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Fetal Testosterone: Developmental Effects on Externalizing Behavior

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Fetal Testosterone: Developmental Effects on Externalizing Behavior

by

Troy A. Webber

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Abstract

Fetal testosterone (FT) exposure influences sexual differentiation and may promote well-established sex differences in externalizing (EXT) behavior. Although puberty may be a critical period for these effects, it is unknown how FT exposure influences EXT as a function of pubertal development. We used a longitudinal, multi-sample design to test the relationships between two proxy indices of FT exposure and EXT as a function of age and pubertal development (approximately ages 6, 9, 11, 14, and 16). Twin data were used to approximate FT exposure (TT-FT) because testosterone is thought to cross the intrauterine membrane and cause variability in co-twin gonadal hormone exposure, with increasing exposure for males and participants with male co-twins. Increasing number of older siblings may also approximate increasing FT exposure (SI-FT), although existing research has yet to disentangle possible postnatal socialization effects from potential FT exposure using this variable. Given that biologically related siblings share a fetal and social environment while non-biologically related siblings simply share a social environment, we tested the independent effect of SI-FT on EXT using a sibling adoption design. Across four independent samples, SI-FT and TT-FT predicted externalizing for males alone. SI-FT predicted EXT over-and-above socialization influences and interacted with pubertal development in two independent samples, with elevated EXT for those in mid-late puberty that were exposed to increased FT. TT-FT predicted EXT differentially as a function of developmental period. Our data are consistent with the notion that exposure to FT promotes sexually differentiated, sexually selected behavior during reproductively relevant periods.
Introduction

The broader construct of externalizing (EXT) is thought to represent individual differences in the tendency to express rather than inhibit impulses (James, 1890/1983). EXT is a multifaceted construct that includes psychological disorders, behavior, and personality traits, such as, attention-deficit/hyperactivity disorder (ADHD), conduct disorder (CD), oppositional defiant disorder (ODD), substance use disorders (SUDs), antisocial personality disorder (ASPD), gambling, sexual risk taking, and sensation seeking (Krueger, 1999; Krueger et al., 2002; Krueger, Markon, Patrick, Benning, & Kramer, 2007). There are well-established gender differences, or sexual dimorphism, in rates of childhood and adult EXT, with a male-female ratio of approximately 3:1 (Beauchaine, Klein, Crowell, Derbidge, & Gatzke-Kopp, 2009; Copeland, Shanahan, Costello, & Angold, 2011; Egger & Angold, 2006; Kessler et al., 1994; Merikangas et al., 2010; Newman et al., 1996; Volkmar, Lord, Bailey, Schultz, & Klin, 2004). While sexual dimorphism in EXT is consistently observed and generally accepted, there is a limited understanding of the basis for these gender differences.

Genetic Contributions to Sexual Dimorphism in EXT

Evolutionary theory, and in particular the theory of sexual selection, has been proposed as a theoretical framework that may partially explain sexual dimorphism in EXT (Martel, 2013). EXT and EXT markers may be genetically programmed, or sexually selected, to enhance reproductive fitness (i.e., likelihood that an organism’s genes will be present in subsequent generations) for a particular sex (Alcock & Crawford, 2008; Andersson, 1994; Darwin, 1957; Pomiankowski & Moller, 1995; Rowe & Houle, 1996). Particularly, EXT (e.g., sensation...
seeking, aggression) may promote intersexual competition in males by enhancing access to mates and control of the resources necessary for reproduction. J. Williams and Taylor (2006) examined sexually selected EXT markers by simulating unpredictable behavior (i.e., risk-taking) in a small number of individuals embedded in a group and found that risk-taking by a minority optimized both individual and group functioning. Robust heritability estimates also support the notion that EXT markers are sexually selected, especially considering that heritability for EXT is greater in males than females and increases throughout development in a similar, sex-typed manner (Blonigen, Hicks, Krueger, Patrick, & Iacono, 2005; Caldwell & Gottesman, 1991; Eley, Lichtenstein, & Stevenson, 1999; Frisell, Pawitan, Långström, & Lichtenstein, 2012; Hicks et al., 2007; Hicks, Krueger, Iacono, McGue, & Patrick, 2004; Kendler, Heath, Neale, Kessler, & Eaves, 1992; Prescott & Kendler, 1999; Rhee & Waldman, 2002). These data and others provide evidence that EXT may be a sexually selected trait.

While potentially a sexually selected phenotype, behavioral genetic methods have observed genetic and environmental effects on EXT markers in both sexes (Hicks et al., 2007; Hicks et al., 2004; Krueger et al., 2002). These environmental influences may muddle the effect of genetics, or sexual selection pressures, on sexual dimorphism in EXT. Indeed, evolutionary theory suggests that heritability is vulnerable to interactive effects with the environment and does not strictly control sexually selected traits or behavior (Ellis & Bjorklund, 2005). Environmental effects may predict additional variance in EXT markers and partially explain why EXT is observed in both males and females. In turn, there may be a confluence of biologically relevant environmental exposure and evolutionarily relevant genetic variability that promotes EXT.

**Fetal Testosterone as an Environmental Marker for EXT**
One biologically relevant, putative environmental influence that may exert effects on the expression of sexually selected markers is exposure to gonadal hormones, especially considering their influence on sexually differentiated behavior (Becker et al., 2005; Collaer & Hines, 1995). Differential exposure to gonadal hormones begins as early as the prenatal period when genetic sex is determined and the sex-determining genetic region of the Y chromosome (Sry) causes the gonads to develop into testes (Breedlove & Hampson, 2002). The testes subsequently release testosterone and other androgenic steroid hormones that masculinize the developing body and brain (Martel, Klump, Nigg, Breedlove, & Sisk, 2009). These differences extend into childhood and adult life when male exposure to testosterone is up to 10 times greater than females (Taieb et al., 2003). Further, there is evidence that hormones instantiate environmental influences on an organism and provide data regarding the environmental context to the somatic and mental state of the individual (Plant, 2001; Terasawa & Fernandez, 2001). As such, exposure to gonadal hormones may be a biologically relevant environmental influence that contributes to sexual dimorphism in EXT.

**Fetal Testosterone and Organizational-hormonal Effects.** The organizational-activational hypothesis of hormonal effects suggests that exposure to gonadal hormones, such as testosterone, exert prominent effects on human development at distinct points of development (Phoenix, Goy, Gerall, & Young, 1959). While organizational effects result in relatively permanent effects on neural and physical development during the prenatal and early postnatal periods, activational effects “switch on” the previously organized physical and neural structures during key developmental periods, such as puberty (Breedlove & Hampson, 2002; Phoenix et al., 1959; Romeo, 2003; Schulz, Molenda-Figueira, & Sisk, 2009; Sisk & Foster, 2004; Sisk & Zehr, 2005). Although originally hypothesized to occur strictly during the prenatal and early postnatal
periods, evidence suggests that organizational effects also occur during critical developmental periods (Schulz et al., 2009).

Exposure to fetal testosterone (FT) exerts organizational effects that masculinize the brain, physiology, and behavior (Breedlove, 1994). The behavior of female rodents exposed to elevated levels of FT show more masculinized patterns, such as delayed mating and impregnation, co-female mounting, and increased aggression (Ryan & Vandenberghe, 2002). Similarly, human females exposed to higher levels of FT have more masculine cerebral lateralization (Cohen-Bendahan, Buitelaar, van Goozen, & Cohen-Kettenis, 2004), otoacoustic emissions (McFadden, 1993), and spatial ability (ColeHarding, Morstad, & Wilson, 1988) than females exposed to lower levels of FT. Evidence from research on females with congenital adrenal hyperplasia, a disease involving increased exposure to adrenal androgens like testosterone, suggest that these effects are most likely due to androgen exposure and not other female-biased characteristics (Berenbaum & Resnick, 1997; Brown, Hines, Fane, & Breedlove, 2002). Exposure to FT has also been linked with markers of childhood and adult EXT in humans, such as more frequent rough-and-tumble play (Auyeung et al., 2009), increased aggression (Bailey & Hurd, 2005), and elevations in what are typically considered dispositional traits like sensation seeking (Fink, Neave, Laughton, & Manning, 2006). Additional evidence suggests that FT promotes other reproductively relevant qualities in males, such as athletic ability (J. T. Manning & Taylor, 2001), sprinting speed (J. Manning & Hill, 2009), body size (Fink, Neave, & Manning, 2003), and number of lifetime and past year sexual partners (Hönekopp, Voracek, & Manning, 2006). Paired with evidence that exposure to FT instantiates environmental conditions, these data suggest that FT exposure may enhance reproductive fitness and provide an adjunct influence to sexually selected genetic influences (Martel, 2013).
Organizational-hormonal Effects on EXT: Fetal Programming. One pathway by which exposure to FT may influence EXT and other reproductively relevant characteristics is via fetal programming, or predictive adaptive response (Ehrhardt, 1985). During fetal programming, the developing fetus receives information about the intrauterine and broader ecological context within which it is being raised and adapts via long lasting changes in structure and function (Glover, 2011; Gluckman, Hanson, & Beedle, 2007; Gluckman, Hanson, & Spencer, 2005). For instance, maternal exposure to harsh or stressful environmental conditions may result in increased levels of circulating maternal hormones (e.g., testosterone, cortisol), particularly considering that male levels of testosterone decrease (Christiansen, 1998; Kreuz, Rose, & Jennings, 1972; Opstad, 1992) while female levels increase (Gray, 1992) under conditions of chronic stress. Maternal hormones are then transferred to the fetus via the placenta and umbilical cord, resulting in elevated intrauterine hormone concentrations (R Gitau, Adams, Fisk, & Glover, 2005; Rachel Gitau, Cameron, Fisk, & Glover, 1998; Sarkar, Bergman, O’Connor, & Glover, 2008). This may provide a signal to the developing fetus that the environment is limited in sexual or energetic resources, or is more broadly stressful (J. T. Manning, Scutt, Wilson, & Lewis-Jones, 1998). The fetus may then use this information to flexibly adjust to the context within which it is developing by promoting characteristics (e.g., EXT) that enhance inclusive and reproductive fitness in harsh or stressful environments via increased production of hormones from the fetus’ own adrenal gland or gonads.

There is well-established support for fetal programming from rodent models. Data suggest that the effect of prenatal stress on behavioral phenotypes is at least partly mediated by the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes,

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1 This occurs because maternal fat cells produce less aromatase – an enzyme responsible for converting androgens into estrogens – under conditions of chronic stress (G. E. Ackerman, Smith, Mendelson, MacDonald, & Simpson, 1981; Naftolin, Ryan, & Petro, 1971, 1972; Thompson & Siiteri, 1974; Weisz, Brown, & Ward, 1982).
which are involved in expression of androgens and glucocorticoids (Herbert et al., 2006; Seckl & Holmes, 2007; Weinstock, 2001). One of the most important and commonly studied stress-related glucocorticoids is cortisol (Burke, Davis, Otte, & Mohr, 2005). While activity and expression of the placental enzyme 11beta-hydroxysteroid dehydrogenase 2 (11b-HSD2) metabolizes maternal cortisol levels and acts as a functional barrier to inter-uterine glucocorticoid transfer, there is evidence for transplacental passage of cortisol between mother and fetus (Osinski, 1960). For instance, although maternal levels of glucocorticoids are up to 10 times higher than fetal levels, there is a significant correlation between naturally occurring maternal glucocorticoids, as well as exogenously administered maternal glucocorticoids, and fetal glucocorticoid levels (Rachel Gitau, Fisk, Teixeira, Cameron, & Glover, 2001; Matthews, 2000; Osinski, 1960; Schneider, Roughton, Koehler, & Lubach, 1999; Weinstock, Poltyrev, Schorer-Apelbaum, Men, & McCarty, 1998). This transfer is increased in response to stress due to down-regulation of the placental enzyme 11b-HSD2 (Mairesse et al., 2007; Welberg, Thrivikraman, & Plotsky, 2005). This transfer may then signal subsequent, simultaneous modulation of cortisol and testosterone release from the HPA and HPG axes of the fetus, which contributes to the development of physiological and behavioral phenotypes (Handa, Burgess, Kerr, & O'Keefe, 1994; Lemaire, Taylor, & Mormède, 1997; Liggins, 1994; Lindsay, Lindsay, Edwards, & Seckl, 1996; Schapiro, 1968; Viau, 2002). Further evidence from non-human primate models also support the mediating role of these axes in fetal programming (Schneider, Moore, Kraemer, Roberts, & DeJesus, 2002).

In humans, the mechanisms underlying fetal programming are less well understood, although human research also supports the influence of HPA and HPG axis functioning. Recent research has observed positive correlations between maternal plasma and amniotic fluid (fetal)
cortisol levels (Sarkar, Bergman, Fisk, O’Connor, & Glover, 2007b) and shown that the correlation is larger as a function of increased maternal stress (Glover, Bergman, Sarkar, & O’Connor, 2009; Sarkar et al., 2008). Other androgenic hormones may also be involved in fetal programming as evidenced by significant covariance between amniotic fluid testosterone levels and amniotic fluid cortisol levels (R Gitau et al., 2005; Sarkar, Bergman, Fisk, O’Connor, & Glover, 2007a; Sarkar et al., 2008). There are multiple pathways by which maternal hormones may be transferred to the developing fetus: 1) both cortisol and testosterone are liposoluble and may cross the fetal membrane through the placenta (Rachel Gitau et al., 1998; Rachel Gitau et al., 2001), 2) cortisol may cross the fetal membrane and influence both cortisol and testosterone production in the adrenal gland or gonads of the fetus (Fujieda, Faiman, Feyes, & Winter, 1982), or 3) some combination of these and other processes. It is likely that both cortisol and testosterone are transferred to the developing fetus considering that, in human females, cortisol and testosterone are simultaneously produced by the adrenal cortex in response to stress (Mazur, Susman, & Edelbrock, 1997; Powell et al., 2002). This is partly due to functional cross-talk between the androgenic and glucocorticoid systems, which are generally produced in the HPA and HPG axes (Handa et al., 1994; Lemaire et al., 1997; Viau, 2002). While data available from human models is more limited than animal models, extant literature suggests that maternal stress and subsequent fetal programming operates via multiple pathways (see Figure 1 for an illustration).

FT and Activational-hormonal Effects. While exposure to FT and sexually selected, genetic influences may interact to promote reproductive fitness via EXT, it is important to consider developmental differences in the influence of FT exposure on EXT – especially considering that EXT shows developmental variations in risk. In particular, there are steep
increases in a variety of psychopathology and problem behavior at puberty (Cicchetti & Rogosch, 2002; Galambos, Barker, & Almeida, 2003; Leadbeater, Kuperminc, Blatt, & Hertzog, 1999; Pellegrini & Long, 2002; Reardon, Leen-Feldner, & Hayward, 2009). This maps on quite well with a critical time for organizational and activational hormonal effects and increased relevance of sexually selected traits. Indeed, male testosterone levels increase 18-fold during puberty and may further organize neural structures or physiological functions, or activate pre-existing physical and neural structures that developed as a result of organizational hormonal effects (e.g., exposure to FT; Susman, Nottelmann, Inoff-Germain, Dorn, & Chrousos, 1987). In other words, exposure to FT may sensitize the brain to changes in gonadal hormones, particularly during periods like puberty in which there are large increases in circulating gonadal hormones.

Organizational- and activational-hormonal effects during puberty have been found for many physical and psychological phenotypes. For instance, increased FT exposure causes variability in responsivity to ovarian hormones release during puberty, which subsequently influences post-pubertal food intake (Bell & Zucker, 1971; Gentry & Wade, 1976; Wade, 1972). Other evidence suggests that increased circulating testosterone during puberty may interact with organizational effects of exposure to FT, such as increased androgen receptor expression at certain skeletal cites, to produce more masculinized facial morphology (e.g., cheekbones, mandibles and chin, forward growth of the bones of the eyebrow ridges; Fink & Penton-Voak, 2002; Grammer, Fink, Møller, & Thornhill, 2003; Kasperk, Wakley, Hierl, & Ziegler, 1997; Thornhill & Gangestad, 1999). Similarly, organizational- and activational-hormonal effects during puberty may act on existing neural structures, such as those in the frontal cortex and subcortical monoaminergic system, that contribute to EXT (Chambers & Potenza, 2003). These
effects may then have downstream, long-term effects on EXT and other sex-typed traits (Romeo, 2003; Schulz et al., 2009; Sisk & Foster, 2004; Sisk & Zehr, 2005).

While there may be organizational- and activational-hormonal effects of testosterone on sex-typed behavior that come “online” during developmentally sensitive windows, there is yet to be a study that simultaneously investigates the effect of exposure to FT on EXT as a function of age and pubertal development. Further, although theory suggests that increased exposure to FT may promote intersexual competition in males (but not females) as a function of elevated EXT, little is known about the effect of exposure to FT on EXT in females. Last, no study has ruled out socialization effects in the relationship between FT and EXT, a potentially important confound that requires attention.

*Multiple Indices of FT Exposure: Pathways.* While exposure to FT can be manipulated in animal models, ethical and practical considerations limit our ability to experimentally control or obtain more direct measures (e.g., amniotic fluid, fetal plasma, and placental testosterone) of FT exposure in humans (R Gitau et al., 2005; Sarkar et al., 2007a; Sarkar et al., 2008). In response, researchers have developed several proxy indices of human FT exposure (Berenbaum, Bryk, Nowak, Quigley, & Moffat, 2009; Brown et al., 2002; Tapp, Maybery, & Whitehouse, 2011; T. J. Williams et al., 2000). Notably, these different indices represent multiple pathways to FT exposure. The most extensively researched and commonly used proxy measure is the second to fourth digit ratio (2D:4D, index finger to ring finger; J. T. Manning et al., 1998). People exposed to higher levels of FT exhibit a lower 2D:4D ratio (i.e., shorter index finger compared with ring finger). The relationship between this index and exposure to FT is thought to occur via fetal programming as well as the combined influence of *Hox* genes on the development of the testes.
and digits (George, Griffin, Leshin, & Wilson, 2013; J. T. Manning et al., 2000; J. T. Manning et al., 1998).

Twin designs, particularly with data on both same-sex (SS) and opposite-sex (OS) twins, have also been proposed as a useful paradigm for testing the effect of exposure to FT on psychological constructs. Like intrauterine position effects observed in rodents, co-twin sex is thought to cause variation in exposure to FT (Miller, 1994). A wealth of research (for a review see Tapp et al., 2011) suggests that a transfer of testosterone occurs across fetal membranes. Samples with both SS and OS twins provide an ordinal index of exposure to FT, with increasing levels of exposure to FT in the following order: SS females, OS females, OS males, SS males. As such, twin samples are particularly useful for studying the effects of FT across gender.

Another proxy index of exposure to FT that has received attention uses birth order of infants (i.e., number of older siblings) to index variability in fetal hormone exposure (Maccoby, Doering, Jacklin, & Kraemer, 1979; T. J. Williams et al., 2000). Two theoretical frameworks may explain how this variable approximates exposure to FT, although a theory developed by V. J. Grant (2007) provides a more empirically supported and parsimonious perspective. This theory suggests that environmental stress leads to increased maternal circulating testosterone levels, which is stored in the female’s follicular fluid – the fluid surrounding the developing ovum as it matures during each menstrual cycle – prior to conception (V. J. Grant & Irwin, 2005; Greenspan, Gardner, & Shoback, 1997; Henderson, McNeilly, & Swanston, 1982; Meinecke, Gips, & Meinecke-Tillmann, 1987). Storage of testosterone in the follicular fluid influences sexual differentiation (male-biased) to enhance the inclusive and reproductive fitness of the offspring in a harsh environment (V. J. Grant, Irwin, Standley, Shelling, & Chamley, 2008). This storage modifies or adapts the ovum during each menstrual cycle to increase the likelihood that it
receives an X- or Y-chromosome-bearing spermatozoon (sperm cell) and may have residual effects on the amount of FT in the post-conception intrauterine environment (Saling, 1991). In such a way, maternal stress may contribute to increased exposure to FT via 1) stored testosterone in the follicular fluid that influences the possibility that offspring will be male, and 2) stored testosterone in the follicular fluid that evokes a release of maternal and fetal hormones (e.g., testosterone), regardless of offspring sex (see Appendix A for a more detailed discussion). These two pathways (via maternal stress – indexed by number of older siblings), transfer across the fetal membrane (twin-type proxy index), and other influences on FT are detailed in Figure 1.2

**Current Study**

The current study utilized a developmentally sensitive design to answer six questions: 1. What effect does exposure to FT have on EXT?, 2. Is the effect of FT exposure on EXT relevant for males and females?, 3. Is this effect observed across multiple indices of exposure to FT?, 4. Is the relationship between exposure to FT and EXT moderated as a function of pubertal status?, 5. Does exposure to FT predict EXT over-and-above socialization effects?, and 6. Is the effect of EXT on FT consistently observed across multiple developmental periods?

Three twin samples and a sibling adoption sample were used to answer questions 1-6. Sample 1 answered question 1 and 2 during childhood and pre/early adolescence using a dummy coding technique that examined levels of EXT for male and female twins reared in utero with either SS or OS twins. Sample 1 also answered question 3 using this index and a sibling proxy index of exposure to FT (with tests of gender invariance). Sample 2 answered questions 1, 2, and 4 during early and middle adolescence using the sibling proxy index of exposure to FT, data on

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2 Notably, the twin-type and sibling proxy indices of exposure to FT represent two different pathways of increased exposure to FT. We recognize that the release of FT may also be driven by other factors, such as maternal reactivity (e.g., personality characteristics) to stress or genetic influences. While testing these possibilities may have important implications on how exposure to FT is implicated in the maternal stress model of EXT, an important first step is identifying the broader influence of exposure to FT on EXT throughout development.
pubertal development, and tests of gender invariance. Sample 3 answered questions 1, 2, and 3 during late adolescence using the dummy coding technique for the twin-type proxy index.

Question 5 was addressed using a family-based adoption design with data on number of older siblings. Using a sample of participants reared with either their adoptive or biological families, the current study predicted EXT from number of older siblings as a function of adoption status. In biological siblings, the effect of number of older siblings on EXT is a product of both fetal (i.e., FT exposure) and social (i.e., socialization effects) environment, whereas in adoptive siblings the number of older siblings-EXT association is a function of socialization alone because they did not share a fetal environment with siblings. In such a way, we examined if exposure to FT predicts EXT over-and-above socialization effects. Last, samples 1-4 were specifically selected because they measured EXT at different developmental periods. Question 6 was answered by collectively examining the pattern of effects of exposure to FT on EXT across the four samples.

We expected to observe significant main effects of exposure to FT on EXT, such that greater exposure to FT would confer enhanced EXT throughout development. Moreover, we expected these effects to be enhanced following puberty, but only for males, given well-established gender differences in circulating testosterone during and after puberty (i.e., potential organizational- and activational-hormonal effects) and the sex-specific relevance of EXT. Last, we predicted that exposure to FT would predict EXT over-and-above socialization effects.
Methods

Participants

Sample 1: Michigan State Twin Registry (MSUTR Sample). To assess the effects of FT on EXT throughout development several samples were selected that measured EXT markers at distinct points in development. Table 1 shows approximate ages at which each measurement occasion was conducted across the four samples. Sample 1 utilized a twin-type and sibling proxy index of exposure to FT to test two pathways of exposure to FT on EXT during childhood and pre/early adolescence. Sample 1 included 160 MZ and 561 DZ (231 SS-DZ, 330 OS-DZ twin pairs) twin pairs from the Michigan Twins Project (MTP) within the Michigan State University Twin Registry (Burt & Klump, 2013; Klump & Burt, 2006). Participants were recruited for the MTP via publicly available birth records in collaboration with the Michigan Department of Community Health and the Michigan Bureau of Integration, Information, and Planning Services. Participants were first assessed at mean age 6.42 (SD = 0.93) and again at mean age 9.24 (SD = 1.05). Wave 2 data were available for 98% of participants that completed wave 1 of data collection. Racial/ethnic composition of the sample was consistent with recruiting region: White (86.3%), Black/African American (4.5%), Hispanic (2.1%), Asian (0.6%), American Indian/Alaska Native (< 1.0%), Multiracial (4.5%), Other (1.3%), and Not Reported (< 1.0%).

Sample 2: Minnesota Twin and Family Study (MTFS Sample). A second sample was used to test the moderating role of pubertal development on the relationship between exposure to FT on EXT in early and middle adolescence (11 and 14 years old, respectively) using the sibling proxy index of exposure to FT. Sample 2 consisted of SS twins participating in the ongoing,
longitudinal Minnesota Twin and Family Study (Iacono, Carlson, Taylor, Elkins, & McGue, 1999). Participants were recruited via birth records and public databases in the state of Minnesota between 1972 and 1984. The MTFS sample included a cohort of 756 same-sex twin pairs with data collected at ages 11, 14, 17, 20, 21, and 24. This study utilized a developmentally sensitive measurement design, such that measures were specifically selected to be developmentally relevant at each measurement occasion. In turn, only the assessments conducted at mean ages 11 and 14 included measures of EXT markers that overlapped with the measures of EXT markers in samples 1, 3, and 4 (i.e., symptoms of CD, ADHD, ODD, and substance use). Only data collected at ages 11 and 14 were analyzed to optimize consistency in measurement of EXT markers across the 4 samples. An enrichment sample of high-risk SS twin pairs was also collected and used for the current analyses. The final sample included 2515 twins with available data for analysis (Keyes et al., 2009). Follow-up rates were high, with a retention rate of approximately 96% (Elkins, McGue, & Iacono, 2007; Hicks, Durbin, Blonigen, Iacono, & McGue, 2012; Klahr, Rueter, McGue, Iacono, & Burt, 2011). Ethnic/racial composition of the sample reflected that of the recruitment area for the birth years sampled, with over 95% of the twins reportedly Caucasian. In total, 66.2% of the twin pairs analyzed were MZ (49.9% female) and 33.8% were DZ (52.6% female).

Sample 3: Colorado Community Twin Study (CTS Sample). Sample 3, a population-based sample of 2365 twins from the Colorado Twin Registry (Rhea, Gross, Haberstick, & Corley, 2006), was utilized to test the effect of exposure to FT on EXT and to determine whether this effect is relevant for males and females. Recruitment involved contact via the Department of

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3 The effect of exposure to FT on EXT was also tested during later developmental periods (17, 20, 21, and 24) using the 11-year-old cohort and another 17-year-old cohort. These analyses utilized measures of EXT markers that were developmentally relevant, such that participants reported on antisocial personality disorder symptoms and substance use, as opposed to symptoms of CD, ADHD, and ODD. Results are presented in Appendix C.
Health and through 170 of the 176 school districts in Colorado. Participants included 464 monzygotic (MZ) twin pairs and 538 dizygotic (DZ) twin pairs (263 SS-DZ twin pairs, 275 OS-DZ twin pairs) with a mean age of 15.94 (SD = 1.73). Racial/ethnic composition of the sample was consistent with recruiting region: White (83.76%), Black/African American (2.16%), Asian (1.44%), American Indian/Alaska Native (1.0%), Hawaiian/Pacific Islander (< 1.0%), biracial (9.09%), and Unknown/Not reported (2.53).

**Sample 4: Sibling Interaction and Behavior Study (SIBS Sample).** It is also important to rule out potential confounds of the relationship between FT and EXT. While samples 1-3 provided convenient proxy measures of exposure to FT, the relationship between these indices and EXT may be confounded by increased exposure to these behaviors, or socialization effects. In other words, growing up with an older sibling close in age may impact variability in EXT because siblings model relevant behaviors (Hicks et al., 2004). We used a sample of 409 adoptive and 208 non-adoptive families recruited in the Sibling Interaction and Behavior Study (SIBS), a classic sibling adoption design, to rule out such an effect. All families included an adolescent sibling pair and one or both parents. Sibling pairs close in age were selected as comparisons, with younger siblings approximately 15 years in age and the older siblings approximately 16 years in age. Adoptive families were recruited from infant placements made by adoption agencies in Minnesota, while non-adoptive families were recruited via publicly available birth records. Eligibility requirements for adoptive families included the following: an adopted adolescent between 11 and 21 who had been placed permanently in the adoptive home prior to the age of 2 years, and a second adolescent in the home who was not biologically related to the adopted adolescent. The second child could have been biologically related to the parents,
or adopted and placed into the home before the age of 2 years. Eligibility for the non-adoptive families included having a pair of full biological adolescent siblings.

In total, the SIBS sample included 1232 adolescents that completed the assessments. Among the 409 adoptive families, there were 124 families in which the second adolescent was a biological child of one or both of the adoptive parents. Average age of placement in the home was 4.7 months (SD = 3.4). In the adoptive families, the gender composition was 96 male/male, 148 female/female, and 163 male/female. The gender composition of the sibling pairs in non-adoptive families was 62 male/male, 68 female/female, and 78 male/female. Additional sample information can be found in McGue et al. (2007).

**Measures**

*Exposure to FT.* Two methods were used to create a proxy index of exposure to FT. Exposure to FT was measured in the MSUTR and CTS samples using a combination of co-twin sex and participant sex (i.e., twin type). Given that intrauterine effects are thought to occur for twins (Miller, 1994; Tapp et al., 2011), co-twin sex and participant sex can be used as a proxy index of exposure to FT. In turn, SS-female (SS-F) twins would be exposed to the lowest levels of FT, and were coded as 1. OS-female (OS-F) twins would be exposed to a higher level of FT and were coded as 2, while OS-male (OS-M) twins would be exposed to even greater levels of FT and were coded as 3. Last, SS-male (SS-M) twins, who would be exposed to the most FT, were coded as 4.

A count of the total number of older biological siblings was used as an alternative proxy measure of exposure to FT in the MSUTR, MTFS, and SIBS samples. Number of older siblings is thought to index variability in exposure to FT, such that a mother’s body has a “memory” for previous fetal environments and alters the fetal development of subsequent children (Maccoby et
al., 1979; T. J. Williams et al., 2000). Previous research has used number of older brothers (two or more) as a proxy index of exposure to FT (T. J. Williams et al., 2000). While the development of the male gonads into testes lends itself to greater exposure to FT in males than females, data in support of predictive adaptive response suggests that females are also exposed to least some degree of FT. Further, females are exposed to (relatively) small amounts of testosterone in utero derived primarily from the fetal adrenal gland and release of androstenedione (Maccoby et al., 1979; Morishima, Grumbach, Simpson, Fisher, & Qin, 1995). Further, residual testosterone from the maternal follicular fluid may influence intrauterine testosterone concentrations for both male and female fetuses (V. J. Grant et al., 2008). Thus, consistent with previous research using older male siblings we created a dichotomous index of exposure to FT that represented the presence of two or more siblings, regardless of sibling gender. Participants with \( \geq 2 \) biological siblings were coded as 2, while participants with < 2 older biological siblings were coded as 1. Given that this proxy index of FT is thought to represent effects of the mother’s womb on exposure to FT, older siblings were only included if they were biologically related to the participant on the mother’s side of the family (either half or full sibling). For the sub-sample of adopted participants in the SIBS sample, a sibling was included regardless of whether they were biologically related to the mother as a means to index socialization effects.

**Externalizing.** A latent EXT factor was created for each measurement occasion in each of the samples. To optimize consistency across samples, only select items were used as EXT markers. Table 2 presents the items included and the measure the item was drawn from for each sample. In the MSUTR sample, items from the Strengths and Difficulties Questionnaire (SDQ; Goodman, 1997) were used. The MTFS sample used interview-based items from a study-specific interview based on the Diagnostic Interview for Children and Adolescents (DICA; Holdcraft,
Iacono, & McGue, 1998) and assessed for lifetime and past 3 year symptoms during wave 1 and wave 2 data collection, respectively. The CTS sample drew upon self-report items from the Tri-dimensional Personality Questionnaire (TPQ; Cloninger, 1987) and interview based diagnostic symptoms counts of substance use disorders and disruptive behavior disorders (i.e., CD, ODD, ADHD) using the Composite International Diagnostic Interview – Substance Abuse Module (CIDI-SAM; L. Cottler, 2000; Robins, Cottler, & Babor, 1986) and Diagnostic Interview Screener for Children (DISC; Shaffer et al., 1993), respectively. The SIBS sample drew upon interview-based responses to the DICA to measure ODD, ADHD, and CD symptoms. To measure any history of substance use, participants completed a computerized substance use module developed by study personnel (Walden, McGue, Burt, & Elkins, 2004). These markers were used to estimate a latent EXT factor. Although from different measures, there was considerable overlap in the items assessed across samples and measurement occasions. Appendix B provides additional information regarding scale, reliability, and validity for the measures included.

**Pubertal Development.** In the MTFS sample, pubertal development was examined as a moderator of the relationship between FT and EXT. Pubertal development was measured at each occasion using the Pubertal Development Scale (PDS), a validated measure of pubertal development (Carskadon & Acebo, 1993; Petersen, Crockett, Richards, & Boxer, 1988). Participants were asked to rate what is happening in their development across 5 items. For male twins, items assessing pubertal development included the following: growth in height, growth of body hair (i.e., underarm hair, pubic hair), skin changes (e.g., pimples), deepening of voice, and

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4 For the interview-based measures of EXT markers, symptoms were dichotomized into present and absent from raw data on absent, sub-threshold, and full-threshold for consistency of measurement across the substance use and other EXT markers. A symptom was coded as present if the participant met sub-threshold criteria given the low base rate of full-threshold symptom endorsement across all samples.
growth of facial hair. For female twins, items included the following: Growth in height, growth of body hair (i.e., underarm hair, pubic hair), skin changes (e.g., pimples), breast growth, and menarche. All items were answered using a 4-point Likert scale (1 = Not yet begun, 2 = Barely started, 3 = Definitely underway, 4 = Seems completed), with the exception of the item assessing menarche (assessed using a scale of “No” = 1 or “Yes” = 4). A sum of the 5 items was used to create a continuous index of pubertal development (Carskadon & Acebo, 1993). Consistent with previous research that has examined the moderating role of exposure to FT on psychological phenotypes, participants were categorized as pre-early puberty (PDS score ≤ 2.4) or mid-late puberty (PDS score ≥ 2.5) because (1) there are substantial phenotypic and genetic effects on EXT at mid-puberty and (2) small sample sizes in some groups did not allow us to examine more than two categories (Culbert, Breedlove, Burt, & Klump, 2008; Culbert, Breedlove, Sisk, Burt, & Klump, 2013; Klump et al., 2012; Klump, Perkins, Burt, McGUE, & Iacono, 2007; Negriff & Susman, 2011).

**Data Analytic Strategy**

*MSUTR and MTFS Samples.* Data from the MSUTR and MTFS samples were analyzed using a series of structural equation models and structural regressions in *Mplus* 7.4 (Muthén & Muthén, 2012). To optimize model fit, latent factors were estimated for each subset of symptoms that were selected as EXT markers. In other words, ADHD, CD, ODD, and substance use indicators were used to estimate latent ADHD, CD, ODD, and substance use factors. These factors were then used to estimate a second-order, latent EXT factor for the MSUTR (Figure 2 and Figure 3)⁵ and the MTFS samples (see Figure 3). These hierarchical structural equation

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⁵ Alcohol, tobacco, and drug use were not developmentally relevant during wave 1 or wave 2 for the MSUTR sample (1 participant endorsed tobacco and drug use), resulting in insufficient variability to estimate a substance use factor using these items. Thus, EXT was estimated using only items reflective of CD, ADHD, and ODD in the MSUTR sample.
models allowed us to examine the impact of FT on EXT using a latent variable framework that accounted for measurement error of the latent factors. Consistent with Mplus guidelines, all observed factor indicators were identified as categorical. Data were nested within twin pair to account for the non-independence of the twin data. Further, these models allowed us to estimate a latent EXT factor for each measurement occasion while accounting for EXT levels at the previous measurement occasion. In such a way, we were able to estimate both the relationship between level of EXT and exposure to FT during wave 1 of data collection and the relationship between residualized change in EXT and exposure to FT. To model common method variance, the longitudinal models were fit with correlated residual variances between each measurement occasion for the observed indicators. Further, an interaction term that represented interactive effects between exposure to FT and pubertal development was included in the prediction of EXT and residualized change in EXT in the MTFS sample (see Figure 3). Model estimation was conducted using the mean- and variance-adjusted weighted least squares (WLSMV) estimator due to the categorical scaling of the EXT markers (Sass, Schmitt, & Marsh, 2014). Model fit was assessed using the Comparative Fit Index (CFI; Bentler, 1990) and Root-Mean-Square Error of Approximation (RMSEA; Browne, Cudeck, Bollen, & Long, 1993). Fit was considered acceptable if CFI ≥ .90 (Hu & Bentler, 1999) and RMSEA ≤ .08 (Browne et al., 1993). In the MSUTR sample, separate models were estimated to predict the effect of twin type and number of older brothers on EXT (see Figures 2 and 3, respectively).

A series of tests of measurement invariance were conducted for the models that utilized number of older brothers as a proxy index of exposure to FT because it does not include information on participant gender (unlike twin-type). Scalar invariance (i.e., factor structure, loadings, and intercepts) was tested, as recommended by Millsap (2012), because non-
polytomous (dichotomous) categorical indicators provide limited information and cause model identification issues when testing metric invariance (i.e., factor structure, loadings). These tests allowed us to determine whether the models fit differently for males and females, and subsequently if the effect of exposure to FT on EXT differed as a function of gender. Multi-group model comparisons by gender and the DIFFTEST function were used in Mplus 7.4 to determine whether the models fit differently for male and female participants (Jöreskog & Sörbom, 1993). First, a relaxed model was fit to the data that freely estimated all parameter estimates for the male and female groups. Next, a constrained model was fit to the data that equated factor structure, loading, and intercept parameters. Thereafter, the DIFFTEST function in Mplus 7.4 evaluated whether the relaxed or constrained model fit the data more appropriately (Muthén & Muthén, 2012). Change in alternative fit indices (e.g., CFI, RMSEA) is not recommended using WLSMV as an estimator because of its sensitivity to sample size and other design factors (Sass et al., 2014). Thus, only change in chi-square using the DIFFTEST option was utilized to evaluate measurement invariance across gender. Models were estimated for males and females separately by freely estimating all model parameters if differences were observed. For the models that included the twin-type proxy index, gender invariance tests were conducted for the measurement (i.e., ADHD, CD, ODD, substance use, and EXT factors) portion of the model and not the structural part (i.e., paths between twin-type and EXT) because gender is inherently measured within the the twin-type variable.

**CTS Sample.** Data from the CTS sample were analyzed using structural equation modeling in Mplus 7.4 (Muthén & Muthén, 2012). A structural equation model was fit to the data using the EXT markers in Table 2. To optimize model fit, latent factors were estimated for each subset of symptoms that were selected as EXT markers. In other words, ADHD, CD, ODD, and
substance use indicators were used to estimate latent ADHD, CD, ODD, and substance use factors. These factors were then used to estimate a second-order, latent EXT factor (see Figure 2). Consistent with M plus guidelines, all observed factor indicators were identified as categorical. Data were nested within twin pair to account for the non-independence of the twin data. The effect of FT on EXT was assessed using a set of dummy coded variables that reflected incremental increases in exposure to FT, with SS-F twins serving as the reference group. In other words, three variables were included that reflected the relative contributions for each category of exposure to FT on EXT. The first, second, and third dummy variables reflected group membership to OS-F, OS-M, and SS-M twin status, respectively. This technique facilitated identification of statistically significant differences in EXT between the reference group and the three dummy coded variables. The predictor variables were treated as dichotomous variables.

Similar to the MSUTR and MTFS samples, WLSMV was selected as the estimator and model fit was assessed using the CFI and RMSEA. Similar to the twin-type model for the MSUTR sample above, tests of gender invariance were conducted for the measurement portion of the model.

**SIBS Sample.** Last, a classic adoption design was used with the SIBS sample to disentangle whether the proxy index of exposure to FT (i.e., number of siblings) predicted EXT over-and-above socialization effects. Number of older siblings (≥ 2 and < 2) was used to measure the effects of socialization and exposure to FT on EXT. The classic sibling adoption design provides a convenient method of measuring these two effects. In the adoption sub-sample, number of older siblings reflects socialization effects on EXT, but not FT effects, because the participant did not share a fetal environment with these siblings. The same variable reflects both socialization and FT effects in the sub-sample of participants reared with their biological siblings because the participants shared both a fetal and social environment with these siblings. Thus, any
differences between the two sub-samples in the effect of older siblings on EXT could be the effect of exposure to FT. Each participant received a score of 1 or 2, indexing < 2 and ≥ 2 older siblings, respectively. This variable represents socialization effects in the adoptive sub-sample and socialization plus FT effects for the biological sub-sample.

Similar to previous analyses, latent ADHD, CD, ODD, and substance use factors were estimated in Mplus 7.4 (see Figure 7). A second-order latent EXT factor was estimated using the first-order factors as indicators. The EXT factor was predicted by number of older siblings, adoption status (1 = biological siblings, 2 = adoptive siblings), and an interaction term for number of older siblings and adoption status. A significant interaction term would suggest that the relationship between exposure to FT (i.e., number of older siblings) and EXT differs for siblings that are biologically and non-biologically related. Given that the biological sub-sample shared a social and fetal environment with their siblings while the adoptive sub-sample shared only a social environment, these differences may represent the effect of exposure to FT on EXT over-and-above socialization effects. A significant interaction term, with the relationship between exposure to FT and EXT larger for biologically related siblings than non-biologically related siblings, would suggest that exposure to FT predicts EXT over-and-above socialization effects, while a non-significant interaction term would indicate that the effect of older siblings on EXT may be due to socialization alone.
Results

MSUTR Sample

Twin-type. A model was fit to the data that examined the impact of exposure to FT on EXT at age 6 and residualized change in EXT at age 9 using the twin-type proxy index (Figure 2a). There was measurement non-invariance of the latent EXT factor for gender, such that a relaxed model fit significantly better than a constrained model ($\Delta \chi^2 = 169.40$, $\Delta df = 26$, $p < .001$). Again, a test of measurement invariance for gender was not conducted for the model that included the twin-type proxy of exposure to FT because this variable reflects gender and accounts for gender effects. Model fit indices were within the acceptable range ($\chi^2 = 833.33$, $p < .001$, CFI = .95, RMSEA = .05). The dummy coded variables that reflected OS-M and SS-M group membership significantly predicted EXT at mean age 6, while the dummy coded variable that reflected OS-F group membership did not. None of the dummy coded variables significantly predicted EXT at mean age 9, suggesting that exposure to FT did not predicted residualized change in EXT at age 9. This model predicted 10.1% and 77.3% of the variance in EXT at age 6 and 9, respectively. Separate models were also estimated for the age 6 and 9 data. The age 6 model fit the data well ($\chi^2 = 294.76$, $p < .001$, CFI = .96, RMSEA = .05) and provided identical results to those obtained in the longitudinal model. This model predicted 10.0% of the variance in EXT. The age 9 model fit the data well ($\chi^2 = 254.62$, $p < .001$, CFI = .96, RMSEA = .05). Results were identical to those in the age 6 data, with the dummy coded variables reflecting

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6 Similar to analyses in sample 1, gender effects were tested with two additional models – one that used a dummy coded variable for SS-F twins and using OS-F twins as the reference group, another that used both SS-F and OS-F twins as the reference group. The patterns of effects were identical to those found above, with both OS-M and SS-M twins exhibiting significantly greater EXT than the reference group.
OS-M and SS-M twins significantly predicting EXT at age 9. This model predicted 8.3% of the variance in EXT at age 9. Consistent with the notion that SS-M twins are exposed to greater FT than OS-M twins or female twins, these findings suggest that there may be an effect of exposure to FT on EXT that is gender specific. Alternatively, these findings may reflect well-established gender differences in EXT, with males exhibiting greater EXT than females (Leadbeater et al., 1999).  

**Siblings.** A model was also fit to the data to examine whether the effect of exposure to FT on EXT occurred via another pathway – namely, using the sibling proxy index (Figure 3). Model fit indices were acceptable ($\chi^2 = 1093.55, p < .001$, CFI = .94, RMSEA = .05). Gender invariance testing indicated that a relaxed model fit the data significantly better than a constrained model ($\Delta \chi^2 = 184.53, \Delta df = 30, p < .001$). Thus, model parameters and fit statistics are presented for the model that estimated parameters separately for males and females. Results revealed that the relationships between exposure to FT and EXT at age 6 and age 9 were non-significant for both males and females. This model accounted for 0.03% and 75.8% of the variance in EXT at age 6 and 9 for males, respectively. The model predicted <0.1% and 76.5% of the variance in EXT at age 6 and 9 for females, respectively. Similar results were obtained from models that estimated EXT at age 6 and 9 separately. The age 6 data fit the data well ($\chi^2 = 330.00, p < .001$, CFI = .96, RMSEA = .06), as did the age 9 data ($\chi^2 = 359.73, p < .001$, CFI = .95, RMSEA = .06). Exposure to FT did not predict EXT in either of these models. The model predicted 0.2% and 0.1% of the variance in EXT at age 6 for males and females, respectively. The model predicted <0.1% and 0.1% of the variance in EXT at age 9 for males and females, respectively. Thus, there were

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7 To test for this possibility, a model was fit with three dummy coded variables: OS-M twins, SS-M twins, and SS-F twins, with OS-F twins representing the reference group. Only the SS-M dummy coded variables significantly predicted EXT ($p < .001$). Another model that used both OS-F and SS-F twins as the reference group and only included dummy coded variables for SS-M and OS-M revealed an identical pattern of results, with only SS-M twins exhibiting elevated EXT.
inconsistent findings for the effect of exposure to FT on EXT at age 6 and 9 across two approaches to measurement.

**MTFS Sample**

To test the relationship between exposure to FT and EXT during early and middle adolescence, a similar model was tested using an alternative proxy index of exposure to FT (see Figure 3). This model utilized longitudinal data collected during early (11) and middle adolescence (14) to determine whether exposure to FT predicts EXT at age 11 and residualized change in EXT from age 11 to 14. An interaction of puberty and FT was also included in the model to test for potential activational hormonal effects. Gender invariance testing was conducted to determine whether these effects were gender specific. Tests indicated gender non-invariance in the models, such that a relaxed model fit the data significantly better than a constrained model ($\Delta \chi^2 = 165.69, \Delta df = 47, p = .001$). Model fit indices for the relaxed model were acceptable ($\chi^2 = 1346.46, p < .001$, CFI = .94, RMSEA = .03). Path estimates for males indicated that exposure to FT significantly predicted EXT at age 11, with more exposure to FT predicting lower EXT at age 11. Exposure to FT did not predict residualized change in EXT from age 11 to 14 for males. Exposure to FT interacted with pubertal status at age 11 and age 14 to predict EXT at age 11 and residualized change in EXT at age 14. Pubertal status negatively predicted EXT at age 11 and residualized change in EXT, with those in early puberty exhibiting greater levels of EXT at age 11 and greater residualized change in EXT at age 14. This model predicted 5.6% and 69.2% of the variance in EXT at age 11 and 14, respectively. In the female model, exposure to FT significantly predicted EXT at age 11, with greater exposure to FT associated with lower EXT. Exposure to FT did not predict residualized change in EXT at age 14. Pubertal status at age 11 and 14 did not predict either EXT at age 11 or residualized change
in EXT at age 14. Neither interaction term significantly predicted EXT. This model predicted 2.1% and 76.6% of the variance in EXT at age 11 and 14, respectively.

To test whether pubertal status moderated the relationship between exposure to FT and level of EXT at age 14 (as opposed to residualized change), separate models were estimated for the age 11 and 14 data. Results were similar to those obtained in the model that included EXT at age 11 and 14. Model fit was acceptable for the age 11 ($\chi^2 = 347.74, p < .001, \text{CFI} = .96, \text{RMSEA} = .03$) and age 14 data ($\chi^2 = 285.63, p < .001, \text{CFI} = .98, \text{RMSEA} = .02$). Figure 4 and 4b provide path estimates and factor loadings for age 11 and 14, respectively. In the model that estimated EXT at age 11, exposure to FT negatively predicted EXT for males and females. Pubertal status negatively predicted EXT for males only. Pubertal status interacted with exposure to FT to predict EXT at age 11 for males, but not females. This model explained 3.1% and 2.4% of the variance in EXT at age 11 for males and females, respectively. As shown in Figure 5, the relationship between EXT at age 11 and pubertal status was significantly larger for males with high exposure to FT than males with low exposure to FT at age 11. The relationship between EXT and pubertal status was not significantly larger for females with high versus low exposure to FT at age 11 (Figure 5). For the model that estimated age 14 EXT, exposure to FT and pubertal status negatively predicted EXT for males but not females. Exposure to FT interacted with pubertal status to predict EXT for males, but not females. This model explained 3.1% and 0.8% of the variance in EXT at age 14 for males and females, respectively. As shown in Figure 5, the relationship between EXT at age 14 and pubertal status was significantly stronger for males with high exposure to FT than those with low exposure to FT. The relationship between
EXT at age 14 and pubertal status was not significantly larger for females with high exposure to FT than those with low exposure to FT (see Figure 5).\(^8\)

To test whether the effect of the sibling index on EXT might be due to maternal harsh or stressful environments and subsequent exposure to FT, two models were estimated that included information on maternal life events prior to twin birth. Consistent with the measurement strategy used in Bemmels, Burt, Legrand, Iacono, and McGue (2008), items were selected from the Life Events interview (Billig, Hershberger, Iacono, & McGue, 1996). Items reflected potentially stressful life events, including financial (e.g., out of work, receiving financial aid, bankruptcy), relationship (e.g., divorce, frequent arguments), and medical (e.g., bad accident, been ill and hospitalized) stress. Events that occurred prior to the maternal age of the twin birth were coded as present (1). All others were coded as absent (0). A sum of the life events items was used to index pre-birth harsh/stressful environments. This item was entered as a covariate in the age 11 and 14 models. Model fit was acceptable for the age 11 (χ\(^2\) = 336.46, \(p < .001\), CFI = .95, RMSEA = .02) and age 14 data (χ\(^2\) = 266.07, \(p < .001\), CFI = .98, RMSEA = .02). For both males and females, the life events index significantly predicted EXT at age 11 (see Figure 6) and age 14 (see Figure 6), while all other variables were non-significant. The age 11 model predicted 12.7% and 7.6% of the variance in EXT for males and females, respectively. The age 14 model predicted 10.4% and 7.6% of the variance in EXT for males and females, respectively. These results add support to the notion that number of siblings indexes exposure to FT via increased maternal stress.

\(^8\) For males, the relationships observed in the age 11 model (see Figure 4) remained significant after controlling for maternal age, while the relationship between FT and EXT at age 11 for females was non-significant (see Figure 4). Similar results were obtained in the age 14 model that controlled for maternal age, such that all paths in the female model remained non-significant, while the interaction term and main effect of pubertal status remained significant in the male model. The relationship between exposure to FT and EXT at age 14 was non-significant when controlling for maternal age in the male model, while maternal age negatively predicted EXT at age 14 for males.
CTS Sample

The CTS sample was used to examine the effect of exposure to FT on EXT during late adolescence using a proxy index that represented four categories of twins with varying degrees of exposure to FT (see Figure 2). There was measurement non-invariance of the latent EXT factor for gender, such that a relaxed model fit significantly better than a constrained model ($\Delta \chi^2 = 121.54$, $\Delta df = 12$, $p < .001$). Model fit for the relaxed model with the proxy indices of FT was acceptable ($\chi^2 = 343.60$, $p < .001$, CFI = .97, RMSEA = .04). There was a significant effect of exposure to FT on the latent EXT factor, but these findings were specific to 1 of the 3 dummy coded variables. The dummy coded variable representing OS-F and OS-M twins did not significantly predict the EXT factor. Alternatively, the dummy coded variable for SS-M twins significantly predicted the EXT factor. In other words, OS-F and OS-M twins did not exhibit significantly different EXT than SS-F twins, but SS-M twins exhibited significantly greater EXT than SS-F twins. This model explained 2.4% of the variance in EXT. While the twin-type model in the MSUTR sample may have reflected well-established gender differences, this pattern suggests that there may be an effect of intrauterine transfer of testosterone between male co-twins. Alternatively, these findings may reflect socialization effects, such that growing up with a male roughly equivalent in age models behavior and contributes to elevated EXT.

SIBS Sample

Given the sex-specific and otherwise developmental period-specific findings in the previous samples, an additional test was used to determine whether the effect of the proxy indices of exposure to FT on EXT exists over-and-above socialization effects (Figure 7). First, tests of gender invariance were conducted to determine whether the model fit significantly different for males and females. Gender non-invariance was detected ($\Delta \chi^2 = 44.96$, $\Delta df = 15$, $p < 0.001$).
such that the relaxed model fit significantly better than the constrained model. Thus, all parameter estimates provided were estimated freely for male and females. Model fit indices for the relaxed model were within the acceptable range ($\chi^2 = 347.17, p < .001, \text{CFI} = .98, \text{RMSEA} = .03$). In the male model, the relationship between exposure to FT and EXT was significant, such that those participants with $\geq 2$ older siblings had higher levels of EXT. A significant effect was also observed for the interaction of adoption status with number of older siblings, such that males with $\geq 2$ biological siblings had higher EXT than those with $< 2$ two biological siblings or either category of adoptive siblings (see Figure 8). This model predicted 3.7% of the variance in EXT. In the female model, exposure to FT, adoption status, and the interaction of adoption status and exposure to FT were all non-significant. Females did not differ in EXT regardless of adoption status or number of older siblings (see Figure 8). This model predicted 2.3% of the variance in EXT. These results are generally consistent with those found in the previous samples, with a small effect of exposure to FT on EXT for males, but not females.9

Similar to the MTFS sample, a model was tested that included maternal harsh/stressful life events as a covariate. An identical measurement strategy to that described above was used to index maternal life events prior to either participating child’s birth. Given that these effects are thought to occur via the maternal intrauterine environment, only siblings that shared a biological mother were included. Model fit was acceptable ($\chi^2 = 242.13, p < .001, \text{CFI} = .97, \text{RMSEA} = .05$). Consistent with the effects observed in the MTFS sample, maternal life events significantly predicted EXT in males ($p = .01$) but not females ($p = .16$). The sibling index of exposure to FT no longer significantly predicted EXT for either males ($p = .87$) or females ($p = .50$). This model

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9 To partially control for any differences in parental rearing environment for biological and adoptive siblings, the same model was estimated including the following covariates: sibling ethnicity, maternal education, and maternal age. The model fit the data well ($\chi^2 = 412.83, p < .001, \text{CFI} = .98, \text{RMSEA} = .03$) Both the FT and the FT-adoption status interaction variables remained significant predictors of EXT (see Figure 9).
predicted 13.9% and 8.9% of the variance in EXT for males and females, respectively. These results add support to the notion that the sibling variable indexes exposure to FT via maternal stress and subsequent intrauterine testosterone exposure.
Discussion

The current study examined the effect of exposure to FT on EXT during childhood and adolescent development. Across two different indices of exposure to FT and in four independent samples, a sex-specific pattern of results emerged wherein higher exposure to FT contributed to increased EXT in males, but not females. These effects were observed over-and-above socialization effects, but only for males during the advanced stages of pubertal development. Effects were not observed in childhood or adolescence for those in the early stages of pubertal development. More specifically, the effect of exposure to FT on EXT was observed for males alone using the twin-type variable but not the sibling variable for either males or females in the MSUTR sample (i.e., childhood and pre/early puberty). In the MTFS sample, pubertal status interacted with exposure to FT to predict EXT at age 11 and 14 in males, such that the relationship between pubertal status and EXT at age 11 and 14 was stronger for males exposed to higher levels of FT. The twin-type variable predicted EXT at mean age 16 in the CTS sample, but only for males reared in-utero with male co-twins. The sibling variable positively predicted EXT at age 16 in the SIBS sample for males, but only when they shared a fetal and social environment with their sibling.

Similar to previous research, these results are consistent with the notion that there are organizational-activational hormonal effects of increased exposure to testosterone on EXT markers for males alone (Liu, Portnoy, & Raine, 2012; Martel, Gobrogge, Breedlove, & Nigg, 2008; Martel & Roberts, 2014). These results are supported within the framework of evolutionary theory, which suggests that sex-specific patterns of phenotypic variability emerge in
response to the pressures of sexual selection. EXT is one phenotype that may be sexually
selected, particularly considering that males exhibit significantly higher levels of EXT than
females throughout development and more prominently during and immediately following
puberty when the traits may be reproductively relevant (J. M. Williams & Dunlop, 1999). The
sex-dependent effects of exposure to FT on EXT observed in the current study are suggestive of
one potential biological contribution to sex differences in EXT.

Although previous research has observed significant relationships between exposure to
FT and EXT markers for children (Martel & Roberts, 2014) and adolescents (Liu et al., 2012;
Martel et al., 2008), results exhibit greater inconsistencies during childhood (Lemiere, Boets, &
Danckaerts, 2010; Robinson et al., 2013). Similarly, the effects of exposure to FT on other
psychological phenotypes are more consistently observed during adolescence and early
adulthood (Culbert et al., 2008; Raevuori et al., 2008) than childhood or later adulthood (Baker,
Lichtenstein, & Kendler, 2009; Lydecker et al., 2012). Results from the current study were
consistent with these data, such that the effect of exposure to FT on EXT was not observed for
both proxy indices of exposure to FT during childhood and pre/early adolescence. Notably, a
significant effect of exposure to FT on EXT was observed for males using the twin-type variable
but not the sibling variable in the MSUTR sample. One plausible explanation for this involves
measurement considerations for the twin-type proxy index of exposure to FT. This proxy index
is created via a combination of twin sex and co-twin sex and, as such, is confounded by the
participant’s sex. In other words, the effect of this variable on EXT may be due to the effects of
exposure to FT or gender. Given that the strength of associations between EXT and OS-M/SS-M
twins were nearly identical, the results of analyses that incorporate this variable must be
interpreted with caution. Considering that this effect was not observed in both proxy indices and
strictly in males using the twin-type variable, it is possible that the results reflect well-established, male-biased estimates of the prevalence of EXT and not the effect of exposure to FT.

Interpreted this way, the pattern of results observed in the current study indicate that the effect of exposure to FT on EXT may be relevant for males during a discrete period of development – namely throughout early, middle, and late adolescence but not childhood and pre/early adolescence. In the MTFS sample, results indicated that pubertal status interacted with exposure to FT at age 11 and 14 in males, such that the relationship between puberty and EXT was stronger for males exposed to higher levels of FT. Notably, Schulz et al. (2009) argues that adolescence is part of a protracted period of sensitivity to androgenic hormones that begins perinatally and extends into late adolescence, particularly considering that organizational and activational hormonal effects are difficult to distinguish (Arnold & Breedlove, 1985). The current findings are consistent with the notion that puberty is a period important for both organizational and activational hormonal effects on development of EXT in males (see Figure 10), particularly considering that the effects were observed during a period of development when males experience a dramatic increase in circulating testosterone (Susman et al., 1987). These findings are also consistent with animal data suggesting that exposure to increased testosterone exaggerates previously established neural networks and preexisting patterns of EXT behavior (Dixson & Herbert, 1977). Similarly, human data suggest that the masculinizing effects of early testosterone exposure lay relatively dormant and only become expressed during puberty when gonadal hormones activate sex-typical behaviors (Culbert et al., 2013).

An alternative explanation regarding the lack of significant associations between age 6 and 9 EXT and one of the two proxy indices in the MSUTR sample may concern measurement
considerations, particularly the parent-report nature of the data. Research demonstrates that not all sources of information are equally useful for the assessment of disruptive behaviors and substance use (Loeber, Green, Lahey, & Stouthamer-Loeber, 1989). For instance, many covert behaviors (e.g., substance use, conduct disorder symptoms) are more valid coming from children themselves, while more overt behaviors (e.g., ADHD and ODD symptoms) are more valid coming from parents or teachers (Hart, Lahey, Loeber, & Hanson, 1994; Loeber et al., 1989). This may account for the virtually non-existent endorsement of substance use variables and inability to estimate a latent substance use factor in the MSUTR sample. It is possible that the effect would be observed across both proxy indices if the latent factors included both parent and twin reports, although there is limited evidence as to why this effect would be observed for one proxy index but not the other using parent report alone. The rates of substance use observed in the current sample are also consistent with very low base rates of substance use for children in elementary school (DeWit, Adlaf, Offord, & Ogborne, 2000; Donovan et al., 2004; Elder et al., 1996; B. F. Grant & Dawson, 1997). With these considerations in mind, the non-significant effects observed at ages 6 and 9 are most likely consistent with gender differences in EXT due to non-hormonal influences and likely reflect a protracted period of sensitivity to androgenic hormones that lay relatively dormant until puberty.

Notably, there was a negative relationship between exposure to FT and EXT at age 11 for males in the MTFS sample. This is consistent with life history theory, which suggests that testosterone slows male offspring growth in humans to extend the prejuvenile period, increase parental investment, augment child trait plasticity, and enhance the practice of reproductively relevant traits to promote competitiveness (Ellis et al., 2012; Geary, 2002; Lee, De Kretser, Hudson, & Wang, 1975; Morris, Jordan, & Breedlove, 2004). Those with low exposure to FT
may exhibit lower EXT because they reach sexual maturity later than age-matched peers with low exposure to FT. Once these individuals reach sexual maturity and experience an increase in circulating levels of testosterone, they exhibit exaggeration of previously established behavioral patterns via activation of neural networks that encourage increased EXT. This finding may also reflect temporary/normative increases in EXT during the transition into adolescence, such that those with low exposure to FT overcompensate during this period while those at high levels of EXT maintain a less dramatic but steady, life-course persistent trajectory (Moffitt, 1993).

Generally, the findings from the current study are most consistent with organizational and activational hormonal effects during puberty and, considering the sexually-selected relevance of EXT during the transition into sexual/reproductive maturity, are supported within the framework of evolutionary theory. This is particularly supported by research suggesting that puberty is a period characterized by sharp increases in EXT and distinct sex hormone-driven changes (Pellegrini & Long, 2002).

Although results from the current study are most consistent with the notion that the effect of exposure to FT on EXT occurs in response to organizational and activational hormonal effects during puberty, it is possible that the effects observed are due to third variables (e.g., socialization). Using a sibling adoption design, the current study observed a pattern of results consistent with the notion that the effect of exposure to FT on EXT exists over-and-above socialization effects. Particularly, male participants exhibited higher EXT if they shared a fetal and social environment with two or more siblings, while participants that shared strictly a social environment with two or more siblings exhibited similar levels of EXT to those with less than 2 biologically related or adoptive siblings. These results suggest that sharing a fetal environment with siblings has an effect on EXT over-and-above sharing a rearing environment with them.
This is the first study to use a sibling adoption design to rule out the effect of socialization on the relationship between exposure to FT and EXT and enhances confidence in using this variable as a proxy index of exposure to FT. Notably, these findings were observed after controlling for relevant covariates (e.g., maternal education, maternal age, ethnicity), although it is possible that some gonadal hormone processes may be shared with other correlated phenotypes (e.g., anxiety, depression).

The particular pathways by which testosterone influences EXT in humans, while not fully understood, may occur via fetal programming (including maternal-fetus transfer), enhanced development of the male testes and subsequent secretion of testosterone, and intrauterine transfer from the male fetus to co-twin fetus (male or female). Sex hormones, like testosterone, promote organizational hormonal effects, which the brain is optimally sensitive to during gestation (see Figure 10; Baum, 1980; Schulz et al., 2009). Researchers argue that maternal exposure to stress and subsequent fetal exposure to testosterone may trigger structural neuroadaptations, such as cerebral lateralization, that affect cellular and behavioral responses to sex hormones and sensitize the brain’s reward system to the reinforcing properties of EXT (Lenz et al., 2012). The Geschwind-Behan-Galaburda hypothesis states that increased exposure to FT interferes with the development of the left hemisphere, thereby favoring the development of the right hemisphere (Geschwind & Behan, 1982; Geschwind & Galaburda, 1985). Recent neuroscience research suggests that increased exposure to FT predicts increased behavioral approach tendencies via reward related-related regions in the caudate, putamen, and nucleus accumbens, particularly in the right hemisphere (Lombardo et al., 2012). Other research has shown that disruption of the right prefrontal cortex via repeated transcranial magnetic stimulation contributes to enhanced EXT. Notably, EXT disorders such as ADHD and alcohol dependence are associated with
cerebral lateralization, particularly right-hemispheric dominance (Denny, 2011; Harburg, 1981; London, Kibbee, & Holt, 1985; McNamara, Blum, O’QUIN, & Schachter, 1994; Nasrallah, Keelor, & McCalley-Whitters, 1983; Sandson, Bachna, & Morin, 2000; Wolfgang Sperling et al., 2010; W Sperling et al., 2000). Together, these studies support the mediating role of right hemispheric reward sensitivity in the relationship between exposure to FT and variability in EXT, but the extant literature is currently limited and research on potentially common pathophysiological mechanisms requires further attention.

It is possible that male-biased increases in EXT and the onset of the effect of FT exhibited during adolescence occur in response to a surge in circulating testosterone that “highjacks” neural rewards circuitry organized by exposure to FT. There is evidence that sex hormones potently modulate the neurotransmitter systems that are associated with the brain’s reward circuitry (Zheng, 2009). For instance, androgens trigger a dopaminergic response in reward-related neural regions, such as the mesolimbic pathway and nucleus accumbens (Alderson & Baum, 1981; de Souza Silva, Mattern, Topic, Buddenberg, & Huston, 2009; Hernandez et al., 1994). The male-biased surge in testosterone during puberty may interact with neural structures already predisposed to heightened reward sensitivity to promote even greater reinforcement of EXT. Although the brain’s sensitivity to the organizational effects of sex hormones decreases substantially by puberty, there is still some degree of sensitivity to sex hormones (see Figure 10). The intersection of this sensitivity and the persistent increase in levels of circulating testosterone during puberty may represent a critical period of responsiveness to both activational and organizational hormonal effects. Notably, males exposed to increased FT are more likely to carry alleles in the androgen receptor gene that respond to testosterone with high transactivational activity, or are more sensitive to testosterone (J. T. Manning, Bundred,
Newton, & Flanagan, 2003). It is possible that males with high exposure to FT may be more genetically sensitive to the activational hormonal effects of testosterone during puberty, or testosterone in general. Androgenic hormones like testosterone may also influence changes in white matter volume during adolescent male brain development via more efficient androgen receptor transcriptional activity and subsequent increases in axonal caliber, but not necessarily myelination (Giedd et al., 1999; Lenroot & Giedd, 2006; Perrin et al., 2008). Taken together, the current results are consistent with previous research suggesting that organizational and activational hormonal effects during puberty – paired with organizational effects during fetal development – may contribute more to sex-linked forms of psychopathology than organizational effects during fetal development alone (Berenbaum & Beltz, 2011).

The current research lends further support to the growing literature on the relationship between exposure to FT and EXT and the larger literature on the influence of psychosocial stress and child development. Specifically, the current findings are suggestive of one potential biological contribution to sexual dimorphism in EXT and have implications for research on epigenetic influences on EXT. In the past decade there has been increased attention to epigenetics – the notion that environmental conditions in early life influence gene function, or expression, and subsequent phenotypic variability (Meaney, 2010). Research suggests that epigenetic influences are important for the development of EXT, particularly for those individuals with life-course persistent trajectories that fail to learn that many forms of EXT are socially unacceptable in adulthood (Tremblay, 2010). Epigenetic influences may impact reward circuitry and subsequent transdiagnostic behavioral phenotypes (e.g., impulsivity) that are present in sexually dimorphic psychological phenotypes (Archer, Oscar-Berman, Blum, & Gold, 2012). Considering that genetic influences on EXT increase during and immediately after
adolescence for males and not females (Hicks et al., 2007), the current findings may be an example of a biological manifestation of the environment that triggers epigenetic effects. Although the extant literature on the epigenetics of EXT is currently limited, this research suggests that the interactions between genetic factors and exposure to testosterone – during gestation, early life, and critical developmental periods like puberty – may be a fruitful area of future research. The notion that these effects may represent epigenetic influences also bear practical implications for treatment of those at high risk for a lifetime of EXT and related maladaptive phenotypes. To minimize the risk that these epigenetic influences have downstream effects it may be important that interventions target mothers and their offspring as close as possible to conception with continued support to the family and child (Petitclerc & Tremblay, 2009). While these interventions will not modify genes, they may impact gene expression and subsequent phenotypic variability in EXT (Tremblay, 2010).

**Limitations and Future Directions**

While the current study has several methodological strengths (e.g., use of longitudinal data, multiple units of analysis, elegant rule out of socialization effects), four sets of limitations qualify the current findings. The most obvious limitation concerns the use of proxy indices of exposure to FT. Previous research suggests that exposure to FT is well represented by several different proxy indices (e.g., finger length ratios, twin-type), although there are more direct measures of exposure to FT (Lutchmaya, Baron-Cohen, Raggatt, Knickmeyer, & Manning, 2004; J. T. Manning et al., 1998; Tapp et al., 2011; van Anders, Vernon, & Wilbur, 2006). Placental and amniotic fluid testosterone levels have been used to investigate hormonal influences on neural reward processing, cognition, psychological disorders, and behavior (Auyeung et al., 2009; Bergman, Glover, Sarkar, Abbott, & O'Connor, 2010; Lombardo et al.,
2012; Seckl & Holmes, 2007). However, these indices are more poorly understood than measures of hormones in fetal plasma, which are becoming increasingly rare due to declining indications for fetal blood sampling (Fisk & Bower, 1993). Like the current study, the overwhelming research on the relationship between exposure to FT and EXT has typically used proxy indices that may represent different pathways to FT exposure. The proxy indices used in the current study have both received adequate attention and have been consistently linked with increased exposure to FT (Maccoby et al., 1979; Tapp et al., 2011). Future research would benefit from replicating these results using more direct measures of exposure to FT that operate via similar pathways. Given previous research indicating that there is variability in fetal testosterone levels based on gestational age (Prince, 2001), this research would provide a more thorough understanding of whether there are critically sensitive periods of fetal development for exposure to FT and downstream effects on EXT. Alternatively, future research may benefit from continued examination of EXT in males with and without congenital adrenal hyperplasia, considering that males with this disease are exposed to higher levels of FT (Brown et al., 2002; Mueller et al., 2010).

The current study was also limited in its ability to detect true activational hormonal effects. The moderating role of puberty was used as a proxy for circulating testosterone and was examined at two points during development that were three years apart. Although this facilitated examination of the moderating role of pubertal status in early/middle puberty (age 11) and middle/late puberty (age 14), additional measurement occasions would facilitate a more sensitive investigation of the timing of the observed effects (e.g., pre-puberty vs. early puberty vs. mid-puberty vs. late puberty). Similarly, future research with measures of exposure to FT and levels of circulating testosterone during puberty may allow researchers to test a specific biological
mechanism underlying the processes observed in the current study. Recent data have shown that the organizational effects of exposure to FT may predispose individuals to lower cortisol reactivity, which contributes to greater aggression and rule-breaking (Portnoy et al., 2015). This highlights the importance of utilizing a multi-systems approach in which interactions between multiple hormones are taken into account. Future research on EXT may clarify the role of organizational and activational hormonal effects during puberty by including measures of exposure to FT, collecting hormonal assays, and following participants with greater regularity throughout adolescence.

Third, the results from the SIBS sample are characterized by the typical limitations of sibling adoption studies. One limitation concerns the influence of parental characteristics on differential phenotypic expression in adopted and biologically related children. However, McGue et al. (2007) showed range-restricted parental disinhibitory psychopathology and SES did not substantially affect estimates of environmental effects on phenotypes. Thus, there is support for using the sibling adoption design to test socialization effects. Another limitation is the possibility of selective placement, as information about the mental health of biological birth parents of adopted participants was not available. However, selective placement usually pertains to physical attributes and if an at-risk child were to be placed in a particularly nurturing environment, environmental effects would have diminished (McGue et al., 2007).

A final set of limitations concerns the multi-sample approach employed in this study. A primary concern is the use of multiple different measures of EXT markers. The current study successfully identified significant overlap in the EXT markers selected for investigation that facilitated comparison across the four samples. However, there were small inconsistencies in the measures these items were drawn from and differential availability of proxy measures of FT
(e.g., twin-type, sibling) that prevented use of methods that simultaneously analyze data across independent samples (e.g., accelerated longitudinal design; Duncan, Duncan, & Hops, 1996). Additionally, the longitudinal samples included in the current study provided limited measurement occasions with the same EXT markers available. This required the current study to conduct multiple statistical tests (increasing the likelihood of type I error), although the consistency of the effects observed in the current study provides evidence against the presence of type I error. While utilizing multiple samples allowed the current study to test the consistency of the effects of exposure to FT on EXT, there are many strengths to true longitudinal designs (e.g., continuity of measurement, establish temporal precedence). Future research on the relationship between organizational/activational hormonal effects and EXT may benefit from utilizing a true longitudinal design that measures FT and EXT at more regular intervals than the current study. Longitudinal studies will promote increased confidence that the differences observed between pubertal groups are in fact reflective of within-person developmental changes. Future research using longitudinal designs may also reduce the influence of measurement inconsistencies and would eliminate the need for multiple studies. Further, results from the current study indicated that exposure to FT predicted residualized change in EXT. Use of a true longitudinal design with more measurement occasions would allow researchers to further investigate whether exposure to FT predicts changes in EXT and identify the precise timing of these effects.

The current research, while qualified by these important limitations, adds support to the growing literature on the relationship between exposure to FT and EXT. Most importantly, the findings presented herein show evidence for the effect of exposure to FT on EXT across multiple indices and tests these effects during childhood and adolescent development. The multi-sample approach also speaks to the consistency of the proxy indices of FT and, contrary to previous
research, suggests that organizational effects during gestation may have less of an impact on EXT than both organizational and activational effects that occur during the transition into puberty and immediately after. This research takes an important step toward identifying the periods most critical for hormonal effects on EXT and suggests that future research may benefit from identifying biological contributions to behavior during multiple periods of development.
### Tables

Table 1: Average ages available per assessment per sample.

<table>
<thead>
<tr>
<th>Age</th>
<th>6</th>
<th>9</th>
<th>11</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTS Sample</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIBS Sample</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>MTFS Sample</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSUTR Sample</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: CTS = Community Twin Study; MSUTR = Michigan State University Twin Registry; MTFS = Minnesota Twin and Family Study; SIBS = Sibling Interaction and Behavior Study
Table 2: Markers of EXT across samples.

<table>
<thead>
<tr>
<th>Item Measure</th>
<th>Item Measure</th>
<th>Item Measure</th>
<th>Item Measure</th>
<th>Item Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Often has temper tantrums or hot tempers</td>
<td>SDQ</td>
<td>Have you lost your temper?</td>
<td>DISC - ODD</td>
<td>Have you lost your temper?</td>
</tr>
<tr>
<td>Restless, overactive, cannot stay still for long</td>
<td>SDQ</td>
<td>When you had to sit still, say for ten minutes, did you always feel restless, as if you wanted to kick your feet or get up and move around?</td>
<td>DISC - ADHD</td>
<td>When you had to sit still, say for ten minutes, did you always feel restless, as if you wanted to kick your feet or get up and move around?</td>
</tr>
<tr>
<td>Often lies or cheats</td>
<td>SDQ</td>
<td>Have you ever lied to get money or something?</td>
<td>DISC - CD</td>
<td>Have you lied to get something in the last year?</td>
</tr>
<tr>
<td>Easily distracted, concentration wander</td>
<td>SDQ</td>
<td>Difficult to keep mind on what you were doing, even for short period of time?</td>
<td>DISC - ADHD</td>
<td>Difficult to keep mind on what you were doing, even for short period of time?</td>
</tr>
<tr>
<td>Sees tasks through to the end, good attention span</td>
<td>SDQ</td>
<td>Ever have trouble finishing things you were supposed to do?</td>
<td>DISC - ADHD</td>
<td>Did you have trouble finishing things you were supposed to do?</td>
</tr>
<tr>
<td>Constantly fidgeting or squirming</td>
<td>SDQ</td>
<td>Are you often fidgety and restless?</td>
<td>DISC - ADHD</td>
<td>Were you often fidgety and restless?</td>
</tr>
<tr>
<td>Steals from home, school, or elsewhere</td>
<td>SDQ</td>
<td>Ever stolen from family? Ever stolen from a store? Ever stolen from anyone else?</td>
<td>DISC - CD</td>
<td>Have you ever stolen without confrontation?</td>
</tr>
<tr>
<td>Generally obedient, does what is asked of him/her</td>
<td>SDQ</td>
<td>Have you refused to do what you were told to do?</td>
<td>DISC - ODD</td>
<td>Have you refused to do what you were told to do?</td>
</tr>
<tr>
<td>Destroys things that belong to others</td>
<td>SDQ</td>
<td>Have you ever broken or damaged somebody else’s things on purpose?</td>
<td>DISC - CD</td>
<td>Have you ever broken or damaged somebody else’s things on purpose?</td>
</tr>
<tr>
<td>Thinks things out before acting</td>
<td>SDQ</td>
<td>I like to think about things for a long time before I make a decision. I usually think about all the facts in detail before I make a decision. When other people demand a quick decision.</td>
<td>TPQ</td>
<td>Do you ever blurt out answers? Do you often have difficulty waiting your turn? Do you often interrupt others?</td>
</tr>
<tr>
<td>Alcoholism</td>
<td>Parent Report of Child</td>
<td>Ever used alcohol</td>
<td>CIDI-SAM</td>
<td>Ever used alcohol</td>
</tr>
<tr>
<td>Drug Abuse</td>
<td>Parent Report of Child</td>
<td>Ever used any drugs</td>
<td>CIDI-SAM</td>
<td>Ever used any drugs</td>
</tr>
<tr>
<td>Tobacco Use</td>
<td>Parent Report of Child</td>
<td>Ever used tobacco</td>
<td>CIDI-SAM</td>
<td>Ever used tobacco</td>
</tr>
</tbody>
</table>

Note: 1 A max was taken of the items to represent a score for that marker of EXT. 2 Reverse coded variables were used.
Figure 1. Illustration of Potential Pathways to Increased Exposure to FT. Harsh environmental stress may facilitate increased exposure to FT via maternal stress that increases maternal testosterone release and cortisol release via down-regulation of the enzyme 11b-HSD2 which metabolizes cortisol in the placenta. These hormones are passed through the placenta and umbilical cord to the developing fetus. Alternatively, testosterone is transferred across the fetal membrane in twins. Other influences include the development of the male testes, fetal adrenal gland, and potentially genetic influences.
Figure 2. Twin Type SEM in MSUTR (left) and CTS (right) Samples. Hierarchical Structural Equation Model of EXT Predicted by Twin Type Dummy Coded Variables in MSUTR and CTS Samples. Unstandardized estimates and standard errors (in parentheses) reported. * = \( p < .05 \), ** = \( p < .01 \), *** = \( p < .001 \).
Figure 3. Sibling SEM in MSUTR (Left) And MTFS (Right) Samples. Hierarchical Structural Equation Model of EXT Predicted by Sibling Index in MSUTR and MTFS Samples. Estimates for the males-only model are shown before the backslash and estimates for the females-only model are shown after the backslash. Unstandardized estimates and standard errors (in parentheses) reported. * = $p < .05$, ** = $p < .01$, *** = $p < .001$. 
Figure 4. FT-EXT by Pubertal Status at Age 11 and 14 in MTFS. Hierarchical Structural Equation Model with Moderation of FT-EXT Relationship by Pubertal Status at Age 11 and 14 in MTFS Sample, with Covarying Age. Estimates for males and females are shown before and after the backslash, respectively. * = p < .05, ** = p < .01.
Figure 5. FT-EXT Moderated by Pubertal Status at Age 11 and Age 14. Relationship Between Exposure to FT and EXT Moderated by Pubertal Status in Males (Left) and Females (Right) at Age 11 (Top) and Age 14 (Bottom). EXT is on the Y-axis and early-late puberty is on the X-axis from left to right.
Figure 6. FT-EXT at Age 11 and 14 Covarying Maternal Life Events. Hierarchical Structural Equation Model of EXT at Age 11 and 14 using FT, Pubertal Status, FT-Pubertal Status Interaction, and Maternal Life Events as Predictors. Unstandardized estimates and standard errors (in parentheses) reported. * = $p < .05$, ** = $p < .01$, *** = $p < .001$
Figure 7. Hierarchical Structural Equation Model using the SIBS Sample. Estimates for the males-only model are shown before the backslash and estimates for the females-only model are shown after the backslash. Adopt = adoption status (1 = biological family, 2 = adoptive family); FT×Adopt = Interaction of FT and adoption status. Unstandardized estimates and standard errors (in parentheses) reported.* = $p < .05$. 
Figure 8. Mean EXT in Males and Females by Adoption Status and FT Index.
Figure 9. Hierarchical SEM using the SIBS Sample and Covariates. Estimates for the males-only model are shown before the backslash and estimates for the females-only model are shown after the backslash. Adopt = adoption status (1 = biological family, 2 = adoptive family); FT×Adopt = Interaction of FT and adoption status. Unstandardized estimates and standard errors (in parentheses) reported. * = $p < .05$, ** = $p < .01$. 
Figure 10. Brain Sensitivity to Organizational Testosterone Throughout Life. The solid lines (Lenz et al. (2012) indicate the degree of brain sensitivity to the organizational effects of sex hormones (modified to Schulz et al., 2009); the dashed line illustrates human male testosterone levels with three distinct perinatal peaks, a mid-gestational peak and two postnatal peaks, as well as a final persistent increase during puberty (McIntyre, 2006).
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significance, and new case incidence from ages 11 to 21. *Journal of consulting and clinical psychology, 64*(3), 552.


APPENDIX A: Relationship between Siblings and Fetal Testosterone Exposure

Sibling Sex/Birth Order as a Proxy Index of Exposure to Fetal Testosterone

While researchers have developed several proxy (indirect) indices of exposure to FT, some indices have been the primary recipients of empirical attention (e.g., 2D:4D ratio). This may rise partly from the fact that these indices do not lack a coherent theoretical framework for understanding the underlying mechanism of these measures (J. T. Manning et al., 1998). One measure that has historically lacked a widely accepted theoretical framework is sibling sex/birth order. Indeed, number of older siblings (typically brothers) has been associated with both direct (Maccoby et al., 1979) and indirect measures of exposure to FT (J. Manning, Martin, Trivers, & Soler, 2002; T. J. Williams et al., 2000). Two theories provide an explanation of the complex relationship between sibling sex/birth order and exposure to FT.

Gualtieri and Hicks (1985) proposed an immunoreactive theory of selective male affiliation, which argued that a larger sibling sex-ratio (more males) lends itself to subsequent male-biased psychopathology via a maternal immune response to male fetuses. Several antigens—a molecule capable of inducing an immune response—may lend themselves to this relationship, although the H-Y antigen and, to a lesser extent, testosterone have received the most attention within this theoretical framework. The male-biased H-Y antigen, which is partially responsible for the development of testes (Müller & Lattermann, 1987), has been consistently shown to cause a maternal immune response (Johansen, Festenstein, & Burke, 1974; Komlos et al., 1990; Müller & Lattermann, 1987) that transfers H-Y antibodies to the developing fetus (Shalev, 1980). Existing research suggests that the maternal immune response is
increasingly potent for each subsequent son (Blanchard & Bogaert, 1996; Bogaert & Skorska, 2011). H-Y antibodies have been proposed to threaten the sexual differentiation of the male fetus, which may result in an adaptive response to protect itself via an overcompensation of testosterone release (Blanchard, 2001; MacCulloch & Waddington, 1981). Maternal antibodies to H-Y antigen may result in subsequent elevations of H-Y antibodies, in turn contributing to accelerated development of the testes and increases in fetal testosterone exposure. Another possibility is that maternal exposure to H-Y antigen results in elevated levels of maternal immune complexes and subsequent maternal stress, although attempts to test this process have proven difficult (Farber, Cambiaso, & Masson, 1981; Gordon, Simpson, & Samelson, 1975; Moseley, 2000). This may result in higher levels of maternal circulating cortisol and testosterone that are then transferred to the fetus.

Alternatively, testosterone may be the relevant fetal antigen that causes a maternal immune response (MacCulloch & Waddington, 1981). An experiment by Bidlingmaier, Knorr, and Neumann (1977) provided evidence that a maternal immune reaction to testosterone contributed to increased serum testosterone levels in male offspring. Findings from this study demonstrate that antibodies to testosterone result in feedback-induced increases in absolute testosterone level. Notably, exposure to FT is related to levels of absolute (circulating) testosterone (Muller et al., 2011), although findings are inconsistent (Hönekopp, Bartholdt, Beier, & Liebert, 2007). If this effect is truly exacerbated for each subsequent child, this may represent a means by which the intrauterine environment is increasingly androgenized, although empirical support for this process is limited. Thus, while this theory provides an attractive theoretical framework for understanding the relationship between number of older siblings and an increase in testosterone exposure, attempts to test it have resulted in inconsistent findings (P.
T. Ackerman, Goolsby, & Paal, 1988). Another challenge is that there is a limited understanding of the mechanisms underlying how testosterone may be involved in this process, how it is “remembered” in the womb, and how the effect increases in potency over time.

More recently, V. J. Grant (2007) proposed a theoretical framework that incorporates a more obvious link between increased fetal exposure to sex hormones (testosterone) and number of older siblings. This theory is largely grounded in research suggesting that hormonally controlled sex-ratios partially mediate the relationship between FT and sexual dimorphism in EXT (increased male births; W. H. James, 2004, 2008a, 2008b; W. H. James, 2010). For the past several decades research has suggested that parental hormone levels control the human sex-ratio (W. H. James, 1986; J. Manning et al., 2002). Research from animal models has consistently shown that increases in exogenous testosterone results in higher primary sex-ratios (increased males; Goerlich, Dijkstra, Schaafsma, & Groothuis, 2009; Krackow, 1995; Rutkowska & Cichoń, 2006; Veiga, Viñuela, Cordero, Aparicio, & Polo, 2004). Similarly, research has observed a relationship between parental levels of circulating hormones and male-biased offspring in humans (DiPietro, Costigan, Kivlghan, Chen, & Laudenslager, 2011; W. H. James, 1996, 2004; Ventura, Gomes, Pita, Neto, & Taylor, 2013). One potential mechanism linking testosterone and sex ratios involves the storage of testosterone in maternal follicular fluid (V. J. Grant, 2009). Research from animal models suggests that controlling maternal levels of follicular testosterone prior to conception leads to an increase in male offspring (V. J. Grant & Irwin, 2005; V. J. Grant et al., 2008). These results suggest that the ovum may be modified or adapted during each menstrual cycle to receive an X- or a Y-chromosome-bearing spermatozoon (sperm cell; Saling, 1991). Thus, maternal stress may influence maternal levels of circulating testosterone, which increases are reflected in levels of testosterone in the follicular fluid prior to
conception. This influences the likelihood that a male offspring is born and the amount of testosterone it is exposed to in utero, regardless of sex. In turn, the more male offspring a mother has, the more likely she is to have experienced stress and subsequent increases in circulating and fetal testosterone.

Notably, the biological mechanism for increasing the likelihood that an offspring is male might fail, resulting in a fetus that did not receive a Y-chromosome bearing sperm cell and is exposed to elevated levels of FT. As such, number of both older male and older female siblings may index increased exposure to FT, although males are exposed to greater levels due to testosterone secreted from the testes. This theory and new data may explains why the sex-ratio is more sensitive to increased exposure to FT, but suggests that the relationship between sex of the older sibling and exposure to FT is merely confounded by increased likelihood of receiving a Y-chromosome-bearing sperm cell. As such, both older male and female siblings may represent an aggregate proxy index of exposure to FT.

While the theory proposed by V. J. Grant (2007) may provide a more parsimonious theory that is consistent with the extant literature, it is possible that the two aforementioned theories are complimentary and can collectively provide a more comprehensive understanding of the complexity that characterizes the relationship between sibling sex-ratio/birth order and exposure to FT. Appendix A Figure 1 (below) provides an illustration of one potential model that incorporates tenets of both these theories. This model shows how maternal stress results in an increased sex-ratio (males births). It also incorporates aspects of the immunoreactive theory, particularly the role of H-Y and testosterone as antigens and their influence on maternal stress. Additional support for incorporating these two theories comes from research suggesting that immunization of females to H-Y antigen can increase the proportion of males in their offspring.
(Blanchard & Klassen, 1997). Other pathways included in this model represent evidence that immune complexes are under the influence of sex steroids, like testosterone (Kiess, Liu, & Hall, 1991).

While this model is suggestive of the complex system underlying the relationship between sibling sex ratio/birth order and exposure to FT, other research that is not included in this figure further complicates the existing data. For instance, maternal and paternal levels of testosterone at conception are related to sex determination of offspring (J. Manning et al., 2002). Thus, not just maternal, but paternal influences may be implicated in this model. Other research suggesting that there are links between levels of testosterone and dominance further complicate the picture (Mazur & Booth, 1998). Under conditions of chronic stress, males levels of testosterone decrease (Christiansen, 1998; Kreuz et al., 1972; Opstad, 1992), but female levels increase (Gray, 1992). Females at either end of a dominance hierarchy are more likely to experience larger degrees of stress and subsequent increases in testosterone. According to V. J. Grant (2007), this could have an effect on the sex determination of their offspring. Together, although there are existing theories that attempt to explain how number of older siblings indexes exposure to FT, there is not one theory that provides a comprehensive explanation. Future research would benefit from further exploration of the complex interactions between these variables and development of a theoretical framework that accounts for this complexity.
APPENDIX B: Externalizing Measures Across Samples

MSUTR Sample

The MSUTR sample primarily measured EXT markers using the SDQ, a 25-item informant-rated questionnaire (Goodman, 1997). Participant’s parents were asked to rate what each twin was generally like using a 3-point Likert scale (0 = Not true, 1 = Somewhat true, 2 = Certainly true). The SDQ has evidenced reliability and validity (Goodman, 1997). Parent’s also reported on twin’s “medical” history, including history of alcoholism, drug abuse, and smoking. Responses to these questions were either affirmative or negative. Thus, each item was coded as “No” (0) and “Yes” (1) for a history of alcoholism, drug abuse, and smoking.

CTS Sample

The TPQ, a self-report measure of personality, was used to assess markers for EXT. Participants were asked to indicate whether a variety of statements describing themselves were “False” (0) or “True” (1). The TPQ has established reliability and validity (Cloninger, 1987).

Diagnostic symptom counts for disruptive behavior disorders, particularly for CD, ADHD, and Oppositional Defiant Disorder (ODD), were also used as EXT markers. These symptoms were measured using the DISC, a semi-structured interview of child psychopathology (Shaffer et al., 1993). Participants were interviewed by trained research assistants and symptoms were rated as “No” (0), or “Yes” (1). The DISC has established reliability and validity (Shaffer et al., 1996; Shaffer, Fisher, Lucas, Dulcan, & Schwab-Stone, 2000).

Last, the CIDI-SAM was used to collect information on twin substance use (L. Cottler, 2000; Robins et al., 1986). Participants were interviewed by trained research assistants regarding
their alcohol, tobacco, and psychoactive drug use, including cannabis, amphetamines, sedatives/hypnotics/tranquilizers, cocaine, opiates, PCP, hallucinogens, and inhalants. The CIDI-SAM has evidenced reliability and validity (L. B. Cottler, Robins, & Helzer, 1989). In order to optimize consistency across samples, we used variables that reflected any history of alcohol, tobacco, or drug use in the participant’s lifetime. No history of use was scored 0, while a positive history of use was scored 1.

MTFS Sample

Items were drawn from an interview of DSM-III-TR symptoms of CD, ODD, ADHD, and substance abuse disorders that was adapted by MTFS staff from the Diagnostic Interview for Children and Adolescents (DICA; Holdcraft et al., 1998; Reich, 2000). Twins and their mothers were interviewed separately by trained research assistants regarding twin CD, ODD, ADHD, and substance use symptoms using the DICA. Previous studies have shown that each informant provides unique and valid information (Burt, Krueger, McGue, & Iacono, 2001). In order to provide the most comprehensive assessment of psychiatric symptoms, a best-estimates approach was used. Specifically, symptoms for CD, ODD, ADHD, and substance use disorders were coded as present if reported by either mother or twin. Those participants without an informant report by the participant’s mother utilized an informant report from the participant’s rearing parent. All data were reviewed by at least 2 graduate students with extensive experience in differential diagnosis. Consensus was reached between diagnosticians regarding the presence or absence of symptoms before assigning symptoms.

SIBS Sample

EXT features were measured using participant and parent reports on the DICA, an interview-based measure of DSM-IV-TR criteria for CD, ODD, ADHD (Reich, 2000). All data
were reviewed by at least 2 graduate students with extensive experience in differential diagnosis. Consensus was reached between diagnosticians regarding the presence or absence of symptoms before assigning symptoms. For the substance abuse disorders, a similar coding system to that used in the CTS and MTFS samples was implemented in the SIBS sample.
APPENDIX C: The Effect of Exposure to FT on Adult Antisocial Behavior

Sample

The MTFS sample includes an 11-year-old and a 17-year-old cohort of male and female twins. Intake and follow-up assessments coincide with major transitions in development. The 11-year-old cohort includes 756 twin pairs with data on antisocial personality disorder symptoms at ages 11, 14, 17, 21, and 24, while the 17-year-old cohort includes 626 twin pairs with data at ages 17, 21, 24, and 27. Follow-up rates were high, with typical retention rates of approximately 90% (Elkins et al., 2007; Hicks et al., 2012; Klahr et al., 2011). Ethnic/racial composition of the sample reflected that of the recruitment area for the birth years sampled, with over 95% of the twins reportedly Caucasian. In total, 896 of the twin pairs are MZ (50.8% female) and 486 are DZ (54.5% female).

Measures

*Exposure to FT.* A dichotomous variable reflecting less than two older siblings (regardless of sibling sex) and greater than or equal to two older siblings was used to represent exposure to FT. This strategy was identical to that used above.

*EXT.* Given that the MTFS sample utilized developmentally relevant measures of psychopathology, we used symptoms of DSM-IV antisocial personality disorder and substance use as markers of EXT. Participants provided self-reports of these markers using the interview-based measures described above in the MTFS sample. The following items were used to reflect antisocial personality disorder symptoms: failure to conform to social norms, deceitfulness, impulsivity, irritability and aggressiveness, reckless disregard for the safety of others, consistent
irresponsibility, and lack of remorse. Similar to the measurement strategy used above, symptoms were coded as present if a participant met sub-threshold or full criteria for a symptom. Measures of alcohol, tobacco, and drug use were collected using the strategy detailed above for the MTFS sample.

Data Analytic Strategy

The effect of FT on EXT in the MSUTR sample was estimated using latent growth modeling (LGM). LGMs provide a flexible framework within which to model longitudinal behavioral data and are capable of fitting models to data that follow non-normal distributions. WLSMV was selected as the estimator due to the categorical nature of the factor indicators. Data were nested within a higher order twin pair to account for the non-independence of the twin data. Four first-order factors were estimated using the EXT markers described above to represent EXT at mean ages 17, 20, 24, and 27. Thereafter, an LGM was fit to determine the shape of change, mean, and variance estimates of the intercept and slope of EXT during late adolescence and adulthood. First, a second-order latent intercept factor was estimated by setting all first-order EXT factor loadings to 1. A second-order latent slope factor was then estimated by setting the first-order latent EXT factor time score loadings to 0, 1, 2, and 3 for ages 17, 20, 24, and 27, respectively. Last, similar to the strategy used above, gender differences in the relationship between exposure to FT and latent intercept and slope of EXT were examined using multi-group comparisons by gender.

Results

Model fit was first assessed via chi-square test of model fit. While the chi-square test statistic indicated that the model did not fit the data well ($\chi^2 = 2865.31$, df = 1663, $p < .001$), it is largely influenced by sample size (calculated via product of sample size – 1 and maximum
likelihood fit function). However, alternative fit indices that are less sensitive to sample size indicated that the model fit the data well (CFI = .97, RMSEA = .02). Parameter estimates for the male model indicated that there was significant covariance between the intercept and slope of EXT (estimate = -.07, s.e. = .03, \( p = .03 \)), but the proxy measure of exposure to FT predicted neither the intercept (estimate = -.01, s.e. = .07, \( p = .91 \)) nor the slope (estimate = -.03, s.e. = .02, \( p = .15 \)) of EXT. Parameter estimates from the female model were similar to those obtained in the male model, such that the covariance between the intercept and slope of EXT was significant (estimate = -.09, s.e. = .04, \( p = .02 \)), while exposure to FT predicted neither intercept (estimate = -.02, s.e. = .07, \( p = .80 \)) nor slope (estimate = .01, s.e. = .02, \( p = .52 \)). Together, these results suggest that exposure to FT may not be relevant for the overall level or change in adult EXT (as measured in the current study) from late adolescence to early adulthood. Moreover, these results are nearly identical for males and females.