Age Determination and Growth of Rainbow Darter (*Etheostoma caeruleum*) in the Grand River, Ontario

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

The accurate determination and validation of age is an important tool in fisheries management. Age profiles allow insight into population dynamics, mortality rates and growth rates, which are important factors in many biomonitoring programs, including the Canadian Environmental Effects Monitoring (EEM) program. Many monitoring studies in the Grand River, Ontario have focused on the impact of municipal wastewater effluent (MWWE) on fish health. Much of the research has been directed at understanding the effects of MWWE on responses across levels of biological organization. The rainbow darter (*Etheostoma caeruleum*), a small-bodied, benthic fish found throughout the Grand River watershed has been used as a sentinel species in many of these studies. Although changes in somatic indices (e.g. condition, gonad somatic indices) have been included in previous studies, methods to age rainbow darters would provide additional tools to explore impacts at the population level. The objective of the current study was to develop a method to accurately age rainbow darter, validated by use of marginal increment analysis (MIA) and edge analysis (EA) and to characterize growth of male and female rainbow darter at a relatively unimpacted site on the Grand River. Rainbow darter were collected from the Grand River at West Montrose on a monthly basis (May 2014 - June 2015). Size (length, weight) and gonad/liver weight were recorded, and left and right sagittal otoliths were collected. Length-frequency distributions were constructed for the darter population in July and October 2014 to assess population structures.

Darters spawn in the Grand River in late April-early May and young-of-the-year (YOY) darters reached a catchable size, using backpack electro-shockers, by July. A distinct YOY cohort was apparent in the July length-frequency distribution; YOY ranged in length from 1.2–2.5 cm. By

October the length-frequency distribution demonstrated that the YOY had started to merge into the other age classes. Direct age determination (using sagittal otoliths) of a subset of the October collections supports that YOY fish are no longer a distinct cohort on the length-frequency distribution, and have assimilated into the rest of the population by this time of year. Direct age determination of fish at this time of the year is therefore necessary to separate age classes.

Examination of rainbow darter otoliths collected monthly was used to validate the use of this structure for accurate age estimation. MIA showed that one annulus was formed per year on sagittal otoliths, and that summer (opaque) growth zone formation began in early summer. EA was able to identify the timing of both summer growth zone and annulus (translucent zone) formation. Summer growth zone formed as early as April, with all fish exhibiting growth by July. Annulus formation was noted in some fish in September, and in all fish by November.

Size-at-age data resulting from the October length-frequency subsampled fish showed differences between male and female rainbow darter. Young fish, both male and female, grow quickly in the first two years (ages 0+ and 1+) and exhibit similar mean length and weight-at-age. Beginning at age 2+ and in each older age group, male rainbow darter become significantly longer and heavier at age compared to females. Additionally, male fish continued to increase significantly in weight each year, with no apparent decrease in weight gain, whereas females did not gain weight significantly after the age of 2+. Estimated von Bertalanffy growth curves for male and female length-at-age relationships further emphasize the difference in male and female growth beginning at age 2+. Furthermore, this model predicted male maximum length to be greater than that of female fish (male: L_{inf}=7.42; female: L_{inf}=6.48). Liver and gonadosomatic indices collected each month indicate increased energy allocation into liver and gonad development in female fish for reproductive purposes, which may account for the difference in male and female size (length/weight) in older age cohorts.

This study has contributed to our understanding of the aging and growth of a small-bodied fish species that is widespread in North America. An accurate and reliable method to age rainbow darter was validated and the knowledge necessary for the addition of growth into biomonitoring studies was established for using rainbow darter as a sentinel species. The ability to accurately estimate age in rainbow darter provides the opportunity to assess growth as an additional population level endpoint in ongoing studies in the Grand River and in other watersheds that are experiencing environmental change.

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Having grown up in Northwestern Ontario, fishing at every possible opportunity with my family, I was accustomed to working with big fish. The members of the Servos Lab introduced me to the small-bodied fish of the Grand River, teaching me all the identification and sampling skills I needed to be successful in the field.

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Chapter 1

Introduction

The ability to accurately estimate age in fish has been an important tool in fisheries management for many years. Age estimation allows insight into population dynamics, mortality rates and growth rates, which are important factors in many biomonitoring programs and fisheries management plans (Beamish and McFarlane, 1987; Gray*, et al.*, 2002). There are various methods of age determination and validation, and the appropriate method is highly dependent on the life-history tactics and physiology of the species under investigation (Campana, 2001). Numerous studies have recently been conducted on the impacts of municipal wastewater on fish in the Grand River Ontario (e.g., Tetreault*, et al.*, 2011). The rainbow darter (*Etheostoma caeruleum*), a small-bodied species widely distributed in the Grand River and its tributaries, has been used as a sentinel species for many of these studies. Despite the large amount of research surrounding this species, a comprehensive study focusing on assessing the impacts of wastewater on fish growth has not been conducted. This is partly due to the absence of a validated method for estimating rainbow darter age in this system. The ability to age this species reliably would provide additional tools to examine how wastewater and other stressors impact rainbow darter populations in this river, which is highly influenced by agricultural runoff and urbanization. It would support the use of this species, as well as other small bodied fish (e.g. other darters), in environmental research and monitoring by creating a baseline of knowledge on their growth that can widely applied. This thesis is focused on filling this important knowledge gap so that better environmental assessments can be done on the impacts of specific and cumulative stressors across watersheds and support the evaluation of effective remedial actions (e.g. wastewater treatment upgrades).

1.1 Fish Age Determination Methods

Numerous methods have been developed to accurately estimate fish age including lethal and non-lethal approaches (Campana, 2001). Often the method chosen is dependent on the species of fish in question, however there are many factors that must be considered when attempting to choose the appropriate method. A common non-lethal method of age estimation is the construction of length-frequency histograms, which can be useful for young, fast-growing fish (Campana, 2001). Length-frequency histograms can provide insight into separate age cohorts, however if assimilation of age groups occur, they can become increasingly difficult to apply (Gray*, et al.*, 2002). One of the most common methods of age estimation is the analysis of periodic growth increments formed on calcified structures such as scales, vertebrae, cliethra, dorsal/pectoral fin rays and otoliths (Sikstrom, 1983; Casselman, 1990; Francis*, et al.*, 2001). Scales and dorsal spines can be obtained without sacrificing the fish, however these structures may not provide an accurate estimation of age (Koenigs*, et al.*, 2015). Scales and otoliths are the most commonly used structures for age estimation. A large amount of research has employed these methods, however the reliability of scales to correctly assess age has been questioned in the past and is species specific (Beamish and McFarlane, 1983; Sikstrom, 1983; Beamish and McFarlane, 1987). Beckman (2002) concluded that scales underestimated age of rainbow darter (*Etheostoma caeruleum*) in southwest Missouri when compared to sectioned or whole otoliths, suggesting that the analysis of otoliths provide a more accurate age estimate.

When attempting to estimate the age of small-bodied fish, researchers are presented with several challenges. These species often grow relatively quickly, limiting the use of length-frequency distributions to young cohorts (Taber and Taber, 1983). Additionally, in times of high stress and food deprivation, resorption of scales has been reported, whereas otoliths grow continuously in similar conditions (Campana, 1983; Campana and Neilson, 1985). The enumeration of periodic growth increments follows the same principal as dendrochronology, the estimation of tree age based upon the knowledge that rings form in a predictable annual pattern (Gutsell and Johnson, 2002). Unlike the formation of growth rings in trees, rings in calcified structures in fish do not always form annually (Beckman and Wilson, 1995) and therefore the validation of the periodicity and timing of zone formation, otherwise referred to as age validation, is necessary (Campana, 2001). Numerous stimuli contribute to the formation of growth zones on calcified structures. Otolith microstructure research has investigated if elemental variation can be used reliably to assess annual and daily incremental growth (Campana, 1999). Annual changes in strontium:calcium ratios have been reported (Radtke and Targett, 1984), however other studies have found no annual correlation (Fuiman and Hoff, 1995). Annual increment formation has been linked to abiotic conditions such as annual variations in water temperature as well and biotic factors including reproductive cycles and feeding habits, each impacting elemental composition through different mechanisms; however a universal stimulus has not been identified (Beckman and Wilson, 1995; Campana, 1999). Differences in the number of annuli formed each year and the timing of annulus formation has been recorded among different populations of the same fish species (Williams et al., 2005; Winker et al., 2010). For example, red throat emperor (*Lethrinus miniatus*) from a southern region of the Great Barrier Reef showed a clear annual periodicity in the formation of opaque zones compared to fish collected from a northern region, which formed annuli in a more ambiguous pattern (Williams et al., 2005). Furthermore, opaque increments formed one month earlier in the southern region compared to the northern site (Williams et al., 2005). The number of growth zones formed on otoliths each year has also been found to differ between populations of the same fish species. Validation of increment formation in asteriscus otoliths collected from a population of common carp (*Cyrpinus carpio*) in Lake Gariep, South Africa, provided evidence of biannual formation of growth zone formation, contradicting previously validated annual formation in astericus otoliths of a population of carp in the Murray-Darling Basin, Australia (Winker et al., 2010). Variations in the timing and periodicity of growth zone formation in multiple populations of a species of fish suggests that validation should be performed as a component of all age and growth studies focusing on a previously unstudied population, however this is often not feasible or practical. Variations can be attributed to a variety of factors, and therefore if a high degree of disparity exists within these factors among populations of fish, validation may be necessary to ensure the accuracy of age estimates.

Darters are a group of small-bodied, benthic fish in the family Percidae that includes approximately 150 species in eastern North America (Paine, 1990). Numerous studies have been conducted to elucidate their life history, including age determination and growth (Table 1.1). In these studies, the main focus was predominantly the construction of length-frequency histograms and subsamples of otoliths or scales were often collected for direct age determination. Non-lethal methods were preferred in studies on threatened species, where the removal of fish was undesirable; aging structures in these studies were often taken from incidental mortalities as a result of fish capture (Finch, *et al.*, 2013) which limits the availability of samples. In many studies, young-of-year (YOY) darters reach catchable size by July for species that spawn in the spring (Layman, 1991; Drake*, et al.*, 2008).

Otoliths have been identified as a reliable structure for accurate age estimation in darters and other small-bodied fish, and they have the ability to provide additional information, such as the variation in size (i.e. length/weight) at age, that cannot be obtained from non-lethal methods such as length-frequency distributions (Beckman, 2002; Robinson*, et al.*, 2010; Simmons and Beckman, 2012).

There are a variety of methods for preparing otoliths for analysis, and the most appropriate method is often dependent on the size and shape of the otoliths being used. For small otoliths that are

Table 1.1 Studies conducted on darter species employing an age estimation technique.

Species	Location	Method of Age Estimation	Age/Size at Sexual Maturity	Spawning Season	Maximum Age Reported	Citation
Bayou Darter *Etheostoma rubrum*	Bayou Pierre System, Mississippi	Length frequency histogram	Not reported	April-June	3	(Slack*, et al.*, 2004)
Bluemask Darter *Etheostoma akatulo*	Collins River, Tennessee	Length frequency histogram	Male: >41 mm Female: >40 mm	May-July	3	(Simmons*, et al.*, 2008)
	Rocky River, Tennessee	Length frequency histogram	Not reported	May-July	3	
Cherokee Darter *Etheostoma scotti*	Hickory Log Creek, Georgia	Scale annuli	Male: 11 mo. Female: 11 mo.	April	2	(Barton and Powers, 2010)
Duskytail Darter *Etheostomapercnurum*	Little River, Tennessee	Scale annuli Length frequency histogram	Male: 1 Female: 1	April-May	2	(Layman, 1991)
Eastern Sand Darter *Ammocrypta pellucida*	Lower Thames River, Ontario	Scale annuli Otolith annuli	Not reported	Not reported	4	(Drake*, et al.*, 2008)
Eastern Sand Darter *Ammocrypta pellucida*	Lower Thames River, Ontario	Scale annuli Otolith annuli	Male: 1+ Female: 1+	April-June	3+	(Finch*, et al.*, 2013)
	Little Muskingum River, Ohio	Scale annuli	Male: 1+ Female: 2+	Not reported	2+	
Florida Sand Darter *Ammocrypta bifasicia*	Blackwater River Drainage, Florida	Length frequency histogram	Male: 43 mm Female: 38 mm	April-July	3	(Heins, 1985)
Johnny Darter *Etheostoma nigrum*	North and South River Systems, Colorado	Length frequency histogram	Not reported	Not reported	3	(Propst and Carlson, 1989)
Least Darter *Etheostoma microperca*	Dinner Creek, Minnesota	Scale annuli	Male: 1 Female: 1	May-June	37 Months	(Johnson and Hatch, 1991)
Missouri Saddled Darter *Etheostoma tetrazonum*	Pomme de Terre River; Niangua River, Missouri	Scale annuli	Male: 2 Female: 1	March-June	4	(Taber and Taber, 1983)
Ontario Channel Darter *Percina copelandi*	Salmon River, Ontario	Otolith annuli	Not reported	April-June	4	(Reid, 2004)
	Trent River, Ontario	Otolith annuli	Not reported	April-June	5	
Orangefin Darter *Etheostoma bellum*	South Fork Green River, Kentucky	Length frequency histogram Scale annuli	Male: 1 Female: 2	May-June	3	(Fisher, 1990)
Savannah Darter *Etheostoma fricksium*	Tinker Creek, South Carolina	Scale annuli Length frequency histogram	Male: 1 Female: 1	February-May	4	(Layman, 1993)

Rainbow Darter *Etheostoma caeruleum*	James River, Missouri	Otolith annuli Scale annuli	Not reported	Not reported	5	(Beckman, 2002)
Spottail Darter *Etheostoma squamiceps*	Big Creek, Illinois	Scale annuli	Male: 1 Female: 1	March-May	3+	(Page, 1974)
	Ferguson Creek, Kentucky	Scale annuli	Male: 1 Female: 1	March-May	3+	
Stippled Darter *Etheostoma punctulatum*	Spring River, Missouri	Scale annuli	Male: 1 Female: 1 (if >49 mm)	February-May	4+	(Hotalling and Taber, 1987)
Tessellated Darter *Etheostoma olmstedi*	Mill River; Wading River; Swift River; Connecticut River, Massachusetts	Scale annuli	Not reported	Not reported	3	(Layzer and Reed, 1978)
Trispot Darter *Etheostoma trisella*	Conasauga River, Tennessee	Length frequency histogram Scale annuli	Male: 1 Female: 1	January-May	2+	(Ryon, 1986)
Vermilion Darter *Etheostoma chermocki*	Black Warrior River System, Alabama	Length frequency histogram Otolith annuli	Not reported	March-June	3	(Khudamrongsawat, *et al.*, 2005)
Waccamaw Darter *Etheostoma perlongum*	Lake Waccamaw, North Carolina	Scale annuli Otolith annuli Length frequency histogram	Not reported	March-June	1+	(Shute, *et al.*, 1982)
Yoke Darter *Etheostoma juliae*	James River, Missouri	Scale annuli	Male: >30 mm Female: 1 (>32 mm)	May	3	(James and Taber, 1986)

thin enough to allow light to pass through, it is possible to use whole otoliths without processing them in any way (Simmons and Beckman, 2012). When the use of whole otoliths is not possible, sectioning, sanding or breaking otoliths can make growth increments more prominent (Campana and Neilson, 1985; Sequeira*, et al.*, 2013).

1.2 Fish Age Validation Methods

Age validation can refer to the validation of the frequency of the formation of a growth increment (i.e. annulus), or absolute age validation, which is only accepted when validation is completed for all age classes (Campana, 2001). In a survey of 500 published studies that dealt with age estimation conducted by Beamish and McFarlane (1983), 170 studies did not attempt validation at all while only 17 studies successfully validated the age of all age classes reported. A number of methods have been developed to validate fish age, however as with age estimation, the method best suited is often dictated by the species being researched, more specifically, their life-history and physiology (Table 1.2). Some methods are designed to validate the age of large, long-lived species while others are better suited for small-bodied, short-lived species, and choosing the appropriate method is paramount to accurately assessing timing and periodicity of growth zone formation in calcified structures such as otoliths (Campana, 2001). Methods such as the release of known-age fish into the wild, and mark-recapture of chemically tagged wild fish, are widely used. These methods, however, rely upon the ability to recapture fish, which can be difficult in species with large geographic home range, mortality due to high predation and short life spans.

The consequences of improper, or the disregard of, age validation can be great and could potentially lead to the misinterpretation of impacts on fish populations or the incorrect management of fisheries. The life history of the highly studied white sucker, *Catostomus commersoni*, was

Table 1.2 Commonly used methods for fish age validation (adapted from Campana, 2001).

Method	Annual (A) Daily (D)	Age Range	Description	Precision	Sample Size Necessary
Bomb Radiocarbon	A	All	- validates absolute age and periodicity of growth increment formation - ideal for long-lived species - measures ^{14}C in otoliths - a proportion of sample fish must have been born prior to 1965 - expensive method	± 1-3 years	20-30
Captive rearing from hatch	AD	All	- validates absolute age and periodicity of growth increment formation - laboratory conditions rarely resemble natural systems, and therefore results seldom mimic those seen in wild fish	± 0 years	> 1
Capture of wild fish with natural date-specific markers	AD	All	- validates periodicity of growth increment formation and sometimes absolute age - relies on a large-scale event that applies a dated mark to all fish, which are infrequent	± 0 years	> 1
Marginal increment analysis	A	All	- validates the periodicity of growth increment formation - examines the growing edge of the aging structure throughout a year to determine when growth increments form - ideal for fast-growing and/or young fish	± 1 year	> 100
Mark-recapture of chemically tagged wild fish	AD	All	- validates periodicity of growth increment formation - uses calcium binding chemicals, such as oxytetracyline, to create a permanent mark on aging structures - the number of growth increments formed after chemical tagging can be compared to time	± 1 year	> 1
Progression of discrete length mode sampled for age structures	AD	0-5 years	- ideal for validating the first 1-2 age classes - length modes cannot overlap - monitor the progression of modes over a year to determine whether modes correspond to age classes	± 0 years	> 100
Radiochemical dating	A	5+ years	- validates absolute age - ideal for long-lived species - measures the occurrence of naturally occurring radioisotopes in otolith cores	± 25-50%	10-50
Release of known age and marked fish into the wild	AD	All	- validates absolute age and periodicity of growth increment formation - requires known-age fish - ideal for short-lived fish (>10 years) - fish spend the majority of their lives in natural conditions	± 0 years	> 1

misunderstood for many years due to the validation of age for only young fish (Beamish and McFarlane, 1983). The commercial fishery worth millions of dollars annually for Pacific Ocean perch (*Sebastes alutus*) off the coast of western Canada was severely impacted when fisheries management plans were designed around the misunderstanding that this species was relatively short-lived and fast-growing when it was not (Beamish and McFarlane, 1983).

Marginal increment analysis (MIA) is a commonly used method of annual increment validation (Campana, 2001). The method is founded on the assumption that if growth increments are formed yearly, the state of completion of the currently forming increment will present as a sinusoidal cycle when plotted against sampling month (Campana, 2001). As Campana (2001) pointed out, MIA can be a challenging method to execute properly due to difficulties associated with viewing the growing edge of structures using variable light sources. MIA is a particularly effective method of age validation in young, fast-growing fish. Caution must be taken when attempting to assign ages to older fish when validation was conducted for younger cohorts only (Campana, 2001). Numerous studies have successfully used MIA to validate the periodicity of otolith growth increment formation in small-bodied species, making it a useful method to employ in darter species (Scheerer and McDonald, 2003; Johnson and Belk, 2004; Houston and Belk, 2006). Edge analysis is a similar validation method to MIA, however it does not include the use of otolith measurements. In place of measurements, edge analysis simply reports the condition of the growing edge of the otolith as either translucent or opaque (Labropoulou and Papaconstantinou, 2000). Some studies have also reported the degree of completion of growth zones (Beckman, 2002), however due to the lack of mathematical support, this is more subjective. As validation methods, both MIA and edge analysis are well suited to assess the season or month of annulus formation, particularly in young, fast-growing fish species (Campana, 2001).

1.3 Monitoring Programs

The ability to accurately estimate age in fish is key to interpreting fish growth and in turn to compare growth and size-at-age of fish from numerous sites. Various monitoring programs use growth as an indicator of energy utilization, including the Environmental Effects Monitoring program (EEM) developed by Environment Canada to assess the impacts of pulp and paper mill and metal mine effluent on receiving environments (Munkittrick*, et al.*, 2002; Munkittrick*, et al.*, 2010). Growth in fish indicates their ability to utilize and store energy acquired from food within the system and is characterized by the change in length or weight over time (e.g., Munkittrick*, et al.*, 2010). Alterations in growth can have implications for survival, age of first reproductive season and condition (e.g., Fraker*, et al.*, 2002; Munkittrick*, et al.*, 2010). Condition (ratio of body weight to length^3) provides valuable information on the quality and availability of food for fish and is often used as a surrogate indicator of energy storage (Gray*, et al.*, 2002; Munkittrick*, et al.*, 2010). It does not, however, provide specific information regarding how fish grow throughout their lives, usually focusing on length-weight relationships in adult fish, and therefore comprehensive growth studies remain a necessary component of many monitoring programs.

A component of the EEM program focuses on fish health and incorporates a variety of indicators including survival (age), weight-at-age, relative gonad and liver weight, and condition (Munkittrick*, et al.*, 2010). A difference (critical effect size) of greater than 25% in many of these parameters, and greater than only 10% in condition factor, between fish collected from an effluent exposed site and a reference site has been proposed to indicate effects (Kilgour*, et al.*, 2005). Kilgour et al., (2005) suggested that changes detected at this level should trigger additional monitoring in following years. Accurate age data is necessary for survival and size-at-age analysis, and therefore the ability to estimate fish age is vital to the incorporation of these endpoints into monitoring programs. This emphasizes the importance of identifying a method capable of accurately estimating age of fish species used in monitoring programs focusing on impact assessment of effluents on aquatic systems and, more specifically, fish health.

The use of small-bodied fish in monitoring programs is becoming more common for numerous reasons mainly related to life history characteristics. Small-bodied fish are often more abundant within a system and less mobile, leading to increased ease of collection using standard fish collection methods (Minns, 1995; Munkittrick*, et al.*, 2002). Larger fish species, which have been the main focus of numerous past and present monitoring studies, are likely more mobile and able to migrate large distances, which may lead to the movement into and out of areas impacted by effluent input (Swanson*, et al.*, 1994). Body size is positively correlated with home range size and therefore small-bodied fish have typically exhibited much more confined home ranges due to limited mobility (Minns, 1995). The more sedentary nature of smaller-bodied fish is a very useful characteristic to consider during the development of a monitoring program, particularly when there are no physical barriers between the sites being compared (Gibbons*, et al.*, 1998b). Small-bodied fish also tend to respond more quickly to environmental changes, which make them ideal for impact assessment (Gibbons*, et al.*, 1998b). A study conducted by Gibbons *et al.* (1998a) demonstrated the use of spoonhead sculpin (*Cottus ricei*) as a useful sentinel species in monitoring the effects of a bleached-kraft pulp mill on the Athabasca River, Alberta. Exposure to effluent led to increased condition, size-at-age and reproductive alterations such as increased gonad size and egg weight (Gibbons*, et al.*, 1998a). In addition, small- bodied fish are often more numerous, easily collected, and are not commercially exploited. This further illustrates the importance and relative ease of incorporating small-bodied fish into monitoring programs. However, caution must be used in the selection of a sentinel species because even small-bodied species of fish, such as darters, may be very mobile during some periods of their life history (K. Hicks, University of Waterloo, personal communication).

1.4 The Grand River Watershed

The Grand River Watershed is the largest watershed in southern Ontario, Canada that drains into Lake Erie. Approximately 70% of the watershed is devoted to agriculture, evenly split between croplands (e.g. corn, soy beans, hay) and livestock (e.g. cattle, chicken) cultivation (Grand River Watershed Water Management Plan, 2014). The remaining 30% of the watershed is shared between urban, forested and wetland areas. The population of the watershed in 2013 was nearing 1 million people, with significant population growth predicted over the next 25 years (Grand River Watershed Water Management Plan, 2014). The largest cities include Kitchener, Waterloo, Guelph, Brantford and Cambridge, all located in the central reaches of the watershed. Currently there are 30 municipal wastewater treatment plants (MWWTP) delivering effluent into the Grand River and its tributaries, which has led to concerns regarding the impact of municipal wastewater effluent (MWWE) on drinking water sources and aquatic ecosystem health (Grand River Watershed Water Management Plan, 2014).

Numerous studies have assessed the impacts of MWWE on fish health in the Grand River (Tetreault*, et al.*, 2011; Tetreault*, et al.*, 2013; Fuzzen*, et al.*, 2015). The potential effects of effluent inputs into aquatic environments are numerous. Increased loading of nutrients can cause eutrophication, which promotes macrophyte and algae growth (Carpenter*, et al.*, 1998; Holeton*, et al.*, 2011) and altered food web dynamics (Loomer*, et al.*, 2015). Increased food availability in these environments may lead to increased fish growth, while alterations in habitat, food quality and toxicity may have negative impacts on overall fish health. Recent studies downstream of MWWTPs in the Grand River Watershed have reported a variety of biological impacts on fish (Tetreault*, et al.*, 2011; Tanna*, et* *al.*, 2013; Bahamonde*, et al.*, 2014). The focus of these studies has been on the diversity of emerging contaminants being released in municipal wastewater, particularly endocrine disruptors, pharmaceuticals and personal care products (Tanna*, et al.*, 2013; Arlos*, et al.*, 2015). Many of these compounds have been previously shown to alter endocrine function and cause effects on growth and reproduction (Jobling*, et al.*, 2002; Mills and Chichester, 2005; Fuzzen*, et al.*, 2015). Reproductive impairment downstream of wastewater treatment plants in the Grand River has been associated with the presence of estrogenic compounds (Tanna*, et al.*, 2013). In particular, high incidence and severity of intersex in rainbow darter has been reported in the Grand River downstream of wastewater treatment plant outfalls (Tetreault*, et al.*, 2011; Bahamonde*, et al.*, 2015; Fuzzen*, et al.*, 2015). Impacts to rainbow darter in response to wastewater exposure have been reported across several levels of biological organization, ranging from changes in gene expression, to altered steroid production and somatic indices (Tetreault*, et al.*, 2011; Bahamonde*, et al.*, 2014). Major treatment plant upgrades have recently been implemented and further upgrades are planned at both the Kitchener and Waterloo MWWTPs (Bicudo, *et al.*, 2016). These upgrades offer a unique opportunity to assess how investments in wastewater infrastructure alter effluent quality and downstream fish health. As nutrient and contaminant loads are changed with treatment improvements, there is potential for changes in fish growth and condition associated with MWWTP outfalls. Condition has been one of the endpoints seen to increase downstream of the wastewater outfalls in the Grand River (Tetreault*, et al.*, 2011) although it has not been consistent across seasons and years (Fuzzen, *et al.*, 2016). The use of growth or size-at-age, however, has not been used as an endpoint in previous studies on the Grand River, but could potentially be a sensitive endpoint for detecting changes in energy use and allocation in fish. Unfortunately, aging of rainbow darter in this system has not been validated, limiting the ability to apply these endpoints in biomonitoring projects and impact assessments. Beckman (2002) validated the timing and periodicity of annulus formation in rainbow darter otoliths in the James River, Missouri, however due to the differences in regional climate, there is evidence to suggest that differences in growth zone formation could exist. Unlike Missouri, southern Ontario experiences a prolonged winter, with regions of the Grand River forming complete ice cover, which could alter rainbow darter feeding and over-wintering habits. Validating the timing and periodicity of growth zone formation in rainbow darter otoliths in the Grand River would address this knowledge gap and would provide additional tools and endpoints to assess any possible impacts on fish in this and other watersheds experiencing similar climates.

The rainbow darter is a small, benthic fish species commonly found in shallow regions of rivers and streams throughout mid-Eastern North America (Stauffer and Hocutt, 1980). The rainbow darter has been used a sentinel species in numerous studies in the Grand River Watershed. High population densities are present across the Grand River Watershed where they primarily occupy fast-flowing, shallow regions of the river. They are relatively easy to collect (using backpack electrofishing) allowing for collection from numerous sites. The majority of rainbow darters captured in riffles have a relatively small home range over most of the year (K. Hicks, University of Waterloo, personal communication). It is a sexually dimorphic species, with males exhibiting bright shades of blue, red and orange during the breeding season while females remain sand-coloured throughout the year and are easily identified by an ovipositor during spawning season. This characteristic allows for easy and reliable identification of each sex in the field. Spawning occurs each year between April and June in riffle areas of the river, with females laying multiple clutches throughout this time, with an average annual fecundity of approximately 300 eggs per female (Fuller, 1998; Fuller, 2003). The rainbow darter therefore represents an excellent species to use in assessing impacts of effluents and environmental change in the Grand

River Watershed. In addition to wastewater, continued rapid urbanization, changes in agricultural practices, and climate change will continue to threaten water quality in the Grand River Watershed (Grand River Watershed Water Management Plan, 2014). The wide variety of stressors may act singly or in a cumulative fashion to impact fish and aquatic ecosystems. Being able to age rainbow darter reliably would provide an additional tool to support future environmental assessments.

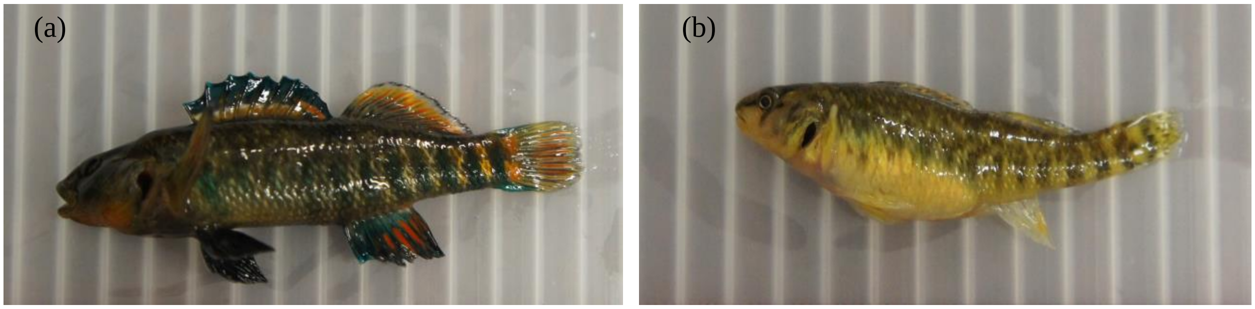

Figure 1.1 Photographs of male (a) and female (b) rainbow darter collected from the Grand River at West Montrose in spring of 2013.

The objectives of the current study were to:

1. Validate the use of sagittal otoliths as an accurate aging structure for rainbow darter by determining the timing and periodicity of growth zone formation; and
2. Characterize growth of rainbow darter and evaluate relationships between age and total length, weight and condition.

Both male and female rainbow darter (approximately 15 of each sex) greater than 4.5 cm in total length were collected from the Grand River monthly between May 2014 and June 2015 at West Montrose, a relatively un-impacted site, upstream of the City of Waterloo. Total lengths (±1 mm) and weights (± 0.001g), as well as liver and gonad weight (±0.001 g) were recorded, and otoliths were removed to estimate age and to determine timing and periodicity of growth zone formation. In July and October 2014 surveys were done to collect fish of all sizes (>100 fish) to construct length-frequency distributions. A sub-sample of rainbow darter from the October collection was sacrificed for direct age determination using otoliths in order to assess size-at-age distributions.

Chapter 2

Age Determination, Validation and Analysis of Growth of Rainbow Darter (*Etheostoma caeruleum*) in the Grand River

2.1 Introduction

Accurate age information is critical in assessments of mortality, growth rate, population structure and population dynamics in fish. The importance of validating the timing and periodicity of growth zone formation in calcified structures of fish has became more apparent after being neglected in numerous studies in the past (Beamish and McFarlane, 1983). Validated ages are imperative to the proper management of fisheries and impact assessment, however age validation has not been completed for many freshwater species apart from popular game fish (Blackwell and Kaufman, 2012; Koenigs*, et al.*, 2015). The term validation can be interpreted differently, and is known as absolute age validation only if the timing of growth zone formation is determined for all ages, which is rarely completed and can be particularly difficult in long-lived species (Beamish and McFarlane, 1983; Campana, 2001). Numerous methods of validation exist, and the method chosen is often dependent on the life history and physiology of the species in question (Table 1.1). Even when validation is done appropriately for a particular species, differences in the timing and periodicity of growth zone formation on calcified structures can differ between populations of the same species, making it especially difficult to apply previous validation data to a new study population (Winker*, et al.*, 2010).

Marginal increment analysis (MIA) is a commonly used method of age validation for short-lived, fast-growing fish species (Table 1.2). The main premise of this method is to use a series of measurements between previously formed and currently growing zones in order to calculate a ratio related to the state of completion of the growing zone (Campana, 2001). When sampling is completed on a regular basis (e.g. monthly) these ratio calculations can provide insight into the timing of growth zone formation. If growth zones form annually, a sinusoidal trend will be apparent when plotted against time (Campana, 2001). Edge analysis is similar to MIA, however no calculations accompany it, and simply the condition of the growing edge is reported as either opaque or translucent (Labropoulou and Papaconstantinou, 2000). State of completion can also accompany edge condition, referring to how much of the zone has formed at a certain time point, however this data is often based on the subjectivity of the person conducting the analysis (Beckman, 2002).

In darter species of the genus *Etheostoma*, age validation has seldom been reported. Beckman (2002) validated the use of otoliths in rainbow darter (*Etheostoma caeruleum*) in southwest Missouri, indicating that annuli were more discernable in sectioned versus whole otoliths and that scales often underestimated fish age. The majority of other studies conducted on species of darters incorporating an age estimation technique did not use validated methods, and used either length-frequency histograms exclusively or were paired with either scale or otolith analysis on a subsample of fish captured to estimate fish age (Table 1.1). The ability to estimate age accurately using a non-lethal method is often preferred, however length-frequency distributions become less reliable in older age cohorts and size-at-age begins to overlap greatly (Khudamrongsawat*, et al.*, 2005; Drake*, et al.*, 2008).

Not only is accurate age estimation vital to the evaluation of life-history tactics and population dynamics of fish, it is important when attempting to assess possible impacts in monitoring programs. The term growth refers to the ability of a fish to utilize and store energy, and is quantified by the change in length or weight over time (Munkittrick*, et al.*, 2010). The

Environmental Effects Monitoring Program (EEM) developed by Environment Canada to assess the impact of pulp and paper mill and metal mining effluents on receiving environments identify growth as a key endpoint in addition to survival, condition and reproductive endpoints (Environment Canada, 2010). The use of small-bodied fish in these monitoring programs is becoming more common, likely due to their relative ease of capture, higher abundance and smaller home ranges (Minns, 1995; Munkittrick*, et al.*, 2002).

The rainbow darter (*Etheostoma caeruleum*) is a small-bodied fish found throughout the Grand River Watershed, Ontario, that has been selected as a sentinel species in an ongoing monitoring study focusing on the impacts of municipal wastewater effluent (MWWE) on fish health (e.g., Tetreault*, et al.*, 2011; Fuzzen*, et al.*, 2015). Numerous impacts have been identified in rainbow darter collected downstream of MWWE outfalls, including decreased sex steroid production and increased incidence of intersex condition in male fish (Tetreault*, et al.*, 2011; Tanna*, et al.*, 2013; Bahamonde*, et al.*, 2015; Fuzzen*, et al.*, 2015). The effect of MWWE and associated nutrient input on fish growth, however, has not been investigated in this system, and therefore a potentially important and sensitive endpoint has not been included. The objectives of this study are to a) validate the timing and periodicity of annuli formation in rainbow darter sagittal otoliths using two common validation methods and b) to characterize the growth of rainbow darter at a reference site and to assess size-at-age relationships. This will create the basis for further studies on the impacts of wastewater effluent and other stressors on growth in rainbow dater in the Grand River as well as other watersheds with similar climate conditions.

2.2 Materials and Methods

2.2.1 Study Site

The site chosen for this study is located near the community of West Montrose approximately 5 km upstream from the city of Waterloo in the Grand River Watershed in southern Ontario (Figure 2.1). The surrounding land has minimal urbanization and is dominated by agriculture, although there are several small wastewater outfalls upstream and a major flood control dam. This site has been used as the rural reference site in numerous ongoing studies in the Grand River (Tetreault*, et al.*, 2011; Fuzzen*, et al.*, 2015). The average summer flow is 5 m^3/s, although it varies greatly throughout the year, with the spring melt causing increased runoff (>100 m^3/s). A TidbiT v2 temperature logger (onset HOBO®) was deployed at the site beginning in May 2014 and was removed in early December and temperature was recorded five times daily (0600, 1000, 1400, 1800, 2200). This provided a detailed water temperature profile of the study site during the 2014 growing season. The two dominant substrate classes at the site are gravel (49%) and cobble (34%) (Tetreault*, et al.*, 2013). Many species of fish can be found in this section of the river, including fantail darter (*Etheostoma flabellare*) and greenside darter (*Etheostoma blenniodes*), however rainbow darter remain a large proportion of fish captured using the backpack electrofishing method (Tetreault*, et al.*, 2013).

2.2.2 Fish Collections

Fish were collected using a backpack electrofishing unit (Smith-Root model LR-20) and 2-3 individuals using dip nets. Captured fish were kept in aerated buckets until sampling could occur in accordance with the University of Waterloo Animal Care Protocol 10-17 and 14-15. For each fish,

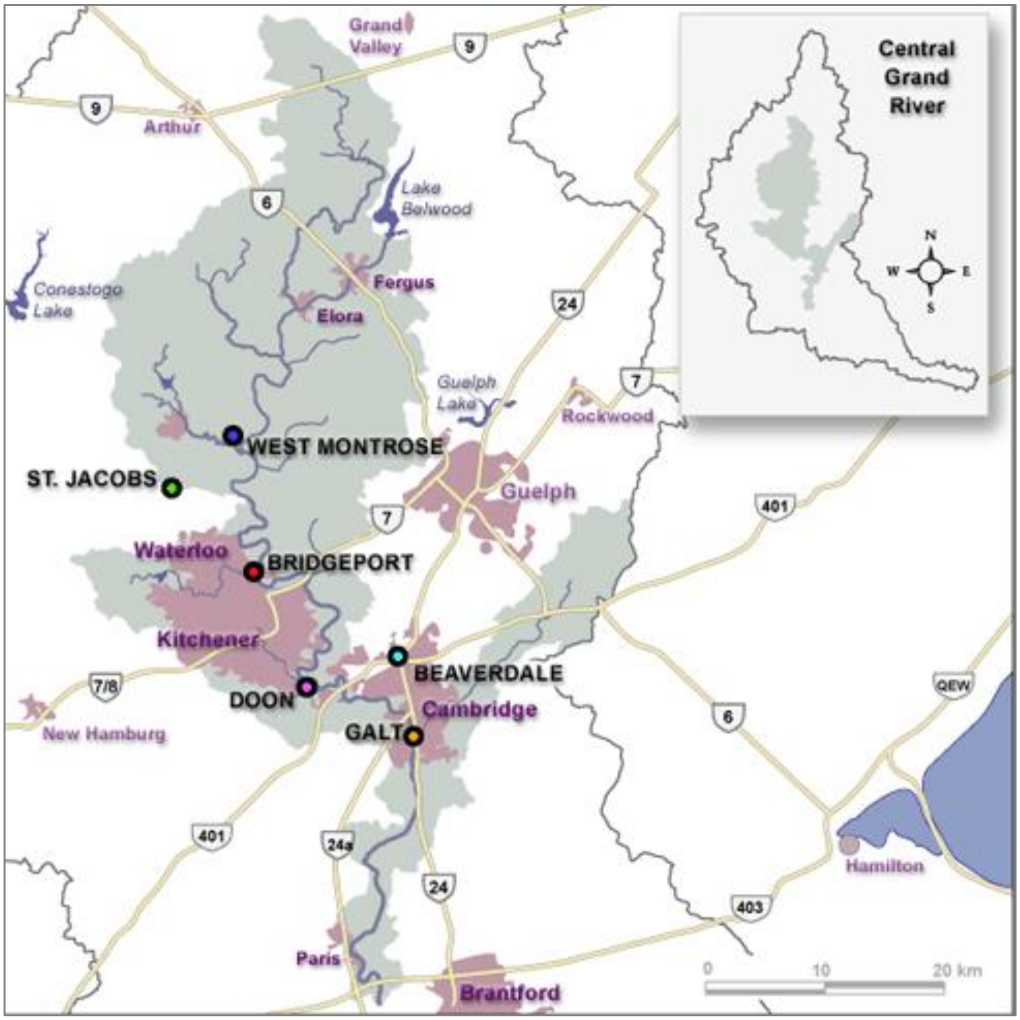

Figure 2.1 Map of the Grand River Watershed indicating the locations of numerous Grand River Conservation Authority flow gauge stations, including West Montrose, in the Central Grand River (figure retrieved from the Grand River Conservation Authority website - https://www.grandriver.ca).

total length (± 0.1 cm) and total weight (± 0.001 g) were recorded. Fish were then sacrificed and gonad weight and liver weight (± 0.001 g) were recorded. Liver (LSI) and gonadosomatic (GSI) indices and condition (K) were calculated using the following equations:

$$GSI = \text{gonad weight (g)} / \text{body weight (g)} \times 100 \qquad \textbf{Equation (1)}$$

$$LSI = \text{liver weight (g)} / \text{body weight (g)} \times 100 \qquad \textbf{Equation (2)}$$

$$K = \text{fish weight} / (\text{total length})^3 \qquad \textbf{Equation (3)}$$

LSI and GSI were calculated for all fish whenever gonad and liver weights were recorded. Fish were then placed into individually labeled Whirlpak™ bags and remained on ice until they were transported to the laboratory at the University of Waterloo. Fish were stored in a -20°C freezer until further analysis could be conducted. Left and right otoliths were removed from each fish, cleaned thoroughly with water, and placed into individually labeled wax paper envelopes. Otolith extractions began with a mid-dorsal cut, starting in the mouth and extending caudally through the braincase. With the braincase now open, the brain tissue was removed. Sagittal otoliths were then located on the lateral surface of the braincase, caudal to the eyes and on each side of the vertebrae.

2.2.3 Age Validation

To validate timing and periodicity of growth zone formation on otoliths, rainbow darters were collected from the Grand River at West Montrose monthly between May 2014 and June 2015. Sampling was not conducted in January, February or March of 2015 due to unsuitable weather/flow conditions. Each sampling event targeted collection of at least 15 male and 15 female rainbow darter greater than 45 mm in length. This size range was targeted to increase the chance that fish were at least 1 year of age, which was necessary for marginal increment analysis.

2.2.4 Fish Growth

To evaluate the population composition, fish growth and young-of-the-year (YOY) growth at the West Montrose site, sample collections targeting >100 individuals were conducted in July and October 2014. All rainbow darters possible were collected to enable the construction of a length-frequency distribution for each of the two sampling events. YOY rainbow darter had reached a catchable size by the July sampling date, and a small mesh (400 µm) dip net was used to ensure all fish shocked were collected. Total length and weight were recorded in July and no fish were sacrificed. To assess fish growth and relationships between age and various metrics including total length, total weight, gonadal and liver somatic indices and otolith length and weight, a subsample of the fish collected were sacrificed for direct age determination (using otoliths) in October 2014. Fish were chosen randomly from aerated buckets for lethal sampling and the first 5 male and female fish (where available) in each 1 mm total length class greater than 40 mm were sacrificed for direct age determination. Two individuals in each 1 mm length class between 30 mm and 40 mm were sacrificed for direct age determination. For all size-at-age analyses, immature fish were randomly assigned a sex. Male and female subsampled fish were separated and length-at-age data was used to estimate von Bertalanffy growth models for each sex using the equation:

$$L_t = L_{inf}\ (1-e^{-k(t-t_0)}) \qquad \textbf{Equation (4)}$$

where L_t is the average length at time, L_{inf} is the asymptotic average length, k is the Brody growth coefficient and t_0 is the time at which average length is zero (von Bertalanffy, 1938). All von Bertalanffy growth modeling was completed using R (version 3.2.1; R Core Team, 2015). The model was fit using the FSA package for R (version 0.3.2; Ogle, 2011). Relationships between both otolith length and weight and fish total length and weight were assessed to characterize the growth of otoliths throughout the life of rainbow darter.

2.2.5 Otolith Preparation and Measurements

Each otolith was weighed to 5 decimal places using an XP205 DeltaRange® (Mettler Toledo) scale. Otoliths were then embedded in CrystalBond 509 (SPI Supplies) on microscope slides with the sulcus on the ventral surface. Otoliths were sanded using various grits of sand papers (1500-12000 grit) to expose the nucleus. All otoliths were flooded with water to rehydrate for 10 minutes prior to being photographed. Samples were viewed under reflected light and photographed using a Leica S6D dissecting microscope mounted with a Leica EC3 camera. Images were viewed in LAS-EZ (Leica) software where they were further enhanced by adjusting exposure and contrast and an accurate scale was added. Once growth zone clarity was optimized, one reader enumerated annuli on two separate occasions. If discrepancies were present, a third reading was conducted and age was assigned. After all otoliths were photographed, photos were viewed using ImageJ software and measurements were taken for marginal increment ratio calculation and total otolith length (mm) was obtained. The plane on which measurements were taken was dependent on the sample, and the plane with the most clearly defined annuli was chosen. Edge analysis was performed for each otolith and the condition (presence of translucent or opaque zone) of the growing edge was recorded. Terminology used in this study is defined in Figure 2.2. The translucent zone, otherwise referred to as an annulus, was defined as a distinct narrow band that was darker in colour compared to surrounding tissue. The nucleus is the center of the otolith and is the point from which otolith

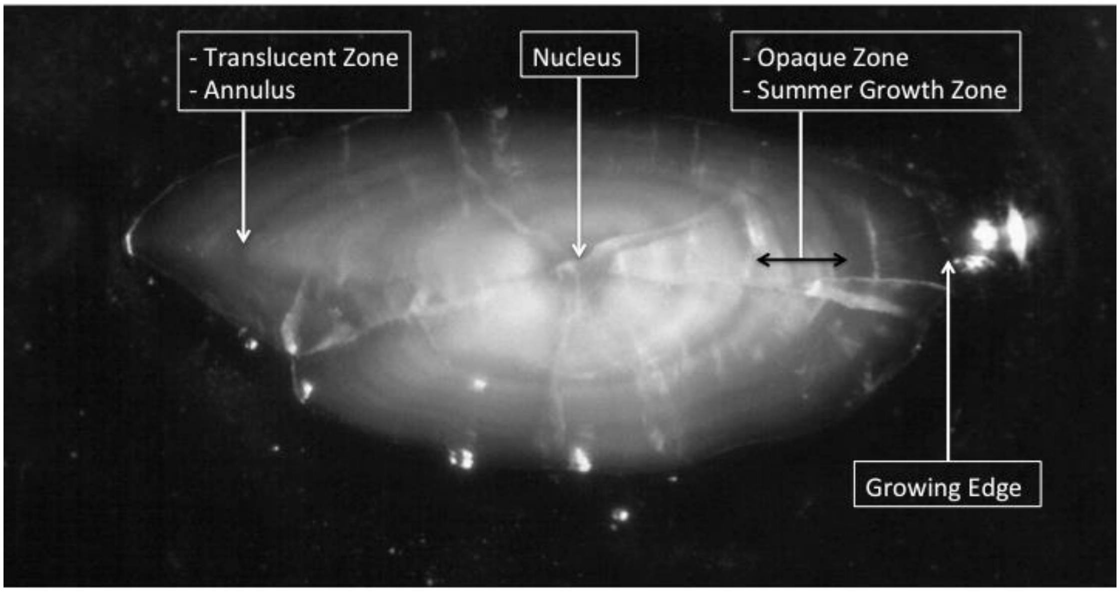

Figure 2.2 Photograph of a rainbow darter otolith identifying key characteristics and defining terminology used in this study.

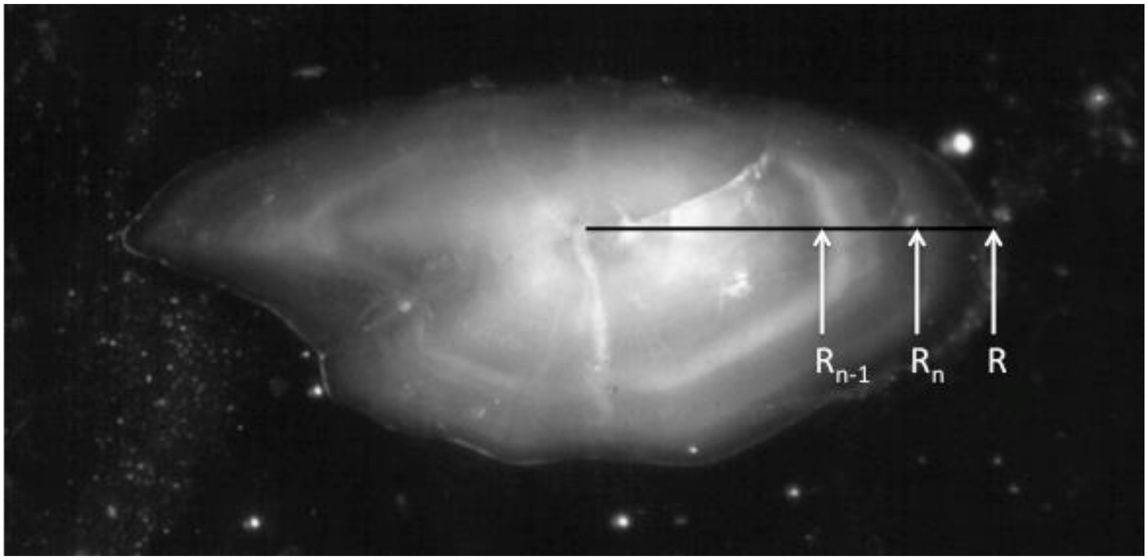

Figure 2.3 Photograph of a rainbow darter otolith identifying the measurements necessary for marginal increment ratio calculation. All measurements are taken from the nucleus and along the same plane.

growth and measurements originate. The opaque zone is also referred to as the summer growth zone and is a band of otolith tissue that presents as a discrete area white in colour compared to surrounding tissue.

2.2.6 Marginal Increment Ratio (MIR) Calculation

Marginal increment ratios were calculated for each otolith using the marginal increment analysis technique for the validation of timing of growth zone formation in otoliths (Campana, 2001; Smith, 2014). Measurements between translucent zones (Figure 2.3) were then used in the equation:

$$MIR = \frac{(R - R_n)}{(R_n - R_{n-1})}$$ **Equation (5)**

where R is the radius of the otolith, R_n is the distance from the center of the otolith to the most recent fully formed annuli, and R_{n-1} is the distance from the center of the otolith to the penultimate annuli (Coelho*, et al.*, 2010). Ratios were calculated for each otolith, and monthly mean ratios for each sex was calculated and plotted against time to determine the timing of growth zone formation. Fish that exhibited opaque growth formation in the spring months were separated from those that had not in order to accurately present the change in MIR at this time of year.

2.2.7 Statistics

Marginal increment ratios (MIR) were tested using a two-way ANOVA to assess differences among months and sexes; data were log transformed to achieve normality. Two-way ANOVA tests were also applied to sex-separated length, weight, and condition-at-age data from rainbow darter collected in October 2014. All pairwise comparisons were done using Tukey post-hoc tests using a significance (α) value of 0.05 (Sigma Plot v12.3, 2011). Monthly fish collections and resulting condition, LSI and GSI data were sex-separated and tested using two-way ANOVAs. Length and weight relationships from October 2014 fish were tested using a linear regression. Linear regressions were also applied to otolith weight and fish length/weight as well as to otolith length and fish length data. All linear regressions were performed using SigmaPlot.

2.3 Results

2.3.1 Study Site

All rainbow darter were collected within a 30 m stretch of river immediately upstream of the West Montrose covered bridge. Water temperatures ranged from 27.2 °C in July to 0.2 °C in late November (Figure 2.4). Temperatures remained between 15.0 °C and 27.2 °C throughout the summer months, and began to drop steadily in September until reaching the lowest recorded temperature in November. Ice had not yet formed on the river at the time the HOBO temperature logger was removed in December.

2.3.2 Age Validation

Monthly sampling yielded a total of 315 rainbow darter ranging in size from 4.5 cm to 7.1 cm and fish ranged from 0+ to 6+ years of age. MIR analysis of fish collected monthly showed that rainbow darter form one annuli per year, with the onset of formation beginning between September and November. A two-way ANOVA revealed a significant interaction between month and sex. Male and female mean MIR differed significantly in August (p=0.009) and October 2014 (p=0.019), but no differences were present between sexes in any other month sampled (Figure 2.5).

Differences could possibly be due to small sample sizes in these months as well as variability in MIR. Sampling biases caused by microhabitat differences and limited fish movement could also attribute to decreased MIR values recorded for male fish in August and October. Differences did exist, however, among months within each sex. Mean MIR for female fish collected in June differed significantly from that in September (p= 0.006), October (p=0.004), November (p=0.019) and December (p<0.001). Mean MIR for male fish in November differed from June (p=0.009), August (p<0.001) and October (p=0.031). Differences in MIR also existed between August and both

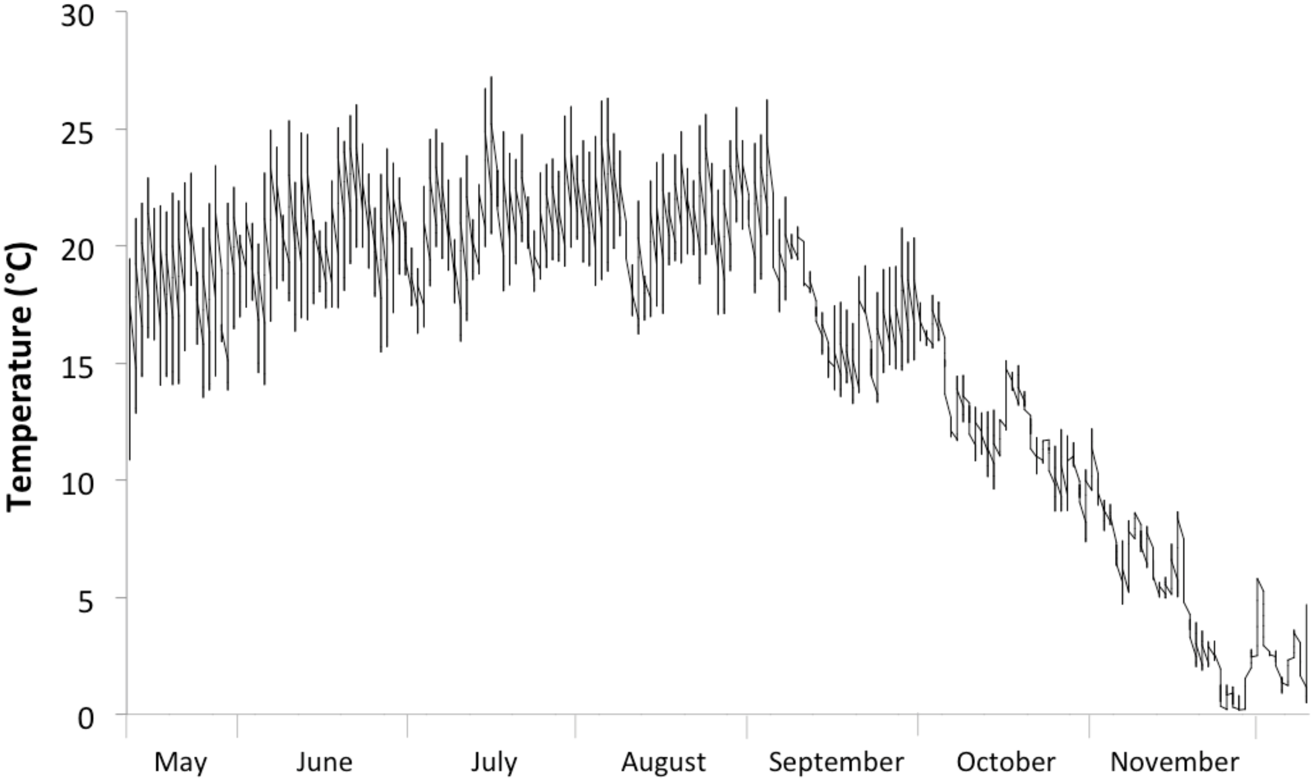

Figure 2.4 Water temperature profile at West Montrose between late May and December 2014.

September (p=0.027) and December (p=0.027). The presence of annuli on the growing edge of otoliths was noted in September in both male and female fish, and all fish sampled in November had begun annuli formation (Figure 2.6). The formation of annuli on otoliths could possible be associated with the decrease in water temperatures concurrently recorded (Figure 2.4) When age cohorts were separated (0+, 1+, 2+, 3+ and 4+), no trend was exhibited in the timing of annulus formation, suggesting that it does not vary with age; additional sampling and increased sample sizes within each age cohort are necessary to further investigate this (Table A.1). Presence of the summer growth zone was identified as early as April and all fish sampled, regardless of sex or age, had begun formation by the July sampling event (Figure 2.7). The timing of opaque zone formation was similar between sexes and among age cohorts; however sample sizes were relatively small which could influence these observations (Table A.2).

2.3.3 Fish Growth

The July 2014 sampling event yielded a total of 133 rainbow darter collected. The minimum length of fish was 1.2 cm and the maximum was 6.7 cm. No fish between 2.6 cm and 3.7 cm were captured (Figure 2.8a). Young-of-the-year fish (0+) were clearly identifiable at this time, ranging between 1.2 cm and 2.5 cm. The October length-frequency sampling event yielded a total of 251 rainbow darter. There was no gap in the length frequency separating the YOY at this time (Figure 2.8b). A subsample of 147 fish was sacrificed for direct age determination using otoliths. Total length of these fish ranged from 3.1 cm to 7.2 cm, and the length-frequency histogram indicates a high degree of overlap in length of each age classes (Figure 2.8b). Overlap of length-at-age of each sex also indicated high overlap (Figure A.1). A weight-frequency distribution was also constructed at this time, however it did not provide any additional separation of age cohorts (Figure A.2). Length and weight increased with age and there was a strong linear relationship between log length and log

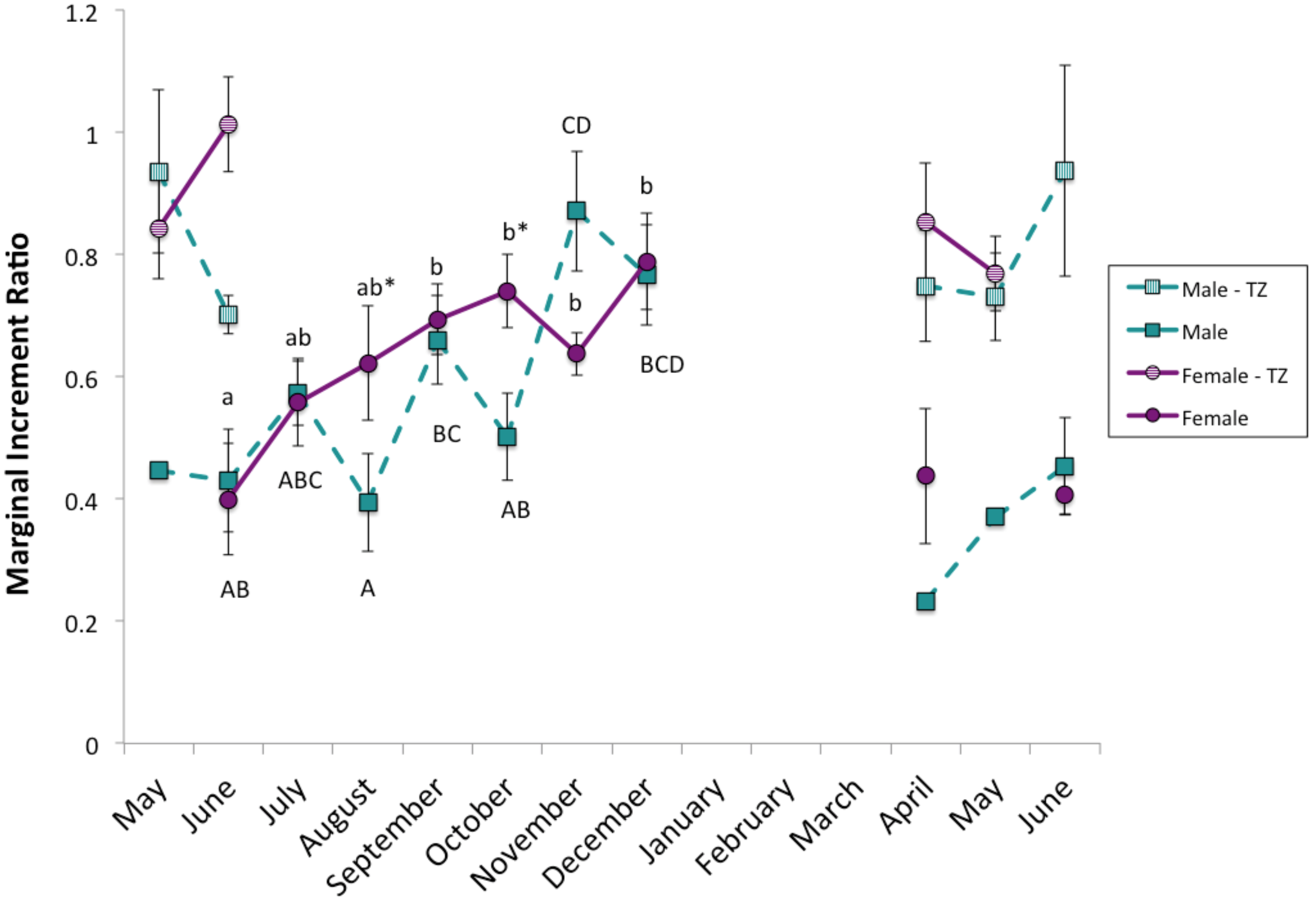

Figure 2.5 Mean marginal increment ratio (±SE) for male and female rainbow darter collected throughout the months of May 2014-June 2015. Sampling was not conducted between January and March 2015 due to adverse weather conditions. Patterned data points represent mean MIR for fish that have not commenced summer growth zone formation, and thus continue to present the translucent zone (TZ) on the growing edge of the otolith. Upper case and lower case letters indicate significant differences among sampling months of male and female fish respectively. The presence of an asterisk (*) indicates a significant difference between male and female fish within the same month.

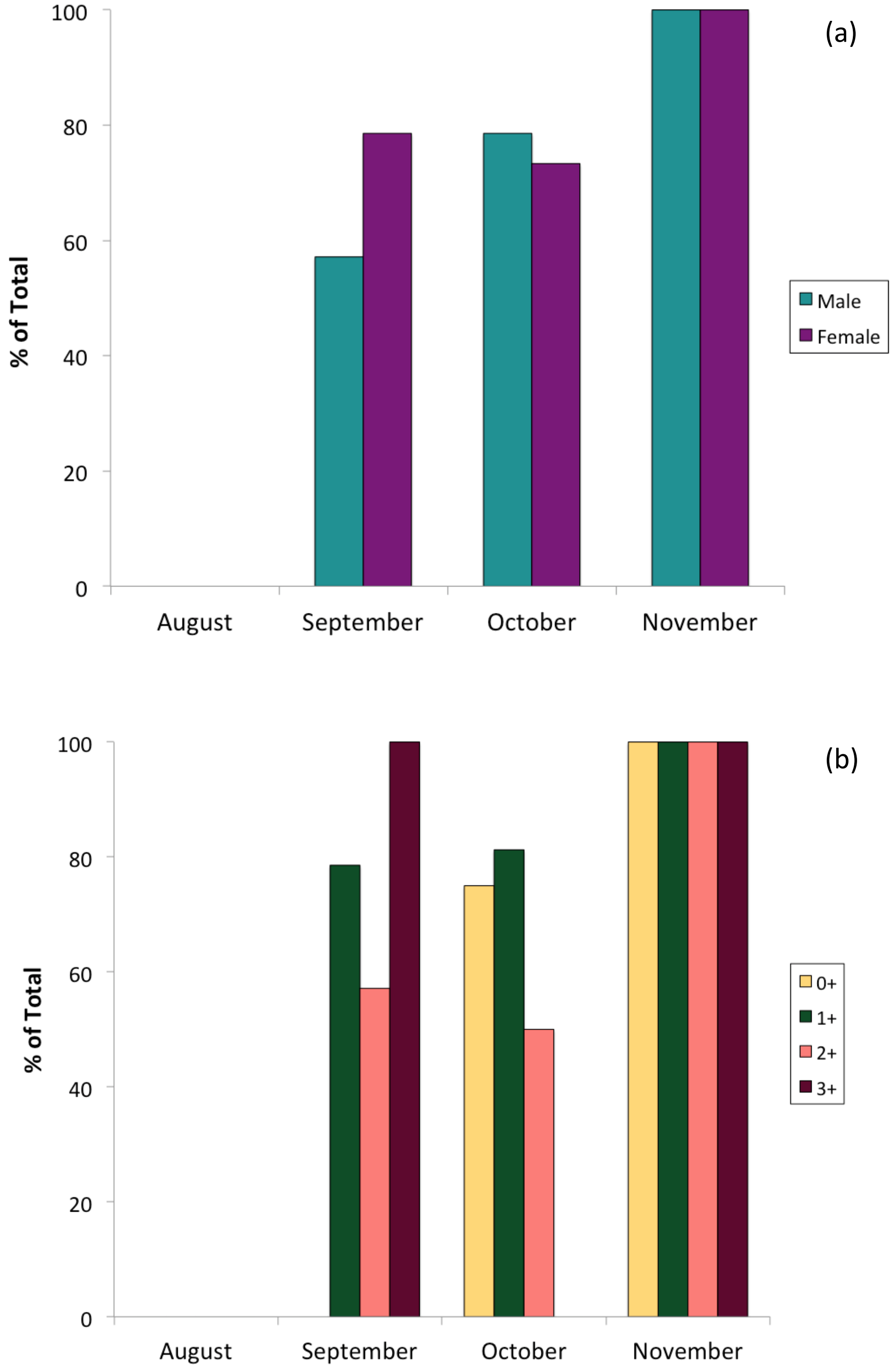

Figure 2.6 Percent of total fish with the presence of a translucent zone (annuli) on the growing edge of otolith in Fall 2014 separated by (a) sex and (b) age cohorts.

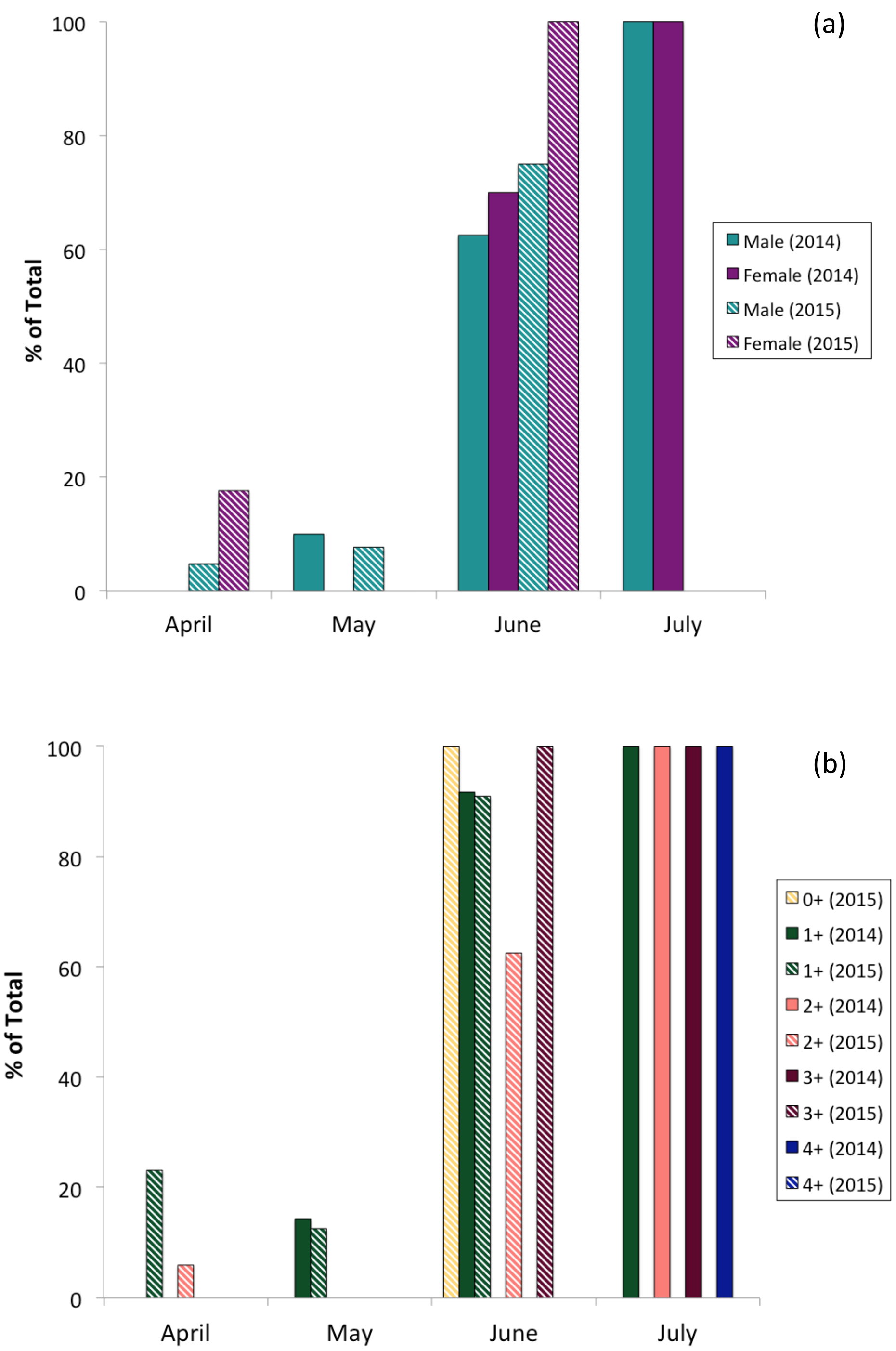

Figure 2.7 Percent of total fish with the presence of summer growth zone formation on the growing edge of otolith separated by (a) sex and (b) age cohorts. Different sampling years are represented by solid (2014) and striped (2015) bars.

weight for all fish collected in October ($r^2 = 0.991$; Figure 2.9). An interaction between fish age and sex on mean length of rainbow darter is apparent (Two-way ANOVA, F = 1.48, p=0.001, d.f =4,133). There is sufficient evidence to suggest that mean length differs between sexes of rainbow darter (Two-way ANOVA, $F_{1,133}$=11.85, p<0.001). Tukey post-hoc tests indicate significant differences between male and female rainbow darter mean length at ages 2+ (p=0.001), 3+ (p=0.022) and 4+ (p=0.017). Mean length of rainbow darter differs significantly due to age (Two-way ANOVA, $F_{4,133}$=153.85, p<0.001). Tukey post-hoc tests indicated differences among age groups within male and female fish categories. Mean length of male fish ages 0+, 1+ and 2+ differed significantly from each other (p<0.05), however these differences became less apparent in older aged fish (Figure 2.10). A similar trend was evident in female fish, with no significant differences in mean total length between age 2+, 3+ and 4+ fish (p<0.05). Female fish age 0+ and 1+ differed significantly (p<0.001) from each other as well as from fish aged 2+, 3+ and 4+ (p<0.05, Figure 2.10). The fit of male and female length and age data to von Bertalanffy growth curve suggest that this model is appropriate for this species (Figure 2.11). Male L_{inf} is larger for male rainbow darter, which further supports the differences seen in male and female length in older age cohorts (Table 2.1). This model supports the increase in length-at-age of male rainbow darter compared to females after the age of 2+ (Figure 2.11).

Table 2.1 Estimates for the von Bertalanffy growth model (±SD) for male and female rainbow darter collected in October 2014.

	Male	Female
n	84	60
L_{inf}	7.42 (± 0.44)	6.48 (± 0.34)
k	0.44 (± 0.09)	0.49 (± 0.13)
t_0	-0.65 (± 0.24)	-0.98 (± 0.39)

A statistically significant interaction exists between fish age and sex impacting mean weight (Two-way ANOVA, F = 8.51, $p<0.001$, d.f =4,133). There is also evidence sufficient to suggest that mean weight differs between sexes of rainbow darter (Two-way ANOVA, $F_{1,133}$=41.37, $p<0.001$). Tukey post-hoc tests performed indicate significant differences between male and female rainbow darter mean weight at ages 2+ ($p<0.001$), 3+ ($p<0.001$) and 4+ ($p<0.001$). Mean weight of rainbow darter differs significantly among age groups (Two-way ANOVA, $F_{4,133}$=151.08, $p<0.001$). Tukey post-hoc tests indicated differences in mean weight among age groups within male and female fish categories (Figure 2.12). Mean weight of male fish differed significantly between all age groups ($p<0.05$). Female mean weight differed significantly between fish aged 0+, 1+ and 2+($p>0.05$) as well as between ages 2+ and 4+ (p=0.007). Mean weight was similar in female fish aged 2+ and 3+ (p=0.846) as well as 3+ and 4+ (p=0.271). No interaction of sex and age was evident acting on fish condition (Two-way ANOVA, F=1.953, p=0.105, d.f.=4,133), however differences between pooled male and female fish were apparent among age groups (Two-way ANOVA, $F_{4,133}$=33.16, $p<0.001$; Figure 2.13). Condition increased significantly from ages 0^+ to 1^+ ($p<0.001$) but did not differ significantly between age groups of older fish (between 2+, 3+ and 4+ fish); differences were apparent between 0+, 1+ and all older cohorts (Figure 2.13). Fish exhibited positive, linear relationships between otolith weight and somatic growth indices. A strong predictive relationship was apparent between otolith weight and total length for both sexes (males: r^2=0.955; females: r^2=0.935; Figure 2.14) and weight (males: r^2=0.961; females: r^2=0.938; Figure 2.15). Otolith length was also strongly related to total fish length (males: r^2=0.869; females: r^2=0.879; Figure 2.16).

Monthly rainbow darter collections provided insight into relationships between numerous growth metrics over time for males and females of all age cohorts. The number of fish of each sex and age cohort (i.e. hatch-year) can be found in Table 2.2. Total length increased throughout the year for all age classes and sexes (Figure 2.17; Figure A.3). For female rainbow darter, length increased with time (months) with the slopes of the lines decreasing as age increased (Figure 2.17; Figure A.3). Male fish mean total length was consistently higher as age increased throughout the year (i.e. months), although small males captured in May and June 2014 altered the slopes of the lines (Table 2.3). The steepest slopes were apparent in fish born in 2014, which were captured in spring 2015. Fish weight over time followed a similar trend as length, with the highest degree of change over time apparent in fish born in 2014 (Figure 2.18; Table 2.3). Female fish showed an increase in weight over the summer but the change in weight (i.e. slopes) throughout the year declined as age increased (Figure 2.18; Table 2.3).

Fish condition increased steadily throughout the year, and was highest in spring, coinciding with the onset of spawning season (Figure 2.19a). Low condition of male and female fish hatched in 2014 compared to older fish suggests that that fish of this age do not participate completely in spawning season (Figure 2.19a). There is insufficient evidence to suggest that mean condition factor differs between sexes (Two-way ANOVA, F=3.430, p=0.065, d.f.=1,282) however evidence suggests there is an effect of an interaction between sex and month (Two-way ANOVA, F=6.281, p<0.001, d.f.=10, 282). Tukey post-hoc tests indicated significant differences between male and female mean condition in May (p<0.001), June (p=0.03) and October (p<0.001) 2014 and also in May (0<0.001) and June (p=0.009) 2015 (Figure 2.19b).

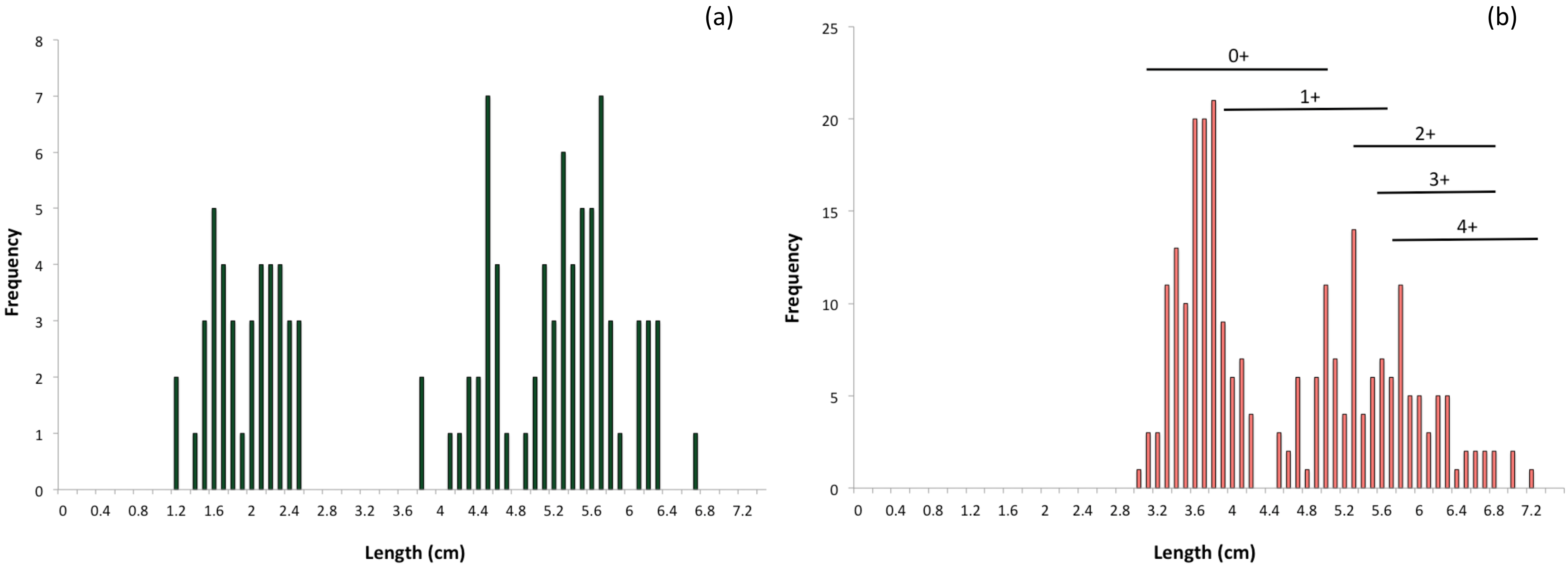

Figure 2.8 Rainbow darter length-frequency histogram constructed in (a) July 2014 (n=133) and (b) October 2014 (n=251). A subsample of fish were sacrificed for direct age determination using otoliths in October 2014 (n=147), which yielded a maximum and minimum length-at-age, represented as a range for each age cohort.

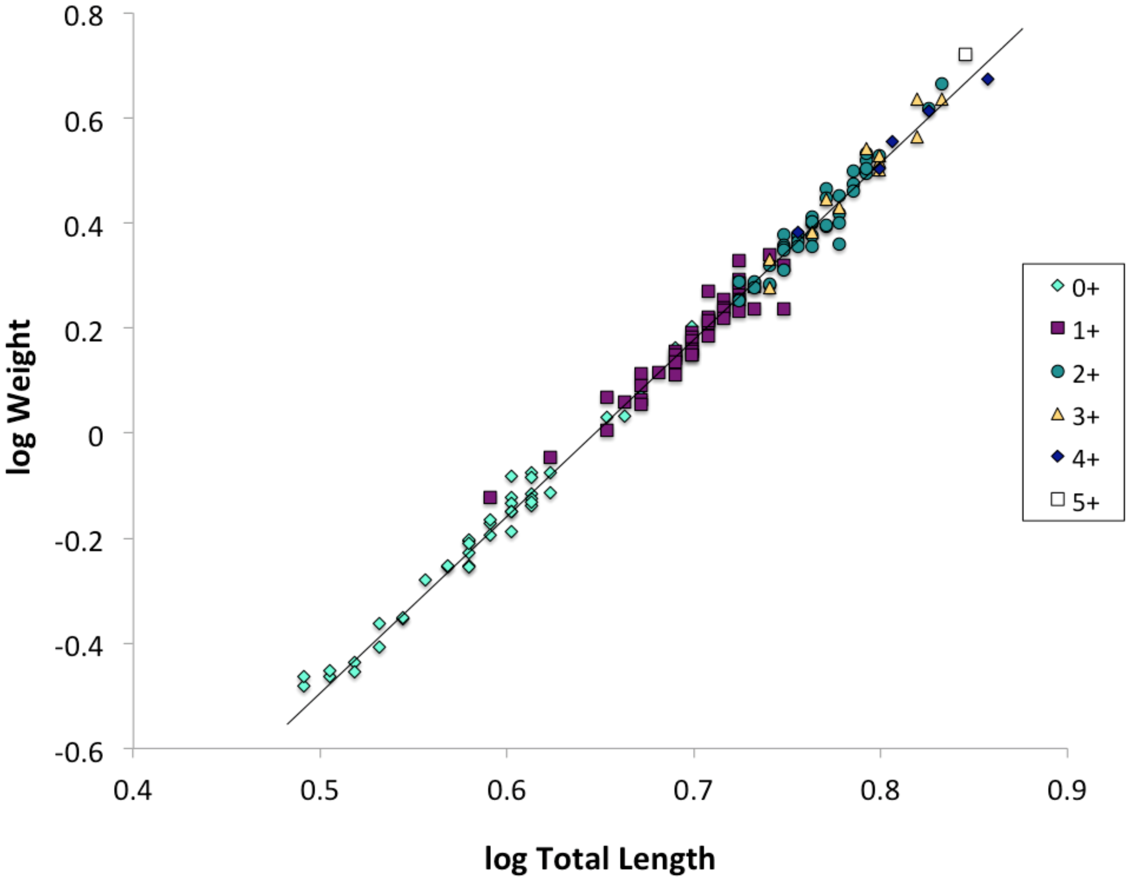

Figure 2.9 Length vs weight for various age cohorts of rainbow darter collected in October 2014. Only fish that underwent direct age determination using otoliths were analyzed (n=147). A strong predictive relationship exists between fish length and weight (Linear regression, r^2=0.991).

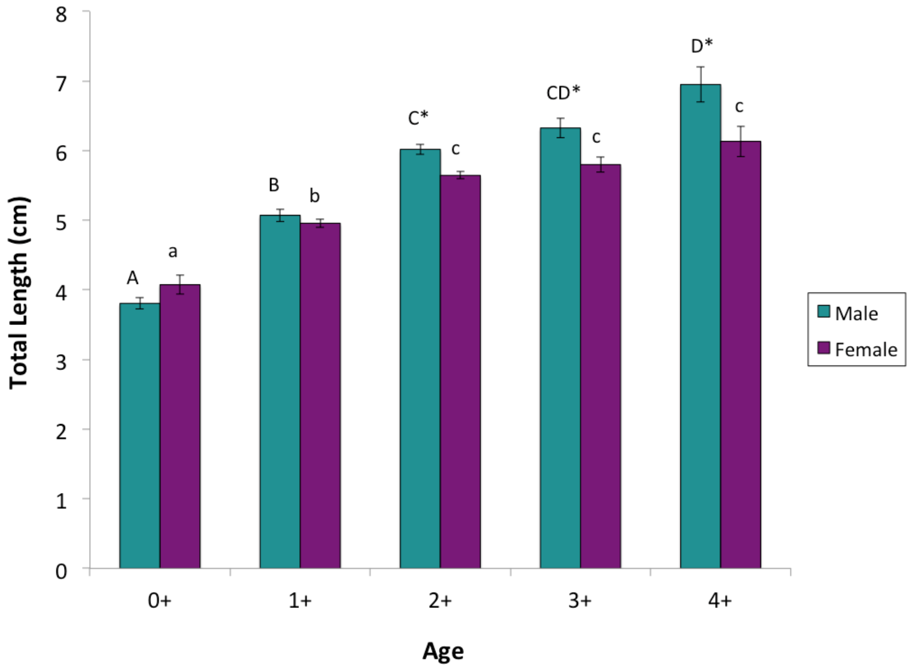

Figure 2.10 Histogram depicting total length (mean ±SE) of male and female rainbow darter of each age cohort in October 2014. Only fish that underwent direct age determination using otoliths were analyzed (n=147). Upper case and lower case letters indicate significant differences among age cohorts of male and female fish respectively. The presence of an asterisk (*) indicates a significant difference between male and female fish within the same age cohort.

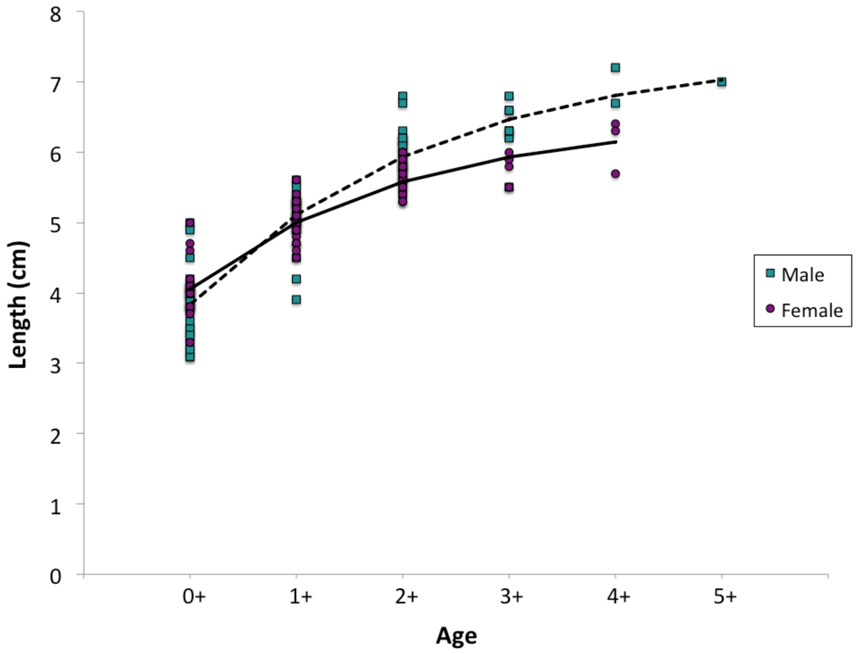

Figure 2.11 Estimated von Bertalanffy growth curves for male (n=84) and female (n=60) fish collected in October 2014.

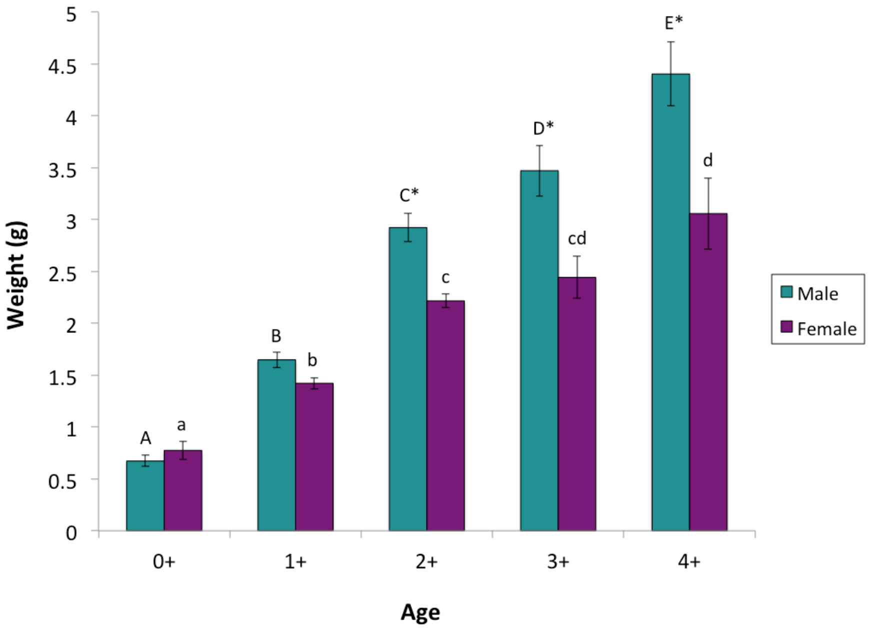

Figure 2.12 Histogram depicting weight (mean ±SE) of male and female rainbow darter of each age cohort in October 2014. Only fish that underwent direct age determination using otoliths were analyzed (n=147). Upper case and lower case letters indicate significant differences among age cohorts of male and female fish respectively. The presence of an asterisk (*) indicates a significant difference between male and female fish within the same age cohort.

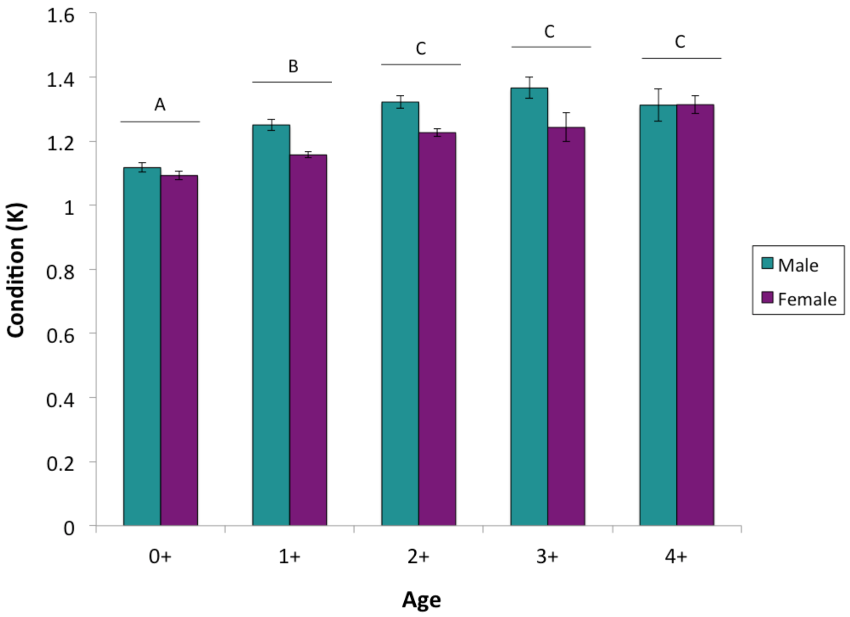

Figure 2.13 Histogram depicting condition (mean ±SE) of male and female rainbow darter of each age cohort in October 2014. Only fish that underwent direct age determination using otoliths were analyzed (n=147). No significant differences were found between male and female fish within an age cohort, and therefore male and female data were pooled for each age group. Upper case letters indicate significant differences among age groups.

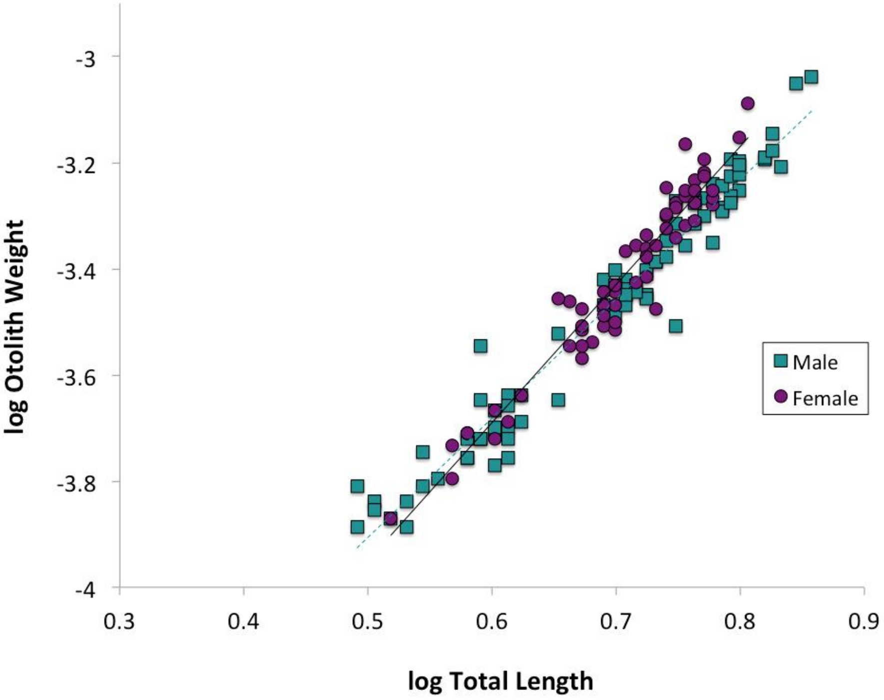

Figure 2.14 Linear regression depicting the relationship between log otolith weight and log total length of male and female rainbow darter collected in October 2014 (n=147). Strong relationships exist for males (Linear regression, r^2=0.955) and females (Linear regression, r^2=0.935).

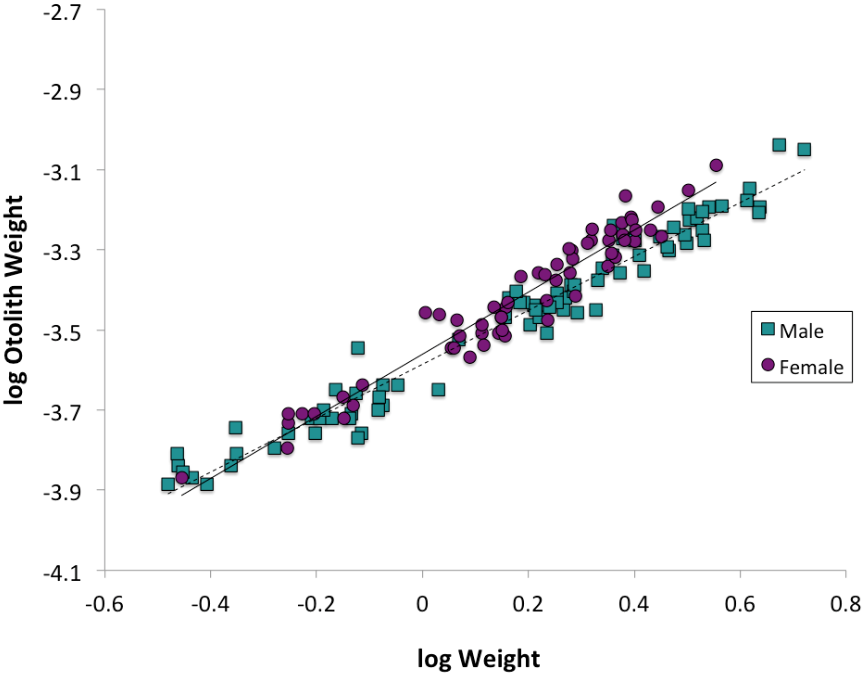

Figure 2.15 Linear regression depicting the relationship between log otolith weight and log weight of male and female rainbow darter collected in October 2014 (n=147). Strong relationships exist for females male (Linear regression, r^2=938) and females (Linear regression, r^2=0.961).

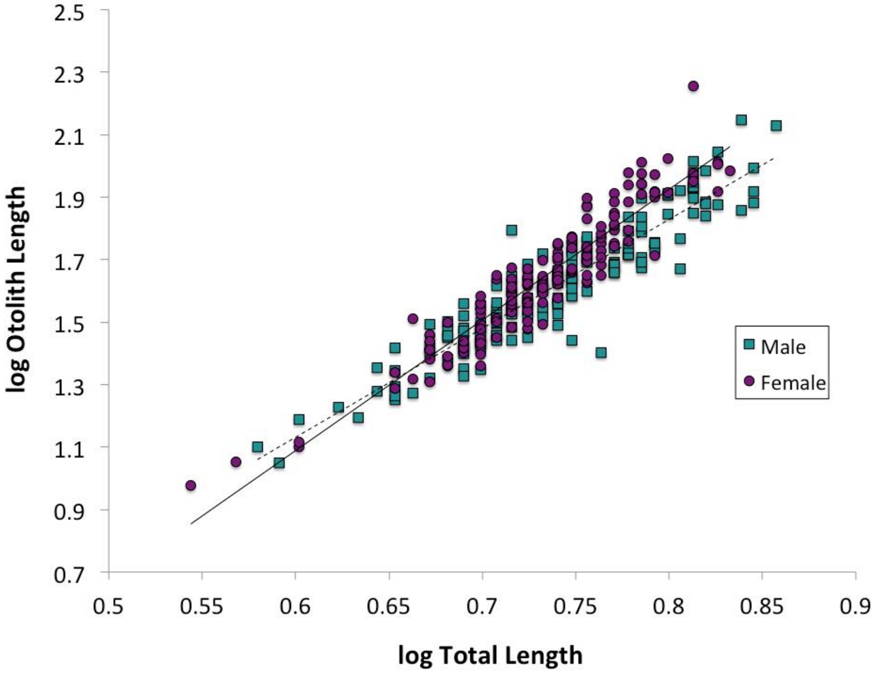

Figure 2.16 Linear regression depicting the relationship between log otolith length and log total length of male (dashed line) and female (solid line) rainbow darter collected between May 2014 and June 2015. Strong relationships exist for males (Linear regression, r^2=0.869) and females (Linear regression, r^2=0.879).

Table 2.2 Number of fish of each sex and hatch-year collected each month between May 2014 and June 2015. The (-) symbol represents a month when sampling was not conducted.

	2014		2013		2012		2011		2010		2009		2008	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
May	0	0	0	0	4	3	6	7	0	0	0	0	0	0
June	0	0	1	0	4	8	4	1	0	1	0	0	0	0
July	0	0	0	0	7	7	8	2	0	3	0	2	0	1
August	0	0	7	4	7	8	1	1	0	1	0	0	0	0
September	0	0	0	0	11	4	1	2	1	4	1	1	0	0
October	0	0	7	6	6	8	2	0	0	0	0	0	0	0
November	0	0	7	1	6	9	2	3	0	2	0	0	0	0
December	0	0	6	2	5	9	2	2	1	1	0	0	0	1
January	-	-	-	-	-	-	-	-	-	-	-	-	-	-
February	-	-	-	-	-	-	-	-	-	-	-	-	-	-
March	-	-	-	-	-	-	-	-	-	-	-	-	-	-
April	6	2	8	5	6	11	1	1	0	0	0	0	0	0
May	0	0	3	5	6	7	4	2	1	1	1	0	0	0
June	4	2	6	5	5	3	0	4	0	1	0	0	0	0

Table 2.3 Slope values fitted to ln length and ln weight data vs time (i.e. months) for male and female rainbow darter of each hatch year between May 2014 and June 2015. N/A is assigned to categories that did not have sufficient sample sizes to fit a line.

	Length		Weight	
Hatch Year	Male	Female	Male	Female
2014	2.59	5.511	2.0851	3.2695
2013	0.09	0.18	0.2152	0.2251
2012	0.34	0.31	0.5133	0.3459
2011	0.68	0.27	1.0146	0.4729
2010	N/A	0.11	N/A	0.3376
2009	N/A	N/A	N/A	N/A
2008	N/A	N/A	N/A	N/A

Mean liver somatic index differs significantly between sexes (Two-way ANOVA, $F_{1,248}$=244.058, p<0.001) and among months (Two-way ANOVA, $F_{8,248}$=136.241, p<0.001). LSI in females was significantly higher than males in September (p<0.001), October (p<0.001), November (p<0.001) and December (p<0.001) 2014 and also in April (p<0.001), May (p<0.001) and June (p<0.001) 2015 as indicated by Tukey post-hoc tests (Figure 2.20b). Mean LSI for both male and female fish were largest in April, and dropped drastically in following months (Figure 2.20b).

Mean gonadosomatic index differs significantly between sexes (Two-way ANOVA, F=1666.918, p<0.001, d.f.=1, 245) and among months (Two-way ANOVA, F=318.168, p<0.001, d.f.=8,245). Mean GSI was significantly higher in females compared to males in all months sampled (p<0.05; Figure 2.21a,b). Low GSI of female fish hatched in 2014 compared to females from previous hatch years suggest that females of this age do not participate fully in spawning (Figure 2.21a). Male and female GSI increases steadily throughout the summer, reaching peak values in early spring, coinciding with spawning season. GSI quickly decreases following this time, however, in response to the conclusion of spawning.

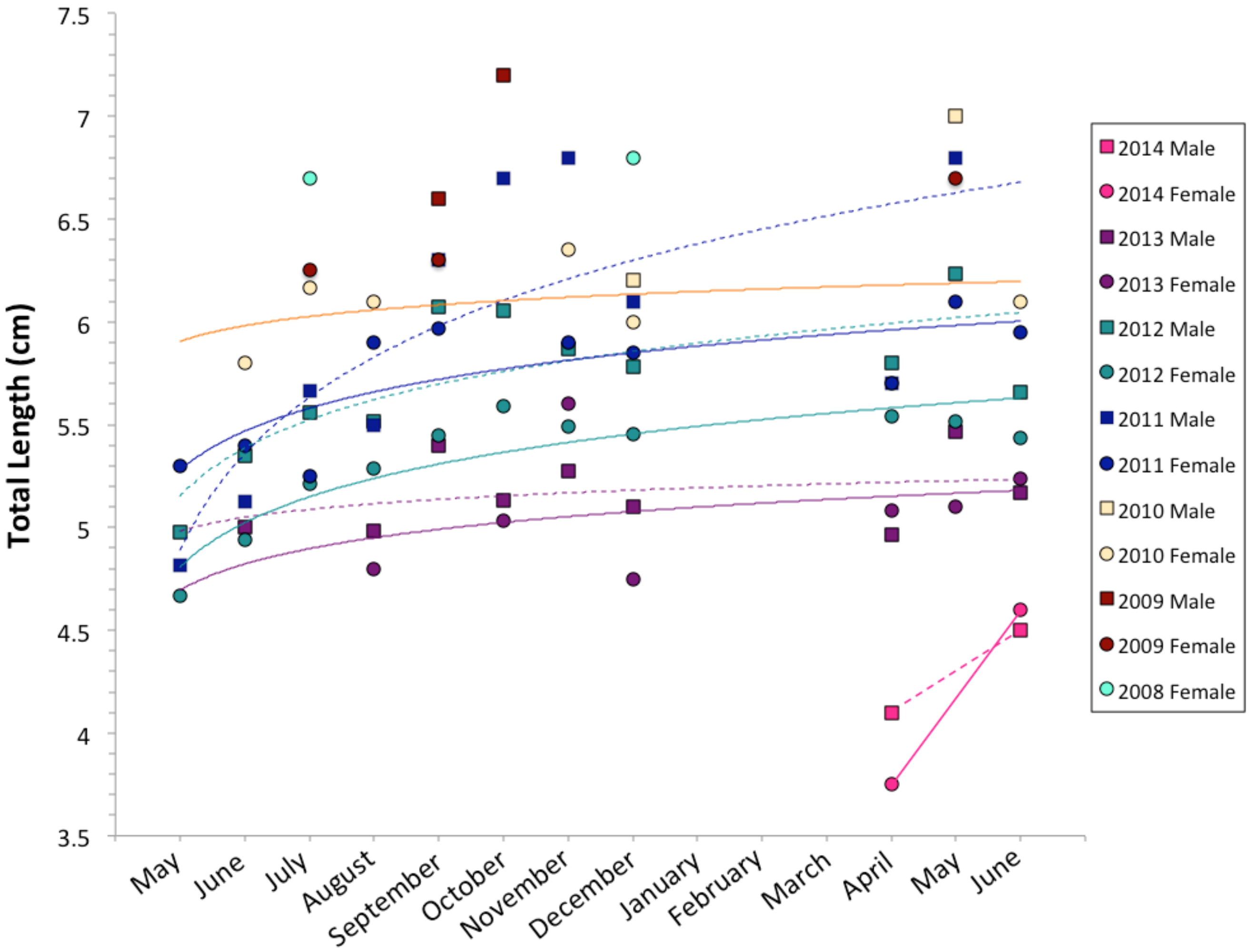

Figure 2.17 Male and female rainbow darter mean total length for fish grouped by hatch year recorded monthly between May 2014 and June 2015. Lines represent log regressions for male and female rainbow darter total length over time. Line colours correspond to year class and males and females are depicted by dashed and solid lines, respectively.

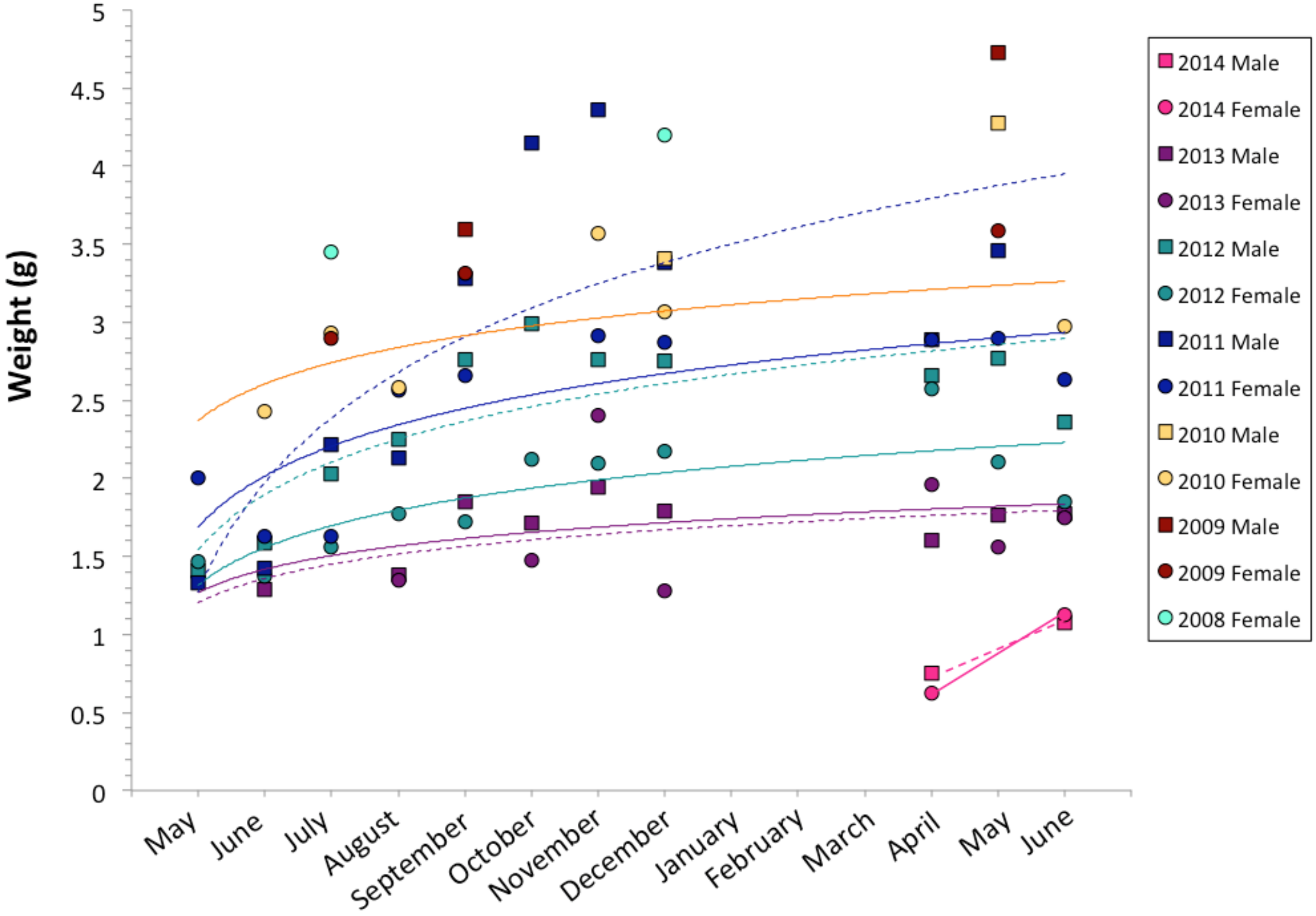

Figure 2.18 Male and female rainbow darter mean weight for fish grouped by hatch year recorded monthly between May 2014 and June 2015. Lines represent log regressions for male and female rainbow darter weight over time. Line colours correspond to year class and males and females are depicted by dashed and solid lines, respectively.

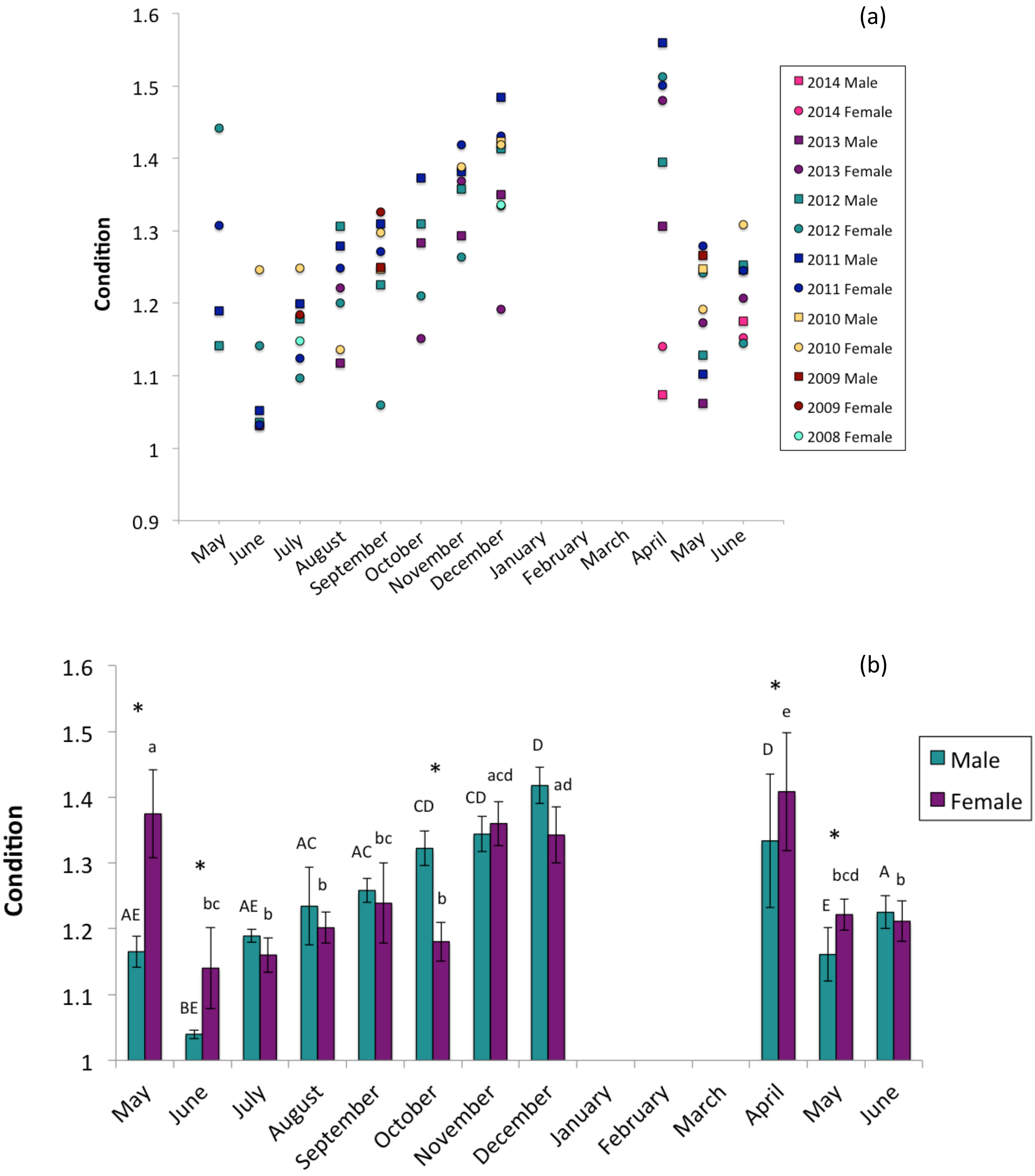

Figure 2.19 Male and female rainbow darter mean condition factor (a) grouped by hatch year and (b) of pooled age classes (±SE) recorded monthly between May 2014 and June 2015.

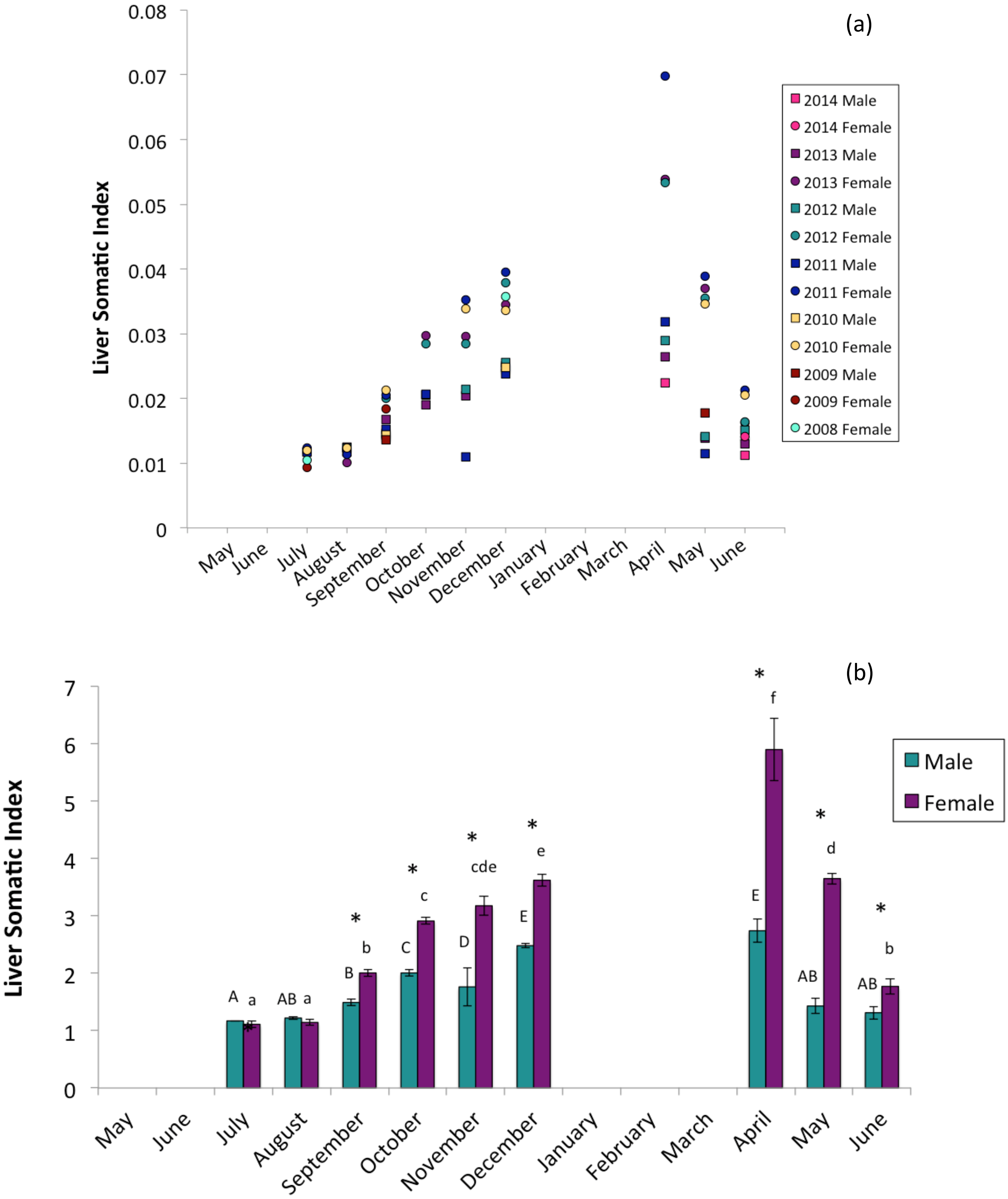

Figure 2.20 Mean liver somatic indices (LSI) for male and female rainbow darter (a) grouped by hatch year and (b) of pooled age classes (±SE) recorded monthly between May 2014 and June 2015. Liver weight was not collected for fish in May and June 2014, and therefore LSI was not calculated.

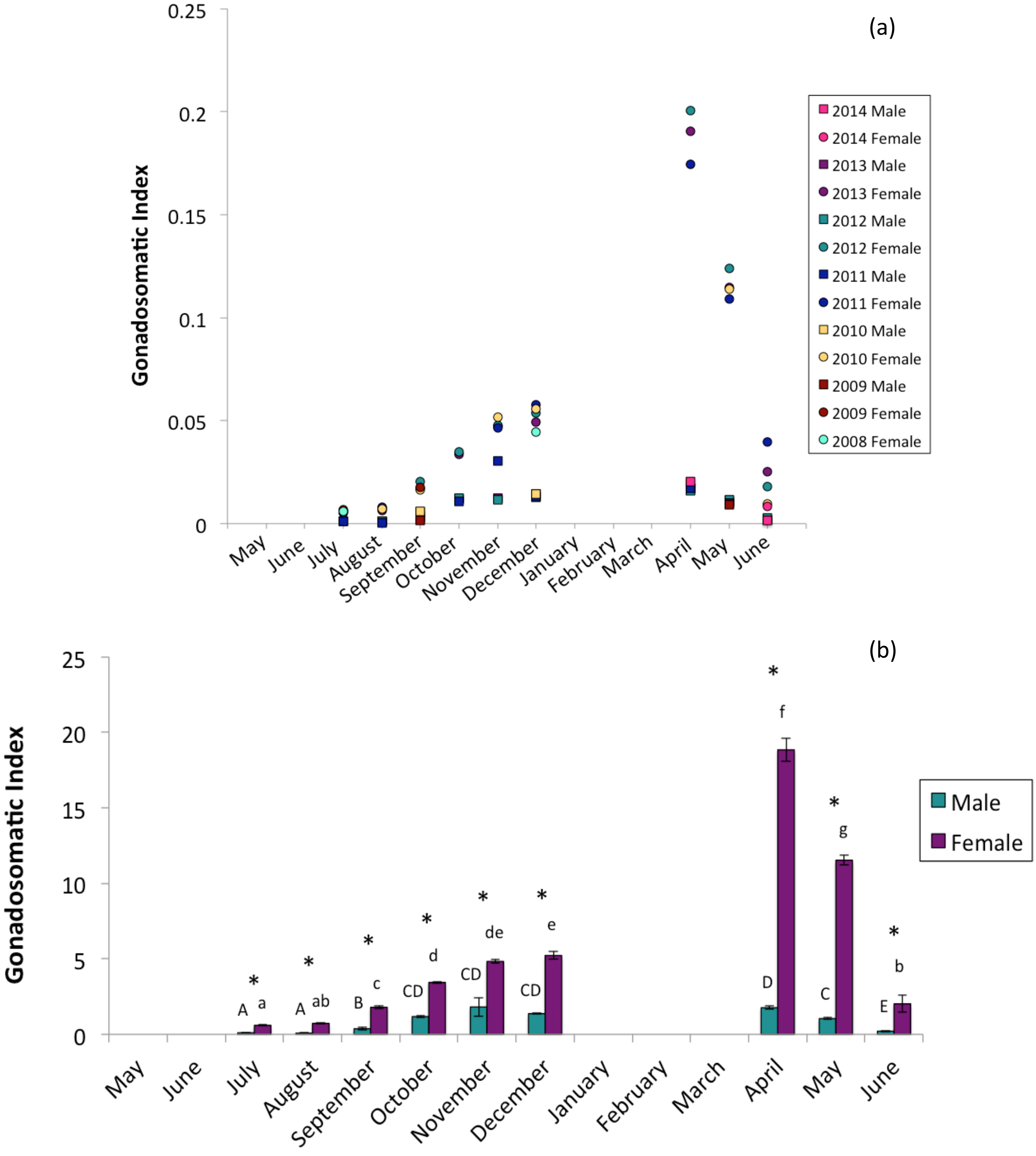

Figure 2.21 Mean gonadosomatic indices (GSI) for male and female rainbow darter (a) grouped by hatch year and (b) of pooled age classes (±SE) recorded monthly between May 2014 and June 2015. Gonad weight was not collected for fish in May and June 2014, and therefore GSI was not calculated.

2.4 Discussion

Rainbow darter in the Grand River form distinct annuli beginning in September, regardless of age or sex. Marginal increment analysis (MIA) and edge analysis (EA) both indicate that each year in the fall a translucent growth zone is formed on sagittal otoliths which can be used to accurately age fish ranging of all ages. Rainbow darter young-of-year (YOY) grow very rapidly in their first year, assimilating into the population by October such that they can no longer be separated from other age classes using length frequency histograms alone. Total length and weight increases over the summer/fall (i.e. growth) in all age classes but is most rapid in the YOY. Although fish of both sexes, grow similarly in their first two years, beginning in their third year males become longer and heavier at age compared to females. These differences in size may be associated with greater energy allocation to reproduction in females as indicated by higher GSI and LSI.

MIA and EA are widely used as validation methods in fisheries research (Campana, 2001). Rainbow darter are well suited for application of these methods as they are short-lived and fast-growing, developing adequate distinction between annuli to allow measurements and edge condition analysis (Campana, 2001). Both MIA and EA provided similar results in this study, however EA also provided the timing of translucent zone formation in the fall in addition to the timing of opaque zone formation. MIA provides more detailed information on the growth of otoliths throughout a full year, however high variability has been noted in numerous species within sex and separated age cohorts (Blackwell and Kaufman, 2012; Smith, 2014). The separation of sexes is not common in MIA studies (Pearson, 1996), however age separation was often done whenever possible (Scheerer and McDonald, 2003; Smith, 2014). Differences in the timing of increment formation between young and older age cohorts was common in these studies, with young fish often beginning formation earlier in the year compared to older fish (Johnson*, et al.*, 1995; Blackwell and

Kaufman, 2012; Smith, 2014). Small sample sizes of each age class in this study did not allow separation, and therefore any differences in the timing of annulus formation were not detectable. Unlike EA, MIA provides a numerical value associated with the size of the growing increment, allowing for statistical analysis. Statistical tests such as ANOVAs and non-parametric Kruskal-Wallis tests have been used on this data in the past, however testing this data is relatively uncommon (Campana, 2001; Caillet*, et al.*, 2006; Blackwell and Kaufman, 2012).

In the Grand River at West Montrose, edge analysis revealed variation in the onset of translucent zone formation, however all rainbow darter begin formation by mid-November. Translucent zones were associated with periods of slow growth (i.e. fall and winter) whereas opaque zones were associated with periods of fast growth (i.e. spring and summer). The timing of translucent zone formation coincides with a decrease in water temperature associated with annual weather changes. The cause of annulus formation on otoliths and other calcified structures used for age determination has not been verified, however correlations have been noted with changes in water temperature, reproductive season and food availability (Beckman and Wilson, 1995). Annulus formation in temperate-climate fishes have been linked to seasonal changes in water temperatures and the associated fluctuations in fish growth (Schramm, 1989; Beckman and Wilson, 1995). Opaque zones in bluegill (*Lepomis macrochirus*), conversely to rainbow darter, were associated with periods of slow growth, and translucent zones accompanied intervals of fast growth (Schramm, 1989). The formation of zones in bluegills was greatly influenced by temperature fluctuations, both in the wild and in experimentally manipulated systems (Schramm, 1989). Rainbow darter did not show an immediate response to decreasing water temperature in September, and the formation of translucent zones was not noted in 100% of fish until mid-November when temperatures had reached the lowest recorded values. Beckman (2002) reported similar findings in rainbow darters collected from the James River in southwest Missouri to those seen in the Grand River, noting the formation of opaque growth zones on sagittal otoliths between July and September. Rainbow darter in the Grand River began formation of summer (opaque) zone formation slightly earlier, beginning in April, however if formation is impacted by water temperature, and as a result the onset of spawning season, timing could vary depending on yearly temperature differences in the spring. The variation in the timing of zone formation in rainbow darter otoliths between Grand River and James River populations suggest that climate may be an influential factor contributing to the onset of formation. Additionally, it implies that caution must be taken when attempting to use previously validated ages in a study of a novel population, particularly when drastic climate differences between sites are evident.

The application of non-lethal aging techniques has been common in fisheries research, however the accuracy of the age estimates have been questioned in the past. Here, it is clear that the use of length-frequency distributions is limited to the YOY cohort, which is further restricted by sampling time. Many small-bodied fish, including darter species, are extremely fast-growing, often reaching sexual maturity and size within two years of hatch (Table 1.1). For spring spawning species of darter, YOY fish reach a catchable length by July, and are capable of integrating completely into the population by October (Layman, 1991; Finch*, et al.*, 2013). The ability to visually separate age cohorts is difficult, however the use of statistical methods such as kernel density analysis can assist in identifying age groups (Simmons*, et al.*, 2008). The same difficulties were apparent when attempting to use length-frequency distributions to assess the growth of slimy sculpin (*Cottus cognatus*), and as a result only the progression of YOY fish size was reported (Gray*, et al.*, 2002). The growth of YOY sculpin wasn't as rapid as rainbow darter, and YOY cohorts could be identified for the majority of the year (Gray*, et al.*, 2002). The growth of vermilion darter (*Etheostoma chermoki*) mirrors that of rainbow darter, exhibiting overlap in age 0+ and 1+ age cohorts beginning in September, with older age cohorts overlapping in length-at-age so greatly that identification was highly unlikely (Khudamrongsawat*, et al.*, 2005). Darter studies conducted in southern Ontario have reported similar findings to those seen here, with YOY fish reaching catchable sizes in July, remaining separate through the summer and integrating into the population by fall (Brown*, et al.*, 2011; Finch*, et al.*, 2013).

The relationship between otolith size and fish size is an important factor when attempting back-calculation of lengths (Campana, 1990). The method of back-calculation of size-at-age is based on the proportionality between fish length and the calcified structure (i.e. scales, otoliths) being used (Campana, 1990). A major assumption of this method is that the distance between features (i.e. annuli) is proportional to fish growth, however this is often not tested before the method is fully employed (Campana, 1990). Rainbow darter have a strong, log-linear relationship between otolith length and fish total length, providing adequate evidence that meet this assumption. This linear correlation has been reported in numerous species including yellow perch (*Perca flavecens*), smallmouth bass (*Micropterus dolomieui*), and white crappie (*Pomoxis annularis*) (Casselman, 1990). Yellow perch exhibited a strong relationship between otolith radii and fish lengths, however relationship slopes among populations differed, further emphasizing the necessity of characterizing this relationship whenever attempting back-calculation (Blackwell and Kaufman, 2012). Additionally, otolith shape, and therefore its growth related to fish length, can differ among populations (Campana and Casselman, 1993). Identifying the relationship between otolith length (or radii) and fish total length allows for the use of the body-proportional method for the back-calculation of size-at-age (Campana, 1990; Wilson*, et al.*, 2009) in rainbow darter. Back calculation using the body-proportional method has been applied in various darter species, further supporting its possible use in rainbow darter in the Grand River (Layzer and Reed, 1978; Drake*, et al.*, 2008). Back-calculation data would provide insight into individual fish growth rates, as opposed to population growth rates (Campana, 1990).

The growth of rainbow darter is rapid in younger fish and decreases as fish age, which follows the same pattern as numerous other darter species (Johnson and Hatch, 1991; Reid, 2004; Finch*, et al.*, 2013). The vermilion darter (*Etheostoma chermocki*) exhibited the fastest growth in their first year compared to growth in older aged fish (Khudamrongsawat et al., 2005). Similarly, savannah darter (*Etheostoma fricksium*) grew quickly in the first 6 months post-hatch, with growth rate declining drastically between 6-12 months of age (Layman, 1993). The mean total length of rainbow darter fry (reared in the laboratory) seven days post-hatch is approximately 0.85 cm (M. Fuzzen, University of Waterloo, personal communication). Field collections indicate that YOY can reach a maximum size of 5.0 cm by October, after only their first growing season. Fast growth within the first year post-hatch is seen in many small-bodied fish species in order to avoid predation and to reach sexual maturity quickly (Paine, 1990). Differences in growth rates between male and female fish has been noted in numerous sexually dimorphic species (Parker, 1992). Depending on life-history characteristics, either sex can present as the larger group (Imsland*, et al.*, 1997; Barton and Powers, 2010). In rainbow darter, growth of male and female fish, both in length and weight, remain similar in the first two years post-hatch. After this time, male fish start to gain weight and grow longer faster than females. This was evident both in the size-at-age comparisons as well as the fitted von Bertalanffy curves for each sex. Many factors contribute to an individual's ability to grow somatically, one of which being the amount of energy spent in the investment of gonadal tissue for spawning (Lambert and Dutil, 2000). Female cherokee darter (*Etheostoma scotti*) have a much higher gonadosomatic index (GSI) than males at the onset of spawning season and males also began to diverge from female weight-at-age beginning around 15 months post-hatch, gaining weight more quickly than females (Barton and Powers, 2010). Higher investment in gonadal development seen in female darters could contribute to their decreased weight gain compared to males, who invest much less energy in gonadal development. Liver size is associated with increased energy storage (Tetreault*, et al.*, 2011), and female rainbow darter invest substantially more resources than males into building up liver size over the fall and winter in order to draw from these energy sources during spawning season. Additionally, male rainbow darter are faced with intraspecific competition during spawning season (Reeves, 1907). Males will spend a large amount of time defending spawning territory against competitive males, and larger males are often more successful in these interactions (Reeves, 1907; Winn, 1958). Fuller (2003) reported that female preference accounted for very little for male spawning success, and in fact it was far more dependent on a male's ability to guard against other males. Increased size has also been correlated to increased luminescence of colours, a secondary sexual characteristic present in male rainbow darter (Zhou*, et al.*, 2014). It is fairly well established in this species that male size is positively correlated with spawning opportunities and success (Fuller, 2003). For these reasons, it is understandable that male rainbow darter grow longer and heavier more quickly than females. The size (length and weight) differences in rainbow darter after the age of 2+ also provide additional rationale for the separation of male and female fish in monitoring studies. Growth differences between male and female darter is highly dependent on the species. Species that do not exhibit sexual dimorphism, such as the eastern sand darter

(*Ammocrypta pellucida*), have been pooled for analysis (Drake, et al., 2008), whereas Missouri saddled darter (*Etheostoma tetrazonum*), which exhibit a high degree of visual dimorphism between sexes, have been separated (Taber and Taber, 1983). Male and female rainbow darter are easily distinguished and growth differences between sexes have been identified, therefore separation for analysis is necessary.

The use of appropriate growth curves and their application to size and age data can provide additional information into the growth patterns of a species. von Bertalanffy growth curves have been used in studies focusing on small-bodied fish, including species of darter (e.g., Finch*, et al.*, 2013; Olson and Martin, 2016). von Bertalanffy growth curves were applied to two separate populations of eastern sand darter (*Ammocrypta pellucida*) located in the Thames River, Ontario and the Little Muskingum River, Ohio (Finch, et al., 2013). Growth curves were then compared to assess differences in growth rates (Finch, et al., 2013). Interestingly, the curves and k (Brody growth coefficient) values differed significantly while the L_{inf} (asymptotic/maximum length) did not differ between populations, equaling 5.55 cm and 5.53 cm for the Thames River and Little Muskingum River populations, respectively (Finch, et al., 2013). This data suggests that growth differed in younger ages but fish reached a similar maximum size regardless of early life growth rates (Finch, et al., 2013). von Bertalanffy growth curves for rainbow darter in the Grand River at West Montrose yielded greater L_{inf} values compared to eastern sand darter, equaling 7.42 cm and 6.48 cm for males and females, respectively. Unlike eastern sand darter, rainbow darter male and female length and weight at older ages differ significantly, and therefore must be separated for analysis. The ability to compare von Bertalanffy growth curves and detect key differences in growth rates of young fish between populations could aid in the application of size-at-age data among populations of rainbow darter in the Grand River, where differences in size may be apparent in younger cohorts while adult fish reach similar maximum sizes.

The ability to accurately age rainbow darters in the Grand River will greatly enhance the current research on the impacts of urbanization, and more specifically the input of municipal wastewater effluent (MWWE) on fish health. Fish growth has been identified in the Environmental Effects Monitoring (EEM) program as an important endpoint when assessing the impact of effluent on the receiving environment (Environment Canada, 2010). Before now, rainbow darter age, and as a result growth, was not estimated in these studies due to the difficulty of otolith extraction, preparation and aging in addition to the absence of a validated method. This study shows that direct age determination using otoliths is an accurate method for estimating age in this species and that the use of length-frequency distributions does not adequately separate ages and therefore cannot be used as an aging method. Male and female fish growth is similar in both length and weight in younger fish, with males becoming longer and heavier at age in older cohorts and the addition of back-calculation of size-at-age would increase the amount of information gained from fish sampling, providing greater insight into the growth of individual fish as well as the population as a whole. This would allow the addition of growth as an endpoint in ongoing and future studies focusing on the impacts of MWWE on fish health and aquatic environments in the Grand River and other watersheds.

Chapter 3

Conclusions and Recommendations

The use of fish growth as an endpoint in impact assessments has been widely accepted, and the ability to accurately age fish greatly enhances the ability to evaluate growth. Monitoring programs, including the Environmental Effects Monitoring (EEM) program developed by Environment Canada, often incorporate measures of fish growth to assess the impact anthropogenic stressors on fish populations (Kilgour*, et al.*, 2005). Municipal wastewater effluent (MWWE) has the ability to impact reproductive endpoints in rainbow darter (Tetreault*, et al.*, 2011) and therefore the assessment of growth would provide additional insight into the effect of effluent on fish health. Increased length and condition of YOY and adult fish downstream of effluent input sites has been identified in rainbow darter and greenside darter in the Speed River, Ontario (Brown*, et al.*, 2011). MWWE has the ability to impact growth rates due to increased nutrient input, which can indirectly elevate food availability within the system (McMaster*, et al.*, 2005). Treatment upgrades have been implemented at the Kitchener municipal wastewater treatment plant (MWWTP) and are currently being constructed at the Waterloo MWWTP, which could alter effluent quality entering the aquatic receiving environment (Bicado, *et al.*, 2016). These upgrades provide the opportunity to assess possible differences in growth among sites before and after implementation. As a component of an ongoing monitoring study, a minimum of 15 male and 15 female rainbow darter have been collected at numerous sites upstream and downstream of the Kitchener and Waterloo MWWTP each fall since 2007 (missing 2009). It is possible that any effects apparent before upgrades were implemented may be altered or diminished in fish collected after these major infrastructure investments go online.

Growth differences in rainbow darter among sites and years can be assessed using various methods. The first is the comparison of length-at-age relationships using ANCOVA tests. This method is commonly used in the EEM program, however is often applied to adult (i.e. older-aged fish) that are no longer growing at a rapid rate and therefore have a strong (log) linear relationship between length and age at a given time point (McMaster*, et al.*, 2002; Munkittrick*, et al.*, 2002). This method has been applied to small-bodied fish species in the past (Gibbons*, et al.*, 1998b), however since rainbow darter grow rapidly both in length and weight within their first year the size-at-age relationship is not linear for this species. A more representative relationship between length and age can be applied using the von Bertalanffy growth equation, and comparisons of the resulting growth curves may be better suited in this species using the analysis of the residual sum of squares (Chen*, et al.*, 1992; Finch*, et al.*, 2013). If sampling was conducted in October to fit von Bertalanffy growth curves, 25 females and 43 males from each site would be necessary to detect a 25% difference in growth, based on power analysis. Power analysis was completed using PS: Power and Sample Size Calculator (version 3.2.1; Dupont and Plummer, 2014), with $\alpha=0.05$, power=0.8 and an effect size of 0.25.

Another method that can be applied to rainbow darter length-at-age data among populations and time points is use of two-way ANOVA tests. This would identify significant differences in mean length-at-age of males and females of the same age class among populations and collection times. A disadvantage to using this testing method is the small sample sizes collected during each sampling event, which could limit its use. The possibility remains, however, to pool samples (with careful consideration of annual variability) collected before and after the upgrades to increase sample sizes sufficiently for testing. Larger sample sizes in the future would allow for this type of statistical testing. Power analysis was performed on length-at-age data collected in October 2014 and the resulting sample sizes can be seen in Table 3.1. Power analysis was completed using PS: Power and Sample Size Calculator (version 3.2.1; Dupont and Plummer, 2014), with α=0.05, power=0.8 and an effect size of 0.25.

Table 3.1 Sample sizes necessary to detect a >25% difference in mean length at age for each sex and age class of rainbow darter collected at West Montrose. Male and female fish length did not differ significantly at ages 0+ and 1+, and were therefore pooled for this analysis.

	0+	1+	2+	3+	4+
Male	59	31	30	40	32
Female			14	13	47

The length-frequency distribution constructed in July 2014 clearly identifies the YOY cohort, which has not yet assimilated into the population. If differences in size-at-age are being investigated among sites, it is possible to use mean YOY length and weight for this testing. This would provide insight into how young fish are growing in their first season and whether differences exist between sites upstream and downstream of MWWTPs and reference sites. Construction of a length-frequency distribution would need to be done in order to ensure adequate separation and identification of YOY cohort. Power analysis conducted on the July 2014 YOY fish indicates that a sample size of at least 36 YOY collected at each site at this time would be able to detect a >25% difference in size between populations. Power analysis was completed using PS: Power and Sample Size Calculator (version 3.2.1; Dupont and Plummer, 2014), with α=0.05, power=0.8 and an effect size of 0.25. Several collections of rainbow darter were made since 2007 that could be used to make these comparisons.

The use of a back-calculation method would provide additional insight into the size-at-age of individual fish, thus increasing the amount of information acquired from each sample. The ability to estimate size-at-age could allow for the comparison of age cohorts in past years. The short lifespan of rainbow darter presents an obstacle when utilizing this method. The maximum age of rainbow darter was found to be 6 years in this study, however few fish over the age of 4 were captured. Back–calculation could potentially use fish captured after the upgrades to estimate their size before upgrades were implemented at the Kitchener MWWTP. Due to the short lifespan and low frequency of fish old enough to provide this information, it may not be possible to use this method for this purpose, although it offers the potential to pool samples collected in the 2 -3 years before and after upgrades to estimate growth of young fish. The Waterloo MWWTP is currently undergoing upgrades, so the possibility remains to use this method to aid in the assessment of size-at-age alterations surrounding these improvements to treatment. Regardless, the use of the back-calculation method would provide more information pertaining to past size-at-age, and would increase our knowledge of how fish grew in past years when samples were not collected or small samples limited the possibility of comparisons. Additionally, this analysis could potentially allow for the increase of sample sizes for comparisons in instances where more statistical power is necessary. Careful consideration of annual variability in environmental factors (e.g. temperature, flow) would be very important for interpretation of the results.

Age validation using marginal increment analysis (MIA) and edge analysis (EA) were successful in determining the timing and periodicity of annuli formation in rainbow darter sagittal otoliths. MIA was able to identify the timing of opaque (summer) growth zone formation, which accompanies the increase in feeding frequency following low temperature and decreased food availability during the winter months. MIA has the ability to identify differences in the timing of opaque zone formation between age cohorts of a fish species, such as lemon sole (*Microstomas kitt*) (Smith, 2014). In this species, it was evident that younger fish began summer growth zone formation approximately one month earlier than older fish (Smith, 2014). Large sample sizes and a long-lived study species allowed for the separation of age classes, which is not as easily done in a species such as rainbow darter. In order to assess whether the timing of zone formation differs between age cohorts in rainbow darter, large sample sizes of each age class would be necessary at each time point. This study identifies the relative timing of zone formation, and therefore sampling events could be focused around these times and would be unnecessary in months where zone formation is not occurring.

This study supports the continuation of sampling of rainbow darter in the fall (i.e. October and November) as there are many advantages. If assessing growth or size-at-age, this time of year is ideal. Gonadal tissue growth in both male and female fish has started but is not yet large, and therefore does not contribute substantially to fish weight. Additionally, annuli in rainbow darter otoliths form at this time of year, and therefore aging of fish using this structure is straightforward. If annuli have not yet formed on the growing edge of the otolith, it can be assumed that within a certain amount of time, depending on when sampling is conducted, an annulus will form. Aging fish with the assumption that the growing edge exhibits the formation of an annulus makes the aging process much easier, faster and more reliable. Lastly, the YOY cohort is easily catchable at this time of year, allowing for complete sampling of all aged fish. Alternatively, spring sampling would yield no YOY fish caught, as they spawn in early April-June. As an asynchronous clutch spawner, gonadal tissue, which can account for as much as 20% and 3% of total weight in females and males respectively, would be highly variable. Recently hatched YOY rainbow darters do not reach a catchable size until early summer (using backpack electrofishing and nets).

The incorporation of a comparison of size-at-age of rainbow darter among sites upstream and downstream of MWWTPs in the Grand River would aid greatly in the understanding of the impacts of MWWE of fish health. Differences in growth has been noted in rainbow darter in the Speed River, a tributary of the Grand River, upstream and downstream of the Guelph MWWTP, and therefore the possibility remains that recent and currently ongoing upgrades at the Kitchener and Waterloo MWWTPs could be detected. These comparisons could be done in various ways, and is dependent on the ages under investigation as well as the number of fish available for analysis. Fall sampling is the ideal timing for rainbow darter collections due to the ease of capture of all aged fish and the relatively small and stable size of gonadal tissue. The addition of this type of analysis would increase our knowledge of how treatment plant upgrades, such as those currently ongoing at the Kitchener wastewater treatment plant, change effluent quality and if this has the ability to subsequently alter growth of rainbow darter downstream of effluent outfalls. This study provided the base knowledge of how rainbow darter grew at a relatively unimpacted site in the Grand River. Further research is necessary to detect whether fish reared in urbanized areas and downstream of MWWE outfalls differ from reference sites.

Appendix A

Table A.1 Number of fish collected of each sex and hatch year and the number collected in fall 2014. The number of fish with the presence of annulus (translucent zone) formation on the growing edge of the otolith is referenced in the (#) column.

		September		October		November	
Hatch Year	Sex	Total	#	Total	#	Total	#
2013	Male	1	0	5	3	7	7
	Female	0	0	6	5	1	1
2012	Male	11	8	7	7	6	6
	Female	4	3	9	6	9	9
2011	Male	1	0	2	1	2	2
	Female	5	4	0	0	3	3
2012	Male	1	0	0	0	0	0
	Female	4	3	0	0	2	2
2011	Male	0	0	0	0	0	0
	Female	1	1	0	0	0	0

Table A.2 Number of fish collected of each sex and hatch year and the number collected in spring 2014 and 2015. The number of fish with the presence of summer (opaque) growth zone formation on the growing edge of the otolith is referenced in the (#) column.

		2014						2015					
		May		June		July		April		May		June	
Hatch Year	Sex	Total	#	Total	#	Total	#	Total	#	Total	#	Total	#
2014	Male	-	-	-	-	-	-	6	0	0	0	6	6
	Female	-	-	-	-	-	-	2	0	0	0	2	2
2013	Male	0	0	1	1	0	0	8	0	3	1	5	4
	Female	0	0	0	0	0	0	5	3	5	0	5	5
2012	Male	4	1	4	4	7	7	6	1	6	0	5	2
	Female	3	0	8	7	7	7	9	0	7	0	3	3
2011	Male	6	0	3	0	8	8	1	0	2	0	0	0
	Female	7	0	1	0	2	2	1	0	2	0	4	4
2010	Male	0	0	0	0	0	0	0	0	1	0	0	0
	Female	0	0	1	0	3	3	0	0	1	0	1	1
2009	Male	0	0	0	0	0	0	0	0	1	0	0	0
	Female	0	0	0	0	2	2	0	0	0	0	0	0
2008	Male	0	0	0	0	0	0	0	0	0	0	0	0
	Female	0	0	0	0	1	1	0	0	0	0	0	0

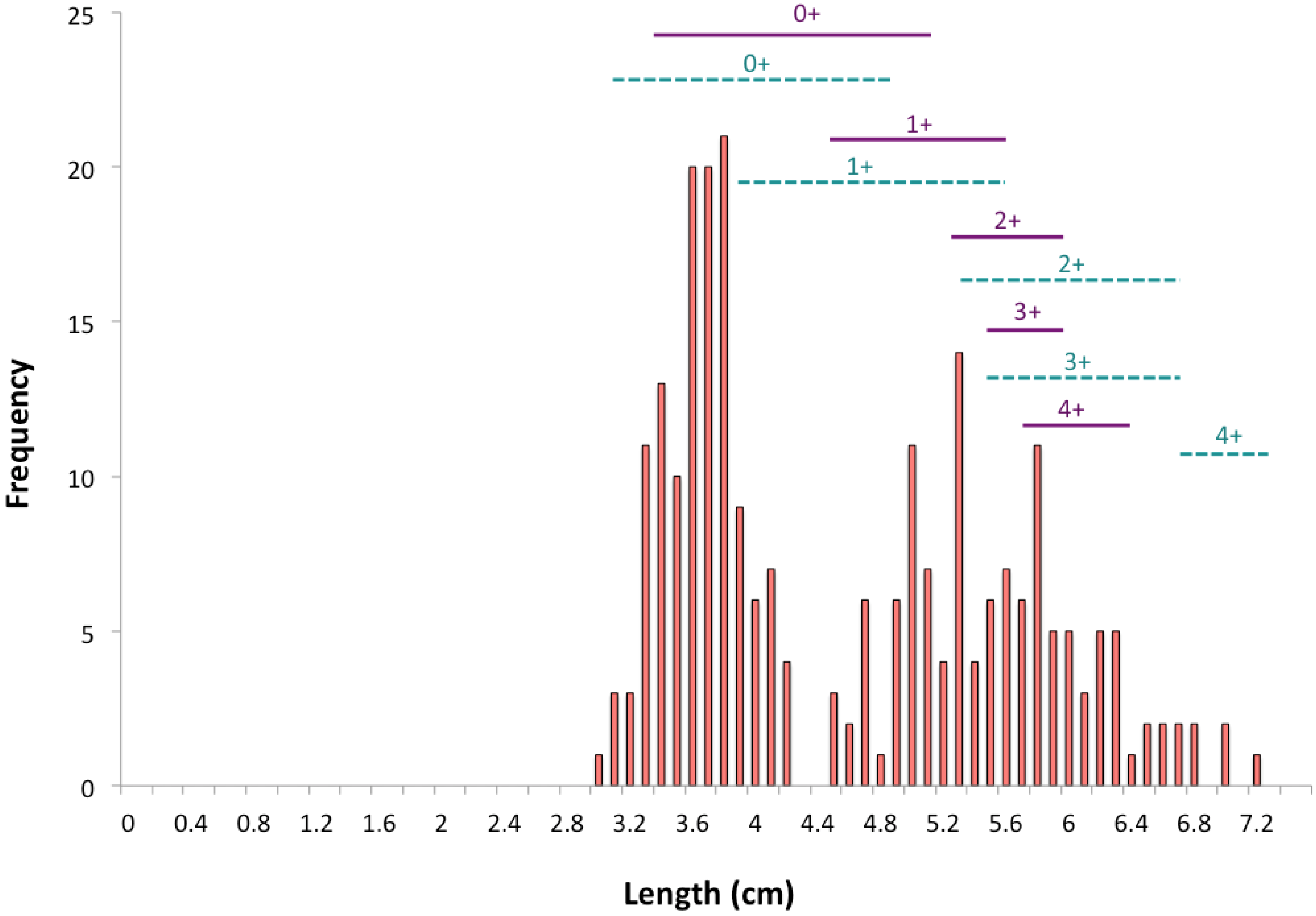

Figure A.1 Length-frequency distribution of rainbow darter in October 2014 at West Montrose (n=251). A subsample of fish were sacrificed for direct age determination using otoliths in October 2014, which yielded a maximum and minimum length-at-age, represented as a range for each age cohort. Length-at-age ranges are represented for female (solid lines; n=60) and male (dashed lines; n=84) fish.

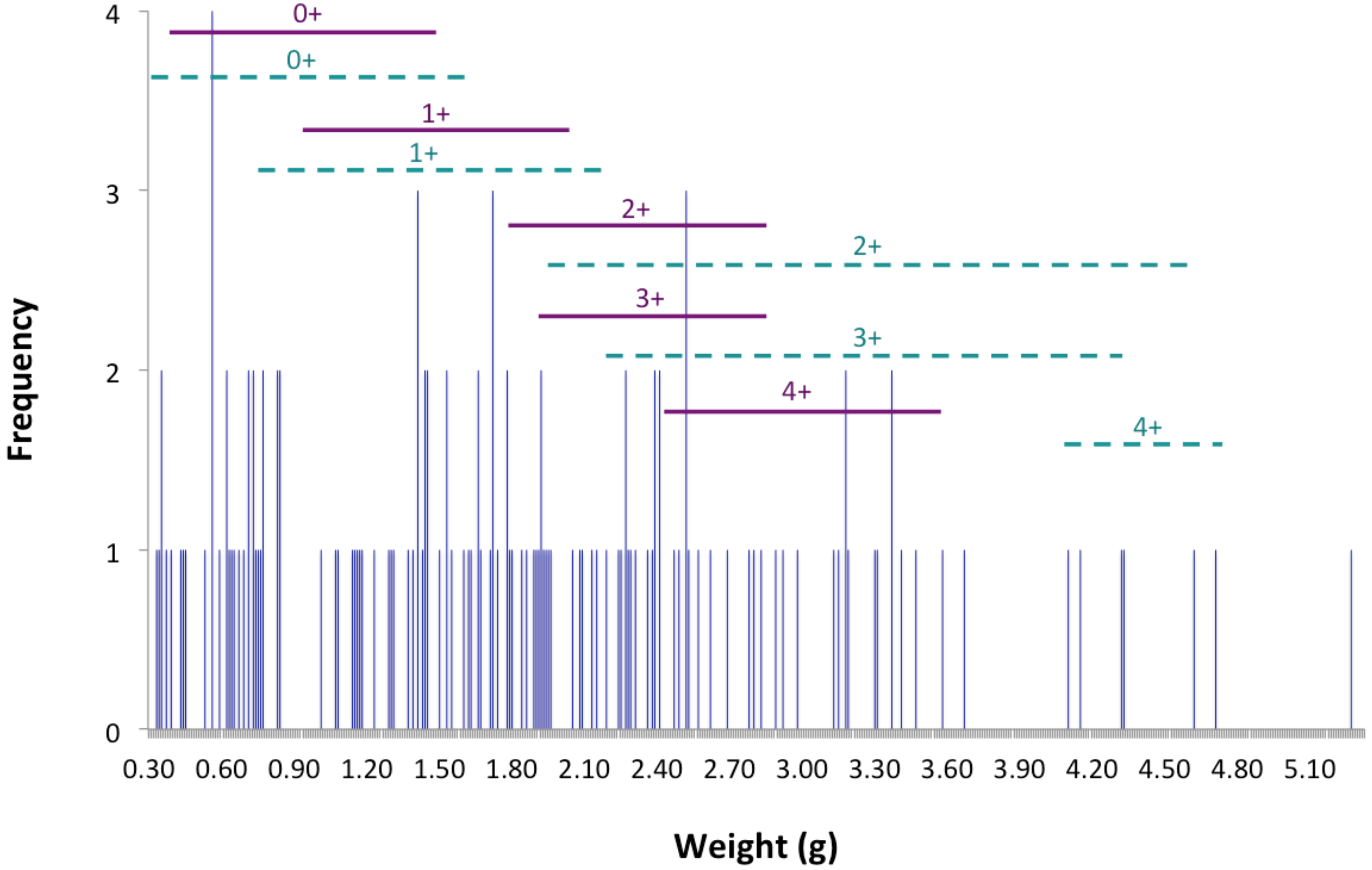

Figure A.2 Weight-frequency distribution of rainbow darter in October 2014 at West Montrose (n=251). A subsample of fish were sacrificed for direct age determination using otoliths in October 2014, which yielded a maximum and minimum weight-at-age, represented as a range for each age cohort. Weight-at-age ranges are represented for female (solid lines; n=60) and male (dashed lines; n=84) fish.

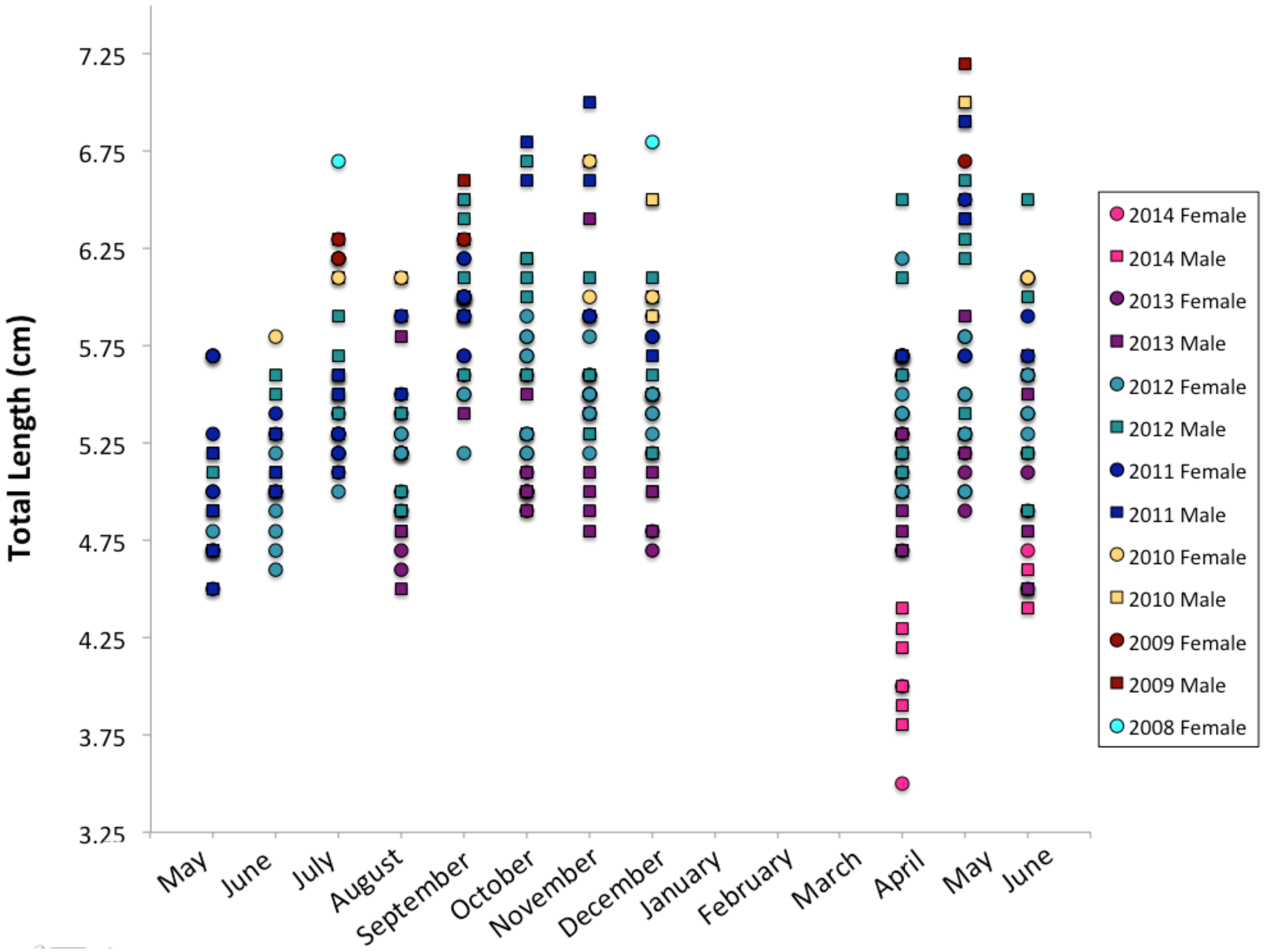

Figure A.3 Total length (cm) of each fish, separated by sex and hatch year, collected between May 2014 and June 2015.

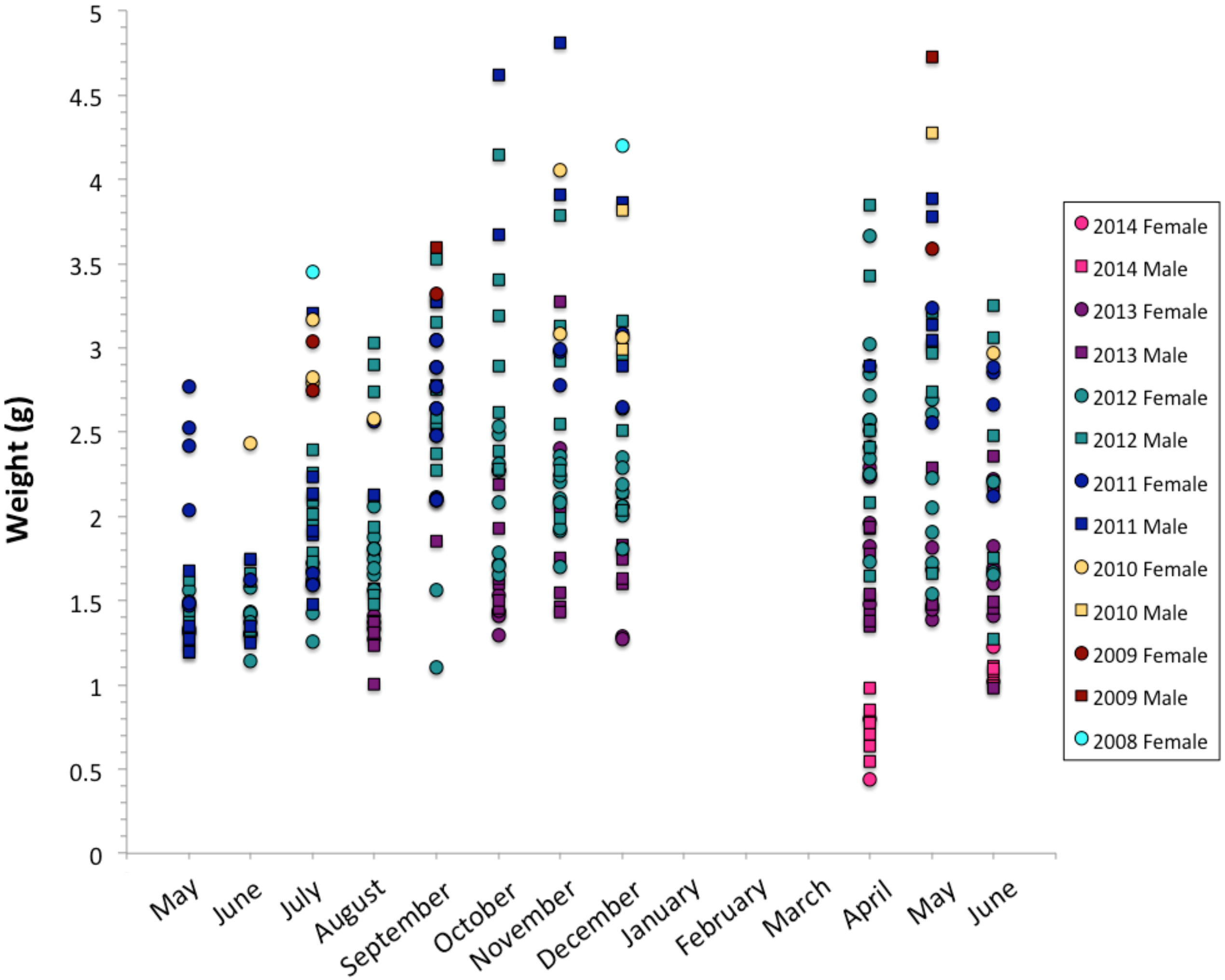

Figure A.4 Weight (g) of each fish, separated by sex and hatch year, collected between May 2014 and June 2015.

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