Conditions for Culturing Biopsy Punches from Ngege (Oreochromis esculentus) Caudal Fins and Applications for Future Conservation Efforts.

by
Melissa Filice

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ABSTRACT

CONDITIONS FOR CULTURING BIOPSY PUNCHES FROM NGEGE (Oreochromis esculentus) CAUDAL FINS AND APPLICATIONS FOR FUTURE CONSERVATION EFFORTS.

Melissa Filice
University of Guelph, 2013

Advisors:
Dr. Gabriela Mastromonaco
Dr. Douglas Fudge

Many factors are known to influence long-term growth potential of primary cell cultures, such as cell culture conditions and culture establishment techniques. This study investigates the relationship between fish cell culture conditions and cell viability as a means of developing cell culture procedures for the conservation of endangered freshwater fish species. The cell culture conditions investigated include: donor age, biopsy size, sampling location, enzymatic dissociation treatments and incubation temperature. Culture quality was assessed for cell viability, oxidative stress, senescence and cell death. The conditions that were most effective for culture quality were 6 mm biopsy punches taken from the proximal area of the caudal fin, digested with collagenase I for 60 minutes, and grown in L-15 medium containing 1x Primocin at 25°C. Viable cell concentrations obtained from fluorescent staining confirmed that cell populations declined to less than half their initial concentration within 7 days in culture. Autophagy and apoptosis may play a role in the resulting cell death inferred by the presence of vacuoles and lysosomal activity observed on advanced cultures. The presence of vacuoles, oxidative stress and senescence among treatment groups are indicative of the inadequacies in the presently evaluated protocols. Further research into the conditions required for culturing healthy cell lines is essential for fish somatic cell banking.
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Completion of this degree would not have been possible without the assistance of my advisory committee, Toronto Zoo staff, family and funding agencies.

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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, performed by staff at the Toronto Zoo, all work reported in the body of this thesis was performed by me.

Assistance with tissue chopping samples in Chapter 3 was performed by research assistant Stacey O’Handley. Anesthesia and euthanasia were performed by veterinarians Dr. Graham Crawshaw, Dr. Pauline Delnatte, and Dr. Simon Hollamby. Animal care and fish catching for sample collection was performed by curatorial keepers, Kyla Greenham and Brian Telford and bacterial isolation and speciation in Chapter 2 was performed by veterinary technician Dawn Mihailovic.
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<td>ABAM</td>
<td>Antibiotic-Antimycotic</td>
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<td>AI</td>
<td>Artificial Insemination</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ART</td>
<td>Assisted Reproductive Technologies</td>
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<td>AZA</td>
<td>American Association of Zoos and Aquariums</td>
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<td>CAT</td>
<td>Catalase</td>
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<td>DDR</td>
<td>DNA Damage Response</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylenediminetetraacetic acid</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ET</td>
<td>Embryo Transfer</td>
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<td>GRB</td>
<td>Genome Resource Bank</td>
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<td>HEPES</td>
<td>Hydroxyethyl piperazineethanesulfonic acid</td>
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<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>IUCN</td>
<td>International Union for Conservation of Nature</td>
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<td>IVF</td>
<td>In vitro Fertilization</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>MMT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SCNT</td>
<td>Somatic Cell Nuclear Transfer</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>SSP®</td>
<td>Species Survival Plan</td>
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<tr>
<td>TFTC</td>
<td>Too Few Too Count</td>
</tr>
<tr>
<td>TUNNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>WAZA</td>
<td>World Association of Zoos and Aquariums</td>
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XTT 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid
CHAPTER ONE

General Introduction
1.1 Introduction

Fishes encompass a taxon containing approximately 27,300 species, equivalent to 48% of all vertebrate species (Clark and May, 2002; Nelson, 2006). This taxon can be further subdivided into three groups: jawless, cartilaginous, and bony fishes, which consist of 58% marine, 41% freshwater and 1% diadromous species (Helfman, 2007). Although marine fishes represent a larger portion of the taxon, freshwater fishes are in greater need of conservation because of the significant global decline in recent years. This is of concern not only for the impact of biodiversity loss on the ecosystem (Suski et al., 2006), but also because they are a main source of sustenance for many countries, and are economically beneficial to trade in the international markets, resulting in a $1.5 billion USD global fisheries industry (Carrizo et al., 2013). Burkhead (2012) compiled data on North American freshwater fishes from 1900-2010, and found that throughout the years 1898 to 2010, the cumulative number of extinctions in fish species and subspecies had risen 25%, resulting in 7.5 extinct fish species per decade. From these data, it was extrapolated that an additional 53-86 species may become extinct by 2050. Although some may argue that extinction is a natural process, the rate at which freshwater fishes are becoming extinct by anthropogenic forces (Burkhead, 2012), such as habitat destruction, introduction of non-indigenous fishes or invasive species, exploitation of fish stocks, and pollution, will be challenging to overcome (Helfman, 2007).

Clark and May (2002) examined two major journals, Conservation Biology (United States) and Biological Conservation (United Kingdom), between the years 1987 and 2001 to determine which taxa (mammals, birds, reptiles, amphibians, and fish) had the greatest number of publications in comparison to the percentage of known species. They found a taxonomic bias in conservation research whereby fishes, comprising 48% of all vertebrate species, made up only 8% of the publications within the 15 years of study, whereas mammals made up 40% of the papers published but only comprised 9% of all vertebrate species (Clark and May, 2002). Since freshwater fishes encompass a greater percentage of vertebrate species, and their population is undergoing a greater rate of decline globally than mammals, greater efforts must be committed from governments and conservation organizations for research and preservation of fishes. Organizations such as the International Union for Conservation of Nature (IUCN) and the World
Association of Zoos and Aquariums (WAZA) have made great efforts to globally assess the number of freshwater species remaining annually in order to chart the rate of decline (Carrizo et al., 2013). In addition, these organizations focus on promoting research and education of fish conservation in an effort to raise awareness and change public perceptions by providing information (Carrizo et al., 2013).

1.2 Lake Victoria Fishes

Located in Eastern Africa, Lake Victoria can be found amongst the seven African Great Lakes. It spans 69,000 km², making it the second largest freshwater lake, and the largest tropical lake in the world (Verschuren et al., 2002; Oweke Ojwang et al., 2010). Lake Victoria has come under major scrutiny since the mid-1900s when 200-300 cichlid species became extinct causing the greatest documented extinction at the fastest rate of all time (Helfman, 2007). Comparatively, other continents have lost significantly fewer species (Asia: 33 species, Central America and Europe: 18-20 species, South America: 11 species, and North America: 27 species; Helfman, 2007).

There have been several causes, primarily anthropogenic, associated with the extinction rate of cichlids in Lake Victoria. These include the introduction of non-native species, such as Nile perch (Lates niloticus) and tilapia (Oreochromis leucostictus and Tilapia zilli), during the 1950-60s (Ogutu-Ohwayo, 1990), overfishing and increased agriculture, as well as a shift in the food web (Oweke Ojwang et al., 2010). The list of potential causes is extensive due to the absence of adequate data, which has made it difficult to directly associate each ecological change to the cause of extinction. In order to investigate this relationship, Verschuren et al. (2002) used paleolimnological data from the last 40 years to investigate Lake Victoria. They found that increased phytoplankton production that began during the 1930s onward occurred alongside the growth of the human population and agricultural industry, causing the cichlid population to decrease (Verschuren et al., 2002). With the human population surrounding Lake Victoria expected to increase to 53 million by 2020, so will the rate of extinction of freshwater species throughout the lake (Verschuren et al., 2002). In the last 20 years two native tilapia species (O. esculentus, O. variabilis) have already become extinct in the lake and can only be found in
satellite lakes (Witte et al., 1992 from Agnese et al., 1999). With fish being a major commodity and source of food for Africans, loss of two commercial fishes and potentially others in the near future will greatly impact the economy and quality of life in surrounding countries. Therefore, conservation and research on Lake Victoria has been deemed to be necessary to protect the remaining species from endangerment and prevent the occurrence of future ecological disasters. This can be seen with the current research and protection of one of Lake Victoria’s most endangered commercial fish species, ngege.

### 1.3 Ngege (Oreochromis esculentus)

1.3.1 General Description

Ngege or Singidia tilapia (*Oreochromis esculentus*) are important commercial fish originating in Lake Victoria that can grow to approximately 50 cm in standard length. Non-breeding males and females cannot be distinguished, as they both have dorsal olive-brown colouration, with creamy silver ventral colouration (Figure 1A; Borstein.com, verified 2013.11.14). During breeding periods, males become red and develop black dorsal fins and markings on the sides (Figure 1B; torontozoo.com, verified 2013.07.11). They are able to breed throughout the year at undefined times, beginning as young as 5 months (Twongo et al., 2013). During breeding, males build macrophyte beds approximately 30 cm in diameter to signal females to begin spawning. Females release approximately 300-1700 eggs, which undergo a gestational period of 14 to 21 days (Twongo et al., 2013; torontozoo.com, verified 2013.07.11). After this point, females brood offspring in their mouths until the young have hatched. Once born, the young become independent at a length of approximately 15 mm and reach adult size at 37 cm with an average life expectancy of 20 years (Twongo et al., 2013; torontozoo.com, verified 2013.07.11).
1.3.2 Life History

The ngege were native commercial fish found predominately in Lake Victoria and Lake Kyoga in East Africa (Ogutu-Ohwayo, 1990). Currently, they are only found in nearby watersheds and satellite basins, propelling their classification from vulnerable in 1996 to critically endangered at present (Balirwa, 1992; Twongo et al., 2013). The causes of this devastation resulted from predation, competition of non-native species, such as the Nile perch (Balirwa, 1992), overfishing in the 1900s (Graham, 1929; Ogutu-Ohwayo, 1990), and a decrease in diatoms food availability (Batjakas et al., 1997; Twongo et al., 2013).

Stress from population decline forced ngege to hybridize with closely related species, *O. niloticus* and *O. variabilis*, to adapt and survive (Welcomme, 1967). In 1995, Mwanja and Kaufman provided evidence for Welcomme's claims by examining random amplified polymorphisms in the DNA of *O. niloticus* and *O. esculentus*. They found that the ngege remaining in Lake Victoria were no longer pure and exhibited signs of a hybridization event with *O. niloticus*. Agense (1999) investigated these results further by examining the ngege in satellite basins and found a high level of differentiation (nei’s distance of 0.141), and therefore purity, between the two species. These results suggest that the pure species can one day repopulate Lake Victoria through proper management and conservation guidelines enforced by the government.

To date, the ngege remain an endangered species and are listed on the American Association of Zoos and Aquariums (AZA) Species Survival Plan (SSP®). The program was
established during the 1990s as a strategy for managing and breeding the remaining ngege population between accredited institutions, in an effort to one day release captive-bred animals back into Lake Victoria. However, the ngege population is far from being as stable as it once was. Lake Victoria still remains an unsustainable environment for many fishes, which is why the conservation of ngege has progressed from basic breeding in captivity to the preservation of its genetic material.

1.4 Biomaterial Banking

For more than 30 years, researchers and organizations have realized the need to collect samples from threatened and endangered species as a means of genetic conservation (Wildt, 2000). The samples collected range from blood, tissues, gametes, embryos, DNA and cells, and remain cryopreserved in genome resource banks (GRBs) located throughout the world. Some of these repositories are proprietary, such as San Diego Zoo Global’s Frozen Zoo®, while other organizations welcome samples from all over the world to be collected in a global consortium, such as the Frozen Ark in the United Kingdom. The main reasons for establishing GRBs are: a) to preserve the genetics of valuable individuals as a form of insurance from any natural or human-perpetuated ecosystem catastrophe, b) to have a distribution of genetic material from the wild and captive populations, and c) to supplement endangered populations with offspring produced through assisted reproductive technologies (ARTs; Holt and Pickard, 1999; Wildt, 2000).

AZA maintains many endangered species in SSPs®, which involves taking some individuals from the wild and bringing them into captivity for breeding and management. However, maintaining animals in captivity is an expensive endeavor, and many species succumb to genetic adaptations towards captivity, which reduce their fitness in the wild and decrease their chances for successful reintroductions (Frankham, 2008). Christie et al. (2012) found that genetic adaptation can occur as rapidly as a single generation in captivity. In this study, they compared lifetime reproductive success (number of returning adult offspring) between wild and first generation (F1) hatchery rainbow trout and found that F1 animals had nearly double the amount of returning adults when spawned in captivity than in the wild, as well as a tradeoff between
fitness in the hatchery to performance in the wild that occurred as juveniles. If genetic adaptation can occur within a single generation, as in the case of rainbow trout, then the longer a species remains in captivity, the greater the occurrence of genetic alterations (Lewis and Thomas, 2001). Biobanking can be used to decrease genetic adaptations by decreasing the generation time animals are held in captivity. Increased genetic diversity can be achieved by collecting samples from wild animals and preserving the genetic material of various individuals from different locations.

The use of ARTs with material held in GRBs has the potential to become a valuable approach for maintaining struggling populations that have difficulty breeding in captivity, such as black-footed ferrets (Dobson and Lyles, 2000) and has been used to supplement captive populations for the aquaculture industry (deMestral Bezanson et al., 2010). Therefore, GRBs allow different strategies for breeding that reduce the cost and risk associated with transporting animals across the globe. ARTs include artificial insemination (AI; deposition of semen (freshly collected or cryopreserved) in the female reproductive tract), in vitro fertilization (IVF; co-incubation of sperm and eggs in vitro to create an embryo that can be transferred or cryopreserved), and embryo transfer (ET; deposition of an embryo into a female uterus) (Bainbridge and Jabbour, 1998). Although sperm and embryos are the most highly used material in GRBs for offspring production, they are limited in their ability to preserve maternal genetics. Cryopreservation of eggs and embryos is complex particularly in fish species where penetration of the yolk sac by a cryoprotective agent is ineffective. This is one of the many reasons why the cryopreservation of somatic cells has become an increasingly popular form of GRB (Bail et al., 2010).

1.4.1 Somatic cell banking

Within the last 20 years, research on the biobanking of fish materials has moved from gametes to somatic cells obtained from fins. The benefits of somatic cells over germplasm are varied. They contain the DNA necessary for many fields of study, such as pathobiology, population genetics and evolutionary biology. They also contain both maternal and paternal genetics, which ensures that valuable alleles are not lost from the population (Hagedorn et al.,
In addition, somatic cells can be collected from individuals that have unknown maturational status or numerous years to reach maturity (Mauger et al., 2006), are exhibiting reproductive dysfunction, have recently died, or are in their post-reproductive phase, unlike gamete collection (Mastromonaco and King, 2007).

Somatic cell banks from fish are generally derived from the fins as seen with *Labeo rohita* (Lakra and Bhonde, 1996; Rathore et al., 2007), *Kryptopterus bicirrhis* (Han et al., 2011), and *Carassius auratus* (Choresca Jr. et al., 2012), to name a few. Fin cell cultures can be retrieved without sacrificing the individual making it ideal for endangered species (Moritz and Labbe, 2008). Furthermore, it is less invasive than gamete collection making it easier to implement in the field (Mastromonaco and King, 2007). Fin cell culture has become a standard for establishing somatic cell banks due to their in vivo regenerative and in vitro proliferative capacities (Akimenko et al., 2003). Mauger et al. (2006) reported that there is no significant difference in goldfish explant ability between anal, pectoral and caudal fin cells indicating that any fin is acceptable for establishing primary cell cultures except the dorsal fin (Mauger et al., 2006). Although culturing fish fins is an attractive opportunity for fish conservation, there are some drawbacks due to the requirement of technical capabilities and financial assistance that may not be available for the massive sampling of wild species (Moritz and Labbe, 2008).

Although the need for somatic cell banks has taken precedence in recent years, many researchers have not diverted from culturing embryonic stem cells (Chen et al., 2004), adult stem cells (Collodi et al., 1992), and reprogrammed somatic cells (Bubenshchikova et al., 2007), due to their proliferative capacity. Although adult stem cells are an intriguing avenue of investigation for many fields, and can be collected non-invasively like somatic cells, they currently play a minimal role in conservation biology. There have been few publications on stem cell isolation, establishment and characterization in wildlife species for conservation purposes (Mastromonaco et al., 2013), especially for endangered fish species. Furthermore, stem cells require a greater financial investment in supplies, technical abilities and rigorous protocols that can lead to a greater risk of failure in establishing adult stem cell lines (Mastromonaco et al., 2013).
1.5 Somatic Cell Nuclear Transfer (SCNT)

SCNT is the transfer of a nucleus from a desired individual into an enucleated oocyte of the recipient individual by electrofusion or intracytoplasmic injection (Bail et al., 2010; Mastromonaco and King, 2007). In fish, nuclear transfer has been practised for over fifty years beginning with the use of embryonic cells as nuclear donor cells (Gasaryan et al., 1979). However, with advancements in technology and protocol optimizations, nuclear transfer can now be performed with more differentiated cells, as seen in the studies of Bubenshchikova et al. (2007) and Siripattarapravat et al. (2009), which used somatic cells from medaka (Oryzias latipes) and zebrafish to produce SCNT offspring.

Lee et al. (2002) were the first to obtain adult zebrafish following the transfer of cultured somatic cells (12 -26 weeks old) into enucleated eggs, unlike previous works that were not able to produce adults from SCNT embryos (Chen et al., 1986). Progress with SCNT continued in fish with the successful production of live medaka from somatic cells and embryonic stem cells (Bubenshchikova et al., 2007; Yi et al., 2009).

Although fish SCNT is possible, the success rate of producing live offspring remains at 1-2% (Siripattarapravat et al. 2009; Luo et al., 2011). This is attributed to the unknown effects of cell cycle synchronization and chromosome aberrations on donor cells (Wakamatsu, 2008), challenges in reprogramming (Luo et al., 2011), and the heterozygosity amongst cell cultures (Lee et al., 2002). However, for SCNT to be a properly implemented and highly practised technique (especially in the aquaculture industry), there needs to be further research on the culture of somatic cells from diverse species and assessment of culture quality for use in SCNT.

1.6 Analysis of cell cultures for GRBs

To ensure the suitability of cells for ARTs it is beneficial to assess the quality of the sample pre- and post-cryopreservation, since cryopreservation is known to affect post-thaw viability of tissue samples (Holt and Pickard, 1999). For sperm, it has become standard practice to assess the motility, morphology and acrosome membrane pre- and post-thaw (Mastromonaco
et al., 2013) as it has been shown to affect the outcome of ARTs both in mammals (Holstein bull; Shojaei et al., 2012) and in fish (red seabream, Pagrus major; Lui et al., 2007). Like sperm, somatic cells have been shown to be affected by environmental and genetic factors that influence their longevity and proliferative capacities (Mastromonaco and King, 2007). However, the same standards of care used for cryopreserving and analyzing sperm for ARTs has not been shown with somatic cell cultures (Mastromonaco et al., 2013). Basic techniques to evaluate cellular parameters can be readily implemented to obtain valuable information on viability and function, including cell cycle analysis, and chromosome normality.

1.6.1 Cell Cycle

The cell cycle is divided in two different phases: interphase, which encompasses S phase (DNA replication and chromosome duplication), G1 (cell growth via metabolism; includes G0, the quiescent state) and G2 (checkpoint before mitosis), and mitosis during which cell proliferation occurs (Karp, 2008). The cell cycle is an important analytical tool for assessing culture quality because the donor nuclear state greatly impacts SCNT outcome (Choresca et al., 2009). Evaluations can easily be performed by purchasing commercial kits, some of which require flow cytometry for accurate quantification (Cheong et al., 2003).

Cell cycle analysis was used to determine nuclear donor status when optimizing protocols to promote cell quiescence (G0 state) prior to SCNT. Campbell et al. (1996) demonstrated that asynchronization between donor nucleus and recipient cytoplasm affected SCNT success. G0/G1 phase is necessary to produce reconstructed embryos with correct ploidy and proper nuclear reprogramming (Choresca et al., 2009; Liu et al., 2004). Various fish studies, have shown that culture conditions, including serum starvation, chemical inhibitors, and confluence, can induce cells to enter the G0/G1 state (Lee et al., 1988; Choresca et al., 2009). In 2006, Hashem et al. were the first to carry out a cell cycle analysis of adult fibroblast cultures from an endangered fish species (goral, Naemorhedua caudatus) to assess their suitability for SCNT. These studies need to be performed on many other endangered fish species to properly assess the value of the cryopreserved material and the effects that culture conditions can have on SCNT success (Han et al., 2011).
In addition to using the cell cycle for assessing synchronization of cell phases, it is also a valuable technique for determining cell proliferation. Bromodeoxyuridine (BrdU) staining kits that detect the incorporation of the thymine analog into newly synthesized DNA (Cecchini et al., 2012) can be used. Healthy cultures will contain actively dividing cells, but cultures with extended proliferation times or high passage number, often have altered viability, chromosomal abnormalities, and reduce SCNT success (Romanov et al., 2001; Mastromonaco et al., 2007). In Lakra et al. (2010), slow growing carp (Cyprinus carpio) cell cultures had 52-54% chromosomal abnormalities approaching passage 50, indicating that the longer cells take to reach confluence (due to reduced proliferation), the higher the proportion of chromosomally abnormal cells.

1.6.2 Chromosome Number

Many fish studies have used chromosome number to characterize their cell lines as with Paralichthys olivaceus (Kang et al., 2003), Puntus sophore (Lakra and Goswami, 2011), Ictalurus punctatus (Zhang et al., 1998) and Caranx melampygus (Zhao and Lu, 2006), to name a few. Although these studies have been beneficial for obtaining the chromosome number of many species and tracking evolutionary changes, it has not been used as a tool to analyze the state of cultures. Identifying the chromosome number pre- and post-cryopreservation is good practice for characterizing cell cultures, and creating a database of chromosomal number for endangered species that have not yet been evaluated, such as O. esculentus. Various studies by Lee et al., (1988), Bols et al. (1994), and Zhu et al. (2004), to name a few, have shown that non-optimal culture conditions, such as a low FBS concentration, glutamine, and seeding density, can lead to decreased cell proliferation and chromosomal abnormalities. These results are concerning because the percentage of aneuploid donor cells have been shown to increase with the duration of the culture, and correlate to the amount of chromosomally abnormal SCNT embryos (Giraldo et al., 2004). Slimane Bureau et al. (2004) found that bovine donor cells from short-term culture cells contained a high of 23% chromosomally abnormal cells which resulted in 55% aneuploid SCNT embryos. Therefore, these results suggest that cells with an altered chromosome number would not be suitable for SCNT.
1.6.3 Viability and Cell Death

The viability of cells should always be assessed for cell cultures, as is routinely carried out with sperm, to determine if they are healthy and worthy of banking in GRBs. A cell is considered viable if it has an intact cell membrane, and remains metabolically active (Stoddart, 2011). Stains, such as trypan blue and calcein AM, work on the principle that viable cells retain intact membranes, and do not allow the stain to enter the cell. Assays, such as MMT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), work by making a viable cell fluoresce if it has metabolic activity (Stoddart, 2011). Cultures can be considered healthy if the percentage of viable cells is greater than dead cells (Stoddart, 2011).

In contrast, cell death is associated with the degradation of the plasma membrane allowing stains like propidium iodide (PI) to penetrate through the plasma membrane and intercalate with DNA as well as the release of lactate dehydrogenase (LDH) into the cell culture environment (Stoddart, 2011). These are the simplest methods of identifying cell death, but since they are very general approaches, they do not provide information on the cell death pathway (apoptosis or necrosis).

In 1972, Kerr et al. were the first to propose apoptosis as the programmed sequence of events responsible for removal of unwanted cells during normal development that can be triggered by stress, end of lifespan, damage beyond repair, or viral infection (Stoddart, 2011). Apoptosis has an early and late phase, each associated with its own morphology and markers. Early apoptosis is identified by changes to the surface of a dying cell that result in its recognition and uptake by phagocytes (Zhang et al., 1997). Also, expression of thrombospondin, loss of sialic acid residues, and exposure of the inner plasma membrane phosphatidylserine to the outside of the membrane have been shown to occur (Zhang et al., 1997). The mid- to late-apoptotic stages result in the activation of caspases, which cleave various cellular proteins as part of the programmed cell death pathway (Stoddart, 2011). This is accompanied by a number of morphological changes including cell shrinkage, membrane permeability, DNA fragmentation, and DNA condensation, the latter two detected by terminal transferase mediated dUTP nick end-
labeling (TUNEL) assay, gel electrophoresis (Stoddart, 2011) and Hoechst staining (Zhang et al., 1997), respectively. These apoptosis-related assays can be difficult for cell cultures that do not have many cells, as the protocols require a minimum of a million cells. In cases with low cell numbers, PI and Hoechst staining are used, but these are not the most accurate for defining apoptosis. One reason is that chromatin condensation occurs in both cells undergoing apoptosis and cell division, making it impossible to differentiate between these two cell states with Hoechst stain.

Necrosis differs from apoptosis because the cell enlarges as organelles begin to swell, resulting in the rupture of the plasma membrane and the loss of intracellular contents (Kromer et al., 2009). Unlike apoptosis, the causes of necrosis are still not defined, but are often associated with excess reactive oxygen species (ROS), lipid degradation, and excess cytosolic calcium pushing the mitochondria into overdrive (Kromer et al., 2009).

Autophagic cell death has recently been defined as a mode of programmed cell death (Tsujimoto and Shimiz et al., 2005; Kromer et al., 2009). Stress associated with temperature, hypoxia and nutrient depletion activate a signal transduction cascade still under investigation, but shown to occur in the absence of the apoptotic Bax/Bak pathway (Wei et al., 2001). Once activated, autophagosomes will fuse with lysosomes and become autolysosomes, where the cellular components will be digested, and produce cytoplasmic vacuoles (Tsujimoto and Shimiz et al., 2005; Kromer et al., 2009). However, autophagic cell death should not be confused with autophagy which promotes cell survival (Henics et al., 1999; Tsujimoto and Shimiz et al., 2005). Both autophagic cell death and cell survival are characterized by the presence of vacuoles and the expression of autophagic proteins such as Atg5, Atg6, and Atg7 (Tsujimoto and Shimiz et al., 2005). However, unlike autophagic cell death, autophagic cell survival has the potential to be reversed (Henics et al., 1999). When cells are stressed or injured from poor establishment techniques and culture conditions, including pH, contamination, and media, the cells will vacuolate by autophagy to try and manage the cellular damage (Henics et al., 1999). Once the stimulus causing cellular injury is removed, the cell can be reversed back to a non-vacuolated state upon a certain threshold (Henics et al., 1999). However, if cells remain vacuolated past the threshold, they too will begin programmed cell death (Henics et al., 1999).
If cultures contain predominately dead cells it may be good to further investigate the method of cell death employed in order to alter establishment techniques or culture conditions to avoid the stresses that activate the many cell death pathways. Although an in-depth analysis of cell death may not be required for the basic identification of culture quality of cells for GRBs, it is good practice to do a viability stain to determine the percentage of live cells, as they are the only cells than can be used for SCNT.

1.6.4 Senescence

Cells are regulated through a multitude of signal transduction cascades that are interconnected in their modes of activation. Cell stressors, such as reactive oxygen species (ROS), DNA damage, telomere dysfunction, and oncogene expression, can activate signaling molecules like p53 to initiate the apoptotic pathway, and in some circumstances cellular senescence (Kuilman et al., 2010).

Cellular senescence is primarily defined as telomere shortening that occurs as a natural part of aging in somatic cells with a definitive proliferative life span (Hayflick limit; Kuilman et al., 2010). When telomeres reach their minimal length, DNA damage response (DDR) signals are triggered and induce phosphorylation of many cell cycle proteins (e.g: CDC25), as well as activation of p53 and p16\textsuperscript{INK4A}-RB pathways, both shown to be agents of the senescence pathway (Campisi, 2005). Upon activation, the cells arrest in G1 in an effort to repair DNA, but when the damage has surpassed the threshold for repair, the cells progress down the apoptotic or senescent pathway (Kuilman et al., 2010).

Senescence is observed by a number of morphological alterations such as an increase in size, and flattening of the cell, both visible by light microscopy (Mastromonaco et al., 2006). It can further be identified by the expression of tumor suppressor factors p53 and p16\textsuperscript{INK4A}-RB, increase in β-galactosidase activity, increased binding of heterochromatin associated proteins in the promoters of several cell cycle regulators (e.g: E2F), and the release of cytokines and chemokines (Kuilman et al., 2010).
ROS is used to define oxygen radicals, but also non-radicals that are oxidizing agents, which can easily be converted into radicals (Halliwell and Whiteman, 2004). ROS are naturally produced by cells as part of cellular respiration and enzymatic reactions (Jos et al., 2009). However, when ROS are in excess, they initiate a stress response that causes DNA and cellular damage (Jos et al., 2009). To minimize the negative effects of ROS, cells contain an antioxidant defense system, including the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione reductase, and glutathione transferase, as well as many non-enzymes, such as glutathione, vitamin E, vitamin C, vitamin A, carotenoids and ubiquinol (Jos et al., 2009). These products are commonly quantified using colorimetric assays to determine basal to high expression levels in culture media to evaluate oxidative stress in cell cultures. Many studies have shown that ROS can activate apoptotic pathways (Wang et al., 2013), as well as cellular senescence (Halliwell and Whiteman, 2004; Lu and Finkel, 2008; Moiseva et al., 2009).

Although the primary trigger for cellular senescence is telomere dysfunction, premature senescence can occur in the absence of telomere dysfunction early in the cell’s lifespan due to stressors like ROS. Primary cell cultures are highly vulnerable to premature cellular senescence because cells are taken from their natural environment in the tissue and forced to grow in two dimensional space with artificial media and growth factors, all of which can cause shock to the cell and elicit responses, such as vacuolation (Leist et al., 1996; Henics et al., 1999 Sherr and DePinho, 2000). In some cases, media can be supplemented with antioxidants or products containing selenium to reduce the production of ROS produced in culture and prevent premature senescence (Tinggi, 2008).

Senescent cells are not valuable for GRBs because they remain arrested in G1/S phase, which is an irreversible state causing chromosome aberrations, altered gene expression and cellular function, making them ineffective for SCNT (Mastromonaco et al., 2006). A study by Mastromonaco et al. (2006) showed that cultures with senescent morphology had decreased chromosome normality (<40% normal cells), which resulted in low blastocyst development compared to highly proliferative cells containing few abnormalities. Therefore, cellular senescence should be examined in cell cultures to ensure that it remains at a basal level and not
overrepresented by cells in culture meant for GRBs as it will affect the outcome of SCNT and embryo production.

1.7 Conclusion

The global decline of freshwater fish stocks has created a need to cryopreserve biological materials from endangered species in an effort to conserve the biodiversity within this taxon. Since maternal gametes in fish are difficult to cryopreserve, somatic cells obtained from fins have become an increasingly popular source as they contain maternal and paternal DNA ensuring valuable traits are not lost from the population (Mauger et al., 2006). The somatic cells stored in GRBs can be used for many conservation studies involving population genetics and phylogenetics, but ultimately for SCNT to supplement endangered populations with genetically valuable offspring. However, the success of SCNT is mainly attributed to the state of the cells used as nuclear donors, which have been shown to be affected by establishment techniques and culture conditions (Mastromonaco et al., 2007). It is because of this potential to create viable offspring that cells, like sperm, should be analysed for quality prior and post cryopreservation in GRB. Examination of cellular parameters including cell cycle, chromosome number, and viability should become standard procedures for biobanking facilities.

1.8 Thesis Objectives

This thesis addresses the question of how cell culture conditions, such as temperature seeding density, and culture media and establishment techniques, such as digestive enzymes and sample selection, affect the outcome of ngege fin cultures. Understanding the relationship between cell culture procedures and viability will enable specialized protocols for fin biopsy cell cultures from endangered freshwater fish species to be developed. The conditions for culturing ngege caudal fin biopsies were examined in three chapters.

In chapter 2, the objective was to determine the effects of antibiotics, biopsy size and temperature on culture confluence. The objective for chapter 3 was to investigate the effects of digestive enzymes and digestion time used to establish primary cell cultures on cell viability.
Finally, chapter 4’s objective was to examine the effects of donor age and biopsy location on the viability of primary cell cultures.

This thesis will set the standard for establishing primary cell cultures of ngege caudal fin biopsies. The ngege was chosen as a model species because it is an endangered freshwater fish from Lake Victoria and has been prioritized by the IUCN for cell culture banking of unaltered fish founders. Although this study focuses on ngege cell culture, the results can be applied to optimize cell culture procedures of other endangered freshwater fish species for conservation and research.
CHAPTER TWO

Optimization of antibiotics, temperature and biopsy size for the establishment of ngege primary cell cultures of the caudal fin
2.1 Introduction

The first fish cell line established in 1962 by Wolf and Quimby (1962) set the standard for fish cell culture procedure. Since then, detailed methods for culturing fish cells have been described by Wolf and Quimby (1969), Wolf (1979), Bols and Lee (1991), Lakra et al. (2011), Lannan (1994), and Ott (2004), to name a few. In general, fish cell culture is not as complex as mammalian and avian procedures (Lannan, 1994). It often requires the use of Leibovitz medium (L-15) and non-specialized incubators maintained at a range of temperatures depending on the fish species. Cold-water fish cells can be grown at 15-21°C, while warm water fish cells require 25-35°C (Lannan, 1994). The optimal temperature to culture fish cells is a few degrees above the species preferred environmental temperature (Alvarez et al., 1991). In contrast, mammalian procedures typically require Dulbecco’s Modified Eagle Medium (DMEM) and 5% CO₂ incubators held at 37°C. However, some laboratories still follow mammalian protocols for fish cell culture, while only adjusting for temperature (Choresca Jr et al., 2012; Han et al., 2011, Rathore et al., 2007).

Modifications to standard cell culture procedures are often necessary when culturing different species, tissues and cell types, as each require different conditions for optimal growth. Some alterations include supplements to basal media, such as specialized fish sera (Clark et al., 1987) and growth factors (Collodi and Barnes, 1990; Chen et al., 2004). However, these additives can be cytotoxic and/or only moderately effective, which is why they are typically not used in fish cell culture (Bols and Lee, 1991).

Antibiotics are one of the most common supplements to basal media, but they too can be cytotoxic (Kuhlmann, 1996; Mirjalili et al., 2005) and can reduce DNA and protein synthesis at higher concentrations (Amonn et al., 1978). Stringent protocols for culturing cells in the absence of antibiotics (Carrel, 1924; Hoshi et al., 1988) and the risk associated with losing valuable cells to contamination has led many researchers to include antibiotics in cell culture despite these consequences. However, antibiotic type and concentration for cell culture are not standardized. Different tissues require different antibiotics depending on their susceptibility to certain microorganisms. Tissues from the interior of the fish, such as organs or embryos, can be
considered sterile if removed with sterile instruments and washed appropriately in a laminar flow hood to minimize contamination (Wolf, 1979). Exterior tissues such as skin, gills and fins are exposed to a multitude of bacteria, fungi, and mycoplasmas in their environment that would require stronger wash solutions and antibiotics (Wolf, 1979). Therefore, antibiotics used in wash and culture solutions should be closely examined when switching between species and tissue types, as the requirement for antibiotics will differ and ultimately affect the outcome of the culture.

In addition to antibiotics, seeding density differs among primary cell cultures depending on tissue type and quantity of sample available to establish the culture. With laboratory fish, the opportunity to sacrifice the individual for research and obtain whole fins is plausible, but the same cannot be performed with endangered species. Thus, samples are often taken from fins because of their high regenerative capacity and accessibility for sampling in the wild (Mauger et al., 2006). In mammals, biopsy punches and darts from ear cartilage and skin have been used to produce cell cultures (Mastromonaco et al., 2006), but biopsy punches are not routinely performed on fish. Moritz and Labbe (2008) were among the first to use biopsy punches on goldfish fins for field scale culturing and cryobanking. However, biopsy punches provide fewer cells for primary cultures than whole fin sampling. Lower seeding densities are not optimal because they exhaust the proliferative ability of the initial cells to form confluent cultures (Mastromonaco et al., 2013), which can ultimately lead to senescence or chromosomal abnormalities compared to whole tissues. However, there are multiple sizes of biopsy punches, ranging from 1 mm to 10 mm diameters, which can yield different seeding densities. Thus, biopsy size for establishing primary fin cell cultures from endangered species requires further examination.

The objective of this study was to determine the effects of antibiotic supplementation, biopsy size and temperature on culture confluence. In experiment one, cultures grown in five antibiotics (penicillin-streptomycin (P/S), antibiotic-antimycotic (ABAM), gentamicin, normocin and primocin) were examined via phase contrast microscopy to detect contamination and measure the confluence of the cultures.
In the second experiment, cultures grown at two incubation temperatures, 25°C and 35°C, were used to determine the temperature most suited for cell growth. Both temperatures are within physiological temperature range, with 35°C being at the maximum, and 25°C being two degrees above environmental temperature. Concurrently with temperature, biopsy sizes of 4 mm, 6 mm and 8 mm diameters were used to establish primary cell cultures, which were examined by phase contrast microscopy to determine the number of cells produced by each biopsy.

2.2 METHODS

All procedures involving animals were approved by the Toronto Zoo Animal Care and Research Committee and by the University of Guelph Animal Care Committee (Protocol # 12R029). The experiments conducted were in accordance with the Animals for Research Act of Ontario and the Canadian Council for Animal Care. All products were purchased from Life Technologies Corporation, Grand Island, NY, USA, unless otherwise stated.

2.2.1 Animals, anesthesia and experimental design

Ngege were maintained at the Toronto Zoo, Scarborough, ON, in 110 US gallon tanks at 23°C, and were sampled at 17 years of age.

In experiment 1, the opportunistic culling of the captive ngege population for management reasons resulted in 3 fish used for antibiotic testing involving 8 treatment groups labeled: 1x Normocin, 2x Normocin, 1x Primocin, 2x Primocin, 4x Primocin, 1x P/S & 1x ABAM, 1x P/S & 2x ABAM, and 1x Gentamicin. Ngege in this experiment were rinsed with 0.9% NaCl (Baxter Corp., Mississauga, ON, Canada) and then injected intravenously with Ketamine at a dose of 50 mg/kg. Once anesthetized, the caudal fins were removed with serological scissors and placed in a specimen container (VWR International, Mississauga, ON, Canada) containing Hank’s Buffered Saline Solution (HBSS), 1% P/S and 2% ABAM, from here on referred to as wash solution. These ngege were later euthanized with 0.2 mL T-61 following fin removal. The 3 caudal fins were placed in separate 100 x15 mm petri dishes (BD
Biosciences, Franklin Lakes, NJ), where they were rinsed 5 times with wash medium and chopped into eight, 1 cm² pieces under a laminar flow hood.

In experiment 2, 6 fish were individually caught with a net individually, anaesthetized with 50 mg/kg ketamine and biopsied with 4 mm, 6 mm and 8 mm biopsy punches (Miltex Inc., York, PA, USA), three times per caudal fin. The fish were placed into a recovery tank for 24-hour observation prior to release into their original tank. Each biopsy punch was placed in its own 15 mL centrifuge tube (BD Biosciences, Franklin Lakes, NJ, USA) containing wash solution. All collected samples were transferred to the cell culture room where the remaining work was performed under a laminar flow hood. The 18 biopsies in experiment 2 were cultured under the same conditions described in experiment 1, with the exception of using only 100 µg/mL Primocin in culture medium, and incubating the cultures at 25°C and 35°C. In this case, the 9 biopsies (3 replicates of 4, 6 and 8 mm biopsies) from the first 3 ngege were incubated at 25°C, while the remaining 9 biopsies were incubated at 35°C.

2.2.2 Cell culture

Fin pieces or biopsies were placed in their own 60 x15 mm petri dish (BD Biosciences, Franklin Lakes, NJ) and further chopped into 1 mm² pieces. Chopped tissues were treated with 0.2 mg/mL collagenase in L-15 for 30 minutes (Mauger et al., 2006) and later inactivated with culture medium [L-15 containing 10% fetal bovine serum (FBS; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada; Mauger et al., 2006) and 100 µg/mL Primocin (except for experiment 1 where multiple antibiotic treatments were tested)]. The final 4 mL solution (containing cell digest, tissue and culture medium) was transferred with a 5 mL serological pipette (BD Biosciences, Franklin Lakes, NJ) into a 12.5 cm² tightly capped cell culture flask (BD Biosciences, Franklin Lakes, NJ, USA). All flasks were incubated at 25°C (Mauger et al., 2006) in Thermo Scientific Heratherm Compact 0.6 ambient incubators (Thermo, Rochester, NY, USA) (except for experiment 1 where multiple temperatures were tested). The medium was changed every fourth day of culture.
2.2.3 Contamination Analysis

Ngege primary cell cultures were examined under an Olympus IX51 inverted phase contrast microscope. Cultures were examined on days 1, 4 and 8 for bacterial and fungal growth. One mL of culture medium was given to the Toronto Zoo’s microbiology laboratory to identify cultures with unknown contaminants. MacConkey and blood agar plates were used for bacterial species isolation, and enterotubes for species identification.

2.2.4 Determination of Cell Number

Pictures were taken on day 8 from 4, 6, and 8 mm biopsy cultures at 25°C and 35°C at 10x magnification using an Olympus SC30 camera directly attached to an Olympus IX51 inverted phase contrast microscope. The number of cells was counted within an area of 6.2x10^4 µm² configured by Image J cell counting parameters (US National Institutes of Health, Bethesda, MD, USA).

2.2.5 Statistical Analysis

Statistical analyses were performed using MiniTab 16 (Mintab Inc., State College, PA, USA) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

The comparison between the number of cells from different biopsy sizes and incubation temperatures were analyzed using one-way ANOVA followed by Tukey post-hoc test. P values less than 0.05 were considered significant.
2.3 RESULTS

2.3.1 Antibiotic Supplementation

All three replicates of 1x Gentamicin, 1x P/S & 1x ABAM, and 1x P/S & 2x ABAM resulted in contaminated cultures between day 1 (the first day after culture incubation) and day 8 (Table 2.1). The bacteria identified in gentamicin cultures were Gram positive Bacillus spp. Fungal contamination observed was filamentous and eventually enveloped the entire culture by the next feeding day. In addition, preliminary trials of antibiotic testing showed that P/S and ABAM culture media were unreliable at maintaining healthy cell cultures because at least one of the three trials developed contamination (Table A.1). Due to the variability in producing healthy cell cultures amongst these antibiotics, it was necessary for other antibiotics such as Primocin and Normocin to be tested alongside the routinely used fish culture antibiotics: P/S, ABAM and gentamicin. This allowed the optimal antibiotic type and concentration for culturing Ngege caudal fin cells to be determined.

The 1x Normocin and 2x Normocin exhibited culture confluence of approximately 10% on day 4 but by day 8, 2x Normocin cells detached from the flask whereas 1x Normocin cells doubled in percent confluence (Table 2.1). Similarly, 1x Primocin and 2x Primocin exhibited culture confluence of approximately 40% on day 4 and by day 8 both cultures grew approximately double with 70% confluence. However, this was not observed in 4x Primocin, as cells only reached 10% confluence by day 8 (Table 2.1).

Cultures that remained viable past 14 days in cultures included 1x Normocin, 1x Primocin, and 2x Primocin. The 1x Normocin treatment group maintained 40% confluence for 21 days before the cells started to detach from the flask. In comparison, the 1x Primocin and 2x Primocin treatment groups maintained 70% confluence for 8 weeks. However, not all the cells in 1x Primocin and 2x Primocin detached from the flask. Approximately 10% of cells remained in culture for an additional 2 weeks but with morphological signs of senescence.
Table 2.1: Observations of contamination and the percentage of culture confluence resulting from the different antibiotic treatments in culture medium using phase contrast microscopy.

<table>
<thead>
<tr>
<th>Reference</th>
<th>1x Antibiotic Concentration</th>
<th>Treatment Groups</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Webber et al., 2012</td>
<td>100 µg/mL</td>
<td>1x Normocin</td>
<td>N</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x Normocin</td>
<td>-</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>Callol et al., 2013</td>
<td>100 µg/mL</td>
<td>1x Primocin</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x Primocin</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4x Primocin</td>
<td>N</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Jensen et al., 2012</td>
<td>P/S: 10,000 IU/mL penicillin and 10,000 µg/mL streptomycin</td>
<td>1x P/S &amp; 1xABAM</td>
<td>+</td>
<td>NC</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>ABAM: 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL of Fungizone®</td>
<td>1x P/S &amp; 2x ABAM</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Mauger et al., 2006</td>
<td>125 µg/ml Amphotericin B, 5 mg/ml Gentamicin</td>
<td>1x Gentamicin</td>
<td>F&amp;B</td>
<td>FD</td>
<td>FD</td>
</tr>
</tbody>
</table>

**LEGEND**

- Percentage of Cells
  - N= No cells have plated but could be seen rounded up in media
  - NC= No cells due to contamination
  - FD= Flasks disposed of due to contamination
  - + = 1 ≥ 10% confluent
  - ++ = 11 ≥ 20% confluent
  - +++ = 21 ≥ 50% confluent
  - ++++ = 51 ≥ 100% confluent

- Contamination
  - - = No contamination observed
  - F = Fungal
  - B = Bacterial
2.3.2 Biopsy Size and Temperature

The number of cells produced from the 4 mm biopsy punches was significantly less (p< 0.0001) at 25°C (18.44 ± 5.05; mean ± SEM; n=9) and 35°C (21.33 ± 10.37; n=9), than the 6 mm biopsy punches at both temperatures and 8 mm biopsy punches at 25°C (Figure 2.1). The number of cells produced from 8 mm biopsy punches incubated at 35°C was not significantly different (p> 0.05) from 4 mm and 6 mm biopsies at 25°C and 35°C, and 8 mm at 25°C. Furthermore, there was no significant difference (p>0.05) between the number of cells produced from 6 mm and 8 mm biopsy punches at 25°C and 35°C (Figure 2.1), which is depicted by their morphologies on day 8 in Figure 2.2.

![Figure 2.1: Mean number of cells counted from an area of 6.2x10^4 μm² using Image J from 4, 6, and 8 mm biopsy cell cultures (n=3), incubated at 25°C and 35°C on day 8 of culture. Error bars indicate standard error, and treatment groups not sharing a letter are significantly different (p<0.0001).](image-url)
Figure 2.2: Morphologies of ngege caudal fin cells derived from biopsies (4, 6, 8 mm) under phase contrast microscopy at 25°C and 35°C on day 8 of culture. The two temperatures have comparable cell growth and morphologies between the 6 mm biopsies and 8 mm biopsies. Four millimeter biopsies (A and B) at 25°C and 35°C are morphologically distinct from 6 mm biopsies and 8 mm biopsies (C-F).
2.4 DISCUSSION

Optimization of primary cell culture procedures is essential for novel cell lines because culture conditions, such as incubation temperature, antibiotics and biopsy size, differ amongst species and tissue types, and can affect cell viability and proliferation. Therefore, this study optimizes the primary cell culture conditions for ngege caudal fin biopsies that have not been previously examined.

2.4.1 Antibiotic Supplementation

Antibiotics are important for many cell culture techniques, but primarily for surface tissues from fish or other aquatic species where a high loss of cultures results from environment-based microorganisms. Furthermore, slow-growing fish cultures (3-4 weeks to confluence) present a challenge in preventing microbial growth from overcoming cellular growth. The most frequently used antibiotics in fish cell culture are P/S, ABAM, and gentamycin because of their ability to inhibit the growth of a broad spectrum of fungi, Gram positive and negative bacteria, and mycoplasmas (Kuhlmann, 1996). However, data obtained in this study suggest that P/S, ABAM and gentamicin did not effectively prevent contamination in ngege cell cultures from day 1 to day 8 of growth. It is possible that the amount of microorganisms on the caudal fin was too great for these antibiotics to eliminate during log phase and/or the microorganisms present consisted of a species outside the antimicrobial spectrum of these common antibiotics. Evidence for these claims can be seen in the 1x P/S & 2x ABAM treatment group. The cultures appeared healthy on day 1, but showed signs of contamination (cloudy medium or fungal spores) on day 4. When left to grow, cultures were consumed with bacteria and fungus by day 8. The described timeline is representative of the bacterial growth phases identified by Lee and Falkow (1990) in Salmonella contaminated mammalian kidney cell cultures. To minimize tissue surface contamination predominately from fecal matter in the water, Wolf (1979) and Lakra et al. (2006) suggest depriving the fish of food the day before culture. Although this procedure may be suitable for laboratory fish, food deprivation was not possible in this study due to standards of animal care at the Toronto Zoo, and cannot be done with fish sampled in the wild.
In contrast, Normocin and Primocin consistently produced healthy fish cell cultures, even though they are routinely used for mammalian cancer and neural cell lines (Dang et al., 2006; Verma et al., 2006; Garrett and Weiner, 2009; Arboleda et al., 2011). Callol et al. (2013) recently used Primocin to culture European eel (Anguilla anguilla) primary cell cultures. In general, Normocin is routinely used to supplement P/S, whereas Primocin does not require the addition of other antibiotics because it targets bacterial DNA gyrase and ribosomal subunits (30S and 50S) of prokaryotes, as well as fungal ergosterol (InVivoGen, accessed 2013.12.11). Therefore, it is an antibiotic containing a broad antimycotic and antibacterial spectrum for cultures prone to contamination in primary stages.

Primocin was examined at 1x, 2x and 4x concentration according to the manufacturer’s claims that it is non-toxic to primary cells. Normocin, however, was examined at 1x and 2x concentration since higher concentrations were found to be cytotoxic according to the manufacturer. In this study, both 1x Normocin and 2x Normocin demonstrated initial cell growth of up to 10% confluence by day 4 in culture. However, by day 8 all the cells detached in the 2x Normocin treatment group, while 1x Normocin grew to 20% confluence. This may have resulted from increased cytotoxicity in the higher Normocin concentration. A similar result was observed in the 4x Primocin treatment group compared to the 1x Primocin and 2x Primocin treatment groups whereby minimal attachment and only 10% confluence were obtained compared to 70% confluence in the latter groups. Duewelhenke et al. (2007) described similar findings in primary human osteoblast cell lines and an epithelial HeLa cell line. They showed that with higher antibiotic concentration cell proliferation was inhibited and cytotoxicity induced. Therefore, 1x Primocin was selected as an appropriate supplement for ngege cell culture media due to its ability to eliminate contamination in primary cell cultures, while maintaining cell growth.

2.4.2 Biopsy Size and Temperature

Maximizing cell plating and growth during the primary culture is important to ensure a healthy long-lived cell line. Basic parameters, including number of viable cells to initiate the culture and proliferative capacity of these cells, must be considered when culture protocols are
being developed. Biopsy size (seeding density) and temperature both play an important role in the initial stages of culture establishment and lifespan (Plumb and Wolf, 1971; Balin et al., 2002; Zhu et al., 2004; Mastromonaco et al., 2006; Mosser et al., 1986).

Biopsy size had a significant effect on culture establishment and growth in this study. Four-millimeter biopsies produced fewer cells to initiate the culture than 6 mm and 8 mm biopsies potentially due to the small amount of tissue available or the higher rate of cell damage associated with mincing and digestion. Cell damage and death was evident by the large number of rounded and floating cells in this treatment group compared to the 6 mm and 8 mm biopsy groups. This morphology is typical of cells in early stages of apoptosis (Rello et al., 2005). Cell detachment and rounding is also common in cells undergoing mitosis (Lancaster et al., 2013), but in this case, the few cells plated by day 8 indicate that mitosis was not likely.

Tissue samples less than 4 mm in diameter have been used to culture primary cells from blastocysts or embryos (Chen et al., 2003; Chen et al., 2004; Sun et al., 1995). These tissues are naturally highly proliferative (totipotent or pluripotent), unlike the differentiated adult fibroblasts, and thus, require only a small amount of tissue to establish a confluent primary culture. Moritz and Labbe (2008) used a 3 mm biopsy punch to establish primary cell cultures of goldfish caudal fins. However, their cultures were derived from digested explants plated as 10-12 explants per well in a 12 well dish; an area of approximately 3.8 cm² compared to the 12.5 cm² surface area of the T-12.5 flasks. Thus, in the current study, cell digests from 4 mm biopsies had a greater area to cover, which may have led to proliferative exhaustion, inhibiting the culture from reaching confluence.

Although 4 mm biopsies resulted in significantly lower cell numbers (p<0.0001) than 6mm biopsies at both temperatures and 8 mm biopsies at 25°C, no significant differences (p>0.05) were observed compared to 8 mm biopsies at 35°C. This was unexpected due to the larger size and cell availability in the 8 mm biopsy. However, examination of cell morphology indicated that cells not in contact with main aggregates looked flat and stretched, which was not observed in the 6 mm biopsies and 8 mm biopsies at 25°C groups. Nelson and Chen (2002) showed that cell contact increases proliferation and cells unable to touch each other spread to
reach nearby cells, ultimately leading to proliferation arrest. Therefore, 8 mm biopsies at 35°C could have experienced growth arrest from an uneven dispersal of cells along the bottom of the flask. Although there were slight differences in the number of cells produced from 6 mm and 8 mm biopsies, the 6 mm biopsy punch is preferred over the 8 mm biopsy punch due to the slightly smaller tissue removed, a benefit when sampling from live animals. Additionally, 6 mm is the standard size of a hole punch (for stationery) if biopsy punches are not available, making it more feasible for sampling in the wild.

In addition to biopsy size, incubation temperature has been shown to affect cell proliferation, as seen in a study by Mosser et al. (1986). In their study, rainbow trout fibroblastic cell line RTG-2 was cultured to investigate cell viability and synthesis of heat shock proteins over a broad range of incubation temperatures (0°C to 35°C). The authors showed although there was a decline in cell proliferation rate with increased deviation from optimal incubation temperature (22°C), cell viability was minimally affected during 7 days in culture (Mosser et al., 1986). This is because cultures grown at 0-28°C were within the cellular endurance zone of RTG-2 cell lines. The cellular endurance zone is a wide range of incubation temperatures that affect cell proliferation rates during a defined period of time (Bols et al., 1992). Similarly, in this study, there were no significant differences (p>0.05) in the number of cells produced and no differences in cell morphology at incubation temperatures of 25°C and 35°C during a 7 day growth period. This could be a result of both temperatures being within the cellular endurance zone of ngege caudal fin cells. However, further research is required to assess cell viability, proliferation, and the synthesis of heat shock proteins, over a broad range of incubation temperatures to truly define the cellular endurance zone of ngege caudal fin cells. Although this study only investigated two incubation temperatures, both of which had no differences in cell morphology and in the number of cells produced, 25°C was preferred over 35°C. In this regard, 25°C is the recommended temperature for growing freshwater fish by Alvarez et al. (1991), Lannan (1994), and Mauger et al. (2006), and is closer to the environmental water temperature (23°C) than 35°C.
2.5 CONCLUSION

Establishing primary cell cultures from ngege caudal fins was possible when 1x Primocin was added to the culture medium to control levels of contamination. A caudal fin biopsy sample 6 mm in diameter was sufficient to initiate primary cell cultures at 25°C and 35°C, with 25°C being the ideal incubation temperature. Although these conditions may enhance our potential for establishing primary cell cultures from ngege caudal fin biopsies, further investigation of cell establishment techniques on cell viability is required.
CHAPTER THREE

The effects of enzymatic treatments and digestion time on cell viability of ngege primary cell cultures
3.1 INTRODUCTION

Cell culture requires living cells that have migrated or dissociated from a three dimensional structure to attach and grow in a two dimensional monolayer. This process can exert stress on the cells, which can impact their long-term growth in vitro. Primary cell cultures can be initiated using two methods: tissue explantation and enzymatic digestion (Wolf, 1979). With explants, small tissue pieces are allowed to adhere to the bottom of a flask so that individual cells can migrate out of the tissue and form a monolayer. Cell digestions, on the other hand, require enzymes to break down the small tissue pieces and release individual or groups of cells. Enzymes commonly used to establish epithelial and fibroblast cultures from fish fins are trypsin and collagenase, as seen in Table 3.1. Trypsin is a strong pancreatic serine protease of animal origin that cleaves peptides on the C-terminal side of lysine and arginine (Worthington Biochemical Corporation, accessed on 2013.07.29) releasing cells from tissues. Due to its potency, however, it may not be suitable for every tissue type. TrypLE™, a trypsin substitute, is animal-free, more gentle on cells, and can be used at room temperature (Invitrogen, accessed on 2013.07.30), making it an attractive option for fish cell culture.

Collagenase is a protease of bacterial origin (MacLennane et al., 1953) and it works by breaking the peptide bonds in collagen that hold tissues together (Invitrogen, accessed on 2013.07.29). Along with being a weaker digestive enzyme, there are seven types of collagenase, unlike trypsin, making each collagenase suitable for a specific tissue type. Although enzymatic digestion is most commonly used, tissues can be broken down by chemical agents, as performed by Khan et al. (1997) using ethylenediaminetetraacetic acid (EDTA), a calcium chelator, to break down the skin and liver of carp and catfish.

Although enzymatic digestion is common practice for fish cell culture, there are concerns regarding diminishing cell membrane integrity with use. This concern is avoided with explants because they closely resemble the structural framework of the in vivo environment (Sobhana et al., 2009). A study by Yoon et al. (2013) showed that explant-derived mesenchymal stem cells (MSC) produced a greater number of viable and proliferative cells than enzyme-derived cultures due to cellular damage that resulted from digestion. However, the major concern with explant-
derived cultures is that they require a greater length of time to reach confluence (Freshney, 2006), increasing the probability of culture contamination. Therefore, it is important to examine establishment techniques, prior to cell culture as they can affect the outcome of the cell culture.

Once a primary cell culture has been established, it can be sub-cultured to produce cell lines (Schaeffer, 1990). The longevity of the cell line can range from a limited to an indefinite number of divisions (Bols et al., 2005), depending on factors such as cell type. Cell lines are used in several fields, including virology (Imajoh et al., 2007), molecular biology (Kawano et al., 2010) and toxicology (Schnell et al., 2009), to name a few. In these cases, cell lines can be used as indicators of cellular changes that are triggered by the agent in question. When considering the use of cell lines for offspring production with technologies such as SCNT, the integrity of the cells plays an important role in embryo outcome. For instance, cell lines that extend past passage 10 are not generally preferred for SCNT (Baguisi, 1999; Polejaeva et al., 2000; Wilmut et al., 1997; Zhang et al., 2009). This is because long-term culture of somatic cells can result in chromosomal aneuploidy (Gomez et al., 2006), senescence (Mastromonaco et al., 2006), ineffective cellular reprogramming of donor cells, and abnormal embryo production, all of which minimize cloning success (Zhang et al., 2009). Although long term cultured cells are not typically used in SCNT, neither are primary cell cultures because they are very heterogeneous in their cell make-up (Bols et al., 2005), which minimizes SCNT success. Lee et al. (2002) showed that embryonic zebrafish primary cells had a lower nuclear transplant success rate (embryo development) than long-term cultured cells (passage 13).

Protocols for initiating primary cell cultures involving temperature, media components (serum), and attachment dishes (plastic ware or glass) are important to examine. Rigorous techniques and harsh protocols can lead to poor cell growth and decreased viability, ultimately leading to the demise of the cells by culture stress (Rodier and Campisi, 2011). As a result, it is important to assure establishment procedures are suitable for the species or cell type by assessing cell health. Some techniques commonly used to evaluate cell health are cell cycle analysis kits, chromosome normality and cell viability assays, such as MTT or XTT metabolic dye assays, resazurin-based assays, and live/dead cell staining (Stoddart, 2001). Understanding the effects that establishment techniques have on cell health may help to produce protocols efficient for fish
cell culture and SCNT. Therefore, the objective of this study was to investigate how cell viability is affected by the digestive enzyme and digestion time used to establish primary cell cultures.

Table 3.1: Commonly used digestive enzymes in primary fish cell culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Digestive enzyme</th>
<th>Tissue</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oryzias Latipes</td>
<td>0.01% trypsin and 0.002% EDTA</td>
<td>Fins (all except non-ventral)</td>
<td>Fibroblast-like</td>
<td>Komura et al., 1988</td>
</tr>
<tr>
<td>Brachydanio rerio</td>
<td>0.2% w/v trypsin and 1mM EDTA</td>
<td>Gill, pelvic fin, caudal fin, viscera</td>
<td>Not stated</td>
<td>Collodi et al., 1992</td>
</tr>
<tr>
<td>Carassius auratus auratus</td>
<td>0.2 mg/mL collagenase</td>
<td>Fins</td>
<td>Epithelial</td>
<td>Mauger et al., 2009</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>10% v/v collagenase I</td>
<td>Caudal fin</td>
<td>Fibroblasts</td>
<td>Choresca et al., 2012</td>
</tr>
<tr>
<td>Scophthalmus maximus</td>
<td>0.2% collagenase II and 0.5% hyaluronidase</td>
<td>Fins</td>
<td>Fibroblasts</td>
<td>Fan et al., 2010</td>
</tr>
<tr>
<td>Kryptopterus bicirrhis</td>
<td>10% collagenase I</td>
<td>Fins</td>
<td>Fibroblast</td>
<td>Han et al., 2011</td>
</tr>
</tbody>
</table>

3.2 METHODS

All procedures involving animals were approved by the Toronto Zoo Animal Care and Research Committee and by the University of Guelph Animal Care Committee (Protocol # 12R029). The experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario and the recommendations of the Canadian Council for Animal Care. All products were purchased from Life Technologies Corporation, Grand Island, NY, USA, unless otherwise stated.
3.2.1 Animals, Anesthesia, and Experimental Design

Ngege were housed under the same conditions as described in Chapter 2. Opportunistic culling of the captive ngege population for management reasons resulted in 3 fish being used for enzymatic digestion treatment. Ngege were rinsed with 0.9% NaCl irrigation and then injected intravenously with Ketamine at a dose of 50 mg/kg. Once anesthetized, the caudal fins were removed with serological scissors and placed in a specimen container containing wash solution described in Chapter 2. These ngege were later euthanized with 0.2 mL T-61 following fin removal. The 3 caudal fins were brought to the lab and the remaining work was performed under a laminar flow hood. Each fin was placed in a separate 100x15 mm petri dish, rinsed 5 times with wash medium and biopsied with a 6 mm biopsy punch 12 times (3 replicates of the 4 treatment groups). The 4 treatment groups were: 0.2 mg/mL collagenase I (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at 37°C for 30 minutes (Mauger et al., 2006) and 60 minutes, and 1x TrypLE™ at room temperature for 30 minutes, and 60 minutes (referred to as C30, C60, T30, and T60, respectively).

3.2.2 Cell Culture

Biopsies were chopped into 1 mm² pieces in their separate 100x15 mm petri dishes. Chopped tissues were digested with one of the 4 treatment groups and later inactivated with culture medium described in Chapter 2. Four milliliters of the final 5 mL solution (containing cell digest, tissue and culture medium) was transferred with a 5 mL serological pipette (BD Biosciences, Franklin Lakes, NJ) into a 12.5 cm² tightly capped cell culture flask (BD Biosciences, Franklin Lakes, NJ, USA). All flasks were incubated at 25°C (Mauger et al., 2006) in Thermo Scientific Heratherm Compact 0.6 ambient incubators (Thermo, Rochester, NY, USA). The remaining 1 mL was used for cell viability and concentration analysis on day 1. The medium was changed every third day of culture.
3.2.3 Subculture

On day 7 the culture medium was aspirated and the monolayers were rinsed with 3 mL wash solution. The wash solution was aspirated and 1 mL of 1x TrypLE™ was added to each flask for 4 minutes. Detached cells from each of the 3 treatment replicates per fish were pooled into a 15 mL centrifuge tube and considered as 1 sample (i.e: Fish 1 had 3 replicates of C30, which were combined to make 1 sample of C30 for fish 1). Three mL of culture medium were added and centrifuged at 50 x g for 5 minutes. The pellet was re-suspended with 5 mL culture medium and 1 mL of the cell suspension was placed into each well of a 4-well dish (Thermo, Rochester, NY, USA). The dishes were incubated at 25°C for 24 hours for senescence, apoptosis and ROS analysis. The remaining 1 mL was used for cell viability and concentration analysis on day 7.

3.2.4 Cell Viability and Concentration

On days 1 and 7, 1 mL of cell suspension from each sample was used for cell viability and concentration assessment. The solution was centrifuged at 50 x g for 5 minutes. The pellet was suspended in 1 mL HBSS and 20 µL were removed for cell counts on a haemocytometer (Hausser Scientific, Horsham, PA, USA). Then a working solution of 2 µM calcein AM and 4 µM EthD-111 in 10 mL PBS was prepared and added to the cell suspension following the manufacturer’s protocols (PromoCell GmbH, Heidelberg, Germany). Twenty µL droplets were placed on a slide, mounted with a coverslip and observed at (10x magnification) using an Olympus 1X51 inverted microscope with a dual fluorescence (red/green) filters. Three hundred live (green) and dead (red) cells were counted. Data were presented as mean ± standard error of the mean (SEM).

3.2.5 Oxidative Stress

Three replicates of each treatment group were analyzed for oxidative stress on day 8. CellROX deep red was added to each well at a final concentration of 5 µM as per the manufacturer’s protocol. The probe was left for 30 minutes at 25°C before being analyzed at 10x
magnification under an Excitation/Emission (Ex/Em) of 530 nm/635 nm. Cells fluorescing red have been oxidized by ROS (Lin et al., 2012) whereas non-oxidized cells remained colourless. A visual analysis of the approximate percentage of cells in each well fluorescing red was recorded. The positive control consisted of cells exposed to 1% hydrogen peroxide added to the culture medium in the well, since it mimics the physiological effects of oxidative stress in vitro (Choi et al., 2009).

3.2.6 Apoptosis

Three replicates of each treatment group were analyzed for apoptosis on day 8. The culture medium from each well was removed and the well was washed three times with HBSS. In 1 mL HBSS, 5 µg/mL Hoechst 33342 (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added to each well and left to incubate for 20 minutes (Allen et al., 2001) at 25°C. Cells were observed under 10x magnification at Ex/Em of 346 nm/497 nm. A visual analysis of the morphological signs of apoptosis such as blebbing and chromatin condensation was carried out (Brewton et al., 2001). The positive control consisted of cells exposed to 1% hydrogen peroxide added to the culture medium in the well.

3.2.7 Senescence

Three replicates of each treatment group were analyzed for senescence on day 8. The culture medium was aspirated and the adhered cells were rinsed twice with 1 mL of 1x PBS. Cells in each well were fixed with a 1x Fixation Buffer and stained with 1 mL Staining Mixture following the manufacturer’s protocol of the Senescence Cell Histochemical Staining Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Cells were incubated at 25°C for 2 hours and then observed under brightfield illumination at 10x magnification. Blue cells were positive for β-galactosidase activity, while colourless cells were considered proliferative or quiescent (Fedorovich et al., 2009). The percentage of senescent cells was counted from 100 cells in replicates of three.
3.2.8 Control Cell Line

Adult zebrafish (*Danio rerio*) caudal fin cell line (AB.9, ATCC® CRL-2298™; ATTC, Manassas, VA, USA) was purchased as a reference for normal cell viability, senescence and oxidative stress response in fish cells. The vial was thawed and grown at 28°C in DMEM containing 15% FBS in a 5% CO₂ incubator as per the manufacturer’s instructions. The same methodologies for cell viability, apoptosis, and senescence were performed on these cells.

3.2.9 Statistical Analysis

Statistical analysis was performed using MiniTab 16 (Mintab Inc., State College, PA, USA) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

A Kruskal-Wallis test was used to compare the viable number of cells produced from each digestive treatment on day 1 and day 7 individually, whereas a Wilcoxon test was performed to compare the day 1 and 7 median number of viable cells. The percentage of senescent cells between C30, C60 and the AB.9 (ATCC® CRL-2298™) control were compared using one-way ANOVA followed by Tukey post-hoc test. P values less than 0.05 were considered significant.

3.3 RESULTS

3.3.1 Cell Viability

On day 1 C30 had the lowest viable cell median (2.0 x 10⁴ cells/mL), which was significantly different (p=0.024) than the other three treatments (Figure 3.1). The AB.9 (ATCC® CRL-2298™) control cell line, in comparison, produced the greatest amount of viable cells between treatments groups with a median of 4.3 x 10⁵ cells/mL.

On day 7, C60 produced the greatest number of viable cells (2.1 x 10⁴ cells/mL), which was significantly different (p=0.012) than the other three treatment groups.
CRL-2298™ control cell line by day 7 had $1.7 \times 10^5$ cells/mL, the highest number of viable cells between the treatment groups (Figure 3.1).

Comparing the median viable cells produced on day 1 ($5.6 \times 10^4$ cells/mL) to day 7 ($8.2 \times 10^3$ cells/mL) showed that there was significantly fewer ($p=0.0062$) viable cells present on day 7 than on day 1 (95% C.I: 15107, 97455).

**Figure 3.1:** Boxplot of median viable cells produced from each digestive treatment (n=3) and control (n=3) on day 1 and day 7. The control is AB.9 (ATCC® CRL-2298™).
3.3.2 Oxidative Stress

Each treatment group produced cell cultures with ROS staining similar to that of the positive control. Between the treatment groups, C60 and T30 had lower percentages than C30 and T60, as seen in Table 3.2. In contrast, AB.9 (ATCC® CRL-2298™) control cell line had less than 5% oxidized cells.
Table 3.2: Percentage of oxidized cells in the different treatment groups and AB.9 (ATCC® CRL-2298™) zebrafish cells.

<table>
<thead>
<tr>
<th>Fish</th>
<th>Treatment Groups</th>
<th>Oxidized Cell Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C30</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C60</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>T30</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Positive Control- H2O2</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>C30</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C60</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T30</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Positive Control- H2O2</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>C30</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C60</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T30</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T60</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Positive Control- H2O2</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>AB.9 (ATCC® CRL-2298™) Control</td>
<td>-</td>
</tr>
</tbody>
</table>

**LEGEND**
- = 0 ≥ 10% oxidized cells
+ = 11 ≥ 50% oxidized cells
++ = 51 ≥ 100% oxidized cells

**NOTE:** Based on visual observation of the stained cells within each well.
3.3.3 Cell Death

On day 7 cell cultures from each treatment group contained vacuoles visible under 10x magnification, that were not observed in the AB.9 (ATCC® CRL-2298™) control cell line (Figure A.1). Examination of nuclei stained with Hoechst 33342, showed that none of the treatment groups demonstrated morphological signs of blebbing as observed in apoptotic positive control cells (approximately 40% of cells undergone apoptotic blebbing; Figure 3.2). The percentage of apoptotic cells among treatments was similar to the percentage of apoptosis observed in the AB.9 (ATCC® CRL-2298™) control cell line (0.03%). However, the staining intensity of ngege fin cultures was brighter than the AB.9 (ATCC® CRL-2298™) control cell line (Figure 3.2)
Figure 3.2: Morphological observation of apoptosis by fluorescence using Hoechst 33342 nuclear stain. A: C60 primary ngege cell culture containing 1% hydrogen peroxide; B: AB.9 (ATCC® CRL-2298™) control cell line; C: C60 primary ngege cell culture. Morphological signs of blebbing are circled in yellow.
3.3.4 Senescence

Cultures from each treatment group contained blue cells due to β-galactosidase activity that were, on average, greater in number than the AB.9 (ATCC® CRL-2298™) control cell line, as seen in Figure 3.3. T30 and T60 had the highest mean (74-88%) of β-galactosidase activity in 2 of the 3 fish. The third replicate of T30 and T60 had less than 300 cells which was not enough to calculate the cell percentage. The amount of senescent cells produced on average between C30, C60 and AB.9 (ATCC® CRL-2298™) control cell line were all significantly different (p<0.0001) from each other, with AB.9 (ATCC® CRL-2298™) producing the least number of senescent cells (15%) on day 8. In addition, a majority of the senescent cells in Figure 3.3 contained vacuoles.

Table 3.3: Mean number of β-galactosidase positive cells counted from 300 cells (n=3) on day 8. The results are presented as mean ± SEM. The control is the AB.9 (ATCC® CRL-2298™) zebrafish cell line.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Fish 1</th>
<th>Fish 2</th>
<th>Fish 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 30</td>
<td>73.0 ± 4.4</td>
<td>77.0 ± 5.3</td>
<td>82.0 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>C 60</td>
<td>60.6 ± 6.5</td>
<td>69.0 ± 2.0</td>
<td>69.3 ± 4.9</td>
<td>15.3 ± 13.6</td>
</tr>
<tr>
<td>T 30</td>
<td>86.3 ± 1.5</td>
<td>74.6 ± 4.2</td>
<td>TFTC</td>
<td></td>
</tr>
<tr>
<td>T 60</td>
<td>80.0 ± 4.0</td>
<td>88.0 ± 2.6</td>
<td>TFTC</td>
<td></td>
</tr>
</tbody>
</table>

LEGEND
TFTC= Too Few To Count (<300 cells)
Figure 3.3: Day 8 brightfield images of cell cultures from A: C30, B: C60, C: T30, D: T60, and E: AB.9 (ATCC® CRL-2298™) control cell line. Senescent cells are blue and a majority of them contain vacuolation.
3.4 DISCUSSION

Conditions used to establish cell cultures from primary cells are critical to the health and normality of the cell lines produced. The data presented in this study show that different digestion protocols produce significantly different results. This can be seen when the number of viable cells on day 1 declined significantly (p<0.0001) by day 7 in all four of the treatment groups. In particular, TrypLE™ performed better early on in the culture as day 1 viable cell concentrations were greater than collagenase. However, by day 7 viable cell concentrations dropped by more than 50% in all treatment groups with the exception of C60. This drastic decline in median viable cell concentrations (from $2.0 \times 10^4$ cells/mL to $8.2 \times 10^3$ cells/mL in C30, $5.6 \times 10^4$ cells/mL to $3.0 \times 10^3$ cells/mL in T30, and $5.0 \times 10^4$ cells/mL to $3.5 \times 10^3$ cells/mL in T60) during the first week in culture suggests that cell death occurred. Although it cannot be confirmed at this time, cell death was potentially the result of the effects of the digestive enzymes during the culture initiation procedures. Treatment groups were selected based on the work by Mauger et al. (2006), which used collagenase to digest caudal fin tissue for 30 minutes at a fish’s physiological temperature. Since $37^\circ C$ is within the thermo-tolerance zone of ngege and is routinely used for mammalian cell culture, it was believed that $37^\circ C$ collagenase would be appropriate to digest ngege caudal fin biopsies. Although TrypLE™ is typically not used longer than 5-10 minutes (depending on the culture medium and cell type) as described by the manufacturer’s protocols (Invitrogen, accessed 2013.12.11), incubation times of 30 and 60 minutes were used to digest caudal fin biopsies to compare to the collagenase treatment groups. However, one cannot rule out other factors in the culture environment, such as media, temperature, contamination, even though they were previously tested. It was expected that TrypLE™ would produce a lower number of viable cells because it is a stronger digestive enzyme than collagenase and that the 60 minute digestion would produce a higher cell concentration. It was not expected that cell viability of 60 minute treatment groups would be better than 30 minute treatment groups because the enzymes had a longer time to cause potential cellular damage. Low viable cell concentrations are a major concern because there is the potential for the remaining viable cells to undergo proliferative exhaustion in order to reach confluence. Similar to the current study, Molinos et al. (2012) showed that different types of collagenase and digestion times affected cell viability and cell concentration. They found that 19
hour digestions of bovine intervertebral disks with collagenase II produced greater cell concentrations and viability than 4 hour digestions (Molinos et al., 2012).

Similar to the treatment groups, AB.9 (ATCC® CRL-2298™) control cell had a higher viable cell concentration on day 1 than what resulted on day 7. Since the control cell line was initiated from a cryopreserved vial, it is typical for a portion of cells to die post-thaw during the first day in culture. In this study, the initial viable cell concentration was taken immediately after thaw (named day 1) and not after 24 hours in culture. This may account for the large discrepancy in cell concentrations between day 1 and day 7. However, the possibility of other potential factors such as culture environment, that affected the treatment groups, cannot be ruled out of causing cell death in the control group.

Staining of nuclei with Hoechst 33342 did not detect morphological signs (<0.03 %) of apoptosis (blebbing) higher than that present in normal cell cultures, but did produce varied staining intensities between ngege caudal fin cells (bright blue), and the AB.9 (ATCC® CRL-2298™) control cell line (dull blue). Although brightly stained nuclei can represent chromatin condensation of an apoptotic cell (Brewton et al., 2001), it can also represent mitosis (Cooper, 2000) and cell differentiation (Golob, 2008). In this study, the ngege cells treated with hydrogen peroxide and C60, both contained the same bright nuclei intensity, unlike the AB.9 (ATCC® CRL-2298™) control cell line. These results suggest that primary ngege cells may have appeared brighter than the AB.9 (ATCC® CRL-2298™) control cell line because they are differentiated and contain heterochromatin, in contrast to the undifferentiated AB.9 (ATCC® CRL-2298™) control cell line containing euchromatin. The inability to differentiate between the cause of chromatin condensation is one of the main reasons Hoechst 33342 staining is not a definitive indicator of apoptosis, unlike assays such as TUNEL and Annexin V. However, it was the most suitable diagnostic tool since low cell concentrations limited the ability to perform many other apoptotic assays. Furthermore, apoptosis naturally occurs among a small percentage of cells in culture that cannot adjust to living in vitro due to nutrient limitations, oxygen availability and cell density (Al-Rubeai and Singh, 1998). This was shown by Qiao and Farrell (1990) who found that the natural rate of apoptosis (0.05%) in primary rat hepatocytes increased (to 3.9 %) with time, based on cell density and attachment dishes used to establish primary cell cultures.
However, in this study, the natural rate of apoptosis (0.02%) taken from the AB.9 (ATCC® CRL-2298™) control cell line was not less than the rate of apoptosis observed in the treatment groups, suggesting that cell death occurred by alternative method of cell death.

Evidence of vacuolation was visible in all cell cultures on day 7, with the exception of the AB.9 (ATCC® CRL-2298™) control cell line. Vacuolated cells are indicative of cell stress and cell death by autophagy (Henics and Wheatley, 1999). When autophagic cell death occurs, there is a release of caspase inhibitors that suppress the apoptotic caspase pathway (Chipuk and Green, 2005), thereby supporting the lack of morphological evidence of apoptosis observed in these cell cultures. To better understand the processes occurring in these cells, further investigation involving organelle staining and electron microscopy is required to accurately define the vacuoles role in autophagic cell death (Tsujimoto and Shimizu et al., 2005), intracellular stress (Mizushima et al., 2008; Denoyelle et al., 2006), or cellular senescence (Chang et al., 1999).

High levels of oxidative stress during the first week of culture (50-90% oxidation in the treatment groups except the control), as evidenced by ROS staining, could have resulted from tissue damage during the initial digestion period, or from nutrient deprivation in the medium (Grezalak et al., 2000; Halliwell and Whiteman, 2004). Oxidative stress is concerning because, as seen in this study, it can lead to irreversible cellular damage and cell death (Chen et al., 2008). Burlacu et al. (2001) showed that the mode of cell death triggered by oxidative stress is influenced by the amount of oxidation. A high degree of oxidative stress caused necrosis, whereas lower levels caused apoptosis. Although the Burlacu et al. (2001) study was done with mammalian cells, Rau et al. (2004) showed that between species of fish and mammals, fish cells (PLHC-1) are significantly more sensitive to oxidative stress than mammal cells, exhibiting reduced viability and increased cytotoxicity. Therefore, the high levels of oxidation present among treatments in this study could have caused cell death aiding in the drastic cell concentration decline.

In addition to cell death, oxidative stress has been associated with premature cellular senescence due to the commonalities in their signal transduction pathways (Chen et al., 1995, Yuan et al., 1995, Sherr and Depino, 2000). Gambino et al. (2013) found that oxidative stress
activates the transcription of p44/p53 and p66, which are cell cycle regulators. Once activated, they down regulate the expression of approximately 200 genes associated with cell proliferation and inhibition of senescence (Gambino et al., 2013). Consequently, these data confirm that oxidative stress can lead to senescent cell cultures. However, oxidative stress may not be the only cause of premature cellular senescence. Cell morphologies observed on days 7 and 8 showed vacuolation in all the treatment groups except the control. This is significant because recent evidence described by Patschan et al. (2008) suggests that autophagy and senescence are interrelated. These authors showed that inhibition of autophagosome production by various drugs decreased the amount of senescent cells produced in culture. Similarly, Young et al. (2009) showed autophagy protein production and the expression of a senescent cell phenotype are connected by negative feedback pathways. These studies suggest that if vacuolation was present as a result of autophagy then a senescent cell morphology would be produced. In this study, therefore, the evidence suggests that premature cellular senescence was a result of oxidative stress, as well as autophagy.

Understanding the effects that cell culture initiation procedures have on cell quality is valuable information for establishing novel cell lines from endangered species. This study provides evidence that digestive enzymes and digestion time used to establish primary cell cultures affect cell viability, and cause cellular stress and damage. Further research into the causes of vacuolation and oxidative stress in these cultures may decrease cell death and allow for optimized protocols to be developed.

3.5 CONCLUSION

Treatments of 0.2 mg/mL collagenase and 1x TrypLE™ at 30 and 60 minute incubation times were used to initiate cell cultures of ngege caudal fin biopsies. C60 had the least amount of cell death, oxidative stress and senescence compared to the other three treatment groups. This indicates that C60 is the most appropriate method to initiate a primary cell culture. Although C60 was the best option among treatment groups, it did not produce healthy cells like the AB.9 (ATCC® CRL-2298™) control cell line. Further examination of vacuoles produced in cell
cultures is required to gain a better understanding of the relationship between vacuolation and establishment techniques.
CHAPTER FOUR

The effects of donor age and biopsy location on cell viability of caudal fin cell cultures
4.1 INTRODUCTION

The development of healthy and proliferative fish cell lines are essential for creating GRBs and achieving success with SCNT. Assessment of cell line health can be done by examining cell viability and replicative capacity that vary between species and tissue type. Developing standard cell culture procedures for endangered species is challenging when the initiating material is not the most suitable tissue for cell culture but is the only available source. Typically, accessibility and invasiveness of the tissue drives the location chosen for sampling from living endangered species. Some concerns with the initiating material include donor age and biopsy location, which have been shown to affect cell viability and proliferation (Bierman, 1978; Eidet et al., 2012).

Within the last 40 years evidence of the relationship between cell donor age and proliferation as well as cell donor age and SCNT success have remained widely controversial. Some researchers state that donor age and in vitro life span are related, while others indicate that donor age has no impact on the replicative lifespan of a cell in vitro. In a study by Bierman (1978), human arterial smooth muscle cells were shown to be inversely proportional to donor age as the cell concentration at confluence and number of passages decreased with older-aged individuals. Similarly, Zaim et al. (2012) found that child donor (0-12 years) mesenchymal cells maintained their replicative fibroblastic morphology for a greater length of time (P15) than adults (25-50 years; P9) and the elderly (over 60 years; P5) under similar environmental conditions. However, Cristofalo et al. (1998) argue that it is not donor age that affects replicative lifespan, but health of the individuals that determines in vitro cell lifespan. In their study, examination of 42 human fibroblastic cell lines derived from fetal (12-20 weeks), young (17-64 years) and old (73-92 years) individuals, all of whom were clinically deemed “healthy”, demonstrated no correlation between donor age and replicative lifespan. The issue of donor age and its relation to the cell cycle becomes increasingly important when the donor cell is used for embryo production. Kasinathan et al. (2001) found that cell donor age affects cell proliferation in vitro as ear biopsies from bovine fetal, and calf cell lines reached 31 passages, whereas the adult cells only reached 18 passages. However, it did not affect embryo production since the cells used for nuclear transfer were in G1 either by the shake-off method or confluence. Therefore, the
The effects of biopsy location on cell line health are other aspects of culture establishment procedures that are generally overlooked when dealing with mammal or whole organ cultures. When working with endangered species this issue becomes more prominent as the sampling location sites may be limited or less than ideal due to accessibility and invasiveness, as previously mentioned. In fish, the caudal fin is the typical area chosen for tissue sampling when establishing cell cultures from a living individual because it is easily accessible and has regenerating potential. The caudal fin is composed of osteoblasts and skeletogenic cells that form rays (Azevedo et al., 2011), which separate from each other in the proxiodistal area to create lepidotrichia. Each lepidotrichium ends with actinotrichia (collagenous fibrils) and are filled with nerves and blood vessels (Azevedo et al., 2011; Santamaria and Becerra, 1991). Parallel lepidotrichium create hemisegments (Becerra et al., 1983), which become covered by mesenchymal and epidermal cells (Yoshinari and Kwakami, 2011). The proximal area of the fin is the thickest portion, mostly containing lepidotrichia in close proximity, and becomes thinner near the distal area due to the functionality of the caudal fin (Becerra et al., 1983). Similar to the caudal fin, murine conjunctiva is comprised of different cell types and variations in thickness depending on the location being sampled. A study by Eidet et al. (2012) showed that different biopsy locations of murine conjunctiva produced variable cell proliferative capacities based on the cell composition of that area. However, they did not investigate whether different biopsy locations affected SCNT. Studies in mammals have shown that cells obtained from different locations throughout the body such as the uterus, ear and liver of cows produced more embryos and live births from transferred nuclei than skin and oviduct cells (Kato et al., 2000). However, no study has examined SCNT success with biopsies from different locations of the same tissue such as the proximal and distal regions of the caudal fin. Therefore, the differences in cell composition and structural morphology throughout the caudal fin make biopsy location a significant area to explore when evaluating culture establishment procedures and related cell line health.
Judging the practicality of establishing fish somatic cell GRBs for SCNT requires extensive evaluation of cell culture conditions. Typically, donor age will remain unknown and biopsy location will be limited in wild fish. If these factors limit cell viability and proliferation, establishing fish cell cultures from living endangered species may not be the most practical method for endangered fish conservation. Therefore, the objective of this study was to evaluate the effects of donor age using young (5 years) and old (17 years) ngege, and biopsy location (proximal, middle, and distal) on the caudal fin on cell viability.

4.2 METHODS

All procedures involving animals were approved by the Toronto Zoo Animal Care and Research Committee and by the University of Guelph Animal Care Committee (Protocol # 12R029). The experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario and the recommendations of the Canadian Council for Animal Care. All products were purchased from Life Technologies Corporation, Grand Island, NY, USA, unless otherwise stated.

4.2.1 Animals, Anesthesia, and Experimental Design

Ngege were housed under the same conditions as described in Chapter 2. Opportunistic culling of the captive ngege population for management reasons resulted in 6 healthy, non-breeding fish used in this study. Three of the ngege were 17 years of age, and 3 were 5 years of age, referred to as old and young, respectively.

Ngege were rinsed with 0.9% NaCl irrigation and then injected intravenously with ketamine at a dose of 50 mg/kg. Once anesthetized, the caudal fins were removed with serological scissors and placed in a specimen container containing wash solution described in Chapter 2. These ngege were later euthanized with 0.2 mL T-61 following fin removal. The 6 caudal fins were brought to the lab and the remaining work was performed under a laminar flow hood. Each fin was placed in separate 100 x15 mm petri dishes, rinsed 5 times with wash medium and biopsied with a 6 mm biopsy punch 9 times (3 replicates of triplicate samples). The
6 treatment groups were biopsy areas on the caudal fin: old-distal (OD), young-distal (YD), old-middle (OM), young-middle (YM), old-proximal (OP), and young-proximal (YP), as seen in Figure 4.1.

![Figure 4.1](image)

**Figure 4.1:** Proximal, middle and distal sampling locations on young and old ngerge caudal fins. Circles (○) represent a 6 mm biopsy punch removed from the caudal fin.

4.2.2 Cell Culture

Biopsies were chopped into 1 mm² pieces in their separate 100 x 15 mm petri dishes. Chopped tissues were digested with 0.2 mg/mL collagenase I for 60 minutes. Four milliliters of the final 5 mL solution (containing cell digest, tissue and culture media) were transferred with a 5 mL serological pipette (BD Biosciences, Franklin Lakes, NJ) into a 12.5 cm² tightly capped cell culture flask (BD Biosciences, Franklin Lakes, NJ, USA). All flasks were incubated at 25°C (Mauger et al., 2006) in Thermo Scientific Heratherm Compact 0.6 ambient incubators (Thermo, Rochester, NY, USA). The remaining 1 mL was used for cell viability and concentration analysis on day 1. The medium was changed every third day of culture.
4.2.3 Subculture

Monolayers were subcultured using 1mL of 1x TrypLE™ for 5 minutes and re-plated into two 4 well dishes on day 7 according to the methodology described in chapter 3. The dishes were incubated at 25°C for 24 hours for senescence, ROS, and organelle staining.

4.2.4 Cell Viability and Concentration

On days 1 and 7, a 1 mL of cell suspension from each sample was removed for cell viability and concentration analysis using Live/Dead Cell Staining Kit protocols described in Chapter 3. Data were presented as mean ± SEM.

4.2.5 Oxidative Stress

Protocols described in chapter 3 involving CellROX deep red at a final concentration of 5 μM were followed to measure the percentage of cells exhibiting oxidative stress.

4.2.6 Senescence

Protocols described in Chapter 3 using the Senescence Cell Histochemical Staining Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) were followed to measure the percentage of senescent cells.
4.2.7 Organelle Staining

Only cell cultures containing vacuolation on day 8 were analyzed with Endoplasmic Reticulum (ER)-Tracker™ Green and LysoTracker®, to examine the source of vacuolation. Culture media from vacuolated wells were aspirated and rinsed twice with 1mL HBSS. A 1 µM working solution of ER-Tracker™ Green dye and a 75 nM working solution of red LysoTracker® were added to HBSS containing wells. The plates were incubated for 30 minutes at 25°C and observed under the Olympus 1X51 inverted microscope using the fluorescence dual (red/green) filter. The percentage of vacuoles present in the ER (green) and lysosomes (red) were presented as mean ± SEM.

4.2.8 Control Cell Line

The adult zebrafish (Danio rerio) caudal fin cell line (AB.9, ATCC® CRL-2298™; ATTC, Manassas,VA,USA) discussed in Chapter 3 was subcultured three times using 1x TrypLE™ prior to use in this study as passage 4. The cells continued to grow at 28°C in DMEM containing 15% FBS in a 5% CO₂ incubator as per the manufacturer’s instructions. The same methodologies for oxidative stress and senescence were performed on these cells.

4.2.9 Statistical Analysis

Statistical analysis was performed using MiniTab 16 (Mintab Inc., State College, PA, USA) and Excel (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

The comparison between the number of cells from different donor ages and biopsy locations on the caudal fin were analyzed using one-way ANOVA followed by a Tukey post-hoc test. A 2-sample t-test was used to compare the difference between the mean number of cells produced on day 1 to day 7. Non-parametric Kruskal-Wallis test was used to compare the number of senescent cells between biopsies of different age groups and sampling location. P values less than 0.05 were considered significant.
4.3 RESULTS

4.3.1 Cell Viability

Treatment groups from the old caudal fins (OP, OM, OD) were not significantly different (p>0.05) from each other on day 1 (Figure 4.2A), but were significantly different (p<0.001) on day 7. OD produced the least mean viable cells (6x10^3 cells/mL) whereas OP produced the greatest mean viable cells (5x10^4 cells/mL) among the old treatments on day 7.

Treatment groups YM and YD from young caudal fins were significantly different (p=0.027) on day 1 (Figure 4.2A) and on day 7 (p<0.001; Figure 4.2B), whereas YP was not significantly different from YM and YD on day 1 (Figure 4.2A), but became significantly different from YD (p<0.0001) on day 7 (Figure 4.2B).

On day 1 there was no significant difference (p>0.05) in the mean number of viable cells between old (2.3x10^5 cells/mL) and young individuals (1.4x10^5 cell/mL). Similarly, on day 7 there were no significant differences in the number of viable cells between old (4.8x10^4 cells/mL) and young individuals (3.3x10^4 cell/mL).

Comparing the mean viable cells produced from each treatment group on day 1 to day 7 showed that there were significantly less (p<0.0001) viable cells produced on day 7 (3x10^4 cells/mL) than on day 1 (13x10^4 cells/mL). YM produced the greatest number of viable cells (2x10^5 cells/mL) and YD the least (7x10^4 cells/mL) on day 1, but by day 7 both proximal treatment groups produced the greatest viable cells and both distal treatment groups produced the least.
Figure 4.2: Mean viable cell counts among treatment groups (n=3) on day 1 (A) and day 7 (B). Error bars indicate standard error, and treatment groups not sharing a letter are significantly different (p<0.05).
4.3.2 Vacuolation Analysis

On day 8 approximately 60-80% of the cells were vacuolated in each of the OP and OM treatment groups and in the negative control (Figure 4.3). Vacuolated cells stained with ER-Tracker™ Green and LysoTracker® Red showed that vacuoles were present in the ER. Among these cultures there was a 10% occurrence of lysosomes present in the ER vacuoles (Figure 4.3: A2). All other treatment groups, as well as the AB.9 (ATCC® CRL-2298™) control cell line, did not produce vacuoles during cell culture.
**Figure 4.3:** ER vacuolation of ngege caudal fin cells treated with H$_2$O$_2$ (A: 20x magnification), OP (B), and OM (C), at 10x magnification on day 8. Figures labeled with 1’s are brightfield images and 2’s are fluorescent images under a dual red/green filter, where red depicts the presence of lysosomes, and green identifies the ER. Arrows (新陈) point towards a vacuole.
4.3.3 Oxidative Stress

Middle and proximal biopsies produced similar amounts of oxidative stress, with less than 5% oxidized cells from young ngege caudal fins and greater than 51% oxidized cells in old ngege caudal fins (Table 4.1). Treatment groups from the distal portion of the caudal fin produced few cells (< 100 cells) to be compared against the middle and proximal treatment groups. Cultures from young individuals as well as the AB.9 (ATCC® CRL-2298™) zebrafish cells, did not exhibit oxidative stress as they contained less than 10% oxidized cells. In contrast, treatments from the old individuals produced levels of oxidation similar to the positive control (>50% oxidation), as seen in Table 4.1.
Table 4.1: Percentage of oxidized cells counted from 300 cells in each treatment group and AB.9 (ATCC® CRL-2298™) zebra fish cells on day 8.

<table>
<thead>
<tr>
<th>Biopsy location on the caudal fin</th>
<th>Fish #</th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal</td>
<td>1</td>
<td>TFTC</td>
<td>TFTC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TFTC</td>
<td>TFTC</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TFTC</td>
<td>TFTC</td>
</tr>
<tr>
<td>Middle</td>
<td>1</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Proximal</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Ngege caudal fin cells treated with H$_2$O$_2$  ++

AB.9 (ATCC® CRL-2298™) zebrafish control  _

**LEGEND**

0 $\geq$ 10% of cells are oxidized = -

11 $\geq$ 50% of cells are oxidized = +

51 $\geq$ 100% of cells are oxidized = ++

TFTC= Too Few to Count (< 100 cells)

**NOTE:** Results are based on visual observations of stained cells within each well.
4.3.4 Senescence

On day 8 old treatment groups produced significantly fewer (p=0.025) senescient cells (19%) than young treatment groups (62%). The percentage of senescient cells between middle and proximal biopsies were also significantly different (p<0.05) from each other. OP produced the lowest median percentage of senescent cells (15%) than OM (24%), compared to YP that produced the highest median percentage of senescent cells (68%) than YM (53%; Figure 4.4). Distal treatment groups had less than 300 cells per well and could not be compared to the other treatment groups. AB.9 (ATCC® CRL-2298™) zebrafish cell line produced the lowest median of senescent cells (8%).

Figure 4.4: Boxplot of the percentage of senescent cells between old and young, proximal and middle biopsy cell cultures (n=3) on day 8. Median values are indicated by a circle (☉). The control is the AB.9 (ATCC® CRL-2298™) zebrafish cell line.
4.4 DISCUSSION

The viability and proliferative capacity of a cell line can be determined by the percentage of living cells in culture and the doubling time. Some characteristics of the sample material that can affect cell viability and proliferation are donor age (Zaim et al., 2012) and biopsy location (Eidet et al., 2012). When examining the relationship between sample characteristics and cell viability in this study, it was found that age had a minor role on cell viability compared to biopsy location. All treatment groups from the old fish produced similar viable cell counts to young treatment groups on day 7, with the distal biopsies producing the least number of viable cells (1.0-1.7 x 10^3 cells/mL) and the proximal biopsies producing the most (9.8-8.7 x 10^3 cells/mL). Similarly, a study by Cristofalo et al. (1998) showed that donor age does not affect cell culture quality as long as the specimens are in similar health. In the case of the ngege, both old and young were maintained under the same controlled environmental conditions, and routinely examined for any behavioural or health issues. Therefore, the similarities in overall health between young and old ngege may explain why there were no significant differences observed between viable cell concentration and donor age.

Biopsy location, on the other hand, did significantly (p<0.001) affect cell viability on day 7. This suggests that the distal portion of the caudal fin may not be the ideal sampling location for establishing cell cultures. This result was expected because the distal end does not contain as many mesenchymal cells as the proximal end, decreasing its proliferative capacity (Yoshinari and Kawakami, 2011). In addition, caudal fin thickness varies from the proximal region, which is the thickest, to the distal region, which is the thinnest, based on its structural components, which include lepidotrichia and actinotrichia. (Becerra et al., 1983). The thickness of the biopsy based on its location, may have been the determining factor in viable cell concentration rather than the cell types that encompass the area. The thicker proximal treatment groups had more tissue layers, making the 60 minute digestion appropriate. In comparison, the distal treatment groups were thinner and may have required a shorter digestion time to minimize the effects of the digestive enzyme on cell viability. Molinos et al. (2012) showed that thick bovine intervertebral disks required a longer digestion time of 19 hours, rather than the shorter standard 4 hour digestion, using collagenase to produce greater viable cell concentration. In contrast, thinner tissues such as
bovine ear cartilage require shorter digestion times of 3-5 hours (Mastromonaco et al., 2006) to produce viable cell lines.

Viable cell concentration also varied between culture days, with day 7 (3×10⁴ cells/mL) having significantly fewer (p<0.0001) viable cells than day 1 (13×10⁴ cells/mL). The decline in cell population may due to initiation procedures being too harsh, which may have affected attachment proteins and cell membrane integrity within the first day of culture, although this is only speculation. Other underlying issues associated with the culture environment that could have caused the cell population decline are nutrient depletion, improper attachment dishes, oxygen availability, and seeding density, all of which incur cell stress that can potentially lead to senescence or programmed cell death (Sherr and DePinho, 2000).

Oxidative stress in culture is typically a result of harsh culture establishment techniques and unsuitable culture materials such as media. In this study, oxidative stress was observed in only the old fish treatment groups, with the exception of the distal treatment as it did not produce enough cells (<100 cells) to be properly analyzed. On average, there were greater than 50% oxidized cells, compared to the AB.9 (ATCC® CRL-2298™) zebrafish control cell line, which only contained approximately 2% oxidized cells. These results differ from studies by Matsuo et al. (2004) and Nag et al. (1996) that have shown that old human cells are more resistant to oxidative stress than young human cells, due to increased levels of glutathione peroxidase and glutathione reductase, which control the presence of ROS. Although oxidative stress pathways were not quantified in this study, it would be beneficial for future studies to examine, because they can compare the difference between old and young donor age samples on the molecular level.

Oxidative stress has been linked to cell death (Chen et al., 2008) and senescence (Gambino et al., 2013), both of which were observed in the old treatment groups in this study. Although the old fish treatment groups on average did not display as many senescent cells as the young fish (14-24% vs 47-57%), they were higher than expected for a healthy cell culture as the AB.9 (ATCC® CRL-2298™) zebrafish control only contained 8% senescent cells in culture. Burova et al. (2013) showed that 90% of cells became senescent on day 7 when treated with
hydrogen peroxide, compared to 5% senescent cells observed in the untreated group. This suggests that oxidative stress could have been a factor in causing premature cellular senescence among old treatment groups.

In addition to senescence, oxidative stress has been linked to cellular vacuolation and autophagic cell death as seen in a study by Szaraz et al. (2010). They showed that the depletion of NADPH in the ER-lumen of HepG2 cells caused vacuolation. Similarly in fish (*Labeo rohita*), Marigoudar et al. (2012) showed that exposure of cells to oxidative stress reagents leads to structural changes in the cell, such as mitochondrial vacuolation. In this study, vacuolated cultures positive for oxidative stress appeared to have vacuoles present in the ER. The ER is an organelle that proteins enter to become folded into complexes prior to release to the Golgi for quality control (Malhotra and Kaufman, 2007). If the proteins remain unfolded in the ER, a stress response is triggered through intracellular signaling pathways. This alters the nucleus to help the cell cope with the ER stress in order to keep it alive (Szaraz et al., 2010). One mechanism triggered by the cell to promote survival is autophagy. It degrades damaged proteins (Funakoshi et al., 2013) and can eliminate polyubiquitinated protein clusters that signal cell death (Ding et al., 2007). If this process fails to keep the cell alive, the cell will begin to die by ER-associated degradation (ERAD) pathway (Malhotra and Kaufman, 2007), apoptosis or autophagic cell death. The latter two are often associated with lysosomal activity (Funakoshi et al., 2013; Guicciardi et al., 2004). Therefore, it is likely that the vacuolated cells associated with lysosomal activity in this study were undergoing cell death. Those that did not exhibit lysosomes in the vacuoles may have just elicited a response to promote cell survival in the old ngege cell cultures. The determinant in whether cells implement autophagy to promote death or survival during ER-stress is dependent upon the status (amount of energy required) of the cell as seen in Ding et al. (2007). This study showed that autophagy helps keep human colon and prostate cells alive, but not normal human cells and murine embryonic fibroblasts that do not express the same polyubiquitinated proteins. To further understand the complexities of vacuolation and its association with ER stress brought on by oxidative stress, future studies must investigate cellular pathways to find the causative links. In addition, immunohistochemistry of molecular factors associated with autophagy and apoptosis is a means of determining the involvement of lysosomes in each cell death pathway (apoptotic or autophagic).
Young treatment groups differed from old in that they did not exhibit oxidative stress or cellular vacuolation, but surprisingly had the highest percentages of senescent cells. Thus, in this study, a direct relationship between oxidative stress and premature cellular senescence cannot be made, and neither can age be related to cell proliferation. A study by Balin et al. (2002) showed that donor biopsy age was not a determinant in the replicative lifespan of a cell in vitro, rather proliferation was affected by establishment procedures and culture conditions, such as cell density and oxygen availability. In addition, Sherr and DePinho (2000) described other factors, such as media composition, plastic ware, and nutrient availability as causing DNA damage and limiting cell proliferation. Therefore, in this study, with old treatment cell lines established 3 weeks prior to the young treatment groups, it is possible that a slight difference in culture conditions could have been introduced on the day the young cells were processed, causing a higher percentage of senescent cells.

4.5 CONCLUSION

Biopsies taken from the proximal, middle and distal regions of young and old ngege caudal fins were used to establish primary cell cultures. Cell viability was greatly affected by biopsy location on the caudal fin, whereas oxidative stress, vacuolation and senescence were affected by donor age. No treatment groups produced viable and proliferative cells as observed in the AB.9 (ATCC® CRL-2298™) control cell line. Therefore, this study shows that tissue sampling can greatly affect the outcome of cell cultures. Further examination of cell signaling pathways is required to gain a better understanding of the relationship between oxidative stress, senescence and tissue sampling procedures.
CHAPTER FIVE

General Discussion
5.1 Major Findings

The global decline of freshwater fishes has prompted an interest in the banking of somatic cells as a method for preserving their genetic material. The limited information on the effects of fish tissue processing techniques on the quality (cell viability and proliferation) of fish cells following in vitro culture has driven the need for this study to be completed.

In Chapter 2, primary cell cultures from ngege caudal fins were established using 6 mm biopsy punches at 25°C using 1x Primocin in the culture medium to eliminate contamination. No differences existed in the number of cells produced between 6 mm and 8 mm biopsy punches or incubation at 25°C and 35°C. Therefore, the smaller hole punch size (6 mm) and the common incubation temperature for freshwater fish species (25°C) were selected as the most suitable option for initiating cell cultures.

In Chapter 3 primary cell cultures from ngege caudal fins were generated using protocols developed in Chapter 2, with the modification of collagenase digestion for 60 minutes at 37°C. It was found that TrypLE™ could significantly reduce viable cell concentrations more so than collagenase. In addition a 60 minute digestion time was able to release more viable cells from the biopsy than a 30 minute digestion time. Therefore, the most suitable condition for initiating viable cell cultures is tissue digestion with collagenase I for 60 minutes.

In Chapter 4, tissue samples from the middle and proximal sections of the caudal fin from both young and old ngege produced the most viable primary cell cultures. Although donor age did not affect cell viability, it may have influenced the occurrence of oxidative stress and cellular vacuolation in ngege cell cultures. Therefore, it is beneficial to consider age and biopsy location of the sample prior to cell culture of endangered fish species.

Although 60 minute collagenase digests in Chapter 3 and proximal fin biopsy punches in Chapter 4 produced the “healthiest” cell cultures in comparison to other treatment groups, there was still evidence of oxidative stress, premature cellular senescence and vacuolated cells. In particular, the percentages of oxidized and senescent cells containing vacuoles in Chapter 4 were
similar to the percentages observed in young fish cell cultures in Chapter 3. However, the same age and shoal of young fish used in Chapter 4 did not have vacuolation or oxidative stress, but maintained a high percentage of senescent cells under the same establishment procedures as young fish in Chapter 3 and old fish in Chapter 4. This variability in culture outcomes using the same establishment procedures suggests that culture conditions, such as media and additives, require further investigation.

5.2 Future Directions

The evidence presented in this thesis strongly suggests that current culture establishment techniques are not effective in creating viable and proliferative primary cell cultures required for somatic cell banking. Future studies are needed to examine the effects of anesthesia and media supplementation on culture quality. Morisset et al. (2004) showed that organic amines present in several drugs, such as Procainamide, can cause vacuolation of rabbit artery smooth muscle cells (SMCs) and COS-1 cells as a result of osmotic swelling in acidic organelles. In the current study, Ketamine, an anesthetic containing organic amines was injected intravenously into each ngege prior to fin removal for biopsy collection. Although a majority of ngege cell cultures were vacuolated, cell cultures grown from young ngege (still anesthetized with Ketamine) did not have vacuolation. Therefore, further examination of the relationship between anesthetic drugs, and viability of fish somatic cell cultures is required.

Growth factors and antioxidants, such as insulin and selenium, have been shown to prevent oxidative stress and increase cell proliferation, which may be required for ngege cell cultures in order to increase proliferation and reduce oxidative stress. A study by Mulvaney and Cyrino (1995) showed that media supplementation with the growth factors Insulin-like Growth Factor type 1 (IGF-1), Fibroblastic Growth Factor (FGF), and Transforming Growth Factor Beta (TGF-β), and use of collagen coated dishes, increased mitogenic activity and cell attachment in primary Channel Catfish cell cultures. In addition, a study by Baker et al. (1998) showed that the addition of 1 nM selenium is essential for producing viable cell cultures and eliminating oxidative stress in hepatoma-derived cell lines. They suggest that the addition of 10-20% serum containing selenium in culture medium is lower than physiological conditions in some species.
and needs to be added to certain cell lines. While these additives are expensive and generally not required for conventional fish cell culture (Bols and Lee, 1991), they have been proven to be effective in other species, and may be crucial in the development of ngege caudal fin cell cultures.

Once healthy cell lines can be produced, it is necessary to examine cryopreservation procedures to ensure cell survival and quality is maintained post-thaw. These preliminary studies would then be applied as proof of concept to one day produce successful SCNT clones. Research in the laboratory of Catherine Labbe using goldfish are examples of the future directions required for fish endangered species somatic cell banking. Labbe’s group was able to optimize protocols for culturing fin explant cell cultures (Mauger et al., 2006), optimize cryopreservation procedures using field procedures (Moritz and Labbe, 2008), and finally perform SCNT on cell cultures produced from those protocols (Le Bail et al., 2010). These studies were performed with the intention of fish somatic cell banking for conservation, as was this thesis. However, their studies focused on goldfish, a laboratory model species that had been previously shown to grow well in culture with standard procedures (Choresca Jr et al., 2009; Choresca Jr., 2012; Kondo and Watabe, 2006; McKenzie and Stephenson, 1970), whereas this study did not have any protocols to refer to. Even though we were unsuccessful at optimizing the conditions required for fish cell culture of caudal fin biopsies, the research by the Labbe laboratory shows that with further investigation, field scale sampling and the production of SCNT clones may be an effective means of conservation.

5.3 Conclusion

This thesis has developed a preliminary model for freshwater fish cell culture using ngege caudal fin biopsies. It was found that 6 mm biopsy punches from the proximal area of the caudal fin, digested for 60 minutes with collagenase, could produce cell cultures in medium containing 1x Primocin at 25°C. Although this procedure was unsuccessful at producing viable and proliferative cell cultures, it has provided valuable information regarding the effects of culture conditions and procedures on culture quality. No other study has examined cell culture parameters using endangered species biopsy punches as a model for field scale collection and
conservation, making this thesis unique. Further examination of culture conditions and cryopreservation protocols is required in the pursuit of developing healthy cell lines for SCNT and fish conservation.
REFERENCES


APPENDIX

Table A.1: Preliminary trials of antibiotics in wash and culture media, using modified procedures from Mauger et al. (2006) and Kwano et al. (2010). Test trials were performed in replicates of three using Tilapia purchased from the grocery store. +/- represent the presence or absence of contamination in cell culture. Protocols that produced contaminated cultures in some replicates but not in others received a + and – to represent the variability in contamination by that antibiotic. Cell growth is deemed low (<30%), moderate (31-60%) and high (60-100%) based on culture confluence, which was judged by observing the entire flask up to two a 2 week period using phase contrast microscopy. During this time some cultures became contaminated the next day of culture (1, 4) while others became contaminated after media changes (tests 2, 5 and 6).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Test</th>
<th>Modifications to the protocol</th>
<th>Contamination</th>
<th>Cell growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mauger et al., 2006</td>
<td>1</td>
<td>None</td>
<td>+</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>- HEPES removed</td>
<td>+/-</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- All L-15 wash solutions were replaced with HBSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>- HEPES removed</td>
<td>-</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- All L-15 wash solutions were replaced with HBSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Fins were rinsed in 70% ethanol for a few seconds prior to rinsing</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Gentamycin was replaced with 1% ABAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kwano et al., 2010</td>
<td>4</td>
<td>None (caudal fin not intestine)</td>
<td>+</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>- Caudal fin instead of intestine</td>
<td>+/-</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 30 min 0.2% collagenase in L-15 containing 1% P/S (instead of explants)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Added 1% ABAM to culture media</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>- Caudal fin instead of intestine</td>
<td>+/-</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 30 min 0.2% collagenase in L-15 containing 1% P/S (instead of explants)</td>
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<tr>
<td></td>
<td></td>
<td>- Added 2% ABAM to culture media</td>
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Figure A.1 Morphologies of A: C30, B: C60, C: T30, D: T60, E: AB.9 (ATCC® CRL-2298™) control cell line on day 3 and day 7, at 10x magnification. Apostrophe (’) denotes day 7. Cells appear flattened and vacuolated on day 7.