VARIATION IN AGE AT MENARCHE AND ADULT REPRODUCTIVE FUNCTION: THE ROLE OF ENERGETIC AND PSYCHOSOCIAL STRESSORS

BY

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DISSERTATION

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Abstract

Reproductive ecology has long examined the flexibility of women's reproductive function in the face of variable environments. The timing of a woman's first menses, or age at menarche, is both used as a proxy of childhood stressors and correlated with adolescent and adult reproductive function. This research seeks to understand the connections between childhood environments, pubertal timing, and adult reproductive function, and I specifically 1) identify a need for including social support measures in studies of pubertal timing through empirical evidence that positive parental-child interactions affect age at menarche, 2) demonstrate that psychosocial and energetic stressors experienced during childhood correlate with pubertal timing and adult reproductive function, 3) compare timing of menarche and variation in reproductive hormones between two populations with similar geographic origins but different subsistence environments, and 4) investigate gene methylation as a potential mechanism mediating the relationship between key stressors and reproductive function.

I first investigated relationships between parent-adolescent communication and age at menarche in a diverse sample of 128 post-menarcheal, American girls aged 12-17. I found that measures of close family relationships, specifically open communication with parents, were correlated with age at menarche in this sample. I further found that mother-adolescent and father-adolescent open communication scores had opposing directional effects on menarcheal timing. These findings suggest that maternal and paternal communication may signal different things about the developmental and reproductive environment. This research underscores the importance of including measures of family support in future studies of adolescent reproductive trait timing, as well as the consideration that positive psychosocial factors, rather than only negative psychosocial factors, may be associated with accelerated menarcheal development.

I further investigated the secular trend of declining ages at menarche in in the rural Beskid Wyspowy region of southern Poland and investigated relationships between childhood stressors, age at menarche, and adult reproductive function. I found that age at menarche has declined over time in rural Poland. Helping on farms and with farm animals as a child were associated with later ages at menarche. Women with a higher number of adverse childhood experiences tended to have earlier age at menarche, although this difference was not statistically significant. Despite different effects on age at menarche, all types of childhood stressors were associated with lower adult E1G concentrations. The results of this study support a model under which developmental conditions affect adult reproductive function, but challenge the use of age at menarche alone as a proxy for childhood stressors.

I compared differences in reproductive traits between women in rural Poland and Polish American women in urban areas of the United States. We found that ages at menarche are earlier in Polish American women. Further, there is a generational effect where Polish American women whose parents were born in Poland did not have significant difference in average ages at menarche compared to women in Poland, but Polish American women whose grandparents were born in Poland had earlier ages at menarche compared to women in Poland. Additionally, we found that the Polish American sample had a significantly longer average luteal phase length compared to the Polish sample. These differences may indicate that, while rural Poland is undergoing many transitions, there are still environmental stressors affecting reproductive function.

Finally, I investigated gene methylation as a potential mechanism mediating the relationship between stress and reproductive function. Epigenetic traits, like gene methylation, are modified by early environmental variables, and, thus, gene methylation is a likely mediator

connecting early life environments with timing of menarche and adult reproductive hormones. I found that methylation at a promoter of the gene coding for aromatase (CYP19A1) along with farming during childhood significantly predicted age at menarche in a rural Polish population. We further identified a potential pathway by which increased cortisol concentrations increases CYP19A1 promoter I.4 methylation, which likely decreases aromatase activity and downstream estrogen and estrone concentrations. This pathway adds another potential pathway helping to explain inter-individual differences in estrogen concentrations. Overall, the results of this research underscore that epigenetic factors like gene methylation play a role in reproductive ecology and may provide a piece of the lacking intermediate structure between early environmental experiences and reproductive traits.

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Chapter 1

Introduction

Research Objectives

I research variation in women's reproductive physiology, and I seek to understand the connection between childhood environments, pubertal timing, and adult reproductive function. My projects combine classic anthropological and cutting-edge epigenetic techniques to explore variation in pubertal timing. I integrate life history survey-based data, degree of methylation at a candidate gene, and reproductive hormone concentration data. My research advances the breadth of knowledge on if, how, and which early environmental variables affect adult reproductive function. I conduct this research in a diverse sample of U.S. adolescents and a comparative sample of women living in Poland and Polish American women living in the United States.

The major contributions of my work are to: 1) identify a need for including social support measures in studies of pubertal timing through empirical evidence that positive parental-child interactions affect age at menarche, 2) demonstrate that psychosocial and energetic stressors experienced during childhood correlate with pubertal timing and adult reproductive function, 3) compare timing of menarche and variation in reproductive hormones between two populations with similar geographic origins but different subsistence environments, and 4) investigate gene methylation as a potential mechanism mediating the relationship between key stressors and reproductive function. In this introduction, I discuss why age at menarche is important, the variables influencing timing of menarche, the effects of timing of menarche, and epigenetic modifications as a potential mechanism linking the environment to reproductive outcomes.

Why is age at menarche important?

Ages at menarche have been declining globally since 1850 (Tanner, 1981). This global secular trend of earlier ages at menarche has been largely attributed to environmental influences, including childhood nutrition and healthcare and lowered energetic and immune stress (Ellison, 2001; Walvoord, 2010). The trend attracted attention in public health due to the association of early menarche with risk of health and reproductive outcomes, including an increased risk of breast cancer (Helmrich et al., 1983; Jasienska, 2001), endometriosis (Nnoaham et al., 2012), and cardiovascular disease (Remsberg et al., 2005). Earlier ages at menarche are also associated with an earlier age at first coitus and first pregnancy (Urdry and Cliquet, 1982; Belsky et al., 1991; Boothroyd et al., 2013), as well as risk for adolescent depression (although see Angold et al., 1999; Allison and Hyde, 2013). The risks of a later age at menarche are less well-studied, but a later age at menarche is associated with increased risk for osteoporosis (Chevalley et al., 2008; Devlin et al., 2010), social anxiety (Siegel et al., 1999), and depression (Stice et al., 2001). Social reactions to female puberty vary globally; however, girls at this age are often subjected to menstrual taboos, dress codes, activity limitations, and increased supervision, any of which might contribute to the associations between age at menarche and risks for depression and anxiety (Siegel et al., 1999; Allison and Hyde, 2013).

In addition to health and social consequences, the timing of menarche is also associated with reproductive function. Adolescent menstrual cycles are quite variable and often anovulatory (ACOG, 2006). A younger age at menarche is associated with reaching a greater frequency of ovulatory cycles faster than girls with older ages at menarche (Apter and Vihko, 1983). Adult estradiol concentrations are also higher for girls with early menarche (Kirchengast and Hartmann, 1994; Emaus et al., 2008). In some populations, age at menarche is also associated

with measures of fertility. Borgerhoff Mulder (1989) found that Kenyan Kipsigis women with a menarcheal onset at ages 12-14 had approximately three more children than those with an age of menarche at 16 or later. Finally, early menarche is also associated with adverse reproductive outcomes including higher offspring mortality among Pumé women in Venezuela (Kramer, 2008) and higher rates of preterm delivery among women in Wuhan, China (Li and Zhou, 1990). The consequences of both earlier and later than average ages at menarche suggest that this age is important and potential useful health marker (ACOG, 2016).

However, there are a few issues with using age at menarche as a health marker. For example, age at menarche varies within and between populations. Average age at menarche is typically deemed normal, with two standard deviations away from the mean indicating either precocious (early) puberty or delayed puberty (Palmert and Boepple, 2001). Table 1.1 displays average ages at menarche in four select countries: The U.S., Poland, Bangladesh, and Great Britain. There are differences between average ages at menarche within and between these populations (Rogers, 2016). The within and between population variation makes it difficult to use age at menarche as a simple reproductive health indicator. This would be especially important in areas with high levels of migration.

Country	Population	Average Age at Menarche	Study Year	Source
USA	non-Hispanic White	12.52	1999-2002	Anderson and Must, 2005
	non-Hispanic Black	12.06	1999-2002	Anderson and Must, 2005
	Mexican	12.09	1999-2002	Anderson and Must, 2005
Poland	Urban	12.56	2000	Popławska et al. 2013
	Rural Poland: Higher SES	12.98	2001	Łaska-Mierzejewska and Olszewska, 2004
	Rural Poland: Lower SES	13.45	2001	Łaska-Mierzejewska and Olszewska, 2004
Bangladesh	Rural	12.8	2005	Rah et al. 2009
	Urban	12.5	2011	Houghton et al. 2014
Great Britain	British-Bangladesh	12.1	2010	Houghton et al. 2014
	White	12.6	2010	Houghton et al. 2014

Table 1.1: Average ages at menarche in different populations living within four select countries: United States, Poland, Bangladesh, and Great Britain. There is considerable variation within and between populations.

Variation in age at menarche: a life history perspective

From a life history perspective the decrease in average ages at menarche has been attributed to tradeoffs in growth and reproduction (Ellison, 2001; Forman and Mangini, 2013). This alternative perspective recognizes the social and biological consequences of variation in pubertal timing, but also argues that both earlier and later maturation can be adaptive in different contexts. A life history framework seeks to understand how evolutionary forces have shaped organisms to use limited resources to optimize survival and reproductive success (Stearns, 1989; Charnov, 1993). Life history theory posits that there are trade-offs between somatic investments in growth, reproduction, and survival (Stearns, 1989). Menarche is one visible, memorable marker of the transition from somatic investment in growth to reproduction and as such is a highly studied life history trait (Ellis, 2004; Ellison et al., 2012). Within this framework, normal variation in age at menarche can be viewed as an adaptive response to varying developmental environments. Two commonly studied sources of this variation include energetic and

psychosocial stressors, which tend to delay and accelerate age at menarche respectively (Ellison, 2001; Ellis, 2004).

The energetics hypothesis posits that menarcheal age should be delayed in environments with low energy status, negative energy balance, or high energy flux, where extending the period of growth might be beneficial in the face of high energetic constraint (Ellison, 2001; Reiches et al., 2013). For example, girls who experienced food deprivation during childhood in France and girls who experienced food insecurity during adolescence in Ethiopia had later ages at menarche (Belachew et al., 2011; Dossus et al., 2012). Girls who participate in intensive sports resulting in negative energy balance have later ages at menarche (Georgopoulos et al., 2010). The energetics hypothesis has been supported in a wide variety of contexts; energetic constraints seem to pull investment away from reproductive effort and towards growth, maintenance, and/or survival.

On the other hand, psychosocial stressors may also pull resources away from reproduction. According to the psychosocial acceleration hypothesis, which also draws on life history theory, age at menarche should be accelerated in risky or uncertain psychological, social, or physical environments where a long lifespan is not assured (Belsky et al., 1991; Ellis, 2004). Experiences like conflict in the home (Jorm et al., 2004) and sexual abuse (Wise et al., 2009; Boynton-Jarrett and Harville, 2012) are associated with earlier ages at menarche. Father absence is also correlated with earlier menarche (Ellis and Garber, 2000; Boothroyd et al., 2013; Jones, 1972) and is particularly well-studied (see Webster et al., 2014). For example, a greater number of years without a father was associated with earlier ages at menarche in a sample of Canadian girls (Surbey, 1990).

Energetic stressors are typically associated with later ages at menarche, and psychosocial stressors are typically associated with earlier ages at menarche. A lack of energetic stress, or

greater energetic availability, would then likely allow for faster growth, larger body size, and earlier ages at menarche (Huss-Ashmore and Johnston, 1985). However, both rich and poor environments and both high socio-economic status and increased social stress can lead to a decreased age at menarche. For example, one might expect poor environmental conditions to be associated with later ages at menarche; however, these conditions alongside high mortality risk is associated with accelerated growth rates and decreased ages at menarche (Walker et al. 2006). Further, a high socio-economic status is associated with a lowered age at menarche in populations where there are disparities between social classes (Adair, 2001), but father absence and low socio-economic status is also associated with earlier ages at menarche (Ellis et al. 2005). Disparate influences can affect the age at menarche in a similar way, which complicates a potential use of age at menarche alone. Figure 1.1 displays energetic and psychosocial variables associated with age at menarche.

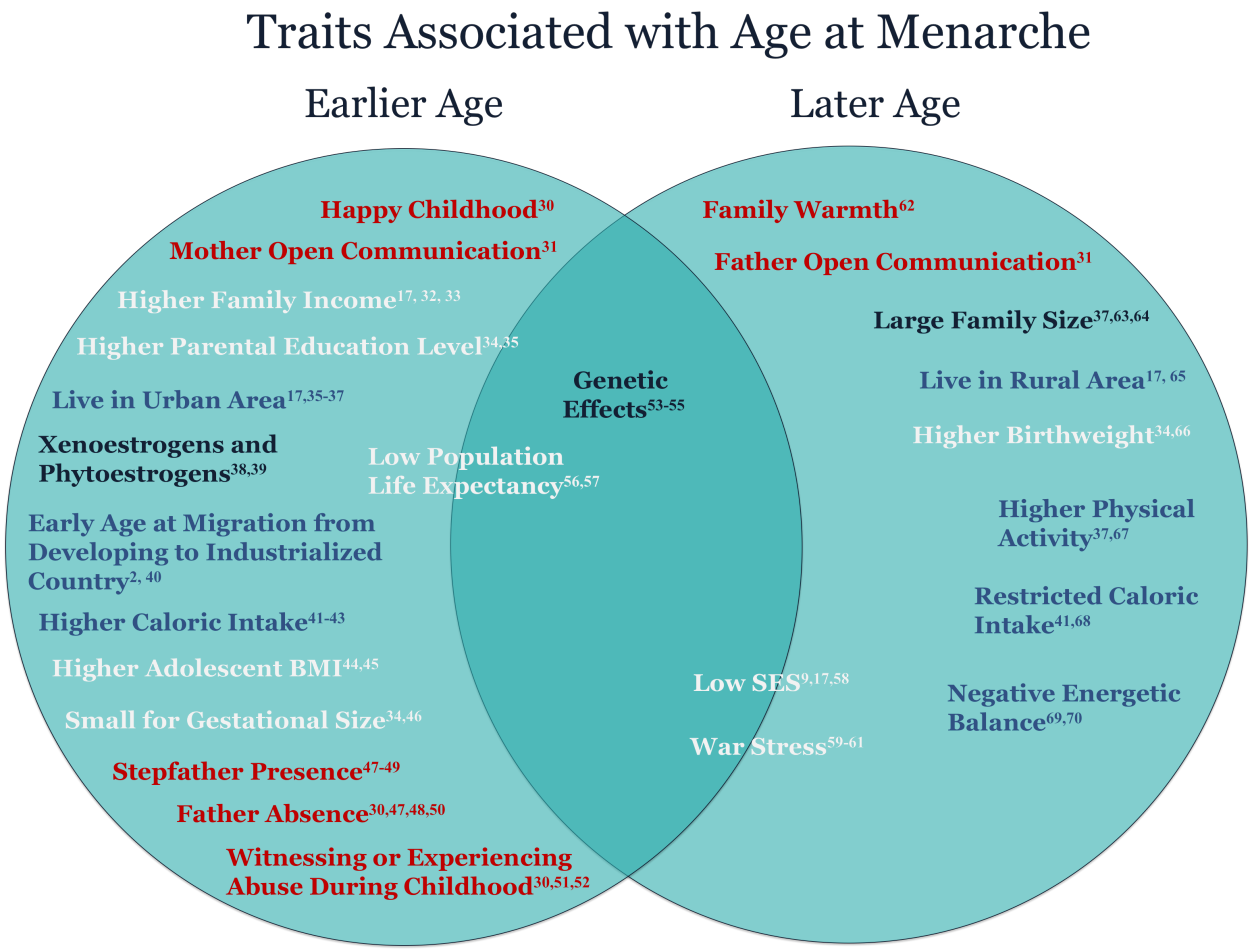

Figure 1.1: Traits associated with earlier and/or later ages at menarche (Figure reproduced from Rogers, 2016). Variables associated with earlier and/or later ages at menarche are color coded as follows: red for psychosocial variables, blue for energetic variables, white for variables that could be considered either psychosocial or energetic.

While the relationship between energetic variables and age at menarche is typically consistent, the relationship between psychosocial variables and age at menarche is less consistent. For example, women who report experiencing a happy childhood had earlier ages at menarche in one study (Jorm et al., 2004), but parental approval and warmth correlated with later ages at menarche in a different study (Graber et al., 1995). As previously mentioned, the relationship between father absence and earlier ages at menarche is particularly well-studied. Most studies use this relationship to provide support for the psychosocial acceleration

hypothesis. I contend that this relationship, and the relationship between psychosocial variables and age at menarche, is likely context-dependent.

Critique of the psychosocial acceleration hypothesis

The relationship between psychosocial variables and age at menarche is less consistent compared to the relationship between energetic variables and pubertal timing, meaning that a psychosocial variable that is associated with age at menarche in one population may not be associated with age at menarche in another group. Overall, there are a few key limitations in studies that provide key evidence supporting the psychosocial acceleration hypothesis. Common issues oft-recognized by study authors is non-random sampling and small sample sizes, as well as other limitations in project design (Sohn, 2017). For example, many studies do not, or cannot, include maternal age at menarche. This is important due to the high heritability estimates of menarcheal timing (Towne et al., 2005). One study investigating the relationship between psychosocial stressors and age at menarche found that controlling for maternal age at menarche resulted in a null relationship between psychosocial stressors and age at menarche (Campbell and Udry, 1995). Another example of an issue affecting key evidence for the psychosocial acceleration hypothesis is the overwhelming focus on father absence in the literature. Such a reliance on father absence as a main piece of evidence for the psychosocial acceleration hypothesis is insufficient as father absence is often ill-defined and there are inconsistent results globally, indicating that father absence is likely a proximate variable for other types of stressors.

First, father absence is often ill-defined, left undefined, or defined differently from study to study (Anderson, 2015). There are many types of father absence and father presence does not necessarily indicate a socially supportive environment. There may be many reasons for father absence including death, divorce, or migrant work. A recent study concluded that different types

of father absence have different relationships with age at menarche; specifically, girls whose fathers were absent due to divorce had earlier ages at menarche, while girls whose fathers were absent due to death or migrant work had later ages at menarche (Shenk et al., 2013). By not defining types of father absence, we may neglect variation in the proximate stressor that father absence indicates.

Second, father absence does not predict age at menarche in developing countries including South Africa, Malaysia, and Indonesia (Sheppard et al., 2014; Anderson, 2015; Sohn, 2017). The majority of studies demonstrating a relationship between father absence and early menarche, come from industrialized nations. High-income countries represent a "WEIRD" group - Western, educated, industrialized, rich, and democratic; these WEIRD countries are over-represented in the literature and not necessarily representative of global trends (Henrich et al. 2010). As an example, no relationship has been found between father absence and reproductive traits in Malaysia (Sheppard et al., 2014). Sheppard et al. (2014) suggest that the culturally-specific role of fathers needs to be taken into account in these types of studies. Further, father absence also did not associate with age at menarche in a representative sample of women in South Africa (Anderson, 2015), and there was no relationship between age at menarche and father absence or mother absence in a large study of 11,138 women in Indonesia (Sohn, 2017).

Thus, age at menarche may only be responsive to father absence in some social contexts in which father absence is actually a proxy for another type of stress. Multiple hypotheses have been put forward to test this idea. Specifically, Romans et al. (2003) found a correlation between father absence and childhood abuse, and they proposed that this association better explains accelerated ages at menarche than paternal absence. Ellis and Garber (2000) found that family interpersonal stress and father absence mediated the relationship between maternal mood

disorders and earlier ages at menarche, proposing instead that parental psychopathology may be driving earlier ages at menarche.

The first studies describing a relationship between father absence and reproductive traits started in the early 1970s (i.e. Jones et al. 1972), coinciding with growing societal concern at the time with the effects of divorce and fatherlessness (Lamb, 2000). Contemporary family studies research moves beyond categorizing fathers as moral guides, primary breadwinners, and masculine role models, and rather recognizes the interactions among the multiple roles a parent can play (Lamb, 2000). Additionally, mother absence does not predict earlier ages at menarche (Bogaert, 2005; Sheppard et al., 2014), and the childcare and social support provided to single fathers differs from the social stigma surrounding single motherhood (Sheppard et al., 2014). The interest in father absence in particular may be one example of subjectivity and personal bias affecting the research process (subjectivity in science discussed in Finlay and Gough, 2000). While some early studies, and now more recent studies (see chapter 2), included positive measures of family warmth, happiness, and social support, the main emphasis of this field has been on social stressors. I contend that it is time to move beyond a simple father absence model, seek what father absence may represent in industrialized societies, and include measures of social support in studies evaluating or engaging with the psychosocial acceleration hypothesis.

Adverse childhood experiences as a measure of psychosocial stress

A growing body of literature focuses on the association between adverse childhood experiences (ACEs) and a variety of outcomes including risk for autoimmune diseases (Dube et al., 2009), ischemic heart disease, and multiple types of cancer (Anda et al., 2010). However, the application of research of ACEs to studies on fertility has been quite limited. A higher number of ACEs indicates a higher psychosocial stress experienced during childhood, and ACEs have been

used in multiple different populations. The ACE questionnaire includes a question about one type of father absence (divorce), but it also includes additional types of stress a child may encounter, making ACEs a more encompassing and standardized questionnaire compared to father presence/absence.

Individual ACEs, specifically sexual and physical abuse, are associated with ages at menarche (Romans et al., 2003; Zabin et al., 2005; Wise et al., 2009; Boynton-Jarrett et al., 2013; Harville and Boynton-Jarrett, 2013) and self-reported infertility (Jacobs et al., 2015). Interestingly physical abuse and sexual abuse might not have the same effect on fertility: while sexual abuse is associated with earlier ages at menarche, physical abuse is associated with both earlier and later ages at menarche (Boynton-Jarrett et al., 2013). This seemingly dichotomous results might return to the likely unnecessary divide between the psychosocial acceleration hypothesis and the energetics hypothesis. It is unlikely that one would only encounter energetic or psychosocial stressors. Indeed, Chisholm et al. (2005) proposed merging the energetics and psychosocial stress hypotheses by hypothesizing that age at menarche is first dependent on energetics. In the absence of extreme energetic constraint, then psychosocial stress may play a larger role (Chisholm et al., 2005). Further research is needed on the relationships between both psychosocial and energetic stressors on age at menarche.

Finally, there is a dearth of research on the physiological mechanisms by which either psychosocial or energetic stressors may affect age at menarche and adult reproductive function. While reproductive ecology has long examined the adaptive flexibility of women's reproduction in the face of variable environments (Wasser and Barash, 1983; Vitzthum, 2008), we have also suffered as a field from not always being able to measure the mechanisms to empirically support assertions of developmental effects, "set points," and other hypothesized links. Epigenetic

mechanisms can be responsive to environmental traits and can modify gene expression (Portela and Esteller, 2010). I propose that epigenetics, or how the environment can interact with the genome to alter gene expression, is poised to explore the mechanism of how life history tradeoffs become embodied.

Epigenetics and life history traits

Age at menarche is highly heritable, but it is also responsive to the environment (Towne et al., 2005). Epigenetics mechanisms change gene function without modifying the nucleotide sequence (Russo et al., 1996) and epigenetic mechanisms may provide an additional layer of control over pubertal timing (Demetriou et al., 2013; Lomniczi et al., 2013; Almstrup et al., 2016). Epigenetic traits could also serve as one mechanism by which environmental cues may be embodied (Gluckman and Hanson, 2006; Feinberg and Irizarry, 2010). Epigenetic traits include histone modification, gene methylation, and non-coding RNAs, and, of these, gene methylation is likely the most studied (reviewed by: Portela and Esteller, 2010). Methylation is the addition of a methyl group to a cytosine, typically located in a cytosine-guanine dinucleotide (CpG). CpG dinucleotides tend to cluster in regions called CpG islands, regions often found in gene promoters. Gene methylation is associated with changes in gene transcription, and methylation at CpG islands is typically associated with gene silencing.

Epigenetic modifications may be one mechanism by which both early life energetic and psychosocial stressors affect timing of menarche or adult reproductive function. Methylation is both heritable and modifiable over the lifespan. For example, Heijmans et al. (2008) found an association between prenatal famine exposure and adult DNA methylation of the *IGF2* gene. Bryan et al. (2013) showed a decrease in methylation across 45 CpG sites after a year-long exercise intervention study in women. Mulligan et al. (2012) linked maternal stress to newborn

birth weight via methylation changes to the *NR3C1* promoter. Epigenetic mechanisms are environmentally responsive and have effects on gene expression. Thus, epigenetics may provide a link between environmental variables and life history traits.

However, few studies to date have investigated the relationship between environmental variables, gene methylation, and timing of menarche. Women with later ages at menarche had higher global DNA methylation as adults in one study assessing methylation of blood leukocytes via the [^{3}H]-methyl acceptance assay (Terry et al., 2008), but the opposite relationship was found with age at menarche in another study assessing methylation of blood lymphocytes using the LUminometric Methylation Assay (Demetriou et al., 2013). In a candidate gene study, a higher degree of methylation at a promoter of the gene *CYP19A1* was associated with earlier breast development in urban American girls who were overweight (Stueve et al. 2014). Another recent study identified changes in DNA methylation that coincide with pubertal development (Almstrup et al., 2016). Prepubertal children had lower gene methylation in open sea and in CpG island shores and selves (Almstrup et al., 2016). The authors found significant overlap in CpG sites associated with pubertal timing and hormone concentrations in boys (Almstrup et al., 2016). Broadly speaking, these studies provide evidence that gene methylation may affect, or be affected by, pubertal timing. They further indicate that there may be interactions between variables such as body weight with gene methylation and pubertal timing and indicate that gene methylation can be associated with both pubertal timing and hormone concentrations.

Thesis Overview

This thesis uses a life history framework to investigate variation in pubertal timing and adult reproductive function. As discussed, the energetics and psychosocial acceleration hypotheses provide explanations for why age at menarche varies within and between

populations. In Chapter 2, I address one limitation of the psychosocial hypothesis: a need to include social support variables. In Chapter 3, I test how the energetics and psychosocial acceleration hypotheses work together to affect age at menarche and adult reproductive function. In Chapter 4, I investigate gene methylation as a potential mechanism connecting childhood environments and age at menarche, as well as adult reproductive function.

In Chapter 2, I investigate if social support is associated with age at menarche. I have worked with adolescent girls since 2013 using an integrated research-educational approach that aims to increase diversity in the sciences (Clancy and Hunter, 2015). On the educational side of this project, Dr. Clancy's lab leads a social science module in a bioengineering science camp for high school girls. We discuss issues related to race and gender, and we challenge the girls to critically evaluate their camp projects from a social science lens. Girls are further invited to join our research project and learn more about their own bodies. We conduct demography and health surveys, multiple psychometric surveys (Barnes and Olson, 1985; Berscheid et al., 1989; Radloff, 1991), anthropometric measurements (Antón et al., 2009), and a subset of girls collect saliva for one menstrual cycle in order to measure hormonal variation. My research focuses on the relationship between parental-adolescent open communication and problem communication, kin social networks, and timing of menarche.

I found that measures of close family relationships, specifically open communication with parents, were associated with age at menarche. This research underscores that psychosocial support variables, rather than simply psychosocial stress, may be associated with pubertal development. I further demonstrated that father-daughter and mother-daughter open communication have opposing effect directions, where father-daughter open communication delays age at menarche and mother-daughter open communication accelerates age at menarche.

Many studies in the U.S. focus on a relationship between father absence and earlier puberty (e.g. Ellis and Garber, 2000; Ellis et al., 2011; Webster et al., 2014). My results provide an alternative explanation that focuses on mother-daughter bonds accelerating age at menarche, rather that father absence alone driving the relationship. These results further add to the growing number of studies challenging conventional explanations on why father absence correlates with life history traits like age at menarche (e.g. Winking et al., 2011; Sheppard et al., 2014b; Sohn, 2017).

In Chapter 3, I seek to further understand the connection between childhood exposures and reproductive function. I conduct this research in the rural, mountainous Beskid Wyspowy region of southern Poland where I found that age at menarche has been decreasing over time since the early 1900's. This transitioning region of Poland, transitioning from an agricultural to market economy, makes it an especially interesting area to research variables affecting age at menarche and further investigate if age at menarche associates with adult reproductive function. Katharine Lee and I recruited women to participate in our research project during the 2014, 2015, and 2017 Mogielica Human Ecology Study Site field seasons. Few studies in reproductive ecology include measures of psychosocial stress and rather focus on immunological and energetic stressors. Women in our study completed the Adverse Childhood Experiences (ACE) questionnaire (Felitti et al., 1998) as a measure of psychosocial stress. I found that ACEs and helping on a farm as a child best predicted age at menarche. Higher ACE correlated with earlier ages at menarche, and farming experiences correlated with later ages at menarche. This finding provides support for the hypothesis that energetic stress delays timing of puberty and psychosocial stress accelerates timing of puberty (reviewed in Chisholm et al., 2005). I further found that farming experiences and ACEs were more predictive than simply age at menarche of differences in average adult urinary estradiol metabolite estrone-3-glucuronide (E1G) during the

follicular, periovulatory, and luteal menstrual cycle phases. These results provide empirical evidence supporting a model by which exposures during childhood affect adult reproductive function (Ellison, 1990; Núñez-de la Mora et al., 2007). This research demonstrates the importance of psychosocial stress on both age at menarche and adult reproductive function.

In Chapter 4, I test an epigenetic mechanism, gene methylation, as a mechanistic link between childhood stress and timing of puberty. Current anthropological models relating childhood environment to adult reproductive traits still lack physiological mechanisms by which to test hypotheses of developmental effects. Gene methylation, one epigenetic process, changes in response to early life experiences, including physical activity (Rönn et al., 2013; White et al., 2013), social stress and support (Weaver, 2011; Gudsnuk and Champagne, 2012; Essex et al., 2013), and nutrition (Delage and Dashwood, 2008; Zhang et al., 2011). These experiences are also implicated as modifiers of menarcheal age (e.g. Ellison, 2003; Anderson and Must, 2005; Boynton-Jarrett and Harville, 2012). Thus, I hypothesized that epigenetic factors both play a role in reproductive ecology and also provide a piece of the lacking intermediate structure between early environmental experiences and reproductive trait timing.

I conducted this research in the same Polish population. I investigated the relationship between childhood farming experiences, promoter methylation of a candidate gene CYP19A1 (aromatase), and age at menarche. CYP19A1 was chosen as a candidate gene because promoter methylation of CYP19A1 is responsive to energetics (Stueve et al., 2014) and could affect estrogen production (Hosseini et al., 2016). I found an interactive relationship between farm work during childhood and CYP19A1 promoter methylation on timing of menarche where CYP19A1 promoter methylation was negatively correlated with age at menarche. In other words, women who farmed children and had lower promoter methylation had the highest ages at

menarche. We further investigate the relationship between life history traits, gene methylation, and adult reproductive hormone variation. I identified a potential pathway by which increased cortisol concentrations increases CYP19A1 promoter I.4 methylation, which likely decreases aromatase activity and downstream estrogen and estrone concentrations. This pathway may help explain differences in estrogen concentrations between individuals. Overall, the results of this research underscore that epigenetic factors like gene methylation play a role in reproductive ecology and may provide a piece of the lacking intermediate structure between early environmental experiences and reproductive traits.

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Chapter 2

Relationship between parent-adolescent open communication and age at menarche in the United States

Abstract

The timing of a woman's first menses, or age at menarche, is one visible, memorable marker of the transition from somatic investment in growth to reproduction. Age at menarche is responsive to childhood environments. Most studies to date focus on the relationship between different types of stress and variation in age at menarche. However, our understanding of the variation in pubertal timing would benefit from a greater understanding of how social support from family and friends may affect age at menarche by either buffering psychosocial or energetic stressors or directly affecting age at menarche. This study investigates relationships between parent-adolescent communication and age at menarche in a diverse sample of 128 post-menarcheal, American girls aged 12-17. We found that measures of close family relationships, specifically open communication with parents, were the strongest predictors of menarcheal timing in this sample. We further found that mother-adolescent and father-adolescent open communication scores had opposing directional effects on menarcheal timing. These findings suggest that maternal and paternal communication signal different things about developmental and reproductive environment. This research underscores the importance of including measures of family support in future studies of adolescent reproductive trait timing, as well as the consideration that positive psychosocial factors, rather than only negative psychosocial factors, may be associated with accelerated menarcheal development.

Introduction

A younger age at first menses, or menarche, is associated with an increased risk for breast cancer, endometriosis, and depression (Valaoras et al., 1969; Helmrich et al., 1983; Nnoaham et

al., 2012; Allison and Hyde, 2013). An older age at menarche is associated with a longer period of adolescent subfecundity and an increased risk for osteoporosis (Apter and Vihko, 1983; Chevalley et al., 2008). Both earlier and later than average ages at first menses are associated with adverse health outcomes, which leads to an understandable medicalizing of the timing of menarche where earlier or later than average ages at menarche are often deemed pathological.

A life history framework offers instead that both earlier and later maturation can be adaptive in different contexts. A life history framework makes it possible to understand how evolutionary forces have shaped organisms to use limited resources to optimize survival and reproductive success (Stearns, 1989; Charnov, 1993; Roff and Fairbairn, 2007). Life history theory posits that there are trade-offs between somatic investments in growth, reproduction, and survival (Stearns, 1989). Menarche is one visible, memorable marker of the transition from somatic investment in growth to reproduction and as such is a highly studied life history trait (Ellis, 2004; Ellison et al., 2012) Within this framework, normal variation in age at menarche can be viewed as an adaptive response to varying developmental environments. Two commonly studied sources of this variation include energetic and psychosocial stressors, which tend to respectively delay and accelerate age at menarche (Ellison, 2001; Ellis, 2004).

However, our understanding of the variation in pubertal timing would benefit from a greater understanding of how and whether social support from family and friends affects age at menarche. Research addressing the relationship between social support and age at menarche is currently quite limited. One study demonstrated a relationship between familial approval and warmth and pubertal timing (Graber et al., 1995), while another found that women who reported experiencing a happy childhood tended to have earlier ages at menarche, though this result was not significant (Jorm et al., 2004). In a third longitudinal study, timing of menarche was

associated with paternal support rather than measures of family conflict (Ellis et al., 1999). Research indicates that many women's health topics tend to focus on negative aspects rather than positive experiences, although there is a growing call to assess the effect of positive experiences and outlook on health outcomes (Seligman, 2008). These examples highlight a need to include measures of social support in overarching hypotheses for variation in age at menarche. Measures of familial social support are one such important area of investigation.

Social support affects many physiological responses in endocrine, cardiovascular, and immune systems, and may serve as a buffer between stress and downstream responses (Uchino et al., 1996; Gunnar, 2017). For example, parental social support decreases stress reactivity for children (Hostinar et al., 2015), and social support buffers the relationship between stress and risk for preterm birth (McDonald et al., 2014). Here we focus on social support specifically within the family unit as recent work indicates that effects of stress on reproductive traits can be buffered by parental-child relationships (Sung et al., 2016).

We focus on kin social networks and parental communication in order to gain insight into the extent to which psychosocial support factors into an adolescent's reproductive life history. In our sample, kin closeness and parent-adolescent communication reflect familial social support. We hypothesize that 1) greater familial social support corresponds to a later age at menarche, and 2) greater social support corresponds to a later age at menarche.

Methods

Participants and Survey Measurements:

Participants included 128 adolescent girls between the ages of 12 and 17 recruited at summer science camps in Champaign County, IL between 2013 and 2017. Recruitment practices are on-going in this integrated research and education project (Clancy and Hunter, 2015b).

Almost all participants who reported family income were at or above the United States median household 2013 income of $51,759, where 73 out of 83 respondents reported family income range of $50,000-$59,000 or more. The educational success of the girls was exceptionally high, with all reported grade point averages being above a 3.0 on a 4.0 scale.

All participants completed a demographic and health survey that included questions about reproductive trait timing. Participants reported both age as well as month and year of menarche. Recall of age at menarche is quite good for adolescent girls (Koprowski et al., 2001). Most girls (n=120/128) reported reaching menarche by the time of the camp, and no girls reported ever being pregnant. Girls who did not report reaching menarche at the time of the camp (n=8) were excluded. Some participants (n=36/120) only reported age and not month at menarche.

Participants reported racial and ethnic backgrounds by selecting one or more from the following categories: Asian, Black, White, or Other and selecting: Hispanic or non-Hispanic. Participants were Asian non-Hispanic (n=28), Asian and White non-Hispanic (n=5), Black non-Hispanic (n=6), White Hispanic (n=5), White non-Hispanic (n=70), other Hispanic (n=5), and other Non-Hispanic (n=1, excluded for low sample size). Some participants (n=7) chose not to report race and/or ethnicity. Participants also reported birth country. Ten participants were born outside of the United States but migrated to the United States at or before the age of 9 years (mean 3.84, SD 3.24, range 6 months – 9 years). Participants born outside of the United States were included as there was not a significant difference in mean ages at menarche for participants born within and outside of the United States.

Participants (n=114/128) completed the Parent-Adolescent Communication survey, which measures openness and problems in communication between adolescents and their parents

via a 20-item, 4-point Likert scale (Barnes and Olson, 1985). Both openness and problems in communication contain 10 questions. Communication problem questions are reverse scored so that a higher score indicates a lack of communication problems. Scores range from 10 (low open communication or problems communicating with caregiver) to 40 (high open communication or lack of problems in communication). Examples of open communication questions include: "I can discuss my beliefs with my caregiver without feeling restrained or embarrassed," and "My caregiver tries to understand my point of view." Examples of communication problem questions include: "I don't think I can tell my caregiver how I really feel about some things," and "Sometimes I have trouble believing everything my caregiver tells me." High scores indicate more open communication or a lack of perceived communication problems.

Participants completed Parent-Adolescent Communication surveys for their two primary caregivers and reported the caregiver relationship (e.g., mother, father, step-parent, grandparent). Based on their responses, we scored mother-adolescent "open" and "lack of problem" communication and father-adolescent "open" and "lack of problem" communication. Responses were excluded if participants reported two same-sex parents (n=1), participants did not report caregiver title or reported a caregiver title other than "Mother" or "Father", or caregivers were not reported as married and cohabitating (divorced: n=5, separated: n=2, other: n=3, not reported: n=4).

Finally, participants (n=108/128) recorded their personal kin and friend social networks on a survey based on the Relationship Closeness Inventory (Berscheid et al., 1989b). Participants first recorded everyone in their social network on worksheets. Space was provided for twenty responses, and girls were told they could write additional names if desired. Participants then recorded their relationship to that person, which we coded as kin, friend, or mentor. Seventeen

participants also chose to list pets. Participants recorded how close they felt to that individual on a 3-point scale from (1) not close, (2) close, and (3) very close. Participants also recorded their frequency of communication with that person or pet on a 3-point scale from (1) not often, (2) often, and (3) all the time. Average kin closeness scores and average overall social network closeness scores were used in this analysis.

Statistics

Parent-adolescent communication scales are stable over adolescence (Lerner and Steinberg, 2009). However, the perception of these communication measures may change over time (Steinberg, 1988). Therefore, we first analyzed the relationship between age and parent-adolescent communication scales and kin social network variables each independently using bivariate linear regression.

In the United States, there are often differences in average ages at menarche for girls of different racial and ethnic backgrounds (Posner, 2006; Braithwaite et al., 2009). We thus also investigated if there were differences in mean ages at menarche for girls of different racial and ethnic backgrounds using one-way analysis of variance (ANOVA). Some groups has small sample sizes, so we thus also compared average ages at menarche between the two largest groups (White non-Hispanic and Asian non-Hispanic girls) using a t-test.

We investigated potential predictors of age at menarche in this sample using the all possible subsets regression procedure. The all possible subsets regression procedure compares linear and multiple regression models that use each predictor term and combination of predictor terms in order to select the best model for a given dataset and selection criteria (Draper and Smith, 1998). This type of model selection is appropriate given the number of included predictors. The following variables were included as original predictors in the model: mother-

adolescent open communication, mother-adolescent lack of problems in communication, father-adolescent open communication, mother-adolescent lack of problems in communication, kin social network average closeness score, and overall social network average closeness scores. The final model was selected using Mallow's C_p Criterion, when C_p was close to the number of included parameters (Mallows, 1973; Draper and Smith, 1998). We controlled for race and ethnicity in the final model. We additionally ran post-hoc power analyses on the variables included in the final models. Statistical analyses were conducted using the R package leaps and JMP Pro 12.0 (SAS Corporation, Cary, NC). A p-value of ≤0.05 was considered statistically significant in all analyses.

Results

All girls reported normal ages at menarche (range 9 – 15.67 years), and the average age at menarche was 12.65 (SD 1.25) years. Girls were within seven years of menarche (mean 2.79, SD 1.42, range 0.08-6.45 years). Table 2.1 displays additional descriptive statistics.

Measure	**Mean (SD)**	**n**
Age at Menarche (years)	12.65 (1.25)	120
Age of adolescent at camp (years)	15.39 (0.98)	122
Gynecological Age (years)	2.79 (1.42)	120
BMI	21.61 (3.22)	114
Parent-Adolescent Communication Scores:		
Mother-adolescent open communication	31.93 (6.14)	114
Mother-adolescent lack of problem communication	27.21 (6.64)	114
Father-adolescent open communication	30.02 (7.23)	110
Father-adolescent lack of problem communication	27.30 (6.77)	110
Social Networks:		
Average of overall social network closeness scores	2.29 (0.34)	108
Average of kin closeness scores	2.47 (0.42)	107

Table 2.1: Descriptive statistics of mean and standard deviation for select variables in the whole sample.

Age at menarche varies for girls of different race and ethnic backgrounds:

In this sample, girls with different race and ethnic backgrounds had significantly different average ages at menarche (one-way ANOVA, p=0.0175). We also found significant differences in average ages at menarche between Asian and White girls, the two categories with the highest sample sizes (p=0.0359). The distribution of ages at menarche for each race and ethnic background is displayed in Figure 2.1, and the averages and sample sizes for each group are displayed in Table 2.2.

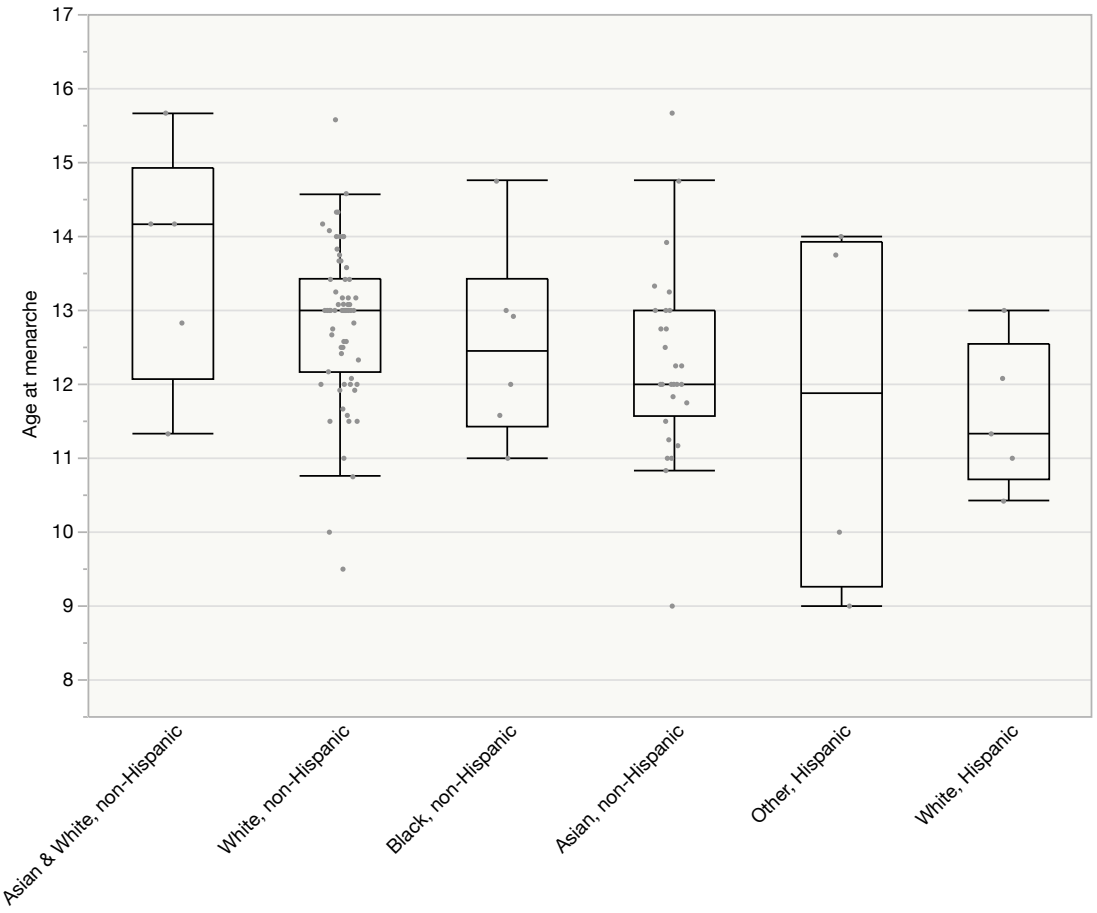

Figure 2.1: Boxplots representing the distribution of ages at menarche for each reported race and ethnicity. Each light grey data point represents an individual.

Group	n	Mean	Std Dev
White, non-Hispanic	67	12.86	1.05
Asian, non-Hispanic	28	12.28	1.28
Black, non-Hispanic	6	12.54	1.33
White, Hispanic	5	11.57	1.00
Asian & White, non-Hispanic	5	13.63	1.63
Other, Hispanic	4	11.69	2.56

Table 2.2: Means and standard deviation of ages at menarche for girls of different race and ethnic backgrounds.

Current age does not predict measures of social support:

We analyzed the relationships between current age and parent-adolescent communication scores and kin social network measures in order to determine if any measure of social support varied by participant age. Age did not significantly predict any measure of kin social support and age within this sample (all $p>0.05$). For example, age did not significantly predict mother-adolescent open communication ($\beta = 0.32$, SE = 0.63, $p=0.6172$), nor did age significantly predict father-adolescent lack of problem communication ($\beta = -0.26$, SE = 0.69, $p=0.7054$). Age also did not significantly predict average kin closeness score ($\beta = 0.01$, SE = 0.05, $p=0.8993$).

Parent-adolescent open communication predicts age at menarche:

All caregiver-adolescent communication scores (mother-adolescent open communication, mother-adolescent lack of problem communication, father-adolescent open communication, and father-adolescent lack of problem communication) and social network closeness scores (average of social network closeness scores and average of kin closeness scores) were included in an all possible subsets regression model in order to select variables that best predicted age at menarche. In this sample, mother-adolescent open communication and father-adolescent open

communication were selected, where mother-adolescent open communication and father-adolescent open communication significantly predicted age at menarche (Table 2.3, regression model p=0.0023, R^2=0.22, adjusted R^2=0.15). Father-adolescent and mother-adolescent open communication had opposing effects on age at menarche in the final linear model (Fig. 2.2). Higher father-adolescent open communication scores predicted a later age at menarche (β = 0.0517, SE = 0.02, p = 0.0333, power = 0.57) and higher mother-adolescent open communication scores predicted an earlier age at menarche (β = -0.0766, SE = 0.03, p = 0.0077, power = 0.77). Figure 2.2 shows leverage plots displaying the different effects that father and mother – adolescent open communication have on age at menarche.

Dependent Variable:	**Age at menarche**			
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	13.3742	0.6382	20.96	<0.0001 ***
Mother-Adolescent Open Communication	-0.0766	0.0281	-2.73	0.0077 **
Father-Adolescent Open Communication	0.0517	0.0239	2.16	0.0333 *
Asian & White, non-Hispanic	1.2730	0.4681	2.72	0.0079 **
Asian, non-Hispanic	-0.2122	0.2694	-0.79	0.4330
Black, non-Hispanic	0.4675	0.5630	0.83	0.4086
Other, Hispanic	-1.0075	0.6057	-1.66	0.0998
White, Hispanic	-1.0038	0.5137	-1.95	0.0538

Table 2.3: The parameter estimates describing the relationship between parent-adolescent communication, race/ethnic background, and age at menarche. (***) indicates a significance level of <0.001, (**) indicates a significance level of <0.01 and (*) indicates significance level of <0.05.

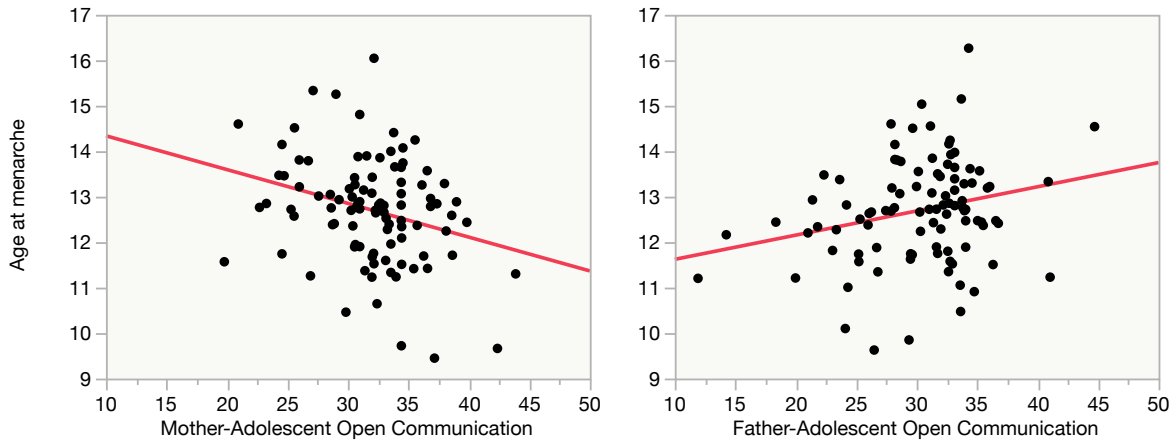

Figure 2.2: Leverage plots displaying the opposite effect directions of parental-adolescent open communication on age at menarche. Mother-adolescent open communication (β = -0.0766, SE = 0.03, p = 0.0077, power = 0.77) and father-adolescent open communication (β = 0.0517, SE = 0.02, p = 0.0333, power = 0.57) were both included as significant predictors of age at menarche in the final regression model (p=0.0023, R^2=0.22, adjusted R^2=0.15).

Discussion

We hypothesized that 1) greater familial social support corresponds with a later age at menarche, and 2) measures of social support correspond to a later age at menarche. Both hypotheses receive partial support. In this sample we found that mother-adolescent open communication and father-adolescent open communication significantly predicted age at menarche. The direction of the effects in the linear model were opposed, where higher mother-adolescent open communication corresponded to an earlier age at menarche, and higher father-adolescent open communication corresponded to a later age at menarche.

We focused on social support specifically within the family unit as recent work indicates that effects of stress on reproductive traits can be buffered by kin social support (Sung et al., 2016). While many studies focus on the relationship between different types of stress and age at menarche, few studies to date have incorporated measures of familial support when investigating the relationship between childhood exposures and age at menarche. Even fewer studies separate

maternal and paternal relationships, except in studies where father absence is used as a proximate measure of childhood psychosocial stress.

Some previous studies also found that social support affects age at menarche. For example, in a sample of mostly white middle- to upper-middle-class adolescent girls, a greater sense of parental approval and less conflict in the home predicted later ages at menarche (Graber et al., 1995). Contrary to these findings, in a sample of white adult Australian women, women who reported having a happy childhood had slightly earlier ages at menarche, but this effect was not significant (Jorm et al., 2004). These studies demonstrate that familial support may affect age at menarche, but the directionality of this relationship may be context-dependent.

While one measure of kin social support, parent-adolescent open communication, predicted age at menarche in this sample, the ages at menarche of participants were still well within the normal range of ages at menarche. There are social and biological consequences for earlier and later ages at menarche (Arim et al., 2011; Allison and Hyde, 2013). For example, in the United States, girls who mature both earlier and later than their peers are at greater risk for bullying from peers and depression (Stice et al., 2001; Herva et al., 2004; Swift, 2017). Further, girls with earlier ages at menarche tend to achieve regular ovulatory cycles far earlier than those with later ages at menarche (Apter and Vihko, 1983).

Life history theory and age at menarche:

A life history perspective recognizes the social and biological consequences of menarcheal timing, but also argues that both earlier and later maturation can be adaptive in different contexts. Menarche is one visible, memorable marker of the transition from somatic investment in growth to reproduction and as such is a highly studied life history trait (Ellis, 2004; Ellison et al., 2012) Within this framework, normal variation in age at menarche can be viewed

as an adaptive response to varying developmental environments. Two commonly studied sources of this variation include energetic and psychosocial stressors, which tend to respectively delay and accelerate age at menarche (Ellison, 2001; Ellis, 2004). Chisholm (1993, 2005) framed the relationship between psychosocial stress and menarcheal age through the lens of life history theory by arguing that measures of family conflict provide a signal of a risky environment. Indicators of risky environments lead to pubertal acceleration in order to maximize reproductive potential when life expectancy is decreased (Chisholm et al., 1993). In this framework, early pubertal timing may be an adaptive response to psychosocially stressful environments.

Much of the work on variables affecting pubertal timing focuses on psychosocial stressors rather than measures of close parent-adolescent relationships. Belsky, Steinberg and Draper (1991) were among the first to propose that psychosocial stress during childhood affects pubertal timing. This idea became known as the psychosocial acceleration hypothesis and is supported mainly in Western, educated, industrialized, rich and democratic (WEIRD) societies, where stressors such as father absence, family conflict, and adverse childhood experiences tend to accelerate age at menarche (e.g. Ellis and Essex, 2007; Pesonen et al., 2008; Wise et al., 2009; Boynton-Jarrett et al., 2013). The absence of a father figure is especially well studied (for a review see Webster et al., 2014). For example, in a sample of Canadian girls, Surbey (1990) demonstrated that not only is early menarche associated with father absence, but also with the number of years of father absence.

Contrary to these findings, father absence does not predict earlier ages at menarche in lower income nations (Sheppard et al., 2014b; Anderson, 2015) and type of father absence (e.g. death, divorce, or migrant work) changes the direction of the association between father absence and age at menarche (Shenk et al., 2013). Moreover, there are alternative explanations for why

father absence may affect age at menarche in some higher income nations or contexts. Specifically, Romans et al. (2003) found a correlation between father absence and childhood abuse, and they proposed that this association better explains accelerated ages at menarche than paternal absence. Alternatively, Ellis and Garber (2000) found that family interpersonal stress and father absence mediated the relationship between maternal mood disorders and earlier ages at menarche, proposing instead that parental psychopathology may be driving earlier ages at menarche. Finally, mother absence does not predict earlier ages at menarche (Bogaert, 2005; Sheppard et al., 2014). The childcare and social support provided to single fathers potentially differs drastically from the social stigma surrounding single motherhood (Sheppard et al., 2014).

These examples and alternative explanations highlight why the social, economic, and cultural context matters for whether and when psychosocial variables affect age at menarche, and more specifically highlight the need to contextualize father absence models. Indeed, Ellis et al. (2011) argued that both stressful and supportive family environments can affect age at menarche and proposed a model of biological sensitivity to context. In Ellis et al's (2011) model, girls take in cues about both the stressful and supportive variables in their environment and respond to both according to their particular context. For example, in a majority white American sample, higher maternal support correlated with later ages at menarche, and socioeconomic status (SES) further separated participants in that girls with higher SES and higher increased maternal support had the latest ages at menarche (Ellis and Essex, 2007). Our study supports such a model by indicating that girls respond to cues of social support variables, although we were limited by a lack of information on social stressors.

Opposing effects of mother-adolescent and father-adolescent open communication on age at menarche:

Our study further indicates that in this sample, different caregivers have opposing effects on adolescent maturational timing where mother-adolescent open communication accelerated age at menarche and father-adolescent open communication delayed age at menarche.

Mother-adolescent open communication predicted earlier ages at menarche in our final regression model, indicating that better mother-daughter relationships corresponded to an earlier age at menarche. From a life history perspective, greater maternal emotional support might indicate future reproductive support. Thus, an earlier transition to reproduction among maternally-supported girls might correspond to greater grandmaternal support, as earlier ages at menarche correlate with earlier ages at first birth in many natural fertility populations (Kramer, 2008). In this sample, open maternal communication may decrease age at menarche by indicating a supportive environment in which to accelerate age at first birth.

In contrast, father-adolescent open communication predicted later ages at menarche in our final regression model. The direction of the relationship we found between father-adolescent open communication and age at menarche is in accordance with the literature on psychosocial stressors. Father absence may accelerate pubertal timing by signaling risky or uncertain environments (Chisholm et al., 2005). In line with this argument, perhaps father-adolescent open communication indicates a safe environment in which to delay pubertal timing and leave time for additional growth.

To our knowledge, only one other study to date has investigated independent maternal and paternal contributions to pubertal timing. In contrast to our study, they found that adolescent reports of lower mother-daughter cohesion, fewer calm discussions with mothers, and more intense conflicts with mothers all accelerated pubertal timing (Steinberg, 1988). They found no relationship between adolescent reports of father-child cohesion or communication on pubertal

timing (Steinberg, 1988). Like our study, this was a sample of American adolescents, but Steinberg (1988) had greater economic diversity and less racial diversity than our sample. Further, pubertal timing was assessed by a home visitor rating facial characteristics, body proportion and shape, and coordination (Steinberg, 1988), rather than self-reported age at menarche. The differences in the study composition or methodology may have contributed to a different result.

The context of variation in age at menarche in this sample

In the context of our study, approximately 42% of participants identified as racial or ethnic U.S. minorities. In our sample race/ethnicity had a significant effect on age at menarche. While our sample was diverse, there were multiple race categories with few participants. In studies that include substantially larger number of participants of different racial identities, socioeconomic statuses, and home environments, race often pulls out as a significant variable (Anderson et al., 2003; Anderson and Must, 2005; Braithwaite et al., 2009). Body fat and body mass index has been hypothesized as one reason age at menarche varies between girls of different backgrounds, but in a large dataset, Anderson and Must (2005) did not find an interactive effect between race/ethnicity and BMI on age at menarche. In our sample, there was large homogeneity in household income (>87% of participants were at or above a median income of $50,000) and caregiver status (all included participants' parents were heterosexual and cohabiting).

In our sample, the two groups with the largest sample sizes were Asian non-Hispanic girls (n=28/128) and white non-Hispanic girls (n=70/128). Asian non-Hispanic girls had earlier ages at menarche compared to white girls, where the average age at menarche for Asian girls was 12.28 years, and the average age at menarche for white girls was 12.86 years. These differences

are quite similar to some former studies. For example, Koprowski et al (1999) reported a median age at menarche of 12.2 years for Asian and Pacific Island girls and 12.8 years for non-Hispanic white girls. Wu et al. (1999) also reported an average age at menarche of 12.2 years for Asian American women born in the U.S. This average age was a little more than one year earlier compared to matched samples born in Asia (Wu et al. 1999). In contrast to these studies, Adair and Gordon-Larsen (2001) found that Asian girls were 1.65 times more likely to have a later age at menarche after controlling for maternal education, family income, age, and residence, while Black and Hispanic girls were 1.55 and 1.76 times more likely to have an earlier age at menarche compared to white girls. Similarly, Nomura et al. (1984) found that Japanese women living in Hawaii had slightly later ages at menarche compared to white women living in Hawaii, although this study did not control for birthplace.

While many studies have investigated differences in average ages at menarche for girls of different race and ethnic backgrounds, few contemporary studies have reported on age at menarche for Asian American girls. In a study that grouped other Hispanics, Asians, Native Americans, Native Hawaiians, and Pacific Islanders together, this group had the largest decline in average ages at menarche from 1988-1994 to 1999-2002 (Anderson and Must, 2005). However, this type of aggregation used in this current study and in many other studies may actually mask variation between Asian American subgroups (Holland and Palaniappan, 2012). Future studies are needed to investigate the secular trend of age at menarche for Asian American girls and general variation between ages at menarche for girls of different race and ethnic backgrounds.

Limitations:

This study is a post-menarcheal sample, and thus it is conceivable that our measures of kin social networks or parent-adolescent communication may change with participant's age. However in our sample, age did not significantly associate with any measure of kin social networks or parent-adolescent communication. Further, parent-adolescent communication tends to be continuous across adolescence, although the content and perceived meaning of these communications may change over time (Lerner and Steinberg, 2009). This evidence suggests that it is appropriate to investigate the parental-adolescent communication scores in relationship to age at menarche in this sample.

A limitation of our study is that we confined our analyses to participants reporting two-parent heterosexual households given the small number of two parent same-sex or one-parent households in the sample, and thus parent absence and other types of childhood stressors were not investigated here. We do not have data to discern maternal age at menarche, nor other childhood energetic and psychosocial measures, which might offer additional explanations and sources of variation. This study is also not a longitudinal study, so we cannot indicate causality. Future work on this sample will include if age at menarche is associated with health or reproductive outcomes.

Conclusion

We found that measures of close family relationships, specifically open communication with parents, were the strongest predictors of menarcheal timing in this diverse sample of adolescents from two-parent households largely at or above the 2013 median U.S. income. We further found that mother-adolescent and father-adolescent open communication scores had opposing directional effects on menarcheal timing. These findings suggest that maternal and

paternal communication signal different messages about developmental and reproductive environment. This research underscores the importance of including measures of family support in future studies of adolescent reproductive trait timing, as well as the consideration that positive psychosocial factors, rather than only negative psychosocial factors, may be associated with accelerated menarcheal development.

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Chapter 3

Childhood stress affects age at menarche and adult reproductive function in a rural Polish sample

Abstract

Age at first menses, or menarche, is used as a proxy of childhood stressors and is also correlated with adolescent and adult reproductive function. Many studies have investigated either the effects of childhood variables on age at menarche, or the effects of age at menarche on reproductive function. However, few studies have investigated the combined effects of childhood environment and age at menarche on adult reproductive function, particularly in transitioning environments like rural Poland where one might expect to see a secular trend in age at menarche alongside changes in economic growth (Colleran 2014).

Here we investigate if menarcheal age is declining in the rural Polish Beskid Wyspowy region. We investigate relationships between menarcheal age and proximate measures of energetic and immune stressors (childhood farming and animal exposures) and psychosocial stressors (adverse childhood experiences, or ACE). We further examine whether childhood stressors are correlated with adult reproductive function (urinary estrone-3-glucuronide (E1G) concentrations).

We find that age at menarche has declined over time in rural Poland. Helping on farms and with farm animals as a child were associated with later ages at menarche. Women with a higher number of adverse childhood experiences tended to have earlier age at menarche, although this difference was not statistically significant. Despite different effects on age at menarche, all types of childhood stressors were associated with lower adult E1G concentrations. The results of this study support a model under which developmental conditions affect adult

reproductive function, but challenge the use of age at menarche alone as a proxy for childhood stressors.

Introduction

The timing of a woman's first menses, or menarche, has declined globally in both rural and urban regions and lower and higher income countries (Tanner, 1981b; Wyshak and Frisch, 1982; Anderson and Must, 2005). This decline in age at menarche, a marker of sexual maturation, has partly been attributed to increases in nutritional and healthcare access and decreases in energetic and immune challenges. Timing of menarche is important as this age is associated with adult health and reproductive outcomes, including breast cancer risk (Helmrich et al., 1983; Kotsopoulos et al., 2005) and reproductive hormone concentrations (Windham et al., 2002; Núñez-de la Mora et al., 2007; Emaus et al., 2008; Clancy et al., 2013). Many studies have investigated either the effects of childhood variables on age at menarche (Graber et al., 1995; Khan et al., 1996; Ellis and Essex, 2007) or the effects of age at menarche on reproductive function (Apter and Vihko, 1983; Ellison, 1996; Posner, 2006; Reiches et al., 2013). However, few studies have investigated the combined effects of childhood environment and age at menarche on adult reproductive function, particularly in subsistence environments transitioning towards an increasing market economy.

The timing of menarche is understood within the context of life history theory, which posits that there are trade-offs in the events related to survival and reproduction over the life cycle (Stearns, 1992; Hawkes and Paine, 2006). Energy used for one purpose cannot be used for another; for example, energy invested in current reproduction cannot be invested in future survival and reproduction (Hill, 1993). Life history theory predicts a trade-off between growth

and reproduction, which is mediated by survival; the more time and energy invested into growth, the greater the risk of not surviving until reproduction (Stearns, 1989).

Several non-exclusive, life-history based hypotheses help explain the ways energetic, immunological, and psychosocial stressors across childhood affect age at menarche (Ellis, 2004). The energetics hypothesis posits that menarcheal age should be delayed in environments with low energy status, negative energy balance, or high energy flux, extending the period of growth in an energetically constrained situation (Ellison, 2001; Reiches et al., 2013). This hypothesis has been widely supported in many contexts. For example, girls who experienced food deprivation during childhood in France and girls who experienced food insecurity during adolescence in Ethiopia had later ages at menarche (Belachew et al., 2011; Dossus et al., 2012). Physical activity, and specifically activities like intensive sports that result in negative energy balance, is also associated with later ages at menarche (Georgopoulos et al., 2010). Immune stress during childhood may similarly delay age at menarche. For example, Tsimane children in Bolivia with higher C-reactive protein, an indication of immune stress, had smaller gains in height over a three-month study period (McDade 2008). Thus energetic and immune constraints have a clear effect on reproductive traits by pulling energy away from reproductive effort and towards maintenance and survival.

However, psychosocial stressors may also pull resources away from reproduction. According to the psychosocial acceleration hypothesis, age at menarche should be accelerated in risky or uncertain psychological, social, or physical environments where a long lifespan is not assured (Belsky et al., 1991; Ellis, 2004). Psychosocial variables associated with earlier ages at menarche include physical and sexual abuse (Wise et al., 2009; Boynton-Jarrett and Harville, 2012), conflict in the home (Jorm et al., 2004), and father absence (Ellis and Garber, 2000;

Boothroyd et al., 2013; Webster et al., 2014). The majority of studies demonstrating support for the psychosocial acceleration hypothesis, and specifically for a relationship between father absence and early menarche, come from industrialized nations and this relationship is often not found in lower-income countries including Malaysia (Sheppard et al., 2014b), South Africa (Anderson, 2015), and Indonesia (Sohn, 2017). Thus, Chisholm et al. (2005) proposed merging the energetics and psychosocial stress hypotheses by hypothesizing that age at menarche is first dependent on energetics. In the absence of energetic constraint, then psychosocial stress may play a larger role (Chisholm et al., 2005). This merged hypothesis has not yet been tested in transitioning environments where energetic constraints have previously been associated with reproductive traits, but economic and social changes have resulted in lower dependency on and participation in physically demanding subsistence farming practices. In this paper, we test the Chisholm et al. (2005) hypothesis in a transitioning region of rural Poland where 68.3% of women in our sample helped with farm work as a child, while only 25.2% of our sample currently work on a farm.

Age at menarche has declined in Poland, and between 1966 and 2012 ages at menarche varied alongside political and socioeconomic changes in the country (Łaska-Mierzejewska and Olszewska, 2007; Gomula and Koziel, 2017). An acceleration in declining ages at menarche was seen after the fall of communism in 1989 and after Poland joined the European Union in 2004 (Gomula and Koziel, 2017). In the rural, mountainous Beskid Wyspowy region of southern Poland, women have later ages at menarche than in the United States (Clancy et al., 2009). Women in the region tend to grow up living and/or working on family farms, and they continue to work on farms and large vegetable gardens into adulthood. Besides farming and gardening, women walk to the village centers, tend animals, and complete substantial domestic work over

the course of their day. Indeed, women in this region take more than twice as many steps per day than the average rural American woman (Lee et al., 2015). Previous work in the region demonstrated that reproductive variables are responsive to energetic constraint, specifically the moderate energetic constraint coinciding with harvest seasons (Jasienska & Ellison, 1998; Jasienska & Ellison, 2004). While much of the previous work in the Beskid Wyspowy region has focused on energetic stressors, few studies have investigated the relationship between psychosocial stress and reproductive traits.

In this study, we first investigated changes in age at menarche in the Beskid Wyspowy region since 1923 in 198 women across five field seasons. We hypothesized that age at menarche has declined over time in this region, in accordance with the global secular trend (Tanner, 1981b; Sørensen et al., 2012) and trends in other Polish regions (Łaska-Mierzejewska and Olszewska, 2007; Wronka and Pawlińska-Chmara, 2009; Woronkowicz et al., 2012; Gomula and Koziel, 2017).

Next, we analyzed urinary reproductive hormone concentrations across the menstrual cycle in fifty-three adult women in order to test multiple additional hypotheses. We hypothesized that an earlier age at menarche is correlated with increased urinary estradiol metabolite estrone-3-glucuronide (E1G) concentrations. Such a finding would be in accordance with the hypothesis that age at menarche is correlated with adult ovarian function (Ellison, 1996), as well as in line previous findings in the region (Clancy et al., 2013).

We investigated if energetic and immune stress is correlated with age at menarche and E1G concentrations. We used farming and farm animal exposures during childhood as proximate measures of energetic and immune stress. These proximate measures were chosen as the traditional farming practices used in rural Poland are also associated with changes in immune

responses in other populations (Stein et al., 2016), and previous work in this Polish region has also demonstrated that energetic expenditure increases coinciding with seasonal farm work decrease ovarian function (Jasienska and Ellison, 2004). In previous studies in other rural Polish regions, children of farmers experienced later ages at menarche than children of non-farmers (Łaska-Mierzejewska and Olszewska, 2004, 2007). We hypothesized that farming exposures during childhood correlate with increased ages at menarche. We further hypothesized that farming experiences during childhood affect adult E1G concentrations where children who grew up on farms would have lower E1G concentrations during adulthood. Such a finding would be consistent with the hypothesis that energetic tradeoffs during childhood have a lasting effect on adult ovarian function (Ellison, 1996).

We also investigated if proximate measures of psychosocial stress (number of adverse childhood experiences [ACEs]), are correlated with age at menarche and adult E1G concentrations. In this sample, we hypothesized that adverse childhood experiences are correlated with earlier ages at menarche in accordance with the psychosocial acceleration hypothesis (Belsky, Steinberg, & Draper, 1991; Chisholm, 1999). No study to date examines the relationship between ACEs and adult reproductive concentrations over the menstrual cycle. Individual ACEs, specifically sexual and physical abuse, are associated with earlier ages at menarche (Wise et al., 2009; Boynton-Jarrett et al., 2013) and self-reported infertility (Jacobs et al., 2015). Experiencing a higher number of ACEs is associated with a variety of outcomes including risk for autoimmune diseases (Dube et al., 2009), ischemic heart disease, and multiple types of cancer (Anda et al., 2010). Thus, we specifically hypothesized that higher number of ACEs correlate with lower E1G concentrations. This finding would be consistent with the

hypothesis that early adverse experiences like child abuse affect adult reproductive function, fertility, and fecundity (Allsworth et al., 2001; Jacobs et al., 2015).

Finally, we investigated interactive effects of childhood environmental variables and age at menarche on adult E1G concentrations. We hypothesized that helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations. We suspect that including farming in our model will have a stronger effect than age at menarche in accordance with our hypothesis that developmental conditions affect both age at menarche and adult reproductive function independently. We suggest that age at menarche is good proximate variable for energetic environments experienced throughout development, and that measuring environmental exposures like farming and adverse experiences will help elucidate which variables and how these experiences affect adult reproductive function.

Overall, our hypotheses can be summarized as follows:

1. Age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland.
2. Working on farms and with farm animals during childhood is associated with later ages at menarche. A higher number of adverse childhood experiences is associated with earlier ages at menarche.
3. Earlier ages at menarche are associated with higher E1G concentrations. Working on farms and with farm animals during childhood is associated with lower E1G concentrations. A higher number of adverse childhood experiences is associated with lower E1G concentrations.

4. Helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations.

Methods

Women (n=123) were recruited at the Mogielica Human Ecology Study Site in the Beskid Wyspowy region of southern rural Poland during the summers of 2014, 2015, and 2017. Women were recruited who were between the ages of 18 and 46, in general good health, not pregnant within the past six months, not breastfeeding within the past three months, not on hormonal contraception, and non-smoking. Participants were recruited in multiple ways: gynecologists, nurses, and a midwife at two local clinics helped recruit women; an announcement was published in the local church bulletin; fliers were left in local businesses including hair salons, book stores, and other types of shops; and local student assistants traveled door-to-door to invite women to participate in the study. Women from multiple villages in the region joined the study. Study protocols were approved by the University of Illinois, Urbana-Champaign Institutional Review Board (#13856).

A subset of participants (n=53/total from 2014-2015 and n=46/total in 2017) collected first morning void urine samples for one full menstrual cycle, filled out daily physical activity and well-being questionnaires (Jasienska et al., 2006b), and recorded their diet five times over their cycle. Participants collected one urine sample per day in a 120 mL cup and used vacuum-sealed tubes (Vacumed ®) to create two 8-10 mL aliquots at home. Participants immediately froze urine aliquots in home freezers. Samples were collected at the end of the menstrual cycle, transported in coolers, kept at -20°C until the end of each field season, and shipped over dry ice back to the University of Illinois, Urbana-Champaign, where samples were stored at -20 or -80°C until processing.

There were two study options, and some women (n=37/total) chose to join a week-long abbreviated version of the study. In the week-long abbreviated version, women completed daily physical activity and well-being questionnaires and recorded their diet three times over that week, but they did not collect any urine samples for hormonal analyses. Sixteen women participated in the project during two or three recruitment periods (e.g., participated in both 2015 and 2017). Of these, ten women enrolled in the full study during two recruitment periods, three women enrolled in the week-long version during a first year and full study during a second year, and three women enrolled in the week-long version during a first year and full study during a second and third year. For women who participated more than once, we used data from study year 2015 in order to maximize hormone data and ensure that no participant responses were included more than once. In order to ensure clarity for readers, figure 3.1 displays the study protocol, and we include the number of participants in our tables and figures.

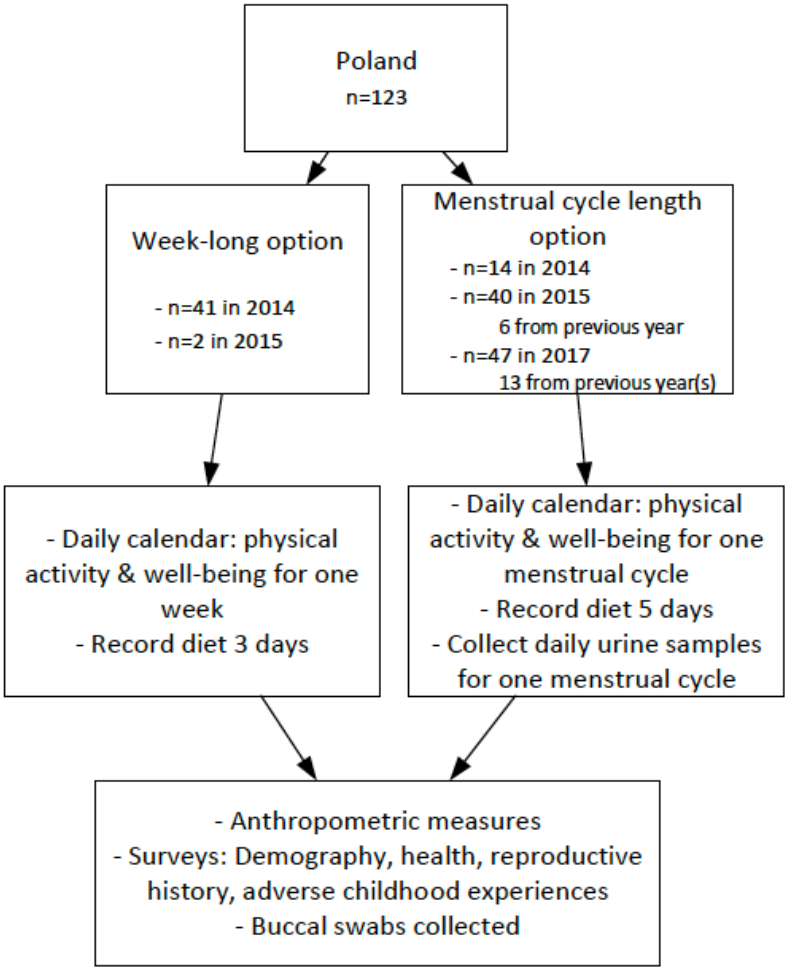


Figure 3.1: The study protocol included two study options: one week-long and one menstrual-cycle length. This figure displays the surveys, measurements, and collected samples for each study option.

Additional age at menarche data from ninety-six women were included in the analyses investigating if age of menarche is changing over time. These participants were recruited in 2005 and 2010, and the recruiting procedures have been described elsewhere (Clancy et al., 2009, 2013). For these participants, year at birth was calculated from the participant's reported age and year of study recruitment.

Surveys and Measurements: All women completed health and demography surveys that included questions about reproductive trait timing and childhood environments. Women were asked about age at menarche via recall, and most women in the study (n=119/123) reported age at menarche. Recall of age at menarche is good for women of ages included in this study (Koprowski et al., 2001; Must et al., 2002). If a woman reported an age range (i.e. age 13 - 14), then an average of those ages was used for analyses. Women were further asked if they lived on a farm during childhood, if they helped on that farm, and what types of animals were present while they were growing up. Women were asked the same questions about their current living environment. Most farming categories were split into yes or no categories by participant response, and these categories included: living on a farm, helping on a farm, owning animals, and caring for animals. We calculated the number of the types of farming animals, which included chickens, ducks, rabbits, sheep, goats, cows, horses, and "other." Other reported animals were counted as farm animals if they did not live inside the home (i.e. pigs or turkeys).

Participants completed a translated version of the Adverse Childhood Experiences (ACE) survey in the privacy of their homes (Felitti et al., 1998). Due to the sensitive nature of these questions, participants were reminded that all surveys and survey questions were optional. Some women (n=24/123) chose not to, or forgot to, complete the ACE survey. Some women (n=19/99) did not answer all ACE survey questions. The majority (n=15/19) of women who partially completed the survey skipped one question (range 1-6 questions skipped). To maximize the amount of data, ACE scores were only excluded if participants skipped one or more questions and had a total score of zero.

We analyzed several components of the ACE score. First, we calculated the total number of reported ACEs, and used the following categories: no reported adverse experiences, one

reported experience, and two or more reported experiences. Subdividing ACEs in a cumulative manner is common in the literature (Felitti et al., 1998; Dube et al., 2003; Cluver et al., 2015), and thus our hypotheses focused on cumulative ACEs. However, studies that did not use the ACE survey, but did include questions about individual ACEs, have demonstrated relationships between certain types of abuse and age at menarche (Mishra et al., 2009; Wise et al., 2009; Boynton-Jarrett and Harville, 2012). Thus, we also examined the relationships between individual ACEs and age at menarche, as well as individual ACEs and adult reproductive function.

Anthropometric measurements were taken upon enrollment of all women according the to the Bones and Behavior Working Group protocol (Antón et al., 2009). Participant weight and body fat percentages were measured using the Tanita© BF-680W body fat monitor. At least two trained researchers assisted with anthropometric measurements to increase accuracy.

Laboratory Protocol: We measured multiple hormones over the course of each participant's menstrual cycle using two types of Quansys multiplex enzyme linked immunosorbent assays (ELISA). Hormone data was generated for samples collected in 2014 and 2015 (n=53). The measured hormones included: the urinary estradiol metabolite (estrone-3-glucuronide, E1G), C-Peptide, tumor necrosis-factor alpha (TNFα), human chorionic gonadotropin (beta-hCG), interleukin 6 (IL-6), cortisol, interleukin 10 (IL-10), C-reactive protein (CRP), follicle-stimulating hormone (FSH), and urinary progesterone metabolite (pregnanediol glucuronide, PdG). All hormones except PdG were run on a custom Quansys 9-Plex ELISA, and PdG was run individually on a 1-plex Quansys ELISA. Sixty-nine total full or half ELISA plates were run for the PdG assay, and seventy-five full or half ELISA plates were run for the 9-Plex. The intra- and inter- assay variation for each measured hormones was as follows: E1G (9.46%, 32.84%), C-

Peptide (5.66%, 25.12%), TNFα (17.97%, 0%), beta-hCG (8.08, 0%), IL-6 (6.70%, 0%), cortisol (10.27%, 20.00%), IL-10 (26.12%, 0%), CRP (4.75%, 17.55%), FSH (5.06%, 34.78%), and PdG (9.46%, 32.19%). While the inter-assay variation is high, it is well within the range typically seen in multiplex assays (Chowdhury et al., 2009; Bastarache et al., 2011). All samples were run in duplicate; if the coefficient of variation (CV) between duplicates was ≥ 15%, then the samples were rerun. E1G was used in this analysis. The final concentration of urinary E1G was corrected by specific gravity using the protocol described in Miller et al. (2004). Some cycles were missing one or more days of urinary collections. When E1G concentrations were available for the previous and following day, then a missing value was estimated by averaging the surrounding E1G concentrations.

Cycles were aligned by mid-cycle drop date according to Lipson and Ellison (1996). Using this method, day of ovulation is determined as the maximum decrease in E1G over two consecutive days. Day of ovulation is marked as day zero, and the follicular and luteal phases are aligned going backwards and forwards, respectively. All cycles were evaluated by two individuals independently (MPR and KML), and consensus was reached using a third opinion (KBHC) for cycles that were not immediately agreed upon. In the event where two potential drop dates were identified, we chose the drop date occurring immediately before a rise in PdG. Four cycles were removed from analyses due to missing data (n=2) or anovulation (n=2), leaving 49 cycles included in the analyses.

Averages of E1G were calculated by cycle phase as follows: follicular phase (days -10 to -1), early follicular phase (days -10 to -6), late follicular phase (days -5 to -1), periovulatory phase (days -3 to 3), early luteal phase (days 0 to 2), early/mid luteal phase (days 3 to 5), late luteal phase (days 6 to 10), and luteal phase (days 1 to 10). These phase partitions are consistent

with the literature (Lipson and Ellison, 1996; Barrett et al., 2013; Clancy et al., 2013). If a participant was missing three or more consecutive days for the follicular or luteal phases, then an average value was not calculated for that phase for that participant. If a participant was missing two or more days for the early follicular, late follicular, periovulatory, early luteal, early/mid luteal, or late luteal phases, then an average value was not calculated for that participant's phase.

Analyses for each hypothesis:

Hypothesis 1: Age at menarche has declined in the Beskid Wyspowy region of southern rural Poland.

Test: We conducted a simple linear regression with year at birth as the predictor variable and age at menarche as the dependent variable. Because there was a low number of women born between 1922 and 1960 (n=22/198), we repeated this analysis with and without participants born before 1960.

Hypothesis 2: Working on farms and with farm animals during childhood is associated with later ages at menarche. A higher number of adverse childhood experiences is associated with earlier ages at menarche.

Tests: We used t-tests to investigate if there are differences in mean ages in menarche for women who did and did not live on or participate in farming activities during childhood. We used one-way analysis of variance (ANOVA) to test if there are differences in mean ages at menarche for women who reported zero, one, and two or more ACEs.

We created a best fit regression model to select predictors of age at menarche using lasso regression. There was multicollinearity between farm-related variables (i.e. growing up on a farm and helping on a farm) in this sample. We thus used a penalized regression technique

(elastic net) that both minimizes the effect of multicollinearity and performs feature selection to create a best fit model (Zou and Hastie, 2005).

Hypothesis 3: Earlier ages at menarche are associated with higher E1G concentrations, working on farms and with farm animals during childhood is associated with lower E1G concentrations, and a higher number of adverse childhood experiences is associated with lower E1G concentrations.

Tests: Repeated measures ANOVA were used to test for differences between E1G concentrations over the menstrual cycle for 1) participants above or below mean age at menarche, 2) childhood farming variables, and 3) 0, 1, or ≥2 ACE scores. Cycle days -9 to 9 were used in repeated measures ANOVA in order to minimize the number of cycles excluded for missing data (Clancy et al., 2013). Follicular (days -9 to -1), periovulatory (days -3 to 3), luteal (0 to 9) phases were analyzed.

Hypothesis 4: Helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations.

Tests: We created a best fit regression model of variables related to averaged E1G concentrations in the luteal, periovulatory, and follicular phases. We used a penalized regression technique (elastic net) lasso regression to control for multicollinearity of age at menarche and farm-related variables. Variables included in the best fit regression model were: current age, current body fat percentage, current waist to height ratio, currently live on a farm, currently work on a farm, number of farm animals currently own, age at menarche, lived on a farm during childhood, helped work on a farm during childhood, number of farm animals during childhood, helped care for animals during childhood, ACE group (0, 1, and 2+ reported experiences), and each individual ACE.

All analyses were run using JMP® PRO 13 and figures were created using the R package ggplot2. Alpha was set at 0.05.

Results

Hypothesis 1: Age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland.

Year at birth was used as the predictor in a simple linear regression to evaluate if age at menarche is changing over time in the Beskid Wyspowy region of southern rural Poland. This regression resulted in an $R^2 = 0.08$ and coefficient on year at birth of -0.03 with associated p-value of <0.0001 (Fig. 3.2). This significant, negative coefficient indicates that age of menarche decreases as birth year increases in this sample. The regression formula and associated parameter estimates are reported in Table 3.1.

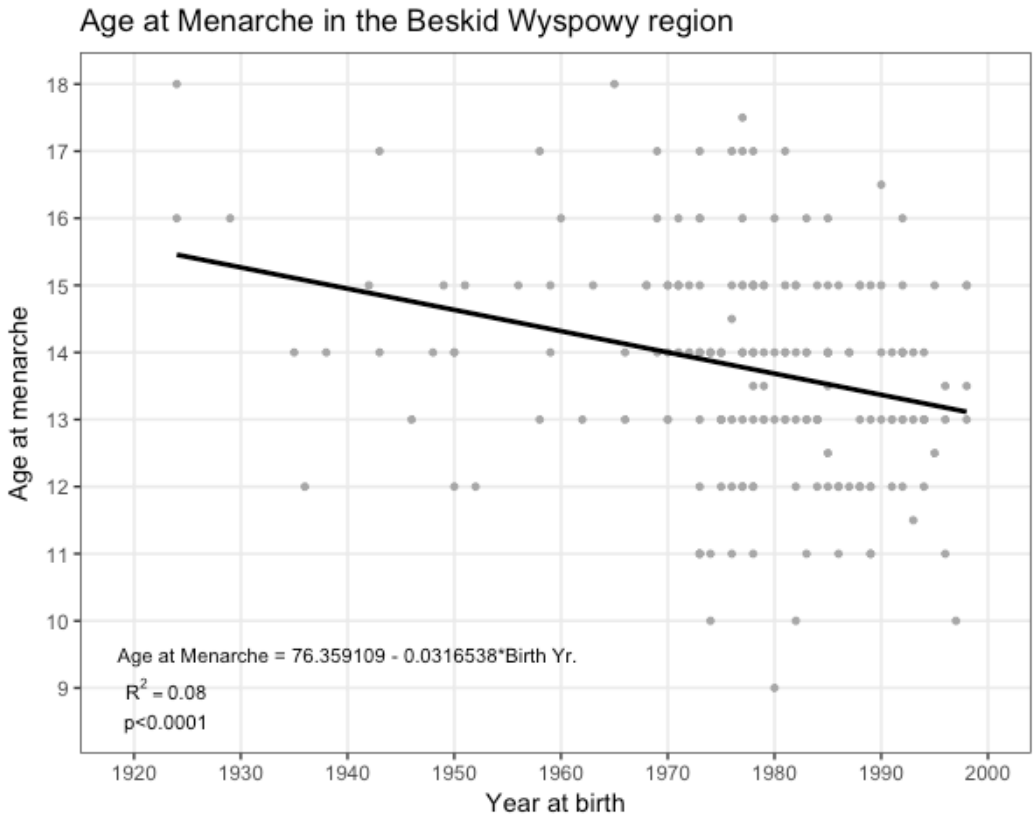


Figure 3.2: Age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland (n=198, β=-0.03, p<0.0001).

Regression equation:	Age at menarche = 76.359109 - 0.0316538*Year at birth			
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	76.3591	15.4446	4.94	<.0001*
Year at birth	-0.0317	0.0078	-4.05	<.0001*

Table 3.1: The regression formula and associated parameter estimates describing the relationship between year at birth and age at menarche.

Because there are few women born before 1960 in this sample (n=22/198), we also tested age at menarche over time with and without those women. Run with only women born after 1960, our second regression yielded an $R^2 = 0.06$ and coefficient on year at birth of -0.05 with

associated p-value of 0.0012. Thus, both analyses suggest a secular trend in age at menarche in this sample.

Hypotheses 2: Working on farms and with farm animals during childhood is associated with later ages at menarche. A higher number of adverse childhood experiences is associated with earlier ages at menarche.

We next considered the potential effects of childhood variables on age at menarche. In our sample, 56.9% (n=70/123) women grew up on farms, 68.3% (n=84/123) women helped with farm work as a child, and 89.4% (n=110/123) women grew up with animals. In contrast, 18.7% (n=23/123) women currently live on farms, 25.2% (n=31/123) women currently help with farm work, and 77.2% (n=95/123) women currently own animals. Table 3.2 displays additional descriptive statistics for this sample.

	Mean(SD)	**Range**	**n**
Age (years)	32.30 (8.00)	18 - 46	123
Age at menarche (years)	13.41 (1.65)	9 – 17.5	119
BMI	25.41 (5.64)	17.3-49.4	123
Waist-to-Hip Ratio	0.79 (0.07)	0.66-1.05	123
Waist-to-Height Ratio	0.49 (0.09)	0.38-0.80	123
Childhood Farming: number of farm animal types	2.72 (2.26)	0-7	123
ACE Score	0.86 (1.26)	0-5	99

Table 3.2: Descriptive statistics of variables included in the study.

The majority of women who completed ACE surveys (n=57/99) reported no adverse experiences. Twenty women in the sample reported one adverse experience, and the most commonly reported adverse experience was substance abuse in the household (Table 3.3). Twenty-two women reported two or more adverse experiences.

Adverse Childhood Experiences (ACE) Category	Yes	No	Skipped Question
Sexual abuse	1	94	4
Member of household went to prison	2	96	1
Parental separation or divorce	3	94	2
Physical Neglect	4	94	1
Physical Abuse	5	92	2
Witnessed abuse of mother or stepmother	9	88	2
Member of household had mental illness or anxiety disorder	10	88	1
Emotional Neglect	10	81	8
Emotional Abuse	14	80	5
Substance abuse in household	27	70	2

Table 3.3: The ten ACE categories listed in order of least to most frequent with number of respondents who answered yes, no, or skipped question.

Mean ages at menarche in this sample differed for multiple farming categories, but not for increasing numbers of adverse childhood experiences (Table 3.4, Fig. 3.3). For example, mean ages at menarche differed for farm animal care where women who cared for farm animals during childhood had a mean age of 13.70 (SD ±1.63) years, while women who did not care for animals had a mean age of 13.11 (SD ±1.46) years ($p=0.05$). Mean ages at menarche did not differ for ACE scores where women with no reported ACEs had an average age of 13.68 (SD ±1.46), women with one reported experience had a mean age of 13.38 (SD ±2.21), and women with two or more experiences had a mean age of 13.11 (SD ±1.46) years ($p=0.41$). Post hoc power analyses of mean ages at menarche for ACE categories revealed a low power of 0.20, while power for all farming categories was above 0.50.

We further tested for differences in mean ages at menarche between women with different ACE scores who did not help on farms during childhood. Mean ages at menarche did not differ between ACE score groups in non-farmers where women with no reported ACEs

(n=12) had an average age of 12.88 (SD ±1.15) years and women with one or more reported experience (n=12) had a mean age of 12.42 (SD ±1.62) years (p=0.43).

		Age at menarche			
		Mean	SD	n	p-value
Living on a farm	Yes	13.53	1.77	67	
	No	13.27	1.56	52	0.4
Helping on a farm	Yes	13.66	1.67	81	
	No	12.89	1.5	38	0.02*
Helping care for animals	Yes	13.57	1.66	99	
	No	12.65	1.42	20	0.02*
Helping care for farm animals	Yes	13.70	1.63	62	
	No	13.11	1.46	57	0.05*
ACE Score	0	13.68	1.46	45	
	1	13.38	2.21	20	
	2 or more	13.11	1.46	22	0.41

Table 3.4: Differences in mean ages at menarche by childhood farming exposures and adverse childhood experiences (ACE).

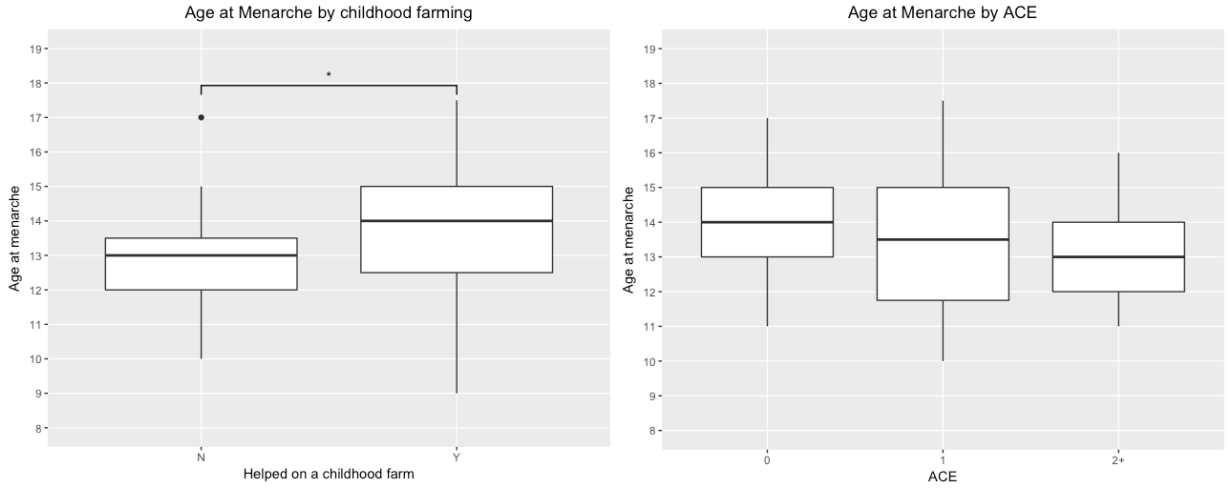


Fig. 3.3: Mean ages at menarche are significantly later for women helped on a farm during childhood (p=0.02). Mean ages at menarche do not significantly differ by number of adverse childhood experiences (p=0.41).

Elastic net regression with AICc validation was performed with all farm and ACE variables as the predictor variables and age at menarche as the dependent variable. The final model of factors that influence age at menarche included helping on a farm as a child and ACE groupings of zero reported ACEs and two or more reported ACEs (Table 3.5).

Dependent Variable: Age at menarche				
AICc	297.06358			
RSquare	0.0885663			
Parameter Estimates for Final Predictor Variables				
Term	**Estimate**	**Std Error**	**Wald ChiSquare**	**Prob > ChiSquare**
Intercept	13.4585	0.3301	1662.1953	<.0001*
ACE Group [0-2+]	0.3163	0.3699	0.7309	0.3926
Childhood: helped on farm [N-Y]	-0.5651	0.3631	2.4224	0.1196
Scale	1.5381	0.1200	164.3826	<.0001*

Table 3.5: Elastic net regression details (AICc and R^2). All predictor variables not listed were zeroed, and the estimates for the predictor variables included in the final model are displayed.

Hypothesis 3: Earlier ages at menarche are associated with higher E1G concentrations. Working on farms and with farm animals during childhood is associated with lower E1G concentrations. A higher number of adverse childhood experiences is associated with lower E1G concentrations.

Variables were independently investigated by E1G over the follicular, periovulatory, and luteal phases using repeated measures ANOVA, and visual representation of average E1G across the menstrual cycle by select variables is displayed in Figure 3.4. E1G concentrations during

almost all menstrual cycle phases were significantly different between women who did and did not participate in farm-related activates during childhood. More specifically, women with more animals and women who helped on farms had lower levels of adult E1G concentrations (helped on a farm: p=0.05, p=0.01, p=0.06 and number of farm animal types: p=0.01, p=0.01, and p=0.01 for the follicular, periovulatory, and luteal phases respectively). There were no significant differences in E1G concentrations for women with earlier and later ages at menarche (p=0.22, 0.40, and p=0.22 for the follicular, periovulatory, and luteal phases respectively). There were no significant differences in E1G concentrations for different ACE categories (scores of 0, 1, or 2 or more, p=0.20, 0.77, and p=0.98 for the follicular, periovulatory, and luteal phases respectively).

Figure 3.4

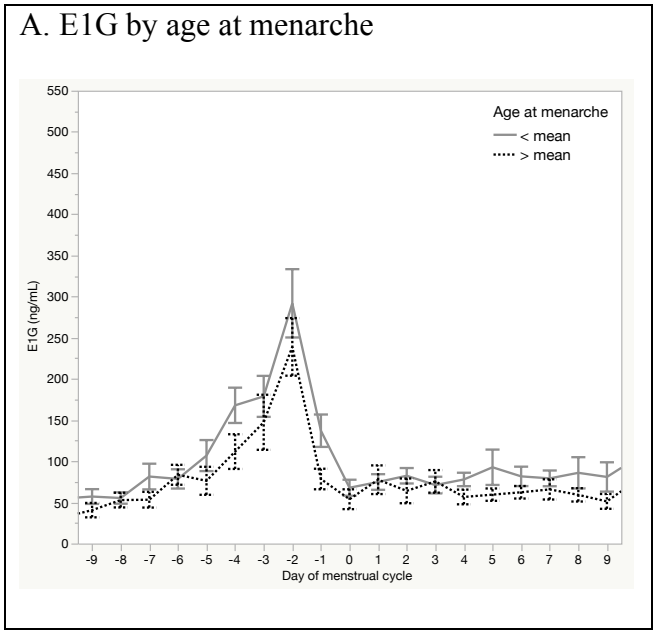
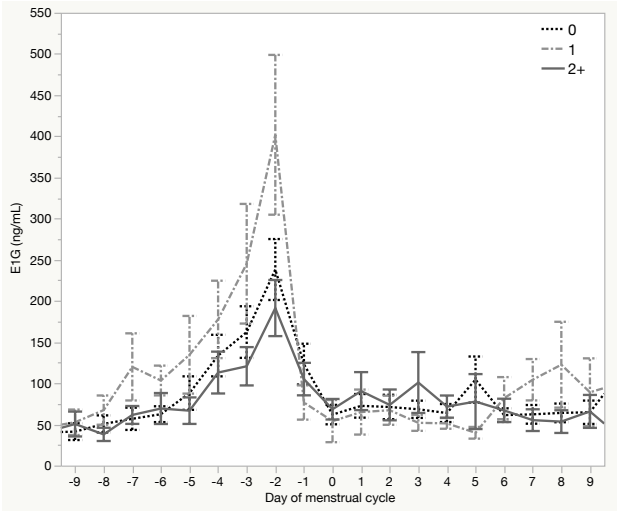


Figure 3.4 (cont.)

B. E1G by adverse childhood experiences



C. E1G by childhood farming

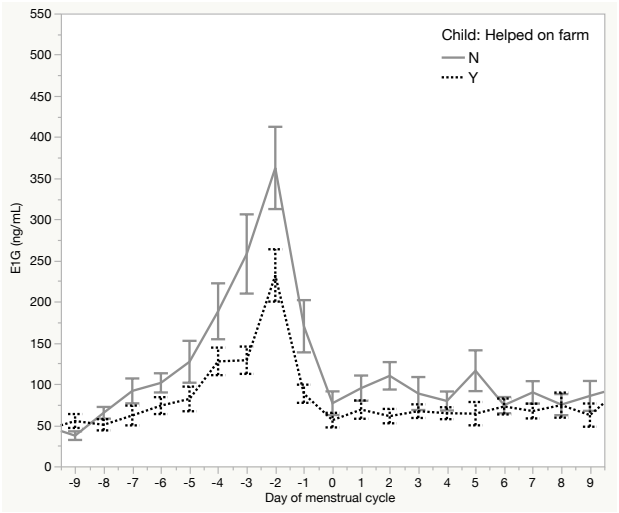


Fig. 3.4: Mean (standard error) of E1G concentration across the menstrual cycle where Day 0 indicates ovulation. There were no significant differences in E1G concentrations for different ages at menarche (Fig 3A) or different ACE score (Fig 3B) for any phase (follicular, periovulatory, or luteal). There were significant differences in E1G concentrations for women who helped on a farm during childhood (Fig 3C, p=0.05, p=0.01, p=0.06 for the follicular, periovulatory, and luteal phases respectively).

Hypothesis 4: Helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations.

We then considered the potential effects of childhood variables and age at menarche on adult E1G concentrations for all menstrual cycle phases. We used elastic net regression to select the subset of predictor variables that should be included in the regression model. The final model AICc and R^2, as well as the variables that were selected for the final models, are displayed in Table 3.6.

Table 3.6

Dependent Variable: Follicular Phase (Days -10 to -1) Average E1G				
AICc	277.23643			
RSquare	0.6832991			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	270.4223	62.3306	18.8228	<.0001*
ACE Group: 0,1,2+ [0-2+]	-3.6645	16.1825	0.0513	0.8209
ACE Group: 0,1,2+ [1-2+]	11.6714	22.1303	0.2781	0.5979
ACE: Physical Abuse [N-Y]	-101.9972	73.2646	1.9381	0.1639
Childhood: helped on farm [N-Y]	20.5949	25.7171	0.6413	0.4232
Childhood: helped care for animals [N-Y]	94.3080	53.2728	3.1339	0.0767
Current waist to height ratio	-156.2749	114.7326	1.8553	0.1732
Scale	37.5544	6.6086	32.2924	<.0001*

Table 3.6 (cont.)

Dependent Variable: Early Follicular Phase (Days -10 to -6) Average E1G				
AICc	244.13226			
RSquare	0.5953829			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	140.43624	25.92897	29.335122	<.0001*
ACE Group: 0,1,2+ [0-2+]	-19.76979	9.7969838	4.0721078	0.0436*
ACE: Mental illness in household [N-Y]	14.199816	8.2876404	2.9356456	0.0866
ACE: Physical Abuse [N-Y]	-33.11122	29.479719	1.2615477	0.2614
Childhood: helped care for animals [N-Y]	49.659096	14.12754	12.35562	0.0004*
Current waist to height ratio	-113.6892	67.391957	2.8459152	0.0916
Scale	21.156391	3.5054379	36.424922	<.0001*
Dependent Variable: Late Follicular Phase (Days -5 to -1) Average E1G				
AICc	327.20025			
RSquare	0.7893934			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	103.1721	107.5842	0.9197	0.3376
ACE Group: 0,1,2+ [0-2+]	-134.7587	51.4543	6.8591	0.0088*
ACE: Substance abuse in household [N-Y]	111.5844	46.2947	5.8096	0.0159*
ACE: Mental illness in household [N-Y]	86.7725	20.7829	17.4322	<.0001*
ACE: Emotional abuse [N-Y]	39.4535	30.3705	1.6876	0.1939
ACE: Physical Abuse [N-Y]	-90.0095	98.3028	0.8384	0.3599
ACE: Emotional neglect [N-Y]	56.1798	34.9524	2.5835	0.108
Childhood: helped on farm [N-Y]	107.1906	21.0100	26.0292	<.0001*
Childhood: helped care for animals [N-Y]	90.1005	46.9015	3.6905	0.0547
Current Age	-1.6897	1.5915	1.1272	0.2884
Scale	49.7491	6.9918	50.6284	<.0001*

Table 3.6 (cont.)

Dependent Variable: Periovulatory Phase (Days -3 to 3) Average E1G				
AICc	320.54085			
RSquare	0.6818685			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	252.6358	105.7460	5.7077	0.0169*
ACE Group: 0,1,2+ [0-2+]	-41.7363	42.4291	0.9676	0.3253
ACE: Substance abuse in household [N-Y]	20.0065	45.9334	0.1897	0.6632
ACE: Physical Abuse [N-Y]	-48.8035	83.0589	0.3452	0.5568
ACE: Emotional neglect [N-Y]	50.0131	27.9168	3.2095	0.0732
Childhood: helped on farm [N-Y]	43.4883	20.6545	4.4332	0.0352*
Childhood: helped care for animals [N-Y]	48.8803	30.8283	2.5140	0.1128
Current waist to height ratio	-24.6632	143.5434	0.0295	0.8636
Current Age	-3.7108	1.2579	8.7030	0.0032*
Scale	41.1313	5.7953	50.3722	<.0001*
Dependent Variable: Early Luteal Phase (Days 0 to 2) Average E1G				
AICc	314.53066			
RSquare	0.7670733			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	221.1297	35.6483	38.4784	<.0001*
ACE Group: 0,1,2+ [0-2+]	-108.7377	17.3843	39.1242	<.0001*
ACE Group: 0,1,2+ [1-2+]	-73.6247	15.6607	22.1018	<.0001*
ACE: Substance abuse in household [N-Y]	55.6163	17.1934	10.4636	0.0012*
ACE: Mental illness in household [N-Y]	-21.69135	22.38601	0.93890	0.3326
ACE: Emotional neglect [N-Y]	30.2469	17.5910	2.9565	0.0855
Childhood: helped care for animals [N-Y]	41.8173	16.4751	6.4425	0.0111*
Currently help work on a farm [N-Y]	44.2673	17.5000	6.3987	0.0114*
Current number of farm animals	17.1842	5.8020	8.7723	0.0031*
Current waist to height ratio	-70.8237	69.5160	1.0380	0.3083
Current age	-3.8528	1.0331	13.9080	0.0002*
Scale	25.8938	4.9495	27.3695	<.0001*

Table 3.6 (cont.)

Dependent Variable: Early/Mid Luteal Phase (Days 3 to 5) Average E1G				
AICc	310.77799			
RSquare	0.627562			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	322.0563	121.3867	7.0392	0.0080*
ACE Group: 0,1,2+ [0-2+]	70.0142	31.6525	4.8928	0.0270*
ACE: Substance abuse in household [N-Y]	-50.4288	29.6758	2.8877	0.0893
ACE: Mental illness in household [N-Y]	-89.8113	42.2759	4.5131	0.0336*
ACE: Sexual abuse [N-Y]	-38.7124	35.4508	1.1925	0.2748
Age at menarche	-3.5885	5.2317	0.4705	0.4928
Currently live on a farm [N-Y]	43.5153	19.4291	5.0163	0.0251*
Current Age	-3.7800	1.0591	12.7393	0.0004*
Scale	31.9962	5.3298	36.0393	<.0001*
Dependent Variable: Late Luteal Phase (Days 6 to 10) Average E1G				
AICc	271.46977			
RSquare	0.7345425			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	336.5907	155.8043	4.6671	0.0307*
ACE: Mental illness in household [N-Y]	-19.4803	42.7587	0.2076	0.6487
ACE: Physical Abuse [N-Y]	-162.8172	82.3954	3.9048	0.0481*
Current number of farm animals	2.1109	14.8542	0.0202	0.887
Current body fat percentage	-0.9422	0.5799	2.6395	0.1042
Current Age	-1.6729	1.7487	0.9152	0.3387
Scale	30.3484	18.3329	2.7404	0.0978

Table 3.6 (cont.)

Dependent Variable: Luteal Phase (Days 1 to 10) Average E1G				
AICc	257.25014			
RSquare	0.5956098			
Term	Estimate	Std Error	Wald ChiSquare	Prob > ChiSquare
Intercept	247.9348	88.5142	7.8460	0.0051*
ACE: Mental illness in household [N-Y]	-28.2383	29.3774	0.9240	0.3364
ACE: Physical Abuse [N-Y]	-42.9302	57.5293	0.5569	0.4555
Current body fat percentage	-1.1081	0.5644	3.8540	0.0496*
Current Age	-2.0879	1.1103	3.5362	0.06
Scale	29.7471	5.9464	25.0251	<.0001*

Table 3.6: Elastic net regression model details (AICc and R^2) for average E1G by each menstrual cycle phase. The dependent variables are highlighted in dark grey. All predictor variables that were zeroed in the final model are not displayed. An (*) indicates that predictor is significant at $p<0.05$.

Some key patterns are identified in these final regression models (Table 3.6). Age at menarche was not selected as a final predictor in any final follicular, periovulatory, or luteal phase models, with the exception of the early/mid luteal phase. At least one type of childhood farming experiences (helping on a farm or helping with farm animals) was selected as a final predictor in the final follicular, periovulatory, and early luteal models. ACE group (0, 1, or ≥2 reported experiences) was selected as a final predictor in all follicular phases, periovulatory, and early and early/mid luteal phases. Individual ACEs were selected as a final predictor in some models; specifically, physical abuse, mental illness in the household, substance abuse, emotional abuse, emotional neglect, and sexual abuse were selected in at least one model. Some final models also included contemporary variables such as current age, body fat percentage, and waist-to-height ratio. Waist-to-height ratio was selected as a final predictor in follicular,

periovulatory, and early luteal models. Current age was selected as a final predictor in late follicular, periovulatory and all luteal models.

Discussion

Reproductive function is sensitive to environmental conditions, and our results indicate that the environment experienced throughout development affects reproductive trait timing and adult steroid hormone concentrations. Childhood environments have been investigated in relationship to age at menarche, and age at menarche, in turn as a proxy for childhood environments, has been investigated in relationship to reproductive function. However, few studies investigate the relationships between childhood environments, age at menarche, and adult reproductive function, particularly in transitioning regions.

We specifically investigated the following hypotheses: 1) Age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland, 2) Working on farms and with farm animals during childhood is associated with later ages at menarche, and a higher number of adverse childhood experiences is associated with earlier ages at menarche, 3) Earlier ages at menarche are associated with higher E1G concentrations, working on farms and with farm animals during childhood is associated with lower E1G concentrations, and a higher number of adverse childhood experiences is associated with lower E1G concentrations, and 4) Helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations.

Our primary findings are as follows: 1) Age at menarche has declined over time in the Beskid Wyspowy region of Poland, 2) energetic, immune, and psychosocial stressors during childhood affect age at menarche in this population, 3) childhood farming practices

independently predict adult E1G across the menstrual cycle, and 4) age at menarche, current age, ACEs, and childhood farming practices affect adult ovarian hormone concentrations.

The Secular Trend of Age at Menarche

Hypothesis 1: Age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland.

Hypothesis one was supported. We found that age at menarche has declined over time in the Beskid Wyspowy region of southern rural Poland at a rate of -0.03 per year from 1923 to 1998. The declining age at menarche matches the secular trend of declining ages at menarche globally, as well as other studies of age at menarche over time in Poland. Comparatively, age at menarche in other Polish rural areas declined at a rate of -0.24 per decade from 1987 to 2001 (Łaska-Mierzejewska and Olszewska, 2007). Age at menarche averaged from seven different Polish regions declined from 13.8 (SD 1.13) years in 1966 to 12.9 (SD 1.15) years in 2012 (Gomula and Koziel, 2017). The secular trend towards earlier ages at menarche is often attributed to increased socioeconomic status, nutritional access, and positive energetic balance (Stearns and Koella, 1986; Okasha et al., 2001; Gluckman and Hanson, 2006). This is likely the case in the Beskid Wyspowy region as the voivodship in which they are located experienced one of Poland's highest GDP growth rate following the Polish transformation (Büttner, 2005).

Our study differs from other studies of age at menarche across Poland, which demonstrate an increase in age at menarche from 1978 to 1989, corresponding to a period of economic crisis and food rationing (Łaska-Mierzejewska and Olszewska, 2007; Gomula and Koziel, 2017). However, age at menarche for children of farmers remained stable during that decade, likely due to a lower dependence on rationed food (Łaska-Mierzejewska and Olszewska,

2007). This is also likely the case in the Beskid Wyspowy region where family farming was common.

While age at menarche in this rural region is declining, the current average age at menarche in our sample was 13.41 years, later than the average age at menarche in Polish cities (12.53 years, (Szwed et al., 2013)) and in the United States (12.43 years (Committee of Adolescent Health Care, 2015)). While a difference of one year may not seem meaningful, it is biologically relevant: a lower age at menarche is associated with higher risk for breast cancer (Gronwald et al., 2006), higher adult estradiol concentrations (Clancy et al., 2013), lower baseline progesterone values (Windham et al., 2002), increased bone density (Chevalley et al 2009, Chevalley et al 2011), and a shortened period of adolescent sub-fecundity (Vihko and Apter, 1984). The decline in age at menarche from women born from 1924 to 1998 in this region may be indicative of political and economic transformations, as well as increases in technological access like farming equipment that decreased the amount of farm work performed by hand. This decline in age at menarche in a population where reproductive traits have been traditionally studied from an energetic perspective (see Jasienska & Ellison, 1998; Jasienska & Ellison, 2004; Jasienska et al. 2006; Nenko & Jasienska, 2013) suggests that it is an interesting time to test new hypotheses about the influence of both energetic and psychosocial stressors on life history traits.

The Environment Experienced during Childhood affects Age at Menarche

Hypotheses 2: Working on farms and with farm animals during childhood is associated with later ages at menarche. A higher number of adverse childhood experiences is associated with earlier ages at menarche.

We found support for hypothesis two: we found that helping on a farm during childhood, helping care for animals during childhood, and helping care for farm animals during childhood all independently associated with age at menarche where farming activities and animal care were associated with older ages at menarche. Within the context of life history theory, age at menarche is expected to be later in energetically constrained situations (Ellison, 2001; Reiches et al., 2013). Investing in growth takes energy, and delaying age at menarche is one way to lengthen the growth investment period. However, it is difficult to separate potential energetic and immune exposures during childhood in this sample, and immune constraints also may delay age at menarche (McDade, 2003; Abrams and Miller, 2011). In our sample, participants reported helping with planting, harvesting, and haying, as well as helping with farm animals by feeding, milking, and cleaning animals, as well as leading animals out to graze. These traditional farming practices are associated with changes in immune responses in other populations (Stein et al., 2016). Previous work in this Polish region has also demonstrated that energetic expenditure increases coinciding with seasonal farm work decrease ovarian function in women despite adequate energy intake (Jasienska and Ellison, 2004). Thus, we contend that growing up on a farm is energetically and immunologically stressful.

Many environments are both energetically/immunologically constrained and psychologically stressful, or operate with energetic plenty and little psychological stress, which creates pushes and pulls on the timing of menarche. Merging the two viewpoints, Coall and Chisholm (2003) proposed that pubertal timing is first dependent on energetics. However, in the absence of energetic constraint, psychosocial stressors should have a stronger effect on age at menarche (Coall and Chisholm, 2003). This hypothesis has never been tested in transitioning environments like rural Poland where many women grew up on farms, but no longer live and

work on farms. While previous reproductive ecology studies in this region have focused on energetic constraints due to farming, none have focused on other types of stressors like psychosocial stress.

In our elastic net regression model for age at menarche, the final selected predictors were childhood farm work and ACE groups zero and two or more reported experiences. According to the psychosocial acceleration hypothesis, which also draws on life history theory, age at menarche should be accelerated in risky or uncertain psychological or social environments where a long lifespan is not assured (Belsky et al., 1991; Ellis, 2004). Support for this hypothesis stems from evidence that physical and sexual abuse (Wise et al., 2009; Boynton-Jarrett and Harville, 2012), conflict in the home (Jorm et al., 2004), and father absence (Ellis and Garber, 2000; Boothroyd et al., 2013; Webster et al., 2014) all predict earlier ages at menarche. In this case, no individual ACE was included in the final model predicting age at menarche. This may be due to the lower power of ACE or the high number of people who chose to skip, or forgot to complete, the ACE survey. However, our ACE survey response rate was 80%, which is similar to others (e.g. Felliti et al. (1998) report a response rate of 70.5%, Dong et al. (2004) report a response rate of 68%). Further, there is likely a dose-response where a higher number ACE exposures affect health outcomes (Felitti et al., 1998; Dube et al., 2003). Thus, it is likely that cumulative ACEs predict earlier ages at menarche as we found in our final regression model.

While childhood farming experiences independently predicted age at menarche, both farming and ACE group were selected as predictors in the final regression model. Overall, our results match both the energetic and psychosocial acceleration predictions: greater energetic and immune stress (farming) predicted later ages at menarche, while greater psychosocial stress (ACEs) predicted earlier ages at menarche.

However, our study provided only partial support for Coall and Chisholm's (2003) hypothesis. Average ages at menarche were lower for women with higher ACE scores, but this difference was not significant in our sample. However, both farming and cumulative ACE scores predicted age at menarche in the elastic net regression model, suggesting that ACEs and farming both affect age at menarche. It is possible that all types of stress push and pull on age at menarche, and in the context of this study, where women are experiencing energetic stress but are not nutritionally deprived, that energetic, immune, and psychosocial stress together affect age at menarche.

Childhood Environment Affects Adult Reproductive Function

The reproductive capacity hypothesis proposes that age at menarche is both a proxy of early environment and correlated with adult reproductive function (Ellison, 1990, 1996). Reproductive function can be measured in multiple ways, including by comparing average concentrations of reproductive hormones within and between populations (Ellison, 1990). The majority of studies to date focus on salivary progesterone, but more recent studies have included estradiol and estrogen metabolites (Núñez-De La Mora et al., 2008). Age at menarche is negatively correlated with adult estradiol concentrations (Windham et al., 2002; Clancy et al., 2013) and adult progesterone values (Núñez-de la Mora et al., 2007). In this study, we investigate not only the relationship between age at menarche as a proxy for early environmental exposures, but also include some potential childhood exposures that may affect adult reproductive function.

Hypothesis 3: Earlier ages at menarche are associated with higher E1G concentrations. Working on farms and with farm animals during childhood is associated with lower E1G

concentrations. A higher number of adverse childhood experiences is associated with lower E1G concentrations.

Our results offer partial support of hypothesis 3. Specifically, we found that adult E1G was significantly lower across the menstrual cycle (follicular, periovulatory, and luteal phases) for women who worked on farms during childhood and women who helped with farm animals during childhood. However, adult E1G was not significantly lower across the menstrual cycle for women with later ages at menarche, nor for women with higher numbers of ACEs. This finding may have been a result of lower sample size for women who reported two or more ACEs.

The frequency of reported adverse childhood experiences was slightly lower for some ACEs in this sample compared to other Polish studies. To the authors' knowledge, no other study in Poland has used the ACE survey, and thus direct comparisons were not possible. However, in a sample of 2,582 women hospitalized for psychiatric disorders, 4% of women reported actual or attempted incest (Sobański et al., 2013), and in a sample of 508 Polish participants who answered an online survey, 4% of Polish female participants reported childhood sexual abuse (Hardt et al., 2010). In comparison, 1% of our samples reported childhood sexual abuse. Additionally, in the same sample of 508 Polish participants, 14% reported childhood physical abuse, 24% reported parental separation during their childhood, 24% violence in family during childhood, 6% reported maternal alcohol problems, and 27% paternal alcohol problems (Hardt et al., 2010; Dragan and Hardt, 2016). Our sample also had 27% of participants reporting adult substance abuse (although we did not distinguish between drugs and alcohol), but our samples had a lower reporting of physical abuse (5%) and parental separation (3%). The lower frequency of ACEs in this sample may be due to an under-reporting of ACEs (Hardt and Rutter, 2004), or an actual lower amount of ACEs experienced.

Previous research in this area of Poland have demonstrated a relationship between age at menarche and adult estradiol in the luteal phase (Clancy et al., 2013). In our sample, we did not find a significant relationship between age at menarche and E1G concentrations in any phase ($p=0.22$, 0.40, and $p=0.22$ for the follicular, periovulatory, and luteal phases respectively). Our finding is consistent with Núñez-de la Mora et al. (2008) who also did not find a relationship between age at menarche and adult estradiol in Bangladeshi, Bangladeshi-British, and white British women. Age at menarche classically has been used as proxy for early environment due to the relationship between energetic stress and age at menarche. However, current research highlighting the pushes and pulls on age at menarche by different types of stress challenge that viewpoint. Here, age at menarche did not predict adult E1G, but the same predictors of age at menarche (specifically farming practices) did predict E1G across the menstrual cycle. Perhaps in this transitioning context, age at menarche may not be the most appropriate proxy of early environments.

Hypothesis 4: Helping on a childhood farm, exposure to farm animals during childhood, number of adverse childhood experiences and age at menarche together affect E1G concentrations.

Finally, our results provided support for hypothesis 4. We used elastic net regression to identify predictors of average E1G in each phase of the menstrual cycle. ACE groups, physical abuse during childhood, childhood farming, childhood animal care, and current waist-to-height ratio predicted average follicular E1G. ACE groups, substance abuse in childhood household, physical abuse as a child, emotion neglect as a child, childhood farming, childhood animal care, current waist-to-height, and current age predicted average periovulatory E1G. Mental illness in childhood household, physical abuse in childhood household, current body fat percentage, and

current age predicted average luteal E1G. Our results suggest that energetic, immune, and psychosocial stressors during childhood affect adult E1G concentrations in this sample.

The reproductive hypothesis more specifically predicts that a later age at menarche, indicative of childhood stress, is associated with decreased reproductive function and lower reproductive hormones (Ellison, 1996). Here, this hypothesis was supported in that that childhood farming in Poland affects both age at menarche and adult reproductive hormone concentrations in the expected direction. Specifically, childhood farming practices predict a later age at menarche and lower E1G concentrations.

However, ACEs also affected both age at menarche and adult reproductive concentrations, but in ways contrary to the expectations of the reproductive capacity hypothesis. Specifically, this measure of psychosocial stress predicts earlier ages at menarche and lower E1G concentrations in the final regression models. By broadening the study to include different types of stressors, we see that multiple types of stress can lead to decreased adult reproductive function, even in the case where they affect age at menarche in opposite directions.

Limitations:

This work was observational and retrospective, and thus our results should be interpreted in that context. We did not survey the frequency and timing of adverse childhood experiences, so we cannot conclusively say if these experiences occurred before or after age at menarche. Moreover, we did not include a measurement of contemporary adverse experiences, which likely also affects ovarian function (Allworth et al. 2007). Future work is needed to understand more about the relationship between all types of stress across the lifespan and reproductive health.

Conclusion

This study is the first to investigate connections between energetic, immune, and psychosocial stress during childhood with age at menarche and adult ovarian function in a transitioning agrarian population. We demonstrate that age at menarche has declined over time in this region in accordance with the global secular trend, although average age at menarche is later in this rural region compared to Polish cities. Helping on farms and with farm animals independently associated with later ages at menarche, which supports the idea that energetic and immune stress during childhood delays age at menarche. Adverse childhood experiences tended to accelerate age at menarche, which supports the hypothesis that psychosocial stress accelerates age at menarche. Despite different effects on age at menarche, all types of childhood stress decreased adult urinary estrogen metabolite concentrations in adult menstrual cycles. The results of this study support a model under which developmental conditions affect adult reproductive function, and we challenge the use of age at menarche alone as a proxy for childhood exposures.

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Chapter 4

The relationship between CYP19A1 promoter methylation and reproductive traits

Abstract

Reproductive ecology has long examined the adaptive flexibility of women's reproduction in the face of variable environments (Wasser and Barash, 1983; Vitzthum, 2008). However, we have suffered as a field from not always being able to measure the mechanisms to empirically support assertions of developmental effects, "set points," and other hypothesized links for which we do not yet have a physiological underpinning. Gene methylation can be responsive to environmental traits and can modify gene expression (Portela and Esteller, 2010). In normal tissue, gene promoter methylation patterns are typically unmethylated and intragenetic regions of DNA are methylated (Jones and Takai, 2001). The modifiable nature of DNA methylation makes it an informative method through which to study how environmental exposures may affect reproductive biology.

Two populations were included in this study: a rural, agricultural Polish population and an urban Polish American population. We first compare differences in ages at menarche and adult reproductive hormone concentrations between these two populations. The rural Polish sample is living in a region transitioning away from subsistence farming (Colleran, 2014). In rural Poland, childhood environmental variables including energetic and psychosocial stressors predicted age at menarche and adult urinary estrone urinary estrone-3-glucuronide (E1G) concentrations (see Chapter 3). While studies have compared reproductive traits between Polish and American samples, no study to date investigates differences in reproductive traits between Polish and specifically Polish American samples.

In this chapter, we investigate the relationship between childhood environments, methylation at a promoter of a candidate gene (CYP19A1), and age at menarche. We further investigate the relationship between life history traits, gene methylation, and adult reproductive hormone variation. We identify a pathway by which cortisol may affect aromatase expression via methylation of CYP19A1 promoter I.4.

Introduction

Age at menarche is highly heritable (Towne et al., 2005; Wells and Stock, 2011), yet a large proportion of the genetic variation remains unexplained (Elks et al., 2010; Wells and Stock, 2011; Almstrup et al., 2016). Epigenetics, the study of covalent modifications of DNA, is one way by which the environment interacts with the genome by altering gene expression without changing the primary DNA sequence (Russo et al., 1996). Gene methylation, one epigenetic process, changes in response to early life variables (Borghol et al., 2012; Tyrka et al., 2012). These include physical activity (Denham et al., 2013; White et al., 2013), psychosocial stress and support (Lam et al., 2012; Suderman et al., 2014), and nutrition (Brait et al., 2009; Zhang et al., 2011). In fact, these early life variables are also identified as modifiers of age at menarche (Dunger et al., 2005; Wise et al., 2009; Kramer and Greaves, 2011). It is thus reasonable to hypothesize that epigenetic factors provide a link between childhood environment and pubertal timing. Here, we investigate the relationship between childhood environments, methylation at a promoter of a candidate gene (CYP19A1), and age at menarche. We further investigate the relationship between life history traits, gene methylation, and adult reproductive hormone variation. We identify a potential pathway by which cortisol may affect aromatase expression via methylation of CYP19A1 promoter I.4.

Reproductive ecology has long examined the adaptive flexibility of women's reproduction in the face of variable environments (Wasser and Barash, 1983; Vitzthum, 2008). However, we have suffered as a field from not always being able to measure the mechanisms to empirically support assertions of developmental effects, "set points," and other hypothesized links for which we do not yet have a physiological underpinning. Gene methylation can be responsive to environmental traits and can modify gene expression (Portela and Esteller, 2010). In normal tissue, gene promoter methylation patterns are typically unmethylated and intragenetic regions of DNA are methylated (Jones and Takai, 2001). The modifiable nature of DNA methylation makes it an informative method through which to study how environmental exposures may affect reproductive biology.

Two populations were included in this study: a rural, agricultural Polish population and an urban Polish American population. We first compare differences in ages at menarche and adult reproductive hormone concentrations between these two populations. The rural Polish sample is living in a region transitioning away from subsistence farming (Colleran, 2014). In rural Poland, childhood environmental variables including energetic and psychosocial stressors predicted age at menarche and adult urinary estrone urinary estrone-3-glucuronide (E1G) concentrations (Chapter 3). While studies have compared reproductive traits between Polish and American samples, no study to date investigates differences in reproductive traits between Polish and specifically Polish American samples. Previous studies indicate that the Polish and American populations should have differences in life history traits including average age at menarche and progesterone and estradiol concentrations; these differences are typically attributed to higher energetic constraint in rural Poland compared to urban United States (Ellison, 2001; Jasienska, 2001; Jasienska et al., 2006b; Clancy et al., 2009). First, we hypothesized that

Polish American women in the United States would have earlier ages at menarche compared to rural Polish women. Second, we hypothesized that Polish American women will have higher concentrations of urinary reproductive hormone metabolite concentrations across the menstrual cycle.

We further investigated the relationship between reproductive traits and promoter methylation at the gene CYP19A1, which codes for the enzyme aromatase. Aromatase is critical for estrogen biosynthesis as aromatase metabolizes androgens into estrogens (Martinez-Arguelles and Papadopoulos, 2010). Methylation of a promoter of the gene CYP19A1 was used in this analysis for multiple reasons. First, decreased methylation of CYP19A1 promoters increases gene activity, aromatase activity, and, likely, estrone and estradiol production (Demura & Bulun, 2008). Methylation at CYP19A1 promoters is also associated with adult polycystic ovarian syndrome (Yu et al., 2013) and endometriosis (Hosseini et al., 2016). Finally, Stueve et al. (2014) found an interactive effect between body mass and CYP19A1 promoter methylation on timing of breast development during puberty. We hypothesized that methylation at this gene interacts with childhood energetic stressors to predict pubertal timing. We additionally hypothesized that CYP19A1 promoter methylation will be associated with adult urinary estrone-3-glucuronide (E1G) concentrations across the menstrual cycle. CYP19A1 promoter methylation might serve as a mechanistic link between childhood environments and adult reproductive hormone concentrations.

In summary, we offer three main hypotheses:

1. Average ages at menarche are earlier in Polish American women compared to Polish women. Reproductive hormone concentrations are higher in Polish American women compared to Polish women.

2. CYP19A1 promoter I.4 methylation is associated with age at menarche. Childhood environmental variables predict degree of methylation at CYP19A1 promoter I.4.
3. CYP19A1 promoter I.4 methylation is associated with adult E1G concentrations.

Methods

Women (n=123) were recruited at the Mogielica Human Ecology Study Site in the Beskid Wyspowy region of southern rural Poland during the summers of 2014, 2015, and 2017. Women (n=47) were recruited in the United States from 2016 – 2017. Women were recruited who were between the ages of 18 and 46, in general good health, not pregnant within the past six months, not breastfeeding within the past three months, not on hormonal contraception, and non-smoking. Polish American women were born in the United States and identified as 1st-3rd generation Polish American. Study protocols were approved by the University of Illinois, Urbana-Champaign Institutional Review Board (#13856). There were two study options in both locations, which are described below and displayed in Figure 4.1. This study is quite extensive, and some women did not complete all sub-sections. In order to ensure clarity for readers, we include the number of participants in our tables. The full study protocols are described in Chapter 3 and updated here to reflect the addition of Polish American women.

Participants were recruited in Poland in multiple ways: gynecologists, nurses, and a midwife at two local clinics helped recruit women, an announcement was published in the local church bulletin, fliers were left in local businesses including hair salons, book stores, and other types of shops, and students traveled door-to-door to invite women to participate in the study. Polish women from multiple villages in the region joined the study.

Participants were recruited in the United States in multiple ways: posted flyers, emails sent to listservs, announcements in local groups, libraries, and classes, and word of mouth. Participants in the United States first completed an online eligibility survey. Eligible participants were contacted, and in-person meetings were set up in order to introduce and begin the study.

A subset of participants (n=53 from 2014-2015 and n=46 in 2017 in Poland and n=31 in the United States) collected first morning void urine samples for one full menstrual cycle, filled out daily physical activity and well-being questionnaires (Jasienska et al., 2006b), and recorded their diet five times over their cycle. Participants collected one urine sample per day in a 120 mL cup and used vacuum-sealed tubes (Vacumed® in Poland and BD Vacutainer™ in the United States) to create two 8-10 mL aliquots at home. Participants immediately froze urine aliquots in home freezers. Samples were collected at the end of the menstrual cycle, transported in coolers, and stored at -20 or -80°C until processing. Samples in Poland were shipped over dry ice back to the University of Illinois, Urbana-Champaign for processing.

There were two study options, and some women (n=37 in Poland and n=13 in the United States) chose to join a week-long abbreviated version of the study. In the week-long abbreviated version, women completed daily physical activity and well-being questionnaires and recorded their diet three times over that week, but they did not collect any urine samples for hormonal analyses.

Surveys and Measurements: All women completed health and demography surveys that included questions about reproductive trait timing and childhood environments. Women were asked about age at menarche via recall, and most women in the study (n=119/123 in Poland and n= 47/47 in the United States) reported age at menarche. Recall of age at menarche is good for women of ages included in this study (Koprowski et al., 2001; Must et al., 2002). If a woman reported an

age range (i.e. age 13 - 14), then an average of those ages was used for analyses. Women were further asked if they lived on a farm during childhood, if they helped on that farm, and what types of animals were present while they were growing up. Women were asked the same questions about their current living environment. Women in the United States were also asked about participation in childhood sports (i.e. years of participation and type of sport).

Participants completed the Adverse Childhood Experiences (ACE) survey in the privacy of their homes (Felitti et al., 1998). Due to the sensitive nature of these questions, participants were reminded that all surveys and survey questions were optional. Some women (n=24/123 in Poland and n=8/47 in the United States) chose not to, or forgot to, complete the ACE survey. Some women (n=19/99 in Poland and n=1/39 in the United States) did not answer all ACE survey questions. The majority (n=15/19 in Poland and n=1/1 in the United States) of women who partially completed the survey skipped one question (range 1-6 questions skipped). To maximize the amount of data, ACE scores were only excluded if participants skipped one or more questions and had a total score of zero. We calculated the total number of reported ACEs and used the following categories: zero to one reported experience and two or more reported experiences. Grouping ACEs in such a cumulative manner is common in the literature (Felitti et al., 1998; Dube et al., 2003; Cluver et al., 2015).

Anthropometric measurements were taken upon enrollment of all women according the to the Bones and Behavior Working Group protocol (Antón et al., 2009). Participant weight and body fat percentages were measured using the Tanita© BF-680W body fat monitor.

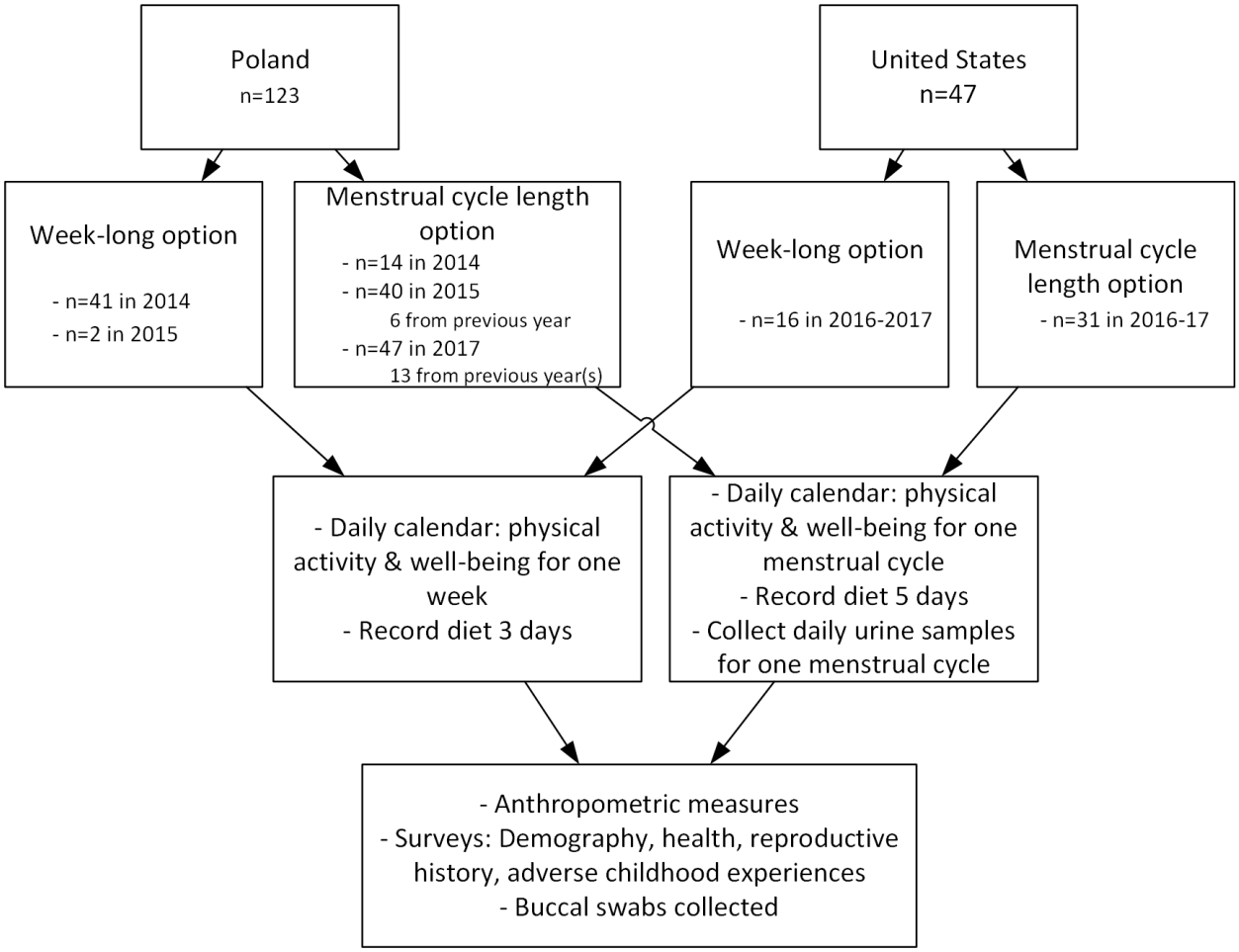


Figure 4.1: The study protocol included two study options: one week-long and one menstrual-cycle length. This figure displays the surveys, measurements, and collected samples for each study option.

Laboratory Protocol (hormone data): We measured multiple hormones over the course of each participant's menstrual cycle using two types of Quansys multiplex enzyme linked immunosorbent assays (ELISA). The measured hormones included: the urinary estradiol metabolite (estrone-3-glucuronide, E1G), C-Peptide, tumor necrosis-factor alpha (TNFα), human chorionic gonadotropin (beta-hCG), interleukin 6 (IL-6), cortisol, interleukin 10 (IL-10), C-reactive protein (CRP), follicle-stimulating hormone (FSH), and urinary progesterone metabolite (pregnanediol glucuronide, PdG). All hormones except PdG were run on a custom Quansys 9-Plex ELISA, and PdG was run individually on a 1-plex Quansys ELISA. Hormone data was

generated for samples collected in Poland in 2014 and 2015 (n=53) and some samples collected in the United States in 2016-2017 (n=30 assays for PdG and n=9 assays for the 9-Plex). There is less data generated for the 9-Plex due to an unfortunate freezer failure resulting in the loss of aliquots of samples from Polish American women.

Sixty-nine total full or half ELISA plates were run for the PdG assay, and seventy-five full or half ELISA plates were run for the 9-Plex. The intra- and inter- assay variation for each measured hormone was as follows: E1G (8.54%, 32.84%), C-Peptide (5.66%, 25.12%), TNFα (17.97%, 0%), beta-hCG (8.08, 0%), IL-6 (6.70%, 0%), cortisol (10.27%, 20.00%), IL-10 (26.12%, 0%), CRP (4.75%, 17.55%), FSH (5.06%, 34.78%), and PdG (9.46%, 32.19%). While the inter-assay variation is high, it is well within the range typically seen in multiplex assays (Chowdhury et al., 2009; Bastarache et al., 2011). All samples were run in duplicate; if the coefficient of variation (CV) between duplicates was ≥ 15%, then the samples were rerun. E1G, PdG, and cortisol concentrations were used in this analysis. The final concentration for all urinary hormones was corrected by specific gravity using the protocol described in Miller et al. (2004). Some cycles were missing one or more days of urinary collections. When concentrations were available for the previous and following day, then a missing value was estimated by averaging the surrounding concentrations.

Cycles were aligned by mid-cycle drop date according to Lipson and Ellison (1996). Using this method, day of ovulation is determined as the maximum decrease in E1G over two consecutive days. Day of ovulation is marked as day zero, and the follicular and luteal phases are aligned going backwards and forwards, respectively. In the event where two potential drop dates were identified, we chose the drop date occurring immediately before a rise in PdG. E1G was not available for all cycles, so some ovulation days for Polish American cycles were estimating

using home luteinizing hormone (LH) test strips. Participants collected an additional urine sample between 10 AM and 8 PM on days 10-18 of their cycle. If multiple days were positive for LH, the first day was used as the best estimate. If no days were positive for LH, we assumed we missed the LH peak, and we did not align that cycle by ovulation day.

Cycles were assessed for anovulation using the cycles method from Kassam et al. (1996). A baseline level of PdG is defined as average PdG concentration for days 6-10, which represents PdG after the clearance of PDG from the previous luteal phase and before the rise of PdG during the current cycle (Kassam et al., 1996). Cycles are considered anovulatory if the ratio of PdG to baseline PdG is greater than or equal to three for at least three consecutive days (Kassam et al., 1996).

Cycles from Polish American participants (n=5) were excluded for anovulation. This left a total of 25 cycles for assessment of variation in PdG. Two of the five anovulatory cycles had 9-plex data available, leaving only seven cycles from Polish American participants available for assessment of E1G and cortisol. Cycles from Polish sample (n=4) were excluded for missing data, pregnancy, or anovulation. This left a total of 49 cycles from Polish sample included in analyses.

Averages of E1G and cortisol concentrations were calculated by cycle phase as follows: follicular phase (days -10 to -1), periovulatory phase (days -3 to 3), and luteal phase (days 1 to 10). Averages of PdG were calculated by cycle phase as follows: luteal (days -1 to -14), early luteal (-10 to -5), and late luteal (-4 to -1). These phase partitions are consistent with the literature (Lipson and Ellison, 1996; Barrett et al., 2013; Clancy et al., 2013). If a participant was missing three or more consecutive days for the follicular or luteal phases, then an average value was not calculated for that phase for that participant.

Laboratory Protocol (epigenetic data): Methylation has high tissue-specificity, and buccal swabs are an informative general tissue collected non-invasively (Lowe et al., 2013). Buccal swabs (n=2-4 Whatman© Omniswabs per participant) were collected for gene methylation analysis. Methylation data from Poland study years 2014 and 2015 was included in this analysis. The CYP19A1 gene is >123 kb long, and there are multiple tissue-specific promoters of this gene (Bulun et al., 2003). Two CpG sites in promoter 1.4 were analyzed via pyrosequencing, and we used additional data from the Illumina Infinium Methylation EPIC array to analyze methylation at CpG sites in promoter II/I.3, promoter I.4, and promoter I.1.

DNA from buccal swabs was extracted using the Qiagen© Mini DNA Kit using the standard procedure. Extracted DNA was then bisulfite converted with the Zymo EZ DNA Methylation-Gold™ Kit using the standard protocol; the bisulfite conversion process deaminates all unmethylated cytosines to uracil. When a bisulfite converted DNA strand undergoes amplification via polymerase chain reaction (PCR), all uracil nucleotides (the unmethylated cytosines) will bind to guanine and thus be later sequenced as thymine. PCR followed by pyrosequencing was used to quantify methylation at CYP19A1 promoter regions. Primers were designed using the Qiagen PyroMark® CpG Assay design software using the sequence for aromatase promoter I.4 (GenBank accession: L21982). The PCR forward primer was AGATTTTTGATTTATGTGGGGTTA, reverse biotinylated primer was TACTCAAACTCCAAAAACTTACCTAAT, and sequencing primer was TGATTTATGTGGGGTTAT. Pyrosequencing was completed using the QIAGEN PyroMark Q24 Advanced System. This method provided the proportion of methylated cytosines at the 8th and 9th CpG sites in CYP19A1 promoter I.4.

Methylation of additional CpG sites for 62 of the Polish samples was assayed using the Illumina Infinium Methylation EPIC array, which provided information on single-base methylation for 865,918 CpG sites across the genome. We specifically used this data to look at CpG sites in additional CYP19A1 promoters. There was no overlap in the promoters included in the Illumina Infinium Methylation EPIC array and the pyrosequenced regions. Quality control of methylation data was performed as outlined in Ratanatharathorn et al. (2017). Dr. Angela Bustamante (UIUC) and I modified the pipeline outlined in Ratanatharathorn et al. (2017) for this study. The Illumina GenomeStudio software, R studio version 1.0.143 (RStudio Team, 2015), and the R packages CpGassoc (Barfield et al., 2012) and WateRmelon (Pidsley et al., 2013) were used to pre-process the data. Specifically, background correction of the data was run in GenomeStudio in order to remove nonspecific signals and technical variation (Wilhelm-Benartzi et al., 2013). Sites with low signal intensity (detection p-value >0.001) or with more than 10% of missing data (n=12,430 in this dataset) were removed from the analysis. Additionally, 44,210 probes characterized as not specific and potentially cross-hybridizing were removed (McCartney et al., 2016). After removing these probes, 809,685 sites were left in analyses. Methylation at these probes was than normalized using the Beta Mixture Quantile normalization (BMIQ). Finally, we extracted only CpG sites located in or near CYP19A1 using the UCSC gene name in the Illumina annotation file. This selection method was used in Clukay et al. (2018) in order to focus analyses on specific candidate genes. This method resulted in methylation data for 60 CpG sites in or around CYP19A1. Some of these sites were located in promoter regions, and promoter methylation was averaged for promoter II/I.3, promoter I.4, and promoter I.1.

Analyses for each hypothesis:

Hypothesis 1: Average ages at menarche are earlier in Polish American women compared to Polish women. Hormone concentrations are higher in Polish American women compared to Polish women.

Test: We tested for differences in ages at menarche between the Polish and Polish American samples by created a standard linear regression model including project location, participant current age, and the interaction between current age and project location. Age was included in the model as there were significant differences in participant ages between the two study locations.

Repeated measures ANOVA were used to test for differences in E1G, PdG, and cortisol concentrations across the menstrual cycle between Polish and Polish American participants. Follicular (days -10 to -1), periovulatory (days -3 to 3), luteal (0 to 10) phases were analyzed for each hormone. Repeated measures ANOVA were used to test for differences in PdG concentrations between Polish and Polish American participants was also assessed using the standard alignment counting backwards from menses. Differences were tested for the luteal phase (days -1 to -14).

Hypothesis 2: CYP19A1 promoter I.4 methylation is associated with age at menarche. Childhood environmental variables predict degree of methylation at CYP19A1 promoter I.4.

Tests: We created a best fit regression model to select predictors of age at menarche in the Polish American sample using a penalized regression technique (elastic net) that both minimizes the effect of multicollinearity and performs feature selection to create a best fit model (Zou and Hastie, 2005). Predictors included in the model were: generation Polish American, helped on a childhood farm, had animals as a child, number of farm animal types during childhood,

participation in childhood sports, ACE group, and physical activity, height and weight at age at menarche in comparison to peers (more, less, similar).

We created multiple linear regression models to test for a relationship between childhood farming variables, adverse childhood experiences, CYP19A1 promoter methylation, and age at menarche in the Polish sample.

Hypotheses 3: CYP19A1 promoter I.4 methylation is associated with adult E1G concentrations.

Tests: We first created examined correlations between methylation at CYP19A1 and average E1G, PdG, and cortisol concentrations. We created a general linear mixed model with cortisol concentration as the dependent variable, CYP19A1 promoter I.4 percent methylation and menstrual cycle phase as the independent variables and individual as a random factor. We created a general linear mixed model with E1G concentration as the dependent variable, CYP19A1 promoter I.4 percent methylation and menstrual cycle phase as the independent variables and individual as a random factor. Finally, structural equation modeling was used to test a pathway where cortisol interacts with CYP19A1 promoter I.4 to predict average E1G concentrations.

Analyses were run using JMP® PRO 13, and alpha was set at 0.05. Structural equation modeling was conducted using the R packages lavaan, semPlot, and OpenMx.

Results

Demographic information for each sample is displayed in Table 4.1.

Table 4.1

Measure	Sample	Mean	Std Dev	n
Age	Poland	32.30	8.00	123
	USA	24.02	7.12	47
Age at menarche	Poland	13.42	1.65	119
	USA	12.77	1.28	47
Body Fat Percentage	Poland	29.40	8.49	123
	USA	29.00	7.24	42
Waist/Height Ratio	Poland	0.49	0.09	123
	USA	0.45	0.06	44
Birth Weight	Poland	3319.72	539.84	72
	USA	3388.39	592.88	23
Number of adverse childhood experiences	Poland	0.94	1.29	90
	USA	1.03	1.68	39
Luteal Phase Length (Days)	Poland	11.27	1.90	49
	USA	13.31	3.81	16
Follicular E1G	Poland	111.19	6I.37	42
	USA	133.75	99.53	5
Periovulatory E1G	Poland	116.84	69.19	49
	USA	152.30	119.58	6
Luteal E1G	Poland	76.62	43.01	47
	USA	86.59	75.35	6
Luteal PdG (aligned by menses, days -1 to -10)	Poland	27578.27	15413.77	46
	USA	29879.13	21142.00	21
Periovulatory PdG (aligned by ovulation day, days -3 to 3)	Poland	14203.48	10773.83	49
	USA	12545.02	13324.65	15
Luteal PdG (aligned by ovulation day, days +1 to +10)	Poland	28279.10	16200.23	48
	USA	32008.95	24150.97	15
Follicular Cortisol	Poland	346.78	190.24	42
	USA	270.88	53.79	6
Periovulatory Cortisol	Poland	310.45	183.22	47
	USA	243.23	62.51	5
Luteal Cortisol	Poland	307.79	145.40	49
	USA	216.66	17.47	5
Ever Smoked	Poland			52/123
	USA			5/47

Table 4.1 (cont.)

Measure	Sample	Mean	Std Dev	n
Helped on a childhood farm	Poland			84
N				39
Y	USA			2
N				45
Had animals as a child	Poland			110
N				13
Y	USA			42
N				5
Currently help on a farm	Poland			31
N				92
Y	USA			3
N				44
Currently have animals	Poland			95
N				28
Y	USA			25
N				22
Played Sports	USA			36
N				12
Played Sports: years played, before age at menarche		3.48	3.56	45
Played Sports: years played, total		7.40	6.19	45

Table 4.1: Demographic information for the Polish and Polish American participants.

Hypothesis 1: Average ages at menarche are earlier in Polish American women compared to Polish women. Hormone concentrations are higher in Polish American women compared to Polish women.

We first investigated potential differences in ages at menarche and hormone concentrations across the menstrual cycle between the Polish and Polish American samples. Polish participants were significantly older than Polish American participants ($p<0.0001$). Polish participants (n=123) had a mean age of 32.30 (SE 0.70), and Polish American participants

(n=48) had a mean age of 24.02 (SE 1.05). Thus, we included age in each of the following results.

Age at menarche: There were significant differences in average ages at menarche between the Polish and Polish American samples (Table 1, p=0.0271). Polish Americans participants had an earlier average age at menarche compared to the Polish participants. Polish participants had an average age at menarche of 13.42 (SD 1.65) years, and Polish American participants had an average age at menarche of 12.77 (SD 1.28) years.

Adult Hormone and Reproductive Variation: There were significant differences in the length of the luteal phase for these two populations (Fig. 4.2, p=0.0251). The Polish sample had an average of 11.27 (SD 1.90) days in the luteal phase, and the Polish American sample had an average of 13.31 (SD 3.81) days in the luteal phase. Additionally, some participants (n=11/49 Polish and n=2/16 Polish American women) had a short luteal phase length (<10 days), an indication of luteal phase deficiency.

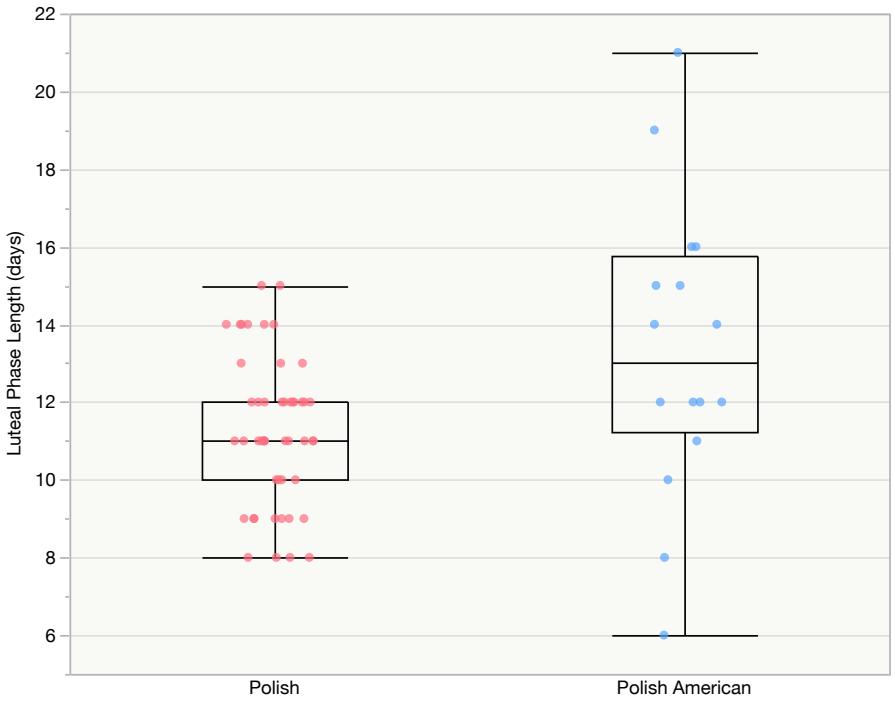


Figure 4.2: There are significant differences in average luteal phase lengths between the Polish (n=49) and Polish American (n=16) samples (p=0.0251). Mean luteal phase length was 11.27 (SD 1.90) days for the Polish sample and 13.31 (SD 3.81) days for the Polish American sample.

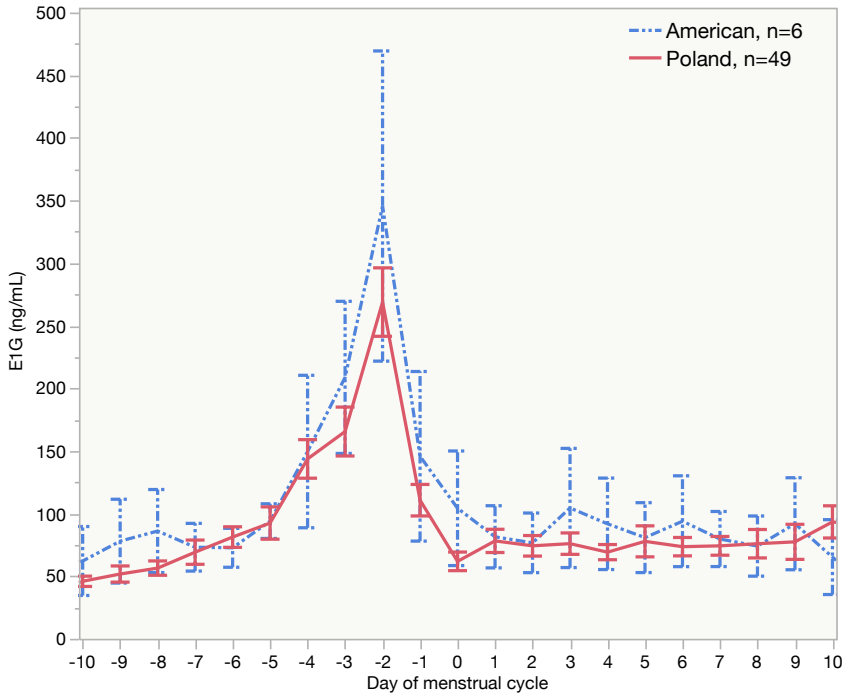
We used repeated measures ANOVA to test for differences in sex steroids E1G and PdG concentrations and glucocorticoid cortisol concentrations between Polish and Polish American samples across phases of the menstrual cycle (Fig. 4.3). There were not significant differences in E1G concentrations between Polish and Polish American samples for any phase. Specifically, there were no differences between E1G concentrations during the follicular phase (aligned by day of ovulation, days -10 to -1, p=0.6852), early follicular phase (days -10 to -6, p=0.6799), late follicular phase (days -5 to -1, p=0.7568), and periovulatory phase (days -3 to 3, p = 0.7357). There were not significant differences in the luteal phase (days 1 to 10, p=0.0935, early luteal phase (days 0 to 2, p=0.9433), early/mid luteal phase (days 3 to 5, p=0.9822), or late luteal phase (days 6 to 10, p=0.1198).

When investigating variation in PdG between populations, it is typically most appropriate and logical to align cycles by counting backwards from the start of the following menstrual cycle (Jasienska and Ellison, 1998; Clancy et al., 2009). When analyzing cycles in this manner, there were not significant differences in PdG concentrations between Polish and Polish American samples for the luteal phase (aligned by menses, days -14 to -1, p=0.7894), mid-luteal phase (days -10 to -5, p=0.4486), and late luteal phase (days -4 to -1, p=0.0976). Due to significant differences in luteal phase length between the two samples, we also aligned PdG by ovulation day and investigated differences in PdG between the Polish and Polish American samples (Fig. 4.3). There were not significant differences in PdG concentrations for the periovulatory phase (days -3 to 3, p=0.7441), luteal phase (days 1 to 10, p=0.6594), early luteal phase (days 0 to 2, p=0.4505), early/mid luteal phase (days 3 to 5, p=0.8367), or late luteal phase (days 6 to 10, p=0.7763).

Finally, there were not significant differences in cortisol concentration between Polish and Polish American samples for any phase (Fig. 4.3). Specifically, there were no differences between E1G concentrations during the follicular phase (aligned by day of ovulation, days -10 to -1, p=0.2464), periovulatory phase (days -3 to 3, p = 0.3334), or luteal phase (days 1 to 10, p=0.5046).

Figure 4.3

3A.



3B.

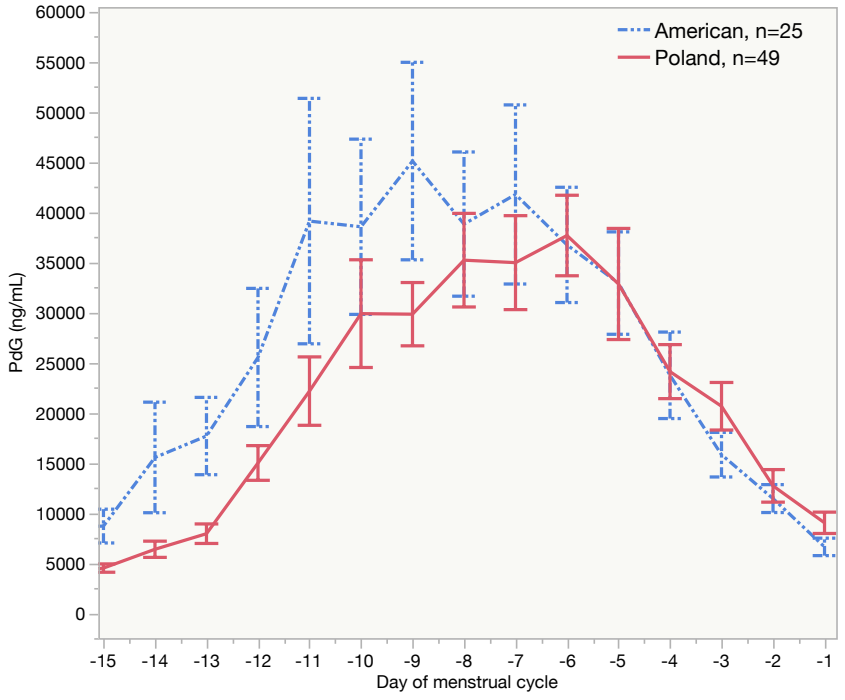


Figure 4.3 (cont.)

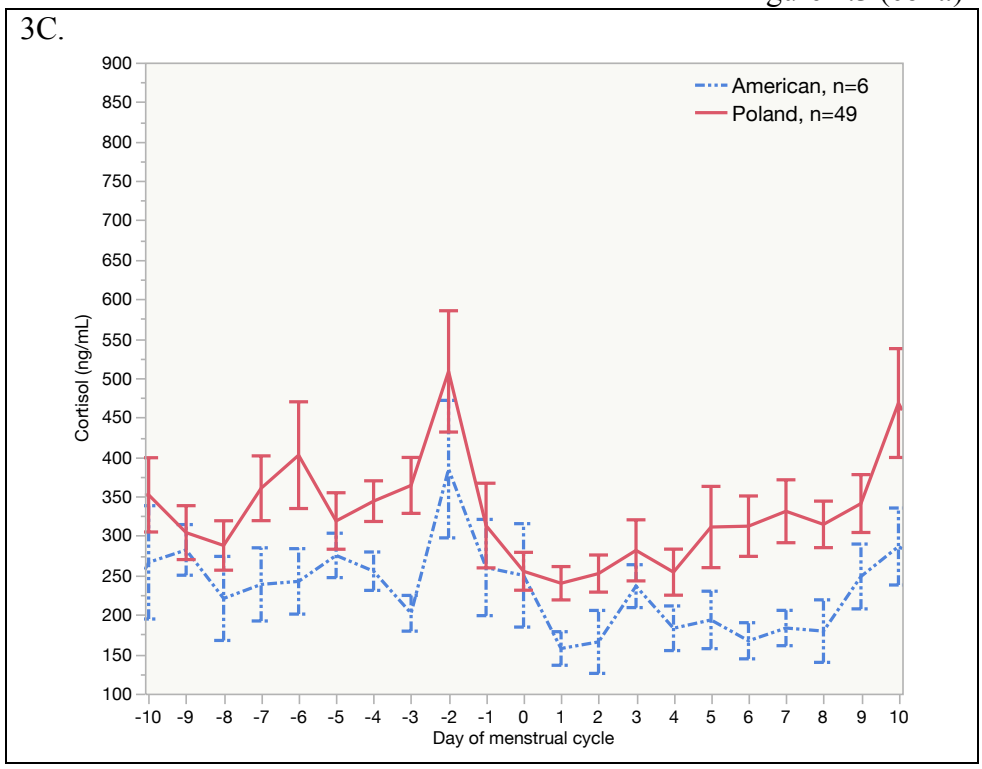


Figure 4.3: There are no significant differences in average E1G concentrations (Fig. 3A), average PdG concentrations (Fig. 3B), or average cortisol concentrations (Fig. 3C) between the Polish and Polish American samples for any phase of the menstrual cycle.

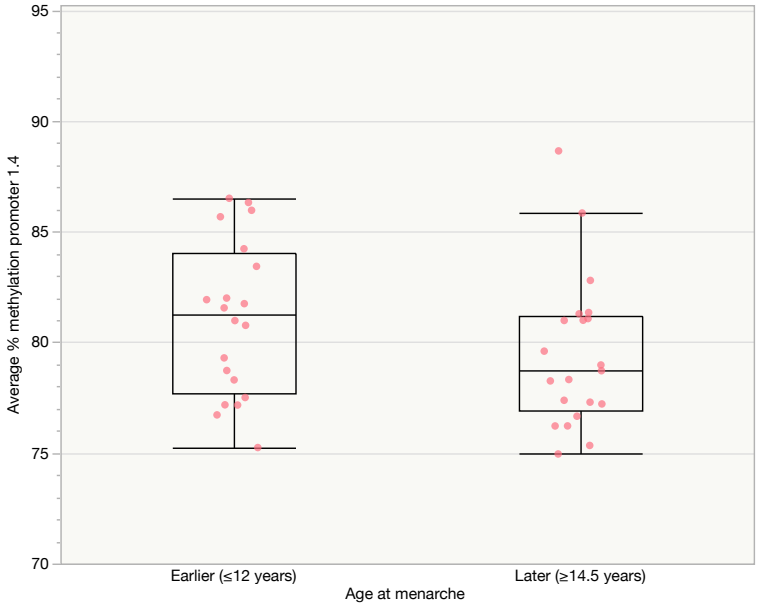
In the last chapter, we found that childhood farming and adverse childhood experiences best predicted age at menarche in the Polish sample (AICc = 297.06, R^2=0.09). Here, we first investigate which childhood variables predict age at menarche in the Polish American sample. I investigated the relationship between age at menarche and early childhood exposures (farming, own animals, played sports during childhood, number of years participate in sports before age at menarche, adverse childhood experiences, generation Polish American, and physical activity, height and weight at age at menarche compared to peers). I found that ACEs did not independently predict age at menarche (p=0.8082), nor did childhood sport participation

(p=0.8105). Using the same elastic net regression technique, none of the variables included in the model significantly predicted age at menarche.

Girls whose parents were born in Poland (n=26) had an average age at menarche of 13.12 (SD 1.29, n=29). Girls whose grandparents were born in Poland and parent(s) born in the United States had a slightly earlier average age at menarche of 12.33 (SD 1.22, n=15). This difference was not significant (p=0.0620). However, Polish American women whose parents were born in Poland did not have a significantly different average age at menarche compared to Polish participants (p=0.3888), while Polish American women whose grandparents were born in Poland did have a significantly earlier average ages at menarche compared to Polish participants (p=0.0126).

Hypothesis 2: Methylation at CYP19A1 promoter I.4 is associated with age at menarche. Childhood environmental variables predict percent methylation at CYP19A1 promoter I.4.

We next examined the relationship between age at menarche and percent methylation at CpG 8 and CpG 9 in CYP19A1 in the Polish sample. We found that women with later (≥ 14.5 years) ages at menarche had slightly, but not significantly, lower percent methylation at CpGs 8 and 9 of the CYP19A1 promoter I.4 compared to women wither earlier (≤ 12 years) ages at menarche (Figure 4.4, p=0.1403).



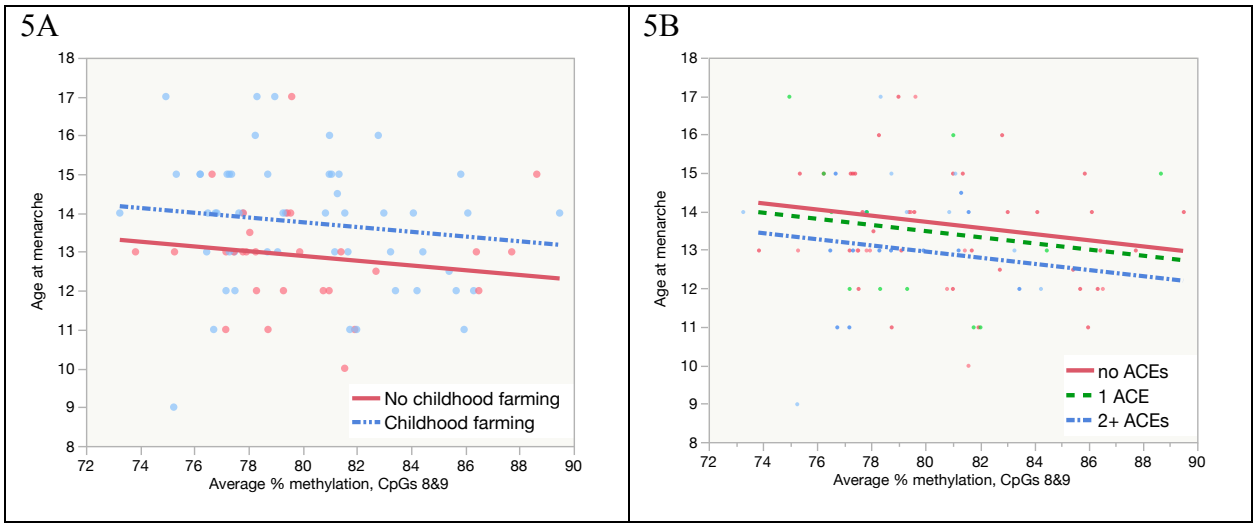
Age at menarche	n	Mean	Std Dev
Later (≥ 14.5 years)	21	79.43	3.42
Earlier (≤ 12 years)	20	81.06	3.50

Figure 4.4: Women with earlier ages at menarche have slightly, but not significantly, increased promoter methylation at CpG sites 8 and 9 of the CYP19A1 promoter (p=0.1403).

In the previous chapter, we found that childhood farming and adverse childhood experiences affect age at menarche in the Polish sample. Here, we investigate combined effects between percent methylation at CYP19A1 promoter I.4 CpG sites 8 and 9 with these variables on age at menarche. We find that childhood farming and percent methylation together significantly predict age at menarche (Figure 5A, p=0.0336, R^2=0.08, n=81). Lower percent methylation at CYP19A1 promoter I.4 and farming during childhood both associated with increased ages at menarche.

Number of adverse childhood experiences and percent methylation at CYP19A1 promoter I.4 CpG sites 8 and 9 did not significantly predict age at menarche in this sample (Figure 5B, p=0.2767, R^2=0.06, n=62). Including methylation at promoter I.1 and promoter II/I.3

did not improve either model, and methylation at these promoters did not significantly predict age at menarche.



Dependent Variable: age at menarche					
R^2	0.08				
Model p-value	0. 0336*				
Parameter estimates					
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	
Intercept	18.2293	3.9084	4.66	<0.0001	*
Childhood farming (No)	-0.4339	0.1836	-2.36	0.0206	*
Average % methylation at CpG 8&9	-0.0613	0.0488	-1.26	0.2127	

Figure 4.5: Helping on a farm during childhood and average percent methylation at CYP19A1 promoter I.4 together significantly predicted age at menarche in the Polish sample (Fig. 5A, p=0.0336, R^2=0.08, n=81). Comparatively, number of adverse childhood experiences and percent methylation at CYP19A1 promoter I.4 CpG sites 8 and 9 did not significantly predict age at menarche in this sample (Fig. 5B, p=0.2767, R^2=0.06, n=62).

Hypotheses 3: Methylation at CYP19A1 promoter I.4 is associated with adult E1G concentrations.

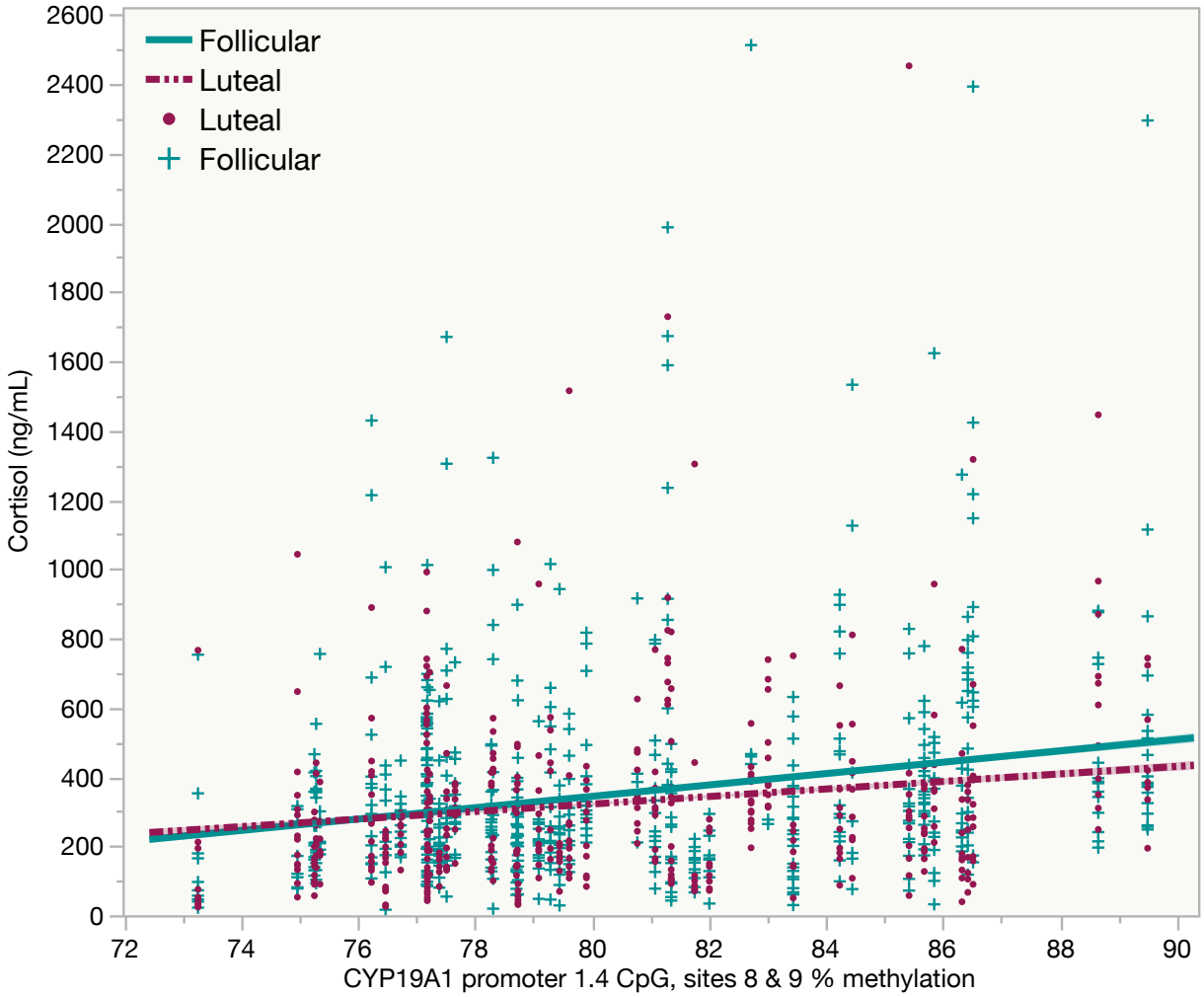
We created a multivariate correlation matrix to display the relationship between measured average hormone concentrations and methylation at all CYP19A1 CpG cites,

CYP19A1 promoter II/I.3, CYP19A1 promoter I.4 (Illumina M-values and percent methylation from pyrosequencing at CpGs 8&9), and CYP19A1 promoter I.1/CpG Island shelf (Table 4.2). Some interesting patterns emerged. First, average methylation was significantly correlated with luteal phase length. Secondly, average cortisol concentrations were significantly positively correlated with average methylation at CYP19A1 promoter I.4 CpG sites 8&9. Finally, follicular and luteal phase cortisol correlated with average E1G concentrations.

We further investigated the relationship between cortisol and CYP19A1 promoter I.4 methylation using a general linear mixed model. Individual was included as a random factor. CYP19A1 promoter I.4 percent methylation was significantly associated with cortisol concentration (Fig. 4.6, $p=0.0125$).

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
1. Follicular Phase E1G	1.0000												
2. Periovulatory Phase E1G	0.9280	1.0000											
3. Luteal Phase E1G	0.7421	0.8530	1.0000										
4. Luteal PdG	0.1188	0.0683	0.2517	1.0000									
5. Follicular Phase Cortisol	0.3544	0.4266	0.3362	0.0507	1.0000								
6. Periovulatory Phase Cortisol	0.2510	0.4066	0.3368	0.1689	0.8595	1.0000							
7. Luteal Phase Cortisol	-0.1435	0.0191	0.1582	0.3515	0.5651	0.7280	1.0000						
8. Average overall CYP19A1 methylation	0.1683	0.0270	0.0267	0.1445	0.1199	0.0614	0.0207	1.0000					
9. CYP19A1 promoter II/I.3 average methylation	-0.1555	-0.1697	-0.0748	0.0585	0.0068	0.0511	0.0775	0.2004	1.0000				
10. CYP19A1 promoter I.4 average methylation	-0.0126	-0.2178	-0.2391	-0.0339	-0.1678	-0.2659	-0.1260	0.3644	-0.0236	1.0000			
11. CYP19A1 promoter I.1 and CpG island shelf average methylation	0.2376	0.1221	0.0055	-0.0731	0.0919	0.1064	-0.0639	0.3170	0.1705	0.2449	1.0000		
12. CYP19A1 CpG sites 8&9 average methylation	0.0034	0.1809	0.0885	-0.1298	0.4678	0.4266	0.4004	0.0557	-0.1633	0.0801	0.0047	1.0000	
13. Luteal Phase Length	0.1833	0.1520	0.1476	-0.0904	0.0141	0.0844	-0.0027	0.3154	-0.1383	0.1059	0.0619	-0.0885	1.0000

Table 4.2: Multivariate correlation matrix displaying relationships between hormone concentrations and select CYP19A1 methylation. Significant pairwise correlations ($p<0.05$) are highlighted in green indicating positive correlations.



Dependent Variable:	**Cortisol Concentration**					
AICc	15230.09					
Log likelihood	15218.01					
Term	**Estimate**	**Std Error**	**df**	**t Ratio**	**Pr (>|t|)**	
Intercept	-783.0580	499.8236	39.4	-1.57	0.1252	
CYP19A1 pI.4 CpG sites 8&9, % methylation	14.8028	5.6561	39.4	2.62	0.0125	*
Phase (Follicular)	30.6989	7.8026	1060.5	3.93	<0.0001	*
Age	-1.8775	3.0809	39.5	-0.61	0.5457	

Figure 4.6: Percent methylation at CYP19A1 promoter I.4 significantly predicated cortisol concentrations. Individual was included as a random factor in the general linear mixed model.

We then investigated the hypothesis that CYP19A1 promoter I.4 methylation is associated with E1G concentrations. We created general linear mixed models for luteal and follicular phase E1G concentrations and included interaction terms between CYP19A1 promoter I.4 methylation and cortisol concentrations. Additional variables that influence adult E1G concentrations were included in the model, and individual was included as a random factor. We found that CYP19A1 promoter I.4 methylation was not a significant predictor of follicular phase E1G (p=0.3129, Table 4.3). Comparatively, CYP19A1 promoter I.4 methylation was a significant predictor of luteal phase E1G (p=0.0033, Table 4.4). The interaction between CYP19A1 promoter I.4 methylation and cortisol concentrations was not a significant predictor of either follicular phase E1G (p=0.1504, Table 4.3) or luteal phase E1G (p=0.2413, Table 4.4).

Dependent Variable	**Follicular Phase E1G Concentration**					
AICc	5596.27					
Log likelihood	5573.69					
Term	**Estimate**	**Std Error**	**DF**	**t Ratio**	**Prob>|t|**	
Intercept	239.3782	206.8841	22.5	1.16	0.2594	
CYP19A1 promoter I.4 CpG sites 8&9 methylation	-2.4982	2.4213	23.0	-1.03	0.3129	
Cortisol	0.0977	0.0172	410.2	5.69	<0.0001	*
(CYP19A1 promoter I.4 CpG sites 8&9 methylation) * (Cortisol)	-0.0054	0.0038	454.8	-I.44	0.1504	
Waist to Height Ratio	68.4016	123.8022	22.6	0.55	0.5860	
Helped on a childhood farm (N)	26.4677	9.7890	23.0	2.70	0.0127	*
Current Age	-0.3997	1.6797	21.5	-0.24	0.8142	
Adverse Childhood Experiences (group: 0 or 1)	18.0278	10.6400	22.6	1.69	0.1040	
Ever Pregnant (N)	4.2046	13.0424	22.3	0.32	0.7502	

Table 4.3: CYP19A1 promoter I.4 methylation was not associated with follicular phase E1G. Only cortisol concentration and childhood farm work were significant predictors of follicular phase E1G. Individual was included as a random factor in the general linear mixed model.

Dependent Variable	Luteal Phase E1G concentration					
AICc	3951.23					
Log likelihood	3928.47					
Term	**Estimate**	**Std Error**	**DF**	**t Ratio**	**Prob>|t|**	
Intercept	582.7140	152.5588	26.4	3.82	0.0007	*
CYP19A1 promoter I.4 CpG sites 8&9 methylation	-5.7110	1.7689	26.5	-3.23	0.0033	*
Cortisol	0.1426	0.0154	331.9	9.28	<0.0001	*
(CYP19A1 promoter I.4 CpG sites 8&9 methylation) * (Cortisol)	-0.0040	0.0034	341.0	-1.17	0.2413	
Waist to Height Ratio	26.8345	91.0147	26.2	0.29	0.7704	
Helped on a childhood farm (N)	6.5906	7.0953	25.6	0.93	0.3616	
Current Age	-2.6024	1.2509	27.2	-2.08	0.0470	*
Adverse Childhood Experiences (group: 0 or 1)	-0.5150	7.8979	27.2	-0.07	0.9485	
Ever Pregnant (N)	22.5537	9.4179	25.7	2.39	0.0242	*

Table 4.4: CYP19A1 promoter I.4 methylation, cortisol concentrations, age, and former pregnancies were significant predictors of luteal phase E1G concentrations. Individual was included as a random factor in the general linear mixed model.

We used structural equation modeling to visualize the relationships among cortisol, CYP19A1 promoter I.4 methylation, and luteal phase E1G. CYP19A1 promoter I.4 methylation negatively correlated with luteal phase E1G concentrations, and cortisol positively correlated with CYP19A1 promoter I.4 methylation (Figure 4.7).

Fig. 7A

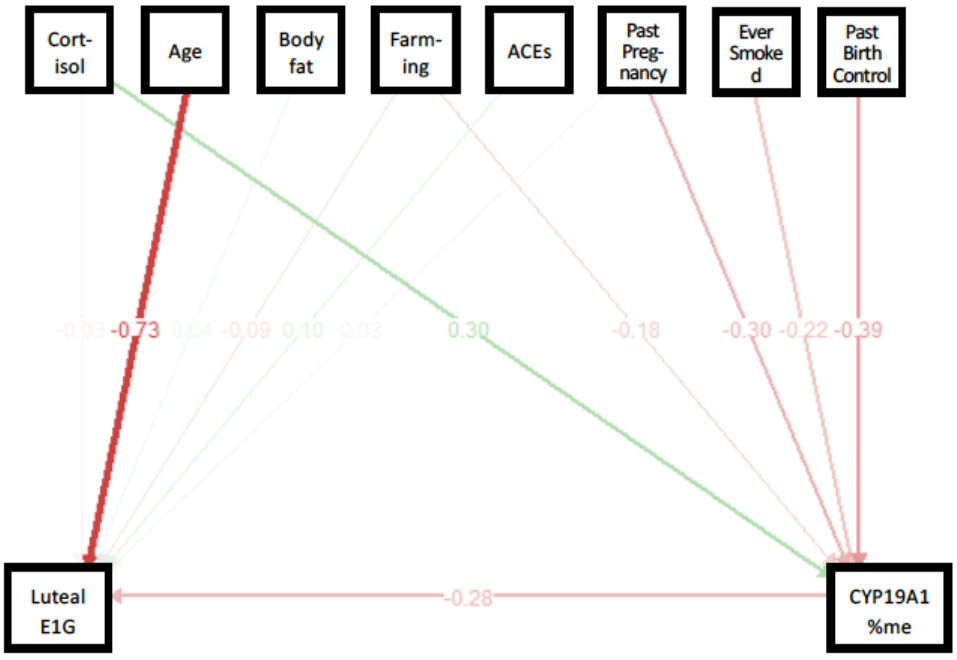


Fig. 7B

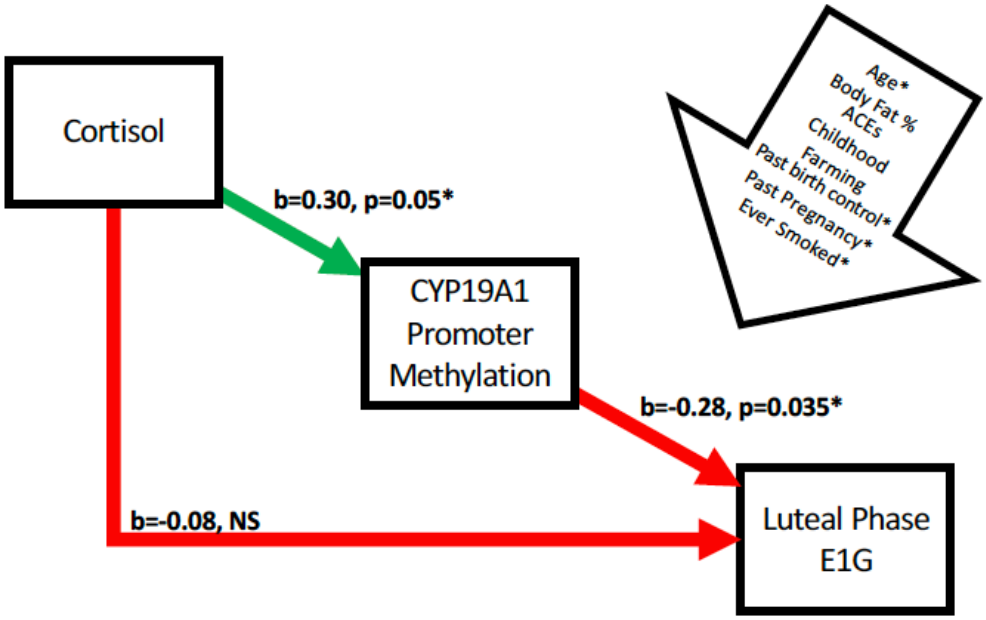


Figure 4.7: A structural equation model displaying the hypothesized relationships between cortisol, CYP19A1 promoter I.4 methylation, and luteal phase E1G. Age, body fat percentage, number of ACEs, childhood farming, past pregnancy, former use of hormonal birth control, and previous smoking were all included in the model. Fig 7A displays the full model and standardized parameter estimates for each relationship. Fig 7B displays a simplified version of the same model in order to visualize CYP19A1 as a mediator of the relationship between cortisol and luteal phase E1G.

Discussion

Reproductive ecology has long examined the adaptive flexibility of women's reproduction in the face of variable environments (Wasser and Barash, 1983; Vitzthum, 2008). Differences in reproductive hormone concentrations between populations has been thought to reflect these flexible reproductive strategies in the face of varying levels of energetic, immunological, and psychosocial stressors. Here we find that while average ages at menarche are significantly different between Polish and Polish American women, there are not significant differences in reproductive hormone concentrations (E1G and PdG) or glucocorticoid concentrations (cortisol). In the Polish American sample, participants whose parents were born in Poland had slightly, though not significantly, later ages at menarche compared with participants whose grandparents were born in Poland. The same variables that predict age at menarche in Poland did not significantly predict age at menarche in the United States.

In addition to comparing reproductive traits between Polish and Polish American women, we also investigated gene methylation as a potential mechanism connecting early life environments and E1G concentrations. Gene methylation, one epigenetic mechanism, is the addition of a methyl group to cytosine at CpG dinucleotides; gene methylation can be responsive to environmental traits and can modify gene expression (Portela and Esteller, 2010). We specifically investigated promoter methylation at CYP19A1, a gene that codes for aromatase. Aromatase is an enzyme that is critical for estrogen biosynthesis. We found that methylation at CYP19A1 promoter I.4 along with farming during childhood significantly predicted age at menarche. We further found that CYP19A1 promoter I.4 methylation significantly positively correlated with cortisol concentrations. We created a path analysis model that presented a hypothetical pathway by which cortisol concentrations affect CYP19A1 promoter I.4

methylation, and CYP19A1 promoter I.4 affects luteal phase E1G concentrations. In this model cortisol positively correlated with promoter I.4 methylation, and promoter I.4 negatively correlated with luteal phase E1G concentrations.

Comparisons between Poland and USA:

We first report that average ages at menarche are significantly later in the Polish sample compared to the Polish American sample. In the Polish American sample, participants whose parents were born in Poland had slightly, though not significantly, later ages at menarche compared with participants whose grandparents were born in Poland. The same variables that predict age at menarche in Poland (see Chapter 3) did not significantly predict age at menarche in the United States. Finally, we report no significant differences in reproductive hormone concentrations (E1G and PdG) or glucocorticoid concentrations (cortisol) between the Polish and Polish American samples. However, women in the United States had significantly later average luteal phases compared to women in Poland.

Previous studies demonstrate differences in progesterone concentrations between rural Polish women and women living in the United States (Ellison, 1994; Jasienska et al., 2000; Jasienska, 2001). Population differences in hormone concentrations typically reflect differences in environment, and, more specifically, differences in energetic and immune stressors (Ellison et al., 1993; Jasienska et al., 2006a; Núñez-De La Mora and Bentley, 2007). The lack of difference we see in each hormone across the menstrual cycle may reflect changes in rural Poland. In previous studies, this region is discussed as a moderately energetically constrained environment where women typically have access to adequate nutrition, and have moderate levels of physical activity during the harvest season (Jasienska and Ellison, 1998, 2004). Typical activities include farming, working with farm animals, and extensive gardening (Jasienska and Ellison, 1998,

2004; Clancy et al., 2013), and women walk significantly more in this region compared to urban women in the United States (Lee et al., 2015). However, this region of rural Poland is undergoing a transition, as evidenced by the increase of wage-labor jobs and decrease of subsistence farming practices (Colleran, 2014), as well as a secular trend in declining ages at menarche (see Chapter 3). These changes may offer one explanation for the lack of difference between Polish and Polish American hormonal profiles.

An alternative explanation is that Polish American women have similarities in genes underlying variation in reproductive hormone variation. Most comparisons in reproductive traits between populations have not specifically focused on immigrant and source populations (for exceptions, see: Núñez-de la Mora et al., 2007; Houghton et al., 2014). In our study, Polish American women whose grandparents were born in Poland had slightly earlier ages at menarche compared to Polish American women whose parents were born in Poland. Interestingly, average age at menarche for Polish American women whose parents were born in Poland was not significantly different from the women born in rural Poland. This finding matches other studies of migrants where Japanese and Chinese Americans have earlier ages at menarche and high rates of breast cancer compared to women in Japan and China (Eaton et al., 1994; Kolonel et al., 2004). Similarly, breast cancer incidence in Polish immigrants to the United States tripled within one generation (Nelson, 2006). Follow-up studies including a larger number of hormonal profiles of Polish American women will help validate the lack of difference in hormone concentrations between populations.

While there were no differences between reproductive hormone concentrations between the two populations, women in Poland had shorter luteal phase lengths compared to women in the United States. Some participants (n=11/49 Polish and n=2/16 Polish American women) had a

short luteal phase length (<10 days), an indication of luteal phase deficiency. Luteal suppression is one of the earliest ovarian responses to ecological, behavioral or constitutional stress (Ellison, 1990; Ellison et al., 1993). Luteal phase suppression may reflect a response to stress resulting in short-term lowered fecundity (Crawford et al., 2017). The shorter luteal lengths of Polish participants may indicate that, while this area is transitioning, there are still environmental stressors affecting reproductive function.

Epigenetics and pubertal timing:

The relationship between methylation at promoters of CYP19A1 and age at menarche was investigated in the Polish sample. We found that women with earlier ages at menarche has slightly, but not significantly, increased promoter methylation at CpG sites 8 and 9 of the CYP19A1 promoter (p=0.1403). Helping on a farm during childhood and average percent methylation at CYP19A1 promoter I.4 together significantly predicted age at menarche in the Polish sample (Fig. 5A, p=0.0336, R^2=0.08, n=81). Comparatively, number of adverse childhood experiences and percent methylation at CYP19A1 promoter I.4 CpG sites 8 and 9 did not significantly predict age at menarche in this sample (Fig. 5B, p=0.2767, R^2=0.06, n=62). Including methylation at promoter I.1 and promoter II/I.3 did not improve either model, and methylation at these additional promoters did not significantly predict age at menarche. This finding indicates that gene methylation may affect pubertal timing, and specifically age at menarche.

Age at menarche is highly heritable, and heritability estimates ranging from to 0.44 – 0.95 (Towne et al., 2005). However, a recent meta-analysis of GWAS studies identified thirty new loci associated with menarcheal timing, which together only explained approximately 7.2-12.2% of the heritability in age at menarche (Elks et al., 2010). Additionally, the secular trend of

declining average ages at menarche globally over time alongside health and nutritional improvements is occurring more quickly than expected under a natural selection (Almstrup et al., 2016). Age at menarche is also quite responsive to the environment (Wells and Stock, 2011). Epigenetic modifications may be one mechanism by which both early life energetic and psychosocial stress affect timing of menarche. Gene methylation at specific loci changes in response to both energetic stress (Zhang et al., 2011; Rönn et al., 2013; White et al., 2013) and psychosocial stress and support (Lam et al., 2012; Mulligan et al., 2012; Non et al., 2016). Additionally, epigenetic modifications resulting from environmental stress can remain stable over the lifetime (Heijmans et al., 2008). It is thus reasonable to hypothesize that epigenetic factors provide a link between childhood environment and pubertal timing.

Few studies to date have investigated the relationship between environmental variables, gene methylation, and timing of menarche. In one study, a higher degree of CYP19A1 promoter II/I.3 methylation was associated with earlier breast development for urban American girls who were overweight (Stueve et al. 2014). Their results were similar to ours in that higher methylation of a CYP19A1 promoter associated with earlier timing of a pubertal marker. Women with later ages at menarche had higher global DNA methylation as adults in one study (Terry et al., 2008), but the opposite relationship was found with age at menarche in another study (Demetriou et al., 2013). This difference may be due to tissue specificity of methylation patterns or differences in methods. Another recent study identified changes in DNA methylation that coincide with pubertal development (Almstrup et al., 2016). Prepubertal children had lower gene methylation in open sea and in CpG island shores and selves (Almstrup et al., 2016). There was significant overlap in CpG sites associated with pubertal timing and hormone concentrations in boys (Almstrup et al., 2016). Broadly speaking, these studies provide evidence that gene

methylation may affect, or be affected by, pubertal timing. They further indicate that there may be interactions between variables such as body weight with gene methylation and pubertal timing and indicate that gene methylation can be associated with both pubertal timing and hormone concentrations.

In this study, we investigated promoter methylation of a candidate gene, chosen based on evidence from published epigenome-wide association studies, gene function, and relevance to reproductive traits (Demetriou et al., 2013; van Veldhoven, 2014). We investigated gene promoter methylation at CYP19A1, which has been associated with energetic traits and pubertal timing (Stueve et al., 2014). The gene CYP19A1 codes for the enzyme aromatase. Aromatase is critical for estrogen biosynthesis in that aromatase metabolizes androgens into estrogens (Martinez-Arguelles and Papadopoulos, 2010). Decreased methylation of CYP19A1 promoters increases gene activity, aromatase activity, and estrone and estradiol production (Demura & Bulun, 2008). The CYP19A1 gene is >123 kb long, and there are multiple tissue-specific promoters of this gene (Bulun et al., 2003). Promoter I.4, promoter I.3, and promoter PII are specific promoters for adipose tissue, including breast adipose tissue. Promoter I.4 is also a specific promoter in skin and fetal tissue (Bulun et al., 2003). Promoter I.4 is located about 73 kb upstream of the aromatase coding region (Bulun et al., 2003). In addition to investigating the relationship between CYP19A1 promoter methylation and age at menarche, we also examined the relationship between CYP19A1 promoter methylation and adult reproductive hormones.

Epigenetics and adult E1G:

We first found that average methylation was significantly positively correlated with luteal phase length. Average cortisol concentrations were significantly positively associated with

average methylation at methylation at CYP19A1 promoter I.4 CpG sites 8&9. Additionally, follicular and luteal phase cortisol positively correlated with average E1G concentrations. In our generalized linear model, CYP19A1 promoter I.4 percent methylation was significantly associated with cortisol concentration ($p=0.0125$). This finding prompted us to investigate the relationship between cortisol, CYP19A1 promoter I.4 methylation, and E1G concentrations. We used structural equation modeling to demonstrate a hypothetical pathway by which cortisol may affect luteal phase E1G concentrations via changes in methylation of CYP19A1 promoter I.4.

Methylation of CpG dinucleotide sites in CYP19A1 promoters likely regulates CYP19A1 transcription (Knower et al. 2010). Lower methylation and increased activity of the CYP19A1 promoter increases aromatase activity and estrone and estradiol production (Demura and Bulun, 2008). We thus anticipated finding a relationship between CYP19A1 promoter methylation and E1G concentrations and were surprised to find a correlation between cortisol concentrations and CYP19A1 promoter I.4 CpG sites 8&9. The relationship between CYP19A1 promoter I.4 and cortisol concentration is plausible as promoter I.4 contains a glucocorticoid response element (GRE); glucocorticoids activate the CYP19A1 promoter I.4 (Demura and Bulun, 2008; Chen et al., 2009). As previously mentioned, there are multiple promoters for CYP19A1, and these promoters are tissue specific. In human placental syncytiotrophoblasts, cortisol treatment increased aromatase expression, and cortisol treatment was associated with changes in methylation and histone acetylation of a CYP19A1 promoter (Wang et al., 2012). In a study of human subcutaneous abdominal adipose tissue, cortisol treatment increased aromatase activity; however, no epigenetic data was assessed in this study (McTernan et al., 2000). It is therefore feasible that cortisol can stimulate aromatase activity via epigenetic interactions, and we suggest further studies are necessary to confirm our finding.

Moreover, we found that CYP19A1 promoter I.4 methylation, cortisol concentrations, age, and former pregnancy were significant predictors of luteal phase E1G concentrations. We used structural equation modeling to demonstrate a hypothetical pathway by which cortisol may affect luteal phase E1G concentrations via changes in methylation of CYP19A1 promoter I.4. We anticipated that methylation of CYP19A1 may influence estradiol concentrations CYP19A1 methylation is associated with aromatase activity and aromatase is important for estradiol biosynthesis. We found a positive relationship between cortisol concentration and methylation of CYP19A1 promoter I.4, and a negative relationship between methylation of CYP19A1 promoter I.4 and luteal phase E1G. This finding is consistent with the literature relating CYP19A1 promoter methylation and aromatase expression. For example, Yu et al. (2013) found that women with PCOS had higher CYP19A1 promoter methylation and lower aromatase expression in ovarian tissue. Similarly, Hosseini et al. (2016) found that women with endometriosis had higher methylation CYP19A1 promoters II/I.3 and I.4 and lower gene expression in cumulus cells. Treating endometrial cells with a demethylating agent enhanced aromatase mRNA (Izawa et al., 2011). Finally, Demura and Bulun (2008) demonstrated that higher methylation at CYP19A1 promoter II/I.3 decreased promoter activity. These studies are consistent with the findings in our study: lower methylation at CYP19A1 promoters is associated with higher aromatase activity in these studies and is associated with increased E1G concentrations in our study.

Study limitations:

This is a retrospective study, and thus results should be interpreted with caution. We ensured that our hypotheses and predicted causal pathways are evidence-based, but we suggest further studies are necessary to confirm our findings. We also do not have data for additional

potential sources of variation. For example, we did not measure exposure to endocrine disruptors, which may affect reproductive traits like age at menarche (Buttke et al., 2012; Wolff et al., 2017) and/or aromatase regulation (Zhao et al., 2017). Finally, DNA methylation can have tissue specificity, and we used a generally informative tissue (buccal cells) to assess methylation at CYP19A1 promoters. That being said, buccal swabs are an informative general tissue collected non-invasively that may be more informative than blood (Lowe et al., 2013).

Conclusion

Reproductive ecology has long examined the adaptive flexibility of women's reproduction in the face of variable environments (Wasser and Barash, 1983; Vitzthum, 2008). While many studies reference the possibility of genetic and epigenetic effects on reproductive traits, few have actually tested the physiological mechanisms by which epigenetic traits may actually produce variation in menarcheal timing and adult outcomes. This study builds on early models to integrate new knowledge and test potential mechanistic links between environments and adult reproductive hormones. We found that methylation at CYP19A1 promoter I.4 (CpG sites 8 & 9) along with farming during childhood significantly predicted age at menarche in a rural Polish population. We further identify a potential pathway by which increased cortisol concentrations increases CYP19A1 promoter I.4 methylation, which likely decreases aromatase activity and downstream estrogen and estrone concentrations. This pathway may help explain differences in estrogen concentrations between individuals.

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Chapter 5

Conclusion

Environmental stressors and social support affect timing of puberty. Pubertal timing, in turn, is associated with adult reproductive function in many studies. In addition to a relationship between pubertal timing and reproductive outcomes, pediatricians also use timing and tempo of puberty as a signal for adolescent environment and adult disease risk (Hillard, 2014). The timing of puberty is typically understood within a life history framework which seeks to understand how organisms optimize their survival and reproductive strategies in different environments. Life history trade-offs occur when there are competing somatic needs for growth, maintenance, reproduction, and survival. Age at first menses, or menarche, is one of the major, visible points at which a girl begins the transition from somatic investment in growth to reproduction (Gluckman and Hanson, 2006b). Childhood environmental variables, like energetic and psychosocial stressors, affect age at menarche in different ways, creating pushes and pulls on pubertal timing. In this research, I investigated multiple aspects of pubertal timing, including how social support measures affect age at menarche (Chapter 2), psychosocial and energetic stressors experienced during childhood correlate with pubertal timing and adult reproductive function (Chapter 3), timing of menarche varies, and reproductive hormones do not vary, between Polish and Polish American women (Chapter 4), and that gene methylation is a potential mechanism mediating the relationship between stress and reproductive function (Chapter 4). The results of this study have implications for reproductive health, and contextualizing age at menarche creates a better understanding of when age at menarche actually might be predictive of adult disease risk.

Energetic and psychosocial stressors tend to respectively delay and accelerate age at menarche (Ellison, 2001; Ellis, 2004). These two types of stress are typically investigated separately. In this project, we both expand research on pubertal timing to include measures of social support from family and friends may affect age at menarche (Chapter 2) and investigate how different types of stress may work together to affect pubertal timing and future reproductive function (Chapter 3). First, I contend that our understanding of the variation in pubertal timing would benefit from a greater understanding of social support from family and friends may affect age at menarche by either buffering psychosocial or energetic stressors or directly affecting age at menarche. Social support is associated with many physiological outcomes and can buffer physiological responses to stress (Uchino et al., 1996; Gunnar, 2017). Parental social support, for example, decreases stress reactivity in children (Hostinar et al., 2015). I found that open communication with parents, as a measure of kin social support, predicted age at menarche in a sample of adolescent girls in the United States. Mother-adolescent and father-adolescent open communication scores had opposing directional effects on age at menarche. Kin social support may provide a signal of surrounding developmental and reproductive environment. I contend that positive psychosocial factors, rather than only negative psychosocial factors, may be associated with earlier menarcheal development in some contexts.

Second, I found that age at menarche has declined over time in the Beskid Wyspowy of rural Poland. Helping on farms and with farm animals as a child were associated with later ages at menarche. Women with a higher number of adverse childhood experiences tended to have earlier age at menarche, although this difference was not statistically significant. Despite different effects on age at menarche, all types of childhood stressors were associated with lower adult E1G concentrations. Chisholm et al. (2005) proposed merging the energetics and

psychosocial stress hypotheses by hypothesizing that age at menarche is first dependent on energetics. In the absence of extreme energetic constraint, then psychosocial stress may play a larger role (Chisholm et al., 2005). The results of my study indicate that it is instead possible for multiple types of stress to push and pull on age at menarche. This rural Poland region is an area of semi-energetic constraint, meaning that women are experiencing energetic stress but are not necessarily nutritionally deprived. In this context, energetic, immune, and psychosocial stressor together all affect age at menarche and further affect adult reproductive function.

Including related populations in different environments is a common anthropological technique harkening to Boas' craniometrics studies (Boas, 1912). Comparing immigrant and county-of-origin populations has been an effective anthropological technique to research environmental effects on phenotypic traits. While Boas particularly challenged the naturalistic viewpoint of his time, the nature verses nurture discussion continues today (see Fox Keller, 2010). Age at first menstruation, menarche, is a reproductive trait with high heritability (Towne et al., 2005). Yet, age at menarche in immigrant populations to the U.S. is often decreased compared country of origin (Kolonel et al., 2004). There are clear genetic and environmental effects on pubertal timing, making age at menarche an interesting test case to explore ideas of evolved environmental responsivity. In this project, I compared differences in reproductive traits between women in rural Poland and Polish American women in urban areas of the United States. While studies have compared reproductive traits between Polish and American samples, no study to date investigates differences in reproductive traits between Polish and specifically Polish American samples. We find that ages at menarche are earlier in Polish American women. Further, there is a generational affect where Polish American women whose parents were born in Poland did not have significant difference in average ages at menarche compared to women in

Poland, but Polish American women whose grandparents were born in Poland. There were no differences in reproductive hormones, nor cortisol concentrations between the two populations.

Physiological mechanisms by which childhood environments affect reproductive traits are often alluded to, but not tested for, in studies connecting stress and reproduction. I investigated gene methylation as a potential mechanism mediating the relationship between stress and reproductive function. Epigenetics can be thought of as above and beyond the genome and, more specifically, is defined as "stable and heritable changes in gene expression that are not caused by changes in underlying DNA sequence" (Russo et al., 1996). One epigenetic mechanism is DNA methylation, the addition of a methyl group to cytosine at CpG dinucleotides (Portela and Esteller, 2010). The amount of DNA methylation at certain CpG sites affects gene transcription and expression (Jones and Takai, 2001). DNA methylation is both heritable and modifiable, and methylation changes in response to environmental cues and experiences (Feinberg and Irizarry, 2010b; Borghol et al., 2012). The modifiable nature of DNA methylation makes epigenetic changes an informative method through which to study the dynamics of genetic and environmental interactions. Changes in DNA methylation are one method of evolved response to environmental stimuli in a manner that directly affects genetic expression. I found that methylation at a promoter of the gene coding for aromatase (CYP19A1) along with farming during childhood significantly predicted age at menarche in a rural Polish population. We further identify a potential pathway by which increased cortisol concentrations increases CYP19A1 promoter I.4 methylation, which likely decreases aromatase activity and downstream estrogen and estrone concentrations. This pathway may help explain differences in estrogen concentrations between individuals. Overall, the results of this research underscore that epigenetic factors like gene methylation may play a role in reproductive ecology and may

provide a piece of the lacking intermediate structure between early environmental experiences and reproductive traits.

Future Directions

The results of this study lead to additional questions. If age at menarche does not associate with adult reproductive hormone concentrations in Polish and Polish American adults, is age at menarche still a good proxy for childhood environments? Why do we find differences in ages at menarche between Polish and Polish American women, but we do not find differences in reproductive hormone concentrations? Third, how do contemporary stressors affect reproductive function? Does reproductive function as indicated by hormone concentrations and luteal phase length affect fertility or fecundity? Does social support or other types of community networks mediate relationships between stressor and reproductive outcomes in these two populations?

Age at menarche is associated with adolescent reproductive function (Apter and Vihko, 1983; Vihko and Apter, 1984). Our results make us question if age at menarche still would be associated with adolescent probability of ovulation and/or menstrual cycle regularity. If not, what better predicts reproductive function in adolescents? Further, we see differences in ages at menarche between girls of different race/ethnic backgrounds. Are there differences in reproductive hormone concentrations, and, if so, are said differences related to 1) social support, 2) energetic or nutritional stress, or 3) psychosocial stress including, but not limited to, adverse childhood experiences (Felitti et al., 1998) and different types of stress and coping (Rowley et al., 2005)?

Overall in this work, I describe results stemming from two larger projects: a combined research and education multi-disciplinary study investigating variation in reproductive traits in adolescent girls and an international study on ecological determinants of reproductive traits in

Polish and Polish American women. During my time at UIUC, I played an integral part in establishing both collaborative projects which have been and will be used for multiple other research questions (e.g. Rodrigues et al., 2018, Lee et al., 2015, 2017, 2018; Ogden et al., 2015; Sanford et al., 2016). I plan to continue to use these collected samples to address new questions. For example, I am currently conducting an epigenome-wide association study to identify novel differentially methylated sites and differentially methylated regions correlated with age at menarche in the Polish sample. I will test for interactions between childhood stressors and methylation at these identified sites. This study will identify novel methylation sites that could connect childhood energetic and psychosocial stress with reproductive physiology. The CpG sites identified in association with age at menarche will also be measured in the Polish American sample in order to compare between these samples. Additionally, I conduct work investigating variation in reproductive hormone concentrations in study of adolescent girls in the United States. Next steps of this project including investigating potential relationships between stress, parent-adolescent communication, and estradiol and progesterone concentrations. In my personal research trajectory, I am interested in how different types of stress affect reproductive health outcomes, and if the effect of stress can be buffered by social support. The results of my dissertation provide evidence that childhood developmental environments, including stressors and social support, can affect reproductive traits. Future work is needed to help elucidate the contextual variation of reproductive traits within and between populations.

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