THE UNIVERSITY of ADELAIDE

Investigating the Effects of High Amylose Wheat on Metabolic, Gastrointestinal and Reproductive Outcomes Using A Mouse Model

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Abstract

High amylose wheat (HAW) is a novel wheat variety with markedly higher levels of amylose, resistant starch and nutrients and a lower glycaemic index compared to commercial standard amylose wheat (SAW) and therefore may confer additional health benefits. While a few studies have indicated that HAW can improve some aspects of metabolic and gastrointestinal health, there was a limited understanding of whether and to what extent these effects varied according to the level of HAW consumed. Furthermore, no studies had determined whether these effects differed between males and females. While improved dietary quality, including increased whole grain intake, has been associated with improved reproductive parameters, the potential impact of HAW on reproductive health had also not been determined. Therefore, the central aim of this thesis was to determine the effects of HAW on metabolic, gastrointestinal and reproductive health outcomes in healthy lean male and female mice.

In Chapter 2, consumption of HAW (35-65% w/w) for eight weeks was associated with a sex-specific effect on several metabolic parameters, including food intake, respiratory quotient (RQ), growth and fat mass. Importantly, these effects were appeared to be dependent on the level of HAW consumed. Specifically, consumption of the lowest level of HAW (35% w/w) was associated with a higher food intake and RQ in female mice whereas consumption of the highest level of HAW (65% w/w) reduced fat mass, particularly abdominal fat mass, in male mice. There was no clear evidence that HAW improved blood glucose or lipid control.

The results presented in Chapter 3 indicated that increased consumption of HAW had effects on several gastrointestinal measures, and these effects were more pronounced in female mice compared to their age-matched male counterparts. The effects observed included increased gastrointestinal weights, enhanced gastric motility and increased

mRNA expression of the intestinal barrier marker *Ocln* and the gut hormone *Pyy*. In both sexes, consumption of higher levels of HAW (50-65% w/w) was associated with increased faecal bacteria load and diversity and a shift in the gut microbiota composition in a manner consistent with improved gastrointestinal health, including increased relative abundance of Bacteroidetes phylum and decreased relative abundance of Firmicutes phylum.

In Chapters 2 and 4, consumption of HAW was associated with positive effects on reproductive health in both male and female mice, including increased testicular weights and an altered pattern of vaginal cytology parameters towards a longer time in estrus/metestrus and less time in diestrus. The positive effects of HAW for female reproductive health were further confirmed by the findings in Chapter 4 where the intake of HAW before and during pregnancy was associated with significantly improved pregnancy rates compared to those consuming SAW diet (94% vs 61%).

Overall, this thesis has provided evidence that consumption of HAW may have benefits on metabolic and gastrointestinal health with dose-dependent and sex-specific effects, and consumption of HAW may improve reproductive function in both males and females. While further studies are required, these results support the potential application of HAW as a functional food to improve health in human consumers.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signature : …

Date : ……15/7/2020……

Publications Arising from This Thesis

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Abbreviations

ANOVA	Analysis of Variance
ART	Assisted Reproductive Technologies
AUC	Area Under the Curve
B2M	Beta-2 Microglobulin
BW	Body Weight
CI	Confidence Interval
CT	Cycle Threshold
CVD	Cardiovascular Disease
ECV	Estimates of Components of Variation
FDR	False Discovery Rate
GCG	Proglucagon
GD	Gestational Day
GI	Glycaemic Index
GIP	Gastric Inhibitory Polypeptide
GLP	Glucagon-Like Peptide
HAW	High Amylose Wheat
HPRT	hypoxanthine-guanine phosphoribosyltransferase
ICSI	Intracytoplasmic Sperm Injection
IVF	*In Vitro* Fertilisation
MUC	Mucin
OCLN	Occludin
PPIA	Peptidylprolyl Isomerase A
PYY	Peptide YY
QIIME	Quantitative Insights into Microbial Ecology
RQ	Respiratory Quotient
RT-QPCR	Reverse Transcription-Quantitative Polymerase Chain Reaction
SAHMRI	South Australian Health and Medical Research Institute
SAW	Standard Amylose Wheat
SBE	Starch Branching Enzyme
SCFA	Short-Chain Fatty Acid
SEM	Standard Error of Mean
SS	Starch Synthase
T2DM	Type 2 Diabetes Mellitus
TILLING	Targeting Induced Local Lesions in Genomes

Thesis Structure

This thesis contains five chapters (Literature Review, 3 Experimental Chapters and General Discussion), two of which are published and one of which was submitted for publication in June 2020. Literature Review (Chapter 1) is divided into two parts. Part 1, which has been published in Food Reviews International, describes existing knowledge of the role of whole grains/high amylose wheat (HAW) on metabolic and gastrointestinal health (the subject of Experimental Chapters 2 and 3). Part 2 focuses on the evidence supporting the potential role of whole grains in improving female reproductive health parameters and introduces the hypothesis that HAW consumption could improve female reproductive health (the subject of Experimental Chapter 4). Experimental Chapter 2 examines the effects of replacing standard amylose wheat (SAW) flour with increasing levels of HAW in diets of male and female mice on metabolic parameters and has been published in Nutrients. Experimental Chapter 3 explores the effects of consuming increasing levels of HAW on gastrointestinal health parameters and gut microbiota composition in male and female mice and has been submitted for publication in Food & Function. Experimental Chapter 4 examines the effects of consuming a high level of HAW before and during pregnancy on fertility and pregnancy outcomes in female mice. Chapter 5 (General Discussion) summarises the findings of the studies, discusses the potential underlying mechanisms, describes the limitations of the studies presented in this thesis and proposes future directions for research.

Chapter 1

Literature Review

1.1 Could High Amylose Wheat Have Greater Benefits on Diabesity and Gut Health than Standard Whole Wheat?

Most materials in this section have been published as:

See Meng Lim, Amanda Page, John Carragher and Beverly Muhlhausler (2019). *Could High-Amylose Wheat Have Greater Benefits on Diabesity and Gut Health than Standard Whole-Wheat?* **Food Reviews International**, 1-13. DOI: 10.1080/87559129.2019.1683743.

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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/6/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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1.1.1 Abstract

Diets which have a low glycaemic index (GI) and high levels of dietary fibre are generally considered to be beneficial for promoting weight loss and improving insulin sensitivity and are therefore recommended for the management of diabesity (the coexistence of obesity and type 2 diabetes mellitus). In addition, high dietary fibre intake is also positively associated with gut health. High amylose wheat (HAW) is a type of wheat which has a lower GI value and contains higher amounts of dietary fibre, including resistant starch, compared with standard wheat, and therefore has potential applications as a functional food for improving metabolic and gut health. The aim of this review is to describe the characteristics of HAW and the current evidence in support of its potential effects on metabolic and gut health, as well as identifying important areas for future research.

Keywords: obesity; type 2 diabetes mellitus; high amylose wheat; gut health

1.1.2 The issue of diabesity

Obesity is a major public health challenge worldwide, with over one-third of the world's adult population being classified as overweight or obese in 2013 [1]. Australia is one of the nations with the highest prevalence of overweight and obesity and according to the latest Australian Health Survey, 63.4% of Australian adults were overweight or obese when the survey was conducted (2014-2015) [2]. Obesity arises from the complex interaction between environmental, socioeconomic and genetic factors. Ultimately, however, weight gain is the result of a chronic imbalance between energy intake and energy expenditure. The consumption of calorie-dense but nutrient-poor foods in

conjunction with low levels of physical activity has been widely recognised as a major factor contributing to the dramatic rise in obesity rates worldwide [3].

Overweight and obese individuals are at an elevated risk of developing a range of debilitating complications that can impact negatively on the quality of life and result in premature mortality. Health consequences of obesity include dyslipidaemia, insulin resistance, osteoarthritis, sleep apnoea, asthma, impaired fertility, cardiovascular disease (CVD), hypertension, some cancers and psychological problems [3]. Type 2 diabetes mellitus (T2DM) is particularly serious comorbidity associated with overweight and obesity [4] and the prevalence of T2DM has increased at an alarming rate, paralleling global overweight and obesity trends. In the latest Australian Health Survey, over 1.2 million Australian individuals were reported to have T2DM [2]. The coexistence of obesity and T2DM in the same individuals is more detrimental to health than either condition alone, since T2DM also confers an elevated risk of developing life-threatening macrovascular (e.g., CVD and stroke) and microvascular (e.g., retinopathy, neuropathy and nephropathy) complications [5].

The strong and interdependent relationship between obesity and T2DM has led to the coining of the term ‘diabesity’ (also known as obesity-associated diabetes) to describe diabetes in the context of obesity [4]. It has been estimated that between 80% and 90% of all diagnoses of T2DM are secondary to overweight or obesity [4]. Both obesity and T2DM make a significant contribution to healthcare expenditure of both industrialised and semi-industrialised nations. In the US, the annual health care cost attributable to obesity was US$190.2 billion in 2005, representing ~21% of total US healthcare expenditure [6]. Global health expenditure on diabetes was US$376 billion in 2010 and is expected to reach US$490 billion by 2030 [7]. According to the Australian Diabetes, Obesity and Lifestyle Study, the total annual direct cost attributable to overweight and

obesity for Australian adults aged ≥30 years was AUS$21.7 billion in 2005 [8], while the amount attributed to diabetes in the same year was AUS$10.6 billion [9]. The significant financial burden of obesity and T2DM has led to an increasing emphasis on identifying effective strategies for preventing and treating these diseases.

1.1.3 Treatment options for diabesity

While a wide range of strategies is used for the treatment and management of obesity and T2DM, the long-term efficacy of most of these approaches remains questionable. Pharmacological approaches for treating obesity and T2DM are available, but most of these have significant side effects, including headache, gastrointestinal discomfort and nausea [10], and are thus not suitable for long-term use. Surgical approaches, such as gastric bypass and sleeve gastrectomy, are an effective treatment option, but are costly and carry the risk of complications from the surgery, and so are typically reserved for individuals with severe obesity who have not responded to other forms of treatment [11].

Diet and lifestyle interventions, particularly low-energy diets and increased physical activity, remain the first-line therapeutic strategies for tackling both obesity and T2DM, and have been shown to be effective in preventing the progression to T2DM in overweight and obese subjects [12]. However, a major challenge with most dietary interventions is the difficulty individuals experience in maintaining weight loss in the long-term. In fact, one review paper noted that only about 20% of overweight individuals were able to successfully maintain a weight loss of ≥10% of their initial body weight for at least one year [13]. Thus, while low-energy diets are often effective in achieving short-term weight loss, most individuals end up regaining any lost weight within a relatively short time frame after the diet ends, and often end up heavier than they were before

dieting. In this context, it remains important to identify diets that are sustainable and can, therefore, assist individuals in maintaining long-term weight loss or avoiding further weight gain.

The low glycaemic index (GI) or glycaemic load diets are one dietary approach that has been widely applied to the management of obesity and T2DM, and have been shown to have beneficial effects on weight loss and insulin sensitivity. The GI of a food is determined by comparing its glycaemic response to that of the same amount of carbohydrate from a standard food (i.e., glucose or white bread) consumed by the same subject. Low GI foods (GI values <55) are digested and absorbed more slowly than high GI foods (GI values >70), and therefore result in longer feelings of fullness after a meal, slowed gastric emptying and improved insulin sensitivity and pancreatic β-cell function [14]. A low GI diet has been shown to be beneficial for weight loss and insulin sensitivity, whereas chronic consumption of a high GI diet has been linked to weight gain, elevated triglyceride and reduced high-density lipoprotein cholesterol concentrations and insulin resistance [15]. As a result, replacing high GI carbohydrate with low GI carbohydrate in the diet has been suggested as a strategy to achieve metabolic benefits, including weight loss and improved insulin sensitivity. Consequently, promoting the consumption of (lower GI) whole grains and whole grain-based foods in the place of (higher GI) refined grains represents a potentially sustainable approach for the management of diabesity.

1.1.4 Whole grains

Whole grains consist of the entire grain, including the bran and germ in their natural ratio, while refined grains only contain endosperm. The germ contains a mixture of lipids, proteins, and some soluble carbohydrates while the bran is composed of mainly fermentable carbohydrates (cellulose, hemicellulose and arabinoxylan) and polyphenolic

lignins [16]. Due to their higher nutrients content in comparison to refined grains, whole grains are generally considered to have a greater nutritional value. A meta-analysis reported that consumption of 40g (and ideally 50g) of whole grains daily is associated with beneficial health effects in human subjects, including protection against obesity and T2DM [17]. Recommendations to consume whole grains also feature in many national dietary guidelines, including the Australian Dietary Guidelines 2013 [18] and Dietary Guidelines for Americans 8th ed. 2015-2020 [19].

1.1.4.1 Anti-diabesity effects

There are several features of whole grains that contribute to their greater health benefits in comparison to refined grains. First, the GI of whole grains is typically lower than refined grains therefore, as indicated above, replacing refined grains with whole grains effectively reduces the GI value of food. For instance, the average GI value of 100% Whole Grain® bread (51) and whole wheat bread (~69) are lower than that of refined wheat bread (~75) [20]. Whole grains also have a higher dietary fibre content compared to refined grains. Whole wheat flour, for example, contains ~280% more dietary fibre than refined wheat flour (13.39g vs. 3.52g/100g) [21]. Dietary fibre is known to have laxative effects and blood lipid and blood glucose-lowering properties [17]. In a prospective cohort study involving 176,117 adults, higher self-reported cereal fibre intake was shown to be inversely associated with T2DM risk (relative risk 0.72, 95% confidence interval [CI]: 0.56-0.93) [22], while in another study intake of fibre derived from whole grains, but not fibre from other foods, was associated with a 17% reduction in the risk of all-cause mortality [23].

The bioactive nutrients present in whole grains, including inorganic nutrients, vitamins, minerals and antioxidant compounds, also contribute to their added health

benefits compared to refined grains. Whole wheat, for example, contains essential amino acids (lysine and tryptophan), vitamins (thiamine and niacin), minerals (phosphorus and iron) and abundant bioactive compounds (alkylresorcinols, benzoxazinoids, phytosterols, tocols, lignans and phenolic acid). The levels of alkylresorcinols (1,3-dihydroxy-5-alkylbenzene derivatives) are particularly high in whole grains: whole wheat contains 489-660 µg of these compounds per gram compared to only 13-47 µg/g in refined wheat [24]. Wheat alkylresorcinols have been shown to promote glucose tolerance and insulin sensitivity in a mouse model of diet-induced obesity by suppressing hepatic lipid accumulation and intestinal cholesterol absorption [25]. Other individual nutrients have also been shown to have favourable metabolic effects; benzoxazinoids and their derivatives have appetite- and weight-reducing effects [26], lignans exhibit antioxidant effects [27] and the phenolic acids (e.g., ferulic acid and vanillic acid) exhibit antidiabetic properties [28]. The different types of bioactive compounds present in whole wheat including their functional properties have been well described in previous reviews [29, 30] and, therefore, will not be discussed in detail here.

1.1.4.2 Gut health

Whole grains have a number of beneficial effects on gut function in comparison to refined grains, including prolonging gastric emptying and increasing stomach distention and small intestinal transit time [31]. These effects are largely due to the higher dietary fibre content of whole grains compared to refined grains, since fibre is one of the most important dietary constituents involved in the regulation of these processes [32]. Dietary fibre also promotes large bowel function and increases colonic transit time, by promoting fermentation by the gut microbiota and through its bulking action [32]. As a result, increased consumption of dietary fibre has been shown to assist in weight loss and

prevention of weight gain, improve glucose tolerance and lower total plasma cholesterol levels [33]. The major forms of fibre in wheat are the insoluble fibre arabinoxylan and soluble fibre β-glucan [34]. Both of these forms of fibre increase faecal bulk, regulate intestinal movement and decrease the amount of glucose absorbed in the small intestine, and consequently reduce circulating cholesterol concentrations [35].

The gut is the largest endocrine organ in the body, secreting more than 30 different peptide hormones. These hormones act either on vagal afferent endings to signal satiety or enter the circulation to target distant organs [36]. The gut hormones have a diverse range of physiological effects. Glucagon-like peptide (GLP)-1, for example, enhances postprandial glucose-dependent insulin release, inhibits glucagon secretion and delays gastric emptying via the vagal pathway and endocrine actions at central sites [36]. Peptide YY (PYY) also promotes satiety both via direct actions on the hypothalamus and by reducing gut motility [37], while ghrelin is orexigenic and promotes appetite via local and systemic actions [38].

The hormones released by the gut depend on the types and amounts of specific nutrients, including carbohydrate, in the diet, which interacts with specialised nutrient receptors on cells within the gastrointestinal tract to facilitate hormone release [39]. For example, glucose released from the digestion of starches, including wheat, binds to the sodium-glucose cotransporter 1. This results in increased release of GLP-1, which in turn acts to suppress gut motility and induce satiety [40]. In addition to the amount and type of wheat consumed, there is evidence that the different physical size of the wheat particles consumed can also impact on gastrointestinal responses [41]. By way of example, one small human trial (n=9) demonstrated that consumption of 55 g of porridge made using coarse (2 mm) wheat particles resulted in significantly lower blood glucose, insulin and

gastric inhibitory peptide (GIP) concentrations in comparison to consuming the same amount of porridge made with smooth (<0.2 mm) wheat particles [41].

The human gut microbiota contains tens of trillions of microorganisms, in which at least 1000 distinct species have been identified [42]. These microbes mainly comprise of bacteria, of which more than 90% belong to either the Firmicutes (60-80%) or Bacteroidetes (20-40%) phylum, although archaea, viruses, fungi and protozoa are also present [43]. There is increasing recognition that shifts in the composition of the gut microbiota have significant effects on human health, including metabolic health, and may contribute to the risk of obesity, insulin resistance and T2DM [44]. Increased abundance of Firmicutes in the gut is generally associated with obesity while the Bacteroidetes are associated with weight loss in most [45, 46], though not all [47], human studies. In addition, increasing gut Bifidobacteria content by supplementing the diet with a prebiotic (oligofructose) has been associated with improved glucose tolerance and glucose-induced insulin secretion and anti-inflammatory effects [48].

The composition of the gut microbiota is highly influenced by dietary intake, with dietary factors estimated to account for ~57% of the variation in gut microbiota, in comparison with only ~12% due to genetic variation [49]. The fermentable carbohydrates, including dietary fibre, resistant starch and oligosaccharides (stachyose, raffinose, and fructooligosaccharides), mainly present in the bran and germ parts of the whole grain, play a particularly significant role in regulating gut microbiota composition, and this may largely explain the positive influence of whole grains on the gut microbiota. These components all appear to have prebiotic effects, which help to increase Bifidobacteria and Lactobacilli in the gut, and this has been demonstrated in human clinical trials as well as animal studies [50].

The fermentation of undigested carbohydrates such as dietary fibres by gut microbiota results in the generation of a number of end-products, of which short-chain fatty acids (SCFAs) are one of the most well-studied. SCFAs are a subset of saturated fatty acids containing six or fewer carbons (C). The most abundant SCFAs are acetate (C2:0), propionate (C3:0), and butyrate (C4:0) [51]. The majority (~ 95%) of these SCFAs are rapidly absorbed by the colonocytes or are released into the circulation. The SCFAs are largely utilised as an energy source to fuel the intestinal epithelial cells [52]. However, some SCFAs also act as signalling molecules for intestinal orphan G protein-coupled receptors, which are involved in the regulation of glucose homeostasis and lipid metabolism, with different SCFAs having different effects [53]. Thus, propionate is mainly involved in promoting hepatic gluconeogenesis, while acetate and butyrate contribute to hepatic lipogenesis and cholesterogenesis [54]. Different bacterial species give rise to different SCFAs; Bacteroidetes mainly produce acetate and propionate and Firmicutes produce predominately butyrate [52]. The polysaccharides from the wheat bran (e.g., cellulose, arabinoxylans, and β-glucan), which escape digestion in the small intestine, may end up being fermented by the microbiota in the large intestine, resulting in the production of individual monosaccharides and additional SCFAs [52]. Animal studies have shown that wheat bran fibre alters intestinal microbiota composition, resulting in increased Lactobacillus counts in the ileum and Bifidobacterium counts in the colon, increased caecal SCFAs concentrations and reduced pH in the colon compared to a control diet without wheat bran [55, 56].

1.1.5 High amylose wheat

High amylose wheat (HAW) was first reported in 2000 by Yamamori and colleagues [57]; since then, it has been increasingly produced in many countries, including Australia. HAW is distinguished from other types of wheat by its higher amylose content. This difference has, in turn, led to suggestions that HAW may confer additional health benefits over and above those provided by other types of whole wheat.

Starch is comprised of the glucose polymers amylose and amylopectin, which mainly differ in their branching and molecular size. The key differences between amylose and amylopectin are shown in Table 1.1. The total starch content in most grains, including wheat, typically comprises 72-75% amylopectin and 25-28% amylose. However, the relative proportions of these polymers can be altered either through normal selective breeding or genetic manipulation. In HAW, the amylose content of wheat can increase to up to ~50% of total starch content through normal selective breeding [58], whereas in some mutant genotypes of wheat, the amylose content can be increased to up to 93% of total starch content [59].

Bird and Regina neatly summarise how the elevation of amylose content in wheat can be attained mainly by manipulating two different mechanisms that control starch biosynthesis: (i) suppression of starch synthase (SS); and (ii) suppression of starch branching enzyme (SBE) [60]. Both SS and SBE have various isoforms; SS includes SSI, SSIIa, SSIIIa, and granule-bound SS, while SBE includes SBEI, SBEIIa, and SBEIIb [61]. SSIIa and SBEIIa are the two predominant isoforms of the enzyme family involved in amylopectin synthesis in wheat, and thus suppression of these enzymes dramatically reduces amylopectin production, resulting in the production of higher amylose grains (Table 1.2) [57, 62-65].

Table 1.1. The key differences between amylose and amylopectin in starch.

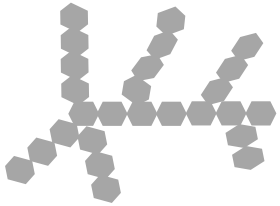
Amylose	**Amylopectin**
	
Mainly α-1,4 linkages	Consist of α-1,6 and α-1,4 linkages
Linear chain; helix	Highly branched molecule; cluster
Long but smaller size than amylopectin	Shorter but much larger size than amylose
500-3000 degree of polymerization	5000-50000 degree of polymerization
Less soluble in water	More soluble in water
Constitutes ~20% of the starch	Constitutes ~80% of the starch
Slowly hydrolysed	Rapidly hydrolysed
Aggregate more rapidly during retrogradation	Aggregate more slowly during retrogradation
Rigid	Soft
Energy storage for long term	Energy storage for short term

Table 1.2. Different levels of high amylose wheat content produced by various methods.

Year	Country	Wheat	Gene	Method	Transgenic/non-transgenic	Amylose (%)	Reference
2000	Japan	Bread	SSIIa	Cross-breeding of natural null mutants of genomes A, B and D	Non-transgenic	30-37	[57]
2006	Australia	Bread	SBEIIa and SBEIIb	RNA interference	Transgenic	>70	[65]
2010	Italy	Durum	SBEIIa	RNA interference	Transgenic	31-75	[64]
2011	Italy	Bread	SSIIa	Cross-breeding of natural null mutants of genomes A, B, and D	Non-transgenic	38	[63]
2012	USA	Bread	SBEIIa	TILLING to identify homozygous alleles A, B and D	Non-transgenic	55	[62]
2012	USA	Durum	SBEIIa	TILLING to identify homozygous alleles A and B	Non-transgenic	47	[62]
2015	Italy	Durum	SBEIIa	TILLING to identify homozygous alleles A and B	Non-transgenic	51-52	[66]
2015	Australia	Bread	SBEIIa and SBEIIb	RNA interference and cross-breeding of null mutants	Transgenic	~85	[67]
2019	USA/Australia	Bread	SBEIIa and SBEIIb	TILLING to identify homozygous alleles A, B and D	Non-transgenic	37-93	[59]

SS: starch synthase; SBE: starch branching enzyme; TILLING: targeting induced local lesions in genomes

1.1.5.1 Potential anti-diabesity effects

To date, no published study has directly evaluated the GI value of HAW as this type of wheat is still novel and has not yet been marketed or grown commercially. Previous studies in other grain varieties have, however, consistently reported negative correlations between the amylose level of the grain and its GI value, such that a higher amylose content is associated with a lower GI [68, 69]. Consequently, the higher amylose content of HAW in comparison to standard wheat would be expected to be associated with a lower GI value. The inverse relationship between the amylose content and GI is due to the fact that the chemically linear amylose chain forms a compact structure that limits enzyme accessibility and amylolysis, thus slowing digestion and reducing postprandial glycaemic and insulinemic response. In contrast, amylopectin is highly branched and less ordered than amylose, and is thus more easily digested and produces a higher glycaemic response. Amylose molecules also aggregate and crystallise more rapidly during retrogradation in cooked starch compared to amylopectin, and are thus resistant to enzyme hydrolysis and are more slowly digested [65]. Therefore, replacing refined grains with HAW may be even more effective than replacement with standard whole grains wheat in reducing the GI value of the food. Directly testing this hypothesis remains an important question to address in future studies.

In addition to the higher amylose content, HAW also has a higher dietary fibre content which contributes by the increased amount of resistant starch compared to standard wheat. Resistant starch is a form of dietary fibre, and is defined as any starch that is not digested by α-amylase in the small intestine and therefore passes to the large bowel to be fermented by microbiota [70]. A recent review published by Bird and Regina describes the health benefits of HAW with particular emphasis on this type of wheat being a superior source of dietary fibre, in particular resistant starch, compared to standard

wheat [60]. This is supported by studies that have assessed dietary fibre content of different types of wheat, including a study that reported one specific type of HAW (of unknown amylose content) contained 2.8-3.6% of resistant starch in dry matter, compared to almost none in standard wheat [71]. Similarly, another study reported that HAW flour (~50% starch as amylose) contained 16.9% of resistant starch in dry matter compared to 1.8-7.3% in flour obtained from other wheat types [58]. The higher amount of resistant starch in HAW flour can be attributed to its higher amylose content, since a previous study identified a positive relationship between resistant starch and amylose content in rice [72]. Since resistant starch is not digested or absorbed in the small intestine, consumption of HAW would also be expected to reduce postprandial glycaemic and insulinemic response in comparison to the consumption of an equivalent amount of standard wheat. This assumption has been supported by a clinical study involving healthy males and females, in which subjects consuming bread made from HAW exhibited less pronounced postprandial glycaemic and insulinemic responses compared to those consuming bread made from standard wheat [73]. In addition, HAW also contains bioactive compounds, vitamins and minerals which have beneficial effects on body weight, insulin sensitivity and/or gut health as similar to other whole grains [29, 30, 74]. However, further studies are needed to determine how the levels compare to those in other commercial wheat types and whether this has the potential to confer additional health benefits.

1.1.5.2 Gut health

Given the significant roles of whole grains in promoting gut function, gut hormone production and microbiota composition, it is expected that the higher levels of amylose and dietary fibres (including resistant starch) in HAW compared to standard

whole-wheat will contribute to additional effects on gut health. To date, however, few studies have directly tested this. In rodents, diets containing high amylose resistant starch have been demonstrated to reduce body fat mass via effects on gut hormone concentrations [75, 76], including increased production (in both the cecum and large intestine) and plasma concentrations of the anorexigenic gut hormones PYY and GLP-1 [75]. There are also indications, however, that the effects of increased dietary intake of resistant starch may differ between lean and obese individuals. In a rodent study, consuming dietary resistant starch from high amylose resistant corn starch (Hi-Maize 260) resulted in decreased fat mass and improved glucose tolerance in lean, but not obese, C57BL/6J mice [77]. Thus, it will be important to evaluate the effects of HAW in both lean and obese humans and animals.

The consumption of HAW also has the potential to favourably alter the composition of the gut microbiota and increase the production of SCFAs by providing more fuel for gut microbiota. Previous studies have demonstrated reduced Firmicutes and increased Bacteroidetes levels in rodents fed a diet containing high amylose corn starch [78, 79]. In addition, supplementing the diet of high-fat diet-fed mice with high amylose corn starch was shown to result in an increased Bacteroidetes:Firmicutes ratio in the gut in comparison with mice fed the high-fat diet alone [80, 81]. Consuming HAW (>70% amylose content) has been reported to lower the pH value (pH 5.90) of the caecal content compared with standard amylose wheat (SAW; pH 6.23) [65]. This study showed that both total SCFAs and all individual SCFAs (acetate, propionate, and butyrate) in the faeces were significantly higher in rats fed with HAW than those fed with SAW. HAW has also been shown to preserve colonic function in rats fed Western-style moderate-fat (19%) and protein (20%) diet by reducing colonic DNA damage and increasing SCFA levels in the digesta [82]. In another study, however, HAW consumption was not

associated with alterations to colon contractility, which suggests HAW does not affect gut motility and transit time [83]. The impact of HAW on the composition of gut microbiota and SCFA production has yet to be determined and represents a critical area for future research.

Even though dietary nutrients, including dietary fibre, present in HAW may impart beneficial effects to health as discussed above there is also the possibility of adverse effects of HAW, particularly at a high level of intake. For instance, metabolic reactions occurring in the colon or distal small intestine, due to unabsorbed polysaccharides such as fructose and fructans that are also present in HAW, could potentially result in increased flatulence, abdominal discomfort/bloating and diarrhoea [84]. It is also likely, however, that these symptoms will vary between individuals. For example, a large proportion of individuals with irritable bowel syndrome have impaired gut transit time and tolerance of intestinal gas load compared with healthy subjects [85], and therefore a HAW diet may not be advisable for this population. However, the impact of different levels of a HAW diet on gut health and whether there may be undesirable side effects associated with higher levels of consumption has yet to be directly tested either in animal models or human subjects.

1.1.6 Public health perspective and potential applications

Wheat is a versatile ingredient for many cereal-based processed products and about 20% of food calories for the world population is supplied from this grain [65]. In Australia, wheat is the largest crop produced yearly: ~21.9 million tonnes of wheat was produced in 2015-2016, which was grown over 11.1 million hectares [86]. In recent years, however, an increasing number of Australian adults are consciously avoiding the consumption of foods containing wheat due to concern about the potential negative health

effects of the gluten components [87]. Since celiac disease (i.e. a medically diagnosed intolerance of gluten), affects less than 1% of the Australian population [88], it is clear that a substantial number of adults are avoiding consumption of wheat-based products predominantly based on perceived negative health effects [87]. In this context, there is a pressing need to re-establish the confidence of Australian consumers in the health benefits that can be obtained from wheat-based products. Consequently, a systematic assessment of the effects of HAW on metabolic- and gastrointestinal health is warranted. If the superior benefits of HAW in aiding weight loss and improving metabolic/gut health are demonstrated, then replacement of standard wheat with HAW in staple foods, such as bread, breakfast cereals and pasta, may potentially offer an alternative approach to treat and/or prevent diabesity without changing existing dietary habits.

1.2 Do Whole Grains and High Amylose Wheat Have A Potential Role in Improving Female Fertility?

I made a major contribution to the conception of this section and the acquisition and interpretation of the studies included in this section. I also drafted the manuscript and revised it according to the recommendations provided by my supervisors.

1.2.1 Abstract

Infertility is a reproductive system disease, which affects 48.5 million couples worldwide. The increasing prevalence of infertility has led to an increasing number of couples utilising assisted reproductive technologies (ART) to assist them to conceive. While the success rates of such treatments are improving, they are nevertheless expensive and are associated with adverse physical and psychological side effects. Consequently, approaches to improve reproductive health and fertility, and thereby avoid the need to access ART, are preferable. Diet quality is known to affect reproductive health and performance and, therefore, improved dietary quality has the potential to improve reproductive outcomes. This review presents current evidence in support of a potential relationship between higher intake of whole grains and improved female reproductive health and fertility and explores the potential for high amylose wheat (HAW) to offer superior benefits to standard wheat varieties for female reproductive health.

Keywords: female; reproductive health; fertility; whole grain; high amylose wheat

1.2.2 Infertility: a global public health issue

Infertility is clinically defined as the failure to achieve conception after one year or more of regular unprotected intercourse [89]. It is almost equally distributed between men and women, and in one-third of cases is attributed to either reproductive health issues in both couples or has an unknown cause [90]. Infertility is associated with significant psychological distress and reduced quality of life for couples. However, there is evidence that this disproportionately affects women [91], who are much more likely to experience social discrimination and stigma due to childlessness [92], although the extent of this varies between countries. Furthermore, unlike men, in whom active sperm production continues throughout adult life, women are born with a fixed number of primordial follicles, whose numbers decrease with age, making women more vulnerable to infertility [93]. According to the 2006-2010 National Survey of Family Growth conducted in the USA, ~9% of men and ~11% of women of reproductive age experienced some form of infertility [94]. Similarly, in a cross-sectional study of 15,162 women and men conducted between 2010 and 2012, the prevalence of infertility was 10.1% among men in the UK compared to 12.5% among women [95]. For these reasons, this review focuses specifically on female reproductive health.

The global prevalence of infertility is also increasing. The number of couples worldwide affected by infertility increased by about 6.5 million, from 42.0 million to 48.5 million between 1990 and 2010 [96]. The progressive rise in infertility has resulted in an increasing number of couples accessing assisted reproductive technologies (ART), such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), in order to conceive. According to the latest International Committee Monitoring ART report, more than 1.6 million ART cycles were performed in the year 2011, resulting in the birth of ~400 thousand babies [97]. In China, which was not included in these figures, it was

estimated that 2 million ART cycles were performed in this same year, resulting in ~500 thousand births [97]. Furthermore, the rising incidence of infertility means that the utilisation of ART is expected to increase further, with one study estimating that the number of ART cycles in Australia will increase by between 34% and 61% over the coming decade [98]. While the efficacy of ART treatments have been improving over time, the process is nevertheless very expensive [99] and is not accessible in all areas [92]. In addition, while sperm collection for ART is largely non-invasive for men, women are required to undertake a series of invasive procedures, including blood collection, hormone injections, surgical ova retrieval and embryo transfer. In addition to being painful, these procedures are also associated with adverse events, including multiple pregnancies, infection, bleeding and psychological distress [100]. As a result, strategies to improve reproductive health and fertility without the need to utilise ART is a more attractive approach.

1.2.3 Whole grains are an important carbohydrate source

Modifiable lifestyle factors, including diet, are known to affect fertility and reproductive performance in both men and women [101]. Generally, eating a nutritionally balanced diet is associated with improved reproductive health and higher rates of fertility [102, 103]. In contrast obesity, consumption of poor quality diets and a sedentary lifestyle can have deleterious effects on a number of fertility parameters, including decreased sperm quality in men [104] and increased risk of ovulatory dysfunction in women [105]. As a result, encouraging men and women planning pregnancy to maintain a healthy diet and lifestyle habit, including maintaining healthy body weight, is likely to be important for improving reproduction health and increasing fertility.

Carbohydrates are the major source of energy in the diet, contributing approximately 50-60% of total daily energy intake in the majority of populations worldwide [106]. Higher quality carbohydrates, such as whole grains, are the preferred sources of dietary carbohydrates in comparison to lower quality carbohydrates such as highly processed grains. While the majority of national dietary guidelines do not include specific advice as to the desirable levels of whole grains in the diet, the majority recommend substituting highly processed grains with whole grains in the diet for improved health (Table 1.3). The superior health qualities of whole grains are due to the fact that they include all three main components (endosperm, germ and bran) in the grain kernel whereas in highly processed grains only the endosperm is present [107]. Consequently, whole grains contain higher amounts of nutrients and phytochemicals, which are mainly concentrated in the bran and germ, and are therefore considered to have greater beneficial effects on health than highly processed grains. This is supported by the results of numerous studies in which higher whole grain intakes have been associated with reduced risks of CVD [108], T2DM [109], excess weight gain [110], and all-cause and disease-specific mortality[108]. While the beneficial effects of increased whole grain intakes for most non-communicable diseases are promising, the role of whole grain consumption on female reproductive outcomes, including potential beneficial effects on fertility and pregnancy success, is yet to be fully investigated.

Table 1.3. Overview of recommendations or guidelines for whole grains intake from multiple regions or countries.

Region/country	Whole grains intake recommendation or guideline	Reference
Arab nation	At least half of all grain serves should be whole grains	Food Dome Dietary Guidelines for Arab Countries 2012 [111]
Australia	At least two-thirds of all grain serves should be whole grains	Australian Dietary Guidelines 2013 [18]
Canada	Eat plenty of whole grains	Canada's Food Guide 2019 [112]
China	Consumption of 50–150 g per day of whole grains and mixed beans	Dietary guidelines for Chinese residents 2016 [113]
Malaysia	At least half of all grain serves should be whole grains	Malaysian Dietary Guidelines 2010 [114]
Netherlands	Eat at least 90 g of brown bread, wholemeal bread or other whole-grain products daily	Dutch Dietary Guidelines 2015 [115]
New Zealand	Choose mostly whole grains and those naturally high in fibre	Eating and Activity Guidelines for New Zealand Adults 2015 [116]
Scandinavia	Consume at least 75 g whole grain per 2400 kcal	New Nordic Diet 2012 [117]
South Africa	Eat starchy foods in the form of minimally processed or whole grains, legumes and root vegetables	Food-Based Dietary Guidelines for South Africa 2013 [118]
UK	Choose whole grains or high fibre versions where possible	Eatwell Guide 2016 [119]
USA	At least half of all grain serves should be whole grains	2015-2020 Dietary Guidelines for Americans [19]

1.2.4 Whole grains and female fertility

While further research is required, a small number of studies have reported positive associations between the intake of whole grains and female reproductive health and fertility, especially in women seeking ART treatment. In one study by Gaskins and colleagues, involving 273 women undergoing IVF treatment, a higher intake of whole grains before treatment was associated with a significantly higher probability of implantation and live birth [120]. Specifically, the study found that a one serving per day (28 g per day) increase in the intake of whole grains was associated with a 33% (95% CI: 1-75%) and 27% (95% CI: -2-65%) higher odds of implantation and live birth, respectively [120]. The study also showed that whole grain intake was positively associated with endometrial thickness in women, with a one serving per day increase in whole grain intake associated with a 0.4 mm (95% CI: 0.1-0.7 mm) increase in endometrial thickness [120]. This is significant, since the endometrial thickness is known to be positively associated with pregnancy and live birth rates in women undergoing IVF. In one study in women undergoing IVF treatment, for example, the miscarriage rate was highest in women with an endometrial thickness less than 7 mm, while those with an endometrial thickness greater than 14 mm had the highest rates of pregnancy and implantation [121].

The positive effects of dietary patterns that include higher whole grain intakes on reproductive health are further supported by the results of several prospective cohort studies. A prospective cohort study of 199 women undergoing IVF/ICSI treatment conducted in the Netherlands, for example, found that higher adherence to the Dutch dietary recommendations (characterised by high intake of whole grains, monounsaturated or polyunsaturated oils, vegetables, fruit, meat or meat replacers, and fish) was associated with a 1.65 fold greater probability of conceiving compared to those with a lower

adherence [122]. Similarly, in the prospective Environment and Reproductive Health study, women with higher adherence to a 'pro-fertility' diet characterised by higher intake of whole grains, supplemental folic acid, vitamin B12, vitamin D, low-pesticide fruits and vegetables, seafood, dairy, and soy foods had significantly higher probabilities of implantation (47%), pregnancy (43%) and live birth (53%) following ART [123].

Consuming a Mediterranean diet pattern (characterised by a high intake of vegetables, fruit, whole grains, legumes, fish, and olive oil) has also been associated with improved reproductive outcomes in several cohort studies. This includes the Environment and Reproductive Health study cited above, in which women in the second through fourth quartiles of adherence to a Mediterranean diet pattern had a significantly higher probability of live birth (0.44, 95% CI: 0.39-0.49) compared with women in the first quartile (0.31, 95% CI: 0.25-0.39) [123]. Similarly, a prospective cohort study of 590 women conducted in China showed that a higher adherence to the Mediterranean diet was associated with an increase in both the number of fertilised oocytes and embryo yield in women undergoing IVF treatment [124], while in a smaller study in Greece, including 244 non-obese women undergoing IVF treatment, women in the highest quartile of adherence to the Mediterranean diet had significantly higher rates of pregnancy (50% vs 29.1%) and live birth (48.8% vs 26.6%) compared to women in the lowest quartile [125]. Similar results were obtained in a case-control study nested in a Spanish cohort of University graduates, such that women with the highest adherence to the Mediterranean-type diet were less likely to experience difficulties getting pregnant compared with those in the lowest quartile (odds ratio: 0.56, 95% CI: 0.35–0.95) [126]. The association between higher dietary quality and reproductive health has not, however, been supported by all studies. For example, a study by Vujkovic and colleagues, which investigated the association between preconception dietary patterns and IVF/ICSI outcomes among

couples in the Netherlands, found no association between consumption of a "health conscious-less processed" dietary pattern (consisting of high intakes of fruits, vegetables, fish, and whole grains and low intakes of snacks, meats, and mayonnaise) and pregnancy success [127].

Taken together, while studies are limited and not all results are consistent, the majority of the studies conducted to date support the hypothesis that increased intakes of whole grains may potentially be associated with improved fertility outcomes in females, especially in those undergoing ART treatments. Most studies have, however, examined the effect of an entire dietary pattern, characterised by higher consumption of a range of foods and food groups (including whole grains) [122-127], and only one study to date has focused specifically on whole grain consumption [120]. Consequently, it is difficult to specifically separate the effects of whole grains from those of other dietary components. In addition, most existing studies have been carried out in couples undergoing ART treatments, and thus the findings may not necessarily apply to the general population. The existing studies have also assessed associations rather than causality and have provided no insights into the underlying mechanisms. Therefore, further studies which examine the effects of whole grains on reproductive health and fertility, particularly in women and couples who are not clinically infertile, are warranted.

1.2.5 Nutrients in whole grains and female fertility

As discussed earlier, whole grains are rich in many nutrients and phytochemicals, including dietary fibre, oligosaccharides (oligofructose and inulin), antioxidant compounds, phytate, phytoestrogens (lignan and plant stanols and sterols), B vitamins, and minerals [128]. Most of these components are found in the germ and bran, and their levels are therefore dramatically reduced during the grain-refining process. It is, therefore,

possible that the increased concentrations of these nutritional factors in whole grains may contribute to beneficial effects on fertility and pregnancy outcomes. Interestingly, a previous study identified a strong positive association between total bran intake, but not germ intake, and implantation, pregnancy, and live birth rates in women undergoing IVF [120], suggesting that components in the bran layer play a major role in conferring the beneficial effects of whole grains on female fertility.

One of the nutrients in whole grains which has been most closely associated with fertility are the B vitamins, including folate (vitamin B9) and vitamin B6 (pyridoxine). Numerous studies have demonstrated that higher dietary folate intakes are associated with improvements in ovarian function [129], oocyte quality [130] and rates of implantation, pregnancy and live birth [131] in women undergoing ART. Similarly, in a prospective study conducted in China, women with higher preconception plasma vitamin B6 concentrations were shown to have reduced odds of early pregnancy loses and higher probabilities of conception and clinical pregnancy [132], and lower risks of clinical spontaneous abortion [133] and preterm birth [134] compared to those within lower concentrations. A plausible explanation for the relationship between these B vitamins and female fertility is that both folate and vitamin B6 participate in many metabolic reactions, including the folate and methionine cycles, which are central to cellular function for the synthesis of DNA, amino acids, and phospholipids [135]. Therefore, both folate and vitamin B6 are critical for supporting the rapid cell growth and proliferation periods that occur in follicular development in the ovaries, and subsequent embryo establishment and development. In addition, since these B vitamins are co-factors in the methionine cycle, lower levels of folate and vitamins B6 and B12 are associated with homocysteine accumulation, which induces various inflammatory responses that have been linked to negative reproductive outcomes [136]. The important roles of B vitamins and

homocysteine metabolism in female reproductive health have been reviewed elsewhere [137, 138], and thus will not be discussed further in this review, but do provide one potential pathway through which increased whole grain consumption could positively influence reproductive health.

In addition to the B vitamins, the antioxidant compounds that are present in whole grains, including the phenolic acids, phytic acid, vitamin E and selenium, may also contribute to improvements in fertility outcomes. These antioxidant compounds inhibit or delay oxidation, either by quenching free radicals or chelating redox metals, and thus reduce the oxidative stress that is associated with inflammation, DNA damage, and oxidation of lipids and proteins. Oxidative stress is known to negatively affect female fertility, and has been implicated in the aetiology of a number of reproductive diseases, including endometriosis and polycystic ovary syndrome, and pregnancy complications, including spontaneous abortion, preeclampsia, preterm labour and intrauterine growth retardation [139]. Different whole grains contain different antioxidant compounds at different concentrations. For example, wheat contains higher levels of ferulic acid; oat contains mainly avenanthramides and β-glucan; barley contains benzoic and cinnamic acid derivatives; rye contains alkylresorcinols; and pigmented rice contains anthocyanins [140]. Therefore, all of these whole grains have the potential to confer protective effects on systematic oxidative stress, which in turn may have beneficial effects on reproductive health. The role of specific antioxidants in reproductive health outcomes is, however, unknown.

Hormonally active compounds in grains, such as lignans, may also positively influence female fertility. Lignans are compounds containing a 2,3-dibenzylbutane structure, which form the building blocks for the formation of lignin found in the plant cell wall. Thus, the bran layer of whole grains is a concentrated source of lignans, whereas

these compounds are not present in highly processed grains [141]. Lignans are found in a variety of whole grains, including whole wheat, whole oats, and rye meal. Support for the potential role of lignans in female fertility is provided by a prospective study which showed that higher female urinary lignan concentrations were associated with a shorter time to pregnancy among couples attempting to conceive [142]. The positive effects of lignans for reproductive health may be a result of their diphenolic structure, which is similar to 17β-estradiol. Therefore, these compounds can bind to estrogen receptors and consequently have the potential to influence hormonal status and increase ovulation rates [142].

Overall, therefore, the available evidence provides biological plausibility and some indirect support for the suggestion that whole grains and whole grain components, including the B vitamins, antioxidant compounds and lignans, could exert beneficial effects on female reproductive health and fertility. However, further studies are required to provide direct evidence to support the relationship, as well as to elucidate the underlying mechanisms.

1.2.6 Low glycaemic index

The glycaemic index (GI) is a relative ranking of carbohydrate-containing foods according to their effect on postprandial blood glucose concentrations in the two hours following consumption. Carbohydrates with a low GI value (≤ 55) are more slowly digested and absorbed and thus produce slower rises in blood glucose and insulin levels, whereas, high GI value (≥ 70) carbohydrates are more rapidly digested and absorbed and thus produce greater increases in blood glucose and insulin levels [143].

Diets with a low GI or glycaemic load (a combination of GI and total carbohydrate intake), have been associated with several potential health benefits, including reduced

risk of weight gain and obesity, reduced blood glucose levels, improved serum lipid profile, and reduced risks of T2DM and CVD [144]. While there are few studies that have investigated the impact of low GI diets on female reproductive health and fertility, existing studies have nevertheless provided some evidence in support of potential positive effects. For example, in the Nurses' Health Study II, a prospective cohort study involving 17,544 women, those with higher adherence to a diet characterised by higher consumption of monounsaturated fats, plant proteins, low GI carbohydrates, full-fat dairy, multivitamins, and iron during the preconception period had a lower relative risk (0.34; 95% CI: 0.23-0.48) of infertility caused by ovulatory dysfunction compared to women with the lowest adherence to this dietary pattern [145]. In addition, in a small randomised controlled trial of 26 overweight or obese women undergoing IVF, consuming a low GI diet was associated with an increase in the number of oocytes retrieved and the rates of pregnancy and live birth [146]. Therefore, a lower GI dietary pattern may also be important for supporting female reproductive health and fertility, but further studies are needed.

1.2.7 High amylose wheat and female fertility

High amylose wheat (HAW) is a novel type of wheat which contains a higher level of amylose compared to commercially available standard amylose wheat (SAW). In SAW, the ratio of amylose to amylopectin is about 1:3, however, in HAW, the level of amylose can be increased significantly, by suppressing the enzymes involved in starch biosynthesis, including the SS and/or SBE, as discussed earlier (section 1.1.5).

HAW has several properties that would be expected to confer additional health benefits when compared to standard wheat varieties. First, HAW is known to have a lower GI compared to SAW, since the amylose is a linear molecule with significantly fewer

branches than amylopectin, and is thus less susceptible to the enzyme hydrolysis, which breaks the molecule down into simple glucose, and consequently more slowly digested and absorbed. In addition, HAW contains a higher amount of resistant starch, which would further reduce carbohydrate absorption, in comparison to SAW. Furthermore, HAW contains higher levels of nutrients, including protein, lipid, minerals and dietary fibre, in comparison to SAW [147].

To date, there are limited studies that have investigated the potential effects of consuming higher levels of HAW, in comparison with standard wheat, on female reproductive health and fertility. However, a recent study by our group found that female mice consuming higher levels of HAW for eight weeks showed an altered pattern of vaginal cytology parameters, suggesting a shift in the estrus cycle towards a longer duration in metestrus and a shorter duration in diestrus compared those consuming the SAW [148]. The observed changes in the distribution of cell populations in the lower reproductive tract may also be indicative of a change in the immune cells present at the mucosal surfaces. The immune cells within the reproductive tract, which are regulated by sex hormones concentrations, such as estrogen and progesterone, provide immunity against pathogens/infections in the lower tract and have an important role in providing immune tolerance for sperm. Consequently, these cells have a key role in influencing a number of physiological events including fertilisation, implantation, pregnancy, and parturition [149]. Further studies specifically focussed on the role of HAW in female reproductive health and immune parameters within the reproductive tract are, however, required.

1.2.8 Conclusion and future direction

Infertility is a global public health issue of which the prevalence is increasing. Dietary quality can significantly impact overall health, including reproductive health and fertility, in both males and females. Consequently, optimising the nutritional status of men and women of reproductive age is likely to be an important strategy for supporting reproductive health. Increasing intakes of whole grain in the diet is likely to be an important component of this approach. This review has provided a summary of the existing evidence supporting the potential benefits of consuming higher amounts of whole grains for female reproductive health, and some of the properties of whole grains that may underlie these effects. While these findings are interesting, and potentially important, it is clear that studies to date are limited, and that further studies are required to establish the potential role of increased whole grain consumption, as well as consumption of novel wheat varieties, including HAW, in improving female immunity.

1.3 Thesis Aims

Diet is a modifiable factor that can greatly influence health and well-being. However, while dietary interventions are effective in reducing weight gain and risk of non-communicable diseases, most individuals experience difficulties adhering to diets that require significant changes to their habitual diets in the long term. As a result, improving the nutritional quality of the food supply, so that improved nutrition can be achieved without individuals having to dramatically change their dietary habits, is likely to be more effective in achieving lasting health benefits.

Carbohydrates contribute approximately 50-60% of total energy intake in the majority of populations worldwide. Wheat is a versatile ingredient for many cereal-based products, including bread, noodles, biscuits and breakfast cereals, which are all important staple foods for many people throughout the world. Therefore, improving the nutritional properties of wheat has considerable potential to increase the nutritional quality of many end-user products and subsequently achieve health benefits for a significant proportion of the world's population.

HAW is a novel type of wheat, which has properties with the potential to deliver additional health benefits compared to commercially available SAW. Existing evidence from clinical and rodent studies has indicated that the consuming of HAW was associated with a reduction in levels of postprandial glucose and insulin and improvements in gastrointestinal health. Given the tight connection between metabolic and reproductive health, HAW may also have benefits for female reproductive health, but no studies have directly assessed this. As a result, we and others have hypothesised that replacing HAW with SAW in wheat products may be associated with improved health outcomes, including improved metabolic, gastrointestinal and reproductive health. The **Central Aim of this thesis was to investigate the effects of higher dietary intakes of HAW, in**

comparison to SAW, on metabolic, gastrointestinal and reproductive outcomes using a mouse model.

A number of previous studies had proposed that the properties of HAW, which including the higher amylose content and lower GI, may have beneficial effects on metabolic health outcomes. However, the level of HAW which may be effective to improve metabolic health outcomes was poorly understood. In addition, it is now widely recognised that metabolic homeostasis is regulated differently in males and females, however, no previous studies had determined whether the effects of HAW differed between sexes. Therefore, **the Aim of Chapter 2 of this thesis was to determine the effect of replacing SAW with increasing levels of HAW (35-65% of the diet by weight) on metabolic health parameters, including fat mass, metabolic rate and fasting glucose concentrations, in male and female mice. Some reproductive markers in both genders, including reproductive organ weights and estrus cycles for females, were also measured in this Chapter.**

Previous rodent studies had reported that consuming a diet containing HAW was associated with improvements in several markers of gastrointestinal health, including increased concentrations of SCFAs in the digesta and reduced caecal pH. However, the effects of increased dietary intake of HAW on functional measures of gastrointestinal health and the composition of the gut microbiota, how these effects vary according to the level of HAW consumed and whether the effects of HAW differ between males and females, was unknown. Therefore, **the Aim of Chapter 3 of this thesis was to determine the effect of replacing SAW in the diet with increasing levels of HAW (35-65% of the diet by weight) on gastrointestinal function and gut microbiota composition in male and female mice.**

Previous epidemiological studies had reported that the higher intakes of whole grains were associated with improvements in female reproductive health and fertility, including increasing the probability of achieving pregnancy and live birth. Furthermore, body weight, body composition and energy metabolism have all been shown to be related to female reproduction, and the increases in ovarian hormone concentrations that occur during puberty and pregnancy influence metabolic pathways and nutrient metabolism. The nutritional properties of HAW suggest that consuming this wheat variety may confer additional beneficial effects on female reproductive health and fertility in comparison to consuming standard wheat. However, no previous studies had directly tested this hypothesis. Therefore, **the Aim of Chapter 4 of this thesis was to determine the effect of consuming a diet containing a high level of HAW prior to mating and during gestation on fertility and pregnancy outcomes in female mice.**

Chapter 2

Sexually Dimorphic Response of Increasing Dietary Intake of High Amylose Wheat on Metabolic and Reproductive Outcomes in Male and Female Mice

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2.1 Abstract

High amylose wheat (HAW) has a higher resistant starch content and lower glycaemic index than standard amylose wheat (SAW), which may be associated with health benefits. This study aimed to determine the effects of replacing SAW with HAW on metabolic and reproductive parameters in male and female mice. Male and female C57BL/6 mice were randomly divided into groups (n = 8/group/sex) and fed either a SAW65 (65% SAW w/w; control), HAW35 (35% HAW w/w), HAW50 (50% HAW w/w) or HAW65 (65% HAW w/w) diet for eight weeks. In males but not females, the HAW65 group had a lower abdominal circumference, relative total fat mass, relative gonadal fat mass and plasma leptin concentration compared to the HAW35 group. There were no differences in fasting blood glucose concentrations or plasma concentrations of cholesterol, triglycerides or non-esterified fatty acids between groups in either males or females. The HAW-fed males had a higher testicular weight and HAW-fed females spent less time in diestrus and a longer time in metestrus compared to the SAW-fed mice. Higher dietary intake of HAW appears to reduce abdominal fat deposition compared to the lower level of HAW in a sexually dimorphic manner. The impacts on reproductive parameters in the HAW-fed mice require further investigation.

Keywords: high amylose wheat; low glycaemic index; metabolic health; reproductive function

2.2 Introduction

Whole grain flours are nutritionally superior to refined flours, since they contain the fibre, vitamins, minerals and phytochemicals that are mainly present in the bran and germ and have been reported to contribute to positive health effects [150]. Recent meta-analyses provide evidence of an inverse association between whole grain intake and risk of non-communicable diseases including cardiovascular disease, type 2 diabetes mellitus, certain cancers and obesity [108, 151]. Higher whole grain intake has also been reported to improve fertility health parameters, notably increasing the probability of embryo implantation and live birth among women undergoing *in vitro* fertilisation [120].

Despite the established benefits of whole grains, most people worldwide are still consuming greater quantities of refined grain products compared to whole grains [152]. In a survey of 12,153 Australian adults conducted between 2011 and 2013, more than 70% failed to meet the recommended daily target intake for whole grains of 48 g [153]. Thus, there remains a need to develop strategies for increasing, and maintaining in the long term, whole grain intake at a population level. The most effective solution is likely to be the incorporation of whole grains into existing staple food products, so that consumers are not required to alter dietary habits in order to increase intake. Bread wheat (*Triticum aestivum* L.) is an important staple cereal grain food for humans throughout the world, but especially in Western-style diets. The transition to increased consumption of wheat and wheat-based products in many Asian populations further contributes to the demand for wheat worldwide [154]. Wheat is used in a wide range of food products, including bread, cookies, pastries, breakfast cereal and noodles. Therefore, any improvements in the nutritional properties of wheat have considerable potential to increase the nutritional quality of a large number of end-user products consumed by many people worldwide.

High amylose wheat (HAW) is a novel wheat type with additional nutritional benefits compared with standard wheat products. It contains higher levels of amylose compared to standard wheat [60], with 38% to 85% amylose [63, 67] compared to ~28% in standard wheat varieties [155]. The higher amylose content is correlated with increased resistant starch [62], with HAW varieties being reported to have a resistant starch content as high as 11.2% compared to negligible amounts (<1%) in standard wheat [62]. The increased linear chain structure of amylose confers greater resistance to digestive enzymes, and thus this wheat has a lower glycaemic index [156]. The greater amount of resistant starch in HAW, which is not absorbed in the small intestine, further reduces the availability of carbohydrate absorption.

Lower glycaemic index and high resistant starch diets have been reported to be associated with reduced risk of metabolic diseases, including obesity, type 2 diabetes mellitus and cardiovascular disease [70, 157, 158]. Consequently, the properties of HAW have led to the suggestion that partial or complete replacement of standard wheat with HAW in staple foods may have beneficial effects on metabolic health, without consumers needing to dramatically change their dietary composition [60]. However, few studies have directly tested the effect of increased HAW consumption on metabolic health outcomes in either humans or experimental animal models. The level of HAW which may be required to improve metabolic health outcomes, and whether the effects of HAW differ between males and females, remain also unclear. Therefore, the aim of this study was to evaluate the effect of replacing standard wheat flour in the diet with increasing levels of HAW on growth, food intake, fat mass, nutrient metabolism and plasma metabolic parameters in male and female mice. Given the established association between metabolic and reproductive health [159], we also sought to examine the effect of the HAW diet on indirect measures of reproductive function in both genders.

2.3 Materials and Methods

2.3.1 Wheat materials

Commercially available standard amylose wheat (SAW; *Triticum aestivum* L. var. Stylet) and HAW were provided by the Waite Research Institute, The University of Adelaide (Glen Osmond, SA, Australia). The HAW was developed through a normal selective breeding process and has ~46% amylose in its total starch content. The nutrient composition of HAW was analysed by the Australian Export Grains Innovation Centre (North Ryde, NSW, Australia) and is detailed in Table 2.1. The dehulled grains of normal bread wheat and HAW were ground to a fine wholegrain flour using a small stone mill (Schnitzer Vario, Offenburg, Germany). The wheat flours were then cooked, in order to reflect how humans typically consume grains. Briefly, each whole wheat flour (HAW, SAW or blend) was mixed with distilled water (1:1.1–1.5 w/v) before being baked at 70 °C and 80% humidity for 1 h, and then cooled overnight at 4 °C to promote retrogradation.

Table 2.1. Nutrient analysis of high amylose wheat used in this study.

Test	%
Moisture	8.80
Total starch	33.30
Amylose	15.46
Amylopectin	17.80
Total dietary fibre	24.90
Insoluble dietary fibre	21.80
Soluble dietary fibre (by difference)	3.10
Resistant starch	4.50
Beta-glucan	1.80
Sugars	3.20
Inulin	7.40

2.3.2 Animal and dietary interventions

All animal procedures were approved by the South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (Project code: SAM294) and were in compliance with the Australian National Health and Medical Research Council's code for the care and use of animals for scientific purposes (8th edition 2013) and South Australia Animal Welfare Act 1985.

Eight-week-old inbred C57BL/6 male and female mice (n = 64) were acquired from the SAHMRI specific pathogen-free and PC2 animal facility breeding colony (Adelaide, SA, Australia) from animals originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). This strain was chosen because it is one of the most widely used inbred strains of laboratory mouse [160], particularly for metabolic studies [161]. Mice of the same sex were group-housed, four to a cage, and maintained within a temperature-controlled environment (22 ± 2 °C) under a 12 h light-dark cycle with *ad libitum* access to food and autoclaved reverse osmosis water throughout the experiment. Mice were randomly assigned to one of four treatment groups (n = 8/group/sex) and received one of four diets containing wheat flour (made in-house) for eight weeks. The four diets replaced some of the carbohydrate components of the AIN-93M formulation with SAW and/or HAW: the SAW65 diet contained 65% SAW (w/w); the HAW35 diet contained 35% HAW (w/w) and 30% SAW (w/w); the HAW50 diet contained 50% HAW (w/w) and 15% SAW (w/w); and the HAW65 diet contained 65% HAW (w/w; Table 2.2). The diets were all vacuum-packed and sent for irradiation at 25 kGy (Steritech, Dandenong, VIC, Australia) prior to storage at -20 °C until use. A sample of each irradiated diet was sent for nutrient analysis by the National Measurement Institute (Port Melbourne, VIC, Australia) and the Australian Export Grains Innovation Centre (North Ryde, NSW, Australia). The nutrient analysis of each diet is presented in Table 2.3.

Table 2.2. Composition of experimental diets.

Ingredient (g)	**SAW65**	**HAW35**	**HAW50**	**HAW65**
Standard amylose wheat	650.00	300.00	150.00	0.00
High amylose wheat	0.00	350.00	500.00	650.00
Maltodextrin	42.97	42.97	42.97	42.97
Sucrose	27.72	27.72	27.72	27.72
Casein	140.00	140.00	140.00	140.00
L-cystine	1.80	1.80	1.80	1.80
Soybean oil	40.00	40.00	40.00	40.00
Cellulose	50.00	50.00	50.00	50.00
Mineral mix, AIN-93M-MX	35.00	35.00	35.00	35.00
Vitamin mix, AIN-93-VX	10.00	10.00	10.00	10.00
Choline bitartrate	2.50	2.50	2.50	2.50
TBHQ, antioxidant	0.008	0.008	0.008	0.008
Total	1000	1000	1000	1000

SAW, standard amylose wheat; HAW, high amylose wheat.

Table 2.3. Nutrient analysis of experimental diets[1].

	SAW65	**HAW35**	**HAW50**	**HAW65**
Energy (kcal/g)	3.76	3.86	3.90	4.05
Moisture (g/100 g)	10.4	8.2	8.1	5.9
Fat (Mojonnier extraction; g/100 g)	5.5	6.4	6.9	7.6
Protein (N × 6.26; g/100 g)	20.9	21.7	21.7	22.5
Ash (g/100 g)	3.1	3.3	3.8	3.5
Carbohydrates (by difference; g/100 g)	60	60	60	61
Calcium (mg/kg)	5000	5000	5300	5300
Iron (mg/kg)	80	72	82	83
Total dietary fibre (%)	13.5	16.2	17.3	18.5
Insoluble dietary fibre (%)	10.4	12.1	12.9	13.6
Soluble dietary fibre (by difference; %)	3.0	4.0	4.5	4.9
Amylopectin (%)	36.2	31.5	29.5	27.5
Amylose (%)	4.5	4.2	3.4	<4.0
Total Starch (%)	40.7	33.6	30.5	27.5
Resistant Starch (%)	1.2	1.6	1.7	1.9
Rapid Digestibility (%)	12.7	11.9	11.6	11.3
Slow Digestibility (%)	32.8	27.5	25.3	23.0

SAW, standard amylose wheat; HAW, high amylose wheat. [1]The fibre, starch and digestibility measures of HAW35 and HAW50 diets were calculated based on the proportions of SAW and HAW in the diet.

2.3.3 Body weight and food intake

The body weight (BW) of each mouse was measured at weekly intervals throughout the study period. Food intake (g/mouse/day) was determined for the first six weeks of the study by calculating the differences between initial and final weight of the food in the cages (including any spilt food in the cages) over a three- or four-day period and dividing by the number of days and number of mice in each cage. Energy intake (kcal/mouse/day) was calculated based on the energy content of the feed, as determined by the nutritional analysis (Table 2.3).

2.3.4 Metabolic measurement

On weeks 7 and 8 of the intervention, individual mice were placed into a metabolic monitoring cage (Promethion, Sable Systems International, Las Vegas, NV, USA). After a 24 h acclimatisation, the energy expenditure, oxygen consumption, carbon dioxide production, water intake and physical activity (ambulatory) of each mouse was recorded over a 48 h period. Respiratory quotient (RQ) was calculated as the ratio of carbon dioxide production over oxygen consumption. Data were analysed and presented separately for the two 12 h cycles [12 h light (sleep) phase and 12 h dark (active) phase] and the average (energy expenditure and RQ) or sum (water intake and ambulatory activity) of these two cycles.

2.3.5 Estrus cycle assessment

After three weeks on their respective diets, the estrus cycle stage of female mice was determined daily, except when mice were housed in metabolic cages. This was achieved by light microscope analysis of cell smears obtained after gently flushing the vaginal opening with saline [162]. The procedure was conducted at the same time of day

(10:00 a.m. to 12:00 p.m.) to reduce variability. The stage of the estrus cycle was identified based on the presence, absence or proportion of leukocytes and cornified epithelial and nucleated epithelial cells [162]. One estrus cycle was considered as the sequence of proestrus, estrus, metestrus and diestrus. Data were analysed and presented as the average number and length of estrus cycles and the number of occurrences of each stage over 24 days from weeks 4 to 7.

2.3.6 Blood and tissue collection

After eight weeks on their respective diets, mice were separated into individual cages and fasted overnight, with coprophagy restricted using a raised wire mesh inserted into the bottom of the mouse cages to allow faecal pellets to drop to the cage floor. On the following morning, mice were anaesthetised by isoflurane inhalation and endpoint BW, abdominal circumference and body length (nose to anus) of each mouse was measured. Blood was collected via the abdominal aorta into EDTA tubes, centrifuged (1000× *g*, 15 min, 4 °C) to separate red blood cells and plasma, and the plasma fraction was snap-frozen in liquid nitrogen and stored at -80 °C for later analysis. Mice were then euthanised by cervical decapitation. Liver, adipose tissues (gonadal, retroperitoneal, mesentery and interscapular depots), kidneys, pancreas, spleen and reproductive organs (testes or uterus and ovaries) were dissected and weighed. All collections were carried out within 1 to 2 h after the beginning of the light phase to minimise possible circadian effects. The majority of female mice were sampled during either the diestrus (72%) or metestrus (19%) stage of the estrus cycle.

2.3.7 Biochemical analyses

Glucose was determined using a blood glucose meter (Accu-Chek, Roche, Basel, Switzerland). Plasma concentrations of total cholesterol (Roche Diagnostics Ltd., Rotkreuz, Switzerland), triglycerides (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and non-esterified fatty acids (Wako Pure Chemical Industries, Osaka, Japan) were measured at the Adelaide Research Assay Facility using a COBAS Integra 400 analytical system (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The minimum detection limit of the assay was 0.1 mmol/L for total cholesterol and triglycerides and 0.05 mmol/L for non-esterified fatty acids. The intra-assay coefficients of variation were 4.3% (total cholesterol), 6.1% (triglycerides) and 11.0% (non-esterified fatty acids). The leptin concentration was determined using the Milliplex xMAP Luminex Assay (Merck Millipore, Temecula, CA, USA). The minimum detection limit for leptin was 0.04 ng/mL and the intra-assay coefficient of variation was 8.3%.

2.3.8 Statistical analysis

Data are presented as mean ± SEM. Data analysis was performed separately on each sex. Two-way ANOVA with Tukey's post hoc test was used where appropriate to determine significant differences in BW, food intake and energy intake over time. Differences between groups in other measures were determined using one-way ANOVA with a Tukey's post hoc test where appropriate. All statistical analyses were carried out using GraphPad Prism software (version 7.04, San Diego, CA, USA). Significance was considered at $p < 0.05$.

2.4 Results

2.4.1 Food intake and energy intake

In males, there were no differences between diet groups in either food intake or energy intake across the first six weeks of the feeding period (Figure 2.1Ai, Bi).

In females, however, the HAW35 group had higher food intake ($p = 0.02$; Figure 2.1Aii) and energy intake ($p < 0.01$; Figure 2.1Bii), across the six-week feeding period, compared to the SAW65 group. The energy intake of the HAW65 females was also higher than the SAW65 group ($p = 0.02$) during this time, but food intake was not different between these two groups.

2.4.2 Energy expenditure, RQ, water intake and ambulatory activity

Overall, both males and females exhibited higher energy expenditure, RQ, water intake and ambulatory activity in the dark phase compared to the light phase. In males, there were no differences in energy expenditure (Figure 2.2A), RQ (Figure 2.2B), water intake (Figure 2.2C) or ambulatory activity (Figure 2.2D) between diet groups.

In females, there were no differences in energy expenditure (Figure 2.2E) or water intake (Figure 2.2G) between diet groups. However, females consuming the HAW35 diet exhibited a higher RQ compared to the SAW65 group ($p = 0.03$; Figure 2.2F) and higher ambulatory activity compared to the HAW65 group ($p = 0.02$; Figure 2.2H), during the light phase only.

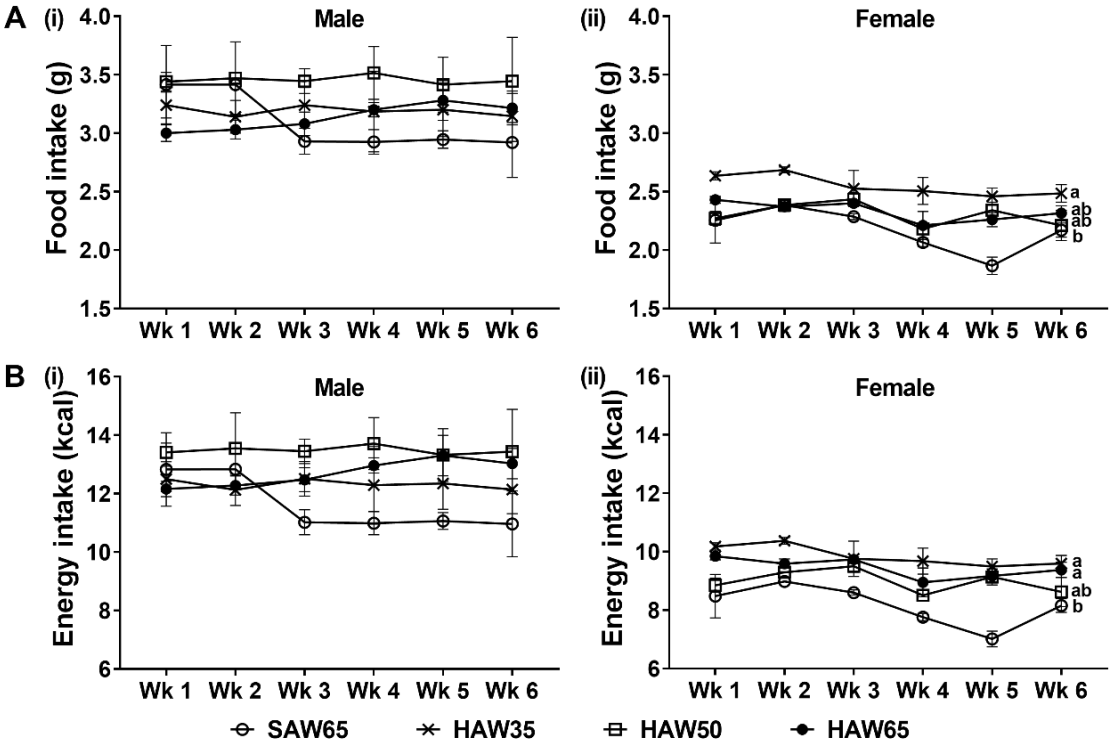

Figure 2.1. Weekly changes of (A) food intake and (B) energy intake in (i) male and (ii) female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) for six weeks. Data are means ± SEM (n = 8 mice/group). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by two-way ANOVA and Tukey's post hoc tests.

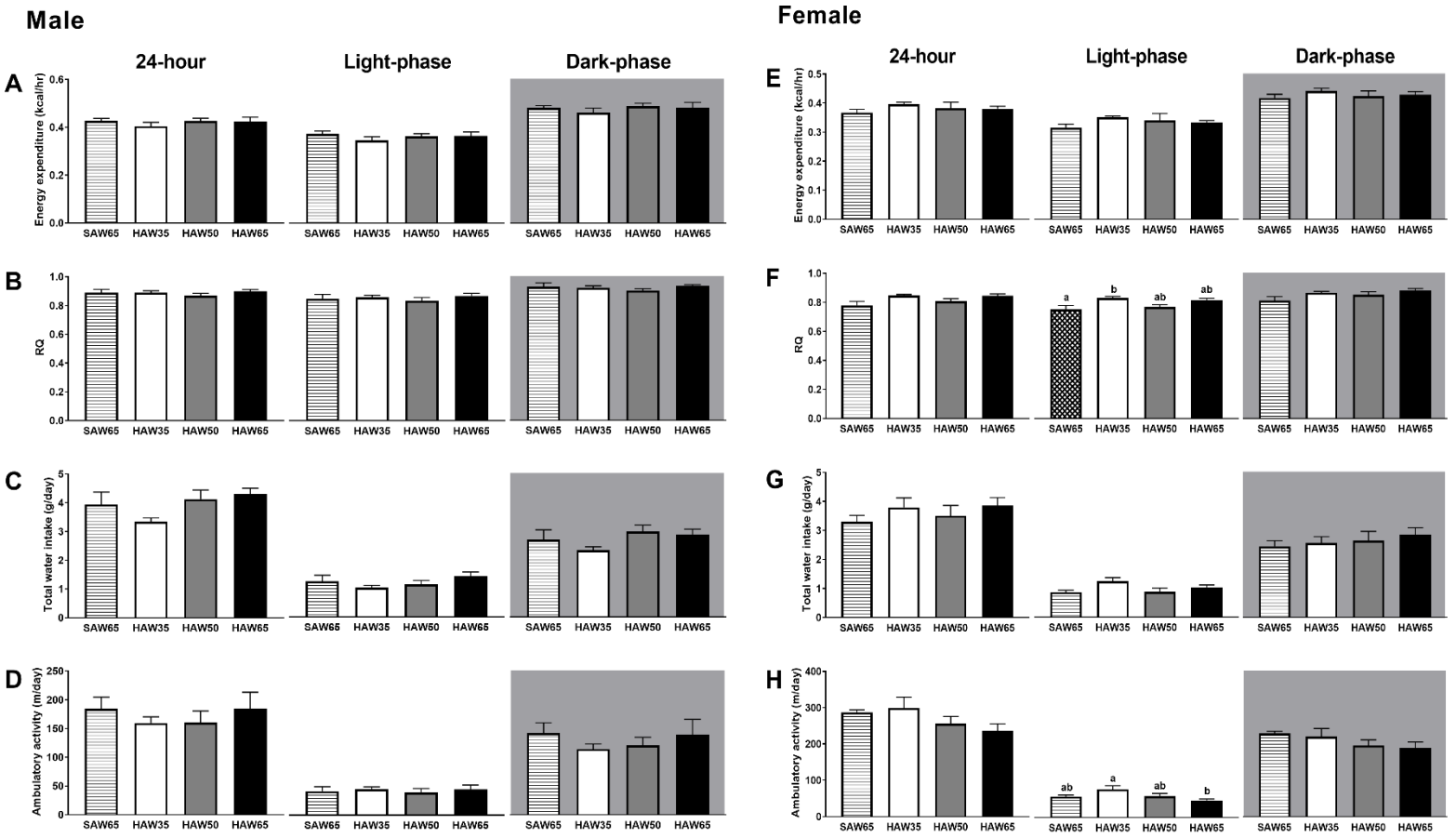

Figure 2.2. Metabolic parameters of mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) in weeks 7 and 8. Twenty-four-hour, light- and dark-phase average energy expenditure (A: male; E: female); average RQ (B: male; F: female); total water intake (C: male; G: female); and total ambulatory activity of (D) male and (H) female mice are shown. Data are means ± SEM (n = 7 or 8 mice/group). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

2.4.3 Body weight, abdominal circumference, nose-anus length, relative fat mass and relative organ weights

Table 2.4 shows BW, abdominal circumference, nose-anus length, relative fat mass and relative organ weights of male and female mice after the eight-week feeding period. In males, mice consuming a diet containing any level of HAW were heavier than those that consumed the SAW65 diet during ($p < 0.05$; Figure 2.3A) and at the end ($p < 0.05$) of the eight-week feeding period. The BW gain across the eight-week feeding period was also higher in all groups of male mice consuming any level of HAW compared to the SAW65 males ($p < 0.05$). Males consuming the diet which contained the highest level of HAW (HAW65) had a lower abdominal circumference compared to the HAW35 males after the eight weeks ($p = 0.04$), but the nose-anus length was not different between groups. Relative total fat mass (24.48 ± 2.50 vs. 40.78 ± 3.00 mg/g BW; $p = 0.01$) and relative gonadal fat mass (9.93 ± 1.35 vs. 18.51 ± 1.59 mg/g BW; $p < 0.01$) were lower in the HAW65 males compared to the HAW35 males. In addition, the HAW35 males had a higher relative gonadal fat mass (18.51 ± 1.59 vs. 11.10 ± 2.00 mg/g BW; $p = 0.02$) compared to the SAW65 males. There were no differences in the relative weights of other fat mass depots (retroperitoneal, mesentery and interscapular) between diet groups. Similarly, no differences were observed in the relative weights of the kidneys, liver, pancreas or spleen between groups (Table 2.4).

In females, there were no differences between diet groups in BW either during (Figure 2.3B) or at the end (Table 2.4) of the eight-week feeding period. However, the amount of BW gained across the eight-week feeding period was higher in the HAW35 females compared to the SAW65 group ($p = 0.03$). There were no differences between diet groups in abdominal circumference, nose-anus length or relative weights of total fat, individual fat depots, kidneys, liver, pancreas or spleen at the end of the feeding period.

Table 2.4. Body weight, abdominal circumference, nose-anus length, relative fat mass and relative organ weights of male and female mice at the end of the eight-week feeding period.

	SAW65	HAW35	HAW50	HAW65
Male				
End point BW[1] (g)	22.64 ± 0.4[a]	25.96 ± 0.26[b]	25.01 ± 0.34[b]	24.56 ± 0.43[b]
Weight gain[2] (g)	1.89 ± 0.21[a]	3.71 ± 0.30[b]	3.71 ± 0.29[b]	3.48 ± 0.33[b]
Abdominal circumference (cm)	7.04 ± 0.10[ab]	7.28 ± 0.11[a]	6.79 ± 0.14[ab]	6.76 ± 0.16[b]
Nose–anus length (cm)	9.16 ± 0.06	9.24 ± 0.10	9.10 ± 0.04	9.08 ± 0.06
Total fat mass (mg/g BW)	28.58 ± 4.95[ab]	40.78 ± 3.00[a]	32.72 ± 2.84[ab]	24.48 ± 2.50[b]
Gonadal (mg/g BW)	11.10 ± 2.00[a]	18.51 ± 1.59[b]	14.42 ± 1.55[ab]	9.93 ± 1.35[a]
Retroperitoneal (mg/g BW)	4.15 ± 0.74	6.55 ± 1.19	4.59 ± 0.94	3.25 ± 0.57
Mesentery (mg/g BW)	8.75 ± 1.64	10.29 ± 0.55	8.02 ± 0.55	7.42 ± 0.58
Interscapular (mg/g BW)	4.58 ± 0.79	5.43 ± 0.46	5.69 ± 0.43	3.89 ± 0.47
Kidneys (mg/g BW)	11.98 ± 0.22	12.81 ± 0.47	12.49 ± 0.22	13.09 ± 0.44
Liver (mg/g BW)	40.40 ± 0.54	38.74 ± 1.80	43.39 ± 0.92	42.42 ± 2.06
Pancreas (mg/g BW)	4.41 ± 0.29	4.53 ± 0.16	3.92 ± 0.45	3.92 ± 0.17
Spleen (mg/g BW)	2.36 ± 0.11	2.60 ± 0.13	2.40 ± 0.10	2.44 ± 0.14
Female				
End point BW[1] (g)	17.04 ± 0.20	18.21 ± 0.38	18.05 ± 0.40	18.01 ± 0.23
Weight gain[2] (g)	1.37 ± 0.43[a]	3.23 ± 0.31[b]	3.00 ± 0.59[ab]	2.56 ± 0.30[ab]
Abdominal circumference (cm)	6.56 ± 0.06	6.59 ± 0.10	6.63 ± 0.06	6.45 ± 0.08
Nose–anus length (cm)	8.55 ± 0.06	8.80 ± 0.06	8.70 ± 0.08	8.60 ± 0.09
Total fat mass (mg/g BW)	27.58 ± 0.99	23.89 ± 2.64	23.39 ± 1.84	23.33 ± 1.04
Gonadal (mg/g BW)	10.82 ± 0.74	8.43 ± 1.22	9.02 ± 1.05	8.52 ± 0.64
Retroperitoneal (mg/g BW)	2.43 ± 0.24	2.40 ± 0.38	2.85 ± 0.32	2.15 ± 0.16
Mesentery (mg/g BW)	8.43 ± 1.28	7.38 ± 0.82	6.89 ± 0.44	8.40 ± 0.52
Interscapular (mg/g BW)	5.89 ± 0.43	5.68 ± 0.63	4.63 ± 0.52	4.26 ± 0.32
Kidneys (mg/g BW)	13.23 ± 0.30	14.26 ± 0.38	13.40 ± 0.48	13.15 ± 0.96
Liver (mg/g BW)	42.61 ± 0.49	40.64 ± 2.04	43.70 ± 1.02	45.08 ± 0.93
Pancreas (mg/g BW)	4.62 ± 0.30	4.31 ± 0.34	4.22 ± 0.42	4.53 ± 0.28
Spleen (mg/g BW)	3.09 ± 0.16	3.93 ± 0.58	3.22 ± 0.39	3.06 ± 0.15

Mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65). [1]Measured before blood and tissue collection at fasted state. [2]Difference between the body weights of week 8 and pre-intervention values. Data are means ± SEM (n = 8 mice/group). Values with different superscripts in a row indicate significant differences between diet groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

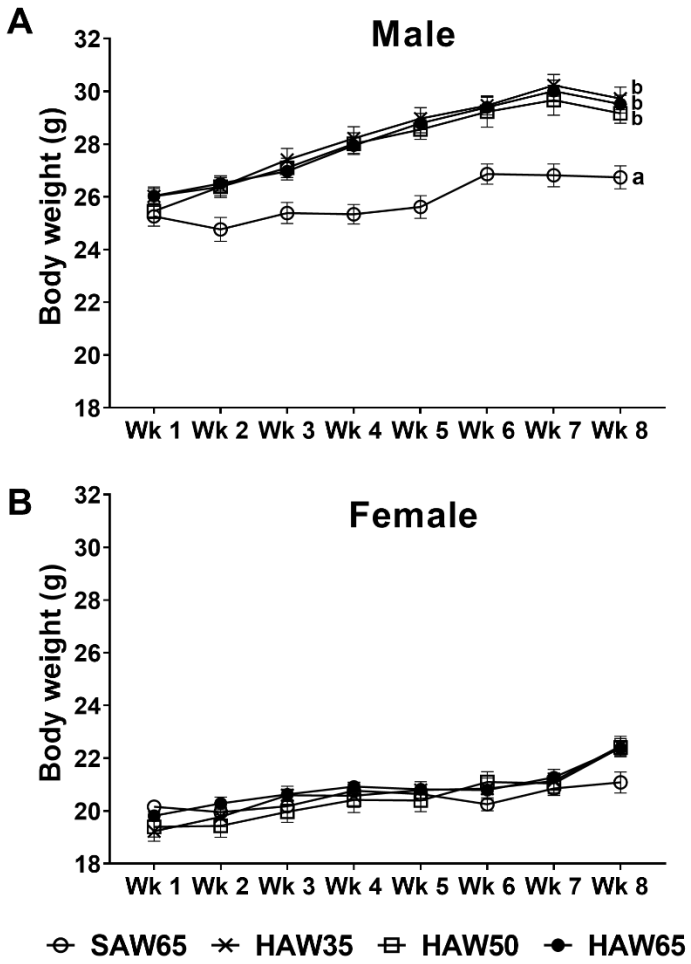

Figure 2.3. Weekly changes of body weight in (A) male and (B) female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) for eight weeks. Data are means ± SEM (n = 8 mice/group). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by two-way ANOVA and Tukey's post hoc tests.

2.4.4 Plasma hormone and metabolite concentrations

In males, there were no differences between diet groups in the fasting blood glucose concentrations or fasting plasma concentrations of total cholesterol, triglycerides or non-esterified fatty acids at the end of the eight-week feeding period (Table 2.5). However, the HAW35 males (0.48 ± 0.12 ng/mL) had higher fasting leptin concentrations compared to both HAW65 males (0.09 ± 0.03 ng/mL; $p < 0.01$) and SAW65 males (0.16 ± 0.06 ng/mL; $p = 0.02$).

In females, no differences were observed between diet groups in the fasting blood glucose concentrations or fasting plasma concentrations of total cholesterol, triglycerides, non-esterified fatty acids or leptin at the end of the experiment (Table 2.5).

Table 2.5. Biochemical analyses of blood/plasma from male and female mice at the end of the eight-week feeding period.

Parameter	SAW65	HAW35	HAW50	HAW65
Male				
Fasting blood glucose (mmol/L)	7.59 ± 0.48	8.15 ± 0.66	8.14 ± 0.69	8.20 ± 0.63
Total cholesterol (mmol/L)	2.54 ± 0.07	2.66 ± 0.23	2.50 ± 0.06	2.35 ± 0.21
Triglycerides (mmol/L)	0.76 ± 0.04	0.85 ± 0.06	0.94 ± 0.06	0.87 ± 0.06
Non-esterified fatty acids (mmol/L)	0.94 ± 0.08	0.84 ± 0.04	0.91 ± 0.08	1.00 ± 0.07
Leptin (ng/mL)	0.16 ± 0.06^{a}	0.48 ± 0.12^{b}	0.21 ± 0.04^{ab}	0.09 ± 0.03^{a}
Female				
Fasting blood glucose	6.58 ± 0.45	7.43 ± 0.47	7.30 ± 0.28	7.11 ± 0.18
Total cholesterol (mmol/L)	1.70 ± 0.03	1.71 ± 0.11	1.61 ± 0.10	1.72 ± 0.05
Triglycerides (mmol/L)	0.74 ± 0.03	0.70 ± 0.03	0.70 ± 0.03	0.71 ± 0.04
Non-esterified fatty acids (mmol/L)	0.81 ± 0.03	0.82 ± 0.06	0.88 ± 0.02	0.80 ± 0.05
Leptin (ng/mL)	0.15 ± 0.03	0.10 ± 0.02	0.14 ± 0.04	0.18 ± 0.03

Mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65). Data are means ± SEM (n = 8 mice/group). Values with different superscripts in a row indicate significant differences between diet groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

2.4.5 Markers of reproductive function

In males, testes weights at the eight-week time point were higher in both HAW35 ($p < 0.01$) and HAW65 ($p = 0.02$) groups and also tended ($p = 0.07$) to be higher in the HAW50 group compared to the SAW65 group (Figure 2.4A).

In females, there were no differences between diet groups in the weights of the uterus (Figure 2.4B) or ovaries (Figure 2.4C) at eight weeks. There were no differences in the average number (Figure 2.5A) or length (Figure 2.5B) of the estrus cycles between diet groups. However, females that were fed on diets containing any level of HAW spent a lower proportion of the assessment period in diestrus ($p < 0.05$; Figure 2.5F) and tended to spend more time in metestrus compared to the SAW65 group (Figure 2.5E). There

were no differences between diet groups in the proportion of time mice spent in either proestrus (Figure 2.5C) or estrus (Figure 2.5D).

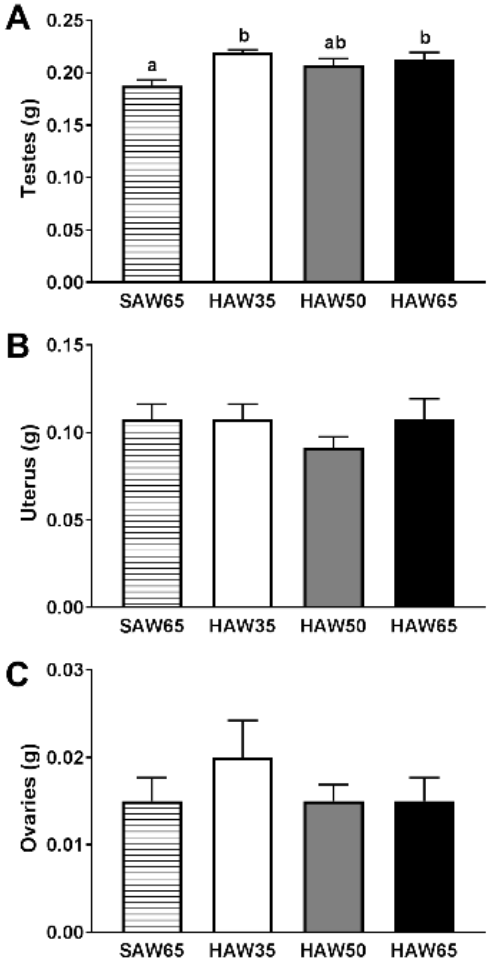

Figure 2.4. Weights of (A) testes or (B) uterus and (C) ovaries in male and female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) at the end of the eight-week feeding period. Data are means ± SEM (n = 8 mice/group). Values with different superscripts indicate significant differences between diet groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

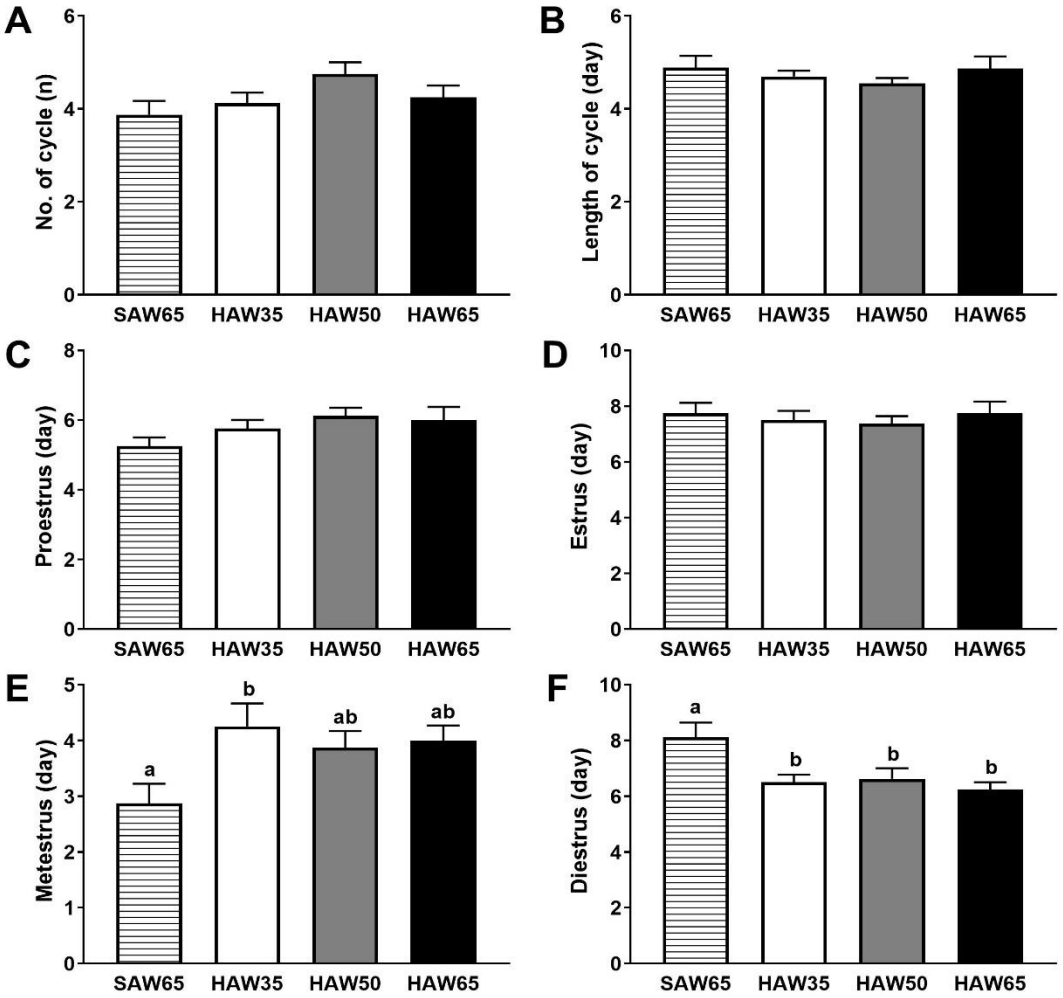

Figure 2.5. Average (A) number and (B) length in days of estrus cycles and a total number of days in (C) proestrus, (D) estrus, (E) metestrus and (F) diestrus in female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) over 24 days from weeks 4 to 7. Data are means ± SEM (n = 8 mice/group). Values with different superscripts indicate significant differences between diet groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

2.5 Discussion

The results of the current study suggest that replacing standard wheat with levels of HAW between 35% and 65% of total diet weight did not produce any substantive effects on fasting blood glucose or plasma lipid profile in either male or female lean mice. There were, however, a number of effects of increased HAW consumption on measures of nutrient metabolism (RQ), growth and body fat mass, which appeared to be dependent on both the sex of the animals and the level of HAW in the diet. We also observed

interesting effects of the HAW diets on reproductive organ weights in males and estrus cyclicity in females, which raises the possibility of effects on the reproductive function which warrant further investigation.

The higher BW observed at the end of the eight-week feeding period in male mice fed on any of the HAW diets is consistent with a previous study in male Wistar rats fed a diet containing 20% HAW [163]. In both the previous [163] and current studies, the increase in BW occurred in the absence of any differences in feed intake, suggesting that it was not a result of any appetite-promoting effects of the HAW diet. Moreover, in the previous study [163], the higher BW of rats fed a 20% HAW diet was attributed to an increase in muscle and bone mineral mass. It is possible that the increased BW in male mice consuming the HAW diets in this study was due to increased accumulation of muscle, and potentially bone mass, however, further studies are required to confirm this directly. An intriguing finding of this study was that, unlike BW, the effects of the HAW diets on fat mass appeared to be dependent on the level of HAW included in the diet, and that fat mass relative to BW was higher in the mice consuming the lowest level of HAW (HAW35) compared to those consuming the highest level (65%). This effect was particularly pronounced for abdominal fat pads, and raises the possibility that higher levels of HAW consumption are required to reduce abdominal adiposity. This is important, given that increased abdominal adiposity is a major risk factor for cardiometabolic disease and mortality [164]. If the positive impact of HAW on fat deposition in males is translated to humans, this has the potential to reduce the risk of cardiometabolic disease, particularly in men who have a greater tendency to accumulate fat around the abdomen than women.

In line with previous studies investigating the effects of a range of dietary interventions [165-167], we observed that the male and female mice exhibited quite

different responses to the consumption of increased amounts of HAW in the diet in relation to effects on food intake, RQ, BW and fat mass. In contrast to the findings of male mice, female mice consuming the lowest level of HAW diet (HAW35) exhibited higher intakes of food and energy compared to those consuming the SAW diet, and this was also associated with increased BW gain. However, fat mass as a proportion of BW tended to be lower in female mice consuming any level of HAW diet compared to the SAW65 group, suggesting that the increase in BW gain was not due to excess fat accumulation. This may suggest an effect on muscle and/or bone mass of HAW.

The higher intakes of food and energy in HAW35 female group were correlated with an increase in RQ during the light phase compared to the SAW65 group, suggesting preferential utilisation of carbohydrate as a fuel source during this period. The increased RQ during the light phase may be due to either an increase in food intake during the light phase or the slower transition and digestion of the HAW diet and therefore delayed provision of carbohydrates to the light phase. The method used for food intake measurement in this study prevented analysis of changes in food intake patterns and, therefore, further studies are required to determine the exact reason for the increased RQ during the light phase, and the impact of this on energy utilization and body fat mass in the longer term.

If the observed effect on RQ in females is translated to humans, it would be expected to be associated with greater utilisation of carbohydrate as an energy source. This, in turn, would provide a more consistent glucose supply, but may also limit the utilisation of endogenous fat stores, which could potentially lead to increase in body fat in the long term when consuming a high-fat diet. The differences in response to diets between males and females were likely due to a number of factors, including sex-specific differences in metabolic control, body fat distribution, sex hormones and taste preferences

[168, 169]. Nonetheless, the current study does clearly demonstrate that the response to the same level of HAW in the diet has different effects on the measured parameters in males and females, and that it is not appropriate to extrapolate results obtained in male mice to their female counterparts.

Dietary fibre plays an important role in maintaining good health and is associated with improvements in glycaemic response and plasma lipid profile [170, 171]. However, consistent with previous findings [163], the HAW diets had no effect on fasting blood glucose concentrations or plasma lipid profiles despite the higher dietary fibre content in the HAW diets compared to the SAW diet. One possible explanation is that both SAW and HAW contain similar types of soluble fibres including arabinoxylans, β-glucans and inulin-type fructans, which have previously been shown to lower plasma cholesterol and improve glucose and lipid metabolism [172, 173]. It is important to note that the mice in this study were lean and metabolically healthy, and this may have limited the potential to produce further improvements in glucose/lipid control. It is possible that more pronounced differences would be observed if mice were metabolically compromised (e.g., diabetic or hyperlipidemic); however, this requires further investigation.

Reproductive health is increasingly recognised to be tightly linked to metabolic health [159], and to be strongly influenced by diet and lifestyle factors [174]. This prompted us to investigate the potential effects of the HAW diet on markers of reproductive function. Clinical and experimental animal studies indicate that testicular volume is positively associated with sperm production and sperm motility [175-178], both markers of male reproductive health, and clinical studies show that this is also related to circulating sex hormone concentrations [177, 178]. This suggests that the higher testicular weights in mice consuming diets containing HAW compared to those consuming the SAW65 diet may be associated with improved sperm characteristics and sex hormone

concentrations. Female mice consuming any level of the HAW diet showed an altered pattern of vaginal cytology parameters, which suggested a shift in the estrus cycle towards a longer period in metestrus and a shorter period in diestrus compared to those consuming the SAW65 diet. Prolonged diestrus is a commonly observed pattern in rodent models of disordered fertility [179], raising the possibility that the HAW diet had a positive impact on reproductive health in females. The changes observed may also be indicative of effects on sex hormone concentrations [180] or immune determinants of reproductive function [181], which were not measured in the current study. Immune cell populations residing in female reproductive tissues change across the estrus cycle in response to ovarian hormone levels, with the largest populations present in tissues and sequestered into the vaginal epithelium at estrus and metestrus stages when estrogen levels are high [182, 183]. Consequently, the longer metestrus phase may reflect altered immune cell populations or function in the reproductive tract, and potentially these are linked with receptivity to pregnancy. Further studies that include more direct and robust measures of reproductive function in males and females will be important to enable us to draw definitive conclusions as to the potential benefits of HAW diets on reproductive performance. The effects of HAW consumption on reproductive parameters observed in this study also raise the possibility that replacing SAW with HAW in human staple foods may provide reproductive health benefits, although it is clear that further studies are required to address this directly.

2.6 Conclusions

This study demonstrates that replacement of SAW flour with increasing levels of HAW in diets of healthy, lean male and female mice was associated with sex-specific effects on growth, fat mass and nutrient metabolism, but in the absence of any effects on

fasting glucose or blood lipid concentrations. The level of HAW also appeared to be important, with different effects on fat deposition, nutrient metabolism and growth observed between the diets containing the lowest and highest levels of HAW. The reason for this is not clear, but these results suggest that considering the level of HAW included in experimental diets is important when comparing studies evaluating the effects of this wheat type. Further research is required to determine the metabolic effects of a HAW diet in metabolically compromised models. The differences in reproductive parameters seen in HAW- compared to SAW-fed male and female mice also require further investigation.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Chapter 3

A High Amylose Wheat Diet Modulates Gastrointestinal Health Parameters and Gut Microbiota in Male and Female Mice

This chapter was submitted for publication as:

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Name of Principal Author (Candidate)	See Meng Lim		
Contribution to the Paper	Performed the experiments, collected, analysed and interpreted the data and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/6/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	16/6/2020

3.1 Abstract

High amylose wheat (HAW) contains more resistant starch than standard amylose wheat (SAW) and may have beneficial effects on gastrointestinal health. However, it is currently unclear whether these effects differ according to the level of HAW included in the diet or between males and females. This study aimed to determine the effects on gastrointestinal health parameters and gut microbiota composition of replacing SAW with different levels of HAW, in male and female mice. Male and female C57BL/6 mice (n=8/group/sex) were fed SAW65 (65% SAW w/w; control), HAW35 (35% HAW w/w), HAW50 (50% HAW w/w) or HAW65 (65% HAW w/w) diet for eight weeks. Female, but not male, mice consuming any amount of HAW exhibited accelerated gastric emptying compared to the SAW65 group ($p < 0.05$). In both sexes, relative colon weights were higher in the HAW65 group compared to the SAW65 group, and in females, the relative weights of the small intestine and caecum were also higher in the HAW65 group. In females only, colonic expression of *Pyy* and *Ocln* mRNAs were higher in the HAW65 group compared to the HAW35 and HAW50 groups. In both sexes, mice consuming higher amounts of HAW (HAW50 or HAW65) had increased faecal bacterial load ($p < 0.01$) and relative abundance of Bacteroidetes phylum and reduced relative abundance of Firmicutes ($p < 0.0001$) compared to those consuming the SAW65 diet. Overall, these data are consistent with a beneficial impact of HAW on gastrointestinal health and indicate dose-dependent and sex-specific effects of HAW consumption.

Keywords: high amylose wheat; resistant starch; gastrointestinal health; gut microbiota

3.2 Introduction

Wheat is one of the most widely consumed cereal grains in the world, with increasing global demand as a result of industrialisation and westernisation [21, 184]. Wheat flour has unique properties and can be processed into a wide range of food products, including bread, noodles, biscuits and breakfast cereals, many of which are dietary staples. Consumption of these wheat-based products makes a major contribution to daily energy intake in many populations worldwide [21]. However, due to cultural and textural preferences, wheat is most commonly consumed in a highly processed and refined form, where the bran and germ fractions have been removed. While refining the grain does not affect the starch content, the fibre content is significantly lower than in whole grains, since this is largely found in the bran layer of the wheat kernel [185].

The lower dietary fibre content of refined grains has significant implications for the health benefits of these products. This is particularly relevant for gastrointestinal health, since dietary fibre plays a critical role in maintaining optimal functioning of the gastrointestinal tract; adequate fibre intake increases faecal weight, promotes regular laxation by accelerating the passage of food through the digestive tract, and reduces the risk of gastrointestinal disorders [186]. Furthermore, there is increasing evidence that the gut microbiota plays an important role in maintaining gastrointestinal health, with alterations to the gut microbiota composition linked with several gastrointestinal diseases, including irritable bowel syndrome and inflammatory bowel disease [187]. Fermentation of resistant starch, defined as a subgroup of dietary fibre that resists digestion in the upper gastrointestinal tract and reaches the colon intact, by colonic bacteria results in the production of metabolites, including short-chain fatty acids, which have been shown to act as signalling molecules for gut hormone release and gastrointestinal function [188]. Despite this, dietary fibre intake in many populations worldwide remains well-below

recommended levels [189, 190]. In Australia, ~60% of children and >70% of adults in the 2011-2012 National Nutrition and Physical Activity Survey did not meet the recommended adequate daily intake of dietary fibre (children: 14-28g; women: 25g and men: 30g) [189]. Therefore, there is a need for new approaches to sustainably increase fibre intake at the population level. Increasing the fibre content of staple foods, such as wheat-based products, offers an attractive solution.

High amylose wheat (HAW) is a novel wheat variety which has up to three times more amylose compared to standard wheat varieties [59]. This high amylose content is associated with higher levels of dietary fibre, mainly in the form of resistant starch, with HAW varieties having a resistant starch content as high as 11.2% compared to negligible amounts (<1%) in standard wheat [62]. The nutritional attributes of HAW underpin the hypothesis that consuming greater amounts of this wheat variety would be associated with improvements in gastrointestinal function [60]. Previous studies conducted in male Sprague-Dawley rats have reported that consuming a diet containing ~50% HAW (w/w) for 2-11 weeks improved indicators of gastrointestinal health in rats, as assessed by an increase in the weight of the large bowel, elevated concentrations of short-chain fatty acids in the digesta and reduced caecal pH [65, 82].

In spite of these encouraging findings, the effects of increased dietary intake of HAW on functional measures of gastrointestinal health and gut microbiota composition, and how these are influenced by the level of HAW in the diet, remain unknown. In addition, whether the effects differ between males and females is also unclear, since to our knowledge all previous studies using animal models to investigate the physiological effects of a HAW diet have been conducted exclusively in males [65, 82, 83]. Therefore, the aim of this study was to determine the effect of replacing standard amylose wheat

(SAW) flour in the diet with increasing levels of HAW on gastrointestinal health parameters and gut microbiota composition in male and female mice.

3.3 Materials and Methods

3.3.1 Animals and dietary interventions

Details of the study design and dietary intervention have been described previously [148]. Briefly, eight-week-old C57BL/6 male and female mice (n= 64) were maintained in a temperature-controlled environment (22 ± 2°C) under a 12-hr light-dark cycle at the South Australian Health and Medical Research Institute (SAHMRI) specific pathogen-free and PC2 animal facility (Adelaide, SA, Australia) with *ad libitum* access to food and purified water. Mice of the same sex were housed in groups of four in ventilated cages (GM500 Mouse IVC Green Line, Tecniplast, Buguggiate, VA, Italy), which contained dust-free laboratory bedding and enrichment (nesting material and rodent tunnels).

The animals were randomly allocated to one of four dietary groups (n= 8/group/sex): the SAW65 group fed a diet containing 65% SAW (w/w), the HAW35 group fed a diet containing 35% HAW (w/w) and 30% SAW (w/w), the HAW50 group fed a diet containing 50% HAW (w/w) and 15% SAW (w/w), and the HAW65 group fed a diet containing 65% HAW (w/w). Table 3.1 shows the composition of each experimental diet. Detailed information on the production of the experimental diets has been published previously [148]. Briefly, all diets were made in-house to the AIN-93M formulation with the exception that a portion of the carbohydrate component replaced by SAW and/or HAW in the proportions indicated above. In order to mimic how humans typically consume grains, the wheat flour was first cooked for an hour at 70°C and 80% humidity to promote gelatinisation, followed by storage at 4°C overnight to promote

retrogradation for resistant starch formation. The HAW used in this study contained ~46% of amylose in its total starch content and was developed through a normal selective breeding process [148]. All animal procedures were approved by the SAHMRI Animal Ethics Committee (Project code: SAM294) and were in compliance with the Australian National Health and Medical Research Council's code for the care and use of animals for scientific purposes (8th edition 2013) and South Australia Animal Welfare Act 1985.

Table 3.1. Modified AIN-93M diets with different levels of wheat flour.

Ingredient (g)	SAW65	HAW35	HAW50	HAW65
Standard amylose wheat	650.00	300.00	150.00	0.00
High amylose wheat	0.00	350.00	500.00	650.00
Maltodextrin	42.97	42.97	42.97	42.97
Sucrose	27.72	27.72	27.72	27.72
Casein	140.00	140.00	140.00	140.00
L-cystine	1.80	1.80	1.80	1.80
Soybean oil	40.00	40.00	40.00	40.00
Cellulose	50.00	50.00	50.00	50.00
Mineral mix, AIN-93M-MX	35.00	35.00	35.00	35.00
Vitamin mix, AIN-93-VX	10.00	10.00	10.00	10.00
Choline bitartrate	2.50	2.50	2.50	2.50
TBHQ, antioxidant	0.008	0.008	0.008	0.008

SAW, Standard amylose wheat; HAW, High amylose wheat.

3.3.2 Assessment of gastric emptying rate

After seven weeks of consuming the experimental diets, all mice were assessed for gastric emptying via a gastric emptying breath testing, using previously described methods [191, 192]. Briefly, mice were fasted overnight in individual cages with wire mesh at the base to restrict coprophagy. On the following morning, mice were placed in individual air-tight containers with the lids of the containers removed between sampling to supply oxygen. After a baseline breath sample collection, the mice were given 0.1 g of baked egg yolk containing 1 μL/g of ^{13}C-labelled octanoic acid (99% enrichment, Cambridge Isotope Laboratories, Andover, MA, USA), which consumed within 1 min.

Breath samples were then collected at 5 min intervals for the first 30 min after food consumption followed by every 15 min until 150 min. After this, the mice were returned to their home cages with free access to water and their respective diets. The $^{13}CO_2$ content of breath samples was analysed with an isotope ratio mass spectrometer (ABCA 20/20 Europa Scientific, Crewe, Cheshire, UK). The measured values were presented as $\Delta^{13}CO_2$ (‰) and area under the curve (AUC; ‰ x min) as described by Uchida and colleagues [193].

3.3.3 Post-mortem and tissue collection

After eight weeks on their respective experimental diets, mice were individually housed in cages with wire-mesh bases and fasted overnight. On the following morning, mice were anaesthetised using isoflurane inhalation (5% induction, 2-3% maintenance) and blood samples were collected from the abdominal aorta. Blood samples were immediately centrifuged (1,000 x *g*, 15 min at 4°C) and plasma was snap-frozen in liquid nitrogen (N_2) and stored at -80°C until analysis. Following blood collection, mice were euthanised by cervical dislocation. Gastrointestinal tract organs, including the stomach, small intestine, caecum and colon were collected, weighed, snap-frozen in liquid N_2 and stored at -80 °C until analysis. Caecal content was also collected, snap-frozen in liquid N_2 and stored at -80 °C until analysis. All collections were carried out within 1-2 hours from the beginning of the light phase, and the majority of female mice were sampled during either the diestrus (72%) or metestrus phase (19%) of the estrus cycle.

3.3.4 Plasma hormone concentrations

The plasma gastric inhibitory peptide (GIP) concentrations were determined, in duplicate, using the Milliplex xMAP Luminex Assay according to the manufacturer's

instructions (Merck Millipore, Temecula, CA, USA). The minimum detection limit for GIP was 0.001 ng/mL and the intra-assay coefficient of variation was 8.43%.

3.3.5 Measurement of caecal content pH

Thawed caecal contents (0.3 g) were homogenised in 0.3 mL of ultrapure water (Milli-Q, Millipore, Bedford, MA, USA) by vortexing, and the pH was measured, in duplicate, using a pH meter (Eutech Instruments pH 510, Singapore) that had been calibrated at room temperature with buffer solutions at pH 4 and pH 7.

3.3.6 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 30 mg of caecum and colon tissues, respectively, using a RNeasy® Mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The RNA quantity and quality were measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). RNA samples with a A260/A280 ratio of 1.9 to 2.1 were considered of acceptable quality. A total of 25 ng/µL of RNA was reverse transcribed and expression of target and housekeeper genes was quantified, in duplicate, using an EXPRESS One-Step SuperScript® qRT-PCR Universal Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and pre-designed TaqMan gene expression probes (Applied Biosystems, Thermo Fisher Scientific, Pleasanton, CA, USA) on the QuantStudio™ 7 Flex real-time PCR System (Applied Biosystems, Thermo Fisher Scientific). The following cycling conditions were used: 50°C for 15 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 15 min. TaqMan probes were used to detect mRNAs encoding peptide YY (*Pyy*; Mm00520715_m1), proglucagon (*Gcg*; Mm01269055_m1), occludin (*Ocln*; Mm00500912_m1), mucin 2 (*Muc2*; Mm01276696_m1), hypoxanthine-guanine phosphoribosyltransferase (*Hprt*;

Mm01545399_m1), beta-2 microglobulin (*B2m*; Mm00437762_m1) and peptidylprolyl isomerase A (*Ppia*; Mm02342429_g1). The NormFinder (https://moma.dk/normfinder-software) stability value was calculated and indicated that *Hprt* and *B2m* provided appropriate stable housekeeper genes for both caecum (0.14) and colon (0.07) samples. Negative controls, containing all reagents but with water instead of RNA, were included for each probe on each plate to confirm the absence of contamination. Data were analysed with a QuantStudio™ real-time PCR System Software v.1.3 (Applied Biosystems, Thermo Fisher Scientific). Cycle threshold (Ct) values of the target genes were normalized to the housekeeper genes and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [194].

3.3.7 Faecal collection, DNA extraction and bacterial quantification

Fresh faecal pellets were collected between 10-11 a.m. with sterile toothpicks and placed into sterile 1.5 mL Eppendorf tubes and then stored at -80°C. At the time of DNA extraction, faecal pellets were weighed, resuspended in 300 µL of phosphate-buffered saline (pH 7.2) by vortexing and pelleted by centrifugation at 10,000 x *g* for 10 min. The supernatant was stored at -80°C, and the pellet underwent DNA extraction using the QIAGEN PowerLyzer PowerSoil kit (QIAGEN, Hilden, Germany), as described previously [195]. Total bacterial load was quantified based on the 16S rRNA abundance by using a SYBR® PCR assay, as described previously [196]. Briefly, each reaction comprised of 1X SYBR® Green, 0.2 µM of each forward and reverse primer, and 1 µL of DNA in a 35 µL total reaction volume. Each reaction was aliquoted into three technical replicates with a volume of 10 µl. The number of 16S rRNA gene copies was normalised against the faecal weight (mg) of each sample.

3.3.8 16S rRNA gene amplicon sequencing and bioinformatics analysis

Barcoded libraries of the bacterial 16S rRNA gene V4 hypervariable region were prepared by using faecal DNA extracts and sequencing was performed on an Illumina MiSeq platform at the David R Gunn Genomics Facility, SAHMRI (Adelaide, SA, Australia). Briefly, amplicons were generated using the modified universal bacterial primer pairs 515F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCC AGCMGCCGCGGTAA-3′) and 806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGGACTACHVGGGTWTCTAAT-3′) with Illumina adapter overhang sequences (indicated by underline), according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol with minor modifications, as described previously [195]. Raw sequence files are publicly available from the Sequence Read Archive database (Bioproject ID: PRJNA630838).

Bioinformatics analyses of paired-end 16S rRNA V4 sequence reads were conducted using the Quantitative Insights Into Microbial Ecology (QIIME) software (v2.2019.4) [197]. Denoising was performed using Dada2, and taxonomic classification of amplicon sequence variants was performed against the SILVA (v132) reference database, based on 16S rRNA V4 hypervariable region of operational taxonomic units that were clustered at 97% similarity. Spurious amplicon sequence variants, including those assigned as mitochondria and chloroplast, were removed. All samples were sub-sampled to the lowest sample read depth of 7,285 sequence reads, at which the rarefaction curve of observed species had reached an asymptote.

3.3.9 Statistical analysis

Statistical analysis for functional gastrointestinal measures and AUC calculation were performed separately for each sex by using the GraphPad Prism software (version

7.04, San Diego, CA, USA). Differences between groups were assessed using one-way ANOVA and Tukey's post hoc analysis where appropriate. Analysis of the gut microbiota was performed separately by sex, as well as in combined data from males and females. Normally distributed microbiota data were analysed using ANOVA with sex and diet as the factors and Tukey's post hoc tests. Non-parametric microbiota data were analysed using Kruskal-Wallis with Dunn's test for post hoc analysis. Microbiota composition was analysed using permutational ANOVA (PERMANOVA) based on the weighted Unifrac distances of samples [198, 199]. Adjusted *p*-values were obtained using the false discovery rate (FDR) method. Significance was considered as $p < 0.05$.

3.4 Results

3.4.1 Gastric emptying rate

In males, there were no significant differences in gastric emptying rates between the diet groups (Figure 3.1Ai and Bi).

In females, the rates of gastric emptying were increased in mice consuming diets containing any level of HAW when compared to those consuming the standard SAW65 diet, as reflected by the elevated $^{13}CO_2$ excretion value (Figure 3.1Aii) as well as the increased AUC for the $^{13}CO_2$ excretion curves ($p < 0.05$; Figure 3.1Bii).

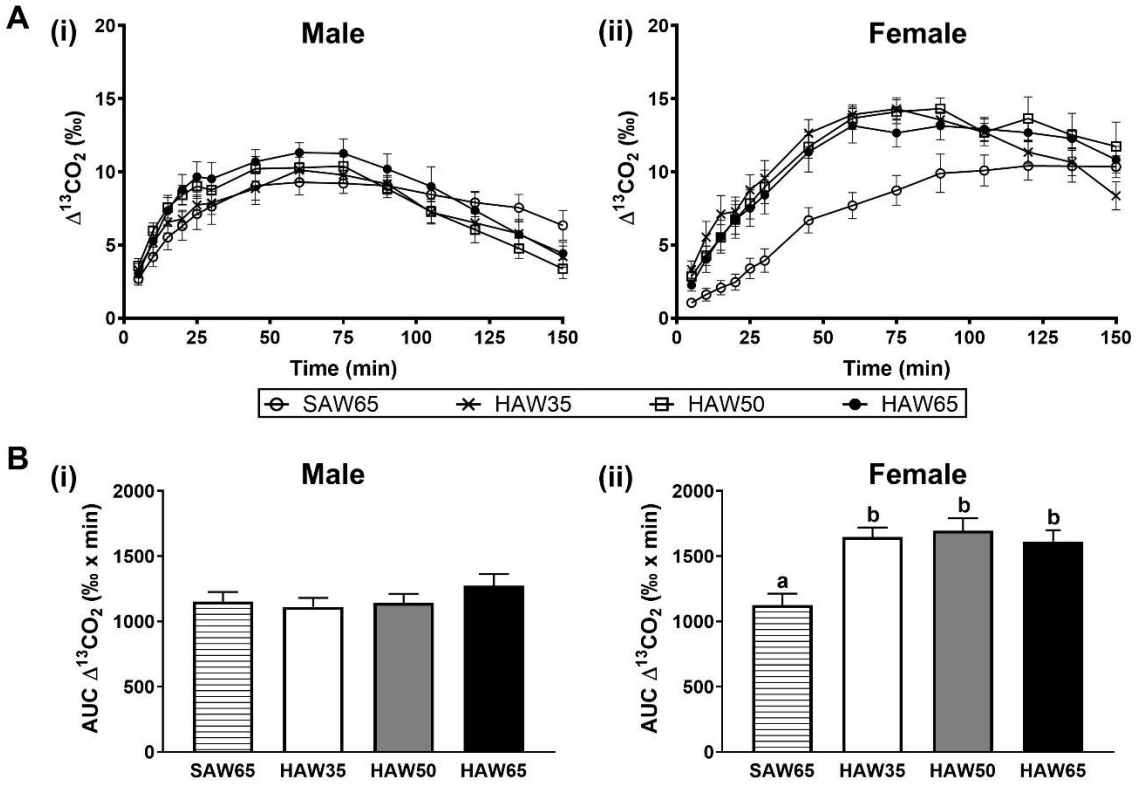

Figure 3.1. Gastric emptying rate expressed as (A) 13CO2 excretion curve and (B) area under the curve (AUC) in (i) male and (ii) female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) as determined by 13C-labelled octanoic breath test after seven weeks of feeding. Data are shown as mean ± SEM (n = 8 mice/group). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc tests.

3.4.2 Gastrointestinal tract organ weights, pH of caecal content and plasma GIP concentrations

In males, relative weights of the small intestine were lower in mice consuming the HAW35 diet when compared to all other diet groups ($p < 0.05$; Table 3.2). Relative colon weights in male mice consuming the HAW65 diet were significantly higher than in males that consumed either the SAW65 ($p < 0.01$) or HAW35 ($p < 0.01$) diet, but relative weights of the stomach or caecum were similar between groups.

In female mice, the relative weights of the small intestine were higher following consumption of the HAW65 diet compared to the SAW65 ($p < 0.01$) or HAW35 ($p = 0.02$) diet (Table 3.2). Female mice that consumed either the HAW65 or HAW50 diet

also had significantly higher relative caecum and colon weights compared to the SAW65 group, however, relative stomach weights were not different between diet groups.

There were no differences in the pH of the caecal content or plasma GIP concentrations between diet groups in either male or female mice (Table 3.2).

Table 3.2. Relative gastrointestinal tract organ weights, caecal content pH and plasma gastric inhibitory peptide concentrations in male and female mice after eight-week feeding.

Parameter	SAW65	HAW35	HAW50	HAW65
Male				
Stomach (mg/g BW)	8.74 ± 1.00	7.82 ± 0.53	8.90 ± 0.65	8.25 ± 0.44
Small intestine (mg/g BW)	30.25 ± 0.72^{a}	26.62 ± 0.67^{b}	29.55 ± 0.96^{a}	30.05 ± 0.60^{a}
Caecum (mg/g BW)	8.47 ± 1.12	9.13 ± 0.92	7.98 ± 0.56	9.92 ± 1.03
Colon (mg/g BW)	5.80 ± 0.29^{a}	5.83 ± 0.44^{a}	6.64 ± 0.26^{ab}	7.69 ± 0.21^{b}
pH of caecum contents	8.07 ± 0.05	7.96 ± 0.09	7.98 ± 0.07	8.10 ± 0.07
Plasma gastric inhibitory peptide (ng/mL)	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
Female				
Stomach (mg/g BW)	9.87 ± 0.67	9.65 ± 0.53	9.03 ± 0.57	10.76 ± 0.42
Small intestine (mg/g BW)	34.53 ± 0.89^{a}	36.24 ± 1.18^{a}	37.05 ± 0.74^{ab}	40.40 ± 0.81^{b}
Caecum (mg/g BW)	8.05 ± 0.47^{a}	9.11 ± 0.80^{ab}	11.22 ± 0.46^{b}	11.35 ± 0.92^{b}
Colon (mg/g BW)	7.12 ± 0.39^{a}	8.35 ± 0.57^{ab}	9.90 ± 0.85^{b}	10.08 ± 0.38^{b}
pH of caecum contents	8.12 ± 0.04	8.00 ± 0.06	8.07 ± 0.07	7.98 ± 0.06
Plasma gastric inhibitory peptide (ng/mL)	0.08 ± 0.02	0.04 ± 0.01	0.06 ± 0.01	0.06 ± 0.01

Mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65). Data are shown as means ± SEM (n = 8 mice/group/sex). Values with different superscripts in a row indicate significant differences between groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc test. BW, body weight.

3.4.3 Caecum and colon gene expression

In the caecum of males, expression of *Pyy* mRNA was lower in mice who consumed the HAW35 diet when compared to the SAW65 diet ($p = 0.02$) whereas *Pyy* mRNA expression in the colon was not different between groups. There were also no differences in mRNA expression of either *Gcg* (that encodes preproglucagon) or the gut barrier markers, *Ocln* and *Muc2*, in either caecal or colonic tissues between diet groups (Table 3.3).

In females, *Pyy* mRNA expression in the colon, but not in the caecum, was higher in mice consuming the HAW65 diet when compared to those consuming the HAW50 diet ($p = 0.01$; Table 3.3), but not the HAW35 or SAW65 diets. Similarly, in the colon, but not the caecum, *Ocln* mRNA expression was higher in those mice that consumed the HAW65 diet when compared to those mice consuming the HAW35 diet ($p = 0.02$). There were no differences in the expression of either *Gcg* or *Muc2* mRNA between diet groups in either the caecum or colon.

Table 3.3. Caecal and colonic mRNA levels of gut hormones and gut barrier markers in male and female mice after eight-week feeding.

mRNA level	SAW65	HAW35	HAW50	HAW65
Male				
Caecum				
Gut hormones				
Peptide YY	0.07 ± 0.01^{a}	0.04 ± 0.01^{b}	0.05 ± 0.01^{ab}	0.06 ± 0.01^{ab}
Proglucagon	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Gut barrier markers				
Occludin	0.46 ± 0.03	0.40 ± 0.03	0.44 ± 0.02	0.41 ± 0.03
Mucin-2	3.34 ± 0.57	3.72 ± 0.70	3.19 ± 0.34	3.29 ± 0.87
Colon				
Gut hormones				
Peptide YY	0.21 ± 0.07	0.28 ± 0.07	0.23 ± 0.07	0.19 ± 0.02
Proglucagon	0.10 ± 0.02	0.11 ± 0.01	0.09 ± 0.02	0.11 ± 0.01
Gut barrier markers				
Occludin	0.60 ± 0.03	0.66 ± 0.05	0.69 ± 0.04	0.66 ± 0.03
Mucin-2	20.20 ± 3.96	19.03 ± 4.55	15.89 ± 3.75	22.43 ± 6.21
Female				
Caecum				
Gut hormones				
Peptide YY	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
Proglucagon	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Gut barrier markers				
Occludin	0.31 ± 0.03	0.31 ± 0.04	0.31 ± 0.04	0.28 ± 0.03
Mucin-2	3.13 ± 0.35	2.40 ± 0.20	2.43 ± 0.29	2.12 ± 0.27
Colon				
Gut hormones				
Peptide YY	0.39 ± 0.06^{ab}	0.39 ± 0.05^{ab}	0.32 ± 0.06^{a}	0.63 ± 0.09^{b}
Proglucagon	0.14 ± 0.01	0.20 ± 0.03	0.16 ± 0.02	0.13 ± 0.01
Gut barrier markers				
Occludin	0.74 ± 0.04^{ab}	0.67 ± 0.04^{a}	0.74 ± 0.07^{ab}	0.91 ± 0.05^{b}
Mucin-2	18.68 ± 4.26	24.83 ± 3.81	25.54 ± 6.16	20.82 ± 8.34

Mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65). Data are shown as means ± SEM (n = 8 mice/group/sex). Values with different superscripts in a row indicate significant differences between groups ($p < 0.05$) as determined by one-way ANOVA and Tukey's post hoc test.

3.4.4 Gut bacterial load and microbiota diversity

Mice consuming either the HAW50 or HAW65 diet had a higher faecal bacterial load when compared to those consuming the SAW65 diet, in both overall ($p < 0.01$; Figure 3.2A) and when males ($p < 0.05$; Figure 3.2B) and females ($p < 0.05$; Figure 3.2C) were separated in the analysis. Further, mice consuming the highest level of HAW (HAW65) also exhibited a higher faecal bacterial load compared to mice consuming the diet containing the lowest level of HAW (HAW35) in both male and female mice.

Mice consuming the diet containing the two highest levels of HAW (HAW50 or HAW65) also exhibited higher alpha diversity measures of microbial richness (observed species; Figure 3.3Ai), evenness (Pielou's evenness; Figure 3.3Bi) and diversity (Faith's phylogenetic diversity; Figure 3.3Ci) when compared to those consuming the SAW65 diet (FDR $p < 0.05$). In addition, mice consuming the lowest level of HAW (HAW35) had a lower gut microbial richness when compared to the HAW65 diet (FDR $p = 0.045$, Figure 3.3Ai) and a lower gut microbial diversity when compared to HAW50 diet (FDR $p = 0.021$, Figure 3.3Ci). When analyses were conducted separately for each sex, there were no differences between diet groups in gut microbial richness (Figure 3.3Aii) or gut microbial evenness (Figure 3.3Bii) in male mice. However, male mice consuming either the HAW50 or HAW65 diet had a higher gut microbial diversity when compared to those consuming the SAW65 diet (FDR $p < 0.001$, Figure 3.3Cii). In females, mice consuming either the HAW50 or HAW65 diet had a higher gut microbial evenness when compared to those consuming the SAW65 diet (FDR $p < 0.05$, Figure 3.3Biii). There were, however, no differences in either gut microbial richness (Figure 3.3Aiii) or gut microbial diversity (Figure 3.3Ciii) between diet groups.

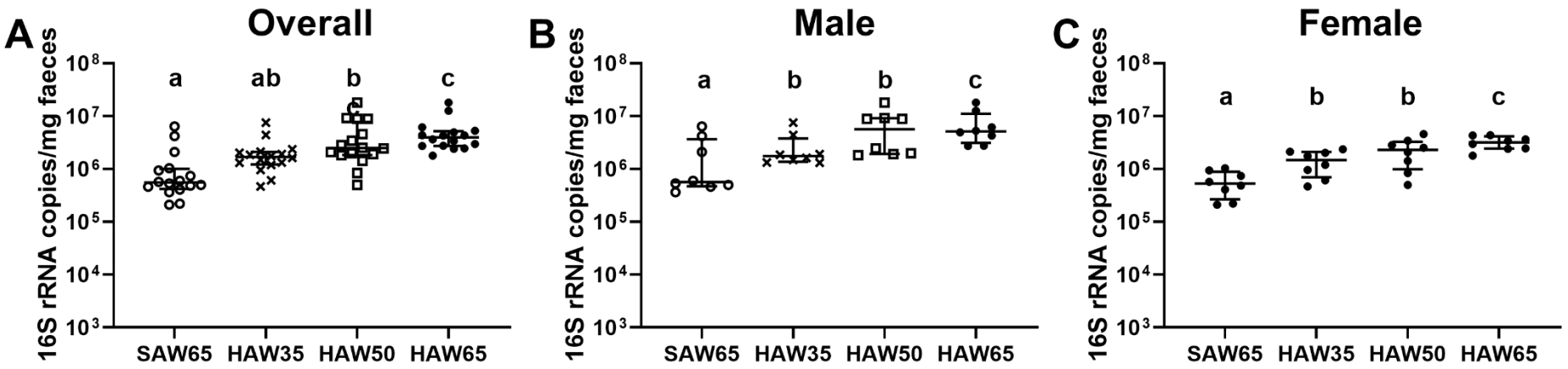

Figure 3.2. Total bacterial load in (A) overall or (B) male or (C) female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) after eight weeks of feeding. Data are shown as median ± IQR (n = 16 mice/group or 8 mice/group/sex). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis and Dunn's post hoc tests.

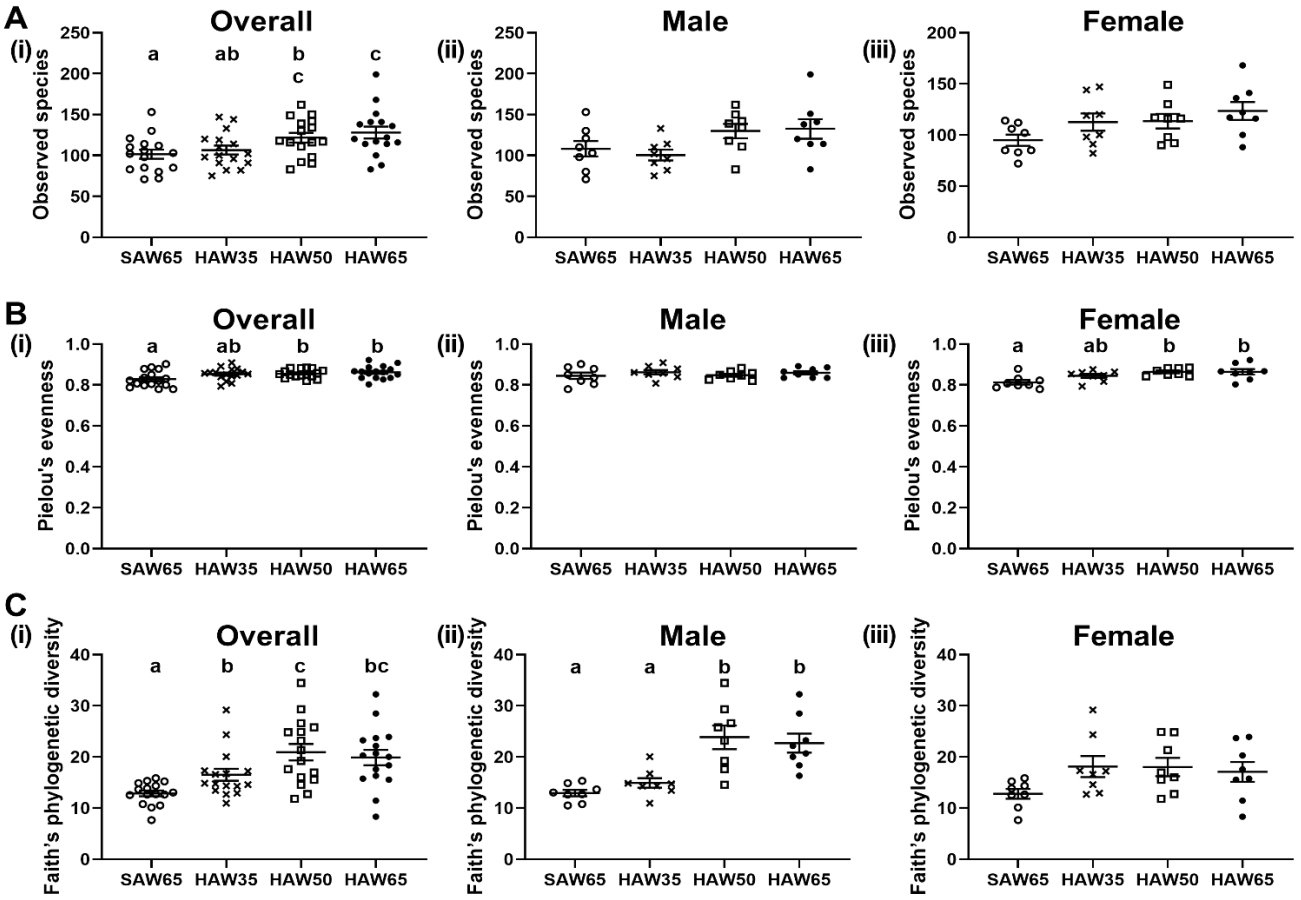

Figure 3.3. Alpha diversity measures of microbial (A) richness (observed species), (B) evenness (Pielou's evenness) and (C) diversity (Faith's phylogenetic diversity) in (i) overall or (ii) male or (iii) female mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65) after eight weeks of feeding. Data are shown as mean ± SEM (n = 16 mice/group or 8 mice/group/sex). Values with different superscripts indicate significant differences between diet groups ($p < 0.05$) as determined by ANOVA and pairwise comparisons tests with false discovery rate correction for *p*-values.

3.4.5 Gut microbiota composition

Analysis of faecal microbiota composition based on weighted Unifrac distances indicated compositional differences between the diet groups (p = 0.021, square root of estimates of components of variation (ECV) = 0.043), with no differences between sexes (p = 0.324, square root ECV = 0.0065) or sex by diet interaction (p = 0.929, square root ECV = -0.0289). At the phylum level, differences between the diet groups were observed in the relative abundances of the two most abundant phyla, Bacteroidetes and Firmicutes (p < 0.0001 for both phyla), and Proteobacteria (p = 0.043), but not for the other phyla, including Actinobacteria, Patescibacteria, Tenericutes, and Verrucomicrobia (p > 0.05; Table 3.4). Specifically, mice consuming diets containing any levels of HAW (35-65% HAW) showed a higher relative abundance of Bacteroidetes, but a lower relative abundance of Firmicutes, in comparison to those consuming the SAW65 diet. These changes were reflected in a lower Firmicutes:Bacteroidetes ratio in mice consuming any level of HAW compared to those consuming the SAW65 diet (p < 0.001). These differences in the relative abundances of Bacteroidetes and Firmicutes and Firmicutes:Bacteroidetes ratio, but not the relative abundance of Proteobacteria, between the diet groups were also observed for both males and females when the analyses were conducted separately in each sex (Table 3.4).

Table 3.4. Phylum-level relative abundances of faecal microbiota in male and female mice after eight-week feeding.

Phylum	**SAW65**	**HAW35**	**HAW50**	**HAW65**
Bacteroidetes	0.223[a] (0.189, 0.262)	0.380[b] (0.320, 0.425)	0.347[b] (0.288, 0.381)	0.361[b] (0.313, 0.425)
Firmicutes	0.713[a] (0.680, 0.717)	0.552[b] (0.499, 0.602)	0.571[b] (0.504, 0.620)	0.556[b] (0.508, 0.582)
Proteobacteria	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0006)	0.0 (0.0, 0.0)
Actinobacteria	0.037 (0.028, 0.053)	0.038 (0.017, 0.057)	0.039 (0.030, 0.052)	0.040 (0.035, 0.054)
Patescibacteria	0.0 (0.0, 0.006)	0.0007 (0.0, 0.002)	0.001 (0.0, 0.003)	0.002 (0.0002, 0.002)
Tenericutes	0.001 (0.0, 0.007)	0.003 (0.0009, 0.005)	0.003 (0.0008, 0.009)	0.005 (0.002, 0.010)
Verrucomicrobia	0.005 (0.0, 0.012)	0.006 (0.0, 0.030)	0.025 (0.0, 0.034)	0.013 (0.0, 0.054)
Firmicutes:Bacteroidetes ratio	3.222[a] (2.636, 3.779)	1.498[b] (1.140, 1.873)	1.638[b] (1.368, 2.111)	1.592[b] (1.221, 1.887)
Male				
Bacteroidetes	0.224[a] (0.189, 0.280)	0.352[b] (0.319, 0.413)	0.369[b] (0.336, 0.381)	0.363[b] (0.346, 0.425)
Firmicutes	0.687[a] (0.647, 0.723)	0.561[b] (0.484, 0.613)	0.538[b] (0.504, 0.574)	0.562[b] (0.517, 0.589)
Proteobacteria	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0009)	0.0 (0.0, 0.0)
Firmicutes:Bacteroidetes ratio	3.131[a] (2.329, 3.779)	1.524[b] (1.211, 1.918)	1.458[b] (1.368, 1.694	1.541[b] (1.221, 1.701)
Female				
Bacteroidetes	0.226[a] (0.187, 0.249)	0.409[b] (0.333, 0.446)	0.289[b] (0.261, 0.387)	0.330[b] (0.282, 0.482)
Firmicutes	0.719[a] (0.688, 0.740)	0.547[b] (0.499, 0.602)	0.619[b] (0.475, 0.670)	0.553[b] (0.427, 0.578)
Proteobacteria	0.0 (0.0, 0.0)	0.0 (0.0, 0.0005)	0.0 (0.0, 0.0004)	0.0 (0.0, 0.0)
Firmicutes:Bacteroidetes ratio	3.222[a] (2.769, 3.984)	1.326[b] (1.140, 1.821)	2.099[a] (1.269, 2.570)	1.712[b] (0.8803, 2.034)

Mice fed diets containing 65% standard amylose wheat (SAW65), 30% SAW and 35% high amylose wheat (HAW35), 15% SAW and 50% HAW (HAW50) or 65% HAW (HAW65). Data are shown as median ± IQR (n = 16 mice/group or 8 mice/group/sex). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis and pairwise comparisons tests with false discovery rate correction for *p*-values.

Pairwise compositional analyses performed between the diet groups indicated that the gut microbiota composition of mice consuming the SAW65 diet differed to that of mice consuming either the HAW50 ($p = 0.029$) or HAW65 ($p = 0.043$) diets. At the genera level, the abundance of nine bacterial taxa, predominantly of the Firmicutes phylum, as well as the Bacteroidetes and Tenericutes phyla, were increased in both the HAW50 and HAW65 diet groups compared to the SAW65 diet group (FDR $p < 0.05$; Figure 3.4). Taxa that were decreased in both the HAW50 and HAW65 diet groups, compared to the SAW65 diet group, were *Clostridium sensu stricto 1*, *Turicibacter*, *Lactococcus*, *Lactobacillus* and *Faecalibaculum*, all of which are under the Firmicutes phylum (FDR $p < 0.05$). The taxa *Dorea*, *Eubacterium coprastanoligenes* and a taxon under the Bacteroidales order increased only in the HAW50 diet group compared to the SAW65 diet group, whereas *Lachnospiraceae GCA-90006657* and *Akkermansia* were increased and *Roseburia* were decreased in the HAW65 diet group compared to the SAW65 diet group (Figure 3.4).

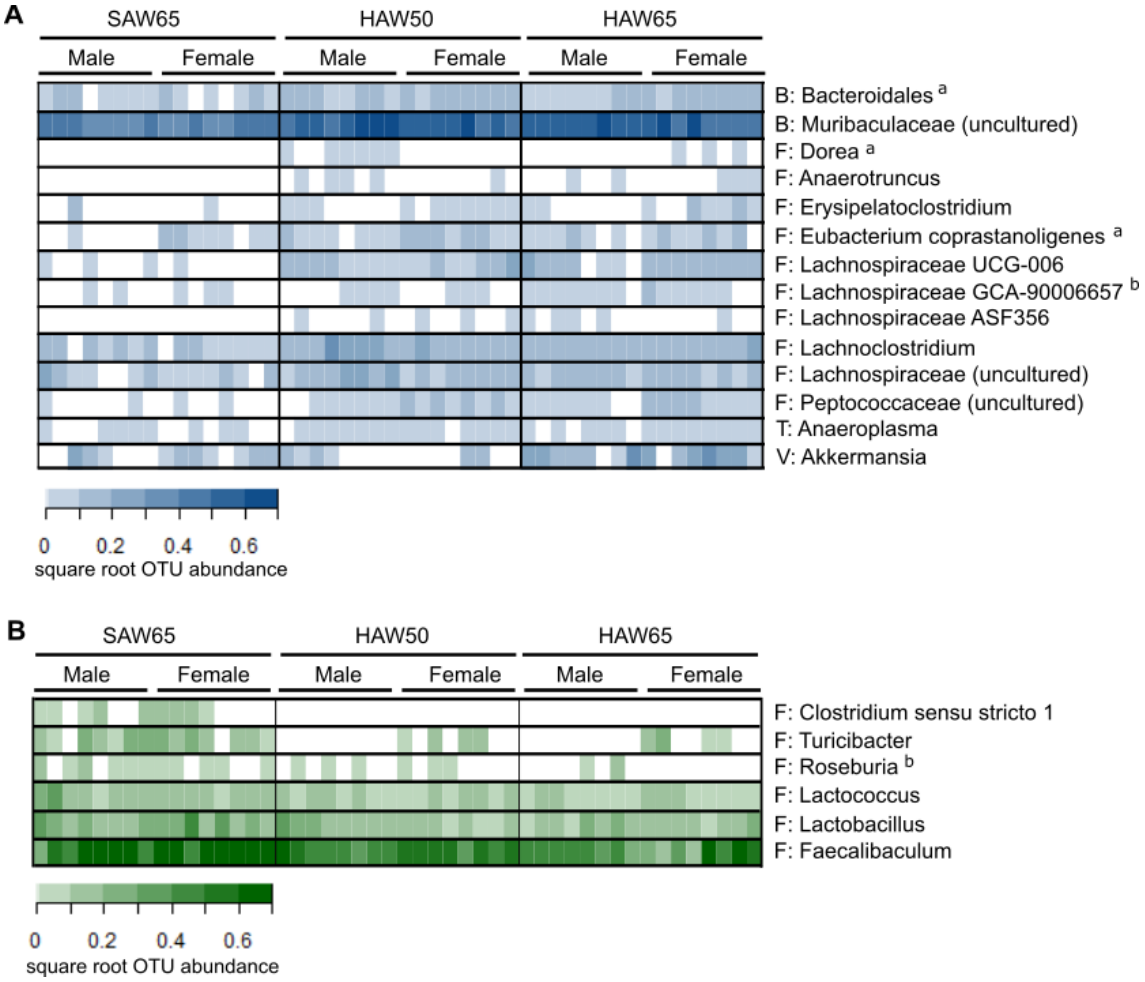

Figure 3.4. Heatmap of bacterial genera, which relative abundance significantly (A) increased or (B) decreased in mice fed diets containing 15% standard amylose wheat (SAW) and 50% high amylose wheat (HAW; HAW50) or 65% HAW (HAW65), in comparison to those consuming diet containing 65% SAW (SAW65) after eight weeks of feeding. Relative abundances of each taxon in male or female mice are based on square root-relative abundances (n = 8 mice/group/sex). Values with different superscripts indicate significant differences between groups ($p < 0.05$) as determined by Mann-Whitney tests and pairwise comparisons with false discovery rate correction for *p*-values. Taxa that were only significant difference between HAW50 and SAW65, or HAW65 and SAW65, are denoted with the superscript 'a' or 'b', respectively. The phyla Bacteroidetes (B), Firmicutes (F), Tenericutes (T) and Verrucomicrobia (V) are denoted to indicate the phylum group of bacterial taxa that were significantly altered.

3.5 Discussion

This study demonstrates that a diet containing HAW at levels between 35% and 65% of total diet weight modulated a number of gastrointestinal parameters, including increasing the rate of gastric emptying, increasing the expression of *Pyy* and *Ocln* and producing significant shifts in gut microbiota composition. The changes in gastrointestinal function and gene expression were more pronounced in female mice, and microbiota changes were present at higher levels of HAW consumption, suggesting that these factors are important to consider when interpreting and extrapolating these findings.

Our finding that female mice consuming any level of HAW exhibited an accelerated rate of gastric emptying compared to those consuming the control diet (SAW65) was unexpected given the higher dietary fibre content in the HAW diets [148]. Foods rich in dietary fibre, especially soluble fibre, have been reported in several previous studies to slow gastric emptying rate [200-202]. This is largely a result of fibre's high water-holding capacity and viscosity, both of which increase gastrointestinal fullness and limit the ability of food particles to enter the duodenum [203, 204]. However, not all studies have reported such effects [205, 206]; one study reported that consumption of 15 g of bran twice daily for four days had no effect on gastric emptying rate in healthy human subjects [205], while other studies reported that healthy human subjects who consumed high fibre products, containing either 5% cellulose or low (2.5%) or high (5%) doses of alginate or a type 3 resistant starch (retrograded amylose) exhibited accelerated gastric emptying rates [206, 207], consistent with our findings. Additional research suggests that the impact of dietary fibre on gastric emptying is also dependent on the type, amount and hydration state of the dietary fibre consumed [208], which may account for the variability in results. Enhanced gastric emptying rates would be expected to be associated with a shorter transit time through the gut. This, in turn, would reduce digestion and absorption

of macronutrients, including fat, and could potentially reduce energy uptake and limit fat deposition. This hypothesis is at least partially supported by our previous finding that female mice consuming diets containing between 35-65% HAW, tended to have lower relative total and gonadal fat mass [148].

The higher relative weights of the small intestine, caecum and colon in female mice consuming HAW diets were consistent with a previous study in which male Sprague-Dawley rats fed a 48% high amylose maize diet (w/w) for 4 weeks exhibited higher small intestine, caecum and colon weights, and had a higher daily faecal output compared to controls [209]. Interestingly, however, in the males in the current study, only the relative weight of the colon was higher in those consuming the highest level of HAW diet (HAW65) when compared to both SAW65 and HAW35 groups, suggesting that effects of our HAW diet on gastrointestinal organ weight were more pronounced in females. It is possible that the increased gastrointestinal organ weights in animals consuming the HAW diets, were due to the increased proliferation of the intestinal epithelial cells, likely stimulated by the fermentation products of dietary fibre, including short-chain fatty acids [210]. This may also explain the greater effects in females compared to males, since males have a reduced colon transit time and thus absorb less short-chain fatty acids than females [211, 212], but this is highly speculative and requires further investigation. Irrespective of the underlying mechanism, however, higher gastrointestinal organ weight is likely to be associated with improved gastrointestinal health, since an increased thickness of the intestinal epithelium can reduce gut atrophy and may enhance nutrient absorption in the small intestine [213].

Diets containing higher amounts of fermentable fibre are typically associated with increased production of short-chain fatty acids, which in turn reduces luminal pH. Given the higher fibre content of the HAW compared to the SW diets, the lack of a difference

in the pH of the caecal content between dietary groups was therefore unexpected. Our finding is, however, consistent with a previous study [82] which also found no difference in caecal content pH in male Sprague-Dawley rats consuming a Western-style diet supplemented with either HAW or SAW for 11 weeks. In contrast, another study reported that consuming a HAW diet for two weeks significantly reduced caecal pH in young male Sprague-Dawley rats [65]. However, the level of amylose in the HAW variety used in this previous study was much higher than in the current study (~70% vs ~46%) and this may account for the different results. It is, therefore, possible that a higher amylose level than occurs in the HAW variety used in our study, which would be expected to result in greater increases in resistant starch content, is required to promote fermentation to a level that is sufficient to reduce luminal pH.

Female mice consuming the diet containing the highest level of HAW (HAW65) exhibited a higher colonic expression of *Pyy* when compared to those consuming a lower level (HAW50). Pyy plays a major role in maintaining energy balance and appetite, influences gut motility, gastrointestinal cell differentiation and proliferation [214], and increased *Pyy* treatment for two weeks has been associated with improvements in several structural markers of gastrointestinal health [215]. Our finding of increased *Pyy* expression is consistent with findings in another study, in which feeding a higher level of resistant starch from Hi-Maize corn starch was associated with increased expression of both *Pyy* and *Gcg* in the colonic epithelial cells in male Sprague-Dawley rats [216]. In response to the Hi-Maize corn starch, however, the increased mRNA expression extended to the caecum [216], possibly due to the higher level of resistant starch in the Hi-Maize corn starch.

The mRNA expression of *Muc2*, the major secretory protein secreted by intestinal goblet cells, was not influenced by the consumption of the HAW diet. This is in agreement

with the results of a previous study by Hedemann and co-workers who found that *Muc2* mRNA expression in the caecum and colon was not different in male Wistar rats that consumed different sources of carbohydrates, including cellulose, pectin, inulin, resistant starch or barley hulls [217]. The maintenance of *Muc2* mRNA expression is important, since the mucus layer acts as an important barrier protecting gut epithelial cells from damage and infection by pathogenic bacteria [218], and reduced *Muc2* mRNA has been associated with several gastrointestinal diseases [219]. While the consumption of the HAW diets did not appear to influence *Muc2* expression, female mice consuming the HAW65 diet had increased *Ocln* abundance when compared to those consuming the HAW35 diet. Occludin is a tight junction protein crucial for maintaining intestinal barrier integrity, and impairments in the intestinal epithelial barriers increase the risk of numerous disorders, including metabolic endotoxemia, inflammatory bowel and celiac diseases [220, 221]. It, therefore, appears that a high HAW diet may have some beneficial effects on intestinal barrier integrity in female mice, however, this requires functional verification. Overall, the findings of this study suggest that the effect of HAW consumption on gastrointestinal gene expression is gene specific and may vary in different regions of the gastrointestinal tract.

In this study, we demonstrated that consumption of the higher levels of HAW, HAW50 or HAW65, was associated with higher faecal bacterial load and alpha diversity of microbiota, including richness, evenness and diversity, suggesting that increased consumption of HAW promotes faecal bacterial growth. This is in line with previous studies, in which higher faecal bacterial load and bacterial diversity are observed when animals are fed diets supplemented with either soluble or fermentable fibre [222, 223]. Furthermore, we found that consumption of the higher levels of HAW, induced a shift in the composition of the gut microbiota towards an increased relative abundance of the

Bacteroidetes phylum and decreased relative abundance of the Firmicutes phylum, ultimately reducing the Firmicutes:Bacteroidetes ratio. This may due to the higher fibre content of the HAW favouring the growth of members of the Bacteroidetes phylum, since fibre is known to be a substrate that favours the growth of these bacteria [224]. The lower Firmicutes:Bacteroidetes ratio may be a marker of improved gastrointestinal health, since previous studies have reported an increased ratio of Firmicutes to Bacteroidetes in patients with either irritable bowel syndrome [225] or inflammatory bowel diseases [226]. An increased ratio of Firmicutes:Bacteroidetes, also increases the efficiency of energy absorption, and has been associated with obesity in both humans [227] and mice [228]. Thus, the lower Firmicutes:Bacteroidetes ratio may also contribute to the lower adiposity we observed in mice consuming the HAW diet in our previous study [148].

The beneficial effects of HAW diets on the microbiota was further supported by the increased relative abundance of several bacteria, including Bacteroidales, Muribaculaceae, Lachnospiraceae, *Anaeroplasma* and *Akkermansia* and reduced relative abundance of bacteria, including *Clostridium sensu stricto 1* and *Turicibacter* in mice consuming diets containing the higher levels of HAW. Members of the order Bacteroidales, have been shown to produce antimicrobial toxins, which may limit the growth of opportunistic pathogens [229]. Several taxa of the Muribaculaceae [230] and Lachnospiraceae families [231], are capable of fermenting plant polysaccharides to produce short-chain fatty acids, which serve as energy sources for the colonic epithelium and maintenance of the gut mucosal barrier [232], while *Anaeroplasma* and *Akkermansia* contribute to improving the integrity of the intestinal barrier [233, 234]. Conversely, overgrowth of *Clostridium sensu stricto 1* and *Turicibacter* are associated with increased frequency of diarrhea [235], risks of necrotic enteritis [236], acute appendicitis [237] and ulcerative colitis [238].

3.6 Conclusion

In conclusion, we have demonstrated that increased consumption of HAW has effects on gastrointestinal weight, gene expression and function, including enhanced gastric motility and increased mRNA expression of the intestinal barrier marker *Ocln* and the gut hormone *Pyy*, particularly in female mice that consumed diets with higher levels of HAW. Furthermore, increased consumption of HAW was associated with increased faecal bacteria load and altered composition of the gut microbiota in both male and female mice in a manner consistent with favourable effects on gut health, particularly at higher levels of HAW intakes. Together, these findings add to the existing evidence that HAW may have beneficial effects for maintaining and improving gastrointestinal health. The findings also indicate that the impact of HAW on a number of gastrointestinal measures is dependent on both the sex of the animal and the level of inclusion in the diet, highlighting the importance of taking these factors into account in the design of future studies in this area.

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Conflict of Interest

J.C. is Director of a company that has licenced the high amylose grain used in this study from the University of Adelaide with the intent of commercialisation. The other authors declare that they have nothing to disclose. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Chapter 4

Higher Intake of High Amylose Wheat Improves Conception Rates, but Not Fetal Growth, in Female Mice

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Contribution to the Paper	Performed the experiments, collected, analysed and interpreted the data and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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4.1 Abstract

High amylose wheat (HAW) is a novel type of wheat which exhibits several nutritional attributes with the potential to improve reproductive health outcomes, however, the effects of consuming higher levels of HAW on reproductive health measures have not been directly investigated. Therefore, this study aimed to compare the effects of consuming diets higher in HAW on reproductive and pregnancy outcomes in female mice. Female C57BL/6 mice (n = 20/group) were fed diets containing either ~63% (w/w) HAW or ~63% (w/w) SAW for four weeks prior to mating until gestational day (GD) 17.5, at which time pregnancy outcomes were assessed. Mice consuming the HAW diet exhibited higher food intake and body weight prior to mating, but not during pregnancy, compared to those consuming the SAW diet ($p < 0.01$). Mice fed the HAW diet spent longer in estrus and less time in diestrus. A greater proportion of females in the HAW group achieved pregnancy post-coitus (94% vs 61%, $p = 0.043$). At GD17.5, dams consuming the HAW diet had higher placenta ($p = 0.003$), but not fetal, weights and a lower fetal-placental weight ratio ($p = 0.004$) compared to those fed the SAW diet. While the observed improvements in conception rates are promising, further studies are required to investigate the short- and long-term impacts of maternal HAW consumption on the offspring.

Keywords: high amylose wheat; female; reproductive health; fertility; pregnancy

4.2 Introduction

Infertility is clinically defined as the inability to conceive after 12 months or more of regular unprotected intercourse [89]. While both men and women are affected, women are more vulnerable, since they are born with a fixed number of primordial follicles whose numbers decrease with age whereas men continue to produce sperm throughout their reproductive life [93]. Infertility is associated with significant psychological distress and reduced quality of life for couples, particularly for women [91, 239]. As a result, couples are increasingly seeking assisted reproductive technologies (ART) to achieve conception and ultimately giving birth to a healthy live baby [240]. While the sperm collection is generally non-invasive for men during the ART, women are required to undergo a series of invasive procedures, including blood tests, hormone injections, retrieval of mature oocytes for fertilisation and embryo transfer, all of which carry side effects, including pain, bleeding, wound infection and psychological distress [100]. In addition, ART treatments are very expensive [99] and not available in all region [92]. Therefore, strategies for improving reproductive health, which reduce the need to access/utilise ART, are preferable.

Diet quality is one of the modifiable factors that have the potential to substantially influence reproductive health and performance [103], and improved dietary quality, therefore, has the potential to improve reproductive outcomes. High amylose wheat (HAW) is a novel type of wheat which may confer additional health benefits compared to commercially available standard amylose wheat (SAW) [60]. HAW is distinguished from standard amylose varieties by its markedly higher amylose content (up to 93% of total starch compared to <30% in SAW) [59], higher levels of resistant starch (11% vs <1%) [62], greater nutrient-density (protein, lipid, minerals and dietary fibre) [147, 241] and lower glycaemic index (GI) [156]. While there was no direct evidence of HAW

improving female reproductive health and performance, higher intakes of whole grains have been previously shown to improve reproductive health parameters, including the increasing probability of embryo implantation and live birth among women seeking ART treatment [120]. The nutrients and bioactive compounds in whole grains, including B-vitamins, antioxidant compounds and lignans, have also been reported to have positive effects on female reproductive function and pregnancy outcomes [131, 142, 242].

In a recent study in our group, focussed primarily on assessing the impacts of HAW on metabolic parameters, we found that adult female mice consuming a higher HAW diet exhibited a shift in their estrus cycle, as indicated by their leukocyte cytology, raising the possibility that consuming higher levels of HAW could influence reproductive parameters and, potentially, exert beneficial effects on female reproductive health [148]. The present study aimed to directly evaluate the effect of consuming a diet containing a high level of HAW prior to mating and during gestation on reproductive and pregnancy outcomes in female mice.

4.3 Materials and Methods

4.3.1 Ethical statement

All procedures involving the use of animals were approved by the Animal Ethics Committee at The University of Adelaide (approval number: S-2019-051) and were conducted in compliance with the Australian National Health and Medical Research Council's code for the care and use of animals for scientific purposes (8th edition) and South Australia Animal Welfare Act 1985.

4.3.2 Experimental diets

The ingredients used in the experimental diets is provided in Table 4.1. The methods used for diet preparation have been described in detail elsewhere [148]. Briefly, both diets were prepared in-house to the AIN-93G formulation, with the exception that the carbohydrate component was replaced with an equivalent amount of either cooked SAW or HAW (both ~63% w/w of the total diet). The HAW used in this study contained ~46% of amylose in its total starch content and was developed through a normal selective breeding process [148].

Table 4.1. Composition of the experimental diets.

Ingredient (g)	Standard amylose wheat	High amylose wheat
Casein	200.0	200.0
L-cystine	3.0	3.0
Standard amylose wheat	629.5	0.0
High amylose wheat	0.0	629.5
Soybean Oil	70.0	70.0
Cellulose	50.0	50.0
Mineral Mix, AIN-93G-MX	35.0	35.0
Vitamin Mix, AIN-93-VX	10.0	10.0
Choline Bitartrate	2.5	2.5
TBHQ, antioxidant	0.014	0.014

4.3.3 Experiment design

Four-week-old C57BL/6 virgin female mice (ARC, Canning Vale, Australia), weighing 9-15 g, were maintained at The University of Adelaide's PC2 animal facility (Kintore Avenue, Adelaide, Australia) under a 12 h light-dark cycle in a temperature and humidity-controlled environment with access to food and water *ad libitum*. The female mice were purchased prior to puberty, since the pubertal period represents a critical period of functional development of the reproductive system [243], and thus exposing the mice to the dietary intervention throughout the pubertal period would maximise the opportunity of detecting any effects of the HAW diet on reproductive outcomes.

Mice were pair-housed in ventilated cages (GM500 Mouse IVC Green Line, Tecniplast, Italy), which contained dust-free laboratory bedding and enrichment (paper nesting material and rodent tunnels). Mice were fed on a standard rodent chow (Teklad 2918, Envigo, USA) for a one-week acclimatisation period. After this period, female mice (n = 20/group) were switched to either a ~63% w/w HAW or ~63% w/w SAW diet for four weeks prior to mating. The body weight of each mouse was recorded weekly during this time. Female mice were maintained on their experimental diets before and during mating, and throughout the gestation period until the end of the experiment. Food intake prior to mating was estimated by calculating the difference between initial and final weights of the food in the cage every three or four days and dividing this figure by the number of days and number of mice in each cage.

4.3.4 Estrus cycle assessment

The estrus cycle stage was assessed in each female mouse daily for 14 days prior mating with males. Briefly, at between 3:00 p.m. and 5:00 p.m. each day, the vaginal opening of females was flushed with 20 µl normal saline (0.9% NaCl) and the vaginal epithelial cell smears were analysed under a light microscope. The stage of the estrus cycle was determined based on the presence, absence or relative proportion of leukocytes and cornified epithelial and nucleated epithelial cells, following standard criteria [244]. One estrus cycle was considered as the sequence of proestrus, estrus, metestrus and diestrus. The average number and length of the estrus cycle and the total number of days in each stage over the 14-day assessment period was calculated for each mouse.

4.3.5 Mating and pregnancy outcomes

Following the four-week feeding period, when female mice were in proestrus, they were mated with sexually mature males of the same strain. Briefly, up to two females who were in proestrus were transferred to a cage with the male mice in the late evening prior to the dark cycle. A total of 10 males were used for mating, and the same males used to mate equal numbers of females in both dietary groups, in order to control for paternal effects. The males were maintained on a standard rodent chow (Teklad 2918, Envigo, USA) throughout the experiment, although they had access to either the HAW or SAW diet during the time when they were co-housed with females.

Mating was confirmed by the presence of a vaginal plug on the following morning, and this was designated as gestational day (GD) 0.5. After detecting a vaginal plug, the females were weighed and transferred to a new cage. If no plug was detected, the females remained in the male cage until either a plug was detected or for a maximum of four nights. The number of days until detection of the plug was recorded for all experimental animals.

4.3.6 Post-mortem and tissue collection

Dams were euthanised on GD 17.5 (the term is ~19 days after mating in this strain [245]), which represents the period of maximal fetal growth and nutrient requirements [243]. Non-pregnant females were euthanised at the same time point as dams but data from these animals is not included in this manuscript. The body weight of each mouse was recorded. Mice were then anaesthetised with isoflurane (5% induction and 2% maintenance) and blood collected into serum tubes via cardiac puncture followed by removal of the heart from the chest cavity. Blood was centrifuged (3000 rpm, 15 min, 4°C) and the serum fraction stored at -80°C for later analysis. Major organs including

ovaries, heart, pancreas, spleen, liver, kidneys and adipose tissue (parametrial fat and perirenal fat) were collected and weighed from the pregnant dams.

The whole gravid uterus was collected and weighed and the number of viable fetuses and resorptions in each uterine horn were recorded. Placentas were then carefully dissected away from the fetuses, and each placenta and fetus were individually weighed. Fetal length, head width and abdominal circumference were measured, and fetuses were killed by decapitation.

4.3.7 Sex hormone analysis

The serum progesterone concentrations were determined using a commercially available mouse/rat enzyme-linked immunosorbent assay kit (Catalog number: 55-PROMS-E01) with an internal control set (Catalog Number: 55-DEV99RC) according to the manufacturer's instructions (ALPCO, Salem, NH, USA). The minimum detection limit for progesterone was 0.156 ng/mL and the intra-assay coefficient of variation was 8.6%.

4.3.8 Statistical analysis

Data are expressed as mean ± SEM for continuous variables and rates for categorical variables. Normal distribution was assessed by the Shapiro-Wilk test. Differences between variables were assessed using an unpaired two-tailed Student *t*-test, Mann-Whitney test or Fisher's exact, as appropriate. For longitudinal variables, a two-way analysis of variance (two-way ANOVA) was used to determine the main effects of diet and time, and the interactions between them, and Bonferroni's multiple comparisons test was used to determine differences between diet groups when there was an observed interaction. Comparisons between diet groups for fetal outcomes, placental weight and

fetal-placental weight ratio using linear mixed models with diet as the categorical predictor, dams included as a random factor and corrected for viable litter size. Significance was considered at $p < 0.05$, and statistical analysis was performed by GraphPad Prism (version 8.2.1, San Diego, CA, USA) or IBM SPSS Statistics (version 26.0, Chicago, IL, USA).

4.4 Results

4.4.1 Food intake before pregnancy and body weight before and during pregnancy

Female mice fed the HAW diet exhibited higher food intakes compared to the SAW diet group over the four weeks prior to pregnancy [$F(1, 68) = 10.75$, $p = 0.0016$; Figure 4.1A].

From the second week after commencing their experimental diets, females fed the HAW diet were heavier than those fed the SAW diet ($p < 0.05$; Figure 4.1B), and the HAW group gained 39% more weight in the period before pregnancy compared to females fed the SAW diet (4.03 g vs 3.09 g).

There was no difference in body weight between the HAW and SAW groups during pregnancy [$F(1, 94) = 2.345$, $p > 0.05$; Figure 4.1C].

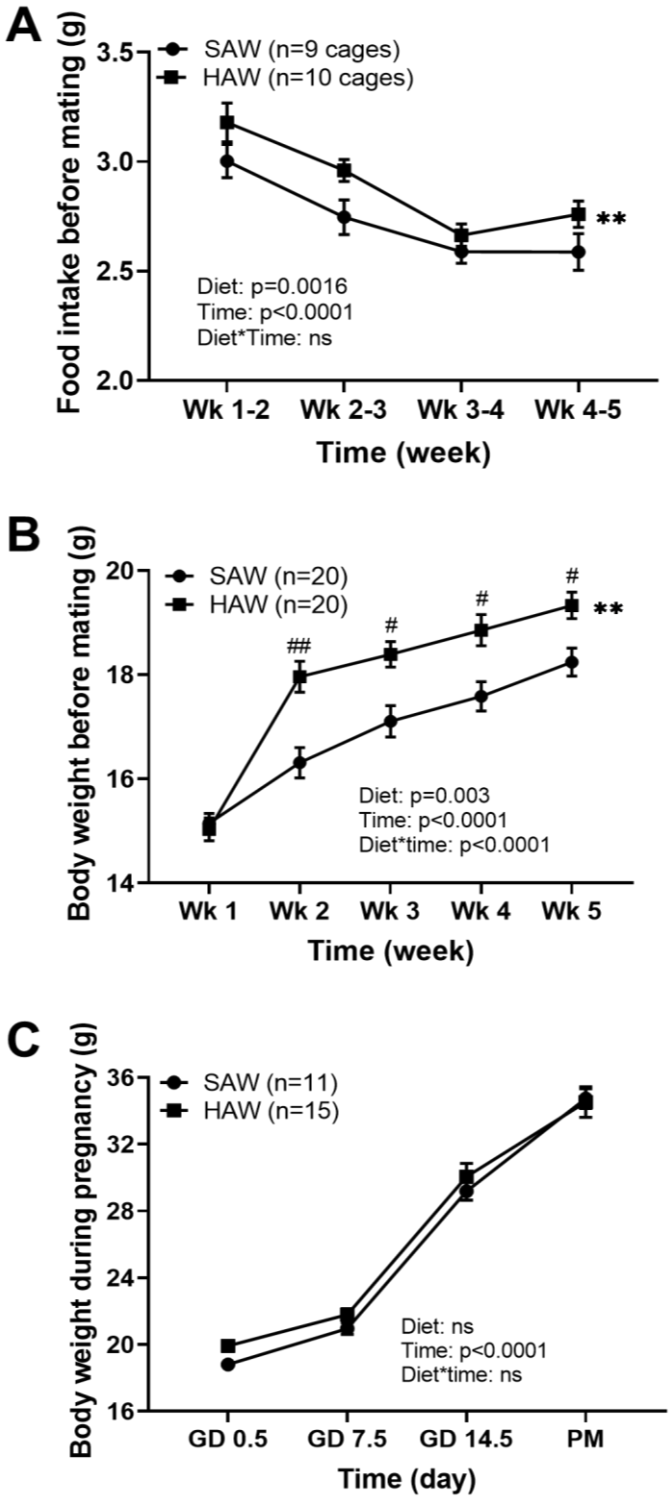

Figure 4.1. (A) Food intake before pregnancy and body weight (B) before and (C) during pregnancy in female mice consuming diet containing either ~63% w/w standard amylose wheat (SAW) or ~63% w/w high amylose what (HAW). Data are mean ± SEM. **p < 0.01, two-way ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, Bonferroni's multiple comparisons test. NS: not significant; WK: week; PM: post-mortem.

4.4.2 Estrus cyclicity before pregnancy

There was no difference in the total number of estrus cycles measured in the mice between the HAW and SAW groups, with an average of two estrus cycles recorded in both dietary treatments (Figure 4.2A). The cycle length was also similar between groups, at approximately five days per cycle (Figure 4.2B). However, females consuming the HAW diet spent a greater number of the 14 days in estrus (p = 0.005; Figure 4.2D) and less in diestrus (p = 0.018; Figure 4.2F) compared to those consuming the SAW diet. There was no difference in the number of days spent in either proestrus (Figure 4.2C) or metestrus (Figure 4.2E) between dietary groups.

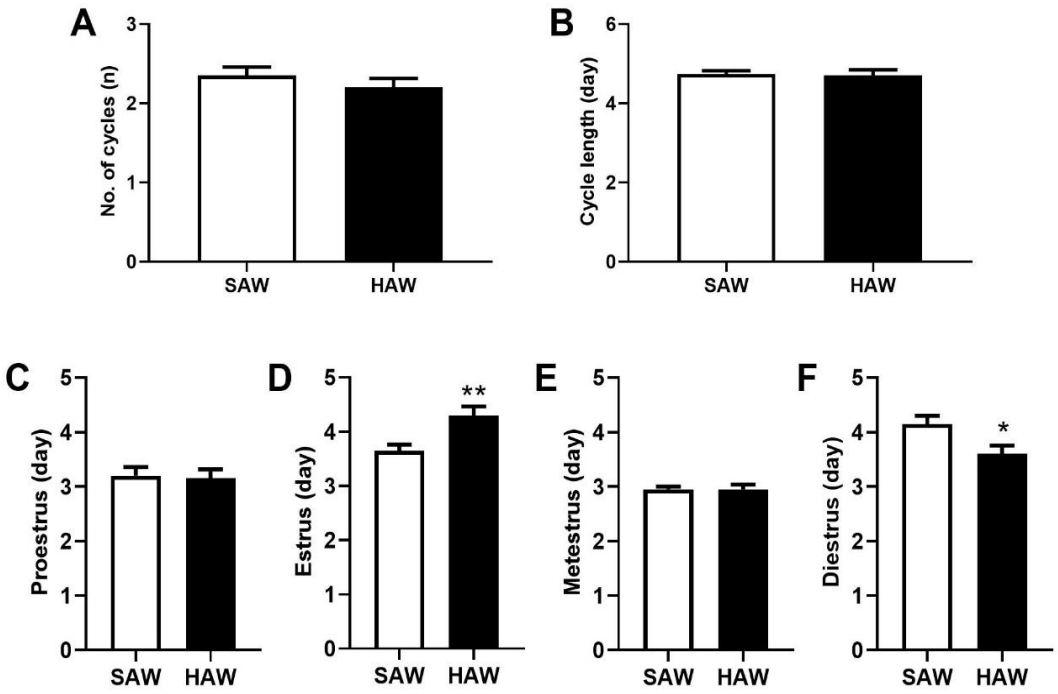

Figure 4.2. Average (A) number and (B) length of the estrus cycle and the total number of days in (C) proestrus, (D) estrus, (E) metestrus and (F) diestrus over the 14-day assessment period in female mice consuming diet containing either ~63% w/w standard amylose wheat (SAW) or ~63% w/w high amylose wheat (HAW) prior to mating. Data are mean ± SEM (n = 20/group). *p < 0.05, **p < 0.01, Mann-Whitney tests.

4.4.3 Mating and pregnancy outcomes

There was no difference between the HAW and SAW groups in the number of mice who exhibited copulatory plugs, or the length of time to plugging (SAW group: 90% of female mice exhibited copulatory plugs, with the first plug an average time of 1.7 days after co-housing with males; HAW group: 80% of female mice exhibited copulatory plugs within an average time of 2 days co-housing with males; Table 4.2). The proportion of mice with a copulatory plug who were subsequently confirmed to be pregnant (pregnancy rate) was, however, significantly higher in the HAW group compared to the SAW group (94% vs 61%, $p < 0.05$).

There were no differences between dietary groups for the majority of measured pregnancy outcomes, including uterine weight, number of implantation sites, viable litter size or resorption rate (Table 4.2). Mice in the HAW group had a higher placental weight compared to the SAW group, whereas fetal body weight tended ($p < 0.06$) to be lower in dams fed the HAW diet. As a result, dams fed the HAW diet exhibited a ~24% lower fetal:placental weight ratio compared to dams fed the SAW diet ($p < 0.01$; Table 4.2). Fetal body length, abdominal circumference and head width were not significantly different between dietary groups.

Table 4.2. Mating and pregnancy outcomes of dams consuming diet containing either ~63% w/w standard amylose wheat (SAW) or ~63% w/w high amylose wheat (HAW) before and during pregnancy.

Variable	**SAW**	**HAW**	***p*-value**
Precoital time (day)[a]	1.72 ± 0.19	2.00 ± 0.30	0.647
Copulation rate (%)[b]	18/20 (90)	16/20 (80)	0.661
Pregnancy rate (%)[c]	11/18 (61)	15/16 (94)	0.043
Whole uterine weight (g)	9.61 ± 0.47	8.61 ± 0.58	0.248
Implantation site (n)	7.91 ± 0.28	7.60 ± 0.496	0.946
Viable litter size (n)	7.82 ± 0.26	7.57 ± 0.53	0.739
Left horn litter size (n)	3.64 ± 0.51	3.21 ± 0.45	0.539
Right horn litter size (n)	4.18 ± 0.42	4.36 ± 0.45	0.784
Resorption rate (%)[d]	1/87 (1.15)	1/114 (0.88)	>0.999
Fetal weight (g)[e]	0.86 ± 0.04	0.75 ± 0.04	0.052
Fetal length (mm)[e]	18.73 ± 0.34	17.96 ± 0.33	0.112
Fetal abdominal circumference (mm)[e]	22.26 ± 0.32	21.48 ± 0.29	0.088
Fetal head width (mm)[e]	5.72 ± 0.08	5.51 ± 0.08	0.084
Placenta weight (g)[e]	0.09 ± 0.00	0.10 ± 0.00	0.003
Fetal:placental weight ratio[e]	9.79 ± 0.52	7.48 ± 0.49	0.004

[a]Time from the first day of couple housing until the visualisation of a copulatory plug. [b](No. of animals observed with copulatory plugs/no. of mated animals)x100. [c](No. of pregnant animals/no. of animals observed with copulatory plugs)x100; [d](No. of resorption/no. of implantation sites)x100. [e]Comparison between diet groups was corrected for viable litter size. Data are means ± SEM (SAW= 11 dams and HAW=15 dams). Significance between the standard amylose wheat (SAW) and high amylose wheat (HAW) groups was determined using Student *t*-test, Mann-Whitney test or Fisher's exact test, as appropriate.

4.4.4 Progesterone concentrations

There was no difference between diet groups in the serum progesterone concentrations on GD 17.5 (Figure 4.3).

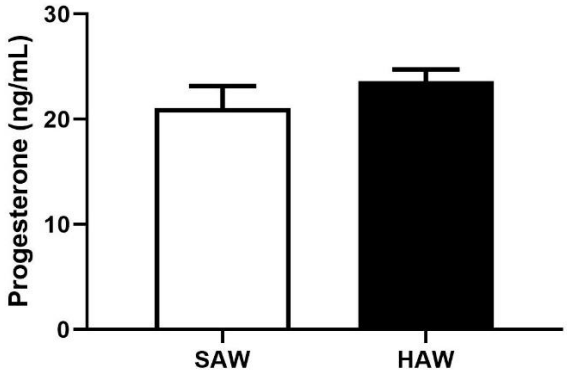

Figure 4.3. Serum progesterone concentrations in female mice consuming diet containing either ~63% w/w standard amylose wheat (SAW) or ~63% w/w high amylose what (HAW) on gestational day 17.5. Data are mean ± SEM. Comparison between groups was determined by Student *t*-test.

4.4.5 Maternal organ weights

There was no difference between diet groups in the weight of the ovaries, heart, pancreas, liver, kidneys or adipose tissue on GD 17.5 (Table 4.3). Dams fed the HAW diet had a 20% higher spleen weight compared to those fed the SAW diet ($p < 0.05$).

Table 4.3. Maternal organ weights on gestational day 17.5.

Organ weight (g)	SAW	HAW	*p*-value
Ovary (left)	0.01 ± 0.00	0.01 ± 0.00	0.160
Ovary (right)	0.01 ± 0.00	0.01 ± 0.00	0.604
Heart	0.13 ± 0.01	0.13 ± 0.00	0.598
Pancreas	0.13 ± 0.01	0.14 ± 0.02	0.539
Spleen	0.10 ± 0.01	0.12 ± 0.01	0.034
Liver	1.70 ± 0.06	1.82 ± 0.04	0.169
Kidney (left)	0.16 ± 0.00	0.16 ± 0.00	0.653
Kidney (right)	0.16 ± 0.01	0.17 ± 0.01	0.656
Parametrial fat	0.55 ± 0.02	0.55 ± 0.03	0.716
Perirenal fat	0.12 ± 0.01	0.14 ± 0.02	0.776

Data are means ± SEM (SAW=11 dams and HAW=14 dams). Significance between the standard amylose wheat (SAW) and high amylose wheat (HAW) groups was determined using Student *t*-test or Mann-Whitney test, as appropriate.

4.5 Discussion

To our knowledge, this is the first experimental animal trial to directly examine the effects of consuming a high HAW diet on female reproductive and pregnancy outcomes. The current study demonstrated that a high intake of HAW before pregnancy was associated with increased food intake and body weight in female mice. Mice consuming the HAW diet also spent longer in estrus and less time in diestrus over the course of the estrus cycle and exhibited increased pregnancy rates when compared to those consuming the SAW diet. The majority of pregnancy outcomes were not different between groups, although mice consuming the HAW diet exhibited a higher placental weight, and a lower fetal:placental weight ratio, when compared to the SAW group.

The increased food intake, observed in the female mice fed the HAW diet before pregnancy, is consistent with our previous work in adult mice fed increasing levels of HAW for eight weeks [148]. Unlike our previous study [148], however, the increased intake of HAW was also associated with increased weight gain and a higher body weight prior to pregnancy. This is not unexpected, given that the HAW diet contained higher levels of energy and nutrients, including protein, lipid and dietary fibre, compared to SAW [147, 148] and, therefore, higher intakes would be expected to promote an increase in both lean and fat mass. It is possible that the difference in effects on body weight observed between this and our previous study was due to the fact that the mice in the current study were juveniles at the start of the dietary intervention, whereas the previous study utilised adult animals [148]. Since the juvenile period is typically associated with a more rapid accumulation of body weight (both lean and fat mass) and increase in bone mineral density [246], the effect of the higher intake of food (and thus dietary energy) was likely to have been exaggerated in these younger animals. In addition, the difference in the sample size between the previous [148] and current studies (n= 8 vs n= 20) could

also be another possible reason for the different results observed for the effects of HAW on body weight.

Interestingly, dams fed the HAW diet did not exhibit a higher body weight during pregnancy, suggesting that they gained less weight during pregnancy compared to those fed the SAW diet. One possibility is that higher dietary intakes of HAW, which is known to have a lower GI, may help to prevent excessive weight gain during gestation. This is supported by a clinical study in which women adhering to a low GI diet from early pregnancy had significantly lower gestational weight gains compared to women consuming a standard (higher GI) diet [247]. Another possible reason is that the female mice fed the HAW diet had sufficient fat reserves and thus did not undergo the same accumulation of body fat during pregnancy compared to the SAW group, consistent with a previous rodent study that identified an inverse relationship between pre-pregnancy body weight and pregnancy body weight gain [248]. It is important to note that we did not assess body composition longitudinally in the mice in the current study and, therefore, it will be of interest to examine the body composition changes before and after a HAW diet intervention in future studies.

In mice, estrus is the phase of sexual receptivity and ovulation, while diestrus is the longest phase of the estrus cycle lasting for ~2 days, during which the animal is not receptive to mating [244]. The difference in the proportion of time spent in estrus and diestrus between the HAW and the SAW groups, in the current study, is consistent with findings of our prior study, in which females fed on diets containing 35-65% HAW spent less time in diestrus and longer in metestrus compared to those fed the SAW diet [148]. Taken together, these results raise the possibility that higher intakes of HAW may be associated with improved reproductive performance. Importantly, these observations are further supported by the finding in the current study that females in the HAW group

exhibited a higher pregnancy rate (as defined by the proportion of successful pregnancies after detection of a copulatory plug) compared to the SAW group. It is possible that this higher pregnancy success was due to the higher body weights of females in the HAW group at the time of mating, since a previous study has shown that dams with higher body weight and lean mass were more likely to achieve a successful pregnancy on their first breeding attempt [249]. Alternatively, it is possible that the greater nutrient density and other nutritional attributes of the HAW diet were an important factor in this improved reproductive performance. The B vitamins (folate and vitamin B6), antioxidant compounds (including ferulic acid) and hormonally active compounds (such as lignans), which are present at higher levels in whole compared to refined wheat [140, 141, 250], have been reported to improve female reproductive health and fertility, including improved ovarian function [129], oocyte quality [130] and rates of maturation, fertilisation and blastocyst formation in oocytes [251].

The observed effects of the HAW diet on the estrus cycle and pregnancy rates in the current study could be due to effects on the maternal immune system. This suggestion is supported, albeit indirectly, by our finding of higher spleen weights in dams fed the HAW diet in late gestation. Increased spleen weight has been consistently observed during pregnancy in previous studies in mice [252-254] and, in addition to expansion of the splenic red pulp to support the production of red blood cells [255], has been linked to an increase in the number of leukocytes [254, 256]. In the current study, the observed differences in the distribution of cell populations (including leukocytes) during the estrus cycle are consistent with shifts in immune cell populations, which has potential benefits for providing immune tolerance for sperm during intercourse [149], and thus play a key role in establishing fertilisation and implantation. Beneficial effects on the immune system could potentially be explained by the higher dietary fibre content of HAW, since

increased consumption of dietary fibre, including fermentable resistant starch, has been reported to favourably modulate the immune system, including increased production of immunoglobulins (antibodies) and Peyer's patches (gut-associated lymphoid tissues) [257]. However, further studies directly investigating the effects of HAW on immune parameters within the reproductive tract are needed.

While there was no effect of the HAW diet on the serum progesterone concentrations and the majority of pregnancy outcomes, including the number of viable fetuses, the number of implantation sites or reabsorptions, the HAW group exhibited a higher placental weight compared to the SAW group. Interestingly, however, the higher placental weight was not accompanied by increased fetal growth, which was unexpected given that placental weight is typically positively correlated with fetal and birth weights [258, 259]. This suggests that while higher intakes of HAW may have promoted placental growth, this was not associated with any accompanying improvements in placental nutrient transfer. The exact reason for this observation is unclear, but it is possible that the trend towards lower fetal growth in the HAW group was related to the lower glycaemic and insulinemic effect of the HAW [73]. Glucose plays a significant role in sustaining fetal growth and development [260], and if maternal glucose concentrations were lower on average in the HAW group this may have reduced fetal growth. This is supported by the results of clinical studies, which have reported lower birth weight and fetal growth rates in women who consumed a low GI diet during pregnancy [261, 262]. While the underlying mechanisms through which HAW may promote placental growth is still unclear, it is likely to be related to the greater nutrient density of the HAW, including higher protein content, since a previous gilt study reported that dietary supplementation with amino acids, including arginine, increased placental weights, in the absence of any effects on litter size and fetal weight [263]. However, further studies are

required to more comprehensively explore the effects of HAW consumption on placental function/efficiency, including placental structure (volume, surface area and shape) and nutrient transport capacity (e.g., expression of glucose and amino acid transporters).

4.6 Conclusion

This study demonstrated that a high intake of HAW in female mice before and during pregnancy improved conception rates and was associated with greater placental, though not fetal, growth. While these data support the potential role of HAW as a functional food to improve female reproductive health and pregnancy success rates, there is a need to exercise caution before proceeding to clinical studies, particularly given the apparently negative effects on fetal growth. Future studies focusing on improving our understanding of the effects of a high intake of HAW on the maternal immune system, and on the short- and long-term outcomes of the offspring, will also be important for the ultimate translation of these research findings.

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Conflicts of Interest

J.C. is Director of a company that has licenced the high amylose grain used in this study from the University of Adelaide with the intent of commercialisation. The other authors declare that they have nothing to disclose. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Chapter 5

General Discussion

5.1 Summary and Implications of Findings

Prior to this thesis, while a small number of studies had supported the suggestion that consuming high amylose wheat (HAW) had potential benefits for metabolic [73, 264] and gastrointestinal health [65, 82, 265], there was limited understanding of whether and to what extent these effects varied according to the level of HAW consumed, and no studies had determined whether these effects differed between males and females. In addition, while improved dietary quality, including increased intake of whole grains, had been associated with improved reproductive health outcomes [120], the potential impact of HAW on reproductive health had also not been investigated. The studies presented in this thesis have added substantial new knowledge to our understanding of the health benefits of HAW and provided new insights into the underlying mechanisms through which HAW consumption affects metabolic, gastrointestinal and reproductive health in males and females.

Metabolic and gastrointestinal health

Across all studies, and in both male and female mice, the consumption of HAW was generally associated with an increase in body weight, and this was most pronounced in adult male (Chapter 2) and in juvenile female (Chapter 4) mice. Importantly, however, and despite differences in the effects of HAW on body weight between adult males and females (Chapter 2), HAW consumption was consistently associated with a reduction in relative fat mass, particularly at the highest level of consumption. This suggests that the body weight gain was due to increased lean mass or bone mineralisation, rather than fat

deposition. Therefore, determining the effect of HAW consumption on body composition and bone mineral content would be a valuable area for investigation in future studies.

Similarly, in both males and females, the consumption of HAW was also associated with an increase in gastrointestinal organ weight, particularly the weight of the colon at the highest level of HAW consumption (Chapter 3). The increased gastrointestinal organ weight could be due to the increased proliferation of the intestinal epithelial cells, which would be expected to reduce gut atrophy and improve gastrointestinal function [213].

The studies presented in this thesis also provided insights into potential mechanisms that could underlie the effects of HAW on body weight, adiposity and gastrointestinal organ weight. Furthermore, the results obtained suggested that some of these mechanisms were similar between male and female animals, while some were different. A summary of potential mechanisms and explanation of how they could account for the observed effects of the HAW diet is summarised below:

a) *Altered gut bacterial load and composition*: In both males and females, higher HAW intakes were associated with significant increases in gut bacterial load and alterations in microbial composition. The changes observed in response to HAW consumption would be expected to enhance gut ecosystem stability and resilience [264]. This would, in turn, be expected to increase the conversion of dietary nutrients into metabolites which promote the proliferation of the intestinal epithelial cells and could thereby account for the increase in gastrointestinal organ weight [264].

b) *Reduced Firmicutes:Bacteroidetes ratio*: This was observed in both males and females at the two highest levels of HAW consumption. Since a lower Firmicutes:Bacteroidetes ratio is associated with a reduced efficiency for energy

absorption [265], this could have resulted in lower uptake of energy (in particular lipids) from the diet, and therefore reduced fat deposition. In addition, a lower Firmicutes:Bacteroidetes ratio is associated with lower lipopolysaccharide levels, and thus inflammation, in the gastrointestinal tract, which would be expected to promote proliferation of the intestinal epithelial cells and, hence, could also contribute to the increased gastrointestinal organ weight [266]. A lower Firmicutes:Bacteroidetes ratio is also associated with weight loss and improved metabolic health in both animal models and humans, and could therefore be associated with improved metabolic health outcomes in the longer term [267].

c) *Increased gastric emptying rate (in female mice only)*: An increased rate of gastric emptying would be expected to be associated with a faster passage of food through the digestive system, which would, in turn, result in less absorption of nutrients in the gut. This could, in turn, reduce energy uptake from the diet and, consequently, reduce fat deposition. A lower fat deposition, particularly abdominal fat deposition, would be expected to be associated with improved metabolic health in the longer term [268].

d) *Increased food intake and respiratory quotient (RQ)*: In female mice, consumption of the lowest level of HAW was associated with a high food intake, which contributed to an increased RQ consistent with increased utilisation of carbohydrate as an energy source (reduced fat oxidation). Since the diet consumed by the mice was high in carbohydrate, this could promote greater storage of glucose as glycogen within the muscle and prevent muscle breakdown and thus account for the increased weight gain in these mice [269].

e) *Increased expression of ocln mRNA*: In female mice, the highest level of HAW consumption was associated with a higher abundance of *ocln* mRNA. Since this

gene encodes a protein that acts to enhance intestinal barrier integrity, this would be expected to reduce gut permeability and thus prevent bacterial and endotoxin translocation, thereby resulting in reduced local and systemic inflammation [270]. This would, in turn, be expected to promote the growth of the intestinal epithelial cells and, hence, could have contributed to the increased gastrointestinal organ weight [266].

f) *Increased expression of pyy mRNA*: This was observed in female mice at the highest level of HAW consumption. Since this gene encodes the gut hormone pyy, which acts to inhibit intestinal motility, this would be expected to increase short-chain fatty acid absorption within the gastrointestinal lumen, and thereby promote proliferation of the intestinal epithelial cells and improve gastrointestinal structural markers [271].

A diagram summarising these potential underlying mechanisms, and how they could account for the phenotypic effects of the HAW diet observed in our study, is shown in Figure 5.1. Given the close relationship between the gut microbiome, the inflammatory environment in the gastrointestinal tract and systemic inflammation, several of the potential mechanisms involve effects on the immune system. Consequently, determining the effect of HAW consumption on immune/inflammatory markers would be a valuable area for future investigation.

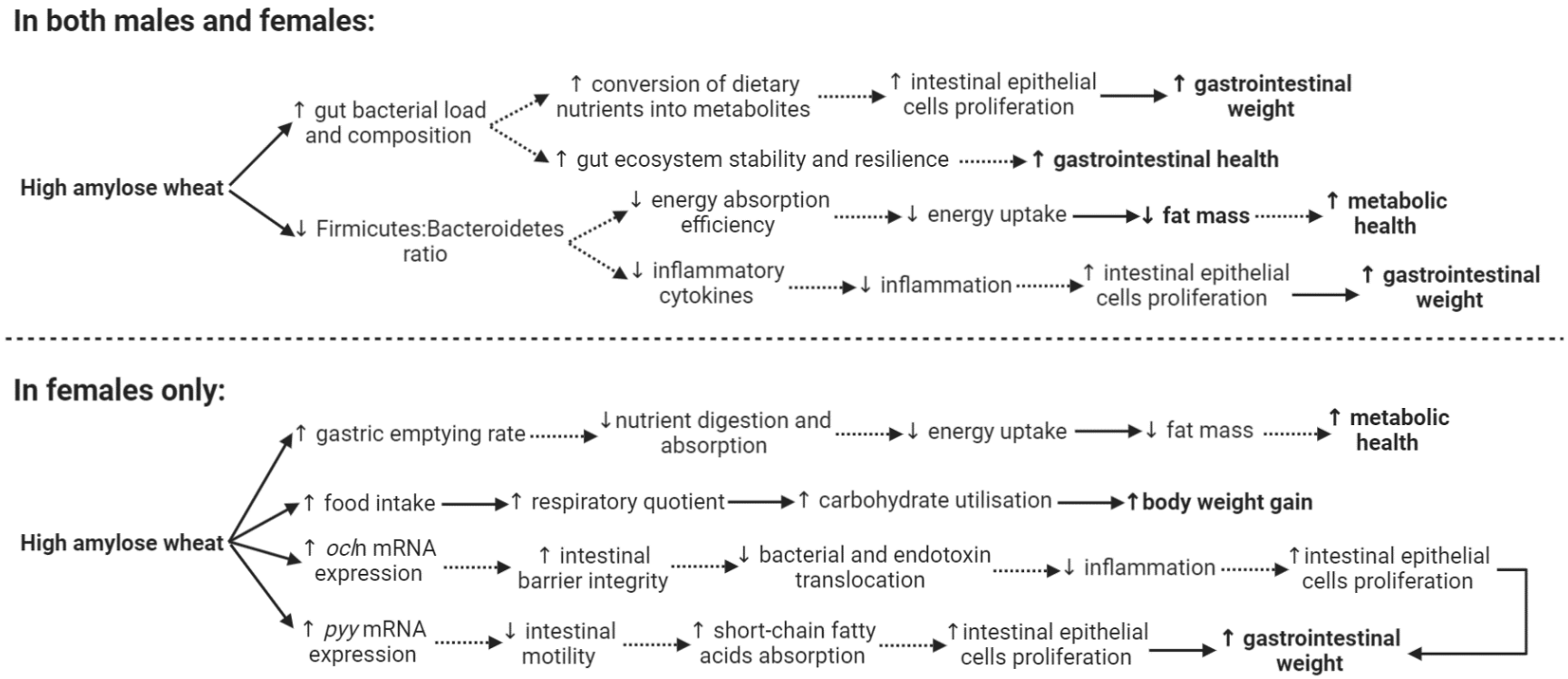

Figure 5.1. Potential underlying mechanisms for the changes on body weight, adiposity and gastrointestinal organ weight in mice consuming high amylose wheat diet. Solid arrow = result presented in this thesis; dashed arrow = proposed mechanism.

Sex differences

The sexually dimorphic responses to HAW consumption for several metabolic (Chapters 2) and gastrointestinal (Chapter 3) parameters could be due to differences between males and females in energy homeostasis, substrate metabolism, body fat distribution and circulating concentrations of sex hormones [168, 169]. In general, the effects of HAW on gastrointestinal organ weights were more pronounced in females compared to males. This could potentially be accounted for by sex differences in gastrointestinal function. For example, compared to females, males tend to mobilise energy stores more rapidly, utilise carbohydrates in preference to lipids as an energy source and store adipose tissue in abdominal areas, all of which could influence how they metabolise HAW [272]. In addition, since females have a higher level of progesterone, which is known to slow down peristalsis in the large intestine, this would, in turn, be expected to be associated with a longer fermentation and increased absorption of short-chain fatty acids that promote the proliferation of the intestinal epithelial cells, and, hence account for the more pronounced effects of HAW consumption in increasing gastrointestinal organ weight in females compared to males [211, 212]. Overall, it will be important in future research to take into consideration the sex of the animal when evaluating the effects of HAW, since it may not be appropriate to extrapolate results obtained in one sex to the other.

Level of HAW differences

In general, the results presented in this thesis suggested that the highest level of HAW consumption was associated with the greatest beneficial effects on metabolic and gastrointestinal parameters in both male and female mice. This was not unexpected, since the diets containing the higher levels of HAW also contained higher levels of the beneficial nutrients present in HAW, including resistant starch, proteins, vitamins and minerals [148]. An

interesting and unexpected finding, however, that the increase in RQ in female mice consuming HAW diets was only observed at the lowest level of HAW. This was largely accounted by the higher food intake in these mice, and the diet with the lowest level of HAW had the highest level of available carbohydrate, which might therefore have promoted the utilisation of carbohydrate as an energy source (Chapter 2). This diet also contained less dietary fibre than the other HAW diets, which could have further contributed to the increased carbohydrate utilisation.

The studies presented in this thesis are the first to directly compare the metabolic and gastrointestinal effects of different levels of HAW and suggest that these effects can differ considerably according to the level of inclusion in the diet. Therefore, the level of HAW consumption should not only be factored into future study designs but also an important consideration when translating the findings into clinical studies.

Reproductive health

Positive effects of HAW consumption on markers of reproductive health were observed in both males and females, including increased testicular weights and a shift in the estrus cycle towards a longer duration in estrus/metestrus, less time in diestrus and improved pregnancy rates (Chapters 2 and 4). The greater nutrient density of the HAW possibly may have contributed to this improved reproductive health and performance, since the B vitamins (folate and vitamin B6), antioxidant compounds (including ferulic acid) and hormonally active compounds (including lignans), all of which are present at higher levels in HAW compared to standard amylose wheat (SAW), have been reported to improve reproductive health and fertility [129, 273]. In addition, the higher dietary fibre of HAW compared to SAW could favourably modulate the immune system through increased production of immunoglobulins (antibodies) and the Peyer's patches (gut associated lymphoid tissues). This, in turn, would be

expected to reduce inflammation, improve ovarian follicle growth and ovulation rates and thus improve reproductive health and fertility outcomes [257, 274]. However, further research is needed to provide direct evidence of the effects of HAW on inflammation and reproductive health and this would be a valuable avenue for further studies.

While consuming HAW before and during pregnancy promoted placental growth, there was no suggestion that fetal growth was improved, suggesting that the placental growth was not accompanied by improvements in placental nutrient transfer. The exact reason for this finding is unclear, but it is possibly due to the lower glycaemic effect of the HAW, which would cause a lower and slower rise in blood glucose [73]. While this could be beneficial for glucose control, a lower average maternal glucose concentration during pregnancy, particularly in late gestation, could also reduce the efficiency of glucose transfer across the placenta (since this occurs via a concentration gradient). This, in turn, could result in lower fetal glucose concentrations and metabolism and thus reduced fetal growth [275]. Further studies that directly examine the effects of HAW on the efficiency of glucose transport across the placenta are needed to verify this hypothesis.

An overall summary of the effects of high amylose wheat on metabolic, gastrointestinal and reproductive outcomes in male and female mice is shown in Figure 5.2.

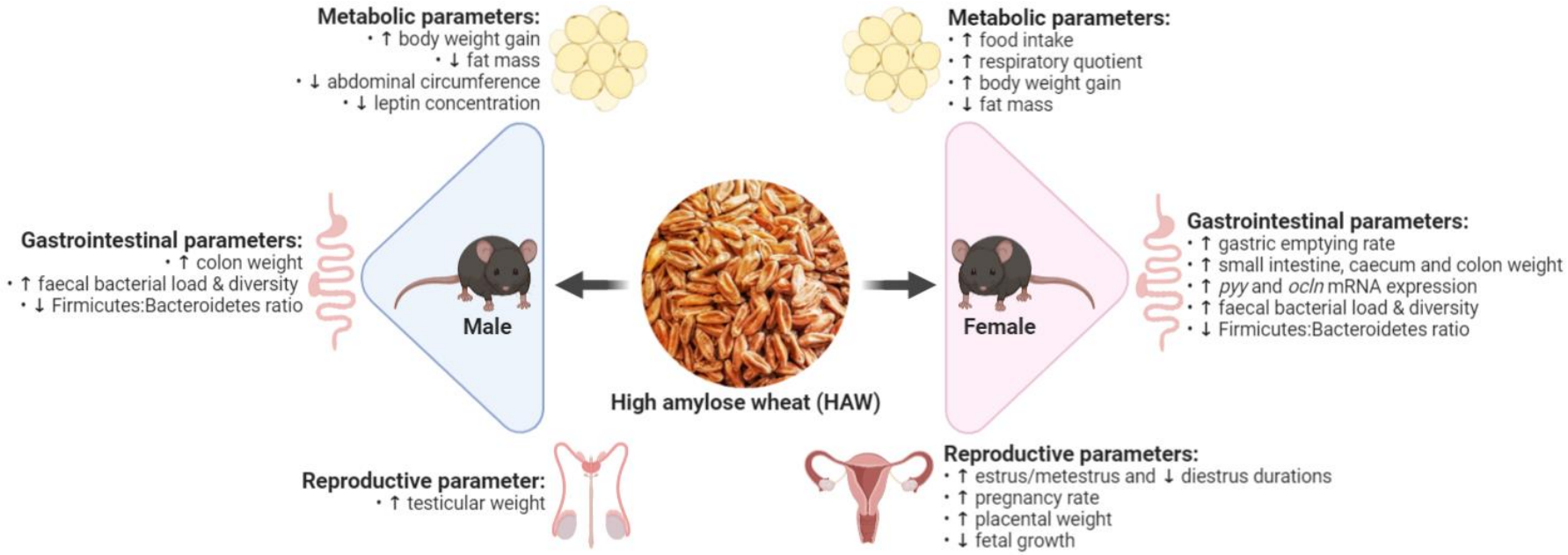

Figure 5.2. Summary of the effects of high amylose wheat on metabolic, gastrointestinal and reproductive outcomes in male and female mice[1].
[1]Figure was produced using Biorender (https://biorender.com).

5.2 Limitations and Recommendation for Future Research

While the results presented in this thesis have improved our understanding of the impact of higher levels of HAW consumption on several aspects of human health, including metabolic, gastrointestinal and reproductive health, there are several limitations and further studies in this area are needed to build on our research findings. The limitations of the current research studies, and recommended areas for future research are described below:

a) The animal trials in this thesis were performed over a relatively short time period. For example, the dietary feeding periods were only up to eight weeks in duration. While this was appropriate for initially exploring the potential benefits of HAW for health outcomes, and to justify longer studies, it does not allow us to draw clear conclusions as to the potential impact of longer periods of HAW consumption on the health aspects identified in this thesis. Therefore, undertaking studies in which the HAW diets are consumed for substantially longer periods (months) will be important to provide further insights as to the potential health benefits (and any negative effects) of consuming this type of wheat over extended periods.

b) The mice utilised in this thesis were lean and metabolically healthy. While this provides information about the impact of HAW in healthy populations, it may not apply to individuals who are metabolically unhealthy. Further research is needed to determine the potential therapeutic effects of HAW in animal models of cardiometabolic diseases, such as diet-induced obese rodent models. Any potential positive outcomes from this model would, in turn, provide an alternative potential management approach for populations with metabolic dysregulation.

c) In both males and females, consumption of HAW was associated with increased body weight gain but not increased fat mass, which leads to the hypothesis that the increased body weight gain could be due to increased lean mass and, potentially, bone mineral mass. However, further research to directly measure body composition by Dual-energy X-ray Absorptiometry is required to ascertain the effect of HAW on lean mass, fat mass, bone mineral density and bone mineral content.

d) Further studies to explore the potential impact of HAW on male reproductive health, particularly sperm production and quality, sex hormone concentrations and the ability to impregnate females, will be important to extend the findings of Chapter 2 and assess the potential benefits of HAW for male reproductive health.

e) Due to delays in reagent and consumable supply chains, which occurred as a result of COVID-19, we were unable to complete the fetal sex determination analyses for Chapter 4. Since previous studies have shown that effects of maternal dietary interventions often vary between male and female fetuses [276, 277], it will be important to include measures of fetal sex (and evaluate effects in male and female fetuses separately) in this work.

f) In Chapter 4, while we have indicated that higher intakes of HAW before and during pregnancy were associated with improved placental growth, the potential effect of HAW on placental function/efficiency is still unclear. Further studies, including analysis of placental structure and nutrient transport capacity such as mRNA expression and protein abundance of glucose and amino acid transporters, will be important to extend our understanding of the effects of HAW on the placenta.

g) Unlike the studies presented in this thesis, all previous animal studies have been conducted exclusively using unprocessed and uncooked HAW [65, 82, 265]. While this has provided evidence of the potential benefits of HAW for health outcomes, it limits the potential to extrapolate the findings to humans as it does not reflect how humans typically consume grains which involve gelatinisation (cooking) and retrogradation (cooling) processes. Therefore, further studies are recommended to take this aspect into consideration in order to extrapolate the findings from animals to humans.

h) Since most grain products are consumed in a highly processed and refined form, principally due to cultural and textural preferences and to increase shelf life of the grain products, it would be worthwhile to investigate the effects of milled HAW flour on the parameters assessed in this thesis. Further studies on this aspect would provide insights as to whether the HAW consumed in refined form could also provide beneficial effects.

i) While the results of this thesis have provided important insight of the effects of HAW on several human health aspects in both males and females, the limitations of preclinical research, including differences that exist between mice and humans such as metabolic rate, glucose and lipid metabolism, feeding behaviour and physiology of the gastrointestinal tract and pregnancy, are important to acknowledge. Therefore, pilot-scale human studies are recommended to validate whether consumption of higher levels of HAW will produce similar positive effects to those identified in this thesis, before the wide-spread application of HAW and its incorporation into functional food products on a large scale.

5.3 Conclusion

The results of this thesis have demonstrated positive effects of HAW consumption on metabolic, gastrointestinal and reproductive health in both male and female mice, although these effects were also dependent on the level of HAW in the diet and the sex of the animal. While there is a need for further studies to confirm the results, and define underlying mechanisms, these findings suggest that replacing SAW with HAW flour offers a promising and practical approach for improving health outcomes in human populations. Consumers could more easily adhere to this approach as it does not require a dramatic change in their dietary intake/eating pattern. Furthermore, since wheat is a versatile ingredient for making various staple foods, including bread, breakfast cereals, biscuits and noodles and demand for wheat products is increasing globally, this dietary intervention could potentially reach broader populations across the world.

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