

SELECTIVE SEROTONIN REUPTAKE INHIBITORS AND RAT BONE

THE EFFECTS OF *IN UTERO* AND POSTPARTUM EXPOSURE TO
SELECTIVE SEROTONIN REUPTAKE INHIBITORS ON BONE PROPERTIES IN
RATS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the
Requirements for the Degree Masters of Applied Science

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MASTER OF APPLIED SCIENCE (2013)
(School of Biomedical Engineering)

McMaster University
Hamilton, Ontario

TITLE: The effect of *in utero* and postpartum exposure to selective serotonin reuptake inhibitors on bone properties in rats

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NUMBER OF PAGES: xiv, 68

ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) are the most common drugs prescribed to treat depression during pregnancy. The influence of SSRI exposure during pregnancy on fetus bone properties is not clearly understood. The overall objective of this thesis project was to investigate the short and long-term effects of *in utero* and postpartum exposure to SSRIs on bone in rats through measuring bone structural and material properties.

Two studies with two types of SSRIs were carried out. Dams in the treated groups were randomly assigned to receive sertraline (10 mg/kg/day, N=5) in the first study and fluoxetine (10 mg/kg/day, N=17) in the second study. Control animals in the studies received saline in a flavoured gelatin vehicle. Rats were exposed to sertraline only during pregnancy, but fluoxetine was administered to the dams during pregnancy and also during breastfeeding. Rat offspring were sacrificed at 3, 7 and 26 weeks of age and left femurs and L6 vertebrae were analyzed for differences in morphology and mechanical properties.

Maternal sertraline exposure resulted in significantly shorter femurs for the offspring at 3 weeks of age. Rat femurs from the sertraline group were also weaker at 3 and 7 weeks of age compared to controls. In comparison, *in utero* and postpartum exposure to fluoxetine did not have a negative impact on bone properties. In fact, the femurs from fluoxetine exposed offspring were significantly stronger at 3 weeks of age compared to the controls

Findings in this project suggest that the type of SSRI used by pregnant woman should be considered as an important factor. Maternal sertraline exposure has a negative effect on offspring bone properties. Considering the fact that various mechanisms are involved in the influence of SSRIs on bone, further studies should be conducted to determine the mechanisms of this influence on bone properties *in utero* and through stages of development.

ACKNOWLEDGMENT

I would like to take this opportunity to express my profound gratitude and deepest appreciation to my supervisor, Dr. Greg Wohl, for his constant support, encouragement and invaluable guidance throughout my research. His supervision has made my foreseen milestones believable and easier to accomplish.

I am obliged to Dr. Alison Holloway and her graduate student, Nicole De Long, for their kind support and cooperation in providing me the rats, doing all the animal handling and facilitating my research. I would also like to thank my dear friend and colleague, Zahra Hosseini, for helping me throughout my project and for designing the femur trays.

A special thank you to my lovely parents, Nahid and Kazem. Mom you're the angel of my life and have always been there for me and helped me get through anything. Dad, I'm proud to have followed your footsteps as an engineer and will never forget your support, help and guidance. To my two wonderful brothers, Hadi and Mohsen, that have always been there for me with their continuous support and love. Last, but certainly not least, to my lovely husband Sajjad for his nonstop encouragement, support and love. Thanks for always being there for me.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	per cent
δ	deformation (displacement)
μm	micrometre
mm	millimetre
$^{\circ}\text{C}$	degrees Celsius
kg	kilogram
mg	milligram
min	minute
N	Newton
SSRI	selective serotonin reuptake inhibitor
BMD	bone mineral density
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	L-5-hydroxytryptophan
TPH	tryptophan hydroxylase
5-HTT	serotonin transporter
GI	gastrointestinal
Lrp5	low-density lipoprotein receptor-related protein 5
CV	cardiovascular
Ad β 2	β 2 adrenergic receptor
OCD	obsessive-compulsive disorder

FDA	Food and Drug Administration
GD	gestational day
PD	postnatal day
CO ₂	carbon dioxide
microCT	microcomputed tomography
Ct.Ar	cortical bone area
I _{xx}	moment of inertia
Y _{post}	distance from centroid to posterior endosteum
μA	micro-ampere
kV	kilovolt
W	watt
FOV	field of view
ROI	region of interest
Tb.Th	trabecular thickness
Tb.N	trabecular number
Tb.Sp	trabecular separation
BV/TV	percent bone volume
GPa	gigaPascal
mJ	milliJoules
g	grams
SD	standard deviation

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Maryam Badv, performed all mechanical testing, analysis of mechanically tested bones, calculations of mechanical and material properties, analysis of micro-computed tomography data, statistical analyses and writing of this thesis. Dr. Greg Wohl provided guidance on testing and analysis and editorial suggestions during writing of my thesis. Design of the sertraline and fluoxetine experiments including animal handling, dosing, and sacrifice was performed by Dr. Alison Holloway and Nicole De Long.

CHAPTER ONE: INTRODUCTION

1.1 Depression, pregnancy and SSRI use

Maternal depression is a common medical condition among pregnant woman. It has been reported that 12 to 15% of woman are at risk of depression during their gestation period [1]. For this reason, the prenatal use of antidepressants is rapidly increasing and making selective serotonin reuptake inhibitors (SSRIs) the most prescribed antidepressant drugs to pregnant woman [2]. The risks associated with prenatal use of these antidepressants to the fetus are still not fully understood and contradiction is seen among results obtained from different studies. Studies have shown that SSRIs cross the placenta [3–5] and are also excreted in breast milk [5–7], meaning that the baby could be exposed to the drug as a fetus *in utero* and also during breast feeding. A number of human studies have reported that SSRI use during pregnancy is associated with birth defects such as craniosynostosis, anencephaly [8], omphalocele [8,9], and craniofacial [10,11] and cardiovascular malformations [12–14], and some studies have suggested an increased risk of congenital malformations in general [15,16]. Other complications such as feeding problems, increased risk of abortions, premature labor and intrauterine fetal death have also been reported in woman using SSRIs during their pregnancy [17–19].

1.2 Serotonin, bone and SSRIs

Serotonin receptors have been found on all major bone cells [20–23], suggesting that bone is responsive to serotonin signaling. The gut and the brain are the two main

sources of serotonin production in the body. Depending on where serotonin is produced, it has been shown to have both negative and positive effects on bone remodeling [22,23]. Gut-derived serotonin has a direct and negative effect on bone formation, while brain-derived serotonin has an indirect and positive effect on both processes of bone formation and bone resorption. Brain-derived serotonin is known to decrease bone resorption and increase bone formation [24]. SSRIs target serotonin transporters and increase the serotonin level in the body by inhibiting serotonin from getting reuptaken back into the pre-synaptic neuron [25]. Since SSRIs interrupt the functioning of the serotonergic system, and the impact of serotonin on bone has been clear, these drugs have also been shown to influence bone remodeling. In this regard, several clinical and animal studies have looked at the association between SSRI use and their effect on bone properties. In adults and also adolescents SSRI use has been associated with lower BMD [26–32] and increased risk of vertebral and non-vertebral fractures [33–40]. SSRI administration to rodents has also been shown to negatively impact bone mechanical properties [41,42] and reduce BMD [31,42,43].

Despite the fact that SSRIs are the most common antidepressants used by pregnant woman and knowing the potential influence of these drugs on bone, not much research has been conducted regarding *in utero* exposure to SSRIs and infant bone health. The few current human studies are limited to only investigating properties such as overall body length, head circumference [19,44,45] and bone density [19] in newborns. Some studies have reported that SSRI exposed infants had smaller head circumferences [19,45] and shorter birth length [19,44,45] compared to the control groups. To our knowledge, no

animal studies to date have looked at the effect of the maternal use of these drugs on offspring bone properties.

1.3 Aim of this project

Overall the influence of SSRI use during pregnancy and lactation on infant bone health is not clear and several short term and long term concerns are remaining. The overall objective of this thesis project was to investigate the effects of *in utero* and post partum exposure to SSRIs on bone properties in rat offspring at three time points after birth: weaning (3 weeks), growth (7 weeks) and early adulthood (26 weeks). The hypothesis was that maternal exposure to SSRI during pregnancy leads to negative effects on material and structural properties of bones in the rat offspring.

CHAPTER TWO: BACKGROUND

2.1 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) was first recognized in 1948. It was known to be released in the process of blood clotting in tissue injuries and act as a vasoconstrictor substance in blood serum [46]. Later on, serotonin was known as a neurotransmitter and currently it is mostly recognized as a mood regulator agent in the body. It is also known to regulate other diverse behaviours such as sleep, appetite, sexual behaviour, anxiety, temperature and movements in the body [47]. Serotonin is synthesized in two steps [20,47]. In the first step, L-tryptophan is hydroxylated into L-5-hydroxytryptophan (5-HTP) by one of the isoforms of tryptophan hydroxylase (TPH), and then in the second step, 5-HTP is decarboxylated by an L-amino acid decarboxylase giving an end product of serotonin [46,48] (Figure 2.1).

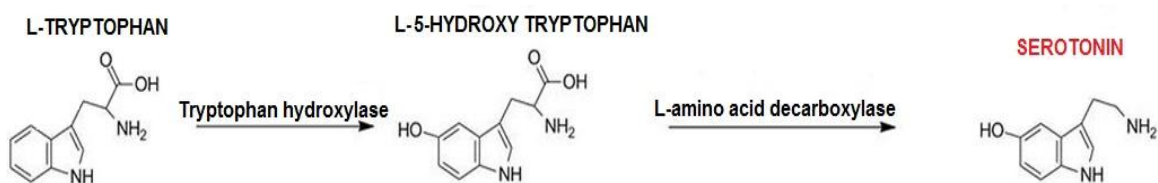


Figure 2.1 The two steps for the synthesis of serotonin

2.2 Serotonin receptors and serotonin transporter

So far, seven families of serotonin receptors have been identified (5-HT₁ to 5-HT₇) and each of these family groups have been shown to have receptor subtypes with different mechanisms of action, resulting in 18 serotonin receptor genes in total [49]. Serotonin

receptors have been found on all major bone cells [20–23]. In addition to serotonin receptors, some studies show osteoclast, osteoblast and osteocyte cell lines also possess the serotonin transporter, 5-HTT which selectively uptakes serotonin into the cells [22,50]. Serotonin transporters are present in the synaptic cleft and regulate the serotonin level in the body by re-uptaking serotonin back into the system.

The findings that bone cells express serotonin receptors and transporters support the idea that serotonin plays a role in bone remodeling and bone cells possess serotonergic pathways that both respond to and regulate serotonin signaling in the body [22].

2.3 Sources of serotonin in the body and their association with bone remodeling

There are two main sources of serotonin in the body: gut-derived serotonin and brain-derived serotonin. Depending which of these sources produces serotonin, it influences bone remodeling in opposite directions [22,23,49].

2.3.1 Gut-derived serotonin

Of the serotonin produced in the body, 95% is synthesized by the enterochromaffin cells in the gut (gastrointestinal (GI) tract). The amino acid catalyzing the rate-limited step in the peripheral production of serotonin is an isoform of tryptophan hydroxylase (TPH) called tryptophan hydroxylase 1 (Tph1) [51]. This reaction is regulated by low-density lipoprotein receptor-related protein 5 (Lrp5) in the gut [24]. Most of the serotonin produced in the gut (>90%) is then taken up by the platelets through a serotonin

transporter and circulated in the cardiovascular (CV) system. The stored serotonin is then released in case of injury and plays a role in blood clotting [23].

Gut-derived serotonin has been shown to have a direct and negative effect on bone formation by decreasing osteoblast proliferation [21,24,52] (Figure 2.2a). In support of this, a study by Yadav et al. [53] showed that mice lacking Tph1 in duodenal cells had higher osteoblast number and rate of bone formation that resulted in greater bone mass.

2.3.2 Brain-derived serotonin

In addition to the 95% serotonin produced in the gut, the remaining 5% of the body's serotonin is produced in the brain. Brain-derived serotonin plays an important role in the function of diverse behaviours such as mood, appetite, sleep, temperature and sex [22]. Tryptophan hydroxylase 2 (Tph2) is the catalyser expressed in brain-derived serotonin. In contrast with gut-derived serotonin, the serotonin produced in the brain has a positive effect on bone remodeling and has an influence on both processes of bone resorption and bone formation involved in bone accrual [23]. This effect is indirect since the serotonin produced in the brain cannot cross the blood-brain barrier [46,51,54]. The positive influence of brain-derived serotonin on bone is negatively regulated by leptin [24]. Serotonin produced in the brain prevents the synthesis of the norepinephrine (noradrenalin) neurotransmitter and hence decreases the sympathetic tone in the body. The decrease in sympathetic tone leads to a decrease in $\beta 2$ adrenergic receptor ($\text{Adr}\beta 2$) signaling. $\text{Adr}\beta 2$ receptor has a negative impact on bone formation and enhances bone resorption, therefore as a result, brain-derived serotonin has a positive indirect effect on bone accrual [24,46,51,55] (Figure 2.2b). In a study by Yadav et al. [55] looking at the

association between serotonin and bone, they showed that bone volume, rate of bone formation, osteoblast number and body weight was decreased and bone resorption was increased in Tph2 knockout mice supporting the fact that brain-derived serotonin has a positive effect on bone accrual.

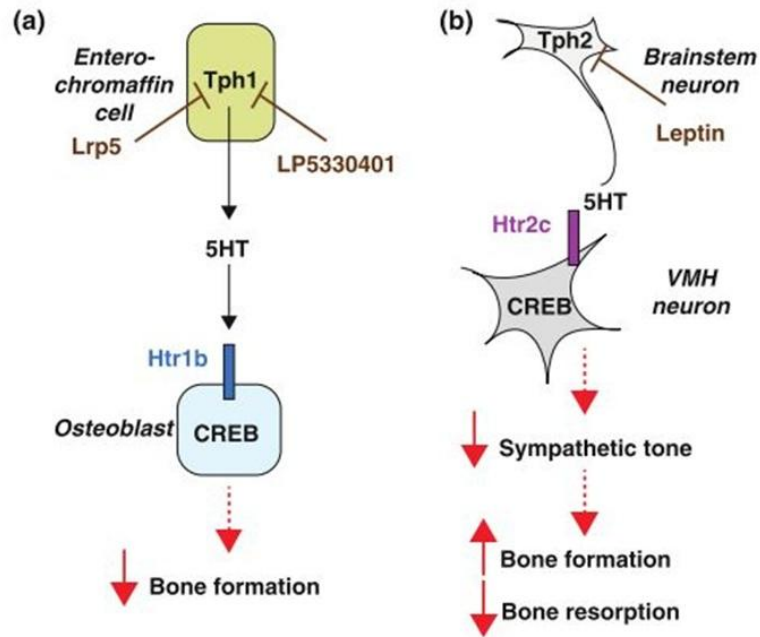


Figure 2.2 a) Gut-derived and b) brain-derived serotonin production procedure and their opposite influence on bone remodeling [24]. Brain derived serotonin has an indirect effect on bone remodeling since the serotonin produced in the brain cannot pass the blood-brain barrier.

2.4 Selective Serotonin Re-uptake Inhibitors

Selective Serotonin Re-uptake Inhibitors (SSRIs) are a class of anti-depressants that are commonly prescribed to patients mostly because of their therapeutic efficacy in treating mild to severe levels of depression [56,57]. They are also considered as safer drugs compared to other classes of antidepressants since they have fewer side effects, and

as a result have improved tolerability in patients [58,59]. The area of action of SSRIs is not only limited to treating depression, they are also prescribed to patients with other kinds of disorders and illnesses such as anxiety, obsessive-compulsive disorders (OCD), eating disorders, panic, insomnia, stroke recovery and sexual disorders [60,61].

2.5 Mechanism of Action of SSRIs

As mentioned earlier, serotonin is a neurotransmitter that is produced in the pre-synaptic neuron and released into the synaptic cleft. With no SSRIs in the system, the serotonin released will either bond with the receptors on pre or post-synaptic neuron or will be re-uptaken back into the pre-synaptic neuron by the serotonin transporters [49,62] (Figure 2.3A). With SSRIs in the system, the serotonin binding sites on the transporters will be blocked and serotonin will no longer be re-uptaken back into the pre-synaptic neuron. This results in the accumulation of serotonin in the synaptic cleft [25] (Figure 2.3B). With an increased level of serotonin in the synapse, the interaction between serotonin and serotonin receptors will increase and this will help relieve disorders such as depression [62,63].

2.6 Types of SSRIs

The class of SSRIs that are currently clinically prescribed to patients to treat depression and related disorders include: citalopram (Celexa®), paroxetine (Paxil®), fluvoxamine (Luvox®), fluoxetine (Prozac®), escitalopram (Lexapro®), and sertraline (Zoloft®) [64]. The antidepressants in this category mostly have similar therapeutic efficacy in treating depression. What makes them different from one another is their

molecular structure and pharmacokinetic properties such as half life, protein binding, and bioavailability [65]. Fluoxetine is known to have the longest half life (1 to 4 days) [66] compared to other SSRIs; for example sertraline has a half life ranging between 22-36 hours [67]. Paroxetine is the strongest SSRI followed by sertraline but it is not a potent site selector in comparison to citalopram having the highest serotonin selectivity. Paroxetine also has the highest bioavailability compared to other SSRIs and completely gets absorbed in the body after administration [66]. Sertraline is the only SSRI that in addition to serotonin also highly binds to dopamine transporters and inhibits dopamine from getting re-uptaken [66][68]. Dopamine is a neurotransmitter that is involved in the synthesis of norepinephrine (noradrenalin). The standard daily therapeutic doses of SSRIs in adults differ between 10 mg (escitalopram), 20 mg (fluoxetine, paroxetine, citalopram) and 50 mg (sertraline, fluvoxamine) [64].

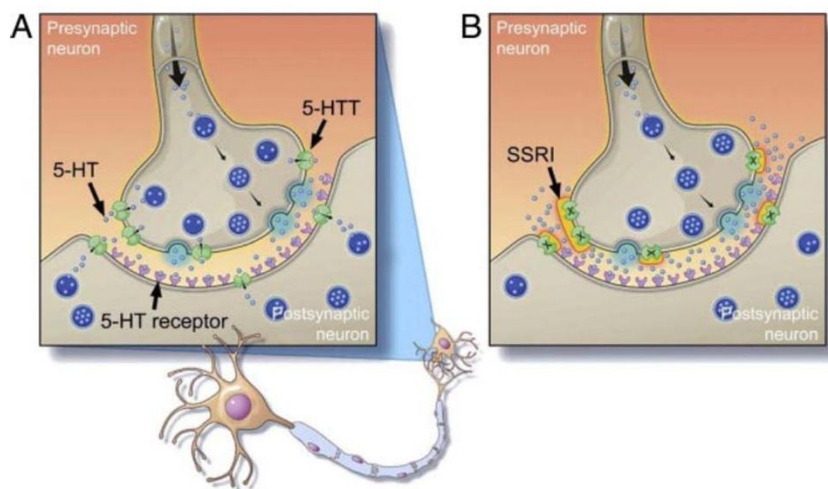


Figure 2.3 Mechanism of action of SSRIs [20]. A) No SSRI in the system. Serotonin is released into the synaptic cleft and bonds with the 5-HT receptors or 5-HT transporter. B) SSRI in the system. SSRIs block the 5-HT transporter and serotonin accumulates in the synaptic cleft increasing the interaction between 5-HT and 5-HT receptors.

2.7 Association between SSRIs and bone

The association between the use of anti-depressants, namely SSRIs and bone has been of great interest during recent years. Several clinical and animal studies have looked into the relationship between the use of SSRIs and their potential effect on bone properties such as bone mineral density (BMD) and risk of fracture [69].

2.7.1 SSRIs and their influence on bone properties

Bone loss leads to lower BMD, increases the risk of osteoporosis and ultimately increases the risk of fracture. There have been a number of cross-sectional and cohort clinical studies looking at the effect of SSRI use on BMD and risk of fracture.

In woman 40 years of age and older, it has been reported that SSRI use has detrimental effect on bone, leading to a significant decrease ($p < 0.05$) in BMD in regions of the hip [26], femoral neck, trochanter and mid-forearm [27] when compared to age matched women that were not exposed to SSRIs. Similar results have been obtained with a population of older men showing significantly lower BMD by 4-5% at the hip and 6% at the lumbar spine compared to non SSRI users [28,29]. In a study of adults aged 50 years and older, without considering gender as a factor, they made a comparison between SSRI users and nonusers and found comparable results [30]. They reported that SSRIs cause reduced BMD in the hip by 4% and in the spine by 2% when compared to adults not using SSRIs [30]. Only two of the mentioned studies had reported the class of the SSRI used by the participants [26][30]. Subjects were exposed to one of the SSRIs including, fluoxetine, sertraline, fluvoxamine or paroxetine.

SSRIs are also used to treat depression in adolescents and children [31]. In a study in this regard of boys aged 7-17 years old, SSRI exposure had negatively altered bone properties by significantly decreasing BMD ($p < 0.05$) at the ultra-distal radius and spine compared to children not taking SSRIs (the type of SSRI not reported) [32]. The differences were still significant after making adjustments for potential confounders in the reported studies.

However, results obtained from studies investigating the association between SSRIs and bone are not consistent. In one study of postmenopausal women no association was found between antidepressant use, including SSRIs (the type of SSRI not reported) and BMD [33]. Similar results were reported in a study on adults aged 17 and older finding no association between antidepressant medication including SSRIs (fluoxetine, sertraline or paroxetine) and bone properties [70]. It is worth mentioning that in both these studies, SSRI users and all other antidepressant users were considered as one group and compared to the controls. Also, since these studies were not specifically directed at looking at the effect of SSRI use on bone health, the sample size of the SSRI exposed participants was small (1 to 3% of the total participants in the study), causing a low power comparison.

Ultimately the most important outcome in terms of bone health is the risk of bone fracture. Many studies claim that there is an association between SSRI use and the increased risk of vertebral and non-vertebral bone fractures. Several studies have reported that in patients aged 50 and older who had previously been treated for hip fractures there was an increased risk of vertebral [33,34] and non-vertebral fractures with SSRI use, including fractures in the hip [34–39], wrist, humerus and pelvis [33,37] when compared

to age matched controls. The association was still evident after making adjustments for different confounders in these studies. These findings were supported in another study on patients with a wider age spectrum, looking at adults aged 18 and older. The risk of hip and femoral fracture was increased in current SSRI users compared to subjects that had never used any kind of antidepressants. However, the risk of hip fracture was reduced about 6 months after discontinuing the drug use [40].

In the respective studies, the class of the SSRI used by patients was not reported in three of the studies [33,34,36] but in the others, fluoxetine, paroxetine, fluvoxamine [35,37–40], sertraline [35,37,38], and citalopram [37] were the classes of SSRIs that were taken by the subjects.

There have also been a number of animal studies investigating the influence of SSRI antidepressants on mice and rat bone properties. Female mice exposed to fluoxetine have been shown to have shorter femurs and smaller cortical bone areas [41] negatively altered trabecular bone architecture [41,43], reduced BMD in the vertebrae [42,43], distal femur and midshaft [31] and also reduced bone strength [41,42] when compared to mice in the control group. Three different doses of 5 [31,43], 10 [41] and 20 (mg/kg/day) [31,42,43] fluoxetine were used in the studies. The serum level of fluoxetine achieved from these doses in mice was shown to be in the same range of the fluoxetine serum level obtained from standard (20 mg/day) and high (40-80 mg/day) doses used in humans in order to treat depression [43,71]. Mice were exposed to the drug for a period of 4 weeks in all mentioned studies.

As in the human studies, there were also contradictions in the animal studies. In one study, administration of fluoxetine (10 mg/kg/day) to mice for a period of 6 weeks was found to increase trabecular bone formation [72]. However, when the mice were ovariectomized, fluoxetine treatment did not prevent the estrogen deficient mice from losing bone and mice in this group had a 50% bone loss. In a study on female rats, fluoxetine administration for long-term (6 months) with a low dose of 5 mg/kg/day did not have significant effects on bone mechanical properties or bone architecture [73]. The rats exposed to fluoxetine did have reduced bone tissue strength compared to the controls, but this was compensated by changes in bone geometry, and ultimately fluoxetine-treated rats had similar bone strength to controls as obtained from three-point bending test [73]. In contrast to all previous animal studies mentioned above, in this study rats were exposed to the drug via gastric intubation instead of being injected.

Regardless of the results, it is evident from the foregoing studies that SSRI use should be considered as an important factor, potentially altering bone properties and their influence on bone should not be neglected.

2.8 Maternal use of SSRIs and pregnancy outcomes

Depression is a relatively common medical condition among pregnant woman and SSRIs are the most common drug prescribed to treat depression during pregnancy [74]. According to the US Food and Drug Administration (FDA), the pregnancy drug labeling category for all of the SSRIs except paroxetine is C, meaning that the maternal use of these drugs *might* not be safe for the fetus (Table 2.1). Paroxetine is the only SSRI that is in the pregnancy drug labeling of D, and its maternal use has been shown to have

detrimental effects on the fetus [6,75]. Studies have shown that SSRIs cross the placental barrier [3–5] and could also be excreted in breast milk [5–7], therefore, the drug could have a direct influence on the fetus *in utero* and on the baby during breast-feeding. Knowing that the fetus's critical developments occur *in utero*, drug exposure during this period could be of greater importance compared to the breast-feeding phase [76,77].

CATEGORY	INTERPRETATION
A	Sufficient and well-controlled studies have not shown an increased risk of fetal abnormalities in any of the three trimesters of pregnancy
B	Animal studies have not reported any detrimental effect on the fetus and there are no sufficient and well-controlled human studies in pregnant women. OR animal studies have shown an adverse effect on the fetus, but sufficient and well-controlled human studies in pregnant women have failed to demonstrate a risk to the fetus during all three trimesters.
C	Animal studies have shown an adverse effect on the fetus but there are no adequate and well-controlled studies in pregnant women. OR No animal studies have been conducted and there are no adequate and well-controlled studies in pregnant women
D	Adequate well-controlled or observational studies in pregnant women have demonstrated a risk to the fetus. However, the benefits of drug use may outweigh the potential risk in some conditions.
X	Adequate well-controlled or observational studies in animals or pregnant women have both reported positive evidence of fetal abnormalities or risks. The use of the product is contraindicated in women who are or may become pregnant

Table 2.1 **FDA pregnancy category drug labeling** [6] The pregnancy drug labeling category for all of the SSRIs is C except for paroxetine. Paroxetine is the only SSRI in the pregnancy drug labeling of D.

Several studies have been undertaken to investigate the relationship between maternal SSRI use and congenital malformations and birth outcomes but their results are inconsistent. A number of studies have reported that SSRI use during pregnancy is associated with birth defects such as craniosynostosis, anencephaly [8] omphalocele [8,9], craniofacial [10,11] and cardiovascular malformations [12–14], and some studies have suggested an increased risk of congenital malformations in general [15,16].

Not much research has been conducted regarding *in utero* exposure to SSRIs and infant bone health, and the few current studies are limited to only investigating properties such as, overall body length, head circumference [19,44,45] and bone density [19] in newborns. In a study by Dubnov-Raz et al. [19] they looked at the relationship between maternal use of SSRIs and neonatal bone density. In this study they found that the newborns in the SSRI group were shorter compared to those in the control group but the results were not statistically significant ($p = 0.07$). The head circumference was significantly smaller ($p < 0.005$) in the SSRI exposed infants compared to the controls. These significant differences were still evident after making adjustments for different confounders such as infant sex, maternal age, previous births and maternal smoking status. No difference was seen in the bone density comparing the two groups. In this study mothers were receiving different classes of SSRI including: citalopram, fluoxetine, escitalopram and sertraline, and a majority of them (92.5%) were using the drug throughout their entire pregnancy. In another study looking at the effect of maternal SSRI use on infant growth, they also found that babies exposed to SSRIs *in utero* had smaller head circumference ($p = 0.08$) and also significantly shorter crown-heel length ($p < 0.01$) compared to controls [45]. Mothers in this study were exposed to paroxetine, fluoxetine or citalopram throughout their entire pregnancy. The number of the subjects using paroxetine (class D antidepressant) was higher than other SSRI users in this study.

In a more recent study, Wisner et al. [44] looked at the effect of treated and non-treated depression during pregnancy on infant growth for a period of one year after birth. Pregnant women were recruited into three groups of: SSRI users, pregnant woman that

had severe depression but were not treated and healthy pregnant woman with no depression history and SSRI use. Babies born to mothers from the SSRI group had shorter birth length compared to other groups but the difference was no longer evident 2 weeks following the birth. In conclusion, they reported no significant differences in weight, head circumference and length when comparing infants of the three groups. In contrast to the last study [45], woman receiving a class D or X antidepressant were excluded from the study. The SSRIs used by the subjects included: fluoxetine, sertraline, citalopram and fluvoxamine [78]. A majority of the subjects were exposed to the drug through gestation (65%) and the rest were either exposed in the first and second trimester (22%) or the second and third trimester (13%).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Animals

All animal experiments were approved by the Animal Research Ethics Board at McMaster University in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous (i.e., no prior pregnancies) 200 to 250 g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 light:dark) and temperature (22° C) with *ad libitum* access to food and water. For all experimental groups (defined in following sections) dams were mated (1:1) with age-matched Wistar rats and were monitored daily for confirmation of breeding (i.e., the presence of sperm in a vaginal flush). The day that a positive sign of copulation was observed was designated as gestational day 0 (GD0). All dams were allowed to deliver normally. At birth, postnatal day one (PND1), litters were aimed to be culled to 8 offspring, preferentially selecting for equal number of male and female offspring to ensure uniformity of litter size between treated and control groups.

3.2 Drug exposure and duration

Two studies with two different SSRI drugs were conducted. The drug exposure experimental design was different between the two studies. The SSRI drugs used were sertraline hydrochloride (Zoloft®, Toronto Research Chemicals, North York, ON) and fluoxetine hydrochloride (Prozac®, Toronto Research Chemicals, North York, ON). In both studies, flavoured gelatin was used as the vehicle for oral dosing (see sections 3.2.1 and 3.2.2 for dose information).

3.2.1 Dams treated with sertraline

In this study, the treatment started on gestational day zero (GD0). Dams were randomly assigned to receive sertraline hydrochloride (10 mg/kg/day, N=5) or saline (N=6) in a flavoured gelatin vehicle. The treatment was carried on for 3 weeks (GD21, i.e., until delivery). After delivery the mother rats were no longer exposed to the sertraline (Figure 3.1).

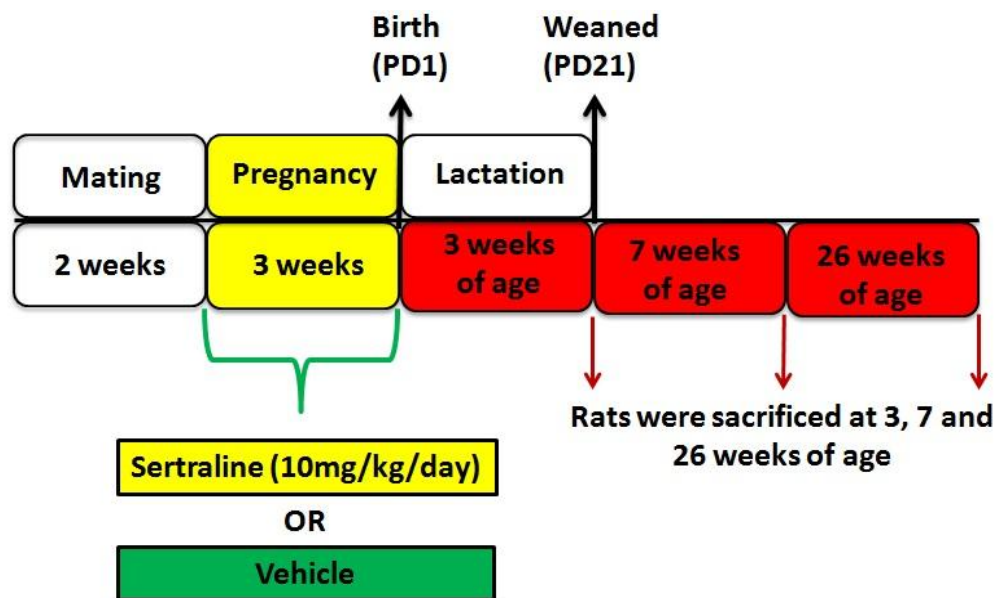


Figure 3.1 The experimental design for rats exposed to sertraline. The dams were exposed to the drug during 3 weeks of pregnancy. The pups were sacrificed at three different age groups of 3, 7 and 26 weeks of age.

3.2.2 Dams treated with fluoxetine

In the fluoxetine study, the treatment started approximately two weeks prior to mating and the dams were randomly assigned to receive either fluoxetine hydrochloride (10mg/kg/day, N=10), or saline (N=9) in a flavoured gelatin vehicle. The treatment was

carried on during pregnancy and also during lactation until the pups were weaned (PD21 = 3 weeks post-delivery). The treatment period was 8-9 weeks in total (Figure 3.2).

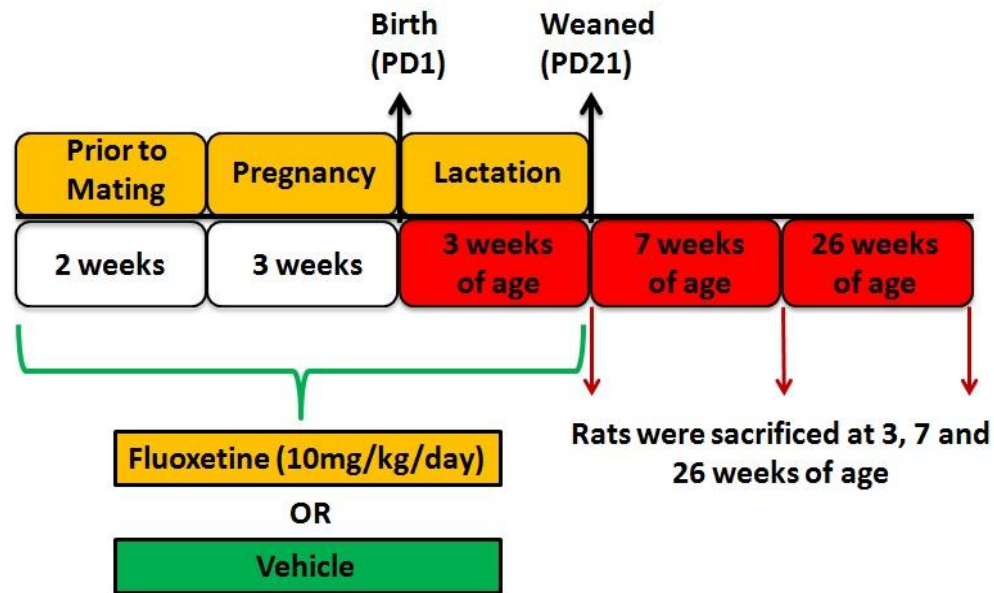


Figure 3.2 The experimental design for rats exposed to fluoxetine. The dams were exposed to the drug before, during and after pregnancy for a period of 8 to 9 weeks. The pups were sacrificed at 3, 7 and 26 weeks of age.

3.3 Sample preparation

All rats were sacrificed through CO₂ asphyxiation in three different age groups of 3, 7 and 26 weeks. Between 7-15 rats were born per litter and litters were culled to approximately 8 offspring (4 males and 4 females). Due to the small litter size and in order to have 1 male and 1 female rat at the age of 26 weeks, the number of pups sacrificed at the age of 7 weeks was less compared to other age groups (Table 3.1). Rat carcasses were placed in a hermetically sealed bag and frozen (-20° C). Prior to dissection, rats were thawed overnight at 4° C. The left femurs, tibias and the L6 lumbar

vertebrae were harvested for bone analysis from rats in all age groups. The soft tissues were removed from the bones by dissection and the bones were separately labeled and wrapped in saline soaked gauze and stored at -20° C (Isotemp freezer, Fisher Scientific). In the process of dissecting the left femurs, the femoral lengths were measured using a caliper (0-150 mm, ± 0.01 mm, Vernier digital caliper).

Female (n)			Male (n)	
Age	Control	Sertraline	Control	Sertraline
3 weeks	6	4	6	5
7 weeks	5	4	5	4
26 weeks	6	4	6	4
Female (n)			Male (n)	
Age	Control	Fluoxetine	Control	Fluoxetine
3 weeks	10	11	12	11
7 weeks	7	8	9	5
26 weeks	11	10	12	9

Table 3.1 **Number of rats at 3, 7 and 26 weeks of age in treated and control groups of two studies.** Due to small litter sizes and in order to have 1 male and 1 female rat at age of 26 weeks, the number of rats collected at 7 weeks of age was less compared to other age groups.

3.4 Calculating the mid-diaphyseal cortical bone area properties

Mid-diaphyseal cortical bone area properties of the femurs were measured using the scans obtained from a microcomputed tomography (microCT) scanner (MicroCT eXplore RS80, GE Medical Systems, Toronto ON). The femurs were removed from the freezer the day before the scanning and left out overnight in a cooler to thaw. In order to obtain the morphological properties of the bones, they were scanned at an isometric resolution of 23 μ m. In order to be able to scan multiple femurs at a time, custom made trays were designed (Figure 3.3) for each age group (i.e., different sized femurs) using AutoDesk

Inventor (version 11, AutoDesk). The trays were made with the rapid prototyping technique using a high definition 3D production system (ProJet HD3000, Agile manufacturing, Inc., Uxbridge, ON). The material used for manufacturing them was an acrylic based polymer (VisiJet EX200).

The trays contained multiple slots that the bones would fit in and each section had its own individual number that was later assigned to the bones. The numbers would show in the reconstructed three dimensional microCT data sets. After filling the trays with the femurs, a saline-soaked gauze was placed on top of each tray covering all bones to prevent them from drying out during the scanning period. The trays were then mounted on top of each other and placed in a container with a lid and completely sealed using a wax film (Parafilm).

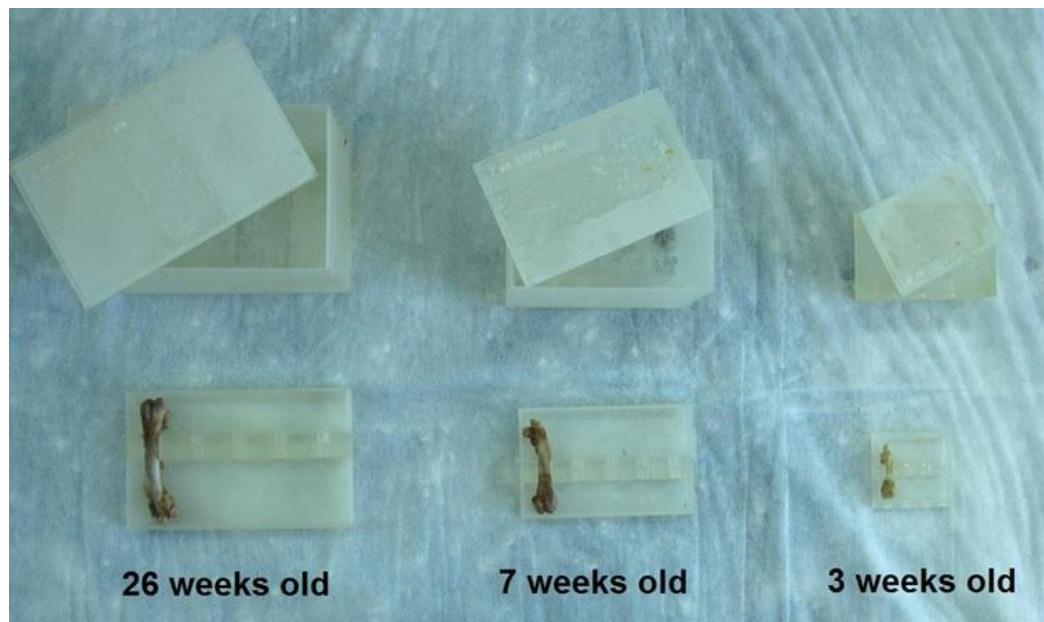


Figure 3.3 Femur trays for different age groups. One bone from each of the 3, 7 and 26 week age groups has been placed on the tray as an example.

Following scanning, the femurs were wrapped in saline soaked gauze, placed in a sealed freezer bag and frozen again (-20° C). The three dimensional data sets were viewed using Microview (GE Healthcare, Waukesha, WI). Since the images contained all bones in one tray, a region of interest was created for each bone individually and the volume of interest was saved as an image dataset for further analyses. Length measurements of the femurs were done using Microview. After saving the individual bone images in DICOM format, they were loaded onto another image analysis software (ImageJ, National Institutes of Health, NIH). After finding the two end points of the femurs and their corresponding slice numbers, the midpoint slice number was calculated and the corresponding image of the cross section at the mid-diaphysis was found. Next, the image was thresholded to segment between bone and everything else (air and soft tissue) and saved as a .txt file containing a matrix of 8 bit bitmap values, either 255 or 0 representing pixels containing bone and pixels not containing bone, respectively. The text files were then loaded into Excel and a custom spreadsheet was used to calculate the mid-diaphyseal cortical bone area (Ct.Ar) and the moment of inertia (I_{xx}). Moment of inertia values were calculated by determining which row of the matrix represented the geometric centroid and summing the square of the distances from this row of all pixels outside of the “centroid row”.

3.5 Mechanical properties - three point bending test

Various interventions might cause changes in structural and material properties of the bone, and the three-point bending test is a reproducible and widely used test

performed on long bones (e.g. femurs and tibias) in small animals in order to determine these properties [79,80].

After collecting all the mid-diaphyseal cortical bone area data using the microCT scans of the femurs, the mechanical properties of the femurs were evaluated by testing them in three-point bending using a material testing system (eXpert 5601, ADMET, Norwood, MA). Twelve hours prior to the test femurs were removed from the freezer to thaw. They were kept wrapped in saline soaked gauze until just before initiating the test. All remaining soft tissue around the femurs was removed before starting the test to insure that there was direct contact between the supports and the bone surface during loading. Custom designed and manufactured fixtures were used for the three-point bending test. Two supports were manufactured from aluminum and capped with stainless steel dowels (2 mm diameter, Spaenaur, Kitchener, ON). An identical dowel was also fixed to the anvil. Femurs were positioned on the two lower supports so that the bending load was applied in the anterior-posterior direction (Figure 3.4 a). The distance between the two lower supports was different between the different age groups and also between male and female femurs due to differences in bone length (Table 3.2). The purpose was to fix the supports at a distance that the middle of the lesser trochanter and the superior surface of the distal condyles would touch the two lower supports and be at a stable position when starting the loading. The loading anvil was set to make contact with the midpoint of the femur. Femurs were held with a preload of approximately 2 N. The femurs were loaded in three-point bending with a displacement rate of 10 mm/min until the femur failed. Force

and displacement data were collected every 0.01 second and saved in a buffer in the ADMET for each femur (Figure 3.4b).

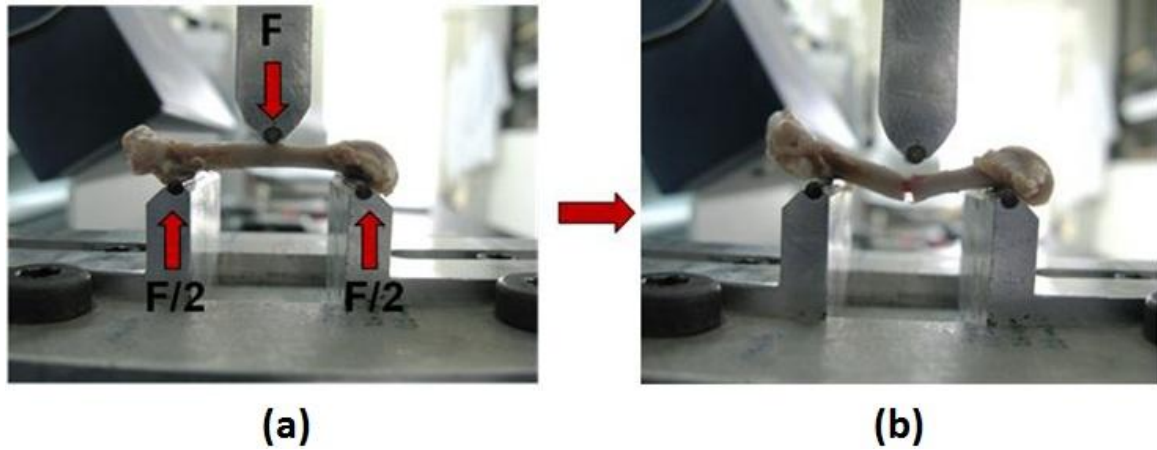


Figure 3.4 Three-point bending test setup. The femurs were loaded until they reached the failure point. (a) The initiation of loading when the top anvil made contact with the midpoint of the femur and a pre-load of approximately 2 N was transferred to the bone. (b) Example of a fractured bone after the loading was stopped.

Distance (mm)	3 weeks	7 weeks	26 weeks
Female	8	16	20
Male	8	18	24

Table 3.2 Distance between the two lower supports in the mechanical testing. The average of the femurs length were almost the same at 3 weeks of age between males and females so the same distance was used for both sexes.

The displacement data provided by the ADMET were determined by the position of the loading platen. After finishing each test the load-displacement data were transferred to a PC computer via the WinCOM software package (ADMET, Norwood, MA). The data were imported into Excel at a later time for further analysis.

3.5.1 Structural Properties

The load-deformation curves were obtained using the data collected from the ADMET and then used to determine different structural properties of the bone (Figure 3.5). Different properties were defined as below:

- Bending stiffness: The slope of the curve in the elastic region.
- Maximum load: The greatest amount of load that the bone could bear before failure.
- Yield load: The load at which the bone begins to undergo plastic deformation.
- Maximum deformation: The deformation at the maximum load.
- Yield deformation: The deformation at the yield load.
- Maximum energy: The area under the curve up to the maximum load.
- Elastic energy: The area under the curve up to the yield load limit.

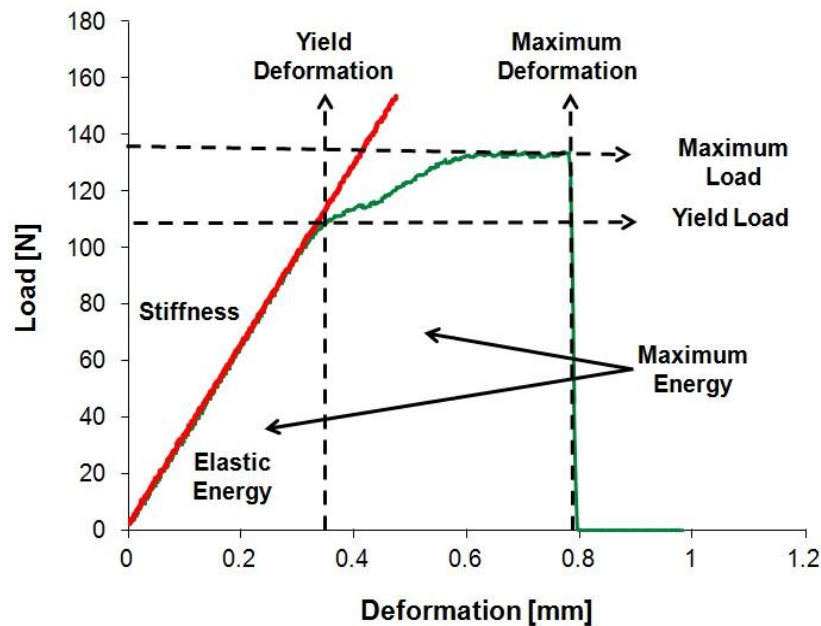


Figure 3.5 The load-deformation curve from three-point bending test performed on a 26 week old rat femur.

3.5.2 Material properties

The mid-diaphyseal cortical bone area measurements from the microCT scans of the femurs and the load and deformation data acquired by the ADMET in the three-point mechanical tests were used to calculate the material properties of the bone. Linear beam theory was used to estimate the elastic modulus of the femurs. (Figure 3.6). Stress and elastic modulus were calculated as below (Figure 3.7).

$$Elastic\ modulus = \frac{F \cdot L^3}{48 \cdot \delta \cdot I_{xx}} = \frac{applied\ load \cdot (support\ distance)^3}{48 \cdot beam\ deflection \cdot moment\ of\ inertia}$$

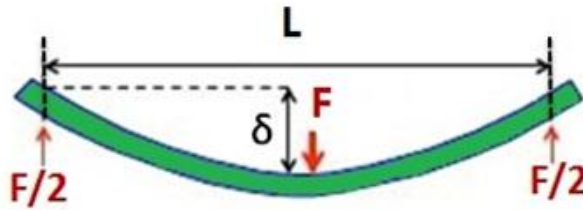


Figure 3.6 A schematic picture of a bending beam and variables used in linear beam theory. L is the distance between the two lower supports, F is the load applied to the midpoint of the beam, δ is the deformation of the beam from the horizontal line (initial position).

$$Stress = \frac{F \cdot L \cdot Y_{post}}{4 \cdot I_{xx}} = \frac{applied\ load \cdot support\ distance \cdot Y_{post}}{4 \cdot moment\ of\ inertia}$$

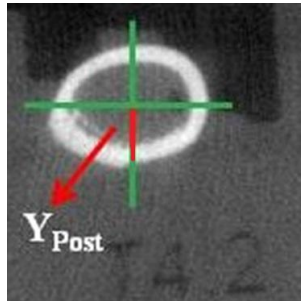


Figure 3.7 Y_{post} in a cross section of the femur at the mid-diaphysis. Y_{post} is the distance between the mid-point and the posterior wall of the cortical section of the bone.

3.6 Cancellous bone analysis

Cancellous bone analysis was performed to measure trabecular bone properties such as porosity, trabecular thickness and bone to tissue volume. MicroCT scans were obtained from the L6 vertebra of all rats using a high resolution x-ray microcomputed tomography system (SkyScan1172, Bruker microCT, Belgium). Similar to the femurs, custom trays were made and 60–90 vertebrae could be scanned at one time depending on the size of the bones (Figure 3.8).



Figure 3.8 Sample preparation for vertebrae scanning. Represented is a picture of trays and containers manufactured for 3, 7 and 26 week old vertebrae.

Twelve hours prior to scanning the vertebrae were removed from the freezer and left at room temperature to thaw. Similar to the femurs, the vertebrae were placed in the trays and prepared for scanning. The specimen stage (52 mm diameter) was used to hold the container and then the stage was held by a chuck in the microCT scanner. The resolution, voltage, current, power and camera were set to obtain optimal scanning for each vertebra size depending on the age group (Table 3.3). Since at 3 weeks of age, the vertebrae were small and bone classification was poor, a lower scanning resolution was used compared to other age groups in order to obtain the best image quality.

	3 weeks	7 weeks	26 weeks
Resolution (μm)	6	12.1	13.1
Voltage (kV)	50	100	100
Current (μA)	201	100	100
Power (W)	10	10	10
Camera (pixel x pixel)	4k (4000x 2667)	2k (2000x 1336)	2k (2000x1336)

Table 3.3 **MicroCT scanning parameters for vertebral scans.** The resolution was set to in order to obtain a high quality image from the vertebrae. Depending on the vertebrae size, each age group had a different scan resolution.

In all scans a 0.5 mm thick aluminum filter was used to filter out the low energy x-rays that cause beam hardening artifact. Beam hardening artifact is caused by the change in the energy of the x-ray beam when passing through an object. The flat-field image was updated before each scan in order to reset the background. This action would make it possible to compare different densities in the sample and it would also avoid ring artifacts. Small dust particles may appear on the camera or filter which will induce ring artifacts in the image.

In order to be able to maximize the number of vertebrae that could fit in one scan, the camera offset mode was activated and images with a width double the standard field of view (FOV) were projected. In this mode, the camera would shift between the left and right position, take two images next to each other and stitch them together automatically.

The three dimensional data sets were loaded into the commercial NRecon software (Skyscan1172, Burkert microCT, Belgium). Each tray of vertebrae was then reconstructed individually and saved as a three dimensional data set for subsequent analyses. After reconstructing, the data sets were loaded into DataViewer (Skyscan1172, Burkert microCT, Belgium). Two steps were performed to obtain the final data set for each individual bone (Figure 3.9).

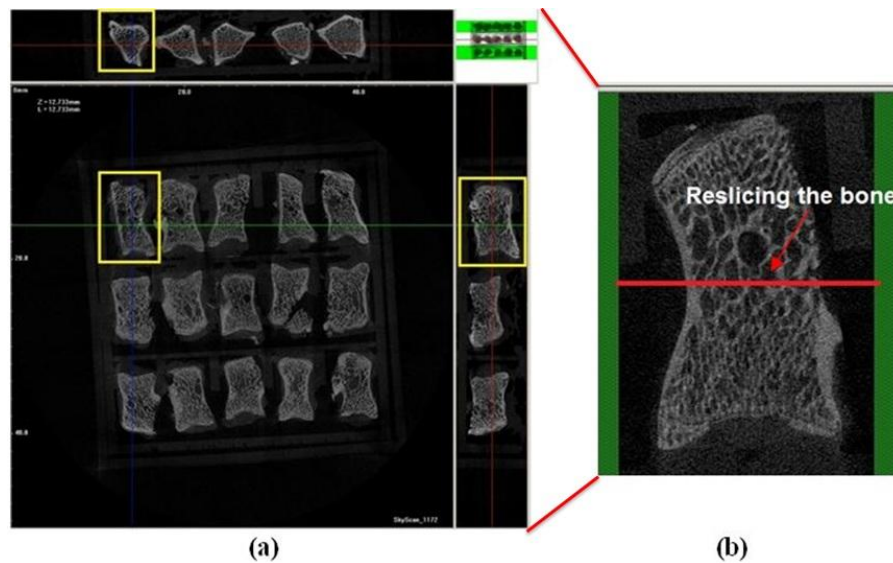


Figure 3.9 Cropping and re-slicing the vertebrae. a) The yellow area shows the bone selected from different projections, giving the ability to make sure the whole bone is captured before cropping. b) The bone is going to be re-sliced from top to bottom to obtain the cranial-caudal view.

In the first step, the volume of interest for each vertebra was cropped and saved as an individual data set. In the second step, images saved from the first step were sliced top to bottom in order to have a “cranial-caudal” view (Figure 3.10) of each vertebra for further analysis. Circular regions of interest (ROI) with different diameters were determined for each age group and gender. Each circular ROI for an age group was selected to achieve the greatest volume of interest that included only cancellous bone (and no cortical bone) through the length of the vertebra for all samples in that age group (Figure 3.10). After going through the slices and finding the mid slice, a region of interest with the biggest possible diameter was found and that ROI was copied through the whole data set, converting the ROI into a cylinder. The selected ROI region was then thresholded. Bone properties such as trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and percent bone volume (BV/TV) [81] were calculated using CTAn (Skyscan1172, Bruker microCT, Belgium).

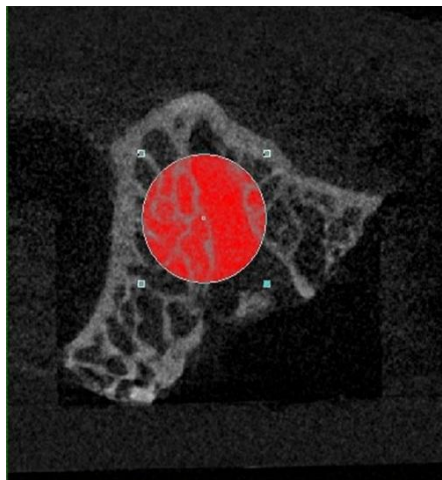


Figure 3.10 Selected Region of Interest (ROI). An image of a mid slice of a microCT scan data set of a 26 week old male vertebra.

3.7 Statistics

After analysing the data collected from different tests, a two way MANOVA with sex and treatment as fixed factors was performed to determine the statistical differences between SSRI treated rats and the controls between different bone properties (SPSS Inc., Chicago IL). A significance level of $p < 0.05$ was used in all statistical analyses.

CHAPTER FOUR: RESULTS

4.1 Rats exposed to sertraline

4.1.1 Body mass (sertraline)

There were no significant differences in body mass between the sertraline group and the controls in any of the age groups. Male rats were significantly heavier than the females ($p < 0.05$) at 7 and 26 weeks of age (Table 4.1). There was no significant sex by treatment interaction for body mass in any of the age groups.

Female - Body Mass (g)					
3 weeks		7 weeks		26 weeks	
Control	Sertraline	Control	Sertraline	Control	Sertraline
48 ± 6	50 ± 4	200 ± 20 [^]	195 ± 12 [^]	341 ± 36 [^]	342 ± 30 [^]

Male - Body Mass (g)					
3 weeks		7 weeks		26 weeks	
Control	Sertraline	Control	Sertraline	Control	Sertraline
50 ± 6	52 ± 5	266 ± 23	264 ± 33	562 ± 58	583 ± 12

Table 4.1 Body mass at 3, 7 and 26 weeks of age (mean ± SD). [^] Significant difference between male and female ($p < 0.05$).

4.1.2 Femoral geometric measurements (sertraline)

The length and mid-diaphyseal cortical bone area were significantly larger ($p < 0.05$) for the femurs from male rats at 7 and 26 weeks compared to female rats. At 3 weeks of age, the overall femur length in the sertraline exposed group was significantly shorter ($p < 0.05$) compared to the controls (Table 4.2, Figure 4.1). At 7 and 26 weeks of age there

was no difference in femoral length between the sertraline and control groups (Table 4.2).

At all age groups, 3, 7 and 26 weeks, there was no significant difference in mid-diaphyseal cortical bone area (Ct.Ar) between the femurs exposed to sertraline and the femurs from the control groups.

Female						
	3 weeks		7 weeks		26 weeks	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
Length (mm)	16.5 ± 0.6	15.7 ± 0.4*	30.1 ± 1.4 [^]	30.0 ± 0.5 [^]	36.5 ± 0.9 [^]	36.8 ± 0.7 [^]
Ct.Ar (mm ²)	1.3 ± 0.2	1.6 ± 0.1	3.3 ± 0.5 [^]	3.1 ± 0.2 [^]	5.2 ± 0.5 [^]	5.4 ± 0.3 [^]

Male						
	3 weeks		7 weeks		26 weeks	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
Length (mm)	16.6 ± 1.0	15.2 ± 0.4*	31.6 ± 0.6	31.6 ± 1.1	41.2 ± 0.8	41.7 ± 0.6
Ct.Ar (mm ²)	1.3 ± 0.3	1.3 ± 0.2	3.8 ± 0.5	3.6 ± 0.4	7.9 ± 0.9	7.3 ± 0.5

Table 4.2 Femoral length and mid-diaphyseal cortical bone area (Ct.Ar) of the femurs (mean ± SD). *Significantly less than control ($p < 0.05$) (also indicated by grey shading). [^]Significant differences between male and female ($p < 0.05$).

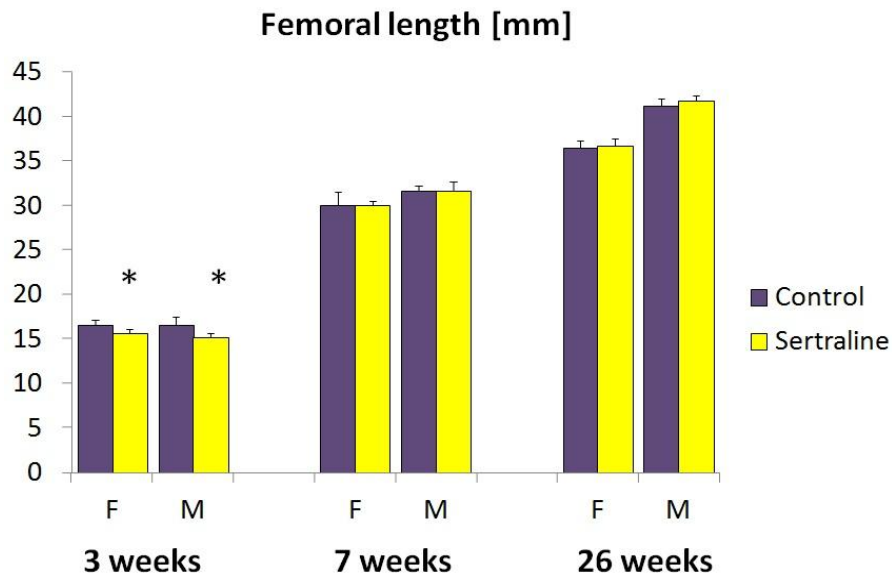


Figure 4.1 Overall femoral length [mm] at 3, 7 and 26 weeks of age. F (female), M (male). Sertraline exposed rats had significantly shorter femurs compared to controls at 3 weeks (* $p < 0.05$).

4.1.3 Three point bending mechanical test (sertraline)

At 3 weeks of age femurs of sertraline exposed rats had significantly lower maximum load and maximum stress ($p < 0.05$) compared to the controls (Table 4.3, Figure 4.2, Figure 4.3). There were no significant differences in other structural or material properties in this age group. At 7 weeks of age femurs from sertraline exposed rats had significantly lower maximum load and stiffness ($p < 0.05$) compared to femurs from the control group (Table 4.3, Figure 4.2, Figure 4.4). No significant difference was found in other properties at 7 weeks of age. Significant sex differences ($p < 0.05$) were seen in 7 and 26 week old rats. At 7 and 26 weeks femurs from male rats had greater yield deformation, elastic energy and maximal energy compared to those from females.

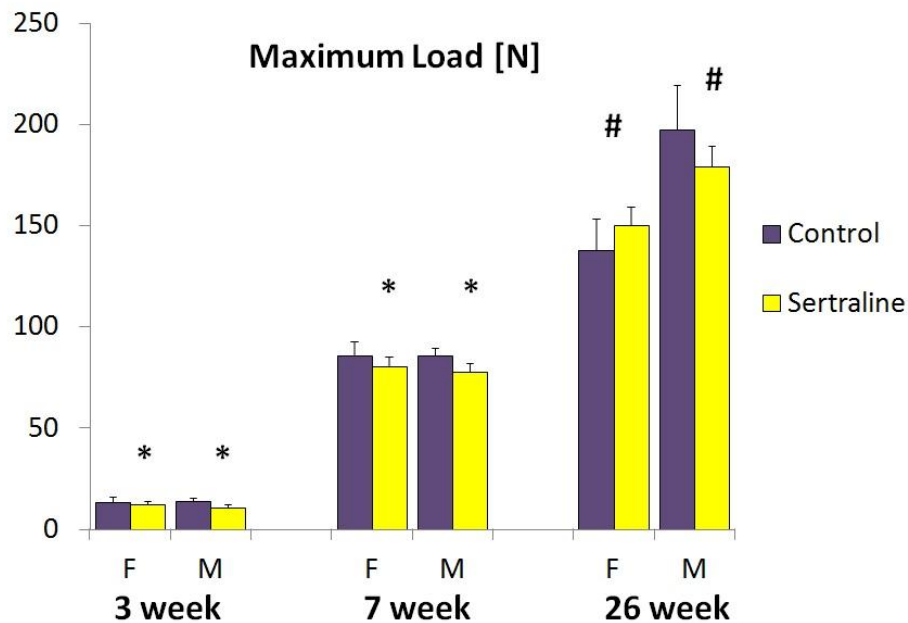


Figure 4.2 Femoral maximum load [N] from three-point bending test. The maximum load was significantly less for femurs from sertraline exposed rats compared to controls at 3 and 7 weeks of age (* $p < 0.05$). There was a significant sex by treatment interaction at 26 weeks (# $p < 0.05$).

Female						
	3 weeks		7 weeks		26 weeks	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
Yield Load (N)	6.6 ± 1.7	5.6 ± 0.3	47.5 ± 4.1	47.2 ± 6.9	103.4 ± 6.7 [^]	112.5 ± 6.7 [^]
Max Load (N)	13.4 ± 2.5	12.2 ± 1.4*	85.5 ± 7.3	80.3 ± 4.8*	137.9 ± 15.6 [^]	150.2 ± 8.8 ^{#^}
Yield Deform (mm)	0.10 ± 0.02	0.08 ± 0.02	0.24 ± 0.02 [^]	0.29 ± 0.07 [^]	0.39 ± 0.04 [^]	0.40 ± 0.04 [^]
Max Deform (mm)	0.67 ± 0.22	0.67 ± 0.12	0.79 ± 0.12	0.81 ± 0.06	0.69 ± 0.08	0.68 ± 0.07
Elastic Energy (mJ)	0.5 ± 0.1	0.3 ± 0.1	6.2 ± 1.0 [^]	7.5 ± 3.2 [^]	20.0 ± 1.0 [^]	20.2 ± 3.1 [^]
Max Energy (mJ)	6.7 ± 2.2	5.9 ± 1.4	45.4 ± 10.4 [^]	43.2 ± 4.7 [^]	57.7 ± 8.2 [^]	57.6 ± 8.7 [^]
Stiffness (N/mm)	48.6 ± 13	43.9 ± 7.8	201.3 ± 13.9	178.1 ± 35*	295.6 ± 41.9	354.3 ± 27.4 [#]
Max Stress(N/mm ²)	49.5 ± 14	30.9 ± 4*	213.9 ± 22.7	206.4 ± 9.7	285.2 ± 54.5	290.6 ± 36.6
Elastic Modulus (GPa)	1.4 ± 0.4	1.0 ± 0.2	7.7 ± 1.6	7.3 ± 1.4	11.0 ± 2.1	10.9 ± 1.6

Male						
	3 weeks		7 weeks		26 weeks	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
Yield Load (N)	6.6 ± 1.4	5.5 ± 0.8	54.6 ± 7.4	49.6 ± 4.6	146.6 ± 19.2	137.1 ± 4.7
Max Load (N)	13.7 ± 1.7	10.5 ± 1.6*	85.7 ± 3.6	77.7 ± 4.0*	197.3 ± 21.9	178.8 ± 10 [#]
Yield Deform (mm)	0.11 ± 0.04	0.09 ± 0.03	0.36 ± 0.08	0.39 ± 0.14	0.50 ± 0.08	0.50 ± 0.03
Max Deform (mm)	0.61 ± 0.20	0.64 ± 0.18	0.94 ± 0.16	1.01 ± 0.13	0.87 ± 0.10	0.84 ± 0.05
Elastic Energy (mJ)	0.5 ± 0.2	0.4 ± 0.1	9.9 ± 2.7	10.5 ± 4.3	37.8 ± 9.3	33.0 ± 3.4
Max Energy (mJ)	5.9 ± 2.6	5.3 ± 2.2	52.8 ± 8.1	51.6 ± 9.4	102.1 ± 23	88.9 ± 16.5
Stiffness (N/mm)	44.1 ± 9.5	39.7 ± 10.6	178.1 ± 14.6	134.8 ± 28*	321.2 ± 35.6	308.9 ± 23 [#]
Max Stress(N/mm ²)	47.3 ± 8.6	36.1 ± 9.8*	197.0 ± 35.2	183.2 ± 42.9	294.3 ± 51.1	296.9 ± 9.8
Elastic Modulus (GPa)	1.2 ± 0.4	1.2 ± 0.3	8.1 ± 2.7	6.9 ± 3.1	9.8 ± 2.2	11.2 ± 1.1

Table 4.3 Mechanical properties from three-point bending test (mean ±SD).*Significantly less than control ($p < 0.05$) (also indicated by grey shading).[#] Significant sex by treatment interaction ($p < 0.05$). [^] Significant difference between male and female ($p < 0.05$).

Also, at 26 weeks of age the femurs from males had greater yield load compared to female femurs (Table 4.3). At 26 weeks there was no significant difference in the mechanical properties between the treated and non-treated groups; however there was a significant interaction between sex and treatment in properties such as maximum load and stiffness ($p < 0.05$). While femurs of female rats from sertraline exposed dams tended to be stronger than controls, the femurs of male rats from sertraline exposed dams tended to be weaker compared the control group (Table 4.3, Figure 4.2 and Figure 4.4).

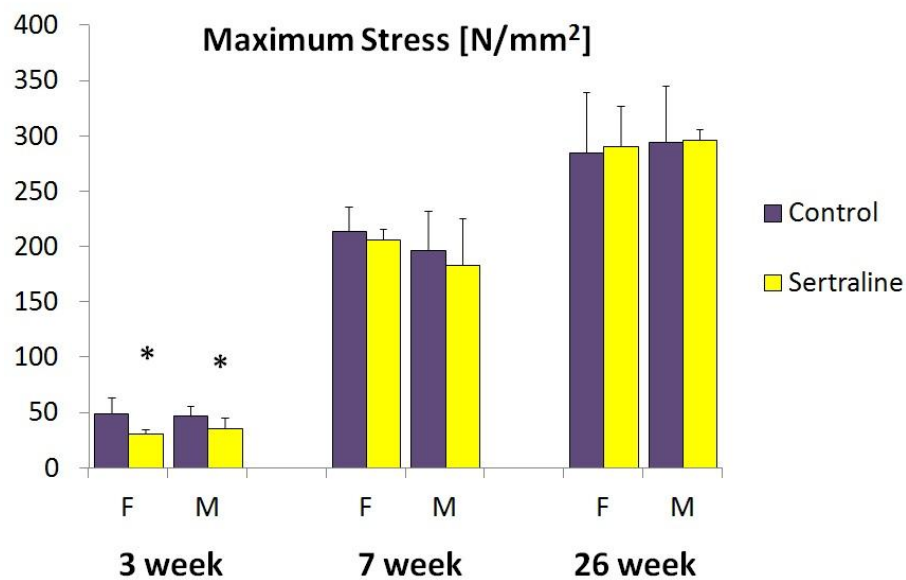


Figure 4.3 Maximum stress [N/mm²] from three point bending test. At three weeks of age femurs exposed to sertraline had significantly lower maximum stress compared to controls (* $p < 0.05$).

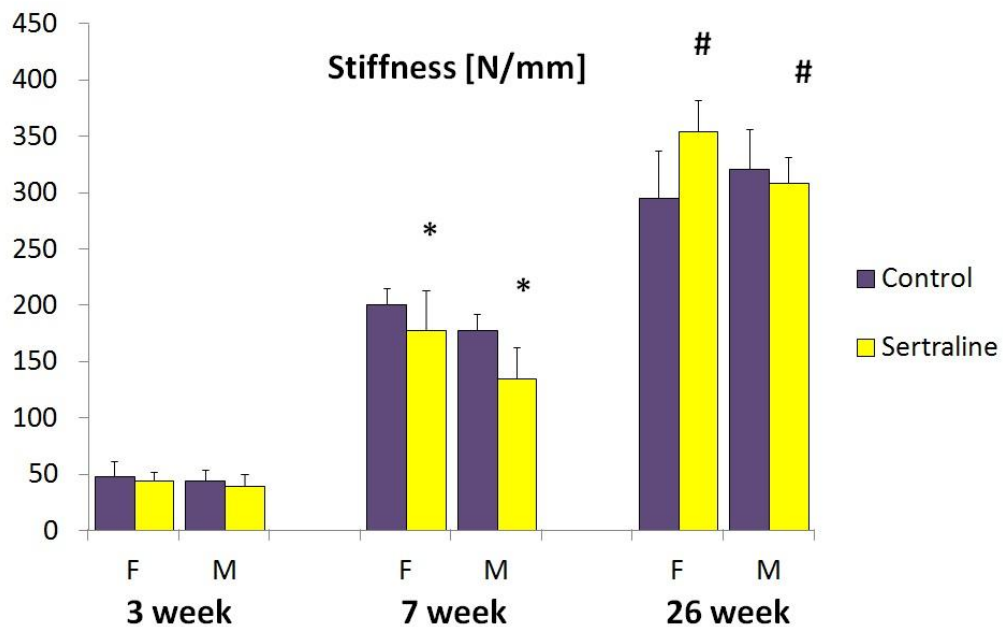


Figure 4.4 Femoral stiffness [N/mm] from three-point bending test. At 7 weeks of age, the femoral bending stiffness was significantly lower in femurs from sertraline exposed rats compared to controls (* $p < 0.05$). There was a significant sex by treatment interaction at 26 weeks of age ($p < 0.05$).

4.1.4 Cancellous bone properties (sertraline)

At all 3, 7 and 26 week age groups, no significant differences were found in cancellous bone properties (BV/TV, Tb.Th, Tb.N, Tb.Sp) between sertraline exposed vertebrae and the controls. Significant sex differences ($p < 0.05$) were seen in some properties at ages 3, 7 and 26 weeks (Table 4.4). At 3, 7 and 26 weeks of age male rats had smaller Tb.N compared to the females. At 26 weeks of age female rats had lower Tb.Sp compared to the male rats.

Female						
	3 week		7 week		26 week	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
BV/TV (%)	14.6 ± 1.8	14.4 ± 1.8	30.9 ± 3.7	27.7 ± 4.5	27.8 ± 3.8	31.5 ± 4.0
Tb.Th (mm)	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
Tb.N (1/mm)	2.7 ± 0.2	2.6 ± 0.3	3.9 ± 0.3	3.6 ± 0.5	3.7 ± 0.5	3.9 ± 0.3
Tb.Sp (mm)	0.27 ± 0.02	0.27 ± 0.02	0.21 ± 0.01	0.22 ± 0.04	0.19 ± 0.02 [^]	0.19 ± 0.02 [^]

Male						
	3 week		7 week		26 week	
	Control	Sertraline	Control	Sertraline	Control	Sertraline
BV/TV (%)	13.2 ± 1.7	12.5 ± 1.3	28.6 ± 2.5	27.0 ± 2.3	27.2 ± 5.2	29.1 ± 3.3
Tb.Th (mm)	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
Tb.N (1/mm)	2.4 ± 0.2 [^]	2.3 ± 0.2 [^]	3.5 ± 0.2 [^]	3.4 ± 0.2 [^]	3.4 ± 0.5 [^]	3.4 ± 0.1 [^]
Tb.Sp (mm)	0.28 ± 0.02	0.27 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.02	0.23 ± 0.02

Table 4.4 Cancellous bone properties of the L6 vertebrae (mean ± SD).
[^]Significance difference between male and female ($p < 0.05$). There were no significant treatment effects on cancellous bone properties.

4.2 Rats exposed to fluoxetine

4.2.1 Body mass (fluoxetine)

At 26 weeks of age, fluoxetine rats were significantly heavier than control rat offspring ($p < 0.05$, Table 4.5, Figure 4.5). There was no significant difference between the fluoxetine and control groups at 3 and 7 weeks of age. At 7 and 26 weeks, male rats were significantly heavier than females ($p < 0.05$, Table 4.5). No sex by treatment interaction was seen at any of the age groups.

Female - Body Mass (g)

3 weeks		7 weeks		26 weeks	
Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
49 ± 7	53 ± 5	194 ± 6 [^]	191 ± 17 [^]	324 ± 19 [^]	349 ± 23^{*,^}

Male - Body Mass (g)

3 weeks		7 weeks		26 weeks	
Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
52 ± 9	54 ± 6	256 ± 39	270 ± 10	575 ± 51	616 ± 52[*]

Table 4.5 Body mass at 3, 7 and 26 weeks of age for fluoxetine experiment (mean ± SD). * Significantly more than control ($p < 0.05$). ^ Significant difference between male and female ($p < 0.05$).

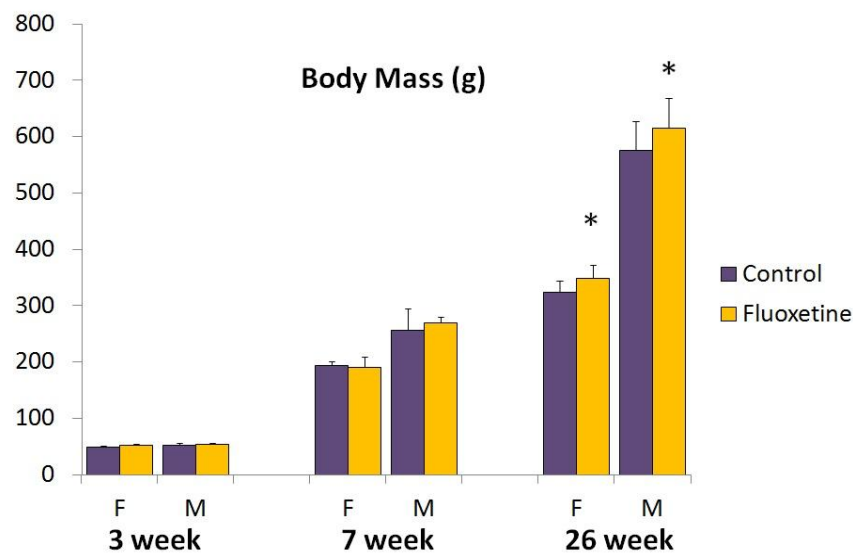


Figure 4.5 Rat body mass (g). At 26 weeks of age, rat offspring from fluoxetine treated dams were significantly heavier than controls (* $p < 0.05$).

4.2.2 Femoral geometric measurements (fluoxetine)

The overall femur length and Ct.Ar were not significantly different between the fluoxetine exposed rats compared to controls at any age (3, 7 and 26 weeks). At 7 weeks of age femurs from fluoxetine exposed rats tended to be shorter compared to the controls

but this difference was not significant ($p = 0.08$). Femurs from the male rats were significantly longer and had significantly greater mid-diaphyseal Ct.Ar than females at 7 and 26 weeks of age ($p < 0.05$) (Table 4.6). No sex by treatment interaction was seen at any of the age groups.

Female						
	3 weeks		7 weeks		26 weeks	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Length (mm)	16.2±0.9	16.4±1.0	30.2±1.0 [^]	29.4±1.1 [^]	37.4±0.7	36.9±0.8 [^]
Ct.Ar (mm ²)	1.3±0.1	1.4±0.1	3.1±0.3 [^]	2.9±0.4 [^]	5.5±0.4 [^]	5.5±0.5 [^]

Male						
	3 weeks		7 weeks		26 weeks	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Length	16.8±1.1	16.4±1.3	32.3±0.8	31.8±1.0	42.5±0.8	42.7±1.1
Ct.Ar (mm ²)	1.3±0.1	1.4±0.2	3.8±0.4	3.6±0.6	7.8±0.5	7.8±0.9

Table 4. 6 Femoral length and mid-diaphyseal cortical bone area of the femurs. (mean ± SD). [^] Significant differences between male and female ($p < 0.05$). There were no significant effects ($p > 0.05$) of fluoxetine treatment on femoral bone geometric measures.

4.2.3 Three point bending mechanical test (fluoxetine)

At 3 weeks of age, femurs from fluoxetine exposed dams had significantly greater yield deformation, yield load and maximum load ($p < 0.05$) compared to femurs from control rats (Table 4.7, Figure 4.6, Figure 4.7 and Figure 4.8). There was no difference in other properties in this age group and no sex by treatment effect was seen. There were no significant differences in the structural and material properties in 7 and 26 weeks of age between the fluoxetine and control groups. No sex by treatment interaction was found in these age groups (Table 4.7). At 7 and 26 weeks femurs from male rats had significantly

greater maximal load compared to the female femurs. At 26 weeks of age, male femurs were also greater in yield deformation, elastic energy and maximal energy compared to femurs from female rats (Table 4.7).

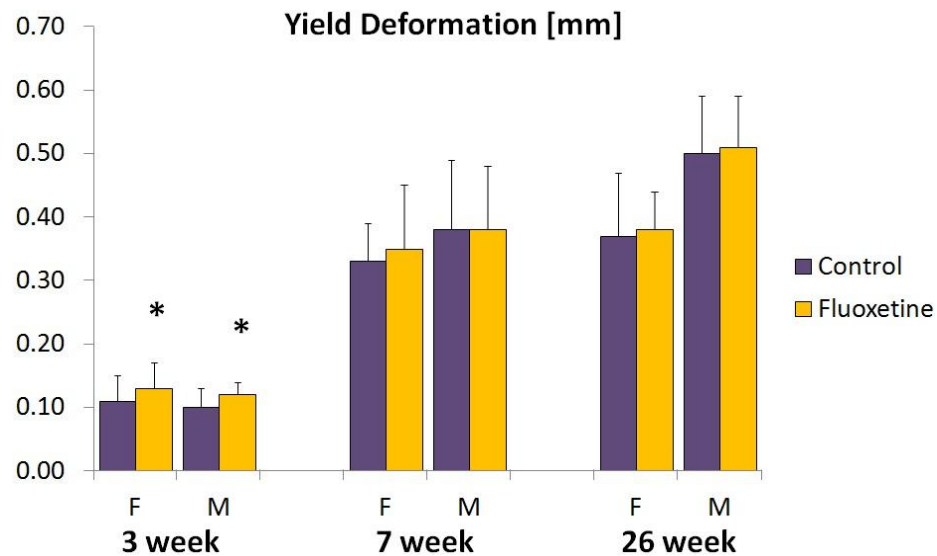


Figure 4.6 Femoral yield deformation [mm] from three-point bending test. Significant differences in 3 weeks are denoted by (* $p < 0.05$).

4.2.4 Cancellous bone properties (fluoxetine)

No significance differences were found in cancellous bone properties between the fluoxetine group and controls at any age group. At 7 weeks of age there was a significant interaction between sex and treatment ($p < 0.05$) in BV/TV (Table 4.8, Figure 4.9). Male vertebrae from the fluoxetine group tended to have greater BV/TV compared to controls while female vertebrae in the fluoxetine group tended to have lesser BV/TV compared to controls. Significant sex differences ($p < 0.05$) were seen at 26 weeks of age (Table 4.8).

The vertebrae from male rats had significantly lower BV/TV and Tb.N compared to the females. Female rats had significantly smaller Tb.Sp compared to male rats.

Female						
	3 weeks		7 weeks		26 weeks	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Yield load (N)	6.7 ± 1.6	7.3 ± 1.6*	45.4 ± 1.9	43.4 ± 6.6	101.4 ± 13.3	107.0 ± 8.9
Max Load (N)	10.6 ± 1.7	11.6 ± 1.7*	63.9 ± 3.5 [^]	63.0 ± 6.4 [^]	141.6 ± 13.4 [^]	140.2 ± 11.4 [^]
Yield Deform (mm)	0.11 ± 0.04	0.13 ± 0.04*	0.33 ± 0.06	0.35 ± 0.10	0.37 ± 0.10 [^]	0.38 ± 0.06 [^]
Max Deform (mm)	0.69 ± 0.20	0.69 ± 0.23	0.88 ± 0.12	0.81 ± 0.08	0.74 ± 0.07	0.75 ± 0.09
Elastic Energy (mJ)	0.6 ± 0.3	0.7 ± 0.3	8.2 ± 0.9	8.6 ± 3.2	19.3 ± 5.2 [^]	20.5 ± 3.1 [^]
Max Energy (mJ)	5.6 ± 1.7	6.0 ± 1.9	39.9 ± 7.8	34.4 ± 6.3	66.5 ± 11.6 [^]	68.1 ± 13.5 [^]
Stiffness (N/mm)	36.5 ± 7.8	39.7 ± 10.5	138.7 ± 13.5	127.4 ± 24.7	305.2 ± 27.9	309.3 ± 32.5
Max Stress(N/mm ²)	40.2 ± 8.7	40.2 ± 5.4	144 ± 15	151.1 ± 17.5	263.1 ± 40.2	252.8 ± 33.8
Elastic Modulus (GPa)	1.4 ± 0.1	1.2 ± 0.3	6.4 ± 1.2	6.6 ± 1.0	10.3 ± 2.4	10.6 ± 1.4

Male						
	3 weeks		7 weeks		26 weeks	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Yield load (N)	6.1 ± 1.0	7.1 ± 1.2*	45.8 ± 2.7	45.9 ± 3.0	136.7 ± 15.8	132.5 ± 10.5
Max Load (N)	9.5 ± 2.0	11.2 ± 2.1*	68.7 ± 8.3	70.2 ± 4.4	178.8 ± 11.0	173.1 ± 16.5
Yield Deform (mm)	0.10 ± 0.03	0.12 ± 0.02*	0.38 ± 0.11	0.38 ± 0.10	0.50 ± 0.09	0.51 ± 0.08
Max Deform (mm)	0.63 ± 0.20	0.68 ± 0.15	0.96 ± 0.13	0.89 ± 0.16	0.94 ± 0.13	0.91 ± 0.05
Elastic Energy (mJ)	0.5 ± 0.2	0.6 ± 0.2	9.9 ± 3.3	10.2 ± 2.6	34.8 ± 8.5	34 ± 5.7
Max Energy (mJ)	4.9 ± 2.0	6.2 ± 1.7	45.7 ± 11.1	40.7 ± 7.4	105.7 ± 16.7	97.0 ± 13.9
Stiffness (N/mm)	34.4 ± 6.6	40.0 ± 7.5	119.3 ± 27.5	116.3 ± 36.6	299.3 ± 32.1	284.1 ± 43.5
Max Stress(N/mm ²)	33.8 ± 7.4	39.4 ± 8.7	130.9 ± 11.6	137.9 ± 30.5	298.7 ± 74 [^]	300.6 ± 95 [^]
Elastic Modulus (GPa)	1.3 ± 0.3	1.3 ± 0.5	5.9 ± 1.4	6.6 ± 4.1	9.7 ± 1.9 [^]	8.9 ± 2.3 [^]

Table 4.7 Mechanical properties obtained from the three-point bending failure test (mean ±SD). * Significantly greater than control ($p < 0.05$) (also indicated by grey shading). [^] Significant difference between male and female ($p < 0.05$).

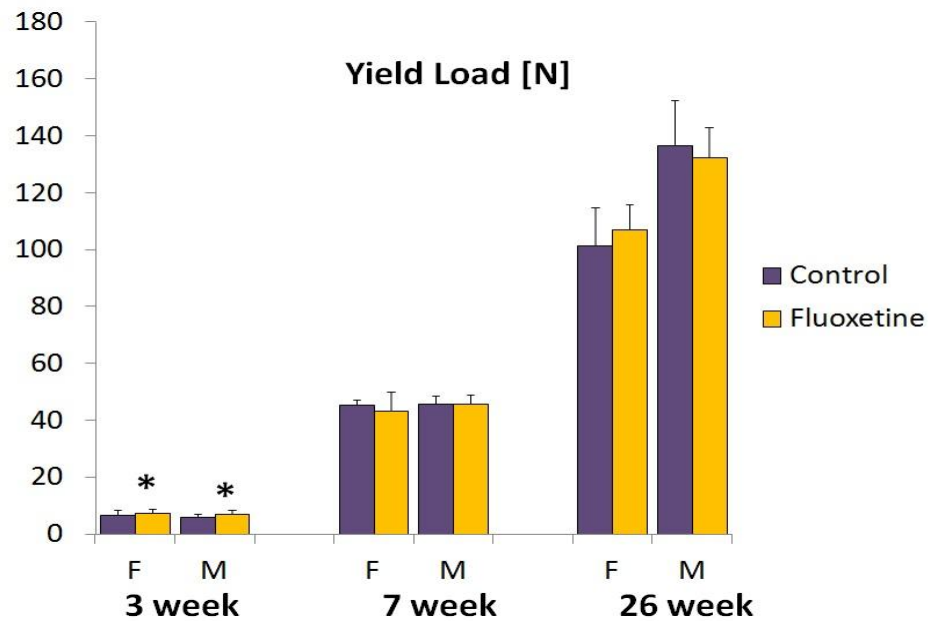


Figure 4.7 Femoral yield load [N] from three-point bending test. * Fluoxetine treated group significantly greater than controls ($p < 0.05$).

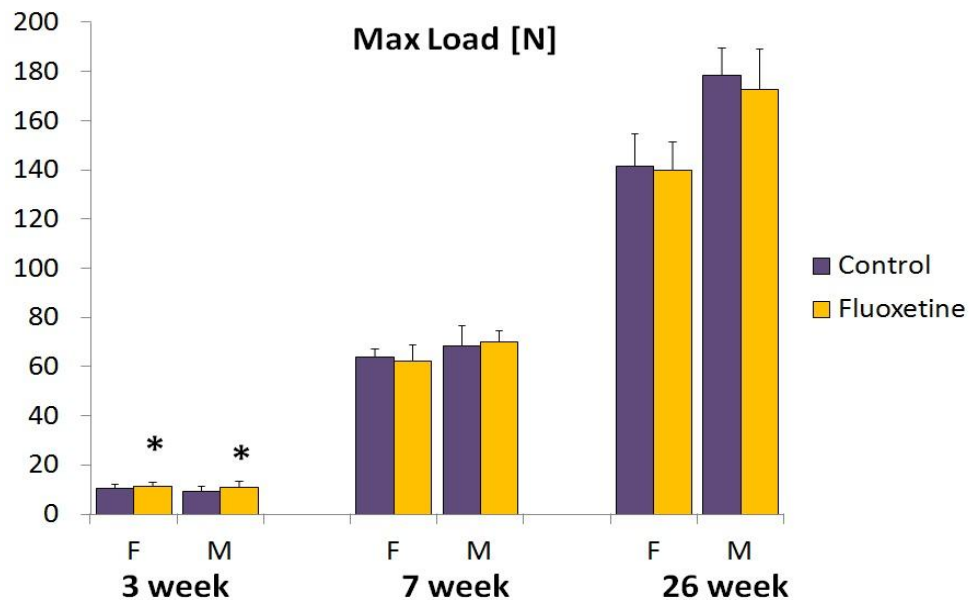


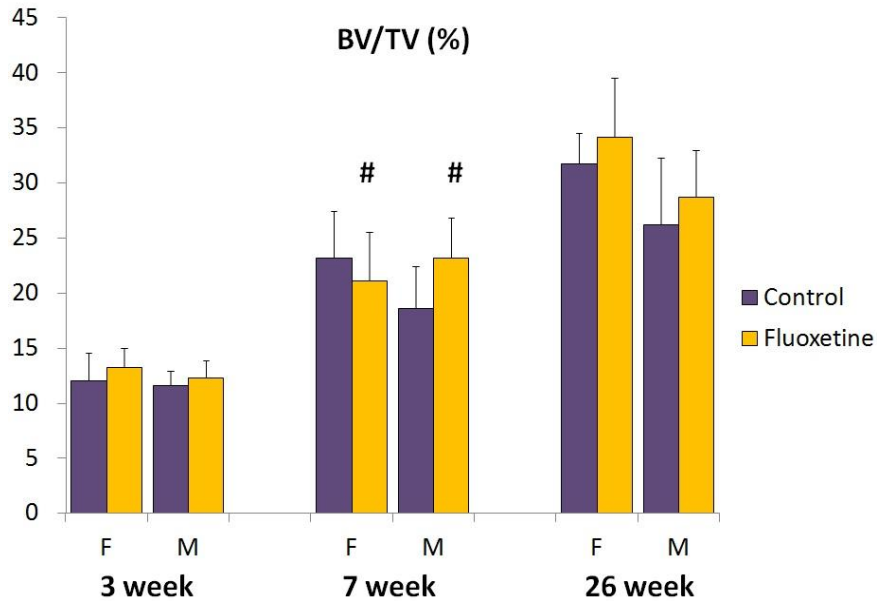
Figure 4.8 Femoral max load [N] from three-point bending test. * Fluoxetine treated group significantly greater than controls ($p < 0.05$).

Female

	3 week		7 week		26 week	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
BV/TV (%)	12.1 ± 2.4	13.2 ± 1.7	23.2 ± 4.2	21.1 ± 4.4[#]	31.7 ± 2.8	34.1 ± 5.4
Tb.Th (mm)	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Tb.N (1/mm)	2.3 ± 0.3	2.5 ± 0.3	3.6 ± 0.5	3.4 ± 0.5	4.0 ± 0.2	4.0 ± 0.4
Tb.Sp (mm)	0.30 ± 0.03	0.29 ± 0.03	0.18 ± 0.02	0.19 ± 0.03	0.17 ± 0.02 [^]	0.18 ± 0.02 [^]

Male

	3 week		7 week		26 week	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
BV/TV (%)	11.6 ± 1.3	12.3 ± 1.6	18.6 ± 3.8	23.1 ± 3.6[#]	26.2 ± 6.0 [^]	28.7 ± 4.3 [^]
Tb.Th (mm)	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Tb.N (1/mm)	2.3 ± 0.2	2.4 ± 0.3	3.0 ± 0.4	3.4 ± 0.3	3.3 ± 0.4 [^]	3.4 ± 0.3 [^]
Tb.Sp (mm)	0.30 ± 0.02	0.29 ± 0.03	0.20 ± 0.03	0.18 ± 0.02	0.22 ± 0.02	0.21 ± 0.02

Table 4.8**Cancellous bone properties of the L6 vertebrae (mean ± SD).**[#] Significant sex by treatment interaction at 7 weeks of age ($p < 0.05$).[^] Significant sex differences at 26 weeks of age ($p < 0.05$).**Figure 4.9** Vertebrae BV/TV (%). Significant sex by treatment interaction at 7 weeks of age is denoted by ([#] $p < 0.05$).

CHAPTER FIVE: DISCUSSION AND CONCLUSION

The purpose of this project was to investigate the effect of *in utero* and postpartum exposure to SSRIs on bone properties in rats at three time points after birth: weaning (3 weeks), growth (7 weeks) and early adulthood (26 weeks). The hypothesis was that SSRI exposure would lead to negative effects on bone material and structural properties of the offspring. The findings of the study were in part supported by this hypothesis but not fully. The disparity in results came primarily from the two different SSRIs that were used in this study (sertraline and fluoxetine). Maternal sertraline exposure resulted in significantly shorter femurs for the offspring at 3 weeks of age. Rat femurs from the sertraline group were also weaker (lower maximal load, maximal stress and stiffness) at 3 and 7 weeks of age compared to controls. In comparison, *in utero* exposure to fluoxetine did not have a negative impact on bone properties. In fact, the femurs from fluoxetine exposed offspring were significantly stronger at 3 weeks of age (greater yield load and maximal load) when compared to the controls. The two studies are not directly comparable due to the differential times of exposure between the two drugs. Nevertheless, the findings indicate that there are differences in the effects of maternal exposure to SSRIs on offspring bone properties.

5.1 Limitations

One of the limitations in this study was the small sample size, especially in the sertraline study. Due to the small litter size and in order to have sufficient number of rats

at 26 weeks of age, the number of rats in each sex group was approximately 5 in the sertraline study and 9 in the fluoxetine study (Table 3.1). Seven week old animals had the lowest sample size in both studies. When fewer animals are in each group, the power to determine the differences between the properties is smaller. In order to increase the sample size and have a higher power for comparison in different bone properties, and also to look at the gender differences, results obtained from female and male animals were combined in each group and a two way MANOVA with sex and treatment as fixed factors was performed. A post hoc power analysis for sample size suggested that in future studies having approximately 20 animals in each sex group would be sufficient to detect differences in the material properties (i.e. max stress and yield stress) and structural properties (i.e. stiffness) at 3 and 7 week old rats based on only one sex.

Due to the differences in the exposure times and also SSRI type, no comparison was made between the results obtained from the sertraline and fluoxetine studies in this project. Studies have shown that SSRIs are excreted into breast milk [5–7] and for this reason in contrast to the sertraline study, offspring rats in the fluoxetine study were also exposed to the drug during breast-feeding. It could be that the SSRI type and the exposure duration are both factors that play a role when looking at the effect of SSRI exposure on bone in general. Since both of these factors were different among the two studies undertaken in this project, general comparisons were only conducted between the overall outcomes of the studies.

Three and four point bending tests are commonly used on long bones of small animals in order to determine mechanical and material properties of the bone. Both these

techniques have their own advantages and limitations. In general, the three point bending test is easier to perform compared to the four point bending test. One of the disadvantages of the three point bending test is the high shear stress that is created near the mid section of the loaded bone. In four-point bending pure bending is transferred between the two points of the bone where the upper loads are applied and this eliminates shear stresses in the midsection. On the other hand, in a four point bending test the two upper forces applied to the bone should be equal and this could be quite a challenge when testing on whole bones in comparison to uniform shaped specimens [82].

Linear beam theory is generally used to calculate material properties from the three-point bending test. One of the assumptions made in this theory is that the aspect ratio of the beam should at least be twenty, meaning that the length of the beam, should at least be twenty times the width [82,83]. If the length of the beam, in our case the bone, is not sufficient enough, the displacement that is caused by loading will mostly be because of the shear stresses and not because of bending itself. The aspect ratios for femurs in our study were approximately 6 in 26 week old rats, 5 in 7 week old rats and 4 in 3 week old rats. The small aspect ratio of rodent bones will result in underestimation of the elastic modulus that is calculated using the resultant deformation data. In order to obtain more accurate results in three-point bending, it would be better to measure the bone strain using a strain gauge that is directly attached to the mid-section of the bone and use the resultant strain values to calculate the elastic modulus [82]. The elastic modulus values calculated for rat femurs using deformation range between 1 to 18 GPa in the literature [84–89]. The underestimation of the elastic modulus caused by the shear stresses might be the reason

for the disparities seen between elastic modulus values reported in the literature.

Nevertheless, the elastic modulus values obtained for rat femurs from the three-point bending test in this project (1-1.4 GPa at 3 weeks, 5.9-8.1 GPa at 7 weeks, 9.7-11.2 GPa at 26 weeks) were reasonable and in agreement with the values reported in the literature that indirectly calculated the elastic modulus using the deformation data [84–89]. The distance between the two lower supports that determines the length-to-width ratio of the bone was calculated according to the rat femur size and gender. The dimensions used in our three-point loading for adult rats were relatively close or similar to the ones used by different groups [85–87,90,91].

5.2 Comparison to other studies

Overall, to our knowledge, no animal studies to date have looked at the effects of maternal and postpartum exposure to SSRIs on offspring bone properties. Animal studies looking at the effect of SSRI use on bone in general differ in several methodological aspects compared to our study, such as, animal model, delivery method, exposure time and type of SSRI used. In human studies not much research has been conducted looking at the effect of maternal use of SSRI on bone properties of the newborn and the few related studies are limited to only investigating properties such as overall body length, head circumference and bone density in newborns. In general, it is hard to make a comparison between the findings in this project and the current related studies because of the several disparities in the methodological aspects.

5.3 Femoral geometric and mechanical properties measurements

5.3.1 Sertraline study

In the sertraline study, the overall femoral length was significantly shorter in the sertraline treated rats compared to the controls at 3 weeks of age. In human studies, babies born to mothers exposed to SSRIs during pregnancy were shorter than those born to mothers who were not exposed to SSRIs [19,44,45]. In two of the studies the differences were statistically significant [44,45] while one study reported a trend that was not statistically significant ($p = 0.07$) between the SSRI group and controls [19].

At 3 weeks of age femurs of sertraline exposed rats were significantly weaker as demonstrated by a lower maximum load and maximum stress compared to the controls. Overall there was a tendency for the sertraline rats in this age group, to have lower material and structural properties compared to the controls. At 7 weeks of age femurs from sertraline exposed rats were also significantly weaker and less stiff compared to the controls. The lack of difference in mid-diaphyseal cortical bone area between sertraline and control rats in this age group suggests that differences in material properties contribute to the weaker bones. At 7 weeks of age, though maximal stress and modulus did show a trend toward being lower in sertraline exposed rats, differences were not statistically significant. At 26 weeks of age, no significant differences were seen in the bone mechanical or material properties between the femurs from sertraline rats and the controls. There was, however, a significant sex by treatment interaction in the structural properties of maximal load and stiffness. While femurs of female rats from sertraline

exposed dams tended to be stronger than the controls, the femurs of male rats from sertraline exposed dams tended to be weaker. This suggests that male rats might be more susceptible to long term effects of *in utero* exposure to sertraline. No studies were found for comparison to this finding since all available animal studies in this regard have only used female rats or mice in their experimental set up. Gender differences were not reported in human studies as well. This effect may be related to the differences in sex hormones between males and females. One of the reported but uncommon side effects associated with SSRI exposure is persistent-SSRI sexual dysfunction, which means the sexual side effects could be evident even after discontinuing the drug use [92]. This condition might be related to having a lower level of serum testosterone [93,94]. Since in men testosterone plays a key role in bone strength, especially after puberty, the decreased level of this hormone could impact bone health in adult men [95,96]. However, more studies should be conducted to clarify the correlation between SSRI use, testosterone level and bone health.

The negative effects of *in utero* sertraline exposure on the offspring bone properties could mostly be associated with the negative influence of gut-derived serotonin on bone formation. Circulating gut-derived serotonin negatively impacts bone formation through binding to serotonin receptors on osteoblasts and decreasing their proliferation [21,24,52]. In humans, the level of circulating serotonin has also been shown to adversely be associated with bone structural and material properties [97]. After SSRI exposure, in this case sertraline, the level of circulating serotonin increases and more serotonin will reach serotonin receptors on bone cells. As a result osteoblast proliferation and ultimately bone

formation would likely decrease more. On the other hand, sertraline also blocks dopamine transporters [66,98] and that could lead to an increase in the concentration of norepinephrine in the brain. This could potentially decrease the positive effect of brain-derived serotonin on bone by increasing the sympathetic tone and as a result decreasing bone formation and increasing bone resorption. Taking into account these two effects of sertraline on pathways of gut and brain-derived serotonin, negative effects seen from *in utero* exposure of sertraline on bone properties could generally be explained.

5.3.2 Fluoxetine study

The *in utero* and postpartum exposure of the rat offspring to fluoxetine did not have a significant effect on the longitudinal bone growth of the femurs at 3 weeks of age. At 7 weeks of age, femurs tended to be shorter in the fluoxetine group compared to the controls but this difference was not significant ($p = 0.08$). Similar to our findings, an animal study on rats found no significant differences in the overall femoral length between the fluoxetine exposed animals and controls [73]. The rats in this study were 8 weeks old when starting the exposure and were exposed to fluoxetine for 6 months.

In contrast to the results obtained from the sertraline study, at 3 weeks of age, femurs from fluoxetine exposed pups were stronger based on significantly greater yield and maximum load ($p < 0.05$) compared to controls. However, no differences were seen in the material and structural properties in the two other age groups of 7 and 26 weeks, and the sex by treatment interaction between male and female rats was not evident in the fluoxetine study. In a study on 8 weeks old rats, long-term administration of fluoxetine for 6 months also did not cause major changes in bone structural properties [73].

5.4 Vertebral versus femoral bone

In cancellous bone measurements on the L6 vertebra of the rats, no significant difference was seen in either of the two studies of sertraline and fluoxetine exposure compared to the control groups. Although similar to the femurs, vertebrae are formed by endochondral ossification, SSRI *in utero* and postpartum exposure did not have an influence on cancellous bone properties of the rat vertebrae. Endochondral and intramembranous ossification are the two main processes involved in bone formation in the skeleton system. The mechanism involved in each of these bone formation processes differ from each other. Intramembranous ossification is responsible for bone formation in flat bones including most of the bones of the skull. Unlike endochondral ossification, cartilage is not involved in this bone forming process [99]. Endochondral ossification occurs in most of the bones in the skeleton specially the long and short bones and starts from the growth plates. Growth plates are composed of hyaline cartilage and are located in the metaphysis at the two ends of long bones. The process of long bone growth happens through the rapid proliferation of the growth plate cells (hypertrophic chondrocytes) in the upper columns, mineralization and apoptosis of hypertrophic chondrocytes at the lower columns and finally replacement of these cells by hard bone tissue via the osteoblasts. The process of bone formation in one place and bone resorption by osteoclasts in another place is why bone maintains its original shape when growing. This process will be continued until the growth plate is completely ossified into bone tissue and this is when a person becomes an adult [99].

5.5 Fluoxetine versus Sertraline

Looking at the results obtained from the two studies, it could be concluded that sertraline and fluoxetine influence bone differently. These differences could be associated with the differences in the pharmacokinetic properties of the two SSRI drugs and their effects on reuptaking other neurotransmitters in addition to serotonin in the body:

First, sertraline is a more potent and more selective SSRI compared to fluoxetine, which has the least serotonin selectivity and potency among SSRIs [66,98]. This means sertraline administration results in blocking more serotonin transporters, and this could lead to a higher concentration of circulating serotonin in the body compared to with fluoxetine use. This could potentially lead to a higher interaction between serotonin and serotonin receptors present in the bone cells and ultimately a greater decrease in osteoblast proliferation and bone formation. In support of this phenomenon circulating serotonin levels in human has shown to adversely be associated with bone structural and material properties [97]. Also, a significant correlation was found between the power of serotonin transporter inhibition and increased risk of fracture; with an increase in the degree of serotonin reuptake inhibition, the risk of hip and femur fracture was also increased among the patients [40]. In one study looking at maternal SSRI use on fetal growth, they found that babies from mothers using SSRIs during pregnancy had smaller head circumferences and also shorter birth length. In this study, most of the participants were taking paroxetine, that is the most potent SSRI [45]. This difference in the power of serotonin reuptake inhibition between sertraline and fluoxetine could be one of the

reasons why in our study, sertraline had a detrimental effect on bone while fluoxetine did not.

Second, norepinephrine inhibition in the brain by serotonin decreases the sympathetic tone and this results in an increase in bone formation and a decrease in bone resorption [24,46,51,55]. By this mechanism, brain-derived serotonin has a positive and indirect effect on bone remodeling [23]. Sertraline is known to be the only SSRI that, in addition to serotonin, potentially blocks dopamine transporters [66,98]. Studies have shown that sertraline leads to an increase in the concentration of dopamine, especially in regions in the brain [68]. An increase in dopamine concentration results in an increase in norepinephrine levels, which in turn causes an increase in sympathetic tone [100]. Dopamine is a neurotransmitter that is involved in the synthesis of norepinephrine (noradrenalin). Taken together, an increase in dopamine concentration, especially in the brain, could potentially inhibit the positive effect of brain-derived serotonin on bone through increasing the concentration of norepinephrine and ultimately increasing the sympathetic tone. The negative effect of elevated levels of dopamine and the positive effect of decreased levels of norepinephrine on bone mass and bone strength has also been supported in animal studies on mice [101–103].

5.6 Exposure time dependency

In this project, the fluoxetine exposed rats were exposed to the drug for a longer period of time (*in utero* and during breast feeding) compared to the sertraline rats. Studies have shown that all SSRIs pass the placenta [3–5], and they are also excreted in breast

milk with different degrees [5–7]. In general the SSRI level observed in breast milk and afterwards in the infant serum has been reported to be low [76]. However, because the infant liver metabolism is not mature, drug accumulation might happen in infants exposed to drugs through breast milk [77]. Fluoxetine has become one of the most commonly prescribed antidepressants during pregnancy [77,104] but not during lactation since the detectable fluoxetine concentration in breast milk is relatively higher compared to other SSRIs and the infant could be exposed to a higher drug dose of fluoxetine, during breastfeeding compared to other SSRIs [1].

Looking at the conflicting results obtained from different animal studies and also the studies conducted in this project, an exposure time dependency could be evident. Studies finding no association or a positive effect between SSRI exposure and bone health had a longer time of drug exposure compared to studies finding a negative association. The exposure time in existing animal studies reporting a negative effect between the fluoxetine exposure and bone was 4 weeks [31,41–43]. In the sertraline study conducted in this project, the rat offspring were exposed to the drug only *in utero*, during the 3 week pregnancy. In comparison, studies finding no association or a positive influence of fluoxetine exposure on bone, including the results obtained in this project from the fluoxetine study, had a longer exposure time ranging from 6 weeks [72] to 6 months [73]. Similar findings have been reported in human studies indicating that the risk of hip fracture is greater among current SSRI users (users that had been exposed to the drug for a shorter period of time) [30,35,39,40].

One mechanism that could explain this happening is that the repetitive administration of SSRIs has shown to down-regulate the β adrenergic receptors [98]. β adrenergic receptors negatively influence bone remodeling by decreasing bone formation and increasing bone resorption [24,46,51,55], so the down-regulation of these receptors could potentially diminish this negative effect on bone. However, more studies should be done to elucidate this association.

5.7 Conclusion and future work

In this thesis project I looked at the effect of *in utero* and postpartum exposure of two types of SSRIs, sertraline and fluoxetine on bone properties. The results obtained from the sertraline study showed that maternal use of sertraline has a negative effect on bone properties of the rat pups at 3 weeks of age, and negative effects were also evident in 7 week old rats suggesting that sertraline could have long term adverse effects on bone properties. In comparison, except for some greater mechanical properties in the fluoxetine group at 3 weeks of age compared to controls, no other association was seen between fluoxetine exposure and bone properties.

Findings in this project suggest that the type of SSRI used by pregnant woman should be considered as an important factor. Due to the differences in the pharmacokinetic properties and also the ability in inhibiting the reuptake of other neurotransmitters (i.e. dopamine, norepinephrine) between the SSRIs, they could have adverse effects on the bone health of the fetus. Exposure time also shows to play a role when looking at the effect of SSRI exposure on bone properties. However, no studies

were found looking at exposure time dependency on offspring bone properties for comparison and further investigation is needed in this area.

In conclusion, maternal sertraline exposure has a negative effect on offspring bone properties. Considering the fact that various mechanisms and pathways are involved in the influence of SSRIs on bone, further studies should be conducted to determine the mechanisms of this influence on bone properties *in utero* and through stages of development. Measuring the serotonin level before, during and after the drug exposure should be considered in future studies in order to determine the influence of each SSRI type on serotonin levels and ultimately how that could have an impact on bone properties. Also, more research should be done investigating the effect of maternal and postpartum SSRI use on the infant's bone, with making adjustments for confounders such as drug type and exposure time.

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APPENDIX I: Animal Handling and Pregnancy Outcomes

The experimental design of the sertraline and fluoxetine studies including animal handling, dosing, and sacrifice were performed by Dr. Alison Holloway and Nicole De Long (McMaster University, Department of Obstetrics and Gynecology, Subdepartment of Reproductive Biology). All the information in this section was provided by Nicole De Long.

For all experimental groups dams were mated (1:1) with age-matched Wister rats and were monitored daily for confirmation of breeding (i.e., the presence of sperm in a vaginal flush). The day that a positive sign of copulation was observed was designated as gestational day 0 (GD0). All dams were allowed to deliver normally. Anywhere between 7-17 pups per litter were delivered. Litters were aimed to be culled to 8 offspring, preferentially selecting for equal number of male and female offspring to ensure uniformity of litter size between treated and control groups. The purpose was to choose 8 healthy looking pups (ones that would survive after weaning). From the ones that would look healthy (pink colour), approximately 8 were randomly chosen within a sex to be culled. After the pups were culled, they were paired in the cages until 7 weeks of age.

There was no difference in the tolerability between the oral treatments of sertraline versus fluoxetine among the mother rats. Litters in both treated groups of sertraline and fluoxetine responded the same to the SSRI drug. Some of the pregnancy outcomes from the two studies are provided in the tables below.

**Birth Phenotype and Fertility Measures in the Sertraline and Fluoxetine studies:
200-250 g Female Wistar rats (mean $\pm$ SD)**

	Control (N=6)	Sertraline (N=5)
Time to Mating (days)	2.5 $\pm$ 1.0	3.3 $\pm$ 1.0
Gestational Length (days)	21.8 $\pm$ 0.5	21.50 $\pm$ 0.3
Pup weight (g)	6.4 $\pm$ 0.7	5.7 $\pm$ 0.5
Sex ratio (M/F)	0.8 $\pm$ 0.1	0.8 $\pm$ 0.3
Survival to weaning (%)	100	100

	Control (N=9)	Fluoxetine (N=10)
Time to Mating (days)	2.3 $\pm$ 0.5	2.2 $\pm$ 0.7
Gestational Length (days)	22.11 $\pm$ 0.48	22.1 $\pm$ 0.31
Pup weight (g)	6.8 $\pm$ 0.3	6.6 $\pm$ 0.3
Sex ratio (M/F)	1.5 $\pm$ 0.6	0.8 $\pm$ 0.1
Survival to weaning (%)	100	100