(PE) exposure.

HYPOTHESIS 2A. Women with higher variance in estradiol (E2), FSH and LH will have more frequent or severe midlife symptoms.

HYPOTHESIS 2B. Women with higher PE exposure/intake will have less frequent or severe midlife symptoms.

BACKGROUND

BIOLOGY OF THE PERIMENOPAUSE: Peripheral *and* central mediation of the menopausal transition

In Western biomedicine, menopause is widely believed to entail a depletion of ovarian follicles followed by a decrease in E2 and an increase in FSH and LH, concomitant with irregular menstrual cycles and hallmark symptoms such as hot flushes, and finally cessation of menses (Klein and Soules 1998). However, this idealized model ignores the substantial inter- and intrapopulational variability observed in recent anthropological studies (Beyene 1986; Lock 1993; Martin, Block et al. 1993) and literature (Prior 1998).

The perimenopause is characterized by changes in the normal feedback loops operating between the ovaries and the neuroendocrine axis. These changes are believed to be driven primarily by decreasing numbers and responsiveness of primordial follicles (Faddy, Gosden et al. 1992; Richardson 1993; Richardson 1995), although there may also be primary alterations in hypothalamic and pituitary control (Reame, Kelche et al. 1996; Wise, Krajnak et al. 1996; Wise, Kashon et al. 1997; Wise, Smith et al. 1999). Although menopause happens to all women, its timing and course vary widely. Examples have been found of women without menses but normal levels of hormones, and of women with high FSH, LH and E2 (Metcalf and Livesey 1985; Longcope, Franz et al. 1986; Ballinger, Browning et al. 1987; Burger 1994; Prior 1998), suggesting that variation is the norm (Johannes and Crawford 1999) and that declining ovarian function may not constitute the complete story (Wise, Smith et al. 1999). Rather we believe that variation within women is most relevant to the experience of symptoms and may reflect central dysregulation of the HPG axis and other areas of the hypothalamus (Wise, Kashon et al. 1997).

In order to understand the effects of PEs on the endocrinology of the menopausal transition, we must first conceptualize the primary pathways involved in the transition. In the PM model (Figure 1, p. 9), symptoms are primarily attributed to the loss of estrogen. This model corresponds to the medically-popular view of menopause as an estrogen deficiency disease requiring hormone replacement therapy (Slemenda, Hui et al. 1987; Matthews 1992; Matthews, Wing et al. 1994). Thus PE may alleviate symptoms by acting directly on autonomic activity, or peripherally via sex hormone binding globulin (SHBG). However, a recent review of vasmomotor symptom (VMS) epidemiology and physiology did not find a conclusive link between low E2 levels and VMS (Kronenberg 1990). A recent prospective study found no correlation between VMS severity and E2 levels (Rannevik, Jeppsson et al. 1995). Results running contrary to the belief that low E2 levels cause menopausal symptoms have often been overlooked or dismissed (Prior 1998). To explain the range of variation observed during the menopausal transition, we must therefore consider the role of the hypothalamus, and the potential central action of PE.

The CM model (Figure 2, p. 9), on the other hand, adds an emphasis on central dysregulation at the level of the hypothalamus occurring simultaneously with declining ovarian function. Central response to ovarian activity is hypothesized to be key in the etiology of VMS and other symptoms. Neurons containing estrogen receptors impinge on areas of the hypothalamus involved in regulation of body temperature and production of gonadotropin releasing hormone (GnRH). Thus central dysregulation may lead to both vasomotor instability as well as variation in gonadotropins.

High E2 levels can occur even with high or rapidly fluctuating gonadotropin levels (Metcalf and Mackenzie 1985; Longcope, Franz et al. 1986; Ballinger, Browning et al. 1987; Burger 1994; Prior 1998), suggesting dysregulation in the HPG axis. Since hot flushes can occur when E2 levels are high (Bider, Ben-Rafael et al. 1989; Gangar, Cust et al. 1989), VMS

are unlikely to be mediated peripherally through E2 levels alone. It has been suggested that estrogen withdrawal, or rapidly decreasing estrogen levels, may provide an adequate explanation for VMS with the addition of the following three corollary conditions (Prior 1998:417):

1) the higher the immediately preceding estrogen level, the greater the likelihood of provoking VMS
2) the shorter the time period of, or the greater the withdrawal slope of estrogen levels, the more intense the VMS
3) previous exposure of the hypothalamus to high estrogen levels (for an unknown duration) is necessary before decreasing estrogen levels would cause VMS

The importance of timing of E2 change, and previous E2 levels, suggests that neuroendocrine factors may be mediating many of the symptoms. This study will collect longitudinal data (repeated measures on individuals) to assess time lag effects, which we expect to be approximately 2 weeks for receptor up- and down-regulation.

CROSS-CULTURAL VARIATION: Japanese women have lower levels of "classic" menopausal symptoms

It has been suggested that only hot flushes, and perhaps atrophic vaginitis, correlate with changing hormones of menopause, and that somatic and psychological symptoms are the result of historical, cultural, and local stressors, or alternatively are secondary to hot flushes (Gannon 1985). The latter hypothesis receives support from cross-cultural studies, yet cross-cultural differences in the incidence of vasomotor symptoms suggest that either these are also influenced by cultural factors, or the relevant biological variables have not been discovered.

In North America, the primary symptoms associated with the perimenopause include vasomotor symptoms such as hot flushes and night sweats (Brenner 1988; Hunter 1990; Matthews 1992; von Muhlen, Kritz-Silverstein et al. 1995), but the incidence of these symptoms is considerably lower in Japanese women than in North American menopausal women (Lock 1993). Lock's research on *kōnenki* in Japanese women found hot flushes and night sweats were reported by 12.3% and 3.8% (respectively) of the sample population (Lock 1993) compared to U.S. women with 34.8% and 11.4% respectively (Avis, Kaufert et al. 1993). In open-ended interviews about menopause, women associated *kōnenki* with stiff shoulders, headaches, dizziness, aching joints, and trouble sleeping, though many women reported hearing about these symptoms, not experiencing them (Lock 1991). Because biological measures were not obtained, these differences between Japanese and North American menopausal women cannot be attributed to cultural differences alone and in fact may be sensitive indices of biological changes during the perimenopause. Results from exploratory field research in 1998 and 1999 (Melby 1999) suggest that rates of hot flushes may have increased since Lock's survey research in 1984, perhaps in part due to westernization of diet and/or medicalization of *kōnenki.* Menopausal Mayan women reported no hot flushes despite similar levels of reproductive hormones compared to Western women, further demonstrating that the experience of the perimenopause is not universal (Beyene 1986; Martin, Block et al. 1993).

In order to avoid confusion, we restrict the term menopause and menopausal symptoms to *etic* Western biomedical symptoms such as hot flushes and night sweats, while using *kōnenki* to refer to the *emic* symptoms such as *katakori* (shoulder stiffness) and *zutsū* (headache) (Lock 1993). Reporting on symptoms experienced in the previous 2 weeks, 51.7% of Japanese women surveyed had *katakori*, while *zutsū* was reported by 27.7% of women (Lock 1993). The combined set of symptoms will be referred to as *midlife* symptoms, and will increase our power to detect midlife symptoms in our sample.

The Japanese experience of the menopausal transition has challenged the Western biomedical model of menopause as a biologically universal experience and suggests that Western notions of menopause are neither ubiquitous nor necessarily associated with industrialization or development. If ovarian depletion of follicles and decrease in E2 cause menopausal symptoms, then all women should have symptoms. If hypothalamic

dysregulation causes menopausal symptoms, then most women should also have symptoms, although their timing and intensity may vary with endocrine fluctuations. The observed cross-cultural variation raises the question of whether the underlying (physiological) process of menopause is universal, and if not, which factors in the local biological and cultural environment influence and explain the observed variation.

REPRODUCTIVE ECOLOGY: Japanese women exhibit reproductive profiles and cancer risk similar to women in developing countries yet share lifestyle factors with women in developed countries

This project utilizes an explicit reproductive ecology approach. Reproductive ecology has made important contributions to our understanding of human reproductive biology by highlighting the influence of ecological and cultural factors on fertility and chronic disease. These studies have demonstrated the marked biological variation among humans in hormonal profiles, reproductive function, and symptomatology throughout the reproductive lifespan (Ellison 1991; Ellison, Panter-Brick et al. 1993; Panter-Brick, Lotstein et al. 1993; Worthman, Jenkins et al. 1993; Campbell and Wood 1994; Ellison 1994; Panter-Brick and Ellison 1994; Short 1994; Vitzhum 1994; Ellison 1995; Ellison 1996). Low luteal phase progesterone levels (Ellison, Lipson et al. 1993) characterize women in many non-Western 'natural fertility' populations, a pattern that has been linked to lower nutrition and increased workload (Ellison, Panter-Brick et al. 1993; Panter-Brick, Lotstein et al. 1993; Worthman, Jenkins et al. 1993; Panter-Brick and Ellison 1994; Wood 1994; Ellison 1995; Ellison 1996; Bentley, Harrigan et al. 1998). A higher incidence of reproductive cancers in North American and Western European populations compared to Asian, African or Eastern European populations (Trowell and Burkitt 1983; Yu, Harris et al. 1991) is associated with lower parity, resulting in higher cumulative lifetime exposure to gonadal steroids, particularly estrogens (Henderson, Ross et al. 1993; Marshall 1993). This pattern spreads as developing countries adopt Western diet and habits (Adlercreutz 1990). Moreover, although first generation immigrants generally exhibit an incidence rate typical of their country of origin, second generation immigrants assume the risk of their host country (Shimizu, Ross et al. 1991).

The Japanese are of particular interest because their reproductive hormonal profiles and cancer risk resemble women in developing countries whereas their ecology is more similar to that of developed countries. They exhibit the low rates of reproductive cancers found in natural fertility populations in spite of the lower parity, higher caloric consumption, and decreased workload typical of industrialized nations. Circulating estrogen levels are low compared to levels in North American women (Goldin, Adlercreutz et al. 1986; Key, Chen et al. 1990; Shimizu, Ross et al. 1990), as are rates of breast, ovarian, and endometrial cancer (Parkin 1989). Genetic differences are unlikely to be responsible for these differences since the incidence of reproductive cancers approaches Western rates when Japanese women emigrate to North America. More likely contributors are lifestyle factors (including diet and exercise) and their effects upon the HPG axis. The same factors may help to explain differences in the experience of menopause in Japan.

DIET AND PHYTOESTROGENS (PE): Phytoestrogens in high quantities may buffer variation in endogenous E2 levels

Lifestyle factors such as exercise and diet (i.e., energy expenditure and caloric intake) are known to influence health and reproductive functioning, but the effects of specific dietary compounds such as PEs have been largely ignored until recently. The choice of what to eat and how to prepare it is profoundly cultural, and is recognized as playing an important role in many aspects of health, from cancer to cardiovascular health and osteoporosis. The Japanese diet is unique in including a high proportion of soy dishes. Soy contains relatively large amounts of the PEs daidzein and genistein (Mazur 1998). Intestinal bacteria convert PEs into weakly estrogenic diphenolic compounds which can influence sex hormone production, metabolism and biological activity, intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, differentiation, cell adhesion and angiogenesis (Adlercreutz and Mazur 1997).

Soy appears in the diet in several forms. *Miso* (soybean paste) is consumed in approximately 50% of Japanese meals, and *tofu* in approximately 27% (measured by number of times of consumption per 100 person-meals) (Tsubono, Takamori et al. 1996). In many Asian diets, up to 1 mg/kg body weight or more of PEs is consumed daily, resulting in plasma concentrations of up to 3 μM (Uehara 1999), 7.5 times greater than levels in U.S. vegetarians (0.4 μM) and 42 times greater than levels in U.S. omnivores (0.07 μM) (Adlercreutz, Honjo et al. 1991). These levels are up to 50,000 times higher than levels of endogenous estradiol. Plasma daidzein concentrations range from 10-235 ng/mL and genistein concentrations range from 25-650 ng/mL (Adlercreutz, Markkanen et al. 1993; Adlercreutz, Fotsis et al. 1994). The high PE concentrations relative to endogenous steroids suggest that PEs may have significant biological effects. However, the Japanese diet is becoming more westernized, including more meat, fat and dairy products and fewer traditional foods such as soy (Japanese Ministry of Health 1996), making it important to carry out this study in the near future.

Phytoestrogens are widely believed to exert protective effects on the reproductive system (Punnonen, Jokela et al. 1987; Horn-Ross 1995; Murkies, Lombard et al. 1995; Fujita 1996; Knight and Eden 1996; Tanaka, Date et al. 1996; Adlercreutz and Mazur 1997; Nestel, Yamashita et al. 1997; Baum, Teng et al. 1998; Nagata, Takatsuka et al. 1998; Rygwelski and Smith 1998; Seidl and Stewart 1998; Tham, Gardner et al. 1998; Nagata, Shimizu et al. 1999). PE consumption has been hypothesized to play a role in decreased reproductive-related cancer risk (Horn-Ross 1995), cholesterol lipid levels (Baum, Teng et al. 1998; Nagata, Takatsuka et al. 1998), cardiovascular risk (Nestel, Yamashita et al. 1997), rates of osteoporosis (Cooper, Champion et al. 1992; Fujita 1996; Adlercreutz and Mazur 1997), and menopausal symptoms such as hot flushes (Nagata, Shimizu et al. 1999) compared to populations which consume relatively few PEs (Adlercreutz and Mazur 1997; Murkies, Wilcox et al. 1998; Rygwelski and Smith 1998; Seidl and Stewart 1998). Although PEs are generally weak estrogens, the high plasma concentrations resulting from ingestion of PE-rich foods like soy appear to partly compensate for their lower estrogenic potency.

Animal studies suggest that PEs augment estrogen action when endogenous estrogen levels are low but are antagonists at high levels of endogenous estrogens (Whitten and Naftolin 1992; Whitten, Russell et al. 1992; Whitten, Russell et al. 1994; Whitten, Kudo et al. 1997). Thus PEs may exert a buffering effect on hypothalamic-ovarian feedback. These effects may occur because PEs influence ovarian estrogen secretion. Low PE levels enhance gonadotropin release whereas higher levels suppress release (Hughes, Chakinala et al. 1991; Hughes, Kaldas et al. 1991). Clinical studies in Western women have produced mixed results, but are consistent with the notion that the pattern of PE actions may vary with endogenous estrogen levels. In premenopausal women, a soy supplement of 0.8 mg/kg increased E2 early in the follicular phase and reduced midcycle LH and FSH (Cassidy, Bingham et al. 1994), while a 3 mg/kg supplement reduced E2 on days 11 and 22 of the cycle (Lu, Anderson et al. 1996). In post-menopausal American women, soy PEs have been found to decrease LH secretion (Van Thiel, Galvao-Teles et al. 1991) and hot flushes (Murkies, Lombard et al. 1995; Albertazzi, Pansini et al. 1998; Whitten and Naftolin 1998), but not all studies have been able to demonstrate a difference from the normal decline in symptoms over time (Murkies, Lombard et al. 1995; Brzezinski and Debi 1999). However, most of these studies were limited by small sample size and short term treatment. Studies of Japanese women, which have produced more consistently positive results, have been larger in size and have focused on women consuming soy over long periods of time. In a study of 50 premenopausal Japanese women, the intake of soy products (as recorded by food-frequency questionnaire) was inversely correlated with E2 on days 11 and 22 of the cycle (Nagata, Kabuto et al. 1997). In post-menopausal Japanese women, only fermented soy product intake was negatively correlated with hot flush severity (Nagata, Shimizu et al. 1999).

None of these studies has considered alternatives to the PM model of menopause. This omission is crucial because the CM model predicts that high or erratic elevations in E2, incorrectly read by the central nervous system (CNS) (i.e., dysregulation), and reflected in high variance in gonadotropins, mediate development of menopausal symptoms. Therefore, buffering effects of PEs may be a function of their ability to reduce or antagonize these

hormonal spikes and compensate for E2 drops during the perimenopause at the level of the hypothalamus. In light of these studies, we hypothesize that the effects of PEs will differ over the menopausal transition as E2 levels change. No studies have examined PEs and relevant hormone measures for any lengthy period of time, particularly in individuals with changing levels of endogenous E2. The menopausal transition, which is hypothesized to be characterized by changing endogenous estrogen levels and hypothalamus-pituitary-gonadal dysregulation, provides a unique opportunity to explore the influence of PEs on regulation of the reproductive endocrine system, and to examine how culture, in the form of dietary choices, can affect biology and health.

STUDY POPULATION: Japanese consume high levels of soy phytoestrogens and hold a concept of midlife transition rather than menopause

Due to dietary practices involving high consumption of soy foods, Japan provides a natural experiment for investigating the effects of PEs (and diet more broadly) on the experience of menopause. Japanese women have low levels of menopausal symptoms (Lock 1993) as well as breast, ovarian and endometrial cancer (Parkin 1989) compared to North American women and Japanese living in Hawaii, suggesting possible beneficial biological factors in their environment (Tham, Gardner et al. 1998). Other potential sources of difference between Western and non-Western women in the experience of menopause are controlled for to some degree in the case of Japan: Western and Japanese women have similar life histories in terms of physical labor and reproductive history compared to natural fertility populations in developing countries.

Soy consumption, while high compared to most other countries, varies regionally throughout Japan. A significant example involves attitudes and preferences for *natto*, or fermented soybeans. Generally, people from the North and East of Japan have a strong taste for *natto*, and those from the South and West dislike it. Attitudes toward *natto* parallel consumption of soy regionally, with the highest average amounts of soy being consumed in the northernmost region (Tohoku) of Honshu (22.4% higher than national average), and the lowest amounts consumed in the Kinki region (12.3% lower than national average) (Japanese Ministry of Health 1996). This regional variation will be exploited in this study to maximize the range of variation in diet with respect to the total PE consumption and bioavailability. Fermentation of soy may increase the bioavailability of PEs (Hutchins, Slavin et al. 1995; Slavin, Karr et al. 1998), thereby demonstrating the importance of food processing as a cultural tool for modifying physiologically-relevant aspects of the environment. For example, one study found that intake of fermented soy products (*natto* and *miso*) but not unfermented *tofu*, was correlated with a decrease in frequency and intensity of hot flushes (Nagata, Shimizu et al. 1999). Given individual differences in metabolism of PEs, measurement of blood concentrations in addition to dietary intake is important (Slavin, Karr et al. 1998) and will be carried out in this study.

Kōnenki has been characterized as a time when the body "loses its balance" (Lock 1993), and is attributed by Japanese to both cultural and biological causes (Melby 1999). While this project will focus on the reproductive ecology of menopause in Japan by focusing on diet, symptoms and hormones, the ambiguities between *kōnenki* and menopause may highlight some interesting biocultural phenomena. Although Western scientists and clinicians may conclude that *kōnenki* is thus separate from menopause, we propose that *kōnenki* may be a more sensitive label for changes occurring in the body during the menopausal transition. Correspondingly, the investigators of the Melbourne Women's Midlife Health Study inferred that "self reported menopausal status is a more sensitive measure of endocrine status" (Dennerstein, Smith et al. 1993) than bleeding patterns. This observation supports the hypothesis that endocrine changes of the perimenopause often precede noticeable changes in menstrual patterns, and that these endocrine changes manifest themselves in symptoms of which women are aware. Similarly, many Japanese understand *kōnenki* to be due to an imbalance of the autonomic nervous system. Lock (1993) quotes one woman who claims that *kōnenki* is due to a hormonal imbalance that can be remedied before the end of menstruation. Since dysregulation in FSH and LH often precedes noticeable changes in menstrual patterns,

kōnenki, and the *emic* understanding of it as reflecting the state of the autonomic nervous system (Lock 1993), may be a more biologically astute concept reflecting the experience of hormonal dysregulation rather than bleeding patterns alone. Although *kōnenki* has important cultural aspects, it may have an equally important biological dimension.

PRELIMINARY STUDIES

MENOPAUSE: Pilot study of perimenopausal neuroendocrine variation

The Laboratory for Comparative Human Biology (Carol M. Worthman, Director) has extensive history in developing and applying non-invasive field-friendly methods for the cross-cultural study of endocrinology across the lifespan (Konner and Worthman 1980; Worthman and Konner 1987; Worthman, Stallings et al. 1990; Beall, Worthman et al. 1992; Angold and Worthman 1993; Worthman, Jenkins et al. 1993; Eaton, Pike et al. 1994; Worthman and Stallings 1994; Dabbs, Campbell et al. 1995; Costello, Angold et al. 1996; Rilling, Worthman et al. 1996; Stallings, Worthman et al. 1996; Worthman and Stallings 1997; Angold, Costello et al. 1998; McDade and Worthman 1998; Angold, Costello et al. 1999). Recently, in collaboration, we have examined variation in neuroendocrine patterns in menopausal women in relation to behavior and well-being (Worthman, Trevathan et al. 1999). We piloted a 4-month study of weekly blood spot self-sampling with daily checklists for symptomatology in 13 pre- and peri-menopausal American women, and found that autonomic symptoms (hot flushes and sweats) were correlated to FSH, LH (r .72, p <.005) and FSH variation (r .72, p .01), but not with E2. The results of this pilot study suggested that blood spot self-sampling with daily checklists was feasible, and indicate attenuated ovarian activity and periodic disruption of HPG feedback regulation during the menopausal transition.

MENOPAUSE: Symptoms and social-cultural context

We have also carried out research in collaboration with Margaret Moloney, Ph.D. (Moloney 2000; Moloney and Melby 2001; Moloney, Melby et al. 2001) on the perimenopause in the U.S., involving extensive interviews, focus groups, and administration of quality of life questionnaires. Finally, in the summers of 1998 and 1999, we carried out feasibility assessments and preliminary field research on *kōnenki*, including interviews and a survey (n = 70, approximately 75% response rate) on views of the symptoms and causes of *kōnenki* in Ishikawa Prefecture (Melby 1999).

PHYTOESTROGENS: Newly developed fluoroimmunoassays for daidzein and genistein

The Laboratory of Reproductive Ecology and Environmental Toxicology (Patricia L. Whitten, director) has extensive history in working with phytoestrogens. Observational as well as experimental studies in a variety of animals ranging from rodents to non-human primates (Whitten 1992; Whitten and Naftolin 1992; Whitten, Russell et al. 1992; Whitten, Lewis et al. 1993; Naftolin, Whitten et al. 1994; Whitten and Naftolin 1994; Whitten, Russell et al. 1994; Whitten, Lewis et al. 1995; Whitten, Lewis et al. 1995; Whitten, Kudo et al. 1997; Brockman, Whitten et al. 1998; Whitten, Brockman et al. 1998; Whitten and Naftolin 1998; Whitten, Stavisky et al. 1998; Patisaul, Whitten et al. 1999; Whitten 1999) have shown that PEs are biologically active at natural dietary levels, affecting brain and behavior as well as the reproductive tract. Both estrogenic and antiestrogenic actions can be observed, some of which occur through negative feedback effects on gonadotropins and gonadal steroids.

Phytoestrogen fluoroimmunoassays measuring daidzein and genistein have been developed for plasma and serum samples (Wang, Lapcík et al. 2000). With the resources of our combined laboratories, we have modified these assays for use with blood spots. These methods are summarized briefly below.

Plasma PE concentrations in Japanese generally range from 10-235 ng/mL and 25-650 ng/mL for daidzein and genistein respectively (Adlercreutz, Markkanen et al. 1993; Adlercreutz, Fotsis et al. 1994). Assay performance characteristics have been optimized for

these ranges. For daidzein they are as follows: working range of assay 0-240 ng/mL; sensitivity 0.6 ng/mL; average % recovery is 101.1%; intrassay CVs (n = 12) = 7.0% (42 ng/mL); interassay CVs = 6.5% (20 ng/mL) and 3.5% (79 ng/mL); cross-reactivity is: genistein 1.1%, equol 0%, endogenous steroids 0%; linearity % recovery ranges from 101-110% for serial dilutions from 1:2 to 1:8; stability is at least 8 weeks at 4°C, room temperature, and 37°C. The genistein assay is similar to that for daidzein with the following differences: a larger range of standards (0-640 ng/mL), decreased sensitivity (5 ng/mL), and cross-reactivity: daidzein 2.5%, equol 0%, endogenous steroids 0%. External validation using the fluoroimmunoassay for plasma samples is now underway in collaboration with Shaw Watanabe and Mariko Uehara (Tokyo University of Agriculture) on blood spot and plasma samples collected from 45 Japanese women. These new methods greatly reduce both the subject burden and financial costs of measuring circulating PE concentrations.

HORMONAL ASSAYS

Blood spot assays for endocrine measures (including FSH, LH, E2, SHBG, T, PRL, ANDY, CORT, and DHEAS) have been developed by the Laboratory for Comparative Human Biology. These assays have been used in many cross-cultural settings including the following: Tibet, Bolivia, Papua New Guinea, Canada, US, and Zimbabwe (Worthman, Jenkins et al. 1993; Worthman and Stallings 1994; Rilling, Worthman et al. 1996; Stallings, Worthman et al. 1996; Stallings, Fleming et al. 1997; Stallings, Worthman et al. 1997; Worthman and Stallings 1997; Beall, Brittenham et al. 1998; Stallings, Worthman et al. 1998).

RESEARCH DESIGN AND METHODS

Figure 1
Peripheral Mediation

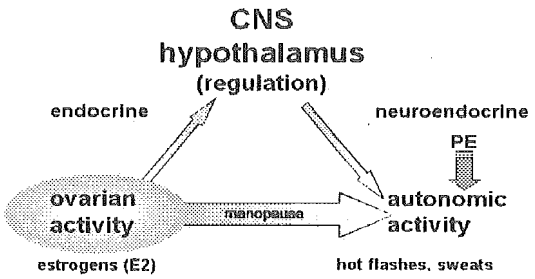

Figure 1: Schematic of menopause according to the Peripheral Mediation model. Ovarian activity (decreased E2) is the primary factor influencing autonomic activity and menopausal symptoms. Feedback between the ovaries and hypothalamus exists, but plays a minor role in the etiology of

Figure 2
Central Mediation

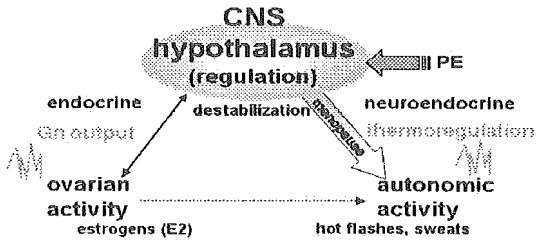

Figure 2: Schematic of menopause according to the Central Mediation model. Dysregulation at the level of the CNS is the primary factor influencing autonomic activity and menopausal symptoms. This model emphasizes central effects of PE.

In order to identify the potential effects of PEs on midlife symptoms, it is first necessary to conceptualize the major pathways involved in the menopausal transition and etiology of symptoms. We do not seek primarily to elucidate the proximal causes of menopause. Rather, we include hypotheses about endocrine factors involved in menopausal symptoms because such physiology is key to addressing our central questions, which concern the role of phytoestrogens (PE) in modulating menopausal symptoms. We hypothesize that central, rather than peripheral, endocrine-neuroendocrine pathways comprise the principal loci for PE effects. Our dual models are intended to help disentangle peripheral from central mediation of PE influence on menopausal symptoms, not to suggest that central and peripheral mediators are mutually exclusive.

We hypothesize that the menopausal transition is characterized by dysregulation primarily at the level of the hypothalamus (Central Mediation Model Figure 2) in addition to declining ovarian function (Peripheral Mediation Model Figure 1), and that high variation in

LH and FSH levels (reflecting high central variability) will correlate with VMS (with a possible further lagged effect of approximately 2 weeks for receptor up- or down-regulation). The PM model hypothesizes that decreased E2 levels cause VMS. The CM model hypothesizes that symptoms are primarily caused by CNS response to variance in E2, due to up- and down-regulation of receptor populations in the hypothalamus, and the ensuing cascade of effects throughout the brain where E receptors are located. When the hypothalamus is destabilized, the typical tight feedback of the HPG axis may collapse leading to simultaneously high levels of E2, LH, and FSH.

As shown in Table 1, during the pre- and perimenopause, when hormone levels are normal (N; characteristic of regulated feedback) symptoms are predicted to be low (L). As a woman enters the perimenopause (and possibly late premenopause), symptoms may emerge. The PM model predicts that increased symptoms will be associated with low estradiol levels whereas the CM model predicts elevations in symptoms will be associated with highly variable gonadotropin and estradiol levels. Postmenopausally, E2 levels are low, and gonadotropins are high, and both models predict symptoms. However, the CM model allows for low symptoms if gonadotropin variance is low. These comparisons show that the two models are best distinguished by the hormonal profiles associated with symptoms in the perimenopausal period.

Table 1. Matrix of predictions for models of the menopausal transition.

Menopausal Status	Model	FSH & LH	E2	Symptoms
Pre	R	N	N	L
	PM	N	N	L
	PM	N-H	L	H
	CM	N	N	L
	CM	HV	N-H, HV	H
Peri	PM	N	N	L
	PM	H	L	H
	CM	N	N	L
	CM	HV	N-H, HV	H
Post	PM	H	L	H
	CM	HV, H	L	H
	CM	LV, H	L	L

Menopausal status will be assigned based on menstrual patterns (see Study Population below). R= Regulated model with HPG feedback characteristic of reproductive years; PM = Peripheral Mediation model; CM = Central Mediation model. For hormones and symptoms: N = normal level; L = low level; H = high level; LV = low variance; HV = high variance.

We further hypothesize that PEs minimize menopausal symptoms by acting at the level of the hypothalamus (buffering hypothalamic estrogen receptors from variation (spikes and crashes) in E2) and leading to fewer regulatory changes in neurotransmitters throughout the brain and thus fewer symptoms associated with destabilization.

We therefore expect to observe decreased variation in gonadotropin levels (an index of central activity) with increased PE levels. Specific hypotheses are outlined below in Table 2, which is essentially the same as Table 1 with the addition PEs as mediating factors. When PE levels are high, symptoms are predicted to be low.

Under the PM model, the added estrogens provided by a PE diet would augment the low estradiol levels produced by ovarian depletion, suppressing menopausal symptoms along with gonadotropin levels. Under the CM Model, PEs would provide a more continuous estrogen signal to the CNS, dampening the symptoms produced by variable estradiol secretion. Following a peripherally-mediated model, some researchers have hypothesized that PE consumption might affect endogenous E2 concentrations via SHBG or other mechanisms, and that FSH and LH would decrease (with PE acting as

Table 2. Matrix of predictions for models of the menopausal transition and phytoestrogens.

Menopausal Status	Model	FSH & LH	E2	PhytoE	Symptoms
Pre	R	N	N	L or H	L
	PM	N-H	L	L	H
	PM	N	L	H	L
	CM	HV	N-H, HV	L	H
	CM	LV	N-H, HV	H	L
Peri	R	N	N	L or H	L
	PM	N	N	L or H	L
	PM	H	L	H	L
	PM	N-H	L	L	H
	CM	N	N	L or H	L
	CM	LV	N-H, HV	H	L
	CM	HV	N-H, HV	L	H
Post	PM	H	L	H	L
	PM	H	L	L	H
	CM	H	L	H	L
	CM	H	L	L	H
	CM	H, LV	L	L	L

Menopausal status will be assigned based on menstrual patterns (see Study Population below). R = Regulated model with HPG feedback characteristic of reproductive years; PM = Peripheral Mediation model; CM = Central Mediation model. For hormones and symptoms: N = normal level; L = low level; H = high level; LV = low variance; HV = high variance.

estrogen replacement therapy with feedback effects on the hypothalamus and pituitary) (Baird, Umbach et al. 1995). However, results did not support these hypotheses, suggesting the need for alternative conceptualizations of perimenopausal endocrinology in studies of PE effects on this transition. These hypotheses will be tested in 120 Japanese women, half from an area with high soy intake and half from an area with low soy intake.

STUDY POPULATION

Japanese women aged 45-55 (menopause occurs in this age range for 60-70% of Japanese women (Shimizu, Kawakami et al. 1996)) are being recruited at public health centers and through friends and acquaintances of participants (snowball method), making this a non-random sample. Approximately 60 women will be recruited from Kyoto prefecture in the Kinki region, which has the lowest average soy consumption in Japan (12.3% lower than national average), and approximately 60 women from Fukushima prefecture in the Tohoku region, which has the highest average soy consumption (22.4% higher than national average) (Japanese Ministry of Health 1996). Sufficient variation in diet is also found in each area (plasma PE concentrations range from 24nM to 3000nM in Tohoku (Uehara 1999) and are expected to be lower and have a smaller range in Kinki), allowing us to control for regional effects. The initial screening will be carried out to select for approximately equal numbers of pre-, peri- and post-menopausal women based on patterns of menstrual bleeding. For this study we define menopausal status following current epidemiological studies (Brambilla, McKinlay et al. 1994): premenopausal = a woman who has had regular periods (no amenorrhea or menstrual irregularity) in last 3 months; perimenopausal = a woman who has experienced at least 3 but less than 12 months of amenorrhea or a self report of increased menstrual irregularity; and postmenopausal = a woman who has had no periods (amenorrhea) in last 12 months. Potential subjects will be excluded if they: have had ovariectomy (both ovaries removed) or hysterectomy (greater than 80% of women in the Takayama study underwent natural menopause and did not take estrogen replacement therapy (Shimizu, Kawakami et al. 1996)); are currently or have recently (in approximately past 10 years) taken birth control pills (or hormone implants) or hormone replacement therapy (since the birth control pill was only approved for use in Japan in 1999 and hormone replacement therapy use is still low, these requirements should not exclude many participants); have an ongoing or recent (in past 6 months) major illness that necessitated hormonal medication.

DATA SAMPLING

Sampling Schedule

Rolling recruitment is being used, and participants will be enrolled for a total of six months. A six month sampling period was chosen in order to obtain estimates of individual variation within and between cycles, obtain longitudinal data (sufficient for perimenopausal women who may have long or short cycles and to assess lag effects), and minimize subject burden.

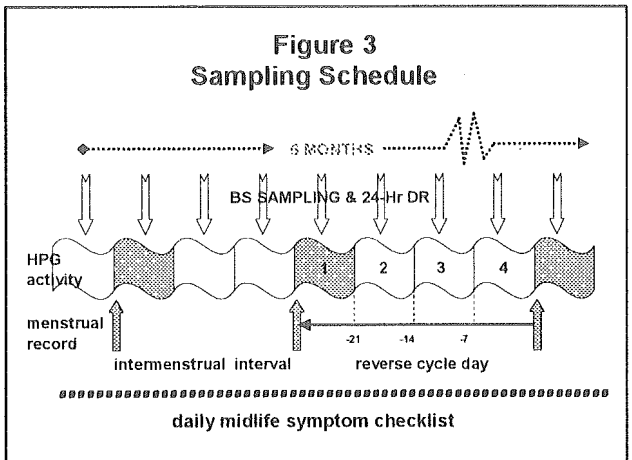

Figure 3: Schematic of sampling schedule. Over the course of six months, individuals will complete a daily midlife symptom checklist, weekly blood spot (BS) sampling and 24-hour dietary recall (DR). Intermenstrual intervals will be calculated using reverse cycle days.

Data collected will include menses and midlife symptoms, blood spot samples for endocrine analyses, and health, dietary, and ethnographic data. Checklists of midlife symptoms will be completed daily by participants throughout the study, and blood spot samples will be collected weekly along with 24-hour dietary recall (DR) (Figure 3). Using the dates of menses recorded on the daily checklist, intermenstrual intervals will

be calculated and reverse cycle days will be used to assign follicular and premenstrual phases (in cycling women). A food frequency questionnaire (FFQ) and a more extensive sociodemographic and health history questionnaire is administered to each participant at the initial interview following enrollment in the study.

Blood Spot Samples

Endocrine parameters examined in this study will be measured in dried blood spots from finger sticks. Women are asked to collect blood spots from finger sticks and do a 24-hour DR (in the form of a simplified dietary survey/checklist) on a weekly basis for a total period of 6 months. Each subject (after receiving instruction, demonstration, and practice)will apply a sterile disposable lancet to her finger, and collect several drops of blood on the supplied, labeled filter paper (#903 Schleicher and Schuell, Keene, NH). On a weekly basis, two samples spaced 20 minutes apart will be obtained to control for hormonal pulsatility. Diabetics perform a similar collection procedure several times a day, and thus this procedure should be minimally inconvenient and painful for participants. Filter paper cards will be allowed to dry during the day, and then stored in Ziploc bags in participants' refrigerators (2-8°C). In order to control for circadian variation in gonadotropin and PE concentrations, each woman will collect blood spots early in the morning. The timing of sampling represents a compromise among the hormones and PEs of interest as peak levels occur at differing times of the day, and follows protocols of other studies investigating PE and endogenous hormone concentrations (Adlercreutz, Markkanen et al. 1993; Adlercreutz, Fotsis et al. 1994).

Our pilot studies have shown that weekly blood sample self-collections are feasible and sustainable and provide a useful index of endocrine function in both pre- and peri-menopausal women (Worthman, Trevathan et al. 1999). A sampling schedule of once a week is a rational compromise balancing accurate estimation of hormone levels with subject burden and study design constraints. This study will be an improvement over the many studies (Prior 1998) that sample once every 6 months with no regard for menstrual cycle day. Although some studies have sampled daily, they rarely last longer than one to two cycles (Prior 1998), and thus cannot provide information on patterns within individuals over the menopausal transition as well as lag effects expected with receptor up- and down-regulation.

Every two weeks, participants will mail blood samples and diary sheets in pre-addressed stamped envelopes to the researcher (Melby). This will enable the researcher to monitor the participation of all individuals, check adequacy of blood spots, address problems, do data entry on a regular basis and send the blood samples via Federal Express mail to the Laboratory for Comparative Human Biology at Emory University where they will be catalogued, and stored at -27°C until analysis. The period of time elapsing between sample collection and freezing will not exceed 3 weeks, and samples will be refrigerated during most of the intervening time period. Samples remain stable at 2-8°C for up to 8 weeks (at room temperature, samples remain stable for a minimum of 2 weeks), and for years at -27°C (Worthman and Stallings 1997).

Midlife Symptom Checklist

Data will also be collected daily on menses and midlife symptoms using a modified, abridged version of the 2-week symptom checklists used by Lock in Japan (Lock 1993) and elsewhere by others (Hunter 1992; Greene 1998). This will be a checklist of core etic (menopausal) and emic (*kōnenki*) symptoms where women can record whether or not they experience a symptom (e.g., hot flush or *katakori*) and estimate the severity. By including both emic and etic symptoms we increase the prevalence of midlife symptoms, and thus our statistical power. The inclusion of symptoms used in several different studies will allow us to construct different menopausal scales (Kaufert, Gilbert et al. 1988; Perz 1997), and thus compare our results with published reports (Alder 1998). Daily checklists have been used successfully in a 6-month collaborative study of menopause by our lab (Worthman, Trevathan et al. 1999) and in a 6-month study on menopause and migraines (Moloney and Melby 2001; Moloney, Melby et al. 2001).

Dietary Data

Three methods of measuring dietary exposure to soy PEs will be utilized in this study: self-administered quantitative food frequency questionnaire (FFQ), 24-hour dietary recall (DR) (for the day preceding blood spot samples), and time-resolved fluoroimmunoassay (TR-FIA) of PE in blood spots. We will use a FFQ developed in Japan (Shimizu, Ohwaki et al. 1999) to estimate average daily food intakes (administered at the beginning and end of the study). This FFQ was validated against 3-day records, 24-hr recalls, and 12 1-day records with 58 males and 59 females, and has been used in subsequent studies where Spearman correlation coefficients comparing intakes of soy products estimated from the FFQ and diet records were 0.71 for total amounts (g) of soy products, 0.70 for *tofu*, 0.76 for *miso*, 0.79 for *natto*, 0.36 for boiled soybeans, and 0.52 for other soy products. Participants will fill out the 24-hr dietary recall weekly, when they do their finger-prick blood spot sampling. Participants will be asked to record all items eaten (or drunk) during the previous 24 hour period, and to estimate the portion size and time at which they were ingested. 24-hr DR will allow us to correlate soy consumption with blood spot phytoestrogen data, assess the effects of fermented vs. non-fermented forms of soy on bioavailability, and to examine variation in dietary behavior and seasonality effects. Following similar studies, individual PE intake will be estimated from data on frequency of intake and portion size using the Standard Tables of Food Composition in Japan (4th revised edition published by the Science and Technology Agency of Japan) and published literature values for typical soy foods (Resources Council 1982; Nagata, Kabuto et al. 1997; Mazur 1998; Nagata, Shimizu et al. 1999; Arai, Watanabe et al. 2000). Due to individual variation in gut microflora and enzyme concentrations (and thus metabolism of soy) it is important to measure PE concentration in the blood. This will be done using our newly developed TR-FIA methods for blood spots (Lapcik, Hampl et al. 1997; Adlercreutz, Wang et al. 1998; Lapcik, Hampl et al. 1998; Lapcik, Hill et al. 1998) described above and in Appendix II.

Ethnographic and Health Data

The broader cultural context in which relationships and associations between PEs, reproductive endocrinology, and symptoms occur will have important implications for applications, interventions, and policy recommendations. To this end, we will collect data on lifestyle factors and life events such as reproductive history, work and health stress, care of elderly parents, retirement of husband, and lack of hobbies which have been observed to affect the experience of *kōnenki* (Lock 1993; Suganuma 1998).

Data on demographics (age, occupation, socioeconomic status, geographical region), exercise, body composition (BMI), personal habits (smoking, alcohol consumption), illness and medication use will be collected in a questionnaire and semi-structured interview at the beginning of the study and from daily midlife symptom checklists. Semi-structured interviews conducted throughout the duration of the study to elicit life events, reproductive history, attitudes toward gender roles, and social relations, will further contribute to an understanding of the many biocultural factors involved in the menopausal transition. After completion of fieldwork, laboratory and statistical analysis will take place at the Laboratory for Comparative Human Biology and Laboratory of Reproductive Ecology at Emory University.

DATA ANALYSIS

Hormonal Analyses

Blood spot samples will be analyzed in the Laboratory for Comparative Human Biology and Laboratory of Reproductive Ecology at Emory University for the following hormones: estradiol (E2) (by radioimmunoassay (RIA)),

Table 3. Hormonal analysis time frames for different menopausal status groups.

Menopausal status	Hormonal Analysis Time Frames
Premenopausal	Follicular phase (FP) Premenstrual/luteal phase (PMP) Intermenstrual interval (II) (approx. 1 month) 6 month (total interval (TI)
Perimenopausal	Follicular phase (FP) Premenstrual/luteal phase (PMP) Intermenstrual interval (II) (variable)* 6 month (TI)
Postmenopausal	Interval (II) (arbitrarily set at 1 month) 6 month (TI)

*By definition, perimenopausal women have variable intermenstrual interval lengths and both short and long cycles.

and follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone binding globulin (SHBG) (by fluoroimmunometric assay (FIMA)). Free (vs. bound) E2 will be estimated from levels of SHBG. These well-validated and field tested methods are described in detail in a recent publication (Worthman and Stallings 1997). Hormonal data will be analyzed on several time scales in the different menopausal status groups (Table 3).

Phytoestrogen Exposure

Soy/phytoestrogen consumption will be estimated in 3 ways: FFQ; 24-hour DR; PE concentration in blood spots. Circulating PE concentrations will be analyzed by our new bloodspot FIAs (Lapcik, Hampl et al. 1997; Adlercreutz, Wang et al. 1998; Lapcik, Hampl et al. 1998) developed in collaboration with Adlercreutz and colleagues whose method for analysis in plasma and serum has been validated against gas chromatography mass spectrometry (GCMS) (Wang, Lapcík et al. 2000). We are collaborating with Shaw Watanabe and Mariko Uehara (Nogyo Daigaku, Tokyo, Japan) to validate our blood spot PE analyses on matched BS and plasma Japanese samples, which are being analyzed by time-resolved fluoroimmunoassay (TR-FIA) (Lapcik, Hampl et al. 1997; Adlercreutz, Wang et al. 1998; Lapcik, Hampl et al. 1998; Wang, Lapcík et al. 2000).

Soy and PE consumption will be estimated from the FFQ and 24-hour DR data on frequency of intake and portion size using the Standard Tables of Food Composition in Japan, 4th revised edition published by the Science and Technology Agency of Japan and published literature values of (average PE)/(unit soy) (Resources Council 1982; Nagata, Kabuto et al. 1997; Mazur 1998; Nagata, Shimizu et al. 1999). The authors of the FFQ, Drs. Shimizu and Nagata at Gifu University, have agreed to assist in analysis of the data. The FFQ and DR estimates will then be compared with the blood spot PE measures, providing internal validation and allowing us to directly evaluate the relationship between PE consumption and circadian levels. These estimates of PE exposure will be compared with data on reproductive hormones and menopausal symptoms to assess whether PE consumption, soy consumption, or other components of the diet are correlated with differences in endocrinology or symptomatology. Average PE concentration/consumption patterns will be used to group women for tests of hypotheses involving between group comparisons. Changes in endocrinology or symptomatology in individual women will be examined in the context of changes in PE consumption.

Midlife Symptom Checklist

Continuous data entry will be done in the field. Using the daily symptom checklist, weekly averages, monthly/cycle interval averages and six-month averages will be calculated for composite symptom indices (Kaufert, Gilbert et al. 1988; Hunter 1992; Perz 1997; Alder 1998; Greene 1998) as well as core individual symptoms of interest (e.g., hot flush, *katakori*) for each subject. The degree to which symptoms of *kōnenki* map onto menopause is an empirical question which will be examined using these data.

Ethnographic and Health Data

Continuous data entry, and preliminary analysis, will be done in the field. Ethnographic data will be analyzed using the qualitative software package, Atlas.ti, to identify themes concerning *kōnenki*, life history and lifestyle factors (such as who gets *kōnenki* and why, marital relations, children, economic stressors, caretaking of elderly parents, friends and social support). Many factors may influence hormone levels and symptom reporting. Variables to be controlled for include: age, BMI (since fat affects amounts of estrogen produced), exercise (each activity level will be assigned an intensity score according to the relative metabolic rate method (Numajiri 1979; Nagata, Kabuto et al. 1997)) sociodemographic variables (e.g. income, occupation, and education); geographic region, reverse cycle day, and reproductive history (e.g. parity, age at menarche). These data will be collected during the extensive initial interview (during which the protocol will be demonstrated and explained), questionnaire, and from later interviews and daily checklists.

Statistical Analysis

Hormonal and survey data will be analyzed using Statistical Analysis Software (SAS) and Stata. Classic descriptive statistics will be used to determine the mean value and range of

variation for each variable. Variables will be treated as continuous and categorical variables. T-tests of means, F-tests of variance, ANOVA, multiple linear regression, and hierarchical linear and logistic regression adjusted for repeated measures will be performed. Concomitant time series analysis to cross-correlate variables of interest with time lags of varying lengths will be used. Diagnostics will be run to test assumptions of statistical models and check for outliers. Tests for normality will be carried out, and data will be transformed if necessary. Regression analyses will allow us to control for PE exposure and other variables (see above section). Logistic regression will allow us to obtain odds ratios to estimate the likelihood of having menopausal symptoms above a certain cutoff (e.g., severe vs. mild) with a certain level of PE exposure, while controlling for other variables.

SPECIFIC HYPOTHESES

Hypotheses, dependent and independent variables, time frames, and statistical tests are summarized in Tables 4 and 5.

HYPOTHESIS 1A. **A peripheral mediation (PM) model will be insufficient to explain hormonal changes in the perimenopause. Additional data from centrally mediated (CM) pathways will be needed to characterize the menopausal transition.**

Gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) will serve as an index of central function, while estradiol (E2) will serve as an index of peripheral function.

- Blood spot (BS) E2 (total and free - estimated from SHBG concentrations) will be higher in peri- than in premenopausal women at times.
- Variance in BS levels of E2, FSH, and LH will be higher in perimenopausal than in premenopausal women.
- Gonadotropin variation will associate with present as well as future symptoms; E2 levels will not associate with current but may predict future symptoms.

Rationale: A recent literature review on perimenopausal endocrinology observed that E2 levels are often normal or high while levels of FSH and LH are normal to high (Prior 1998). This suggests that many perimenopausal symptoms are not due solely to decreased E2 levels consistent with a PM model, but rather are responses to increases or variation in E2 mediated by central dysregulation of the HPG axis (Worthman, Trevathan et al. 1999). To our knowledge, no study of menopausal symptom physiology has considered lag effects expectable from priming and up- or down-regulation impact of gonadal steroids (and chronic PE exposure).

Method and Analysis: Estradiol levels, FSH, and LH will be measured at weekly intervals using well-validated RIAs and FIMAs for blood samples obtained from women by finger-pricks. Total and free E2 will be estimated by measuring levels of SHBG in samples (Rilling, Worthman et al. 1996). Daily diaries will be used to identify ovarian cycles based on intermenstrual intervals using reverse cycle days.

Menstrual cycles will be divided into follicular phase (FP) and premenstrual phase/luteal phase (PMP) based on the number of weeks preceding menses. Hormone concentrations will be averaged for the follicular phase, premenstrual phase, intermenstrual interval, and six-month sample period for each subject. The standard deviations of the resultant means will be used as an estimate of variation during the time periods.

Estradiol levels for each time period will be compared between pre- and peri-menopausal women using 2-way ANOVA. A sample size of 40/group gives a power of 80% to detect differences comparable to that previously reported between pre- and perimenopausal women (Prior 1998). Variance in BS levels of E2, FSH, and LH will be compared between pre- and perimenopausal women for the 4 time periods (FP, PMP, II, and TI) using F-tests. A sample size of 40/group gives a power of 90% to detect differences in variance observed between pre- and perimenopausal women (Lenton,

Sexton et al. 1988). To evaluate the prospective data for time-lagged effects, we will use concomitant time series analysis to cross-correlate symptom frequency and intensity with endocrine status at time lags 0, +1, and +2 weeks. We have used this data analytic strategy to examine relationships of symptoms to mood and stress in our pilot study on menopausal transition with Burleson and Trevathan.

Possible Results and Interpretation

- Higher BS E2 levels (total &/or free) in perimenopausal compared to premenopausal women will support the CM model.
- Lower BS E2 levels in perimenopausal compared to premenopausal women will support the PM model.
- Higher variance in BS levels of E2, FSH, and LH in perimenopausal compared to premenopausal women will support the CM model. Smaller (or no difference in) variance measures in perimenopausal women will lead to rejection of the CM model. The PM model does not make predictions about variance of hormone levels.
- Detection of time-lagged effects of gonadotropin variance will support a CM model, as will the presence of such effects for E2 in the absence of concurrent E2 effects.

HYPOTHESIS 1B. **Variance in gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) will be negatively correlated with phytoestrogen (PE) exposure.**

- Variance in FSH and LH will be lower in women with higher PE exposure/soy consumption.
- SHBG means will be higher in women with higher PE exposure/soy consumption.
- Variance in FSH and LH will be negatively correlated with soy/PE intake and blood spot concentrations in women of all three menopausal status groups.
- SHBG levels in all three menopausal status groups will be positively correlated with PE intake and blood spot concentrations.

Rationale: Negative feedback effects of a soy PE on LH release have been demonstrated experimentally in rodents (Hughes, Chakinala et al. 1991; Hughes, Kaldas et al. 1991), and there is evidence that soy consumption also can suppress gonadotropin levels in pre- and post-menopausal women (Cassidy, Bingham et al. 1994; Nagata, Kabuto et al. 1997; Xu, Duncan et al. 1998; Duncan, Underhill et al. 1999). Both elevations (Cassidy, Bingham et al. 1995; Petrakis, Barnes et al. 1996) and reductions in E2 levels have been reported in premenopausal women (Lu, Anderson et al. 1996; Xu, Duncan et al. 1998; Duncan, Underhill et al. 1999). PEs may also affect the bioavailability of endogenous reproductive hormones by altering SHBG concentrations (Adlercreutz, Hockerstedt et al. 1987; Rosner 1991; Mousavi and Adlercreutz 1993; Loukovaara, Carson et al. 1995; Nagel, vom Saal et al. 1998), although this has been disputed. Administration of exogenous estrogens resulted in a dose-related increase in plasma SHBG with a maximum of about a four-fold increase from baseline (Mandel, Geola et al. 1982; Rosner 1991). Examining SHBG will allow us to test the degree to which the effects of PEs in the presence of endogenous estrogen are due to modulation of free E2 levels by effects on levels of SHBG (Rilling, Worthman et al. 1996). The use of DR data on sampling days, as well as FFQ data, in conjunction with PE analyses will enable us to distinguish between effects of PEs in soy as opposed to other components.

Method and Analysis: Estimates of PE exposure obtained from averages of FFQ, 24-hour DR, and weekly blood spots will be compared across all subjects using Pearson's correlation coefficients. The contribution of individual foods to blood spot PE concentration will be assessed using stepwise multiple regression. High/low levels of PEs and soy consumption will be based on above and below median level.

Variance in BS levels of FSH, and LH will be compared between low (L) and high (H) PE exposure groups women using an F-test. Variance in BS levels of FSH, and LH will be compared between L and H PE exposure groups and the three menopausal status

groups using 2-way ANOVA. Variance in BS levels of E2, FSH, and LH will be compared between pre- and perimenopausal women for the 4 time periods (FP, PMP, II, and TI) using F-tests. A sample size of 40/group gives a power of 90% to detect differences in variance observed between pre- and peri-menopausal women (Lenton, Sexton et al. 1988). SHBG means for each time period will be compared between L and H PE exposure groups women using 2-way ANOVA. SHBG means will be compared between L and H PE exposure groups and the three menopausal status groups using 2-way ANOVA. A sample size of 20-40/group gives a power of 80% or more to detect differences reported in the literature for Japanese postmenopausal women (Nagata, Kabuto et al. 1997).

Means of SHBG and measures of variance for FSH, and LH will be regressed against the three estimates of PE exposure using stepwise multiple regression within each of the three menopausal status groups and for all women.

Possible Results and Interpretation:

The buffering model of PEs acting at the level of the hypothalamus to diminish impact of varying E2 levels during menopause will be supported by the following:

- Lower variance in FSH and LH in women with higher PE exposure/soy consumption
- A negative correlation between variance in FSH and LH and PE intake

The above model will be rejected if the following is observed:

- No difference in variance in FSH and LH in women with higher PE exposure/soy consumption
- No correlation between variance in FSH and LH and PE intake

The hypothesis that PEs increase SHBG levels will be supported by the following:

- Higher mean SHBG levels in women with higher PE exposure/soy consumption
- Positive correlation between SHBG levels and PE exposure

The hypothesis that PEs increase SHBG levels will be rejected if the following is observed:

- No difference or lower mean SHBG levels in women with higher PE exposure
- No correlation or negative correlation between SHBG levels and PE exposure

If soy consumption (as measured from DR and FFQ) is correlated with lower variance in FSH and LH, and/or higher mean SHBG levels, but PE exposure (as measured in blood spots or estimated from surveys) is not, we will conclude that a non-PE component of soy (e.g., protein) may be responsible for the hormonal differences observed.

Table 4. Summary of hypotheses 1A & 1B and statistical tests.

A B *Hypothesis*	Dependent Variables	Test groups/ Independent Variables	Time frame	Statistical Test
1A	Hormonal Profile	Menopausal status		
Central Mediation	E2 (total & free) levels	Pre vs. Peri-menopausal women	FP, PMP, II, TI	2-way ANOVA T-test for means
	Variance in E2, FSH, LH	Pre vs. Peri-menopausal women	FP, PMP, II, TI	F-test for variance
		Pre vs. Peri-menopausal women (& control variables)		Multiple linear regression
		Pre vs. Peri-menopausal women	as above, lag +1	

			or +2 wk.	
1B	Hormonal Profile	Phytoestrogen level		
Inc. PE → Dec var in Gonadotropins	Variance in FSH & LH	H vs. L – all menopausal groups H vs. L – by menopausal status	II, TI II, TI	F-test for variance 2-way ANOVA
	SHBG levels	H vs. L – all menopausal groups H vs. L – by menopausal status	II, TI II, TI	2-way ANOVA T-test for means
	SHBG levels & Variance in FSH & LH	Continuum: 3 menopausal groups and all women (& control variables)	II, TI	Multiple linear regression

HYPOTHESIS 2A. **Women with higher variance in E2, FSH and LH will have more frequent or severe midlife symptoms**

- Symptom frequency and severity will be positively correlated with variance in BS E2, FSH and LH.
- Symptom frequency and severity will not be correlated with mean values of E2 (total or free).

Rationale: The menopausal transition appears to be characterized by central mediation/dysregulation in addition to declining ovarian function (Wise, Kashon et al. 1997; Prior 1998; Wise, Smith et al. 1999). The CM model attributes symptoms to central effects of fluctuating E2 and the rates of change, presumably mediated by receptor up- and down-regulation. These central effects are expected to correlate with gonadotropin levels (Ballinger, Browning et al. 1987; Sherwin 1994). Since hot flushes can occur when E2 levels are high (Bider, Ben-Rafael et al. 1989; Gangar, Cust et al. 1989), ovarian depletion is unlikely to be the only cause of VMS, necessitating the addition of other pathways to the model of the menopausal transition.

Method and Analysis: Several indices of menopausal symptom frequency and severity will be calculated according to previously reported scales (Kaufert, Gilbert et al. 1988; Lock 1993; Greene 1998) to allow us to compare our results to published literature reports. Women will be divided into 2 groups of L and H hormone levels/variance (using the median as the cutoff) to allow us to perform t-tests and 2-way ANOVA. Menopausal index score (and individual symptom (e.g., hot flushes, *katakori*) frequency and/or severity) will be regressed on variance in E2, FSH and LH for the two time periods (II and TI) using stepwise multiple regression controlling for variables such as PE exposure, menopausal status, region, and age (see Data Analysis section above). Similarly, menopausal index score (and individual symptom frequency and/or severity) will be regressed on mean E2 (total and free) for the two time periods (II and TI) using stepwise multiple regression. Logistic regression will be performed to obtain odds ratios for symptomatology. In a previous study we have used logistic regression with similar sample sizes (n=35) and observed significant relationships (Stallings, Worthman et al. 1996).

Possible Results and Interpretation

The CM model will be supported by the following:

- A positive correlation between menopausal symptom score (composite or individual symptom) and variance in E2, FSH, and/or LH

The PM model will be supported by the following:

- A negative correlation between menopausal symptom score (composite or individual symptom) and mean E2 levels

HYPOTHESIS 2B. **Women with higher PE exposure/intake will have less frequent or severe midlife symptoms**

- Menopausal symptom frequency/severity will be lower in women with high PE exposure/soy intake.
- Symptom frequency and severity will be negatively correlated with PE exposure/intake.

Rationale: Epidemiological research has suggested that women consuming greater amounts of soy, or having higher levels of urinary PEs, have fewer hot flushes (Nagata, Shimizu et al. 1999). Experimental studies in which women ingest soy supplements over a short period, have also supported a beneficial role for some component of soy in reducing hot flushes (Murkies, Lombard et al. 1995). But other studies have not been able to show a difference from the normal decline in symptoms over time (Murkies, Lombard et al. 1995; Brzezinski and Debi 1999).

Method and Analysis: As noted for Hypothesis 2A, several indices of menopausal symptom frequency and severity will be calculated to allow comparison of our findings with previous reports. Comparisons for menopausal symptom frequency and severity will be made for L and H PE groups (determined using 3 methods and divided into high/low groups based on above and below median level) using t-tests. Menopausal index score (and individual symptom (e.g., hot flushes, *katakori*) frequency and/or severity) will be regressed on PE exposure/soy consumption (using blood spot measures, 24-hour DR and FFQ) and means for two time periods (II and TI) using stepwise multiple regression controlling for variables such as PE exposure, menopausal status, region, and age (see Analysis section above). Logistic regression will be performed to obtain odds ratios for symptomatology.

The power to detect a 0.5 correlation is 98% with samples of 60, and greater than 99% with a sample of 120. A previous study (Nagata, Shimizu et al. 1999) found a significant correlation between hot flushes and soy consumption using a food frequency questionnaire. By comparing measures and indices of midlife symptoms to PE concentration in blood, we expect to improve the power of the study to support the hypothesis compared to many studies (presumably due to cost and methodological limitations) that rely on soy consumption alone, which may be limited by inter-individual variation in PE absorption and metabolism.

Possible Results and Interpretation

- Negative correlation of PE exposure/ intake to menopausal symptom indices or measures of severity/frequency will support a model of PEs acting to decrease symptoms of perimenopause.
- If dietary intake of soy (as assessed by FFQ and DR) is negatively correlated with menopausal symptoms, but PE blood spot levels are not, this will support the interpretation that a non-PE soy component(s) is responsible for lower menopausal symptoms.

Table 5. Summary of hypotheses 2A & 2B and statistical tests.

C D *Hypothesis*	Dependent Variables	Test groups/ Independent Variables	Time frame	Statistical Test
2A	Menopausal symptoms	**Hormonal Profile**		
Dec. gonadotropin	Frequency and/or	L vs. H groups	II, TI	2-way ANOVA

variance → Dec. menopausal symptoms	severity of symptoms (composite indices & individual symptoms)	of: Variance in E2, FSH, & LH		T-test for means
	Frequency and/or severity of symptoms (composite indices & individual symptoms)	L vs. H groups of: Mean E2 (total & free) levels 3 menopausal groups & all women	II, TI	2-way ANOVA T-test for means
	Frequency and/or severity of symptoms (composite indices & individual symptoms)	Variance in E2, FSH, & LH Mean E2 (total & free) levels (control variables)	II, TI	Multiple linear regression & logistic regression
2B	Menopausal symptoms	Phytoestrogen level		
Inc PE → Dec menopausal symptoms	Frequency and/or severity of symptoms	H vs. L – all menopausal groups H vs. L – by menopausal status	II, TI II, TI	T-test for means 2-way ANOVA
		Continuum 3 menopausal groups & all women (& control variables)	II, TI	Multiple linear regression & logistic regression

SIGNIFICANCE OF THIS RESEARCH

Greater understanding of effects of aging and diet on the biology and experience of menopause: In order to improve women's health during and after menopause, a thorough understanding of the biological and social factors influencing endocrinology and symptomatology is required. The Japanese and their unique diet may provide clues to health and longevity for women in industrialized nations with reproductive histories and lifestyles that depart greatly from those of our evolutionary heritage. As the Japanese diet becomes more westernized (including more fat, meat and dairy, and less soy) (Japanese Ministry of Health 1996), rates of cancer and heart disease, and possibly menopausal symptoms, are increasing (Parkin 1989; Wakai, Suzuki et al. 1995; Nagata, Kawakami et al. 1997). It is important to study the effects of diet on aspects of health while enough variation exists, and so that public health policies and recommendations can be made before health is compromised.

Focus on diet and exogenous hormones (phytoestrogens): Specific dietary compounds such as phytoestrogens are often overlooked in studies of menopause and reproductive ecology in general because of the technical and financial challenges. Nutrition, caloric intake, and energy expenditure have been widely studied and have been found to affect levels and patterns of reproductive hormones, particularly in non-Western populations (Ellison, Panter-Brick et al. 1993; Campbell and Wood 1994; Wood 1994; Ellison 1995). However, the subtler complicated actions of ingested chemicals have been ignored. In the West (and increasingly in Japan as well) concern about the effects of specific chemical constituents of our environment, particularly pesticides and other pollutants, has increased (Daston, Gooch et al.

1997). Japan, with its high soy consumption patterns (Adlercreutz, Honjo et al. 1991; Adlercreutz, Markkanen et al. 1993), provides a natural experiment by which we can begin to explore the effects of exogenous estrogenic compounds on endogenous hormones as well as symptomatology. Our laboratories possess great expertise and are uniquely poised to carry out such a study at minimal cost, both financially and to the participants.

Innovative field methods: Finger stick blood spot methods are minimally invasive, amenable to field collection by participants, and have proven useful for cross-cultural studies of reproductive ecology and human development (Worthman and Stallings 1997). Blood spot phytoestrogen fluoroimmunoassay measures overcome the considerable financial and methodological limitations of collection of plasma by allowing repeated measurement of phytoestrogens in a non-clinical setting, as well as avoiding the costly measurement by gas chromatography mass spectrometry. Furthermore, blood spot hormonal measures surmount the limitations of assignment of menopausal status based on bleeding patterns alone. Hormone levels will be used as more accurate biomarkers of endocrine dynamics and menopausal status than bleeding patterns. These methods will allow more frequent sampling and a more comprehensive biological characterization of the variation inherent in the menopausal transition.

Explicit biocultural approach: The cultural organization of the life course profoundly affects the meanings and experience of menopause (Gannon 1985; Beyene 1989; Du Toit 1990; Lock 1993). Cultural factors hypothesized to play a role in the experience of menopause (and quality of life during this period) include: cultural attitudes towards the menopause (Martin 1988; Avis and McKinlay 1991), meanings assigned to menopause (e.g., natural and normal, deviant, or illness) (Estok and O'Toole 1991), attitudes toward childrearing and women's roles (Sanchez Perruca, Civeira Murillo et al. 1989), attitudes toward symptoms of menopause (Robinson 1996), social support and extended family (Rousseau and McCool 1997), social status, socioeconomic status, education level, career, religious beliefs and relationships with husbands/partners (Lock 1993; Suganuma 1998).

While cultural influences on menopause have been investigated, biological factors have been relatively ignored. Diet is one example of a cultural factor with the potential to profoundly influence the biology and experience of menopause. The choice of what to eat is profoundly cultural, and in the case of phytoestrogens, may affect the biological activity of endogenous hormones. However, a focus on diet alone would be just as limited as previous exclusively cultural approaches. Thus, this study will use both biological data and cultural data to explain the variation in menopausal physiology as well as symptomatology.

Practical use of hormone replacement therapy for women's health and longevity:
Concern over the negative consequences of decreased estrogen levels has led to the characterization of menopause as a deficiency disease, and to widespread recommendations for hormone replacement therapy (HRT). While HRT may be indicated for some women, not all women are candidates for such treatment. An understanding of the effects of phytoestrogens on menopausal symptoms and health will also enable creative prescriptions combining lifestyle changes (e.g., exercise, soy consumption) with use of HRT to minimize side effects and maximize health.

RESEARCH PROGRESS REPORT
as of 25 January 2002

As of January 25, 2002, 35 women are enrolled in the study and filling out the daily checklist, collecting weekly finger-prick blood spot samples, and doing weekly 24-hour dietary recall. Before beginning research every woman is screened to determine if she is eligible. If she meets the participation criteria, the researcher (Melby, usually accompanied by a research assistant) meets with her for approximately 2 hours to carry out the initial interview and orientation. At this meeting, which generally takes place at the participant's house, a public health center, or Kyoto Furitsu Ika Daigaku, detailed explanations of the methods are

given, the finger-prick blood spot method is demonstrated and then practiced, as are the daily checklist and dietary recall. Height, weight, triceps skinfold, elbow breadth, and upper arm circumference are also measured.

Based on answers to questions about recent menstrual history the current sample population is comprised of 11 premenopausal, 17 perimenopausal, and 7 post-menopausal women. A total of 48 women have been screened (12 premenopausal, 21 perimenopausal, and 15 postmenopausal). Of these 48 women, 2 declined to participate for unspecified reasons, 2 declined participation due to family illness, 1 was rejected because she was taking HRT, 3 were rejected because they had had a hysterectomy, and 1 was rejected because she lived in a neighboring prefecture. At the time of writing this report, 4 women are still considering participation.

The initial questionnaire covers the following topics: demographics, health history, reproductive history, FFQ, and general well-being and recent life events. Data from these questionnaires is currently being entered, and preliminary analysis will begin once all data is received.

Initial ethnographic interviews on daily life (done after 1 month of data is received – thereby enabling the researcher and participant to review methods, problems, and clarify items on the daily checklist and dietary recall) have been carried out with 8 women.

PROJECT SCHEDULE

The research schedule for this project is outlined in Table 6 and entails approximately 2 years of data collection in Kyoto and Fukushima prefectures in Japan. Phase II (Fukushima) projected start dates Laboratory analysis will begin at Emory University in Atlanta in August of 2002, and continue through to the end of 2003. Data entry and analysis will be ongoing and preparation of manuscripts for publication will primarily be done in 2004.

Table 6. Project schedule.

Phase I	8/1/01-11/ 30/01	Kyoto	Train research assistants; translate all questionnaires, consent forms, etc. Identify recruitment options
	12/1/01-2/28/02	Kyoto	Initial interviews, orientation, enroll 60+ women
	12/1/01-8/31/02		Data collection; in-depth interviews; data entry and preliminary analysis
Phase II	6-9/02-9-12/02	Fukushima	Initial interviews, orientation, enroll 60+ women
	6-9/02-2-5/03		Data collection; in-depth interviews; data entry and preliminary analysis
Phase III	8/1/02-7/31/03	Atlanta	Laboratory analysis (done by 1-2 technicians) – begin with E2, FSH, LH, SHBG
	8/1/03-7/31/04		Laboratory analysis of remaining hormones and daidzein and genistein (6months) (done by Melby and technicians); survey and ethnographic analysis, publication of results (6 months)

REFERENCES

Adlercreutz, H. (1990). "Western diet and Western diseases: some hormonal and biochemical mechanisms and associations." Scandinavian Journal of Clinical & Laboratory Investigation - Supplement 201: 3-23.