Original Research Article

Relationships Between Biomarkers of Inflammation, Ovarian Steroids, and Age at Menarche in a Rural Polish Sample

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Objectives: To test the hypothesis that life history trade-offs between maintenance and reproductive effort would be evident through inverse associations between levels of a biomarker of inflammation [C-reactive protein (CRP)], and ovarian hormones. Associations between CRP and age at menarche were also explored.

Methods: Urinary CRP, salivary progesterone, and estradiol were measured over one menstrual cycle from rural Polish women (n = 25), representing a natural fertility sample. Age of menarche was assessed through interview recall methods. We used minimum second-order Akaike Information Criteria as a means of multiple regression model selection, and repeated measures ANOVA to test cycle-dependent hypotheses.

Results: Comparisons of individuals in high and low CRP tertiles revealed that those with high CRP had significantly lower progesterone (luteal \( P = 0.03 \), mid luteal \( P = 0.007 \)) but not estradiol (follicular \( P = 0.21 \), luteal \( P = 0.15 \)) concentrations through the menstrual cycle. However, when the age at menarche was included in the analysis, both age at menarche and urinary CRP were negatively associated with estradiol (\( R^2 = 0.44 \), \( P = 0.0007 \)). Age at menarche and estradiol were the strongest negative predictors of CRP (\( R^2 = 0.52 \), \( P = 0.0001 \)).

Conclusions: Inflammation itself may suppress ovarian function, or indicate immune challenges that lead to ovarian suppression. The timing of menarche may also influence adult inflammatory sensitivity and ovarian hormone concentrations. This lends support to existing models of trade-offs between maintenance and reproduction in women.

Ovarian function sensitivity to ecological stresses has received significant attention, with most studies supporting the life history prediction that levels of ovarian steroids vary in response to energetic conditions, reflecting predicted trade-offs between present and future reproduction (Ellison, 2003; Jasienska and Ellison, 1998; Voland, 1998). However, investment in current dependent offspring and future reproductive opportunities is contingent on survival. Therefore, there should be an inverse relationship between reproductive and maintenance effort. This has been investigated in human males (Muehlenbein and Brubiescas, 2005) but has not received similar attention in women.

Life history trade-offs between reproduction and immune challenges, a portion of maintenance effort, have been studied in other animals. For instance in polygynous mating systems in birds, males have lower immunocompetence than females because of their greater allocation to mating effort, where in monogamous systems male and female immunocompetence is equivalent (Hasselquist, 2007). Lee (2006) integrates seemingly discordant results on the life history trade-offs between reproduction and immune function across several studies by showing the differential effects of components of the immune system. Induced innate immune defenses and induced adaptive, cell-mediated immune defenses that trigger a systemic inflammatory response may pull significant resource toward the immune system, where other kinds of constitutive and induced immune defenses may not, leading to variable relationships depending on the immunological parameter measured (Lee, 2006). Systemic inflammation can also indicate an environment with higher psychosocial stressors or poor socioeconomic environment high in pathogens (Aiello et al., 2009); dealing with these stressors could draw resources towards somatic maintenance and away from reproduction. As a result, many regard the inflammatory response as an indicator of maintenance effort (e.g., McDade, 2005; McDade et al., 2008a).

Many challenges to survivorship result from environmental and psychosocial stressors that elicit acute and chronic immune responses (McDade, 2001). C-reactive protein (CRP) levels are often used to assess inflammatory responses and therefore represent an important facet of immune function as a part of maintenance effort. (McDade et al., 2007, 2008b). CRP is an acute-phase protein that is part of the innate immune system and tends to indicate inflammation (Doronzo et al., 2005; Honda et al., 2006; Ridker, 2009; Rutter et al., 2004; Williams et al., 2004) but also covaries with psychosocial stressors (Coussons-Read, 2007; Lewis et al., 2010; McDade et al., 2006). CRP is produced in the liver, but is also produced by adipose tissue in response to proinflammatory stimuli such as adipocytes (Calabrò et al., 2008); so, energetic

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markers like body mass index and percent body fat are often positively correlated with CRP (Guzelmeric et al., 2007; McDade et al., 2006, 2008b). CRP also has two forms: a native, pentameric form and a modified, monomeric form (Eisenhardt et al., 2009; Heuertz et al., 2005). The pentameric form tends to indicate cardiovascular disease risk and the initiation of broad host pathogen defense, but the pentameric form must break down into monomeric subunits to orchestrate localized, tissue-based inflammation effectively (Heuertz et al., 2005; Verma et al., 2004). Therefore, pentameric CRP could represent the potential for an immunological event, where monomeric CRP could indicate maintenance effort regarding actual localized, immunological events.

Adult CRP is associated with current and past challenges: in a Philippine sample of males and females, lower birth weight was associated with higher adult CRP, yet microbial exposure in early childhood was correlated with lower adult CRP (McDade et al., 2009a). Where low birth weight can put an individual at higher risk for metabolic syndrome and elevated CRP, the opposite relationship between CRP and early microbial exposure suggests that these immune challenges influence adult immune system sensitivity. Further, as the adaptive immune system has been primed in early childhood among those with higher microbial exposure, it may take less effort to stave off illness at an older age. As a further example, in a lowland Bolivian population, children with higher CRP had smaller gains in height over three months (McDade et al., 2008b). The height differences in children with high versus low CRP levels were greater if they were two to four years old, a critical growth period, or if they had lower body fat as assessed by skinfold measurements (McDade et al., 2008b). CRP was also moderately related to infection and symptoms of infection in this population. Finally, Blackwell et al. (2010) found associations between CRP and stature in a sample of children from the Shuar in southeast Ecuador, a population that lives with a high degree of helminth exposure and thus a significant pathogen load. In children 2–7 years, higher CRP was associated with shorter stature, but in children 8–15 years, it was associated with greater stature. Again, one sees a shift in the relationship between CRP and ecological variables across developmental stages from infancy to adolescence. The importance of these maintenance effort effects may reach beyond adolescence and influence the developmental trajectories of adult reproductive function.

Developmental trajectories of reproductive function are set not only by maintenance effort, but by life history strategies concerning the timing of reproductive maturity. Pubertal timing responds to different kinds of environmental stressors. This has led to nonmutually exclusive hypotheses about the impact of environmental stress on age at menarche. First, certain psychosocial stressors—measured by self-report, psychometric scales, and cortisol—should drive menarche early (Ellis, 2004; Ellis and Garber, 2000; Ellis et al., 2011). Father absence, mother psychopathology, and stepfather presence (Ellis and Garber, 2000), as well as high extrinsic mortality (Allsworth et al., 2005; Walker et al., 2006), are associated with earlier menarche. Delayed menarche is seen in individuals who experience food insecurity (Belachew et al., 2011) and in young, underfed, or elite athletes (Warren and Perlroth, 2001). Indirect evidence for the impact of energetic constraint on timing of menarche is demonstrated by the downward secular trend in age at menarche that is largely associated with a shift toward a positive energy balance and improved socioeconomic conditions (Graham et al., 1999; Laska-Mierzejewska et al., 1982; Okasha et al., 2001; Wattigney et al., 1999). However, these psychological and energetic factors co-occur regularly, and thus some caution is warranted.

Still, the impact of immune challenges or maintenance effort more broadly on the timing of menarche has been rarely examined. Allostatic load (i.e., movement out of homeostasis, or activation of the stress response) was higher when menarche occurred before the age of 10 (Allsworth et al., 2005), and in a study of the relationship between CRP and risk of breast cancer, higher CRP was correlated with an earlier age at menarche (Zhang et al., 2007). The fact that systemic inflammation is part of the mechanism to draw resource to immune function, yet also can disrupt the normal tissue remodeling inherent in much of female reproductive function (Clancy, 2009, 2012), adds another layer of complexity to our understanding of the reproduction versus maintenance life history trade-off. Ovulation and menstruation (Critchley et al., 2001; Espey, 1980, 1994; Fleming et al., 2006; Maybin et al., 2011; Tsafirri, 1995; Wander et al., 2008) as well as implantation (King and Critchley, 2010; King et al., 2003; Lea and Sandra, 2007) are inflammatory events due to extensive tissue remodeling. Several studies have examined CRP across the menstrual cycle to understand how this biomarker is affected by ovarian and uterine inflammatory events. Jilma et al. (1997) found that CRP was significantly higher in single blood samples of women at midcycle and the mid luteal phase compared with the follicular phase, and that these increases were correlated with progesterone concentrations. However, Wander et al. (2008) found that CRP was positively associated with progesterone, but negatively with estradiol, in a sample of spontaneously cycling women. Conversely, Wunder et al. (2006) found no associations between CRP and menstrual cycle phase.

In this study of largely agricultural women at the Mogielica Human Ecology Study Site in southern Poland, we test two hypotheses. First, when maintenance effort is high, ovarian function is suppressed, and we predict higher CRP will be correlated with lower ovarian hormones independent of age at menarche. Second, the timing of reproductive maturation influences adult sensitivity to environment, particularly regarding allocations to ovarian function and maintenance effort, and we predict that a later age at menarche will be correlated with lower CRP, estradiol, and progesterone. We also advance an alternate hypothesis that age at menarche variation is a proxy of past events rather than a driver of adult ovarian function. This research will contribute to the body of work that seeks to understand life history trade-offs between maintenance and reproduction, particularly in the context of a nonindustrial population.

METHODS

Study site and participants

The Polish population at the Mogielica Human Ecology Study Site is a rural, family owned farming population that uses traditional farming techniques where women participate fully (with the exception of horse handling; Jasienska and Ellison, 1998, 2004). The steep slopes of the Beskid Wyspowy mountain range where they live and
farm impedes the use of modern farming technology, and most work is done by hand. The harvest season in this area requires a moderate to high workload. Further, even those relatives, neighbors and friends who do not own farms themselves are usually involved in farmwork in July and August. The Polish population of Mogielica was chosen because it is a largely natural fertility population that has been shown to exhibit significant ovarian function variation in response to negative energy balance (Jasienska and Ellison, 1998, 2004). Moreover, compared with populations living in tropical conditions, parasite exposure is modest thereby allowing for a conservative assessment of the role of chronic inflammation on ovarian function.

Participants were healthy premenopausal women between the ages of 20 and 40 (n = 25, mean 28.92, SD 5.34 years), living in a village within the Mogielica Human Ecology Study Site in Poland (Jasienska and Ellison, 2004). Women were screened by interview for any major health problems, particularly reproductive or endocrine disorders like endometriosis or fibroids, or any autoimmune disease. Participants had not used hormonal contraceptives for at least 3 months, had not been pregnant or breastfeeding for at least 6 months, and were nonsmokers. Nineteen participants had children (73%; mean 1.73, SD 1.31 children for the whole group) and were employed outside the home (73%), with typical jobs being hairdressers, cashiers, and secretaries. Many participants lived on farms, but even those participants that were not farm owners tended to spend time in the harvest months (July–August) helping family or neighbors with their crop.

The agricultural Polish population was studied during the harvest season, which is their period of highest physical activity and has been documented to have the greatest degree of ovarian suppression of the year (Jasienska and Ellison, 2004). Participants were recruited over the summer of 2005 through word of mouth and home visits, as well as through the approval and help of the local doctor and priest. Participants provided written informed consent and were monetarily compensated for their involvement. The Yale University (New Haven, CT) Human Subjects Committee approved protocols for the primary investigation (fieldwork, reproductive hormone analysis). The University of Illinois, Urbana-Champaign (Urbana, IL) Institutional Review Board approved protocols for the secondary investigation (CRP laboratory and statistical analysis).

Field protocol

Participants collected seven morning void urine samples in 4-ounce polystyrene containers pretreated with sodium azide as a preservative, using the same concentration as Lipson and Ellison (1987) over the course of one menstrual cycle. Samples were collected for four consecutive days in the follicular phase (days 3–6 from first menses) and three consecutive days in the luteal phase (days 21–23). Participants also collected saliva samples every day in tubes pretreated with sodium azide over the course of one menstrual cycle to capture adequately ovarian hormone variation (Jasienska and Jasieniak, 2008). Both urine and saliva samples were kept at room temperature until the subject’s participation had ended. After that, samples were frozen at −20°C.

Anthropometric measurements were taken on the day the participant entered the study, and on the day they returned their samples at the end of the investigation. Height was measured to the nearest tenth of a centimeter using a stadiometer, weight to the nearest tenth of a kilogram, and body fat to the nearest percent using a Tanita Body Fat Monitor/Scale (Jebb et al., 2000). Medical measuring tape was used to measure bust, ribs, waist, and hips circumferences to determine energy distribution using the protocol of Jasienska et al. (2004). Age at menarche was determined via participant recall, which is an imperfect but relatively accurate method. Younger women are able to recall their age at menarche with much greater accuracy (Koo and Rohan, 1997; Koprowski et al., 2001; Must et al., 2002) than women into middle age (Cooper et al., 2006). Because age at menarche is later in this sample than industrial population samples (Clancy et al., 2009), and participants were 20–40 years old, the time of recall was in the range of other studies that have shown good accuracy in recalled age at menarche.

Laboratory protocol

Radioimmunoassays were performed in the Yale Reproductive Ecology Laboratory according to previously established methods (Lipson and Ellison, 1987, 1994; O’Rourke, personal communication). Estradiol and progesterone were measured from saliva samples using an Iodine 125 radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX), modified for saliva. Alignment of menstrual cycles for analysis and determination of ovulation was based on the mid cycle drop of estradiol, defined as the second of the two consecutive days (following estradiol peak) between which the greatest decrease in estradiol occurred (see Lipson and Ellison, 1996 for full description of method). Quality control, curve, and nonspecific binding values were within appropriate limits. Estradiol quality control coefficients of variation were the following (n = 14 assays): interassay high quality control 11%; low quality control 16%; intraassay variation 13%. Progesterone quality control coefficients of variation were the following (n = 17 assays): interassay high quality control 7%; low quality control 12%; intraassay variation 7%. Estradiol and progesterone concentrations were calculated using RIA AID radioimmunoassay analysis software for PC (Robert Maciel Associates, Arlington, MA).

Human CRP concentrations can be analyzed from serum samples using enzyme immunoassays, but urine is infrequently assayed directly for CRP. To achieve this, Klein et al. (2010) modified previously existing protocols for the isolation and measurement of CRP from human ascites fluid (Volanakis et al., 1978) and rat urine (Ortiz et al., 2009). Disposable affinity columns (Pierce; Product #29920) were prepared with 1 ml immobilized p-aminophenyl phosphoryl choline gel (Pierce; Product #20357) according to manufacturer directions. After equilibration with 3 ml of a high calcium binding buffer solution (1–2 mM Tris, 1–2 mM NaCl, and 10 mM CaC12), a 1 ml urine sample was added to the column and incubated at room temperature for 75 min. Columns were then drained of sample and washed with 5 ml of the high calcium buffer solution. After washing, 3 ml of a high EDTA elution buffer (1–2 mM Tris, 1–2 mM NaCl, and 10 mM EDTA) was incubated in the column for 15 min. Eluent for each sample was collected in filtered centrifuge tubes (Millipore, Product #UFC50VL96). Samples were centrifuged
for recovery of the concentrated protein as instructed by the manufacturer and were stored at −20°C between elution and analysis.

Within two weeks of elution, samples were assessed for CRP with an ELISA kit from Helica Biosystems (Product # 961CRP011H-96) designed for use with human serum. Results of the assay were determined using a Biotek ELX808 plate reader and Gen5 Software. A standard curve provided with the assay kit was used to determine concentration of the samples.

As urine concentration greatly varies both between and within individuals, optical density was determined for each sample with an ATAGO Pal-3 refractometer and converted to a measure of specific gravity. Specific gravity was used to adjust the data for the dilution of urine in a population-specific way according to Miller et al. (2004). The average specific gravity for the Polish population was 1.020.

Statistical analyses

Ovarian hormone concentrations were normally distributed, which justified our use of parametric methods and allowed us to avoid log transforming the values. The urinary CRP concentrations were log transformed to normalize the distribution, and data points below the detectable limit of the assays were removed. The percentage of points below the detectable limit was 43.3%, and these values were fairly evenly distributed among participants. CRP concentrations that read below blank is common (Whitcomb and Schisterman, 2008), and there are three ways this is ordinarily handled: by leaving the values as is but assigning participants into low, medium or high CRP categories (Mitani et al., 2005; Visser et al., 1999), by assigning the values an arbitrary low number (usually 0 or 0.0001 pg/ml; Fichtlscherer et al., 2000; McCade et al., 2011b) or by removing the values (Whitcomb and Schisterman, 2008). We ran several of our analyses with and without the concentrations below detection limits, and the results were the same. Importantly, the participants grouped into low, medium, and high CRP tertiles remained the same.

Due to the timing of consecutive urine collections in the follicular and luteal phases, the CRP concentrations represented cycle averages outside of ovulation and menstruation, both of which are inflammatory processes (Brännstrom et al., 1994; Espey, 1980; King and Critchley, 2010). Further, no difference was found between average follicular and luteal CRP concentrations (paired t-test, P = 0.82). Therefore, median CRP was used as the main CRP variable, which is methodologically consistent with other published work (Dufaux et al., 1984; McCade et al., 2009b; Zee and Ridker, 2002).

Our two main predictions involve relationships between CRP and ovarian hormones, as well as CRP and age at menarche. We initially tested these predictions using simple linear regressions, which allowed us to test the relationship between CRP and ovarian hormones as continuous variables, and were used to analyze the association between CRP with estradiol and progesterone across different phases of the cycle. For estradiol aligned by midcycle drop date (day 0), early follicular phase was defined as days −10 to −6, late follicular phase as days −5 to −1, periovulatory phase as −3 to 3, early luteal phase as days 0–2, early/mid luteal phase as days 3–5, and late luteal phase as days 6–9. For progesterone aligned by second menses (day 0), early luteal phase was defined as days −13 to −10, mid luteal phase as days −9 to −5, and late luteal phase as days −4 to −1. To account for methodologies used in other publications that compare CRP and reproductive hormones values sampled on the same day (Jilma et al., 1997; Wander et al., 2008), we narrowed correlations of CRP concentrations and the salivary progesterone and estradiol values only to the days when CRP was sampled. We compared concentrations of CRP and ovarian hormones only when measured on the same day, which means there are no more than 7 samples per participant, since that’s the number of times we measured urine.

We also used stepwise multiple regressions with minimum second-order, corrected Akaike Information Criterion values (AICc) as a means of model selection in models where CRP, estradiol, and progesterone were the dependent variable, respectively. AICc is preferred when the sample size is small (Motulsky and Christopoulos, 2003). We then ran multiple regressions on those models with the lowest AICc values.

Furthermore, we used repeated measure ANOVA to compare levels of progesterone and estradiol in women with high, medium, and low CRP levels. For that purpose, participants were sorted into tertiles based on the median of all of their samples for CRP concentrations (low CRP n = 8, medium CRP n = 8, and high CRP n = 9). For repeated measures analyses, estradiol concentrations were aligned by midcycle drop date, and days −9 to 9 were analyzed because they were the broadest number of days with individuals that had consecutive values, without daily values missing due to phase length or sample loss. For estradiol repeated measures, follicular (days −9 to −1), luteal (days 0 to 9), periovulatory (−3 to 3), and all (−9 to 9) phases were analyzed. For progesterone, based on midcycle drop date alignment, follicular (days −9 to −1) and periovulatory (−3 to 3) phases were analyzed; for start of the next cycle alignment, luteal (days −13 to −3), and mid luteal (days −9 to −5) were analyzed. Day −2 and −1 were excluded so as not to have to exclude any participants.

One subject in the high CRP tertile was missing several consecutive reproductive hormone values and was excluded for a final group size of 8 in the midcycle drop alignment. Three participants were missing a single reproductive hormone measurement. To maximize sample size for repeated measures only the average of the hormone concentration on either side of that day was used to estimate the missing value. These values were not used for the regression analyses.

We used JMP 10 (SAS, Cary, NC) to determine the best multiple regression models and run them. Prism 5 (GraphPad Software, La Jolla, CA) was the statistical software package used for all other analyses. Alpha was set at 0.05.

RESULTS

To test the hypothesis that ovarian function is suppressed when maintenance effort is high, we ran linear regression comparisons between CRP and ovarian hormones. CRP was negatively correlated with mid luteal progesterone (\( R^2 = 0.15, P = 0.05 \), Fig. 1) and late luteal progesterone (\( R^2 = 0.19, P = 0.03 \), Table 1). CRP was also
negatively correlated with late follicular estradiol (\(R^2 = 0.25, P = 0.01\)), periovulatory estradiol (\(R^2 = 0.18, P = 0.04\)), early/luteal estradiol (\(R^2 = 0.30, P = 0.005\)), and late luteal estradiol (\(R^2 = 0.17, P = 0.05\)); a negative relationship between CRP and early luteal estradiol (\(R^2 = 0.12, P = 0.10\)) was not significant. Further, CRP was negatively correlated with age at menarche (\(R^2 = 0.18, P = 0.03\)).

We then compared estradiol and progesterone in low and high CRP tertile groups. Compared with the low CRP tertile group, participants in the high CRP tertile had significantly lower progesterone concentrations through the entire luteal phase (repeated measures ANOVA, \(F_{1,15} = 6.07, P = 0.03\), Fig. 2) and the mid luteal phase (\(F_{1,15} = 9.79, P = 0.007\)) but not the follicular phase (\(F_{1,15} = 1.53, P = 0.23\)). Compared with the low CRP tertile group, participants in the high CRP tertile had lower estradiol concentrations through the follicular phase, but these differences were not significant (repeated measures ANOVA, \(F_{1,15} = 1.75, P = 0.21\), Fig. 3). Comparisons of estradiol between the two groups in the periovulatory (\(F_{1,15} = 1.85, P = 0.19\)) and luteal phases (\(F_{1,15} = 2.37, P = 0.15\)) were also not significant. However, post-hoc power analysis revealed that statistical power was low in the estradiol group comparisons (33%).

To account for methodologies used in other publications that compare CRP and reproductive hormones sampled on the same day, we ran linear regressions of all CRP concentrations and the salivary progesterone and estradiol values only on the 7 days CRP was sampled. CRP and progesterone were significantly negatively correlated (\(R^2 = 0.05, P = 0.05\)) but CRP and estradiol were not (\(R^2 = 0.01, P = 0.36\)). When these comparisons were made within menstrual cycle phases, CRP and progesterone remained significantly negatively correlated in the luteal phase (\(R^2 = 0.15, P = 0.01\)) but not the follicular phase (\(R^2 = 0.01, P = 0.81\)), and relationships between CRP and estradiol were not statistically significant in either phase (\(R^2 = 0.01, P = 0.86\) follicular, \(R^2 = 0.03, P = 0.28\) luteal).

To test the hypothesis that the timing of reproductive maturation influences adult sensitivity to environment, particularly regarding allocations to ovarian function and maintenance effort, we calculated stepwise multiple

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**Table 1. Linear regressions of reproductive variables with median CRP**

<table>
<thead>
<tr>
<th>Variable</th>
<th>(R^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (aligned by midcycle)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early follicular phase (−10−6 days)</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>Late follicular phase (−5−1 days)</td>
<td>0.25</td>
<td>0.01</td>
</tr>
<tr>
<td>Periovulatory phase (−3−3 days)</td>
<td>0.18</td>
<td>0.04</td>
</tr>
<tr>
<td>Early luteal phase (0−2 days)</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>Early/mid luteal phase (3−5 days)</td>
<td>0.30</td>
<td>0.005</td>
</tr>
<tr>
<td>Late luteal phase (6−9 days)</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>Progesterone (aligned at midcycle for follicular and early luteal phase, start of next cycle for mid luteal, and late luteal phase assessments)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular phase (−9−1 days)</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>Early luteal phase (0−2 days)</td>
<td>0.07</td>
<td>0.22</td>
</tr>
<tr>
<td>Mid luteal phase (−9−5 days)</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Late luteal phase (−4−1 days)</td>
<td>0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>0.18</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Reproductive hormone values are averaged across the days reported within each phase (see methods). All slopes were negative.
regression models using AICc. BMI was initially included as an independent variable to control for adiposity in all models, but each time was removed because BMI did not correlate with any dependent variables and always increased AICc values (see Table 2). When estradiol was the dependent variable, age at menarche and urinary CRP were the strongest, negative predictors ($R^2 = 0.44$, $P = 0.0007$). When progesterone was the dependent variable, the best model included age at menarche only, where other variables did not improve the model ($R^2 = 0.12$, $P = 0.05$). When urinary CRP was the dependent variable, age at menarche and estradiol were the strongest, again negative predictors ($R^2 = 0.52$, $P = 0.0001$).

CRP was not correlated with BMI ($P = 0.80$), body weight ($P = 0.95$), percent body fat ($P = 0.80$), waist circumference ($P = 0.81$), waist to hips ratio ($P = 0.60$), or age ($P = 0.31$). A summary of the main variables compared in this study can be found in Table 3.

### DISCUSSION

Our first hypothesis stated that when maintenance effort is high ovarian function is suppressed, and we predicted that higher CRP would be correlated with lower ovarian hormones. Correlation and multiple regression tests found significant, negative relationships between CRP and progesterone, and CRP and estradiol. Using more sophisticated repeated measures ANOVA analysis, we found that the relationship between CRP and estradiol was not significant in any phase; however, post-hoc power analysis revealed insufficient statistical power. The slightly different relationships using repeated measures versus linear regressions present a challenge in interpreting our results. While estradiol and CRP appear to covary in some ways, our sample size limits more sophisticated analyses. Further, this is some indication that this relationship could be bidirectional, as multiple regression AICc best fit models included CRP and estradiol in models where the other was the dependent variable: with progesterone the best fit model included only age at menarche.

However, a significant negative relationship does hold for all other tests between CRP and progesterone in the luteal phase. Two nonexclusive possibilities exist for this relationship: the hypothesis proposed that high inflammation indicates maintenance effort and allocation away from reproduction, or that progesterone suppresses inflammatory activity. Progesterone may be inherently anti-inflammatory, as its removal can initiate inflammatory reactions and its administration quiets inflammation (Beckley and Finn, 2007; Finn, 1998). Progesterone administration also reduces inflammation in traumatic brain injury rodent models (He et al., 2004; Pettus et al., 2005). From a broader adaptive standpoint, some anti-inflammatory action during the luteal phase is important to increase the chances of fertilization and implantation, since inflammatory activities would increase with the presence of foreign bodies (Finn, 1998), although estradiol may have an anti-inflammatory, suppressive effect as well (Salem et al., 2000). However, follicular and luteal CRP concentrations were not different in this sample, and so CRP was not necessarily suppressed in the luteal phase in these participants. A recent study of industrialized women in western New York found that CRP increased through the luteal phase in spontaneous nonconceptive cycles (Gaskins et al., 2012), which suggests that systemic inflammatory activity is not necessarily lower through the luteal phase.

Our second hypothesis stated that the timing of reproductive maturation influences adult sensitivity to environment, particularly regarding allocation of resources to ovarian function and maintenance effort: from this, we predicted that a later age at menarche would be correlated with lower CRP, estradiol, and progesterone. We arrived at this hypothesis from work by McDade et al. (2009a) that shows that low adult CRP is correlated with greater immune challenges in childhood, and Zhang et al. (2007) that demonstrated a negative relationship between adult CRP and age at menarche. Therefore, we expected that later menarche and lower ovarian hormone concentrations would correlate with lower CRP concentrations, as these all reflect greater environmental challenges (i.e., immune challenges, energetic constraint) in childhood.

We found that age at menarche was the strongest predictor of CRP, estradiol and progesterone in multiple regression models, and was significantly negatively correlated in all linear regressions. In fact, the rather robust relationship between age at menarche and these variables was one of the more interesting findings from this study and consistent with recent work on stress and age at menarche (Allsworth et al., 2005; Zhang et al., 2007). We cannot determine from these data whether the timing of reproductive maturation influences adult sensitivity, or whether it serves as a proxy for childhood and pubertal environment and thus only indicates rather than drives responsiveness to ecological stressors. However, the
timing of age at menarche has been found to correlate with adolescent and adult reproductive functioning. Girls who achieve menarche at younger ages have regular ovulatory cycles faster and earlier than those with later menarche (Vihko and Apter, 1984). Girls with earlier ages at menarche also tend to have higher reproductive hormone concentrations in adulthood (Emaus et al., 2008; Vihko and Apter, 1984).

The negative relationship of estradiol concentrations and age at menarche to CRP could be the result of only one of these factors having a causative link to CRP, or due to the presence of an additional factor that influences these reproductive variables and CRP. These variables could also all vary with ecological stresses in a similar way. Alternately, low CRP in adulthood could be associated with a later age at menarche in this population due to interindividual variation in immune challenges through development. Rural Polish women have variable exposure to farm animals, as some keep horses for fieldwork, dogs for protection, or chickens and cows for food, while others keep none at all. Therefore, future research could disentangle these relationships further by examining differences in pathogen exposure, animal feces exposure, or frequency of diarrheal episodes in infancy and childhood. The evidence that early life psychosocial stressors also affect adult CRP suggests this is an additional pathway to consider (Miller et al., 2008; Slopen et al., 2010; Taylor et al., 2006).

Two methodological issues may provide insight into the relationships found here through the menstrual cycle and with reproductive hormones: the form of the CRP measured, and the timing of its collection. Because this study measured urinary and thus monomeric CRP, we are measuring a downstream event from the initial acute phase reaction characterized by an increase in serum pentameric CRP: pentameric CRP dissociates into monomeric subunits at local inflammatory sites. Monomeric CRP is the tissue-based form of CRP, and it orchestrates inflammation by binding to immune complexes and facilitating clearance of debris via phagocytes. Its more specific effects include enhancing monocyte chemotaxis and adhesion (Nurden, 2011): these are related to monomeric CRP’s general function of serving as an intermediary, or opsonin, for phagocytosis. Monomeric CRP also can generate reactive oxygen species, chemically reactive oxygen-containing molecules that can lead to oxidative stress (Nurden, 2011). Monomeric CRP is not necessarily directly correlated with its native, pentameric form (Verma et al., 2004), and unlike the native form its actions appear to be explicitly and singly proinflammatory. Pentameric CRP predominates in serum samples, and current ELISAs cannot distinguish between pentameric and monomeric CRP. Therefore, these results may not be directly comparable to any other work on CRP and life history trade-offs in the literature to date, yet these analyses represent an exciting measure that may allow more concrete analysis of proinflammatory phenotypes.

The timing of urine collection in the original study was to measure C-peptide concentrations (Clancy et al., 2009), and designed to promote ease of collection for participants, and thus four consecutive early mid follicular days were sampled (days 3–6 from first day of menses), and three consecutive early mid luteal days (days 21–23). In designing this secondary analysis of urine samples for CRP, we hypothesized that these two time periods represented times of low inflammatory activity of reproductive origin, as menses would be nearly over and the implantation window not yet, or only just begun for most participants. This could mean variation in CRP concentrations in this study reflects maintenance effort rather than reproductive inflammatory activity. Either way, a larger sample size is necessary to do more sophisticated modeling, our proposed hypothesis regarding allocation between maintenance and reproduction does not exclude a confounding relationship in the other direction, relating to progesterone’s anti-inflammatory properties.

These results are compelling because this is the first investigation of CRP both using daily measures of ovarian hormones across the menstrual cycle and in a largely agricultural population. This is notable because CRP responds differently to markers of energetics and immune function among different subsistence types. Over a woman’s lifetime, living in a population of energy surplus or constraint modifies the developmental trajectories that lead to adult variation in her reproductive hormones and physiology (Jasienska, 2003, 2013). Therefore testing these relationships in a nonindustrial population, one with at least moderate energy constraint, allows us to see a broader spectrum of nonpathological variation that is more reflective of global populations than is currently available.

McDade et al. (2009b) found, at the same level of adiposity and skinfold measurement Filipino participants had lower CRP than American participants. Differences in microbial exposure (McDade et al., 2009a) and ethnicity (McDade et al., 2006, 2011a; Shah et al., 2010) each may impact population CRP concentrations as well. In this study, our sample of rural Polish women has a significantly later age at menarche, lower C-peptide concentrations, and lower progesterone concentrations than an age-matched American sample (Clancy et al., 2009). Further, during the harvest season rural Polish women also participate in moderate physical activity, which has been found in other populations to be negatively associated with CRP (Albert et al., 2004; Bassuk and Manson, 2005; Ford, 2002; Kasapis and Thompson, 2005). While bouts of intense exercise increase CRP concentrations, an overall pattern of higher physical activity tends to have an anti-inflammatory effect (Kasapis and Thompson, 2005). Thus, this data are consistent with the moderate energy constraint and suppressed luteal function previously documented in this rural Polish population (Clancy et al., 2009; Jasienska and Ellison, 1998, 2004) and expected with the spectrum model that postulates suppression of luteal before follicular function (Ellison et al., 1993).

These results may also help explain population variation in ovarian hormones when energetic constraint is not found. Núñez-de la Mora et al (2007) found that Bangladeshi women who immigrated to the UK after menarche had significantly lower progesterone concentrations than those who immigrated before menarche, but estradiol concentrations were not affected (Núñez-de la Mora et al., 2008). Interestingly, no differences in energy status or availability were found. The authors suggested that differences in resource allocation to maintenance (in this case, most likely, immune function) may explain why women who immigrated to the UK after pubertal maturation would have lower progesterone concentrations, as stressors in their prior environment could have contributed to lowering their reproductive function set point.
This work that so nicely demonstrates the impact of immigration on reproduction, may be also demonstrating the impact of greater nonenergetic challenges in the prior environment. Further, recent work parsing relationships between estradiol, progesterone, gonadotrophins, and menstrual cycle characteristics reveal that ovarian hormones operate semi-independently of one another, despite consistency across the menstrual cycle (Barrett et al., 2013). Different relationships between age at menarche, ecological factors, and ovarian hormones in this study may support Barrett et al’s suggestion that estradiol and progesterone may be differentially influenced by acute and chronic stressors. In fact, the stronger relationship between age at menarche and progesterone is consistent with previous work that demonstrates stronger population variation in progesterone than estradiol, likely from environmental differences during development (e.g., Ellis et al., 1993; Jasienska and Jasienski, 2008; Jasienska and Thune, 2001).

This study is limited in a few important ways. First, the study of monomeric CRP is new and its functions not fully understood (Boncler and Watala, 2009; Eisenhardt et al., 2009; Nurden, 2011; Sjowall et al., 2004; Verma et al., 2004). Therefore, conclusions about what monomeric CRP represents in terms of maintenance or more specifically immune effort must be tentative. Further, the design of this study does not allow for definitive testing on the direction or existence of a causal relationship between age at menarche, CRP, and reproductive hormones. Finally, this study’s sample size was not large: statistical power for most analyses was sufficient, but sophisticated multivariate modeling was not permitted.

What is exciting about this work is that it is the first to begin to assess relationships between maintenance effort and female reproductive function in a largely agricultural population. As research continues to develop, it may be that monomeric CRP becomes a powerful tool to assess proinflammatory phenotypes and immune effort. Future research should assess relationships between pentameric and monomeric CRP, assess microbial exposure of participants, and if possible sample larger numbers of women. This would allow for a better understanding of the ecological factors that may produce variation in the different forms of CRP and how they relate to reproductive hormones. Our research demonstrates correlations between age at menarche, CRP, and reproductive hormones that will drive future hypotheses about the nature of causality between developmental trajectories, anti-inflammatory hormones, and maintenance effort.

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