrogates to produce SBT. By identifying the right surrogate for SBT and developing the specific know-how with respect to optimal germ cell management, SBT seed could be produced in a fast maturing small host. This would completely overcome the need for large, expensive broodstock facilities and long term holding of broodstock, while ensuring a continuous supply of SBT seed, which is much needed for larval rearing research and development and commercialization. This project relates to the overall investment in closing of the life-cycle of SBT.

1.2 Objectives

1. To develop and optimise protocols for the collection and dissociation of SBT germ cells for transplantation
2. To assess and optimise cryopreservation procedures of SBT germ cells
3. To investigate and establish potential surrogate host for SBT
4. To develop molecular tools for the identification of SBT germ cells in surrogate host
5. To develop strategies for sterilization of surrogate host to obtain only SBT eggs and sperm for transplanted fish

2. Methods

2.1 To develop and optimise protocols for the collection and dissociation of SBT germ cells for transplantation

2.1.1 Collection of SBT testis material

Testis material was collected from SBT during commercial harvests from sea-cages offshore of Port Lincoln, South Australia. The testis material was then transferred into approximately 100 mL of 10mM phosphate buffered solution (PBS) (Sigma, P4417), which was kept on ice until arriving back at the laboratory approximately four hours after collection.

2.1.2 Optimisation of the dissociation protocol

The testis material was removed from PBS and weighed with and without the mesenteric fat. It was then dissected into small pieces (between 300 and 1000 mg), transferred into a glass cavity slide with small volume of PBS (between 2 and 3 mL) and minced with Wecker scissors. Once the testis material had been minced to a paste-like consistency, it was transferred into a 15 mL tube and rinsed twice in Leibovitz's-15 (L-15) media (Gibco Invitrogen, 11415-064) containing 1% Penicillin/Streptomycin (Sigma, P0781). Rinsing consisted of pipetting the solution up and down gently in the 15 mL tube to mix the cells before it was centrifuged at 200 g for 5 min at 4^{o}C. The supernatant was discarded between each rinse. The cells were then transferred into a 12 well plate with the corresponding dissociation media for incubation. Different dissociation enzymes, concentrations of enzymes and amount of minced testis material were examined, as described in Table 1.

Table 1. Summary of the different dissociation enzymes, concentrations of enzymes and amount of minced testis material examined in the three experiments involved in optimising the dissociation of SBT testis material

Experiment	Dissociation Enzymes	Concentration of enzyme	Amount of minced testis tissue (mg.ml^{-1})	Incubation temperature and time	Percoll gradient
1	Collagenase/ dispase	2 mg.mL^{-1} /600U	30mg	2h at 30^{o}C	No
2	Collagenase/ dispase or trypsin	4mg.mL^{-1} /1200U or 0.0583	30, 50, and 100mg	2h at 30^{o}C	No
3	Collagenase/ dispase or trypsin	2mg.mL^{-1} /1000U or 0.0583	100, 200, 300, 400 and 500mg	3h at 25^{o}C	Yes

In Experiment 1, the efficacy of three different working concentrations of collagenase/dispase to dissociate the tissue were examined: a) 2 $mg.mL^{-1}$ of collagenase and 600 U of dispase; b) 4 $mg.mL^{-1}$ collagenase and 1200 U of dispase and: b) 10 $mg.mL^{-1}$ of collagenase and 6000 U of dispase (units of dispase are expressed as Standard Japanese Units). 30 $mg.mL^{-1}$ of SBT testis material was examined here. In Experiment 2, the efficacy of collagenase/dispase combined (4 $mg.mL^{-1}$ and 1200 U, respectively) or trypsin (0.0583 $mg.mL^{-1}$) were examined for the dissociation of three different amounts of tissue for dissociation (30, 50 and 100 $mg.mL^{-1}$). Experiment 3 examined varying amounts of SBT testis material for dissociation (100, 200, 300, 400 and 500 $mg.mL^{-1}$), using enzymes trypsin or a combination of collagenase/dispase. The working concentration of trypsin was 0.0583 $mg.mL^{-1}$ and for the combined collagenase/dispase, the concentration was 2 $mg.mL^{-1}$ and 1000 U, respectively.

Once the corresponding dissociation media was added to the rinsed tissue, as described above, the tissue was incubated according to Table 1. Whilst incubating, the cell suspension was gently mixed by pipetting slowly up and down every 30 min for 30 sec. Following the incubation, the dissociation media and tissue were filtered at 150 and 50 µm (CellTrics filter, Partec GmbH, Germany), sequentially. The cell suspension was then slowly pipetted into a 15 mL centrifuge tube containing a Percoll gradient (the Percoll gradient separates cell types so a cell suspension enriched in type-A spermatogonia could be obtained) (Figure 1). The Percoll gradient was prepared by diluting Percoll Plus (GE Health, 17-5445-01) with PBS to 20% and 30% concentrations. Two mL of the 30% Percoll was pipetted into the 15 mL centrifuge tube before 1 mL of the 20% Percoll was very slowly pipetted on top (Figure 1A). One mL of the cell suspension was then slowly pipetted onto the 20% Percoll layer and the Percoll gradient was then centrifuged at 800 g for 30 minutes at 4°C (Figure 1B). The Spermatogonia enriched fraction was then carefully pipetted from within the 20% Percoll/PBS layer before performing two washes with 3 mL of L-15 media containing 1% Penicillin/Streptomycin. The cells were counted using a haemocytometer under a compound microscope (Olympus BX51) before and after the Percoll gradient, to assess the effectiveness of the gradient for each treatment group.

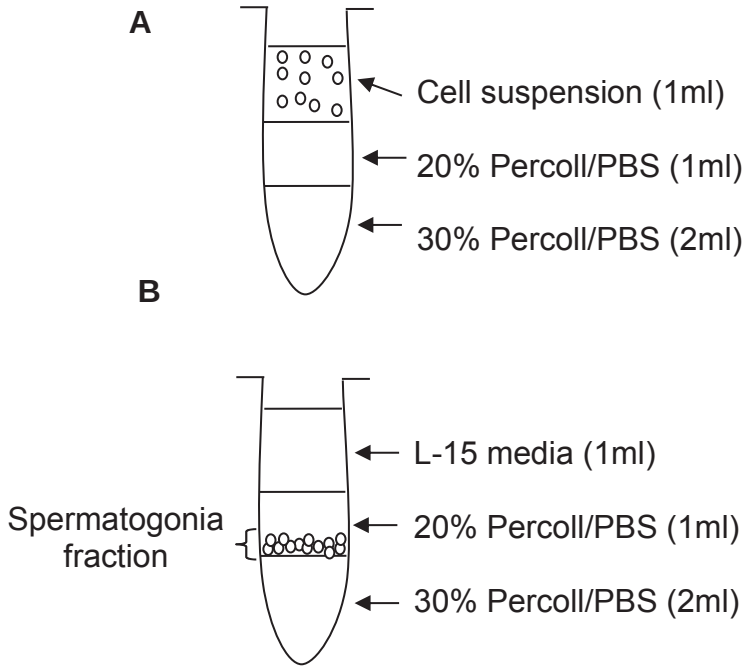

Figure 1. Illustrative representation of the Percoll gradient process before (A) and after (B) centrifuged at 800g at 4°C for 30 minutes.

The cells in the resulting cell suspension from the Percoll gradient was then rinsed twice in L-15 media containing 1% Penicillin/Streptomycin before being counted for each treatment group using a haemocytometer under a compound microscope (Olympus BX51).

2.1.3 Assessment of the effect size and reproductive maturity of SBT on testicular cell numbers and viability

Testis material was sampled every month from SBT during their commercial production season, as described in Section 2.1.1. Material was collected from fish ranging in size from 17 to 71 kg (n = 84) between June and October, 2012 and between April and May, 2013 (Table 1). Water temperature during the sampling period was obtained from Australian Southern Bluefin Tuna Industry Association, which was logged every 15 min from a sea cage offshore of Port Lincoln, South Australia. Mean daily water temperature (ºC) ranged between 13.4 and 20.0ºC during 2012 sampling period, and 17.6 and 19.9ºC during 2013 sampling period (Figure 2).

Table 1. Details of Southern Bluefin Tuna (*Thunnus maccoyii*) sampled for maturation assessments during commercial harvests between June and October, 2012 and April and May, 2013.

Fish No.	Month/ Year sampled	Fish whole wet weight (kg)	Fork length (cm)	Gonad weight without fat (g)	GSI	Size Class	Maturation class	Cell count (cells.mL^{-1})	Cell Viability (%)
1	June '12	59.6	142	40.63	0.068	L	3	67.5	92.4
2		33.7	119	18.84	0.056	M	2	42.0	90.8
3		43.7	131	23.47	0.054	L		15.0	89.4
4		40.6	128	20.67	0.051	M	2	35.0	92.3
5		36.0	117	14.72	0.041	M	2	48.0	92.5
6		26.3	108	4.02	0.015	S		10.0	76.7
7		25.4	105	6.30	0.025	S	1	6.0	89.0
8		29.7	112	12.53	0.042	M	1	13.0	85.4
9		42.1	127	25.53	0.061	L		26.0	89.7
10		20.9	100	5.70	0.027	S	2	15.0	94.9
11		23.9	103	7.16	0.030	S	1	8.0	79.3
12		55.1	145	52.55	0.095	L	4	108.0	88.4
13	July '12	25.0	103	4.89	0.020	S		8.0	87.2
14		41.8	127	22.12	0.053	L		70.0	95.1
15		31.7	117	14.38	0.045	M	2	27.3	93.9
16		46.5	128	26.47	0.057	L	2	100.0	95.7
17		22.1	104	3.38	0.015	S	1	9.8	90.0
18		22.6	104	4.02	0.018	S	1	10.8	91.0
19		43.0	127	15.41	0.036	L	1	41.8	94.9
20		31.7	113	9.06	0.029	M	1	22.8	91.9
21		36.5	123	12.06	0.033	M	1	7.3	89.8
22		42.7	124	17.01	0.040	L	1	19.5	94.6
23		23.3	103	7.24	0.031	S	2	6.0	96.4
24		41.5	126	20.45	0.049	L		41.5	92.7
25	Aug '12	37.9	120	17.13	0.045	M	2	69.3	90.4
26		22.9	109	7.39	0.032	S	1	6.8	79.0
27		47.9	132	26.05	0.054	L		86.5	91.0
28		58.0	144	36.32	0.063	L	1	20.5	90.2
29		34.5	117	20.82	0.060	M	2	142.0	72.9
30		28.6	109	8.95	0.031	M		13.5	85.5
31		18.3	93	3.91	0.021	S	1	9.5	95.0
32		46.8	131	20.39	0.044	L		23.8	88.7
33		30.5	111	4.03	0.013	M	1	5.0	80.4
34		16.7	92	3.68	0.022	S	1	2.5	75.4
35		46.4	129	27.93	0.060	L		28.8	84.7
36		57.7	142	50.10	0.087	L	2	57.3	80.0
37	Sept. '12	19.5	100	5.10	0.026	S	1	249.3	48.9
38		30.3	118	13.93	0.046	M		40.0	30.9
39		35.0	116	12.00	0.034	M	1	46.3	19.6
40		24.8	109	8.19	0.033	S	2	30.0	26.6
41		26.5	108	9.45	0.036	S		71.0	64.3
42		31.7	120	15.15	0.048	M	2	40.0	86.8
43		38.9	122	28.83	0.074	M	2	195.8	92.5
44		25.6	105	8.91	0.035	S		23.5	83.9
45		65.1	139	78.53	0.121	L	2	3268.0	87.1
46		71.0	145	79.66	0.112	L	2	2740.0	91.1
47		67.2	144	71.60	0.107	L	2	364.0	90.8
48		45.4	129	38.33	0.084	L	2	932.0	91.8
49	Oct '12	19.83	100	5.26	0.027	S	2	249.3	48.9
50		50.37	132	63.76	0.127	L	3	3268.0	87.1
51		44.68	129	22.60	0.051	L		2740.0	90.2
52		26.73	109	7.92	0.030	S		30.0	83.5
53		34.43	116	12.06	0.035	M	1	40.0	96.0
54		54.63	140	106.60	0.195	L	3	364.0	89.4
55		25.22	107	7.04	0.028	S	1	71.0	87.8
56		28.52	112	11.77	0.041	M	1	46.3	98.0
57		48.9	136	128.86	0.264	L	3	932.0	95.4
58		31.79	116	10.14	0.032	M	1	40.0	80.6
59		30.27	113	23.67	0.078	M		195.8	96.1
60		24.79	103	10.84	0.044	S		23.5	84.4
61	April '13	45.4	125	22.01	0.048	L	2	34.3	83.7
62		17.2	94	2.55	0.015	S	2	6.3	82.5

Table 1. Continued

Fish No.	Month/ Year sampled	Fish whole wet weight (kg)	Fork length (cm)	Gonad weight without fat (g)	GSI	Size Class	Maturation class	Cell count (cells.mL^{-1})	Viability (%)
63	April '13	35.8	118	10.21	0.029	M		7.0	55.5
64		20.3	100	6.23	0.031	S	1	1.8	55.6
65		29.8	112	9.62	0.032	M	2	7.0	83.8
66		30	111	11.31	0.038	M	3	22.0	93.0
67		31.2	117	12.85	0.041	M	1	3.0	55.6
68		20.6	99	3.96	0.019	S	1	8.5	73.0
69		43.6	129	27.17	0.062	L	3	4.5	93.8
70		32.1	118	9.74	0.030	M		1.8	55.6
71		43	126	23.23	0.054	L	3	54.3	91.1
72	May '13	35	121	15.61	0.045	M	2	9.3	86.5
73		24.2	104	5.78	0.024	S	1	7.0	78.6
74		21.7	103	6.47	0.030	S	1	5.0	46.5
75		41.7	126	25.95	0.062	L	2	74.5	92.8
76		26.7	103	8.96	0.034	S	1	36.3	90.4
77		35.2	119	16.57	0.047	M		20.8	89.2
78		30.2	114	12.22	0.040	M	2	19.8	90.9
79		31.8	115	11.14	0.035	M	2	27.3	88.4
80		23.7	102	5.91	0.025	S		27.0	72.2
81		37.4	119	115.27	0.308	M		40.5	91.3
82		39.5	121	18.66	0.047	M		20.5	38.0
83		41.3	123	15.81	0.038	L	2	10.8	53.6
84		45.3	129	18.23	0.040	L	2	8.5	66.5

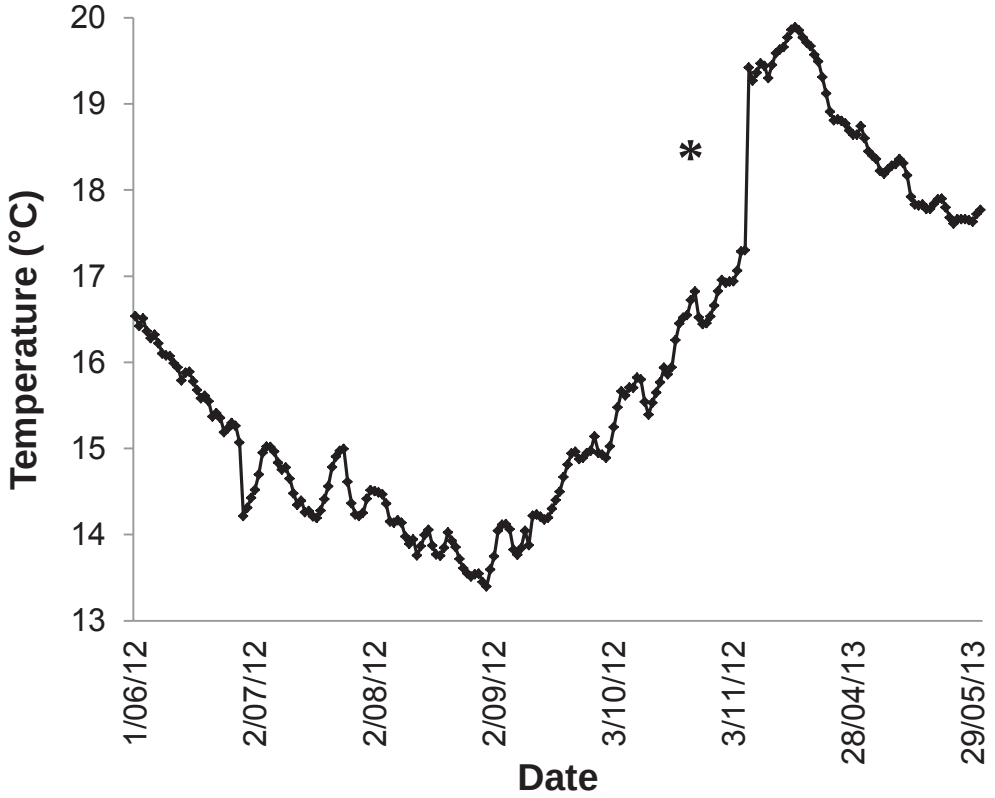

Figure 2. Mean (± SE) daily water temperature (°C) logged from sea cages near Port Lincoln, South Australia between June and October in 2012 and April and May in 2013. * Denotes missing data.

A cross section of testis material was sampled from the middle of one lobe from each fish and placed in Bouins fixative (Sigma, HT10131) and stored overnight at 4ºC. The following day, the sampled testis tissue was washed thoroughly in 70% ethanol and subsequently stored in 70% ethanol at 4ºC until required for histological analysis (see Section 2.1.3.1). The remaining testis material was then divided into 1 g pieces and minced and washed as described in Section 2.1.2. The testis material was then incubated in 2 mL of 40 $mg.mL^{-1}$ Collagenase H (Roche, 11074059001) and 33.3 $mg.mL^{-1}$ Dispase II (Roche, 04942078001) in L-15 media containing 5% foetal bovine serum (FBS) (Invitrogen, 10437-077) and 1% DNase 1 (Roche, 11284932001) for 3h at 25ºC. During the enzymatic dissociation, gentle pipetting was applied to physically disperse any remaining intact portions of the testis. The resultant cell suspension was filtered through 150 µm and 50 µm filters (CellTrics, Partec GmbH, Germany) to eliminate non dissociated cell clumps before being washed two more times in L-15 media containing 1% Penicillin/Streptomycin. The cell suspension was then resuspended in 1 mL of L-15 media containing 1% Penicillin/Streptomycin and slowly pipetted into a 15 mL centrifuge tube containing a Percoll gradient. Once the Percoll gradient was performed, the donor cells were then resuspended in 3 mL of L-15 media containing 1% Penicillin/Streptomycin, 1% FBS and 1% DNase 1 and stored at 4ºC for up to 48h.

At 0 and 24h after dissociation, the viability of the cells was assessed using the Trypan blue exclusion method (Kobayashi et al. 2007). Specifically, the resultant cell suspension for each fish was immediately incubated in the presence of Trypan Blue dye (Sigma, T8154) for 2 minutes prior to counting the cells on a haemocytometer. Cell survival was assessed by examining the cells ability to exclude the Trypan blue dye. If the cell was able to exclude the blue dye from its cell membrane, it was classed as a viable cell. Alternatively, if a cell appeared to have a blue cell cytoplasm, it was classed as non-viable.

2.1.3.1 Histological analysis

The testis material stored in 70% ethanol was embedded in a paraffin wax and sectioned (7 µm thick) using a microtome. These sections were stained with Harris haematoxylin and eosin stains, permanently mounted using DePex (BDH Chemicals), examined and digitally imaged using a compound microscope (100 to 400× magnifications, Olympus BX51) equipped with a digital camera (Olympus, Camedia C-7070). The stage of reproductive maturity was then assigned to each

sample classified on an ordinal scale of 1 to 5 according to the criteria previously described in Bubner et al. (2012) for SBT.

2.1.4 Statistical analysis

Results are presented as mean values ± standard error. Statistical analyses were performed using SPSS statistics package volume 20. A two-way analysis of variance (ANOVA) was used to analyse significant differences between means ($P < 0.05$). All correlation analysis between cell numbers, viability, size and reproductive maturity of SBT was performed using a general linear model ($P < 0.05$).

2.2 To assess and optimise cryopreservation procedures of SBT germ cells

The cryopreservation of SBT testis material was assessed and optimised in two separate experiments. The first experiment directly examined the suitability of a protocol developed by Professor Goro Yoshizaki's research group from the Tokyo University of Marine Science and Technology, for the cryopreservation of Pacific Bluefin Tuna (*Thunnus orientalis*) (PBT) testis material. The second experiment examined different variations of the protocol for the cryopreservation of SBT testis material (see Table 2). For both experiments, testis material was collected from SBT, as described in Section 2.1.1.

Table 2. Summary of treatment groups examined in experiment two of the cryopreservation trials

Treatment no.	Biocell used	Temp when in Biocell (^{o}C)	Time in Biocell (min)	Plunged into N_2	Concentration of DSMO (%)
1	Yes	-80	90	Yes	9
2	Yes	-80	90	Yes	5
3	Yes	-20	90	Yes	9
4	No	-	-	Yes	9

In the first experiment, the freshly collected testis material from one SBT (Fish 1, Table 3) was divided into 500 mg pieces that were minced separately in a glass cavity slide using Wecker scissors. The minced tissue was then transferred into 2 mL cryogenic vials with 1 mL of cryopreservation media (1.5% bovine serum albumin (Sigma, A2153) and 100 mM Trehalose Dihydrate (Sigma, T9531) in Leibovitz's L-15 media, 9% of the cryoprotectant dimethyl sulfoxide (DMSO) (Sigma, D4540)). The vials were then placed into pre-chilled (4ºC) Biocell bio freezing vessels (Nihon Freezer Co. Ltd, Japan) and transferred into -80ºC for 90 min before being plunged into liquid nitrogen. The remaining testis material was stored at 4°C overnight in PBS.

Table 3. Summary of Southern Bluefin Tuna (*Thunnus maccoyii*) (SBT) sampled for cryopreservation trials

Fish No.	Experiment No.	Length (cm)	Weight (kg)	Gonad weight (g)	GSI	Maturation stage
1	1	148	70.9	61.21	0.086	2
2	2	126	42.8	24.51	0.057	2
3	2	129	43.0	26.38	0.061	2
4	2	139	56.6	48.72	0.086	2
5	2	148	70.9	131.22	0.185	4

*Denotes gonad weight without fat, GSI = Gonadosomatic index

In the second experiment, the testis material of four SBT (SBT 2, Table 3) was again divided into 500 mg pieces in the laboratory and minced separately in a glass cavity slide using Wecker scissors. The minced tissue was then transferred into 2 mL cryogenic vials with 1 mL of cryopreservation media (1.5% bovine serum albumin and 100 mM Trehalose Dihydrate in Leibovitz's L-15 media) containing two different amounts of the cryoprotectant DMSO (5 and 9%). The vials were then placed into chilled Biocell bio freezing vessels that were either transferred into -20 or -80ºC for 90min before being plunged into liquid nitrogen. The last treatment was plunged directly into liquid nitrogen without the use of a Biocell. A summary of the different treatment groups is shown in Table 2.

After overnight storage in liquid nitrogen, the testis material was thawed at 15ºC for 3 min, washed once with Leibovitz's L-15 media containing 1% penicillin/streptomycin that was pre-chilled to 4ºC, and subsequently dissociated using the optimised protocol described in Section 2.1.3. The testis material stored at 4°C overnight was also dissociated using this protocol concurrently. At 0 and 24h the cell viability of the resultant cell suspension was assessed using the Trypan blue exclusion method for each treatment group, as described previously.

2.2.1 Statistical analysis

Results are presented as mean values ± standard error. Statistical analyses were performed using SPSS statistics package volume 20. A two-way analysis of variance (ANOVA) was used to analyse significant differences between means ($P < 0.05$).

2.3 To investigate and establish potential surrogate host for SBT

2.3.1 YTK as a potential surrogate host for SBT

Five transplantation experiments examining the suitability of YTK took place at Cleanseas Tuna's Hatchery in Arno Bay, South Australia between August 2012 and September 2013.

2.3.1.1 Testicular cell preparation

Testicular cells for transplantation were prepared from testis material collected from SBT as described in Section 2.1.1. Following collection, the testis material was divided into 1 g pieces and minced with Wecker scissors in a glass cavity slide before being dissociated using the protocol described in Section 2.1.3. Following dissociation, the number of viable cells per 1 g of testis material was counted using 15 µL of the cell suspension on a Neubauer counting chamber after Trypan Blue exclusion and extrapolating the results. For visualisation, the remaining cells were then stained with the fluorescent membrane dye PKH26 (Sigma, PKH26GL). Approximately 10 million cells were suspended in 400 µL of diluent C (an iso-osmotic aqueous solution provided with the PKH26 dye) with 4 µL of dye and incubated for 5 min. The suspension was then centrifuged at 100 g for 5min at 4ºC. The supernatant was discarded and 3 mL of L-15 media containing 1% Penicillin/Streptomycin was then added to the cells. The resultant cell suspension was then washed twice more, by centrifuging the cell suspension two more times, discarding the supernatant and re-suspending the cells in L-15 media containing 1% Penicillin/Streptomycin. The cells were stored on ice for up to 48h prior to transplantation.

2.3.1.2 Germ cell transplantation

Transplantation needles were prepared by pulling thin walled glass capillaries (Narishige, G-100) using an electric puller (Narishige, PC-10). The tips of the needles were sharpened with an electric grinder (Narishige, EG-400) until the opening of the needle reached 40-60 µm. Recipient larvae (6 to 11 DPH) were sourced from stocks reared in 280, 3000 or 8000 L tanks. For transplantation, the larvae were anaesthetized with 0.0075% ethyl 3-aminobenzoate methanesulfonate salt (Sigma, A5040) in seawater, which contained 0.1% bovine serum albumin. They were transferred onto a petri dish coated with 3% agar using a wide bore 3 mL plastic transfer pipette. Donor cells were then transplanted into the anaesthetised larvae with a micromanipulator (Narishige, M33-01R) and microinjector (Narishige, IM-9B) attached to a dissection microscope (Olympus, SZH10). After transplantation,

recipient larvae were transferred from the petri dish to a recovery tank filled with seawater, before being transferred into a larval 100 or 280 L rearing tank. Control larvae (i.e. non-transplanted larvae) were also stocked at this point into separate larval rearing tanks.

2.3.1.3 Rearing of transplanted larvae

Different larval rearing systems were used in each transplantation experiment, where various modifications were made over time to enhance larvae survival post-transplantation (see Table 4).

Table 4. Summary of larval rearing conditions, origin and transplantation age of larvae for transplantation trials that took place at Cleanseas Tuna's YTK hatchery in Arno Bay, South Australia between August 2012 and September 2013. Larvae were transplanted over a five day period.

Trial No.	Date	Origin of larvae	Age (DPH) when transplanted	Number of larvae transplanted	Larval Rearing Tank Size	System type
1	Aug '12	280L tanks	6 – 11	2691	280L	Flow through
2	Sept '12	280L tanks	6 – 11	3173	280L	Flow through
3	Nov '12	280L tanks	6 – 11	2370	100L	Re-circulation
4	June '13	3000L R&D tanks	6 – 10	1680	100L	Re-circulation
5	Sept '13	8000L commercial tanks	6 – 10	2171	100L	Flow through

Larval rearing tanks were either part of a flow-through system, or part of a recirculation system where the out-going water was collected back into a biofilter. Specifically, in the flow through system tanks were supplied with filtered seawater (sand and 25 µm sock filtered) with a daily water exchange rate of 450% at 0 DPH, which increased to 600% by 30 DPH. One air dissolving stone was placed in each tank and dissolved oxygen ($mg.L^{-1}$ and % saturation), water temperature and pH were monitored three times daily. Flux, salinity and ammonia were monitored at the start and end of the experiment. The tanks were cleaned daily using a siphon hose, the water outlet screens were cleaned every second day and the surface of the water was skimmed up to five times daily to remove any build-up of foreign particles, dead rotifers, *Artemia* and excess protein. Two fluorescent tube lights (35W each) above each rearing tank provided a surface light intensity of 255 lux at the centre of each rearing tank and a 12h light (09:00 to 21:00) and 12h dark photoperiod was used. A 300 W aquarium heater (280 L tanks, Aqua One, Ingleburn, Australia), or a 2.5 kW coil heaters (850 L tank, Hotco, Australia), were placed in the tanks to assist in maintaining constant temperature. In the recirculation system, the biofilter recirculated between an 850 L sump tank, a 350 L tank that contained small plastic

beads that promoted the growth of ammonia stripping bacteria, and a protein skimmer to rid the water of excess protein. The biofilter's 850 L sump tank was heated by two coil heaters (2.5 kW, Hotco, Australia). The larval rearing tanks were supplied with water from the biofilter's 850 L sump tank that flowed through a 5 and 1 µm sock filter as well as an ultra-violet light filter. The exchange rate in the 100 L larval rearing tanks was 1000% daily water exchange throughout the trial. Aeration, light regime, water quality monitoring, tank cleaning and maintenance, water temperature control for the transplantation trials were the same as the stocking density trial in Section 2.1. Nanopaste and live feeds (rotifers and *Artemia*) were added to all larval rearing tanks in the transplantation trials as described in Section 2.1. From 18DPH, the larvae were fed two types of artificial dry food (200 – 300 µm Start L and 300 – 400 µm Wean S, INVE, Belgium) until the end of the experimental periods. The number of surviving larvae was assessed at the conclusion of the experiments.

2.3.1.4 Assessment of SBT donor testicular cells in recipient YTK larvae

Migration and colonisation of the PKH26 labelled SBT donor testicular cells in the host YTK gonad was evaluated by observing the transplanted fish under a fluorescent microscope (BX50, Olympus) at 18 and 28 days post transplantation (DPT) (29 and 39 DPH respectively). To facilitate examination of the gonad in the body cavity of the larvae, the head and digestive organs were removed. The gonad was initially observed in the body cavity before fixing the larvae using a rapid fixative (Ufix, Sakura, Japan) for 3 min, followed by 2 rinses in PBS to assist the removal of the gonad. Once the gonad was removed, it was placed on a Frontier glass microscope slide (FRC-01, Matsunami, Japan) with a coverslip and further evaluated by observing it under a fluorescent microscope. Digital images of the migration and colonisation of the SBT donor cells were recorded using a digital camera (UC50, Olympus) attached to the microscope (Olympus BX51) and examined using image software (AnalySIS, Version 5, Olympus).

2.3.2 Alternative species as a potential surrogate hosts for SBT

The suitability of other species of the Scombrid family as alternative potential surrogate hosts for the SBT was explored. These species included the Mackerel Tuna (*Euthynnus affinis*), Australian Bonito (*Sarda australis*), Frigate Mackerel (*Auxis thazard*), Blue Mackerel (*Scomber australasicus*) and Skipjack Tuna (*Katsuwonus pelamis*). These fish species are not available from aquaculture facilities anywhere in Australia and therefore larvae for transplantation experiments needed to be produced

by the research group, following the collection and spawning of broodstock from the wild.

2.3.2.1 Broodstock tank

A purpose built 100m^3 fiberglass tank (8.2 x 2.2 m) was constructed at the Bribie Island Research Centre (BIRC) to hold the alternative species broodstock fish (Figure 3A-B). The interior of the tank was pre-painted with orange (Munsell Value-4) epoxy paint and black adhesive vinyl tape was put on the interior surface in a grid pattern (0.35 x 0.35 cm squares), as recommended by Professor Goro Yoshizaki, to prevent the broodstock fish from colliding with the tank wall. The tank was connected to a flow-through seawater system and an aeration system. A 2 m high working platform and stairs were built to allow easy and safe access to the water level (Figure 3C-D). In addition, shading of the tank was also installed to control algal growth and photoperiod for the broodstock. An egg collection device was installed at the water outlet, to collect floating eggs in case of spontaneous or induced spawning. An adjustable external standpipe was fitted, to maintain minimum water level while draining the tank, reducing the chance of accidental water drainage of the entire tank (Figure 3E-F). A quote for a recirculating water system was requested to enable better control over water temperature and water quality parameters; however, while this would have contributed greatly to the control over spawning, at $36,000 it was well beyond the project budget.

Figure 3. External (A) and internal (B) view of the purpose built 100 m^3 fiberglass tank (8.2 x 2.2 m) constructed at the Bribie Island Research Centre to hold broodstock fish of alternative surrogate host species for Southern Bluefin Tuna. Additional stairs (C) and working platform (D) to allow easy and safe access to tank were built, as well as egg collection tank and external standpipe (E, F – top view).

2.3.2.2 Transport unit - Tuna Tube

The first transport unit that was constructed to assist in transporting the wild caught fish onshore was based on a design that has been used by other research groups in the past to successfully transport live Scombrid fish, such as Leaping Bonito, Skipjack Tuna and Mackerel Tuna and is commonly referred to as a 'Tuna Tube'. The unit consists of a single 300 L tank (1 x 0.6 x 0.5 m) with 6 vertical tubes (18 cm diameter x 50 cm high each) installed in the tank, held together by an aluminium frame (Figure 4). During transportation, the fish are positioned inside the vertical tubes. At the bottom of each tube a plastic funnel is connected to a water flow system to allow water to flow over the gills of the fish while they are being transported. The amount of water flow to each tube can be controlled using a valve at the base of the funnel. Uptake of fresh seawater to the vertical tubes is facilitated through a 1" water pickup located at the boat's transom, with a head height of 1 m. Next to the water pickup, a 2200 gallons per hour (gph) bilge pump has been mounted to continue to supply fresh water to the tubes when the boat is idle. There were also another two 1500 gph bilge pumps that can be connected to the water flow system inside the tank to recirculate the water from the tank to the tubes, in case there was a problem with the auxiliary pump and pickup intake. The transport unit was designed so it is totally portable and can be moved between different boats, depending on their availability and the targeted fishing area.

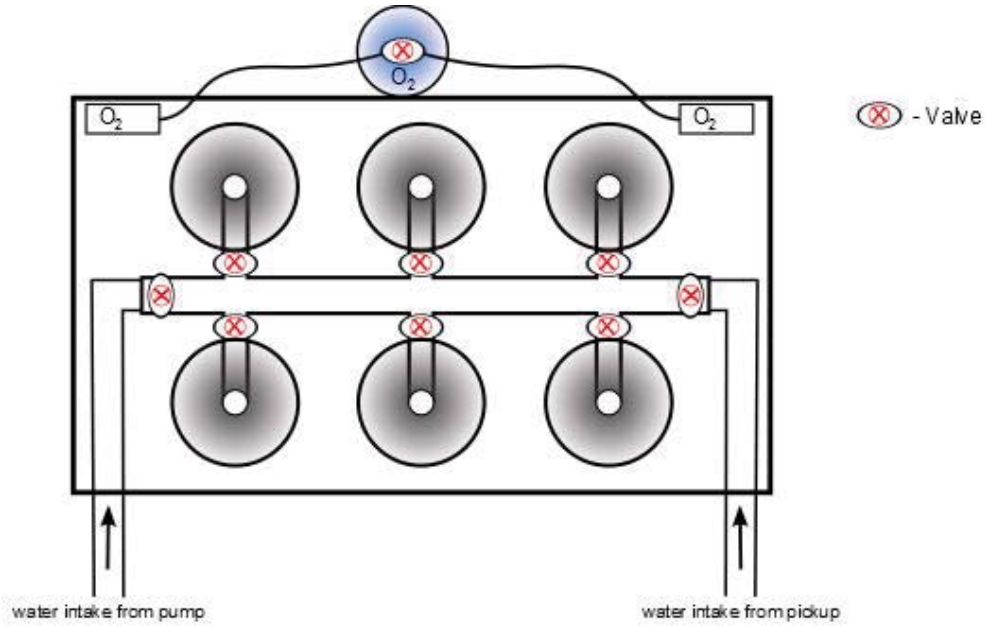

Figure 4. Diagram of the transport unit that has been constructed to move wild fish caught offshore to the onshore facility at Bribie Island Research Centre as broodstock of alternate surrogate host species.

2.3.2.3 Transport unit - Modified Tuna Tube

Modifications were made to the Tuna Tube transport unit to eliminate problems that were found when using the unit during the first six fishing trips. Soft, high density foam that becomes very smooth when wet was added to the inside of the tuna tubes to help protect the skin of the fish during transport (Figure 5A). Two gas dissolving stones, a 5 L oxygen tank and regulator were also mounted on the transport unit to allow oxygen to be added to the water during transport of the fish if it was required (Figure 5A), additional holes have been drilled at the bottom of the funnels to improve water flow through the tubes (Figure 5B) and improvements in the fresh water pickup were also made (Figure 5C).

Figure 5. Tuna tube apparatus. **A** – Six tubes attached to an aluminium frame and placed inside a 300 L tank. Note the oxygen on the bottom of the picture and the water inlet pipes at the top. **B** – Funnel at the bottom of the tube, directs the water over the snout of the fish. **C** – One of two 1500gph bilge pumps connected to the tubes water system that recirculates the water from the tank to the tubes.

2.3.2.4 Transport unit – On-board transport tanks

Associate Professor Yutaka Takeuchi, a project collaborator and mentor from the Tokyo University of Marine Science and Technology in Japan, recommended using round 500 L tanks for transportation of live little tuna (1-2 kg). Following his recommendations, the research group examined the feasibility of using a bigger fishing vessel, capable of holding 500-1000 L water tanks. The fishing vessel FRV 'Tom Marshall' was equipped with an ocean water pump to provide a constant supply of fresh water and a second, smaller boat can be towed to allow easier approach to

the feeding fish schools. A round, covered water tank was installed on deck, 1.70m in diameter, together with an oxygen supply.

2.3.2.5 Fishing for broodstock

A total of 16 fishing trips were made to source broodstock of the listed target species. Details of these fishing trips are outlined in Table 4.

Table 4. Fishing trip number, date, fishing vessel, departure and landing location, fishing area and transport unit used during fishing trips conducted to obtain broodstock of alternative species as a potential surrogate hosts for SBT

No.	Date	Fishing vessel	Departure location	Fishing area	Transport unit
1	19/12/2011	'Makaira' DAFF fishing vessel	Spinnaker Sound Marina boat ramp	Shipping channel east of Bribie Island	Tuna Tubes
2	11/05/2012				
3	20/02/2012	Triton IV fishing charter boat, from South Queensland Charter services	Mooloolaba Marina	Gneering shoal (S26 38.609, E153 10.250) at the north to Hamilton Patches reef (S26 50.300, E153 13.250) at the south, east of Caloundra.	
4	01/03/2012				
5	24/04/2012		Spinnaker Sound Marine		
6	20/05/2012				
7	23/01/2013	FRV 'Tom Marshall' (DAFF)	Scarborough	Moreton Bay	Onboard transport tank
8	18/04/13	'Makaira' DAFF fishing vessel	"Spinnaker Sound Marina" boat ramp (S27°04.177', E153°08.032')	Shipping channel east of Bribie Island, including Skirmish Passage (S26°59.751', E153°11.126')	Modified Tuna Tubes
9	26/04/13				
10	14/05/13				
11	17/05/13				
12	24/05/13				
13	07/06/13				
14	26/06/13				
15	30/07/13				
16	06/08/13				Onboard transport tank

During fishing trips 1 to 6, fishing equipment and techniques used included metal slugs (10-20 g) that were cast towards the surface of feeding schools, using medium-light spinning rods and reels and trolling medium sized hard bodied lures. Feeding schools were visualized by presence of tern and seagull schools that were feeding on the same bait schools. Feeding fish were approached carefully in order to avoid any disturbance to the fish and the bait. Hooked fish were brought to the surface as soon as possible, landed using a "fish friendly" rubber net, unhooked using minimal contact with the skin of the fish and placed in the vertical tubes in the transport unit, head first. Water pumps were running water over the gills of the fish immediately after they were put in the tubes.

During fishing trips 8-12, to optimise the chance of catching the target species, these fishing trips required prior spotting of any of the target species, the right weather and sea conditions and the availability of DAFF staff with coxswain license to drive the boat. Special casting plastic lures that were shipped from Japan were used on these fishing trips. The lures varied in size (35-60 mm) and in colour (reflecting white/blue/silver), to specifically match the baitfish that the tuna are feeding upon (see Figure 6). Single hooks were used with barbs crimped off to ensure easy and fast release of the fish into the tubes when caught and to minimise handling and potential injury to the fish. Again, feeding schools were visualized by presence of tern and seagull schools that were feeding on the same bait schools. Feeding fish were approached carefully so the fish and bait schools were not disturbed. Hooked fish were brought to the surface as quickly as possible, landed using a PVC sling lubricated with K·Y water based gel, unhooked while maintaining minimal contact with the fish skin and placed head first into the tubes. Water pumps were used to run water over the gills of the fish as soon as they were put into the tubes.

Figure 6. Variety of fishing lures trialled to capture potential broodstock.

Landing point of the boat and live fish was dependent on surf conditions. To reduce transport time and when surf conditions allowed (i.e. swell height under 1 m), the boat approached the beach closest to the BIRC between fishing trips. A team member waited at the beach with a Recreational Off-highway Vehicle (ROV) and the transport trailer at the parking lot nearby. The pumps were disconnected from the tubes, which were unloaded from the boat and quickly loaded on the ROV and then drove up to the trailer. The tubes were transferred to the trailer into a tank with fresh, oxygenated ocean water and the pumps were re-installed and connected to a battery. The trailer was then driven to BIRC and the fish were transferred into the assigned 100 m^3 broodstock tank. Overall, the transportation from the boat to the fish tank took less than 15 minutes, out of which the fish were in the tubes without water circulation for no more than 2 minutes. This was only possible in fishing trips 8-12; with the landing of fish from fishing trips 1 to 6 a further 1.5 hours from BIRC.

2.3.2.6 Spawning of broodstock

According to Rogers et al. (2009), Blue Mackerels spawn during the winter months in east Australia waters (June-November), peaking at October. Given the season and the developmental stage of the gonads (spermiating testis, GSI>5%) of the Blue Mackerels that were successful captured and held at BIRC, two trials were carried out in attempt to induce spawning with GnRHa slow release implants, to secure larvae for transplantation experiments. We used EVAc implants and aimed to achieve a final dose of ~50-75 µg LHRHa/kg body weight. A total of 12 fish were treated. Fish were monitored daily for 10 days post transplantation.

2.4 To develop molecular tools for the identification of SBT germ cells in surrogate host

2.4.1 Cloning and sequencing of candidate genes expressed specifically in SgA cells

Total RNA was extracted from type A spermatogonia (SgA) enriched culture of germ cells stored in RNAlater solution (Sigma). Germ cells were isolated by enzymatically dissociating spermiating SBT testis (fish weight >55 kg) and discontinuous Percoll gradient was used to enrich SgA population in the culture. The cells were centrifuged at 4000 rpm for 3 minutes and RNAlater supernatant was discarded by pipetting. Total RNA was isolated using 1 ml of RNAZol RT (RN 190, Molecular Research Center), according to the manufacturer protocol. Total RNA was isolated from three samples and was sent for RNA-Sequencing Transcriptome analysis (coverage of 5 GB per sample, performed at BGI, China). The transcriptome data was assembled

and revealed the full length mRNA of *Dead-End*, a novel germ cell protein, *Piwil1/2* and partial sequences of gonadal soma-derived growth factor (*GSDF*), anti-Mullerian hormone (*AMH*) and Activin receptor type 1B/2A, all of them important genes and factors involved in germ cell differentiation and maturation. In addition, important house-keeping genes such as elongation factor 1a (*EF1a*) and 18S were detected. The *Dead-End* nucleotide sequence was found to be poorly conserved with other species, (apart from a short conserved segment of 150 nucleotides), with regions that have only 64% bases identity between Southern Bluefin Tuna and the Nile Tilapia (*Oreochromis niloticus*), the closest annotated perciform fish (see Figure 7). This result explains the previous difficulty the research group has had to detect the gene using degenerate primers, designed on the basis of conserved regions.

However, this low homology makes the *Dead-End* gene a perfect candidate as a species-specific marker, to distinguish between donor and host cells.

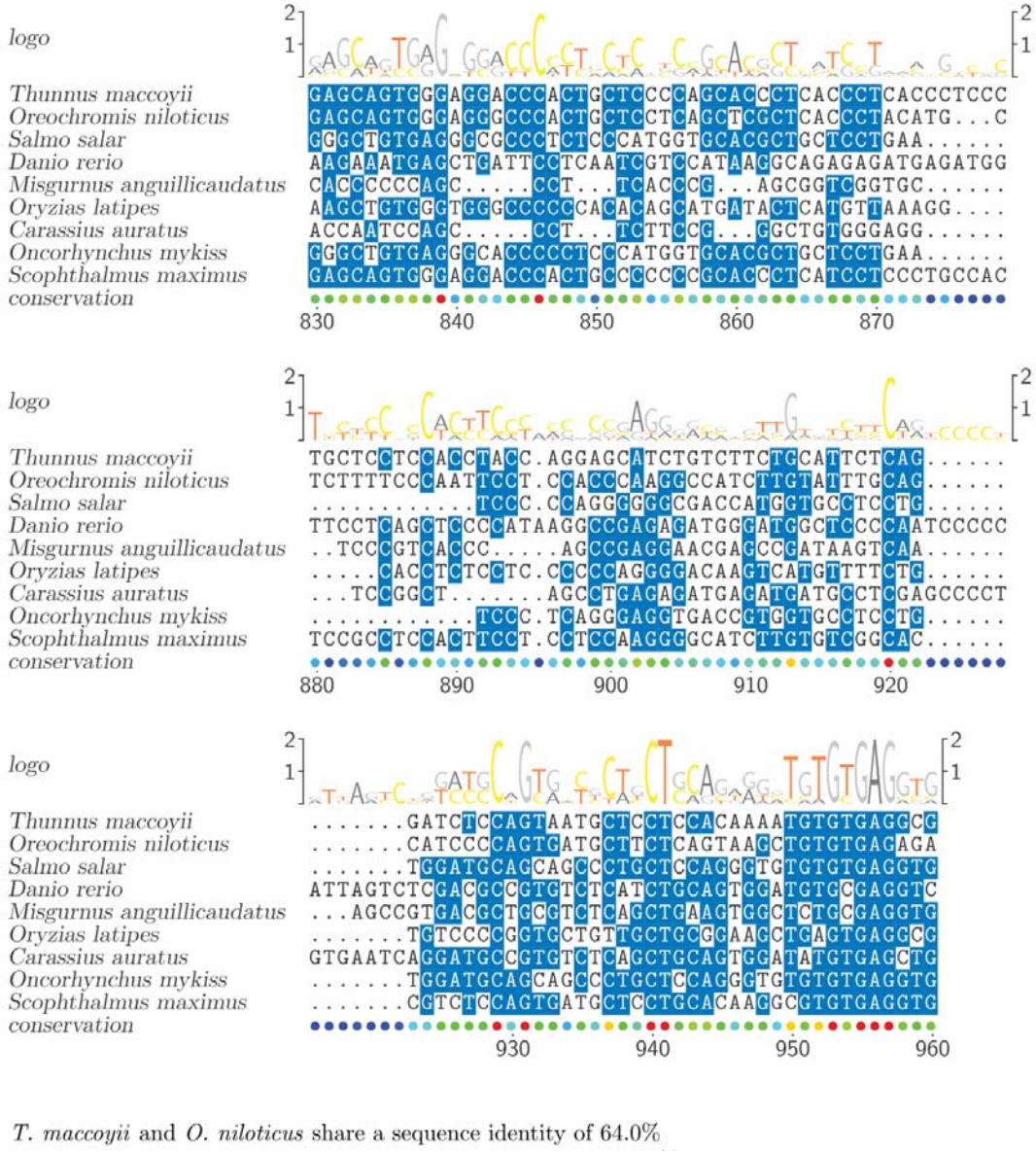

Figure 7. Sequence alignment of a poorly conserved region of the *Dead-End* gene, comparing Southern Bluefin Tuna (*Thunnus maccoyii*) and eight other fish species. The size of the nucleotides in the logo correlates to the frequency of each nucleotide among all sequences. Conservation colour coding (bottom line) denotes red colour for high conservation and blue to low conservation (hot to cold).

2.4.2 Identification and localisation of SgA cells

Specific PCR primers, flanked with bacterial SP6 and T7 promoters' sequences, were designed, ordered and used to amplify gene specific PCR products. DIG-labelled probes were synthesised, using these PCR products as templates. These probes were used in *in-situ* hybridisation experiments, to determine localised gene expression in tuna gonads and to identify SgA cells. Tuna testes were sampled from Longtail Tuna (*Thunnus tonggol*) and SBT, fixed in 4% paraformaldehyde (PFA) for 24 hours, and then dehydrated in graduated ethanol washes and embedded in

paraffin blocks. The blocks were sectioned, and the sections were dried on slides and used as template for *in-situ* experiments. All solutions prior to probe hybridisations were maintained RNase free, using either 0.1% DEPC treated MilliQ water or the addition of RNase Inhibitor (Protect RNA, R7397, Sigma Aldrich). Using a closely related tuna species allowed us to determine the necessary divergence in the nucleotide sequence, needed to distinguish between the donor and the host cells after germ cell transplantations.

2.4.3 Detection of SBT cells in transplanted YTK larvae

PCR assay to detect SBT genomic DNA (gDNA) in transplanted gonads has been developed. Specifically, transplanted YTK larvae and fingerlings were sampled 39, 47, 75 and 165 days post transplantation. The gonads were dissected, lightly fixed and stored at RNase free 70% ethanol. Whole genomic DNA was extracted from the gonads by modified protein salting-out method. In brief, the gonad was dried from ethanol and placed in 550 µl of cell lysis solution (50 mM Tris- HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl). SDS solution was added to a concentration of 1% and cells were further lysed by incubation with 1µl Proteinase K solution (20 mg/ml) for 2 hours at 50°C. Three hundred µl of 5M NaCl were added to the lysate and the sample was vortexed and centrifuged (13,000 rpm, 10 minutes) to precipitate the proteins. The supernatant liquid phase was mixed with 900 µl of freezer cold 100% isopropanol, incubated for 2 hours at -20°C and centrifuged (13,000 rpm, 5 minutes). The resulting DNA pellet was washed with 700 µl of 70% ethanol, dried for 15 minutes and dissolved overnight in 100 µl DNAse free water at 4°C. DNA concentration and quality (Optical Density OD260/OD280 ratio) were measured using NanoDrop ND-1000 (NanoDrop Technologies). DNA samples were diluted to a 25 ng/µl concentration and stored at -20°c for further analysis.

In order to distinguish between YTK and potential SBT gDNA, we have designed PCR primers for the 3' Untranslated Region (UTR) of the *Vasa* gene, which is poorly conserved between the two species. Location of the primers and conservation between the sequences can be seen in Figure 8 and shows that the design was such that no cross-amplification should occur between YTK primers to SBT gDNA and vice versa. The primers were tested and PCR conditions were optimised to verify amplification specificity.

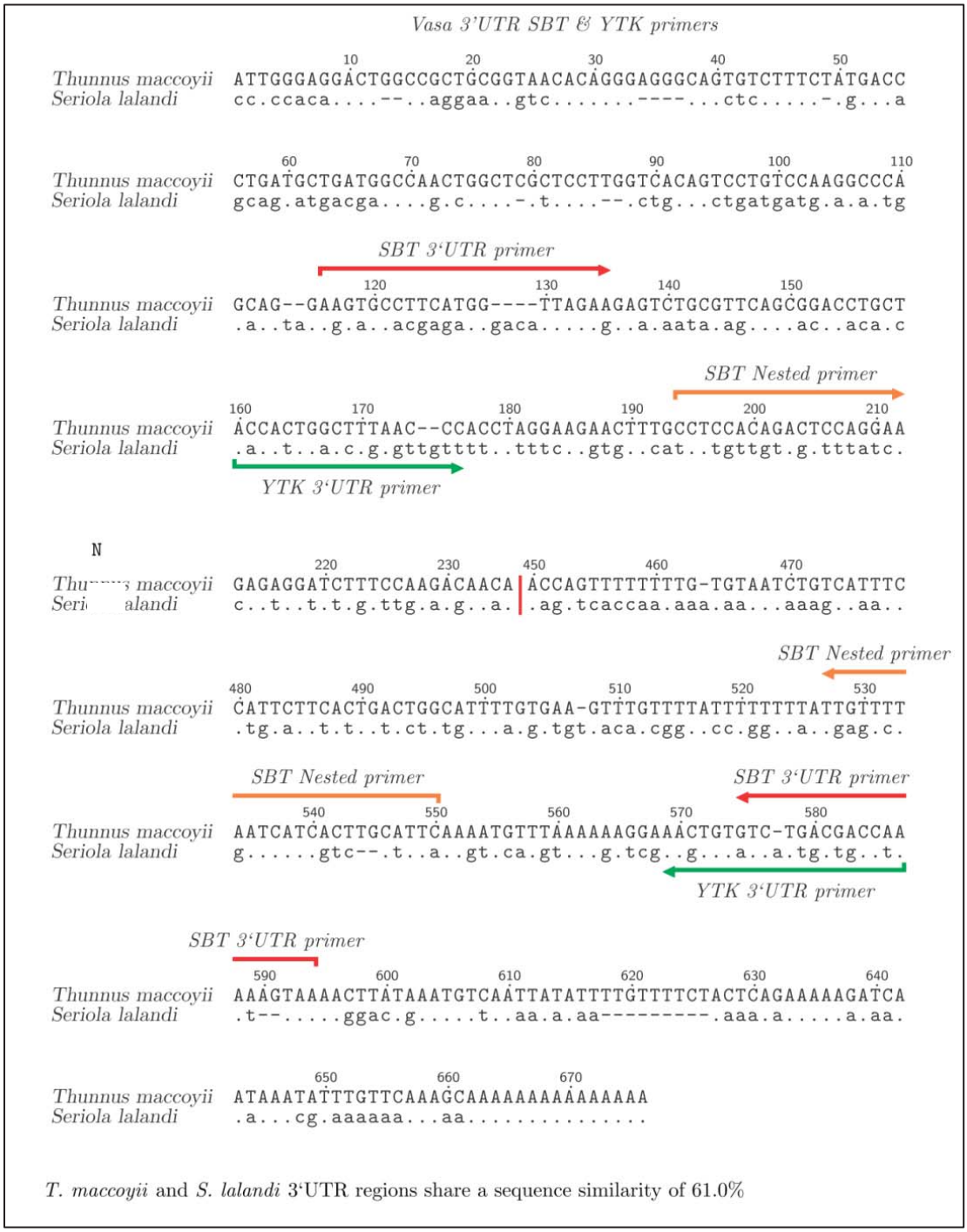

Figure 8. Yellowtail Kingfish and Southern Bluefin Tuna *Vasa* 3'UTR sequences and designed primers. Sequence alignment of the *Vasa* gene in Southern Bluefin Tuna and Yellowtail Kingfish and primer pairs' locations are presented.

2.5 To develop strategies for sterilization of surrogate host to obtain only SBT eggs and sperm for transplantation

2.5.1 Inducing triploidy in YTK larvae

To prepare for future transplantations, a preliminary trial towards inducing triploids in YTK larvae was conducted. Specifically, fertilised YTK eggs were collected from a YTK broodstock tank 5-10 minutes post-fertilisation. The fertilised eggs were immediately placed in a 5°C water bath (i.e. to apply cold shock), after which they were transferred to an egg incubator*. The eggs were separated into three treatments groups, which varied in the length of the cold shock treatment (i.e. 5, 10, and 15 minutes). The eggs were then left to hatch in the incubator and sampled at two days post hatch (prior to first feeding) and stored in 70% ethanol at 4°C. The hatched larvae were individually analysed for ploidy level, by dissociating the larva in Citric acid/0.1% Tween 20 solution, followed by enzymatic digestion in 3% Trypsin solution, nuclei DNA content labelling with Propidium Iodide (PI) and sorting of the cells on a Flow Cytometer (BD Accuri™ C6 System, BD Biosciences). PI labelled cells were counted at the FL2 optical filter, at wavelengths of 585/40 nm. The results were analysed to distinguish between peaks representing diploid cells with normal DNA content (2n) and triploid cells with increased DNA content (3n).

*We have chosen to use cold shock and not pressure shock for two reasons. The first is that this method was applied successfully in Prof Yoshiazki's laboratory for a number of species and is very simple to operate. The second is that we do not have the required pressure equipment at Arno Bay, and hence it was not feasible to apply.

3. Results

3.1 To develop and optimise protocols for the collection and dissociation of SBT germ cells for transplantation

3.1.1 Optimisation of dissociation protocol

When examining different concentrations of the dissociation enzyme in Experiment 1, there was no significant difference in the mean number of cells dissociated when using 2, 4 or 10 mg.mL^{-1} of collagenase/dispase (7.0 x 10^4 ± 2.55, 9.0 x 10^4 ± 0.84, 4.4 x 10^4 ± 1.21 cells.mL^{-1}, respectively) (df = 2, F = 1.843, P = 0.200). In Experiment 2, the interaction between enzyme type and amount of testis material was not significantly different between the treatment groups examined (F (1, 35) = 0.15, P = 0.701) (Figure 9). There was no significant difference in the number of cells produced when examining enzyme type (F (1, 35) = 3.75, P = 0.061); however, there was a significant difference in the number of cells produced when examining the amount of testis material dissociated (F (2, 35) = 22.27, P < 0.001) (Figure 9). Specifically, a significantly higher number of cells were produced when 100 mg.mL^{-1} of tissue was dissociated compared to 30 or 50 mg.mL^{-1} of testis material (P < 0.001) (Figure 9).

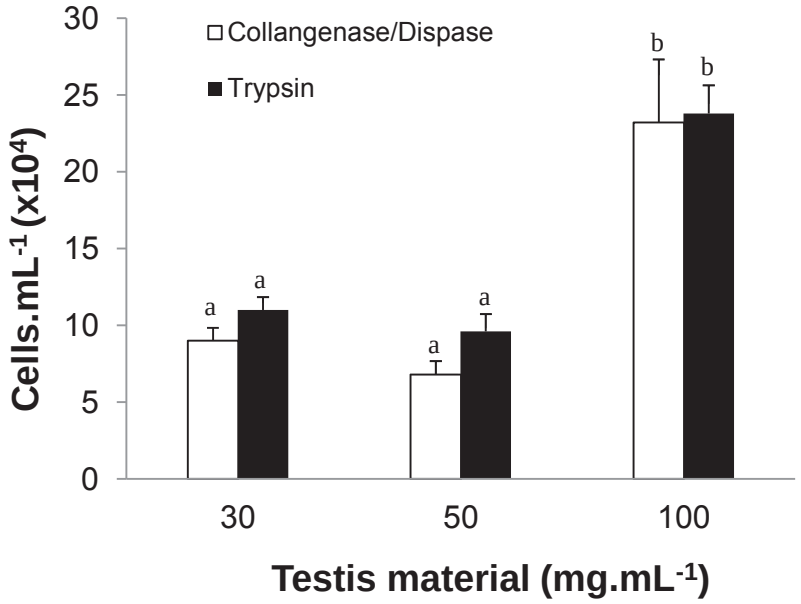

Figure 9. Mean number (± SE) of testicular cells produced from three different amounts of Southern Bluefin Tuna testis material (30, 50 and 100 mg.mL^{-1}) dissociated with collagenase/dispase (4 mg.mL^{-1}) or trypsin (0.0583 mg.mL^{-1}). Means with different superscripts are significantly different (P < 0.01) (n = 4).

In Experiment 3, where the amount of tissue dissociated was examined, the number of cells dissociated also generally increased as the amount of tissue dissociated increased (Figure 10). The highest number of cells were produced when 300, 400

and 500 mg.mL^{-1} of testis material was dissociated with collagenase/dispase prior to the Percoll gradient, respectively. Before the Percoll gradient was performed, cell numbers ranged between 21.3 and 928.0 cells.mL^{-1} (x10^4) for collagenase/dispase and 28.1 and 461.0 cells.mL^{-1} (x10^4) for Trypsin (Figure 10A). Post Percoll, cell numbers ranged between 10.0 and 88.0 cells.mL^{-1} (x10^4) for collagenase/dispase and 1.0 and 11.0 cells.mL^{-1} (x10^4) for Trypsin (Figure 10B). When comparing the number of cells between groups after the percoll gradient, the highest number of cells were also produced when 300, 400 and 500 mg.mL^{-1} of testis material was dissoiated with collagenase/dispase, respectively (Figure 10B). Statistical analysis of this data was not performed due to an insufficient number of replicate groups.

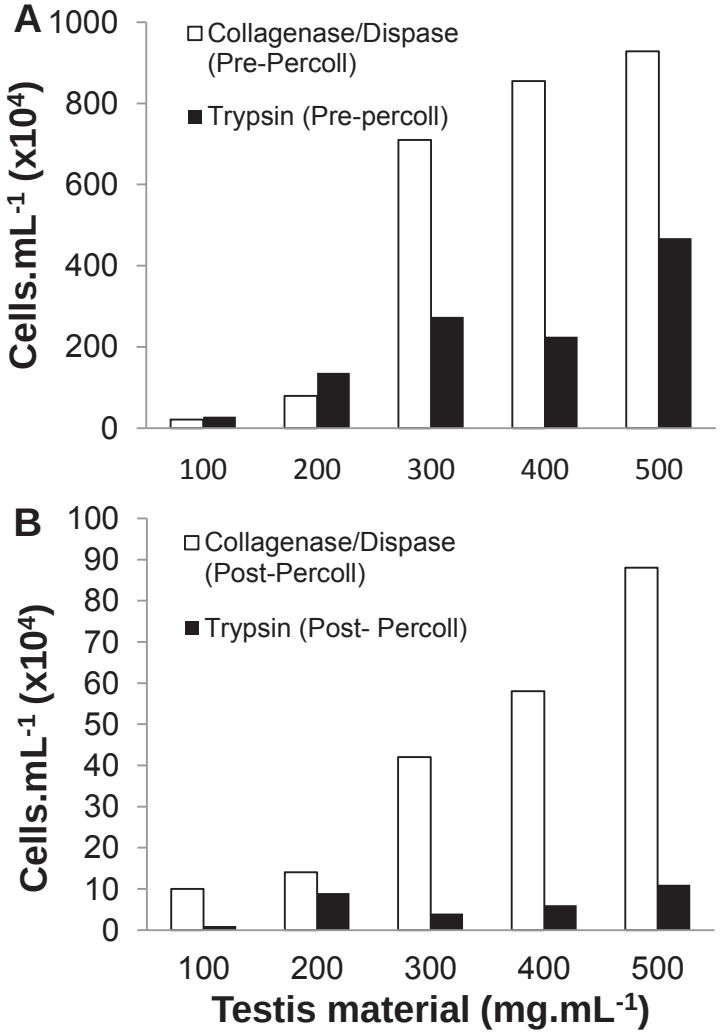

Figure 10. Total number of testicular cells dissociated from different amounts of Southern Bluefin Tuna testis material (100, 200, 300, 400, and 500 mg.mL^{-1}) with Collagenase/Dispase (2 mg.mL^{-1}) or Trypsin (0.0583 mg.mL^{-1}) before (A) and after (B) a Percoll gradient.

3.1.2 Assessment of the effect of size and reproductive maturity of SBT on testicular cell number and viability

The total cell number (cells.mL^{-1}) generally increased with increasing SBT weight and fork length, with moderate positive correlation evident between the variables respectively (r = 0.34 and 0.29 respectively, n = 84, P < 0.01) (Figure 11A, B). Cell viability was quite variable across the range of SBT weight and fork length examined (Figure 11C, D); however, moderate positive correlations were evident in both cases (r = 0.35 and 0.32 respectively, n = 84, P < 0.01) (Figure 11C, D).

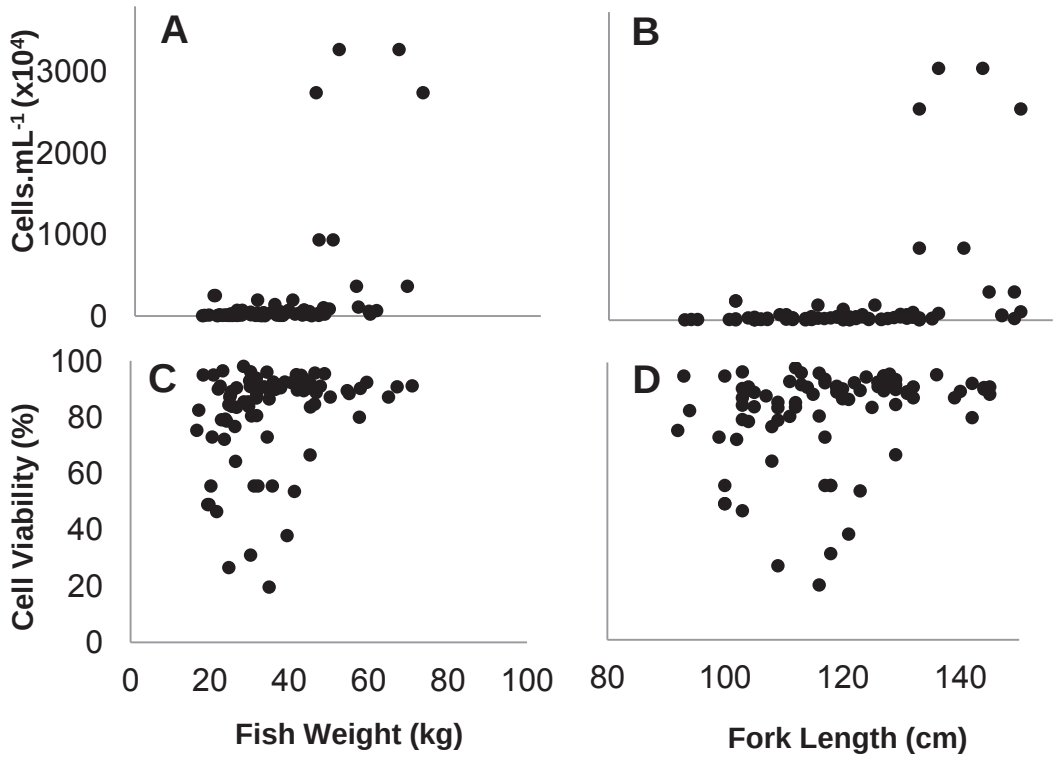

Figure 11. Cell number and viability of 1000mg of dissociated testis material collected from Southern Bluefin Tuna of different weights (A, C) and fork lengths (B, D) during June to October 2012 and April and May in 2013 (n = 84).

Of the 62 SBT sampled for histological analysis, 26 were classified as stage 1 of reproductive maturation (Figure 12A, B), 28 as stage 2 (Figure 12C, D), seven as stage 3 (Figure 12E, F) and one was as stage 4 (Figure 12G, H).

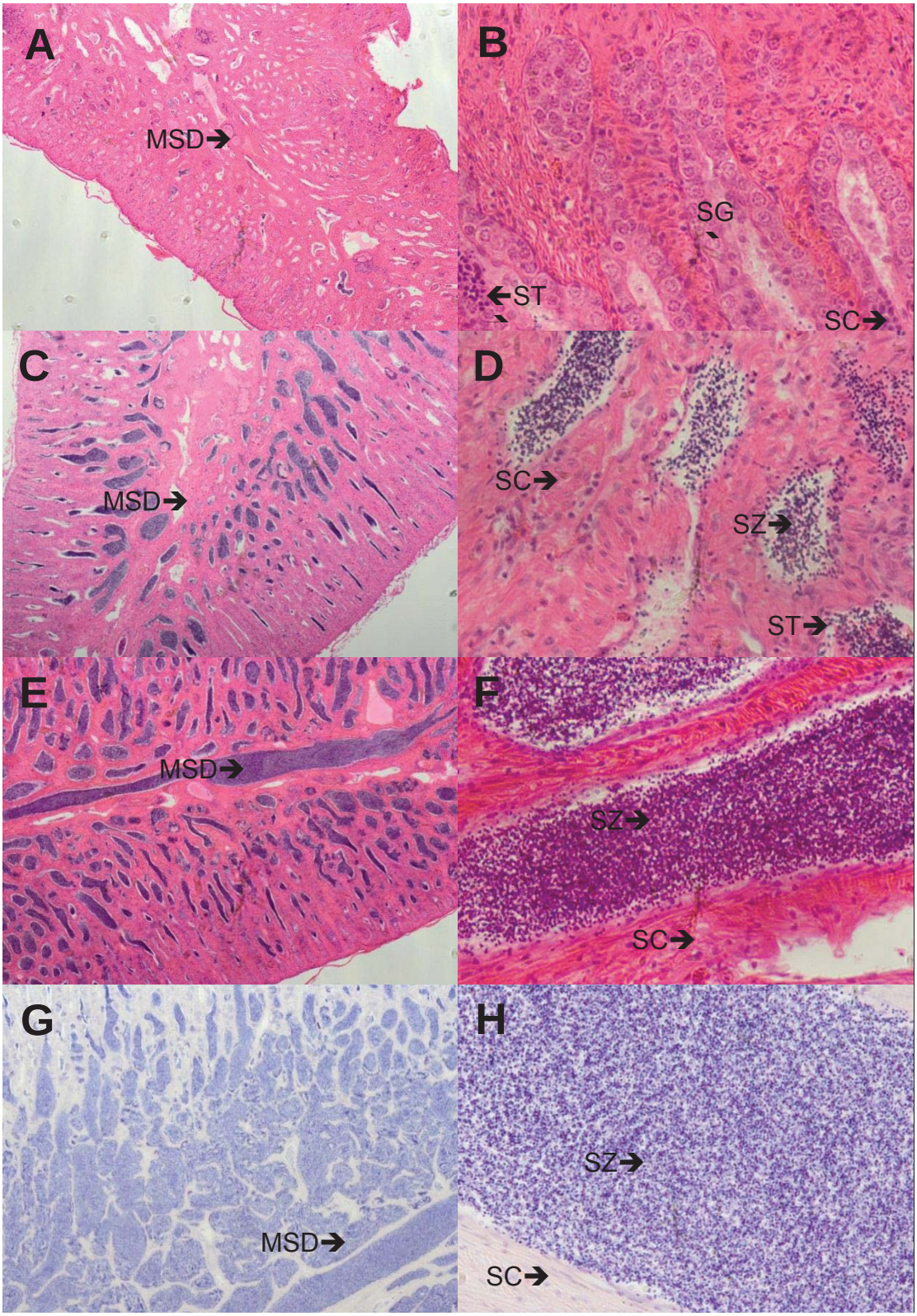

Figure 12. Histological sections of testis material from Southern Bluefin Tuna at stage 1 (A, B), stage 2 (C, D), stage 3 (E, F) and stage 4 (G, H) of reproductive maturity (SG = spermatogonia, SC = spermatocytes, ST = spermatids, SZ = spermatozoa, MSD = main sperm duct). Magnification x40 (A, C, E, G), x400 (B, D, F, H). Stages 1 and 2 are classes as immature and stage 3 and 4 are classed as mature.

Total cell numbers (cells.mL^{-1}) obtained in the dissociation experiments generally increased with increasing gonadosomatic index (GSI) and stage of reproductive maturation, with a strong and moderate positive correlations evident between the variables, respectively (r = 0.68 and 0.32 respectively, n = 84, P < 0.01) (Figure 13A, B). A moderate positive correlation was also detected between cell viability and GSI (r = 0.26, n = 84, P < 0.01) (Figure 13C); however, there was no statistical correlation evident between cell viability and stage of reproductive maturation (P = 0.33) (Figure 13D).

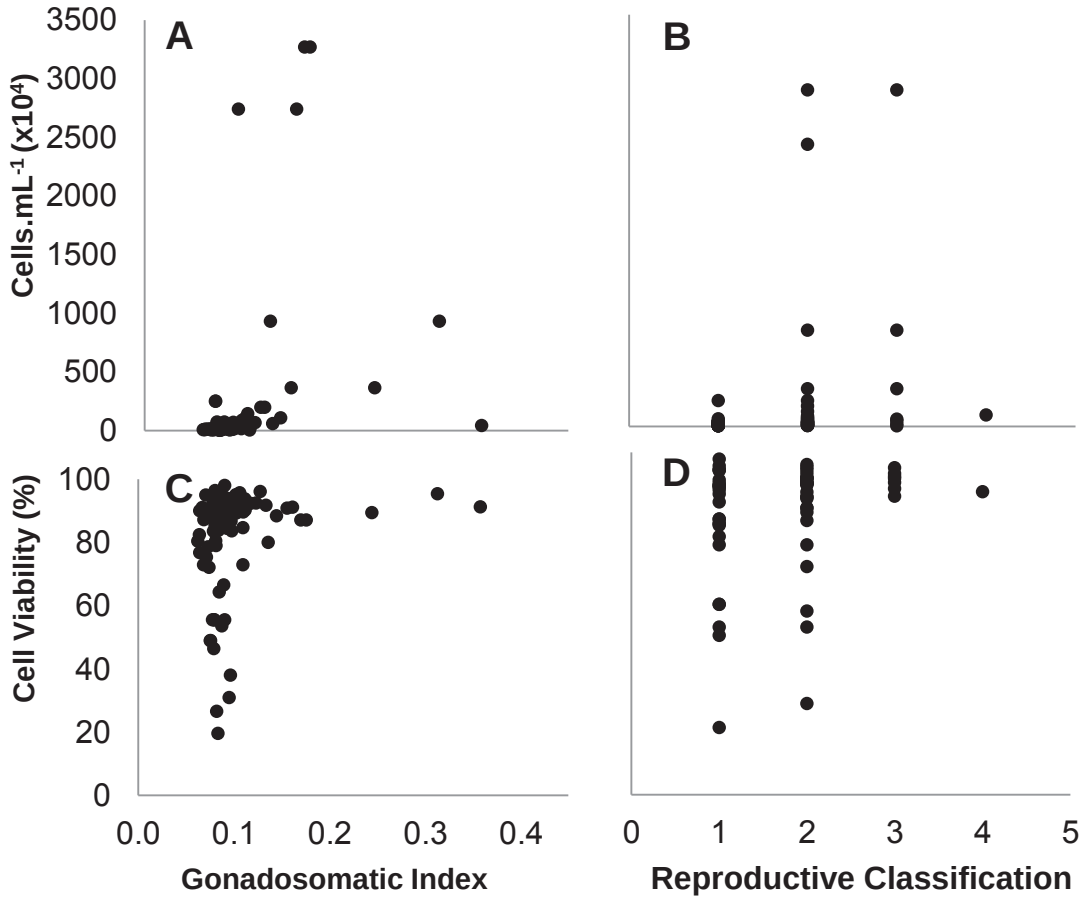

Figure 13. Testicular cell number and viability dissociated from 1000 mg of testis material collected from Southern Bluefin Tuna with different gonadosomatic index (A, C) and stage of reproductive maturation (B, D) sampled during June to October 2012 and April and May in 2013 (n = 84).

Cell numbers ranged between 1.8 x 10^4 cell.mL^{-1} in April 2013 to 3.27 x 10^6 cell.mL^{-1} in September 2012 (Figure 14). There was a significant difference in cell numbers dissociated between different months during the commercial SBT harvest season (df = 6, F = 3.641, P = 0.003). Specifically, testis material that was dissociated in September had significantly higher cell numbers than June, July and August in 2012 and April and May in 2013 (P = 0.010, 0.010, 0.011, 0.009, and 0.007, respectively)

but not October, 2012 (P = 0.058) (Figure 14A). There was a significant difference in cell viability of the cells dissociated between different months during the commercial SBT harvest season (df = 6, F = 4.378, P = 0.001). Specifically, testis material that was dissociated in September had significantly lower cell viability than June, July and October in 2012 (P = 0.021, 0.002, and 0.013, respectively) but not October, 2012 and April and May, 2013 (P = 0.114, 0.883, and 0.846, respectively) (Figure 14B).

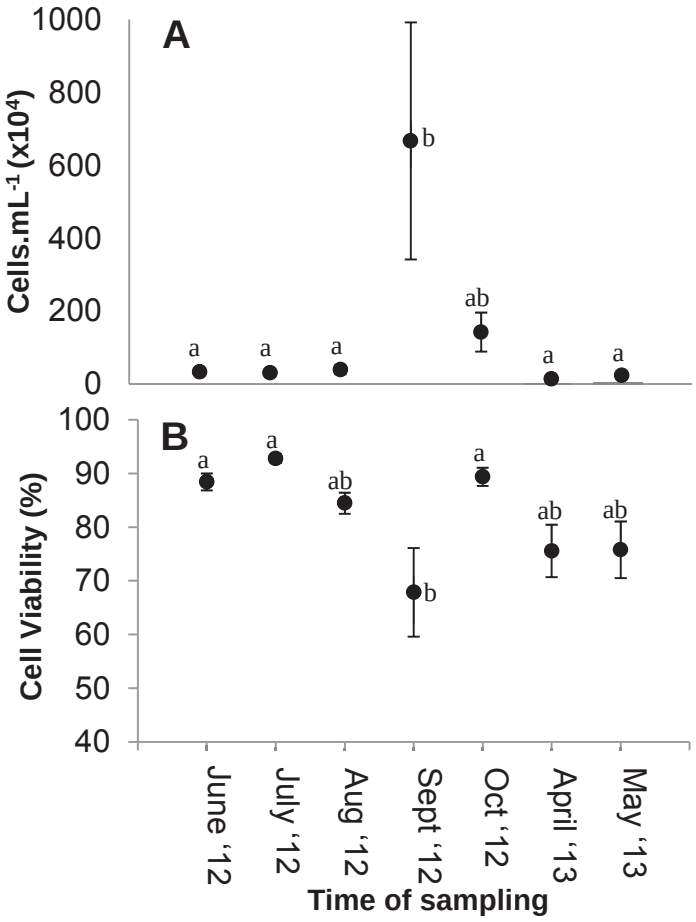

Figure 14. Testicular cell number (A) and viability (B) dissociated from 1000 mg of testis material collected from Southern Bluefin Tuna (*Thunnus maccoyii*) sampled during June to October 2012 and April and May in 2013 (n = 84).

3.2 To assess and optimise cryopreservation procedures of SBT germ cells

In the first experiment that directly examined the suitability of a cryopreservation protocol developed for PBT, the mean number of testicular cells dissociated from fresh tissue (98.94 x 10^4 ± 3.50 mL^{-1}) was significantly higher compared to the number of cells dissociated from the cryopreserved tissue (11.63 x 10^4 ± 11.70 mL^{-1}) (df = 1, F = 51.140 P < 0.001) (Figure 15A). The viability of the cells prepared from

fresh and cryopreserved tissue decreased from 0 to 12h and 12 to 44h after dissociation. There was a significant difference between fresh tissue and cryopreserved tissue at 0, 12 and 44h (P = 0.002) (Figure 15B). However, the only significant difference detected was between cells prepared from fresh tissue at 0, 12 and 44h and cryopreserved tissue at 44h (P = 0.002, 0.011 and 0.013, respectively) (Figure 15B).

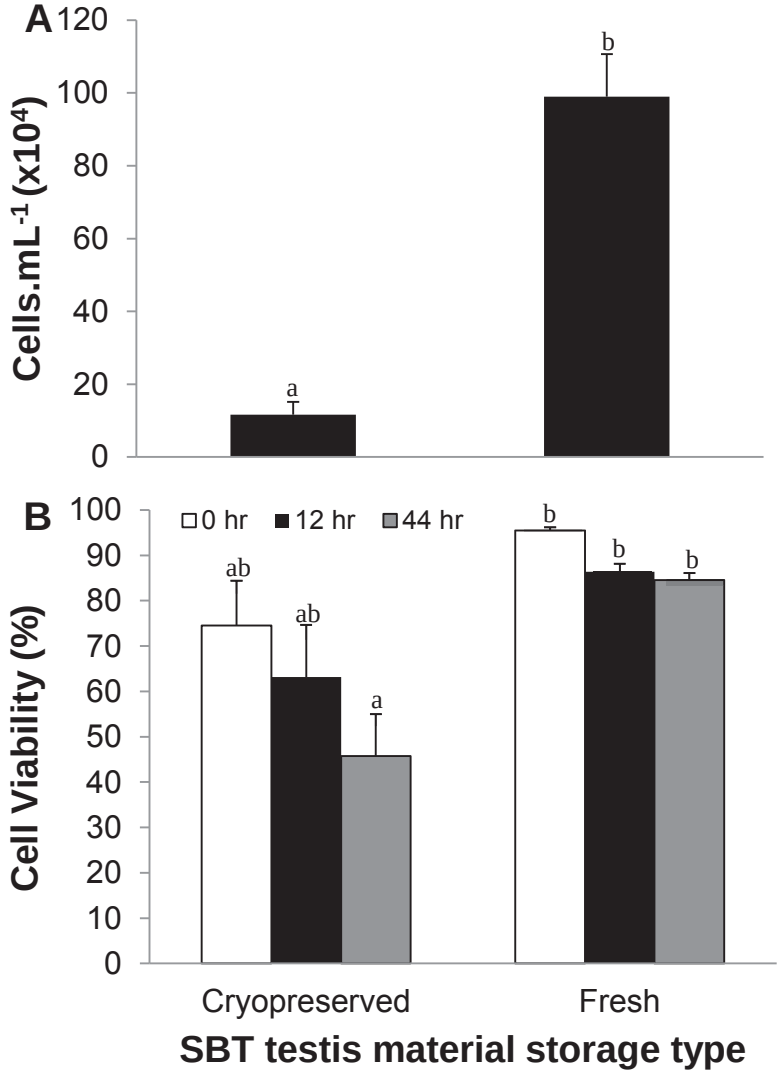

Figure 15. Mean (± SE) number (A) and viability (B) of cells dissociated from 1000mg of cryopreserved and fresh Southern Bluefin Tuna testis material. Cell viability was assessed at 0, 12 and 44 hours post dissociation (n = 4). Means with different superscripts are significantly different (P < 0.05).

In the second experiment that examined different concentrations of DMSO in the cryopreservation media and different cooling regimes, the number of cells dissociated from Treatment 3 (i.e. tissue cooled at -80°C for 90 min in 9% DMSO) ($21.17x10^4$ ± 9.63 $mL.^{-1}$) was significantly higher compared to all other treatment

groups (P = 0.030, 0.041 and 0.031 respectively) (Figure 16A). Cell viability showed a similar trend, with cell viability also significantly higher in Treatment group 3 compared to all other groups (P < 0.001, P = 0.001 and P < 0.001, respectively) (Figure 16B).

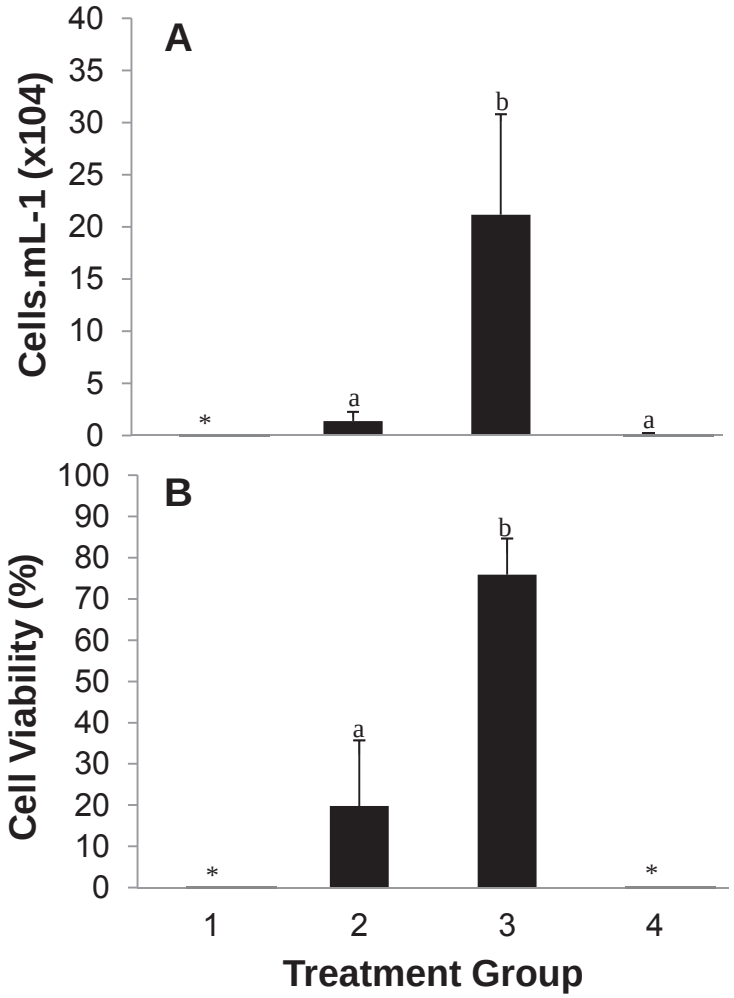

Figure 16. Mean (± SE) number (A) and viability (B) of cells dissociated from 1000 mg of cryopreserved Southern Bluefin Tuna testis material using three different cooling methods and two different concentrations of cryoprotectant agent (DMSO) (n = 4): 1) tissue stored directly into liquid nitrogen, 9% DMSO; 2) tissue stored at -80°C for 90 minutes (min) before transferred into liquid nitrogen, 5% DMSO; 3) tissue stored at -80°C for 90 min before transferred into liquid nitrogen, 9% DMSO and; 4) tissue stored at -20°C for 90 min before transferred into liquid nitrogen, 9% DMSO. Means with different superscripts are significantly different (P < 0.05). * denotes a zero value

3.3 To investigate and establish potential surrogate host for SBT

3.3.1 YTK as a potential surrogate host for SBT

Approximately 12,000 YTK larvae were transplanted with donor SBT testicular cells during five different transplantation experiments conducted in 2012 and 2013 (Table

5). Mean body weight and GSI of donor SBT across all five experiments (n = 14) was 57.0 kg ± 2.9 kg and 0.145 ± 0.013, respectively. Survival of the transplanted larvae ranged from 0% in Experiment 1 at 18 DPH to 9.2% in Experiment 4 at 64 DPH (Table 5). PKH26 labelled donor cells were observed in the developing gonad of transplanted YTK in Experiments 2, 4 and 5 (Table 5). Specifically, PKH labelled cells that displayed red fluorescence were observed in the developing gonad in the peritoneal cavity of the transplanted larvae (Figure 17) and in the developing gonad after removal from the peritoneal cavity (Figure 18). This suggests that transplanted donor testicular cells had successfully migrated and colonised in the recipient's gonads. These cells were seen in the developing gonads of 50% of the transplanted larvae in Experiment 2 (n = 2), 33.3% (n = 12) and 37.5% (n = 8) of recipient larvae in Experiment 4 and 16.7% (n = 6) in Experiment 5 (Table 5).

Table 5. Summary of transplantation experiments where donor Southern Bluefin Tuna (*Thunnus maccoyii*) cells were transplanted into recipient Yellowtail Kingfish (*Seriola lalandi*) larvae.

Exp No.	No. of transplanted larvae	Age (DPH) at transplantation	Survival (%) of transplanted larvae	Survival (%) of control larvae	Observation of donor cells in larvae	Successful colonisation and migration of donor cells
1	845	7 - 8	0	0	n/a	n/a
	1041	8 - 9	0	4.5	n/a	n/a
	805	9 - 10	0	0	n/a	n/a
2	1050	8 - 9	0.19	0.06	Yes	50% (n=2)
	1093	8 - 9	0.09	10	No	0 (n=1)
	1030	8 - 9	0	10	n/a	n/a
3	1010	6 - 7	0	n/a	No	n/a
	1210	9 - 10	0	n/a	No	n/a
	150	11	0	n/a	No	n/a
4	680	6 - 7	9.11	15.14	Yes	33.3% (n = 12)
	1000	9 - 10	9.2	14.60	Yes	37.5% (n = 8)
5	850	6 - 7	0	1.65	No	n/a
	1321	9 - 10	0.9	6.96	Yes	16% (n=6)

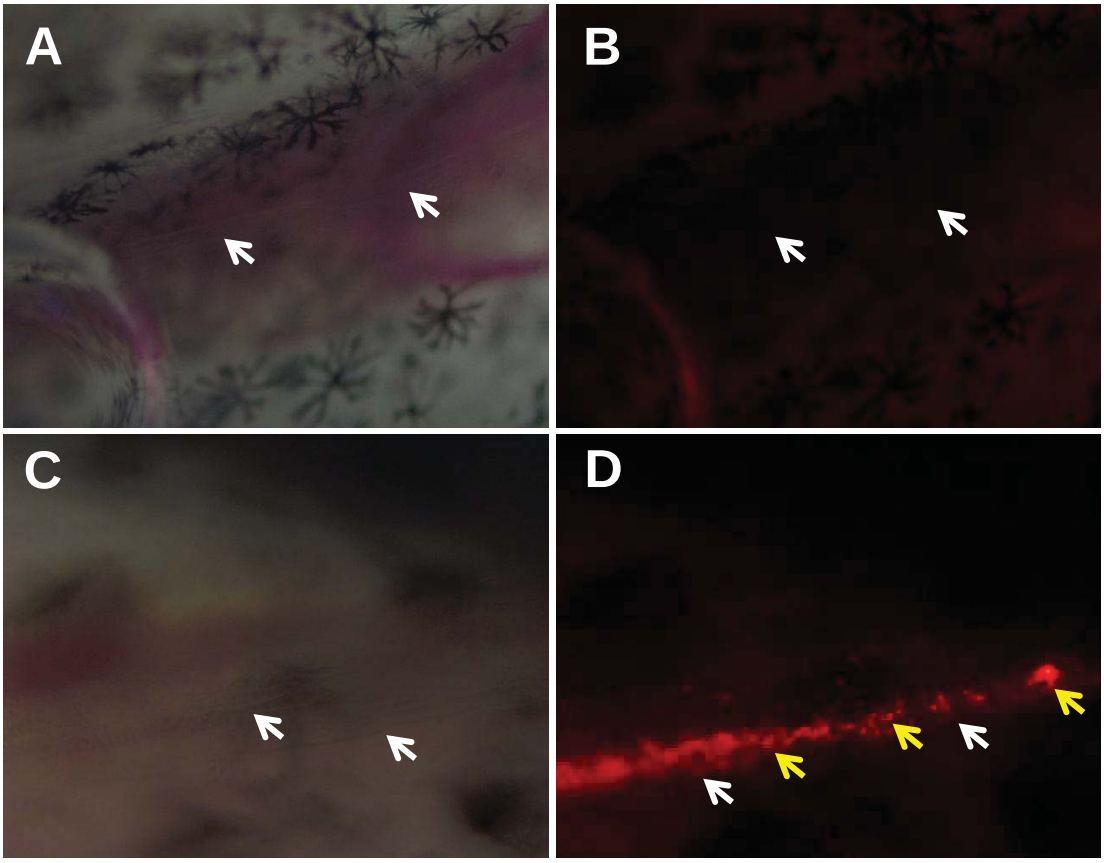

Figure 17. Incorporation of transplanted PKH26-labelled Southern Bluefin Tuna testicular cells in the genital ridge of recipient Yellowtail Kingfish (YTK). Bright field (A, C) and fluorescent field (B, D) of the ventral view of the peritoneal cavity of non-transplanted YTK (A, B) and transplanted YTK (C, D) at 18 days post transplantation (29 DPH). Anterior end of each fish is located top right. White arrows indicate location of the genital ridges attached to the peritoneum. Yellow arrows indicate fluorescently labelled donor derived cells. Magnification x100

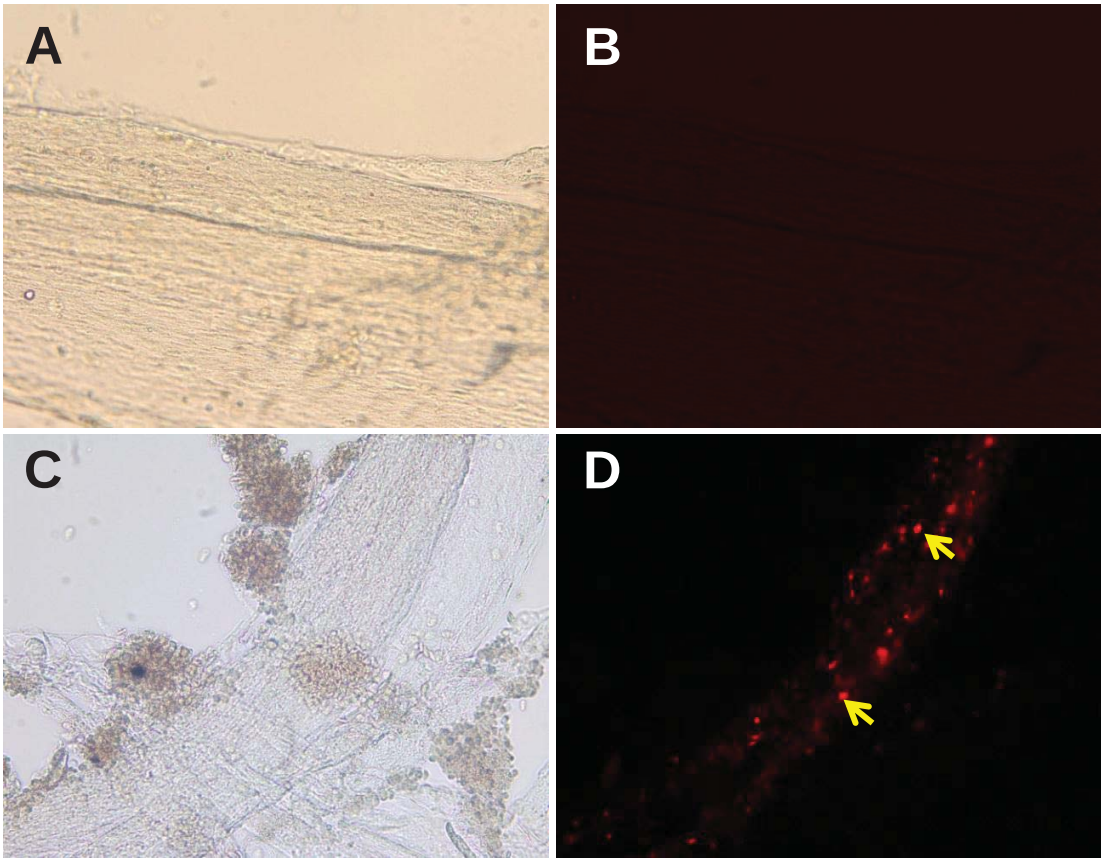

Figure 18. Incorporation of transplanted PKH26-labelled Southern Bluefin Tuna (*Thunnus maccoyii*) testicular cells in the removed gonad of recipient Yellowtail Kingfish (*Seriola lalandi*) (YTK). Bright field (A, C) and fluorescent field (B, D) of removed gonad of non-transplanted YTK (A, B) and transplanted YTK (C, D) at 28 days post transplantation (39 DPH). Yellow arrows indicate fluorescently labelled donor derived cells. Magnification x400

3.3.2 Alternative species as a potential surrogate hosts for SBT

3.3.2.1 Capture of alternative species as a potential surrogate hosts for SBT

The number and species of fish captured during the 16 fishing trips varied and are summarized in Table 6.

Table 6. Fishing trip number, date and outcome of trips to obtain broodstock of alternative species as a potential surrogate hosts for SBT (i.e. fish species captured, the number of fish, surviving fish).

No.	Date	Fish species captured	No. of fish captured	No. transferred into the broodstock tank alive	Survival duration in the broodstock tank
1	19/12/2011	-	-	-	-
2	11/05/2012	-	-	-	-
3	20/02/2012	-	-	-	-
4	01/03/2012	Mackerel Tuna Skipjack Tuna	5 1	0 0	-
5	24/04/2012	-	-	-	-
6	20/05/2012	-	-	-	-
7	23/01/2013	-	-	-	-
8	18/04/2013	Mackerel Tuna Longtail Tuna	4 1	2 Sacrificed for tissue collection	11 months
9	26/04/2013	Mackerel Tuna	4	0	
10	14/05/13	Mackerel Tuna Leaping Bonito	2 3	2 3	1-3 days 2 fish for 2 days and 1 fish for 6 months
11	17/05/13	Longtail Tuna	1	Sacrificed for tissue collection	
12	24/05/13	-	-	-	-
13	07/06/13	-	-	-	-
14	26/06/13	-	-	-	-
15	30/07/13	-	-	-	-
16	06/08/13	Blue Mackerel	28	28	1 month

The six fish were caught during fishing trip 4 were caught within 1.5 hours, between 7:35am and 8:50am. However, during the two hours between catching the fish and bringing them ashore at the Bribie Island boat ramp, all the fish had died. The fish were examined to establish sex, and total fish weight, total fish length and gonad weight were measured to calculate gonadosomatic index and determine length to weight formula (see Table 7 and Figure 19). Difficulties in locating suitable fish in the wild, problems associated with the transport unit and the delays in transferring the captured fish onshore have all attributed to the low success rate of bringing fish onshore in the first six fishing trips. No fish were caught during fish trip 7, but the overall performance of the team and equipment was satisfactory for future trips. During fishing trips 8 to 12 the fish that did not survive the transport to BIRC were sacrificed, examined for their sex and gonad developmental stage and were sampled in 4% PFA and RNAlater for RNA extraction and *in-situ* hybridisation analyses, as described in Section 3.4. Fish were found suitable for transportation in the tuna tubes at a body weight smaller than 1 kg, at which point they were still sexually immature and required a period of fattening in the tank to grow to over 2 kg, the known size for puberty in *E. affinis.*

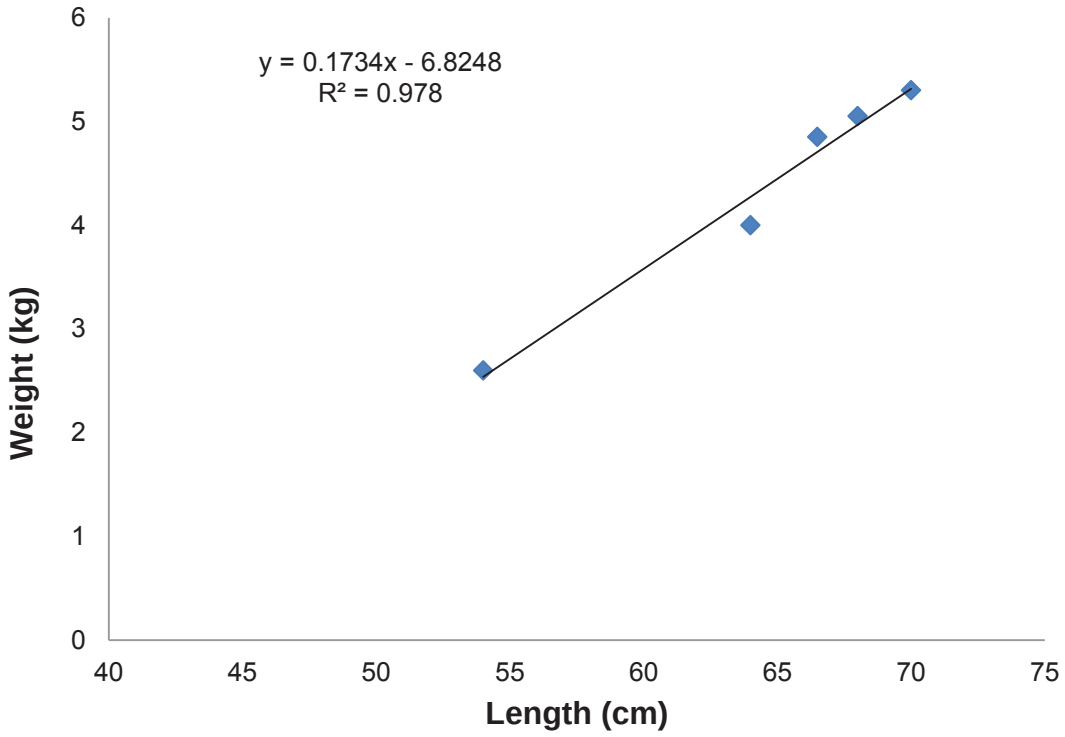

Figure 19. Mackerel Tuna (*Euthynnus affinis*) length to weight formula, as calculated from caught fish.

Table 7. Fish measurements used to determine sexual developmental stage and length to weight formula, obtained from fish caught on fishing trips to obtain broodstock of alternative species as a potential surrogate hosts for SBT. M and F in the remarks denotes male and female respectively.

Species	Length [cm] TL	Weight [kg]	Gonad Wt [gr]	Remarks	GSI [%]
Euthynnus affinis	54	2.6	4.26	M - Immature testis	0.1638
Katsowonis pelamis	48.5	2.45	9.97	M	0.4069
Euthynnus affinis	64	4	51.36	F - Mature ovaries	1.284
Euthynnus affinis	66.5	4.85	34.12	M - Dark gonads	0.7035
Euthynnus affinis	70	5.3	55.18	M - Mature testis	1.0411
Euthynnus affinis	68	5.05	41.14	M - Mature testis	0.8147
Thunnus tonggol		3.35	4.6	M - Immature testis	0.1373
Euthynnus affinis		0.78	0.42	Immature gonads	0.0538
Euthynnus affinis		0.68	0.357	Immature gonads	0.0525
Scomber australasicus		0.06	3.51		5.85

Once in the tank at BIRC (see Figure 20), some of the fish started feeding within three days of capture and started recovering. Fish that refused to feed did not

survive. The fish were fed on thawed blue bait (50-80 mm long) and white bait (40-60 mm long) injected with antibiotics (OTC, 50-80 mg/kg body weight/day, for 10 days). Two days after the introduction of the first batch of fish, live young mullet and herring were introduced to the tank to induce predation activity of the tuna. Skin infection was evident on the fish from the second day in the tank; however, the surviving fish that were feeding have recovered and their skin healed within the first week in the tank. After the acclimatisation of the fish, they were fed approximately 10% of their body weight per day. The tuna tank was cleaned and the sump was drained on a regular basis. The survival of these fish was an important breakthrough in the project to secure live broodstock.

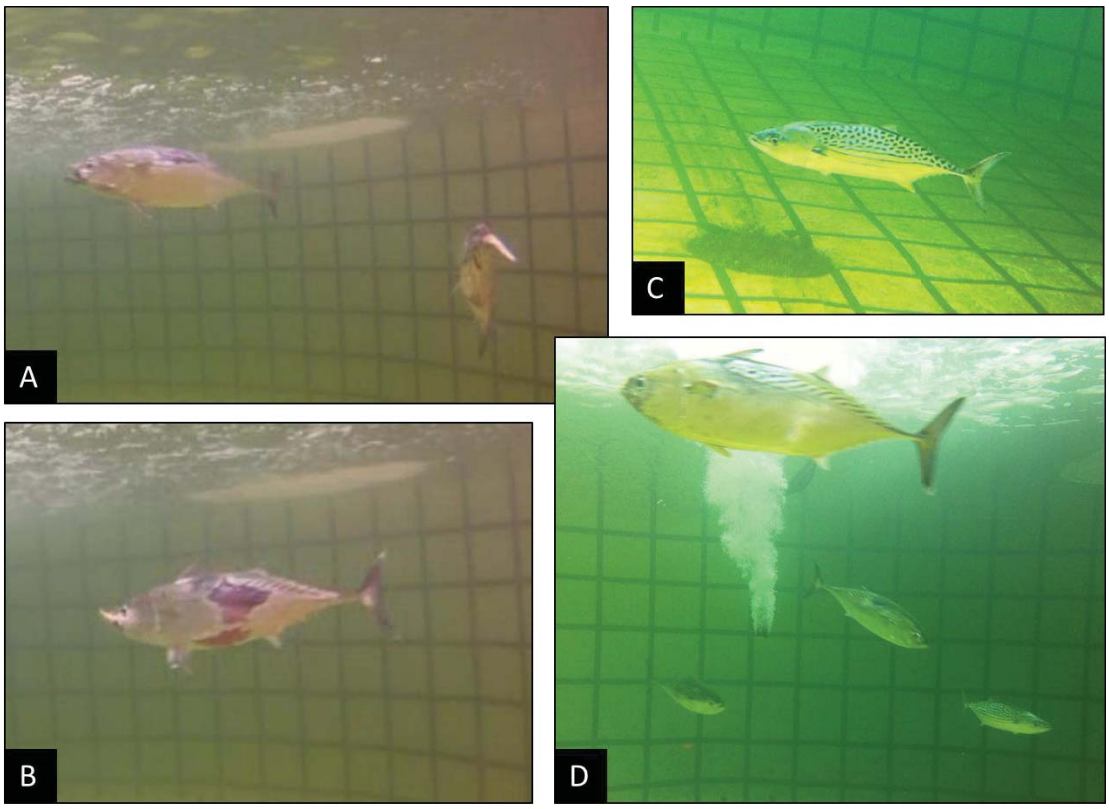

Figure 20. Mackerel Tuna and Leaping Bonito in the broodstock tank at Bribie Island Research Centre (BIRC). **A** – Mackerel Tuna feeding on thawed sardines. **B** – Damaged skin on Mackerel Tuna following transfer to onshore tank, which is likely to have resulted from handling abrasion or bacterial infection. **C** – Leaping Bonito. **D** – Fish in tank immediately after fishing.

During fishing trips 13-15, no fish were spotted, as it was at the end of the tuna migration season (usually December-May). However, fishing trip 16 was a major success with the capture of 28 Blue Mackerels. The ease of capture and handling, and their small size, make them ideal candidates as surrogate broodstock. The fish started feeding the next day post capture and were fed 30-50 mm long “whitebait”,

fortified with antibiotics (OTC, 50-80 mg/kg body weight/day) for 10 days. The fish were feeding well and seemed to adapt to the tank conditions; however, after approximately three weeks post capture (at the 26/08/13), there was a fish mortality in the tank. Post mortem examination revealed presence of gill parasites, and so the remaining fish were treated with formalin (100 ppm for 1 hour), followed by a second treatment 4 days later. Following those treatments, two more fish perished, but the remainder seemed to recover and feed well (300 g/day).

3.3.2.2 Spawning of alternative host broodstock

No spawning of Blue Mackerel broodstock captured on fishing trip 16 was visible as a result of the GnRHa treatment, although the gonads of even the small fish were well developed (Figure 21).

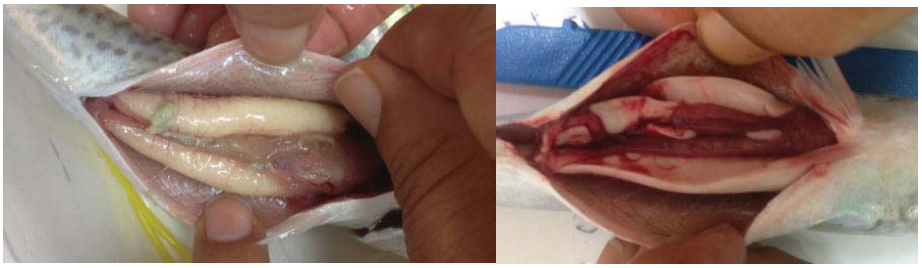

Figure 21. Reproductively developed gonads of Blue Mackerels, *Scomber australasicus*. Left picture displays a female with developed ovaries. In the right picture, a male with well developed testis

A spawning induction trial was planned in an attempt to induce spawning of the two remaining Mackerel Tuna in the tank at BIRC by oral administration of GnRH analogue, at a dose of 6 mg/kg body weight/day. However, both fish died prior to this happening of unknown causes. Post mortem examination of one of the fish that was found in non-degraded state, revealed it was a sexually undeveloped male (GSI=0.042%), which grew to 1.4kg and total length of 45.6cm.

3.4 To develop molecular tools for the identification of SBT germ cells in surrogate host

3.4.1 Cloning and sequencing of candidate genes expressed specifically in SgA cells

A full list of the genes that were discovered by degenerate primers PCR and transcriptome analysis can be seen in Table 8. For each gene, specific PCR primers were designed to amplify partial regions, usually 300-1500 base pairs (bp) long, that can be used both as template for synthesis of *in-situ* hybridisation probes and as

species specific markers. Currently, *Vasa* and *Dead-End* are used extensively as germ cell markers however, the other genes will be used in future analyses to describe in detail their expression and localisation in SBT testis. A deeper analysis of the transcriptome database will take place, using bioinformatics tools, to compare these genes against genomic databases from other closely related species (such as the recently published genome of the Pacific Bluefin Tuna, *Thunnus orientalis*). A comparative transcriptome analysis from RNA of SBT testis and ovaries from different maturation stages will be conducted in late 2014. The results will shed light on differential expression patterns between the different control groups and may lead to the discovery of SBT sex specific markers.

Table 8. Germ cell and house-keeping genes expressed in testis and germ cell development. Current status in the cloning and sequencing of each one in both surrogate host and donor species is detailed.

Gene	Species[1]	Method	Sequenced
Vasa	*K. pelamis*, *S. lalandi*, *T. maccoyii*	3', 5' RACE, Transcriptome	Fully
Dead-End	*T. maccoyii*	Transcriptome	Fully
Piwil1	*E. affinis*, *K. pelamis*, *T. maccoyii*	Transcriptome	Fully
Piwil2	*K. pelamis*, *T. maccoyii*	Transcriptome	Fully
Nanos1	*E. affinis*, *T. maccoyii*	PCR, Cloning	Partially
Notch1	*T. maccoyii*	PCR, Cloning	Partially
GFRa1	*T. maccoyii*	PCR, Cloning	Partially
GSDF	*T. maccoyii*	Transcriptome	Partially
Activin 1B/2A	*T. maccoyii*	Transcriptome	Partially
AMH	*T. maccoyii*	Transcriptome	Partially
EF1a	*T. maccoyii*	Transcriptome	Partially
18S	*T. maccoyii*	Transcriptome	Partially

[1] *T. maccoyii* – southern bluefin tuna, *S. lalandi* – yellowtail kingfish, *E. affinis* – Mac tuna, *K. pelamis* – Skipjack tuna.

3.4.2 Identification and localisation of SgA cells

The expression of *Dead-End* and *Vasa* genes in cross sections of SBT testis is shown in Figure 22. It is visible that *Dead-End* is expressed mainly at the head of the cysts which reside at the peripheral edge of the testis (marked with arrows), whereas *Vasa* is expressed as well in cells that extend deeper into the middle of the testis cross section in dense clusters (marked with triangles). This expression profile, along with the known literature demonstrates that *Dead-End* can be used as a SgA specific marker, whereas *Vasa* is expressed strongly in SgA cells, but also in SgB and Spermatocytes. Another conclusion from this experiment is that SgA cells reside at the peripheral area of the testis. We will therefore use this part of the testis to dissociate and isolate SgA cells for transplantations.

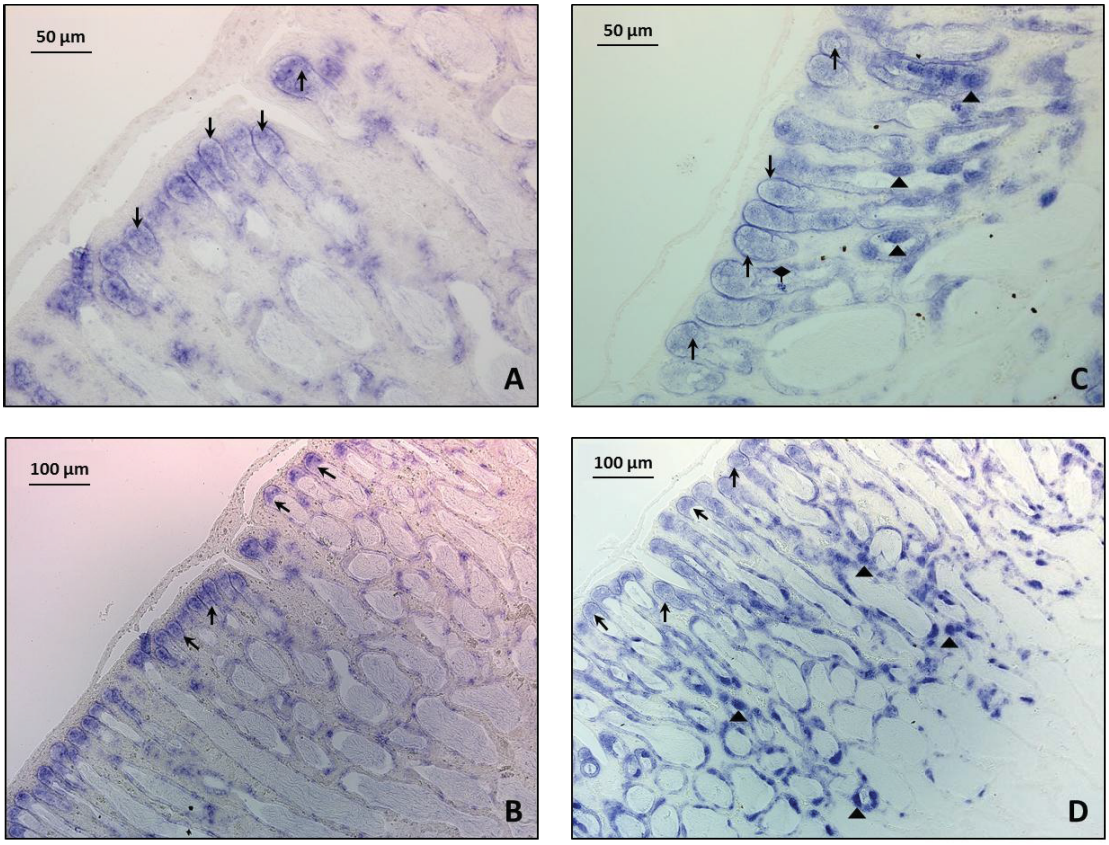

Figure 22. Expression of *Dead-End* and *Vasa* in Southern Bluefin Tuna testis. Expression of *Dead-End* (**A, B**) and *Vasa* genes (**C, D**) in a cross section of Southern Bluefin Tuna testis. Black arrows point at SgA cells organised in cysts, black triangles point at SgB cells and black diamond points at Spermatocyte cells.

The same experiment in Longtail Tuna (*T. tonggol*) testis, using the same probe, showed similar results, indicating that both genes are conserved between the species and that these probes cannot be used to distinguish between donor and host cells among species from the *Thunnus* genus. The protocol for *in-situ* hybridisation was optimised for testis cross sections and transplanted larvae gonads.

3.4.3 Detection of SBT cells in transplanted YTK larvae

PCR of *Vasa* 3'UTR showed presence of SBT gDNA in 5 out of 12 transplanted YTK larvae gonads (75 dpt) and none in any of the control larvae (Figure 23); the amplified products were sequenced and proved to be of SBT origin (>99% nucleotide identity). However, the positive controls of YTK primers with control larvae gDNA were not yet convincing and the PCRs are being repeated with optimised conditions to verify the reliability of these preliminary results. These results are qualitative only and do not give any indication about the amount of SBT DNA present.

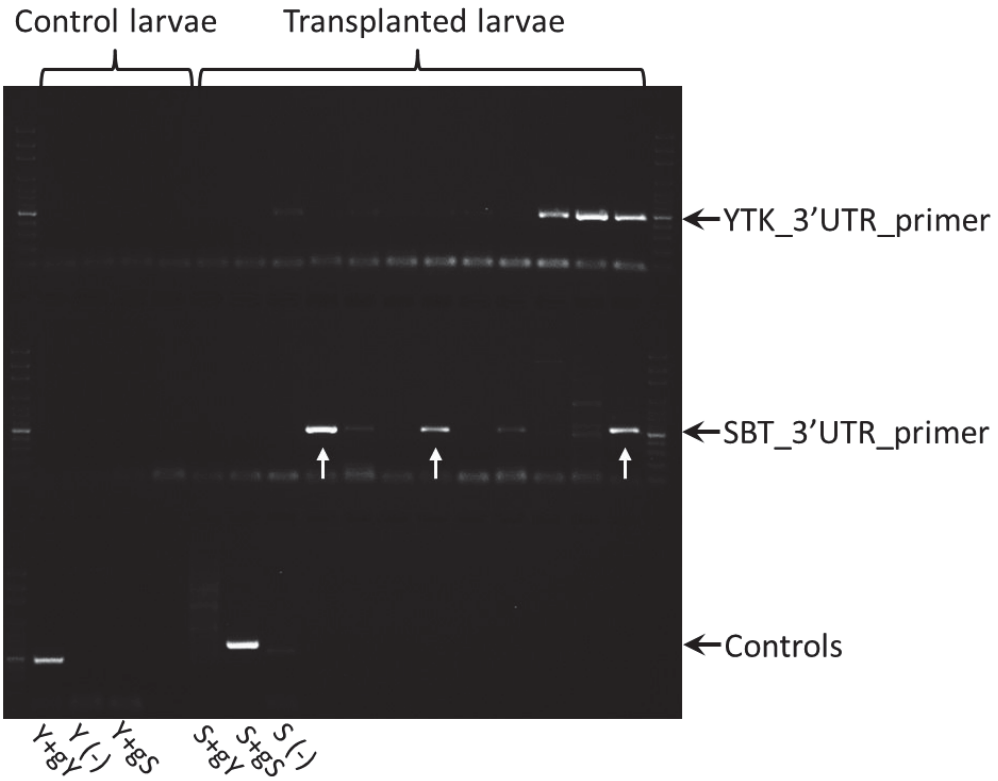

Figure 23. PCR of 3'UTR *Vasa* gene in Yellowtail Kingfish gonads DNA. 12 samples of transplanted and 4 non transplanted control YTK larvae gonad DNA were used as templates for PCR with YTK specific primers (upper row), SBT specific primers (second row) and controls: Y+gY – YTK primers with YTK gDNA, Y(-) – No Template Control (NTC), Y+gS – YTK primers with SBT gDNA and S+gY – SBT primers with YTK gDNA, S+gS – SBT primers with SBT gDNA, S(-) – NTC .Left lane – 100 bp DNA size marker.

Another PCR assay, using the more specific SBT *Dead-End* gene, was not able to amplify products from DNA of transplanted YTK gonads at 165 dpt, even after secondary amplification with nested primers (Figure 24). This assay is currently being repeated for transplanted YTK, sampled at earlier days post transplantation (at 39 and 75 dpt), to verify the exact time point post transplantation, at which donor SBT cells could be detected.

A complimentary approach for the detection of SBT germ cells in transplanted YTK gonads is through *in-situ* hybridisation of species-specific probes in gonads cross sections. We have optimised the protocol and verified through positive and negative controls that the *Vasa* 3'UTR probes are germ cells specific and species-specific, i.e., SBT probe is specific to SBT tissue, but does not produce signal on YTK tissue and vice versa (Figure 25). To date, 5 transplanted YTK gonads at 165 dpt were cross sectioned and analysed using this method, but no positive signals of SBT cells were found.

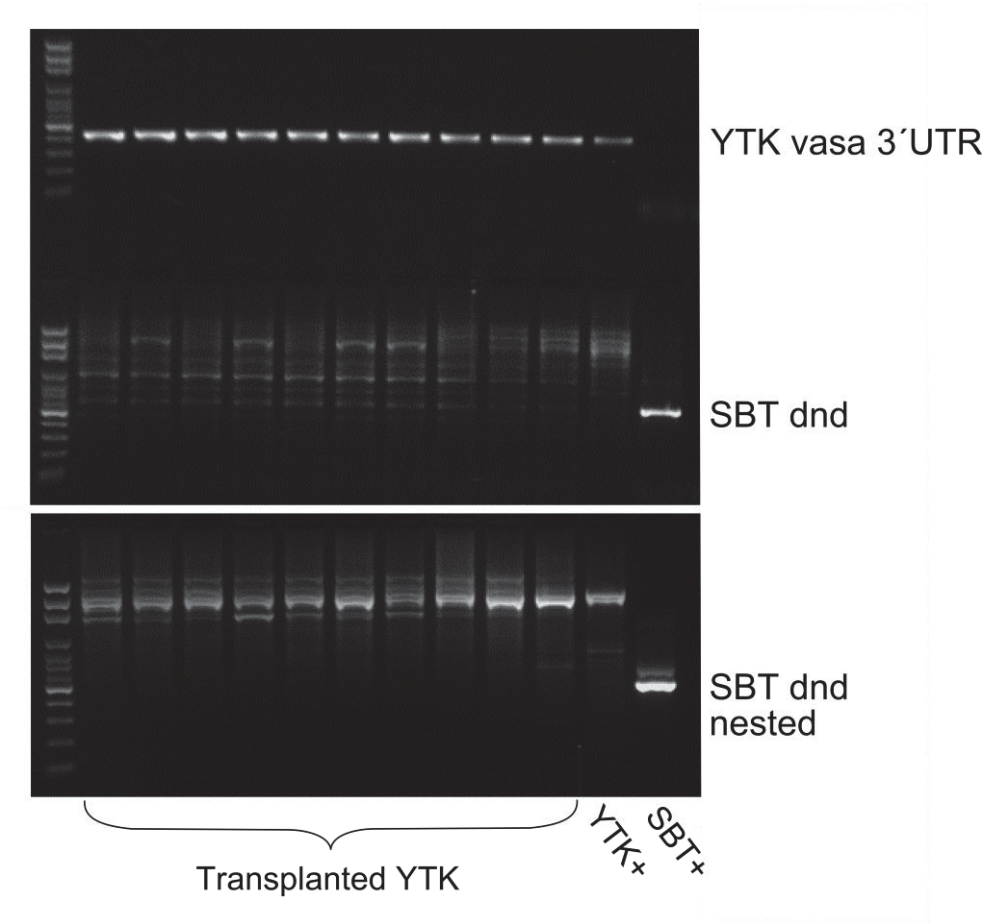

Figure 24. PCR of *Dead-End* gene in Yellowtail Kingfish gonads DNA. 10 samples of transplanted YTK gonad DNA (165 dpt), non-transplanted YTK (YTK+), and SBT (SBT+) controls were used as templates for PCR with YTK specific *Vasa* 3'UTR primers (upper row), SBT specific *Dead-End* primers (second row) and SBT specific nested *Dead-End* primers (bottom row) .Left lane – 100 bp DNA size marker.

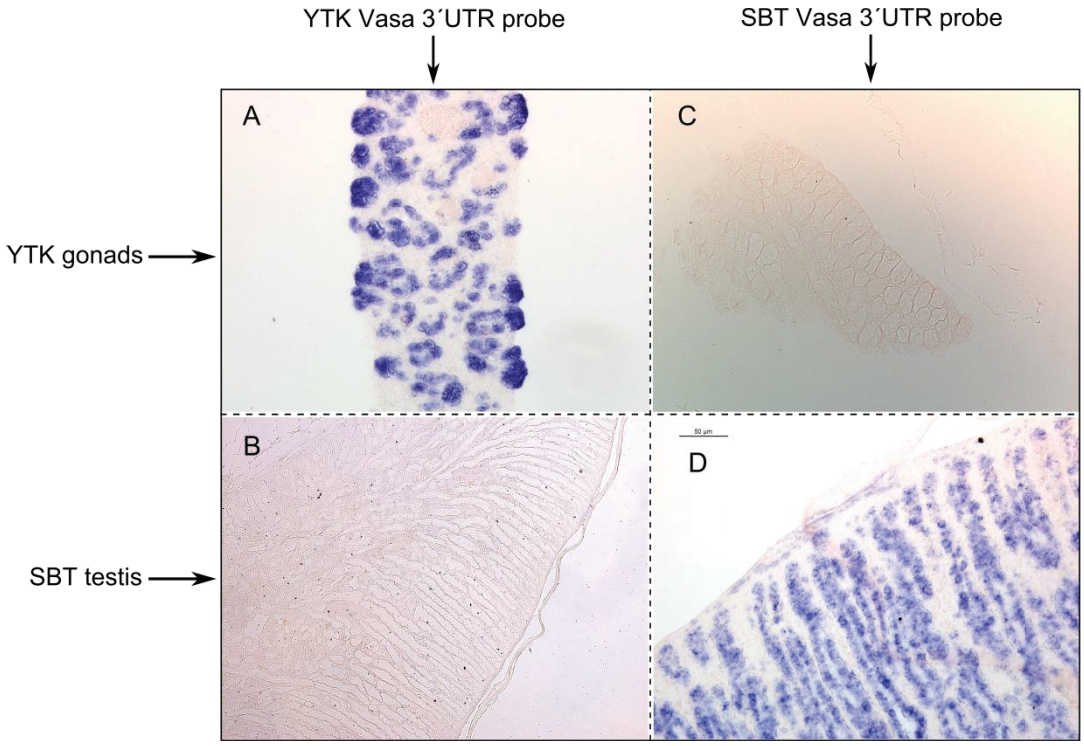

Figure 25. *in-situ* hybridisation controls. Specificity of the *Vasa* 3'UTR probes is demonstrated. **A**, **B** – labelling of YTK gonad and SBT testis cross sections (respectively) with YTK specific probe. **C**, **D** – labelling of YTK gonad and SBT testis cross sections (respectively) with SBT specific probe. Sections are not presented to scale, to provide a similar point of view despite the differences in tissue sizes.

3.5 To develop strategies for sterilization of surrogate host to obtain only SBT eggs and sperm for transplantation

3.5.1 Inducing triploidy in YTK larvae

An initial triploid induction trial in YTK larvae was performed as detailed in section 2.5.1. Spawned YTK eggs were separated into three treatment groups, differing in the duration of the cold shock treatment (5, 10 and 15 minutes). The eggs were incubated until hatched.

The cold treated eggs displayed poor hatching and survival rates, especially in the 10 minutes cold shock group, with only 3 surviving hatched larvae. Flow cytometer analysis of the hatched larvae from treated and non-treated (control) eggs, showed no evidence for triploid cells containing increased DNA content (3n), when compared to cells from control diploid larvae. Number of larvae analysed was n=14, 3 and 5 for each treatment duration group (5, 10 and 15 minutes).

4. Discussion

Germ cell transplantation could be a way to overcome current issues associated with the management and breeding of Bluefin Tuna broodstock (Takeuchi et al. 2009; Yazawa et al. 2010). It would allow smaller bodied, faster maturing surrogates to produce Bluefin Tuna eggs and sperm. The current project examined a number of aspects associated with development of surrogate technology, with a focus the optimisation of the dissociation and cryopreservation of SBT testis material, suitability of YTK and alternative species as a surrogate broodstock for SBT, the development of molecular tools for the identification of SBT germ cells in surrogate hosts and the development of strategies for sterilization of the surrogate host to obtain only SBT eggs and sperm.

4.1 To develop and optimise protocols for the collection and dissociation of SBT germ cells for transplantation

4.1.1 Optimisation of dissociation protocol

To optimise the dissociation protocol of SBT testis material for transplantation, different dissociation enzymes, concentrations of enzymes and the proportion of minced testis material to dissociation media were examined. Although there is limited literature on the optimisation of the dissociation process itself, previous studies have reported the use of either combined enzymes collagenase/dispase or trypsin for the dissociation of testis material (Morita et al., 2012; Takeuchi et al. 2009). The use of trypsin as the dissociation enzyme produced 9.4 $x10^4$ type-A spermatogonia cells when dissociating 1mg.mL^{-1} of testis material from 3-month-old Nibe Croaker (Takeuchi et al., 2009). When 100mg.mL^{-1} of testis tissue from 10-month-old Japanese Yellowtail was dissociated using the combined dissociation enzyme collagenase/dispase, approximately 10^7 testicular cells were produced.

The results from the Experiment 2 found no significant difference in cell numbers when examining the two dissociation enzymes collagenase/dispase and trypsin when dissociating 2, 4, 10, 30, 50 and 100mg.mL^{-1} of SBT testis tissue. There was also no significant difference in cell numbers between the three different working concentrations of the enzyme collagenase/dispase (2, 4 and 10mg.mL^{-1}) when dissociating 30mg of testis material. However, there was a significant difference in cell numbers when larger amounts of tissue were dissociated. Specifically, significantly higher number of testicular cells were produced when 100mg.mL^{-1} of tissue was dissociated with either trypsin or collagenase/dispase compared to 30 and 50mg.mL^{-1}. This was further supported in Experiment 3 when cell numbers dramatically increased, as the amount of testis material increased. Statistical analysis of this data was not performed due to insufficient replicate numbers; however, the results clearly indicated a trend of increasing cell numbers with increasing tissue amount (from 100 to 500 mg of testis tissue per ml of media). The results of this experiment also showed that the dissociation enzyme collagenase/dispase was a more effective enzyme than trypsin before and after the Percoll gradient was applied. These results differ to those in Experiment 2 when smaller amount of tissues were used and no significant differences were found between the two dissociation enzymes. The results of Experiment 3 also showed that cell numbers dropped dramatically after the Percoll gradient was applied in each treatment, which was expected as the Percoll gradient acts as a cell separation technique by using density

gradient centrifugation and removes cells considered not typical of type-A spermatogonia size.

In summary, the results established a protocol that enhanced the number and viability (i.e. up to 10 million cells.mL^{-1}) of testicular cells dissociated from SBT testis material available for transplantations into potential surrogate hosts. This method enabled us to transplant a maximum of 1321 recipient larvae over two days using 10.5x10^{6} cells (see Section 3.3.1, Table 5). Generally, time was the limiting factor in regards to the number of transplantations that could be carried out rather than the amount of cells available. Previous germ cell transplantation research has stated that a 15nL injection, transplants around 20,000 testicular cells into the recipient larvae (Morita et al., 2012). This is the same ballpark number of cells used in this study.

4.1.2 Assessment of the effect of size and reproductive maturity of SBT on testicular cell number and viability

The SBT commercial harvest season typically runs between April and October each year, which is the timeframe that fresh SBT testis tissue is available to sample for dissociation and subsequent transplantation. This part of the project examined the quantity and viability of testicular cells dissociated from different sizes and stages of reproductive maturity of SBT that were sampled every month of the commercial harvest season. The SBT sampled ranged in weight and fork length from 17 to 71 kg and 92 to 145 cm, respectively. Both SBT weight and fork length had a positive correlation with increasing cell numbers and viability, ranging between 1.8 and 3268.0 cells.mL^{-1} (x10^{4}) and 19.6 and 98 % viability, respectively. One of the reasons larger fish produced a higher number of viable cells could be due to the fact that a larger testis broke down and dissociated easier than a smaller testis when mincing with the Wecker scissors. Increasing GSI and stage of reproductive maturation also had a positive correlation with increasing cell number. GSI was also positively correlated with cell viability. Ideally, these factors, along with fish weight and fork length should be targeted when sampling SBT testis material to obtain testicular cells for transplantation. However, the reality of sampling at sea with time constraints and harsh weather conditions, assessing the GSI and using gross observations to assess approximate stage of reproductive maturation could prove to be difficult, but possible if adequate equipment and trained personnel were available. A previous study on the stage of reproductive maturity of production SBT showed that 92% of production fish sampled were classed as being sexually immature (i.e. either stage 1 or stage 2 of reproductive maturity) (Bubner et al., 2012) and therefore, it is likely that sexually

mature testes are difficult to get during the limited commercial harvest period. However, in any case attempts to sample larger fish should be made, which would be amiable downstream for germ cell transplantation technology.

This part of the project also found that SBT testis material sampled in September produced significantly higher amount of testicular cells than the majority of the other months. Cell viability from SBT testis material sampled in September was; however, significantly lower compared to testis material sampled in June, July, and October in the same year. The reason for this is unclear, and therefore it may be possible that there was an artefact associated with the processing of the cells in September. Sampling SBT testis material over future commercial harvest seasons would need to be conducted to validate the results found here.

4.2 To assess and optimise cryopreservation procedures of SBT germ cells

As stated previously, SBT testis material is only available during the commercial harvest season, which means that its availability is limited throughout the year. Cryopreservation technology could assist in supplying a readily available source of SBT testis material for germ cell transplantations year-round, take the pressure off that is associated with having to coordinate the availability of donor tissue with the presence of suitable host larvae, and allow the use of this technology to be applied in other geographical locations, as cryopreserved material can be easily transported.

The results of the present study showed that SBT testis material can be successfully cryopreserved, thawed and dissociated to produce viable testicular cells for transplantation. The first experiment assessed the suitability of a cryopreservation protocol developed for PBT. The results showed that cryopreserved SBT testis material did produce viable testicular cells, however, the number of cells was lower than that of fresh SBT testis tissue (i.e. tissue that was not cryopreserved). This may have been due to two main issues that arise for cells cooled below freezing temperature: 1) water crystallizes to form ice and; 2) salt concentrations rise which can rupture and cause serious damage to the cells (Gosden, 2011; Mocé and Vicente, 2009; Watson, 2000). When the viability of cells was assessed overtime using the Trypan blue exclusion method, the viability of the testicular cells dissociated from the cryopreserved tissue did not significantly decrease up to 44 h post-thawing and dissociation; however, the viability of these cells was significantly lower at this time compared to the viability of the cells dissociated from the fresh

tissue at 0, 12 and 44 h post-thawing. This suggests that testicular cells dissociated from cryopreserved SBT testis material degrades faster compared to testicular cells that are dissociated from fresh SBT testis material.

Among the various factors that affect the success of a cryopreservation method, is the balance between the bio-toxicity of the cryoprotectant and the protection afforded during the freezing and thawing steps (Fabbrocini et al., 2000). The results shown in this study suggest that fresh SBT testis material would be preferred for transplantation trials because significantly more cell numbers can be dissociated from fresh testis tissue compared to cryopreserved testis tissue. Also, viability assessments of the testicular cells, examined using the Trypan blue exclusion method, found that cells dissociated from fresh tissue does not degrade as quickly as the cell viability of the cells dissociated from cryopreserved testis tissue. However, in the instance where fresh SBT testis material is not available, cryopreserved testis material may be an adequate alternative. The study by Lee et al. (2013) found that thawed type-A spermatogonia derived from cryopreserved rainbow trout testis material did not lose their transplantability after 939 days of cryopreservation. Functional eggs and sperm were successfully derived from frozen testicular germ cells through the transplantation of those germ cells into sterile triploid rainbow trout hatchlings. This is an encouraging finding that gives confidence about future transplantation of SBT testicular cells that are dissociated from cryopreserved material. The viable SBT testicular cells produced from cryopreserved tissue in the current study indicated that the cryoprotective agent DMSO was effective in protecting the cells against damage whilst freezing. Further improvements to the cryopreservation protocol may assist in obtaining a larger amount of viable SBT testicular cells and would be worthy of investigation in the near future. This would reduce the time required to prepare the cells for transplantation, as it eliminates the need to collect samples at sea as the tissue would be readily available.

The second of the cryopreservation experiments aimed at optimising the established cryopreservation protocol by slowing the cooling rate to eliminate the formation of ice crystals, reducing the toxicity of the cryoprotectant, and plunging the SBT testis material straight into liquid nitrogen. The results show that the original protocol developed by Professor Goro Yoshizaki's research group yielded significantly higher cell numbers and viability than all other treatments examined. Plunging the testis tissue straight into liquid nitrogen rather than into -80ºC in a Biocell to control the cooling rate yielded zero cells. This is most likely due to the formation of ice crystals

in the cell membrane that typically occur when cells are rapidly frozen, which can rupture and cause serious damage to the cells (Gosden, 2011; Mocé and Vicente, 2009; Woods et al., 2004). This indicates that controlling the cooling rate has a decisive role in determining the survival of cryopreserved and thawed cells. Cooling the testis material at a slower rate by using a -20ºC freezer did produce a small amount of testicular cells; however, the amount of cells was significantly lower than cooling the testis material using a -80ºC freezer, which could be due to the slower cooling rate allowing the toxicity of the DMSO to extensively dehydrate the cells and upon thawing, causing the cells to swell and rupture (Woods et al., 2004).

Previous studies on the cryopreservation of sperm found that sperm quality after freezing-thawing improved when the concentration of cryoprotective agents were lowered from 8 to 4% (Mocé and Vicente, 2009). However, this was not the case in the present study when a lower concentration of DMSO was used (from 9% to 5%), as it may not have offered enough protection against intracellular ice formation during freezing. Although DMSO at higher concentrations are potentially toxic to cells, previous studies have shown that DMSO concentrations between 12.0 and 17.5% have been used successfully to cryopreserve sperm (Mocé and Vicente, 2009). Therefore, future studies to further optimise the cryopreservation of SBT testis material could examine higher DMSO concentrations.

Although the results of the current study showed that SBT testis material can be successfully cryopreserved and thawed to produce viable testicular cells, such cells have not yet confirmed to successfully migrate and colonise in the YTK transplanted hosts, which would need to be confirmed to establish the true success of the cryopreservation technique for SBT.

4.3 To investigate and establish potential surrogate host for SBT

4.3.1 YTK as a potential surrogate host for SBT

The previous components of this study (small-scale larval rearing of YTK, dissociation and cryopreservation of SBT testis material) lead into the key segment, which investigated the suitability of YTK as a surrogate broodstock for SBT. During 5 transplantation experiments (between 2012 and 2013), 12,085 YTK larvae were transplanted with SBT testicular cells. The current study confirmed that intraperitoneally transplanted SBT testicular cells were incorporated into the developing gonads of recipient YTK larvae, and this has been achieved between a

donor and recipient that are from different families (SBT – Scombridae and YTK – Carangidae). Specifically, PKH26-labelled SBT donor testicular cells that were transplanted into the peritoneal cavity of YTK recipients between 6 and 11 DPH were observed to be incorporated into the genital ridges of YTK recipient larvae when examined at 21 and 28 DPT (Figure 16 and Figure 17). The findings confirm the ability of the transplanted SBT testicular cells to migrate towards the genital ridge of the YTK recipient was not suppressed by the xenogeneic environment. The colonization rate was between 33.3 and 37.5%, which was low compared to other studies, where 70% colonisation was observed in Chub Mackerel (*Scomber japonicus*) recipients in the xenogenic environment and 92.6% in the Japanese Yellowtail (*Seriola quinqueradiata*) in the allogeneic environment (Morita et al., 2012; Yazawa et al., 2010). However, it was still a big step forward in examining the suitability of YTK as a surrogate host for the SBT.

The initial three experiments had very poor survival (<0.2%) of transplanted YTK larvae. In these three experiments, the control larvae had a survival rate that ranged from 4.5% and 10%. The final two transplantation experiments achieved better survival by sourcing larvae from Cleanseas Tuna's YTK production or R&D larval rearing, which typically achieves swim bladder inflation above 95%. The survival rates of these two experiments achieved survival up to 9%. The poor survival rate of the transplanted and control larvae in the initial three experiments could have be attributed to a number of factors including: water temperature fluctuations, inadequate water flow and water dynamics, poor quality eggs or larvae, low swim bladder inflation, bacterial blooms in larval rearing tanks and the potential stress of the transplantation process on the larvae. However, poor swim bladder inflation in the YTK larvae is the most probable major contributor to the poor survival. Out of the five transplantation experiments, the two experiments that achieved the highest larvae survival were achieved when larvae were sourced from the industry partner Cleanseas Tuna Ltd (Experiments 4 and 5). These YTK larvae were considered superior quality compared to those reared by the research group in the other experiments (Experiment 1, 2, and 3), with swim bladder inflation in excess of 95%. This highlights the importance of sourcing good quality YTK larvae for such experiments. It was not possible to source YTK larvae from Cleanseas Tuna's production run for the initial three experiments as the availability of SBT testis material and YTK larvae did not align. The optimal survival rate of the transplanted YTK larvae in this study were similar to previous germ cell transplantation research on the Japanese Yellowtail (*S. quinqueradiata*), where the survival of transplanted

larvae was 8.0 and 10.9% for larvae of 4.8 and 5.2mm in length, respectively (Morita et al., 2012). The survival rate of the Japanese Yellowtail did increase with the size of the larvae, with survival rates increasing to 15.9 and 29.6% for larvae 5.6 and 6.0mm in length, respectively (Morita et al., 2012). Strategies to increase the survival rate of transplanted YTK larvae are paramount if future research is to build on the findings of this study. Enhanced survival rate of transplanted recipients may be achieved by transplanting larvae at an older age (>11 DPH). However, they would need to be <15 DPH, as previous histological analysis of YTK gonads found that at 15 DPH endogenous PGC had settled at the genital ridge and had started to become enclosed by somatic cells, which suggests larvae at this age may no longer be suitable for transplantation as the primordial gonad had started to develop in the larvae (Bubner, 2011). Although the study by Morita et al. (2012) found higher survival in the Japanese Yellowtail when the larvae were transplanted at a longer length (suggesting an older age), the incorporation rate of the donor cells into the recipient gonad decreased with the length of the transplanted larvae.

The use of antibiotics could be another strategy to enhance the survival of transplanted YTK larvae. Antibiotics have been shown to result in a five-fold increase in survival with increased growth and less intestinal bacterial growth in the striped trumpeter larvae and could be a possible way to improve the survival of transplanted YTK larvae (Battaglene et al., 2006). While not recommended as a general strategy for commercial larval rearing, could make a significant contribution to labour intensive small-scale rearing such as transplantation work.

While the survival of the transplanted larvae was low, the current study now has approximately 100 YTK surrogate fish that are over 10 months old. Molecular analysis on the YTK surrogates is currently being carried out. Preliminary results of Polymerase Chain Reaction (PCR) analysis found that gonads sampled from 5 month old transplanted YTK surrogates contained SBT DNA, therefore providing evidence that the transplanted SBT testicular cells have colonised within the YTK gonads. Future in-situ hybridisation analysis are currently taking place to provide visual evidence of the transplanted SBT testicular cells within the YTK gonad and see if they have actually proliferated or only migrated and colonised. These fish are now being held by Cleanseas Tuna and in another 2 years' time, when the YTK surrogates reach sexual maturity, we will be able to assess whether any SBT derived sperm or eggs are produced.

Future implications

The success of this germ cell transplantation technology coupled with CST resolving the technology for SBT larval rearing will enable the acceleration of a selective breeding program for SBT by using a faster maturing surrogate such as YTK. To implement a traditional genetic breeding program for SBT, a 12 year generation time would be required to select for improved traits such as; improved growth rates, improved resistance to disease and parasites, and enhanced post-harvest product quality. Using YTK as a surrogate host for SBT would greatly decrease the generation time needed to select for desirable traits. Transplanting testicular cells derived from SBT with desirable traits into YTK larvae, we can see the benefit of selected traits within three years. This means that over the course of one generation of SBT under a traditional genetic breeding program (12 years), surrogate broodstock technology could achieve 3-4 generations in improvements in selected lines. The benefits from germ cell transplantation technology would greatly enhance the aquaculture industry from an economic and product quality perspective.

As highlighted previously, germ cell transplantation technology can greatly reduce the generation time of specific aquaculture species. In the current study, if the YTK indeed carry SBT germ cells we potentially reduced the generation time of SBT from 12 to 3 years. This could further be reduced by transplantation of donor testicular cells into adult recipients, such as the study by Lacerda (2012) who reported donor derived spermatozoa within 9 weeks of transplantation into the adult Nile Tilapia (*Oreochromis niloticus*). However, this research is still in its developmental stages, and is still progressing to be able to create donor derived gametes.

Another approach to enhance the germ cell transplantation procedure is the sterilisation of the surrogate host species so that only gametes of the donor species are produced. Recent studies by Okutsu et al. (2007) investigated xenogenic transplantation of Rainbow Trout (*Oncorhynchus mykiss*) spermatogonia into sterile triploid Masu Salmon (*Oncorhynchus masou*) larvae. This study resulted in offspring populations consisting only of the donor trout that were generated through the fertilisation of eggs and sperm from surrogate salmon parents. The benefits of using a sterile host are producing solely donor-derived offspring which results in less time, money and energy being wasted in raising non-target offspring (Okutsu et al., 2007). The sterilisation of YTK surrogates will result in an increase in seed production of SBT, as gonad production is solely generating donor-derived gametes (Okutsu et al., 2007). This could potentially be applied to future germ cell transplantation research in

SBT and assist in closing the life-cycle for aquaculture. Currently the University of the Sunshine Coast Genecology Research Centre is investigating the conditions for the generation of YTK triploids and if proliferation of SBT donor cells can be confirmed in the YTK host, future transplantations will be carried out into triploid YTK larvae

4.3.2 Alternative species as a potential surrogate hosts for SBT

It has been very challenging to source scombrid fish to establish broodstock for generating surrogate larvae for SBT. We have managed to develop the appropriate fish holding contraption and when fish were sourced close to Bribie Island, to successfully transport them into the facility. However, we have been unable to reliably source fish in the vicinity of the Island, and any sources that were further away could not be transported alive the greater distance. The Blue Mackerel were the only scombrid species that both transported well and handled well in tank conditions. We have been able to anaesthetise the fish without experiencing any mortality, and the fish have schooled and fed almost immediately after being introduced into the tank. Treatment with GnRHa to induce spawning resulted in an increase in gonad size, and this August we plan to source a larger number of Blue Mackerel fish and provide them with a number of spawning induction treatments to establish a reliable supply of surrogate larvae, before we move on to create triploids for this species. The evolutionary distance between the Blue Mackerel and SBT is 83 million years compared with 160 million years which separate YTK from SBT, making it a more suitable host.

4.4 To develop molecular tools for the identification of SBT germ cells in surrogate host

Developing a toolbox of molecular tools for the identification of SBT germ cells was crucial for their specific labelling and localisation. It enables us to detect SBT germ cells in the donors' testes, follow their migration pathway and destination after transplantation and distinguish between the transplanted cells and the host endogenous germ cells. These molecular tools are based on gene and species specific probes and PCR primers and required the establishment of germ cells specific gene library and detection methods. In this study we have been able to identify and describe for the first time germ cells specific genes and their expression in SBT testis. We have used these genes to develop probes that identify type A Spermatogonia germ cells, as well as species-specific probes for both PCR and *in-situ* hybridisations. Once established, these probes could be readily optimised for use with any other alternative surrogate host species. The analysis of the transplanted

YTK has confirmed that SBT germ cells migrated and colonised in the YTK gonad, however we have not been able to show proliferation of the SBT cells in the YTK gonad. This is still under investigation.

4.5 To develop strategies for sterilization of surrogate host to obtain only SBT eggs and sperm for transplantation

Producing and utilising triploid fish as surrogate hosts is an effective strategy for achieving sterilisation of its own gametes so it can produce gametes of the donor species only (i.e. host cannot produce its own viable eggs and sperm). It is an essential step towards commercialisation of the surrogate technology as it allows great control over the host's gonad development. Triploid induction via temperature shock (heat shock for cold water species and cold shock for temperate and tropical water species) is relatively easy, cost effective and produces high rate of triploids, when optimal conditions are established per species. The results from the preliminary trial conducted with YTK in the project suggest that a more extensive research is needed to determine the optimal conditions (time after spawning, temperature and duration of the cold shock) to ensure high rates of triploid larvae, along with improved survival rates; however, this step will only became relevant when we confirm SBT gametes proliferate in the YTK gonad.

5. Benefits and Adoption

We have established the technology and the know-how for carrying out germ cell transplantation including cryopreservation, cell preparation and molecular tools, which can be used for SBT, but also provide the basis for other species where the technology is relevant. Currently the knowledge is being adopted at the USC lab for the giant grouper aquaculture sector in Australia as part of an ACIAR funded project in collaboration with the Northern Fisheries Centre, RIA1 in Vietnam and SEAFDEC in the Philippines. This technology is not ready to be adopted by the tuna industry at this stage, as more R&D is required to develop the technology to the point where it could be applied to commercial objectives (see section 6 below)

6. Further Development

We now have YTK transplanted with SBT germ cells, however we have not yet been able to determine if SBT germ cells proliferate in the YTK gonad, and this investigation is still on going. We have also been able to establish protocols for the capture of alternative small tunas, raising them in the tank, and in August we will do a trial to spawn Blue Mackerel, which is likely to be a more suitable species, in terms of evolutionary distance from SBT, to be a surrogate for SBT. However this potential is less immediate than YTK for example as its own aquaculture still remains to be developed.

7. Planned Outcomes

Public Benefit Outcomes

We have established knowledge and protocols for the harvesting and preparation of SBT germ cells, and their cryopreservation. We have protocols and experience in preparing and transplanting cells into larvae, and doing small scale larval rearing. We also have the molecular tools for distinguishing between SBT and YTK cells. This information is available for anyone who wants to set up surrogate technology. There are multiple potential applications for surrogacy methods in fish, for aquaculture and conservation for example. This project has effectively transferred technology, developed initially in Japan, to Australia and it is hoped that practical and commercial applications will arise from this following further R&D.

Private Benefit Outcomes

The private benefits from this project include training 2 graduate students in surrogate technology, setting up the platform for implementing surrogate technology at Cleanseas tuna, with further R&D.

Linkages with CRC Milestone Outcomes

This project operated under the following CRC milestone outcomes:

Outcome 1- Substantial increase in the production and profitability of selected wild-harvest and aquaculture species.

Output 1.1- Technically verified new aquaculture production systems on a commercial scale.

Milestone 1.1.3- Key researchable constraints successfully addressed in at least two new production systems.

We have identified the development of surrogate technology for Southern Bluefin Tuna as a researchable question that could lead to substantial increase in production and profitability in aquaculture systems in general and SBT in particular, and have researched this in two systems, using YTK and other smaller tunas as a host.

8. Conclusion

This project has established key components that form a platform for germ cell transplantation technology for SBT and potentially other commercially important species. The project established a dissociation and cryopreservation method for SBT testicular material that will enable year-round access to transplantable testicular cells for transplantation. In addition, the current project also demonstrated that the somatic microenvironment of the YTK larval gonads supported the survival, migration and colonisation of testicular cells derived from SBT. This highlights the fact the germ cell transplantation technology is potentially applicable between a donor species (SBT – *Scombridae*) and recipient species (YTK – *Carangidae*) that are of different families. This research forms the foundation to a major breakthrough that could assist in closing the life-cycle of SBT for aquaculture and could potentially replace the need to maintain large, late maturing Bluefin Tuna broodstock in captivity, which is a major challenge for the sustainable aquaculture of SBT.

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10. Appendices

Appendix 1: Intellectual Property

Appendix 2: Staff and Students

The staff engaged in this project were:

University of the Sunshine Coast

Professor Abigail Elizur

Dr Scott Cummins

Flinders University

Dr Erin Bubner

Queensland Department of Primary Industries and Fisheries

Dr Peter Lee

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The students engaged in this project were:

University of the Sunshine Coast

Mr Ido Bar

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