Growth of rainbow darter *(Etheostoma caeruleum)* in the Grand River watershed

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.
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Abstract

Assessment of growth is an important tool in fisheries assessment and management and provides valuable insight into biological responses to stressors in biomonitoring programs. A small-bodied fish species, rainbow darter (*Etheostoma caeruleum*), has been used as a sentinel species for numerous studies on the impacts of municipal wastewater effluent (MWWE) on fish health in the Grand River. The Waterloo wastewater treatment plant (WWTP) has undergone major infrastructure upgrades that were completed in 2017 and resulted in improved effluent quality. Previous studies have documented changes in a variety of endpoints in rainbow darter including condition and somatic indices such as gonadosomatic index (GSI) and liver somatic index (LSI), but limited research has occurred on population level endpoints such as growth. The objective of this study was to compare growth of rainbow darter upstream and downstream of the Waterloo WWTP before and after the WWTP was upgraded in 2017.

Fish were collected during the fall of 2014, 2018 and 2019 and fish length, weight, liver weight and gonad weight were recorded, and sagittal otoliths were extracted to age fish. Parameters from von Bertalanffy growth model and somatic indices were used to characterize growth differences between sexes and across sites and years. The back-calculation method was validated for rainbow darter in the Grand River, and the biologically modified Fraser-Lee equation was used to estimate past length for the 2014 fish population as an approach to increase sample sizes. Growth and size-at-age consistently differed between males and females, with male fish growing to a larger size at a faster rate than female fish across most sites and years. Fish growth was also faster at the two downstream sites relative to the upstream reference sites across years, but there was no consistent effect that could be associated with the WWTP, although fish growth and size-at-age were observed to increase post-upgrades. Although no significant differences in LSI and GSI between sites were evident, there was variation in LSI and GSI across years with slight increases in LSI and GSI post-upgrades.

This study has increased our understanding of fish growth in an urbanized watershed. The use of the von Bertalanffy growth model for rainbow darter is a reliable and accurate method to assess a more sensitive population level endpoint. The use of a back-calculation method to obtain past length-at-age is a powerful tool that can be utilized to understand rainbow darter growth in previous years when sample sizes are low. This study supports the incorporation of estimates of growth rate in biomonitoring programs with small bodied fish, such as rainbow darter, to detect subtle, population level impacts in aquatic ecosystems.

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Chapter 1
Introduction

Age and growth determination are important tools in fisheries assessment and management. Growth rates, mortality rates and productivity are all derived from age data and hence, age determination is vital to understanding individual and population level effects (Beamish & McFarlane, 1987). There are a variety of species specific methods that are employed for age determination, depending on the life history and physiology of the fish species in question (Campana, 2001). Age, in conjunction with other fish metrics (length/weight), are used in many biomonitoring programs including the Canadian Environmental Effects Monitoring (EEM) program for important endpoints, such as growth, for assessment of potential impacts of stressors and contaminants in the receiving aquatic environment (Gray et al., 2002; Munkittrick et al., 2002).

The Grand River receives various inputs from agricultural and urban sources, but municipal wastewater treatment plants (MWWTPs) continue to be one of the major sources of contamination in the watershed (Cooke, 2006). The Kitchener and Waterloo MWWTPs are the largest treatment plants in the watershed, and both have recently undergone major infrastructure and treatment process upgrades in 2013 and 2017, respectively (Bicudo et al., 2016). Impacts of municipal wastewater effluent (MWWE) on fish health have been studied extensively in the Grand River (Fuzzen et al., 2016; Hicks et al., 2017; Tetreault et al., 2011) and a small-bodied fish species, rainbow darter (*Etheostoma caeruleum*), have been used as a sentinel species in these studies. Prior to infrastructure upgrades, rainbow darter downstream of MWWTPs exhibited altered steroid production, changes in gonadal development and high rates of intersex, in addition to changes in condition (Tetreault et al., 2013, Fuzzen et al., 2016). In a tributary of the Grand River (Speed River), differences in fish growth, species distribution and abundance were noted downstream of the wastewater treatment plant in Guelph (Brown et al., 2011). Despite comprehensive research on this species in the Grand River, limited population level impacts have been explored, due in part to the difficulty of aging small bodied fish. Age determination not only reveals characteristics of an individual fish, but also provides valuable information about a given population's age structure and growth. A recent study has validated the method of estimating age of rainbow darter in the Grand River using sagittal otoliths (Crichton, 2016), which created an opportunity to use archived samples to explore effects of wastewater on fish growth.

1.1 Age Determination

To determine fish age, a variety of lethal and non-lethal aging methods are commonly used. One of the most convenient and widely used non-lethal approaches is the use of length-frequency histograms; these are often used for fast growing, younger fish (Campana, 2001). Although length-frequency histograms provide an overview of the population structure, they can be difficult to apply for short lived species as age classes may overlap/merge, making separation difficult (Gray et al., 2002). This is the case for rainbow darter, where most young-of-year (YOY) have merged into the general population by October and cannot be separated by the use of histograms (Figure A. 1). In addition, this method does not provide information regarding past size-at-age and/or growth rate. Other non-lethal approaches include use of structures such as scales and spines, but these have been questioned in the past due to considerable underestimation of age. While using scales does not require sacrificing the fish, it has been reported to be inaccurate. Age of rainbow darter in the Missouri River was underestimated using scales when compared to using otoliths (Beckman, 2002). This has been reported for other darter species as well as bluefish, lake whitefish and yellow perch among others (Muir et al., 2008; Robillard et al., 2009; Robillard & Marsden, 1996). Underestimation of age can be attributed in part to scale reabsorption during stressful periods of food shortage (Simkiss, 1974). In addition, scales do not always form distinct annuli, making it difficult to age these structures (Beamish & McFarlane, 1983).

Many calcified structures are used for estimating age of fish, including scales, otoliths, vertebrae, spines, pectoral fins, fin rays, opercular bones, dentary bones, and cleithra (Maceina et al., 2007). Of all these, the most commonly used lethal acquired structure is otoliths. Otoliths are paired ear bones located on either side of the brain that aid fish in detecting movement and orienting themselves (Quist et al., 2012). There are three pairs of otoliths: sagittal, lapilli and asterisci, of which sagittal otoliths are the largest and hence, preferred for aging (Brown et al., 2004). The use of otoliths for age determination is a lethal and time-consuming, but accurate because otoliths form during the embryonic stage and are not reabsorbed during times of stress (Popper et al., 2005). Opercular bones are also used for age determination, the accuracy of this aging structure varies among fish species. For example, immature lake trout age does not vary depending on the aging structure (scales, otolith, opercular bone) but for mature lake trout, scales significantly underestimate age whereas, opercular bone and otoliths ages are comparable (Sharp & Bernard, 1988). For other freshwater fish species, opercular bones can underestimate age when compared to otoliths (Ma et al., 2011). In general, opercular bones are a better alternative to scales, but overall otoliths are known to be a far more accurate and precise structure for age determination (Donald et al., 1992; Le Cren, 1947; Ma et al., 2011). Using calcified structures such as otoliths to age fish is therefore a powerful tool in fisheries assessment and management (Beckman, 2002).

All of these calcified structures display alternating opaque and translucent bands corresponding to daily, seasonal or annual growth cycles. This is much like dendrochronology, age determination of trees from the counting of rings that form annually (Black et al., 2005). Otoliths however, do not always form marks annually and hence, age validation within a system is required (Beamish & McFarlane, 1983). Previously, a study has looked at periodicity and timing of zone formation in rainbow darter in the Grand River using marginal increment and edge analysis. Results validate the method of aging rainbow darter using sagittal otoliths (Crichton, 2016). It was reported that the summer growth zone, which is an opaque zone under reflected light, is a white band that forms from April to July. The annulus, or the translucent zone under reflected light is narrower and darker than the opaque zone and forms during the winter months (Figure 1. 1). The summer growth zone (opaque) is a period of faster growth whereas the winter growth zone is a period of slower growth (translucent). Regardless of age or sex, an annulus starts to form in September for rainbow darter and this fall translucent ring can be used to age fish reliably (Crichton, 2016). While the exact cause is unknown, it is speculated that changes in water temperature, reproductive season and food availability might be implicated in triggering formation of a growth zone (Beckman & Wilson, 1995). For rainbow darter in the Grand River, onset of annulus formation did not correspond with decreases in water temperature in the fall (Crichton, 2016).

Otoliths can be prepared for analysis in various ways to allow for accurate reading and the methods are dependent on the shape and size of the otoliths. Whole otoliths can be used but sectioned otoliths are often preferred (Hoyer et al., 1985) as they provide clearer growth marks and make aging and growth measurements easier. Sectioned otoliths are often sanded down or broken to expose the nucleus and growth zones, allowing for a more accurate reading (Hoyer et al., 1985). For some species, both whole and sectioned otoliths are equally accurate (Gettel et al., 1997) so it may be more convenient to use whole otoliths. The accuracy of whole or sectioned otoliths being dependent on the species may be attributed in part to whether a given species is fast growing or slow growing as whole otoliths work better for slow growing species (Gettel et al., 1997). For rainbow darter, whole otoliths that are sanded down have been demonstrated to be ideal for age estimation (Beckman, 2002; Crichton, 2016).

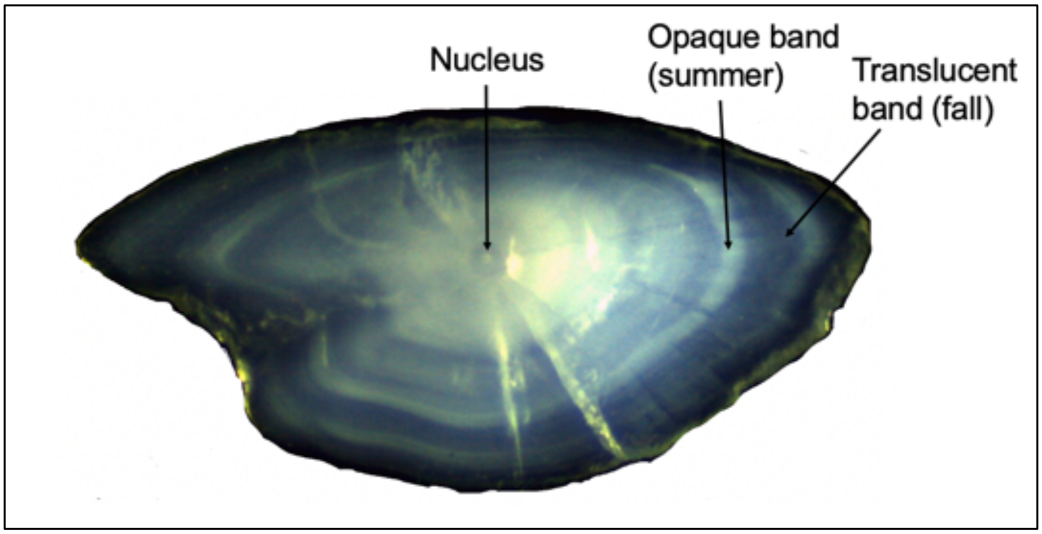

Figure 1. 1. A photograph of a rainbow darter sagittal otolith with its labelled growth zones.

1.2 Growth Determination

Growth is defined as a change in length or weight of an individual and growth rate is defined as change in size over time (Schreck & Moyle, 1990). Growth and growth rate is an important endpoint that aids in understanding life history and population dynamics of a fish species. It provides information about an individual, population and their surrounding environment (Kilgour et al., 2005; Munkittrick et al., 2009). Fish growth represents energy storage from food in an ecosystem and changes in growth can both represent adverse effects and have implications for survival and reproductive success (Fraker et al., 2002; Munkittrick et al., 2010). Therefore, accurate and reliable growth rate calculations allow for better management and conservation of fish populations.

Fish growth and growth rate are influenced by many factors such as water temperature, food availability and habitat quality (Soderberg, 2017). Greenside darter (*Etheostoma blennioides*) downstream of a tertiary treated treatment plant have been shown to have increased condition and length in a tributary of the Grand River (Brown et al., 2011). While it is difficult to pinpoint one factor in the natural environment, many explanations have been put forth. It is speculated that the warm wastewater effluent may stimulate metabolism leading to increased growth (Brown et al., 2011). Food availability also increases downstream of the WWTP due to the nutrients present in wastewater which may provide fish with extra energy for storage (Kilgour et al., 2005). It is possible that these factors work in combination to have cumulative effects on fish growth, growth rate and condition. Changes in the growth of rainbow darter associated with effluent outfalls may therefore be important indicators of fish health and environmental change.

Length-frequency analysis, growth models and back-calculation can all be used to determine growth rate (Drake et al., 2008; Le Cren., 1947). Length-frequency distributions can be used to estimate growth/growth rate by following the progression of age classes in the histograms over time. However, this method is not suitable for species such as rainbow darter where age classes overlap greatly. Growth models relate the age of fish to their length/weight and the resulting analysis produces an equation that describes growth (Jones, 2000). This equation has associated parameters that allow for comparisons of fish growth among populations as well as within populations (Quist et al., 2003). The three most commonly used growth models are the Gompertz, logistic and the von Bertalanffy growth model (Quist et al., 2012). The Gompertz growth model is sigmoid shaped and is often used for larval and juvenile fish growth (Campana & Jones, 1992). It is ideal for fish species that exhibit slow growth rate initially; the common form of the equation is

$$L_t = L_{inf}\,(e^{-e^{[-G(t-t_o)]}}) \qquad \text{Equation 1}$$

where L_t is the length at time t, L_{inf} is the asymptotic length, G is the instantaneous growth rate at t_o and t_o is the inflection point on the S-shaped curve where growth rate begins to decline (Quist et al., 2012). A very similar growth model is the logistic growth model that has the following form

$$L_t = L_{inf}\,/[1+ e^{-G(t-to)}] \qquad \text{Equation 2}$$

where G is the instantaneous growth rate at the origin of the curve and all other parameters are as above (Equation 1). Often, the resulting curves of both of these models look very similar but a key difference is that growth above and below the inflection point is symmetrical for the logistic model but not for the Gompertz model (Quist et al., 2012). The most popular growth model, however, is the von Bertalanffy growth model that has been widely used in fisheries science for over 100 years (Haddon, 2011). A common form of the equation is

$$L_t = L_{inf} \, (1 - e^{-k(t - t_o)}) \qquad \text{Equation 3}$$

where L_t is the length at time t, L_{inf} is the asymptotic length, k is the growth coefficient and t_0 is time when length is zero (von Bertalanffy, 1938). The Brody growth coefficient represents how fast asymptotic size is attained and L_{inf} is not the maximum length for an organism but rather the asymptote for average length-at-age for the model (Francis, 1988). t_o, despite being defined as the time where fish length is zero has no biological basis and is simply an extrapolated artificial modeling artifact (Francis, 1988). Hence, only the two parameters L_{inf} and k can be compared to detect any differences in growth between populations. Choosing a suitable growth model for the data is important for correctly characterizing fish growth. The Gompertz and logistic growth models are not suitable for rainbow darter because initial growth is fast, and the data does not mimic a sigmoid shape. The von Bertalanffy growth model is appropriate for rainbow darter and has been successfully used to compare growth between sexes in the Grand River (Crichton, 2016). The von Bertalanffy growth model revealed that both sexes grew similarly for the first two years of their life after which they tend to deviate (Figure 1. 2) where male fish continue to increase in length and weight while female rainbow darter showed little growth as they presumably invest their energy into gonad development (Crichton, 2016). This study was conducted at a reference site and relative growth of rainbow darter population at an effluent exposed site is unknown. However, there does appear to be an increase in condition at sites downstream of wastewater outfalls in the Grand River, so it is possible that there are differences in growth between upstream and downstream sites (Fuzzen et al., 2016; Tetreault et al., 2011).

However, small sample sizes of fish collected historically present a challenge for adequate characterization of fish growth in previous years.

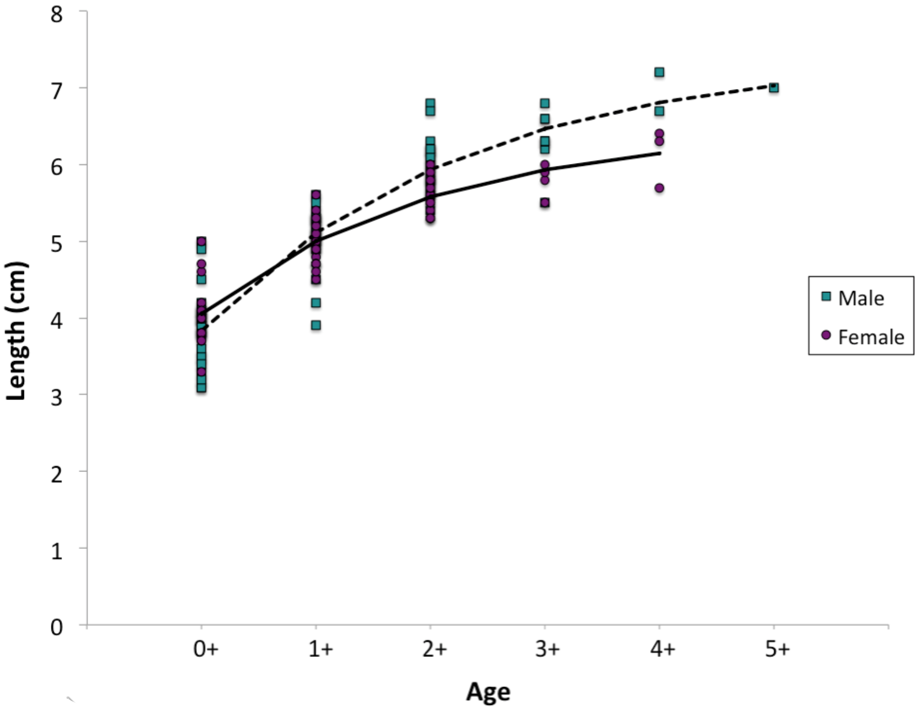

Figure 1. 2. Estimated von Bertalanffy growth curves for male and female rainbow darter collected in the fall of 2014 at a reference site upstream of the WWTP in the Grand River (From Crichton, 2016).

1.3 Back-calculation of fish length

Back-calculation is a technique that is based on the relationship between fish length and radius of a given hard structure, and it is used to obtain past length-at-age (Ricker, 1992). This technique requires measuring the radius of an otolith/aging structure as well as the distance between every annulus and the nucleus; this distance is then related to fish length (Campana & Jones, 1992). After measurements are obtained, a variety of methods are utilized to calculate past length-at-age. The two types of methods extensively discussed in a review by Francis (1990) are direct regression and proportional methods. Direct regression techniques are strongly advised against, as they ignore variation in size among individuals and tend to overestimate past length-at-age (Francis, 1990). Instead, proportional methods that assume a proportional relationship between otolith and fish length are recommended (Ricker., 1992; Francis, 1990). Proportional methods such as the Fraser-Lee and Dahl-Lea can be used to derive past length-at-age. The Dahl-lea method is used when the relationship between otolith radius and fish length is linear and passes through the origin (i.e. otolith growth is directly proportional to body growth) (Francis, 1990). Fish length at previous ages of an individual can then be calculated as

$$L_i = L_c\ (S_i/S_c) \qquad \text{Equation 4}$$

where L_i is the past length of an individual when *i*th increment was formed, S_i is the radius of otolith when *i*th increment was formed, L_c is the length of the individual at capture, and S_c is the radius of the otolith at capture (Ricker, 1992). This method is commonly applied when fin rays, spines or otoliths are being used (Schramm et al., 1992). It was observed that the relationship between radius length and fish length rarely passed through the origin for many species. The Fraser-Lee method was developed to account for growth of these fish species; past length-at-age for this method can be calculated with

$$L_i = ((L_c - a)/S_c)*S_i + a \qquad \text{Equation 5}$$

where *a* is the intercept the line passes through and all other parameters are as defined above. Many interpretations of *a* have been proposed and debated over the years but often the *a* value that gives the best estimate of L_i is used (Campana & Jones, 1992; Schreck & Moyle, 1990). A standardized *a* value can be found for common fish species but unfortunately, such values do not exist for rainbow darter. Since this value does not have any biological meaning, a biologically modified equation has been developed by Campana (1990).

$$L_i = L_c + [(S_i - S_c)(L_c - L_o)]/(S_c - S_o) \qquad \text{Equation 6}$$

L_o refers to length of fish and S_o is the size of otolith at the biological intercept (Campana, 1990; Campana & Jones, 1992). Often, the smallest individual fish in a given sample is used for setting the biological intercept. There are many variations of these equations and more than 20 back-calculation models are described thoroughly in Vigliola and Meekan (2009) and the appropriate model has to be determined for the fish species and study design in question. While past length-at-age could be calculated for various purposes, it is most often used to increase length-at-age data for computing growth curves (Vigliola & Meekan, 2009). Regardless of the model chosen or the purpose of back-calculation, relationships between otolith radius and fish size (length/weight) must be tested to ensure proportionality.

1.4 The Grand River watershed

The Grand River watershed is the largest watershed in southern Ontario that drains into Lake Erie. Surrounding areas are dominated by agriculture and urbanization and the river receives contaminants from these sources, leading to poor water quality in some sections of the river (Cooke, 2006). This is apparent in the central Grand River, where inputs from agricultural tributaries (e.g. Conestogo river) and urban areas add substantial amounts of ammonia, nitrate, phosphorous and chloride, degrading water quality considerably (Cooke, 2006; Srikanthan, 2019). Currently, there are approximately a million human inhabitants in the watershed, and this is predicted to increase significantly over time, adding to water quality concerns (Grand River Management Plan, 2014).

There are a total of 30 WWTPs discharging into the Grand River, which raises concerns regarding habitat quality for aquatic organisms (Grand River Management Plan, 2014). The Kitchener MWWTP and Waterloo MWWTP are the two largest wastewater treatment plants that discharge into the Grand River, and both have recently undergone major infrastructure and process upgrades to improve effluent quality in 2012 and 2017, respectively (Bicudo et al., 2016). Impacts of wastewater on fish health have been documented across different levels of biological organization (e.g, gene expression, histology, physiology, somatic indices) in response to wastewater effluent exposure (Tetreault et al., 2013; Hicks et al., 2017b; Fuzzen et al., 2016). Fish condition was seen increase downstream of the WWTPs, but this is not observed consistently across seasons and years (Tetreault et al., 2011, Fuzzen et al., 2016). Furthermore, contaminants of emerging concern (CECs), such as pharmaceuticals and endogenous hormones, have been reported to be associated with feminization of male fish (intersex) in the Grand River (Fuzzen et al., 2016; Hicks et al., 2017a). Several of these responses, including intersex and impacts on steroid production in rainbow darter, have improved in response to treatment upgrades (Marjan et al., 2018; Hicks et al., 2017b). Potential growth differences have not been considered previously and this population level endpoint can be an important indicator of subtle impacts of wastewater effluent on fish health.

1.5 Biomonitoring programs

Fish growth is an important endpoint that provides valuable insight into individual and population dynamics. Size-at-age is used in many biomonitoring programs including the Canadian Environmental Effects Monitoring (EEM) program, as an indication of energy utilization and storage (Munkittrick et al., 2009). The EEM program is an environmental monitoring framework developed by Environment Canada to determine whether metal mines and paper and pulp mills that meet effluent discharge regulations still have remaining effects on organisms in the receiving environment (Kilgour et al., 2005; Munkittrick et al., 2002). This framework has been continuously modified over the years with gonadosomatic index (GSI), liver somatic index (LSI), size-at-age and condition all being incorporated to better assess impacts of effluent on fish health (Munkittrick et al., 2002). Growth is the change in length or weight, whereas condition is the ratio of weight to length or how fat the fish is. Both parameters provide valuable information including indications of food availability and habitat quality and can be used to understand how fish store and use energy from their food sources (Gray et al., 2002; Kilgour et al., 2005). Differences of 25% or greater in age and growth of fish (size-at-age) from exposed sites in comparison to reference sites are considered to be ecologically relevant and should trigger additional monitoring (Kilgour et al., 2005; Munkittrick et al., 2009). A difference of only 10% for condition is usually used as an indicator of an important effect (Kilgour et al., 2005; Munkittrick et al., 2009). While condition can be an important and quick method to utilize, it is not very informative of potential changes in patterns of growth throughout the life of fish. Condition factor can also change with age and therefore confound the interpretation. Therefore, age and growth (i.e. size-at-age) are vital in these frameworks to better monitor potential impacts in response to stressors in the environment. The EEM supports the use of small bodied sentinel species in monitoring programs for various reasons such as low mobility, increased sensitivity and ease of capture. As Crichton (2016) demonstrated the ability to age rainbow darter samples, this has allowed for additional metrics to be used to support greater understanding of impacts of wastewater effluent on fish health in the Grand River.

1.6 Rainbow darter as a sentinel species

Rainbow darter (*Etheostoma caeruleum*) are a small bodied benthic fish species in the family Percidae (Ray et al., 2006). They are mostly found in streams and rivers in eastern North America, with large numbers found in tributaries of the Great Lakes and the Ohio River valley (Ray, et al., 2006). Rainbow darter prefer fast-moving shallow riffles with gravel or rocky substrates and have a small home range (~5m) often confined to one riffle (Hicks & Servos, 2017; Harding et al., 1988). As a sexually dimorphic species, male and female rainbow darter have different coloring and size with male fish displaying shades of red, orange and blue and growing to a larger size while female fish are sand coloured and smaller in comparison. Fish spawn multiple times during the reproductive season between April and June every year where colourful and larger sized male have higher chances of reproductive success (Fuller., 2003). Rainbow darter are mainly insectivores and their diet includes caddisflies, aquatic larvae and fish eggs changing seasonally with water temperature (Adamson & Wissing, 1977). All of these characteristics (small home range, abundant, etc.) make rainbow darter an excellent model species to study the impacts of MWWE on fish health. The nutrient rich, warmer effluent can change available food types and abundances in a system, in addition to being a source of contaminants. Therefore, MWWE can inhibit or increase fish growth depending on the effects of a given stressor or a combination of multiple ones interacting to bring about changes in fish growth. This has implications for fish growth at the downstream sites and considering the massive cost of treatment infrastructure, it is increasingly important to develop and utilize methods to detect impacts of wastewater effluent on key aspects of fish health, aiding in management decisions and potential investment in treatment upgrades.

The objectives of this study were to:

1. Compare and characterize growth rate (growth coefficient (k), maximum length (L_{inf})) of rainbow darter upstream and downstream of the Waterloo wastewater treatment plant pre- and post-upgrades (2014, 2018 and 2019);
2. Validate the biologically modified Fraser-Lee back-calculation method and use it to obtain past length-at-age of rainbow darter at a downstream site to assess fish growth pre-upgrades (2014);
3. Characterize and evaluate relationships between age and somatic indices (length, weight, condition, GSI, LSI), and,
4. Characterize any differences between abiotic conditions (temperature, discharge, etc.) of sites and relate it to qualitatively to any changes in fish growth upstream and downstream of the Waterloo WWTP.

A minimum of 43 male and 25 female rainbow darters of all sizes were collected upstream and downstream of the WWTP in the fall of 2018 and 2019 for age determination; 43 male and 25 female fish were deemed necessary by a power analysis to detect a 25% difference in growth rate as recommended in the EEM program. Archived samples from 2014 include 22 male and 15 female fish at the downstream (DSW) site requiring the use of back-calculation method to increase sample sizes to estimate past length-at-age. Data from a reference site (Ref 2) in 2014 is from Crichton (2016) and comprises 145 individuals (84 male and 61 female). Total length and total weight were recorded for all fish, but gonad and liver weight were measured for only a subsample of fish collected (25 male and 20 female). Use of archived samples and additional post-upgrades sampling created an opportunity to explore how effluent quality can alter fish responses, including growth. Otoliths were extracted from pre- and post-upgrade years and aged to compute von Bertalanffy growth curves in conjunction with the biologically modified Fraser-Lee back-calculation model to investigate the effects of MWWE on rainbow darter growth.

Chapter 2
Growth of rainbow darter (*Etheostoma caeruleum*) in the central Grand River

2.1 Introduction

Age and growth both provide unique information about individuals and the population of a given fish species. Age provides insight on characteristics of individual fish and age structure of the population, in addition to mortality rates and recruitment dynamics (Buktenica et al., 2007). Growth augments this information, integrates environmental conditions and genetic factors and reflects characterization of individual fish and populations (Quist et al., 2012). While both endpoints provide unique and valuable insight for the ecology and management of fishes, a combination of the two provides a more holistic assessment of fish populations (Buktenica et al., 2007). Age determination is critical for computing the von Bertalanffy growth curves with age and length data plotted for each population. This model is widely used in fisheries management and remains a high priority for fisheries scientists because age and growth information apply to most aspects of fisheries science including population vital rates (Quist et al., 2012). However, little work has been done comparing fish populations in reference to stressors and contaminants in the natural environment using this model in the Grand River. Crichton (2016) validated the method of aging rainbow darter using sagittal otoliths using the marginal increment analysis and edge analysis. In addition, the von Bertalanffy growth model was shown to be a good fit for this species and differences in growth rate between male and female fish were characterized at a reference site (Crichton, 2016). The use of back-calculation as a technique to estimate past size-at-age was recommended by Crichton (2016) to increase sample sizes, but this has not been explored for rainbow darter and the method needs to be validated to be used for analysis.

There are many methods to estimate past length-at-age, but the Fraser-Lee method is ideal for species with otolith radius and fish length relationship that does not pass through the origin. The biologically modified Fraser-Lee method is preferred as the values are biologically derived and a complete proportionality between otolith and body size is not required (Campana, 1990). However, the use of this method and its applicability for darter species had not been investigated.

Age and growth are important in the frameworks of monitoring programs for assessing potential impacts of contaminants on fish health. To study the impacts of effluent from pulp and paper mills and metal mining, Environment Canada developed The Environmental Effects Monitoring (EEM) Program wherein it identified growth as an important endpoint along with condition, liver size and reproductive endpoints (Environment Canada, 2010). The use of small-bodied fish like rainbow darter are often preferred for their small home range, high abundance and easy capture using the backpack electrofishing method (Munkittrick et al., 2002).

The small-bodied fish species, rainbow darter (*Etheostoma caeruleum*), are widely distributed in the Grand River and have been used as a sentinel species for understanding impacts of WWTP effluent on various metrics of fish health (Fuzzen et al., 2016; Hicks et al., 2017). These studies have demonstrated impacts of MWWE on almost every level of biological organization ranging from decreased sex steroid production to high incidence of intersex in male fish, to changes in community assemblages (Marjan et al., 2018; Hicks et al., 2017; Tetreault et al., 2012). However, a lack of endpoints related to age and growth in most of these studies have left a considerable gap in our understanding of impacts of MWWE on fish health. The addition of nutrient-rich, warmer effluent to the river can impact fish growth (McMaster et al., 2005) and a recent study has validated a method of evaluating rainbow darter growth in the Grand River allowing for assessing potential population level impacts in this system (Crichton, 2016). The objectives of this study were to a) characterize and compare growth rate of rainbow darter from 2014 (pre-upgrades), 2018 and 2019 (post-upgrades) at multiple sites in the Grand River in reference to the Waterloo WWTP, b) to compare growth rate and size-at-age relationships of rainbow darter between the pre- and post-upgrade years and c) to validate the use of the biologically modified back-calculation method to estimate past length-at-age of rainbow darter in the Grand River. This study will also validate the use of von Bertalanffy growth curves to evaluate impacts of wastewater effluent and other contaminants on fish growth in the Grand river and other aquatic ecosystems.

2.2 Materials and Methods

2.2.1 Study sites

The sampling sites for this study were selected to be consistent with previous studies conducted on the Grand River as part of a long-term effort to assess the effects of wastewater in the Grand River (Tetreault et al., 2011; Fuzzen et al., 2016; Hicks et al., 2017). The study sites are located in the upper portion of the Grand River watershed and represent a gradient from an intensive agricultural landscape to a highly urbanized area moving downstream. The three sites are located upstream of the Waterloo Wastewater Treatment Plant (WWTP) and one is located one km downstream of WWTP effluent outfall. The two most upstream sites (Ref 1 and Ref 2) are located outside the City of Waterloo in a highly agricultural environment while reference site (Ref 3) is located 5 km above the WWTP outfall in an urban area (Figure 2. 1). The Reference 3 site is located downstream of the confluence of the Conestogo River which is a nutrient rich tributary of the Grand River. Substrate type consists mainly of gravel and cobble at the two upstream (Ref 1, 2) and downstream (DSW) sites, whereas Reference 3 consists of rock and silt (Tetreault et al., 2013).

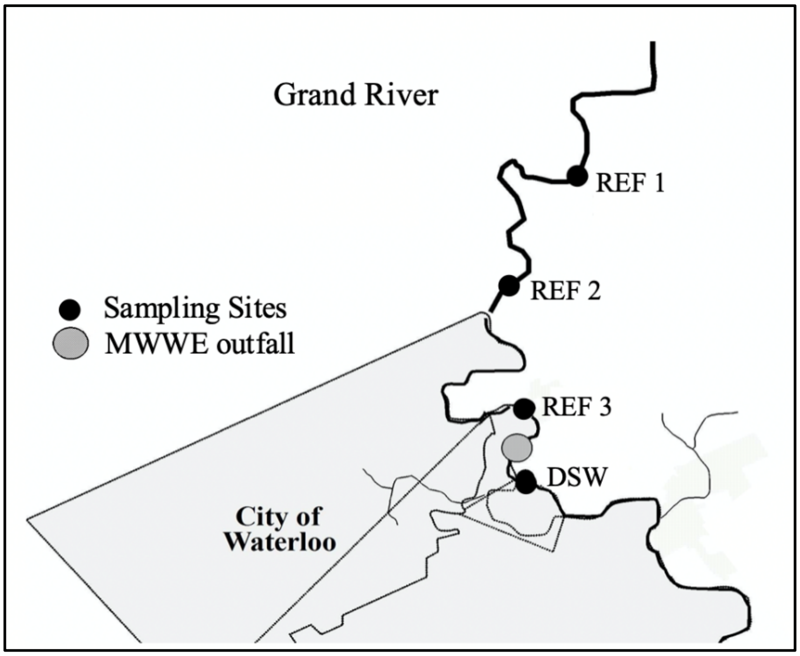

Figure 2. 1. Map of the sampling sites along the Grand River, Ontario in relation to the Waterloo wastewater treatment plant. Adapted and modified from Fuzzen et al., 2016.

Data loggers were deployed at four sites in the summer of 2019 to record water temperature and specific conductivity. TidbiT v2 temperature loggers (onset HOBO) recorded hourly water temperature, from beginning of June to mid-November at each site. HOBO conductivity loggers were also utilized during this time period to better understand effluent movement and water quality at the different sites. A total of twelve temperature loggers and three HOBO conductivity loggers were positioned at the downstream (DSW) site across the width of the river and going downstream to characterize the site. Two temperature loggers and one conductivity logger were deployed at each of the three reference sites. Temperature and conductivity were similar across these loggers and were averaged for each site for data analysis and plotted (Figure 2. 4 and Figure 2. 5). Data where loggers appeared to be out of water were manually removed. In addition, selected nutrients (ammonia, nitrite, nitrate) were measured as part of the water survey that accompanies fish sampling and was conducted by Maxxam Labs (Waterloo, Ontario). Average flow data for the Grand River at West Montrose (Reference 2, station 02GA034) were downloaded from the Environment and Climate Change Canada Historical Hydrometric dataset on March 19, 2020 (Water Survey of Canada 2018). River flow for Bridgeport (DSW, station 68) were downloaded from the GRCA monitoring data, available under the Grand River Conservation Authority's Data License v2.0 on January 5, 2020 (Grand River Conservation Authority, 2020).

2.2.2 Fish collection

Many species of fish including several darter species, such as greenside darter (*Etheostoma blenniodes*) and fantail darter (*Etheostoma flabellare*), are found in this section of the river (Hicks et al., 2017). Rainbow darter (*Etheostoma caeruleum*) are one of the most abundant fish species captured consistently across the sites using backpack electrofishing (Tetreault et al. 2013). Rainbow darter were collected in the fall at all the sites in all years using backpack electrofishing (Smith Root LR-24) and dip nets. Fish total length (± 0.1 cm) and weight (± 0.001 g) were measured before fish were euthanized by a blow to the head and spinal severance. Liver weight (± 0.001 g) and gonad weight (± 0.001 g) were measured and liver somatic index (LSI), gonad somatic index (GSI) and Fulton's condition factor (K) were calculated using the following equations:

$$\text{LSI} = (\text{liver weight (g)} / \text{body weight (g)}) \times 100 \qquad \text{(Equation 7)}$$

$$\text{GSI} = (\text{gonad weight (g)} / \text{body weight (g)}) \times 100 \qquad \text{(Equation 8)}$$

$$K = \text{body weight (g)} / (\text{total length (cm)})^3 \times 100 \qquad \text{(Equation 9)}$$

Fish bodies were individually placed in a labelled Whirlpack bag and transported to the laboratory on ice where they were frozen at -20º C until further analysis. All fish handling protocols were approved by the University of Waterloo Animal Care Committee (AUPP # 40318 and 40315).

2.2.3 Otolith preparation

Otoliths were extracted, mounted and sanded in order to be photographed for age determination. A cut from the mouth through the braincase was made and brain tissue was removed to expose the sagittal otoliths located laterally on either side. They were cleaned with Milli-Q water and placed in labelled wax paper envelopes. Otoliths were mounted onto microscope slides using Crystal Bond with their sulcus on the dorsal surface. To expose the nucleus and annuli, otoliths were sanded using various grits of fine sandpaper (1500-12000). An image of the slide was taken using a Leica S6D dissecting microscope attached to a camera after being flooded with water for 10 minutes. Otolith length from tip to the growing edge was measured using the Leica Microscope software (LAS-EZ) and all other measurements (radial distances) were made using Fiji software (Schindelin et al., 2012). Each otolith was aged twice on separate occasions by the same reader and any discrepancy was resolved with a third reading. A subsample of otoliths from Reference 2 site from 2014 and 2018 were weighed to five decimal places using the XP205 DeltaRange® scale (Mettler Toledo). Aging and otolith preparation from fish collected in 2014 at the Reference 2 site were completed by Alexandra Crichton and data are reported in Crichton (2016).

2.2.4 Back-calculation measurements

A series of measurements were recorded for calculation of past length-at-age for fish collected in 2015, 2018 and 2019. Otolith radius and the distance between the nucleus and each annulus was measured on two separate occasions by the same reader (Figure 2. 2). These measurements were used to calculate past length using the biologically modified Fraser-Lee and Dahl-Lea methods. Back-calculated length and observed length and their associated growth curves were compared using analysis of residual sum of squares at both a reference (Ref 2) and downstream site to determine the accuracy of the biologically modified Fraser-Lee method. The biological intercept was set using the smallest aged fish from both sites (Ref 2, DSW) in 2019 with fish length and otolith radius set as 2.3 cm and 0.27 cm, respectively. A total of 28 rainbow darter (14 male and 14 female) otoliths from 2015 at the downstream (DSW) site were used to back-calculate length-at-age in 2014. Fish length and otolith radius at the biological intercept for 2015 fish was set to 4.4 cm and 0.38 cm, respectively. This technique was then utilized to obtain past length-at-age for fish in 2014 at the downstream (DSW) site from fish collected in 2015.

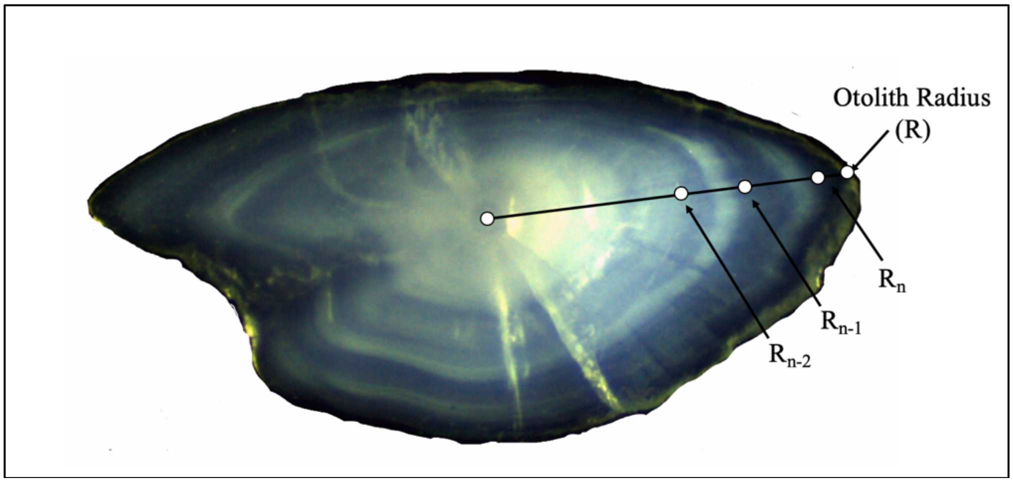

Figure 2. 2. A photograph of an otolith indicating measurements taken for back-calculation. R_n refers to the most recently formed translucent band (annulus).

2.2.5 Statistics

von Bertalanffy growth curves were computed using the FSA package (R Core Team, 2020; Ogle et al., 2020). The von Bertalanffy growth curves and parameters for sexes, sites and years were compared using the analysis of residual sum of squares (ARSS) and likelihood ratio tests following procedures outlined in Ogle (2016), Chen et al. (1991) and Kimura (1980). Residual sum of squares (RSS) with its associated degrees of freedom (DF) were calculated in R for every growth curve computed (pooled and individual growth curves of groups being compared). The F-statistic was then calculated as

$$RSS = \frac{\frac{(RSSp - RSSs)}{DFp - DFs}}{\frac{RSSs}{DFs}} \qquad \text{(Equation 10)}$$

where RSS_p = RSS of pooled growth curve, RSS_S = sum of individual RSS for groups being compared and DF_p and DF_s being degrees of freedom associated with RSS_p and RSS_s, respectively. All ARSS comparisons were made at a significance (α) value of 0.05. For growth curves that significantly differed, likelihood ratio tests compared their associated parameters to discern how growth differs among groups. In addition, two-way ANOVAs were used to assess differences between sites in mean length, weight, condition factor, LSI and GSI on sex-separated data. Separate two-way ANOVAs to assess differences among years were conducted for each somatic index and revealed no differences between the two post-upgrades years. Therefore, 2018 and 2019 data were pooled, and analyses were conducted for pooled and 2014 data corresponding to post- and pre-upgrade years, respectively. All pairwise comparisons used Tukey's post-hoc tests with an alpha value of 0.05. Linear regressions were performed for all length and weight relationships including otolith length and weight. All analyses were conducted in R version 3.5.2 with the exception of residual sum of squares which were carried out manually in an Excel spreadsheet. FSA, tidyverse and fishmethods packages were used for computation of von Bertalanffy growth models, likelihood ratio tests and graphs.

2.3 Results

2.3.1 Water quality

Numerous parameters were used to characterize sites and assess potential differences between sites in terms of water quality. Selected nutrients (nitrite, nitrate, ammonia) were measured in river water at the four sites pre- and post-upgrades (Figure 2. 3, Table 1). These were selected as they have been shown to effect fish growth and survival (Kilgour et al., 2005). Increased concentrations of ammonia and nitrite were observed in 2014 at the downstream (DSW) site but there were minimal differences among the four sites in 2018 and 2019 (Figure 2.3). Nitrate concentration is elevated at the downstream (DSW) site in 2014 but this difference is more pronounced post-upgrades in 2018 and 2019 (Figure 2. 3). There were also differences in other water quality parameters including chloride and conductivity with consistently higher conductivity and chloride concentrations at the downstream (DSW) site both pre- and post-upgrades (Table 1). Temperature and conductivity data loggers revealed differences between the four sites during the summer and fall of 2019. Water temperatures ranged from 10° C in June to 0° C in November when data loggers were removed. The Reference 3 site and downstream (DSW) site were consistently warmer than the two upstream reference sites (Figure 2. 4). The most upstream site, Reference 1, is downstream of the Shand Dam which releases cold water and explains the consistently lower temperatures compared to all other sites. Furthermore, specific conductivity (µS/cm) values were higher for the downstream (DSW) site compared to all of the upstream reference sites (Figure 2. 5). This difference was more pronounced pre-upgrades to the WWTP (Table 1, Hicks, 2017). River flow during the three years at the Reference 2 and Downstream (DSW) sites indicated higher flows at the downstream (DSW) site (Figure 2. 6). This is expected as the river receives considerable input from a major tributary of the Grand River (Conestogo River) upstream of the Reference 3 site.

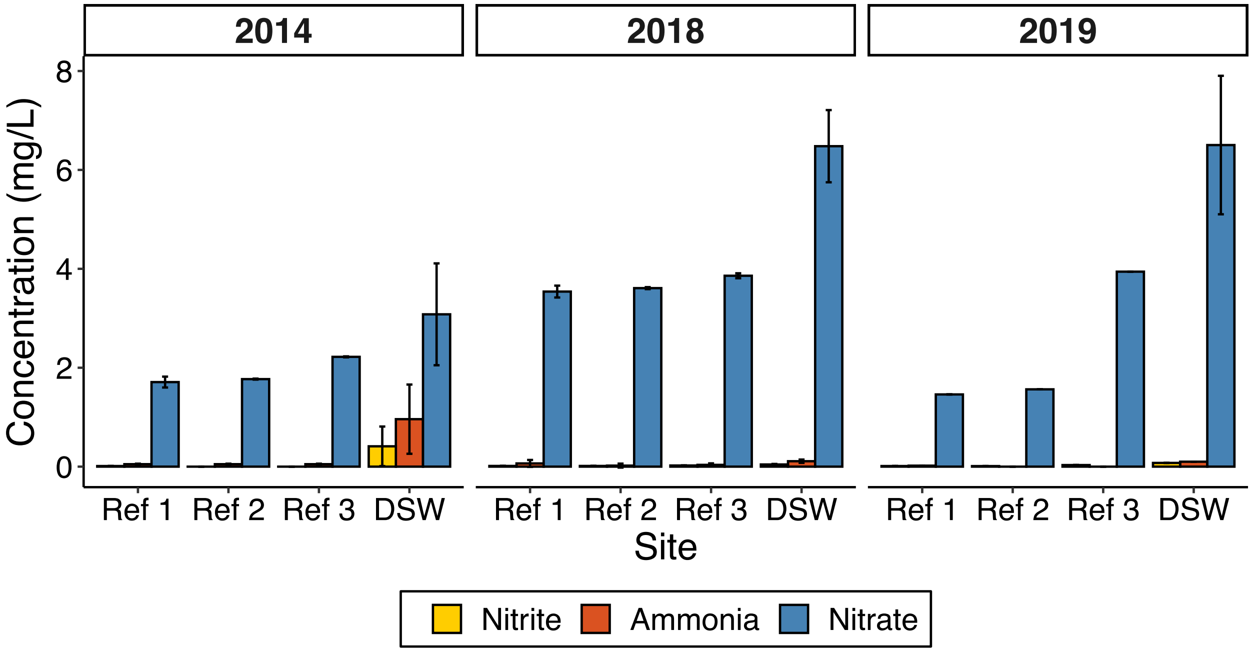

Figure 2. 3. Concentration of selected nutrients (mean ± SD) as measured in river water at the four sites (Ref 1, Ref 2, Ref 3, DSW) during the fall of 2014, 2018 and 2019.

Table 1. Additional water quality parameters at the four sites (Ref 1, Ref 2, Ref 3 and DSW) from fall sampling in 2014, 2018 and 2019. Mean concentration of dissolved chloride is in mg/L (± SD) and conductivity was measured in µmho/cm (± SD).

Year	**Parameter**	**Ref 1**	**Ref 2**	**Ref 3**	**DSW**
2014	Chloride	27 ± 0.6	28 ± 0.6	33 ± 0.6	73 ± 26.5
	Conductivity	593 ± 5.8	597 ± 5.8	660 ± 0.1	807 ± 106
2018	Chloride	31 ± 0.6	31 ± 0.6	36 ± 0.6	105.7 ± 16.9
	Conductivity	513 ± 5.8	513 ± 5.8	563 ± 5.8	793.3 ± 56.8
2019	Chloride	27.9 ± 2.3	27.7 ± 0.5	54.0 ± 32.9	77.7 ± 17.1
	Conductivity	535.5 ± 7.5	533.5 ± 3.5	629.5 ± 5.1	821.6 ± 70.2

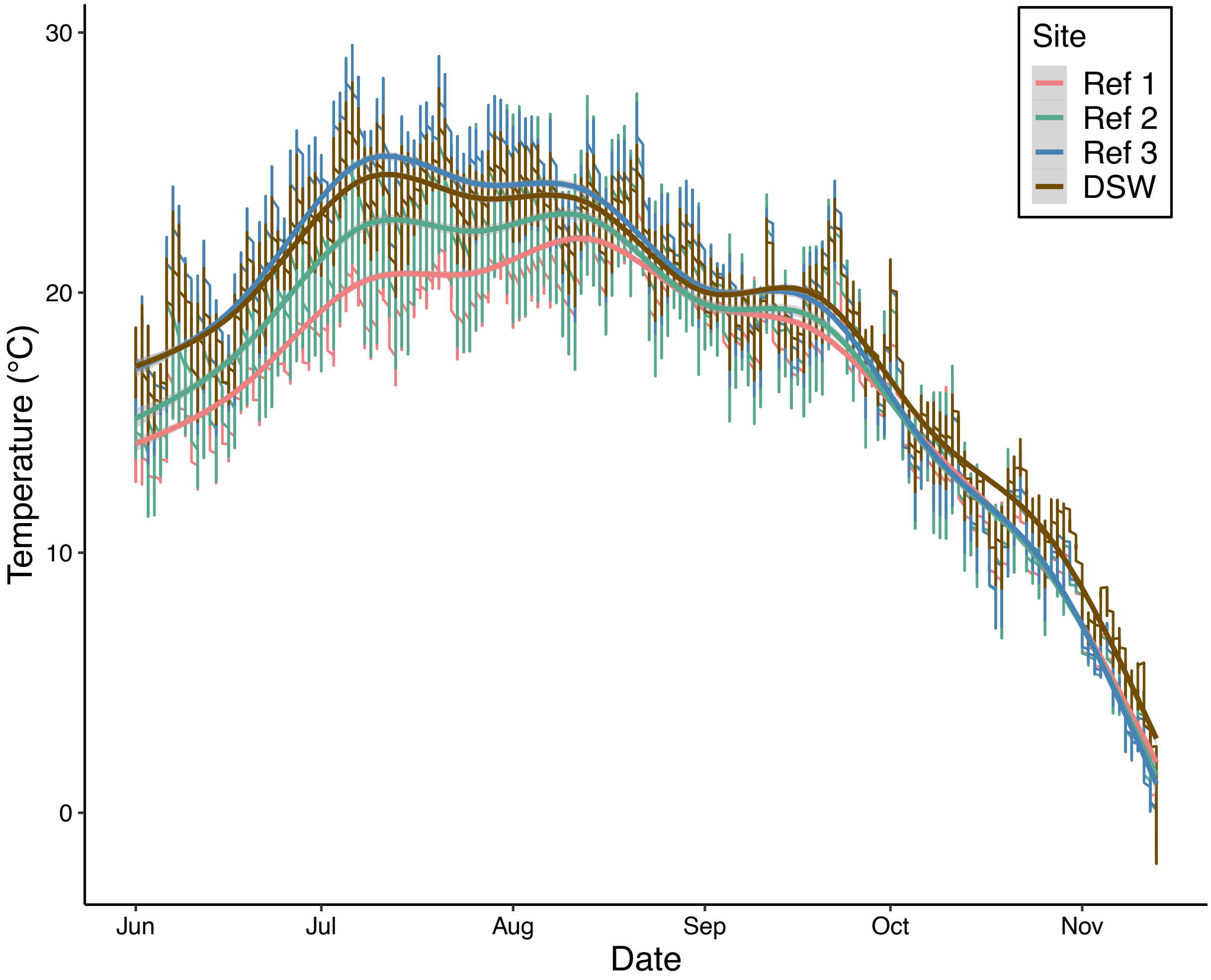

Figure 2. 4. Water temperature profiles of the four sites (Ref 1, Ref 2, Ref 3, DSW) between June and mid-November 2019 indicates higher temperatures at the Reference 3 and DSW sites.

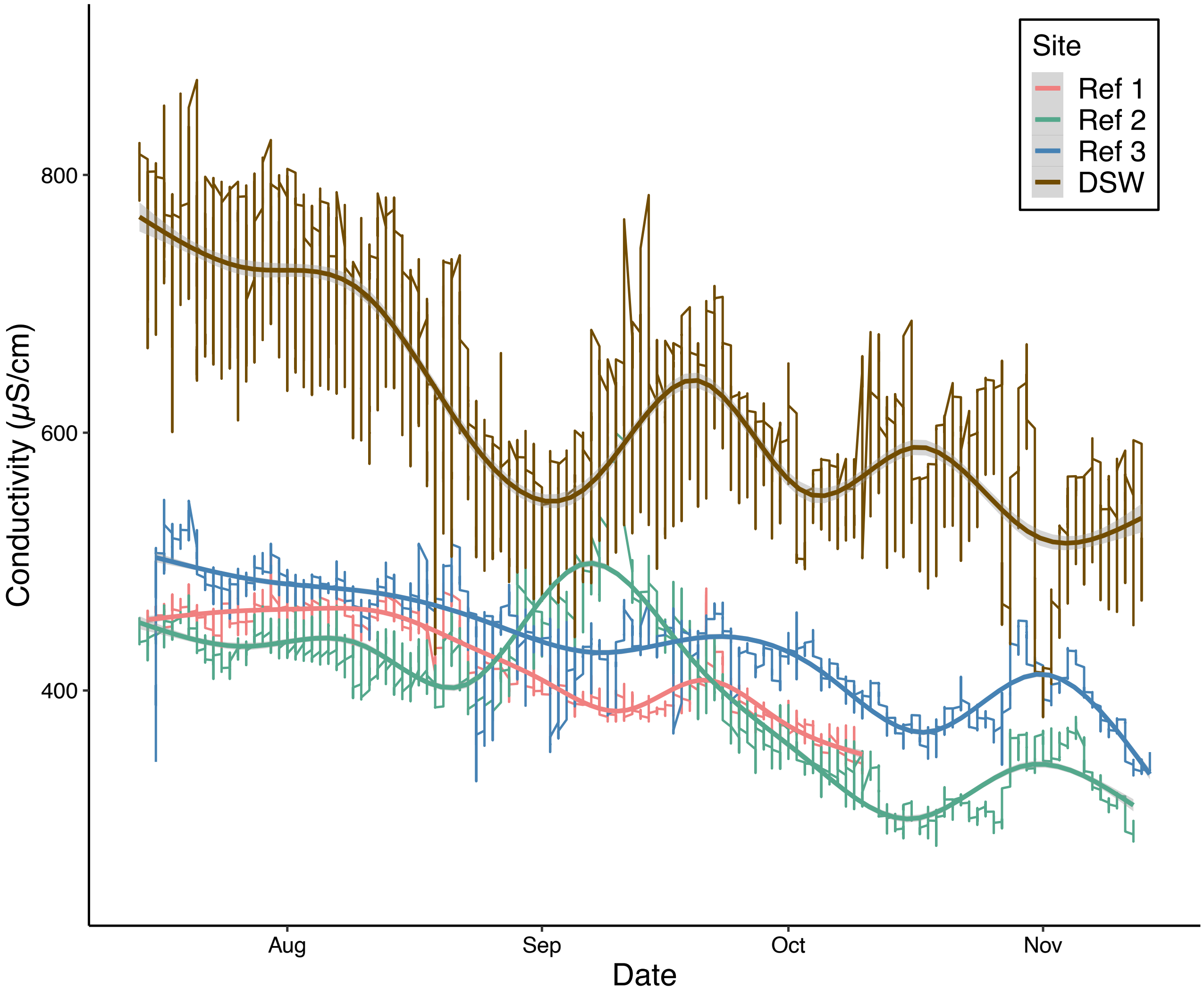

Figure 2. 5. Water conductivity (µS/cm) at the four sites (Ref 1, Ref 2, Ref 3, DSW) between late June and mid-November 2019 indicates higher conductivity at the DSW site compared to all reference sites.

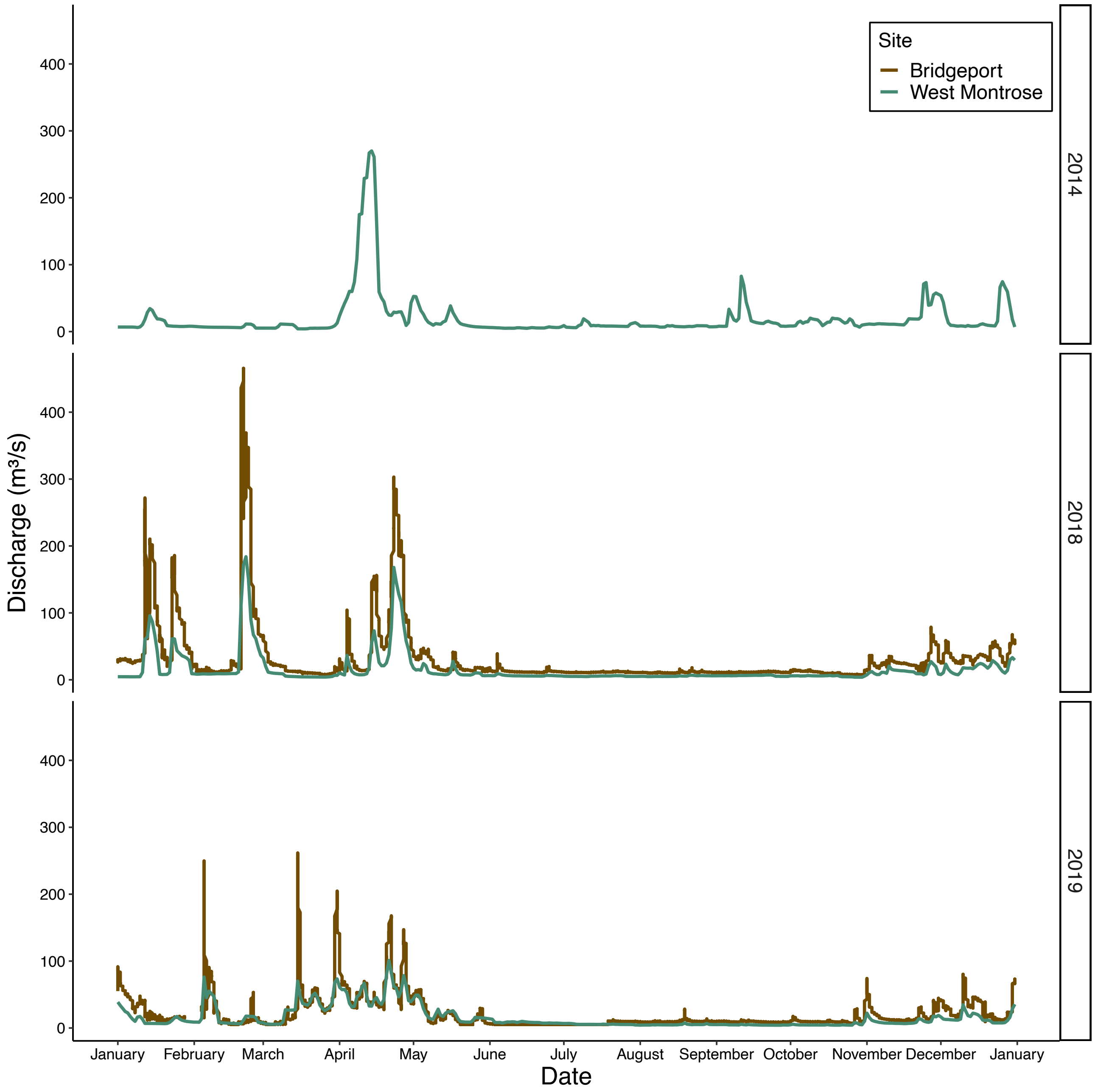

Figure 2. 6. Water flow (m^3/s) at West Montrose (Ref 2) and Bridgeport (DSW) indicates higher river discharge at the DSW site in 2018 and 2019. Data from Bridgeport (DSW) was not available from 2014.

2.3.2 Otolith growth and Back-calculation

A key assumption of using a back-calculation method and assessing growth from otoliths is that otolith growth is proportional to fish growth (Campana, 1992). This assumption need to be met in order to estimate past length-at-age to increase sample sizes for growth curves. Fish from Reference 2 site were used for otolith length and weight measurements. Linear regressions were performed to assess the relationship between otolith growth and fish growth. A linear regression between otolith length and total fish length indicates a strong predictive relationship for both male and female rainbow darter (r^2= 0.868 and 0.879, respectively) (Figure 2. 7). Similarly, linear regression between log body weight and log otolith weight indicated a strong relationship with r^2 values of 0.939 for male and 0.963 for female fish (Figure 2. 8). Linear regression between otolith weight and total fish length also indicated a strong relationship for both male and female rainbow darter (linear regression, male: r^2 = 0.956 and female: r^2 = 0.935, Figure A. 2.). While otoliths from the downstream (DSW) site were not weighed and otolith length was not measured, a strong relationship between otolith radius and fish length suggests similar relationships exist at the downstream (DSW) site (Linear regression, male: r^2 = 0.786 and female: r^2 = 0.816, Figure A. 3). These relationships indicate a strong proportional relationship between otolith growth and fish growth.

Back-calculated lengths can introduce biases in the analyses and must be compared with observed length to assess whether this method is suitable for its purpose. Fish collected in 2019 were used to back-calculate length for the 2018 populations at the Reference 2 and downstream (DSW) sites (Figure 2. 9). These growth curves were compared using ARSS which revealed no significant differences between back-calculated and observed growth curves ($F_{(3,110)\ \text{Ref 2}}$= 0.09,

$F_{(3,142)\ DSW} = 1.56$, $p>0.05$). There were also no apparent differences in the associated growth parameters of the von Bertalanffy growth model (Table A. 1). Therefore, back-calculation is a suitable technique that can be used to increase sample sizes for the von Bertalanffy growth curves for a given year.

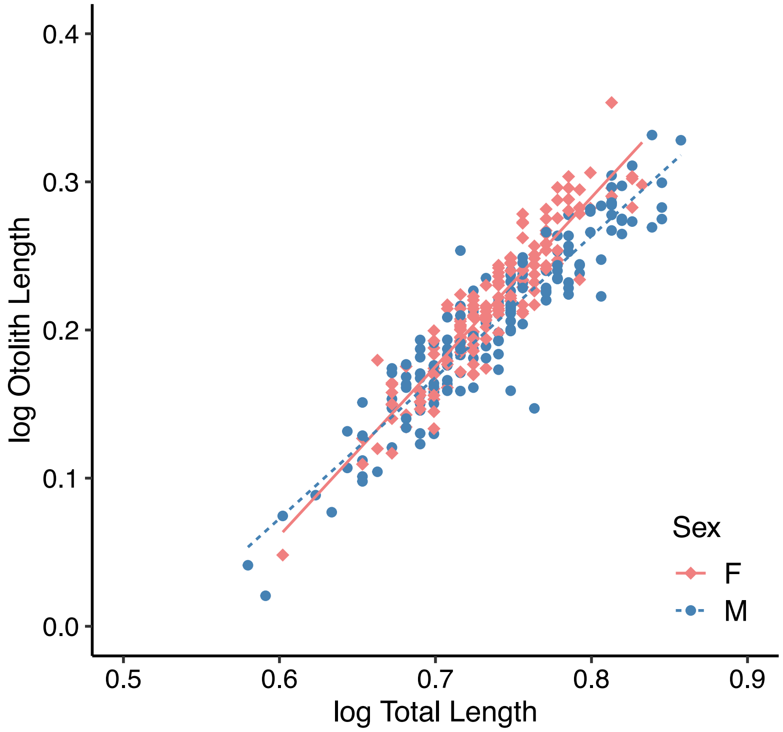

Figure 2. 7. Linear regression between log 10 transformed otolith length and total length of male and female rainbow darter (N = 216) exhibits a strong relationship for male ($r^2 = 0.868$) and female rainbow darter collected at a reference site (Ref 2) ($r^2 = 0.879$).

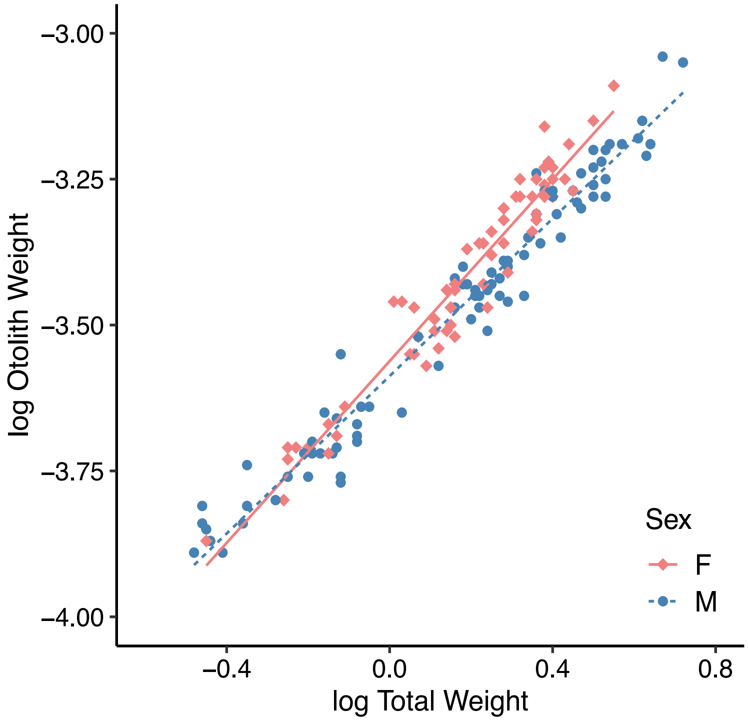

Figure 2. 8. Linear regression between log 10 transformed body weight and otolith weight indicates a strong relationship exists for both male and female fish (N = 216) at a reference site (Ref 2) (Linear regression, Male: $r^2 = 0.939$ and Female: $r^2 = 0.963$).

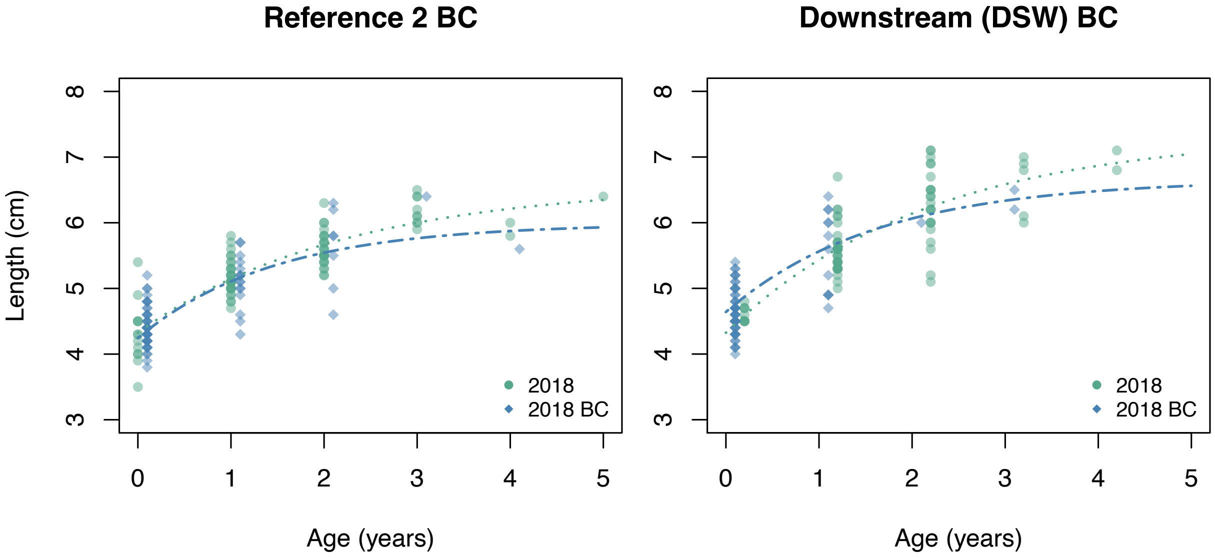

Figure 2. 9. von Bertalanffy growth curves for 2018 fish populations at the Reference 2 and downstream (DSW) sites exhibit similar observed and back-calculated (BC) lengths at each age. ARSS revealed no significant differences between back-calculated and observed growth curves ($p>0.05$).

2.3.3 Fish Growth

The von Bertalanffy growth curves were computed for both male and female rainbow darter at the specified sites for each year and associated parameters and curves were compared using likelihood ratio tests and ARSS, respectively, to assess differences in fish growth between sexes, sites and years. Growth curves of fish from 2014 were computed and an analysis of residual sum of squares (ARSS) revealed a significant difference between the two sites (Ref 2, DSW) for both male and female fish ($F_{(3,114)\ \text{Male}} = 4.38$; $F_{(3,87)\ \text{Female}} = 5.18$, $p<0.05$) (Figure 2. 10, Table A. 2). Subsequent likelihood ratio tests (LRT) indicated significant differences in all growth parameters (K, L_{inf}, t_o) between sites of female ($p<0.001$) but not male rainbow darter (Table 2. 1). Male and female growth curves were significantly different at each site (ARSS: $F_{(3,142)\ \text{Ref 2}} = 5.88$; $F_{(3,59)\ \text{DSW}} = 2.98$, $p<0.05$), however, likelihood ratio tests revealed no differences between growth parameters at the downstream site (Figure 2. 11, Table A. 4). In contrast, male and female rainbow darter growth in 2018 was significantly different only at the downstream (DSW) site ($F_{(3,69)} = 3.12$, $p<0.05$) (Figure 2. 13, Table 2. 2). Fish growth was significantly different between the three sites in 2018 ($F_{(6,138)\ \text{Male}} = 7.11$; $F_{(6,75)\ \text{Female}} = 2.37$, $p<0.05$). To discern which sites were different, additional pairwise ARSS were conducted. The results indicated no differences in male growth curves between Reference 3 and downstream (DSW) sites ($F_{(3,88)} = 2.14$, $p<0.05$) whereas, Reference 2 site differed significantly from Reference 3 and the downstream (DSW) site (ARSS: $F_{(3,91)} = 4.39$ and $F_{(3,97)} = 18.51$, $p<0.05$). In contrast, growth was only significantly different between Reference 2 and downstream (DSW) site for females (ARSS: $F_{(3,50)} = 8.09$). However, there were no differences in any of the growth parameters for both male and female fish (LRT, $p>0.05$;Table A. 2).

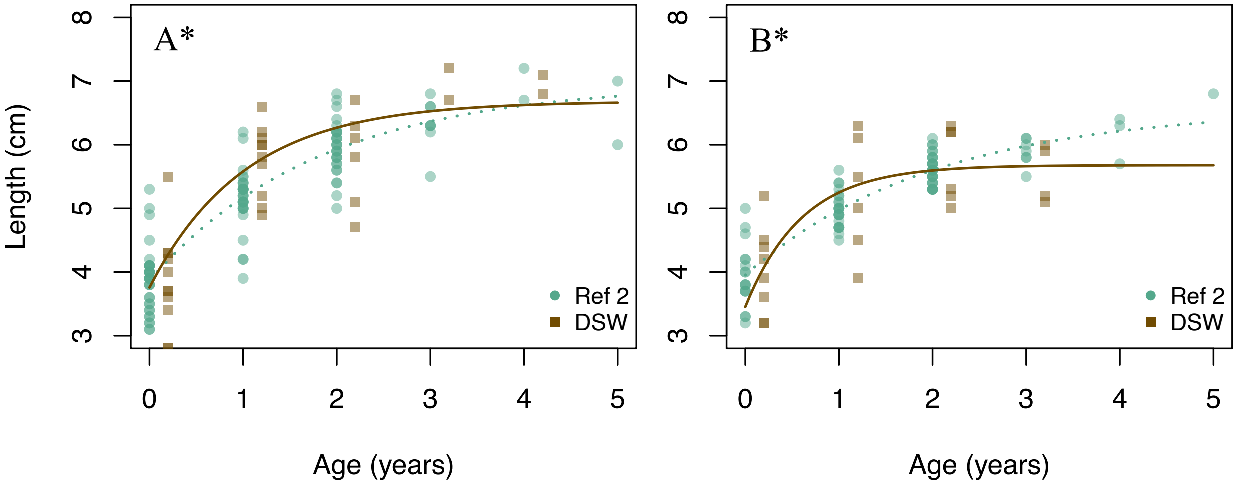

Figure 2. 10. von Bertalanffy growth curves for (A) male (n=117) and (B) female (n=90) rainbow darter collected in the fall of 2014 at the Reference 2 and downstream (DSW) sites. Asterisks indicate significant differences between the two sites for both male and female fish (ARSS, p<0.05).

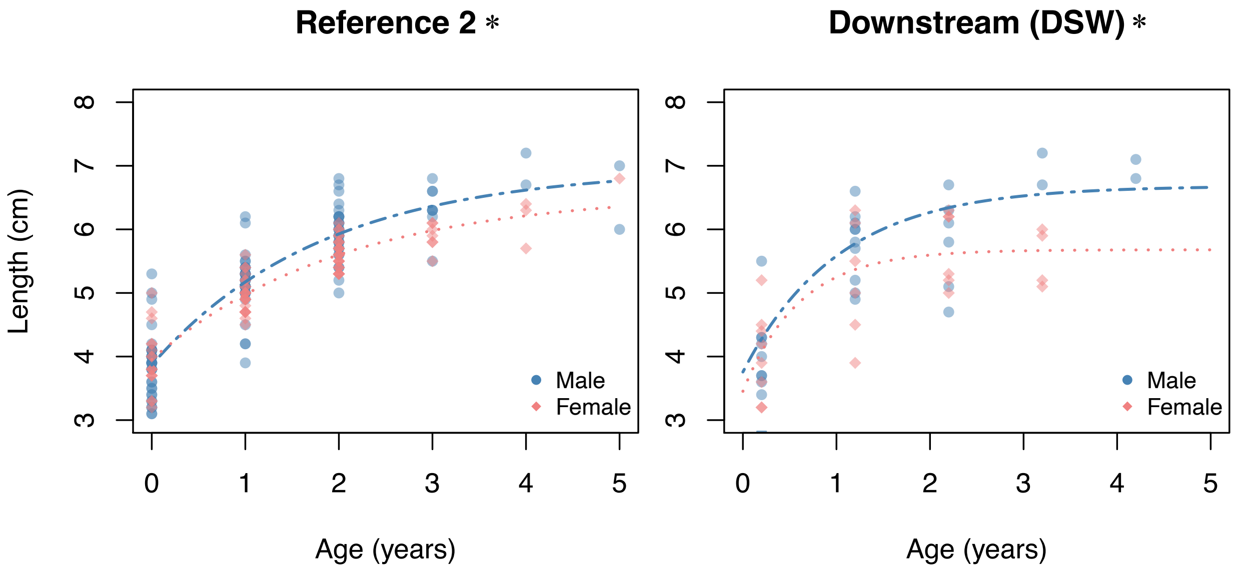

Figure 2. 11. Estimated von Bertalanffy growth curves for male and female fish collected in 2014; asterisks indicate significant differences in growth between the sexes at both sites (ARSS, p<0.05).

Table 2. 1. Estimated von Bertalanffy growth parameters (± SD) for fall 2014 populations at each site for male and female fish. Bolded numbers indicate differences among sites for the

specific parameter while asterisks indicate differences between male and female rainbow darter at the given site.

	Reference (Ref 2)		Downstream (DSW)	
	M	F	M	F
L_{inf}	6.9 ± 0.31*	**6.6 ± 0.28**	6.7 ± 0.44	**5.7 ± 0.41**
k	0.54 ± 0.11*	**0.49 ± 0.10**	0.98 ± 0.38	**1.64 ± 1.33**
t_0	-1.5 ± 0.21*	**-1.9 ± 0.29**	-0.9 ± 0.29	**-0.6 ± 0.43**

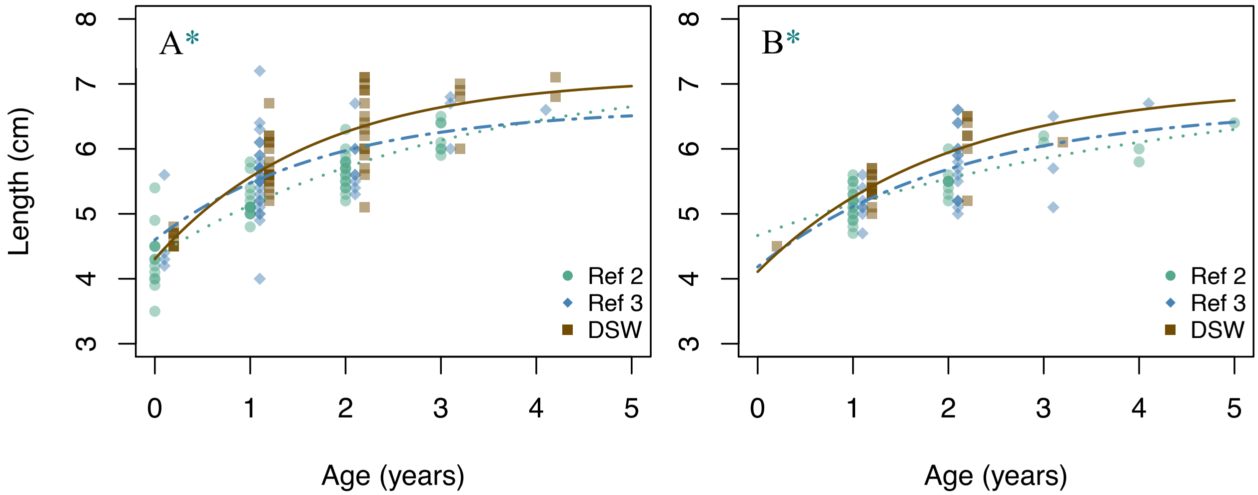

Figure 2. 12. von Bertalanffy growth curves fitted to length for (A) male (n=141) and (B) female (n=79) fish collected at the three sites (Ref 2, Ref 3, DSW) in 2018. Asterisks indicate significant differences between the Reference 2 and the other two sites for males (A) and downstream (DSW) site for females (B) (ARSS, p<0.05).

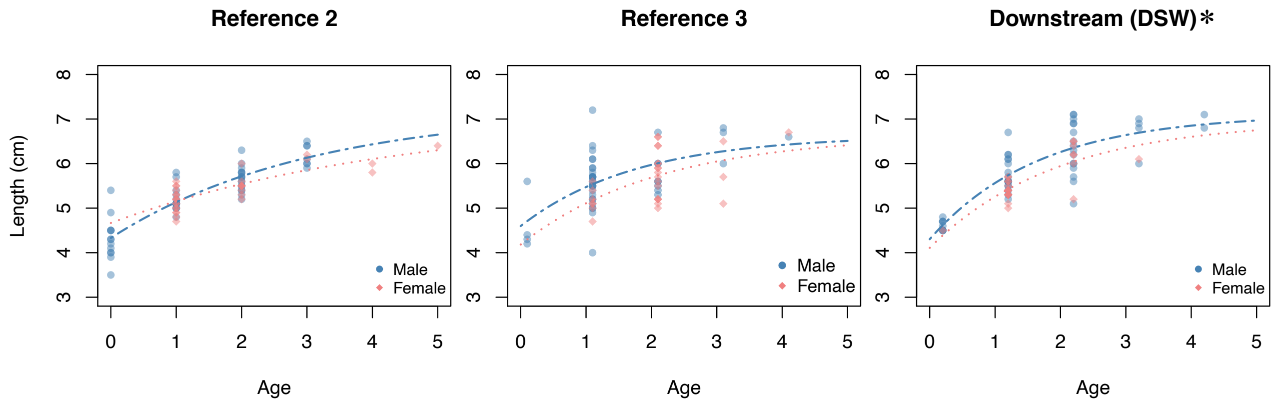

Figure 2. 13. von Bertalanffy growth curves for male and female rainbow darter collected at each site in 2018. Presence of an asterisk indicates growth between male and female fish differed only at the downstream site (ARSS, p<0.05).

Table 2. 2. von Bertalanffy growth parameters for fall 2018 populations at each site for male and female rainbow darter (± SD). Asterisks indicate significant differences between male and female growth parameters at the given site.

	Reference (Ref 2)		Reference 3 (Ref 3)		Downstream (DSW)	
	M	F	M	F	M	F
L_{inf}	7.2 ± 0.99	7.1 ± 1.59	6.6 ± 0.67	6.6 ± 1.46	7.1 ± 0.39*	7.0 ± 0.84
k	0.33 ± 0.17	0.22 ± 0.25	0.56 ± 0.37	0.47 ± 0.71	0.60 ± 0.19*	0.51 ± 0.33
t_0	-2.8 ± 0.88	-4.8 ± 3.9	-2.1 ± 1.27	-2.1± 3.38	-1.6 ± 0.45*	-1.7± 0.99

Male and female fish growth curves were assessed for potential differences among the four sites (Ref 1, Ref 2, Ref 3, DSW) from 2019 using ARSS. Initial analysis revealed that at least two curves differ significantly for male (ARSS, $F_{(9,225)} = 4.03$, $p<0.05$) but not female $F_{(9,146)} = 1.95$, $p>0.05$) fish (Figure 2. 14). Pairwise ARSS and likelihood ratio tests for males revealed significant differences between the most upstream reference site (Ref 1) and all other sites (Table A. 2). All growth parameters (L_{inf}, k, t_0) of the von Bertalanffy model also differed significantly between Reference 1 and other reference sites (Ref 2 and Ref 3) ($p<0.001$) but only growth coefficient (K) was significantly different between Reference 1 and downstream (DSW) site ($p<0.001$, Table A. 2). While Reference 3 male growth curves were not significantly different from downstream (DSW) site (ARSS, $p>0.05$), likelihood ratio test found growth parameters to be significantly different between the two sites ($p<0.001$). Growth curves of male fish at the Reference 2 site were significantly different from fish at the Reference 3 site (ARSS: $F_{(3,111)} = 2.93$, $p<0.05$) and downstream (DSW) site ($F_{(3,107)} = 7.97$, $p<0.05$, Table 2. 3). However, growth parameters only differed between Reference 2 and downstream (DSW) site (LRT, $p<0.01$, Table A. 2). Additionally, ARSS and likelihood ratio tests were conducted at each site to compare growth between sexes of rainbow darter. It was revealed that male and female growth curves and parameters differed significantly at all four sites in 2019 (Table A. 4, Figure 2. 15).

To assess whether fish growth differed between the two post-upgrade years, growth curves at each site were compared for both sexes (Table A. 2). Both ARSS and likelihood ratio tests revealed no differences in growth between years at each site for both male and female fish (Table A. 3). Pre- and post-upgrades comparisons were conducted by comparing 2014 and 2019 growth as well as 2014 and 2018 growth at the two sites (Ref 2 and DSW). ARSS revealed no significant differences at the reference site between the three years but growth curves for the downstream site differed significantly between 2014 and 2019 for both male and female rainbow darter (ARSS: $F_{(3,85)\ \mathrm{Male}} = 7.70$; $F_{(3,58)\ \mathrm{Female}} = 2.71$, $p<0.05$, Table A. 3). In contrast, only male growth curves were significantly different at the downstream site between 2014 and 2018 ($F_{(3,76)} = 2.74$, $p<0.05$). This was followed by likelihood ratio tests which found no significant differences between growth parameters pre- and post-upgrades (Table A. 3).

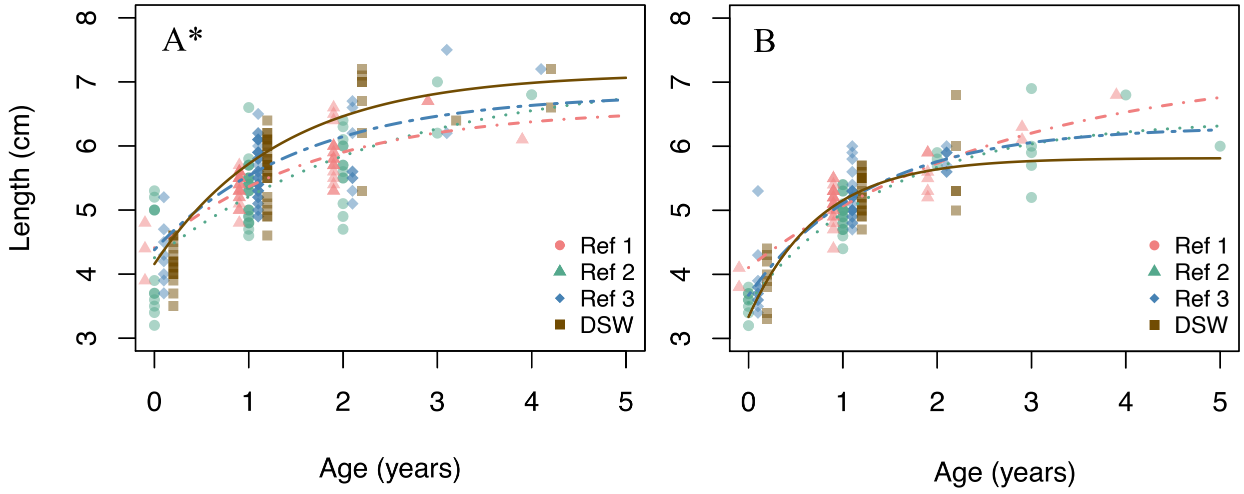

Figure 2. 14. Estimated von Bertalanffy growth curves for (A) male (n=223) and (B) female (n=149) rainbow darter collected in 2019 at the four sites (Ref 1, Ref 2, Ref 3, DSW). Presence of an asterisk indicates significant differences between at least two curves (ARSS, p<0.05).

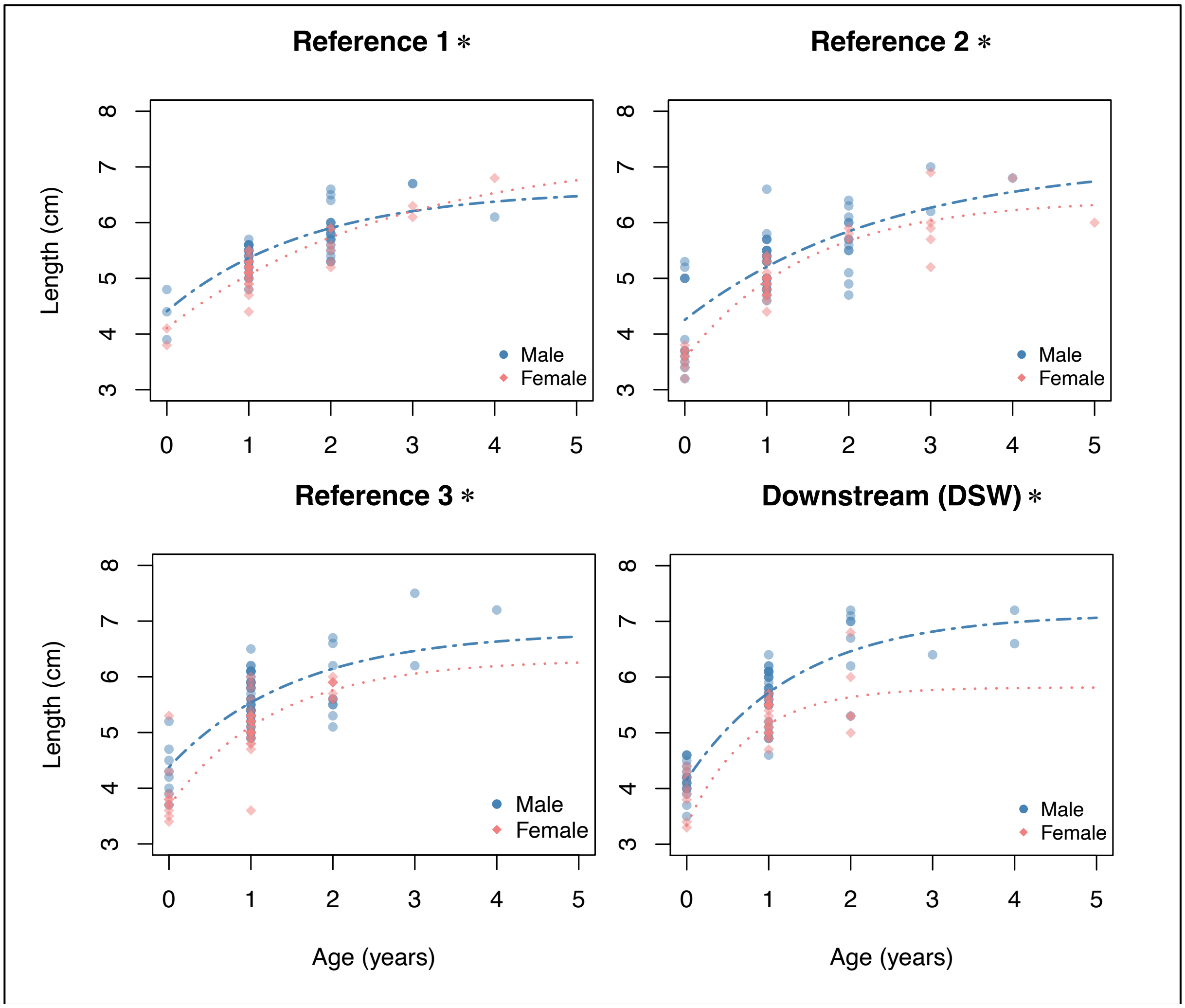

Figure 2. 15. Estimated von Bertalanffy growth curves for male and female rainbow darter at the four sites (Ref 1, Ref 2, Ref 3, DSW) collected during the fall of 2019. Asterisks indicate significant differences between the two curves at the given site (ARSS, p<0.05).

Table 2. 3. Estimated von Bertalanffy growth parameters (± SD) for fall 2019 populations at each site (Ref 1, Ref 2, Ref 3, DSW) for male and female rainbow darter. Asterisks indicate significant differences between sexes at the respective site.

	Reference 1 (Ref 1)		Reference 2 (Ref 2)		Reference 3 (Ref 3)		Downstream (DSW)	
	M	F	M	F	M	F	M	F
L_{inf}	6.9 ± 0.8*	7.3 ± 0.8	7.2 ± 0.9*	6.4 ± 0.2	6.6 ± 0.7*	6.3 ± 0.5	7.1 ± 0.4*	5.8 ± 0.3
k	0.39 ± 0.2*	0.36 ± 0.2	0.33 ± 0.2*	0.67 ± 0.1	0.56 ± 0.4*	0.78 ± 0.3	0.6 ± 0.2*	1.32 ± 0.6
t_0	-2.8 ± 1.3*	-2.3 ± 0.8	-2.8 ± 0.9*	-1.2 ± 0.2	-2.1 ± 1.3*	-1.1± 0.3	-1.6 ± 0.5*	-0.64 ± 0.3

2.3.4 Somatic Indices

The number of fish captured during the fall of 2014, 2018 and 2019 varied as did the number of fish that were sacrificed (Figure 2.16). Fish length ranged from 3.0 cm to 7.2 cm with mean (± SD) length of 5.7 (±0.9), 5.5 (±0.7) and 5.2 (±0.8) cm for 2014, 2018 and 2019, respectively. The observed median length (± SE) was 5.2 (±0.6), 5.5 (±0.4) and 5.3 (±0.4) for 2014, 2018 and 2019, respectively. A strong linear relationship between log length and log weight was observed ($r^2 = 0.935$, Figure 2. 17) and the relationship is plotted for Reference 2 site, but similar relationships are present across years and sites (Table A. 5). The equation for the relationship is log weight (g) = -2.1 + 3.3*(log length(cm)) indicating isometric growth of rainbow darter with a regression coefficient of 3.3. The r^2 for 2014, 2018 and 2019 were 0.971, 0.927 and 0.935, respectively.

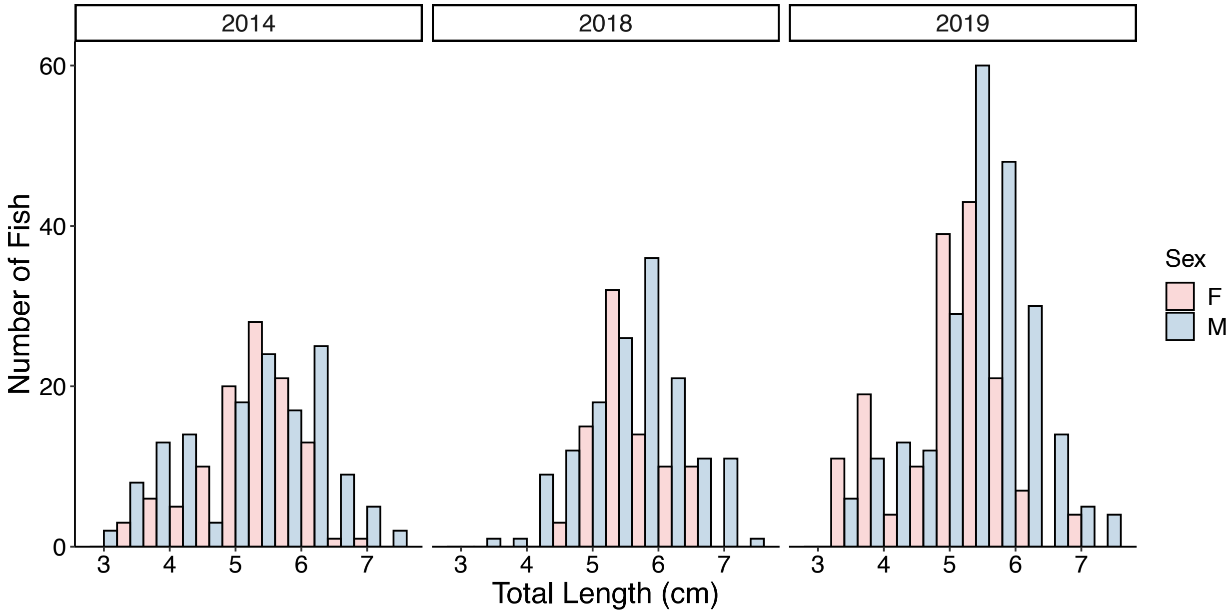

Figure 2. 16. Length frequency distributions for male and female fish collected during the three years from a reference (Ref 2) and downstream (DSW) site (pooled).

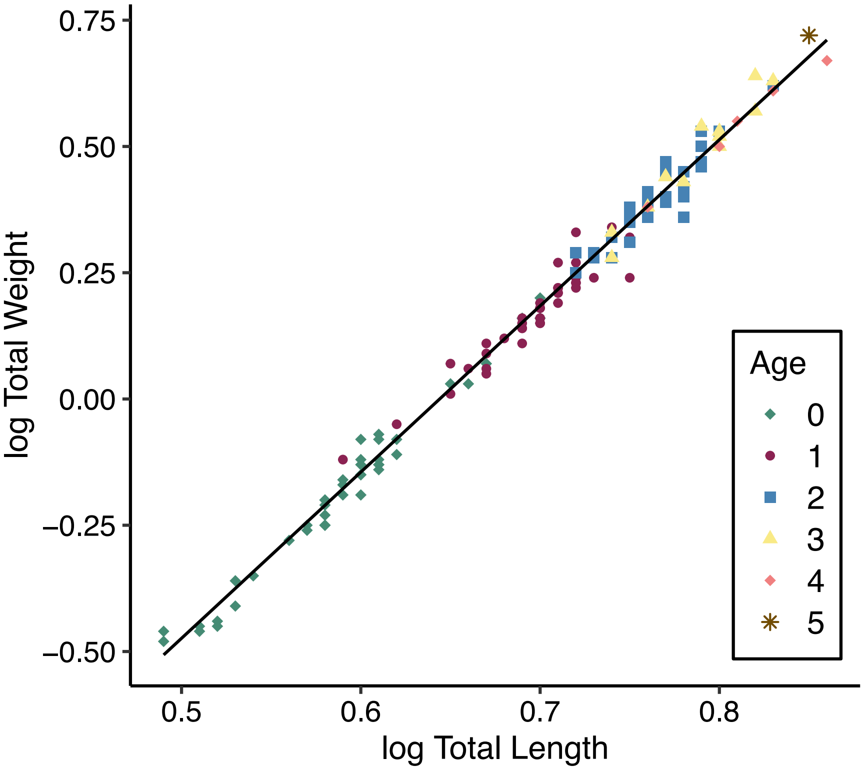

Figure 2. 17. Log 10 transformed length vs. weight for fish collected in 2019 at the Reference 2 site for all observed age cohorts of rainbow darter. There is a strong relationship between fish length and weight (Linear regression, $r^2 = 0.935$).

All somatic indices were compared for male and female rainbow darter separately to assess whether there were any significant differences between sites in the 2014, 2018 and 2019 populations. To evaluate potential differences between the three years, a series of two-way ANOVAs were conducted for each somatic index. The results indicated an effect of year on the eachn index but a follow-up Tukey's test found no significant differences between 2018 and 2019 fish populations for any index while GSI and LSI differed significantly between 2014 and post-upgrade years ($p<0.05$, ANOVA table: Table A. 8). The results from ARSS, likelihood ratio tests and two-way ANOVAs found no significant differences in growth between 2018 and 2019 populations and, therefore, data were pooled for comparison of all somatic indices.

A two-way ANOVA indicated a significant interaction of age and sex acting on fish length ($F_{(5, 509)} = 2.49$, $p<0.05$) for 2018 and 2019 pooled data. Mean length was significantly different between sexes of rainbow darter at ages 1+, 3+ and 4+ (Tukey's post hoc, $p<0.05$). There were also differences among age groups in mean length; female and male mean length significantly differed between 0+, 1+ and all older ages (Tukey's post hoc, $p<0.05$). In addition, male fish length was also observed to be different between 2+ and older ages (Tukey's test, $p<0.05$). To explore potential differences in fish length across sites, additional two-way ANOVAs were conducted for male and female rainbow darter. There was an interaction between age and site on mean length of male rainbow darter from 2018 and 2019 ($F_{(9,304)}=1.72$, $p<0.05$). Tukey's post hoc test indicated differences between both reference sites (Ref 2 and Ref 3) as well as between Reference 2 and downstream (DSW) site ($p<0.01$). However, male length did not differ between Reference 3 and downstream site (Tukey's post hoc, $p>0.05$). Mean length among the three sites was significantly different at 1+ and 2+ ($p<0.05$) with no differences at any other age. There were also significant differences in mean length of males among age groups; 0+ differed from all other ages at all three sites while 1+ were different from 3+ and 4+ at the two reference sites and from all older ages at the downstream site (Tukey's post hoc, $p<0.05$, Figure 2. 18). In contrast to male rainbow darter, a two-way ANOVA found no interaction between age and site for female rainbow darter ($F_{(7,185)}=1.178$, $p=0.3$) in years post-upgrades. However, mean length for females did differ among age groups and across sites. Female length was significantly different between the two reference sites, Reference 2 and downstream (DSW) site at ages 1+, 2+ and 3+ but not between Reference 3 and downstream (DSW) site (Tukey's post hoc, $p<0.01$).

Female length of 0+ and 1+ was significantly different from all other ages at the three sites ($p<0.0001$, Figure 2. 19).

In contrast to post-upgrades fish population, there was no interaction between age and sex in 2014 ($F_{(5,203)} = 1.255$, $p=0.28$) but main effects of age ($F_{(5,204)} = 153.7$, $p<0.0001$) and sex ($F_{(1, 204)} = 15.59$, $p<0.001$) were apparent. Female and male rainbow darter differed significantly at all ages except 0+ (Tukey’s post hoc, $p<0.001$). Female and male mean length differed among 0+,1+ and older ages in addition to 2+ and 4+ for males (Tukey’s post hoc, $p<0.001$). A two-way ANOVA indicated a significant interaction between age and site on mean length of male rainbow darter ($F_{(4,113)}=2.847$, $p<0.05$). Subsequent post-hoc tests indicated no significant differences between the two sites (Ref 2 and DSW) but there were differences among age groups at both sites (Tukey’s post hoc, $p<0.05$; Figure 2. 20). Fish aged 0+ and 1+ differed significantly from all other ages as did 2+ and 4+ aged fish (Tukey’s post hoc, $p<0.001$). In contrast to males, there was no significant interaction between age and site on mean length of females ($F_{(3,86)}=2.034$, $p<0.05$). A main effect of age was evident ($F_{(5,87)} = 46.62$, $p<0.001$) with 0+ and 1+ aged female fish significantly differing in mean length from all other ages (Tukey's post hoc, $p<0.001$). Consistent with male fish, there were no differences in mean length of female rainbow darter between the two sites in 2014 (Tukey’s post hoc, $p>0.05$; Figure 2. 21).

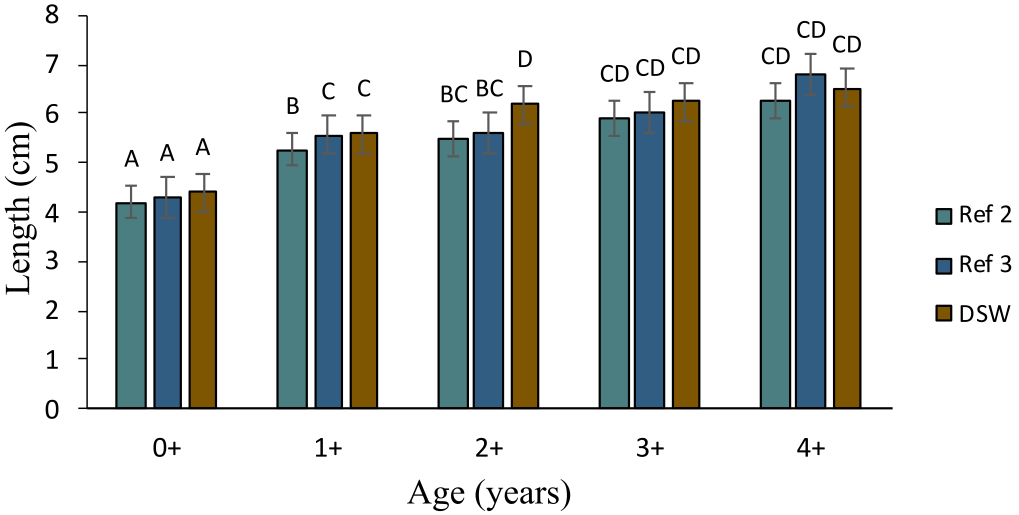

Figure 2. 18. A bar chart representing total length (mean ± SE) of male rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate differences in mean length among age groups and sites.

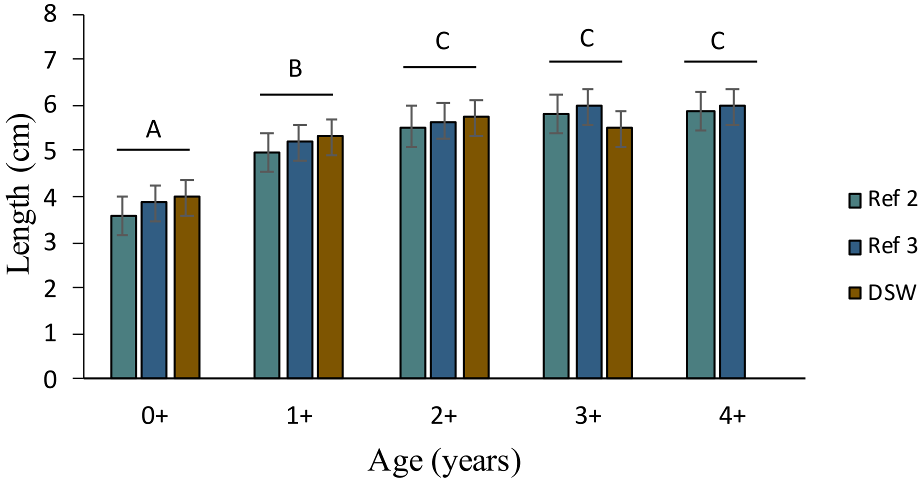

Figure 2. 19. A bar chart representing total length (mean ± SE) of female rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters over bars indicate differences among age groups for pooled sites.

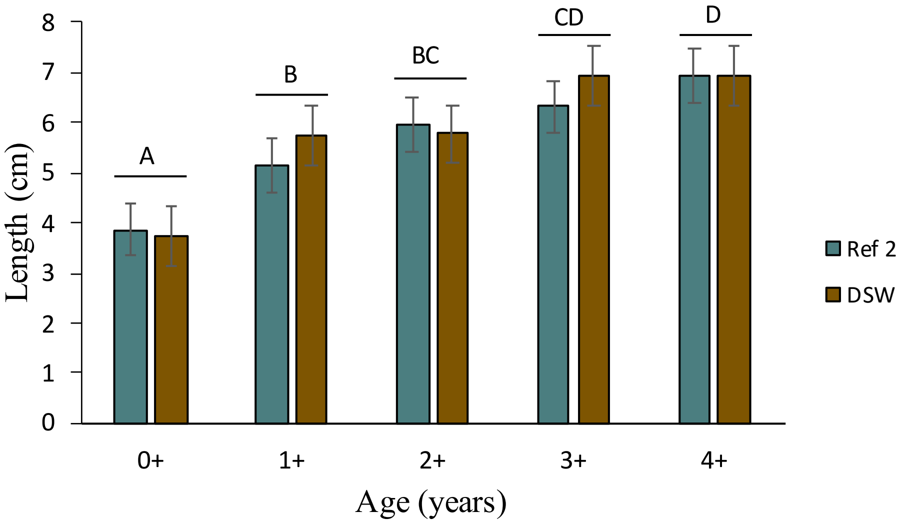

Figure 2. 20. A bar chart representing total length (mean ± SE) of male rainbow darter for each age cohort at the two sites from fish collected in 2014. Letters indicate differences among age groups for pooled sites.

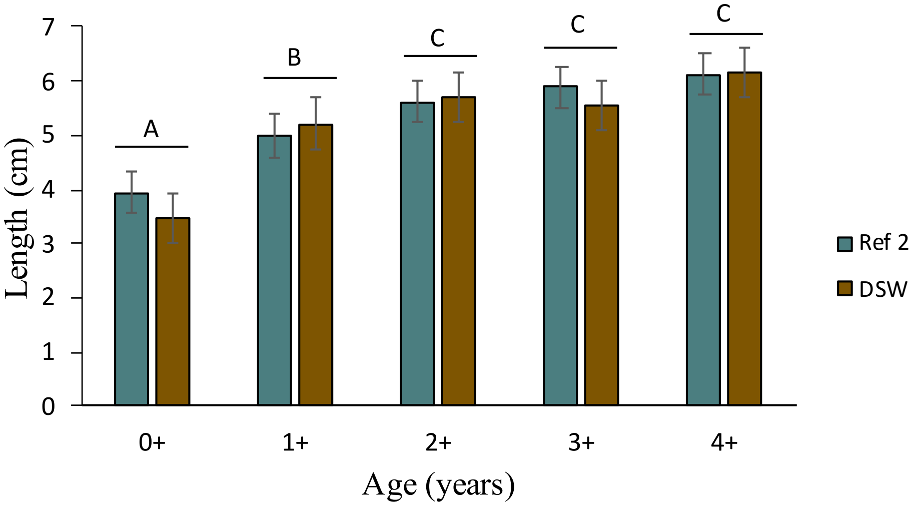

Figure 2. 21. A bar chart representing total length (mean ± SE) of female rainbow darter for each age cohort at the two sites from fish collected in 2014. Letters indicate differences among age groups for pooled sites.

A statistically significant interaction between age and sex on mean weight of rainbow darter in 2018 and 2019 was evident (Two-way ANOVA, $F_{(5,509)}$=2.317, p=0.04). Pooled male and female fish weight indicated significant differences in weight between all age groups

(Tukey's post hoc, $p<0.01$). Mean weight differed significantly between sexes of rainbow darter at 1+, 3+ and 4+ fish (Tukey's post hoc, $p<0.001$). Two-way ANOVAS conducted to examine differences between sites revealed a significant interaction between age and site on mean weight of male ($F_{(9,304)}=2.11$, $p=0.02$) but not female ($F_{(7,185)} =1.588$, $p=0.14$) fish. Tukey's test indicated that mean weight of males differs significantly between the reference sites (Ref 2 and Ref 3) and downstream (DSW) site while the two reference sites did not differ in mean weight ($p<0.01$, $p>0.05$). Mean weight also differed significantly among age groups for male fish at the three sites; 0+ aged fish differed significantly from all other ages at the three sites while 1+ differed only from 3+ and 4+ at the two reference sites but from all other ages at the downstream (DSW) site ($p<0.001$, Figure 2. 22). Additionally, male fish weight was significantly different between 2+ and older fish (3+,4+) at the two reference sites (Ref 2 and Ref 3) ($p<0.01$). Mean weight of female fish also differed significantly among age ($F_{(5,185)} = 55.96$, $p<0.001$) groups and between sites ($F_{(2,185)} = 4.1$, $p=0.01$). Tukey's post hoc test indicated significant differences in female body weight between Reference 2 and downstream (DSW) site but no differences between other sites were apparent ($p<0.001$). Female weight was significantly different among 0+, 1+ and all older ages at all three sites ($p<0.05$, Figure 2. 23).

In the 2014 rainbow darter population, no significant interaction between age and sex acting on mean weight was apparent (Two-way ANOVA, $F_{(5,81)} = 0.525$, $p = 0.75$). However, there was evidence of differences in weight among age groups ($F_{(4,86)} = 7.09$, $p<0.001$) and between sexes of rainbow darter ($F_{(1,86)} = 4.38$, $p=0.03$). Mean weight was significantly different between male and female rainbow darter at ages 1+, 2+ and 3+ (Tukey's post hoc, $p<0.05$). 0+ aged fish differed significantly from 3+ and 4+ while 1+ were different from 2+ and 3+ ($p<0.05$).

There was no significant interaction between age and site on mean weight of male ($F_{(3,38)}$ = 1.163, p=0.33) and female ($F_{(3,35)}$ = 0.424, p=0.737) fish in 2014. Main effects of age and site were apparent but subsequent post hoc tests indicated no differences in fish weight between the two sites (Ref 2 and DSW) for both male and female rainbow darter (p>0.05). However, differences among age groups were apparent for both males and females with 0+ and 1+ male being significantly different from all other ages while 0+ and 1+ only differed from 3+ and 4+ for females (p<0.01, Figure 2. 24, Figure 2. 25).

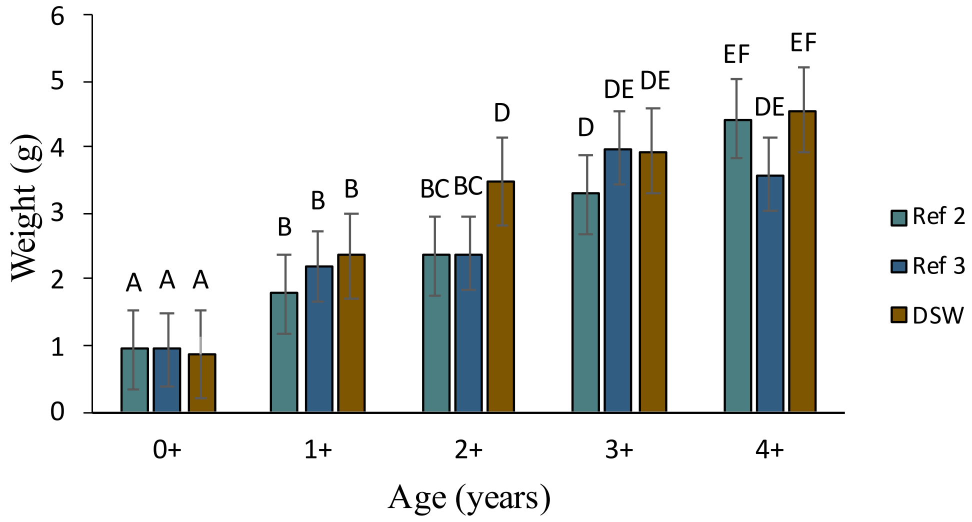

Figure 2. 22. A bar chart representing total weight (mean ± SE) of male rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences among age groups and between sites for male rainbow darter.

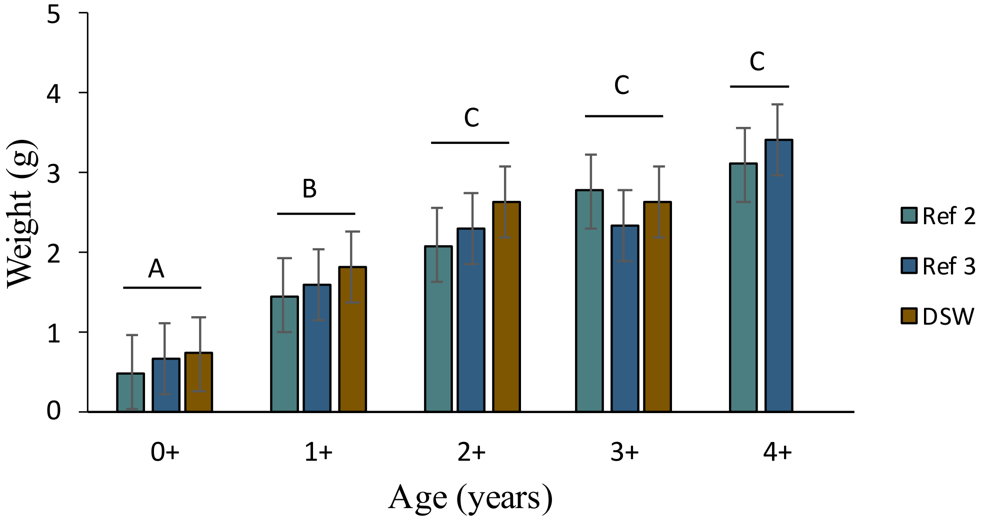

Figure 2. 23. A bar chart representing total weight (mean ± SE) of female rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters over bars indicate significant differences among age groups for pooled sites.

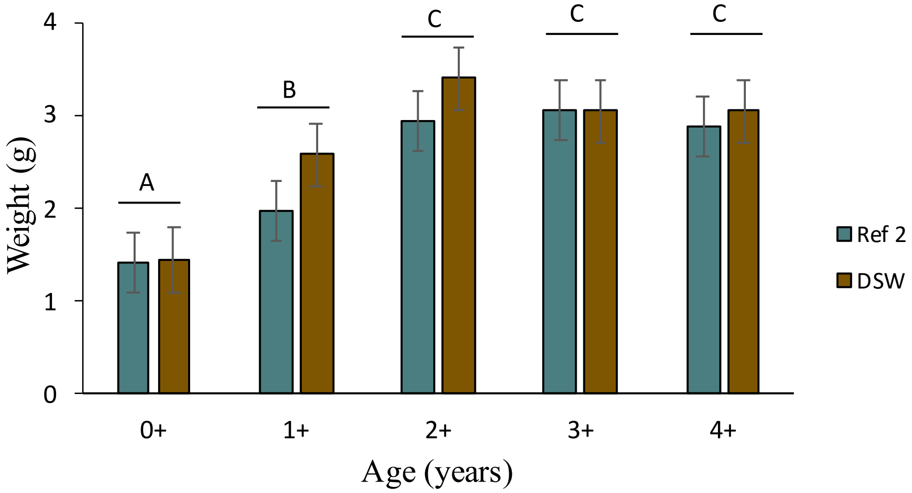

Figure 2. 24. Total body weight (mean ± SE) of male rainbow darter at each observed age at reference 2 and downstream (DSW) sites from 2014. Letters indicate significant differences among age groups for pooled sites.

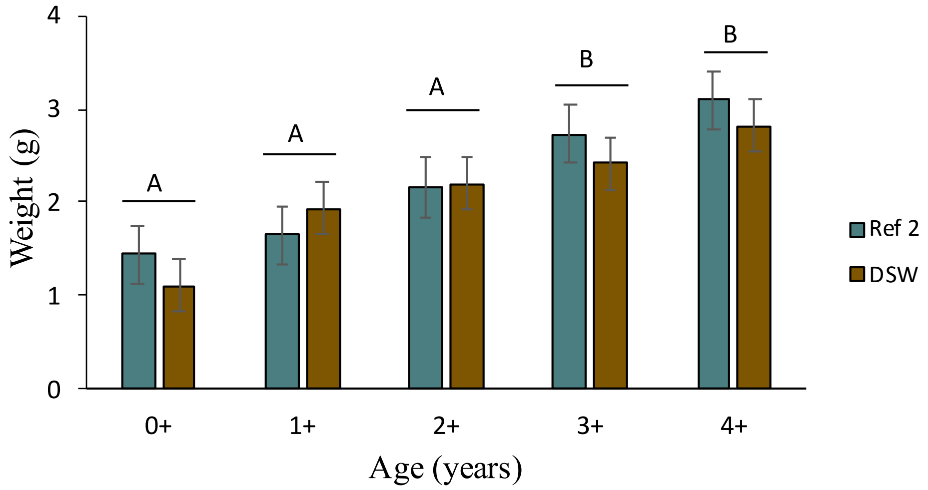

Figure 2. 25. Total body weight (mean ± SE) of female rainbow darter at each observed age at Reference 2 and downstream (DSW) sites from 2014. Letters indicate significant differences among age groups for pooled sites.

There was no interaction between age and sex on fish condition in 2018 and 2019 (Two-way ANOVA, $F_{(5,509)} = 0.941$, p=0.4). However, fish condition did differ significantly among age groups ($F_{(4,514)} = 20.34$, p<0.001) and between male and female rainbow darter ($F_{(1,514)} = 38.94$, p<0.001). Fish condition was different between the sexes of rainbow darter at every age (Tukey's post hoc, p<0.001). Tukey's test indicated fish condition for 0+ cohort differed significantly from all other age groups and between 1+ and 2+ (p<0.01). Two-way ANOVAs indicated a significant interaction between age and site on male ($F_{(9,304)} = 3.183$, p=0.001) and female ($F_{(7,185)} = 2.598$, p=0.01) fish condition. Tukey's post hoc tests indicated no differences in fish condition between sites for both male and female rainbow darter (p>0.05) but there were differences among age groups with 0+ differing from all other ages for both male and female fish (p<0.001, Figure 2. 26, Figure 2. 27).

Consistent with 2018 and 2019, no interaction between age and sex acting on fish condition was apparent in 2014 ($F_{(5,81)} = 0.640$, p = 0.67). Fish condition did however, differ significantly among sexes of rainbow darter at every age ($F_{(1,86)} = 14.23$, p=0.0003) and among age groups ($F_{(4,86)} = 3.812$, p=0.003). Tukey's test indicated fish condition was different between 1+ and 3+ age cohorts ($p<0.01$). Additional two-way ANOVAs conducted found no interaction between age and site on fish condition of male ($F_{(3,38)} = 0.986$, p=0.4) and female ($F_{(3, 35)} = 0.812$, p=0.4) rainbow darter in 2014. There were also no differences in fish condition between the two sites (Ref 2 and DSW) or among age groups for both male ($F_{(1,41)\ \text{site}} = 2.157$, p=0.15, $F_{(5,41)\ \text{age}} = 1.930$, p=0.11) and female ($F_{(1,38)\ \text{site}} = 0.012$, p=0.9, $F_{(5,38)\ \text{age}} = 2.11$, p=0.08) rainbow darter (Figure A. 9, Figure A. 10).

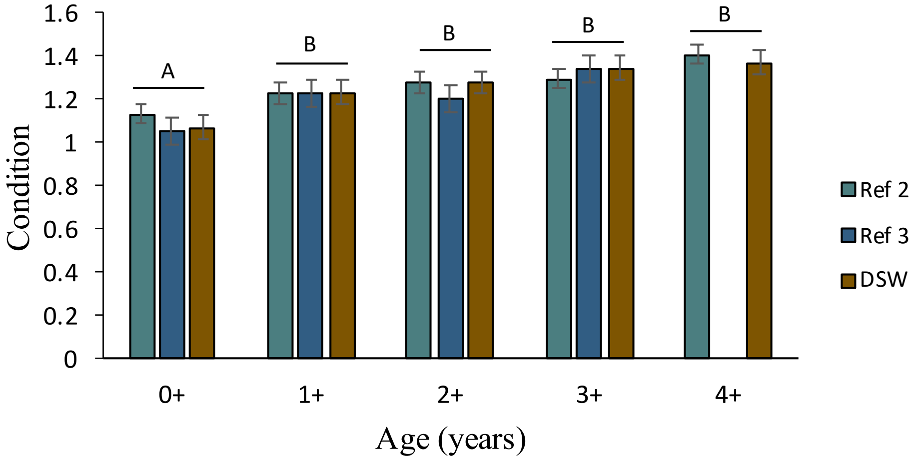

Figure 2. 26. A bar chart representing condition factor values (mean ± SE) of male rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences among age groups for pooled sites.

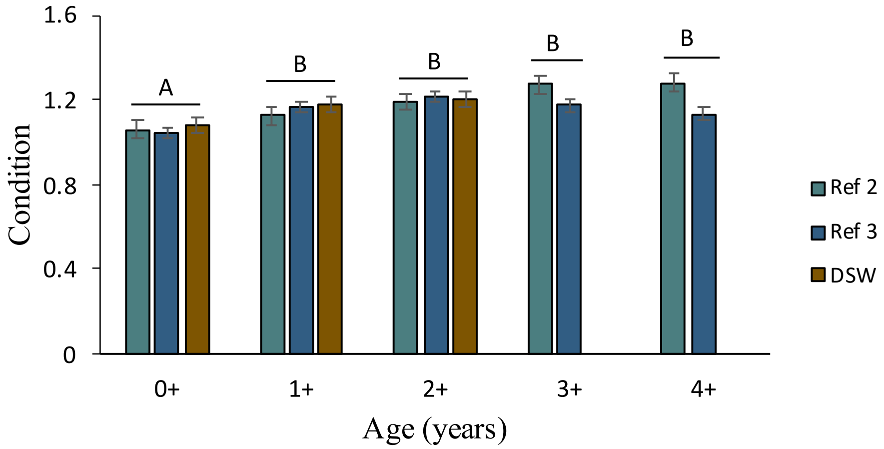

Figure 2. 27. A bar chart representing condition factor values (mean ± SE) of female rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences among age groups for pooled sites.

No interaction between age and sex on mean LSI for 2019 and 2018 population was evident (Two-way ANOVA, $F_{(4,335)} = 0.553$, p=0.6), however, differences among the different age cohorts were apparent ($F_{(5,339)} = 6.914$, $p<0.001$). Pooled male and female mean LSI were different between 0+ and 1+ and 1+ and 2+ and 3+ fish ($p<0.01$). LSI values were also different between male and female rainbow darter at every age ($F_{(1,339)} = 119.5$, $p<0.001$). There were no interactions between age and site on mean LSI for male ($F_{(9,204)} = 1.431$, p=0.17) and female ($F_{(2,118)} = 15.019$, $p<0.001$) rainbow darter. Tukey's post hoc test indicated no differences in LSI between the three sites (Ref 2, Ref 3 and DSW) of male rainbow darter. However, there were differences among age groups for males between 1+ and 3+ aged fish (Tukey's test, $p<0.05$; Figure 2. 28). In contrast, there was no effect of age on mean LSI of female fish ($F_{(4,118)} = 1.476$, p=0.2, Figure 2. 29). There were significant differences in LSI of female fish between sites ($F_{(2,118)} = 15.02$, $p<0.001$) with both reference sites (Ref 2, Ref 3) being significantly different from each other as were Reference 3 and downstream (DSW) site (Tukey's post hoc, $p<0.01$).

LSI values did not differ between Reference 2 and downstream (DSW) site ($p=0.3$). There was no interaction between age and sex on mean LSI values for 2014 fish population ($F_{(5,81)} = 1.49$, $p=0.2$) but LSI did differ between male and female rainbow darter at all ages ($F_{(1,81)} = 10.54$, $p=0.001$). There was no interaction between age and site on mean LSI for both male and female rainbow darter ($F_{(3,38)\ \mathrm{male}} = 0.247$, $p=0.86$, $F_{(3,35)\ \mathrm{female}} = 1.307$, $p=0.29$). There were also no differences among age groups or sites for both male and female fish (Figure A. 11, Figure A. 12, $p>0.05$).

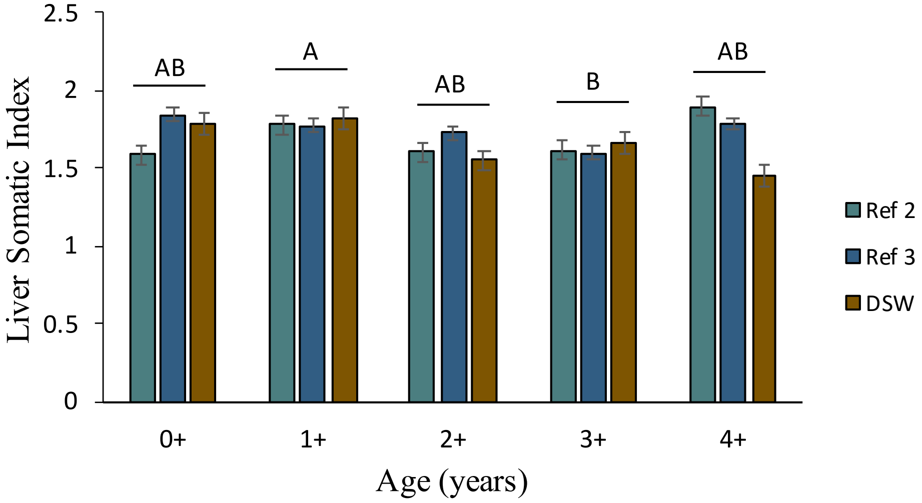

Figure 2. 28. A bar chart representing liver somatic index values (mean ± SE) of male rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences among age groups for pooled sites.

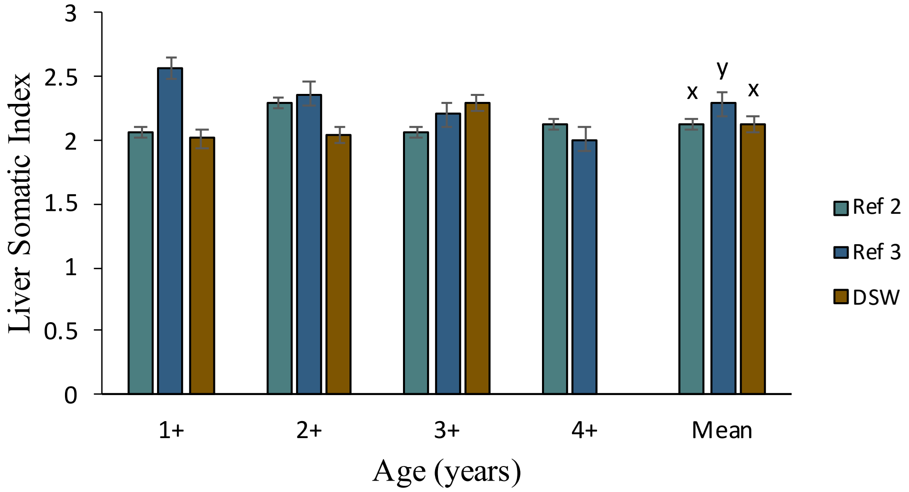

Figure 2. 29. A bar chart representing liver somatic index values (mean ± SE) of female rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences between sites for pooled age groups.

Mean gonad somatic index (GSI) for male and female rainbow darter were also compared for 2018 and 2019 population indicating a significant interaction between age and site on GSI (Two-way ANOVA, $F_{(4, 335)} = 2.858$, p=0.02). Male and female GSI differed significantly at every age except 0+ (Tukey's post hoc, $p<0.001$). Tukey's test also indicated significant differences among 0+, 1+ and all other age groups ($p<0.01$). GSI was also significantly different between 2+ and 3+ aged fish as well as 3+ and 4+ ($p<0.01$). There was a significant interaction between age and site on GSI for male ($F_{(9,204)} = 2.234$, p=0.02) but not female ($F_{(5,113)} = 1.336$, p=0.2) rainbow darter. Tukey's post hoc tests found that male GSI differed between the two reference sites (Ref 2, Ref 3) and Reference 2 and downstream (DSW) site at age 2+ ($p<0.05$) while Reference 3 and downstream (DSW) site did not differ ($p>0.05$). There were also differences among age groups at the downstream (DSW) site; male GSI was significantly different between 0+ and all other age groups and between 1+ and 3+ fish. (Tukey's test, $p<0.05$) while there were no differences between age groups at the reference sites ($p>0.05$) (Figure 2. 30).

Female GSI was not different among age groups or across sites (p>0.05, Figure A. 13). There was no interaction between age and sex on mean GSI for 2014 fish ($F_{(5,81)} = 2.131$, p=0.06). However, main effects of age ($F_{(4,81)} = 3.06$, p=0.01) and sex ($F_{(1,81)} = 212.2$, p<0.001) were apparent. There were significant differences in GSI between male and female fish at every age with the exception of 0+ (Tukey's post hoc, p<0.01). Two-way ANOVAs found no significant interaction between age and site on GSI of male ($F_{(3,38)} = 1.227$, p=0.3) or female ($F_{(3,35)} = 2.81$, p=0.06) fish. No differences in GSI between sites were apparent for both male and female rainbow darter (p>0.05) (Figure A. 14, Figure A. 15). There were differences in male GSI among age groups with 1+ being different from all older age groups (Tukey's post hoc, p<0.01) but no differences among age groups exist for females (p=0.6).

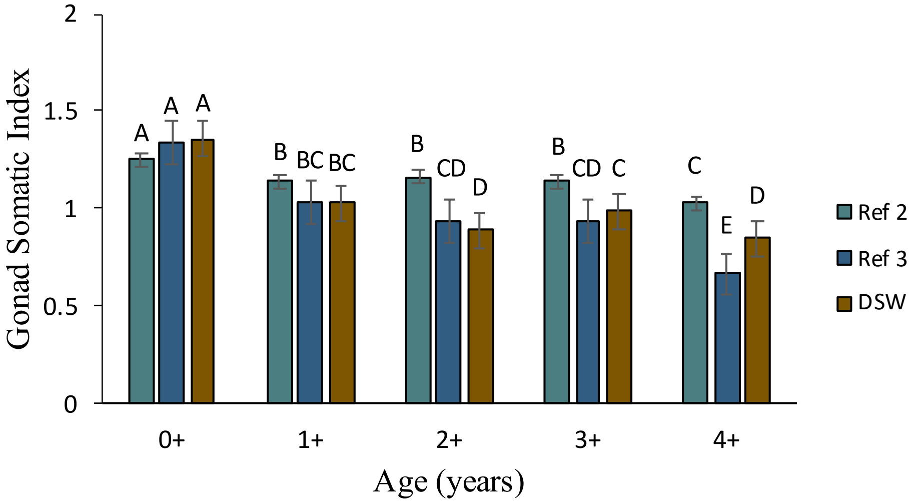

Figure 2. 30. A bar chart representing gonadosomatic index values (mean ± SE) of male rainbow darter for each age cohort at the three sites from fish collected in 2018 and 2019. Letters indicate significant differences in LSI between sites and among age groups for male fish.

2.4 Discussion

Growth curves and parameters (K, L_{inf}) differed between male and female rainbow darter, which is consistent with several studies previously conducted on the Grand River and elsewhere (Crichton, 2016; Beckman, 2002). Estimated von Bertalanffy growth curves of male and female fish differed with males growing to a larger size-at-age and at a faster rate than females at all sites across all years with the exception of reference sites (Ref 2 and 3) in 2018. In contrast, growth parameters were statistically different between male and female fish only at Reference 2 site in 2014, downstream (DSW) site in 2018 and at all sites in 2019. This could be attributed to a lack of young-of-year (0+ age class) caught in 2014 and 2018. While not all differences were significant, male size-at-age (length, weight, condition) was always larger than female fish at all ages except 0+. Differences between male and female size-at-age and growth are fairly common in sexually dimorphic species (Parker, 1992). In rainbow darter, male and female growth and size-at-age is similar in the first two years after which male rainbow darter continue to grow but females do not. Growth is also observed to be faster in the first two years with male and female fish attaining up to 86% of their length by age 2 after which growth decreases. This pattern is prevalent in many species of darters; for example, male and female bayou darter (*Etheostoma rubrum*) in southwestern Mississippi were observed to grow rapidly in their first year reaching similar length but in their second year and onwards, males exhibit a larger size-at-age than females (Knight & Ross, 1992). Likewise, male tessellated darter (*Etheostoma olmstedi*) and orangefin darter (*Etheostoma bellum*) grow more rapidly attaining a larger length-at-age compared to female fish (Layzer & Reed, 1978; Fisher, 1990). Initial rapid growth is understood to be an evolutionary strategy for small-bodied, short lived fish species to increase survival (escape predation) and gain sexual maturity quickly (Paine, 1990). Allocation of energy and resources to reproduction can account for differences in somatic growth between male and female rainbow darter as female fish have substantially higher GSI than males. This is true for orangefin darter (*Etheostoma bellum*) where female fish invest their energy into gonad development having on average 10 times the GSI during the spawning period than males (Fisher, 1990). Male orangefin darter, on the other hand, spend more energy on aggressively establishing and defending their territory during spawning (Fisher, 1990). Similar behavior is observed in rainbow darter and it was found that guarding ability determined mating success more than female choice did (Fuller, 2003). There was a strong positive correlation between body size and the ability to defend one's territory and thus, larger size is selected for in intraspecific interactions and determines mating success (Fuller, 2003). Female rainbow darter also have higher LSI compared to males and it has been speculated that females store their energy in their livers during the fall and winter to be able to use this energy reserve during spawning (Tetreault et al., 2011; Crichton, 2016). It is fairly well established that faster growth rate and higher gonad weight represent higher energy expenditure while higher condition and liver weight is thought to signify higher energy storage (Gibbons & Munkittrick, 1994). Interestingly, male GSI is lower than females at every age and is seen to decrease with age (Figure 2. 30); this further supports the idea that female rainbow darter invest more energy into reproduction to maximize their clutch size while male increase their fitness by investing in somatic growth. The differences in growth and size-at-age necessitates separation of sexes for all analyses but this is dependent on the species at hand. Highly sexually dimorphic species like rainbow darter and orangefin darter have been separated for analyses while species that are not sexually dimorphic such as eastern sand darter (*Ammocrypta pellucida*) and channel darter (*Percina copelandi*) have been pooled for analysis (Drake et al, 2008; Reid, 2004; Fisher,1990). Male and female rainbow darter exhibit differences in size and growth and it is therefore recommended that they be separated for analysis.

Fish growth is influenced by a variety of factors including water temperature, water quality, river discharge and substrate type. As WWTPs are a major source of nutrients to aquatic systems, it has been speculated that fish growth should be different at sites downstream of effluent discharge (DSW). Fish growth between Reference 2 and downstream (DSW) site was consistently different with faster growth rate (k) and larger size-at-age observed at the downstream (DSW) site in all years. However, faster growth at the downstream (DSW) site in 2014 cannot be attributed to the wastewater effluent as the Reference 3 site was not included in the analysis. Reference 3 site exhibited similar growth to the downstream (DSW) site and differed from other reference sites in 2018 and 2019. This indicates no significant impact of the MWWE on fish growth post-upgrades; however, size-at-age and growth for fish at the downstream (DSW) site were always larger than any other site. Several physical characteristics of Reference 3 and downstream (DSW) sites are similar with temperature being a key one. Throughout the summer months, recorded temperatures at both sites were higher compared to other sites reaching upwards of 28ºC. Water temperature is known to have a governing effect on fish growth; a laboratory study found that growth rate and size of orange-throat darter (*Etheostoma spectabile*) was substantially larger for fish in 26ºC water than fish at lower temperatures of 20ºC (similar to Ref 1 and 2) (West, 1996). Reference 1 site had the lowest temperatures and exhibited slower growth and size-at-age than all other sites indicating a potential effect of temperature on fish growth. In addition, higher discharge and substrate type can greatly influence fish growth (Drake et al., 2008). Eastern sand darter (*Ammocrypta pellucida*) exhibited faster growth at higher annual discharge and silt free substrate (Drake et al., 2008) further supporting conducive conditions for faster growth at the downstream (DSW) site. However, comparable growth between Reference 3 (silty) and downstream (DSW) site alludes to a smaller effect of substrate on fish growth. Many studies on the Grand River have reported changes in health indices (K, LSI, GSI) downstream of wastewater effluent presumably due to nutrient sources that promote algae/biofilm growth and/or toxicity (Fuzzen et al., 2016; Bahamonde et al., 2015). Nutrient concentrations (nitrite, nitrate, ammonia) at the downstream (DSW) site are higher compared to other sites across years but nitrite and ammonia concentrations are seen to decrease significantly post-upgrades while nitrate increases two-fold. Increased concentrations of potentially growth limiting nutrients nitrate and phosphorous promote growth of primary producers: aquatic plants and algae thereby increasing production of invertebrates and fish populations through bottom-up effects (Kilgour et al., 2005). However, this can also decrease available dissolved oxygen for fish and other aquatic life affecting their survival. Downstream (DSW) site had visibly more biofilm on substrate as well as vegetation compared to upstream reference sites. In contrast, nitrite and ammonia can have a toxic effect on fish health increasing mortality and stunting growth (Yeom et al., 2007; Lewis et al., 1986). Although upgrades to treatment processes at the Waterloo WWTP have reduced nitrite, ammonia and total estrogenicity of the effluent, which are closely tied to improvements in reproductive impacts (intersex, hormone production) in rainbow darter, the final effluent still contains a mixture of compounds including venlafaxine, carbamazepine and other pharmaceuticals at concentrations similar to those measured pre-upgrades (Marjan et al., 2017; Srikanthan, 2019). Antidepressants such as venlafaxine and fluoxetine have been shown to alter fish behavior and thus growth in several fish species (Corcoran et al., 2010). Laboratory and field studies showed decreased territorial aggressive behavior of male bluehead wrasse (*Thalassoma bifasciatum*), reduced ability to capture prey in striped bass and decreased feeding rates in fathead minnow exposed to various concentrations of fluoxetine (Corcoran et al., 2010). It can therefore be speculated that at the downstream (DSW) site fish growth could be reduced due to stressors that are altering fish behavior that is key to survival and growth despite other favourable conditions (nutrients, etc.). It is also possible that adverse contaminant effects are masked by increases in nutrients and thus, growth. Faster growth at the Reference 3 site compared to other reference sites can be explained by higher temperatures, increased nutrients from agricultural tributaries (Conestogo River) and lack of contaminant stressors compared to the downstream (DSW) site. Therefore, fish growth is determined by different factors that can work in synergistic, antagonistic or additive ways that are site and species-specific and cannot be attributed to a particular stressor or factor. Further research is needed to elucidate drivers of among-site and among-year differences in growth quantitively.

In addition to growth curves, it is important to consider somatic indices to explore differences in growth. Mean length and weight for fish post-upgrades did not differ between Reference 3 and downstream (DSW) site except for female weight. In contrast, there were no differences in mean length or weight pre-upgrades in 2014. Condition factor was not significantly different between sites pre- and post-upgrades. In a wastewater monitoring framework proposed by Kilgour et al. (2005), fish condition was classified as a crude, less sensitive and ecologically relevant measure than growth. It is then expected that differences in growth can be detected when there are no differences in condition between groups, which is consistent with the findings of this study. In addition, male rainbow darter LSI did not differ upstream and downstream of the WWTP, but smaller gonad sizes were observed at the downstream (DSW) site post-upgrades. Female fish, in contrast, had higher LSI at the Reference 3 site but no differences in GSI were apparent across age groups. Compared to 2014, LSI decreases for both male and female fish collected in 2018 and 2019 while GSI decreases in males but increases in females. This is not an effect of upgrades to the WWTP as this was also observed at the reference sites and could reflect natural/annual variability due to other factors such as temperature or discharge. There is also no effect of age on GSI and LSI for rainbow darter which is expected as gonadal recrudescence and regression occurs every year (Bahamonde et al., 2016); an exception to this is an apparent decrease in GSI of male fish with age which corresponds to utilization of energy towards growth. These results are inconsistent with other studies that have noted increases in growth, condition and LSI downstream of WWTPs (McMaster et al., 2005; Jefferies et al., 2008; Yeom et al., 2007). Longnose dace (*Rhinichthys cataractae*) exhibited larger livers, length and condition downstream of a WWTP in the Red Deer River, an agricultural and urbanized watershed (Jefferies et al., 2008). Likewise, Yeom et al. (2007) reported larger liver size for female pale chub (*Zacco platypus*) only. An increase in liver size at an impacted site can reflect high nutrient concentrations or chemical stress (detoxification) (Jefferies et al., 2008). As mentioned earlier, large liver size and increased condition together indicate high energy storage implying no significant benefit/effect of nutrients from wastewater effluent on fish growth (Gibbons & Munkittrick, 1994). Changes in GSI are study-specific with some studies detecting an increase while others a decrease or no difference in fish gonad size at sites receiving wastewater effluent (Yeom et al., 2007; McMaster et al., 2005; Jefferies et al., 2008). Jefferies et al. (2008) reported reduced male gonad size at exposed sites for male longnose dace while Yeom et al. (2007) reported larger gonad size at an exposed site for pale chub (*Zacco platypus*); however, these differences were not significantly different indicating increase in LSI/condition did not translate into higher reproductive output. Gonad size of male longnose sucker were reported to decrease at downstream sites during the second year of the study, but no differences were detected in the first year (McMaster et al., 2005). Therefore, changes in GSI can vary temporally depending on effluent characteristics and site conditions for a given period of time. For male rainbow darter, gonad size is smaller at the downstream (DSW) site pre- and post-upgrades (Tetreault et al., 2011; Fuzzen et al., 2016). Persistent reduction of gonad sizes of male rainbow darter at the downstream (DSW) site indicates impacts of wastewater on reproductive health of these fish. A lower GSI can also be reflective of early sexual maturation and/or a younger population at an impacted site but there is little evidence to support these hypotheses for rainbow darter. Overall, an increase in fish condition and LSI does not always correspond to an increase in reproductive output and can represent metabolic disruption (Kilgour et al., 2005). It is expected that eutrophication as a result of wastewater effluent or agricultural and urban runoff increases food availability within a system increasing growth and reproductive output of fish (Kilgour et al., 2005). This pattern does not reflect adverse effects and should only be a concern if a critical effect size of 25% or greater is observed (Kilgour et al., 2005). Any differences in growth or somatic indices in this study were not above the critical effect size suggesting very little impact of wastewater effluent on fish growth in the central Grand River. However, adverse effects of wastewater effluent on fish were more pronounced at sites downstream of the Kitchener WWTP and it is possible that cumulative effects have a deleterious effect on the population structure further downstream.

The back-calculation method used to obtain past length-at-age is a powerful technique that allows for understanding growth of individuals and populations. It is recommended that this method be validated within each system to ensure accuracy of estimated lengths (Francis, 1990). The validation process consists of three components, 1) a radial measurement is the same as the time when the annulus was formed (i.e., the radius does not change); 2) the time of annulus formation is correct; and 3) the measurements and formulas that are used relate otolith size to body size accurately (Francis, 1990). The first two parts are difficult to validate but various methods have been suggested to assess them. To determine whether the timing of annulus formation is correct, marginal increment analysis (MIA) and edge analysis should be conducted to validate the periodicity and timing of annulus formation (Francis, 1990; Campana., 2001). A previous study utilized MIA and edge analysis and determined the timing of the fall annulus formation was between September and November in rainbow darter (Crichton, 2016). The last step to validate back-calculation method involves comparing back-calculated lengths to observed lengths. This comparison was conducted for observed lengths in 2018 and back-calculated lengths from 2019 at the Reference 2 and downstream (DSW) sites; no significant differences were apparent in mean length-at-age or growth curves/parameters between back-calculated and observed lengths (Figure A. 16). The Dahl-Lea and Fraser-Lee back-calculation methods were compared for rainbow darter and it was revealed that the Dahl-Lea method significantly underestimated length at every age while the Fraser-Lee method estimated length that was similar to those observed in the population (Figure A. 16). Another method to decipher accuracy of back-calculated lengths is to compare the standard deviations of observed and back-calculated lengths (Francis, 1990) where large differences indicate problems with the model. Standard deviations for mean length were similar between observed and back-calculation methods across age groups indicating no gross errors in the model (Figure A. 16, Table A. 9). In addition, body proportional hypotheses must analyze relationships between otolith growth and fish growth to determine whether the assumption of proportionality holds true (Francis, 1990). Although, the biologically modified Fraser-Lee does not require complete proportionality, otolith length and weight was compared with fish length and weight. The assumption of proportionality was satisfied as strong correlations between otolith growth and fish growth were apparent. In addition to validation of the method, back-calculated lengths should also be assessed for the presence of Lee's phenomenon as it can be indicative of problems with the model (Ricker, 1969). Lee's phenomenon refers to the observation that back-calculated lengths for an age cohort get smaller when the fish they are obtained from get older (Ricker, 1969). On the contrary, it has also been observed that back-calculated length at a given age become larger with increasing fish age; this is known as reverse Lee's phenomenon. Back-calculated lengths exhibited a slight tendency towards the reverse Lee's phenomenon (Table A. 10) with an average increase of 1 mm in back-calculated length with increasing age. Ricker (1969) put forth several explanations for Lee's phenomenon; 1) an incorrect mathematical model was used for back-calculation of lengths, 2) biased sampling favours younger, faster-growing individuals or older, slow growing individuals and 3) length-selective mortality eliminates faster-growing individuals from the population resulting in capture of slow growing individuals with their smaller back-calculated lengths. In the case of rainbow darter captured in 2019, it is very unlikely that there was a size bias during fish collection and the reverse Lee's phenomenon was also evident in the otoliths (annulus measurements) indicating the problem does not stem exclusively from the Fraser-Lee calculations. Moreover, individual growth histories did not provide evidence for slow growth in older individuals or faster growth in younger individuals; fish exhibited similar growth trajectories regardless of age. A more likely explanation could be length-selective mortality that favours larger sized fish; this was the case for tessellated darter that exhibited the reverse effect of Lee's phenomenon with the cause being attributed to differential mortality that favours fast growing individuals (Layzer & Reed, 1978). Length-selective mortality was also observed at a site downstream of WWTP effluent in Miho Stream in Korea and the authors attributed the cause to ammonia toxicity and habitat degradation (Yeom et al., 2007). This supports the validation of the back-calculation method for each population since effects can be site-specific. However, similar relationships between fish and otolith growth and observed and back-calculated lengths at both reference 2 and downstream (DSW) sites indicate no site-specific differences that could be attributed to the presence of reverse Lee's phenomenon. Generally, back-calculated length matched closely with observed lengths, the increase in back-calculated length with age group was minimal and the von Bertalanffy growth model was fitted to length adequately proving this method can accurately and reliably estimate past length-at-age for rainbow darter in the Grand River.

The utilization of fish otoliths to age rainbow darter in the Grand River adds to our understanding of impacts of urbanization and wastewater effluent on fish growth. Growth and size-at-age are classified as highly sensitive and ecologically relevant population level endpoints for studying impacts of industrial or municipal wastewater effluent (Kilgour et al., 2005). Prior to this study, growth of rainbow darter upstream and downstream of a wastewater treatment plant pre- and post-upgrades has not been studied due to limited archived samples and the difficulty of aging these small-bodied fish. This study demonstrated that the von Bertalanffy growth model and the biologically modified Fraser-Lee back-calculation equation are a reliable and accurate method for comparing growth of male and female rainbow darter across sites and years. In addition, somatic indices and site characteristics greatly enhance our ability to characterize and interpret any growth differences. Male and female rainbow darter growth differs at older ages with males attaining length and weight throughout their life. There were no consistent differences between reference and downstream sites indicating a minor effect of the Waterloo WWTP on fish growth. This study supports the use of growth as an endpoint in monitoring programs to assess impacts of point and non-point sources of pollution on fish health in aquatic environments.

2.4.1 Limitations of data

There are potential limitations of this study that should be considered for future research. A comparatively smaller sample size at the downstream site and a lack of samples from Reference 3 site in 2014 reduces our ability to detect growth differences and attribute them to the WWTP and to the upgrades. Although growth curves utilizing back-calculated lengths were significantly different between the Reference 2 and downstream (DSW) sites in 2014, differences could be underestimated due to a bias of the back-calculation method. In addition, back-calculated lengths assume it is representative of the population but fish that have survived to a certain age do not account for fish mortality. This is to say that 0+ fish from 2014 that have survived to 4+ in 2018 is not the same as all the 0+ fish in 2014 (Francis, 1990). Another potential bias results from a tendency to sacrifice larger sized fish for reproductive endpoints (hormone production, intersex). It is also important to note that fish growth is a continuous process that occurs over years and therefore, post-upgrades fish populations that were sampled include fish growth that occurred in pre-upgrade years. To assess fish growth that occurs only post-upgrades, the earliest sampling period would be during the fall of 2022. It should also be noted that rainbow darter can move in and out of the effluent limiting their exposure; this is especially prevalent during periods of spawning where smaller fish exhibit more movement while larger male rainbow darter guard their territories (Hicks et al., 2017b; Fisher et al., 1990). This implies spatial and temporal differences in movement and thus, exposure of fish depending on their size.

Chapter 3
Conclusion and Recommendations

Growth determination using the von Bertalanffy growth model greatly enhances our understanding of fish health at the individual and population level. The Environmental Effects Monitoring framework includes age and growth to assess impacts of industrial and municipal wastewater effluent on fish populations (Kilgour et al., 2005). Fish downstream of Waterloo and Kitchener WWTP have been impacted at various levels of biological organization ranging from molecular to community level with more pronounced adverse effects downstream of the Kitchener WWTP due to impact of cumulative effects (Tetreault et al., 2013; Hicks et al., 2017b; Marjan et al., 2018). Upgrades to the Kitchener WWTP were associated with reduction of many adverse impacts such as rapid reduction in intersex incidence in rainbow darter (Hicks et al., 2017b). Treatment upgrades to the Waterloo WWTP in 2017 provided a unique opportunity to understand effects of effluent quality on fish growth. A minimum of 25 male and 20 female fish were consistently collected each fall upstream and downstream of the Waterloo WWTP since 2013 as part of ongoing monitoring studies on the Grand River. A method to reliably and accurately age rainbow darter in the Grand River was validated using otoliths allowing for assessment of growth pre- and post-upgrades (Crichton, 2016).

This study compared growth across sites and years in reference to the Waterloo WWTP effluent and revealed significant differences between reference sites as well as reference (Ref 2) and the downstream (DSW) site. Although, there was no significant effect of the WWTP effluent on fish growth post-upgrades, growth rate and size-at-age of fish at the downstream (DSW) site were always higher than other sites indicating the conducive conditions for growth associated with the wastewater effluent including increased temperature, nutrients and higher discharge. In addition, somatic indices revealed differences in energy utilization between sexes, sites and years. Male rainbow darter grow to a larger size at a faster rate while females invest more energy into reproduction evident by higher LSI and GSI values. Furthermore, no consistent differences between sites immediately upstream and downstream of the WWTP suggest similar energy utilization and expenditure despite varying site conditions. However, none of the differences exhibited between sites or years were above the ecologically relevant difference of 25% as recommended by the Environment Effects Monitoring (EEM) program. This is indicative of no adverse effects of the Waterloo WWTP on the rainbow darter population downstream of the WWTP. However, it is critical to understand the influence of temperature and flow on endpoints so these can be incorporated into models and reduce variability in endpoints being measured. The application of the von Bertalanffy growth model is suitable for fish species like rainbow darter that exhibit non-linear growth. To statistically compare growth of different populations, the analysis of residual sum of squares (ARSS) and likelihood ratio tests are appropriate methods to decipher any growth differences.

The use of back-calculation is an excellent approach to estimate past length-at-age that can be utilized to increase sample sizes for a given year for computation of the von Bertalanffy growth curves. The biologically modified Fraser-Lee method is a far more accurate method than Dahl-Lea and should be used for rainbow darter after basic assumptions such as proportionality are met. This method would allow for assessment of growth from archived samples collected in the fall from upstream and downstream of the Kitchener WWTP. However, ideal sampling time for fish species that spawn multiple times such as rainbow darter is few weeks prior to spawning

(Barret & Munkittrick, 2010). A review by Barret & Munkittrick (2010) revealed that differences in growth between sites were underestimated when fish were not sampled according to the recommended time according to their life history. However, sampling a few weeks prior to spawning period is not always possible in the Grand River due to high flow and ice cover conditions in April and May. Fish were collected in several years during the spring and fish growth could be analyzed to determine whether there are seasonal differences apparent. However, small sample sizes and inconsistent sampling across the years limits the usability of this approach. Additionally, the fall annulus provides a more reliable method of aging and low gonad weight to body weight ratio in the fall makes for a better assessment of somatic growth. A far better method to explore seasonal and spatial differences in growth is the use of increment width as a surrogate for fish growth (Stocks et al., 2011; Herdter et al., 2017). Increment width was used to compare growth of red snapper (*Lutjanus campechanus*) between reference and sites exposed to an oil spill in the Gulf of Mexico across years (Herdter et al., 2017). This study also utilized the biologically modified Fraser-Lee equation to increase sample sizes for the von Bertalanffy growth curves in addition to comparing increment width. This supports the appropriateness of this method for quantifying potential growth differences between sites with varying environmental conditions. Increment width is a far less biased method for comparing age-specific growth. Measuring width of growth zones can provide insight into growth differences between summer and winter growth. It is possible that temperature differences between reference and downstream site are more pronounced in the winter when warmer effluent is discharged into the receiving environment. The use of back-calculation technique in conjunction with von Bertalanffy growth model or the use of increment width can provide valuable insight into individual and population growth rates in previous years.

The use of sagittal otoliths of rainbow darter and the von Bertalanffy growth model for age and growth determination greatly enhances our understanding of population health at sites exposed to wastewater effluent. Fish sampling during the fall is ideal and large sample sizes are required to detect differences of 25%. This study used commonly used fisheries models and techniques to understand potential impacts of agricultural runoff, urbanization and municipal wastewater effluent on fish growth on a small-bodied sentinel species. Further research can utilize similar methods to those used in this study for examining impacts of stressors in aquatic ecosystems on fish growth in other watersheds.

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Appendix A

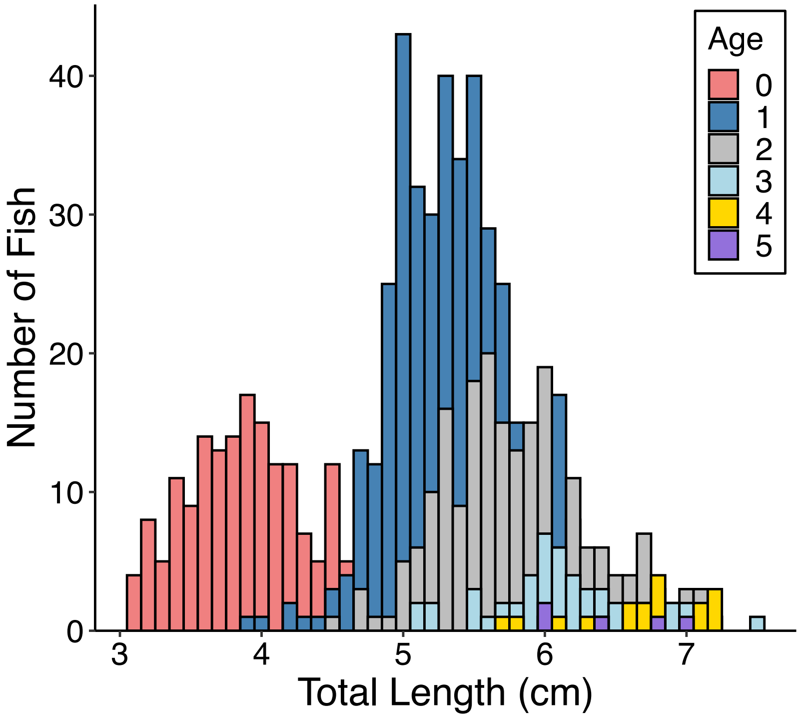


Figure A. 1. Length-frequency distribution for 2018 and 2019 pooled populations indicates significant overlap between age groups requiring aging of individual fish for growth determination.

Table A. 1. Growth parameters from von Bertalanffy growth model for back-calculated past length-at-age and observed length-at-age for the 2018 populations at the reference 2 and downstream (DSW) sites from fish collected in 2018 and 2019.

	Reference (Ref 2)		Downstream (DSW)	
	Observed	BC	Observed	BC
L_{inf}	6.6 ± 0.30	5.1 ± 0.24	7.4 ± 0.51	6.7 ± 0.66
k	0.45 ± 0.11	0.60 ± 0.39	0.46 ± 0.15	0.61± 0.33
t_0	-2.4 ± 0.45	-1.8 ± 0.80	-1.9 ± 0.52	-1.9 ± 0.72

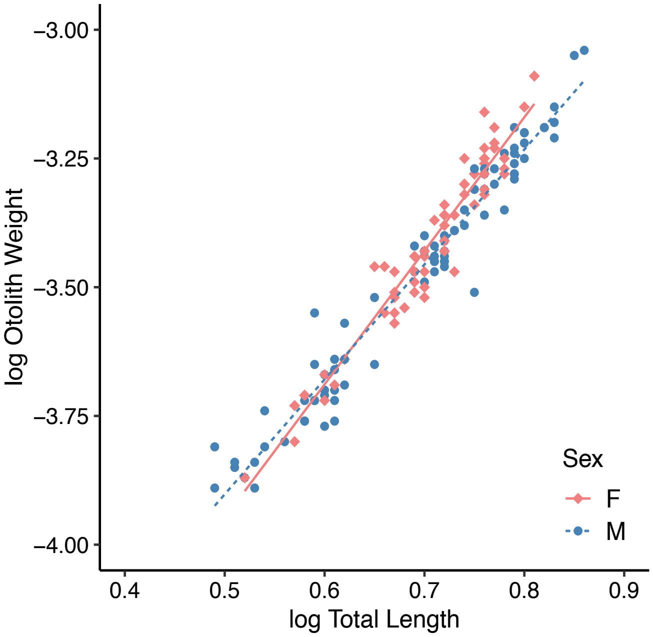


Figure A. 2. Linear regression between log otolith weight and log total length indicates a strong predictive relationship for both male and female rainbow darter (male: $r^2 = 0.956$ and female: $r^2 = 0.935$).

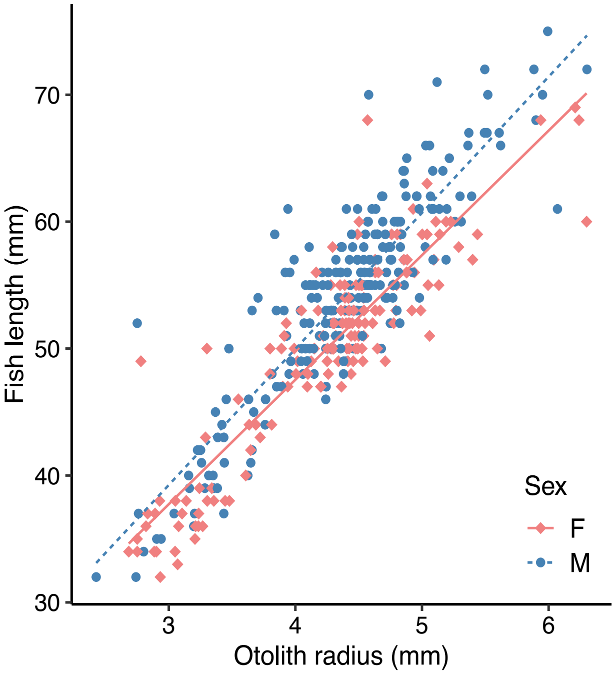


Figure A. 3. Linear regression between otolith radius and fish length exhibits a strong relationship for both male and female rainbow darter collected in 2019 at the downstream (DSW) site (Linear regression, male: $r^2 = 0.786$ and female: $r^2 = 0.816$).

Table A. 2. Summary statistics for von Bertalanffy growth curves and parameters for rainbow darter populations at different sites in 2014, 2018 and 2019. Results of ARSS are presented next to the years. An asterisk indicates a statistically significant difference between the growth curves being compared. Likelihood ratio test (x^2) also compares the overall curve in addition to the various parameters; significant differences are indicated by bolded p values.

Hypothesis	χ2	P	Hypothesis	χ2	P
2014 Males: RSS: $F_{(133)} = 3.48$ *	3.51	0.32	2014 Females: RSS: $F_{(100)} = 5.18$ *	17.4	**<0.001**
$H_0 = L_\infty$	0.35	0.55	$H_0 = L_\infty$	14.0	**<0.001**
$H_0 = k$	0.06	0.81	$H_0 = k$	13.7	**<0.001**
$H_0 = t_0$	0.65	0.42	$H_0 = t_0$	14.7	**<0.001**
2018 Males: RSS: $F_{(138)} = 7.11$ *	27.74	**<0.001**	2018 Females: RSS: $F_{(75)} = 2.37$ *	6.06	0.11
$H_0 = L_\infty$	0.05	0.98	$H_0 = L_\infty$	0.28	0.59
$H_0 = k$	2.63	0.27	$H_0 = k$	1.04	0.31
$H_0 = t_0$	3.36	0.19	$H_0 = t_0$	0.73	0.39
2019 Males: RSS: $F_{(225)} = 3.94$ *	25.8	**<0.001**	2019 Females: RSS: $F_{(146)} = 1.95$	5.63	0.78
$H_0 = L_\infty$	18.8	**0.001**	$H_0 = L_\infty$	4.48	0.21
$H_0 = k$	16.8	**0.002**	$H_0 = k$	2.83	0.42
$H_0 = t_0$	15.0	**0.002**	$H_0 = t_0$	1.55	0.67
2019 Males Ref 1 vs. Ref 2 vs. Ref 3: RSS: $F_{(164)} = 4.38$ *	19.54	**0.003**	2019 Males Ref 1 vs. Ref 2: RSS: $F_{(104)} = 5.45$ *	**14.9**	**0.002**
$H_0 = L_\infty$	16.6	**<0.001**	$H_0 = L_\infty$	**14.5**	**<0.001**
$H_0 = k$	13.8	**0.001**	$H_0 = k$	**13.2**	**<0.001**
$H_0 = t_0$	11.8	**0.003**	$H_0 = t_0$	**12.1**	**0.001**
2019 Males Ref 2 vs. Ref 3: RSS: $F_{(111)} = 2.93$ *	4.38	0.22	2019 Males Ref 1 vs. Ref 3: RSS: $F_{(113)} = 5.63$ *	15.28	**0.002**
$H_0 = L_\infty$	0.16	0.69	$H_0 = L_\infty$	13.1	**<0.001**
$H_0 = k$	0.05	0.82	$H_0 = k$	10.3	**0.001**
$H_0 = t_0$	0.02	0.89	$H_0 = t_0$	9.06	**0.003**
2019 Males Ref 1 vs. DSW: RSS: $F_{(109)} = 7.90$ *	13.25	**0.004**	2019 Males Ref 2 vs. DSW: RSS: $F_{(107)} = 7.97$ *	**7.69**	**0.053**
$H_0 = L_\infty$	9.14	**0.003**	$H_0 = L_\infty$	3.36	0.067
$H_0 = k$	2.20	0.14	$H_0 = k$	6.12	**0.013**
$H_0 = t_0$	1.60	0.21	$H_0 = t_0$	6.83	**0.009**
2019 Males Ref 3 vs. DSW: RSS: $F_{(116)} = 2.36$	5.65	0.13			
$H_0 = L_\infty$	5.25	**0.02**			
$H_0 = k$	5.59	**0.01**			
$H_0 = t_0$	4.99	**0.03**			

Table A. 3. Summary statistics for comparison of von Bertalanffy growth curves and parameters between the three years using analysis of residual sum of squares and likelihood ratio tests.

Hypothesis	$\chi2$	***P***	**Hypothesis**	$\chi2$	***P***
2018 vs. 2019 Male: Reference 2 RSS: $F_{(101)} = 0.37$	1.23	0.75	2018 vs. 2019 Female: Reference 2 RSS: $F_{(63)} = 2.58$	7.89	0.06
2018 vs. 2019 Male: Reference 3 RSS: $F_{(101)} = 0.50$	1.70	0.64	2018 vs. 2019 Female: Reference 3 RSS: $F_{(65)} = 0.06$	0.27	0.97
2018 vs. 2019 Male: DSW RSS: $F_{(103)} = 2.42$	7.43	0.06	2018 vs. 2019 Female: DSW RSS: $F_{(56)} = 2.12$	6.74	0.08
2014 vs. 2018 Male: Reference 2 RSS: $F_{(154)} = 2.70$*	9.57	0.06	2014 vs. 2018 Female: Reference 2 RSS: $F_{(104)} = 2.70$	6.43	1.0
2014 vs. 2018 Male: DSW RSS: $F_{(76)} = 2.74$*	12.15	**0.007**	2014 vs. 2018 Female: DSW RSS: $F_{(46)} = 2.81$	9.05	**0.02**
2014 vs. 2019 Male: Reference 2 RSS: $F_{(155)} = 1.85$	5.79	0.12	2014 vs. 2019 Female: Reference 2 RSS: $F_{(111)} = 2.98$	8.60	0.06
2014 vs. 2019 Male: DSW RSS: $F_{(85)} = 7.70$*	0.93	0.82	2014 vs. 2019 Female: DSW RSS: $F_{(58)} = 2.71$*	0.28	0.96

Table A. 4. Summary statistics for analysis of residual sum of squares and likelihood ratio test comparing von Bertalanffy growth curves and parameters between male and females at all the sites over the years. Significant differences are highlighted by asterisk (ARSS) and bolded p values (likelihood ratio tests).

Hypothesis	$\chi2$	**P**	**Hypothesis**	$\chi2$	**P**
2014 Male vs Female: Reference 2 RSS: $F_{(180)} = 5.88$ *	17.60	**<0.001**	2014 Male vs. Female: DSW RSS: F(53) = 2.98 *	5.80	0.12
2018 Male vs Female: Reference 2 RSS: $F_{(78)} = 1.35$	4.23	0.24	2018 Male vs Female: Reference 3 RSS: $F_{(66)} = 1.44$	4.70	0.19
2018 Male vs Female: DSW RSS: $F_{(69)} = 3.12$ *	9.55	**0.02**	2019 Male vs. Female: Reference 1 RSS: $F_{(79)} = 4.98$ *	12.65	**0.005**
2019 Male vs. Female: Reference 2 RSS: $F_{(86)} = 4.74$ *	13.9	**0.003**	2019 Male vs. Female: Reference 3 RSS: $F_{(100)} = 6.42$	18.46	**<0.001**
2019 Male vs. Female: DSW RSS: $F_{(90)} = 8.91$ *	24.79	**<0.001**			

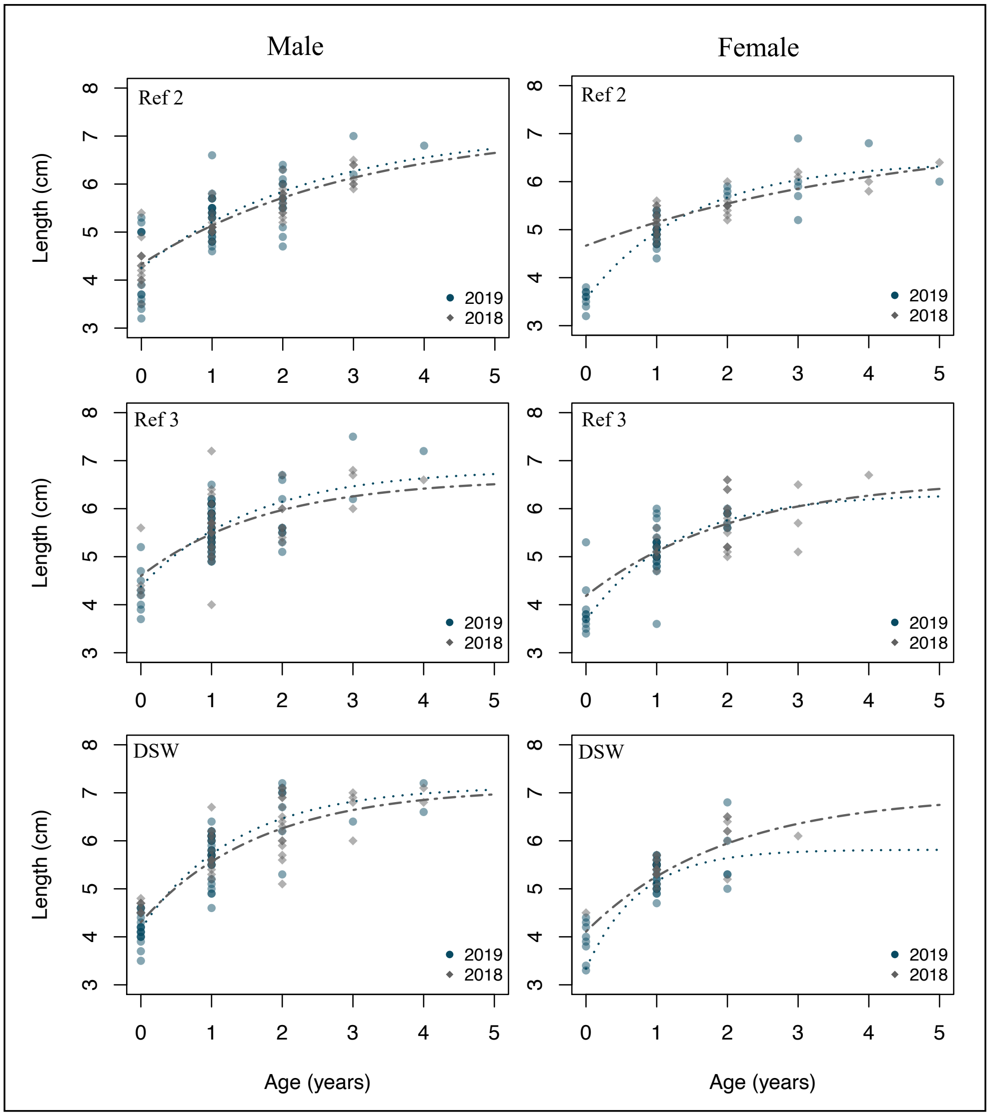


Figure A. 4. von Bertalanffy growth curves for male (left) and female (right) fish from the three sites (Ref 2, Ref 3, DSW) for fish collected in 2018 and 2019 indicate no differences in growth between the two years (ARSS: p>0.05).

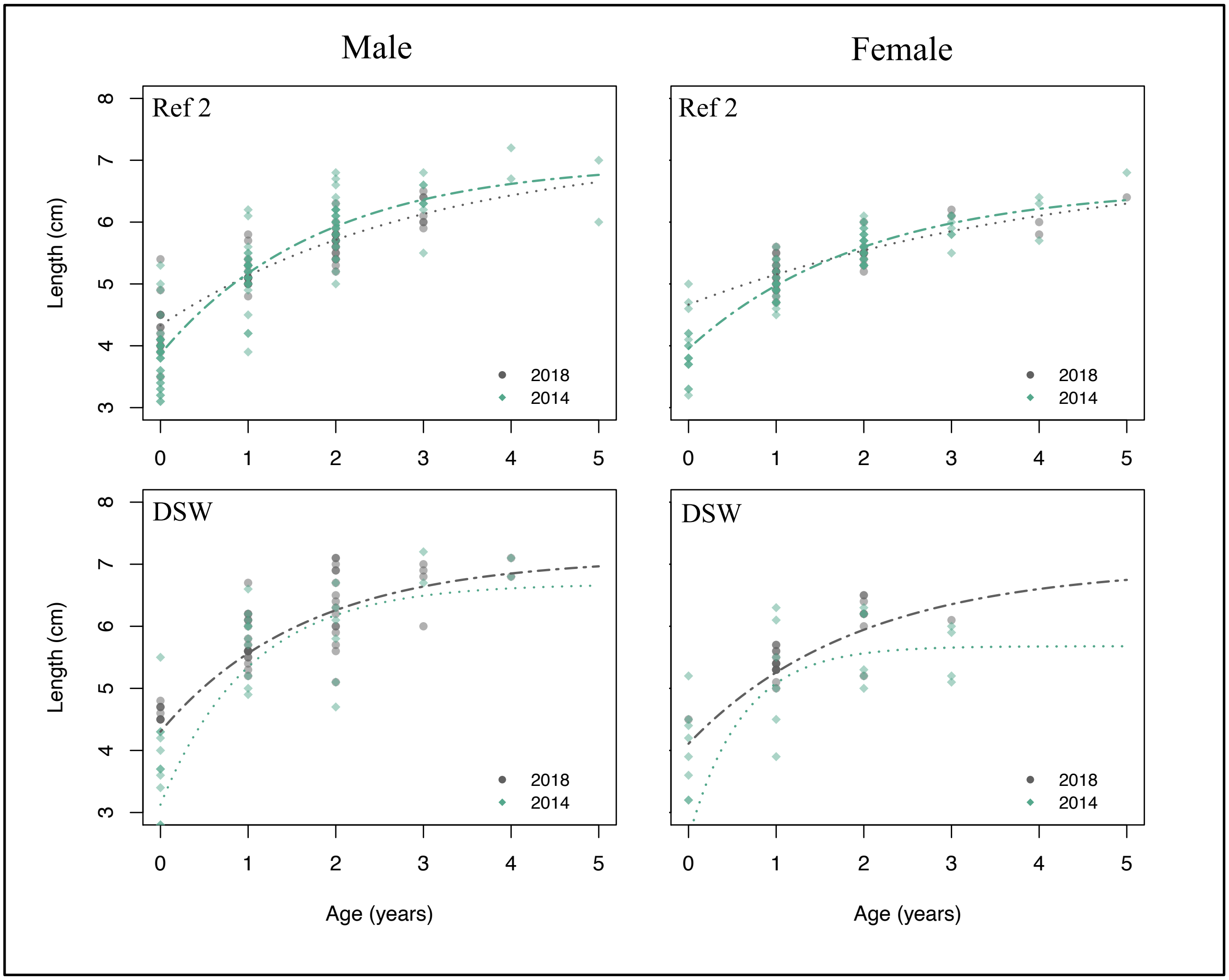


Figure A. 5. Estimated von Bertalanffy growth curves for pre- and post-upgrades comparison for male (left) and female (right) rainbow darter at the three sites (Ref 2, Ref 3, DSW) for fish collected in 2014 and 2018 indicate significant differences between years only at the downstream (DSW) site for male fish (ARSS: $p<0.05$).

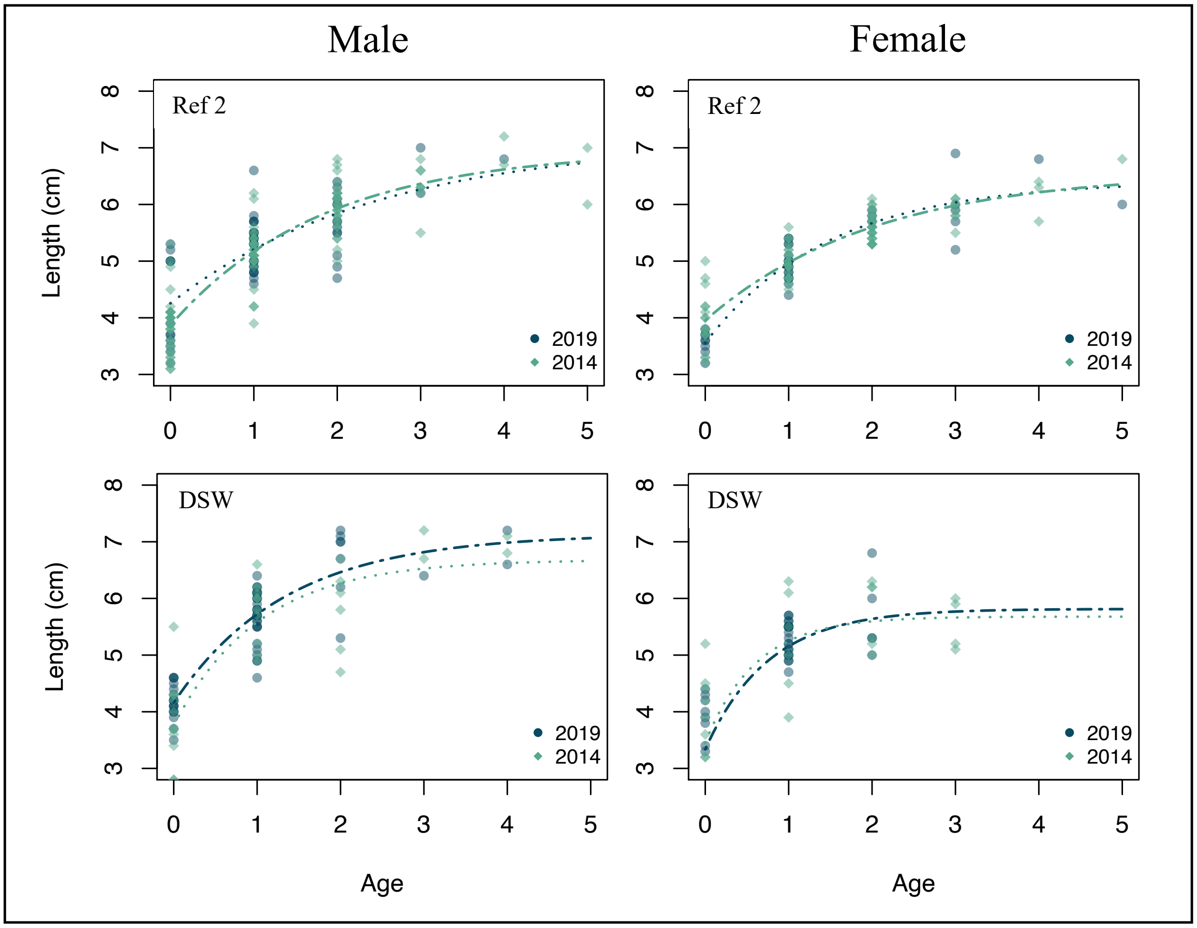


Figure A. 6. Estimated von Bertalanffy growth curves for pre- and post-upgrades comparison for male (left) and female (right) fish at the reference (Ref 2) and downstream (DSW) sites indicate significant differences for male and female fish only at the downstream site.

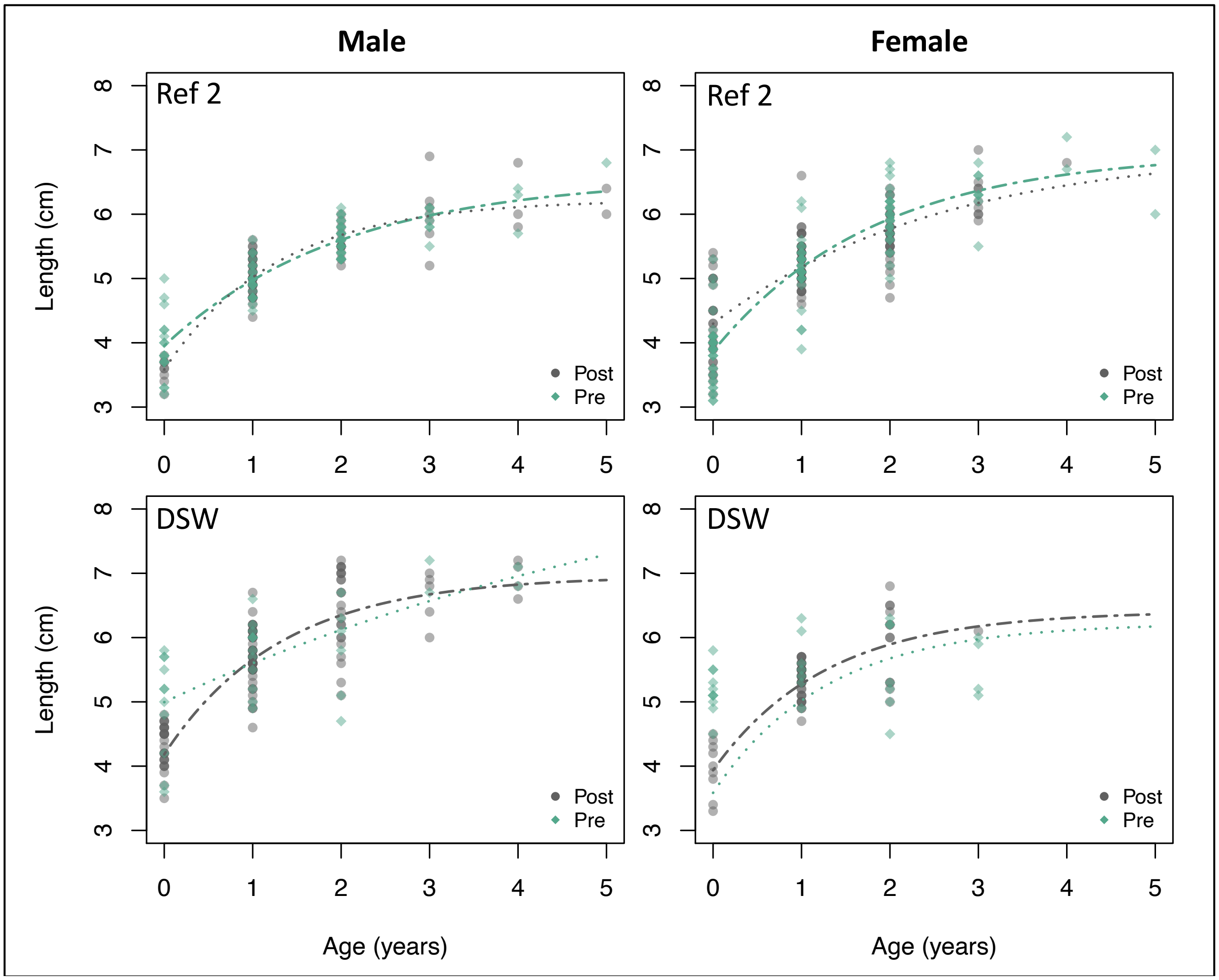


Figure A. 7. Estimated von Bertalanffy growth curves for pre- and post-upgrades comparison with pooled 2018 and 2019 data for male (left) and female (right) rainbow darter at the two sites (Ref 2, DSW). There were no differences in growth pre- and post-upgrades (LRT).

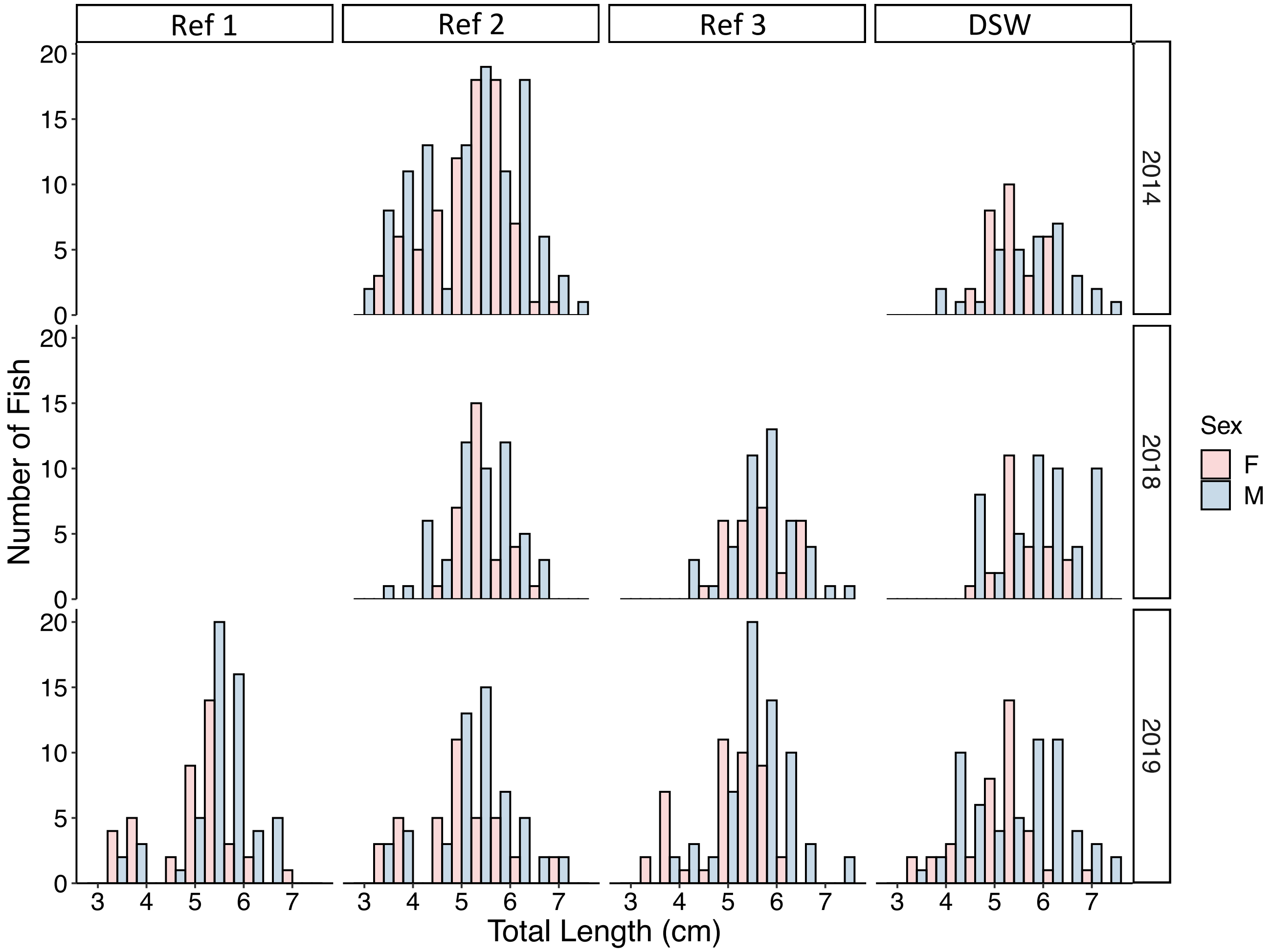


Figure A. 8. Length frequency distribution for fish collected during the three years at the various sites for both male and female rainbow darter.

Table A. 5. Linear regression results for log length and log weight relationships at the different sites for male and female rainbow darter show a strong relationship between fish length and weight.

Year	Sex	Ref 1	Ref 2	Ref 3	DSW
2014	M	-	0.962	-	0.982
	F	-	0.958	-	0.979
2018	M	-	0.959	0.917	0.978
	F	-	0.912	0.875	0.944
2019	M	0.973	0.950	0.945	0.984
	F	0.944	0.971	0.953	0.906

Table A. 6. Mean, median and standard deviation values for fish length for male and female rainbow darter population from the fall of 2014, 2018 and 2019.

	Mean		Median		St dev	
	M	F	M	F	M	F
2014	5.8	5.5	5.7	5.5	0.60	0.47
2018	5.7	5.5	5.7	5.5	0.64	0.47
2019	5.4	5.0	5.5	5.1	0.80	0.76

Table A. 7. Mean, median and standard deviation values for fish length for male and female rainbow darter population at the Reference 2 and downstream (DSW) sites from fall of 2014, 2018 and 2019.

	Mean		**Median**		**St dev**	
2014	**M**	**F**	**M**	**F**	**M**	**F**
Ref 2	5.7	5.5	5.5	5.5	0.56	0.42
DSW	6.0	5.6	6.0	5.5	0.67	0.57
2018	**M**	**F**	**M**	**F**	**M**	**F**
Ref 2	5.5	5.4	5.5	5.4	0.79	0.40
Ref 3	5.8	5.7	5.7	5.6	0.55	0.53
DSW	5.9	5.6	6.0	5.5	0.79	0.44
2019	**M**	**F**	**M**	**F**	**M**	**F**
Ref 1	5.5	5.2	5.5	5.2	0.60	0.57
Ref 2	5.2	4.9	5.3	5.0	0.83	0.73
Ref 3	5.5	5.0	5.5	5.1	0.70	0.77
DSW	5.4	5.1	5.6	5.2	0.99	0.73

Table A. 8. Results from multiple two-way ANOVA with factors age and year for various somatic indices along with Tukey's post hoc hoc tests to assess which years were different.

Endpoint	Test	Variation	DF	SS	MS	F	*p*
Length	Main test	Age	6	156.08	26.013	106.738	<0.0001
		Year	2	1.42	0.708	2.904	0.05
		Age: Year	9	8.16	0.906	3.718	0.0001
	Tukey's post hoc		Diff. of means	*p*			
		2018-2014	0.02	0.93			
		2019-2014	0.08	0.40			
		2019-2018	0.10	0.12			
Weight	Main test	Age	6	192.39	32.06	67.494	<0.0001
		Year	2	5.53	2.76	5.818	0.003
		Age: Year	9	13.38	1.49	3.129	0.001
	Tukey's post hoc		Diff. of means	*p*			
		2018-2014	0.04	0.89			
		2019-2014	0.16	0.15			
		2019-2018	0.20	0.07			
Condition Factor	Main test	Age	6	1.958	0.3264	31.242	<0.0001
		Year	2	0.189	0.0946	9.057	0.0001
	Tukey's post hoc		Diff. of means	*p*			
		2018-2014	0.02	0.14			
		2019-2014	0.005	0.88			
		2019-2018	0.01	0.2			
LSI	Main test	Age	6	1.20	0.19	0.17	<0.985
		Year	2	65.0	32.49	28.42	<0.0001
	Tukey's post hoc		Diff. of means	*p*			
		2018-2014	0.99	<0.0001			
		2019-2014	0.81	<0.0001			
		2019-2018	0.17	0.24			
GSI	Main test	Age	6	13.8	2.308	1.385	0.22
		Year	2	50.3	25.167	15.103	<0.0001
		Age: Year	9	34.5	3.829	2.298	0.01
	Tukey's post hoc		Diff. of means	*p*			
		2018-2014	0.78	<0.0001			
		2019-2014	0.89	<0.0001			
		2019-2018	0.11	0.79			

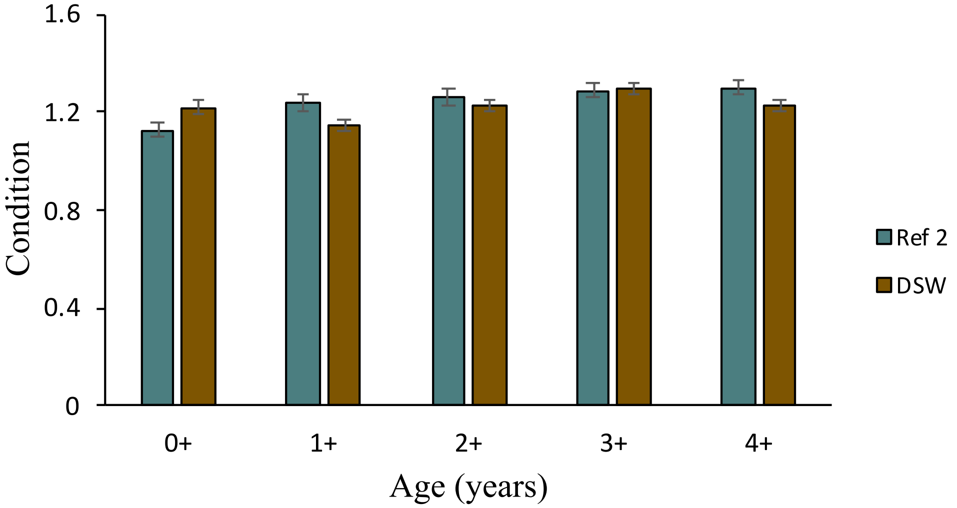


Figure A. 9. Mean condition factor (± SE) for male fish collected in 2014 at the two sites (Ref 2 and DSW) for each age group indicate no differences between sites or among age groups.

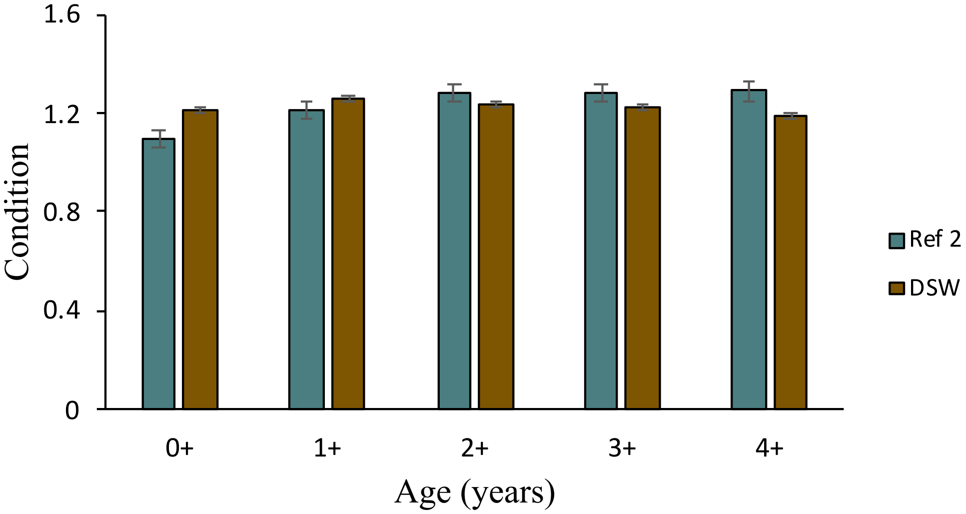


Figure A. 10. Mean condition factor (± SE) for female fish collected in 2014 at the two sites (Ref 2 and DSW) for each age group indicate no differences between sites or among age groups.

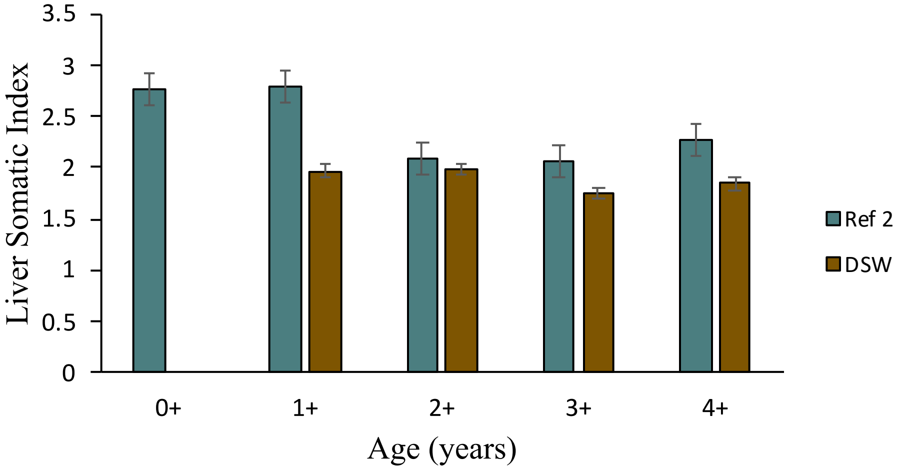


Figure A. 11. Mean LSI values (± SE) for male fish collected in 2014 at the two sites (Ref 2 and DSW) for each age group indicate no differences between sites or among age groups.

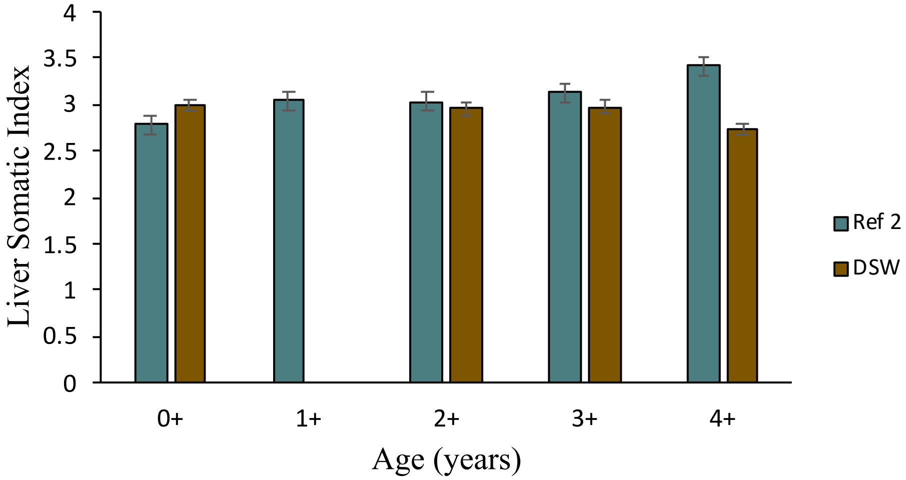


Figure A. 12. Mean LSI values (± SE) for female fish collected in 2014 at the two sites (Ref 2 and DSW) for each age group indicate no differences between sites or among age groups.

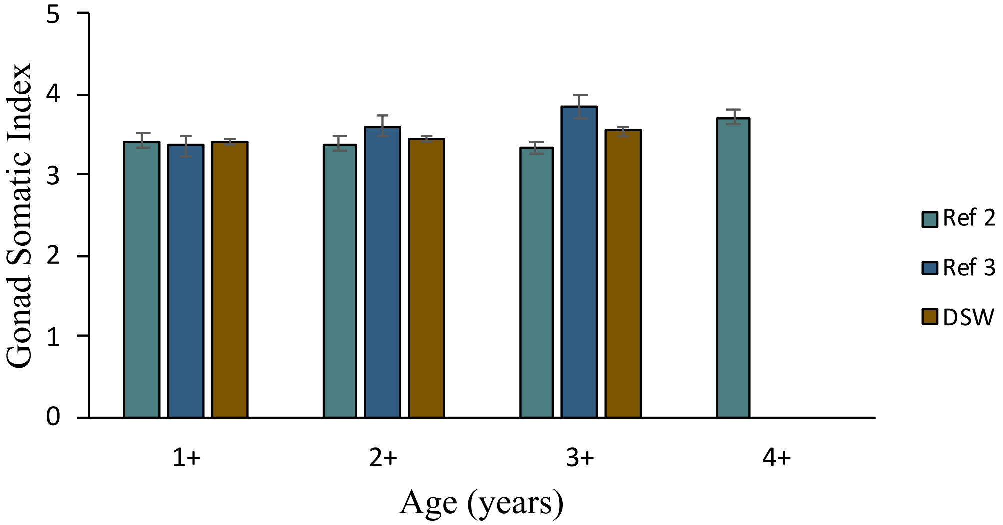


Figure A. 13. Mean GSI values (± SE) for female rainbow darter collected in 2018 and 2019 at the three sites (Ref 2, Ref 3, DSW) for each age group indicate no differences between sites or among age groups.

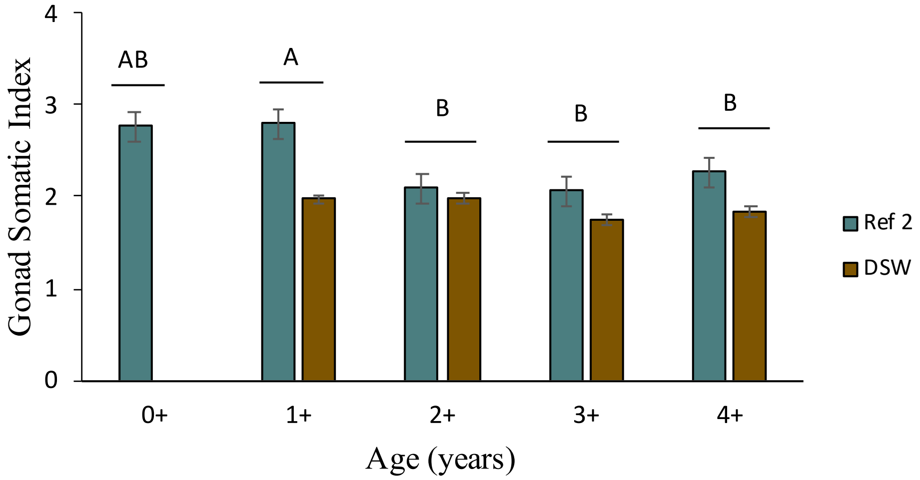


Figure A. 14. Mean GSI values (± SE) for male rainbow darter collected in 2014 at the two sites (Ref 2, DSW) for each age group. Letters indicate significant differences among age groups for pooled sites.

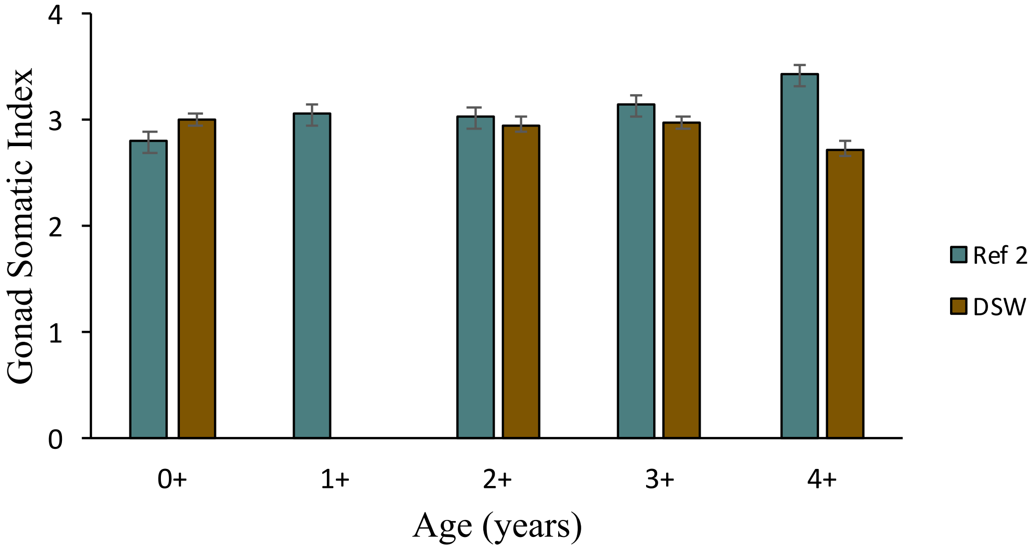
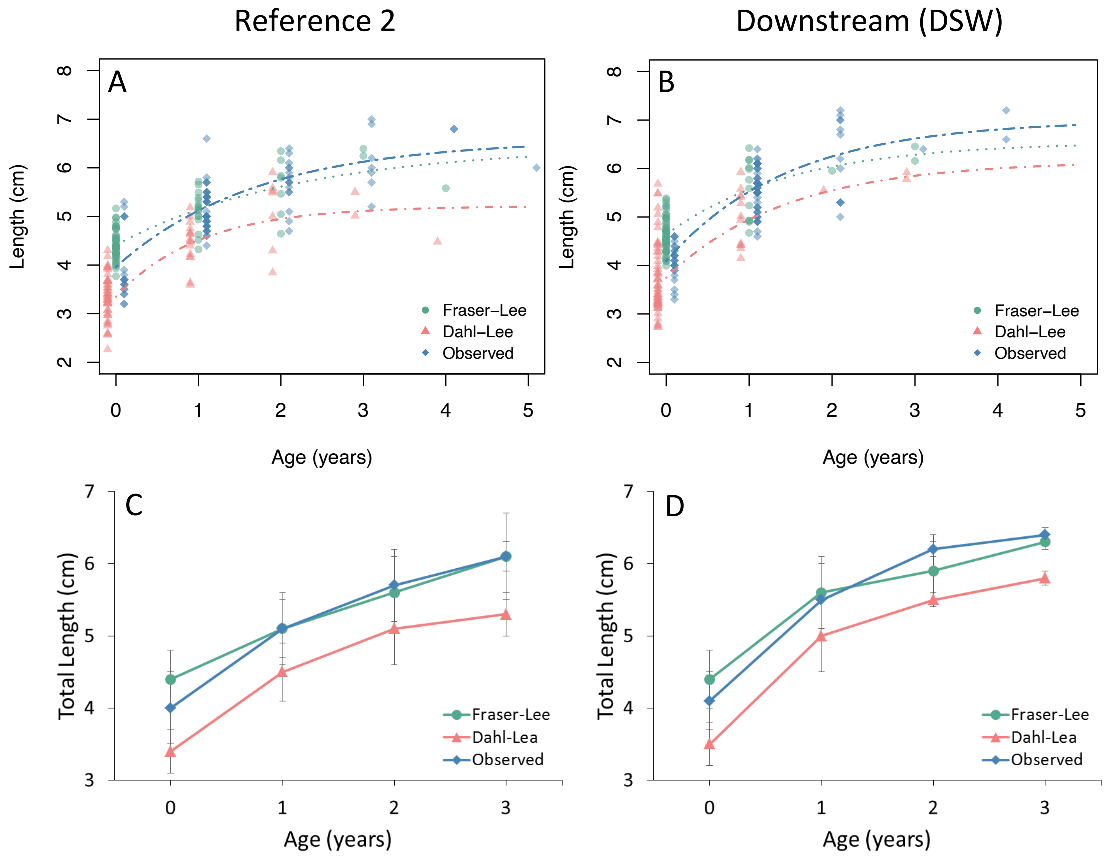


Figure A. 15. Mean GSI values (± SE) for female rainbow darter collected in 2014 at the two sites (Ref 2, DSW) for each age group indicate no differences between sites or among age groups.

Table A. 9. Standard deviation of mean length for each age at the Reference 2 and downstream (DSW) sites from observed and back-calculated methods indicate no significant differences in standard deviation across age, method or site.

		Reference 2			Downstream (DSW)	
Age	Observed	Fraser-Lee	Dahl-Lea	Observed	Fraser-Lee	Dahl-Lea
0	0.5	0.3	0.3	0.5	0.4	0.5
1	0.5	0.3	0.4	0.4	0.4	0.5
2	0.5	0.4	0.5	0.5	0.5	0.5
3	0.6	0.2	0.3	0.3	0.3	0.6



E)

	Reference (Ref 2)			Downstream (DSW)		
	Observed	Fraser-Lee	Dahl-Lea	Observed	Fraser-Lee	Dahl-Lea
L_{inf}	6.6 ± 0.35	6.5 ± 0.65	5.2 ± 0.32	7.0 ± 0.51	6.5 ± 0.59	6.2 ± 1.01
k	0.58 ± 0.15	0.44 ± 0.22	0.97 ± 0.38	0.68 ± 0.15	0.66± 0.35	0.69± 0.50
t_0	-1.6 ± 0.32	-2.6 ± 0.86	-1.1 ± 0.32	-1.3 ± 0.21	-1.8 ± 0.69	-1.4 ± 0.67

Figure A. 16. Comparison of back-calculated lengths and observed lengths for the 2018 populations at the Reference 2 (left) and downstream (DSW)(right) sites. von Bertalanffy growth curves for Fraser-Lee and Dahl-Lea back-calculated lengths and observed lengths at the Reference 2 (A) and downstream (DSW)(B) sites indicate comparable growth curves for Fraser-Lee method and observed population while Dahl-Lea equation significantly underestimates length at each age. Mean back-calculated and observed lengths (± SD) at each age at the Reference 2 (C) and downstream (D) indicates suitability of the Fraser-Lee method for rainbow darter; back-calculated lengths using the Fraser-Lee method are similar to the observed lengths while lengths obtained from the Dahl-Lea equation underestimate length at each age. E) von Bertalanffy growth parameters from observed and back-calculated lengths reveal similar growth rate (k) at the downstream (DSW) site for the three curves while maximum length (L_{inf}) is underestimated using the Dahl-Lea equation but not the Fraser-Lee method.

Table A. 10. Back-calculated (BC) length for the 2018 population from fish collected in 2019 indicate the reverse of Lee's phenomenon with BC length increasing with increasing age.

	Back-calculated length-at-age (cm)			
	0	1	2	3
Age	2018 year-class			
1	4.6	5.1	5.5	-
2	4.7	5.2	5.6	-
3	4.7	5.3	5.7	6.0
4	4.8	5.4	5.8	6.1

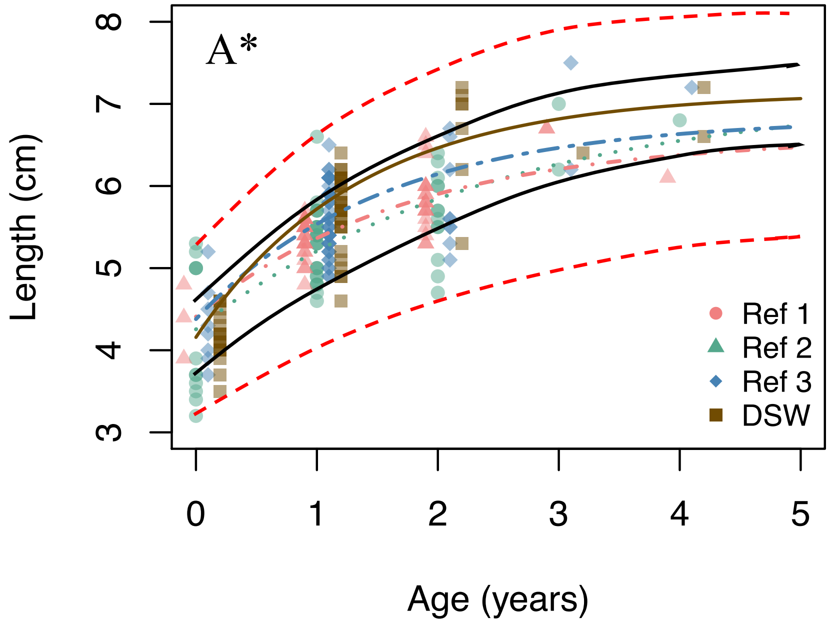


Figure A. 17. von Bertalanffy growth curves for male rainbow darter collected in 2019 at the four sites (Ref 1, Ref 2, Ref 3, DSW). The black solid and red dashed lines represent a difference of 10% and 25%, respectively, in mean length from the Reference 2 site.

Table A. 11. GPS coordinates of the four sites where fish were sampled.

GPS coordinates	Ref 1	Ref 2	Ref 3	DSW
Latitude (N)	43°37'52.1"	43°35'9.8"	43°30'17.2"	43°28'25.4"
Longitude (W)	80°26'33.5"	80°28'42.4"	80°28'30.6"	80°28'25.5"