RGC Ref.: **X-HKUST602/14**
(please insert ref. above)

The Research Grants Council of Hong Kong
SFC/RGC Joint Research Scheme
Completion Report

(Please attach a copy of the completion report submitted to the Scottish Funding Council by the Scottish researcher)

Part A: The Project and Investigator(s)

1. Project Title

Application of an ion-sensitive microprobe to investigate and compare the contribution to homeostatic Ca^{2+} regulation by the scales of diadromous sea trout (*Salmo trutta*) and freshwater zebrafish (*Danio rerio*).

2. Investigator(s) and Academic Department/Units Involved

	Hong Kong Team	Scottish Team
Name of Principal Investigator *(with title)*	Prof. Andrew L. Miller	**Dr. Chevonne Angus**
Post	Professor	**Joint Head Marine Science**
Unit / Department / Institution	Division of Life Science and State Key Laboratory for Molecular Neuroscience, HKUST, NT, Hong Kong	**Marine Science Centre, NAFC, UHI, Scalloway, Shetland Isles, Scotland, UK.**
Contact Information	Email: almiller@ust.hk Phone: 852 2358 8631	Email: chevonne.angus@uhi.ac.uk Phone: 44 01595 772306
Co-investigator(s) *(with title and Institution)*	-	-

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted)*
Project Start date	01/01/15	NA	
Project Completion date	31/12/15		
Duration *(in month)*	12 months		
Deadline for Submission of	31/12/16		

Completion Report			

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. To extend our limited understanding of the roles played by the elasmoid scales of a freshwater and a diadromous fish species - the zebrafish (*Danio rerio*) and the sea trout (*Salmo trutta)*, respectively - focusing on the regulation and maintenance of Ca^{2+} homeostasis when faced with varying internal and external calcemic challenges.
2. We plan to carry out these experiments in as near to an *in vivo* situation as experimentally possible by using living, exfoliated scales maintained in controlled cultured conditions.
3. Scales will be collected from either wild sea trout (along with blood and water samples) trapped by NAFC staff at different sites in the Shetland Isles along their inshore migration route as they make the transition from a euhaline to fresh water environment, and from zebrafish raised in the zebrafish facility at HKUST, Hong Kong.
4. We propose to expose scales collected from both species to varying Ca^{2+} challenges, where their culture medium will contain different amounts of free Ca^{2+} (i.e., hypo-, iso-, and hypercalcemic with respect to blood plasma levels) in order to compare the Ca^{2+} dynamics generated by their scales.
5. We will also explore the role played by the estrogen 17β-estradiol and parathyroid hormone with regards to regulating the Ca^{2+} exchange between scales and their bathing medium under varying Ca^{2+} challenges.
6. Ca^{2+} fluxes into and out of designated quadrants of scales on both the episquamal and hyposquamal sides, as well as the lateral edges, will be measured using a unique non-invasive, extracellular, Ca^{2+}-sensitive microprobe, via a methodology known as the Scanning Ion-Selective Electrode Technique (SIET), which has a sensitivity in the sub pmol/cm^2/sec range, and with a spatial resolution that is determined by the probe-tip size, which can be as small as 3-5 μm in diameter.
7. Following the generation of precise Ca^{2+} flux maps, scales will be fixed for post-SIET analysis, where via immunohistochemistry-based labelling we propose to correlate the inward or outward fluxes of Ca^{2+} with the cell and tissue types that make up an elasmoid scale, as well as with anatomical features like circuli and possible sites of mineralization and resorption.
8. We plan to explore the possibility that fish scales may serve as a non-mammalian model for the study of human bone with regards to mineralization and resorption, as well as injury, disease and regeneration.

5.2 Revised Objectives: Nil

Date of approval from the RGC: NA

Reasons for the change: NA

1.
2.
3. ....

6. Research Outcome

Major findings and research outcome
(maximum 1 page; please make reference to Part C where necessary)

Thanks to SRC/RGC funding, in addition to a MASTS Visiting Fellowship to support Prof. Miller, we have firmly established a successful and productive collaboration between the NAFC Marine Centre, UHI, and the The Hong Kong University of Science and Technology (HKUST). In addition to these two main partners, we have also recruited additional expert collaborators from the School of Biosciences, University of Essex, UK; the Bone Metabolism Unit, at the Ospedale San Raffaele, Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy; the Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Università di Reggio Emilia, Università degli Studi di Modena e Reggio Emelia, Modena, Italy; the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI, Canada; the National Marine Fisheries Service, Northeast Fisheries Science Center, Woods Hole, MA, USA; and the College of Arts and Sciences, University of New England, Biddeford, ME, USA. When added to the resident scientists and staff at the NAFC Marine Centre, we believe that we have assembled the nucleus of a first-class, international team of researchers with the experience and expertise to tackle a challenging and potentially rewarding research project. Further details regarding these collaborations are outlined below. Thanks to the support that we have already received, we have been able to gather a significant amount of new and exciting data that are currently being analysed and written-up for submission to peer-reviewed journals. These data will also provide essential support in our current and future efforts to raise new funds to support a continuation of this project.

Potential for further development of the research and the proposed course of action
(maximum half a page)

Future Plans: We have submitted one proposal and are currently preparing additional grant applications in order to raise funds to continue and expand the fish scale collaboration between the NAFC and HKUST in the summer of 2016 and beyond. These include applications to:

(a) The Hong Kong Research Grants Council (RGC) General Research Fund. We currently have a grant under review by the HK RGC where we have requested funding for the continued support for the HKUST-based zebrafish-related portion of the SFC/RGC fish scale project. Our challenge now is to raise funds to support the NAFC side of the collaboration.
(b) We are currently preparing a resubmission to the Wellcome Trust Seed Awards in Science. These 2-year, one-off, grants of up to £100,000 would be a perfect way to keep the HKUST/NAFC-based work going.
(c) The study of human bone physiology and degeneration that results from long-exposure to microgravity is an area of current intense research. We propose, therefore, to also submit an application to the European Space Agency (ESA) to seek support for our proposition that fish scales might act as a cost-effective model to study low-gravity effects on bone. A colleague based in the US, Dr. Paola Pajevic-Deviti, located at the Endocrine Unit of Massachusetts General Hospital, has obtained support through NASA via the National Institutes of Health Biomedical Research on the International Space Station (BioMed-ISS) to study the effects of microgravity on bone density loss. We feel, therefore, that it might be worth exploring whether the ESA might be willing to support the establishment of a team based at the NAFC to explore the use of fish scales as a substitute to study the effects of microgravity on bones.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

The elasmoid scales of fish, which are part of the dermal skeleton, show a significant degree of similarity with the skeletons of mammals in terms of bone architecture, matrix proteins and molecular signalling (Pasqualetti *et al*., 2012a). Although it has been proposed for many years that teleost scales represent a significant internal reservoir of Ca^{2+} (~20% of total body Ca^{2+}; Takagi *et al*., 1989), little was known about their physiological function and how they might contribute to plasma Ca^{2+} balance and how Ca^{2+} deposition and mobilization are regulated. Teleost scales also contain bone forming osteoblasts and reabsorbing osteoclasts (Suzuki *et al*., 2009) similar to those found in mammalian bone. Furthermore, fish scales contain numerous organic and inorganic components, mainly collagens and hydroxyapatite which potentially have a commercial value through use in manufacturing foods, cosmetics and biomedical products (Lin *et al*., 2010). We have explored Ca^{2+} dynamics and correlate it with the detailed anatomy of living scales removed from zebrafish and sea trout in order to extend our understanding of the scale metabolism under different calcemic stresses and to assess whether elasmoid scales might be used as a model to study human bone metabolism, disease, repair and regeneration.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project

(Please attach a copy of each publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.))

The Latest Status of Publications				Author(s) ***(bold** the authors belonging to the project teams and denote the corresponding author with an asterisk*)*	Title and Journal/ Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Year of publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*						

-

-	-	-	Yes	Jacky T.S. Hung, Alessandro Rubinacci, Joseph Kunkel, Alan M. Shipley, Paola Pajevic-Devieti, **Chevonne Angus,** Sarah E. Webb and **Andrew L. Miller***	**The use of the elasmoid scales of zebra fish as a model to study mammalian Ca^{2+} homeostasis, and related diseases**		No	Yes	-

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered *(Please attach a copy of each delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)*

Month/Year/ Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
21st - 24th July/2016/ Zunyi, China.	**The zebrafish scale as a possible model for studying mammalian bone/plasma Ca^{2+} exchange.**	**The 11th Symposium on Calcium Signaling in China (SCSC).**	2016	Yes	Yes	Yes
1st- 4th October/ 2016/ Singapore.	**The use of the elasmoid scales of zebrafish as a model to study mammalian Ca^{2+} homeostasis and related disease**	**The 7th Asia Oceania Zebrafish Meeting (AOZM).**	2016	Yes	Yes	Yes
September 29th - October 3rd, 2015 -University of Strathclyde, Glasgow, Scotland, UK.	**Application of a non-invasive scanning ion-sensitive microprobe to investigate the contribution made to homeostatic Ca^{2+} regulation by the scales of sea trout (*Salmo trutta* morpha *trutta*).**	**2015 Marine Alliance for Science and Technology for Scotland (MASTS) Annual Scientific Meeting**	2016	Yes	Yes	Yes

3rd – 7th November/ 2014/ Suzhou, China	**The zebrafish scale as a possible model for studying mammalian bone/plasma Ca^{2+} exchange.**	**Cold Spring Harbor Asia Conferences: Bone and Cartilage: from Development to Human Diseases.**	2016	Yes	Yes	Yes
3rd – 7th November/ 2014/ Suzhou, China	**The zebrafish scale: a model for studying the molecular regulation of mammalian bone/plasma Ca^{2+} exchange; the immediate bone wounding response; and bone regeneration and repair.**	**Cold Spring Harbor Asia Conferences: Bone and Cartilage: from Development to Human Diseases.**	2016	Yes	Yes	Yes

10. Student(s) trained *(Please attach a copy of the title page of the thesis.)*

Name	Degree registered for	Date of registration	Date of thesis ~~submission~~/ graduation
Mr. HUNG Tin Shing Jacky	MPhil, HKUST	09/13	04/16
Mr. CHAN Yin Seng Harvey	MPhil, HKUST	02/13	03/15
Ms. WATERS Angel Waters.	BSc., University of New England, Maine, USA	09/14	08/18

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

Establishment of UK Collaborators: **Drs. Jamie Stevens and Andrew King:** We would also like to report the successful initiation of a complementary collaboration with Drs. Jamie Stevens and Andrew King, from the School of Biosciences at the University of Essex. As part of a sea trout population genetics survey funded partially by the Atlantic Salmon Trust, Drs. Stevens and King visited the NAFC between the 30th September and the 3rd October 2015, and collected samples (via fin-clipping) for DNA-fingerprinting by electrofishing the burns from the East

(Laxo Burn) and West (Strom Burn) coast sample sites selected by us for the sea trout scale project. These data will enable them to investigate whether the East and West coast populations of Shetland sea trout interbreed. The fin-clipped samples collected by Drs. Stevens and King were supplemented by additional scales provided from our own sampling activities, as fish scales also provide a suitable source of marker DNA. Once analysed by the team from Exeter, the data collected will hopefully result in additional complementary publications resulting from our joint SRC/RGC funding.

Establishment of International Collaborations:

(a) Drs. Alessandro Rubinacci and Carla Palumbo: Dr. Alessandro Rubinacci MD, from the Bone Metabolism Unit, at the Ospedale San Raffaele, Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy, is a recognized international expert in the field of mammalian bone physiology, morphology, injury, repair and regeneration. He is acting as a collaborator on the Shetland sea trout scale project. He visited the NAFC between the 8th and 15th October, 2015, in order to obtain a first-hand view of the project laboratory and our daily operations. Dr. Rubinacci's major role in the collaboration will be to advise us with regards to all possible translational aspects of the fish scale-derived data. His focus is mainly on the question of whether fish scales might be a used as a non-mammalian model to study human bone physiology and metabolism. Dr. Rubinacci has also donated some major pieces of equipment to the NAFC "fish scale laboratory", including a second vibration-free isolation table, and a set of Narishige micro-manipulators. In addition to recruiting Dr. Rubinacci's participation in the project, we have also enrolled a colleague of his, Dr. Carla Palumbo, from the Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Università degli Studi di Modena e Reggio Emelia, Modena, Italy. Dr. Palumbo is an expert electron microscopist, with a particular interest in bone osteocytes. In addition to collecting scale samples in the field for our own SIET-based examination, and for the population genetics study being conducted by Drs. Stevens and King from the University of Essex, we also collected additional samples for Dr. Palumbo. These were fixed then sent to her at the Università degli Studi di Modena e Reggio Emelia, for EM-based examination. Having high resolution TEM and SEM images of the scales collected from fish obtained from fresh, brackish, and salt water sampling sites, will provide additional information to complement our SIET-based studies as well as our immunolabelling and fluorescent confocal imaging data.

(b) Dr. David Groman: Dr. Groman of the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI, Canada, is a recognized expert of fish heath, disease, and most relevant in our case, of fish blood analysis. He is currently collaborating with us regarding the detailed analysis of blood samples taken from every fish trapped/caught during the field work undertaken in Shetland. Our initial primary focus will be on determining the resting [Ca^{2+}] in the blood plasma of fish taken from our three different calcemic environments: hypo-, iso-, and hyper-calcemic. Knowing these values will add an important factor to our overall analysis. Subsequent analysis will determine the levels of Ca^{2+}-related regulatory proteins present in the fish blood.

(c) Dr. Richard S. McBride: Another key collaboration established was that with Dr. McBride of the National Marine Fisheries Service, Northeast Fisheries Science Center, Woods Hole, MA, USA. Dr. McBride is a recognized expert on the biology of diadromous fishes and of sclerochronology. Sea trout scale samples collected, but not used for SIET-based analysis, were fixed and will be send to Dr. McBride for analysis. This will help us to confirm the age of the trout sampled as well as establish their history with regards to their diadromous behaviour. As otoliths were also collected from a number of fish we sampled, age data obtained from the scales will be compared with those derived via analysis of the otoliths. This will allow us to compare scale-derived aging with that obtained from otoliths.

Student training:
We regarded student training to be an essential component of the project. Thus, in addition to NAFC staff who assisted with the project (see next section) and were trained to maintain and operate the SIET equipment, three international students also participated in the sea trout scale project. Their travel and living expenses were partially supported by SRC/RGC funds. These individuals were, from Prof. Miller's lab at the Division of Life Science, HKUST, Hong Kong, Mr. Jacky T.S. Hung and Mr. Harvey Y.S. Chan, and from the College of Arts and Sciences, University of New England, Biddeford, ME, USA, Ms. Angel Waters. Mr. Chan spent 4 months at the NAFC, while Ms. Waters and Mr. Hung visited for 6 weeks each. Training students in the specialist techniques required is seen as an important factor in continuing and expanding the project.

Support from NAFC: This was considered to be one of the most important aspects that contributed to the success of the collaborative project. The Joint Heads of Department of the NAFC Marine Centre, Drs. Chevonne Angus (also a Co-PI) and Beth Mouat, could not have been more supportive of the project. An excellent lab was provided on the ground floor of the John Goodlad Centre, along with an office for Prof. Miller, as well as access and space in the Hatchery building for housing our Parr and Smolts. NAFC vehicles were also provided for transporting our fish trap to sampling sites. Ms. Leanna Henderson, a laboratory technician in the Fisheries Section, was assigned to work on the project, and was trained on the maintenance and operation of the SIET system. This was a crucial assignment, and Ms. Henderson proved to be an excellent and highly skilled researcher who quickly mastered the complex and quirky nature of ion-sensitive microelectrodes. Dr. Paul Macdonald (section leader of the Fisheries Section) provided much appreciated advice and problem solving skills. Further excellent support in the lab, field, and hatchery, was provided by Mr. Gregg Arthur (Hatchery Manager/Aquaculture Scientist, and Section Leader of the Aquaculture Section) and Saro Saravanan (a member of the Training & Skills Development Staff). Much appreciated technical support was provided by Geoff Young (Facilities Technician). Finally our field work, especially setting and moving the fish trap, relied very heavily on the help of just retired NAFC stalwart, Mr. Kenny Gifford, and the brains and brawn of two NAFC summer interns, Mr. Mark Jones and Mr. Callum Tait.

Support from Mr. Alan M. Shipley, founder and CEO of Applicable Electronics LLC: We are most grateful to Mr. Shipley for loaning us a complete SIET rig and all the required supporting equipment for the duration of the project. This highly specialized equipment enabled us to measure the extremely small Ca^{2+} fluxes in the extracellular medium bathing fish scales. Mr. Shipley visited the NAFC for an extended period during April and May 2015 and helped to install the SIET rig and train staff and students in its operation. This equipment is still located at the NAFC and we are currently negotiating with Mr. Shipley to see if he is willing to turn his generous loan into an even more generous donation.

Support from the Shetland Anglers Association (SAA): During the course of the project we built-up an excellent working relationship with the SAA, and especially with Mr. Alec Miller, the Association Secretary. Expert advice was provided with regards to trap setting locations, and we worked hand-in-hand with the members so as not to disrupt or interfere with their sporting activities. The Association also provided access to their fresh water brood-stock cages in the Upper Loch of Brouster so that Drs. Stevens and King could collect fin-clip samples from sea trout held in the facility.

Support from Mr. Jim Smith, owner of the Arisdale Smolts hatchery in Yell: We are also very grateful for the kind support provided by Mr. Jim Smith the owner of the Arisdale Smolts hatchery in Burravoe, Yell, Shetland Isles. Mr. Smith provided, free of charge, a number of sea trout Smolts that we transported down to the hatchery at the NAFC. Thus, along with Parr taken from the Laxo Burn, we could compare the Ca^{2+} dynamics of Parr and Smolts, with adult sea trout taken from salt, brackish, and fresh water.

Support from Mr. Gordon Williamson, hotelier and gillie: We supplemented the wild sea trout taken in our traps by fly fishing in the Voes, brackish pools, and fresh water burns at our

collecting sites. This was found to be a necessary component in obtaining sufficient numbers of fish from the three calcemic environments on both the East and West sides of Shetland. We were greatly assisted in this endeavour by Mr. Williamson, who accompanied Prof. Miller on most days, regardless of the weather, in order to try and secure scales from two sea trout per collecting day. Over the course of the 2015 sea trout season (in Shetland this runs from 25th February till the 31st October), the scales of a total of 27 experimental sea trout were collected and examined via the SIET back in the NAFC laboratory. This amounted to a total of ~10 fish from each calcemic regime, i.e., salt, brackish, and fresh water, and where ~5 fish were taken from each regime on the East coast and ~5 from each one on the West coast. Taking into account the difficulties and challenges involved, this was considered a reasonably sized data set to work with.

Support from Mr. Jon Beer, angler, author, and President of the Wild Trout Trust: During September 2015 (16th - 20th), Jon Beer visited Shetland to gather material for his proposed article on the sea trout scale project for "Trout & Salmon" magazine. Mr. Beer is an expert angler and a keen naturalist. Furthermore, he has visited the Shetland Islands to fish for trout on several previous occasions. Thus, when he volunteered to help-out with our sample collection activities, his offer was gratefully accepted. Indeed, some of the data that we will eventually publish will be derived from sea trout that he caught during his visit.